UNIVERSITY OF ADELAIDE LIBRARY
31. 8. 93

EFFECT OF EXOGENOUS EPIDERMAL GROWTH FACTOR ON THE NORMAL AND ULCERATED COLON IN RATS

A thesis submitted to the University of Adelaide,
South Australia,
for the degree of Doctor of Philosophy

by

KAREN ANN RIBBONS. BSc.(Hons.) - Flinders University

Department of Obstetrics and Gynaecology,
University of Adelaide,
South Australia. 5000

May 1993

Awarded 1993

How can you get very far,
If you don't know Who You Are?
How can you do what you ought,
If you don't know What You've Got?
And if you don't know Which To Do
Of all the things in front of you,
Then what you'll have when you are through
Is just a mess without a clue,
Of all the best that can come true
If you know What and Which and Who.

From: The Tao of Pooh, Benjamin Hoff, 1982

This thesis is dedicated to

my husband

Simon Paul Thistlewood

and my parents

Edna Alice and Harry Frank Ribbons

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference had been made in the text.

I give consent to this copy of my thesis, when deposited in the Univeristy Library, being available for photocopying and loan.

Signed: Date

21/5/93

(Karen Ann Ribbons)

BSc. (Hons)

ACKNOWLEDGMENTS

For her guidance and encouragement throughout this PhD. I am indebted to my supervisor and mentor Dr. Leanna Read, Director, Child Health Research Institute, South Australia. For their advice, assistance and friendship my utmost thanks is also extended to Dr. Anne Martin, Mr. Callum Gillespie and Mr. Gordon Howarth. I would also like to extend my gratitude to Dr. Carlos George-Nascimento, Chiron Corporation, Emeryville, California, for providing me with recombinant human EGF throughout this project. I also wish to thank Mrs. Julie Goldfinch, Manager, Animal Care facility, Adelaide Children's Hospital, for providing outstanding animal surgery facilities, and Dr. Anthony Bourne, Pathologist, Adelaide Children's Hospital, for his learned advice and allowing me access to the histological equipment and services provided by the Department of Histopathology. For his support and clinical insight, my thanks is also extended to Mr. Andrew Ford, Surgeon, Department of Pediatric Surgery, Adelaide Children's Hospital.

Unless otherwise stated, all of the work described in this thesis was performed by the candidate. Acknowledgment for work performed by others is given in the methods section of the appropriate chapters.

PUBLICATIONS AND SCIENTIFIC PRESENTATIONS DERIVED FROM WORK PRESENTED IN THIS THESIS

PUBLICATIONS

Karen A. Ribbons, G.S. Howarth, K.B. Davey, C.G. Nascimento and L.C. Read
Effects of subcutaneous and intraluminal epidermal growth factor on colonic growth in adult rats.
Submitted to Growth Factors - March 1993

Karen A. Ribbons, G.S. Howarth, K.B. Davey, C.G. Nascimento, W.D. A. Ford, A.J.Bourne and L.C. Read
EGF does not enhance re-epithelialization of acetic acid-induced colonic ulcerations in rats.
Submitted to Gastroenterology - March 1993

SCIENTIFIC PRESENTATIONS

Gastroenterological Society of Australia, Annual Scientific meeting, Adelaide, South Australia, May 1991.
K.A. Ribbons, L.C. Read, G.S. Howarth and K.B. Rice
Development of experimentally induced colonic ulcerations in the rat for use in assessment of growth factors as wound healing agents.

Australian Society of Medical Research, Adelaide meeting, August 1991.
K.A. Ribbons, L.C. Read, G.S. Howarth and K.B. Davey
The effect of subcutaneously and luminally delivered epidermal growth factor in the normal adult rat colon.

Gastroenterological Society of Australia, Damage and Repair in the G.I tract, Canberra, A.C.T., September 1992.
K.A. Ribbons, L.C. Read, G.S. Howarth and K.B. Davey
An assessment of the ulcer-healing properties of epidermal growth factor in acetic acid-induced colitis in the rat.

Endocrine Society of Australia, Annual Scientific meeting,Adelaide, South Australia, September 1992.
K.A. Ribbons, L.C. Read, G.S. Howarth and K.B. Davey
Subcutaneously but not luminally delivered epidermal growth factor is mitogenic to the normal adult rat colon.

TABLE OF CONTENTS

	Page
THESIS ABSTRACT	1
CHAPTER ONE LITERATURE REVIEW	3
Introduction	3
The Pathophysiology of Inflammatory Bowel Disease	3
Introduction	3
Etiology of Inflammatory Bowel Disease	4
Normal colon morphology	4
Disease symptoms	5
- Ulcerative colitis	5
- Crohn's Disease	6
Colon cancer	7
Biochemical and functional changes in the colonic mucosa associated with inflammatory bowel disease	8
- Colonic proliferation	8
cell proliferation in the normal colon	8
colonic proliferation in colitis	9
- Mucosal permeability	10
- Electrolyte balance	11
short chain fatty acids	12
- Colonic mucin	12
Mediators of colonic inflammation	13
- T cell activation	13
- Neutrophil activation	14
- Macrophage activation	15
- Eicosanoids	15
- Summary	17
Current treatments used in inflammatory bowel disease	17
- Drug therapy	18
Aminosalicylates	18
Corticosteroid therapy	20
- Surgery	22
- Nutrition and Nitrogen balance	23
- New dug therapies	24
5-ASA	24

Corticosteroids 25
Immunomodulators / immunosuppresents 25
Antimicrobial agents 26
- Summary 26
Peptide growth factors and ulcer healing 27
Introduction 27
General characteristics of growth factor action 28
Endogenous EGF associated with the gastrointestinal tract 29
- Isolation and distribution 29
- Receptor localization 30
- Function 31
Development of the gastrointestinal tract 31
Ulcer healing action of EGF 33
Summary 35
Experimental models of inflammatory bowel disease 35
Introduction 35
Naturally occurring colitides 36
Experimentally induced colitides 37
- Carrageenan 37
Mode of action 38
Comparison to human inflammatory bowel disease 40
- Acetic acid 41
Mode of action 41
Comparison to human inflammatory bowel disease 41
- Other experimentally induced colitides 42
Summary 42
Overall Conclusions 43

CHAPTER TWO SCOPE OF THESIS 44

CHAPTER THREE THE EFFECTS OF EXOGENOUS EGF ON THE NORMAL ADULT RAT COLON 46
Abstract 46
Introduction 47
Methods 50
Animals and Experimental Design 50
- Recombinant human EGF 50
- Subcutaneously delivered EGF 51
- Luminally delivered EGF 52
Analytical measurements 52

Histological measurements 53
- Quantitative morphology 53
Immunohistochemical localization of PCNA 54
Measurement of PCNA labelling index and PCNA labelled crypt fraction 54
Stability of EGF in the colonic lumen 57
Measurement of EGF in urine and plasma 59
Statistical Analysis 60
Acknowledgments 60
Results 61
The effects of subcutaneously delivered EGF 61
Stability of EGF in the colonic lumen 66
The effects of luminally delivered EGF 69
Discussion 71

CHAPTER FOUR DEVELOPMENT AND VALIDATION OF AN ANIMAL MODEL OF COLITIS 76
Abstract 76
General Introduction 78
Carrageenan-induced colitis 79
Introduction 79
Methods 80
- Acid hydrolysis of carrageenans 80
- Animals and experimental design 81
Comparison of the colitis-inducing properties of 3 types of carrageenan. 81
Determination of the optimal dose of iota carrageenan required to induce colonic ulcerations 81
- Statistical Analysis 82
- Acknowledgments 82
Results 83
- Comparison of the colitis-inducing properties of 3 types of carrageenan. 83
- Determination of the optimal dose of iota carrageenan required to induce colonic ulcerations 85
Discussion 89
Acetic acid-induced colitis 90
Introduction 90
Methods 92
- Experimental animals 92
- Experimental trials 92

Exposure of the colonic serosa to acetic acid 92
Exposure of the colonic lumen to acetic acid 93
exposure of the entire colonic lumen to acetic acid 93
exposure of the distal colonic lumen to acetic acid 94
expousre of a segment of the distal colonic lumen to acetic acid 96
- Analytical measurements 97
Protein and DNA content of mucosal and muscularis samples 97
Na+/K+ ATPase activity 98
Histological measurements 99
- Statistical Analysis 99
- Acknowledgments 100
Results 101
Exposure of the colonic serosa to acetic acid 101
Exposure of the colonic lumen to acetic acid 101
exposure of the entire colonic lumen to acetic acid 101
the effect of exposing the distal colonic lumen to acetic acid 106
time course recovery trial 106
an assessment of intestinal permeability using 51Cr-EDTA 110
exposure of a 2cm segment of the distal colonic lumen to acetic acid 112
Discussion 117

CHAPTER FIVE THE EFFECTS OF EXOGENOUS EGF ON THE ULCERATED RAT COLON 121
Abstract 121
Introduction 122
Methods 124
Animals and Experimental Design 124
- Induction of colitis 124
- Subcutaneous and Luminal EGF delivery 125
- Stability of EGF in the colonic lumen of ulcerated rats 128
Analytical measurements 129
Measurement of EGF in urine and plasma 130
Statistical Analysis 130
Acknowledgments 131
Results 132
Plasma and urinary concentrations of EGF 134
Stability of EGF in the colonic lumen of ulcerated rats 134

Effect of 4 and 6 days treatment with EGF on repair of the damaged segment 138
Responses in the colonic segment adjacent to the ulceration
- a) Effects of acetic acid 142
- b) Effects of subcutaneous and luminally administered EGF 148
The effect of subcutaneous EGF infusion on the undamaged colonic segment 149
Discussion 152

CHAPTER SIX FINAL DISCUSSION 158

APPENDIX A. HISTOLOGICAL METHODS 166

APPENDIX B. STATISTICS SUMMARY TABLES 168

BIBLIOGRAPHY 175

THESIS ABSTRACT

Crohn's disease and ulcerative colitis are chronic forms of colitis for which the etiology is unknown and there is no known cure. Improvement in the existing treatments is still under clinical evaluation and there is a need for new effective therapies. The major emphasis of this thesis was to evaluate the potential therapeutic application of peptide growth factors, in particularly epidermal growth factor (EGF), in the treatment of colonic ulcerative conditions.

While the effect of exogenous EGF on ulcer healing, growth and maintenance of the upper gastrointestinal tract has been reported, its effect on ulcer healing in the colon has received little attention. In this thesis the responsiveness of the normal colon to EGF was assessed. A suitable model to assess effects of EGF on colonic ulcer repair was developed and used to assess the effects of exogenous EGF on the ulcerated colon.

The responsiveness of the normal adult colon to exogenous EGF delivered by continual subcutaneous infusion and by intraluminal bolus administration was measured. A 7 day continual subcutaneous infusion of EGF delivered by mini-osmotic pumps (200µg/kg/day), induced growth of the proximal colonic mucosa as shown by a significant increase in the mucosal wet weight, protein content and colonic circumference, as well as an increase in the number of cells per crypt and mucosal area above that of vehicle-treated control animals. Muscularis mass was also increased in the proximal colon where the wet weight and protein content was significantly elevated. Similar trends were observed in the distal colon although the magnitude of the response was lower than that seen in the proximal colon. In contrast, intraluminal EGF administration (1.6mg/kg/day) given as twice daily bolus injections, had no effect on colonic mucosal or muscularis growth in either the proximal or distal colon. The lack of effect of luminal EGF would not appear to be due to luminal degradation as radiolabelled EGF remained stable in the proximal and distal colonic

lumen for at least four hours. These findings suggest that the normal colon is responsive to exogenous EGF with systemic route being more effective than luminal delivery in eliciting a mitogenic response.

The assessment of new therapeutic therapies of colitis conditions in humans is necessarily limited in scope due to ethical constraints, therefore a suitable animal model for assessing the colonic ulcer healing effects of EGF was developed. Intraluminal application of acetic acid to a segment of the distal colon of adult rats, produced a distinct colonic lesion, which was reproducible, measurable and resolved over a 14 day period. A range of biochemical, physiological and histological markers of colonic damage were assessed for their ability to quantitate the severity of the induced colonic lesion.

The same treatment regimes and doses used to assess the effect of exogenous EGF in the normal rat colon were used to measure its effect on the acetic acid damaged colon. EGF treatment was commenced at the time of at the acid insult and assessed after 4 and 6 days. Neither luminal nor subcutaneous EGF administration enhanced re-epithelialization of the ulcerated colon after 4 or 6 days treatment, although a slight increase in mucosal hyperplasia was observed in the luminal EGF group in the region adjacent to the ulceration where the crypt length was increased by 20% above that of vehicle-treated controls. The edema of the mucosa and submucosa layers in this region was significantly suppressed by subcutaneous EGF treatment, resulting in a reduction of the thickness of these layers by 20% and 42%, respectively, compared to vehicle-treated animals.

In conclusion, although EGF stimulates proliferation of the intact mucosa, it does not accelerate re-epithelialization of the acetic acid damaged colonic mucosa. Nevertheless, the reduction in edema and thickness of the submucosal and muscularis externa layers at the margins of the colonic lesion may confer some benefit to the damaged colon.

CHAPTER 1. LITERATURE REVIEW

INTRODUCTION

This review includes information published prior to June 1989 at the time of commencement of experimental work performed for this thesis. More recent work published since that time will be cited in the relevant discussion sections of each chapter and in the final thesis discussion. Three major topics will be discussed in this historical review including a summary of the major pathophysiological features of inflammatory bowel disease and the current therapy regimes used in disease treatment. Drug therapies used are continuously under clinical review and new forms of therapy are being developed. The potential therapeutic efficacy of growth factors, in particularly epidermal growth factor (EGF), as ulcer healing agents will be discussed. Finally, a review of animal models available for use in assessing new therapeutic agents for inflammatory bowel disease will be given.

The Pathophysiology of Inflammatory Bowel Disease

Introduction

Inflammatory bowel disease is the collective term used to describe ulcerative and Crohn's colitis. Idiopathic ulcerative colitis and Crohn's disease are chronic ulcerative conditions of the colon although a clear understanding of the etiological events which initiate the pathophysiological changes in these diseases still remains unknown. Both conditions result in altered morphology and function of the colonic mucosa with chronic and acute inflammatory states. A complex interplay exists between inflammation and altered morphology of the colonic mucosa. The morphological and functional changes which occur in the colonic mucosa in inflammatory bowel disease to be discussed in this review include

alterations in crypt cell proliferation, mucosal permeability, electrolyte balance, colonic mucin profile and inflammatory mediators. Changes in these parameters have been investigated in an attempt to further understand the etiology of inflammatory bowel disease as well as provide a range of disease markers which can be used in diagnosis and treatment. Current treatment regimes can maintain patients in a remission phase but there is no known cure for ulcerative colitis or Crohn's disease. Improvement in the existing treatments is still under clinical evaluation and there is a need for new effective therapies.

Etiology of Inflammatory bowel disease

Ulcerative colitis was first described by Wilks and Moxon in 1875 (Cello and Meyer 1978) and a later identification of a regional enteritis condition was made by Crohn in 1930. Various disease inducing-agents have been proposed based on the nature of disease symptoms and epidemiological data. Epidemiological data showing a higher incidence in Jewish populations and in families where the disease is present is suggestive of genetic involvement but is still inconclusive (Weterman and Pena 1984, Gilat et al.1987). The autonomic nervous system has also been implicated from evidence of a possible phsycosomatic inducer of mucosal damage which may influence the onset and severity of the disease (Cello and Meyer 1978). This has been observed to effect the onset of symptoms in colitis patients and is not considered as a major etiological factor. Based on the inflammatory nature of these diseases, infectious and immunological theories have been suggested but to date no single infectious agent has been attributed to the etiology of idiopathic colitis conditions.

Normal colon morphology

The wall of the colon consists of four tissue layers, serosa, muscle coat, the sub-mucosa and the mucosa. The colonic mucosa contains epithelium arranged in a series of "test-tube shaped" crypts (Shamsuddin et al. 1982), inter-dispersed with lamina propria. Variation in the morphology of the crypts has been noted along the length of the colon (Sunter et al. 1979). In the guinea pig the density and depth of crypts increases towards the rectum, while in the rat, crypts are long in the proximal colon and become shorter distally.

Within the crypt epithelia three major cell types can be identified; columnar, mucous (goblet) cells and endocrine cells. The columnar epithelial cells line the colonic luminal surface between the crypts while the goblet cells, the most abundant cell type in the crypt, produce mucin which is released into the colonic lumen when the goblet cells become hyperdistended. Endocrine cells are situated on the basement membrane of the crypt. The rectum contains a large number of endocrine cells generally organised into aggregates (Shamsuddin et al. 1982). Lymphoid cells of the colon are aggregated in lymphoid follicles, which are situated in the submucosa or in the lamina propria.

Disease symptoms

Ulcerative colitis

The symptoms of ulcerative colitis vary in their severity, duration and in the effected region, the most common including bloody faeces, diarrhea, urgency, tenemus, weight loss and often abdominal cramps, all of which are intermittent and/or consistent (Roth 1980). Sixty to seventy percent of patients have intermittent symptoms with remissions between attacks. Due to the variation in the disease status three classifications of the disease have been made (Cello and Meyer 1978). Mild colitis, the most common condition inflicting 60% of patients diagnosed, involves a segmentally distributed colitis usually restricted to the distal colon. Patients experience very mild diarrhea and rectal bleeding, which can go undiagnosed. Moderate colitis is detected in 25% of patients and involves more severe symptoms than the mild disease, including low grade fever, diarrhea and intervals of weight loss. The colitis symptoms may be expressed in a series of attacks and an increase in the severity of the disease can occur. The rarest form of the disease, occurring in 15% of colitis patients, is severe colitis in which there is a sudden onset of disease symptoms, with more consistent rectal bleeding and diarrhea. Patients also experience high fever, abdominal cramps and hypoalbuminemia, the latter resulting from an increase in the loss of colonic serum albumin. As well as variations in the severity of the disease symptoms, the distribution of the diseased region of the colon also varies. Segments of the entire colon can be effected as well as the terminal ileum. The most common disease condition is a proctocolitis involving the rectum and the anal margin.

The most common positive diagnosis of ulcerative colitis is morphological disturbance of the colonic mucosa. This assessment is made by sigmoidoscopy and analysis of rectal biopsies. In ulcerative colitis, the inflammatory disease is restricted to the mucosa and the submucosa with the deeper muscle layers usually not being involved. The primary lesion detected is cryptitis, which involves the aggregation of polymorphonuclear cells at the crypt tip and subsequent degeneration of the crypt epithelia. Electron micrographs of the mucosa show shortening of the epithelial microvilli, endoplasmic reticulum dilation and mitochondrial swelling accompanied by lysosomal infiltration (Shields et al. 1985). Crypt abscesses are indicative of an ulcerative colitic condition. A state of chronic inflammation is present, which results in damaged mucosa and necrosis of the crypt structure with polymorphonuclear infiltration into the crypts. A significant decrease in the goblet cell number in the crypts is also observed (Filipe and Dawson 1970). These changes are followed by a reparative process with the deposition of granulation tissue and collagen deposits in the lamina propria. These microscopic changes account for the most prominent features of the disease, blood in faeces is caused by mucosal ulceration and friable granulation tissue while diarrhea has been attributed to colonic disruptions in electrolyte balance (Cello and Meyer 1978).

Crohn's colitis

A great deal of overlap in symptoms and morphological disturbances exists between ulcerative and Crohn's colitis. Common symptoms to both diseases are diarrhea, abdominal pain and anorexia, although rectal bleeding commonly observed in ulcerative colitis is rarely seen in Crohn's colitis. The disease progresses with time although 10-20% of patients remain asymptomatic for up to 20 years. Crohn's colitis is a chronic inflammatory disease which can involve the entire alimentary canal from mouth to anus with the distal ileum being the most commonly region affected (Donaldson 1989). Unlike ulcerative colitis, in which the rectum is nearly always affected (Korelitz 1985), Crohn's disease rarely involves the rectum. Taylor (1980) described the microscopic appearance of Crohn's colitis as involving confluent linear ulcerations with deep microscopic fissures, and as in ulcerative colitis,

inflammatory infiltrates were present. Unlike ulcerative colitis, the inflammation extends through all layers of the gut wall and can involve mesentary and lymph nodes and submucosa becomes edematous producing a thickening of the intestinal wall.

Colon cancer

A possible major complication associated with inflammatory bowel disease is colonic cancer. In severe (universal) colitis the incidence of colonic cancer is increased by 20-30 times that of the normal population (MacDermott 1985). The increased cancer risk was observed to be dependant on the duration and severity of ulcerative colitis. A recent cohort study by Gyde et al. (1988) examined the incidence of colorectal cancers in over 800 ulcerative colitis patients in Europe and the U.K. They observed that the incidence of colon cancer in colitis patients was 8 times greater than that of the normal population and in patients with severe colitis the incidence increased to 19 times of the normal population. Unlike Macdermott (1985) who claimed that a negative correlation exists between disease remission and cancer incidence, Gyde et al. (1988) found that colonic cancer developed in patients around the age of 50 years independent of disease duration. Macdermott (1985) also reported that colo-rectal cancers in the colitis patients were widespread along the length of the colon whereas in non-colitis patients the cecum and recto-sigmoid regions were the usual sites involved. In an earlier study the incidence of all cancers in ulcerative colitis patients was observed to be 8% higher compared to the total population, at 25 years after the initial diagnosis (Prior and Gyde 1982). These studies suggest that in ulcerative colitis patients the incidence of colonic cancer involving the entire colon is increased as is the incidence of cancer in general. In Crohn's disease a 20-fold greater risk of colon cancer has been reported in patients with a 20 year history of disease symptoms (McIllmurray and Langman 1975).

Biochemical and functional changes in the colonic mucosa associated with inflammatory bowel disease

Colonic proliferation

Given the increase in the incidence of colonic cancer in inflammatory bowel disease patients, factors which render these patients more susceptible to developing colonic dysplasia have been investigated. Changes in the proliferative profile of the colonic mucosa have been measured which are characteristic of a pre-neoplastic state and may in part explain the increased incidence of colonic carcinoma in ulcerative colitis patients (Serafini et al. 1981).

Cell proliferation in the normal colon

The proliferative region in the colon is normally restricted to the lower three quarters of the crypt. Cells migrate from the base of the crypt column towards the lumen and are then shed from the mucosal surface (Lipkin 1985). The size of crypts and the number of cells in the crypt column varies along the length of the colon, with crypts in the cecum and ascending (proximal) colon being significantly shorter than those in the descending (distal) region (Sunter et al. 1979). The proliferative status of normal human colonic explants has been studied in biopsy samples (Usugane et al. 1982) although no regional comparison of changes in the proliferative status along the length of the colon have been reported in the human. In animal models, the distribution of proliferating cells within the crypt has been measured using range of techniques, including the uptake of DNA nucleotide analogues such as ^{3}H-thymidine into the tissue, thereby localizing cells in S-phase of the cell cycle, or metaphase arresting agents such as vincristine to measure the rate of cell production and estimate the cell cycle time. Sunter et al. (1979) reported that in the rat, the average cell cycle time ranged from 25 hours in the cecum to 58 hours in the distal colon. In the cecum the proportion of cells in S phase was higher (38%) than in the the distal region (18%). Regional variations were also measured in the position of the proliferative compartment, with the proliferative zone being lower in the crypt in the distal region compared to the

proximal region. Despite regional variations in the size and position of the proliferative compartment along the length of the colon, the cell cycle time of the first five cells in the crypt cell column at all regions was slower than that of other cells in the crypt column, and may represent a stem cell population at the base of the crypt. Taken together, these studies highlight the complexity and regional variation of cell proliferation in the colon and the need to define the anatomical location of colonic samples used in colonic proliferation studies.

As well as anatomical location, the proliferation of cells in the colon is influenced by a number of factors including microbial metabolism, luminal nutrients, fecal material and hormonal and growth factors (Stragand and Hagemann 1977). The mitogenic effect of luminal factors on colonic proliferation has been illustrated in studies where the exposure to luminal contents was modified by fasting and diversion of the fecal stream (Delvaux et al. 1979, Butler et al. 1988). The type of luminal contents also effects colonic mucosal growth. (Ryan et al. 1979, Goodlad and Wright 1983, Sakata and von Engelhardt 1983). Short chain fatty acids (acetic, propionic and butyric) produced by colonic anaerobic bacterial metabolism of polysaccharides have been implicated as mitogens to the colonic mucosa.

Colonic proliferation in colitis

A significant increase in cell proliferation in human ulcerative colitis biopsies has been detected by several techniques including incorporation of tritiated thymidine and metaphase arrest (Eastwood and Trier 1973, Serafini et al 1981, Allan et al. 1985). Associated with colonic mucosal hyperplasia is a displacement of the proliferative compartment in the crypt. Dividing cells were detected higher up in the crypt column, nearer to the surface epithelia than that observed in the same region in normal animals (Serafini et al. 1981, Eastwood and Trier 1973). These changes in colonic proliferation are dependant on the stage of colitis activity. In active colitis, colonic mucosal cell proliferation is 45% faster than that of biopsies from patients in remission which was in turn 14% faster than observed in normal colonic mucosa (Allan et al. 1985). However the displacement of mitotic figures within the crypt was observed in active colitis and also in periods of remission (Serafini et al. 1981). The upward displacement of the proliferative compartment

suggests that the cellular migration rate is increased. The presence of a higher proportion of 'immature' cells at the luminal surface may effect mucosal function and metabolic processes. Moreover, similar abnormalities in colonic proliferation have been measured in biopsies from colonic adenomas (Deschner 1988). This suggests that abnormal proliferative profile observed in inflammatory bowel disease may render the patient more susceptible to developing colonic carcinoma although the factors which induce mucosal hyperplasia and an upward shift in the proliferative compartment in colitis still remain unknown.

Mucosal permeability

Hypoalbuminenemia is experienced by severe colitis sufferers resulting from a loss of serum albumin (Roth 1980). The elevated loss of serum protein occurs across the diseased colon resulting in a reduction in serum albumin levels (Lennard-Jones et al 1975). The loss of serum proteins into the colonic lumen can be directly monitored by measuring the presence of blood antigens in the faeces. One such metabolite, alpha-1-antitrypsin is the major protease inhibitor in human serum is excreted undegraded into the faeces when protein leakage occurs (Durie 1985). Fecal alpha-1-antitrypsin has been detected in high concentrations in patients with acute diarrhea and in children with protein-losing enteropathy (Hill et al. 1981, Thomas et al. 1981, Durie 1985). Elevated levels have also been measured in the faeces of ulcerative colitis and Crohn's colitis patients. A positive correlation between alpha-1-antitrypsin and disease activity has been measured in ulcerative colitis although in Crohn's colitis the levels measured were variable between patients and no correlation with disease activity was measured (Griffiths et al. 1986, Biemond et al. 1984).

Changes in the barrier function of the colonic epithelia to macromolecules has also been detected in inflammatory bowel disease using a range of non-absorbable markers. Markers such as lactulose, mannitol, ^{51}chromium-labelled ethylenediaminetetra acetate (^{51}Cr-EDTA) and polyethylene glycols, have been used to assess the extent of epithelial permeabilization in inflammatory bowel disease (Murphy et al. 1989, Ramage et al. 1988a, 1988b, Maxton et al. 1986). These markers cross the mucosa entirely by passive non-mediated diffusion and are not metabolized after absorption, being fully excreted in the urine

(Maxton et al. 1986, Krugliak et al. 1989). Hence, urinary recovery of these markers enables an estimate of intestinal permeation to be made.

Electrolyte metabolism

The colonic and rectal epithelium is polarised with luminal contents carrying a net a negative charge compared to blood electrolytes (Edmonds and Pilcher 1973). Regional variations in ion exchange and absorption have been measured with the proximal colon having a greater capacity for electrolyte uptake than distal segments (von Engelhardt and Rechkemmer 1986). Alterations in water absorption and electrolyte balance in colitis are suggested by the changes in stool frequency and consistency experienced by colitis sufferers, with diarrheal symptoms dependent on the extent and region of the colon effected (Rao et al 1988).

Changes in electrolyte balance have been measured in isolated colonic loops in colitis patients derived from coloctomy. A reduction in electrolyte uptake was measured from the colonic lumen across mucosa in active diseased segments compared to normal colon although ion transport across the diseased mucosa into the lumen was increased (Duthie et al. 1964, Harris and Shields 1970). The potential difference across the isolated colonic mucosa has been measured in colonic biopsies. Such studies demonstrated that the polarity of the mucosa is altered in acute colitis conditions with the potential difference approaching zero (Rask-Madsen and Dalmark 1973). Disruptions in the electrolyte balance and ion transport in the colonic mucosa may contribute to the accumulation of luminal fluid in colitis-associated diarrhea.

Changes in the mechanisms which regulate ion flow have also been measured in colitis and diarrheal conditions. Colonic Na^+/K^+ATPase activity was reduced in colonic biopsies to 1/4 the activity expressed in the normal colon (Rachmilewitz et al. 1984). Alterations in enzyme activity have also been measured in other diarrheal conditions. A similar reduction in enzyme activity to that observed in colitis was measured in postenteritis

syndrome, although in small intestinal biopsies from infants with toddler diarrhea the activity was increased (Tripp et al. 1980).

Short chain fatty acids

Short chain fatty acids produced by colonic bacterial metabolism are the major anions found in colonic fluid (von Engelhardt and Rechkemmer 1986, Cummings 1981). The pH of the colonic lumen is maintained just below 7 by the buffering action of luminal bicarbonate produced by the colonic epithelium. Changes in the balance of bicarbonate ions and short chain fatty acid metabolism has been described in ulcerative colitis. Roediger et al. (1984) monitored rectal bicarbonate output by rectal dialysis in healthy volunteers and patients with active ulcerative colitis. In acute colitis bicarbonate output and luminal pH was reduced. Moreover, stimulated bicarbonate output by butyrate was reduced by 80% in active colitis compared to normal controls. These findings suggest that the buffering capacity of the colon facilitated by bicarbonate release is impaired in chronic colitis.

Colonic mucin.

Colonic mucin is a glycoprotein which is secreted by goblet cells within the crypt epithelia and adheres to and covers the intestinal surface. The mucin layer provides the epithelia with a protective barrier against fecal and food material as well as digestive enzymes (Allen 1983, Sakata and Engelhardt 1981a and 1981b). Therefore changes in this layer may render the mucosa vulnerable to damage. Comparisons between normal colonic biopsy samples and those from ulcerative colitis, Crohn's colitis and colonic tumours have indicated that changes in the mucin glycoprotein profile occur in these diseases , including an increased mannose content, total carbohydrate content and regional glycosylation pattern, and the proportion of sialated to sulphated mucin (Cope et al. 1988, Rhodes et al. 1985, Podolsky and Isselbacher 1983 and 1984, Reid et al. 1984). Recent studies have also identified changes in the protein moiety of colonic mucin in colitis conditions (Hertzog and Linnane 1988) . Although the functional implication of these biochemical alterations in colonic mucin is not fully understood, the changes have been likened to those occurring in a

pre-cancerous state as an increase in o-acetyl substitution for sialic acid has been measured in biopsies from colonic carcinomas (Culling et al. 1977, Agawa et al. 1988).

Mediators of colonic inflammation.

A chronic inflammatory state exists in ulcerative and Crohn's colitis characterised by a cellular infiltrate containing lymphocytes (B and T cells), neutrophils, eisonophils, mast cells and macrophages.(MacDermott and Stenson 1988). An altered regulation of inflammation has been proposed as a potential factor involved in the chronicity of inflammatory bowel disease. Modified metabolism of the cell types associated with the inflammatory infiltrate and the expression of mediators involved in the inflammatory response have been measured in ulcerative colitis and Crohn's disease, which may provide an insight into the injurious effect of the inflammatory response as well as provide potential mechanisms for targeting new therapies for inflammatory bowel disease.

T Cell activation

An impaired immune response characterised by the production of serum antibodies against colonic epithelial cells and cell-mediated cytotoxic events have been identified in inflammatory bowel disease and have been linked to the inflammatory response observed (For a review see MacDermott and Stenson 1988). Serum from inflammatory bowel disease patients contains antibodies directed against epithelial cells and a preferential production of IgG and IgA class antibodies have been detected on colonic epithelial cells from colitis patients (Brandtzaed et al. 1974). The colon antibodies however, are not disease specific, and a similar response has been observed in a range of inflammatory states (MacDermott and Stenson 1988).

The total number of lymphocytes in intestinal specimens from patients with active inflammatory bowel disease is four times that observed in the normal colon (Brandtzaed et al. 1974). A cascade of events involving the activation of T and B lymphocytes has the potential of releasing cytoxic factors which contribute to colonic epithelial cell damage. The direct immune-inflammatory inducing response remains unclear, however cytokines

produced by activated T lymphocytes and macrophages, have been suggested as potential mediators of the inflammatory response. Recently interleukin-1 and tumor necrosis factor have been used to induce an inflammatory bowel-like condition in rats (Butler et al. 1989) with interleukin 1 levels in this model correlating with disease activity. An enhanced interleukin-1 production has also been detected in mononuclear cells from patients with active inflammatory bowel disease (Mahida et al. 1989). Interleukin-1 displays a range of pro-inflammatory response including an enhanced antibody production by B lymphocytes (Falkoff et al. 1983) an enhancement of cellular adhesion and translocation of neutrophils to site of inflammation (Mantovani and Dejana 1989) and an increased eicosanoid production (MacDermott and Stenson 1988).

Neutrophil function

The anti-oxidant properties of peripheral blood neutrophils has been measured in Crohn's and ulcerative colitis patients (Verspaget et al. 1988). A significant reduction in superoxide dismutase activity was observed in colitis patients compared to normal subjects and the enzyme activity was negatively correlated to disease activity. The impaired anti-oxidant properties of the neutrophils would give rise to a higher concentration of toxic reactive oxygen radicals thereby producing tissue damage. Reactive oxygen radicals have also been implicated in an impairment of the formation of mucin and effecting the barrier function against luminal toxins (Grisham et al. 1987). On the basis of these findings Grisham and Granger (1988) proposed that oxygen radicals play an integral role in the initiation and the inflammatory and ulcerative responses in colitis. Formation of oxygen radicals by colonic epithelium following an ischemic event would result in a mucin coat with a reduced barrier function thereby facilitating the entry of bacterial endotoxins from the colonic lumen into the lamina propria where oxygen radical production would induce a cascade of events resulting in mucosal ulceration.

While no differences in the activity of neutrophil proteases has been observed between normal and inflammatory bowel disease patients, the acid proteases are potential mediators of tissue damage. Acid proteases can be released from cells following cell death

from leukocytes engaged in phagocytosis (Janoff 1972). The aggregation of neutrophils in the colon may induce an increased local level of protease production resulting in colonic tissue damage.

Macrophage activation

The normal role of macrophages is in the removal of dead or damaged cells, and the synthesis of inflammatory mediators (such as cytokines and prostaglandins), interferon and proteases (Tanner et al. 1984). An increased level of circulating monocytes, from which macrophages are derived, has been measured in inflammatory bowel disease and in Crohn's colitis large numbers of macrophages are associated with the granulomatous reaction. (Mee and Jewell 1980). Activated macrophages in the colonic tissue also produce oxygen derived free radicals which may induce tissue damage, as outlined above. The activity of lysosomal enzymes N-acetyl-B-glucoaminidase, and B-glucuronidase in macrophages from inflammatory bowel disease is increased, and in ulcerative colitis is correlated to disease activity (Mee and Jewell 1980).

Eicosanoids

Eicosanoid is the collective term for a range of lipid mediators derived from arachidonic acid metabolism. Arachidonic acid is released from cellular phospholipids by a range of phospholipases and is metabolised through either the cyclo-oxygenase pathway to produced prostaglandins, prostacyclin and thromboxane, or the lipoxygenase pathway producing hydroxyperoxyeicoatetetraenoic acids (HPETE), hydroxyeicosatetreaenoic acids (HETE) and leukotrienes (Lauritsen 1989). Eicosanoid metabolites are released from damaged cell membranes therefore tissue collection and extraction may stimulate or alter the extent of eicosanoid release (Macdermott and Stenson 1988). Despite this limitation, the major eicosanoid products produced in the gastrointestinal tract have been identified and include the cyclo-oxygenase products, prostaglandins and thromboxanes, produced by a range of cell types including intestinal epithelium, mast cells, macrophages and platelets (Macdermott and Stenson 1988) which display a range of biological functions., together with lipoxygenase products synthesized by inflammatory cells such as neutrophils and

macrophages; 5-HETE and leukotriene B_4 (For a review see Macdermott and Stenson 1988).

Eicosanoid content or the activity of enzymes involved in biosynthesis of eicosanoid metabolites, have been measured in normal and inflammatory bowel disease patients (Bukhave et al. 1988, Hawkey and Rampton 1985, Sharon et al 1978). In colonic biopsies from patients with the active form of colitis elevated prostaglandin E2, leukotriene B4 and 5-HETE levels were detected compared to normal colon biopsies (Sharon et al. 1978, Sharon and Stenson 1984). These findings were supported by in vivo analysis of rectal diasylates from Crohn's disease and ulcerative colitis patients in which the level of both cyclo-oxygenase and lipoxygenase metabolites correlated with disease activity (Lauritsen et al. 1988, Lauritsen 1989).

The biological consequence of the level and type of eicosanoid metabolites in relation to the clinical manifestation of inflammatory bowel disease has been questioned. The edema in ulcerative and Crohn's colitis may in part be due to an increased vascular permeability and vasodilation induced by increased release of prostaglandin E_2 (Hawkey and Rampton 1985). Moreover, due to the mitogenic and cytoprotective properties of the prostaglandins the increased prostaglandin release may be a response to mucosal damage and ulceration (Johansson and Kollberg 1979, Hawkey and Rampton 1985). The ability of the prostaglandins to act as a major inflammatory mediator in inflammatory bowel disease has been discounted as blocking endogenous prostaglandin production does not result in any clinical improvement in disease symptoms (Gould et al. 1981). In contrast lipoxygenase metabolites are considered to have a greater role in mediating the inflammatory response in inflammatory bowel disease (Sharon and Stenson 1984). Lipoxygenase products such as leukotriene B4 are likely to amplify or modulate the inflammatory response due to their effect on neutrophil translocation and aggregation (Sharon and Stenson 1984, MacDermott and Stenson 1988, Nielsen et al. 1988).

Summary

Ulcerative colitis and Crohn's disease are chronic ulcerative conditions with an unknown etiology. Biochemical and functional changes are observed in the colonic mucosa of colitis patients together with phases of acute and chronic inflammation. A precise cause / effect relationship between the altered mucosal morphology and inflammatory response has not been elucidated although an interplay does exist between the two processes. Eicosanoids may in part attenuate the altered electrolyte balance observed in colitis resulting in tissue edema and diarrhea. Impairment of the barrier function of colonic mucin may render the mucosa more susceptible to damage by luminal contents, while the alteration in the mucosal proliferative profile increases the susceptibility of the mucosa becoming dysplastic. The underlying mechanism which initiates these events and causes the chronicity of these diseases remains unknown. The changes in colonic mucosal function and inflammation discussed here provide an insight into some of the factors responsible for the disease symptoms observed in colitis and may be useful markers for assessing the therapeutic efficacy of new treatments.

Current treatments used in inflammatory bowel disease

The regime of treatments used in inflammatory bowel disease is dictated by the severity, location and nature of the disease. The three major types of therapy used are drug treatments, including aminosalicylates and the corticosteroids, dietary modifications and in severe cases surgical procedures are used. Other drug treatments including immunosuppresents and antibiotics are used in adjunct with the afore mentioned therapies. Corticosteroids are the first form of drug therapy used for treatment of active disease while aminosalicylates are used as maintenance therapy. Overall, the treatment protocol used usually consists of a combination of drug therapies and in severe colitis the damaged segment can be removed by surgical means. While these treatments can suppress inflammation and maintain patients in remission the drug therapies do not cure patients of inflammatory bowel disease. Current drug therapies are also restricted in their use by a range of symptomatic and hypersensitive adverse reactions to the treatments. In light of

these limitations therapies are under constant clinical evaluation and new therapies for the treatment of these conditions are still being sought.

Drug therapy

Aminosalicylates

Administration of sulphasalazine (salicylazosulphapyridine) is the most common therapy used for maintaining patients in remission in ulcerative colitis as well as inducing remission in patients with mild to moderate colitis. Its usefulness in the treatment of Crohn's colitis is still under investigation and while no prophylactic effects have been reported in Crohn's patients, some success in the treatment of patients with mild to moderate ileal involvement has been reported (Goldstein et al. 1987). Sulphasalazine was first developed in Sweden in the 1940's to deliver sulphonamides and salicylates in the treatment of rheumatoid arthritis. The use of sulphasalazine in the treatment in inflammatory bowel disease occurred serendipitously when arthritis patients with colitis showed an improved colitis following treatment (Svartz 1942, in Hoult 1986).

When given orally, sulphasalazine is absorbed intact across the small intestine and is returned to the colon in bile. In the colonic lumen the molecule is cleaved by azo-reducing bacteria into sulphapyridine and 5-aminosalicyclic acid (5-ASA). Sulphapyridine is absorbed from the colon and is metabolised by acetylation or hydroxylation and then excreted in the urine. In contrast, 5-ASA is poorly absorbed, having a long half-life in the lumen, and is eventually acetylated and excreted in the faeces (Hoult 1986). Therefore in the colonic lumen 3 compounds exist sulphasalazine, sulphapyridine and 5-ASA, one or all or which may be therapeutically beneficial. The active moiety of sulphasalazine was investigated by Azhad Khan and co-workers (1977) by administrating a daily rectal enema of sulphasalazine, sulphapyridine or 5-ASA to patients with mild or moderate active ulcerative colitis. After 2 weeks of treatment the histopathology of the colitis was improved in 30% of patients receiving sulphasalazine or 5-ASA compared to a 5% improvement rate in sulphapyridine-treated patients. Similar findings were made by van Hees and co-workers (1980) in ulcerative proctitis patients where 5-ASA was more effective in inducing remission

than sulphapyridine or sulphasalazine. 5-ASA enemas have also been shown to induce remission in 91% of distal colitis patients (Campieri et al. 1981). These findings, taken together with the rapid azo-reduction of sulphasalazine in the colonic lumen, suggest that 5-ASA is the active therapeutic moiety of sulphasalazine. Due to the requirement of colonic bacteria to facilitate azo-reduction to release 5-ASA the use of sulphasalazine in the treatment Crohn's colitis may be restricted to patients with colonic involvement. Contrary to this, sulphasalazine has been shown to improve the clinical and radiological status in 25% of Crohn's disease patients with small intestinal involvement (Goldstein et al.1987). This suggests that sulphasalazine or circulating sulphapyridine may exert some therapeutic benefit to Crohn's patients.

A number of theories have been proposed to explain the mode of action of sulphasalazine in maintaining and inducing disease remission in inflammatory bowel disease. A range of characteristics of drug therapy including alteration in gut microflora, colonic fluid and electrolyte uptake, B cell number and lymphocyte and neutrophil function have been observed (Hoult 1986). Recently, the most widely investigated mechanism of sulphasalazine action has been its ability to modulate arachidonic acid metabolism thereby modifying eicosanoid production (Lauritsen 1989). Modulation of both the cyclo-oxygenase and lipoxygenase pathways by sulphasalazine and its metabolites has been investigated, although the precise mechanism which produces the therapeutic benefit of this drug is still not fully understood.

As low levels of prostaglandin E_2 are observed during periods of remission, sulphasalazine may act by blocking the cyclo-oxygenase pathway. However this is unlikely as *in vitro* studies suggest that sulphasalazine is a weak cyclo-oxygenase inhibitor (Sharon et al. 1978) and administration of agents which block prostaglandin production, such as indomethacin, do not result in improvement of the disease state (Bondesen et al. 1987). A modulation of the lipoxygenase pathway has also been hypothesised as a potential mode of action of sulphasalazine. Both sulphasalazine and 5-ASA inhibited 5-HETE and leukotriene B_4 synthesis in human neutrophils, and in Crohn's and ulcerative colitis biopsies reduced

the production of 5 and 15-HETE and leukotriene B_4 by 50% (Sharon and Stenson 1985). As treatment produces a reduction in both prostaglandin E_2 and leukotriene B4 in patients responding to therapy the possibility that the changes observed in eicosanoid production may be a secondary response to the therapeutic effect has been suggested (Lauritsen 1989).

The major factor limiting the dosage and use of sulphasalazine are the side effects associated with therapy experienced by up to a third of patients (Taffet and Das 1983). Two major types of side effects have been described, symptomatic effects which are positively correlated to the dose of sulphasalazine used include nausea, headache, malaise, hemolytic anemia, reticulocytosis and methemoglobinemia, and non-dose related, hypersensitive reactions including skin rash, and pulmonary and hepatic dysfunction (Azad Khan et al. 1980, Taffet and Das 1983, Das 1989). The symptomatic effects are the most common with 50% of patients experiencing side effects displaying at least one of the afore mentioned symptoms (Taffet and Das 1983). Similar symptomatic symptoms to those observed in sulphasalazine treatment have been detected in patients undergoing sulphonamide therapy (Azad Khan et al 1980), therefore the level of serum sulphapyridine has been investigated as the possible moiety contributing to sulphasalazine related side effects. One method of overcoming the adverse side effects associated with sulphasalazine treatment has been to desensitize the patient against sulphasalazine by stopping treatment and to re-introduce the drug at an increasing dose. This has proven successful in patients experiencing symptomatic effects (Das et al. 1973). However in patients experiencing severe hypersensitive reactions continuing treatment with sulphasalazine is not recommended (Taffet and Das 1982 and 1983)

Corticosteroid therapy

Corticosteroids are used effectively in the treatment of a wide spectrum of inflammatory conditions. In ulcerative and Crohn's colitis they are the mainstay therapy used for treating acute attacks of colitis (Rhodes and Heatley 1983). Since their introduction as therapeutic agents in inflammatory bowel disease treatment in the 1950's the improvement in the mortality rate in patients with severe active disease has increased from

35% to less than 5% in 1987. While the advancement in other drug treatments, nutritional status, and surgical procedures has occurred over this time, the improvement in survival rate has largely been attributed to corticosteroid therapy (Jewell 1989). Unlike aminosalicylate therapy, corticosteroids have not been shown to elicit any prophylactic effect in inflammatory bowel disease (Jewell 1989). The anti-inflammatory steroids used are analogues of the endogenous steroid hydrocortisone, together with adrenocorticotrophic hormone (ACTH) which has been used to stimulate the adrenal cortex to elevate endogenous cortisol levels (Flower 1988, Jewell 1989).

The two most common corticosteroids used in inflammatory bowel disease therapy are cortisone and prednisone which are hydroxylated in the circulation to form cortisol and prednisolone. When administered orally, they are rapidly absorbed across the small intestine with peak blood levels of cortisol and prednisolone achieved within 3 hours of treatment, which is then rapidly absorbed by the colon and rectum. Water soluble analogues of cortisol and prednisolone have been developed which allow topical delivery to the colon and rectum by enema and are only partially absorbed from the lumen into the the circulation. Combinations of oral, intravenous and topical forms of corticosteroids are used in inflammatory bowel disease therapy depending on the distribution and severity of the disease. Oral prednisone, prednisolone and retention steroid enemas are commonly used in the treatment of proctitis and mild to moderate ulcerative colitis, whereas in Crohn's disease with ileal involvement, oral prednisolone treatment has shown positive effects (Jewell 1989).

The exact mechanism by which corticosteroids reduce inflammation is not fully understood, although a number of responses to corticosteroids have been reported which may enhance the anti-inflammatory effect including; a reduction in capillary permeability, decreased migration of macrophages and poylmorphonuclear cells into the inflamed region, suppression of macrophage function and an inhibition of cell-mediated immunity (Meyers and Janowitz 1985). One of the most investigated mechanisms of anti-inflammatory action of corticosteroids has been their ability to modulate arachidonic acid metabolism and thereby

modify eicosanoid production (Flower 1988). Analysis of rectal diasylates from patients with active ulcerative colitis with rectal involvement show a reduced production of prostaglandin E_2 and leukotriene B_4 following topical treatment with prednisolone (Lauritsen 1989). In a series of experiments using the perfused guinea pig lung Flower (1988) and co-workers identified a substance that was released in a dose-dependent fashion by macrophages following glucocorticoid treatment. The substance denoted lipocortin, was subsequently sequenced and purified and shown to directly inhibit the activity of phospholipase A_2. As well as inhibiting eiocosanoid production corticosteroids also inhibit the release of other substances from inflammatory cells including histamine. Phospholipase A_2 inhibition has also been suggested as the possible mechanism by which corticosteroids modulate histamine release (Scheimer 1985).

As previously mentioned, the corticosteroids used as anti-inflammatory agents are derivatives of endogenous glucocorticoid hormones and therefore also display the catabolic action of endogenous steroids. Corticosteroids increase hepatic gluconeogenesis resulting in an enhanced catabolism of muscle, skin, lymphoid, adipose and connective tissues. Following long term corticosteroid treatment these catabolic actions can manifest in severe side effects such as myopathy, bone marrow depression and diabetes (Swartz and Dluhy 1978, Flower 1988). Corticosteroids also display sodium retaining properties and can result in compromised cardiac function. In contrast to its anti-inflammatory effect in inflammatory bowel disease, corticosteroids can also be an irritant to the gastrointestinal tract with patients experiencing nausea and vomiting and 10-15% of patients can develop peptic ulcerations which can perforate and result in peritonitis (Swartz and Dluhy 1978, Jewell 1989). These effects are associated with long term high dose treatment regimes with the incidence and severity of symptoms being reduced when the dosage is reduced or terminated (Swartz and Dluhy 1978).

Surgery

Surgical parameters in colitis involve ileorectal anastomoses variations in which the diseased region is removed. Resection of the colon in ulcerative colitis is the most drastic

form of treatment and is usually performed in chronic colitis where continuous symptoms are experienced and are not relieved by drug therapy. In ulcerative colitis removal of the entire colon and rectum results in positive curative responses (Korelitz 1985, Smith 1989). A total proctocolectomy involves externalising the distal ileum via a stoma and is the most effective curative treatment. There is a slight risk of obstruction and prolapse and patients can feel discomfort and embarrassment. Other procedures involving an ileorectal anastomosis leave the sphincter intact or strip away the rectal mucosa prior to re-attachment to the distal ileum, although such manipulations are not curative as rectal disease can re-appear. Modifications to the total proctocolectomy have been developed which involve forming an internal reservoir in the ileum such as a Kooch pouch which is emptied by insertion of a catheter into the end of the pouch (Smith 1989). While these procedures are usually only used in severe ulcerative colitis the prognosis following surgery is usually positive and mortality resulting from surgery is low. In contrast, in Crohn's colitis surgery is performed to alleviate severe symptoms which are not benefited by other therapies. No curative effects of surgery are observed and the recurrence rate of the disease is high (Shorb 1989).

Nutrition and Nitrogen balance

Nutrition regimes have also been used in the treatment of chronic colitis patients. Vogel et al. (1974) observed that a large proportion of chronic colitis patients, who had undergone colonic resection, experienced relapse of disease symptoms. Vogel et al. (1974) attributed the relapse, in part, to a negative nitrogen balance and by intravenous hyperalimentation was able to enhance a positive nitrogen balance and improve the status of the patient. The symptoms of weight loss and anorexia frequently observed suggest that colitis is a catabolic condition (Powell-Tuck et al. 1984). A catabolic state will exist if protein breakdown exceeds synthesis or when nitrogen intake is reduced. In ulcerative colitis protein losses occur in the lumen due to a leakage of plasma proteins through the damaged colonic mucosa and therefore faecal nitrogen levels increase (Roth 1980). Urinary nitrate levels are increased in severe colitis, and correlate with disease activity suggesting that protein turnover is increased. Klein et al. (1988) also observed an increase in urinary

nitrogen levels in colitis patients which were comparable to those observed in elective surgery patients. While increased urinary nitrate suggests an increase in protein turnover is occurring in colitis the observations need to be interpreted with caution as urinary nitrogen can be increased by a variety of factors. Drug therapies such as the use corticosteroids increase muscle protein catabolism and would elevate urinary nitrogen levels. Problems also arise in determining the basal energy expenditure and nitrogen status of the patient before the disease onset (Klein et al. 1988).

New drug therapies

5-ASA

Although sulphasalazine is effective in maintaining patients in remission its use in the treatment of inflammatory bowel disease is restricted due to the adverse reactions experienced by some patients. Side effects have been in large attributed to the serum sulphapyridine moiety and as eluded to earlier, 5-ASA has been recognized as the major therapeutically active component. From these findings a new range of drugs are being developed which lack sulphapyridine and use other means of delivering 5-ASA to the colonic lumen. Truelove (1988) describes three new types of drugs which have been designed to target 5-ASA to the colonic lumen and produce minimum side effects these include: mesalazine; resin coated 5-ASA, osalazine; which contains 2 5-ASA molecules linked by an azo bond, ipsalazide (4-aminobenzoglycine-ASA) and balsalazide (4-aminobenzoyl-B-alanine- ASA). The release of 5-ASA into the intestinal lumen by mesalazine preparations is facilitated by an acrylic based resin which can be degraded at pH 5.6 or 7 allowing 5-ASA to be delivered to either the distal small intestine or the colonic lumen, respectively. By this mechanism 5-ASA may be used in treating small intestinal involvement in Crohn's disease (Brogden and Sorkin 1989). Both mesalazine and osalazine have been used in maintaining and inducing remission in mild and moderate ulcerative colitis with comparable success to that achieved using the same dose of sulphasalazine (Riley et al. 1988, Ireland et al. 1988). Few side effects to both forms of treatment were observed, with less than 15% of patients receiving mesalazine experiencing headache and nausea which were not dose related (Riley et al. 1988). To date no clinical trials using ipsalazide and

balsalazide have been performed, though animal and human studies suggest that the fecal excretion of 5-ASA is similar to that observed during sulphasalazine administration (Chan et al. 1983).

Corticosteroids

As in sulphasalazine therapy a range of corticosteroids are being developed to produce fewer side effects. Topical application of corticosteroids is effective in the treatment of distal ulcerative coltis and results in fewer deleterious effects than oral of systemic administration (Jewell 1989). A range of corticosteroids which undergo extensive hepatic first pass metabolism, therby producing fewer side effects, have been developed and are as effective as topical prednisolone in the treatment of active distal ulcerative colitis (Mulder et al 1989, Danilsson et al. 1987).

Immunomodulators / Immunosuppresents

Immunomodulating agents such as Cyclosporin A act via mediating T cell activation by inhibiting interleukin-2 production and suppressing lymphocyte proliferation (Peltekian et al. 1987). Immunomodulating agents such as cyclosporin A, a fungal metabolite, have been assessed as potential therapeutic agents in Crohn's colitis (Allison and Pounder 1987, Peltekian et al. 1987) and positive effects to the drug have been observed although, to date, clinical trials have been small and uncontrolled. A limiting factor of cyclosporin treatment is the potential development of hematologic and renal toxicity, and hence careful patient monitoring for the onset of adverse effects is required (Peltekian et al. 1987).

The potential therapeutic action of immunosuppresents in inflammatory bowel disease has also been assessed. Suppression of lymphocyte proliferation is achieved by adminsitration of agents such as 6-mercaptopurine and azathoprine. The active modality of both these compounds is thioinosinic acid which is incorported into the DNA of dividing lymphocytes inhibiting their proliferation.(Kirk and Lennard-Jones 1982). The efficacy of azathoprine treatment has been assessed in a number of clinical trials in patients with chronic ulcerative colitis and Crohn's disease patients unresponsive to steroid therapy (Jewell and

Truelove 1974, Kirk and Lennard-Jones 1982, Pelkekian et al. 1987). Positive benefical effects were observed in Crohn's disease patients although the use of immunosuppresive agents is limited by the potential of developing adverse side effects such as leukopenia, infections and pancreatitis (Swartz and Dluhy 1978).

Antimicrobial agents

The antimicrobial component of sulphasalazine, sulphapyridine, provides little benefical effects to ulcerative colitis patients. Other broad spectrum antibiotic agents such as metronidazole may be beneficial in ulcerative colitis patients susceptible to infectious complications such as in toxic megacolon and patients undergoing colonic resection. In Crohn's disease patients with small intestinal involvement, the effects of metronidazole have been assessed as small intestinal strictures in Crohn's disease can result in intestinal statis and bacterial overgrowth (Ursing and Kanne 1975). However no significant improvement in small intestinal Crohn's disease was observed and drug administration was associated with a range of side effects such as nausea, anorexia and fatigue (Ursing and Kanne 1975, Ursing et al.1982). In contrast, positive effects of metronidazole have been observed in Crohn's patients with anal lesions where prolonged therapy significantly reduced the severity of the lesions (Brandt et al. 1982). Unfortunately, a high proportion of patients undergoing prolonged therapy experienced severe side effects such as a loss of motor neural activity.

Summary

Current treatment regimes used in ulcerative colitis and Crohn's disease provide positive relief for the patient although do not result in curing the condition. Aminosalycilates and corticosteroids are used in remission maintenance and treatment of active colitis, respectively. Both therapies reduce intestinal inflammation with mode of action of both drugs being linked to a modulation of eicosanoid release. The major factor limiting the use and dosage of these drugs is a high incidence of side effects experienced by patients. As a consequence, treatments are under constant clinical assessment and new forms of therapies are currently being developed which produce fewer adverse reactions. Surgical manipulations are the most drastic form of treatment, with complete removal of the colon and

rectum resulting in curing ulcerative colitis and providing positive relief to Crohn's patients not responding to drug therapy.

Peptide growth factors and ulcer healing

Introduction

As previously mentioned the pathophysiology of inflammatory bowel disease is comprised of an altered colonic mucosal morphology together with acute and chronic inflammatory responses. A complex interplay exists between these two major facets of colitis conditions, with some mediators of colonic inflammation being suggested as potential causal factors of mucosal ulceration, and in turn the altered mucosal morphology attenuates the inflammatory response. As the etiology of these diseases remains unknown no effective curative drug treatment has been developed. Instead, current drug therapies are effective in maintaining or inducing a remission phase of the diseases, acting via a modulation of the inflammatory responses. Unfortunately these therapies are associated with a broad spectrum of side-effects and new therapies are being developed which produce fewer deleterious effects. With an increased understanding of the action of mediators of the inflammation, new therapies are being developed to modulate their action and reduce the activity of the disease.

The emphasis of current drug therapy has been in the treatment of the inflammatory response in colitis, with little emphasis being made on treating the colitis condition by directly enhancing ulcer healing. In the upper gastrointestinal tract a range of ulcer healing agents have been developed including cimetidine (Szabo and Bynum 1988), solcoseryl (Brzozowski et al. 1987) and colloidal bismuth subcitrate (De-Nol) (Wieriks et al. 1982). In the development of new therapies for ulceration Poulsen (1988) stipulates that if an agent is

to be considered cytoprotective or beneficial to ulcer healing in the stomach and duodenum it must be available to the stomach and duodenum, be able to bind to the intact or damaged mucosa and finally it should have an effect on mucosal protection or ulcer healing if administered exogenously. As these criteria are relevant to the development of new therapies for ulcer healing in the upper gastrointestinal tract they are also applicable to the treatment of colonic ulcerations. A range of agents that are currently being assessed experimentally for the treatment of gastroduodenal ulcerations have the potential for use in the treatment of colonic ulcerations. Of these agents, the peptide growth factor, epidermal growth factor (EGF), fulfils the criteria stated by Poulsen (1988) of a potential ulcer healing agent in the upper gastrointestinal tract and it is the major aim of this thesis to assess its ulcer healing properties in the colon. Accordingly, this section will address the properties of EGF with particular emphasis on its ulcer healing actions and mitogenic activities in the gastrointestinal tract, in particularly the colon.

General characteristics of growth factor action

The actions of polypeptide growth factors as cellular mitogens have been extensively investigated. Growth factors have an endocrine-like structure and function but have an apparent paracrine mode of action. Stimulation of anabolic processes in target cells is modulated through binding to the extracellular domain of specific membrane-bound receptors. The receptors are plasma membrane glycoproteins and binding the growth factor to the receptor induces endocytosis of the receptor-ligand complex into the cell cytoplasm (James and Bradshaw 1984). Tyrosine-specific protein kinase activity, has been associated with several growth factor receptors, including insulin-like growth factor 1, platelet-derived growth factor and epidermal growth factor, and is thought to be activated by ligand binding resulting in autophosphorylation of the receptor and other cellular proteins (Carpenter et al. 1978). In addition, the internalized receptor-growth factor complex generates a cascade of events which induces cell growth including nutrient uptake, ion transport, DNA and RNA synthesis and protein synthesis (Carpenter and Cohen 1976, Hoober and Cohen 1967, Arsenalut and Menard 1987, Dembinski and Johnson 1985). The internalization of the ligand and receptor complex also provides a means of down regulating the cellular responses

to the growth factor by reducing the number of available binding sites on the cell surface (James and Bradshaw 1984, Carpenter and Cohen 1981).

Endogenous EGF associated with the gastrointestinal tract

Isolation and distribution

The mitogenic action of EGF on the gastrointestinal tract was first observed by Cohen (1962), who purified a low molecular weight, heat stable polypeptide as a contaminant of nerve growth factor preparations derived from male mouse submaxillary gland. Cohen (1962) demonstrated that the contaminant accelerated eyelid opening and incisor eruption in new born mice. The physiological responses observed in newborn mice were due to a stimulation of epidermal growth and keritinization. The factor responsible was isolated and denoted epidermal growth factor. EGF is a 53 amino acid polypeptide containing 3 intramolecular disulphide bonds between cysteine residues which are required for biological activity (Savage and Cohen 1972, Taylor et al. 1972). EGF is synthesized as a large 1217 amino acid precursor molecule, which contains 7 EGF-like sequences and a portion which shares 77% homology to the EGF receptor. The RNA for the precursor molecule has been detected in the submaxillary gland and in the distal convoluted tubules of the kidney. The precursor form of EGF is cleaved to the 53 amino acid form in the submaxillary gland. However in the kidney, the high molecular weight precursor is not processed and exists as a membranous protein thought to be involved in ion transport (Scott et al. 1985). The precursor molecule and a range of molecular weight species of EGF have been detected in human urine and a range of body fluids (Tsukumo et al. 1987, Pesonen et al.1987) suggesting that precursor molecules are processed and secreted giving rise to biologically active EGF.

EGF has been purified from a number of species and found to have a relatively low conservation of sequence. However, the receptor-binding region is highly conserved between species, so EGF is equally active in a range of species, for example 33 of 48 amino

acid residues of human EGF are found in coresponding positions in the rat (Morrison et al. 1985) and rat EGF competes for binding of human radiolabelled EGF in human carcinoma cell lines and elicits the same mitogenic effect in mouse 3T3 cells (Morrison et al. 1985).

Other EGF-like molecules have been isolated. Transforming growth factor-alpha (TGF-α) is a single chain polypeptide which shares 20% structural homology to mouse EGF and 8% homology to human EGF and binds to the EGF receptor (Massague 1983). Similar mitogenic effects on epidermal cells and keritinization, as seen with EGF, are observed when TGF-α has been administered to experimental animals. Moreover, TGF-α has been suggested to be a potent mitogen in developing animals (Tam 1985).

EGF has been detected at a number of sites along the length of the gastrointestinal tract in adult humans, including the submandibular gland, duodenum, jejunum and kidney (Hirata and Orth 1979, Poulsen et al. 1986). It is also present in a range of fluids associated with the gastrointestinal tract including saliva, Brunner's gland secretions, gastric juice (Gregory et al. 1979) and human breast milk (Read et al. 1986), and has been detected in the gut lumen from the stomach to the distal colon (Cartliage and Elder 1989). The concentrations of EGF extracted from body tissues are low and EGF does not occur in a free form in plasma but rather, it circulates within platelets. It is likely therefore, that EGF is produced at low concentrations by tissues and acts by an autocrine or paracrine mechanism, rather than as a circulating endocrine factor (Hirata and Orth 1979, Carpenter and Cohen 1981).

Receptor localization

The EGF receptor is a 170KDa glycosylated phosphoprotein and has been localized on a wide variety of cell types including corneal cells, human fibroblasts, lens cells, human glial cells, human epidermoid cells, 3T3 cells, granulosa, vascular, endothelial and epithelial cells (Carpenter and Cohen 1979). The receptor has a tight specificity for EGF and both high and low affinity receptors have been identified in a range of tissues (James and Bradshaw 1984). EGF receptors have been detected along the length of the gastrointestinal

tract in the fetal animal and throughout the postnatal period in the gastric mucosa, small intestine and colon of the mouse, rat and human (Pothier and Menard 1988, Nexo et al. 1980, Adamson and Meek 1984, Gallo-Payet et al.1987, Forgue-Lafitte et al. 1984, Menard et al. 1987). The localization of EGF receptors on the microvillus membrane in the rat small intestine (Thompson 1988) suggests that luminal EGF present in fluids associated with the gastrointestinal tract may have a direct effect on the intestinal mucosa.

Function

The function of EGF in the gastrointestinal tract has been widely investigated with most studies performed using *in vitro* techniques such as isolated cells or organ cultures. In contrast, *in vivo* studies have been hampered due to the difficulty in purifying sufficient amounts from natural sources. However, with the advent of recombinant DNA technology, EGF is now commercially available in quantities to allow an assessment of its effect on *in vivo* systems.

Development and growth of the gastrointestinal tract

The presence of EGF and its receptor in fetal tissues and amniotic fluid suggests that the growth factor may play a role in the development of the gastrointestinal tract. EGF is trophic to gut epithelia and enhances maturation and cellular proliferation (Weaver and Walker 1988). *In vitro* studies of cells isolated from fetal gastric mucosa, small intestine and colon, from a range of species, suggests that EGF enhances cell growth and differentiation indicated by an increase in DNA synthesis and modulation of hydrolytic enzyme activity (Mulvihill et al. 1986, Beaulieu et al. 1985, Menard et al.1988). The mitogenicity of EGF to the fetal gastrointestinal tract is supported by *in vivo* studies in which overall gut growth is enhanced when exogenous EGF is administered to fetal monkeys (Read et al. 1989).

The EGF present in breast milk provides a source of EGF to the suckling infant (Read et al 1986), although the effects of EGF in the postnatal period remain unclear and vary depending on the route of administration. Enterally administered EGF significantly

increases DNA synthesis in the colon of neonatal rats (Pollack et al. 1987). As milk derived EGF may have a low rate of absorption from the intestinal lumen, these the mitogenic effects could be exerted via receptors on the luminal surface of the gut (Read et al. 1987, Thompson 1988). The neonatal gastrointestinal tract is also responsive to systemic EGF. Gastric mucosal growth has been induced in the neonatal mouse, although its effect on intestinal differentiation remains contentious (Dembinski and Johnson 1985, James et al. 1987).

In the adult, EGF is considered to play a role in the maintenance and protection of the upper gastrointestinal tract. Anti-secretory and cytoprotective activities of EGF were first observed by Sandweiss in 1945 (Gregory 1975) when EGF in human urine inhibited gastric acid secretion and aided healing of gastric ulcers in dogs. Gregory (1975) showed that urogastrone, a known inhibitor of gastric acid secretion, and EGF were in fact the same compound. EGF modulates gastric secretion by inhibiting release of hydrochloric acid from parietal cells (Weaver and Walker 1988) and occurs as a response to parenteral but not oral EGF. In humans, elevated gastric acid secretion in response to a number of stimuli, is inhibited by 0.25μg EGF / kg body weight, whereas in other species such as in the dog 0.1-1.0μg/kg EGF will inhibit acid release (Koffman et al. 1982).

When administered orally, EGF has been shown to display cytoprotective effects in rats and cats (Konturek and Radecki 1981, Konturek and Brzozowski et al. 1981, Kirkegaard et al. 1983, Gysin et al. 1988). EGF infusion protects against gastric and duodenal lesions induced by a range of injurious agents such as acetic acid, cysteamine, ethanol, cold water stress and indomethacin. Concomitant administration of EGF and the injurious agent results in a decreased size of gastric and duodenal ulcerations and is accompanied by an elevation in DNA synthesis.

Salivary EGF is considered to act as an endogenous cytoprotective agent. Skov-Olsen et al. (1984) illustrated the protective action of salivary EGF against cysteamine-HCl induced gastric ulcerations in sialoadenectomized rats. Removal of the submandibular glands resulted in a reduction in the gastric EGF content, and the development of gastric

lesions, however concomitant administration of EGF, at a physiological dose, resulted in a significantly reduced area of the stomach displaying ulcerations.

The mechanism by which cytoprotection is elicited remains unclear, but it is likely to be distinct from its action in inhibiting acid secretion as both oral and systemic EGF induce cytoprotection whereas only systemic administration induces an anti-acid secretory response (Konturek and Radecki 1981, Konturek and Brzozowski et al. 1981). The cytoprotective effect of salivary EGF may in part be due to its effect on maintaining the gastric mucosal mucus coat (Sarosiek et al. 1988). Deprivation of salivary EGF produces a reduction in the thickness of gastric mucus, which was restored to normal following intragastric EGF administration, suggesting that EGF enhances the production of the protective mucus barrier.

Ulcer healing action of EGF

In addition to displaying cytoprotective functions in the upper gastrointestinal tract, EGF also accelerates gastric and duodenal ulcer healing. Ulcer healing is a process involving wound contraction and epithelialization (Hase et al. 1989). EGF has been associated with a number of parameters which enhance ulcer healing. The ulcer healing effect of EGF has been assessed by inducing chronic ulcerations using injurious agents such as serosally applied acetic acid or by subcutaneous cysteamine-HCl administration (Konturek and Brzozowski et al. 1988, Konturek and Dembinski et al. 1988, Skov Olsen et al. 1986a and 1986b). Oral administration of 30μg/kg body weight/day enhances healing of chronic cysteamine-induced duodenal ulcerations. As this dosage of EGF does not inhibit gastric acid secretion it was suggested that the ulcer healing effect of EGF was due to a direct effect on the duodenal mucosa (Skov Olsen 1986b). This was supported in studies performed by Konturek and co-workers (1988) in which the same dose of oral or systemic EGF used to induce cysteamine duodenal ulcer healing, reduced the period required for healing gastric and duodenal acetic acid ulcerations from 14 to 7 days. Ulcer healing was associated with an elevated weight, RNA and DNA content, of the duodenal and gastric

mucosa adjacent to the ulcerative lesion (Konturek and Brzozowski et al. 1988, Konturek and Dembinski et al. 1988). These studies suggest that the ulcer healing effect of EGF is modulated via a promotion of mucosal growth and subsequent re-epithelialization of the ulcerated mucosa.

The ulcer healing effect of EGF has also been linked to its ability to enhance the formation of granulation tissue. The area of gastric ulcerations induced by serosal acetic acid is reduced and angiogenesis of granulation tissue is enhanced following 10 days treatment with 100μg/kg/day of synthetic human EGF (Hase et al. 1989). An enhancement of angiogenesis is considered beneficial in the enhancement of wound and ulcer healing (Hase et al. 1989). EGF also enhances the formation of collagen, thereby potentially modulating ulcer contraction (Kumegawa et al. 1982).

EGF has been implicated as the effector system of ulcer healing drugs such as sucralfate (Nexo and Poulsen 1987) and De-Nol (Konturek and Dembinski et al. 1988). Both agents form a coating across the denuded tissue providing a physical barrier between the ulceration and the lumen. Both sucralfate and De-Nol bind EGF at acidic pH without effecting its receptor binding ability. Localization studies of the bound material show an aggregation of EGF at the site of the ulcerative lesion (Nexo and Poulsen 1987, Konturek and Dembinski et al. 1988). These studies suggest that these agents may act by targeting endogenous to the ulcerative site.

It is not known if EGF is directly involved in the pathogenesis of inflammatory bowel disease, for instance a lack of endogenous EGF expression, reduced EGF receptor numbers or ligand binding affinity has not been measured in colitis patients. Therefore it is not known if EGF would be a useful therapy in the treatment of inflammatory bowel diseases, although its ability to enhance the healing of ulcerations in the upper gastrointestinal tract suggests that it could be a useful adjunct to other therapies for inflammatory bowel disease.

Summary

In the upper gastrointestinal tract endogenous EGF enhances intestinal growth and maturation in fetal and neonatal animals. When administered parenterally, EGF inhibits gastric acid secretion, whereas orally administered EGF acts as a cytoprotective agent to the gastric and duodenal mucosa. As well as protecting against ulceration, EGF accelerates ulcer healing. Ulcer healing is enhanced by the ability of EGF to increase mitogenesis of the mucosa adjacent to ulcerative lesion, angiogenesis, collagen formation and mucin production. EGF has also been implicated in the development of the colonic mucosa, although to date, no studies have assessed the ulcer healing properties of EGF on the colonic mucosa and this is the major emphasis of this thesis.

Experimental models of Inflammatory bowel disease

Introduction

Due to ethical considerations, the study of the pathophysiology and treatment of ulcerative colitis in human patients is necessarily limited in scope. The alternative is to use experimental models in which more invasive studies can be employed, notably cell cultures and animal models. Cell cultures, involving primary colonocytes, established cell lines and organ culture have been used extensively in various studies of colonic metabolism. Whitehead (1987) has recently described a cell line which forms organoid structures in culture which are functionally organized like crypts and are viable in culture for up to 21 months. In general however, colonocyte cultures have limited usefulness in the study of

inflammatory bowel disease as a result of poor viability and morphological integrity (Roediger and Truelove 1979, Ahnen et al.1987, Burger et al. 1985, Moyer 1984).

As an alternative experimental tool, animal models of colitis either naturally occurring or experimentally induced are considerably more complex than cultured cells, but offer the advantage of a more physiological model for testing of new therapies. Strober (1985) stipulated a range of characteristics that should be possessed by animal models of inflammatory bowel disease. Ideally, the animal model should have the same causal factors, pathology and pathophysiology and clinical spectrum as human inflammatory bowel disease. The colitis should occur in an animal that is accessible and reasonably inexpensive, with a defined genetic background and similar immune system to humans and can be manipulated as to its dietary intake, immunological status and exposure to infectious agents. As the etiology of inflammatory bowel disease remains unknown models which exactly mimic the human disease do not exist. At the time of writing this literature review, several natural and experimental models of ulcerative colitis had been reported which share some of the features of the human disease.

Naturally occurring colitides

Colitis-like diseases have been diagnosed in a number of species including monkeys, dogs, horses and pigs (For reviews see MacPherson and Pfeiffer 1976, Strober 1985 and Onderbonk 1985). Lushbaugh et al. (1985) detected many acute colitis conditions in captive marmoset colonies. These observations were made over a 20 year period and were most commonly seen in *Saguinus fusciollis illigeri* and *Callithrix jacchus*. The symptoms were similar to human colitis showing a range of severities and locations of the disease. Unfortunately, the most susceptible species identified by Lushbaugh et al. (1985) are endangered, making them unsuitable as an experimental animal model. The most extensively studied animal colitis is that occurring in the boxer dog. Canine colitis shows the symptoms of loose stools, occult blood and mucus in faeces, occurring in periodic phases as is the case in the human disease. Histologically, the mucosa degenerates into

distinct ulcerations with neutrophil infiltrates in the lamina propria. However, unlike human ulcerative colitis no crypt abscesses are detected and the majority of damage is in the submucosa and the lamina propria with the distribution and symptoms being likened to Crohn's colitis. The canine colitis is thought to be caused by bacteria such as *Chlamydia*. Colitis observed in pigs and hamsters has also been linked to bacterial infections (Strober 1985). Porcine enteritis is detected in 1% of slaughtered swine (Strober 1985). This condition shares some similar histological characteristics with human ulcerative colitis including deep ulcerations and necrosis of the colonic mucosa. In hamsters a proliferative ileitis has been observed in enclosed colonies which differs from ulcerative colitis in that it only involves the terminal ileum (Amend et al. 1972). Overall, the incidence of naturally occuring colitides is low, and in general the species in which they occur are endangered or are expensive to maintain as an experimental model.

Experimentally induced colitides

Due to difficulties in obtaining a suitable natural model of colitis a number of injurious agents have been used to induce a colitis comparable to the human condition. While these colitides do not exactly mimic the human condition they share features of the disease which may be used to assess new therapeutic agents. Agents such as carrageenan, acetic acid and agents which alter the immune status of the animal will be discussed as they induce a reproducible colitis condition with a low mortality rate. The procedures used are quick and easy to perform and can be applied to laboratory animals such as the rat and mouse which are inexpensive to maintain.

Carrageenan

Carrageenans are composed of a series of sulphated polygalactose units of high electronegativity which bind proteins, in particularly casein and have gel-forming properties. Native carrageenans are extracted from red seaweed (E. spinosum and E. cottoni) with the polygalactose units existing in 3 forms, 2 which have gelling properties, iota and kappa, and a non-gelling form lambda carrageenan (Benitz et al. 1973, Ishioka et al.1987). These edible gums are used as thickening agents in the food industry and in the 1960's were used

in peptic ulcer treatment due to their ability to reduce peptic activity (Benitz et al. 1973). Their use in peptic ulcer therapy was ceased due to findings in a range of species including primates, that carrageenan ingestion induced a colitis-like condition (Ishioka et al 1987). The clinical features of carrageenan-induced colitis included weight loss, diarrhea, occult blood and mucus in the faeces, accompanied by colonic mucosal ulceration, crypt abscesses, a decreased goblet cell number and inflammation (Benitz et al. 1973). Hence, many of the histological features of the carrageenan-induced colitis are comparable to human ulcerative colitis.

The sulphur content of carrageenan appears to be an important factor in its ulcerogenic properties in view of the fact that a range of sulphated polysaccharides with a comparable molecular weight and sulphur content have also been found to cause colonic ulcerations. For example, oral administration of amylopectin sulphate (48kDa, 19% sulphur content) derived from potato starch and sodium dextran sulphate (54kDa, 19% sulphur content), a synthetic polysaccharide, both induce ulcerations with similar clinical and morphological characteristics to those observed following acid-hydrolysed carrageenan treatment (Ishioka et al 1987).

Mode of action

Dose response studies of carrageenan and sulphated amylopectin (Watt and Marcus 1973) in rabbits have indicated that the incidence and extent of colonic ulceration is increased with dosage and duration of exposure to the sulphated polysaccharides. The direct effect of these polyanionic compounds on inducing epithelial damage has been measured. The injurious properties of degraded carrageenan has been investigated *in vitro* using a small intestinal epithelial cell preparation (Ling et al. 1988). Addition of acid hydrolysed-carrageenan to the epithelial culture altered the cell junction and cell membrane integrity in a time and dose-dependent fashion, suggesting that the impairment of epithelial barrier function initiates the colitis produced by carrageenan administration.

Inter-species variation in the location and severity of ulcerations has been observed by a number of investigators with the most susceptible species being the guinea pig and rabbit (Sharrat et al. 1971). The distribution of carrageenan has been monitored in pigs, dogs, ferrets, monkeys, guinea pigs and rabbits using toluidine blue staining to localize iron-labelled carrageenan (Sharrat et al. 1971). A substantial accumulation of carrageenan was observed in the cecum of the guinea pig and rabbit, suggesting that the greater susceptibility of these herbivorous species to carrageenan-induced ulceration may be attributed to their developed cecum. Nevertheless, the injurious action of carrageenan is not restricted to the cecum and proximal colon. In guinea pigs and rabbits long term administration of a 1% acid hydrolysed carrageenan solution also induced ulcerations in the distal colon and rectum (Kitano et al. 1986). In non-herbivorous species such as the rat, treatment with 1,2 and 5% acid hydrolysed carrageenan in the drinking water for up to 2 years resulted in a colitis with similar histological features as the human disease, including squamous epithelium in the distal rectum and blood in the faeces and at the anal margin (Marcus and Watt 1971, Fabian et al. 1973, Ishioka et al 1987). In the rat, metachromatically stained carrageenan has been detected in rectal macrophages following oral administraton implicating macrophage metabolism as a potential mechanism of carrageenan-induced ulceration (Ishioka et al 1987, Fabian et al. 1973). The uptake of carrageenan by macrophage lysosomes in colonic submucosa and lamina propria has been monitored, with and inhibition of lysosomal endocytosis inhibiting the ulcerogenic effect of carrageenan (Abraham et al. 1974). Lysosomal enzyme release from macrophages following carrageenan uptake has been implicated in the formation of subcutaneous granulomas and hydrolytic enzymes release may induce epithelial intestinal damage in the colon (Fabian et al.1973, Abraham et al. 1974). In contrast, in the rhesus monkey no such macrophage uptake of carrageenan has been detected, and the mechanism of carrageenan action in this species still remains unknown (Benitz et al. 1973)

Colonic microflora have also been implicated in the uptake and metabolism of carrageenan, although the precise role in enhancing the ulcerogenic action of carrageenan still remains a contentious issue. Fabian et al. (1973) observed no alteration in macrophage

uptake of carrageenan in animals treated with antibiotics such as neomycin, whereas in guinea pigs pre-treated with metronidazole no ulcer inducing effects of carrageenan were observed (Onderbonk 1985). In these studies variation in the effect of antibiotic treatment may be attributed to the type or dose of antibiotic used.

In human ulcerative colitis the incidence of colon cancer is increased compared to the normal population (Gyde et al. 1988, McIllmurray and Langman 1975). Prolonged treatment with a high dose of carrageenan for up to 2 years results in an increased incidence of colorectal tumors in rats (Ishioka et al.1987, Fabian et al. 1973). This feature of carrageenan-induced colitis may enable investigation of the pre-cancerous nature of colitis to be studied.

Comparison to human inflammatory bowel disease

The profile of clinical features resulting from carrageenan treatment in the rat and guinea pig is comparable to that observed in human ulcerative colitis, including weight loss, diarrhea and occult blood and mucus in the faeces (Ishioka et al 1987, Anver and Cohen 1976). Moreover, similar histological characteristics between carrageenan induced ulceration and human ulcerative colitis are observed including superficial erosion, crypt abscesses, mucosal distortion, a decreased goblet cell number, edema, and neutrophil and mononuclear infiltrates. Despite these pathological similarities, differences in the onset and development of the experimentally induced ulceration and human ulcerative colitis exist. Although the etiology of ulcerative colitis is unknown, it cannot be attributed to a single infectious agent and granuloma formation does not usually occur. In contrast, carrageenan-induced colitis, particularly in herbivorous species, involves a granulomatous response leading to the subsequent mucosal ulceration. The distribution of the ulceration also varies between the experimentally induced colitis and the human disease. In herbivorous species, carrageenan induced ulcerations are first observed in the caecum with lower colon and rectal involvement associated with a prolonged treatment regime, whereas in human ulcerative colitis the rectum and lower colon are the more commonly effected regions (Watt and Marcus 1973).

Acetic acid

Chemical irritants such as acetic acid, which damage the intestinal mucosa, have been used to induce ulcerations in the upper gastrointestinal tract. Topical application of acetic acid onto the serosa of the duodenum and stomach fundus induces ulcerations which appear three to five days after the acid insult persist up to eighty days (Okabe et al. 1971). This method produces a reproducible and well defined ulcerative lesion and has been used extensively in the assessment of new therapies for gastric and duodenal ulcerations (Konturek and Dembiski et al.1988, Konturek and Brzozowski et al. 1988). Intraluminal instillation of acetic acid into the colon produces an acute inflammatory state and mucosal ulceration. Animals present with diarrhea, intestinal ulceration and weight loss within three days of the acid insult (MacPherson and Pfeiffer 1976). Histological features of the colitis induced include; mucosal and submucosal edema, mucosal ulceration, leukocytic infiltration of the mucosa, submucosa and muscularis layers and cryptitis (Sharon and Stenson 1985).

Mode of action

The method by which intraluminally administered acetic acid induces colonic inflammation and mucosal injury still remains speculative. The protonated form of the organic acid is required for colitis induction as administration of hydrochloric acid at the same pH or sodium acetate does not produce colonic ulceration (Zeitlin and Norris 1983). It has been suggested that the lipophylic acid enters the intracellular compartment of the epithelial cell and produces acetate ions and protons. This intracellular acidification would in turn injure the epithelial cells and induce an inflammatory response.

Comparison to human inflammatory bowel disease

Acetic acid induced-colitis shares many of the histological features of human ulcerative colitis including mucosal and submucosal ulceration and goblet cell depletion, as well as sharing the same clinical features of blood and mucus in the faeces, and weight loss (MacPherson and Pfeiffer 1976,1978). The acute inflammatory response induced has similar characteristics to that observed in the human disease. Mucosal samples collected from rats 24 hours after exposure to 5%(v/v) acetic have a similar arachidonic acid

metabolism to that observed in human ulcerative colitis biopsies. The concentration of leukotriene B4 was comparable to that derived from active ulcerative colitis biopsies (Sharon and Stenson 1985). Although despite a similar acute inflammatory response being observed between the two colitis states, the pattern of inflammatory events differs. In acetic acid-colitis the inflammation is induced and resolved rapidly whereas in human ulcerative colitis both acute and chronic inflammatory states are observed.

Other experimentally induced-colitis models

A range of other inducing agents have been used to produce a colitis-like condition including vascular impairing agents, drugs, irradiation and alteration of the immune status of the animal (For a review see MacPherson and Pfeiffer 1976). The immune status can be altered by exposure to bacterial antigens which produces a granulamatous inflammation in a range of species including rabbits, dogs and rats. Other agents such as dinitrochlorobenzene and more recently trinitrobenzenesulfonic acid (TNBS) have been used to invoke an immune response by acting as a hapten and binding to tissue proteins (Rabin 1980, Morris et al. 1989). Both these agents induce a granulomatous inflammatory response and severe transmural ulcerations. By administering a mucosal damaging agent such ethanol with TNBS granuloma formation and ulcerations have been observed up to 8 weeks after the initial insult. The TNBS / ethanol model has a low mortality rate and histological characteristics of the induced colitis has been likened to that observed in Crohn's disease (Morris et al. 1989).

Summary

Naturally occurring colitis conditions occur in a range of species, although the frequency is low and the animal species inflicted are endangered or very expensive to maintain. These factors limit their use as an experimental model in the investigation of new therapies of inflammatory bowel disease. A range of agents have been used in a number of animal species to experimentally induce ulcerative colitis-like symptoms. Orally administered degraded carrageenan and sulphated polysaccharides induce colitis in monkeys, guinea pigs, rabbits and rats, while chemical irritants such as acetic acid have been used to

produce a colonic lesion in rats. The clinical and histological profile of the induced colitis are comparable to human ulcerative colitis. Agents which alter the immune status have been developed to produce an experimental colitis comparable to Crohn's disease. These models provide a potential means of studying changes in the morphology and biochemistry of the colonic mucosa in colitis, as well as providing a way of testing various new therapeutic agents for inflammatory bowel disease.

Overall conclusions

Ulcerative colitis and Crohn's disease are chronic ulcerative disease with an unknown etiology. The pathophysiology of the these diseases includes an altered morphology and function of the colonic mucosa with acute and chronic inflammatory responses. Current drug therapies can maintain patients in a remission phase of the disease but do not cure the condition. Drug therapies are under constant clinical review and new therpeutic agents are being developed to modulate the inflammatory response. A novel approach to treating these diseases would be to investigate mechanisms of enhancing colonic ulcer healing and thereby promoting disease remission. Epidermal growth factor enhances ulcer healing in the upper gastrointestinal tract although its ulcer healing effects in the colonic mucosa have not been previously assessed. A major factor limiting the assessment of new drug therapies is the availability of appropriate animal models of inflammatory bowel disease. Colitis-like conditions can be induced using a range of injurious agents and the resulting colonic lesion shares some of the histopathological features of the disease which can be used for assessing new therapies for inflammatory bowel disease.

CHAPTER 2. SCOPE OF THESIS

Drug therapies for ulcerative colitis and Crohn's disease can maintain patients in a remission phase of the disease but do not cure the condition. A novel approach to inducing a remission phase of these diseases is to develop agents which enhance the healing of the colonic ulceration. Epidermal growth factor is a potent ulcer healing agent in the upper gastrointestinal tract and may also represent part of the natural process of ulcer repair although its use in treating colonic ulcerations has not been investigated. It is not known if a deficiency of EGF is involved in the etiology of colonic inflammatory bowel disease nor if EGF accelerates ulcer healing in the lower gastrointestinal tract.

It is hypothesised that EGF will be useful in the treatment of inflammatory bowel disease by enhancing re-epithelialization of the ulcerated colon.

The hypothesis will be tested by:

a) Examining the effect of exogenous EGF on the normal adult colon.

EGF has been implicated in the growth and maturation of the fetal and neonatal colonic mucosa however to date, the effect of EGF on the normal adult colon has not been investigated. The ability of exogenous EGF to elicit a mitogenic effect on the normal adult colon will be assessed to determine the responsiveness of the adult colon to the growth factor. A comparison the responsiveness of the colon to two routes of EGF administration, continual subcutaneous infusion and intraluminal bolus injections, will be made using biochemical and quantitative histological techniques.

b) Developing an appropriate animal model of inflammatory bowel disease for testing the ulcer healing properties of EGF in the colon.

At the time of commencing this thesis several experimentally induced animal models of colitis had been described in the literature which produced colitis-like conditions. Carrageenan and luminal acetic acid administration had been reported to produce a colitis which was reproducible, easy to perform and had a low mortality rate. Both these models will be assessed as potential *in vivo* systems for determining the colonic ulcer healing effect of EGF. The criteria used for assessing these models will be their ability to produce a colonic mucosal lesion which is reproducible, can be quantitated and provides a recovery window adequate for the assessment of the effects of EGF. The technique must also be easy to perform and have a low mortality rate.

In the upper gastrointestinal tract, EGF has been shown to enhance ulcer healing by a number of mechanisms, however the major aspects of ulcer healing induced by EGF in the colon to be measured here will be its ability to enhance re-epithelialization of the ulcerative lesion and mitogensis of the mucosa adjacent to the ulcerative segment. To enable these aspects to be measured a range of biochemical, histological and functional assays will be developed to enable a quantitative assessment of growth factor action to be made.

c) Measuring the ulcer healing properties of EGF in the experimental colitis model.

Using the animal model developed, the ability of EGF to enhance re-epthelialization of the ulcerated colon will be assessed. The responsiveness of the colonic segment adjacent to the ulcerative lesion to EGF will be measured using similar techniques used in the normal colon and EGF study. Two methods of EGF administration, a continual systemic infusion and intraluminal bolus doses,will be assessed for their mitogenic and ulcer healing abilities.

CHAPTER 3. THE EFFECTS OF EXOGENOUS EGF ON THE NORMAL ADULT RAT COLON

Abstract

The effect of exogenous epidermal growth factor (EGF) on colonic growth was assessed in normal adult male rats. Comparisons were made between 2 routes of EGF administration and between the responsiveness of the proximal and distal colonic regions to EGF. A 7 day continual subcutaneous infusion of 200μg EGF/kg/day delivered by mini osmotic pumps, induced growth of the colonic mucosa as shown by a significant increase in the mucosal wet weight, protein content and colonic circumference, as well as an increase in the number of cells per crypt and mucosal area above that of vehicle-treated control animals. Muscularis mass was also increased in the proximal colon where the wet weight and protein content were significantly elevated. Similar trends were observed in the distal colon although the magnitude of the response was lower than that seen in the proximal colon. In contrast, luminal administration of 1.6mg EGF/kg/day, given as 2 daily bolus 1ml injections via a luminally implanted cannula into the proximal or distal colon, had no effect on colonic mucosal or muscularis growth. The lack of effect of luminal EGF would not appear to be due to luminal degradation as radiolabelled EGF remained stable in the proximal and distal colonic lumen for at least four hours. The results indicate that the colon is responsive to subcutaneously delivered EGF, with the proximal colon more responsive than the distal region, whereas EGF may not be active on the normal colon when presented from the luminal direction.

Introduction

The effect of EGF on the gastrointestinal tract has been widely investigated, although most studies have focused on the response of the upper gastrointestinal tract, and its effect on the colon and rectum have received little attention. Accordingly, in this chapter the proliferative responsiveness of the normal adult rat colon to exogenously administered EGF was assessed.

In the upper gastrointestinal tract, the mitogenic action of EGF has been clearly demonstrated. The intestinal lumen is exposed to EGF contained in saliva, Brunner's gland secretions, gastric juice and milk, and EGF has been detected in the lumen and mucosa throughout the upper gastrointestinal tract (Gregory et al. 1979, Heitz et al. 1978, Read et al. 1986, Schaudies et al. 1989, Cartliage and Elder 1989). These endogenous sources of EGF have been shown to display both cytoprotective and mitogenic properties in the gastrointestinal tract in a range of *in vivo* studies (Konturek and Dembinski et al. 1988, Konturek and Brzozowski et al. 1988, Tepperman et al. 1989, Sarosiek et al. 1988). Receptors for EGF have also been identified along the length of the gastrointestinal tract (Gallo-Payet et al. 1987, Forgue-Lafitte et al. 1984), further supporting the notion that EGF has a role in the maintenance and development of the gastrointestinal tract . The upper gastrointestinal tract is also responsive to EGF given by either the systemic or luminal route of administration, although the optimal route of delivery for enhancing growth and repair varies depending on the status of the gastric / intestinal mucosa. While luminal EGF exerts cytoprotective and ulcer healing effects on the gastroduodenum (Skov Olsen et al.1986a and 1986b, Konturek and Brzozowski et al. 1988 and 1990), it has little effect on mucosal atrophy induced by parenteral nutrition (Goodlad et al. 1987). In contrast, continuous intravenous infusion of EGF has been shown to reverse intestinal atrophy (Goodlad et al. 1985, 1987,1992) as well as enhancing epithelialization of small intestinal patch defects (Thompson et al. 1990).

Much less attention has been given to the colon as an EGF target tissue. Receptors for EGF have been located in the colon (Menard et al. 1987, Koretz et al. 1990, Koenders et

al. 1992), as illustrated by high levels of radiolabelled-EGF binding detected in isolated colonocytes from the neonatal and adult mouse (Menard et al. 1987) as well as in the adult human colonic mucosa (Koretz et al. 1990, Koenders et al. 1992).

Endogenous EGF has been implicated in the growth and development of the colonic mucosa (Pollack et al. 1987). Milk derived-EGF has been shown to increase the colonic mucosal DNA content in suckling rats (Pollack et al. 1987) and EGF has been detected in the colonic lumen of adult humans, although the role of EGF in the lower gastrointestinal tract, particularly in the adult is still not fully understood.

Few studies have investigated the responsiveness of the colon to exogenous EGF. The mitogenic effect of exogenous EGF on the intact colonic mucosa has been shown in normal adult male mice, where a subcutaneous injection of EGF increased DNA tritiated thymidine incorporation in the colon and rectum within 12 hours (Scheving et al. 1980). Systemic EGF has also been shown to increase colonocyte proliferation in colonic mucosal atrophy, induced by parenteral nutrition and diversion of the fecal stream (Goodlad et al. 1987, Foster and Whitehead 1990). The reduction in the proportion of cells undergoing metaphase, induced by a diversion of the fecal stream, was restored to normal levels following a continual subcutaneous infusion of EGF (Foster and Whitehead 1990). Similarly, the reduced crypt cell production rate and colonic wet weight resulting from parenteral nutrition, was restored to values comparable to those of orally fed animals in rats receiving parenteral nutrition supplemented with EGF (Goodlad et al. 1987). As seen in the upper gastrointestinal tract, the responsiveness of the colonic mucosa to EGF differs depending of the route of growth factor administration. In contrast to the mitogenic effect of systemic EGF on colonic atrophy, intraluminal EGF has no effect on colonocyte proliferation in this model (Foster and Whitehead 1990). To date, the responsiveness of the damaged and normal colonic mucosa to luminally delivered EGF has received little attention.

In this chapter, assessment of the mitogenic effect of exogenous EGF on the normal colon involved measuring the responsiveness to both systemic and intraluminally delivered

EGF. Systemic administration was by a continual subcutaneous infusion of EGF using implanted osmotic minipumps filled to deliver a dose of EGF which is within the range previously used by other investigators to exert mitogenic effects in the upper gastrointestinal tract (Goodlad et al. 1983, 1987,1992). For studies on growth responses to intraluminal EGF, the growth factor was administered in a separate study into the proximal or distal colon as two bolus doses per day. Bolus doses in a large volume, rather than slow continuous infusion through osmotic pumps was considered more likely to ensure exposure to the entire colonic surface to the growth factor. An eight-fold higher total daily dose was used in these experiments than in subcutaneous infusion studies in an attempt to compensate for any fluctuations in the luminal concentration due to degradation and wash out of luminal contents.

As EGF has been previously shown to increase colonic wet weight, DNA content and the proliferative status of the colonic mucosa (Scheving et al. 1980, Foster and Whitehead 1990, Goodlad et al. 1987, 1992), the response of the normal colon to exogenous EGF was assessed in the current study using analytical measurements of wet weight, protein and DNA contents of the colonic mucosa and muscularis externa, together with quantitative histological measurements. To determine the effect of EGF on the proliferation of the colonic epithelia a marker was selected which would allow an estimate of the proliferative status and size of the zone of proliferation to be measured. A range of methods for estimating proliferative status have been applied to the colonic mucosa including the detection of radioactive and synthetic thymidine analogues such as tritiated-thymidine and bromodeoxyuridine, together with metaphase arrest techniques using vincristine sulphate (Butler et al.1988, Sunter et al. 1979, Foster and Whitehead 1990). Instead of detecting the uptake of thymidine analogues, proliferating cells can be labelled by detecting antigens which are produced in a cell-cycle specific manner. One such antigen, proliferating cell nuclear antigen (PCNA) is expressed by cells in S and G1 phases of the cell cycle (Bravo et al. 1987) and using immunohistochemical techniques the proportion of cells undergoing proliferation or having 'proliferative potential' in the crypt can be measured. In this chapter

detection of cells expressing PCNA was used to determine the size and position of the proliferative zone in the colonic crypt.

As well as assessing the responsiveness of the colon to exogenous EGF the stability of EGF in the colonic lumen was also determined. In previous studies (Rao et al. 1991), regional variation in the stability of intraluminally administered EGF has been detected in the upper gastrointestinal tract. Immunoreactive and receptor active EGF was detected in the circulation of suckling rats following infusion of EGF into the jejunal and ileal lumen (Rao et al. 1991) compared to a negligible level of EGF detected in the circulation following infusion into the stomach lumen. Luminal processing of exogenous EGF was also detected with carboxy-modified forms of EGF present in the small intestinal lumen and in the wall of the stomach (Rao et al. 1991). In contrast, a detailed investigation of the uptake and survival of EGF in the colonic lumen has not been previously performed.

Methods

Animals and Experimental Design

Recombinant human EGF

Recombinant human EGF was provided by Chiron Corporation (Emeryville, U.S.A.) and stored as a lypholized powder at -20°C prior to use. For the preparation of EGF solutions of known concentrations, lypholized EGF was weighed and added to a volume of 10mM HCl in 0.9% NaCl (w/v) and the optical density was measured at 280nm on a Shimadzu spectrophotometer (UV-120-02, Kyoto, Japan). The concentration of the solution (mg/ml) was calculated as the optical density (at 280nm) divided by the specific absorptivity of recombinant human EGF (3.021) (George-Nascimento, Chiron Corporation, personal communications). All EGF solutions were steralized by injecting through a 0.22μm filter unit (Millex-GV, Millipore Corporatoin, Bedford,U.S.A.). EGF solutions used to fill osmotic minipumps were prepared on the day of loading the pump, while solutions used for bolus injections were stored at -20°C in sterile tubes prior to use. The biological activity of the solution was indicated by its receptor and antibody binding abilities

such that 80% and 100% of the recombinant human EGF bound to placental membrane and anti-EGF antibody preparations respectively.

Subcutaneously delivered EGF

Male Sprague Dawley rats weighing 190g were housed in individual metabolism cages and fed a high-carbohydrate, powdered diet (Tomas et al. 1984). After a 3-day acclimatisation period in the metabolism cages, rats were randomised into control (n=7), or EGF treatment (n=8) groups. Each rat was anaesthetized with an intraperitoneal injection of 4.2ml/kg body weight of 90mg/ml methohexitone sodium and 60mg/ml pentabarbitone sodium. An osmotic minipump (Alzet model 2001, Alza Corporation Palo Alto, U.S.A.) was inserted into the supra scapular region to deliver recombinant human EGF (Chiron Corp. Emeryville, U.S.A.) at a rate of 200μg/kg body weight / day for 7 days (EGF-treated group), or vehicle (0.9% (w/v) NaCl) in the case of control animals. Prior to pump insertion, a 1ml blood sample was taken from the tail vein of each animal and centrifuged at 2000g for 10 minutes. Plasma was collected and stored frozen at -20°C. Food intake and body weight were measured daily during the 3-day acclimatisation period and the 7-day experimental period.

At the end of the 7-day experimental period, animals were stunned and decapitated. Trunk blood was collected and plasma was separated and frozen as for tail vein blood. A suture was placed in the colonic mesentery adjacent to the middle colic vein before the cecum and colon were excised rapidly with the terminal end of the colon taken as the site adjacent to the pelvic bone. The colon was divided into the proximal region from the caecal-colonic junction to the middle colic vein, and the distal colon from the middle colic vein to the terminal end. All dissections and length measurements were performed on a chilled glass slab to keep the tissues cold and avoid any stretching of the intestine. The lengths of the proximal and distal colon were recorded before both regions were cut open longitudinally. A 1cm section from the proximal end of each region was weighed, placed serosal side down on filter paper and flooded with Methacarn fixative, after which the section was transferred to a beaker of Methacarn for a further 2 hour fixation period. The remaining tissue was

rinsed in ice-cold 0.9%(w/v) NaCl, blotted dry and weighed before the mucosa and submucosa were scraped from the underlying muscularis layer using a glass slide. The combined scraping of mucosa and submucosa is referred to subsequently as mucosa. The mucosal scrapings and muscularis samples were weighed and stored at -70°C in 3ml of 10mM $NaPO_4$ (pH 7.4) or 3ml of 2M NaOH, respectively.

Luminally delivered EGF

Rats were maintained in metabolism cages, with daily measurements of food intake and body weight as described in the subcutaneous EGF trial. Instead of subcutaneous delivery, EGF or vehicle was administered via the colonic lumen over a 7-day experimental period, through a silastic cannula implanted into the lumen of the proximal (Study A) or distal colon (Study B). The cannula was inserted into each animal under the same anaesthetic conditions described in the subcutaneous EGF trial. It was anchored to the colonic wall using a purse string suture and externalised through the supra scapular region. Two 1ml bolus injections of 200μg EGF/ml in 0.9% NaCl were given at 12 hourly intervals, resulting in a total dose of 1600μg EGF/kg body weight/day and control animals received bolus 1ml injections of 0.9% (w/v) NaCl. At the end of the 7-day experimental period, animals were killed for tissue collection. The proximal colon from Study A and the distal colon from Study B was collected and processed as described in the subcutaneous EGF trial.

Analytical measurements

Mucosal scrapings were thawed and homogenized for 20 seconds at 15,000 rpm at 4°C using an Ika Ultra-Turrax (Janke and Kunkel, Germany). An aliquot of the homogenate was digested in a final concentration of 0.5M NaOH at 4°C for 48 hours. Muscularis samples were thawed, homogenized as for mucosa, and an aliquot was diluted to a final concentration of 0.5M NaOH. Protein content of the digests was determined using a modified Lowry method as described by Dulley and Grieve (1975), while the content of DNA was measured using the diphenylamine reaction as described by Burton (1956). Both

methods were adapted for use on microplates, and optical density measurements were made using a Titertek Multiskan microplate reader (Eflab, Finland).

Histological measurements

In the previous chapter, Bouin's fluid was shown to provide adequate preservation of colonic tissue to perform semi-quantitative and quantitative histological measurements. In this chapter a further comparison between fixatives was carried out to determine the optimal conditions for immunohistochemical detection of nuclear PCNA. Three consecutive 1cm segments were dissected from the distal colon of a male Sprague Dawley rat (250g). Each segment was slit open, placed serosal side down on filter paper and flooded with Bouin's, Carnoy's or Methacarn fixative (see Appendix A), then transferred to a beaker of the appropriate fixative for a further 12 hour, 12 hour,or 2 hour fixation period, respectively. 2μm sections of paraffin embedded tissues from each fixation treatment were cut and stained with hematoxylin and eosin or immunohistochemically stained for PCNA (see below). The suitability of each fixative was determined by the quality of preservation of colonic tissue and the level of nuclear preservation. Using the criteria that a distinct brown stained nuclei must be present in PCNA positive cells with a minimal level of brown cytoplasmic staining, a 2 hour fixation using Methacarn fixative was chosen for all subsequent PCNA-staining.

Quantitative morphometry

Transversely orientated, Methacarn-fixed tissues, were embedded in paraffin and 4-5μm sections were taken at 3 tissue levels at 200μm intervals. Sections were stained with hematoxylin and eosin and viewed using a Zeiss Jenaval light microscope (Jena, Germany). Quantitative morphometric measurements were made from images acquired with a JVC colour video camera and digitized by a PRISM Image Analysis software system (Dapple Systems Inc, Sunnyvale, U.S.A.) coupled to an Apple Macintosh II cx computer. Histological measurements made included; the tissue circumference measured along the submucosa-muscularis externa interface, mucosal thickness from the luminal surface to the base of the muscularis mucosae, muscularis thickness was measured as the thickness of the muscularis externa layers, the total number of cells per crypt column and the PCNA labelling

index and PCNA labelled crypt fraction. Area measurements were derived from the product of the layer thickness and the colonic circumference.

Immunohistochemical localization of PCNA

Nuclear PCNA staining was performed using a modified method of Eldridge et al.(1990). Methacarn-fixed sections were deparaffinized in xylene, re-hydrated in graded alcohols and then incubated with anti-PCNA monoclonal antibody (IgM clone 19A2) (Path /Mark Coulter Clone, Coulter Immunology, Hialeah U.S.A.) for 90 minutes at a final dilution of 1/300. The antibody was diluted in phosphate-buffered saline (pH 7.4) (Sigma Chemical Co., St. Louis, U.S.A.) containing 1% Tween (w/v), 1% (w/v) bovine serum albumin and normal horse serum (1.32%w/v). To amplify the antibody binding, slides were incubated with biotinylated horse anti-mouse IgM serum for 60 min (Vector laboratories, Burlingame U.S.A.) at a 1/200 dilution, followed by a 30 min incubation with streptavidin-peroxidase conjugate (Biogenex, San Ramon, Cal. USA) diluted 1/10 in phosphate-buffered saline (pH 7.4). Visualization of the bound peroxidase was achieved using the diaminobenzidine-H_2O_2 reaction. All incubations were performed at room temperature and slides were rinsed between each incubation with phosphate-buffered saline (pH 7.4) containing 1% Tween (w/v). After completion of PCNA staining, sections were counterstained with hematoxylin (12.5%, w/v) for 1 minute.

Measurement of PCNA labelling index and the PCNA labelled crypt fraction

To determine the frequency of PCNA-labelling, all epithelial cells were designated as PCNA-positive or negative on the basis of specific brown nuclear immunostaining, and the 'labelling index' was calculated for each colon sample as the percentage of crypt cells labelled with PCNA. The distribution of PCNA-labelling was analysed by calculating the 'PCNA-labelled crypt fraction'. For this purpose, each cell in a crypt column was designated a position number from the base to the luminal surface, with position one at the crypt base (Figure 3.1). For each position in the the crypt, the number of PCNA-positive cells was recorded, and expressed as percentage of the total number of cells at that position. This percentage was plotted against cell position generating a PCNA labelling distribution

curve (Figure 3.2), and the 'PCNA-labelled crypt fraction' was calculated for each colon sample as the cell position number at which half-maximal PCNA-labelling occurred, divided by the number of cells per crypt column.

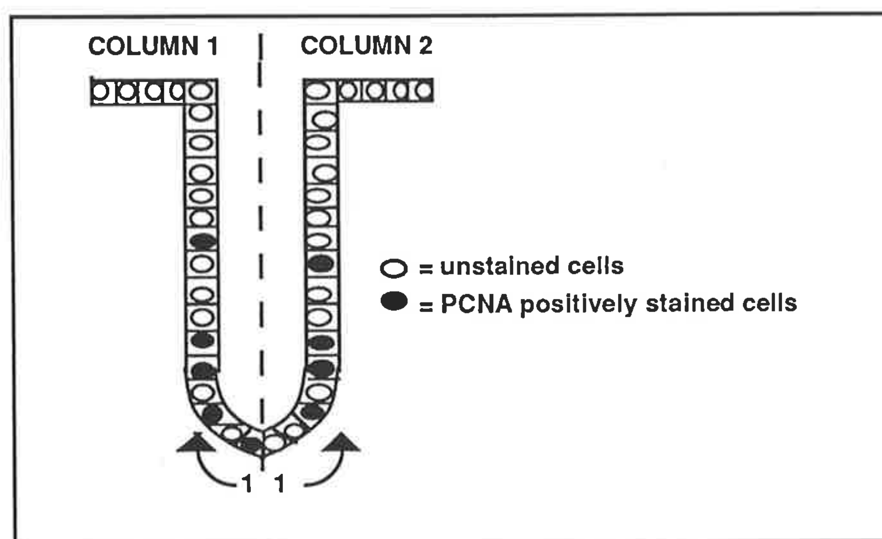


Figure 3.1 Diagrammatic representation of a colonic crypt showing PCNA positively labelled cell nuclei. Cell counts were made per crypt column with cell position 1 at the base of the cell column.

To determine the optimal number of crypt cell columns to count for measurement of the total cells per column, PCNA labelling index and PCNA labelled crypt fraction, the progressive mean and coefficient of variation (standard error as a percentage of the mean) of these parameters were calculated for 5-30 crypt columns (Table 3.1). Measurements were made only in crypts when the entire crypt was intact from the base at the muscularis mucosae to the luminal surface. Between 25 and 30 crypt column counts, the mean value of each parameter was unchanged and the coefficient of variation was steady (Table 3.1). Therefore 30 well-orientated crypt columns were routinely counted in measurement of the total cells per column and the PCNA labelling index and labelled crypt fraction.

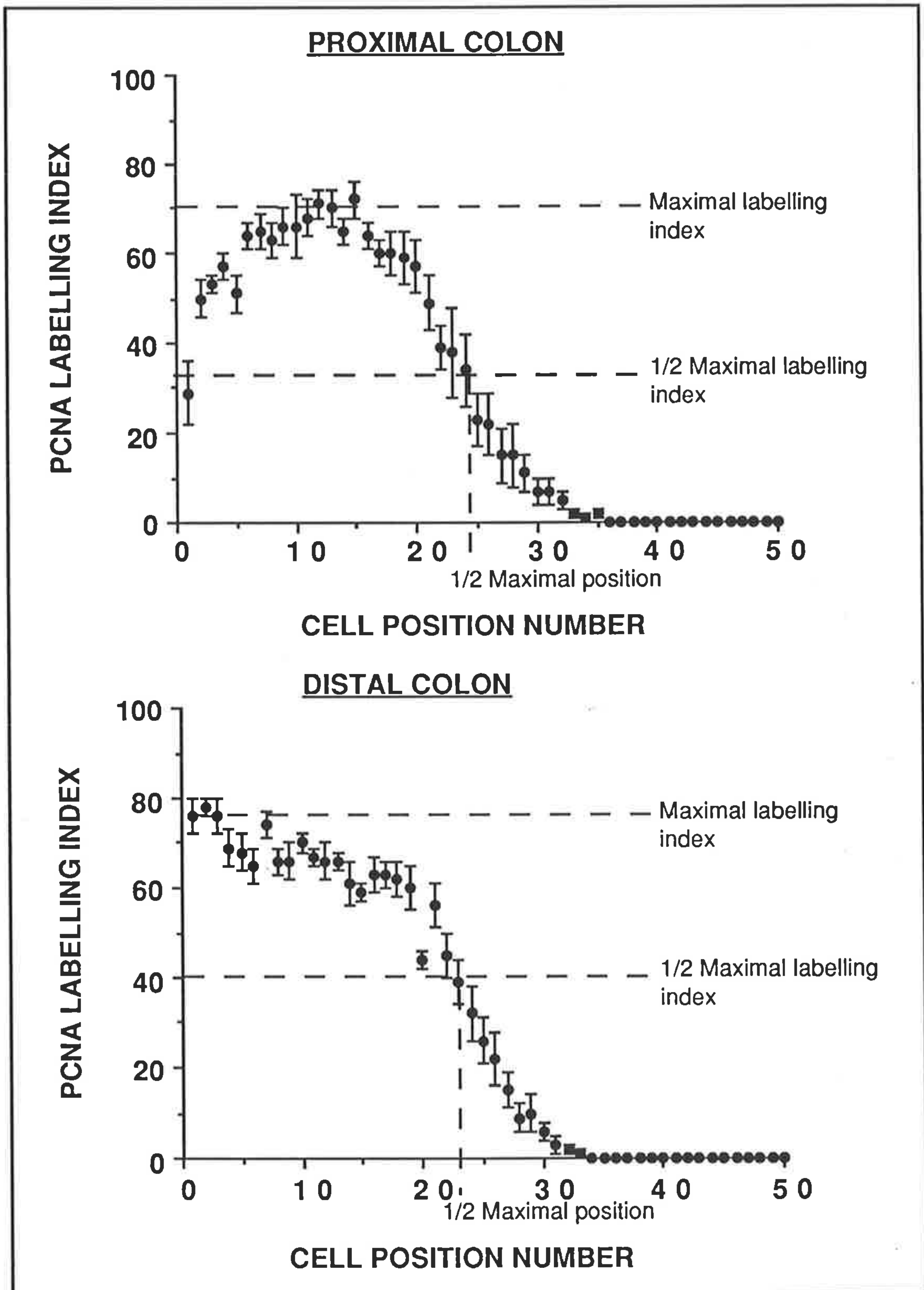


Figure 3.2 Labelling index distribution curves from the proximal and distal colon of the normal rat. Data are expressed as mean±SEM from vehicle treated animals (n=7).

Table 3.1. Validation of PCNA labelling indices

No. of crypt columns	Total cells/ column		Labelling index (%)		Labelled fraction (%)
	Mean	Co-efficient of Variation (%)	Mean	Co-efficient of Variation (%)	
5	54.6	3.3	45.8	6.5	56
10	55.3	4.5	46.2	7.1	58
15	54.3	6.1	45.7	9.7	65
20	53.2	8.1	46.9	9.7	64
25	52.3	8.3	46.9	9.3	63
30	52.3	8.9	46.4	9.5	63

Stability of EGF in the colonic lumen

The rate of degradation of radiolabelled EGF in the proximal and distal colon was measured *in vivo* to determine the stability of luminally delivered EGF. Male Sprague Dawley rats (250g) were anaesthetised with 8ml/kg body weight of urethane (180g/l) and body temperature was maintained by placing animals onto a heated blanket. The colon was exposed through a mid-line incision and 3-0 silk sutures were placed around the colon at the point of the cecal/colonic junction, the middle colic vein and at the position adjacent to the pelvic bone, thereby isolating the proximal and distal colon.

Iodinated recombinant human EGF was prepared according to the method described by Read et al. (1986) was provided by Mr C. Gillespie (Child Health Research Institute, North Adelaide). A 1ml bolus of 0.9%(w/v) NaCl containing 200μg EGF and ^{125}I-labelled EGF (aproximately 50ng, 200,000 cpm) was injected through a 27g needle into the proximal end of each loop. A thermometer was inserted into the abdomen to monitor the temperature of the intestinal preparation, and the abdominal incision was partially closed using wound clips.

Rats were maintained under anaesthesia for one hour (n=4) or four hours (n=4) before the colonic loops were excised, and the animal was killed under anaesthesia by cutting the diaphragm. The distal end of each loop was slit open and luminal contents were flushed out with 5ml of 10mM HCl in 0.9% NaCl. The luminal flushings were centrifuged for 10 mins at 2000g and the supernatant was collected. The mean radioactivity in the luminal flushings was calculated by counting 3 aliquots of the supernatant using an LKB 1261 Multi G gamma counter (Turku, Finland). Each loop of tissue was minced and then homogenized for 30 secs at 15,000 rpm at 4°C using an Ika Ultra-turrax (Janke and Kunkel, Staufen, Germany) and the mean tissue radioactivity was calculated by counting 3 aliquots of the homogenate. The percentage recovery of radioactivity in the colonic lumen and tissue was calculated as the total radioactivity recovered in the luminal flushings or intestinal homogenate, divided by the total radioactivity infused into the colonic loop.

The proportion of radioactivity in the luminal flushings and tissue representing intact EGF was estimated by three methods, trichloroacetic acid precipitability, immuno-binding and receptor-binding activity. Trichloroacetic acid-precipitability of samples was determined by adding 250μl of 25% (w/v) trichloroacetic acid and 200μl of a 4mg/ml solution of bovine serum albumin to a 100μl volume of luminal flushing, tissue homogenate or infusate. After a 30 min incubation at 4°C, precipitates were centrifuged for 20 min at 4800g. The percentage of trichloroacetic acid-precipitable radioactivity was calculated as the radioactivity in the pellet divided by the total radioactivity in the sample aliquot.

To measure immuno-binding, approximately 5000cpm of luminal flushing, tissue homogenate or infusate was incubated overnight at 4°C with rabbit anti-human EGF polyclonal antiserum at a final dilution of 1:40000 in a total volume of 200µl. Antibody-bound radioactivity was precipitated by adding anti-rabbit IgG polyclonal antibody (a final dilution of 1:100) in the presence of a 1:40 final dilution of normal rabbit serum. These concentrations of primary and secondary antibodies were shown in preliminary experiments to precipitate approximately 80% of the ^{125}I-labelled EGF-infusate, the maximum that could be achieved.

Receptor-binding activity was determined using human placental membranes, prepared using the method of Nexo et al. (1981). Approximately 5000cpm of luminal flushings, tissue homogenate or infusate were added to 154µg of placental membrane protein in a total volume of 300µl of 50mM Tris/HCl (pH 7.4) containing 2.5mg/ml bovine serum albumin. After a 60 minute incubation at room temperature, samples were centrifuged for 30min at 4800g at 4°C and the percentage of radioactivity bound to receptors was calculated as radioactivity in the pellet divided by the total radioactivity in the sample aliquot. The concentration of placental membranes was determined in preliminary experiments to bind the maximal amount of radiolabelled EGF-infusate (80%).

Measurement of EGF in urine and plasma

Immunoreactive EGF was measured in plasma and urine collected at the commencement and completion of the experimental period. Radioimmunoassays were performed using a modified method of Read et al. (1986). Briefly, 50µl of the sample or EGF standards (0.05 to 50ng) were incubated in triplicate overnight at 4°C in a total volume of 300µl containing rabbit anti human EGF polyclonal antiserum at a final dilution of 1:300 000, and ^{125}I-labelled EGF(0.67ng, 5000 cpm), such that 35-45% of the added tracer bound to the antibody in the absence of added sample or competing EGF. Antibody-bound radioactivity was precipitated using sheep anti-rabbit IgG polyclonal antibody, at a final dilution of 1:100, and a 1:40 dilution of normal rabbit serum. The lowest detection limit of this radioimmunoassay was 0.5ng in the 300µl incubation volume.

Statistical Analysis

Data are expressed as mean ± SEM, unless otherwise stated. Statistical comparisons were made between groups using Student's unpaired-t tests.

Acknowledgments

The experimental design, all animal surgery, sample collections, histological measurements, stability studies and EGF radioimmuno-assays were performed by the candidate. The calculation of the PCNA labelling index and labelled crypt fraction were performed by the candidate, although immunohistochemical staining and counting of positively labelled cells was performed by Mr. G. Howarth. Iodonation of recombinant human EGF and EGF radioreceptor assays were performed by Mr. C. Gillespie, with the human placental membranes used in EGF radioreceptor assays prepared by Mr. G. Howarth. Cutting of paraffin-embedded histological specimens and hematoxylin and eosin staining was performed by Mrs K. Davey.

Results

The effect of subcutaneously delivered EGF

Following 7 days constant subcutaneous infusion with 200μg EGF/kg/day the concentration of EGF in the blood remained below 1ng/ml, the detection limit of the radioimmunoassay, although a significant increase in urinary EGF concentration from below 1ng/ml prior to EGF infusion to 4.46±0.64 ng/ml at the end 7 days.EGF infusion. This suggests that the infused material was cleared rapidly from the circulation.

Body weight gain was similar in the vehicle (47.9±2.0g) and EGF-treated groups (43.9±2.5g) during the 7-day treatment period, despite a significantly lower average feed intake in the EGF treated animals (75±2g/kg body weight/day) compared to vehicle treated control animals (84±2g/kg body weight/day), suggesting that food utilization in the EGF-treated group was more efficient than in vehicle-treated controls. The total wet weight of the colon was significantly greater in the EGF-treated group (1.34g±0.1g) compared with vehicle-treated controls (0.93g±0.04g) ($P<0.001$). The increase in colonic weight was not due to a lengthening of the gut, since EGF-treated and control animals had similar colonic length (14.3±0.3cm in EGF-treated vs 13.7±0.4cm in control animals). Rather, the wet weight per cm significantly increased from 76.8±1.8mg/cm in control animals to 106.7±5.4mg/cm ($P<0.001$) in the EGF-treated group, indicating a greater cross sectional mass.

The mechanism responsible for the increased cross-sectional mass in the colon of EGF-treated rats was examined further by determining the wet weight, protein and DNA content of the mucosa and muscularis externa layers as well as histological changes. In the proximal colon, EGF treatment induced a 52% increase in the wet weight of the combined mucosa and submucosa layers (referred to as mucosa) compared with controls (Figure 3.3). The protein content per cm of mucosa was 64% higher (Figure 3.3), with no change in DNA content per unit length. Accordingly, the protein / DNA ratio increased from 68.5±9.1g/g in the control group to 104.7±6.9g/g in the EGF treated group ($P<0.01$). Similar trends were apparent in the mucosa of the distal colon with a comparable increase in the protein content,

although the wet weight per cm was not significantly elevated, and unlike the proximal colonic mucosa, no significant increase in the protein/DNA ratio was observed.

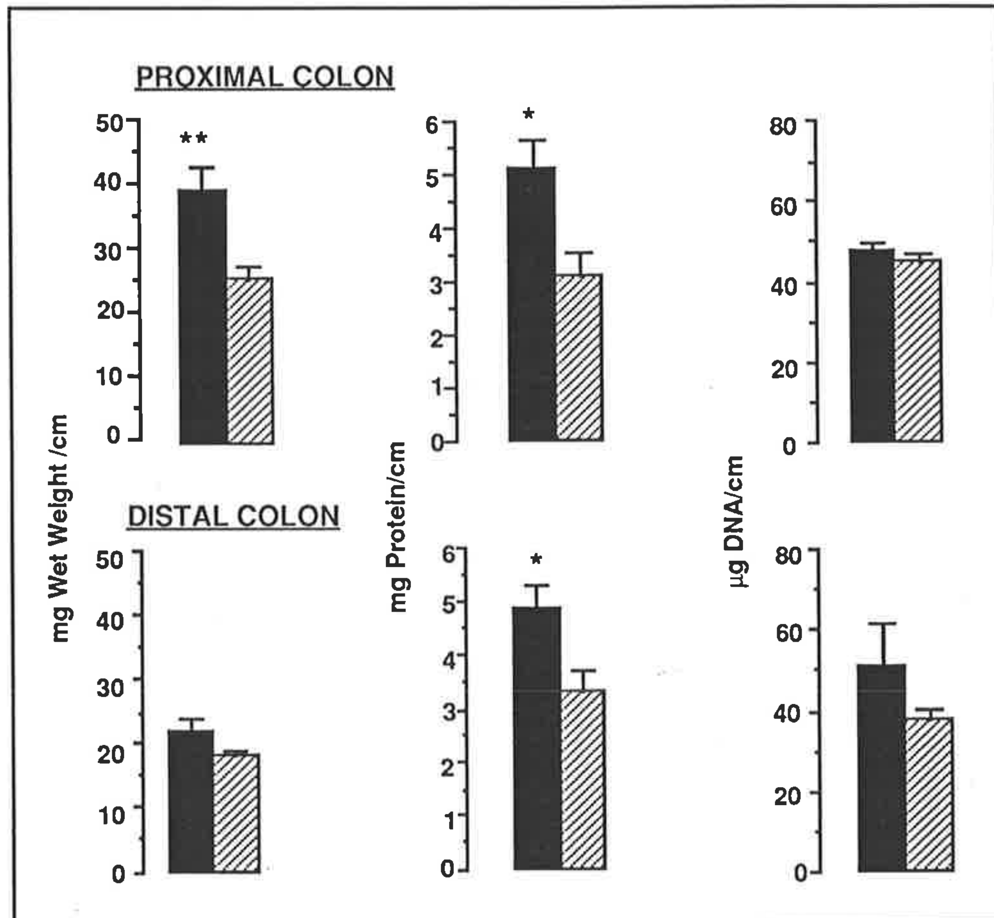


Figure 3.3 Mucosal wet weight, protein and DNA content per cm of colon from normal rats treated subcutaneously with EGF (solid bars) or vehicle (hatched bars). Data are shown for the proximal colon and distal colon, and are expressed as mean ± SEM of EGF (n=8) and vehicle (n=7) treated animals. Significance levels shown are for comparisons between the two groups such that * $P \leq 0.05$, ** $P \leq 0.01$, ***$P \leq 0.001$.

The muscularis layer was also affected by subcutaneous infusion of EGF. In the proximal colon, EGF increased muscularis wet weight and protein content per cm by 31% and 63% respectively above the control values (Figure 3.4), with no significant change in the DNA content per cm and the protein/DNA ratio was unchanged between the control (39.5±3.8(g/g)) and EGF-treated group (37.7±1.9(g/g)). However, in the distal colonic muscularis layer, no statistical difference in wet weight, protein or DNA contents was observed between the control and EGF treatment groups.

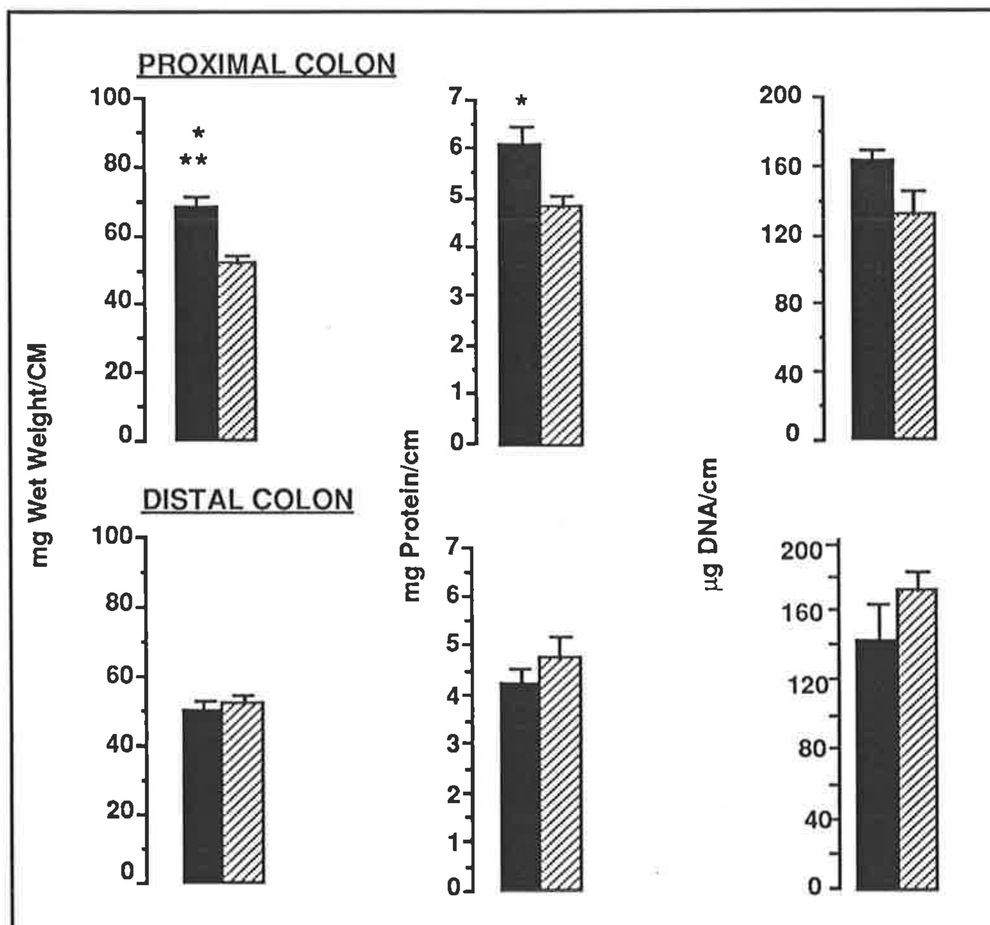


Figure 3.4 Muscularis wet weight, protein and DNA content per cm of colon from normal rats treated subcutaneously with EGF (solid bars) or vehicle (hatched bars). Data are shown for the proximal colon and distal colon, as described in Figure 3.3.

Because EGF had comparable effects on the wet weight of the mucosal and muscularis layers of the proximal colon, the mucosal weight expressed as a percentage of the total colonic weight (mucosal + muscularis weights) was similar in control (32.5±2.1%) and EGF-treated rats (35.5 ±2.0%). In the distal colon, however, the EGF response was more pronounced in the mucosal layer, so a significantly higher ratio was observed in EGF-treated rats (29.9±1.3%) compared with controls (25.8±0.7%) ($P<0.01$).

Histological analysis of the colonic mucosa supported the biochemical evidence that subcutaneous administration of EGF induced mucosal growth, particularly in the proximal colon. The circumference, mucosal thickness and cross-sectional area of mucosa of the proximal colon were significantly elevated by 27%, 28% and 69% respectively in the EGF group above that of vehicle-treated controls (Table 3.2). Less pronounced responses were

apparent in the distal region, where EGF-treatment induced an 18% increase in the thickness of the mucosal layer with no significant changes in the colon circumference or the mucosal cross-sectional area. By histological analysis, the muscularis layers appeared less responsive to EGF than the mucosa. EGF treatment resulted in a 16% reduction in the thickness of the muscularis externa, although cross-sectional area was unchanged as a result of the greater intestinal circumference in EGF-treated rats. There was no effect of EGF on muscularis externa thickness or cross-sectional area in the distal colon (Table 3.2).

In association with an increase in the mucosal thickness the mean number of cells per crypt column of EGF-treated rats increased by 35% ($P< 0.001$) in the proximal colon and 20% ($P<0.01$) in the distal colon above that of the control group (Table 3.3). This suggests that EGF increases proliferation of the colonic epithelia which in turn leads to a lengthening of the crypt and an increased mucosal width.

The PCNA labelling index and labelled crypt fraction were measured to determine if EGF treatment modified either the total number of crypt cells capable of cell division or their position in the crypt. The proliferative profile varied considerably between the proximal and distal colon. The shape of the labelling index distribution curves differed between the two regions (Figure 3.2). In the distal colon, maximal PCNA labelling was achieved within the first 5 cell positions and a high labelling index was maintained in the first half of the crypt after which there was a rapid decline in PCNA labelling. In contrast in the proximal colon, basally located cells had a low PCNA labelling index and the maximal PCNA labelling index occurred between cell positions 10 and 20 after which PCNA labelling was rapidly reduced. The overall PCNA labelling index and PCNA labelled crypt fraction was slightly higher in the distal colon compared to the proximal region (Table 3.3). Despite the fact that EGF treatment increased the total number of cells per crypt, this was not associated with a change in the PCNA labelling index or in the region of the crypt occupied by positively labelled PCNA cells (Table 3.3). These results provide evidence that subcutaneous EGF induces colonic crypt hyperplasia while maintaining a normal proliferative profile.

Table 3.2. Quantitative histological measurements of the mucosa and muscularis externa layers of the colon of normal rats.

	Proximal Colon		Distal Colon	
	Vehicle	EGF	Vehicle	EGF
Circumference (mm)	6.7 ± 0.2	8.5 ± 0.5**	8.9 ± 1.0	8.8 ± 0.8
Mucosal thickness (μm)	250 ± 20	320 ± 20**	280 ± 20	330 ± 10**
Mucosal area (mm^2)	1.6 ± 0.1	2.7 ± 0.2***	2.4 ± 0.1	2.9 ± 0.2
Muscularis thickness (μm)	225 ± 12	188 ± 8*	160 ± 10	180 ± 10
Muscularis area (mm^2)	1.5 ± 0.1	1.6 ± 0.1	1.4 ± 0.2	1.6 ± 0.1

Data are expressed as mean ± SEM of vehicle (n=7) and EGF (n=8) derived from measurements taken at 3 tissue levels. Area measurements were calculated as the product of the circumference and the thickness of the mucosal or muscularis layer. Significance levels were determined for comparisons made between the vehicle and the EGF group using student's t tests.
*** $P \leq 0.001$,** $P \leq 0.01$,* $P \leq 0.05$,

Table 3.3. Crypt cell counts and PCNA- labelling indices of proximal and distal colon of normal rats treated with subcutaneous EGF or vehicle

	Proximal Colon		Distal Colon	
	Vehicle	EGF	Vehicle	EGF
Total cells / crypt column	37 ± 1.4	50 ± 2.3***	35 ± 0.6	42 ± 1.6**
Labelling index (%)	41 ± 0.5	41 ± 0.8	45 ± 1.0	48 ± 0.7
PCNA Labelled crypt fraction (%)	60 ± 1.6	60 ± 1.0	64 ± 1.0	64 ± 0.7

Data are expressed as mean ± SEM of vehicle (n=7) and EGF (n=8) treated groups. Data are derived from measurements taken from 30 crypt cell columns. Significance levels are the same as those used in Table 3.2. Labelling index (%) is defined as the number of PCNA labelled cells per total cells counted and the PCNA labelled crypt fraction (%) is defined as the proportion of the crypt occupied by PCNA labelled cells.

Stability of EGF in the colonic lumen

Prior to determining the effect of chronic luminal infusion of EGF on colonic growth, the stability of EGF in the lumen was assessed. In preliminary experiments TCA-precipitability, EGF immunoreactivity and EGF receptor binding activity were compared as measures of the proportion of intact radiolabelled EGF in luminal flushings from 3 animals collected one hour after the infusate was injected into the colonic lumen. Results from TCA-precipitability indicated that the luminal flushings contained 84.2±9.4% (mean±SEM) intact material, which was similar to estimates from immunoreactivity (80.2±11.3%) or receptor binding (85.7±10.6%) measurements. As the results obtained from TCA-precipitability did not vary from those derived by the other methods, TCA-precipitability alone was used to determine the time-course of degradation of ^{125}I-labelled EGF in the colonic lumen. In preliminary experiments the three methods for measuring the intactness of radiolabelled EGF were also compared in colonic tissue homogenate samples. However, due to interference of

tissue homogenates with EGF receptor binding and antibody binding these methods were considered inappropriate for estimating the intactness of radiolabelled EGF in colonic tissue.

The stability of EGF in the colonic lumen was assessed by determining the rate of disappearance of ^{125}I-labelled EGF injected into the colonic lumen as well as the TCA-precipitability of ^{125}I-labelled EGF in luminal flushings. The total recovery of the infused material was estimated as the sum of the radioactivity recovered in the luminal wash and the colonic tissue, expressed as a percentage of the radioactivity infused. At one hour, over 84% of the infused radioactivity could be recovered from the combined luminal flushing and colonic tissue, in either the proximal or distal infusion studies. Even at 4 hours, greater than 70% of the infused radioactivity was recovered from the lumen and colonic tissue combined (Table 3.4), indicating that EGF was not absorbed rapidly from the colon into the general circulation. At 1 hour, most of the radioactivity remained in the lumen (80.7% in the proximal colon, 88.7% in the distal colon), with less than 10% transferred to the colonic tissue (Table 3.4). After 4 hours the recovery from the lumen was markedly reduced in the proximal colon, and a slight reduction in radioactivity was seen the distal colonic lumen. Conversely, a significant elevation in the amount of radioactivity was observed in the proximal colonic tissue after 4 hours (Table 3.4), suggesting that an increase in the uptake of ^{125}I-labelled EGF from the lumen into the tissue had occurred in the proximal colon after 4 hours.

The intactness of ^{125}I-labelled EGF recovered from the colonic tissue or lumen was estimated to be greater than 84% by TCA-precipitability at either 1 or 4 hours in both regions of the colon. Taken together with the fact that over 70% of the infused radioactivity remained in the lumen or colonic tissue 4 hours after infusion, this suggests that EGF was not degraded rapidly in the colonic lumen of the proximal or distal colon.

Table 3.4. Stability of EGF in the colonic lumen

	Proximal Colon		Distal Colon	
Time of exposure (hrs)	1	4	1	4
% infused radioactivity recovered in the luminal wash	80.7 ± 1.7	18.3 ± 9.3	88.7 ± 2.4	63.7 ± 6.0
% infused radioactivity recovered in the colonic tissue	3.8 ± 0.9	75.8 ± 9.7	9.9 ± 2.7	7.2 ± 0.7
% total recovery	84.5	94.1	98.6	70.9
% TCA precipitable radioactivity in luminal wash	93.3 ± 6.7	85.3 ± 11.2	84.7 ± 10.6	96.4 ± 2.4
% TCA precipitable radioactivity in colonic tissue	92.9 ± 2.3	86.9 ± 11.6	84.3 ± 3.8	90.5 ± 4.1

Data are expressed as the mean ± SEM of 4 animals in each group.
The recovery of infusate in the luminal wash and colonic tissue at the end of the exposure period is expressed as a percentage of the counts in the infusate. The percentage of TCA precipitable material was calculated as the cpm in the pellet expressed as a percentage of the total cpm in the sample aliquot (see text for methodology details).

The effects of luminally delivered EGF on normal rats

Body weight gain did not significantly differ between the vehicle-treated control group and animals administered intra-luminal EGF via the proximal colon (36±7g vs 50±7g) or the distal colon (33±5g vs 26±3g). Similarly, comparable feed intakes were observed between control groups and treatment groups in animals receiving bolus injections into the proximal colon (71±3 vs 70±2g/kg body weight/day) and the distal colon (66±2 vs 70±1g/kg body weight/day). EGF administered into the lumen of the proximal colon (Study A, Table 3.5) had no effect on wet tissue weight, protein or DNA content of either the mucosa or muscularis layer. Similarly, luminal EGF had no effect on these parameters when infused into the distal colon (Study B, Table 3.5). Thus in contrast to the marked effects of subcutaneous EGF, on the protein, DNA and wet weight of the mucosal and muscularis layers of the colon luminally administered EGF was without effect. Only biochemical analyses were used in this study because of the strong correlation between biochemical and histological responses seen in the subcutaneous trial.

Table 3.5. The effect of luminally delivered EGF on the proximal and distal colon of normal rats

A. Proximal colonic administration study

	Proximal colonic Mucosa		Proximal colonic Muscle	
	Vehicle	EGF	Vehicle	EGF
Wet weight (mg/cm)	31.6 ± 2.5	29.5 ± 2.4	56.8 ± 4.0	50.1 ± 2.6
Protein (mg/cm)	3.10 ± 0.1	3.10 ± 0.2	7.58 ± 0.7	6.86 ± 0.5
DNA (μg/cm)	58.5 ± 5.1	49.6 ± 2.5	152 ± 8.7	111±13.6

B. Distal colonic administration study

	Distal colonic Mucosa		Distal colonic Muscle	
	Vehicle	EGF	Vehicle	EGF
Wet weight (mg/cm)	22.3 ± 2.6	22.3 ± 3.7	68.4 ± 6.7	62.6 ± 4.6
Protein (mg/cm)	2.64 ± 0.4	2.45 ± 0.5	6.81 ± 0.8	5.8 ± 0.9
DNA (μg/cm)	58.6± 6.0	54.5 ± 13.7	285.1 ± 46.3	282.7 ± 13.8

Data are expressed as mean ± SEM of the proximal colon delivered vehicle (n=5) or EGF (n=4) and in the distal colon groups (n=5) . No significant difference ($P \leq 0.05$) was observed between the EGF and vehicle treated groups in either region of the colon.

Discussion

In this study biochemical and histological evidence suggests that 7 days of subcutaneous EGF infusion increases growth of the colon. Thus EGF induced a 44% increase in total colonic weight due mainly to a greater circumference and cross-sectional area rather than a lengthening of the colon. The proximal colon was the most responsive region, with proportional increases in the wet weight of the mucosal and muscularis layers. Increased protein content but not DNA content in the mucosa of both the proximal and distal colon suggested a hypertrophy of the mucosa and histological evidence was obtained for an increased number of cells per crypt as well as an increased mucosal thickness. Taken together these data suggest that EGF induced epithelial cell hyperplasia together with cellular hypertrophy. The histological analysis performed in this study was restricted to estimating the number of cells per crypt by measuring the cell number per crypt column, although it must be remembered that the colonic crypt is a three dimensional structure and the total number of crypt cells is also contributed to by the cells per crypt width. The effect of EGF on the crypt length and diameter has been measured in the hypoplastic small intestine and colon of rats receiving parenteral nutrition (Goodlad et al. 1992). In the small intestine, EGF induced crypt hyperplasia by increasing both the cell number per crypt column and the number of cells across the crypt diameter, whilst in the colon, EGF-induced hyperplasia was only associated with an increase in the number of cells per crypt column.

Despite the fact that subcutaneous EGF treatment increased the number of cells per crypt column of both the proximal and distal colon, no effect of EGF on either the total proportion of PCNA labelled cells or their position in the crypt was observed. One possible explanation of this observation is that EGF is not mitogenic in the colon. If this is the case, the increased number of cells per crypt column could be attributed to a reduced rate of cells being sloughed off the luminal surface of the crypt or due to a slower cell migration in the crypt column. No evidence of a reduction in epithelial cell migration rate induced by EGF has been previously reported. On the contrary, the increased rate of formation of a single layer of epithelium across the denuded acetic acid damaged gastric and duodenal mucosa

following EGF treatment (Skov Olsen et al.1986b), suggests that EGF enhances epithelial migration.

A second possible explanation of the observed response to subcutaneous EGF is that EGF is mitogenic to the colon although PCNA labelling did not detect the changes in colonocyte proliferation. Other studies have shown that growth of the colonic mucosa induced by EGF is associated with a significant increase in tritiated thymidine incorporation into the DNA suggesting that EGF increases the mitotic cell pool (Arsenault and Menard 1987). Detection of thymidine analogue incorporation into the DNA enables cells exclusively in S phase to be located and allows an estimate of the rate of crypt cell production to be made, whereas PCNA is produced by cells in S and G1 phases of the cell cycle and immunohistochemical detection of cells expressing the antigen tags cells which are proliferating or have 'proliferative potential' at a single time point. Hence, any changes in the rate of S phase induced by EGF may not be detected by PCNA labelling.

Although PCNA labels a higher proportion of crypt cells than tritiated thymidine labelling techniques (Yamada et al. 1992), the PCNA labelling distribution was similar to that previously reported using tritiated thymidine (Sunter et al. 1979, Wright and Alison 1984). While other studies have shown that EGF increases the number of cells in metaphase and S phase in the colonic mucosa (Goodlad et al. 1992), no positional analysis of these cells within the crypt has been reported. The proliferative zone of the colonic mucosa normally occupies the lower three quarters of the crypt and any change in this zone could indicate an induction of abnormal growth. For example, an upward shift of this zone, with an increased number of cells in S phase at the luminal surface, has been observed in pre-cancerous tissue adjacent to colonic adenomas (Sizeland et al. 1991). Thus it appears that EGF did not induce an abnormal proliferative zone in the colon.

In this study, the low PCNA labelling index detected in the basally located cells of the proximal colon may correspond to a population of non-proliferating cells or cells with a slower cell cycle compared to the rest of the crypt (Sunter et al. 1979). It is possible that the

hyperplastic effect of subcutaneous EGF treatment resulted in the non-proliferating cells being replaced by proliferating cells and hence the position of the PCNA labelled crypt fraction would remain unchanged, however if this was the case an increased PCNA labelling index would have been detected. As maximal PCNA labelling was observed between positions 10 and 20, a hyperplasia of the proximal colon may induce a downward migration of cells, thereby potentially resulting in an underestimate of the size of PCNA labelled crypt fraction (Yamada et al. 1992). In contrast, in the distal colon the maximal PCNA labelling was observed in the basally located cells, and other studies have shown that all cells in the lower two thirds of the distal colon are undergoing proliferation (Wright and Alison 1984).

The increased number of cells per crypt column in the absence of an accompanying increase in PCNA labelling at day 7 of treatment, may have also occurred if EGF was mitogenic only during the early treatment period. This explanation seems unlikely, due to the reported acute and long term mitogenic effects of EGF treatment detected in parenterally fed animals. Intravenous EGF increased colonic proliferation, as indicated by an increased number of arrested metaphase figures, within 18 hours after the initiation of treatment (Goodlad et al. 1987). In the same model of colonic atrophy, an increased number of arrested metaphase figures was also detected 10 days following the commencement of EGF infusion (Goodlad et al 1987), and after 8 days a significant increase in tritiated thymidine incorporation compared to saline treated control animals was observed (Goodlad et al. 1992). Responses to EGF are also detected in acute and chronic ulceration models in the upper gastrointestinal tract so that EGF treatment reduced the area of aspirin-induced gastric lesions three hours after the commencement of treatment (Konturek and Brzozowski et al. 1981), and in acetic acid-induced gastric and duodenal ulcerations a reduction in the size of the induced lesions was detected after 7 days of EGF treatment (Konturek and Brzozowski et al. 1990).

As the mucosal scraping sample is comprised of cells from the colonic crypts, lamina propria, muscularis mucosae and submucosa the hypertrophy observed may be contributed to by cells from one or all of these layers. It is also possible that the increased wet weight

and protein content may be contributed to by non-cellular material and reflect an edemic response. All of the published studies investigating the mitogenic effect of EGF on the colon have restricted their observations to the responsiveness of the colonic mucosa, whereas in the current study we have observed that EGF can elicit enlargement of the colonic muscularis layers as well as the mucosa. Biochemical analysis indicated hypertrophy and/or edema of the muscularis layers, but this was not reflected by an increased cross sectional area of the muscularis externa.

This study did not address the question of whether colonic growth induced by EGF occurred via a direct or indirect mechanism. However, EGF receptors have been identified in normal colo-rectal mucosa (Menard et al. 1987) suggesting that the growth response could have been activated by a direct receptor-mediated mechanism. Exogenously-delivered EGF also has the potential to mediate the release of other factors which may in turn induce mitogenesis. In cultured keratinocytes and a gastric carcinoma cell line, EGF added to the culture medium enhanced TGF-α mRNA expression (Coffey et al. 1987, Yoshida et al. 1990). Hence, the increased crypt cell number observed in the current study following treatment with exogenous EGF may be mediated by TGF-α-induced mitogenesis. Indeed, TGF-α has been detected in the normal human gastrointestinal tract with the high levels present in colonic tissue extracts (Cartliage and Elder 1989), suggesting that it may be an endogenous growth factor involved in the growth and maintainance of the colon.

The ability of luminally applied EGF to induce colonic growth was assessed to determine if the responsiveness of the colon to EGF is influenced by the route of growth factor administration. Luminal EGF induced none of the biochemical changes seen in response to subcutaneous EGF. Instability in the luminal environment is unlikely to account for the result in view of the demonstration that ^{125}I-labelled EGF remained essentially intact in the colonic lumen for at least four hours, a time-frame in which EGF has been shown to initiate DNA synthesis of cultured epithelial cells (Mullin et al. 1988). Alternative explanations for the lack of effect of intraluminal EGF could be that rapid washout of EGF from the colonic lumen reduced the luminal concentration below the effective level, or that

receptors were not accessible to intraluminal EGF. While receptors for EGF have been identified in the adult colonic epithelium (Menard et al. 1987, Koenders et al. 1992), their localisation on the epithelial cell has not been determined. Moreover, the effect of the mucin barrier on access of luminal EGF to the colonic epithelium *in vivo* is unknown. However, these explanations are not consistent with the report of Reeves et al. (1991) that daily rectal enemas of EGF for 168 days, at a dose more than 10000-fold lower than that used in the current study, was able to significantly elevate crypt cell production rates in the distal colon. These findings suggest that long term infusion of a low dose of EGF may be more effective in inducing colonic growth than a short term administration of a high EGF dose, although the mechanism which enables an increased potency of EGF by this treatment regime remains unclear.

In conclusion, these studies suggest that the normal colon is responsive to exogenous EGF and that the systemic route appears considerably more effective in eliciting a mitogenic response than intraluminal EGF administration. However, studies performed in the upper gastrointestinal tract suggest that this result does not necessarily infer that luminal EGF will be ineffective in the healing of the ulcerated colon. For example, while luminal EGF does not enhance cell growth in the intact small intestine (Goodlad et al 1987) it does accelerate gastric and duodenal ulcer healing (Konturek et al.1990). Factors such as changes in the luminal mucin layer, EGF receptor distribution and stability of EGF in the colonic lumen may alter the responsiveness of the ulcerated colonic mucosa to EGF.

CHAPTER 4. DEVELOPMENT AND VALIDATION OF AN ANIMAL MODEL OF COLITIS

Abstract

The appropriateness of two experimental colitis models, induced by oral carrageenan and acetic acid, were evaluated for their use in assessing the colonic ulcer-healing properties of EGF. The optimal type and dose of oral carrageenan required to induce a colitis-like condition in adult rats, together with a comparison of the severity and distribution of colonic lesions induced by serosal and intraluminal acetic acid administration was made. The severity of the induced colonic damage was assessed using a range of clinical, biochemical, histological and physiological markers of colitis severity. In addition, a preliminary trial was performed to assess the colonic ulcer-healing properties of EGF using the intraluminal acetic acid model.

Both carrageenan and acetic acid induced colonic mucosal lesions in adult rats. Oral administration of 3% iota carrageenan induced a patchy, mild colitis with a phasic onset of clinical symptoms due to the cyclic nature of the carrageenan intake. Acetic acid induced severe penetrating ulcerations when applied to the colonic serosa, and a milder colitis when flushed through the colonic lumen. The disruption to the colon induced by intraluminal acetic acid was mainly localized to the distal colon, as reflected by an increase in mucosal and muscularis wet weight by 41% and 2.3 fold, respectively and a 59% reduction in the distal mucosal DNA content compared to sham-operated control animals. In an attempt to enhance the uniformity of exposure to the injurious agent, the intraluminal acetic acid model was modified by restricting the exposure of the acid to the distal colon. The damage induced to the distal colon was maximal at 24 hours with the mucosal wet weight and protein content increased by 200% and 50%, respectively. This was accompanied by a reduction in DNA content and Na^+/K^+ATPase activity by 54% and 80%, respectively compared to control values. The colonic lesions were patchy in distribution along the length of the distal colon,

being interdispersed with intact colonic mucosa. By 5 days, some damage to the distal colon was still apparent and by 14 days the damage was resolved.

In an attempt to reduce the patchiness of the colonic lesions induced by intraluminal acetic acid exposure the model was modified further by restricting the area of the distal colonic lumen exposed to the injurious agent to a 2 cm segment. Colonic damage and recovery were assessed by measuring the extent of re-epithelialization of the ulcerated segment using quantitative histological techniques. A fulminating, reproducible colitis was produced in which there was near complete obliteration of the colonic mucosa with a small percentage of epithelium remaining at the margins of the segment. Re-epithelialization of the colonic lesion was not apparent until 6 days after the acid insult and by 14 days significant epithelial coverage had occurred.

In conclusion, the intraluminal administration of acetic acid to a segment of the distal colon was considered the most appropriate model for assessing the colonic ulcer healing properties of EGF. Using this model, the severity and recovery of the induced colonic lesion can be quantitated by measuring the percentage of epithelial coverage of the acid-exposed segment using histological techniques.

General Introduction

The incidence of naturally occurring animal models of colitis is low, and species they occur in are often expensive to maintain and are in some cases endangered. Therefore, to assess new therapeutic agents for inflammatory bowel disease an experimental animal model needs to be developed that mimics the human condition. To date, no colitis model has been described which completely fulfils the criteria of the 'ideal' colitis model specified by Strober (1985), as discussed in Chapter 1. Instead, a range of injurious agents has been described to produce colitis-like conditions that share some of the clinical, histological and biochemical characteristics of the human disease. In addition to mimicking some aspects of the natural disease condition, the following criteria were also considered important for a model to test the colonic ulcer healing properties of EGF: The agent must induce a colitis condition in an inexpensive, easy to handle and readily available species as large numbers of animals will be required for assessing new therapies. Secondly, the colitis induced must provide an appropriate recovery period for assessing the ulcer healing properties for EGF. In the upper gastrointestinal tract, healing of acetic acid-induced gastroduodenal ulcerations was accelerated following 7-9 days treatment with EGF (Konturek and Dembinski et al. 1988, Hase et al. 1989) On the basis of these criteria, two methods of inducing an experimental colitis, carrageenan and acetic acid, were assessed as potential models for examining the colonic ulcer healing effects of EGF. Both these agents produce a colitis-like condition in the rat, which is an accessible and inexpensive species to maintain. The chemical irritant acetic acid has been reported to induce an acute damage to the colon (Sharon and Stenson 1985). In contrast, long term oral administration of carrageenan has been used to induce a more prolonged colitis condition (Ishioka et al. 1987). In this chapter, the distribution and chronicity of the colonic lesions induced by both agents was assessed using a range of biochemical, histological and physiological markers of colitis severity.

CARRAGEENAN-INDUCED COLITIS

Introduction

Oral carrageenan administration has been used to induce a colitis-like condition, which shares some of the clinical and histological features of human ulcerative colitis, in a range of species including monkeys, guinea pigs, rabbits and rats (Benitz et al. 1973, Langman et al. 1985, Watt and Marcus 1973, Ishioka et al. 1987). Although the guinea pig is the most common species used for inducing a carrageenan colitis condition, a number of studies have been performed which show that oral carrageenan can induce a colitis condition in the rat. Extensive studies performed by Ishioka et al. (1987) using intragastric and intracolonic administration of acid hydrolyzed carrageenan to adult rats induced colonic lesions and, in high doses, colonic polyps. Similar findings were made by Marcus and Watt (1971) in which colonic lesions were induced in neonatal rats administered oral carrageenan.

Iota carrageenan derived from the red seaweed *E.spinosum* is the most common form of carrageenan used in experimental colitis induction, although kappa carrageenan has been used in guinea pigs with some success (Marcus and Watt 1971,1974, Langman et al. 1985). In the native form, high molecular weight carrageenans (100-800kDa) are poor inducers of colonic ulceration. A number of studies, in a range of species, have shown that acid hydrolysed carrageenans display more potent ulcer-inducing properties compared to the native forms (Marcus and Watt 1971,1974, Ishioka et al 1987, Fabian et al. 1973). Acid hydrolysis produces a carrageenan of a lower molecular weight (30kDa) and increased water solubility while maintaining a similar sulphur content (18-40%) (Watt et al. 1979).

The severity of the colonic ulceration induced by acid hydrolysed-carrageenan is dependent on the concentration used and the duration of exposure. In all species examined, no ulcer-inducing effect has been observed at doses lower than 1%(w/v) (Macpherson and Pfeiffer 1976). In guinea pigs and rabbits, colonic ulceration results from 2% or 5%(w/v) carrageenan in the drinking water for 6 and 3 weeks respectively (Watt et al. 1979, Watt and

Marcus 1973), whereas in rhesus monkeys, a 1% or 2%(w/v) solution induced ulceration observed after either 7 and 11 weeks of treatment (Benitz et al. 1973). At concentrations higher than 2%(w/v), the effective dosage of carrageenan ingested by the animal may be reduced due to the solution becoming less palatable. The maximal concentration that can be administered in the drinking water is also limiting due to the gel-forming properties of the carrageenans. This problem has been overcome by administering 5% and 10% solutions using more invasive procedures such as by gastric cannulation or rectal infusion (Ishioka et al.1987, Jensen et al. 1984).

To assess the colitis-inducing properties of carrageenan in the adult rat, it was necessary first to determine the type and dosage required to produce colitis-like symptoms. Initially, three types of carrageenan were compared using the clinical manifestations of colitis conditions including diarrhea, weight loss and occult blood in the faeces as indicators of disease activity. After choosing the appropriate type of carrageenan, the optimal oral dose required to produce a quantitative colonic lesion was assessed by grading the severity of the histopathological features of the resulting colitis.

Methods

Acid hydrolysis of Carrageenans

Kappa, iota (Sigma Chemical Co, St.Louis, U.S.A.) and E.Cottini food grade carrageenan (Davis Germantown, Vic., Australia), were acidified using the method described by Watt et al. (1979). Carrageenan powder (1g) was mixed with 1ml of 10M HCl and left at room temperature for 1 hour to hydrolyse. Water was then added to dissolve the powder and the solution was neutralized to pH 7 using 2M NaOH. The volume was adjusted to give the desired final weight/volume percentage of degraded carrageenan, and the solutions were stored at 4°C for subsequent administration to rats.

Animals and Experimental design

Ethics approval for the assessment of the colitis-inducing ability of orally administered carrageenan in adult rats was granted by the Women's and Children's Hospital Ethics Committee.

Comparison of the colitis-inducing properties of 3 types of carrageenan

Twelve male Sprague Dawley rats (250g) were housed in individual meshed bottom cages to prevent coprophagy and to allow monitoring of changes in the fecal consistency, as well as the presence of occult blood in the faeces. Animals were provided a 2% (w/v) solution of acid hydrolysed solution of kappa (n=3), iota (n=3), E.Cottini food-grade (n=3) carrageenan or water (n=3) ad libitum and given free access to standard laboratory rat chow (Milling Industries Limited, Adelaide, Australia) over a 60 day treatment period. Fresh carrageenan solutions were prepared on every alternate day over the treatment period. The faeces was tested for the presence of occult blood with Hemotest tablets every second day for the duration of the experiment, together with the body weight and fluid intake.

Determination of the optimal dose of iota carrageenan required to induce colonic ulcerations

Based on the findings from the previous trial, iota carrageenan was chosen as the appropriate type of carrageenan for inducing a colitis-like condition in rats. Three concentrations of acid hydrolysed iota carrageenan were compared for their ulcer inducing properties. Male Sprague Dawley rats (250g) were provided 2%(w/v) (n=3), 3%(w/v) (n=3) or a 5%(w/v) (n=3) solution of acid degraded carrageenan instead of drinking water over a 50 day treatment period, with control animals receiving water alone (n=3) . Body weight, fluid intake and occult blood measurements were performed as described in the previous trial.

In addition to the measurements used in the first trial, histological analysis was carried out to determine the distribution and severity of the colonic lesions induced by carrageenan. For this purpose, animals were stunned and decapitated at the end of the

experimental period. The middle colic vein was located through a mid-line incision and a 4-0 suture was placed around the colon adjacent to the vein before the colon was removed with the terminal end taken at the point adjacent to the pelvic bone. The colon was slit open longitudinally and placed serosal side down on filter paper and flooded with Bouin's fixative. Bouin's fixative was chosen on the basis of a preliminary trial comparing the quality of haematoxylin and eosin staining, the level of structural definition and contrast and general tissue preservation of Bouin's, Formalin and Carnoy's fixed colonic tissue from normal rats. After flooding with Bouin's, the colon was divided into two segments at the point of the suture. Both segments were rolled longitudinally to form coils of intestine and transferred to a beaker of Bouin's fixative for a further 24 hour fixation period. The coils of colon were dehydrated using a Titertek automated tissue processor (Miles Scientific, Naperville, U.S.A.), embedded in paraffin and 10µm sections were taken at 200µm intervals using a Riechert-Jung Biocut 2035 microtome (Heidelberg, Germany). Sections were stained with hematoxylin and eosin and viewed using a Zeiss Jenaval light microscope (Jena, Germany). A qualitative assessment of the severity of the colonic lesion in proximal and distal colon from each treatment group was made, with damage designated as mild, moderate or severe.

Statistical Analysis

Data are expressed as mean ± SEM unless otherwise stated. Statistical comparisons between groups were made using one-way Analysis of Variance and Fishers PLSD post hoc tests.

Acknowledgments

The experimental design and all animal handling, sample collections and assessment of histological samples were performed by the candidate. Cutting of paraffin-embedded histological specimens and hematoxylin and eosin staining was performed by Mrs. K. Davey (Child Health Research Institute, North Adelaide, South Australia).

Results

Comparison of the colitis-inducing properties of 3 types of carrageenan

The average daily fluid intake was significantly elevated in animals receiving iota carrageenan compared to the control, kappa and food grade carrageenan treatment groups by 2, 1.5 and 1.6 fold respectively, over the 60 day treatment period (Table 4.1). Although carrageenan administration did not effect the average weight gain over the 60 day period (Table 4.1), a phasic change in body weight was observed in the iota carrageenan group with a loss in body weight between day 12 and day 16 and a cessation in weight gain between day 46 and 48 (Figure 4.1). Fluctuations in the daily intake of iota carrageenan were also observed with a minimal daily intake of 28mls at day 14 and a maximal intake of 94mls on day 28 of the treatment period. Clinical symptoms indicative of a colitis-like condition, including occult blood in the faeces and diarrhea, were only observed in the iota carrageenan treatment group. The symptoms were first detected between 8 and 10 days after commencing treatment and remained for a 5 day period (Figure 4.1) with a second onset of symptoms occurring on day 48 and persisting until day 55. On the basis of these findings, iota carrageenan was chosen as the appropriate form and a dose-response study was performed to identify the optimal dose of iota carrageenan.

Table 4.1 Daily body weight gain and fluid intake of rats administered water, kappa, iota or food grade carrageenan

	Control	kappa carrageenan	iota carrageenan	food grade carrageenan
N	3	3	3	3
Average body weight gain (g/day)	2.9±0.2	2.0±0.2	2.1±0.3	2.1±0.3
Average Fluid intake (mls/day)	27.6±2.3	37.0±2.9	55.2±5.9 *	33.7±2.0

Data are expressed as mean±SEM over the treatment period
*P<0.05 (versus control group)

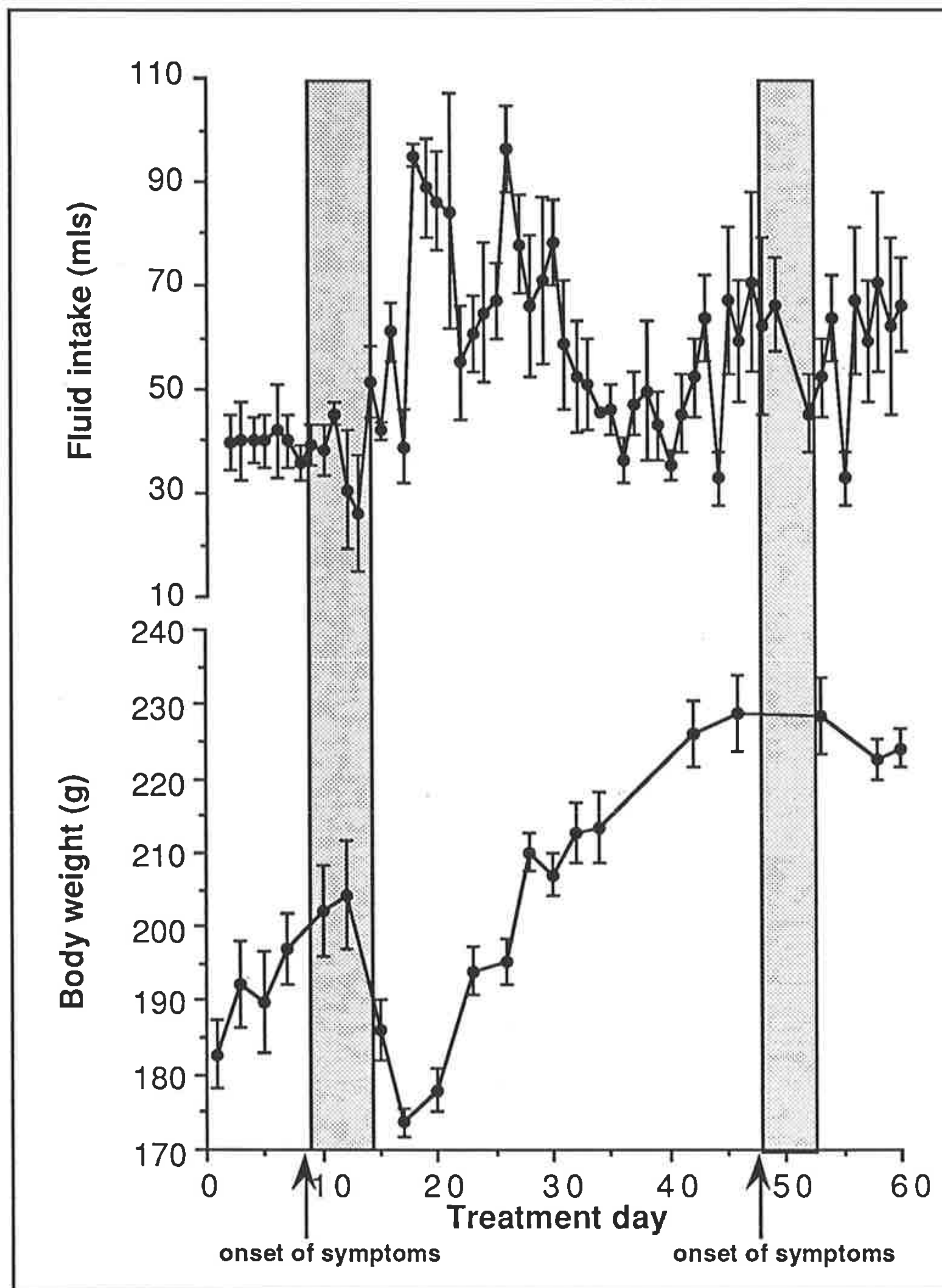


Figure 4.1 Mean ± SEM fluid intake and body weight for animals administered iota carrageenan. Hatched regions correspond to the presentation of diarrhea and occult blood in the faeces.

Determination of the optimal dose of iota carrageenan required to induce colonic ulcerations

Iota carrageenan was administered to rats in the drinking water at 0, 2, 3 or 5%. The 5% carrageenan solution was poorly tolerated so that rats consumed very little fluid and lost weight severely. For this reason, treatment with 5% iota carrageenan was ceased at day 7 and the group was deleted from the trial. The mean daily body weight gain from all other groups was higher than in the previous trial. The 2% dose did not affect the average body weight gain over the 45-day treatment period whereas 3% iota carrageenan reduced weight gain to 64% of the control values (Table 4.2) The average daily fluid intake was significantly increased in animals receiving 2% or 3% iota carrageenan by 1.8 and 1.9 fold respectively, compared with control animals receiving water only (Table 4.2). As observed in the previous trial, daily iota carrageenan consumption fluctuated over the treatment period (Figure 4.2) although the phasic fluid intake observed in the 2% group was less evident in the 3% iota carrageenan group.

Table 4.2 Daily body weight and fluid intake of rats administered water, 2% or 3% iota carrageenan

	Control	2% iota carrageenan	3% iota carrageenan
N	3	3	3
Body weight gain (g/day)	14.1±0.9	12.3±0.6	5.0±1.7*
Fluid intake (mls/day)	34.2±1.0	61.5±7.4*	66.6±2.7*

Data are expressed as mean±SEM over the 45 day treatment period *P<0.05 (versus control group)

Occult blood and diarrhea were observed 8-10 days after the commencement of treatment in both carrageenan-treated groups. In the 2% treatment group, symptoms had

resolved by day 14 and did not re-occur during the 45-day treatment period. In the 3% treatment group symptoms were more prolonged and occurred more frequently. The initial onset of symptoms was observed between 8 and 10 days after treatment commenced, persisting over a 10 day period, and re-occurred at day 38.

Histological assessment of the severity of the carrageenan-induced colitis was made at the end of the 45-day treatment period. In animals receiving 2% acid hydrolysed iota carrageenan, no visible disruption to the colonic mucosa was observed in the proximal or distal colon. Since clinical symptoms were apparent between days 10 and 18 in this group, the normal histological appearance at day 50 suggests that either the damage was very mild or had resolved by day 50. In the 3% carrageenan treatment group a mild colitis was still apparent in the proximal colon at day 50. The lesions induced were patchy in distribution, with areas of mild inflammation and edema in the submucosal and mucosal layers together with some epithelial denudation, interdispersed with regions of apparently normal colonic tissue (Plate 4.1).

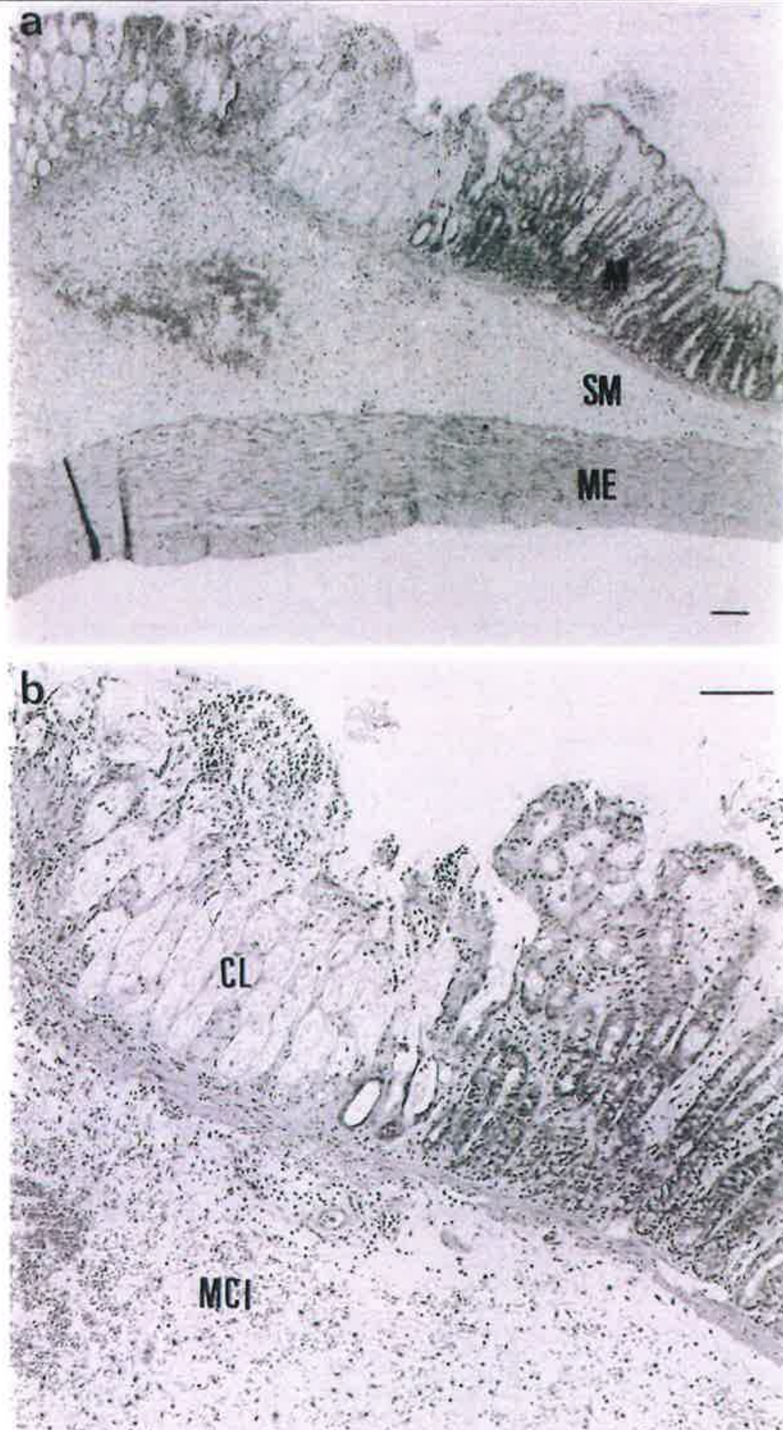
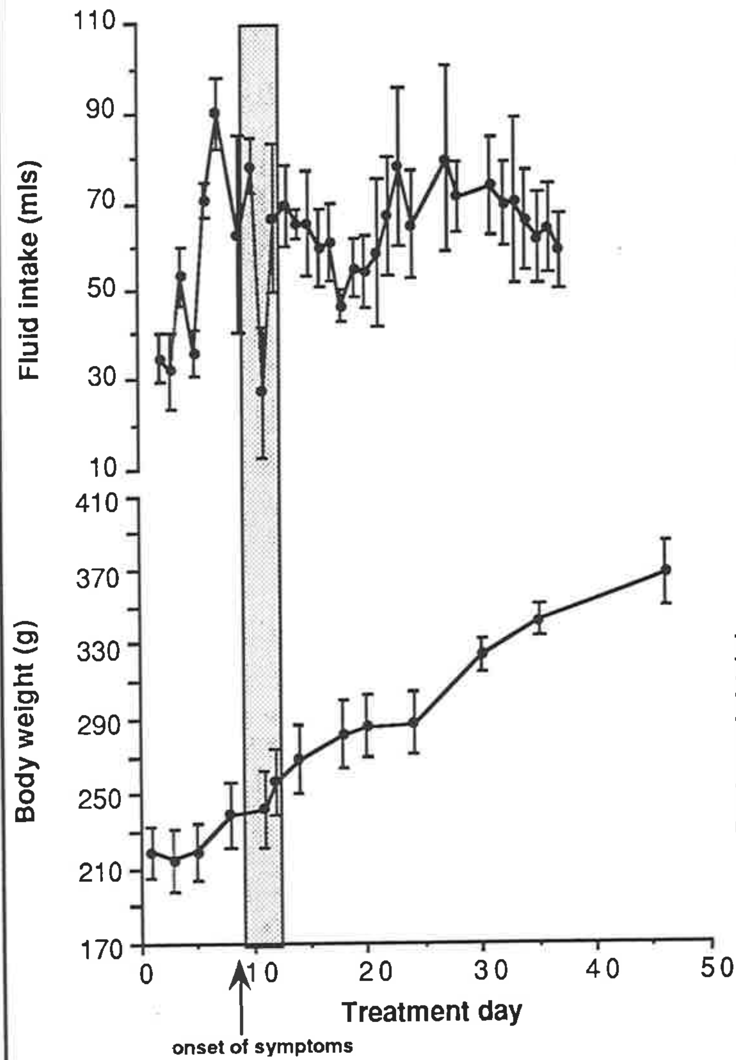
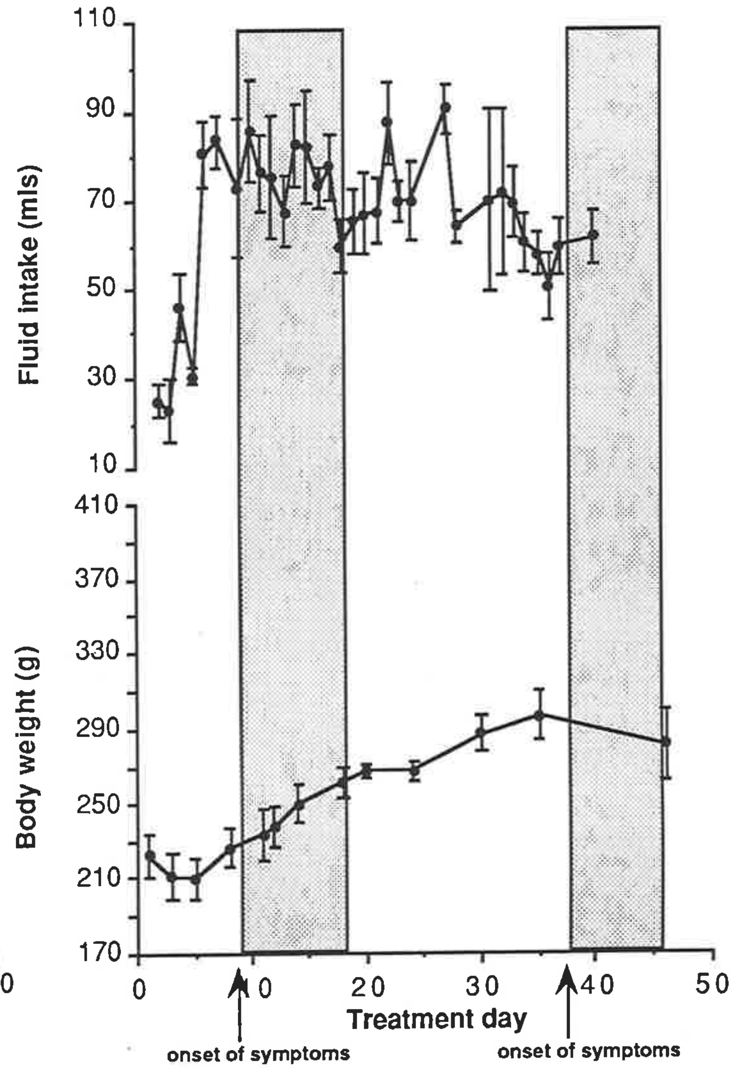


Plate 4.1a and b Proximal colonic segment showing a mild colitis-like condition induced by 50 days oral treatment with 3% iota carrageenan. A loss of crypt structures (CL) were observed and the lesions induced were patchy and interdispersed with intact mucosa (M). Areas of mild inflammation, as indicated by a mixed cellular infiltrate (MCI) in the submucosa (SM) were detected, with the muscularis externa (ME) appearing normal. Calibration bar = 100μm

Figure 4.2 Mean fluid intake and body weight for animals administered 2% and 3% iota carrageenan. Hatched regions correspond to the presentation of occult blood in the faeces and diarrhea.



2% IOTA CARRAGEENAN
Fluid intake (mls)
110
90
70
50
30
10
Body weight (g)
410
370
330
290
250
210
170
0
10
20
30
40
50
Treatment day
onset of symptoms



3% IOTA CARRAGEENAN
Fluid intake (mls)
110
90
70
50
30
10
Body weight (g)
410
370
330
290
250
210
170
0
10
20
30
40
50
Treatment day
onset of symptoms
onset of symptoms

Discussion

Of the three types of carrageenan administered to rats, iota carrageenan was the only one that induced clinical symptoms of colitis including weight loss, occult blood in the faeces and diarrhea. The maximal concentration of iota carrageenan that could be administered orally for long periods was limited to 3%, firstly because of problems with the gel-forming properties of carrageenan at concentrations higher than 5%, and secondly by the poor palatability of 5% iota carrageenan. The volume of 5% iota carrageenan consumed by rats could potentially be increased by gavage feeding the carrageenan solution. However, due to viscosity of the solution and the volume to be administered, this procedure was considered inappropriate. While 3% iota carrageenan induced a colitis which shared some of the clinical and histopathological features of human colitis, it was considered unsatisfactory as an experimental model for several reasons. The colitis was very mild and patchy making it difficult to quantitate repair by biochemical, histological and functional methods. The patchiness was in part related to the variability in intake of carrageenan associated with changes in drinking pattern. This occurred because the animals would reduce their fluid intake as they became symptomatic and then increased their intake when they recovered thereby creating a cyclical pattern of the dose of carrageenan consumed. While this phasic nature of the induced-colitis can be compared to the interdispersed active and remission phase observed in human colitis, it introduced considerable variability, and made it difficult to regulate the dose of carrageenan being consumed. The variability in the onset of symptoms also made it difficult to predict the optimal time for growth factor administration.

In conclusion, while oral carrageenan induced a mild colitis which shared some of the histopathological features of the human disease, it was considered inappropriate for assessing the colonic ulcer healing properties of EGF. A model was sought which produced a more severe, quantitative colitis-like condition.

ACETIC ACID-INDUCED COLITIS

Introduction

In the upper gastrointestinal tract, both serosal and intraluminal routes of administration of acetic acid have been used to induce ulcerations. Studies involving serosal application of acetic acid have only been reported for the upper gastrointestinal tract, including the stomach and duodenum (Okabe et al. 1971), whereas luminally delivered acetic acid has been used to produce a colitis-like condition in the colon of experimental animals (MacPherson and Pfeiffer 1978, Sharon and Stenson 1985). In this section, colonic lesions induced by both routes of administration of acetic acid were assessed.

The method of serosal acetic acid application developed by Okabe et al. (1971) involves applying acetic acid onto the gut serosa of anaesthetised animals, with the acid contained within the confines of a metal mould placed on the serosal surface. After a fixed exposure period the mould is removed and the acid is blotted from the tissue. A major benefit of this model is that it permits control over the duration of exposure as well as the size of the region exposed to the injurious agent. The ulcerations are rapidly induced, persist up to 100 days, and cover a discrete region, providing a useful model for assessing the mechanisms of ulcer repair and new therapeutic agents (Konturek et al. 1988).

Intraluminal acetic acid induces a milder colonic lesion than that achieved by serosal application, and results in an ulcerated condition which shares some of the histological features of human ulcerative colitis (MacPherson and Pfeiffer 1978, Sharon and Stenson 1985) . The acetic acid is injected through the colonic wall, and then flushed out of the lumen after a defined period. The severity of the ulceration and the occurrence of intestinal perforation is dependent on the strength of the acid solution instilled as well as the duration of acid exposure. Previous studies have used concentrations of 4% to 10%(v/v) with the exposure time prior to evacuating the acid from the lumen varying from an instantaneous

removal to sixty seconds (MacPherson and Pfeiffer 1978, Sharon and Stenson 1985, Rolandelli et al. 1988). In this section, intraluminal administration of acetic acid was performed using the method described by Sharon and Stenson (1985). The colitis condition induced using this method has been reported to share a similar inflammatory response to human inflammatory bowel disease, with respect to arachidonic acid metabolism, and shares the histopathological features of human colitis of mucosal edema, neutrophil infiltration and mucosal ulceration (Sharon and Stenson 1985).

The exposure of the colonic lumen to acetic acid as described by Sharon and Stenson (1985), induced lesions across a large area of the colon, therefore enabling analytical biochemical measurements to be made on colonic mucosal scrapings. Estimates of mucosal and muscularis wet weight, protein and DNA contents were used to assess the severity of the induced colonic lesion.

In an attempt to enhance the uniformity of exposure to the injurious agent, the size of the colonic segment exposed to acetic acid was reduced by firstly administering acetic acid to the distal colonic lumen and secondly by applying the acid to a 2cm segment of the distal colon. The severity of the damage induced by exposing the distal colonic lumen to acetic acid was assessed by analytical measurements performed on mucosal scrapings together with the activity of the colonic enzyme Na^+/K^+ ATPase. In human colitis, it has been proposed that intestinal fluid accumulation and disruption of normal electrolyte balance resulting from the reduction in enzyme activity may contribute to diarrhea associated with active colitis (Rachmilewitz et al. 1984, Tripp et al. 1980). Alterations in intestinal permeability was also assessed by measuring the urinary excretion of a luminally administered non-absorbable permeability marker ^{51}Cr-EDTA which has been used in other studies to measure the extent of epithelial permeabilization in inflammatory bowel disease (Murphy et al. 1989, Ramage et al. 1988, Maxton 1986).

The severity of the colonic lesion induced by exposing a 2cm segment of the distal colonic lumen to acetic acid, was assessed using quantitative histological measurements of the extent of epithelialization of the segment.

Methods

Experimental Animals

Ethics approval for the studies on serosal and luminally administered acetic acid in adult rats was granted by the Women's and Children's Hospital Ethics Committee. In all experiments, male Sprague Dawley rats (250g body weight) were housed in metabolism cages to prevent coprophagy, with water and standard laboratory rat chow (Milling Industries, South Australia) provided *ad libitum* unless otherwise stated. Prior to surgery, rats were anaesthetised with an intraperitoneal injection of 4.2ml/kg body weight solution containing 90mg/ml methohexitone sodium and 60mg/ml pentabarbitone sodium.

Experimental Trials

Exposure of the colonic serosa to acetic acid

Colonic ulcerations were induced by serosal application of acetic acid using the method described by Okabe et al. (1971) for inducing duodenal ulcerations. Four rats were anaesthetised, the colon was exposed through a mid-line incision and a metal mould (5mm diameter) was firmly placed onto the colonic serosal surface 2cm distal to the cecal / colonic junction. Acetic acid (100%(v/v), 70μl) was added to the mould and left for 30 seconds, after which the mould was removed and the acid was blotted from the colonic serosa with cotton gauze. The mid-line incision was closed and animals were placed in individual cages to recover with water and rat chow provided *ad libitum*. Twenty four hours after the acid insult the colonic region exposed to the acid was examined.

Exposure of the colonic lumen to acetic acid

Exposure of the entire colonic lumen to acetic acid.

Colitis was induced by intraluminal acetic acid using a modification of the method described by Sharon and Stenson (1985). Rats were fasted for 24 hours prior to surgery with water provided *ad libitum*. Animals were anaesthetised and through a mid-line incision, the colon was located and a 4-0 suture was placed around the proximal colon at the cecal / colonic junction to prevent the acetic acid entering the cecum. A 2ml bolus of 5% (v/v) acetic acid was injected through a 27g needle into the proximal colon. After 30 seconds the acid was removed from the lumen by injecting a 5 ml bolus of air into the colonic lumen. The suture was removed and the mid-line incision was closed. Sham operated (control) animals received a 2ml bolus of 0.9% NaCl into the colonic lumen instead of the acetic acid.

The severity and extent of colonic damage induced by the acetic acid instillation was assessed by comparing control (n=4) and acetic acid-treated rats (n=8) 24 hours after treatment. Animals were stunned and decapitated. A suture was placed in the colonic mesentery adjacent to the middle colic vein before the cecum and colon were rapidly excised with the terminal end of the colon taken at the site adjacent to the pelvic bone. The colon was divided into the proximal region from the cecal / colonic junction to the middle colic vein, and the distal colon from the middle colic vein to the terminal end. All dissections and length measurements were performed on a chilled glass slab to keep the tissues cold and avoid any stretching of the intestine. The lengths of the proximal and distal colon were recorded before both regions were cut open longitudinally and the tissue was rinsed in ice-cold 0.9%(w/v) NaCl. The colonic segments for the four control animals and four of the acetic acid -treated rats were blotted dry and weighed before the mucosa and submucosa were scraped from the underlying muscularis layer using a glass slide. The mucosal scrapings and muscularis samples were weighed and stored at -70°C in 3ml of 10mM $NaPO_4$ (pH 7.4) or 3ml of 2M NaOH, respectively prior to being assayed for protein and DNA content.

The remaining four acetic acid-treated rats were used to determine the histological features of the induced-colitis. For this purpose, coils of the proximal and distal colon were prepared as described in the carrageenan-colitis trial, and fixed in Bouin's fluid. 10µm sections of paraffin-embedded intestinal coils were cut and sections were stained with hematoxylin and eosin.

Exposure of the distal colonic lumen to acetic acid

In a subsequent trial, the intraluminal acetic acid model was modified in an attempt to enhance the uniformity of the exposure of the colonic lumen to the injurious agent. To reduce the amount of faeces in the colonic lumen, a 48 hour fast was incorporated into the protocol prior to the acetic acid insult, a procedure that was found to remove most faecal material from the intestine. The liquid rehydrating solution, Vytrate, was provided *ad libitum* as a nutritional supplement during the 48 hour fast. Preliminary experiments showed that Vytrate supplementation reduced the loss of body weight during fasting from 45g to 35g.

The second major modification of the acetic acid model involved reducing the colonic region exposed to the injurious agent by administering acetic acid exclusively to the distal colon. After fasting, each rat was anaesthetized and the middle colic vein was located through a midline incision. Without occluding any blood vessels, a 4-0 suture was placed around the colon adjacent to the vein and a 2ml bolus of 5% (v/v) acetic acid was injected through a 27g needle into the colonic lumen at the proximal end of the distal colon. Control (sham operated) animals received a 2ml bolus of 0.9% NaCl into the colonic lumen instead of acetic acid. After 15 seconds the acid was flushed from the lumen, the suture was removed from the colon and the mid-line incision was closed.

In a preliminary experiment, a comparison was made between two methods for removal of acetic acid from the lumen. In the first method, the acid was removed by injecting a 2ml bolus of air into the proximal end of the distal colon, while the second method included a 2ml bolus flush of 0.9% NaCl after the air. No difference in the severity

of the damage induced in the distal colon was observed between either flushing method at 24 hours after acid exposure. From these findings, air flushing was considered an appropriate method of removing residual acetic acid from the distal colonic lumen.

The extent of colonic damage induced by distal acetic acid application was assessed 24 hours after acid exposure. Acetic acid exposed (n=5) and sham operated (n=5) control animals were stunned and decapitated. Proximal and distal colonic segments were dissected and mucosal scrapings were performed. 1ml of buffer containing 250mM sucrose, 20mM Tris, 2mM EDTA and 2mM $MgSO_4.7H_2O$ (pH 7.6),was added to mucosal samples, while 1ml of 2M NaOH was added to muscularis samples. All samples were stored at -70^oC prior to subsequent determination of the protein and DNA content of the mucosa and muscularis and mucosal Na^+ / K^+ ATPase activity.

A time course study was performed to further characterise the extent of colonic damage induced by acetic acid and to determine the rate of ulcer healing. For this purpose, rats were killed at 5 (n=15) and 14 (n=5) days after acid exposure with sham operated-control animals (n=11) receiving 0.9% NaCl into the distal colonic lumen. Acetic acid exposed rats (n=6, n=5) and control animals (n=6, n=5) at 5 or 14 days respectively, were anaesthetised, the colon was removed, and samples of the proximal and distal colon were collected for determination of mucosal and muscularis protein and DNA content and mucosal Na^+ / K^+ ATPase activity. At 5 days, 5 acetic acid exposed rats were used to determine the level of epithelialization of the acid exposed distal colon. For this purpose coils of the distal colon were prepared as described in the carrageenan-colitis trial and fixed in Bouin's fluid.

The urinary excretion of a non-absorbable permeability marker,^{51}Cr-EDTA, was determined in acetic acid-exposed (n=4) and sham operated (n=4) rats over a 4 day period. A 1ml bolus of demineralized water containing 0.831mg of Cr-EDTA and 350μCi of ^{51}Cr-EDTA (Australian radioisotopes, Lucas Heights, Australia) was injected through a 27g needle into the stomach fundus at the time of acetic acid exposure. The mid-line incision was closed and animals were placed into metabolism cages for quantitative collection of

urine. Standard laboratory rat chow (Milling Industries, South Australia) and water provided *ad libitum*. Urine was collected 24 hours after injecting the marker, and subsequently at 24 and 8 hour intervals over a 4 day period. The total urine volume excreted at each collection time was noted, two 2ml aliquots were removed, and the radioactivity was measured on an LKB 1261 Multi G gamma counter (Turku Finland). The radioactivity of a 1ml sample of the infusate injected into the stomach, was measured concurrently with each sample, to determine any changes in the specific activity in the marker over the 4 day period, and the radioactivity of the samples were adjusted accordingly.

Exposure of a segment of the distal colonic lumen to acetic acid

In a further attempt to obtain a more uniform damage over the entire area exposed to the acid, the method was modified further so that a defined 2cm segment of the distal colon was exposed to the injurious agent, rather than the entire distal colon.. Rats were fasted 48 hours prior to surgery, anaesthetized, and the middle colic vein was located through a mid-line incision. Scovall clamps were placed across the colon 1cm and 3cm distal to the vein, thereby occluding the lumen of a 2cm distal colonic segment. A 700μl bolus of 5%(v/v) acetic acid was injected through a 27g needle into the colonic lumen at the proximal end of the segment. Sham-operated animals received a 700μl bolus of 0.9% NaCl into the colonic lumen instead of acetic acid. This volume of acid was adequate to fill the segment without causing distension of the colonic wall. After a 60 second exposure period, the distal-most clamp was removed and the acetic acid was flushed from the colon by injecting a 2ml bolus of air followed by an 8ml bolus of 0.9% NaCl into the proximal end of the segment. The proximal clamp was then removed. For subsequent identification of the acid-exposed intestinal segment, a 6-0 silk suture was placed in the colonic mesentery adjacent to the position of each clamp, and the mid-line incision was closed. Animals were returned to metabolism cages and were allowed free access to water and standard laboratory rat chow (Milling Industries, South Australia).

A time-course study was performed to characterize the ulcerative damage and to determine the rate of ulcer healing. To assess the uniformity of the induced colonic lesion

histological methods were used to quantitate the epithelial coverage of the acid-exposed segment. For this purpose, rats were killed at 1 (n=8), 2 (n=8), 4 (n=8), 5 (n=5), 6 (n=4), 7 (n=5) and 14 (n=6) days after administration of acetic acid. On the appropriate day, each rat was anaesthetised and the colon was removed through a mid-line incision. The acid-damaged region of the distal colon, bordered by the two suture points was isolated. In addition, a 1cm segment was taken adjacent to the proximal end of the acid damaged segment to measure the effect of acetic acid exposure at the margins of the acid-exposed segment . Both segments were cut open longitudinally, placed serosal side down on filter paper and flooded with Methacarn fixative prior to being placed in a beaker of Methacarn for a 2 hour fixation period.

Analytical measurements

Protein and DNA content of mucosal and muscularis samples

Mucosal scrapings and muscularis samples were thawed and homogenized at 4°C for 20 seconds at 15000rpm using an Ika Ultra Turrax (Janke and Kunkel, Germany) to generate a homogeneous tissue suspension. An aliquot of the mucosal samples was removed and diluted to a final concentration of 1M NaOH and digested at 4°C for 48 hours. Mucosal and muscularis samples were diluted to a final concentration of 0.5M NaOH for the measurement of the protein content by the Lowry method as modified by Dulley and Grieve (1975). Protein content was determined by adding 150μl of reagent mix containing 2.56% (w/v) Na_2CO_3, 2.56% (w/v) Na tartrate and 1.28% (w/v) $CuSO_4.5H_2O$ to a 50μl volume of the mucosal or muscularis digest in a 300μl microplate well. The well contents were mixed and 50μl of 25%(v/v) Folin reagent was added. After a 60 minute incubation at room temperature the optical density at 750nm was measured using a Titertek Multiskan microplate reader (Eflab, Finland). Bovine serum albumin (Sigma Chemical Co., St. Louis. U.S.A) was used as a standard. The DNA content was measured by the method of Burton (1975) after DNA had been extracted from mucosal and muscularis digests in 0.2M perchloric acid. In brief, 100μl of the DNA extract was added to 200μl of acetic acid containing 1.5%(w/v) diphenylamine, 1.5%(v/v) H_2SO_4 and 0.105μl of acetylaldehyde. After a 17 hour

incubation in the dark at 30°C, the optical density was measured at 620nm using calf thymus DNA (Sigma Chemical Co., St. Louis. U.S.A) as a standard.

Na^+/ K^+ ATPase activity

The activity of Na^+/K^+ATPase, a key enzyme in colonic electrolyte balance, was measured in colonic mucosal samples to determine its usefulness as a marker of colonic ulcer severity. Na^+/K^+ATPase activity was determined by measuring the total ATPase (Mg^{++} $Na^+$and K^+ stimulated) activity and subtracting the Mg^{++}stimulated ATPase activity using a modification of the method described by Tripp et. al (1980). Total ATPase activity was determined by conversion of ATP to inorganic phosphate (Pi) in the presence of Mg^{++}, $Na^+$and K^+, by incubating 200-250μg of mucosal protein in a total volume of 1200μl containing 30mM Imidazole, 0.15M Na_4EDTA, 6.5mM $MgCl_2$, 140mM NaCl, 13mM KCl and 4.4mM ATP (pH 6.6) for 15 minutes at 37°C. The reaction was terminated by adding 25% $HClO_4$ at 4°C and tubes were spun at 5000g for 10 minutes to remove precipitated proteins. Under these conditions 6-8% of the substrate (ATP) was converted to inorganic phosphate (Pi). Mg^{++}stimulated ATPase activity was determined in the same manner by addition of 1mM ouabain to inhibit Na^+/K^+ATPase activity. Both total and magnesium-stimulated ATPase activity were proportional to the concentration of mucosal protein up to 300μg of mucosal protein per reaction tube. A linear increase in activity at 37°C was observed for at least 30 minutes (results not shown). The amount of Pi liberated into the supernatant was determined using a modification of the Fiske-Subbarow method described by Rosenthal and Matheson (1973). To a 600μl aliquot of the reaction supernatant 100μl of 2.5M H_2SO_4, 100μl of 2.5% $(NH_4)_6$ $MO_7O_{24}.4H_2O$ and 200μl of 1% Elon in 3% $Na_2S_2O_5$ were added and the reaction mixture was incubated for 10 minutes at room temperature. The optical density was measured at 700nm on a Shimadzu spectrophotometer (UV-120-02, Kyoto, Japan) and Pi levels were determined using KH_2PO_4 as a standard. In each assay, a reagent blank containing all reagents except the sample homogenate, and a sample blank containing all reagents except ATP, were included to determine the level of non-ATPase induced ATP degradation and the inorganic phosphate content of the sample,

respectively. The level of Pi obtained from each of these blanks was deducted from the total and Mg^{++}stimulated ATPase Pi levels. The Na^{+}/K^{+}ATPase activity of each sample was calculated by subtracting the Mg^{++}stimulated ATPase activity from the total ATPase activity and expressed as nmol Pi/mg mucosal protein/hour. The activity of purified ATPase (Sigma Chemical Co., St. Louis. U.S.A) was also determined in each assay.

Histological measurements

Where samples were collected for histological evaluation, Bouins-fixed coils of the proximal and distal colon were transversely orientated, while Methacarn-fixed tissues were orientated longitudinally, to allow measurement of the length of the segment covered by epithelium. All samples were embedded in paraffin. 4-5µm sections were taken.at 6 tissue levels at 200µm intervals in the acetic acid damaged colon and at 3 tissue levels at 200µm intervals in the 1cm segment adjacent to the acid exposed distal colonic segment. Sections were stained with hematoxylin and eosin and viewed using a Zeiss Jenaval light microscope (Jena, Germany). Quantitative measurements were made from images acquired with a JVC video camera and digitized by a PRISM Image Analysis software system (Dapple Systems Inc., Sunnyvale, U.S.A.) coupled to an Apple Macintosh II cx computer. The extent of ulceration was assessed by measuring the percentage length of the segment covered by epithelium calculated by measuring the total length of the segment and the length of the segment covered by intact epithelium. Intact epithelium was defined as crypt-like structures including both immature and mature crypts. In the 1cm segment adjacent to the 2cm acid-exposed colonic segment the thickness of the mucosa (from the luminal surface to the base of the muscularis mucosae), the submucosa (from the base of the muscularis mucosae to the muscularis externa) and the muscularis externa layers were each expressed as a mean thickness derived from measurements taken at 3 tissue levels.

Statistical Analysis

Data are expressed as mean ± standard error of the mean unless otherwise stated. Statistical comparisons between sham-operated and acetic acid exposed groups, were made using Student's unpaired t-tests. The difference in the urinary excretion rate of ^{51}Cr-EDTA

in acetic acid and sham-operated animals over a 4 day period was compared using Wilcoxon signed rank tests.

Acknowledgments

The experimental design, all animal surgery and analytical procedures were performed by the candidate. Cutting of paraffin-embedded histological specimens and hematoxylin and eosin staining was performed by Mrs. K. Davey (Child Health Research Institute, North Adelaide, South Australia)

UNIVERSITY OF ADELAIDE LIBRARY

Results

Exposure of the colonic serosa to acetic acid

Serosal application of acetic acid in the stomach and duodenum has been reported to induce a reproducible ulceration of defined size which can persist for up to 100 days (Okabe et al.1971). The method did not prove appropriate for the colon. At 24 hours after serosal acid exposure, extensive adhesions between the damaged colon and surrounding organs was observed in all animals, and in 3 out of 4 animals the colon had perforated. Problems were also encountered in trying to contain the acid within the metal mould during the exposure period, thereby making it difficult to induce a colonic lesion of a known size. On the basis of these findings, the colonic damage induced by serosal application of acetic acid was considered too severe for assessing new therapeutic agents for colonic ulcerative conditions. Therefore a model was sought which induced a less severe, non-perforating colonic lesion.

Exposure of the colonic lumen to acetic acid.

Exposure of the entire colonic lumen to acetic acid

Exposure of the colonic lumen to acetic acid was considered a more appropriate method of experimental ulcerations because it has been reported to induce a colitis over the entire colonic mucosal surface, without significant perforation of the colonic wall (Sharon and Stenson 1985). Histological analysis of the acetic acid exposed colon showed that a colitis-like condition was induced with mucosal disruption and inflammation, mainly in the transverse and distal colon. A widespread loss and dilation of crypts was seen and inflammatory cells, including neutrophils, eosinophils and lymphocytes were observed in the submucosal layer. (Plate 4.2) The colonic lesions were patchy and interdispersed with intact colonic mucosa (Plate 4.3)

The technique of luminal acetic acid exposure caused a wide spread ulceration, and afforded the opportunity to determine the effect of ulceration on wet weight, protein and DNA content of the mucosa and muscularis layers using intestinal scrapings. Twenty four hours after the acid insult the wet weight of the proximal colon was increased, as indicated by a significant 50% increase in the wet weight of the muscularis externa and a more

variable, non-significant 2-fold increase in the wet weight of the mucosa of acetic acid animals compared to the control group. In contrast, the DNA content of mucosa was slightly reduced compared with control animals, and a significant reduction in DNA content was observed in the proximal colonic muscularis although the mucosal and muscularis protein content was unchanged by acetic acid treatment (Table 4.3). These findings suggest that the increase in wet weight may have been due to the presence of blood and non-cellular material such as exudate and fluid. In the distal colon, the wet weight per cm of the mucosa and muscularis significantly increased by 41% and 2.3 fold, respectively above that of control animals, which was accompanied by a 59% reduction in DNA content of the mucosa (Table 4.3). In the muscularis of the distal colon, the increase in wet weight was associated with a significant, 2.4 fold increase in the protein content per cm although the DNA content per cm was variable and remained unchanged compared to control animals.

The biochemical parameters measured suggest that a the majority of disruption to the colon following acetic acid exposure occurs in the distal colon. While changes in the mucosa and muscualris of the proximal colon were detected, the responses were variable. The variability in the analytical measurements observed in both the proximal and distal colon may be due to either a variable inter-animal response to acetic exposure or due to a variable exposure to the injurious agent.

Table 4.3 The effect of intraluminal acetic acid administration on the colonic mucosa and muscularis

	PROXIMAL COLON		DISTAL COLON	
	Control	acetic acid	Control	acetic acid
MUCOSA				
Wet Weight (mg/cm)	22.5±2.7	49.2±16.3	16.8±1.9	23.7±1.4 *
Protein (mg/cm)	2.3±0.1	2.2±0.2	1.9±0.2	1.7±0.5
DNA (µg/cm)	110.7±7.6	89.3±13.9	110.9±13.9	44.9±12.6 **
MUSCULARIS				
Wet Weight (mg/cm)	49.2±4.4	73.8±2.8**	43.9±4.3	103.0±4.4****
Protein (mg/cm)	7.3±1.2	7.5±0.7	5.3±0.3	12.0±2.8*
DNA (µg/cm)	195±30.1	83.6±3.5**	146±20.7	154±44.5

Data are expressed as mean±SEM for 4 animals per group
Comparisons between the control and acetic acid groups are made using two-tailed Student's t-tests *P<0.05, ** P<0.01,****P<0.0001.

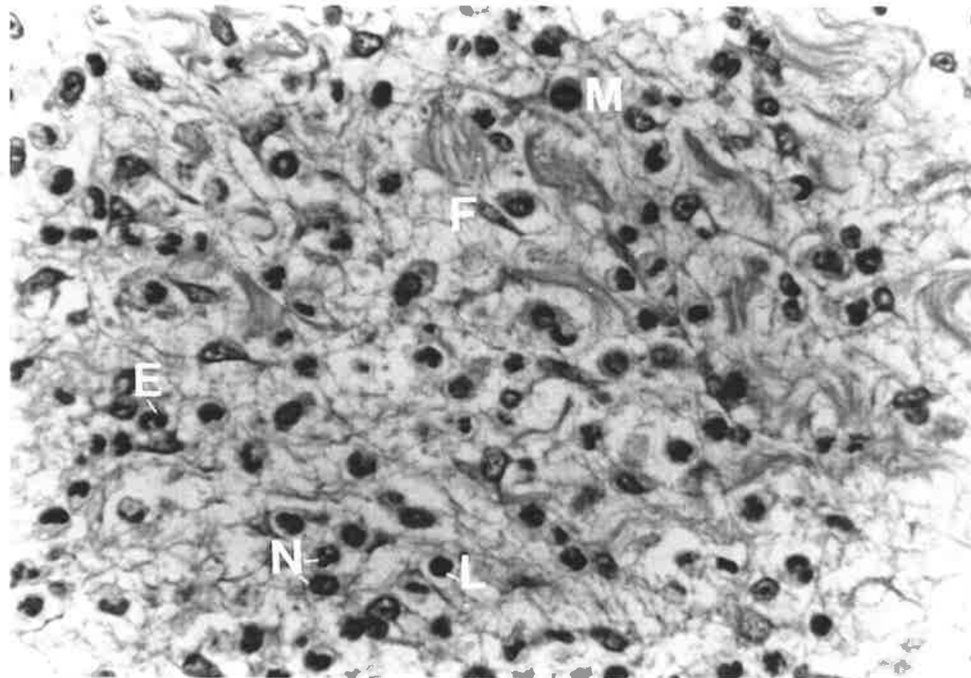


Plate 4.2 Inflammatory infiltrate comprised of neutrophils (N), eosinophils (E), lymphocytes (L), fibroblasts (F) and macrophages (M) detected in the colonic submucosa following intraluminal administration of acetic acid.

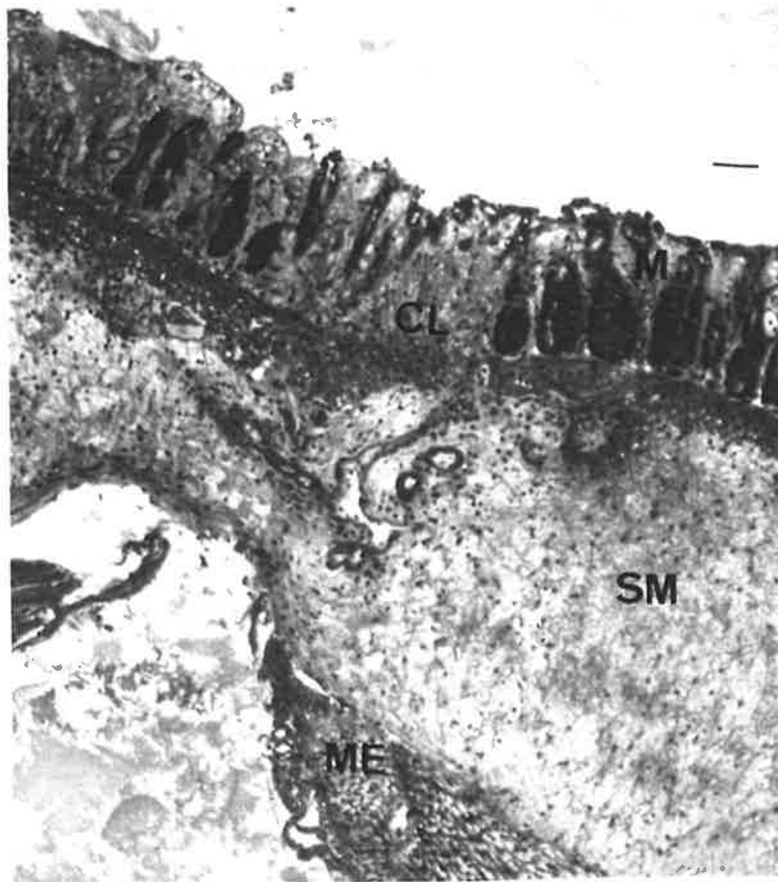


Plate 4.3 Segment of the transverse colon showing patchy mucosal lesions induced by exposing the entire colonic lumen to acetic acid. Regions of crypt loss (CL) were interdispersed with intact colonic mucosa (M). Inflammatory infiltrate and edema was present in the submucosa (SM) and muscularis externa (ME) layers.
Calibration bar = 100μm

The effect of exposing distal colonic lumen to acetic acid

In an attempt to enhance the uniformity of the exposure of the colonic mucosa to the injurious agent, the intraluminal acetic acid model was modified by including a 48 hour fast prior to acid treatment to ensure removal of all fecal material from the colon, and by restricting the area of acid exposure to the distal colon. In the acetic acid-exposed distal colon, a substantial disruption to the colonic mucosa was observed 24 hours after acid exposure. The mucosal wet weight and protein content were significantly increased by 200% and 50%, respectively, above that of control animals while the DNA content and Na^+/K^+ ATPase activity were significantly reduced by 54% and 80%, respectively (Table 4.4). The wet weight of the distal colonic muscularis was significantly increased by 66%. This was probably due to fluid accumulation, since the DNA content of the muscularis remained unchanged, while protein content was increased to a variable extent.

Although exposure of the distal colonic lumen to acetic acid did not cause a marked disruption upstream in the unexposed proximal colon (Table 4.4), the non-significant increase (19%) in mucosal wet weight and decrease (37%) in Na^+/K^+ ATPase activity in this region suggest that some adaptive response to distal colonic damage has occurred.

Time course recovery trial

In a separate trial, rats were killed at longer time points after acid exposure to characterise the time course of colonic repair. In the distal colon, the elevation in mucosal wet weight observed at 24 hours was still seen at 5 days, together with a reduction in Na^+/K^+ ATPase activity to one third of that measured in normal animals (Table 4.5). On the other hand, the marked reduction in mucosal DNA content observed at 24 hours after the acid insult was nearly resolved by 5 days. In the distal colonic muscularis, the wet weight, protein and DNA content per cm were increased by 3.7, 2 and 3.8 fold above that of control values at 5 days, respectively, a response that may have reflected muscle contraction and/or infiltration with inflammatory cells.

In the proximal colon, mucosal and muscularis wet weights were significantly increased by 33% and 44% respectively, above control values, even though the proximal colon was not directly exposed to acid. The increase in wet weight was not associated with an increase in protein and DNA content, suggesting tissue edema rather than a cellular origin.

By 14 days, the alteration in the biochemical parameters measured in the distal and proximal colon resulting from acetic acid exposure were no longer apparent, suggesting that the damage had resolved (Table 4.5).

Analysis of the histological samples indicated that at 5 days after acetic acid insult a patchy colitis was induced in the distal colon with complete obliteration of the mucosa in some regions leaving only 55.4±13.0% of the total length of the distal colon covered by epithelium. Although the elevation in mucosal DNA between 24 hours and 5 days after the acid insult suggested that the mucosal damage induced by acetic acid was markedly resolved at 5 days, this was not supported by the histological findings which suggested that a large area of the acid exposed segment was still denuded at 5 days. The increase in the DNA content of the distal colonic mucosa measured in EGF-treated animals may therefore have reflected an increase in cell proliferation in the colonic mucosa only at the margins of the ulcerative lesions, or it may be due to an increased number of other cell types in the mucosal scraping sample such as inflammatory cells, but was not indicative of an increase in the extent of re-epithelialization of the colonic lesions.

Table 4.4 The effect of luminal administration of acetic acid into the distal colon on the mucosal and muscularis layers of the proximal colon (no direct exposure to acetic acid) and the distal colon (directly exposed to acetic acid) 24 hours after the acid insult.

	PROXIMAL COLON (Unexposed to acetic acid)		DISTAL COLON (Directly exposed to acetic acid)	
	Control	acetic acid	Control	acetic acid
MUCOSA				
Wet Weight (mg/cm)	27.3±4.3	32.6±8.8	17.8±1.8	33.7±1.7*
Protein (mg/cm)	2.8±0.7	2.4±0.2	2.3±0.2	3.6±0.3
DNA (µg/cm)	83.9±16.7	70.5±7.4	91.7±8.1	41.7±11.2*
Na/K ATPase (nmol Pi /mg pr /hour)	1.6±0.4	0.6±0.2	1.6±0.1	0.3±0.1*
MUSCULARIS				
Wet Weight (mg/cm)	72.8±8.8	77.3±5.8	55.2±4.7	91.9±6.9*
Protein (mg/cm)	7.4±1.3	6.2±4.7	5.8±0.6	8.5±1.2
DNA (µg/cm)	116.2±15.5	171.0±30.2	159.3±15.8	152.2±18.5

Data are expressed as mean±SEM for control (n=5) and acetic acid exposed (n=5) animals.
*P<0.05 (versus control group)

Table 4.5 Colonic mucosal and muscularis responses 5 and 14 days after flushing acetic acid into the distal colon

Time after acid insult	5 days		14 days	
	Control	acetic acid	Control	acetic acid
DISTAL COLON (Directly exposed to acetic acid)				
MUCOSA				
Wet Weight (mg/cm)	28.3±1.3	46.9±6.3*	24.6±2.7	33.5±7.0
Protein (mg/cm)	3.0±0.2	3.7±0.3	2.2±0.2	2.3±0.2
DNA (µg/cm)	76.8±12.1	65.4±12.1	62.9±9.6	48.3±2.7
Na/K ATPase (nmol Pi /mg pr /hour)	1.2±0.2	0.8±0.1	0.6±0.2	1.1±0.4
MUSCULARIS				
Wet Weight (mg/cm)	63.0±3.7	169±24.3**	72.6±14.8	106±22.7
Protein (mg/cm)	6.5±0.4	12.8±0.9****	8.9±0.8	12.1±2.3
DNA (µg/cm)	86.0±6.4	326±43.1**		
PROXIMAL COLON (No direct exposure to acetic acid)				
MUCOSA				
Wet Weight (mg/cm)	28.4±1.8	37.9±3.4*	27.2±2.2	34.1±8.3
Protein (mg/cm)	2.9±0.1	3.0±0.1	2.3±0.1	2.2±0.1
DNA (µg/cm)	58.6±11.9	67.4±9.4	76.5±5.1	78.4±1.7
Na/K ATPase (nmol Pi /mg pr /hour)	3.8±0.9	3.2±0.4	2.7±0.5	2.9±0.4
MUSCULARIS				
Wet Weight (mg/cm)	59.0±2.3	86.9±10.6*	61.5±2.1	68.2±7.4
Protein (mg/cm)	5.0±0.2	6.5±0.8	7.9±0.4	9.7±0.6
DNA (µg/cm)	90.6±6.9	125±18.9	95.6±7.2	117±19.5

Data are expressed as mean±SEM for the control group at 5 days (n=6) and 14 days (n=5) and the acetic acid-treated animals at 5 days (n=6) and 14 days (n=5) after the acid insult.

An assessment of intestinal permeability using luminally delivered ^{51}Cr-EDTA

As an estimate of intestinal permeability, the amount of ^{51}Cr-EDTA excreted in the urine over a 4 day period was expressed as a percentage of the amount administered into the stomach of control and acetic acid-treated rats. In both groups, excretion was maximal 24 hours after the marker was administered and then declined progressively over the 4 day period (Figure 4.3(a)). Only a small percentage of the administered dose of ^{51}Cr-EDTA (6% in controls, 14% in acetic acid-treated) was excreted into urine over the 4 day period. There was a high inter-animal variability in the urinary recovery of ^{51}Cr-EDTA over the 4 days and no significant difference between the two groups was detected, despite the trend for an increased ^{51}Cr-EDTA excretion in acetic acid animals compared to control animals at all time points (Figure 4.3(b)).

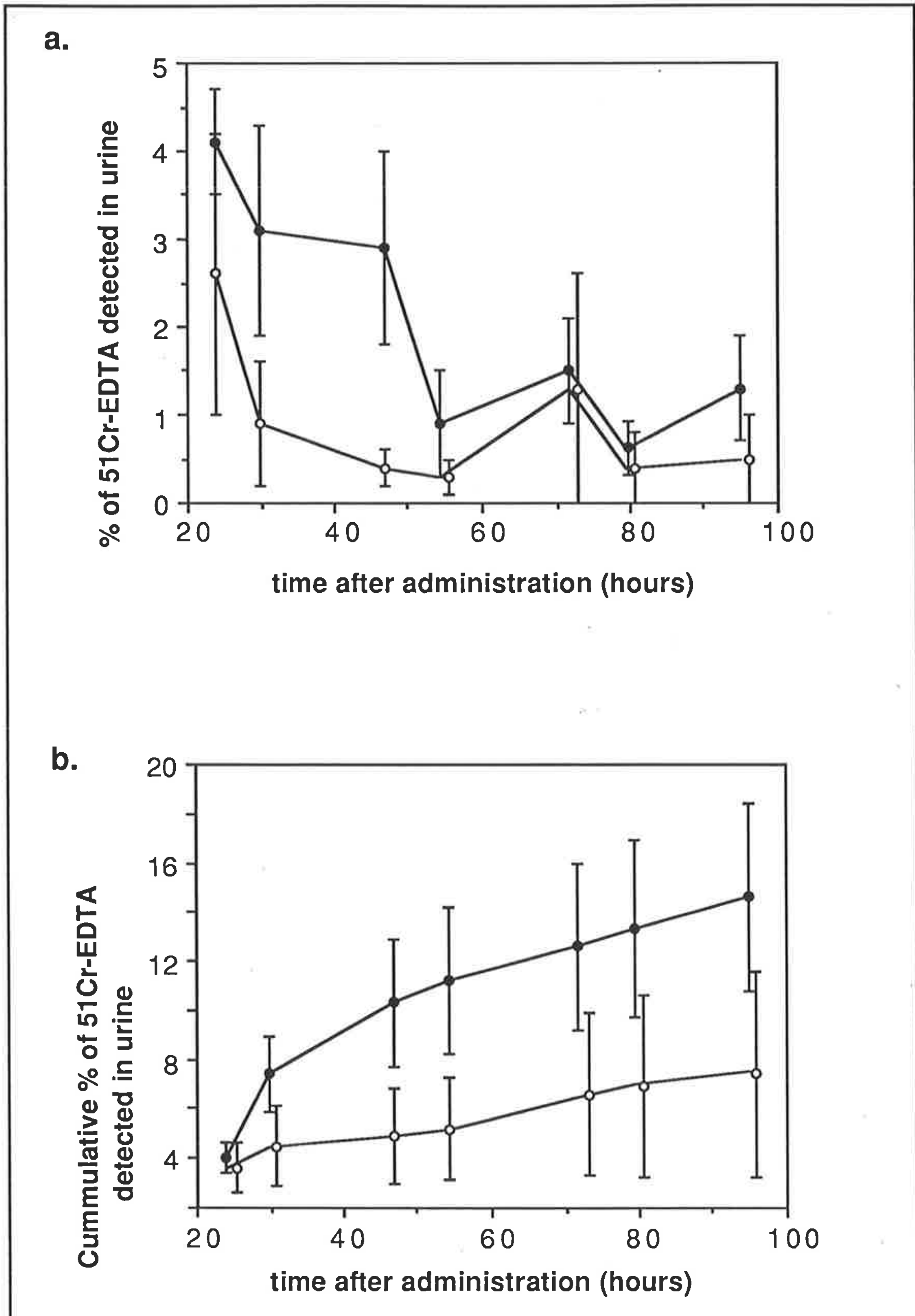


Figure 4.3 (a) Percentage of 51Cr-EDTA administered in the urine and (b) cumulative urinary excretion over a 4 day period in control (—○—) and acetic acid exposed rats (—●—).

Exposure of a 2cm segment of the distal colonic lumen to acetic acid

In an attempt to produce a more extensive obliteration of the colonic mucosa in the segment exposed to the acetic acid, the region exposed to the acid was restricted to a consistent 2cm segment. Exposure of the distal colonic segment to an intraluminal bolus of acetic acid produced a severe mucosal lesion, as shown by a marked reduction in the percentage length of epithelium covering the segment 1 day after the acid insult (Figure 4.4). Unlike the patchy colonic lesions induced by exposing the entire distal colonic lumen to acetic acid, exposure of a distinct segment of the distal lumen produced a fulminating colitis in which there was near complete obliteration of the colonic mucosa. Low levels of epithelium remained at the margins of the 2cm segment, amounting to less than 20% of the total length of the segment. Significant re-epithelialization was not detectable by 5 days after the acid insult, after which time there was a rapid, but variable increase in coverage by epithelium, so that by day 7, 60% of the segment length was re-epithelialized with either immature or mature crypt structures. By 14 days, 80% of the acid-exposed segment was re-covered by epithelium.

While this technique afforded increased reproducibility in terms of histological assessment of re-epithelialization, the short length of the acid exposed-bowel, together with tissue friability effectively precluded the scraping of mucosa from the muscularis for biochemical analysis.

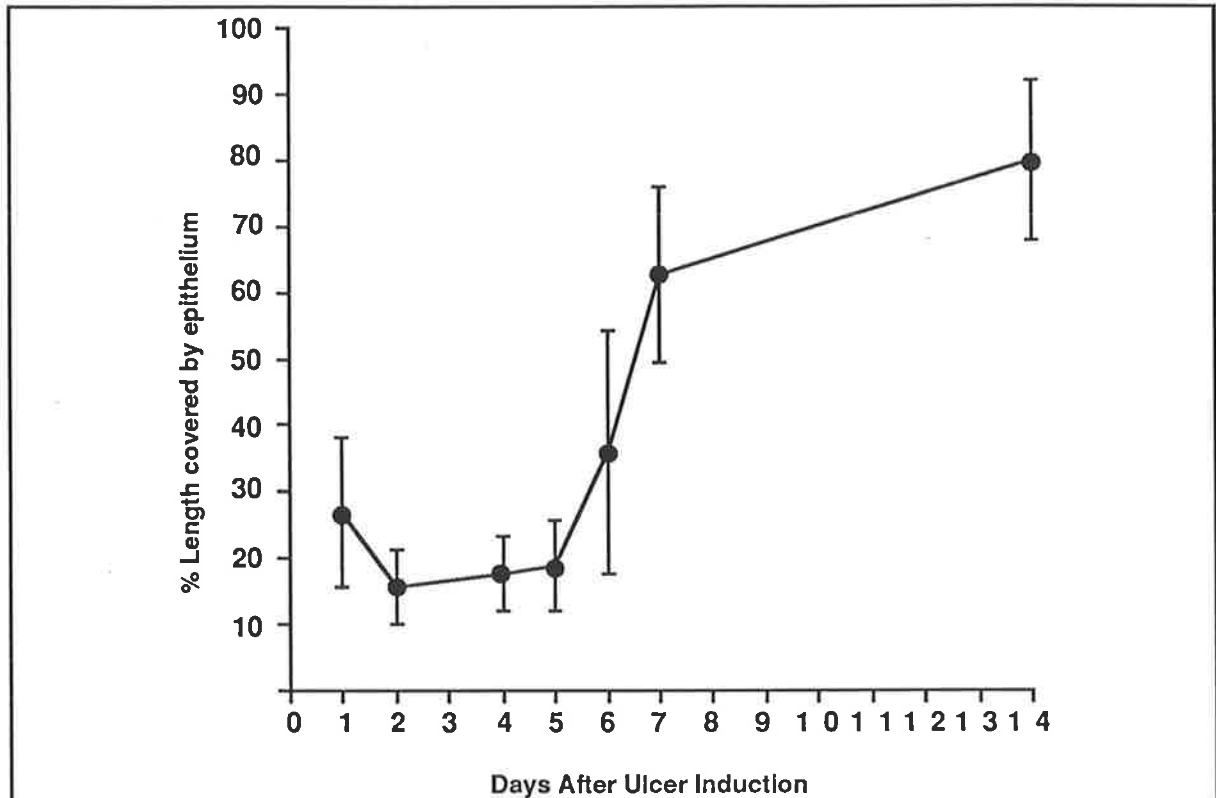


Figure 4.4 The effect of luminal acetic acid administration on the % epithelium covering the acid exposed segment 1-14 days after administration. Data are expressed as mean±SEM at each time point

As acetic acid denuded all but the margins of the damaged area, it was assumed that re-epithelialization was initiated by growth and migration of epithelium from the colonic mucosa adjacent to the ulceration and indeed, migration of a single layer of epithelium was observed in some samples (Plate 4.4). For this reason, adaptive changes in the colonic segment adjacent to the ulceration were measured. The thickness of the mucosa in the adjacent segment increased by 34% between 1 and 5 days after the acid insult (Plate 4.5), after which the thickening was slightly reduced and by 14 days it still remained 22% higher than at day 1 (Figure 4.5). In the submucosa of the adjacent segment, an acute inflammatory response accompanied by edema was associated with a 3-fold increase in the thickness of this layer 1 day after acid exposure (Figure 4.5) (Plate 4.5). The submucosal thickness then reduced rapidly so that by 6 days it had returned to near-normal. The thickness of the muscularis externa increased progressively between 2 and 5 days following acid exposure, a response associated with visual evidence of edema. After 5 days, muscle thickness

increased only slightly more, but in appearance was less edematous and appeared to contain a greater density of muscle fibres per unit length.

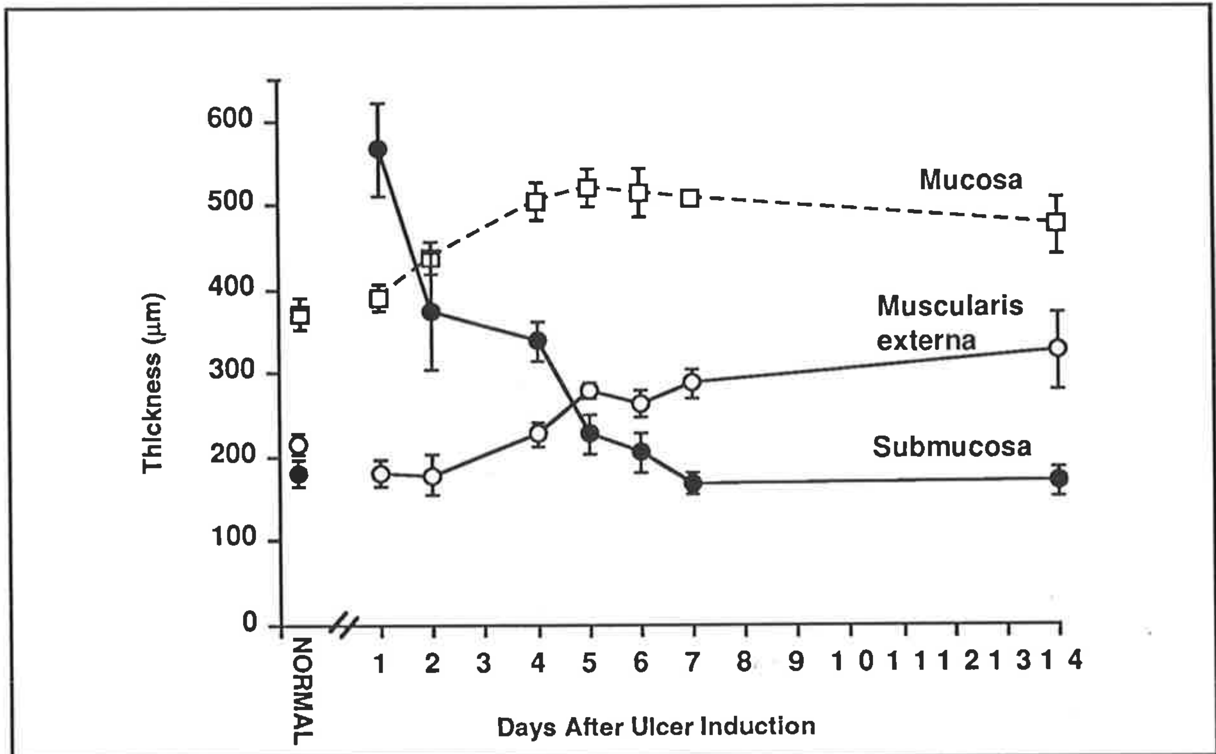


Figure 4.5 The effect of luminal acetic acid administration on the thickness of the mucosa (--□--), submucosa (—●—) and muscularis (—○—) externa layers of the colonic segment adjacent to the acid exposed segment 1-14 days after administration. Data are expressed as mean ± SEM at each time point. Normal values are derived from sham-operated animals (n=7).

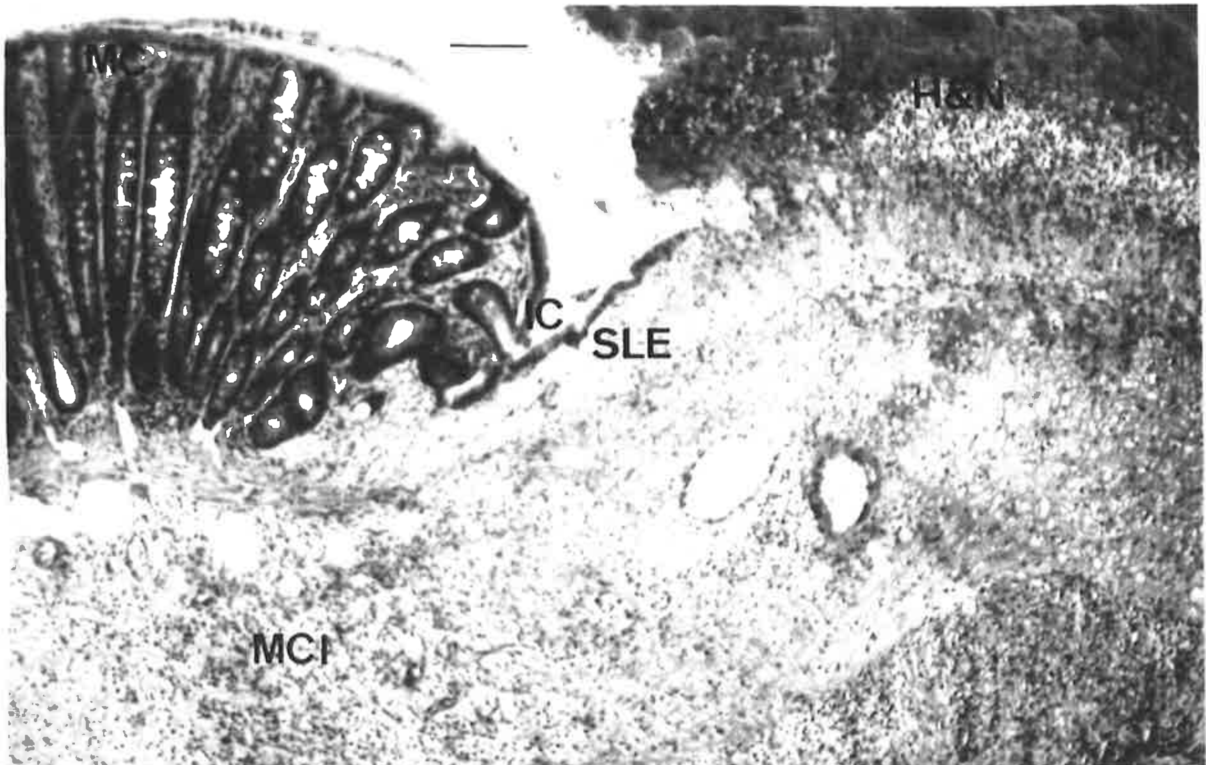


Plate 4.4. Acetic acid exposed distal colonic segment 4 days after acid insult, showing mixed cellular infiltrate (MCI) in the submucosa and muscularis externa layers and haemorrhage and necrosis (H&N) of the colonic mucosa. A single layer of epithelium (SLE) is seen migrating across the denuded mucosa which forms immature crypts (IC) and later mature crypt structures (MC).
Calibration bar = 100µm

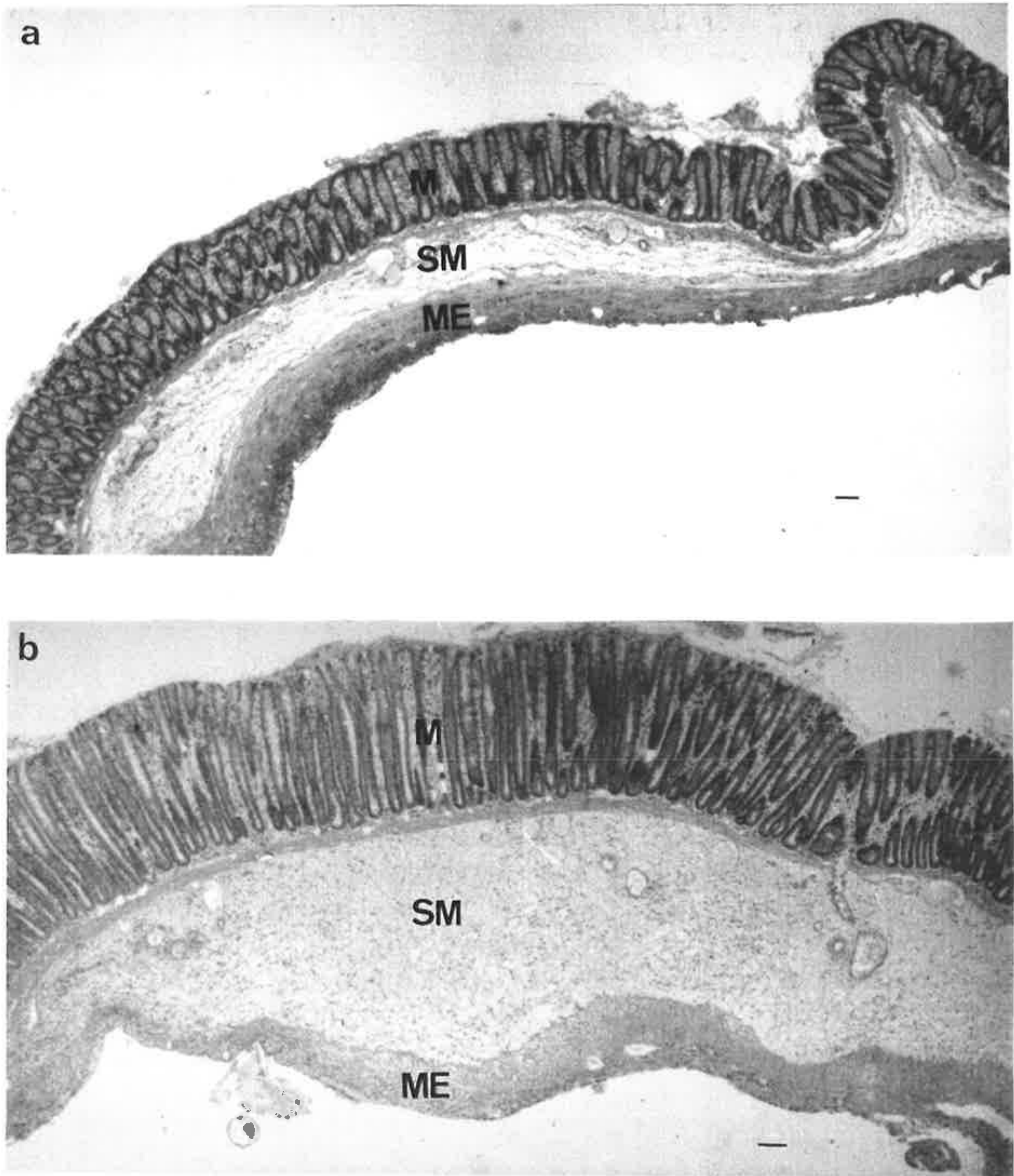


Plate 4.5. Distal colonic segment adjacent to the acid exposed region showing the colonic mucosal (M), submucosal (SM) and muscularis externa (ME) layers from (a) sham-operated control and (b) vehicle-treated ulcerated rats.
Calibration bar = 100μm

Discussion

The chemical irritant acetic acid induced an immediate and severe damage to the colonic mucosa, unlike the more latent and variable onset of colitis-like symptoms observed following oral carrageenan administration. Serosal application of acetic acid was considered inappropriate as an experimental model because of the high proportion of penetrating colonic ulcerations. Chronic ulcerations induced by serosal acetic acid in the upper gastrointestinal tract have been previously used in the assessment of the cytoprotective and ulcer healing effects of EGF (Skov-Olsen et al. 1986a and 1986b, Konturek and Brzozowski et al. 1988). Gastric lesions induced by serosal acetic acid application penetrate the stomach wall and adhere to the adjacent liver wheras in the duodenum, non-perforating ulcers of a reproducible size and depth have been reported (Okabe et al.1971). The induced lesions in both organs heal within 80 days of the acid insult. However, when applied to the serosa of the colon, acetic acid damage resulted in perforation of the colon and due to difficulties in generating ulcers of a defined size the method was considered inappropriate for assessing the colonic ulcer healing properties of EGF.

Exposure of the entire colonic lumen to acetic acid induced a colitis-like condition which shared the histopathological features of ulcerative colitis of an acute inflammatory response and denudation of the colonic mucosa. The induced colonic lesions were patchy, with damage being more prominent in the distal region as indicated by both histological and biochemical features. The patchiness of the damage may be in part attributed to non-uniformity of the intraluminal bolus of acid in exposing the entire colonic mucosal surface, while the greater severity of damage induced in the distal colon may have reflected more residual acid in this region. To enhance the uniformity of acid exposure the model was modified firstly to remove any residual fecal material, which may hamper the exposure of the colonic mucosa to the acid, and also by reducing the area of the lumen exposed to the injurious agent. Two methods were used to restrict the region of acid exposure. Firstly, the acid was infused directly into the distal colon and secondly, acetic acid was injected into a defined 2cm segment of the distal colon isolated temporarily from the remainder of the colon by clamps. While both methods of exposing the colonic lumen to acetic acid induced a

colitis-like condition which displayed characteristics beneficial for assessing the colonic ulcer-healing effects of EGF, the distribution of the induced lesions limited the types of analytical parameters which could be used for assessing ulcer severity. Both methods induced a colitis condition in the rat which was easy to perform, reproducible and provided an adequate recovery window for assessing the ulcer healing properites of EGF. Exposure of the entire distal colon to acetic acid induced colonic lesions over a large area and was amenable to performing analytical biochemical measurements on mucosal scrapings. Mucosal wet weight, protein and DNA contents were altered by acetic acid exposure, although the observed changes did not reflect the severity of the colonic lesion detected by histological analysis. Due to the mixed cell population present in mucosal scraping samples, and the patchiness of the colonic lesions, the changes in the biochemical parameters may have reflected changes in the intact mucosa at the ulcer margins or reflect changes in inflammatory infiltrates but were not indicative of the extent of re-epithelialization of the colonic lesions.

Na^+/K^+ ATPase enzyme activity was measured in colonic mucosal samples from acetic acid-exposed rats to determine its usefulness as a marker of colonic electrolyte balance and ulcer severity. Na^+/K^+ ATPase has been implicated as a modulator of intestinal Na^+, water and glucose absorption (Charney and Donowitz 1978) and in the colon a reduction in enzyme activity is apparent in patients with diarrheal conditions including active ulcerative colitis (Ramakrishna and Mathan 1988, Rachmilewitz et al. 1984, Tripp et al. 1980). A comparable reduction in Na^+/K^+ ATPase enzyme activity to that observed in biopsies from children and adults with active ulcerative colitis (Tripp et al. 1980, Rachmilewitz et al. 1984) was measured in rats exposed to intraluminal acetic acid.

To assess changes in intestinal permeability in the acetic acid-induced colitis model a method was sought which was non-invasive and did not interfere with the action of the injurious agent used in the model. A range of non-absorbable markers including lactulose, mannitol, ^{51}Cr-EDTA and polyethylene glycols, have been used to assess the extent of epithelial permeabilization in inflammatory bowel disease (Murphy et al. 1989, Ramage et al.

1988, Maxton et al. 1986). As these markers cross the mucosa entirely by passive non-mediated diffusion and are not metabolized after absorption, (Maxton et al. 1986, Krugliak et al. 1989) urinary recovery of these markers enables an estimate of intestinal permeation to be made. While a trend for a higher urinary excretion of ^{51}Cr-EDTA was observed in the acetic acid-exposed animals, no significant increase above levels excreted by normal animals was observed over the 4 day period following colitis-induction. The maximal level of the marker excreted was detected 24 hours after the acid insult and represented less than 5% of the administered dose, suggesting that either a minimal disruption in intestinal permeability is induced by acetic acid treatment, or that ^{51}Cr-EDTA administered into the stomach is not sensitive in detecting changes in colonic permeability.

Hence, while exposure of the distal colonic lumen to acetic acid induced a patchy colitis which produced an altered wet weight, protein and DNA content and Na^+/K^+ activity, these parmaters did not reflect the extent of epithelialization of the induced colonic lesions illustrated by histological analysis. The low sensitivity of luminal ^{51}Cr-EDTA in detecting changes in colonic permeability also made this marker inappropriate for assessing ulcer severity. It appeared therefore that the most informative marker of the distribution and severity of the induced colonic lesions was quantitative histological measurement of the extent of epithelialization of the damaged colon.

Unlike the patchy colonic lesions induced by exposing the entire distal colonic lumen to acetic acid, exposing a defined 2cm segment of the colonic lumen to acetic acid induced a discrete colonic lesion which had considerable advantages in terms of histological analysis, as the percentage length of the acetic acid segment covered by epithelium as well as changes in the morphology of the colon at the ulcer margins could be measured. On the other hand, performing mucosal scrapings on the damaged colon for biochemical analysis was precluded due to the size of the segment and friability of the tissue. Denudation of the acetic acid exposed colonic segment occurred rapidly and the segment became re-epithelialized over a 14 day period following the acid insult. During this period two phases of ulcer healing were observed. Re-epithelialization of the colonic lesion was not evident in the first 5 days

following exposure to the injurious agent. Following this lag phase there was a rapid onset of re-epithelialization and by 14 days near complete re-epithelialization of the denuded colon had occurred.

As the denudation of the colonic mucosa was restricted to a defined region with intact mucosa present at the margins of the lesion, this model enables the processes involved in re-epithelialization of the ulceration and adaptive responses to acetic acid exposure in the colonic mucosa adjacent to the ulceration to be examined. In the colonic segment adjacent to the ulceration, an increase in mucosal thickness was induced and maintained over the 14 day period examined. Inflammation and edema was evident in the submucosa and muscularis externa layers which was also induced and resolved rapidly.

In conclusion, exposure of the entire colonic lumen to acetic acid induces a patchy mucosal damage. By restricting the exposure of the acetic acid to the distal colonic lumen a more consistent denudation of the mucosa was produced. While the biochemical parameters measured reflected a disruption to the colonic mucosa had been induced, they were not indicative of the extent of epithelial coverage in the acid exposed region, a parameter which was more accurately determined using histological measurements. By further restricting the area of the colonic lumen exposed to the injurious agent, by exposing a defined segment of the distal lumen to acetic acid, a distinct colonic lesion was induced. The induced lesion was reproducible, and using quantitative histological techniques, enabled an assessment of re-epithelialization of the denuded colon and changes in the morphoogy of the colon surrounding the colonic lesion to be made. The colitis condition induced by this method was easily produced and provided an adequate recovery window for assessing the colonic ulcer healing properties of EGF.

CHAPTER 5. THE EFFECT OF EXOGENOUS EGF ON THE ULCERATED RAT COLON

Abstract

The effects of subcutaneously and luminally delivered epidermal growth factor on acetic acid-induced colitis were studied in the rat. Colonic damage and recovery were assessed by measuring the extent of re-epithelialization in the ulcerated segment as well as changes in the colonic morphology and proliferative status in the region adjacent to the ulceration. In addition, the effect of EGF treatment was assessed in a colonic segment distant from the damaged site. Application of acetic acid to the distal colonic lumen induced mucosal ulceration and inflammation which resolved by 14 days. In the region adjacent to the ulceration, at 4 days after the acid insult, the mucosa was hyperplastic compared with sham operated animals, and the submucosa became thickened due to the presence of inflammatory infiltrate and edema. Luminal EGF (1.6mg/kg bw/day) or subcutaneous EGF (200µg/kg bw/day) administration did not enhance re-epithelialization of the ulcerated colon after 4 or 6 days treatment. A slight increase in mucosal hyperplasia was observed in the luminal EGF group in the region adjacent to the ulceration where the crypt length was increased by 20% above that of vehicle-treated controls. The edema of the mucosa and submucosa layers in this segment was suppressed by subcutaneous EGF treatment resulting in a reduction of the thickness of these layers by 20% and 42%, respectively, compared to vehicle-treated animals. In conclusion, this study shows that exogenous EGF does not enhance re-epithelialization of acetic acid-induced colonic ulcerations but does modulate the associated edematous response.

Introduction

In Chapter 3 it was shown that exogenous EGF elicited a mitogenic response in the normal colon when administered by the subcutaneous but not the luminal route. Biochemical and histological evidence suggested that both the proximal and distal colonic mucosa were responsive to EGF and that epithelial hyperplasia and possibly hypertrophy was induced. On the basis of these findings it was anticipated that EGF would also be effective in enhancing growth of the colonic epithelia and in turn healing of the acetic acid damaged colon.

In the upper gastrointestinal tract, the responsiveness to EGF varies depending on the status of the mucosa, although both systemic and intraluminal EGF have been used to enhance ulcer healing (Konturek and Brzozowski et al. 1988, Konturek and Dembinski et al.1988). An enhancement of ulcer healing by exogenous EGF is accompanied by an elevation in DNA synthesis in the intact mucosa at the ulcer margins (Konturek and Brzozowski et al. 1988, Konturek and Dembinski et al.1988) and an increase in cell migration across the denuded intestine (Skov Olsen 1986b). Endogenous EGF has also been implicated in mucosal protection and ulcer healing as suggested by the the reported cytoprotective properties of salivary EGF (Konturek and Dembinski et al. 1988, Skov Olsen et al. 1986a). While the effect of exogenous EGF on colonic ulcerations has not been previously investigated, the recent demonstration of an EGF-producing cell lineage which emerges at the margins of intestinal ulcerations along the length of the gastrointestinal tract (Wright et al.1990a), suggests that this endogenous source of EGF may act as an ulcer healing agent to colonic ulcerations. Although the binding of EGF to colonic lesions has not been investigated, an enhancement of binding of radiolabelled EGF to the intestinal mucosa at the margins of experimentally induced gastroduodenal ulcerations has been shown (Poulsen 1988, Konturek and Brzozowski et al. 1990).

In this chapter the effect of EGF on ulcer healing was assessed at 4 and 6 days after the induction of the ulceration. These time points were selected because they corresponded

to two distinct phases of ulcer healing as shown in Chapter 4, with day 4 representing a time well before re-epithelialization had been implemented, and day 6, the time at which re-epithelialization was beginning. A longer period of treatment with EGF was not considered appropriate because substantial re-epithelialization had already occurred by day 7 therefore reducing the window for detecting EGF responses. The effect of both luminally and subcutaneously delivered EGF on ulcer healing was assessed in the same study using the same doses and delivery systems applied to normal animals in Chapter 3. Although the normal rat colon was not responsive to luminal EGF, the demonstrated efficacy of enteral EGF in stimulating ulcer repair in the upper gastrointestinal tract (Konturek and Dembinski et al. 1988, Konturek and Brzozowski et al. 1988) suggests that the damaged colon may be responsive to luminally applied EGF.

To determine the effect of EGF on enhancing healing of the ulcerated colon, quantitative histological techniques were used to measure the rate of epithelialization of the acetic acid-exposed distal colon and the ability of EGF to enhance mitogenesis at the ulcer margins. In the upper gastrointestinal tract ulcer healing induced by EGF is associated with an increased DNA synthesis at the ulcer margins (Konturek and Dembinski et al. 1988). In the current study, the effect of acetic acid exposure as well as exogenous EGF on the proliferation of the colonic epithelia at the ulcer margins was assessed using immunohistochemical detection of proliferating cell nuclear antigen (PCNA).

As well as inducing a denudation of the distal colonic mucosa, luminal acetic acid exposure also evokes a thickening of the submucosal and muscularis externa layers at the ulcer margins contributed to by visual evidence of edema and inflammatory infiltrates, as shown in the time course recovery trial performed in Chapter 4. To further characterise the severity of the inflammatory response occurring in the acetic acid exposed colon, a semi-quantitative scoring system of the extent of edema and inflammatory infiltrate was applied in the EGF trial.

In Chapter 3 the stability of EGF in the colonic lumen of normal animals was demonstrated. In this chapter, similar techniques were applied to determine the uptake and intactness of EGF in the ulcerated colonic lumen. Although little uptake of radiolabelled EGF from the distal colonic lumen was observed in normal animals, the rate of uptake of EGF from the lumen of colitic animals.may be altered given the reduction in mucin production (Poldosky and Isselbacher 1984,Cope et al. 1988) and altered permeability of the colonic epithelia (Olaison et al. 1989) observed in human colitis conditions.

Methods

Animals and Experimental Design

Induction of Colitis

The method for inducing a colitis-like condition in the rat was the same as the method developed in Chapter 4 in which a 2cm segment of the distal colonic lumen was exposed to an intraluminal bolus of acetic acid. In brief, Scovall clamps were placed across the colon 1cm and 3cm distal to the vein, thereby occluding the lumen of a 2cm distal colonic segment. A 700µl bolus of 5%(v/v) acetic acid was injected through a 27g needle into the colonic lumen at the proximal end of the segment. Sham operated animals received a 700µl bolus of 0.9% NaCl into the colonic lumen instead of acetic acid. After a 60 second exposure period the distal-most clamp was removed and the acetic acid was flushed from the colon by injecting a 2ml bolus of air followed by an 8ml bolus of 0.9% NaCl into the proximal end of the segment. The proximal clamp was then removed. For subsequent identification of the acid-exposed intestinal segment, a 6-0 silk suture was placed in the colonic mesentery adjacent to the position of each clamp, and the mid-line incision was closed. Animals were returned to metabolism cages and were allowed free access to water and standard laboratory rat chow (Milling Industries, South Australia).

Subcutaneous and Luminal EGF delivery

Rats were treated with EGF for 4 or 6 days following induction of ulceration with acetic acid, with subcutaneous and luminal routes of EGF administration compared in the same study. Colonic ulceration was induced in 39 male Sprague Dawley rats and 14 animals were sham operated. A 1ml blood sample was taken from the tail vein of each animal immediately after induction of anaesthesia, and centrifuged at 2000g for 10 minutes. Plasma was collected and stored frozen at -20°C. The methods used for administering exogenous EGF to ulcerated rats were the same as those used in the normal rat study in Chapter 3. Subcutaneous administration was achieved via osmotic minipumps (Alzet model 2001, Alza Corporation Palo Alto, U.S.A.) implanted in the supra-scapular region, while luminal administration was via a cannula surgically implanted in the colonic lumen. A silastic cannula plus an osmotic minipump were implanted into all animals at the same time as acetic acid administration. The silastic cannula was implanted into the lumen of the distal colon 1cm proximal to the acid-exposed segment, anchored to the colonic wall using a purse string suture and externalised through the supra scapular region. At the completion of surgery animals were returned to metabolism cages for daily measurement of food intake and body weight. Urine samples collected over the 24 hour period prior to the commencement of treatment and on the final treatment day, were stored frozen at -20°C prior to determining EGF content by radioimmunoassay.

The preparation of EGF solutions was the same as that described in Chapter 3. The doses of EGF given to ulcerated animals were also identical to those administered to normal rats in Chapter 3 such that the subcutaneous EGF-treatment group (n=13) received recombinant human EGF (Chiron Corp. Emeryville, U.S.A.) via the osmotic pump at a dose rate of 200μg/kg body weight/day while all other groups were implanted with pumps containing 0.9%NaCl(w/v). The luminal EGF treatment group (n=13) received 12 hourly injections of 200μg EGF in 1ml of 0.9% NaCl(w/v) via the implanted cannula and all other groups had bolus injections of 1ml 0.9%NaCl(w/v). The vehicle-treated control group (n=13) and sham operated animals (n=13) received 0.9%NaCl(w/v) via both routes of administration (Figure 5.1).

Animals from each experimental group were killed by decapitation at 4 or 6 days after acetic acid administration. Plasma was obtained from trunk blood and frozen as for tail vein blood. After the colon was removed, the acetic acid-exposed distal colonic segment was located and its length was measured before the damaged segment was excised. In addition, a 1cm segment adjacent to the proximal end of the damaged region (referred to as the adjacent segment) was taken for determination of the proliferative responses at the margins of the exposed segment. Finally, a 1cm piece of tissue was taken 2cm from the cecal /colonic junction, approximately 7cm from the ulceration (referred to as the undamaged segment), to assess the effect of EGF on proliferation of an undamaged region of the colon. The three segments were opened longitudinally and fixed in Methacarn as described in the time-course recovery trial.

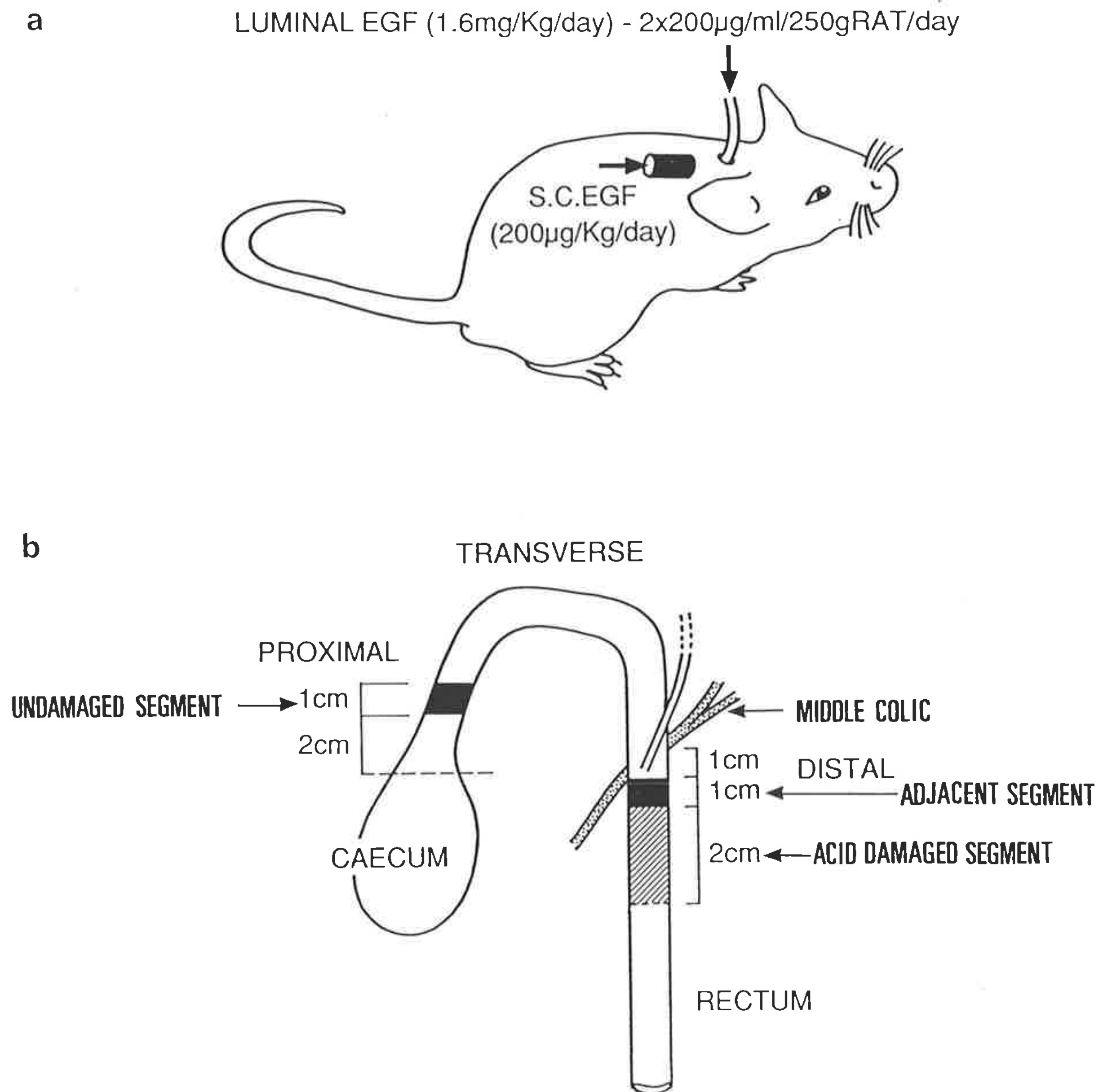


Figure 5.1 (**a**) Routes and doses of exogenous EGF administered to acetic acid-exposed rats and (**b**) the location of colonic segments sampled for histological analysis in the EGF trial.

Stability of EGF in the colonic lumen of ulcerated rats

The rate of degradation of ^{125}I-labelled EGF in the proximal and distal colon of rats 4 and 6 days after acetic acid exposure was measured *in vivo* as described in Chapter 3 to determine the stability of EGF in the lumen of the ulcerated colon. Iodonated EGF, prepared by the method of Read et al. (1986), was provided by Mr. C. Gillespie (Child Health Research Institute, North Adelaide). Acetic acid colitis was induced in 19 male, 250g Sprague Dawley rats. At 4 (n=8) or 6 (n=11) days after acetic acid exposure animals were anaesthetised with 8ml/kg body weight of urethane (180g/l) and body temperature was maintained by placing animals onto a heated blanket. As in the study in normal rats in Chapter 3, loops of the proximal (undamaged) and distal colon (containing the ulcerated segment) were were isolated by placing 3-0 silk sutures around the colon at the point of cecal/colonic junction, the middle colic vein and at the position adjacent to the pelvic bone. A 1ml bolus of 0.9%(w/w) NaCl containing 200µg unlabelled EGF and 53ng ^{125}I-labelled EGF (200,000cpm) was injected through a 27g needle into the proximal end of each loop. A thermometer was inserted into the abdomen to monitor the temperature of the intestinal preparation, and the abdominal incision was partially closed using wound clips. Animals were maintained under anaesthesia for one or four hours before the loops were exised and luminal flushings and tissue collections were carried out as in the EGF stability trial performed on normal rats described in Chapter 3.

To estimate the amount of radioactivity that had been transferred from the colonic lumen into the circulation a 1ml blood sample was collected from the vena cava of each animal prior to sacrifice. The cpm was measured and the total blood volume per animal was calculated as 6% of the body weight (Garcia 1957). The distribution and stability of ^{125}I-labelled EGF in the proximal and distal colon were determined by measuring the cpm and TCA precipitability of luminal flushings and tissue homogenates as described in Chapter 3.

Analytical Measurements

The severity of the colonic ulceration and the extent of morphological changes associated with acetic acid exposure were measured in the time-course study and EGF trial using histological techniques. Methacarn fixed tissues were embedded in paraffin with the acetic acid exposed segment orientated longitudinally to allow measurement of the length of the segment covered by epithelium. The adjacent and undamaged segments were embedded transversely. 2μm sections were taken at 1mm intervals at 6 tissue levels in the acetic acid-exposed segment, and at 3 tissue levels in the adjacent segment and in the undamaged segment. Sections were stained with hematoxylin and eosin and viewed using a Zeiss Jenaval light microscope (Jena, Germany).

A semi-quantitative histological score was developed to determine the extent of edema and amount of inflammatory infiltrate in the submucosal layer of the acetic acid exposed segment, and the submucosal and muscularis externa layer of the adjacent segment. Histological grading was determined in a blinded fashion. The extent of edema was graded as follows: 0, no edema; 1, mild edema; 2, moderate edema; and 3, severe edema containing few stromal cells and extensive areas of collagen and fluid. Inflammatory infiltrate was graded as follows: 0, no inflammation; 1, mild inflammation; 2, moderate inflammation; and 3, severe inflammation with large numbers of inflammatory cells (Plate 5.1).

Quantitative morphometric measurements were made from images acquired with a JVC video camera and digitized by a PRISM Image Analysis software system (Dapple Systems Inc., Sunnyvale, U.S.A.) coupled to an Apple Macintosh II cx computer. In the acetic acid-exposed segment, the percentage length of the segment covered by intact epithelium was calculated by measuring the total length of the acetic acid exposed segment and the length of the segment covered by intact epithelium. Intact epithelium was defined as crypt-like structures including both immature and mature crypts. In the adjacent segment the mean crypt length and the number of cells per crypt column were measured in 30 crypts well-orientated from their base at the muscularis mucosae to the luminal surface on the basis of the validation trial performed in Chapter 3. The thickness of the mucosa (from the

luminal surface to the base of the muscularis mucosae), the submucosa (from the base of the muscularis mucosae to the muscularis externa) and the muscularis externa layers were each expressed as a mean thickness derived from measurements taken at 3 tissue levels. Immunohistochemical detection of proliferating cell nuclear antigen (PCNA), was performed as described on the adjacent segment by the method described in Chapter 3. The PCNA labelling index and the PCNA labelled crypt fraction was derived from the frequency of positive PCNA labelled cells counted in 30 well orientated crypt columns per colon sample based on the results from the validation trial performed in Chapter 3.

Measurement of EGF in urine and plasma

Immunoreactive EGF was measured in plasma and urine collected at the commencement and completion of the experimental period using the method described in Chapter 3.

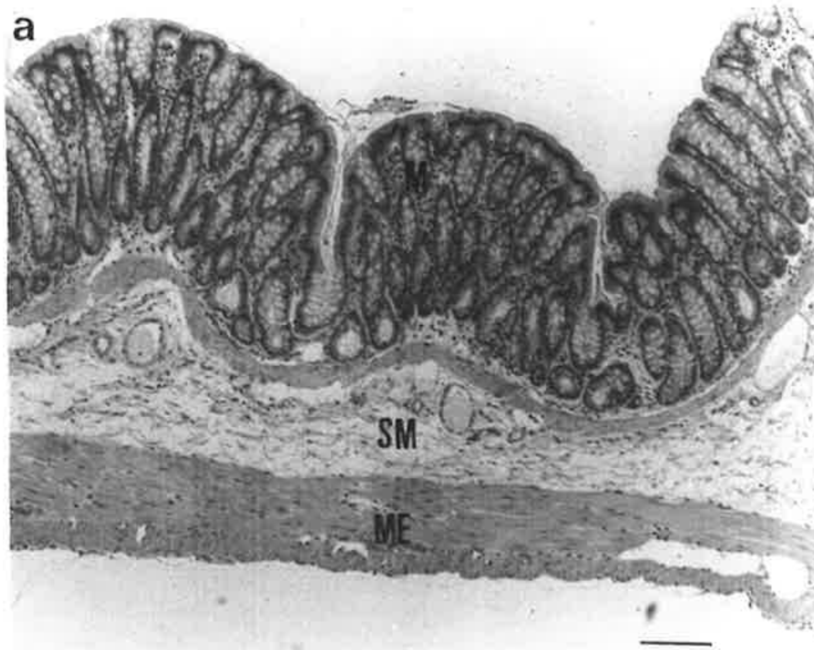
Statistical Analysis

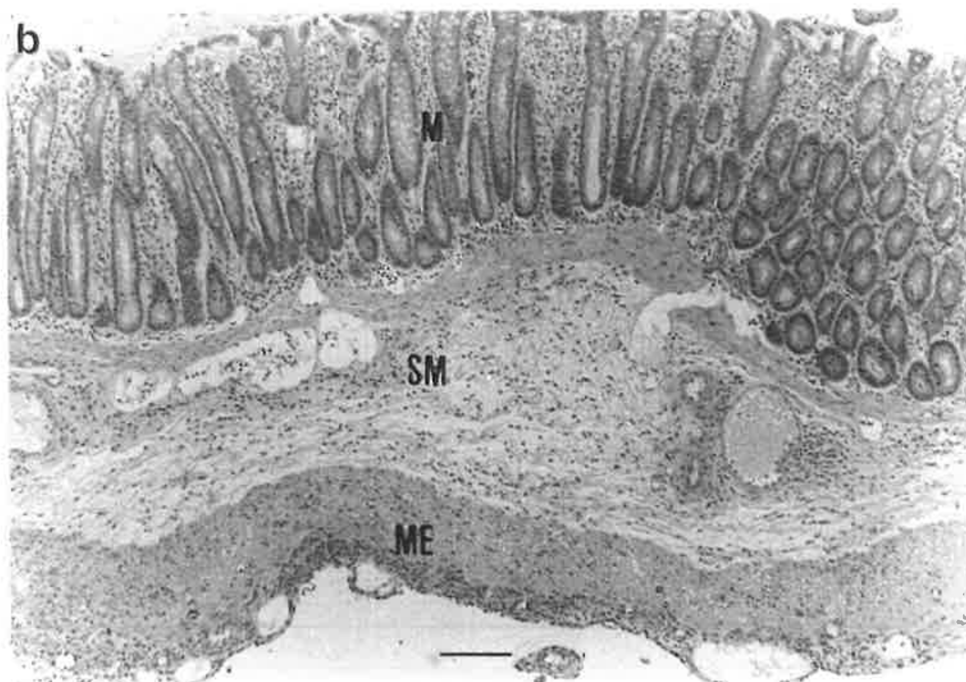
Data are expressed as mean ± standard error of the mean, unless otherwise stated. Differences between groups were analysed by a two-way analysis of variance in which the data was classified by two factors, treatment group and time of treatment. A significant interaction effect between the two factors was detected in the crypt length and submucosal thickness measurements of the adjacent segment, so that 4 and 6 day data were analysed separately by one-way analysis of variance (see Appendix B for statistics tables). No effect of the time of treatment or any interaction effect was detected in all other parameters measured and hence the data from 4 and 6 days was pooled for statistical comparison (for pooled mean±sem data see Appendix B). Where a significant effect of treatment was apparent by ANOVA comparisons between treatment groups were made using Fisher's PLSD post hoc tests.

Acknowledgments

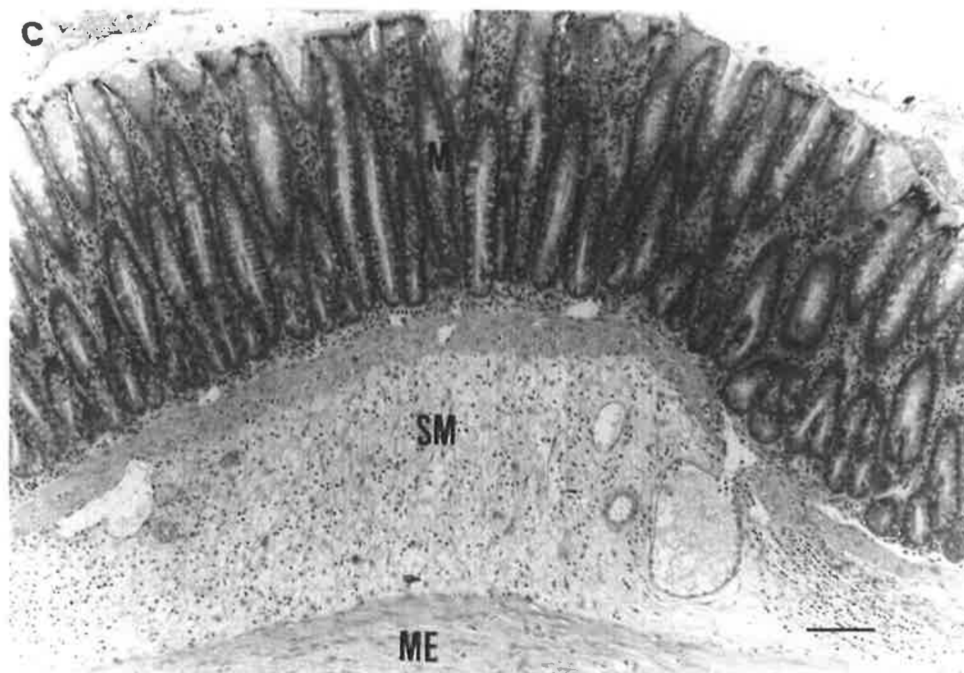
The experimental design, all animal surgery, sample collections, quantitative histological measurements, stability studies and EGF radio-immunoassays were performed by the candidate. The calculation of the PCNA labelling index and labelled crypt fraction were performed by the candidate, although immunohistochemical staining and counting of positively labelled cells, together with the semi-quantitative scoring of the extent of edema and inflammatory infiltrates were performed by Mr. G. Howarth. Iodonation of recombinant human EGF was performed by Mr. C. Gillespie. Hematoxylin and eosin staining was performed by the candidate, although cutting of paraffin-embedded histological specimens was performed by Mrs. K. Davey.

Plate 5.1(a) - Adjacent segment of the distal colon from a sham-operated rat 4 days after surgery showing the mucosal (M), submucosal (SM) and muscularis externa (ME) layers. Submucosal edema grade=0, inflammation grade=0. **(b-d)** - Sections of the distal colonic segment adjacent to the acetic acid-damaged segment 4 days after the acid insult. Submucosal edema grade and inflammation grade is given below each plate.
Calibration bar=100μm

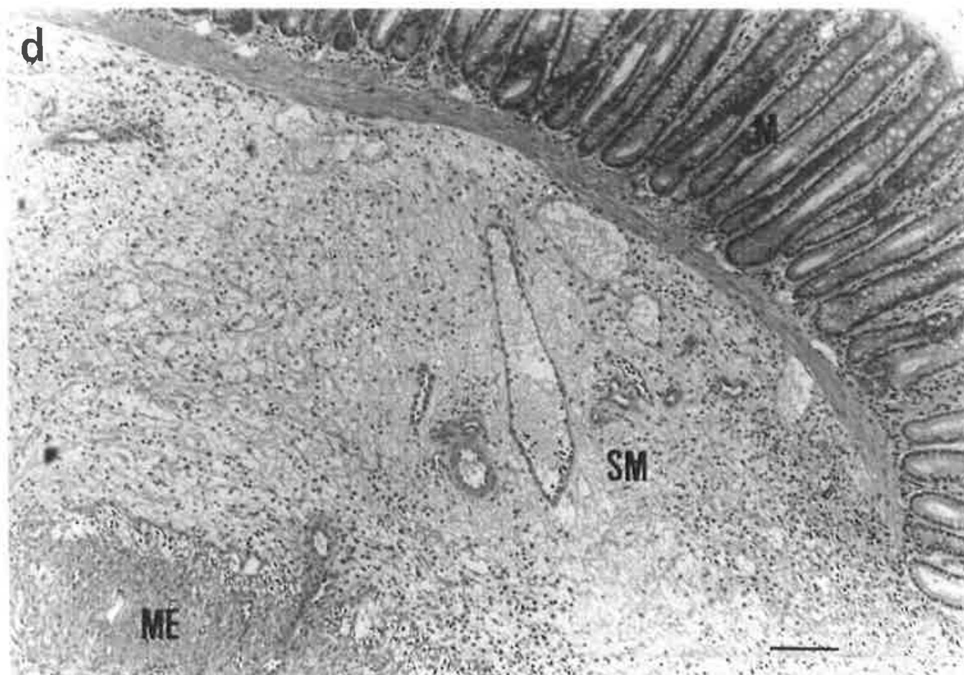




Submucosal edema grade=1, Submucosal inflammation grade=1



Submucosal edema grade=2, Submucosal inflammation grade=1



Submucosal edema grade=3, Submucosal inflammation grade=1

Results

Plasma and urinary concentrations of EGF

Plasma EGF was not detectable in control animals and subcutaneous or luminal EGF treatment did not increase the plasma EGF above detectable levels. Although I attempted to measure urinary EGF levels there was a lack of parallelism between the EGF standard curve of the radioimmunoassay and serial dilutions of urine from ulcerated animals. This suggested that the urine of ulcerated animals contained components which interfered with the binding of the antibody to radiolabelled EGF prohibiting an accurate measure of urinary EGF content by radioimmunoassay. However in Chapter 3, I showed that an identical dose and infusion protocol increased urinary EGF levels 4-fold, although plasma EGF was not significantly elevated, a result that was presumed to reflect a rapid clearance of EGF from plasma to urine.

Stability of EGF in the colonic lumen of ulcerated rats

The stability of EGF in the colonic lumen of acetic acid-treated rats was determined at 4 and 6 days post-acetic acid exposure, the end-points chosen to assess the effects of EGF on ulcer repair. In each experiment, a bolus of ^{125}I-labelled EGF was infused simultaneously into a loop of proximal colon, which had no direct exposure to acetic acid and into a loop of distal colon, which had been exposed to acetic acid 4 or 6 days earlier. At 4 days after the acetic acid insult, 57.5% and 29.7% of the infused radioactivity was recovered from the proximal colon unexposed to acetic acid after one and four hours respectively (Table 5.1). Most of the radioactivity was recovered from the lumen with only 6.2% and 4.0% transferred to the colonic tissue at one and four hours. In the distal colon directly exposed to acetic acid, a much lower proportion of the infused material was recovered at either one hour (27.2%) or four hours (15.7%) after administering the ^{125}I-labelled EGF into the colonic lumen, suggesting that the infused material was rapidly cleared from the lumen of the acid damaged distal colon. The distribution of radioactivity in the distal colon was comparable to that seen in the proximal colon, with the majority of the radioactivity present in the colonic lumen rather than colonic tissue. It would therefore

appear that radiolabelled EGF was transferred rapidly from the lumen to the circulation, rather than retained in the colonic tissue.

The intactness of ^{125}I-labelled EGF recovered from the colonic tissue or lumen was estimated by TCA precipitability. On this basis, the ^{125}I-labelled EGF recovered from the colonic tissue and lumen of the proximal colon was estimated to be greater than 90% intact at one hour and decreasing to 72.6-77.3% intact at four hours after infusion into the colon (Table 5.1). EGF was apparently degraded more rapidly in the distal ulcerated colon, so that 77.9% and 84.2% of the infused radioactivity was TCA precipitable in the luminal wash and colonic tissue respectively at one hour. The proportion of intact material declined even further by four hours to 33.2% and 62.6% TCA precipitable in the luminal wash and tissue respectively The distribution and clearance of ^{125}I-labelled EGF infused into the colonic lumen at 6 days after the acid insult was comparable to theat observed at 4 days with the exception that recovery of radioactivity from the proximal undamaged colon one hour after the infustion (32.9%) was substantially lower than that recovered at 4 days after the acid insult (57.5%) (Table 5.2). A comparable decline in TCA precipitability of ^{125}I-labelled EGF between 1 and 4 hours after infusion to that seen at 4 days after the acid insult was observed at 6 days.

Less than 4% of the infused radioactivity was measured in the blood at 1 and 4 hours after intraluminal ^{125}I-labelled EGF infusion in either the 4 or 6 day experiments. The lack of correlation between the progressive loss of ^{125}I-labelled EGF from the colon and appearance in blood, presumably reflects rapid clearance from the circulation into other tissues.

Table 5.1 Stability of EGF in the colonic lumen of rats 4 days after acetic acid insult

	Proximal Colon (not exposed to acetic acid)		Distal Colon (exposed to acetic acid 4 days prior to stability study)	
Hours after ^{125}I-labelled EGF Infusion	1	4	1	4
N	4	4	4	4
% infused radioactivity recovered in the luminal wash	51.5±10.6	26±8.0	20.5±7.1	11±2.8
% infused radioactivity recovered in the colonic tissue	6.2±1.6	4.0±1.4	7.0±1.6	4.7±1.0
% total recovery	57.5	29.7	27.2	15.7
% TCA precipitable radioactivity in luminal wash	94.9±3.1	77.3±6.1	77.9±5.8	33.2±5.1
% TCA precipitable radioactivity in colonic tissue	90.7±5.4	72.6±9.5	84.2±5.1	62.6±8.8

Data are expressed as the mean ± SEM
The recovery of infusate in the luminal wash and colonic tissue at the end of the exposure period is expressed as a percentage of the counts in the infusate. The percentage of TCA precipitable material was calculated as the cpm in the pellet expressed as a percentage of the total cpm in the sample aliquot.

Table 5.2 Stability of EGF in the colonic lumen of rats 6 days after acetic acid insult.

	Proximal Colon (not exposed to acetic acid)		Distal Colon (exposed to acetic acid 6 days prior to stability study)	
Hours after ^{125}I-labelled EGF Infusion	1	4	1	4
N	5	6	5	6
% infused radioactivity recovered in the luminal wash	27.2±9.3	19.0±5.7	17.7±9.7	11.5±0.3
% infused radioactivity recovered in the colonic tissue	5.7±1.2	6.0±2.1	11.2±4.9	8.0±1.4
% total recovery	32.9	25	28.9	19.5
% TCA precipitable radioactivity in luminal wash	91.3±4.9	53.9±13.0	83.8±6.3	53.3±1.4
% TCA precipitable radioactivity in colonic tissue	92.5±2.6	83.2±11.0	92.6±3.0	70.2±5.9

Data are described as in Table 5.1

Effect of 4 and 6 days treatment with EGF on repair of the damaged segment

Comparison between the sham group, not exposed to acetic acid, and the vehicle-treated control group shows that acetic acid exposure significantly suppressed body weight gain, particularly during the first 4 days post-acid insult.(Table 5.3). This was associated with a significantly lower average feed intake in all acetic acid-exposed groups compared to sham operated animals.

Table 5.3 The effect of acetic acid exposure and EGF treatment on daily body weight and feed intake.

	Sham	Vehicle	S.C. EGF	Luminal EGF
4 DAYS				
N	7	7	7	7
Average Body weight Change (g) over the treatment period	16.9±4.5 *	-5.4±6.1	-5.9±6.7	-1.2±4.5
Average Feed intake (g/kg body weight)	60.9±2.7 *	33.3±3.8	27.4±6.3	26.2±3.6
6 DAYS				
N	7	6	6	6
Average Body weight Change (g) over the treatment period	24.1±4.1 *	7.3±3.2	-0.9±5.7	4.0±11.9
Average Feed intake (g/kg body weight)	64.7±6.5 *	47.6±3.4	39.0±4.3	34.5±7.9

Data are expressed as mean ± SEM. N represents the number of animals in each treatment group. Body weight change and feed intake are shown for the duration of the treatment period.

As in the time-course trial described in Chapter 4, severe disturbances in gross colonic morphology were apparent in the exposed segment 4 or 6 days after acetic acid administration in vehicle-treated controls. The length of the exposed region, defined by mesenteric sutures placed at the margins of the region at the time of acetic acid treatment, was measured at the time of killing the animal to determine if significant contraction of the 2cm damaged segment occurred over the 4 and 6 day experimental periods. In the sham-operated animals the mean length of the distal colonic segment bordered by the 2 marker sutures was 1.76±0.10cm. No significant difference in segment length was apparent at either 4 or 6 days in any of the acetic acid exposed groups so that the mean segment length of the combined 4 and 6 day groups was 2.70±0.05cm, 1.93±0.06cm and 2.05±0.07cm in the vehicle, subcutaneous and luminal EGF groups respectively.

In acetic acid-exposed control (vehicle-treated) rats the extent of epithelial denudation at 4 days was similar to that at 6 days, with 10.7% and 9% of the segment covered by epithelium, respectively (Table 5.4). This suggested a more severe ulceration than observed in the time course trial performed in Chapter 4, where some re-epithelialization, albeit variable between animals, was apparent at 6 days. Although subcutaneous EGF treatment appeared to induce a slight increase in the percentage length of the damaged segment covered by epithelium at 6 days, the response was variable between animals so that no significant effect of subcutaneous EGF compared with vehicle-treated controls was observed at either 4 or 6 days. A similar lack of enhanced re-epithelialization of the ulcerated colon was also observed following luminal EGF treatment at both 4 and 6 days.

Table 5.4. The effect of acetic acid exposure and EGF treatment on the percentage length of the distal colonic segment covered by epithelium

	Sham	Vehicle	S.C. EGF	Luminal EGF
4 DAYS				
N	7	7	7	7
% Epithelium	100	10.7±4.8	10.4±2.2	8.3±4.4
6 DAYS				
N	7	6	6	6
% Epithelium	100	7.9±3.9	16.7±16.7	9.5±4.5

Data are expressed as mean ± SEM. N represents the number of animals in each treatment group. All ulcerated groups contain significantly less epithelium than the sham operated group at both 4 and 6 days ($P<0.0001$).

In the submucosa of the acetic acid exposed segment, a severe edematous response and large number of inflammatory infiltrate cells were observed at both 4 and 6 days. A median severity score of 3 was achieved for the vehicle-treated group, with a slightly improved median score of 2 in both subcutaneous and luminal EGF-treated animals. Damage of the muscularis externa layer associated with adhesions to surrounding intestinal tissue prevented an accurate estimate of the severity of edema and immune infiltration in this layer. The presence of adhesions also prevented an accurate measurement of the thickness of the submucosa and muscularis externa layers of the acetic acid-exposed segment (Plate 5.2).

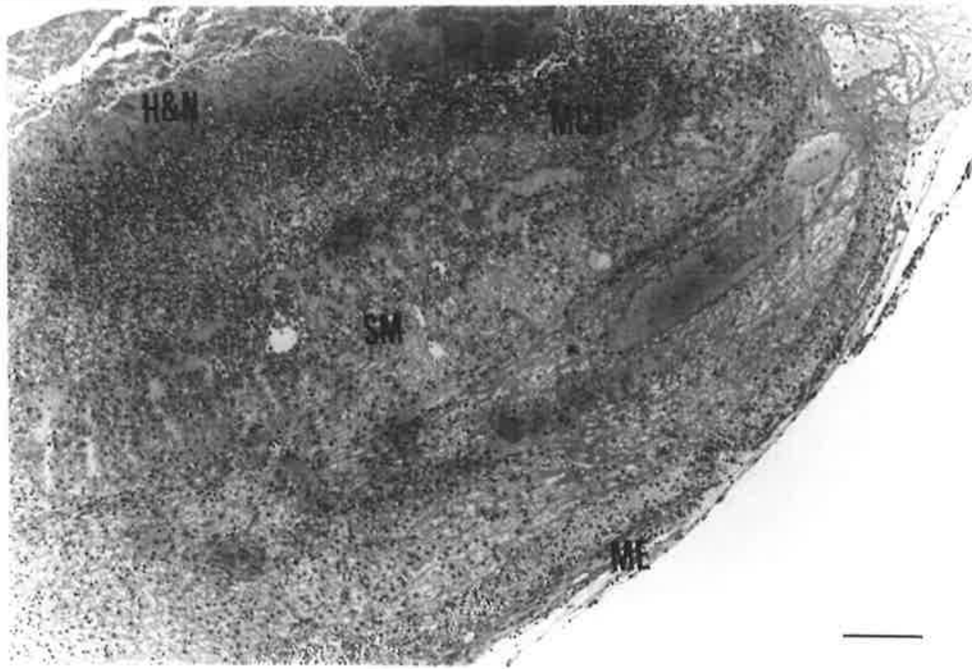


Plate 5.2 Acetic acid damaged colonic segment 4 days after the acid insult showing hemmorhage and necrosis (H&N) in the denuded mucosal layer and mixed cellular infiltrate (MCI) in the submucosal (SM) and mucosal layers. Damage to the muscularis externa (ME) layers, due to fragility of the segment and adhesions with other organs, prohibited an accurate measurement of the muscularis externa and submucosal layers of the acid damaged segment to be made.

Responses in the colonic segment adjacent to the ulceration:

(a) Effects of acetic acid

Acetic acid exposure increased the total mucosal thickness of the segment adjacent to the margins of the exposed region by approximately 50% at both 4 and 6 days compared with sham operated rats (Figure 5.2a) ($P<0.001$, 4 and 6 day means pooled, see Table B.4). Since the mucosal thickness measurement included multiple layers, namely the crypt epithelia, muscularis mucosae and lamina propria, more detailed histological measurements of the crypt epithelia were made to characterise the mucosal response to acetic acid. Acetic acid exposure resulted in a marked epithelial hyperplasia characterised by a 70% increase in the crypt length ($P<0.001$, 4 and 6 day means pooled, see Table B.6) and a corresponding increase in the cell number/crypt ($P<0.001$) at both 4 and 6 days after the acid insult (Figure 5.2b and 5.2c). This was associated with changes in the size and distribution of the proliferative zone of the colonic crypts in the distal colon. Thus, the PCNA labelling index of all acetic acid-exposed groups at both 4 and 6 days after the acid insult was significantly increased ($P<0.001$,4 and 6 day means pooled, see Table B.11) above that of sham-operated animals, indicating that a higher proportion of crypt cells were capable of cell division in the mucosa at the margins of the damaged colonic segment than in the normal colon. The shape of the PCNA labelling distribution curves also varied between the sham-operated controls and acetic acid-exposed vehicle group (Figure 5.3). In the control animals, maximal PCNA labelling was achieved in crypt cells at positions 1-10 in the crypt column and maintained up to cell position 20 (approximately 40% of the crypt column) after which the labelling index declined rapidly. At the margins of the ulcerated distal colon of acetic acid-exposed animals, the maximal PCNA labelling index was maintained up to cell position 40 (approximately 55% of the crypt column) prior to a rapid reduction in the labelling index. Changes in the distribution of PCNA labelled cells in the crypt were reflected by a significant increase in the magnitude of the PCNA labelled crypt fraction in all acetic acid exposed groups compared to sham-operated controls (Table 5.5)

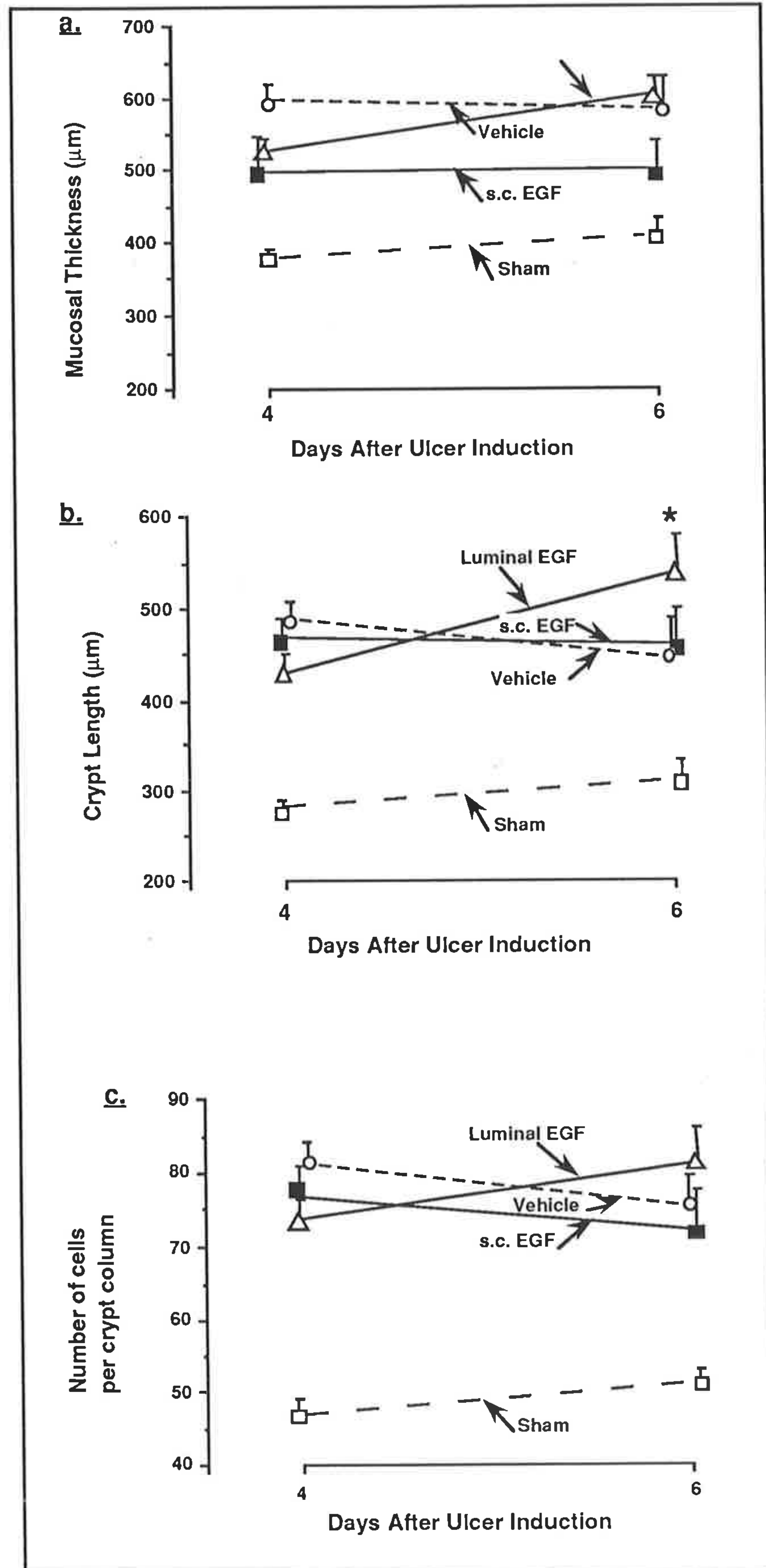


Figure 5.2 The effect of EGF treatment on the (a) mucosal thickness, (b) crypt depth and (c) number of cells per crypt column of the colonic segment adjacent to the ulceration. Data are expressed as mean + SEM for the sham-operated (——□——), vehicle (-- o ---), s.c. EGF (——■——) and luminal EGF (——▲——) treatment groups. * P<0.05 (vs vehicle).

Table 5.5. PCNA labelling indices of the colonic mucosa adjacent to the ulceration.

	Sham	Vehicle	S.C. EGF	Luminal EGF
<u>4 DAYS</u>				
Labelling Index(%)	50±1.6	57±0.5	57±1.1	56±1.4
PCNA Labelled Crypt Fraction (%)	63±1.9	73±1.5	69±1.3	68±2.4
<u>6 DAYS</u>				
Labelling Index(%)	49±1.1	53±1.7	57±1.0	55±1.0
PCNA Labelled Crypt Fraction (%)	62±3.4	68±1.8	70±2.3	72±1.5

Data are expressed as mean ±SEM. Data are derived from measurements taken from 30 crypt columns. Labelling index is defined as the number of PCNA labelled cells per total cells counted and the PCNA labelled crypt fraction is defined as the proportion of the crypt occupied by PCNA labelled cells. All ulcerated groups have a significantly higher PCNA labelling index ($P<0.0001$) and PCNA labelled crypt fraction ($P<0.01$) than the sham-operated group at both 4 and 6 days.

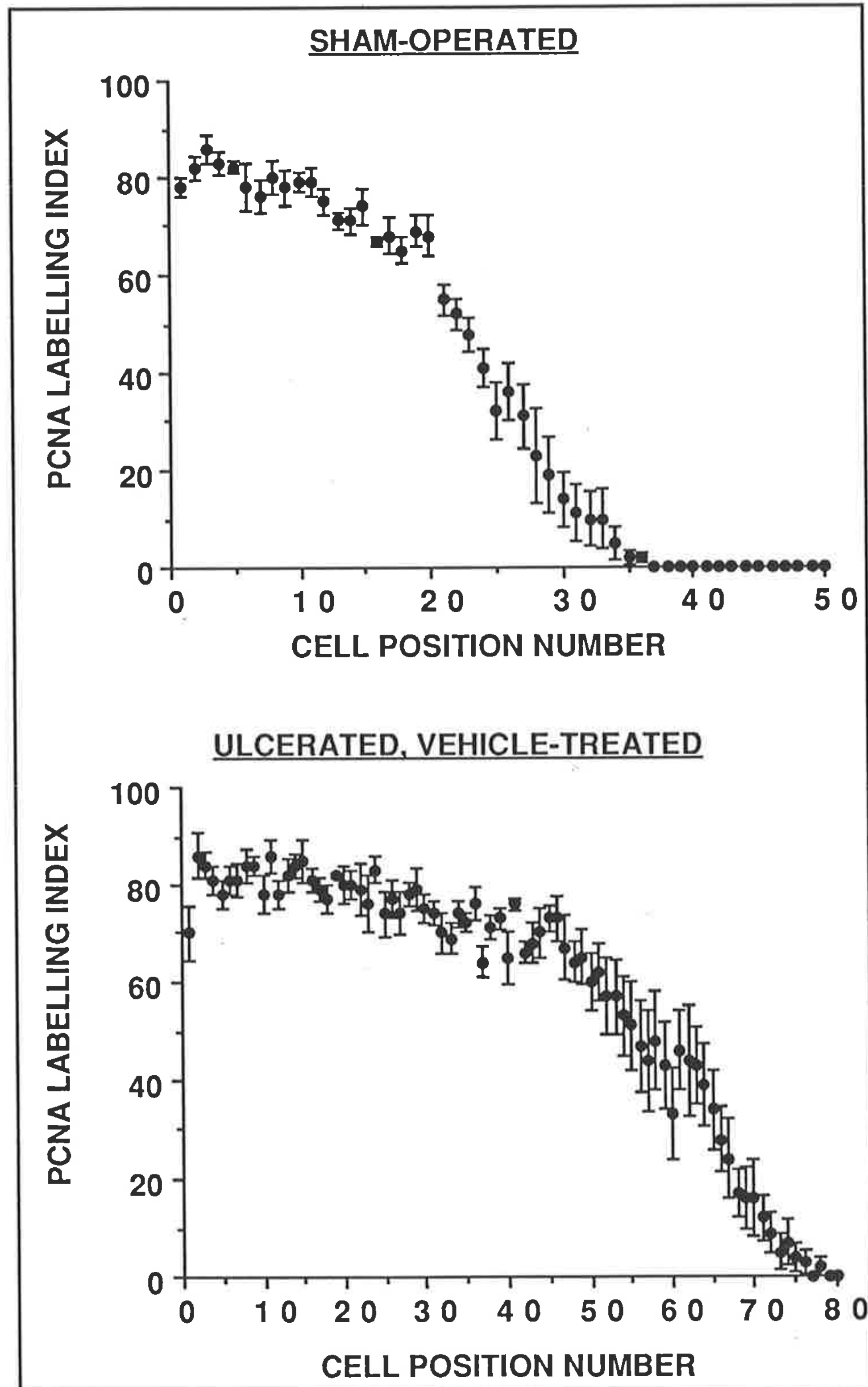


Figure 5.3 PCNA labelling index distribution curves from the segment adjacent to the ulceration of sham operated (n=7) and vehicle-treated (n=7) animals 4 days after the acid insult.

As well as increasing the width of the adjacent segment mucosa, acetic acid exposure altered the thickness and morphology of the submucosal and muscularis externa layers in the same region. Four days after the induction of colitis the thickness of the submucosal layer in the vehicle-treated (control) group was increased 2.4-fold above that of sham-operated animals ($P<0.01$) (Figure 5.4a). The increased submucosal thickness could be attributed to severe edema (median histology score of 3 at day 4) together with mild inflammatory infiltrate (median score of 1 at day 4). By day 6, submucosal thickness (Figure 5.4a) and edema (median score of 0) had returned to normal, although the mild inflammatory infiltrate (score of 1) remained. Muscularis externa thickness was significantly increased by 21% in the vehicle-treated group above that of the sham-operated control group ($P<0.01$, 4 and 6 day means pooled, see Table B.18). This thickening was due to a mild inflammatory infiltrate and edema in this layer, with a median histology score of 1 given for both parameters at 4 and 6 days.

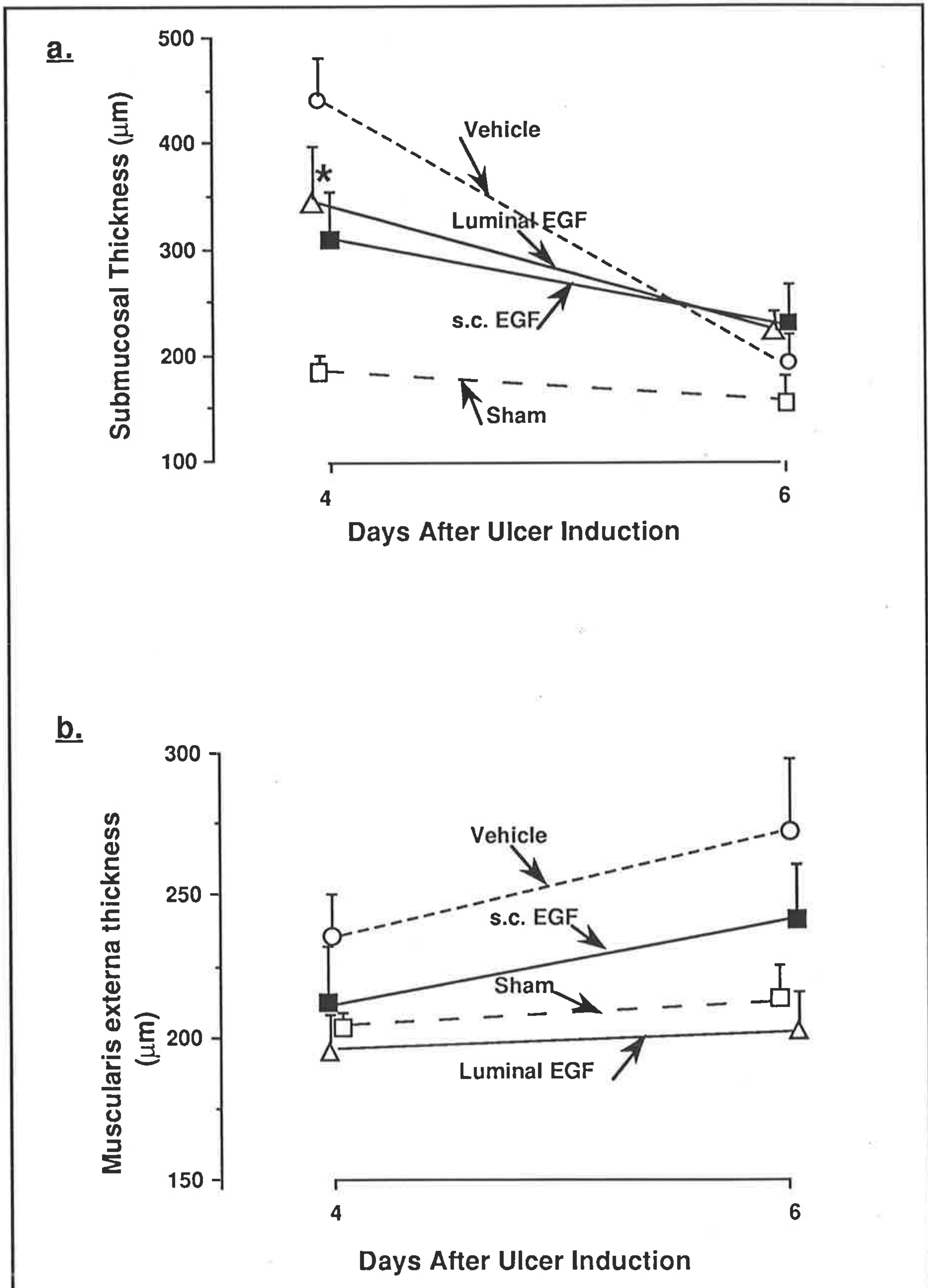


Figure 5.4 The effect of acetic acid-induced ulceration and EGF treatment on the thickness of the (a) submucosa and (b) muscularis externa layers of the colonic segment adjacent to the ulceration. Data are expressed as mean+SEM for the sham-operated (— —□— —), vehicle (-- o---), s.c. EGF (——■——) and luminal EGF (——△——) treatment groups at 4 or 6 days. * P<0.05 (vs vehicle group).

In summary, acetic acid exposure induced an increase in the thickness of the mucosal layer of the adjacent segment at 4 and 6 days after acid exposure, due in part to crypt epithelial hyperplasia. The thickness of the submucosal layer was also increased at 4 days due mainly to edema with some associated inflammatory infiltrate, although the thickening was resolved by 6 days. An increase in the muscularis externa thickness also resulted from acetic acid treatment at 4 and 6 days which was contributed to by edema and inflammatory infiltrate.

Responses in the colonic segment adjacent to the ulceration:
(b) Effects of subcutaneous and luminally administered EGF

The adaptive responses to acetic acid exposure observed in the colonic segment adjacent to the ulceration were attenuated by subcutaneous EGF treatment. Approximately 50% of the increased mucosal thickness induced by acetic acid was prevented by subcutaneous EGF infusion at both 4 and 6 days (Figure 5.2a). However, this effect was not accompanied by any reduction in the crypt length, cell number per column (Figures 5.2b and 5.2c) or PCNA labelling indices (Table 5.5). This suggests that the attenuation of the thickening of the mucosal layer was due to a decreased thickness of the lamina propria or muscularis mucosae layers, rather than the crypt epithelium, so that subcutaneous EGF was attenuating an inflammatory or edematous response rather than reducing crypt proliferation. As further support for an attenuated inflammatory response, subcutaneous EGF also reduced the acetic acid-induced thickening of the submucosal layer by approximately 40% at 4 days (Figure 5.4a), and caused a slight reduction in the width of the muscularis layer at both 4 and 6 days (Figure 5.4b). The reduction in submucosal thickening was mirrored by an attenuation of the edematous response as shown by a reduction in the severity grade from 3 (severe) in the vehicle group to 2 (moderate) in the subcutaneous EGF group. No change in the severity of the submucosal inflammatory infiltrate or the severity of the muscularis edema and infiltrate was observed in EGF-treated animals compared to the vehicle-treated control group.

In contrast to subcutaneous EGF treatment, luminal EGF had no significant effect on the mucosal thickness at 4 or 6 days (4 and 6 day means pooled, see Table B.4), although there was a trend towards a decreased mucosal thickness of the luminal EGF-treated group at 4 days compared with vehicle-treated control rats (Figure 5.2a). Despite the lack of effect of luminal EGF on mucosal thickness, there was a 20% increase in the crypt length between the luminal EGF-treated and vehicle-treated groups ($P<0.05$) accompanied by an 8% increase in the number of cells/crypt column at 6 days (Figures 5.2b and 5.2c). This was not accompanied by a change in the proliferative compartment as no change was observed in the PCNA labelling index or the PCNA labelled crypt fraction (Table 5.5). Thus, the similarity in mucosal thickness between the vehicle and luminal EGF-treated groups may have reflected a counteracting effect between a reduction in inflammation of the lamina propria or muscularis mucosae and an increased crypt length in the EGF-treated group.

The effects of luminal EGF on the submucosa were similar to the responses to subcutaneous EGF, so that both routes of EGF administration suppressed the acetic acid-induced thickening of the submucosa at 4 days, and caused a moderate reduction in edema, without affecting the mild inflammatory infiltrate seen in vehicle-treated animals (Figure 5.4c). In the muscularis externa layer, luminal EGF was more potent than subcutaneous EGF, such that the muscularis thickness was reduced to that of sham-operated animals (Figure 5.4b, 4 and 6 day means pooled see Table B.18), although no change in the severity of the edema or inflammatory infiltrate was seen.

The effect of subcutaneous EGF infusion on an undamaged colonic segment proximal to the acid-exposed segment.

The thicknesses of the mucosa, submucosa and muscularis externa layers were also measured in a colonic segment approximately 7cm proximal to the damaged site to determine if the undamaged colon underwent any adaptive responses. As this region was not directly exposed to the luminal EGF, comparisons were made between the sham-operated, vehicle-treated and subcutaneous EGF-treatment groups only. No significant interaction or time

effects were detected by two-way analysis of variance so 4 and 6 day means were pooled (see Tables B.20, B.22, B.24). The acetic acid-induced colitis did not significantly alter the thickness of the mucosal, submucosal or muscularis layers of this colonic segment although there was a trend for a reduction in the muscularis externa layer (Figure 5.5). The effects of EGF on the undamaged segment contrasted with the responses in the region adjacent to the damaged site. Rather than a decrease in mucosal thickness, the mucosal width was increased in subcutaneous EGF-treated animals by 14% and 23% ($P<0.05$) above the vehicle-treated and sham-operated groups, respectively (Figure 5.5a). EGF treatment also increased the thickness of the muscularis externa layer by 20% compared with vehicle-treated animals, although the effect was not significant (Figure 5.5c). In contrast to the marked reduction in the submucosal thickness seen in the adjacent segment, no effect of EGF was measured in the submucosal layer of the undamaged region (Figure 5.5b).

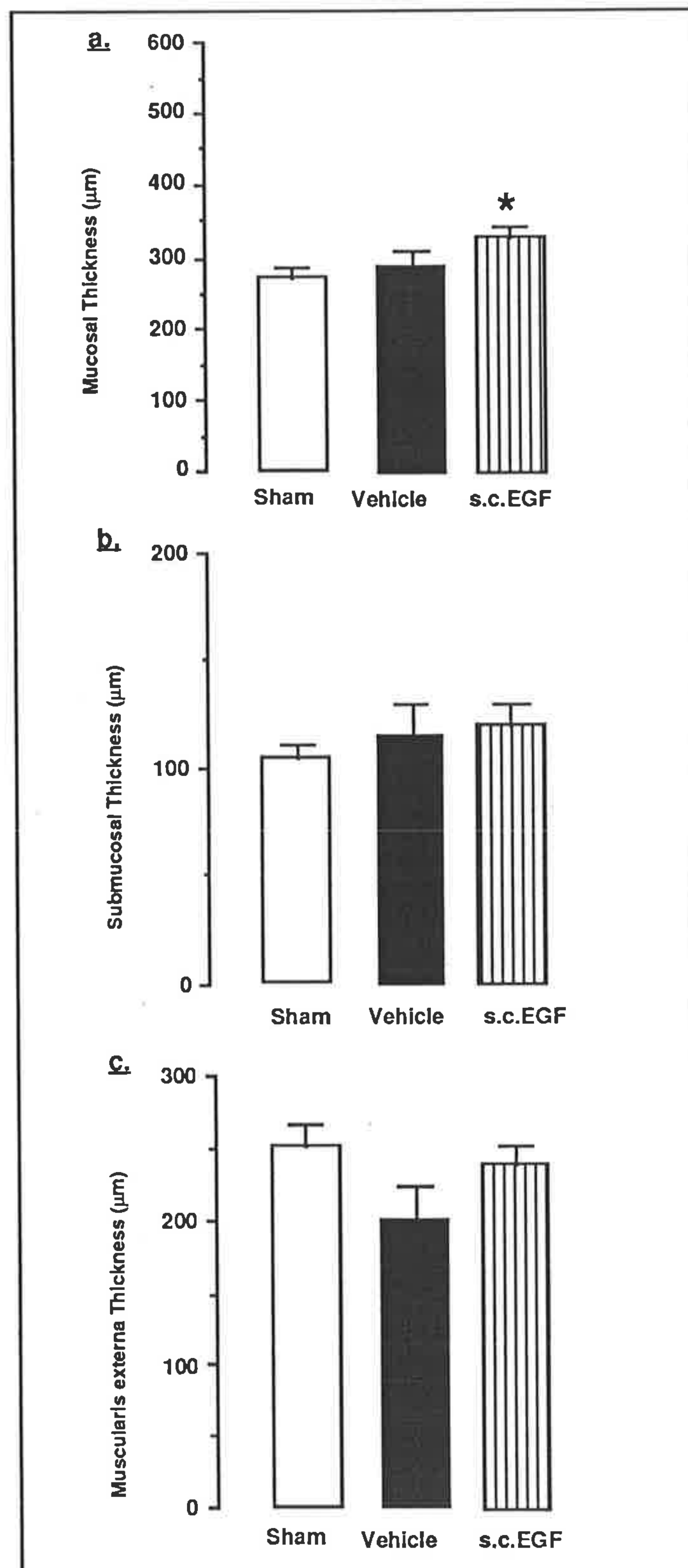


Figure 5.5 The effect of acetic acid induced ulceration and EGF treatment on the thickness of the (a) mucosa, (b) submucosa, and (c) muscularis externa layers of the undamaged colonic segment, upstream from the acetic acid-exposed segment. Data are expressed as mean + SEM for sham (unfilled bar), vehicle-treated (filled bar), s.c. EGF-treated groups (striped bar) of data from 4 and 6 days combined. (*)-$P<0.05$ vs vehicle group.

Discussion

While the cytoprotective and ulcer healing properties of EGF have been reported in experimentally induced ulcerations in the upper gastrointestinal tract, the effect of EGF in colonic ulcerations has not been previously investigated. To address this problem a reproducible model of colonic ulceration was developed in the rat, as described in Chapter 4. Intraluminal acetic acid exposure produced a colonic lesion which spontaneously healed over a 14 day period and induced a thickening of the mucosa, submucosal and muscularis externa layers at the margins of the lesion. The thickening of the mucosal layer was contributed to by hyperplasia of the crypt epithelia at the ulcer margins as indicated by an increased crypt length and number of cells per crypt column. Associated with this hyperplastic state was an upward shift in the crypt proliferative compartment as characterised by an increased PCNA labelled crypt fraction, measured at 4 and 6 days after the acid insult. Similar changes in the crypt proliferative compartment have been observed in colon biopsies from ulcerative colitis patients where the crypt cell production rate has been found to increase by 65% above that of normal individuals (Allan et al. 1985), and there is an upward shift in the proliferative compartment as indicated by an increased proportion of cells in S phase in the upper third of the crypt (Serafini et al. 1981). Thickening of the submucosal and muscularis externa layers was contributed to by a marked edemic response and inflammatory infiltrate cells. Mucosal edema is also observed in active ulcerative colitis, which has in part been attribted to the release of soluble mediators of inflammation (MacDermott and Stenson 1988, as discussed in Chapter 1). As shown in Chapter 4, the type of inflammatory cells present following the acetic acid insult namely lymphocytes, macrophages, neutrophils and eosinophils, are also observed in active ulcerative colitis (Roediger 1988, Haggitt 1990, Yamada et al. 1991) where a diffuse inflammation is detected throughout the mucosa and in severe cases can extend into the muscualris mucosae and involve the submucosa (Haggitt 1990). Following acetic acid exposure, the inflammatory response was induced and resolved rapidly so that by 6 days after the acid insult edema of the submucosal layer was reduced. This rapid resolution of the inflammatory response differs from that observed in human colitis in which episodes of both chronic and acute inflammation are seen (Haggitt 1990).

In this chapter, the effect of subcutaneously or luminally delivered EGF on repair of acetic acid ulceratons was determined. Neither systemic nor luminal EGF increased the re-epithelialization of the ulcerated colon, a result that was surprising, considering the potent ulcer healing properties of EGF in acute or chronic ulcerations in the stomach and duodenum (Konturek and Dembinski et al. 1988, Skov-Olsen et al. 1986b, Konturek and Brzozowski et al. 1988). Thus, subcutaneous or oral administration of EGF at doses 7 to 50 fold less than those used in the current study, have been shown to reduce the area of acute ulcerations produced by serosal application of acetic acid in the gastro-duodenum (Konturek and Dembinski et al. 1988, Konturek and Brzozowski et al. 1988) while 25 days of oral EGF administration at a 5 fold lower dose than was used here significantly increased healing of chronic cysteamine-induced duodenal ulcers (Skov-Olsen et al. 1986b).

The apparent lack of effect of EGF in this study may have occurred if the growth factor is only able to accelerate ulcer repair once the re-epithelialization process has been initiated. If so, a significant effect may have been observed if EGF was administered for longer than the 6 day period chosen. In fact, the experimental design was intended to address this problem, such that the 6 day time period was chosen on the basis of the preliminary time course trial in which re-epithelialization of the denuded segment had commenced by 6 days after the acid insult. Unfortunately in the EGF trial, re-epithelialization was not evident at 6 days after the acid insult, presumably these results reflect variability in the onset of re-epithelialization in the acetic acid model.

Differences in the mechanism of ulcer repair in the two regions of the gut are unlikely to account for the disparity in response to EGF. In the upper gastrointestinal tract, ulcer repair involves two key processes, mucosal proliferation adjacent to the ulceration, and migration of a single layer of epithelium across the denuded area (Konturek and Dembinski et al. 1988, Skov-Olsen et al. 1986b, Takeuchi and Johnson 1986), both of which are enhanced by EGF (Konturek and Dembinski et al. 1988, Skov-Olsen et al. 1986b, Thompson et al. 1990). A similar mechanism of repair in the acetic acid damaged colon is

suggested by the observed migration of epithelium over the denuded area in many sections as well as the obvious proliferative response in the adjacent mucosa, characterised by increases in the crypt depth, number of epithelial cells per crypt and the PCNA labelling index. Yet, despite this apparent similarity in mechanism, the ulcer healing properties of EGF apparent in the gastro-duodenum were not replicated in acetic-acid induced colonic ulceration.

Alternatively, if the rate of epithelial migration or of mucosal proliferation at the ulcer margins were not the rate-limiting steps in the repair of colonic ulcerations, then exogenous EGF might not be expected to stimulate repair. Certainly, acetic acid exposure itself induced a marked hyperplasia in the adjacent mucosa, which was not enhanced further by subcutaneous EGF. Although luminal EGF induced a significant increase in crypt length, together with a small increment in the number of epithelial cells per crypt, these changes may not have been substantial enough to enhance the rate of ulcer repair. In support of this argument, EGF-induced stimulation of repair of intestinal patch defects has been shown to disappear when proliferation of the adjacent mucosa is stimulated by performing an intestinal resection upstream (Thompson et al. 1990).

It is possible that endogenous EGF plays a role in the marked acetic acid-induced hyperplasia at the margins of a colonic ulceration. Increased concentrations of immunoreactive EGF have been measured at the site of gastroduodenal ulcerations (Konturek and Dembinski et al. 1988), and a recent report has described a cell lineage which develops adjacent to the site of gastric, duodenal and colonic ulcerations, starting as a bud at the base of the crypts, and then forming a gland structure which secretes EGF (Wright et al. 1990a). Thus, although levels of endogenous EGF were not measured in the current study, it is possible that sufficient amounts were released at the site of acetic acid damage to obviate any response to exogenously applied EGF.

The inability of EGF to accelerate repair of colonic ulcerations is also surprising in view of the fact that the colonic mucosa is a known target for the mitogenic actions of EGF

(Goodlad et al. 1992) with EGF receptors being localized in the colonic mucosa (Schaudies et al. 1989). The observation that subcutaneous EGF treatment increased mucosal width of an uninvolved colonic region distant from the site of acetic acid damage suggests that the doses of EGF used in this experiment were sufficient to stimulate mucosal growth. Moreover, the studies described in Chapter 3 showed that in normal rats, subcutaneous EGF administered for 7 days by an identical protocol induced a marked proliferative response in both the proximal and distal colonic mucosa, characterised by an increased mucosal thickness and an increased number of cells per crypt column in both the proximal and distal colon.

Despite the lack of effect of EGF on the re-epithelialization of the acetic acid-damaged region, luminal EGF did induce a mitogenic, albeit mild, effect on the colonic mucosa at the ulcer margins. This contrasts with the lack of effect of luminal EGF in the distal colon of normal animals. This disparity in colonic responsiveness to luminal EGF is a phenomena also observed in the upper gastrointestinal tract where luminally delivered EGF enhances DNA synthesis at the margins of the gastroduodenal ulcerations (Konturek and Dembinski et al. 1988), yet has no effect on increasing epithelial proliferation in the intact mucosa of the hypoplastic intestine (Goodlad et al. 1987). The increased responsiveness to EGF in the damaged intestine may be due in part to an increased accessibility to EGF binding sites as a result of acetic acid damage to the protective mucin layer. This is supported by studies performed in upper gastrointestinal tract ulcerations where it has been demonstrated that binding of radiolabelled EGF infused into the intestinal lumen is higher at ulcerated sites than in the intact mucosa adjacent to the lesions (Konturek and Dembinski et al. 1988). An increased accessibilty to EGF receptors in the damaged colon is also suggested by the luminal stability studies in which the total recovery of the infused material in the colonic lumen and tissue combined was lower in acetic acid treated animals compared with that in the colon of normal animals, (Chapter 3). The more rapid clearance of EGF from the colonic lumen after acetic acid exposure is consistent with a greater accessibility to tissue receptors in the damaged versus normal colon. An interesting observation of the study was the enhanced clearance of EGF from the proximal colon of rats that had been treated with acetic acid in the

distal colon. Based on its macroscopic and histological appearance, the proximal colonic mucosa not directly exposed to acetic acid appeared intact, but nevertheless the clearance of EGF from the colonic lumen of this region was also increased above normal levels. It is possible that some backwash of acid from the distal colon into the proximal segment occurred. Hence,the increased uptake of EGF from the proximal colonic lumen may be due to the presence of micro-ulcerations and distortion of cellular tight junctions which were undetectable by light microscopy (Olaison et al. 1990). Indeed, similar alterations in colonic permeability were made by Fedorak et al. (1990) in which colonic lesions induced by luminal acetic acid, appeared healed despite a persisting net colonic secretion. It is also possible that as a response to distal colonic damage induced by acetic acid exposure, adaptive changes were initiated in the undamaged proximal colon, upstream from the damaged site, resulting in altered uptake of EGF from the colonic lumen in this region. Indeed in Crohn's disease patients with colonic ulceration, an increase in intestinal permeability was detected in the apparently intact small intestine (Olaison et al. 1989).

In addition to inducing a mild mitogenic effect at the ulcer margins, luminal EGF decreased submucosal edema and significantly attenuated muscularis externa thickening. Moreover, subcutaneously delivered EGF markedly reduced the edematous response in the submucosa and possibly muscularis externa. This effect of EGF has not been reported previously in upper gastrointestinal tract ulcers and may therefore reflect a response that is specific to the colon. The mechanism by which EGF reduces colonic edema remains unclear but may relate to effects on vascular permeability. While EGF has been shown to enhance vascular endothelial cell proliferation (Hase et al.1989) no evidence has been reported to suggest that EGF directly modulates vascular permeability. While attenuation of tissue edema may be due to a reduced blood flow resulting from EGF treatment, the vasoactive properties of EGF still remains a contensious issue with both vasocontrictive (Harris et al. 1988) and vasodilatory effects (Gan et al. 1987) of EGF reported.

In conclusion, although exogenous EGF stimulates proliferation of the intact mucosa, it does not accelerate re-epithelialization of the acetic acid-denuded colonic mucosa.

Nevertheless, the reduction in edema and thickness of the submucosal and muscularis externa layers at the ulcer margins may confer some benefit to the damaged colon.

CHAPTER 6. FINAL DISCUSSION

One of the major factors hampering the development of new drug therapies for chronic colitis conditions is the availability of appropriate animal models. The development of the acetic acid model of colitis in the rat as described in Chapter 4, produces a colitis-like condition which is easy to induce and produces a distinct, reproducible and quantitative colonic lesion. However, unlike the chronic nature of mucosal damage observed in Crohn's disease and ulcerative colitis, the mucosal injury induced by acetic acid and indeed by other caustic agents, such as ethanol, resolve rapidly after the injurious insult. As a complex interplay exists between colonic inflammation and mucosal damage, an abnormality in the colonic epithelium or an aberrant inflammatory response may account for the chronic nature of these conditions.

Differences in the chronicity of the mucosal injury in the human colitis condition compared to the acetic acid colitis model, may be due to a range of factors including differences in the metabolism or type of inflammatory infiltrate cells or due to an aberrant immune response. The major cell type found in the inflammatory infiltrate following acetic acid insult are neutrophils (Yamada et al. 1991). While neutrophil infiltrate is also observed in human colitis, the role of these inflammatory cells in inducing mucosal damage differs between the experimental and natural conditions. In the acetic acid model, the mucosal damage is apparently not directly triggered by a neutrophil-mediated mechanism as other studies have not detected a reduction in the severity of acetic acid-induced mucosal injury in neutropenic animals (Yamada et al. 1991). The inflammatory response in the acetic acid model also resolves rapidly, so that any damaging effect of neutrophils on the colonic mucosa would be short lived. In contrast, in human colitis an impaired neutrophilic oxidative system has been identified which may contribute to mucosal injury (Verspaget et al. 1988). The aggregation of neutrophils can result in the production of toxic levels of oxygen metabolites which is normally counteracted by the release of enzymes such as

superoxide dismutase. However, in colitis patients the neutrophil content of this enzyme is low (Verspaget et al. 1988), and the resulting abundance of reactive oxygen metabolites may result in tissue damage (Grisham and Granger 1988).

Since the commencement of this thesis, several new models of inflammatory bowel disease have been described which use a variety of agents to induce colonic damage by modifying the inflammatory / immune response. Intracolonic instillation of agents which enhance the aggregation and adherance of neutrophils have been used to induce a colitis-like condition. Chemotactic agents such as n-formyl-methionyl-lecyl phenylalanine (n-FMLP) display similar actions to those formed by colonic bacteria, with receptors to such neutrophil chemotactic agents being detected on colonic granulocytes (LeDucl and Nast 1990). Based on the colitis-inducing properties of n-FMLP, an increased colonic permeability to neutrophil chemo-attractants has been suggested as a potential pathogenic mechanism involved in the development of inflammatory bowel disease (LeDucl and Nast 1990). Colitis-like conditions have also been induced by stimulating B and T cell activation. A chronic colitis condition has been described in rats by inducing animals to produce antibodies against the colonic mucosa (Axelsson and Ahlstedt 1990). Similarites with the human condition are suggested by the demonstration of inappropriate antibody production, with antibodies to surface antigens on colonic epithelium identified in a number of patients (Hibi et at. 1983). Methods of enhancing T cell activation include intracolonic administration of haptens such as trinitro-benzene sulfonic acid (TNBS) which bind to tissue proteins, and stimulate cell-mediated immunity. Unlike the rapid restoration of the colonic mucosa in colitis conditions induced by caustic agents such as acetic acid, TNBS in ethanol produces a colonic lesion which remains unhealed up to 8 weeks after the injurious insult (Morris et al. 1989), associated with an inflammatory infiltration of neutrophils, lymphocytes, mast cells as well as the formation of granulomas. In this model, mucosal injury has been associated with neutrophil infiltration, as inhibition of neutrophil adherence reduced the extent of epithelial injury induced by TNBS (Wallace et al. 1992). Indeed, the mechanism by which TNBS colitis is induced has been likened to the onset of mucosal injury in human colitis which may involve an immunological hapten (Fidler 1985, Yamada et al. 1992). Hence the type of the

inflammatory infiltrate, chronicity of the inflammation and colonic lesion, together with the nature of induction of TNBS experimental colitis, suggest that this model is more comparable to human colitis than the acetic acid model, which induces an acute colonic lesion and inflammatory response, and may therefore provide a more appropriate system for assessing new therapeutic agents for colitis conditions. Despite these major benefits, inter-animal variability in the severity and duration of the colonic lesion induced by TNBS would appear to be greater than that seen in acetic acid-induced colitis (Yamada et al. 1992). Hence, while these models share characteristics similar to the human disease they do not completely mimic the human condition or represent all the features of the 'ideal' model of colitis as outlined by Strober (1985).

The reproducible and quantitative colonic lesion induced by acetic acid exposure, provided a model for assessing factors which may accelerate repair through an enhanced rate of re-epithelialization of the damaged colon. Any such factors could provide adjuncts to therapies for colitis conditions. It was hypothesised that EGF would enhance re-epithelialization of the ulcerated colon in an analogous manner to its potent cytoprotective and ulcer healing properties in the upper gastrointestinal tract. However, while exogenous EGF was found to enhance growth of the crypt epithelium in the intact colonic mucosa, no enhancement of colonic ulcer healing was detected. The rapid resolution of the acetic acid-induced colonic lesion, characterised by marked hyperproliferation at the margins of the ulcerated region, described in Chapter 5, suggests that the ulcer healing process is proceeding at near-maximal rate in this model. In light of other studies demonstrating the emergence of an EGF-producing cell lineage at gastrointestinal ulcer margins (Wright et al. 1990), it was further suggested that endogenous EGF may be implicated in the mechanism of repair of acetic acid-induced ulcerations. Alternatively, other growth factors may be implicated. Conversely, chronic wounds, such as occur in natural colitis, or even the semi-chronic TNBS-model, may be deficient in growth factors. Indeed this is suggested by studies in which fluids collected from chronic wounds differ from those obtained from healing wounds in the level of peptide growth factors and their ability to enhance DNA synthesis in fibroblast cultures (Schultz et al. 1991). This hypothesis could be tested by a

comparison of the endogenous expression of EGF and other peptide growth factors or their receptors in acute and chronic colitis conditions, using techniques such as immunoassay, immunohistochemistry, in situ hybridization and measurement of tissue mRNA levels.

While EGF has been implicated as a potent cytoprotective and ulcer healing agent, other factors that are released or co-expressed with endogenous EGF may enhance the ulcer healing process. Wright et al.(1990) detected a cell lineage at the site of gastrointestinal lesions which secretes EGF and pS2 polypeptide, a cysteine-rich protein, with a 'trefoil' disulphide loop structure, highly homologous to pancreatic and human spasmolytic peptides. These 'trefoil' peptides may directly enhance ulcer healing, as pancreatic spasmolytic peptide is mitogenic to epithelial cells *in vitro* (Thim 1989). Synthesis of hyaluronic acid is also increased with EGF expression and the expression of a range of other growth factors including platelet-derived growth factor and basic fibroblast growth factor (Heldin et al. 1989). When administered exogenously, hyaluronic acid is a potent enhancer of wound healing as observed in the damaged cheek pouch where cell proliferation and migration are enhanced by hyaluronic acid treatment (King et al. 1991). Whether factors such as the spasmolytic peptides and hyaluronic acid represent agents which act synergistically with endogenous EGF to enhance ulcer healing, or whether they have an obligatory role in EGF action remains unclear. However, an assessment of the effect of administration of such agents to the damaged colonic mucosa is worthy of investigation.

The almost complete denudation of the colonic mucosa induced by acetic acid exposure may also reduce the responsiveness of the damaged colon to EGF by obliterating cells containing the EGF receptor. While an increased level of EGF binding has been detected in experimentally induced ulcerations in the upper gastrointestinal tract, the distribution of EGF receptors in the acetic acid damaged colon has not been measured. Although stability and uptake studies of radiolabelled EGF from the normal and acetic acid-ulcerated colonic lumen was performed in this thesis, the distribution of EGF binding was not measured. In future experiments, autoradiographic localization of luminally administered radiolabelled EGF or immunohistochemical detection of the EGF receptor in

the normal and ulcerated colonic mucosa would enable an assessment of the availability and distribution of EGF binding sites in the acetic acid model to be made.

EGF may also require the presence of other growth factors to induce colonic repair. Growth factors such as platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) have been associated with cell chemotaxis, mitogenesis and angiogenisis occurring during the wound healing process (Dijke and Iwata 1989) and may confer some benefit to the healing of the ulcerated colon. Indeed in chronic incisional wounds in diabetic rats, administration of PDGF and FGF(basic) enhanced wound healing (Greenhalgh et al. 1990). When EGF and PDGF are co-administered, the two factors act synergistically to enhance mitogenisis of fibroblasts (Dijke and Iwata 1989) and administration of exogenous PDGF *in vivo* has been shown to enhance granulation tissue formation (Sprugel et al. 1987). Hence the concurrent administration of EGF and PDGF may elicit a more beneficial effect on colonic ulcer healing than when EGF alone is given. FGF(basic) is a potent mitogen to a range of cell types including fibroblasts, endothelial and epithelial cells and both FGF(basic) and FGF(acidic) stimulate the proliferation of normal and neoplastic colonic cells (Burgess and Sizeland 1990). Moreover, FGF(basic) is the major peptide growth factor extracted from porcine colonic mucosa and may therefore represent the endogenous growth factor involved in the growth and maintenance of the colon (Burgess and Sizeland 1990). In light of this finding, and given its mitogenic effect on fibroblasts and colonic epithelial cells, FGF(basic) administration by itself or in combination with EGF may be more appropriate treatment to enhance colonic ulcer healing. For this reason, an assessment of the endogenous levels of FGF in the normal and ulcerated colon to determine if a deficiency in FGF expression contributes to the chronicity of colonic ulcerations, as well as the effect of exogenous FGF administration on colonic healing is worthy of investigation. Actions of other growth factors such as transforming growth factor-alpha (TGF-α) and hepatocyte growth factor (HGF) suggest that these factors may also serve to enhance colonic ulcer healing. TGF-α elicits its biological response by binding to the EGF receptor and shares similar biological activities to EGF. Due to its release by virally transformed cells (Azano et al. 1983), detection of TGF-α has been considered to be a marker of malignancy. However

its expression by activated macrophages (Dijke and Iwata 1990) and the mucosa along the length of the gastrointestinal tract of normal humans (Cartliage and Elder 1989), suggests that TGF-α may play a role in gastrointestinal growth and maintenance. TGF-α has also been implicated as an endogenous wound healing agent due to its presence in wound fluids (Schultz et al. 1991) and in ascites fluid in cirrhotic patients (Sporn and Roberts 1986). HGF is a potent stimulator of hepatocyte proliferation and has been implicated as a factor involved in liver regeneration (Matsumoto and Nakamura 1991). In addition to its role in the growth and maintenance of the liver, HGF has also been purified from platelets and has been detected in salivary gland and the duodenum using immunohistochemical techniques (Zarnegar et al. 1990). Moreover, its scatter factor-like activity of enhancing movement and motility of epithelial cell lines, and its mitogenic effect on renal epithelial cells and epidermal cells (Matsumoto and Nakamura 1991), suggests that HGF may exert both proliferative and chemotactic effects in a range of cell types. These HGF-mediated effects suggest that this growth factor may enhance re-epithelialization of ulcerative lesions. While the action of HGF *in vivo* is still not fully understood and large quantities of HGF are currently not available to perform large scale *in vivo* studies, future studies investigating the effect of HGF and colonic ulcer healing would be of interest.

The lack of effect of EGF on healing of colonic ulcerations can not be attributed to the healing of ulcerations by EGF being a response specific to the gastric and duodenal epithelium as EGF is also a potent enhancer of re-epithelialization of lesions in other organs. Alkali burns of the cornea produce a chronic ulceration where there is a recurrence of epithelial erosions (Singh and Foster 1987). Topical application of EGF to these non-healing wounds increases the rate of re-epithelialization, although it does not prevent re-current erosions as cell attachment is not enhanced (Singh and Foster 1987). Like the proliferative effect of EGF on the damaged intestinal epithelium, healing of the damaged corneal epithelium is also associated with an increased proliferation of the epithelium at the margins of the lesion (Kitazawa et a. 1990). Similar differences in responsiveness to luminal EGF detected in the intact normal epithelium and the damaged colon, are also observed in the corneal epithelium where topical EGF enhances proliferation at the margins

of the wound but not in the normal intact corneal epithelium (Kitazawa et a. 1990). Wound healing properties of EGF have also been illustrated in chronic skin wounds. Non-healing skin wounds, which do not respond to conventional therapies, can be susceptible to invasive infection and result in amputation (Brown et al. 1991). Twelve-hourly topical application of EGF over a minimum 3 week period to non-healing skin ulcerations and incisional wounds in diabetic patients, produced an enhancement of wound healing (Brown et al. 1991). In EGF-treated wounds, granulation tissue formation was enhanced after which there was an ingrowth of epithelial cells migrating from the wound margin forming an epithelial sheet followed by wound contraction and healing (Brown et al. 1991).

Despite the apparent lack of effect of exogenous EGF on re-epithelialization of the ulcerated rat colon, the attenuation of tissue edema resulting from EGF treatment, suggests that EGF may modulate the colonic inflammatory process induced by ulceration. While the effect of EGF on the inflammatory and immune response induced by acetic acid exposure was not the major area of interest in this thesis, the effect of EGF on colonic inflammation warrants further investigation. Indeed unlike its cellular mitogenic modalities, EGF has been shown to reduce proliferation of lamina propria lymphocytes *in vitro* (Elitsur et al. 1991) and if this occurred in the ulcerated colon *in vivo* it would result in a suppression of the inflammatory response and subsequent epithelial damage. Activation of lamina propria T-cells has been implicated in the pathogenesis of inflammatory conditions, and as discussed by Targan et al.(1991), a defect in the regulation of mucosal immunity has been suggested as a potential pathogenic factor in inflammatory bowel disease. Activation of the mucosal immune system initiates the release of cytokines, interleukins and chemotactic factors which in turn triggers the release of neutrophils and eosinophils leading to the subsequent production of arachidonic acid metabolites and oxygen radicals (Targan et al. 1991). Epithelial damage resulting from T-cell activation has been illustrated in small intestinal explants (MacDonald and Spencer 1988) and in organ culture of human fetal colon explants, where an activation of lamina propria T-cells enhances epithelial cell proliferation and decreases goblet cell numbers (Evans et al. 1992), both of which are histopathological features detected in human inflammatory bowel disease. Hence, the suppression of T-cell

proliferation by EGF may confer some benefit to the inflammed colon. In addition, suppression of pro-inflammatory factors released upon T-cell activation, may also provide a potential therapeutic mechanism for inflammatory bowel disease. Indeed, antagonists to the interleukin-2-receptor are currently being assessed in clinical trials as potential therapeutic agents in inflammatory bowel disease (Dinarello and Wolff 1993).

In conclusion, *in vivo* studies in the adult male rat performed in this thesis illustrated that exogenously administered EGF was capable of enhancing epithelial hyperplasia in the intact colonic mucosa but did not increase the extent of re-epithelialization of the acetic acid damaged colon, despite the fact that EGF increased epithelial hyperplasia at the margins of the induced colonic lesions. While these findings suggest that administration of exogenous EGF to the damaged colon does not enhance the ulcer healing process, limitations of the acetic acid model make an extrapolation of these results to the appropriateness of EGF therapy in human inflammatory bowel disease difficult. Indeed future experiments investigating the effects of EGF in a more chronic colonic ulcerative model are warranted. Moreover the administration of EGF in combination with other growth factors or agents which enhance the attachment of newly formed epithelium may be more beneficial to the ulcerated colon than when EGF alone is given.

APPENDIX A - Histological Methods

Histological Fixatives

Bouin's Fluid

per 100ml of fixative: 75ml Saturated picric acid solution
25ml Formaldehyde
5ml Glacial acetic acid

Methacarn Fixative

per 2 Litres of fixative: 1200mls Methanol
600mls Chloroform
200mls Glacial acetic acid

Carnoy's Fixative

per 2 Litres of fixative: 1200mls Ethanol
600mls Chloroform
200mls Glacial acetic acid

After fixation, tissues were transferred to 70% (v/v) ethanol prior to tissue processing.

Tissue Processing

Tissue processing was performed on a Titertek automated tissue processor (Miles Scientific, Naperville, U.S.A.) under vacuum prior to embedding tissue in paraffin wax using a Titertek embedding centre (Miles Scientific, Naperville, U.S.A.). Fixed tissue was exposed to the following solvents:

1 hour in 70% Ethanol
1 hour in 85% Ethanol
1 hour 45 minutes in 95% Ethanol
4 hours in Absolute Ethanol
3 hours in Chloroform
4 hours in paraffin wax at 60°C

Histological Stains

Mayer's haematoxylin staining was used to counterstain PCNA immunohistochemical staining, and Mayer's haematoxylin / Eosin staining was used as a routine histological stain throughout this thesis.

Mayer's Haematoxylin

Per Litre of stain:
2g Haematoxylin
1 Litre Distilled water
50g Ammonium Alum Sulphate
200mg Sodium iodate
1g citric acid
50g Chloral hydrate

Haematoxylin was dissolved in water, using gentle heat, and the remainder of the ingrediants were added in the sequence shown above. The stain was then cooled and filtered prior to use.

Eosin

Per 100ml of stain:
5g Eosin
100ml of Distilled water
1ml 2% Acetic acid

Haematolxylin / Eosin staining

Slides were de-waxed in Xylene and graded alcohols prior to being immersed in filtered haematoxylin for 30 seconds, then transferred to running tap water for two minutes. To enhance the haematoxylin staining, slides were then placed in a 25% Lithium carbonate solution for 10 seconds. Slides were then immersed in eosin for 30 seconds, and rehydrated through graded alcohols and Xylene and mounted under galss coverslips with Depex mounting medium.

APPENDIX B - Statistics summary tables

ACETIC ACID EXPOSED SEGMENT

Percentage Epithelialization

Table B.1 2-Way Analysis of Variance Table

Source	df	Sum of Squares	Mean Square	F_{value}	P_{value}
Time	1	18.164	18.164	0.072	0.7901
Treatment	3	82391.7	27463.9	1.08E2	0.0001
Time x Treatment	3	138.62	46.218	0.183	0.907
Residual	45	11394.9	253.220		

Table B.2 4 and 6 day pooled means

Sham	100±0.00
Vehicle	9.42±3.06
s.c. EGF	13.3±7.45
lum. EGF	8.86±3.02

ADJACENT SEGMENT

Mucosal Thickness

Table B.3 2-Way Analysis of Variance Table

Source	df	Sum of Squares	Mean Square	F_{value}	P_{value}
Time	1	3849.7	3849.7	0.441	0.509
Treatment	3	353603.2	117867.7	13.51	0.0001
Time x Treatment	3	3798.6	1266.2	0.14	0.932
Residual	45	3925.2	8722.6		

Table B.4 4 and 6 day pooled means

Sham	392.0±14.3
Vehicle	589.7±23.6
s.c. EGF	499.7±31.7
lum. EGF	585.6±27.6

Number of cells per crypt column

Table B.5 2-Way Analysis of Variance Table

Source	df	Sum of Squares	Mean Square	F_{value}	P_{value}
Time	1	1.658	1.658	0.021	0.885
Treatment	3	8056.9	2685.6	34.17	0.0001
Time x Treatment	3	411.7	137.2	1.74	0.1711
Residual	45	3536.6	78.59		

Table B.6 4 and 6 day pooled means

Sham	48.9±1.3
Vehicle	78.3±2.6
s.c. EGF	74.8±3.0
lum. EGF	77.2±2.7

Crypt Length

Table B.7 Two-Way Analysis of Variance Table

Source	df	Sum of Squares	Mean Square	F_{value}	P_{value}
Time	1	10453.7	10453.7	2.26	0.139
Treatment	3	332078.7	110692.9	23.98	0.0001
Time x Treatment	3	41262.1	13754.0	2.98	0.0413
Residual	45	207697.7	4615.5		

Table B.8 4 Day Treatment One-Way Analysis of Variance Table

Source	df	Sum of Squares	Mean Square	F_{value}	P_{value}
Treatment	3	185093.2	61697.7	21.31	0.0001
Residual	24	70074.0	2919.7		

Table B.9 6 Day Treatment One-Way Analysis of Variance Table

Source	df	Sum of Squares	Mean Square	F_{value}	P_{value}
Treatment	3	186542.8	62180.9	9.5	0.0004
Residual	24	137623.7	6553.5		

PCNA Labelling Index

Table B.10 Two-Way Analysis of Variance Table

Source	df	Sum of Squares	Mean Square	F_{value}	P_{value}
Time	1	22.248	22.248	2.30	0.136
Treatment	3	379.645	126.548	13.09	0.0001
Time x Treatment	3	45.67	15.224	1.575	0.2085
Residual	45	434.839	9.664		

Table B.11 4 and 6 day pooled means

Sham	49.94±0.93
Vehicle	55.56±1.01
s.c. EGF	56.74±0.71
lum. EGF	55.57±0.83

PCNA Labelled Crypt Fraction

Table B.12 Two-Way Analysis of Variance Table

Source	df	Sum of Squares	Mean Square	F_{value}	P_{value}
Time	1	0.835	0.835	0.028	0.868
Treatment	3	569.1	189.7	6.27	0.0012
Time x Treatment	3	165.17	55.06	1.822	0.1568
Residual	45	1360.1	30.23		

Table B.13 4 and 6 day pooled means

Sham	62.52±1.89
Vehicle	70.57±1.33
s.c. EGF	69.25±1.21
lum. EGF	69.89±1.55

Submucosal Thickness

Table B.14 Two-Way Analysis of Variance Table

Source	df	Sum of Squares	Mean Square	F_{value}	P_{value}
Time	1	185239.2	185239.2	23.9	0.0001
Treatment	3	162633.2	54211.0	7.02	0.0006
Time x Treatment	3	86301.6	28767.2	3.72	0.0179
Residual	45	347670.0	7726.0		

Table B.15 4 Day Treatment One-Way Analysis of Variance Table

Source	df	Sum of Squares	Mean Square	F_{value}	P_{value}
Treatment	3	235805.1	78601.7	7.09	0.0014
Residual	24	266028.79	11084.5		

Table B.16 6 Day Treatment One-Way Analysis of Variance Table

Source	df	Sum of Squares	Mean Square	F_{value}	P_{value}
Treatment	3	185093.2	61697.7	21.13	0.0001
Residual	24	70074	2919.7		

Muscularis Externa Thickness

Table B.17 Two-Way Analysis of Variance Table

Source	df	Sum of Squares	Mean Square	F_{value}	P_{value}
Time	1	5472.3	5472.3	3.23	0.0789
Treatment	3	21983.4	7327.8	4.33	0.0092
Time x Treatment	3	2380.6	793.5	0.47	0.7055
Residual	45	76173.0	1682.7		

Table B.18 4 and 6 day pooled means

Sham	207.9±6.2
Vehicle	252.6±14.4
s.c. EGF	225.1±13.9
lum. EGF	200.8±9.5

UNDAMAGED SEGMENT- UPSTREAM FROM THE DAMAGED SEGMENT

Mucosal Thickness

Table B.19 Two-Way Analysis of Variance Table

Source	df	Sum of Squares	Mean Square	F_{value}	P_{value}
Time	1	5094.144	5094.144	1.315	0.2596
Treatment	3	25135.752	12567.876	3.243	0.0514
Time x Treatment	3	4852.286	2423.143	0.626	0.5407
Residual	45	131749.331	3874.980		

Table B.20 4 and 6 day pooled means

Sham	269.34±15.33
Vehicle	290.17±19.97
s.c. EGF	330.18±15.27

Submucosal Thickness

Table B.21 Two-Way Analysis of Variance Table

Source	df	Sum of Squares	Mean Square	F_{value}	P_{value}
Time	1	593.26	593.26	0.406	0.5285
Treatment	3	1855.009	927.505	0.634	0.5366
Time x Treatment	3	5508.982	2754.491	1.883	0.1677
Residual	45	49735.110			

Table B.22 4 and 6 day pooled means

Sham	104.10±7.58
Vehicle	116.41±14.09
s.c. EGF	120.85±9.60

Muscularis Externa Thickness

Table B.23 Two-Way Analysis of Variance Table

Source	df	Sum of Squares	Mean Square	F_{value}	P_{value}
Time	1	337.934	337.934	0.147	0.7041
Treatment	3	799.547	399.774	0.174	0.8415
Time x Treatment	3	2680.449	1340.225	0.582	0.5644
Residual	45	78339.210	2304.09		

Table B.24 4 and 6 day pooled means

Sham	252.03±15.49
Vehicle	250.15±10.51
s.c. EGF	241.28±11.43

BIBLIOGRAPHY

Abraham R., Fabian R.J., Golberg L., Coulston F. (1974)
Role of lysosomes in carrageenan-induced cecal ulceration.
Gastroenterology 67, 1169-1181

Adamson E. and Meek J. (1984)
The ontogeny of epidermal growth factor receptors during mouse development.
Developmental Biology 103, 62-70

Agawa S., Mutto T., Morioka Y. (1988)
Mucin abnormality of colonic mucosa in ulcerative colitis associated with carcinoma and/or dysplasia.
Diseases of the Colon and Rectum 31, 387-389

Ahnen D.J., Warren G.H., Greene L.J., Singleton J.W., Brown W.R. (1987)
Search for a specific marker of musosal dysplasia in chronic ulcerative colitis.
Gastroenterology 93, 1346-1355

Allan A., Bristol J.B., Williamson R.C.N. (1985)
Crypt cell production rate in ulcerative proctocolitis: Differential increments in remission and relapse.
Gut 26, 999-1003

Allen A. (1983)
The structure of colonic mucus.
IN Colon, Structure and function. Bustos-Fernandez (ed.). Plenum Press N.Y.43-55

Allison M.C. and Pounder R.E. (1987)
Cyclosporin for Crohn's disease.
Alimentation Pharmacology ad Therapeutics 1, 39-43

Amend N.K., Loeffler D. G., Ward B.C., Van Hoosier G.L. (1972)
Transmission of enteritis in the Syrian hamster.
Laboratory Animal Science 26, 566-572

Anver M.R. and Cohen B.J. (1976)
Animal Model: Ulcerative colitis induced in guinea pigs with degraded carrageenan.
American Journal of Pathology 84(2), 431-434

Anzano M.A., Roberts A.B., Smith J.M., Sporn M.B. and DeLarco J.E. (1983)
Sarcoma growth factors from conditioned medium of virally transformed cells is composed of both type -α and type -β transforming growth factors.
Procedings of the National Academy of Sciences U.S.A. 80, 6264-6268

Arsenault P. and Menard D.(1987)
Stimulatory effects of epidermal growth factor on deoxyribonucleic acid synthesis in the gastrointestinal tract of the suckling mouse.
Comparative Biochemistry and Physiology 86B(1), 123-127.

Axelsson L.G. and Ahlstedt S. (1990)
Characteristics of immune-complex induced chronic experimental colitis in rats with a therapeutic effect of sulphasalazine.
Gastroenterology 25, 203-209

Azhad Khan A.K., Howes D.T., Piris J. and Truelove S.C. (1980)
Optimum dose of sulphasalazine for maintenance treatment in ulcerative colitis
Gut 21, 232-240

Azhad Khan A.K., Piris J., Truelove S.C. (1977)
An experiment to determine the active therapeutic moiety of sulphasalazine.
Lancet 2, 892-895

Beaulieu J.F., Menard D. and Calvert R. (1985)
Influence of epidermal growth factor on the maturation of the fetal mouse duodenum in organ culture.
Journal of Pediatric Gastroenterology and Nutrition 4, 476-481

Benitz K.F., Golberg L., Coulston F. (1973)
Intestinal effects of carrageenans in the rhesus monkey (Macaca mulatta).
Food and Cosmetics Toxicology 11, 565-575

Biemond I., Selby W.S., Jewell D.P., Klasen E.C.(1984)
Alpha-1-Antitrypsin serum concentration and phenotypes in ulcerative colitis
Digestion 29, 124-128

Bondesen S., Rasmussen S.N., Rask-Madson J., Nielsen O.H., Lauristen K., Bonder V., Hansen S.H. and Hvidberg E.F. (1987)
5-Aminosalicylic acid in the treatment of inflammatory bowel disease.
Acta Medica Scandinavia 221, 227-242

Brandt L.J., Bernstein L.H., Boley S.J. (1982)
Metronidazole therapy for perineal Crohn's disease: A follow up study.
Gastroenterology 83, 383-387

Brandtzaed P., Baklien K., Fausa O., Hoel P.S. (1974)
Immunohistochemical characterization of local immunoglobin formation in ulcerative colitis.
Gastroenterology 66, 1123-1136

Bravo R, Frank R, Blundell PA, Mathews MB and Stillman B.(1987)
The cell-cycle regulated proliferating cell nuclear antigen is required for SV40 DNA replication *in vitro.*
Nature 326;471-475

Bravo R.(1986)
Synthesis of the nuclear protein cyclin (PCNA) and its relationship with DNA replication.
Experimental Cell Research 163, 287-293

Brogden R.N. and Sorkin E.U. (1989)
Mesalazine: A review of its pharmacodynamic and pharmacokinetic properties and therapeutic potential in chronic inflammatory bowel disease.
Drugs 38(4), 500-523

Brown G.L., Curtsinger L., Jurkiewicz M.J., Nahai F., Schultz G. (1991)
Stimulation of healing of chronic wounds by epidermal growth factor.
Plastic and Reconstructive Surgery 88(2), 189-194

Brzozowski T., Radecki T., Sendur P., Gustaw P., Konturek S.J. (1987)
Prevention of acute gastric mucosal lesions by solcoseryl.
Hepato-Gatroenterology 34, 86-89

Bukhave K., Lauritsen K., Laursen L., Rask-Madsen J. (1988)
Role of eicosanoids in diarrheal diseases.
Digestive Diseases and Sciences 6, 149-158

Burger H.J., Hauber G., Scholte W., Schwenk M.(1985)
Comparison of isolated cells from liver, colon and kidney of the guinea pig.
Cell Physiology 17, 271-278

Burgess A.W. and Sizeland A.M. (1990)
Growth factors and the gut.
Journal of Gastroenterology and Hepatology S1, 10-21

Burton K.(1956)
A study of the conditions and mechanism of the diphenylamine reaction for colorimetric estimation of deoxyribonucleic acid.
Biochemistry Journal 62, 315-323

Butler L.D., Layman N.K., Cain R.L. (1989)
Interleukin-1-induced pathophysiology: Induction of cytokines, development of histopathologic changes and immunopharmacologic intervention.
Clinical Immunology Immunopathology 53, 400-421

Butler R.N., Lawson M.J., Goland G.J. Jarret I.G. and Roberts-Thompson I.C. (1988)
Proliferative activity in the proximal and distal colon of the rat after fasting and refeeding.
Immunology and Cell Biology 66, 193-198

Campieri M., Lanfranchi G.A., Bazzochi G., et al. (1981)
Treatment of ulcerative colitis with high-dose 5-aminoslicyclic acid enemas.
Lancet 2, 270-271

Carpenter G. and Cohen S.(1976)
Human epidermal growth factor and the proliferation of human fibroblasts.
Journal of Cell Physiology 88, 227-237

Carpenter G. and Cohen S. (1979)
Epidermal growth factor.
Annual Review of Biochemistry 48, 193

Carpenter G. and Cohen S. (1981)
EGF: Receptor interactions and the stimulation of cell growth.
IN Receptor Regulation, Receptors and Recognition Series B. Lefkowitz R.J. (Ed.) 13-

Cartlidge SA and Elder JB.(1989)
Transforming growth factor α and epidermal growth factor levels in normal human gastrointestinal mucosa.
British Journal of Cancer 60, 657-660.

Cello J.P. Meyer J.H.(1978)
Ulcerative colitis.
IN Gastrointestinal diseases V2- pathophysiology, diagnosis, management. Sleisenger M.H. and Fordtran J.S. (eds.). W.B. Saunders N.Y.1597-1653,

Charney A.A. and Donowitz M. (1978)
Functional significance of intestinal Na^{+}-K^{+}-ATPase: *in vivo* ouabain inhibition.
American Journal of Physiology 234(6), 629-636

Coffey R.J., Derynck R., Wilcox J.N., Bringman T.S., Goustin A.S., Moses H.L., Pittelkow M.R. (1987)
Production and autoinduction of transforming growth factor-α in human keratinocytes.
Nature 328, 817

Cohen S. (1962)
Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the newborn animal.
Journal of Biological Chemistry 237, 1555-1562

Cope G.F., Heatley R.V., Kelleher J., Axon A.T.R. (1988)
In Vitro mucus glycoprotein production by colonic tissue from patients with ulcerative colitis
Gut 29, 229-234

Culling C.F., Reid P.E., Worth A.J., Dunn W.L.(1977)
A new histochemical technique of use in the interpretation and diagnosis of adenocarcinomas and villous lesions in the large intestine.
Journal of Clinical Pathology 30, 1056-1062

Cummings J.H. (1981)
Short chain fatty acids in the human colon.
Gut 22, 763-779

Danilsson A., Hellers G., Lyrenas E. (1987)
A controlled randomized trial of budesonide versus prednisolone retention enemas in active distal ulcerative colitis.
Scandinavian Journal of Gastroenterology 22, 987-992

Das K.M., Eastwood M.A., McManus J.P.A. and Sircus W. (1973)
Adverse reactions during saliclazosulfapyridine therapy and the relation with drug metabolism and acetylator phenotype.
The New England Journal of Medicine 289(10), 491-495

Das K.M. (1989)
Sulfasalazine therapy in inflammatory bowel disease.
Gastroenterology Clinics of North America 18, 1-19

Delvaux G., Caes F., Willems G. (1979)
Influence of diverting colostomy on epithelial cell proliferation in the colon of rats.
European Journal of Surgical Research 15, 223-229

Dembinski A.B. and Johnson L.R. (1985)
Effect of epidermal growth factor on the development of rat gastric mucosa.
Endocrinology 116, 90-94

Deschner .E.(1988)
Cell proliferation and colonic neoplasia.
Scandinavian Journal of Gastroenterology 23(S151), 94-97

Donaldson R.M.J.R. (1989)
Crohn's disease.
IN Gastrointestinal disease, pathophysiology, diagnosis and management
Sleizenger M.H., Fordtran J.S. (eds.)
Saunders N.Y. 1327-

Dijke P.T. and Iwata K.K. (1989)
Growth factors for wound healing.
Biotechnology 7, 793-798

Dinarello C.A., Wolff S.M. (1993)
The role of interleukin-1 in disease.
New England Journal of Medicine 328, 106-113

Dulley J.R. and Grieve P.A. (1975)
A simple technique for eliminating interference by detergents in the lowry method of protein determination.
Analytical Biochemistry 64; 136-139

Durie P.R.(1985)
Intestinal protein loss in fecal α1- antitrypsin
Journal of Pediatric Gastroenterology 4(3) 345-347

Duthie H.L.,Watts J.M., Dombal D.E., Goligher F.T.(1964)
Serum electrolytes and colonic transfer of water and electrolytes in chronic ulcerative colitis.
Gastroenterology 47(8) 525-530

Eastwood G.L., Trier J.S.(1973)
Epithelial cell renewel in cultured rectal biopsies in ulcerative colitis.
Gastroenterology 64(3), 383-390

Edmonds C.J., Pilcher D.(1973)
Electrical potential difference and soduim and potassium fluxes across rectal mucosa in ulcerative colitis.
Gut 14, 784-789

Eldridge S.R., Tilbury L.F., Goldworthy T.L., Butterworth B.E.(1990)
Measurement of chemically induced cell proliferation in rodent liver and kidney : A comparison of 5-bromo-2' deoxyuridine and (^{3}H) thymidine administration by injection or osmotic pump.
Carcinogenesis V11(12), 2245-2251

Elitsur Y., Majumdar A.P.N.,Sakr W.A. and Luk G.D. (1991)
Epidermal growth factor regulation of DNA synthesis in human colonic lamina propria lymphocytes.
Digestive Diseases and Sciences 36(3), 335-340

Evans C.M., Philips A.D., Walker-Smith J.A., MacDonald T.T. (1992)
Activation of lamina propria T cells induces crypt epithelial proliferation and goblet cell depletion in cultured human fetal colon.
Gut 33, 230-235

Fabian R.J., Abraham R., Coulston F., Golberg L.(1973)
Carrageenan-induced squamous metaplasia of the rectal mucosa in the rat.
Gastroenterology 65, 265-276

Falkoff R.J.M., Muraguchi A., Hong J.X., Butler J.L., Dinarello C.A., Fauci A.S. (1983)
The effects of interleukin 1 in human B cell activation and proliferation.
Journal of Immunology 131, 801-805

Fedorak R.N., Empey L.R., MacArthur C. and Jewell L.D. (1990)
Misoprostol provides a colonic mucosal protective effect during acetic acid-induced colitis in rats.
Gastroenterology 98, 615-625

Fidler J.M. (1985)
Induction of hapten-specific immunological tolerance and immunity in B lymphocytes. VII Corelation betwen trinitrobenzenesulfonic acid administration, serum trinitrophenyl content, and level of tolerance.
Cell Immunology 94, 285-291

Filipe M.I., Dawson I. (1970)
The diagnostic value of muco-substances in rectal biopsies from patients with ulcerative colitis and Crohn's disease.
Gut 11, 229-234

Flower R.J. (1988)
Lipocortin and the mechanism of action of the glucocorticoids.
British Journal of Pharmacology 94, 987-1015

Forgue-Lafitte M.E., Kobari L.K., Gespach C., Chamblier M.C., and Rosselin G.(1984)
Characterization and repartition of epidermal growth factor-urogatrone receptors in gastric glands isolated from young and adult guinea pigs.
Biochimica and Biophysica Acta 798, 192-198.

Foster H McA and Whitehead R.H.(1990)
Intravenous but not intracolonic epidermal growth factor maintains colonocyte proliferation in defunctioned rat colorectum.
Gastroenterology 99, 1710-1714

Gallo-Payet N., Pothier P. and Hugon J.S.(1987)
Ontogeny of EGF receptors during postnatal development of mouse small intestine.
Journal of Pediatric Gastroenterology and Nutrition 6, 114-120.

Gan B.S., MacCannell K.L. and Hollenberg M.D. (1987)
Epidermal growth factor-urogastrone causes vasodilation in the anesthetized dog.
Journal of Clinical Investigation 80, 199-206

Garcia J.F (1957)
Changes in blood, plasma and red cell volume in the male rat, as a function of age.
American Journal of Physiology 190(1), 19-24

Gibson P.R. and Pavli P. (1992)
Pathogenic factors in inflammatory bowel disease.
Digestive Diseases and Sciences 10, 17-28.

Gilat T., Hacohen D., Lilos P., Langman M.J.S.(1987)
Childhood factors in ulcerative colitis and Crohn's disease
Scandinavian Journal of Gastroenterology 22, 1009-1024

Goldstein F., Farquar S., Thornton J.J., Abramson J. (1987)
Favorable effects of sulfasalazine on small bowel Crohn's disease: A long term study.
The American Journal of Gastroenterology 82(9), 848-853

Goodlad R.A., Lee C.Y. and Wright N.A. (1992)
Cell proliferation in the small intestine and colon of intravenously fed rats: Effects of urogastrone-epidermal growth factor.
Cell Proliferation 25, 393-404

Goodlad R.A., Wilson T.J.G., Lenton W., Gregory H., McCullagh K.G. and Wright N.A.(1987)
Proliferative effects of urogastrone-EGF on the intestinal epithelium.
Gut 28S1, 37-43

Goodlad R.A. and Wright N.A. (1983)
Effects of addition of kaolin or cellulose to an elemental diet in intestinal cell proliferation in the mouse.
British Journal of Nutrition 50, 91-98

Gould S.R., Brash A.R., Conolly M.E.and Lennard-Jones J.E. (1981)
Prostaglandins, Leukotrienes Medicine 6, 165-182

Greenhalgh D.G., Sprugel K.H., Murray M.J. and Ross R. (1990)
PDGF and FGF stimulate wound healing in the genetically diabetic mouse.
American Journal of Pathology 136(6), 1235-1246

Gregory H. (1975)
Isolation and structure of urogastrone and its relationship to epidermal growth factor.
Nature 257, 325-327

Gregory H., Walsh S and Hopkins C R. (1979)
The identification of urogstrone in serum, saliva and gastric juice.
Gatroenterology 77, 313-318.

Griffiths A.M., Drobnies A., Soldin S.J., Hamilton J.R.(1986)
Enteric protein loss measured by fecal α1-antitrypsin clearance in the assessment of Crohn's disease activity: A study of children and adolescents
Journal of Paediatric Gastroenterology and Nutrition 5(6) 907-911

Grisham M.B. and Granger D.N. (1988)
Neutrophil-mediated mucoal injury: Role of reactive oxygen metabolites.
Digestive Diseases and Sciences 33(3), 6-15

Grisham M.B., von Ritter C., Smith B.F., LaMont J.T., Granger D.N. (1987)
Interaction between oxyradicals and gastric mucin.
American Journal of Physiology 253, G96-G96

Gyde S.N., Prior P., Allan R.N., Stevens A., Jewell D.P.,Truelove S.C., Lofberg R., Brostrom O., and Hellers G.(1988)
Colorectal cancer in ulcerative colitis: a cohort study of primary referrals from three centres.
Gut 29, 206-217

Gysin B., Muller R.K.M., Otten U. and Fischili A.E. (1988)
Epidermal growth factor content of submandiblar glands is increased in rats with experimentally induced gastric lesions.
Scandinavian Journal of Gastroenterology 23, 665-671

Haggit R.C. (1990)
Differential diagnosis of colitis.
IN Gastrointestinal Pathology. International Academy of Pathology Monograph.
Goldman H., Appelman H., Kaufman N. (eds.)
U.S.A. 325-355

Harris J., Shields R.(1970)
Absorption and secretion of water and electrolytes by the intact human colon in diffuse untreated proctocolitis
Gut 11, 27-33

Harris R.C., Hoover R.L., Jacobson H.R. and Badr K.F. (1988)
Evidence for glomerular actions of epidermal growth factor in the rat.
Journal of Clinical Investigations 82, 1028-1039

Hase S., Nakazawa S., Tsukamoto Y., Segawa K. (1989)
Effects of prednisolone and human epidermal growth factor on angiogenesis in granulation tissue of gastric ulcer induced by acetic acid.
Digestion 42, 135-142

Hawkey C.J. and Rampton D.S. (1985)
Prostaglandins and the gastrointestinal mucosa: Are they important in its function, disease or treatment.
Gastroenterology 89, 1162-1188

Heitz PU, Kasper M, Van Noorden S, Polak JM, Gregory H and Pearse AGE.(1978)
Immunohistochemical localization of urogastrone to human duodenal and submandibular glands.
Gut 19, 408-413.

Heldin P., Laurent T.C., Heldin C.H. (1989)
Effects of growth factors on hyaluronan synthesis in cultured human fibroblasts.
Biochemistry Journal 258, 919-922

Hertzog P.J.and Linnane A.W. (1988)
Intestinal mucin antigens associated with colorectal caracinoma.
IN Proceedings of the Biology of the Colonic Mucosa Conference,
Royal Melbourne Hospital, Australia 1988.

Hibi T., Aiso S., Ishikawa M. (1983)
Circulating antibodies to the surface antigens on colon epithelial cells in ulcerative colitis.
Clinical Experimental Immunology 54, 163-168

Hill R.E., Hercz A., Corey M.L., Gilday D.L., Hamilton J.R.(1981)
Fecal clearance of α1-antitrypsin: A reliable measure of enteric protein loss in children.
The Journal of Paediatrics 99(3), 416-418

Hirata Y. and Orth D.N. (1979)
Epidermal growth factor (Urogastrone) in human tissues.
Journal of Clinical Endocrinology and Metabolism 48(4), 667-672

Hoober J.K. and Cohen S. (1967)
Epidermal growth factor 1. The stimulation of protein and ribonucleic acid synthesis in chick embryo epidermis.
Biochemica et Biophysica Acta 138, 347-356

Hoult J.R.S. (1986)
Pharmacological and biochemical actions of sulphasalazine. Mode of Action
Drugs 32(S1), 18-26

Ireland A., Mason C.H., Jewell D.P.(1988)
Controlled trial comparing olsalazine and sulphasalazine for the maintenance treatment of ulcerative colitis.
Gut 29, 835-837

Ishioka T., Kuwabara N., Oohashi Y., Wakabayashi K. (1987)
Induction of colorectal tumors by sulfated polysaccharides.
CRC Critical Reviews in Toxicology 17(3), 215-244

James R.and Bradshaw R.A. (1984)
Polypeptide growth factors.
Annual Review of Biochemistry 53, 259-292

James P.S., Smith M.W., Tivey D.R and Wilson T.J.G. (1987)
Epidermal growth factor selectively increases maltase and sucrase activities in neonatal piglet intestine.
Journal of Physiology 393, 583-594

Jensen B.H., Anderson J.O., Poulsen S.S., Skov-Olsen P., Norby-Rasmussen S., Hansen S.H., Hvidberg E.J. (1984)
The prophylactic effect of 5-aminosalicyclic acid and asalosulphypyridine on degraded carrageenan-induced colitis in the guinea pig.
Scandinavian Journal of Gastroenterology 19(3), 299-303

Jewell D.P. (1989)
Corticosteroids for the management of ulcerative colitis and Crohn's disease.
Gastroenterology Clinics of North America 18(1), 21-34

Jewell D.P. and Truelove S. (1974)
Azathioprine in ulcerative colitis: Final report on a controlled therapeutic trial.
British Medical Journal 4, 627

Johansson C., Kollberg B. (1979)
Stimulation by intragastrically administered E_2 prostaglandins of human gastric mucus output.
European Journal of Clinical Investigation 9, 229-232

King S.R., Hickerson W.L., Proctor K.G. and Newsone A.M. (1991)
Beneficial actions of exogenous hyaluronic acid on wound healing.
Surgery 109(1), 76-84

Kirk A.P. and Lennard-Jones J.E. (1982)
Controlled trial of azathioprine in chronic ulcerative colitis.
British Medical Journal 284, 1291-1292

Kirkegaard P., Skov-Olsen P., Poulsen S.S. and Nexo E. (1983)
Epidermal growth factor inhibits cysteamine-induced duodenal ulcers.
Gastroenterology 85, 1277-1283

Kitano A., Matsumoto T., Hiki M., Hashimura H., Yoshiyashi K., Okawa K., Kuwajima S., Kobaysahi K. (1986)
Epithelial dysplasia of the rabbit colon induced by degraded carrageenan.
Cancer Research 46, 1374-1376

Kitazawa T., Konoshita S., Fujita K., Araki K., Watanabe H., Ohashi Y. and Manabe R. (1990)
The mechanism of accelerated corneal epithelial healing by human epidermal growth factor.
Investigative Opthalmology and Visual Science 31(9), 1773-1778

Klein S., Meyers S., O'Sullivan P., Barton D., Leleiko N., Janowitz H.D. (1988)
The metabolic impact of active ulcerative colitis-energy expenditure and nitrogen balance.
Journal of Clinical Gastroenterology 10(1), 34-40

Koenders P.G., Peters W.H.M., Wobbes Th. Beex L.V.A.M., Nagengast F.M. and Benraad Th J. (1992)
Epidermal growth factor receptor levels are lower in carcinomatous than in normal colorectal tissue.
British Journal of Cancer 65, 189-192

Koffman et al. (1982)
Effect of urogastrone on gastric secretion and serum gastrin in patients with duodenal ulceration.
Gut 23, 951-956

Konturek S.J., Brzozowski T., Dembinski A., Warzecha A., Drozdowicz D. (1988)
Comparison of soloseryl and epidermal growth factors (EGV) in healing of chronic gastroduodenal ulcerations and mucosal growth in rats.
Hepato-Gastroenterology 35, 25-29

Konturek S.J., Brzozowski T., Drozdowicz D., Dembinski A., Nauert C. (1990)
Healing of chronic gastroduodenal ulcerations by antacids. Role of prostaglandins and epidermal growth factor.
Digestive Diseases and Sciences 35(9), 1121-1129

Konturek S.J., Brzozowski T., Piastucki I., Dembinski A., Radecki T., Dembinska-Kiec A., Zmuda A., Gregory H. (1981)
Role of mucosal prostaglandins and DNA synthesis in gastric cytoprotection by luminal epidermal growth factor.
Gut 22, 927-932

Konturek S.J., Dembinski A., Warzecha Z., Brzozowski T. and Gregory H. (1988)
Role of epidermal growth factor in healing of chronic gastroduodenal ulcers in rats.
Gastroenterology 94, 1300-1307

Konturek S.J., Dembinski A., Warzecha Z., Bielanski W., Brzozowski T. and Drozdowicz D. (1988a)
Epidermal growth factor (EGF) in the gastroprotective and ulcer healing actions of colloidal bismuth subcitrate (De-Nol) in rats.
Gut 29, 894-902

Konturek S.J., Pawlik W., Mysh P., Gustaw P., Sendur R., Mikos E., and Bielanski W. (1990)
Comparison of organ uptake and disappearance half-time of human epidermal growth factor and insulin.
Regulatory Peptides 30, 137-148

Konturek S.J., Radecki T., Brzozowski T., Piastucki I., Dembinski A., Dembinska-Kiec A., Zmuda A., Gryglewski R. and Gregory H. (1981)
Gastric cytoprotection by epidermal growth factor. Role of endogenous prostaglandins and DNA synthesis.
Gastroenterology 81, 438-442

Korelitz B. (1985)
General observations on inflammatory bowel disease.
IN Inflammatory bowel disease: Experience and Controversey
Korelitz B.I.and Sohn N.(eds.)
Grune and Stratton U.S.A. pp 3-7

Koretz K., Schlag P. and Moller P. (1990)
Expression of epidermal growth factor receptor in normal colorectal mucosa, adenoma and carcinoma.
Virchows Archiv A Pathol Anat 416, 343-349.

Krugliak P., Hollander D., Ma T.Y., Tran D., Dadufalza V.D., Katz K.D., Le K. (1989)
Mechanisms of polyethylene glycol 400 permeability of perfused rat intestine.
Gastroenterology 97, 1164-1170

Kumegawa M., Hiramatsu M., Yajima T., Hatakeyama K., Hoseda S., Namba M. (1982)
Effect of epidermal growth factor on collagen formation in liver-derived epithelial clone cells.
Endocrinology 110 607-612

Kusugami K., Youngman K.R., West G.A. (1989)
Intestinal immune reactivity to interleukin-2 differs among Crohn's disease, ulcerative colitis and controls.
Gastroenterology 97(1)

Langman J.M., Rowland R., Vernon-Roberts B. (1985)
Carrageenan colitis in the guinea pig: pathological changes and the importance of ascorbic acid deficiency in disease induction.
Australian Journal of Experimental Biology and Medical Science 63(5), 545-553

Lauritsen K. (1989)
Drug treatment and formation of eicosanoids in patients with chronic inflammatory bowel disease.
Danish Medical Bulletin 36(4), 378-393

Lauritsen K., Staerk Laursen l., Bukhave K. and Rask-Madsen J. (1988)
Use of colonic eicosanoid concentrations as predictors of relapse in ulcerative colitis: double blind placebo controlled study on sulphasalazine maintenance teatment.
Gut 29, 1316-1321

LeDuc L.E. and Nast C.C. (1990)
Chemotactic peptide-induced acute coliits in rabbits.
Gastroenterology 98, 929-935

Lembach K.J. (1976)
Enhanced synthesis and extracellular accumulation of hyaluronic acid during stimulation of quiescent human fibroblasts by mouse epidermal growth factor.
Journal of Cell Physiology 89, 277-288

Lennard-Jones et al. (1975)
Assessment of severity of colitis: a preliminary study.
Gut 16, 579-584

Ling K.Y., Bhalla D., Hollander D. (1988)
Mechanism of carrageenan injury of IEC18 small intestinal epithelial cell monolayers.
Gastroenterology 95, 1487-1495

Lipkin M. (1985)
Growth and development of gastrointestinal cells.
Annual Review of Physiology 47, 175-197.

Lushbaugh C., Humason G., Clapp N. (1985)
Histology of colitis: Saguinus oedipus oedipus and other marmoset.
Digestive Disease and Sciences 30(12), 45S-51S

Macdermott R.P. (1985)
Review of clinical aspects of cancer of the colon in patients with ulcerative colitis.
Digestive Diseases and Sciences 30(12), 1145-1185

Macdermott R.P. and Stenson W.F. (1988)
Alterations of immune system in ulcerative colitis and Crohn's disease.
Advances in Immunology 42, 285-328

MacDonald T.T. and Spencer J.(1988)
Evidence that activated mucosal T cells play a role in the pathogenesis of enteropathy in human small intestine.
Journal of Experimental Medicine 167, 1341-1349

MacPherson B.R. and Pfeiffer C.J. (1976)
Experimental Colitis.
Digestion 14, 424-452

MacPherson B.R. and Pfeiffer C.J. (1978)
Experimental production of diffuse colitis in rats.
Digestion 17, 135-150

Mahida Y.R., Wu K., Jewell D.P. (1989)
Enhanced production of interleukin 1B by mononuclear cells isolated from mucosa with active ulcerative colitis and Crohn's disease.
Gut 30, 835-838

Mantovani A., Dejana E. (1989)
Cytokines as communication signals between leukocytes and endothelial cells.
Immunology Today 10, 370-375

Marcus R. and Watt J. (1971)
Colonic ulceration in young rats fed degraded carrageenan.
Lancet 2, 765

Massague (1983)
Epidermal growth factor-like transforming growth factor.
Journal of Biological Chemistry 258, 13614-13620

Matsumoto K. and Nakamura T. (1991)
Hepatocyte growth factor: Molecular structure and implications for a central role in liver regeneration.
Journal of Gastroenterology and Hepatology 6, 509-519

Maxton D.G., Bjarnson I., Reynolds A.P., Catt S.D., Peters T.J., Menzies I.S. (1986)
Lactulose, 51Cr-labelled ethylenediaminetetra acetate, L-rhamnose and polyethylene glycol 400 as probe markers for assessment in vivo of human intestinal permeability.
Clinical Science 71, 71-80

McIllmurray M.B., Langman M.J.S. (1975)
Large bowel cancer: causation and management
Gut 17, 815-820

Mee A.S., Jewell D.P. (1980)
Monocytes in inflammatory bowel disease: Monocyte and serum lysosomal enzyme activity.
Clinical Science 58, 295-300

Menard D., Arsenault P., and Pothier P. (1988)
Biological effects of epidermal growth factor in human fetal jejunum.
Gastroenterology 94, 656-663

Menard D., Pothier P. and Gallo-Payet N. (1987)
Epidermal growth factor receptors during postnatal development of the mouse colon.
Endocrinology 121(4), 1548-1554

Meyers S. and Janowitz H.D. (1985)
Systemic conricosteroid therapy of ulcerative colitis.
Gastorenterology 89, 1189-1199

Morris G.P., Beck P.L., Herridge M.S., Depew W.T., Szewczuk M.R., Wallace J.L.(1989)
Hapten-induced model of chronic inflammation and ulceration in the rat colon.
Gastroenterology 96, 795-803

Morrison J.R., Lloyd C.J., Grego B., Burgess A.W., Nice E.C. (1985)
Rat epidermal growth factor: complete amino acid sequence. Homology with the corresponding murine and human proteins. Isolation of a form truncated at both ends with full *in vitro* biological activity.
European Journal of Biochemistry 153, 629-637

Moyer M.P.(1984)
Human colon cells: Culture and *in vitro* transformation.
Science 224, 1445-1447

Mulder C.J., Rondos A.A., Wilkink E.H., Tytgat G.N. (1989)
Topical corticosteroids in inflammatory bowel disease.
Netherlands Journal of Medicine 35, 527-534

Mullin J.M and McGinn M.T.(1988)
Epidermal growth factor-induced mitogenesis in kidney epithelial cells (LLC-PK1).
Cancer Research 48, 4886-4891

Mulvihill S.J., Stone M.M., Fonkalsrud E.W., Debas H.T. (1986)
Trophic effect of amniotic fluid on fetal gastrointestinal development.
Journal of Surgical Research 40, 291-296

Murphy M.S., Eastham E.J., Nelson R., Pearson A.D.J., Laker M.F. (1989)
Intestinal permeability in Crohn's disease.
Archives of Disease in Childhood 64, 321-325

Nexo E., Hollenberg M.D., Figueroa A., Pratt R.M. (1980)
Detection of epidermal growth factor-urogastrone and its receptor during fetal mouse development.
Developmental biology 77(5), 2782-2785

Nexo E.and Poulsen S.S. (1987)
Does epidermal growth factor play a role in the action of sucralfate?
Scandinavian Journal of Gastroenterology 127, 22-45

Nexo E., Lamberg S.I. and Hollenburg M.D.(1981)
Comparison of a receptor binding assay with a radioimmunoassay for measuring human epidermal growth factor-urogastrone in urine.
Scandinavian Journal of Clinical Laboratory Investigations 41, 577-582

Nielsen O.H., Bukhave K., Ahnfelt-Ronne I., Rask-Madsen J. (1988)
Source of endogenous arachidonate and 5-lipoxygenase products in human neutrophils stimulated by bradykinin and A231287.
Gut 29, 319-324

Okabe S., Roth J.L. and Pfeiffer C.J. (1971)
A method for experimental, penetrating gastric and duodenal ulcers in rats.
Digestive Diseases and Sciences 16(3), 277-284

Olaison G., Sjodahl R., Leandersson R. and Tagesson C.(1989)
Abnormal intestinal permeability pattern in colonic Crohn's disease.
Scandinavian Journal of Gastroenterology 24, 571-576

Olaison G., Sjodahl R., Tagesson C. (1990)
Abnormal intestinal permeability in Crohn's disease.
Scandinavian Journal of Gastroenterology 25, 321-328

Onderbonk A.B. (1985)
Experimental models for ulcerative colitis.
Digestive Diseases and Sciences 30 (12), 405-455

Peltekian K.M., Williams C.N., MacDonald A.S. (1987)
Open study of cyclosporin A (CY A) in Crohn's disease (CD).
Gastroenterology 94, A137

Pesonen K., Viinikka L., Koskimies A., Banks A.R., Nicolson M. and Perheentupa J. (1987)
Size heterogeneity of epidermal growth factor in human body fluids.
Life Sciences 40, 2489-2494

Podolsky D.K., Isselbacher K.J. (1983)
Composition of human colonic mucin-selective alteration in inflammatory bowel disease
Journal of Clinical Investigations 72,142-153

Podolsky D.K., Isselbacher K.J. (1984)
Glycoprotein composition of colonic mucosa: Specific alterations in ulcerative colitis.
Gastroenterology 87, 991-998

Pollack P.F., Goda T., Colony P.C., Edmond J., Thornburg W., Korc M. and Koldovsky O.(1987)
Effects of enterally fed epidermal growth factor on the small and large intestine of the suckling rat.
Regulatory Peptides 17, 121-132.

Pothier P. and Menard D. (1988)
Presence and characteristics of epidermal growth factor receptors in human fetal intestine and colon.
FEBS letters 228(1), 13-117

Poulsen S.S., Nexo E., Skov Olsen P., Hes J., Kirkegaard P. (1986)
Immunohistochemical localization of epidermal grwoth factor in rat and man.
Histochemistry 85, 389-394

Poulsen S.S.(1988)
On the role of epidermal growth factor in the defence of the gastrododenal mucosa.
Scandinavian Journal of Gastroenterology 22 (S128), 20-23

Powell-Tuck J., Day D.W., Buckell N.A., Wadsworth J., Lennard-Jones J.E.(1982)
Correlations between defined sigmoidoscopic appearances and other measures of disease activity in ulcerative colitis.
Digestive Diseases and Sciences 27(6), 533-537

Prior P.and Gyde S.N.(1982)
Cancer morbidity in ulcerative colitis
Gut 23, 490-497

Rabin B.S. (1980)
Immunological model of inflammatory bowel disease. Animal model.
American Journal of Pathology 99(1), 253-256

Rachmilewitz D., Karmeli F., Sharon P.(1984)
Decreased colonic Na-K-ATPase activity in active ulcerative colitis.
Israel Journal of Medical Sciences 20, 681-684

Ramage J.K., Stanisz A., Scicchitano R., Hunt R.H., Perdue M.H. (1988)
Effect of immunologic reactions on rat intestinal epithelium. Correlation of increased permeability to Chromium 51-labeled ethylenediamine-tetra acetic acid and ovalbumin during acute inflammation and anaphylaxis.
Gastroenterology 94, 1368-1375

Ramakrishna B.S. and Mathan V.I. (1988)
Absorption of water and sodium and activity of adenosine triphosphatases in the rectal mucosa in tropical sprue.
Gut 29, 665-668

Rao R.K., Koldovsky O., Grimes J., Williams C. and Davis T.P. (1991)
Regional differences in gastrointestinal processing and absorption of epidermal grwoth factor in suckling rats.
American Journal of Physiology 261(24) G790-G798

Rao S.S.C., Holdsworth C.D., Read N.W.(1988)
Symptoms and stool patterns in patients with ulcerative colitis.
Gut 29, 342-345

Rask-Madsen J.and Dalmark M. (1973)
Decreased transmural potential difference across the human rectum in ulcerative colitis.
Scandinavian Journal of Gastroenterology 8, 321-326

Read L.C., Gale S.M.and George-Nascimento C. (1987)
Intestinal absorption of epidermal growth factor in newborn lambs.
IN Human Lactation 3. The Effects of Human Milk on the Recipient Infant. Goldman A.S and Brady R.O.(eds). 119

Read L.C., Tartantal A. and George-Nascimento C. (1989)
Effects of recombinant human epidermal growth factor on the intestinal growth of fetal rhesus monkeys.
Acta Paediatric Scandinavian Supplement 351, 97-103

Read LC, Summer L, Gale SM, George-Nascimento C, Ballard FJ and Wallace JC. (1986)
Immunological, receptor-binding and biological properties of synthetic-gene recombinant human epidermal growth factor: Comparison with the natural growth factor from human urine and milk. Journal Endocrinology 109, 245-250.

Reeves J.R., Richards R.C. and Cooke T.(1991)
The effects of intracolonic EGF on mucosal growth and experimental carcinogenesis.
British Journal of Cancer 63, 223-226

Reid P.E., Culling C.F.A., Dunn W.L., Ramey C.W., Clay M.G. (1984)
Chemical and histochemical studies of normal and diseased human gastrointestinal tract.1. A comparison between histologically normal colon; colonic tumours;ulcerative colitis and diverticular disease of the colon.
Histochemical Journal 16, 235-251

Rhodes J.M., Black R.R., Gallimore R., Savage A. (1985)
Histochemical demonstration of desialation and desulphation of normal and inflammatory bowel disease rectal mucosa by faecal extracts.
Gut 26, 1312-1318

Rhodes J. and Heatley R.V. (1983)
Medical treatment: Topical and systemic corticosteroids.
IN Inflammatory Bowel Diseases
Allan R.N., Keighley M.R.B., Alexander-Williams J. and Hawkins C. (eds)
Churchill Livingston, N.Y.221-226

Riley S.A., Mani V., Goodman M.J., Herd M.E., Dutt S., Turnberg L.A.(1988)
Comparison of delayed release 5 aminosalicyclic acid (mesalazine) and sulphasalazine in the treatment of mild to moderate ulcerative colitis relapse.
Gut 29, 669-674

Roediger W.E.W. (1988)
What sequence of pathogenic events leads to acute ulcerative colitis?
Disease of the Colon and Rectum 31,

Roediger W.E.W., Lawson M.J.K., Wok V., Kerr Grant A., Pannall P.R.(1984)
Colonic bicarbonate output as a test of disease activity in ulcerative colitis.
Journal of Clinical Pathology 37, 704-707

Roediger W.E.W., Truelove S.C. (1979)
Method of preparing isolated colonic epithelial cells (colonocytes) for metabolic studies.
Gut 20, 484-488

Rolandelli R.H., Saul S.H., Settle R.G., Jacobs D.O., Trerotolo S.O., Rombeau J.L. (1988)
Comparison of parenteral nutrition and enteral feeding with pectin in experimental colitis in the rat.
American Journal of Clinical Nutrition 47, 715-721

Rosenthal S.L. and Matheson A. (1973)
ATPase in isolated membranes of Bacillus Subtilis.
Biochimica et Biophysica Acta 318; 252-261

Roth J.L.A. (1980)
Diagnosis and differential diagnosis of chronic ulcerative colitis and Crohn's colitis.
IN Kirsner J.B. and Schorter R.G. (eds.) Inflammatory bowel disease. Lea and Febiger Philadelphia

Ryan G.P., Dudrick S.J., Copeland E.M. and Johnson L.R. (1979)
Effects of various diets on colonic growth in rats.
Gastroenterology 77, 658-663

Sakata T., von Engelhardt W. (1981a)
Luminal mucin in the large intestine of mice; rats and guinea pigs.
Cell and Tissue Research 219, 629-635

Sakata T., von Engelhardt W. (1981b)
Influence of short-chain fatty acids and osmolality on mucin release in the rat.
Cell and Tissue Research 219, 371-377

Sakata T. and von Engelhardt W (1983)
Stimulatory effect of short chain fatty acids on the epithelial cell proliferation in rat large intestine.
Comparative Biochemistry and Physiology 74A, 459-462

Sarosiek J., Bilski J., Murty V.L.N., Slomiany A. and Slomiany B.L.(1988)
Role of salivary epidermal growth factor in the maintenance of physiochemical characteristics of oral and gastric mucosal mucus coat.
Biochemical and Biophysical Reasearch Communications 152(3), 1421-1427.

Savage C.R. and Cohen S.(1972)
Proliferation of corneal epithelium induced by epidermal growth factor.
Experimental Eye Research 15, 361-366

Schaudies RP, Grimes J, Davis D, Rao RK and Koldovsky O.(1989)
EGF content in the gastrointestinal tract of rats: effect of age and fasting/feeding.
American Journal of Physiology 256, G856-G861.

Scheimer R.P.(1985)
The mechanism of anti-inflammatory steroid action in allergic disease.
Annual Review of Pharmacology and Toxicology 25, 381-412

Scheving L.A., Yeh Y.C., Tsai T.H. and Scheving L.E. (1980)
Circadian phase-dependent stimulatory effects of epidermal growth factor on deoxyribonucleic acid synthesis in the duodenum, jejunum, ileum, caecum. colon. and rectum of the adult male mouse.
Endocrinology 106, 1498-1503

Schultz G., Rotatori D.S., Clark W. (1991)
EGF and TGF-α in wound healing and repair.
Journal of Cellular Biochemistry 45, 346-352

Scott J., Patterson S., Ra L., Bell G.I., Crawford R., Penschow J., Niall H.and Coghlan J., (1985)
The structure and biosynthesis of epidermal growth factor precursor.
Journal of Cell Science S3, 19-28

Serafini E.P., Kirk A.P. Chambers T.J.(1981)
Rate and pattern of epithelial cell proliferation in ulcerative colitis.
Gut 22, 648-652

Shamsuddin A.M., Phelps P.C., Trump B.F. (1982)
Human Large Intestinal Epithelium: Light microscopy, hitochemistry, and ultrastructure.
Human Pathology 13, 790-803

Sharon P., Ligumsky M., Rachmilewitz D., Zor U. (1978)
Role of prostaglandins in ulcerative colitis. Enhanced production during active disesae and inhibition by sulfasalzine.
Gastroenterology 75, 638-640

Sharon P. and Stenson W.F. (1984)
Enhanced synthesis of leukotriene B4 by colonic mucosa in inflammatory bowel disease.
Gastroenterology 86,453-460

Sharon P. and Stenson W.F. (1985)
Metabolism of arachidonic acid in acetic acid colitis in rats. Similarity to human inflammatory bowel disease.
Gastroenterology 88, 55-63

Sharrat M., Grasso P., Carpanini F., Gangolli S.D. (1971)
Carrageenan ulceration as a model for human ulcerative colitis
Lancet 192,193

Shields H.M., Bates M.L., Goldman H., Zuckerman G.R., Mills B.A., Best C.J., Bair F.A., Goran D.A., DeSchryver-Kecskemeti K. (1985)
Scanning electron microscopic appearance of chronic ulcerative colitis with and without dysplasia.
Gastroenterology 89, 62-67

Shorb P.E.(1989)
Surgical therapy for Crohn's disease (1989)
Gastroenterology Clinics of North America 18(1), 111-128

Singh G. and Foster C.S. (1987)
Epidermal growth factor in alkali-burned corneal epithelial wound healing.
American Journal of Opthalmology 103, 802-807

Sizeland A., Bol S. and Burgess A.W.(1991)
The action of epidermal growth factor (EGF) is limited to specific phases of the cell cycle in an EGF dependent colonic cell line.
Growth Factors 4, 129-143.

Skov-Olsen P., Poulsen S.S., Kirkegaard P. and Nexo E. (1984)
Role of submandibular saliva and epidermal growth factor in gastric cytoprotection.
Gastroenterology 87, 103-108

Skov-Olsen P., Poulsen S.S., Therkelsen K. and Nexo E. (1986a)
Effect of sialodenectomy and synthetic human urogastrone on healing of chronic gastric ulcers in rats.
Gut 27, 1443-1449

Skov-Olsen P., Poulsen S.S., Therkelsen K. and Nexo E. (1986b)
Oral administration of synthetic human urogasrone promotes healing of chronic duodenal ulcers in rats.
Gastroenterology 90, 911-917

Smith L.E. (1989)
Surgical therapy in ulcerative colitis.
Gastroenterology Clinics of North America 18(1), 99-110

Sprugel K.H., McPherson J.M., Clowes A.W., Ross R. (1987)
Effects of growth factors *in vivo*. 1. Cell ingrowth into porous subcutaneous chambers.
American Journal of Pathology 129, 601-613

Stragand J.J. and Hagemann R.F.(1977)
Effect of luminal contents on colonic cell replacement.
American Journal of Physiology 233, E208-211

Strober W. (1985)
Animal models of inflammatory bowel disease - an overview.
Digestive Diseases and Sciences 30(12), 3-10

Sunter J.P., Wright N.A. and Appleton D.R. (1979a)
Cell population kinetics in the epithelium of the colon of the male rat.
Virachows Archives B Cell Pathology 26, 275-287

Swartz S.L. and Dluhy R. (1978)
Corticosteroids: Clinical pharmacology and therapeutic use.
Drugs 16, 238-255

Szabo S., Bynum T.E. (1988)
Review: Alternatives to the acid-orientated approach to ulcer disease: Does 'cytoprotection' exist in man? A new classification of antiulcer agents.
Scandinavian Journal of Gastroenterology 23, 1-

Taffet S.L. and Das K.M. (1982)
Desensitization of patients with inflammatory bowel disease to sulfasalazine.
The American Journal of Medicine 73, 520-524

Taffet S.L. and Das K.M. (1983)
Sulfasalazine. Adverse Effects and Desensitization.
Digestive Disease and Sciences 28(9), 833-842

Takeuchi K.and Johnson L.R.(1986)
Effect of cell proliferation on healing of gastric and duodenal ulcers in rats.
Digestion 33, 92-100

Talha S. and Harel L.(1985)
Early stimualtion of ATP turnover induced by growth factors. Synergistic effect of EGF and insulin and correlation with DNA synthesis.
Experimental Cell Research 158, 311-320

Tam J.P. (1985)
Physiologcial effects of transforming growth factor in the newborn mouse.
Science 229, 673-

Tanner A.R., Arthur M.J.P., Wright R.(1984)
Macrophage activation, chronic inflammation and gastrointestinal disease.
Gut 25 760-783

Targan S.R., Deem R.L., Shanahan F.(1991)
Role of mucosal T-cell-generated cytokines in epithelial cell injury.
Immunology Research 10, 472-478

Taylor J.M., Mitchell W.M. and Cohen S. (1972)
Epidermal growth factor - physical and chemical properties.
Journal of Biological Chemistry 247, 5928-5934

Taylor K.B.(1980)
Ulcerative colitis and Crohn's disease of the colon: symptoms, signs and laboratory aspects.
141-149

Tepperman B.L., Soper B.D. and Morris G.P.(1989)
Effect of sialoadenectomy on adaptive cytoprotection in the rat.
Gastroenterology 97, 123-129.

Thim L. (1989)
A new family of growth factor-like peptides. 'Trefoil' disulphide loop structures as a common featrue in breast cancer associated peptide (pS2), pancreatic spasmolytic polypeptide (PSP), and frog skin peptides (spasmolysins).
FEBS Letters 250,85-90

Thomas D.W., Sinatra F.R., Merrit R.J. (1981)
Random fecal α1-antitrypsin concentration in children with gastrointestinal disease.
Gastroenterology 80, 776-782

Thompson J.F. (1988)
Specific receptors for epidermal growth factor in rat intestinal microvillus membranes.
American Journal of Physiology 254, G429-G435

Thompson J.S., Brag L.E., Saxena S.K.(1990)
The effect of intestinal resection and urogastrone on intestinal regeneration.
Archives of Surgery 125, 1617-1621

Tomas F.M., Murray A.J., Jones L.M.(1984)
Modifications of glucocorticoid-induced changes in myofibrillar protein turnover in rats by protein and energy deficiency as assessed by urinary excretion of N5-methylhistine.
British Journal of Nutrition 51, 323-337

Tripp J.H., Muller D.P.R. and Harries J.T. (1980)
Mucosal (Na^+-K^+)-ATPase and adenylate cyclase activities in children with toddler diarrhoea and postenteritis syndrome.
Pediatric Research 14; 1382-1386

Truelove S.C. (1988)
Evolution of Olsalazine.
Scandinavian Journal of Gastroenterology 23(S148), 3-6

Tsukumo K., Nakamura H., Sakamoto S. (1987)
Purification and Characterization of high molecular weight human epidermal growth factor from human urine.
Biochemical and Biophysical Research Communications 145, 126-133

Usugane M., Fujita M., Palmer R., Friedman E., Augenlicht L. (1982)
Cell proliferation in explant cultures in human colon .
Digestion 24, 225-233

van Hees P.A.M., Makker J.H., van Tongeren J.H.M. (1980)
Effect of sulphapyridine, 5-aminosalicyclic acid, and placeboe in patients with idiopathic proctitis; a study to determine the actice therapeutic moiety of suphasalazine.
Gut 21, 632-635

Verspaget H.W., Pena A.S., Weterman I.T., Lamers C.B.H.W. (1988)
Diminished neutrophil function in Crohn's disease and ulcerative colitis identified by decreased oxidative metabolism and low superoxide dismutase content.
Gut 29 223-228

Vogel C.M., Corwin T.R., Baue A.E. (1974)
Intravenous hyperalimentation. In the treatment of inflammatroy disease of the bowel.
Archives of Surgery 108, 460-467

von Engelhardt W. and Rechkemer G. (1986)
Absorption of inorganic ions and short-chain fatty acids in the colon of mammals.
IN Intestinal Transport.
Gilles-Ballien M. and Gilles R.(eds.), Berlin
27-45

Wallace J.L., Higa A., McKnight G.W., MacIntyre D.E. (1992)
Prevention and reversal of experimental colitis by a monoclonal antibody which inhibits leukocyte adherance.
Inflammation 16(4), 343-354

Watt J. and Marcus R. (1973)
Progress Report: Experimental ulcerative disease of the colon in animals.
Gut 14, 506-510

Watt J., Maclean C., Marcus R. (1979)
Degradation of carrageenan for the experimental production of ulcers in the colon.
Japanese Journal of Pharmacology 31; 645-646

Weaver L.T. and Walker W.A. (1988)
Epidermal growth factor and the developing human gut.
Gastroenterology 94, 845-

Westermark B. (1990)
Review: The molecular and cellular biology of platelet-derived growth factor.
Acta Endocrinology 123, 131-142

Weterman I.T.and Pena A.S.(1984)
Familial incidence of Crohn's disease in the Netherlands and a review of the literature.
Gastroenterology 86, 449-452

Whitehead R.H., Jones J.K., Gabriel A. and Lukies R.E. (1987)
A new colon carcinoma cell line (LIMI863) that grows as organoids with spontaneous differentiation into crypt-like structures *in vitro*.
Cancer Research 47, 2683-2689

Wieriks J., Hespe W., Jaitly K.D., Koekkoek P.H., Lavy U. (1982)
Pharmacological properties of colloidal bismuth subcitrate (CBS, DE-NOL).
Scandinavian Journal of Gastroenterology, 17(S80), 11-16

Wright N. and Alison M. (1984)
The biology of epithelial cell populations.
Clarendon Press, Oxford 684-686

Wright N.A., Pike C., Elia G. (1990)
Induction of a novel epidermal growth factor-secreting cell lineage by mucosal ulceration in human gastrointestinal stem cells.
Nature 343, 82-85

Wright N.A., Poulsom R., Stamp G.W.H., Hall P.A., Jeffrey R.E., Longcroft J.M., Rio M.C., Tomasetto C. and Chambon P. (1990)
Epidermal growth factor (EGF/URO) induces expression of regulatory peptides in damaged human gastrointestinal tissues.
Journal of Pathology 162, 279-284

Yamada K., Yoshitake K., Sato M. and Ahnen D.J. (1992)
Proliferating cell nuclear antigen expression in normal, preneoplastic and neoplastic colonic epithelium of the rat.
Gastroenterology 103, 160-167

Yamada T., Marshall S., Specian R.D.and Grisham M.B. (1992)
A comparative analysis of two models of colitis in rats.
Gastroenterology 102, 1524-4534

Yamada T., Zimmerman B.J., Specian R.D. and Grisham M.B. (1991)
Role of neutrophils in acetic acid-induced colitis in rats.
Inflammation 15(5), 399-411

Yoshida K., Tsujino T., Wataru Y., Kameda T., Toshiake S., Nakayama H., Toge T., Tahara E. (1990)
Induction of growth factor-receptor and metalloproteinase genes by epidermal growth factor and / or transforming growth factor-α in human gastric carcinoma cell line MKN-28.
Japanese Journal of Cancer Research 81, 793-798

Zarnegar R., Muga S., Rahija R. and Michalopoulos G. (1990)
Tissue distribution of hepatopoetin-A: A heparin-binding polypeptide growth factor for hepatocytes.
Proceedings of the National Academy of Sciences U.S.A. 87, 1252-1256

Zeitlin I.J. and Norris A.A. (1983)
Animal models of colitis.
IN Mechanism of gastrointestinal inflammation. BSG / SKand F International workshop.
Stansteak Abbotts (ed.) 70-73