Chemotherapy-induced mucositis: Mechanisms of damage, time course of events and possible preventative strategies.

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Declaration

'This work contains no material which has been accepted for the award of any other degree of 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 has been made in the text.'

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April 2004

Abstract

Mucositis is a major oncological problem affecting large numbers of patients undergoing treatment for cancer. It can be produced by both chemotherapy and radiotherapy and can affect all areas of the gastrointestinal tract. This thesis has concentrated on several key gaps in current scientific knowledge that required investigation to enable a complete understanding of the cellular mechanisms associated with gastrointestinal mucositis. The different aspects of this thesis approach these fundamental knowledge gaps through a series of discrete research chapters which, when combined, provide evidence of the similarity in response to chemotherapy of the differing regions of the gastrointestinal tract. The research chapters investigated; (1, 2) the effects throughout the gastrointestinal tract of chemotherapeutic agents, Methotrexate (MTX) and Irinotecan, (3) the possible ameliorating potential of the cytokine Interleukin-11 in reducing the side effects of chemotherapy, (4) the expression of pro- and anti-apoptotic proteins and transcription factors along the gastrointestinal tract in normal human patients and (5) the time-course of development of oral mucositis in human patients.

Previous research has shown that cancer chemotherapy side effects within the gastrointestinal tract are predominantly due to apoptosis occurring in the crypts of the intestine. It is however unknown, whether there is a relationship between chemotherapy dose, apoptosis, p53/p21 expression and intestinal crypt cell proliferation. This thesis determined that low dose MTX (0.5 mg/kg) caused a high peak of apoptosis but minimal crypt cell hypoproliferation in the rat small intestine, and a lower peak of apoptosis with no crypt cell hypoproliferation in the colon. Higher doses of MTX (1.5; 2.5 and 5.0 mg/kg) caused lower peaks of apoptosis but severe crypt cell hypoproliferation in the small intestine. A change in p53 expression did not precede early high levels of apoptosis in the small intestine, whereas p53 upregulation was increased with late levels of apoptosis.

As mucositis is not specific to any one cytotoxic agent, a further aim of this thesis was to investigate if other cytotoxic drugs caused intestinal mucositis in a similar fashion to that described for MTX. Irinotecan is a commonly used chemotherapy agent, that causes severe mucositis in many patients. This thesis confirmed that Irinotecan acted in a similar fashion to MTX in the small intestine by causing severe small intestinal damage, with increased apoptosis and crypt cell hypoplasia. However, results also showed that

Irinotecan induced severe colonic damage with excessive mucus secretion, both of which are unusual for cytotoxic drugs.

A number of factors, such as interleukin-11 (IL-11), have been shown to have to be anti-mucotoxic potential – particularly for small and large intestinal symptoms. However, before they can be tested for their efficacy in humans, they must be shown to be safe, and not interfere with cytotoxic treatment or cause tumour growth. This part of my thesis aimed to determine if IL-11 ameliorated gastrointestinal mucositis. Results from this initial study also showed that administration of IL-11 caused no change in small intestine weight indicating IL-11 did not have any direct trophic effect on the small intestine. Following administration of MTX, IL-11 was able to ameliorate small intestinal mucositis by maintaining intestinal weight intestinal morphometry, despite being unable to prevent apoptosis. Although MTX induced hypoproliferation of crypt cells, IL-11 improved this reduction by helping to maintain villus area.

Our laboratory has previously shown that apoptosis occurs in the crypts of the small intestine shortly after the administration of chemotherapy and this may be directly related to the damage that is subsequently seen. Bcl-2 and other family members are either anti-apoptotic (Bcl-2, Bcl-xL, Bcl-w, Mcl-1) or pro-apoptotic (Bax, Bak, Bad, Bim) genes. Although there is still some uncertainty, the ratio of anti-apoptotic to pro-apoptotic proteins (eg Bcl-2:Bax) is believed to regulate apoptosis. The expression of both pro- and anti- apoptotic proteins, as well as the transcription factors, p53 and p21, are unknown in the normal human gastrointestinal tract. Therefore this thesis investigated the expression of 8 Bcl-2 family members and 2 transcription factors through seven regions of the gastrointestinal tract (oesophagus, antrum, duodenum, ileum, caecum, colon and rectum). Results clearly showed that for the majority of Bcl-2 family members, there was a lower level of expression within the oesophagus. The exception for this was the expression of Bcl-xL which showed no significant difference from other regions of the tract. Conversely, when the expression of the transcription factors p53 and p21 were examined it was found that p53 had a highly variable response. p21 also showed highly variable expression but as a general trend, decreased throughout the tract with highest levels observed in the oesophagus and lowest levels in the rectum.

Whilst there have been reports on treatment and prevention options of oral mucositis after chemotherapy, very little research has been conducted on the mechanisms behind oral mucositis. To date there are no conclusive data on the morphological changes in the

human oral mucosa following chemotherapy, furthermore, the time course of histological changes and the correlation with clinical symptoms and oral ulceration are not known. The final part of this thesis aimed to investigate the changes that occur in the oral mucosa of patients receiving chemotherapy and correlate these with chemotherapy "naïve" controls. The results from this study showed that the ultrastructural changes in the oral mucosa occur early after chemotherapy, and are not always correlated with histological changes as seen under a light microscope. Apoptosis within the basal cells of the oral mucosa occurred at day 1, 2, and 3 after chemotherapy and could be confirmed through transmission electron microscopy and TUNEL assay. In addition, expression of pro- and anti-apoptotic proteins and two transcription factors, p53 and p21, were examined. There was high variation in the expression of these, likely to be attributable to architectural changes. Therefore this study confirmed that, like the small intestine, changes occur early, with symptoms appearing later.

This thesis has provided tangible evidence to suggest that the entire gastrointestinal tract follows a similar pattern of development of mucositis. Results presented herein show that for the chemotherapeutic agents tested, a similar mode of action was reported in causing small intestinal mucositis. This is an important finding in developing and targeting appropriate preventative strategies. Apoptosis was also found to have a key role in mucositis and was detected in the oral cavity, small intestine and large intestine following chemotherapy. From this thesis it can be concluded that, although apoptosis was present following administration of chemotherapy, there was not always histological evidence of mucositis. Furthermore, results have also shown that cytokines such as IL-11, may have an important role to play in ameliorating mucositis. Further studies are now warranted in this area to optimise dose schedule and to clarify their mode of action.

Acknowledgments

I extend my thanks and gratitude to my two supervisors, Dr. Dorothy Keefe and Dr. Adrian Cummins for giving me the opportunity to undertake this PhD, reading the many drafts of this thesis and always providing constructive criticism. In addition I must thank Professor Ian Olver, for his support over the years. Thanks also to my fellow PhD students, Joanne Bowen and Randall Grose for their friendship and support over the years.

To Dr. Richard Logan, thanks for always finding time in your busy clinic schedule to see the patients for the oral mucositis study, and for always being available to discuss the results. To Dr. Tabitha Healey for helping to recruit the patients, a very sincere thank you.

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Thanks must also go to my entire family for always encouraging me and for always believing that I could do it. Special thanks go to Mum, Dad and Catherine, who listened to the many gripes over the last couple of years. Finally, to my husband, Glenn, I have finally finished!!

Publications Arising From This Thesis

1. **Gibson, R.J.,** Bowen, J.M., Inglis, M.R.B., Cummins, A.G., and Keefe, D.M.K., (2003). Irinotecan causes severe small intestinal damage, as well as colonic damage, in the rat with implanted breast cancer, *Journal of Gastroenterology and Hepatology,* 18(9): 1095-1100.

2. **Gibson, R.J.,** Keefe, D.M.K., Thompson, F.M., Clarke, J.M., Goland, G.J. and Cummins, A.G., (2002). Effect of interleukin-11 on ameliorating intestinal damage after methotrexate treatment of breast cancer in rats, *Digestive Diseases and Sciences,* 47(12): 2751-2757.

3. Keefe, D.M.K., **Gibson, R.J.,** and Hauer-Jensen M., (2004). Gastrointestinal mucositis, *Seminars in Oncology Nursing,* 20(1): 38-47.

Publications: Submitted for Publication or in Preparation

4. **Gibson, R.J.,** Bowen, J.M., Cummins, A.G., and Keefe, D.M.K., (2003). Relationship between dose of methotrexate, apoptosis, p53/p21 expression and intestinal crypt proliferation in the rat, *Clinical and Experimental Medicine,* (submitted).

5. **Gibson, R.J.,** Cummins, A.G., Bowen, J.M., Logan, R., Healey, T., and Keefe, D.M.K., (2004). Ultrastructural changes occur early in the basal layer of the oral mucosa after cancer chemotherapy, *Clinical Cancer Research,* (in preparation).

Contributions made by Co-authors

Dr. Dorothy M.K. Keefe

Gibson, R.J., **Keefe, D.M.,** Thompson, F.M., Clarke, J.M., Goland, G.J., and Cummins, A.G., (2002). Effect of Interleukin-11 on ameliorating intestinal damage after methotrexate treatment of breast cancer in rats, *Dig Dis Sci.,* 47:2751-2757.

Gibson, R.J., Bowen, J.M., Inglis, M.R.B., Cummins, A.G., and **Keefe, D.M.K.,** (2003). Irinotecan causes severe small intestinal damage, as well as colonic damage, in the rat with implanted breast cancer, *J Gastroenterol Hepatol.,* 18:1095-1100.

Gibson, R.J., Bowen, J.M., Cummins, A.G., and **Keefe, D.M.K.,** (2004). Relationship between dose of methotrexate, apoptosis, p53/p21 expression and intestinal crypt proliferation in the rat, *Clin Exp Med.,* (submitted).

Gibson, R.J., Cummins, A.G., Bowen, J.M., Logan, R. Healey, T., and **Keefe, D.M.,** (2004). Apoptosis occurs early in the basal layer of the oral mucosa after cancer chemotherapy, *Clin Can Res.,* (in preparation).

Dr. Keefe was my principal supervisor and therefore was listed as a co-author on all publications arising from this thesis. She helped to design and interpret the results from the series of experiments, as well as gain funding for the project. In addition, she read multiple drafts of the papers.

Dr. Fiona M. Thompson

Gibson, R.J., Keefe, D.M., **Thompson, F.M.,** Clarke, J.M., Goland, G.J., and Cummins, A.G., (2002). Effect of Interleukin-11 on ameliorating intestinal damage after methotrexate treatment of breast cancer in rats, *Dig Dis Sci.,* 47:2751-2757.

Dr. Thompson was responsible for the experimental analysis for a section of work that is not included in my thesis.

Dr. Julie M. Clarke

Gibson, R.J., Keefe, D.M., Thompson, F.M., **Clarke, J.M.,** Goland, G.J., and Cummins, A.G., (2002). Effect of Interleukin-11 on ameliorating intestinal damage after methotrexate treatment of breast cancer in rats, *Dig Dis Sci.,* 47:2751-2757.

Dr. Clarke was listed as a chief investigator on a grant relating to work that was not included in my thesis. She was involved in the experimental design and analysis of this aspect of the paper.

Mr. Gary Goland

Gibson, R.J., Keefe, D.M., Thompson, F.M., Clarke, J.M., **Goland, G.J.,** and Cummins, A.G., (2002). Effect of Interleukin-11 on ameliorating intestinal damage after methotrexate treatment of breast cancer in rats, *Dig Dis Sci.*, 47:2751-2757.

Mr. Goland was involved in technical aspects of the study, not relating to my thesis. In addition he provided assistance with animal trials.

Dr. Adrian Cummins

Gibson, R.J., Keefe, D.M., Thompson, F.M., Clarke, J.M., Goland, G.J., and **Cummins, A.G.,** (2002). Effect of Interleukin-11 on ameliorating intestinal damage after methotrexate treatment of breast cancer in rats, *Dig Dis Sci.*, 47:2751-2757.

Gibson, R.J., Bowen, J.M., Inglis, M.R.B., **Cummins, A.G.,** and Keefe, D.M.K., (2003). Irinotecan causes severe small intestinal damage, as well as colonic damage, in the rat with implanted breast cancer, *J Gastroenterol Hepatol.*, 18:1095-1100.

Gibson, R.J., Bowen, J.M., **Cummins, A.G.,** and Keefe, D.M.K., (2004). Relationship between dose of methotrexate, apoptosis, p53/p21 expression and intestinal crypt proliferation in the rat, *Clin Exp Med.*, (submitted).

Gibson, R.J., **Cummins, A.G.,** Bowen, J.M., Logan, R. Healey, T., and Keefe, D.M., (2004). Apoptosis occurs early in the basal layer of the oral mucosa after cancer chemotherapy, *Clin Can Res.*, (in preparation).

Dr. Cummins was my co-supervisor. Together with Dr. Keefe he helped to design and interpret the results from this series of experiments, as well as gain funding for the project. In addition, he read multiple drafts of the paper.

Ms. Joanne Bowen

Gibson, R.J., **Bowen, J.M.,** Inglis, M.R.B., Cummins, A.G., and Keefe, D.M.K., (2003). Irinotecan causes severe small intestinal damage, as well as colonic damage, in the rat with implanted breast cancer, *J Gastroenterol Hepatol.*, 18:1095-1100.

Gibson, R.J., **Bowen, J.M.,** Cummins, A.G., and Keefe, D.M.K., (2004). Relationship between dose of methotrexate, apoptosis, p53/p21 expression and intestinal crypt proliferation in the rat, *Clin Exp Med.*, (submitted).

Gibson, R.J., Cummins, A.G., **Bowen, J.M.,** Logan, R. Healey, T., and Keefe, D.M., (2004). Apoptosis occurs early in the basal layer of the oral mucosa after cancer chemotherapy, *Clin Can Res.*, (in preparation).

Ms. Bowen is a fellow PhD student in the laboratory. She helped to conduct the animal work, as well as providing assistance with interpretation of results and reading multiple drafts of the manuscript.

Mr. Mark Inglis

Gibson, R.J., Bowen, J.M., **Inglis, M.R.B.,** Cummins, A.G., and Keefe, D.M.K., (2003). Irinotecan causes severe small intestinal damage, as well as colonic damage, in the rat with implanted breast cancer, *J Gastroenterol Hepatol.*, 18:1095-1100.

Mr. Inglis undertook a small aspect of research relating to the toxicity of the buffer in which the Irinotecan is administered as a student research project.

Dr. Richard Logan

Gibson, R.J., Cummins, A.G., Bowen, J.M., **Logan, R**. Healey, T., and Keefe, D.M., (2004). Apoptosis occurs early in the basal layer of the oral mucosa after cancer chemotherapy, *Clin Can Res.*, (in preparation).

Dr. Logan was the Oral Pathologist who obtained the biopsies for this study.

Dr. Tabitha Healey

Gibson, R.J., Cummins, A.G., Bowen, J.M., Logan, R. **Healey, T.,** and Keefe, D.M., (2004). Apoptosis occurs early in the basal layer of the oral mucosa after cancer chemotherapy, *Clin Can Res.*, (in preparation).

Dr. Healey was the Medical Oncologist who did much of the early recruiting of patients for this study.

Additional Studies and Publications

During my candidature, I was involved in several other studies, not presented in this thesis. These have resulted in co-authorship on several other manuscripts. I am first author on a manuscript currently in preparation, detailing our laboratory's work on keratinocyte growth factor, and a co-author on an invited review of mucositis. In addition to this, I am also a co-author on a paper submitted by Joanne Bowen, another PhD student in the Mucositis Laboratory, detailing the effects of chemotherapy on the expression of pro- and anti-apoptotic proteins in the intestine. I was involved in helping her with the animal work detailed in this manuscript. Reference are made to these papers in the general discussion and these are referenced accordingly.

1. Bowen, J.M., **Gibson, R.J.,** Keefe, D.M., and Cummins, A.G., (2003). Cytotoxic chemotherapy upregulates pro-apoptotic Bax and Bak expression in small intestinal crypts. Pathol (submitted).

2. **Gibson, R.J.,** Bowen, J.M., Cummins, A.G., and Keefe, D.M.K., (2003). Keratinocyte Growth Factor offers protection from irinotecan-induced diarrhoea in rats with breast cancer, (in preparation).

Thesis Explanation

The format of this thesis is as follows: literature review, five distinct research chapters, and then general discussion. During the course of my candidature, two research chapters were published; therefore each chapter is written as a publication each complete with introduction, materials and methods, results and discussion. Repetitions of the introduction and materials and methods occur only as necessary for the format of the papers.

Abbreviations

MTX	Methotrexate
CPT-11	Irinotecan Hydrochloride
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
IL-1	Interleukin-1
IL-11	Interleukin-11
IL-15	Interleukin-15
DA	Dark Agouti
KGF	Keratinocyte Growth Factor
TNF-α	Tumour Necrosis Factor α
EGF	Epidermal Growth Factor
TGF-β	Transforming Growth Factor β
5-FU	5-Fluouracil

Chemotherapy-induced mucositis: Mechanisms of damage, time course of events and possible preventative strategies

Literature Review

1.1 Introduction

Mucositis is a major oncological problem, caused by the cytotoxic effect of cancer chemotherapy. By definition mucositis suggests that inflammation is present however recent studies have shown that this is not necessarily the case (1, 2); rather the name simply refers to the damage that occurs to the mucous membranes of the body (1, 2). Mucositis affects the entire gastrointestinal tract and causes pain and ulceration in the mouth and small and large intestines. In addition, it causes abdominal bloating, vomiting and diarrhoea (2-4). Mucositis occurs in approximately 40% of patients after standard doses of chemotherapy, and in 100% of patients undergoing high dose chemotherapy and stem cell or bone marrow transplantation (2-4). The frequency and severity of mucositis varies depending on the type of cancer (and therefore the treatment regimen) and on the patient's age with the very young and the very elderly being most affected (4). Despite the severity and prevalence of mucositis, there is no known treatment for established symptoms, with management currently limited to pain relief and maintenance of good oral hygiene. Susceptibility to toxicity has been attributed to the high rate of proliferation, as cytotoxic drugs used to treat cancer also cause apoptosis in these cells (2, 5, 6). However, changes in anti-apoptotic and pro-apoptotic protein expression are more likely to explain such susceptibility (7, 8). This review will examine in detail the relationship between cancer chemotherapy and mucositis throughout the gastrointestinal tract.

1.2 The Gastrointestinal Tract

The gastrointestinal tract extends from the mouth through to the anus and contains some of the highest proliferating cells in the body (9). Five basic functions are conducted by the gastrointestinal tract: (1). Ingestion of nutrients, (2). Passage of nutrients, (3). Digestion of nutrients, (4). Absorption of nutrients, and (5). Defecation of waste (10).

1.2.1 Oral Mucosa

The oral cavity is the start of the gastrointestinal tract, and is lined by the oral mucosa, which is a stratified squamous epithelium (11, 12). This epithelium may or may not be keratinised; this is highly dependent on regions of high friction. Underlying the epithelium is the lamina propria, which is composed of dense collagenous tissue (11, 12). Within the epithelium there are four layers of cells: the basal cell layer (which contain the stem cells), and the prickle cell layer which are found in both keratinised and non-keratinised tissue. Keratinised epithelium have a granular layer followed by a keratinised layer, whereas non-keratinised epithelium has an intermediate layer and a superficial layer (12).

1.2.2 Intestinal Anatomy and Histology

The small intestine can simplistically be divided into two units: proliferative units known as crypts and functional units known as villi (13). The epithelium lining the small intestine is simple columnar epithelium and is constantly replaced as cells are sloughed into the lumen (14). The large intestine is also composed of simple columnar epithelium; however there are no villi postnatally (10). All intestinal cells arise from stem cells found in the base of the crypts with each cell dividing four to six times before reaching its ultimate destination (14). Within the intestinal epithelium, there are four main cell lineages namely columnar, mucin-secreting (Goblet cells), endocrine and Paneth cells.

1.2.3 Crypt Structure and Function

Within the murine small intestinal crypts there are approximately 250 cells arranged in a flask-like structure (14, 15). When the crypts are 'opened up' they are approximately 22 cells high and 16 cells wide at the widest point (Fig 1.1) (14, 16). The gastrointestinal epithelium is one of the fastest proliferating tissues in the body and in order to maintain this high proliferative rate, the crypt has many active cycling cells (5, 15). Renewed cells are produced in the basal two thirds of the small intestinal crypts and this zone is called the proliferative zone (15, 17). All the cells (columnar, mucin-secreting, endocrine and Paneth cells) of the small intestinal epithelium are derived from stem cells which are located within the proliferative zone (16, 17). The exact number of stem cells in each crypt and how they control their proliferation is not yet known (18). Despite this, the general consensus is that there are somewhere between 4 and 16 stem cells per crypt (19-22) and that they divide without maturation approximately once every 12-32 hours with the resulting daughter cells migrating up the crypt and onto the villus (23). Additionally, there are thought to be somewhere between 30 and 40 clonogenic cells (cells which can function

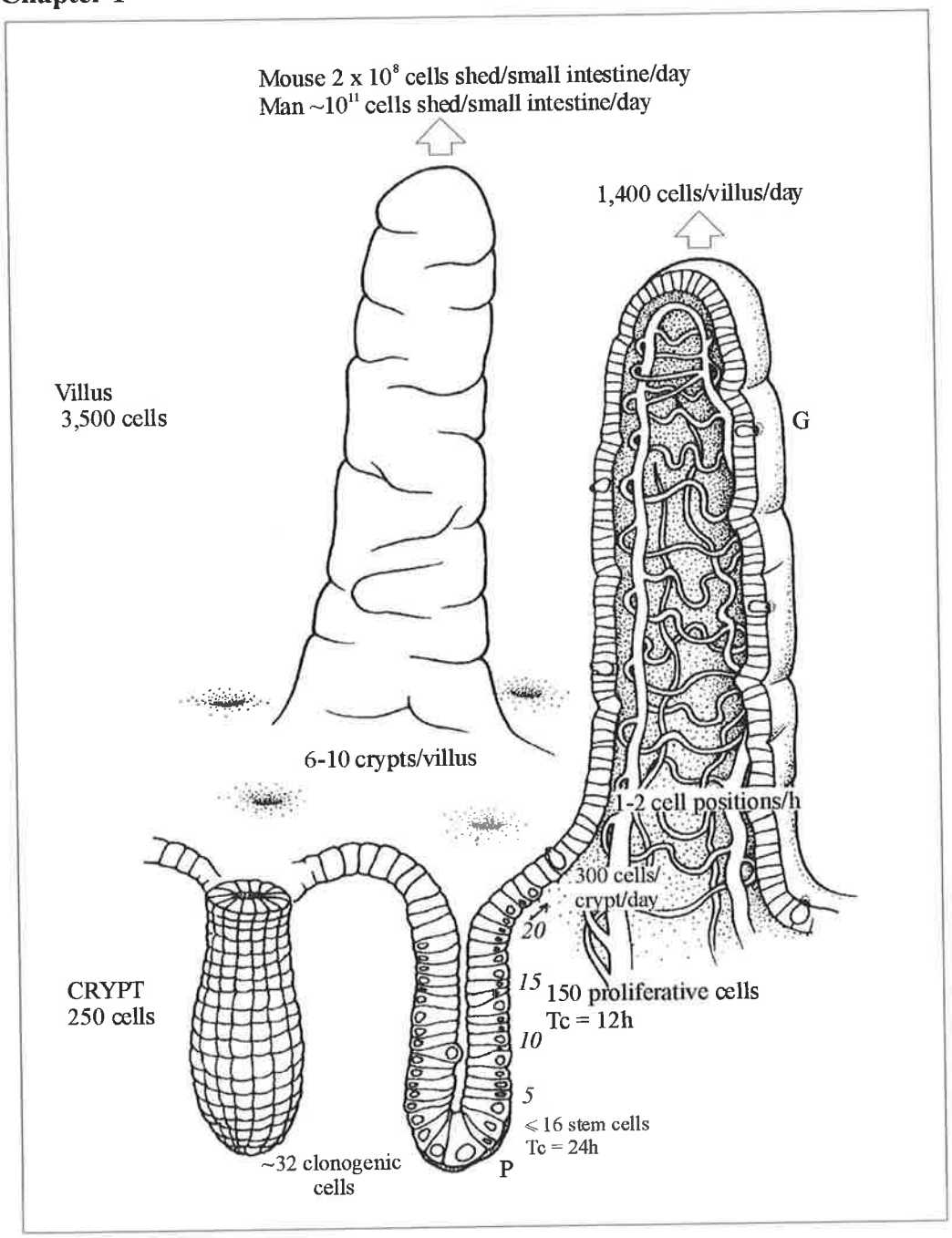


Figure 1.1 A diagrammatic representation of the three dimensional gross architecture, histological cell organisation and kinetic hierarchy of the mouse ileum.Redrawn with permission from Potten (Development, 110:1001-1020, 1990) as in Keefe (MD Thesis, The University of Adelaide,1998).

In longitudinal sections, the crypt cells can be identified by their position (1-20). The capillary network is shown on the right.

P = Paneth Cells
Tc = Cell Cycle Duration
G = Goblet Cells

as stem cells in a crisis) (24). Evidence has been presented that crypt clonogenic cell number is highly dependent on the amount of damage that the crypt has undergone, the more crypt damage induced resulting in more cells being recruited into the clonogenic cycle (24).

1.3 Mucositis

1.3.1 Cancer Chemotherapy and Mucositis

Cytotoxic drugs are used to treat malignancies to eradicate neoplastic cells. Unfortunately these drugs may result in side effects, as they cannot distinguish between normal and cancerous cells. The efficacy of chemotherapy treatment relies on two principles: firstly the sensitivity of cancerous cells to chemotherapy and secondly, the ability of the body's normal, healthy cells to recover more quickly than cancer cells (1). Rapidly dividing cells, like those of the gastrointestinal tract and bone marrow, are generally more susceptible to chemotherapy, but they also have a greater recovery capacity. The maximum dose of chemotherapy is limited by its toxicity in normal tissues, the so-called "dose-limiting toxicity" (1). Until recently, the most common dose-limiting toxicity was suppression of the bone marrow, with chemotherapy doses usually unable to reach levels where gastrointestinal toxicity was a major issue. However, with the development of colony-stimulating factors (eg granulocyte macrophage colony stimulating factor, GM-CSF), which stimulate bone marrow recovery, higher doses of chemotherapy are now administered, which has not only led to an increase in the efficacy of chemotherapy, but also the prevalence of gastrointestinal mucositis, which has now become the main dose-limiting factor (1).

1.3.2 Chemotherapy and Apoptosis

Chemotherapy is a relatively recent therapeutic option for cancer – only having been in use for approximately 50 years (25). However it has only been in the last decade with rapid advances in molecular analysis, that some of the mechanisms by which chemotherapeutic drugs induce cell death have been elucidated (25). A study conducted in the late 1980s (26) examined the effect of a DNA inhibitor, etoposide, and found that it induced internucleosomal DNA degradation, suggesting that etoposide may have caused apoptotic cell death. This led to extensive research on the apoptotic capabilities of many chemotherapeutic agents (27-29). From these studies, it has been confirmed that many

chemotherapeutic drugs, including cyclophosphamide, 5'-fluorouracil and adriamycin, induce apoptosis (30). In addition to causing apoptotic cell death in the tumours, many chemotherapeutic agents have been demonstrated to cause widespread apoptosis in normal tissues that undergo rapid proliferation, leading to the conclusion that elevated levels of apoptosis are responsible for many of the adverse side effects of chemotherapy treatment (31).

Apoptosis is distinguished from the other predominant form of cell death – necrosis – by characteristic morphological features, including cell shrinkage, nuclear blebbing, the condensation of chromatin, fragmentation of the nucleus and the lack of associated inflammation (Fig 1.2) (32, 33). There are two different pathways by which cells can undergo apoptosis. The first pathway is receptor-linked and is an extrinsic pathway from the activation of death receptors such as Fas/CD95 ligand, TNF receptor and TRAIL (33). The second pathway is induced by DNA damage. This is an intrinsic pathway that induces mitochondrial release of cytochrome C and subsequent activation of caspases in the cytoplasm (33). Cancer chemotherapy is thought to activate the DNA-damage induced or intrinsic pathways but may also upregulate death receptors and their ligands, contributing to the extrinsic pathway (33, 34).

1.3.3 Spontaneous Apoptosis

Apoptosis occurs spontaneously in intestinal crypts, removing normal, healthy cells (35). Estimations have been made that approximately 1 apoptotic cell is seen in every 5 histological crypt sections, (which is equivalent to less than 1%), and this is generally believed to occur in order to maintain the balance of stem cells within the crypts (17, 35). Although this spontaneous apoptosis occurs in both the small and large intestines, significantly more is seen in the small intestine, most probably due to the presence of pro- or anti-apoptotic genes (35).

Different classes of cytotoxic agents act at different levels in the small intestinal crypt cell hierarchy (36-38). There are three main categories of cytotoxic agents: (1) Drugs such as doxorubicin and bleomycin which act at cell positions 4-6 (2): Actinomycin-D and cyclophosphamide which act at positions 6-8 (3): Methotrexate and 5-fluouracil which act at positions 8-11 (36-38). This may have implications for the severity of mucositis, and also the potential methods of prevention (39).

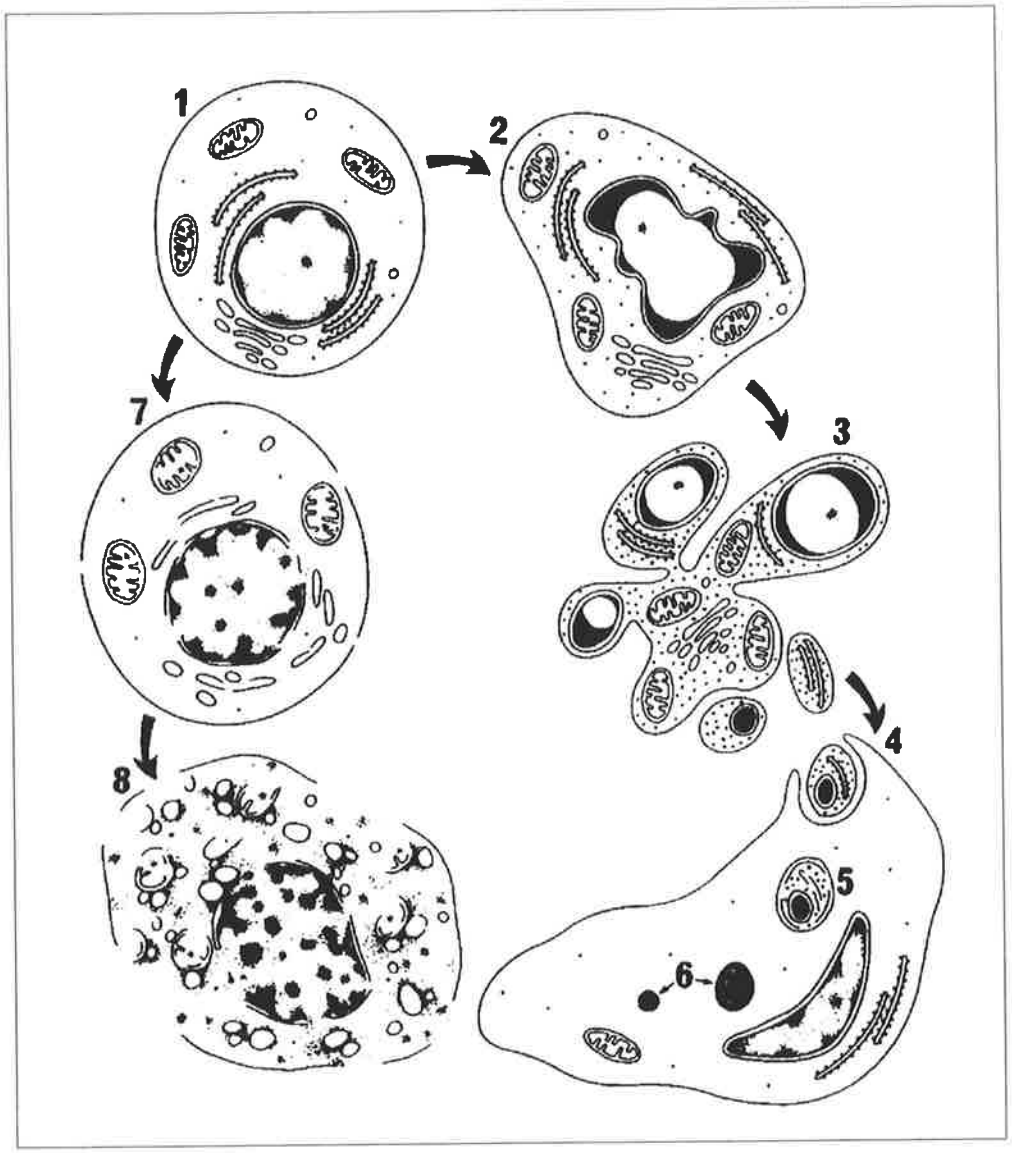


Figure 1.2 Diagram illustrating sequence of ultrastructural changes in apoptosis (2-6_ and necrosis (7 and 8) (From Kerr, Winterford et al., 1994).

1. Normal cell.
2. Early apoptosis is characterised by compaction and margination of nuclear chromatin, condensation of cytoplasm, and convolution of nuclear and cell outlines.
3. At a later stage, the nucleus fragments, and protuberances that form on the cell surface separate to produce apoptotic bodies.
4. Apoptotic bodies are phagocytosed by nearby cells.
5. Apoptotic bodies are degraded within lysosomes.
6. Apoptotic bodies are degraded within lysosomes.
7. The development of necrosis is associated with irregular clumping of chromatin, marked swelling of organelles and focal disruption of membranes.
8. Membranes subsequently disintegrate, but the cell usually retains its overall shape until removed by mononuclear phagocytes.

1.3.4 Apoptosis and Mucositis

Initial studies of the effect of methotrexate (MTX), a commonly used chemotherapeutic agent, on the human small intestine identified pyknotic bodies in cells lining the crypts, now known to be apoptotic cells (5, 6). These cells were observed within 6 hours of MTX administration and were still present at 48 hours. Further studies conducted by Keefe and colleagues (2) confirmed the presence of these apoptotic cells in the human small intestine following treatment with chemotherapy. Their study showed small intestinal apoptosis increased seven-fold 1 day after chemotherapy and was followed by a reduction in intestinal morphometry (villus area, crypt length mitotic count and enterocyte height) 3 days later, which coincided with patient symptoms of mucositis. The authors concluded that small intestinal apoptosis leads to crypt hypoproliferation and that the two are directly related to mucositis.

1.3.5 Chemotherapy Dose and Levels of Apoptosis

A gene that is commonly mutated in cancer is the p53 gene (15). Normally expressed in low levels, p53 becomes upregulated when DNA damage occurs, moving to the nucleus where it then acts to regulate several other genes, including $p21^{waf1/cip1}$ (15, 40, 41). Upregulation of p53 can have a number of consequences for the cell following DNA damage. It can either initiate G1 arrest, allowing the cell time to repair itself or undergo apoptosis, or it can directly initiate apoptosis (15, 42). It has also been suggested that low levels of p53 can induce differentiation (41). When p53 is upregulated, it activates the transcription of $p21^{waf1/cip1}$, a cyclin dependent kinase (cdk) inhibitor (42). $p21^{waf1/cip1}$ binds with other cellular components to form a quaternary complex which is necessary for normal functioning of DNA polymerase (42), thereby inducing cell arrest in the G1 phase. $p21^{waf1/cip1}$ is necessary to obtain cell cycle arrest that is p53-dependent in response to DNA damage (Fig 1.3) (43).

Several studies have shown that the expression of p53 within the small intestinal epithelium increases following γ-irradiation, supporting the hypothesis of recognition of DNA damage and subsequent induction of apoptosis (41, 44, 45). Interestingly these studies showed that the apoptotic response which occurs within 3-6 hours of radiation damage occurs specifically around the proposed stem cell region of the crypt, i.e. positions 4-5 was p53 dependent, whereas the apoptotic response at 12-24 hours after radiation damage was p53 independent (41, 44, 45). The importance of p53 dependent apoptosis

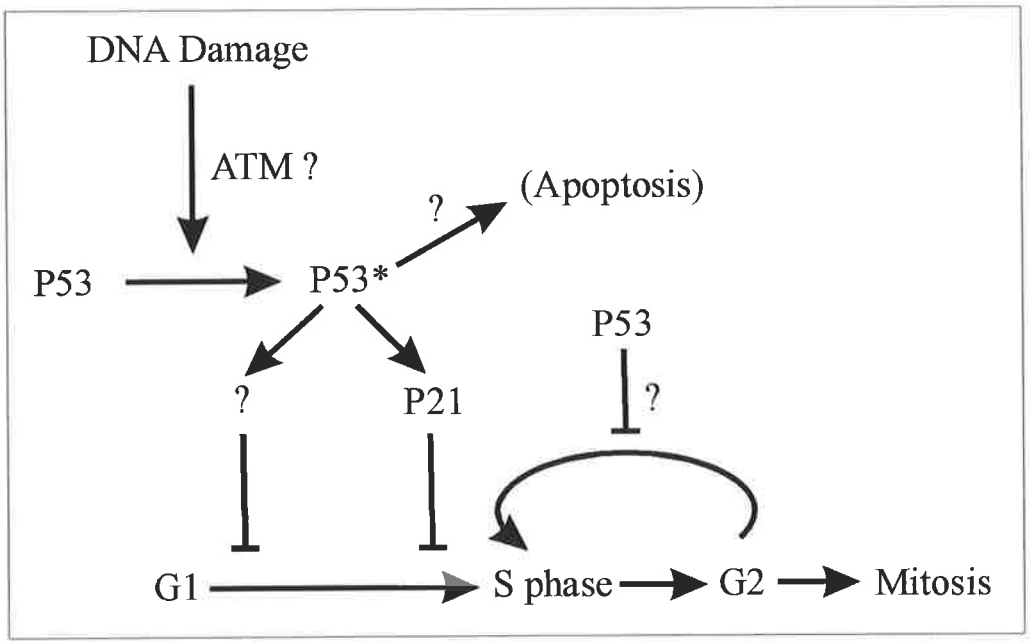


Figure 1.3 A current view of the integrated actions of p21and p53. DNA damage leads to stabilisation and activation of activated p53 protein (p53*) induces transcription of p21 $^{CIP1/WAF1}$ and other genes involved in cell arrest and DNA repair. Under certain conditions that are not completely understood, p53 activation can lead to apoptosis (From Deng, Zhang, et al., 1995).

was shown by p53 knockout mice which are relatively resistant to apoptosis caused by radiation damage (45). Further studies investigated the role played by p53 in spontaneous intestinal apoptosis and concluded that levels of spontaneous apoptosis seen in p53 null mice were not significantly different from wild-type, indicating that p53 is not important for spontaneous apoptosis (Table 1.1) (45). This supports the observation that p53 null mouse embryos undergo normal development (46).

Apoptosis in the large intestine, caused by radiation therapy, occurs at a lower rate than in the small intestine. The apoptotic cells are not associated with the proposed stem cell region (47). It is hypothesised that these differences between the small and large intestines are due to differences in the expression of Bcl-2, an anti-apoptotic gene family member (48). It is further proposed that when DNA damage is detected in the large intestine, p53 upregulation leads to initiation of cell repair, rather than initiation of apoptosis (Table 1.1) (47).

It has been assumed that apoptosis is a direct determinant of subsequent damage to the small intestine caused by cancer chemotherapy. However, a study by Pritchard and colleagues (1998) showed that the severity of apoptosis does not correlate with subsequent tissue damage. They found that p53 inhibits proliferation through $p21^{waf\text{-}1/cip1}$ transcription factor. Thus, decreased proliferation, as well as apoptotic cell loss, contributes to crypt cell loss. Additionally, they highlighted the deficiency of measuring only one variable (eg apoptosis) at any one time point, showing that it is not always an accurate indicator of the ultimate outcome for tissue damage. Thus, there still remains some controversy, although apoptosis is related to intestinal damage caused by cytotoxic agents.

	Spontaneous Apoptosis		
	Observation	**Conclusion**	**Consequences**
SI	AI at stem cell location approx 10% Apoptosis is p53 independent	Apoptosis removes excess stem cells Apoptosis is part of stem cell homeostatic mechanism	Stable stem cell population leads to stable crypt
LI	Apoptosis is a very rare event, not concentrated at stem cell location Bcl-2 expressed in the stem cells Apoptosis increased in stem cells in Bcl-2 null mice	Occasional excess stem cells not removed Bcl-2 overrides stem cell homeostatic process	Excess stem cells persist and numbers may increase with time Hyperplastic crypts
	Damage-induced apoptosis		
	Observation	**Conclusion**	**Consequences**
SI	Cells at stem cell location exquisitely sensitive to damage Approx. 6 hypersensitive cells per crypt Damage p53 dependent	Very sensitive damage detection mechanism No attempt to repair Damaged cells efficiently removed	SI is protected against DNA damage (mutation) and carcinogenesis
LI	Lower levels than in SI Apoptosis distributed throughout the crypt BCL-2 expressed in stem cells AI increased in stem cell position in Bcl-2 null mice	Damaged cells not removed Cell death prevented by Bcl-2	Damaged or misrepaired cells may persist as initiated cells leading to higher risk of cancer Tumours arising in Bcl-2-expressing cells continue to express it, making them difficult to kill (chemoresistant)

Table 1.1 Summary of the observations and conclusions regarding apoptosis in the intestinal epithelium and their likely implications. SI = small intestine; LI = large intestine; AI = apoptotic index. From (15).

1.3.6 Methotrexate

Methotrexate (MTX) is a cytotoxic agent that was introduced clinically more than fifty years ago to treat many cancers (49). Its mechanism of action is by inhibiting dihydrofolate reductase and the synthesis of DNA (5, 50, 51). Side effects from administration of this cytotoxic agent include diarrhoea, anorexia, with associated malabsorption, malnutrition and dehydration (49).

MTX predominantly causes damage to the small intestine. Past studies have detailed the response of both the rat and human small intestinal epithelia following treatment with MTX and have shown reductions in mitoses and shortened villi (5, 51). These distinct morphological changes to the epithelium have been reported within three hours of MTX treatment (5, 51). In addition, these early studies identified discrete spherical bodies, which we now know to be apoptotic bodies (5, 51). More recent research has shown that apoptosis peaks approximately six hours following cytotoxic treatment (including MTX) in both the rat and human (1, 2, 52, 53). This apoptosis leads to a subsequent crypt hypoplasia with the loss of the regenerating crypt cells which leads on to villus atrophy (1, 2, 52, 53). Once the MTX insult has passed, the small intestinal epithelia is able to regenerate and heal (1, 2, 52, 53).

1.3.7 Irinotecan

Irinotecan hydrochloride (or CPT-11) is a relatively new chemotherapeutic agent used to treat solid tumours, acting on malignant cells by inhibiting DNA topoisomerase I (54-57). A major problem of this cytotoxic agent is that it causes severe diarrhoea and leukopenia, which severely limit the dose of administered drug (55-57).

As diarrhoea is a well recognised side effect of CPT-11 treatment, the histological changes that occur in the gastrointestinal tract in response to Irinotecan administration have been examined in several animal studies (54, 56, 57). A study investigating the effect of intraperitoneal irinotecan on the mouse ileum and caecum reported that 100 mg/kg administered daily for 4 days caused vacuolation of the epithelium, blood vessel dilatation and infiltration of polymorphs in the ileum, and hyperplasia of the epithelium with accompanying goblet cell metaplasia in the caecum (56). The authors concluded that diarrhoea resulted from malabsorption of water and electrolytes and mucin hypersecretion. (56). In contrast, Araki and colleagues (1993) concluded that the diarrhoea was due to

haemorrhagic enterocolitis, caused by high levels of the SN-38, the active metabolite of irinotecan which was retained within the intestines (54).

A detoxified form of irinotecan, SN-38 glucuronide, may be processed to SN-38 by β-glucuronidase activity of the microflora of the intestine (57). It is SN-38 that causes intestinal cytotoxicity (57). The administration of antibiotics to the animals inhibited β-glucuronidase in the intestinal flora and markedly reduced diarrhoea, which argues that irinotecan toxicity is in part due to the colonic microflora. Thus, there may be several mechanisms by which irinotecan-induced diarrhoea occurs.

A study in humans using intensive high-dose loperamide, a common anti-diarrhoeal agent to control diarrhoea, reported that Irinotecan treatment can be made tolerable (58). The protocol involved patients taking 2 mg of loperamide for every diarrhoeal episode (defined as one or many successive loose stools lasting until the current diarrhoea was effectively controlled by loperamide), taken every 2 hours beginning 8 hours or more following irinotecan administration. Only one patient failed to have his diarrhoea controlled when following this protocol, indicating that loperamide at clinically high doses was effective in controlling diarrhoea induced by irinotecan treatment (58). Another Phase I study examined the effect of granulocyte colony-stimulating factor (G-CSF) treatment of patients after both irinotecan and cisplatin (59) and showed that G-CSF reduced both the incidence and severity of treatment related diarrhoea. Both of these studies, whilst describing the effects of possible control agents for the diarrhoea, did not examine the histological changes in the gastrointestinal tract after irinotecan administration and were therefore unable to assess the mechanisms by which the control agents exert their function.

A rat model with similar dose-limiting toxicities to that seen in patients, was developed by Cao, *et al.*, (1998) in order to evaluate the protective effect of interleukin-15 (55, 60). This cytokine was chosen for investigation as a previous report had implied that IL-15 may be involved in the response of the gastrointestinal tract to damage caused by chemotherapy (61). Animals treated with IL-15 (100 μg) at varying schedules were protected from diarrhoea and death caused by Irinotecan toxicity, with the best protection being found in animals that received the maximum dose (60). Despite this encouraging finding, the authors were unable to define the mechanism by which IL-15 protects the gastrointestinal tract from damage, although they suggested that this cytokine, like IL-11, may act by inhibiting intestinal apoptosis and/or stimulating intestinal cell growth.

1.4 Oral Mucositis

Oral mucositis as an entity was first described approximately 18 years ago and was used to describe the oral mucosal damage that was caused by both chemotherapy and radiotherapy (62, 63). The term 'oral mucositis' is a separate entity from other oral lesions, having a different pathogenic background (62, 63). Oral mucositis is a well recognised phenomenon and is a frequent side effect of cancer chemotherapy (4, 64, 65). It is a significant non-haematological complication causing pain and ulceration (62). Like the remainder of the gastrointestinal tract, the oral mucosa has epithelial cells which rapidly divide and mature (64). Currently there is no known treatment for oral mucositis, with management being limited to pain relief and maintenance of good oral hygiene (Table 1.2).

1.4.1 Biological Aspects of Oral Mucositis

Whilst there has been research reported on the treatment and prevention options of oral mucositis after chemotherapy (62, 66) and on possible preventative agents, little research has been reported on the mechanisms behind oral mucositis. Several papers have been published suggesting distinct phases of damage (63, 65, 67, 68), although these have not specifically examined the time-course of damage following chemotherapy. All analysis of oral mucositis has been based on a purely observational basis, and whilst there have been a number of hypotheses as to the mechanisms behind oral mucositis, the actual pathophysiology remains undefined (4). Currently, the most widely accepted hypothesis is based on the mechanisms of both the development and resolution of oral mucositis, and is still highly speculative (4, 67, 69, 70), This hypothesis is based on the assumption that mucositis occurs in four distinct phases: inflammatory/vascular, epithelial, ulcerative/microbiological and healing (4, 65, 67, 69, 70). Additionally each phase is assumed to be independent of the others and is caused by a cascade of actions that are initiated by cytokines and growth factors, action of the cytotoxic drug on the oral mucosa and the patient's bone marrow status (4, 67, 69, 70). The hypothesised phases of oral mucositis are detailed below.

The inflammatory/vascular phase is thought to occur rapidly, within 24 hours after cytotoxic treatment (67). Administration of chemotherapy causes cells to release cytokines including interleukin-1 (IL-1), which initiates an inflammatory response and tumour necrosis factor alpha (TNF-α), which causes tissue damage. Both of these cytokines may be the initiating event for oral mucositis (67).

The inflammatory/vascular phase is followed by the epithelial phase, which occurs between 4 and 5 days after treatment (67). Cytotoxic agents directly inhibit DNA replication and mucosal cellular reproduction, ultimately leading to a decrease in basal epithelial proliferation (4, 66, 67). Consequently, as not all cytotoxic drugs affect DNA synthesis (67) there is a higher probability that patients on particular treatment regimes will be more susceptible to oral mucositis than others. Evidence supporting the notion that basal epithelial damage leads to oral mucositis comes from studies in children, who are known to have a high basal cell proliferation. These children are three times more likely to develop oral mucositis than older adults, who have a slower basal cell proliferation (71).

The reduction in cellular proliferation of the oral mucosa causes mucosal atrophy, breakdown of collagen and frank mucosal ulceration, which is most frequent over areas that are exposed to trauma caused by teeth movements, such as the lips and buccal mucosa (68). This marks the beginning of the ulcerative and bacteriological third phase. Clinically for patients this is the most symptomatic phase, and usually presents approximately one week following treatment (67). These ulcers and their locations make the oral mucosa highly susceptible to infection (4, 66, 68). Bacteria are often found in the ulcers, and are different from those found in normal healthy patients (67).

Past:	Management was limited to treatment of oral pain and concomitant infection
Present:	Phase 1 and Phase 2 trials have resulted in the development of a panel of agents used for prevention and treatment of mucositis. None of these agents seem particularly effective.
Future:	There is a need for: (1). Further elucidation of the pathophysiology of mucositis. (2) Optimisation of clinical trial methodology including common criteria for assessment, large patient samples and use of randomised placebo-controlled design. (3). New Agents

Table 1.2 Past, present and future in management of mucositis in cancer patients. From (72).

The fourth and final phase of oral mucositis is the healing phase and refers to the oral mucosa resuming normal basal cell proliferation and differentiation as well as controlling the oral bacterial flora (67). The length of this final phase is related to the duration of the condition, however it most likely does not relate to the intensity. Additionally, anything which adversely affects wound healing will also affect this final phase (67). Although oral mucositis is recognised as being a major side effect of treatment for cancer, no detailed studies have been conducted in humans to investigate this hypothesis.

1.4.2 Animal Models of Oral Mucositis.

The lack of knowledge of oral mucositis also comes from the lack of an appropriate animal model in which to define the mechanisms. Sonis and colleagues established the currently accepted hamster model of mucositis (73), however this model is flawed by the lack of associated tumour and lack of spontaneous chemotherapy-induced mucositis. Briefly this model of mucositis uses male golden Syrian hamsters, as unlike other rodents, they have a buccal cheek pouch which is susceptible to chemotherapy. Mucositis is induced in this model by the administration of 5-fluorouracil (5-FU) at 60 mg/kg on three days (days 0, 5 and 10). The buccal pouch mucosa is superficially irritated (mechanically scratched) on days 1, 2 and 3, resulting in mucositis in most of the animals (73). In humans, however, the oral mucosa does not need to be superficially irritated in order to induce mucositis, and so this model cannot be compared with the clinical setting. Additionally, superficial irritation may also result in wound healing mechanisms being initiated.

There are potentially many anti-mucotoxics which may prevent mucositis in this animal model including Epidermal Growth Factor (EGF) (74) and IL-11 (75, 76). However, when they have been translated to the clinical setting they have been found to not work, strongly suggesting that there are different mechanisms occurring in the hamster from those that occur in the human. These observations also reiterate the inability of using this animal model as a basis for human studies.

1.4.3 Prevention and Treatment of Oral Mucositis

A number of agents have been clinically studied to assess their ability to prevent or aid in the treatment of oral mucositis. These include GM-CSF (64, 77), capsaicin (78), sucralfate (79, 80), prostaglandins (81), silver nitrate (82-84), cryotherapy (85, 86), allopurinol (87),

beta-carotene (88, 89) and TGF-β (69, 90, 91). However, detailed discussion into these agents is outside the scope of this thesis. Please refer to the references for full details.

1.5 Oesophageal Mucositis

Anatomically the oesophagus extends from the upper oesophageal sphincter to the lower oesophageal sphincter, these sphincters delineating it from the oropharynx and the stomach respectively (92, 93). The oesophageal mucosa is composed of nonkeratinised epithelium, lamina propria and muscularis mucosae, all of which protect the underlying tissue from damage during the passage of food and drink (10). Chemotherapy damages dividing and differentiating cells, causing the epithelium to become thin and ulcerated (93). Additionally chemotherapy alters the proliferative rate of connective tissue cells within the lamina propria, initiating a cascade of reactions that include an increase in vascular permeability and inflammatory infiltrate (93). These lead to fibrosis and tissue ischaemia, which decrease the role of the oesophagus in protection (93-97). Very little information exists on oesophageal mucositis, and many symptoms are most likely to be misdiagnosed with gastro-oesophageal reflux disease, *Candida* oesophagitis, cytomegalovirus oesophagitis, or oesophagitis induced by herpes virus (98). However a study by Keefe and colleagues found minimal macroscopic changes at endoscopy (1, 2).

1.6 Intestinal Mucositis

The pathophysiology of intestinal mucositis has been described in both animal models and patient trials. In a rat model of mucositis, the time-course of apoptosis and intestinal damage were investigated over a 5 day period following MTX treatment (52). The earliest change seen was a 28-fold increase in apoptosis within intestinal crypts at 6 h after MTX administration and this gradually improved over subsequent days. This correlates with the findings of Keefe and colleagues (2000) and also agrees with previous studies that showed pyknotic bodies in crypts of humans after MTX, even though apoptosis was not a widely recognised phenomenon at the time (5, 6). In humans, the natural history of intestinal mucositis was described by Slavin and colleagues (1978) (99). In the first week following chemotherapy, normal highly undifferentiated cells are observed, while in the second week after chemotherapy, cellular necrosis, decreases in cellular proliferation, abnormal villi architecture and infections are observed within the intestine before recovery is initiated and intestinal function returns to normal (99).

1.7 Dark Agouti Rat Mammary Adenocarcinoma Model (DAMA)

A model was developed in the female dark agouti (DA) rat to study simultaneously the effect of potential anti-mucotoxics and of chemotherapy in the small and large intestines (1). This model is unique in that the mammary adenocarcinoma arose spontaneously in the rat, and therefore mimics the human situation in which tumours arise spontaneously. It has been used for examining of the effect of keratinocyte growth factor (KGF) and methotrexate on small intestinal mucositis (53), as well as for studies into malnutrition following chemotherapy (100, 101) and neuroprotection by glutamate (102).

1.8 Protection from Mucositis

Many agents, including KGF, TGF-β and IL-11, have been assessed in preventing or ameliorating mucositis by manipulation of intestinal stem cells (103). Although these studies have shown significant improvement in mucositis (103), they did not test whether these agents enhanced tumour growth or interfered with the efficacy of chemotherapy. For the purposes of this thesis, IL-11 only will be discussed.

1.8.1 Interleukin-11

Interleukin-11 was first cloned as a stromal-cell derived multi-functional cytokine (104). It is a member of a family which also includes interleukin-6, leukaemia inhibitory factor, ciliated neurotrophic factor, oncostatin M and cardiotrophin (105, 106). Over the past decade, there has been extensive research conducted into the function and action of IL-11, and these have been assessed in early clinical trials for patients with varying malignancies (107-109). Additionally this cytokine has been the subject of research into other diseases such as arthritis (110), Crohn's disease and ulcerative colitis (111, 112) and short-bowel syndrome (113, 114).

IL-11 has also been the focus of much research into the protection and recovery of stem cells found within the haematopoietic system (115-117). IL-11 stimulates proliferation of haematopoietic stem cells (115) as well as stimulating haematopoietic recovery following high-dose chemotherapy treatment (116). Additionally IL-11 has been shown to have non-haematological effects and is produced in varying locations throughout the body, including the alveolar cells of the lung, chondrocytes, synoviocytes and thyroid carcinoma cells (118-120). Thus, IL-11 may have a role in the stem cells found in the small intestinal crypts (121-123).

IL-11 and its receptor IL-11Rα are expressed within the gastrointestinal epithelium (124-126). Furthermore, *in vitro* studies using two intestinal stem cell lines, IEC-6 and IEC-18, have shown that IL-11 directly interacts with the gastrointestinal epithelial cells, more specifically interacting with aspects of cell proliferation (122, 127). Animal studies have highlighted the pleiotrophic nature of IL-11 in numerous small intestinal pathologies including those induced by cytotoxic treatment. Keith Jr and colleagues (1994) used two different models of gastrointestinal pathology, and concluded that there was a strong beneficial effect of IL-11, which often led to the reduction or resolution of the pathology, although they made no attempt to elucidate the mechanisms behind this. Another study conducted by Du, *et al.,* (1994) (118-120), examined the functional significance of IL-11 in mice that were treated with combined chemotherapy/radiotherapy. The results from their study clearly demonstrated that mice receiving this cytokine had an increased survival rate from their control counterparts, as well as an elevated mitotic index within the crypt epithelial cells, indicating a more rapid recovery time (118-120). In another study, IL-11 increased survival of mice treated with combined chemotherapy/radiation treatment, aided the recovery of small intestinal villi and increased cell proliferation in crypts (123). This was a comprehensive study and analysed different administration schedules of IL-11, including treatment pre, pre and during, and post cytotoxic exposure. This study did not however evaluate escalating doses of IL-11 and was unable to ascertain whether increasing the dose resulted in corresponding increases in protection (118-120). Furthermore, none of the above studies used tumour-bearing animals and were therefore unable to evaluate the effect of IL-11 on the tumour, nor the confounding effect of IL-11 and chemotherapy/radiotherapy on the tumour.

The importance of ensuring that IL-11 does not increase tumour growth has been recognised by research groups (105, 128). The effect of IL-11 on human tumours was investigated by Soda *et al.,* (1999), who, using a human tumour colony-forming assay, determined that IL-11 stimulated tumour growth in two out of 66 tumours, and inhibited tumour growth in 16 of the 66 tumours (105). This strongly suggests that IL-11 is unlikely to stimulate tumour growth in most common tumours including lung and colon (105). Another group investigated whether IL-11 was produced and regulated by breast cancer cells (128). It was reported that in the 13 different cell lines examined, two expressed IL-11 at the protein and mRNA levels, whilst a further 5 were not growth modulated by IL-11. There was no evidence of IL-11 in the remaining cell lines (128). Whilst there is no

evidence suggesting that IL-11 has any effect on causing tumour growth, this has yet to be fully evaluated in an animal model.

1.9 Specific Aims:

Whilst extensive literature exists on oral mucositis and possible preventative/treatment strategies for the condition, there is relatively little information on the mechanisms behind the damage caused by cancer chemotherapy. Additionally, very limited information exists on mucositis for the remainder of the gastrointestinal tract. Therefore the specific aims of this PhD were:

1. To investigate the effect varying doses of chemotherapy have on the small and large intestines of the DA rat with breast cancer
2. To investigate the mechanisms of Irinotecan-induced (CPT-11) small and large intestinal damage
3. To investigate the role of IL-11 (a potential anti-mucotoxic drug) on small intestinal mucositis in the DA rat with breast cancer
4. To compare and contrast pro- and anti-apoptotic protein expression along the gastrointestinal tract by determining their relative expression in normal human patients
5. To investigate and document human oral mucositis

Relationship between dose of methotrexate, apoptosis, p53/p21 expression and intestinal crypt proliferation in the rat

2.1 Introduction

Mucositis is an important complication of chemotherapy that is poorly understood. The condition occurs in 40% of patients after standard dose chemotherapy (3, 4) and in 100% of patients undergoing high dose chemotherapy and stem cell or bone marrow transplantation (4). Mucositis increases morbidity of patients undergoing cancer treatment, prolongs hospital stay, increases re-admission rates and is occasionally fatal (4). Currently, there is no known treatment or prevention, with management limited to pain relief, maintenance of good oral hygiene, antiemetics and anti-diarrhoeal medications.

Initial studies investigating the effect of methotrexate (MTX) on the rat small intestine described pyknotic bodies in crypts, now known to be apoptotic bodies (5, 6). Further animal studies investigating the effects of other cytotoxic drugs demonstrated that these agents also induce apoptosis in intestinal crypt cells within 24 h of treatment (37). Apoptosis also peaks 7-fold in the crypts of the small intestine in humans after cancer chemotherapy (2); this is followed by hypoplastic villus atrophy at 2 to 4 days after treatment. These data suggest that apoptosis inevitably leads onto crypt hypoproliferation.

Cytotoxic drugs increase p53 after inducing DNA damage. When upregulated, p53 either initiates G2/G1 arrest, allowing the cell time to repair itself, or it initiates apoptosis (15, 42). p53 activates transcription of p21, a cyclin dependent kinase inhibitor of cell proliferation (42). p21 binds with other cellular components to form a quaternary complex which is necessary for normal functioning of DNA polymerase (42) thereby inducing cell arrest in the G1 phase. p21 is necessary to induce cell cycle arrest that is p53-dependent in response to DNA damage (43).

Previously, it has been assumed that apoptosis determines subsequent damage to the small intestine caused by cancer chemotherapy. However, a study by Pritchard and colleagues (39) showed that the severity of apoptosis did not correlate with subsequent tissue damage. They found that p53 inhibited proliferation through the $p21^{waf\text{-}1/cip1}$ transcription factor responsible for decreased proliferation. This hypoproliferation, as well as apoptotic cell loss, contributed to crypt cell loss and intestinal damage. Additionally, they highlighted the deficiency of measuring only one variable (i.e., apoptosis) at any one time point,

demonstrating that this is not always an accurate indicator of tissue damage. Thus, there still remains some controversy regarding the exact role that apoptosis plays in the development of intestinal damage caused by cytotoxic agents.

The purpose of this study was to further investigate the relationship between apoptosis, p53 and p21 protein expression and crypt cell hypoproliferation over a range of doses of MTX using the laboratory DA rat implanted with breast cancer. The changes in p53 and p21 protein expression in the jejunal apoptotic response were also examined.

2.2 Materials and Methods

2.2.1 Preparation of Breast Cancer Cell Suspension

This study was approved by the Animal Ethics Committee of The Institute of Medical and Veterinary Sciences, Adelaide and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Teaching (1997).

The mammary adenocarcinoma used in this model arose spontaneously in the 1970s and was propagated by passage through female DA rats. This tumour has been used routinely in our laboratory for several years and was donated by Dr. A. Rofe (Institute of Medical and Veterinary Sciences, Adelaide, South Australia). Three female donor rats were injected s.c. on both flanks with 0.2 ml (2.0×10^7 cells per ml) tumour inoculum 11 days prior to the beginning of the study. These cells subsequently formed a tumour from which cells were harvested. The animals were culled by CO_2 asphyxiation and cervical dislocation, and subcutaneous tumours were dissected from the rats and placed into sterile Phosphate Buffered Saline (PBS) for further passage.

2.2.2 Preparation of Tumour Inoculum

Diced pieces of tumour were homogenised and filtered through sterile gauze. The tumour cell suspension was spun at 500 g for 3 mins each time; subsequently the pellet resuspended in fresh PBS and spun a further 4 times. A viable cell count was carried out using 0.5% w/v trypan blue.

2.2.3 Experimental Design

Seventy female DA rats (weighing approximately 150 g) were implanted with tumour inoculum of 4.0×10^6 cells in 0.2 ml phosphate buffered saline (PBS) s.c. into each flank and the tumours were allowed to grow for 9 days. Tumour bearing animals were used in this study to ensure that tumours do not have any adverse effect on the gastrointestinal tract, nor alter the expression of the two transcription factors under investigation. Rats were randomly divided into eleven groups of 6 animals. The first group of animals acted as controls, and received no treatment. Two other control groups received two daily i.m. injections of saline (control) and were killed 6 and 24 h post treatment. The eight remaining groups were treated with two daily i.m injections of either 0.5, 1.5, 2.5 or 5.0

mg/kg MTX and killed at either 6 or 24 hours post treatment. MTX is a commonly used chemotherapeutic agent in the clinical setting, particularly with breast cancer, thus it was appropriate to use.

Body weight was recorded each day and organ weight measured at autopsy as mucositis may change body and organ weight (129). The gastrointestinal tract from the pyloric sphincter to the rectum was removed, flushed with 0.9% (w/v) saline and the wet weight of the small and large intestines recorded. Two cm samples of small intestine (jejunum) at 25% of the length of the small intestine from the pylorus were collected. One was placed into Clark's fixative (60% w/v ethanol: 40% acetic acid) and another in 10% formalin for analysis. Additionally two cm samples of the colon were collected and one each placed in Clark's fixative and 10% formalin. The subcutaneous tumours were removed and weighed. Cross sections of non-necrotic tumour were dissected and placed into 10% formalin.

2.2.4 Intestinal Morphometry

Small sections (1 cm) of jejunum were opened onto cardboard, fixed in Clark's fixative for 24 h and stored in 70% ethanol. The stored tissues were rehydrated and hydrolysed in 1M HCL for 7 mins at 60°C. After two washes in double distilled water, the tissues were stained with Schiff's reagent (Sigma, St Louis, USA) for 45 mins before being microdissected as previously described (130). The tissues were examined following mounting in 45% acetic acid using a calibrated microscope graticule. Measurements were taken of the villus length, and apical and basal widths of 15 villi and the lengths of 15 crypts. Villus length, apical villus width and basal villus width were recorded and villus area was calculated using a trapezoid approximation (130). Mitotic counts per crypt were also recorded, following squashing of microdissected crypts. In this way, mitotic figures are clearly identified (131). Villus area and crypt length correlate with villus and crypt epithelial cell populations, respectively (131). Small (1 cm) pieces of colon were processed in the same way and the colonic crypt length and mitotic count per crypt were recorded. All measurements were conducted in a blinded fashion.

2.2.5 TUNEL Assay

Sections of jejunum and colon (1 cm each) were fixed for 24 h in 10% formalin prior to being dehydrated by passing through graded ethanols and infiltrated with, and embedded in, paraffin wax. 4 μm sections of jejunum and colon were cut using a rotary microtome

and stained for the presence of apoptosis via the TUNEL assay. The method for labelling the apoptotic cells has been described by our group previously (53). Briefly the assay was performed by using *In Situ Cell Death Detection Kit AP* (Roche, Mannheim, Germany). After dewaxing, the slides were rehydrated. Slides were immersed in a 0.1% Triton X-100 in 0.1% sodium citrate buffer for 8 mins at room temperature. Following two rinses in PBS, slides were placed in a TUNEL buffer solution (150 mM Tris; 0.7 M NaCaCo; 10 mM $CoCL_2$; 10% BSA and sterile H_20) for a further 10 mins at room temperature. Slides were immediately placed into the reaction mixture and incubated in a humidified chamber for 3 h at 37°C. Following three rinses in PBS, slides were incubated with Converter-AP in a humidified chamber for 60 mins at 37°C, after which they were rinsed a further two changes of PBS. Fast red chromogen (Roche, Germany) was applied for 15 mins. Slides were rinsed and counterstained with haematoxylin. Apoptosis in the jejunum and colon could then be counted and expressed as apoptotic bodies per crypt. All counts were conducted in a blinded fashion.

2.2.6 Immunohistochemistry for Detection of p53 and p21 within the Jejunum.

Two consecutive 4μm paraffin sections of jejunum were cut, dewaxed in xylene and rehydrated through a graded series of alcohols. One section was designated as the negative control, the other as the experimental section. Slides were washed 2 x 5mins in PBS prior to being subjected to heat mediated antigen retrieval in citrate buffer (pH 6.5). Slides were quenched in 3% hydrogen peroxide in methanol for 1 min to deactivate endogenous peroxidase activity, before non-specific antigens were blocked by normal serum for 20 mins. Finally, endogenous avidin and biotin was eliminated using a commercial avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA USA). Slides were incubated overnight at 4°C with the primary antibody (p53 Mouse Monoclonal Novocastra, NCL-p53-240, 1:50 dilution; $p21^{waf1/cip1}$ Rabbit Polyclonal Santa Cruz Biotechnology, sc-397, 1:900 dilution) on the experimental section, and PBS only on the negative section. The following morning, slides were incubated with a universal linking antibody, before the reaction was amplified with Ultra-Streptavidin conjugated to horseradish peroxidase and visualised with diaminobenzidine chromogen. Slides were counterstained with Lillie-Mayer's Haematoxylin, dehydrated through a graded series of alcohols and mounted with PIX mounting medium. Both positive and negative control sections were included in each run. Positive sections included breast carcinoma for p53 and liver for p21, whereas negative sections had the primary antibody step omitted.

2.2.7 Quantitative Immunohistochemistry

In order to quantify staining, an algorithm which calculated the cumulative signal strength or energy of a digital file of the image in question was used (132, 133). It was assumed that all staining in both the experimental and negative control image was important (132, 133). Digital photographs were taken of identical intestinal crypt sections of experimental and control sections (Fig 2.1) using a Nikon 800 research microscope (x63 dry lens) and SPOT-RT camera (Diagnostic Instruments Inc, Michigan, Detroit). A 49x49 pixel area over the nucleus of individual jejunal crypt enterocytes was selected in both experimental and control sections (excluding the goblet cells), using Image Pro Plus (Media Cybernetics, Silver Spring MD) (Fig 2.1). These were saved as TIFF files, which allowed for LZW compression without the loss of any data (132, 133), before being converted to image matrices, and normalised using functions in Matlab (Mathworks Inc. Natick, MA USA) (132, 133). The intensity of staining was expressed as the pixel energy difference in cumulative signal between the control and experimental image matrices.

2.2.8 Statistical Analysis

All statistical analysis were conducted using the Peritz' F Test, which is a robust measure of differences between group means (134).

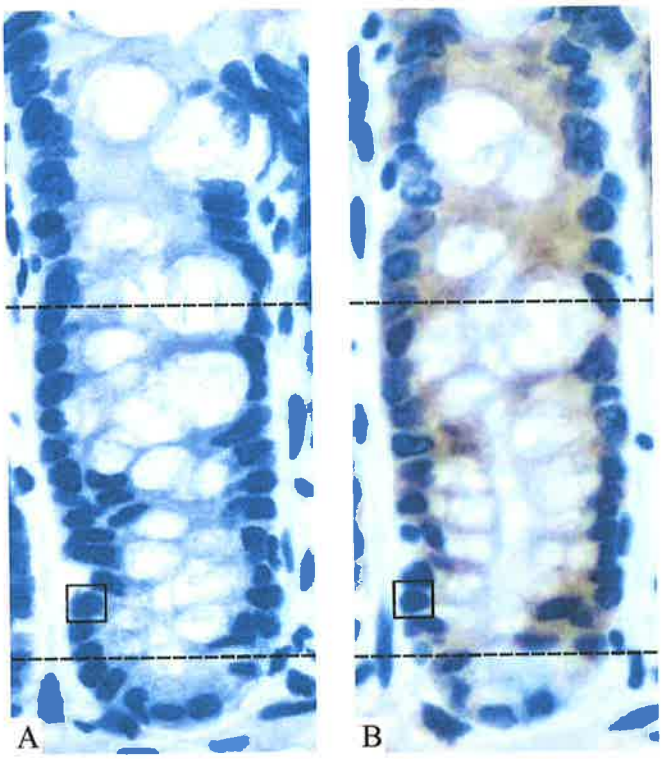


Figure 2.1 Photomicrographs of a serial A) unstained control and B) stained experimental (mouse monoclonal p53 antibody) section of rat jejunum taken at 600 x magnification overlayed with a 49 x 49 area of interest box.

2.3 Results

2.3.1 Response to Treatment

At autopsy, final body weights as well as the weight of the small and large intestines and the tumour were recorded. Body weights were not affected by any dose of MTX at either 6 or 24 h after treatment (Table 2.1). At 6 h after MTX treatment, there was no significant difference between the wet weight of the small intestine for any treatment group, however at 24 h post treatment, the small intestinal weight was significantly reduced in animals that received 2.5 and 5.0 mg/kg MTX compared to untreated animals ($p < 0.034$ and $p < 0.050$ respectively) (Table 2.1). The wet weight of the colon was not significantly affected by MTX at any dose over either time point (Table 2.1). As expected, tumour weights did vary with the dose of MTX. The higher the dose of MTX the lighter the tumours were at both 6 and 24 h after treatment (Table 2.1). Control animals had tumours which increased over the two time-points.

2.3.2 Small Intestinal Morphometry

At 6 h after treatment, low dose (0.5mg) MTX caused only a minor reduction in villus area and crypt length (Fig 2.2), that was not significantly different from untreated control animals (Villus area p = NS; crypt length p = NS). However, mitotic count per crypt decreased from 5.5 ± 0.3 to 1.6 ± 0.2 ($p < 0.025$) (Fig 2.3). There was also no significant difference observed between low dose MTX and control animals in villus area and crypt length at 24 h (Fig 2.2). However, higher doses of MTX (1.5 mg/kg and above) showed severe significant reduction in villus area (1.5 mg/kg $p < 0.034$; 2.5 mg/kg $p < 0.025$; 5.0 mg/kg $p < 0.05$) and crypt length (1.5 mg/kg $p < 0.05$; 2.5 mg/kg $p < 0.034$; 5.0 mg/kg $p < 0.042$) at 6 h. At 24 h there were still severe significant reductions in villus area (1.5 mg/kg $p < 0.05$; 2.5 mg/kg $p < 0.034$; 5.0 mg/kg $p < 0.042$) and crypt length (1.5 mg/kg $p < 0.05$; 2.5 mg/kg $p < 0.034$; 5.0 mg/kg $p < 0.042$). These reductions were not dose-dependent, with no significant damage being seen between these groups (Fig 2.2).

2.3.3 Colon Morphometry

MTX did not significantly affect colonic crypt length or mitotic count at either time point investigated (Table 2.2).

Treatment Group	Body Weight	Small Intestine	Large Intestine	Tumour
True Controls	185.28 ± 8.70	5.81 ± 0.47	1.30 ± 0.09	6.47 ± 0.43
0.5mg MTX 6 h	187.25 ± 6.61	6.37 ± 0.28	1.40 ± 0.16	9.96 ± 0.88
1.5mg MTX 6 h	177.63 ± 4.92	5.96 ± 0.17	1.24 ± 0.08	6.85 ± 1.08
2.5mg MTX 6 h	179.87 ± 8.98	5.49 ± 0.18	1.41 ± 0.16	6.59 ± 0.84
5.0mg MTX 6 h	183.14 ± 5.51	5.83 ± 0.20	1.70 ± 0.17	5.19 ± 0.57
Saline 6 h	189.22 ± 6.51	6.31 ± 0.15	1.26 ± 0.14	10.04 ± 0.67
0.5mg MTX 24 h	185.63 ± 5.89	6.15 ± 0.31	1.16 ± 0.05	14.18 ± 1.06
1.5mg MTX 24 h	179.98 ± 5.62	5.53 ± 0.24[b]	1.20 ± 0.06	6.14 ± 0.54
2.5mg MTX 24 h	176.38 ± 3.71	5.15 ± 0.15[a]	1.20 ± 0.06	3.69 ± 0.53
5.0mg MTX 24 h	176.64 ± 5.13	5.40 ± 0.28	1.14 ± 0.07	3.04 ± 0.24
Saline 24 h	196.39 ± 10.91	6.54 ± 0.27[a b]	1.27 ± 0.06	16.77 ± 0.94

Table 2.1: Body weight, weight of the small intestine, weight of the large intestine and tumour weight at autopsy in female DA rats treated with MTX. Data are given as mean ± SE (g).

a = $p < 0.0500$; b = $p < 0.0336$

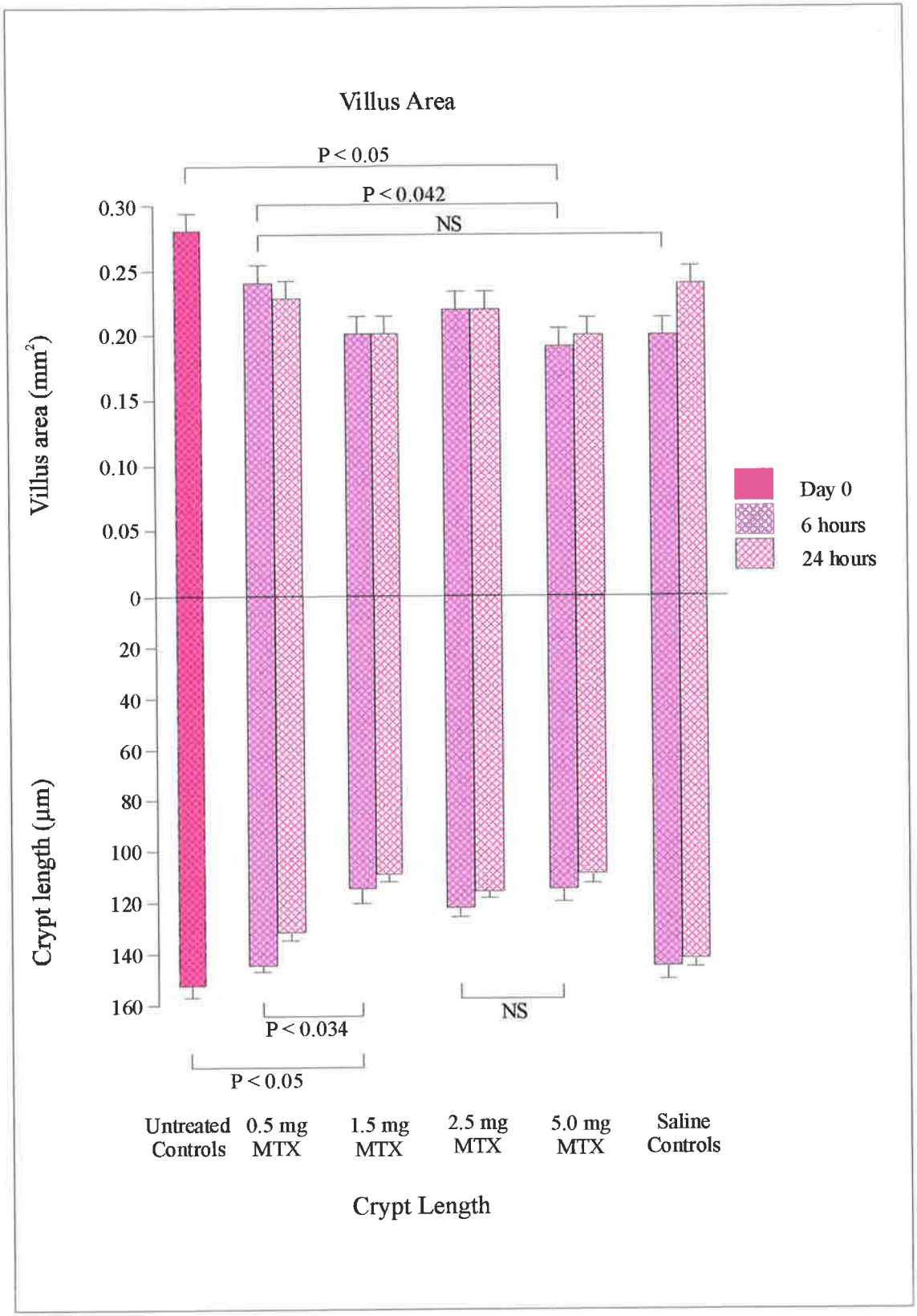


Figure 2.2 Effect of varying doses of MTX on small intestinal morphometry (villus area and crypt length) over 2 time points in rats with breast cancer. Data are expressed as mean +/- SEM.

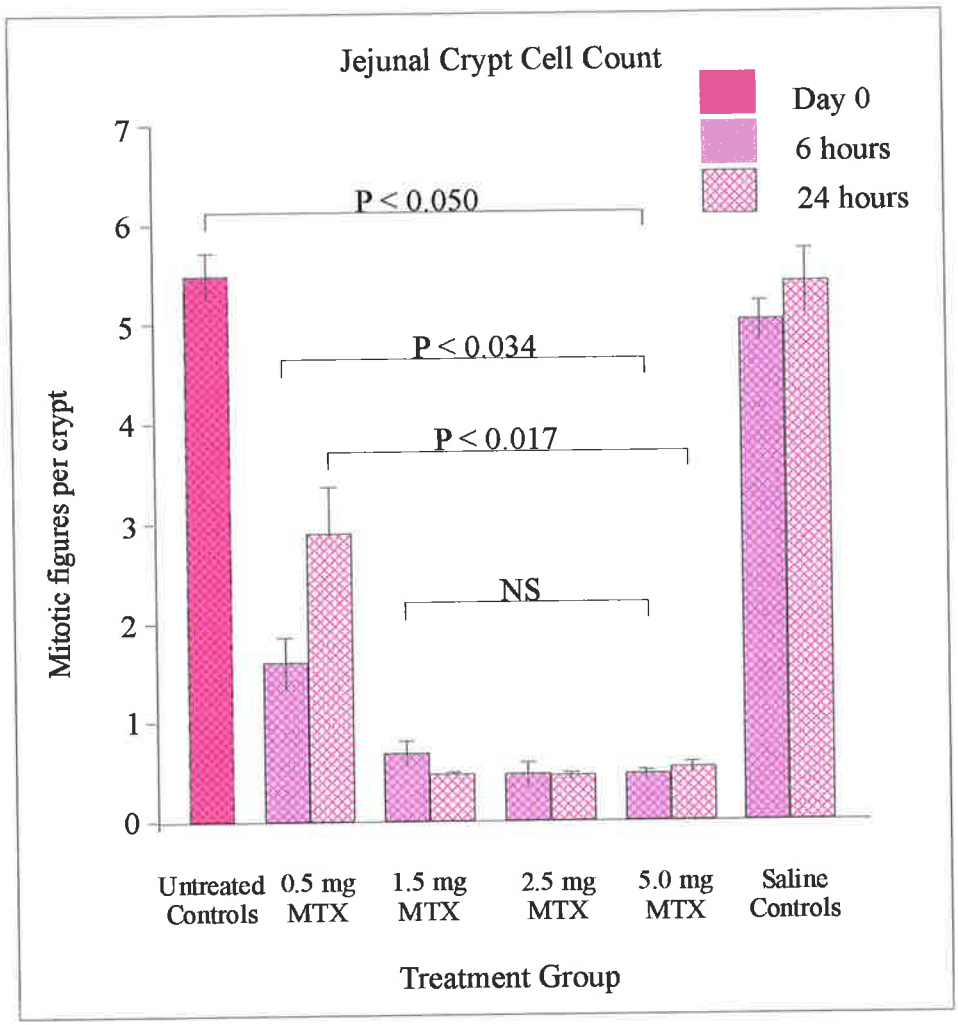


Figure 2.3 Effect of varying doses of MTX on crypt cell mitoses over two time points in rats with breast cancer. Data are expressed as mean +/- SEM.

Treatment Group	Crypt Length	Mitoses
Untreated controls	301.62 ± 18.40	2.35 ± 0.23
0.5mg MTX 6 h	303.22 ± 13.55	2.67 ± 0.20
1.5mg MTX 6 h	298.90 ± 10.92	2.13 ± 0.23
2.5mg MTX 6 h	304.36 ± 8.01	4.49 ± 2.99
5.0mg MTX 6 h	312.46 ± 10.50	1.59 ± 0.18
Saline 6 h	285.25 ± 14.23	2.28 ± 0.40
0.5mg MTX 24 h	315.72 ± 6.98	2.38 ± 0.31
1.5mg MTX 24 h	314.98 ± 5.50	1.87 ± 0.12
2.5mg MTX 24 h	310.77 ± 10.54	1.87 ± 0.20
5.0mg MTX 24 h	311.83 ± 16.90	1.84 ± 0.25
Saline 24 h	317.93 ± 8.16	2.67 ± 0.42

Table 2.2: Table showing colonic crypt length and mitotic count per crypt for varying doses of MTX. Data are given as mean ± SE (g). There was no significant difference between any of the groups.

2.3.4 Apoptosis

Jejunum

At 6 h after treatment, low dose (0.5 mg/kg) MTX caused a higher level of apoptosis (3.01 per crypt), whereas higher doses of MTX caused lower levels of apoptosis in the jejunum (1.5 mg/kg 1.32; 2.5 mg/kg 0.99; 5.0 mg/kg 1.04 per crypt respectively) when compared to controls (0.08 per crypt) (Fig 2.4). By 24 h after treatment, low dose MTX dramatically reduced apoptosis (0.43 per crypt) whereas the higher doses still had relatively elevated levels (1.5 mg/kg 0.96; 2.5 mg/kg 0.68; 5.0 mg/kg 0.66 per crypt respectively. At both time points, there was no significant difference recorded between the higher (1.5 mg/kg; 2.5 mg/kg and 5 mg/kg) treatment groups (Fig 2.4).

Colon

In contrast to the jejunum, apoptosis was approximately 10-fold lower in the colon. The degree of apoptosis increased with the dose of MTX in the colon; however none of these increases were significant (Fig 2.5).

2.3.5 p53 Expression

Low dose MTX (0.5 mg/kg) showed no change from controls in levels of p53 protein expression 6 h after treatment, correlating with the peak incidence of jejunal apoptosis. When the later time point of 24 h post treatment was examined, p53 levels increased 2-fold ($p < 0.05$), corresponding to the sharp decline in apoptosis. In contrast, the higher doses of MTX increased p53 levels at 6 h and these remained elevated at 24 h with no significant difference detected between them (Fig. 2.6).

2.3.6 p21 Expression

There was no apparent change in the expression levels of the p21 protein after any dose of MTX at either 6 or 24 h (data not shown).

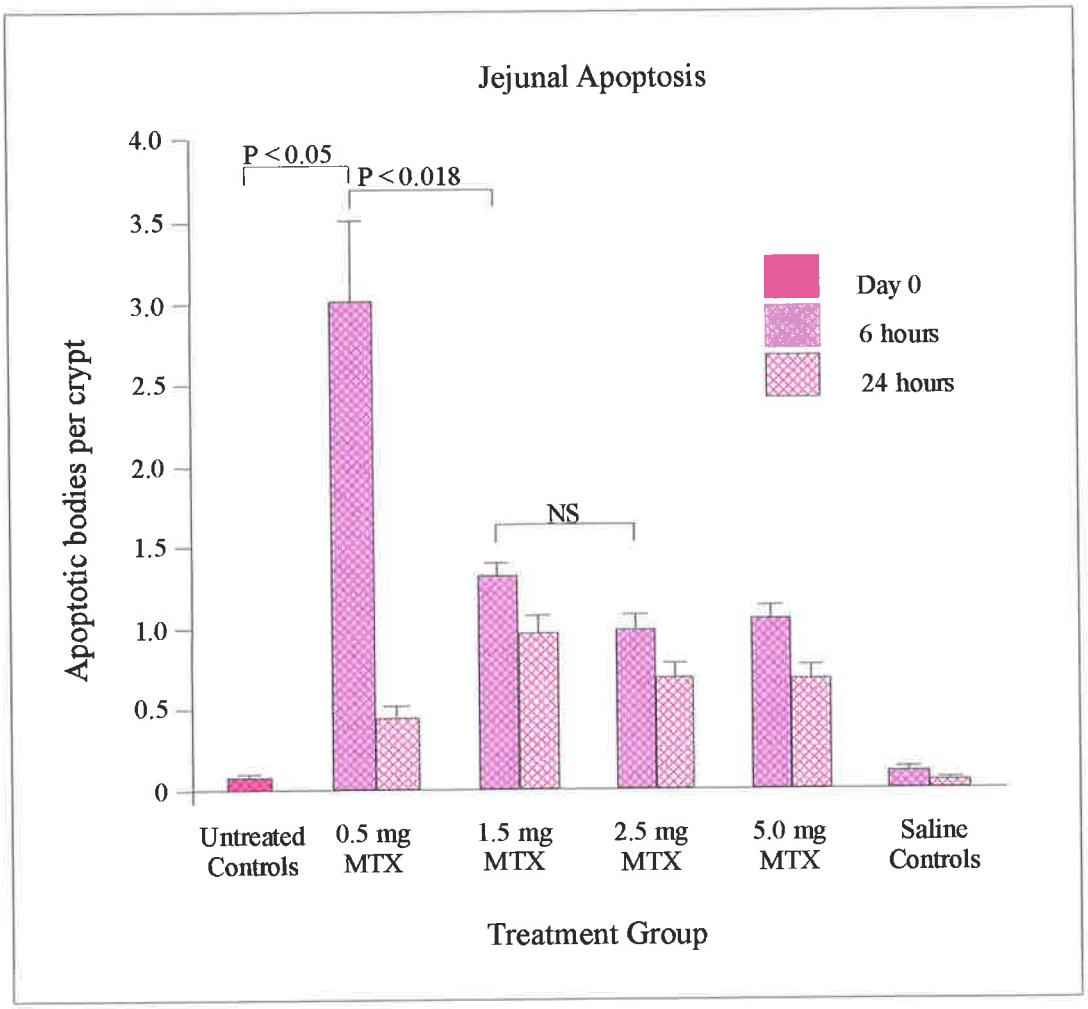


Figure 2.4 Effect of varying doses of MTX on apoptosis of jejunal crypt cells over two time points in rats with breast cancer. Data are expressed as mean +/- SEM.

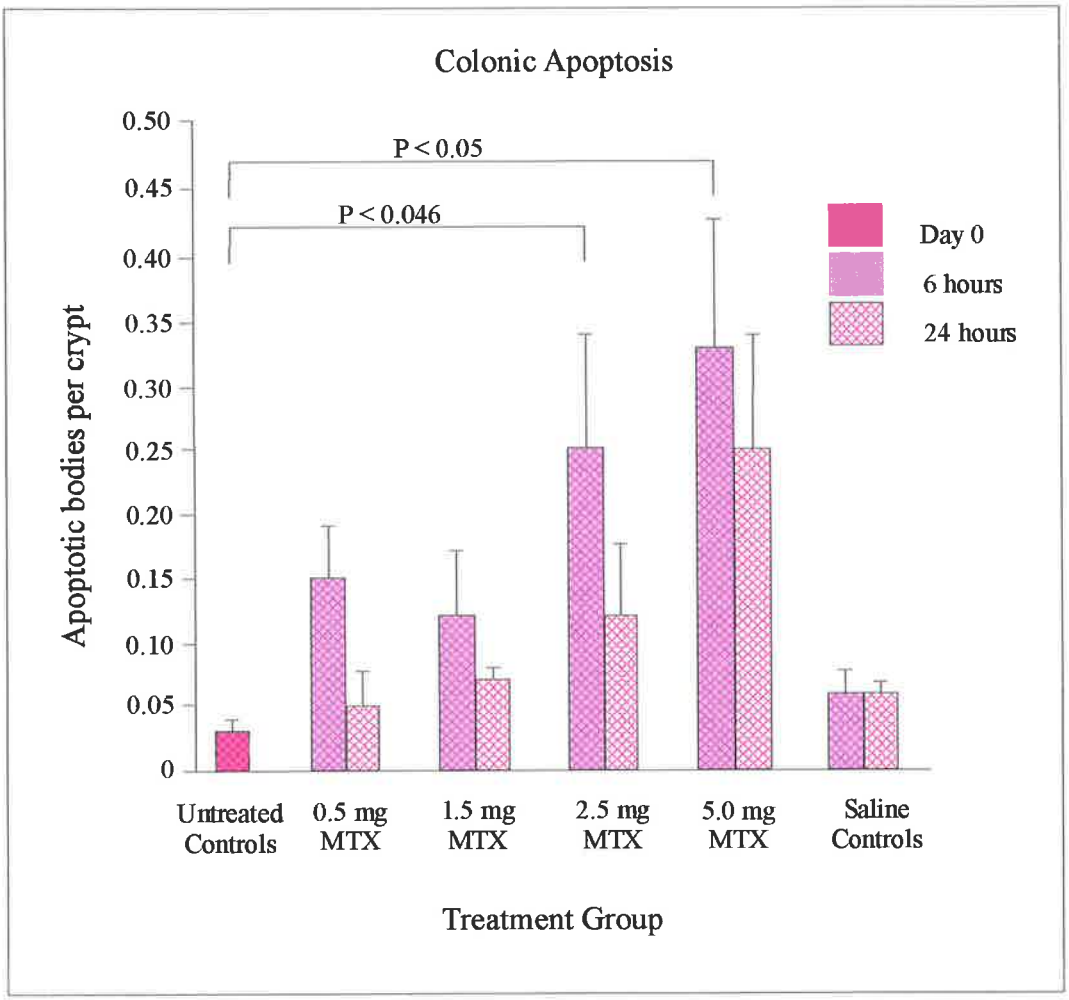


Figure 2.5 Effect of varying doses of MTX on apoptosis of colonic crypt cells over two time points in rats with breast cancer. Data are expressed as mean +/- SEM.

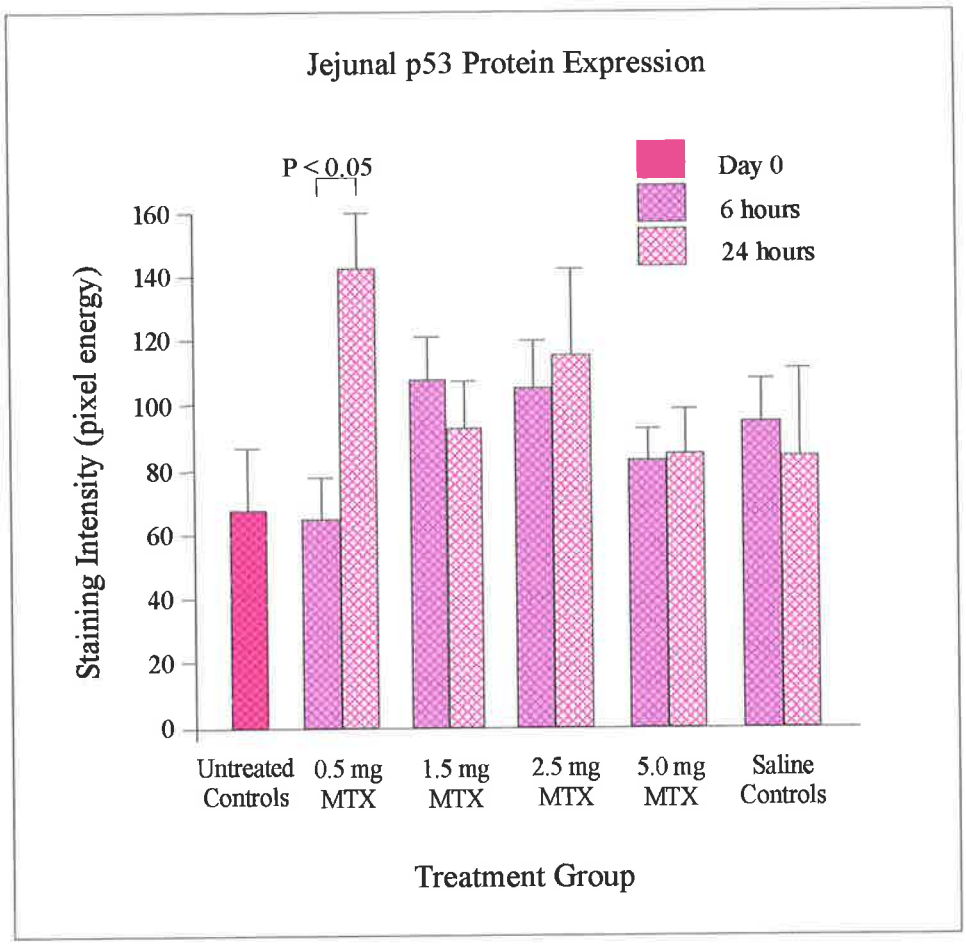


Figure 2.6 Effect of varying doses on MTX on p53 protein expressionin jejunal crypt cells over two time points in rats with breast cancer. Data are expressed as mean +/- SEM.

2.4 Discussion

In this study, the effect of dose of MTX on the sensitivity of the jejunum and colon of tumour-bearing rats to apoptosis and intestinal damage was investigated. Tumour-bearing animals were used to ensure that the tumour load did not cause a change in gastrointestinal response nor a change in the protein expression of p53 and p21. The present study has shown that low dose MTX (0.5 mg/kg) in the jejunum caused a high peak of apoptosis but minimal crypt cell hypoproliferation. In the colon however, apoptosis was lower and dose dependent, and was not accompanied by crypt cell hypoproliferation. A change in p53 expression did not precede the high level of apoptosis that were observed at 6 hours following 0.5 mg/kg MTX in the jejunum; however at 24 h there was an increase in p53 expression that may be associated with later levels of apoptosis. These data are similar to those described by Pritchard and colleagues (39) who showed that a low (40 mg/kg) 5-flourouracil dose caused apoptosis but no subsequent intestinal damage, whereas high (400 mg/kg) 5-flourouracil caused apoptosis and significant intestinal damage (39). The present study extends this research by firstly investigating relationships of p53 and p21 expression to jejunal apoptosis and secondly by examining the colonic response to varying chemotherapy doses.

Previous studies have shown that apoptosis in the large intestine occurs at a 10-fold lower rate than in the small intestine (14, 15). The data presented here show that, despite there being no tissue damage following MTX treatment, there is accompanying colonic apoptosis, occurring at a much lower rate than that seen in the jejunum. Other research in this thesis (detailed in Chapter 3) has shown that a cytotoxic agent, irinotecan, causes apoptosis in the colon, however this increase in apoptosis is accompanied by severe tissue damage. The results from this study differ however, in that there was an increase in apoptosis with increasing doses of chemotherapy, without accompanying tissue damage. Previous research by Ijiri and Potten (36, 37, 135) have clearly demonstrated that different chemotherapeutic agents act at different locations within individual crypts. As the colonic stem cells are thought to be located at the base of the crypts in cell position 1 and 2, this study highlights that whilst cytotoxic drugs may cause colonic apoptosis, this apoptosis does not necessarily lead to subsequent tissue damage, as the colonic stem cells are not targeted.

Several studies have shown that the expression of p53 in the small intestinal epithelium increases following γ-irradiation, supporting the hypothesis of recognition of DNA

damage, p53 upregulation, and subsequent induction of apoptosis (41, 44, 45). The present study has shown that low dose MTX (0.5 mg/kg) caused minimal disruption to jejunal tissue architecture, despite having very high levels of apoptosis and no change in levels of p53 protein expression at 6 h after treatment. These results are somewhat surprising as it has been previously suggested that lower doses of cytotoxic agents such as 5-FU may stabilise the p53 protein, not only allowing induction of apoptosis, but also allowing the transcription of p21 which inhibits progression of the cell cycle at G1 (14, 15), and that these actions preserve tissue integrity. Whilst the results from this study confirmed that there was minimal change in jejunal tissue architecture, despite high levels of apoptosis, it did not confirm that changes in p53 and p21 expression were responsible. It may be that there are other proteins involved in the response. The later time point of 24 h after treatment was also examined, and it was found that p53 expression increased 2-fold and appeared to correspond to a sharp decline in apoptosis in this treatment group. In contrast at both 6 and 24 h after treatment with higher doses of MTX (1.5, 2.5 and 5/0 mg/kg), rats showed significant disruption to jejunal tissue architecture, increased levels of apoptosis, and increased p53 expression. For all doses of MTX and both time points investigated, we were unable to demonstrate any significant change in the expression levels of the p21 protein.

A confounding factor in the analysis of p53 and p21 was the double dose of chemotherapy administered on two consecutive days. This was necessary because previous work found that a single dose of methotrexate causes an unreliable level of intestinal mucositis that is difficult to reproduce between experiments (51). Despite the fact that single dose chemotherapy may not cause gross architectural changes, it is likely that it causes changes at the protein expression level. Therefore, when measuring protein expression in order to determine their relationship to mucositis, administration of a single dose of chemotherapy would be optimal with protein expression measurements carried out early after treatment. It is therefore possible that changes in p53 protein expression which may have occurred following the first chemotherapy injection were unable to be detected. In addition, the sampling times of 6 and 24 hours after treatment may have missed an early peak of p53 protein expression. A recent study, using radiation has shown that p53 induction already occurred by 2 h after treatment (136) and another even more recent study suggested that p53 induction occurred by 40 min after treatment (137).

It therefore can be concluded that low dose MTX in the jejunum leads to a short intense early peak of apoptosis, minimal crypt damage and no initial change in p53 protein

expression. Increasing the dose of MTX initially leads to a longer duration of apoptotic activity, more severe crypt cell damage and increased p53 expression. p21 remains constant at all doses during the times studied. The colon is resistant to damage by MTX.

Irinotecan causes severe small intestinal damage, as well as colonic damage, in the rat with implanted breast cancer

3.1 Introduction

Irinotecan hydrochloride (CPT-11) is a relatively new chemotherapeutic agent used to treat solid tumours by acting on malignant cells through inhibition of DNA topoisomerase I (54-57). A major problem of this cytotoxic agent is severe diarrhoea and leukopenia, which severely limits the dose of administered drug (55-57).

Irinotecan causes two types of diarrhoea; first an early secretory diarrhoea which is cholinergic in nature and can be prevented by the administration of atropine prior to irinotecan administration, and second a delayed diarrhoea which is the subject of the present study. The mechanism of the delayed diarrhoea following irinotecan administration remains uncertain. Previous studies investigating irinotecan have differed greatly in proposed mechanisms behind the delayed diarrhoea. Whilst some studies have suggested that cytotoxicity of irinotecan causes a gastrointestinal enterocolitis with accompanying water, electrolyte and mucus secretion from the colon (54), others have suggested that irinotecan interferes with the normal intestinal bacterial flora, causing increased intestinal β-Glucoronidase activity and this is responsible for the delayed diarrhoea (57). These mechanisms are unusual for cytotoxic drugs, with previous studies indicating that chemotherapeutic agents in general cause apoptosis in small intestinal crypts with accompanying villous atrophy and crypt hypoplasia (2, 52, 53). Despite the many varied suggestions on how irinotecan causes large intestinal damage with delayed diarrhoea, there is little information on how it affects the small intestine.

The aims of this study were therefore: (1) to investigate the effect of dose of irinotecan on diarrhoea and (2) to describe the time-course of apoptosis, intestinal damage, change in goblet cells and mucin, in both the small and large intestines, in order to elucidate the possible mechanisms behind the delayed diarrhoea.

3.2 Materials and Methods

3.2.1 Passage of Tumour

This study was approved by the Animal Ethics Committee of The Institute of Medical and Veterinary Sciences, Adelaide and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Teaching (1997).

The mammary adenocarcinoma used in this model arose spontaneously in the 1970s and was propagated by passage through female DA rats. This tumour has been used routinely in our laboratory for several years and was donated by Dr. A. Rofe (Institute of Medical and Veterinary Sciences, Adelaide, South Australia). Three female donor rats were injected s.c. on both flanks with 0.2 ml (2.0×10^7 cells per ml) tumour inoculum 11 days prior to the beginning of the study. These cells subsequently formed a tumour from which cells were harvested. The animals were culled by CO_2 asphyxiation and cervical dislocation, and subcutaneous tumours were dissected from the rats and placed into sterile Phosphate Buffered Saline (PBS) for further passage.

3.2.2 Preparation of Tumour Inoculum

Diced pieces of tumour were homogenised and filtered through sterile gauze. The tumour cell suspension was spun at 500 g for 3 mins each time; subsequently the pellet resuspended in fresh PBS and spun a further 4 times. A viable cell count was carried out using 0.5% w/v trypan blue.

3.2.3 Experimental Plan

All experiments described were conducted in female dark agouti (DA) rats, weighing approximately 150 g. All animals received breast cancer inoculum as described above. Irinotecan was used as the chemotherapeutic agent.

Sixty four DA rats were randomly assigned to receive either irinotecan or buffer control. Rats received 0.01 mg/kg subcutaneous atropine (to reduce any cholinergic reaction to irinotecan) prior to administration of either 100, 150 or 200mg/kg intraperitoneal irinotecan or vehicle control. Irinotecan (kindly supplied by Pharmacia) was administered in a sorbitol/lactic acid buffer (45 mg/ml sorbitol/0.9 mg/ml lactic acid pH 3.4), required for activation of the drug, on days designated 0 and 1. Control rats received buffer only.

Groups of rats were killed via CO_2 asphyxiation at 6, 24, 48 and 96 h after second treatment, and tissue was collected for analysis. The gastrointestinal tract (from the pyloric sphincter to the rectum) was dissected out, flushed with room temperature isotonic saline, and wet weights of small and large intestines were recorded. Two centimetre samples of small intestine (taken at 25% of the length of the small intestine from the pylorus) were collected and placed in Clarke's fixative (60% v/v ethanol: 40% v/v acetic acid) for intestinal morphometry, and in 10% formalin for histological examination and TdT-mediated dUTP nick end labelling (TUNEL) staining. The tumours were removed and weighed. Small (approximately 3 cm) cross-sections of non-necrotic tumour were dissected and placed into 10% formalin for histological examination and TUNEL staining. In addition the spleen and liver were removed, weighed and fixed in formalin for histological examination.

3.2.4 Diarrhoea Assessment

All animals were checked three times daily and diarrhoea recorded. This was graded as 0 = no diarrhoea, 1 = mild diarrhoea (staining of anus), 2 = moderate diarrhoea (staining over top of the legs and lower abdomen) and 3 = severe diarrhoea (staining over legs and higher abdomen, often associated with continual oozing).

3.2.5 Histological Examination

Samples throughout the gastrointestinal tract, liver, spleen and tumours were taken and fixed in 10% formalin for routine histological examination. Once fixed, samples were processed and embedded in paraffin, prior to 4μm sections being cut. Sections were dewaxed, rehydrated and stained in Mayer's haematoxylin for 10 mins. After differentiation in 1% acid alcohol and blueing in Scott's tap water, sections were counterstained in eosin, before being dehydrated and mounted. Examination of all sections was performed by specialist veterinary pathologist, Dr. John Finnie, in the Institute of Medical and Veterinary Sciences, Adelaide, South Australia.

3.2.6 Intestinal Morphometry

Sections of jejunum and colon (sites corresponding to 25% of the jejuno-ileum and 50% of the colon length) were opened onto cardboard, fixed in Clarke's fixative for 24 h and stored in 70% ethanol at room temperature prior to use. The stored tissue was rehydrated through a graded series of ethanols and hydrolysed in 1M HCL for 7 mins at 60°C. After

two washes in double distilled water, the tissue was stained with Schiff's reagent (Sigma, St Louis, USA) for 30 mins before being microdissected using a cataract knife and stereomicroscope. The tissue was mounted in 45% (v/v) acetic acid and measurements were taken, using a calibrated graticule, of the villus length and apical and basal widths of 15 villi (jejunum) and the lengths of 15 crypts (jejunum and colon) (130). Villus area was calculated as a trapezoid approximation (130). Villus area and crypt length correlate with villus and crypt epithelial cell populations, respectively.

3.2.7 Measurement of Apoptosis

Sections of jejunum and colon (sites corresponding to 25% of the jejuno-ileum and 50% of the colon length) were fixed for 24 h in 10% formalin and stored in 70% ethanol. Tissue was dehydrated, embedded in paraffin wax and histological 4 μm sections were cut. The method for labelling the apoptotic cells was performed by using *In Situ Cell Death Detection Kit AP* (Roche, Mannheim, Germany). After dewaxing, the slides were rehydrated, prior to immersion in a 0.1% TX-100 in 0.1% (w/v) sodium citrate buffer for 8 mins at room temperature. Following two rinses in PBS, slides were placed in a TUNEL buffer solution (150mM Tris; 0.7M NaCaCo; 10mM $CoCL_2$; 10% BSA and sterile H_20) for a further 10 mins at room temperature. Slides were immediately placed into the reaction mixture and incubated in a humidified chamber for 3 h at 37°C. Following three rinses in PBS slides were incubated with Converter-AP in a humidified chamber for 60 mins at 37°C, after which they were rinsed a further 2 changes of PBS. Fast red chromogen (Roche, Germany) was applied for 15 minutes. Slides were rinsed and counterstained with haematoxylin. Apoptotic bodies were counted per crypt per 4 μm section.

3.2.8 Alcian Blue- PAS Analysis of Goblet Cells

Following fixation in 10% formalin, and embedding of tissue, 4 μm sections of the gastrointestinal tract were cut and rehydrated, before being immersed in Alcian blue solution for 5 mins at room temperature. Following two washes in tap water and distilled water, sections were placed in a 1% aqueous periodic acid solution, before being immersed in freshly prepared Schiff's reagent for 15 mins. Sections were washed in running tap water for 8 mins before being lightly counterstained (15 s) with Lillie Mayer's Haematoxylin. Sections were dehydrated and coverslipped.

3.2.9 Statistical Analysis

Group means were compared using the Peritz' F Test, which is a robust multiple comparison test of group means where group size is not necessarily equal (138).

3.3 Results

3.3.1 Response to Treatment

No control animal recorded diarrhoea. In animals that received 100 mg/kg irinotecan, no diarrhoea was observed in the first 24 h, however mild diarrhoea developed after this time point. At 96 h there was no mortality observed in animals that received this dose. Animals receiving 150 mg/kg irinotecan also had no diarrhoea observed within the first 24 h, however after this time point, mild to moderate diarrhoea was observed and by 96 h 50% of animals receiving this dose had died. Seventy five percent of rats that received 200 mg/kg irinotecan had mild to moderate diarrhoea initially, and this progressed to severe diarrhoea in most animals. At 96 h there was 100% mortality in animals that received this high dose (Fig 3.1). Autopsy revealed that all animals that died prematurely had lost approximately 26% of their pre-treatment bodyweight, and had perforated duodena leading to peritonitis. The pathology that was underlying and precipitating duodenal perforation was as follows: Animals suffered from paralytic ileus. Upon inspection of their abdominal cavities, they were found to contain fluid and finally there were focal areas of perforation and adherent peritoneum or omentum.

3.3.2 Histology

Pathological changes caused by irinotecan were most marked in the colon with dilated crypts, cellular debris and hypersecreted mucus over the luminal mucosal surface. Only minimal inflammation was observed. The small intestine, most notably the jejunum, showed patchy apoptosis of individual or grouped enterocytes deep within the crypt. Occasional villous atrophy was observed.

Control animals periodically showed mild histopathological changes within the liver and spleen, suggesting that the sorbitol/lactic acid buffer used for the drug administration has a systemic effect. However, a subsequent study comparing the effects of the buffer with isotonic saline showed no significant differences between these two organs (data not shown).

Irinotecan at all doses decreased the weight of the spleen (Table 3.1). Histological examination of the spleen revealed acute congestion, but no depletion of lymphoid tissue. Irinotecan decreased liver weight at 96 h, however upon pathological examination there was no disruption to the architecture.

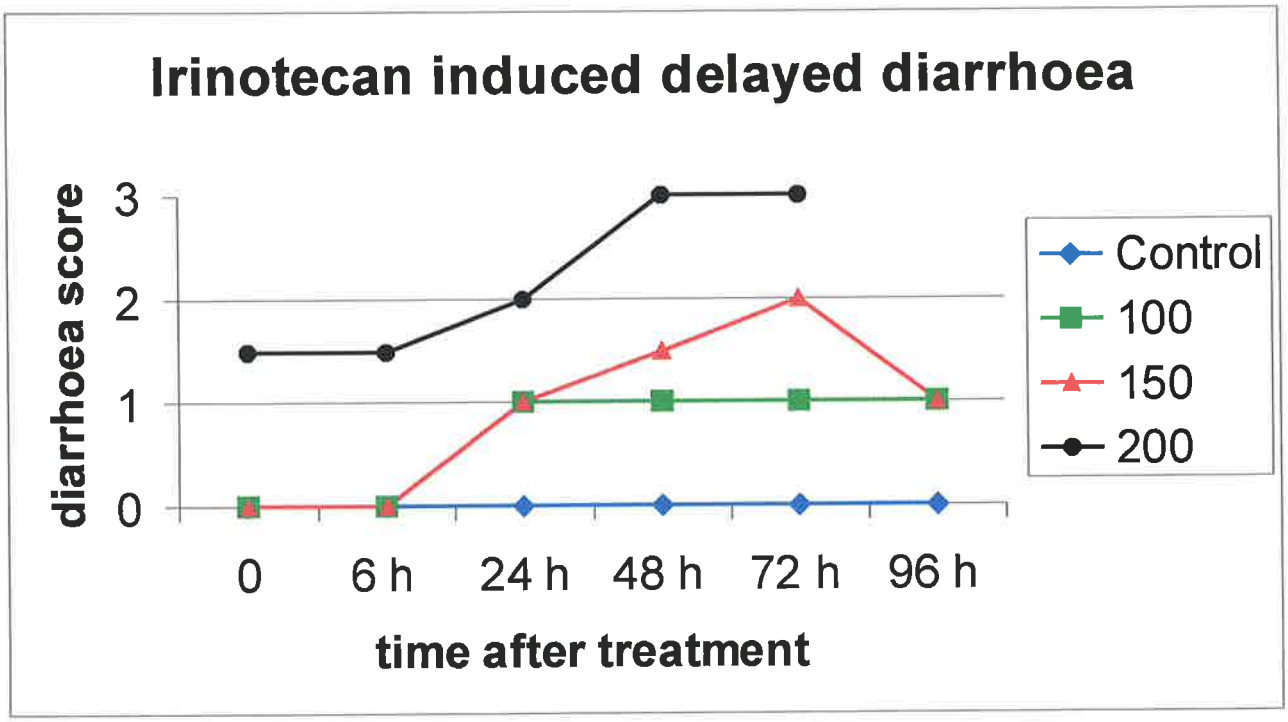


Figure 3.1 Effect of dose on irinotecan induced delayed diarrhoea over time. No diarrhoea was observed in control animals, animals receiving 100 mg/kg developed only mild diarrhoea, whilst those receiving 150 mg/kg and 200 mg/kg developed moderate to severe diarrhoea, with the latter group having 100% mortality by 96 h. (Scale 0 = no diarrhoea; 1 = mild diarrhoea; 2= moderate diarrhoea; 3 = severe diarrhoea).

Time Point	Treatment Group	Small Intestine	Large Intestine	Spleen
Kill 6 h	Control	6.28 (± 0.17)	1.23 (± 0.05)	0.40 (± 0.01)
	100mg/kg CPT-11	4.49 (± 0.24)	1.02 (± 0.06)	0.28 (± 0.005)
	150mg/kg CPT-11	4.84 (± 0.17)	1.08 (± 0.05)	0.28 (± 0.01)
	200mg/kg CPT-11	5.27 (± 0.14)	1.25 (± 0.16)	0.26 (± 0.02)
Kill 24 h	100mg/kg CPT-11	4.05 (± 0.21)	1.01 (± 0.04)	0.29 (± 0.01)
	150mg/kg CPT-11	4.59 (± 0.39)	1.15 (± 0.05)	0.30 (± 0.04)
	200mg/kg CPT-11	4.91 (± 0.57)	1.09 (± 0.09)	0.20 (± 0.01)
Kill 72 h	100mg/kg CPT-11	5.22 (± 0.56)	1.43 (± 0.03)	0.26 (± 0.04)
	150mg/kg CPT-11	4.32 (± 0.3)	1.42 (± 0.06)	0.25 (± 0.03)
	200mg/kg CPT-11	3.7 (± 0.22)	1.27 (± 0.06)	0.15 (± 0.01)
Kill 96 h	Control	5.64 (± 0.21)	1.40 (± 0.06)	0.56 (± 0.02)
	100mg/kg CPT-11	7.46 (± 0.34)	1.59 (± 0.12)	0.34 (± 0.03)
	150mg/kg CPT-11	5.00 (± 0.91)	1.14 (± 0.12)	0.17 (± 0.02)
	200mg/kg CPT-11	All animals died prior to this timepoint		

Table 3.1: Tabular representation of autopsy weights over time of animals (n = 4) in each treatment group. Data are expressed in grams (mean ± SD).

3.3.3 Intestinal Morphometry

3.3.3a Jejunum

Irinotecan caused maximal villus and crypt hypoplasia at 24 h (crypt hypoplasia 100 mg/kg vs control $p < 0.025$) and rebound crypt hyperplasia at 72 h (100 mg/kg vs control $p < 0.034$) (Fig 3.2).

3.3.3b Colon

Colonic crypt length was unchanged in all treatment groups at 6 and 24 h. Irinotecan did not cause crypt hypoplasia and caused minor crypt hyperplasia but only at the 100 mg/kg dose (data not shown). The colon was examined for crypt length and crypt cell count. At 72 and 96 h rats treated with 100 mg/kg irinotecan showed a significant increase in crypt length when compared to control animals $p < 0.0418$ and $p < 0.050$ respectively (data not shown).

3.3.4 Intestinal Apoptosis

Irinotecan caused a peak of jejunal apoptosis at 6 h (100 mg/kg and 200 mg/kg $p < 0.050$), which decreased at later time intervals, but remained elevated over levels seen in control animals (Fig 3.3). A similar peak of apoptosis occurred in the colon at 6 h (100 mg/kg $p < 0.042$; 200 mg/kg $p < 0.040$) which decreased more rapidly than that in the small intestine (Fig 3.4).

3.3.5 Goblet Cell Analysis

The number or appearance of goblet cells in the small intestine did not alter after irinotecan. In contrast, goblet cells in the large intestine had a more diverse staining pattern of mucins after irinotecan. At all doses given, there was a marked increase in mucus production, although there was no obvious increase in goblet cell numbers at 6 h. By 24 h goblet cell numbers had started to decrease, occurring simultaneously with severe architectural changes. However, mucus secretion continued to increase. By 96 h complete loss of normal architecture, decreases in goblet cell numbers and mucus hypersecretion were observed in all treated animals (Fig 3.5).

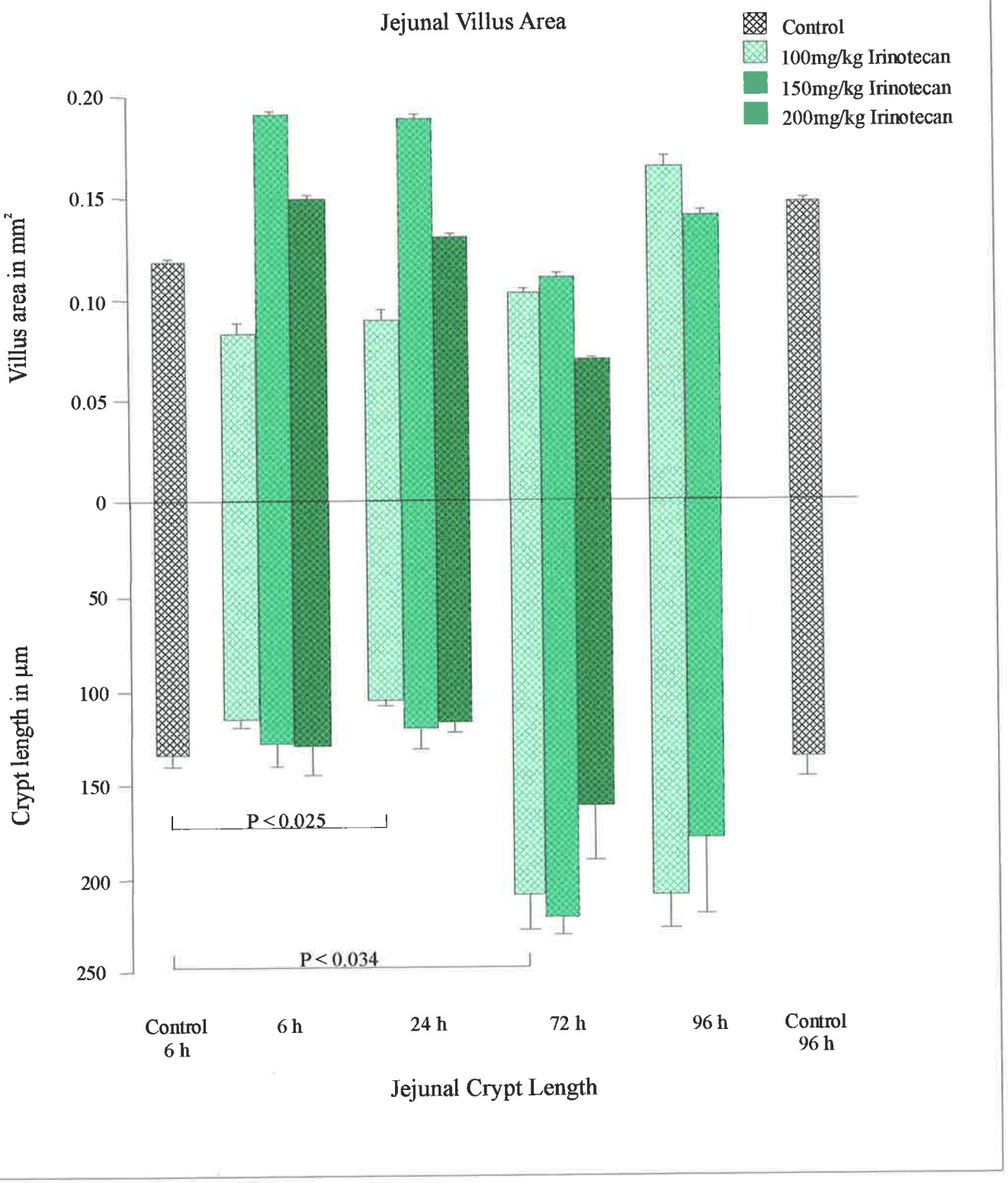


Figure 3.2 Effect of 100 mg, 150 mg and 200 mg/kg Irinotecan given i.p. over 2 days on intestinal morphometry in rats with breast cancer. Crypt hypoplasia was maximal at 24 h after treatment ($P < 0.025$) and rebound crypt hypoplasia was maximal at 72 h ($P < 0.034$).

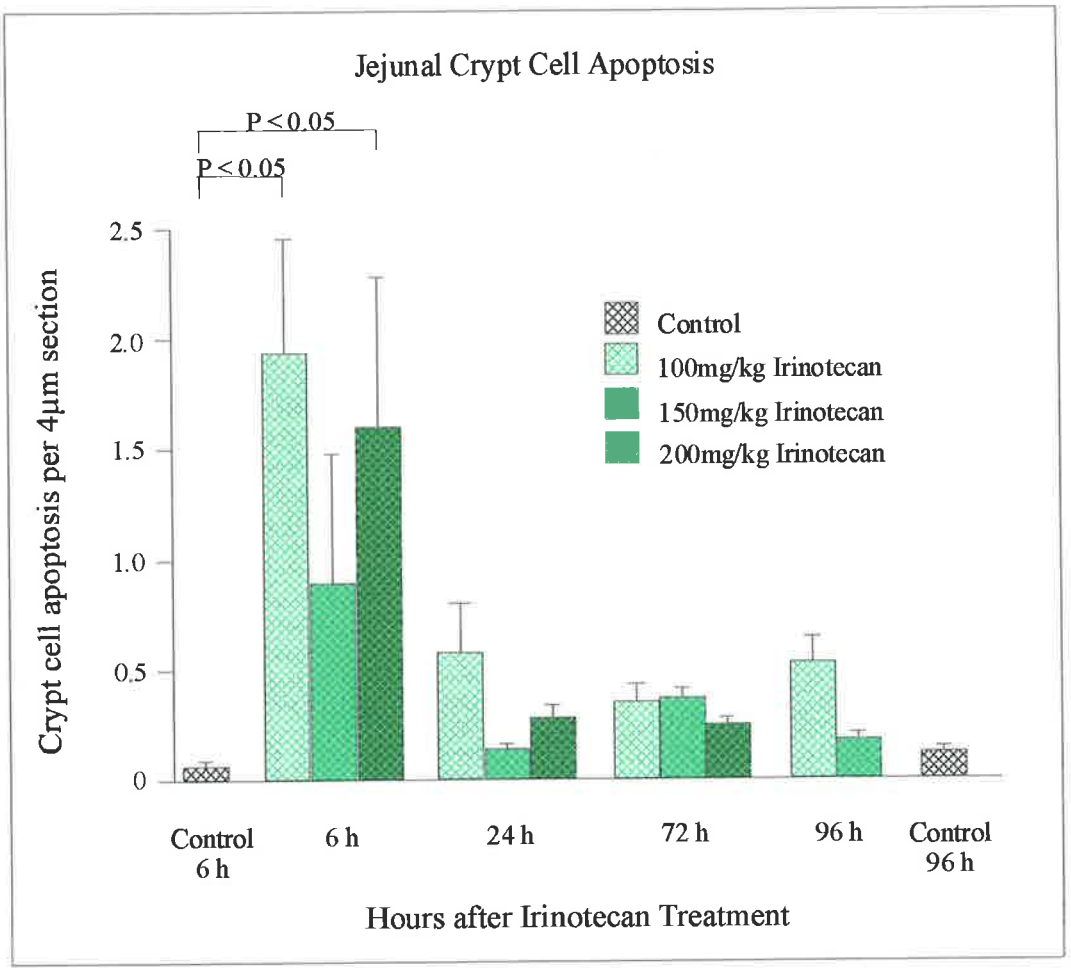


Figure 3.3 Effect of 100 mg, 150 mg and 200 mg/kg irinotecan given i.p. over 2 days on jejunal crypt cell apoptosis in rats with breast cancer. Apoptosis was maximal in all doses 6 h following treatment (100 mg and 200 mg/kg $P < 0.05$).

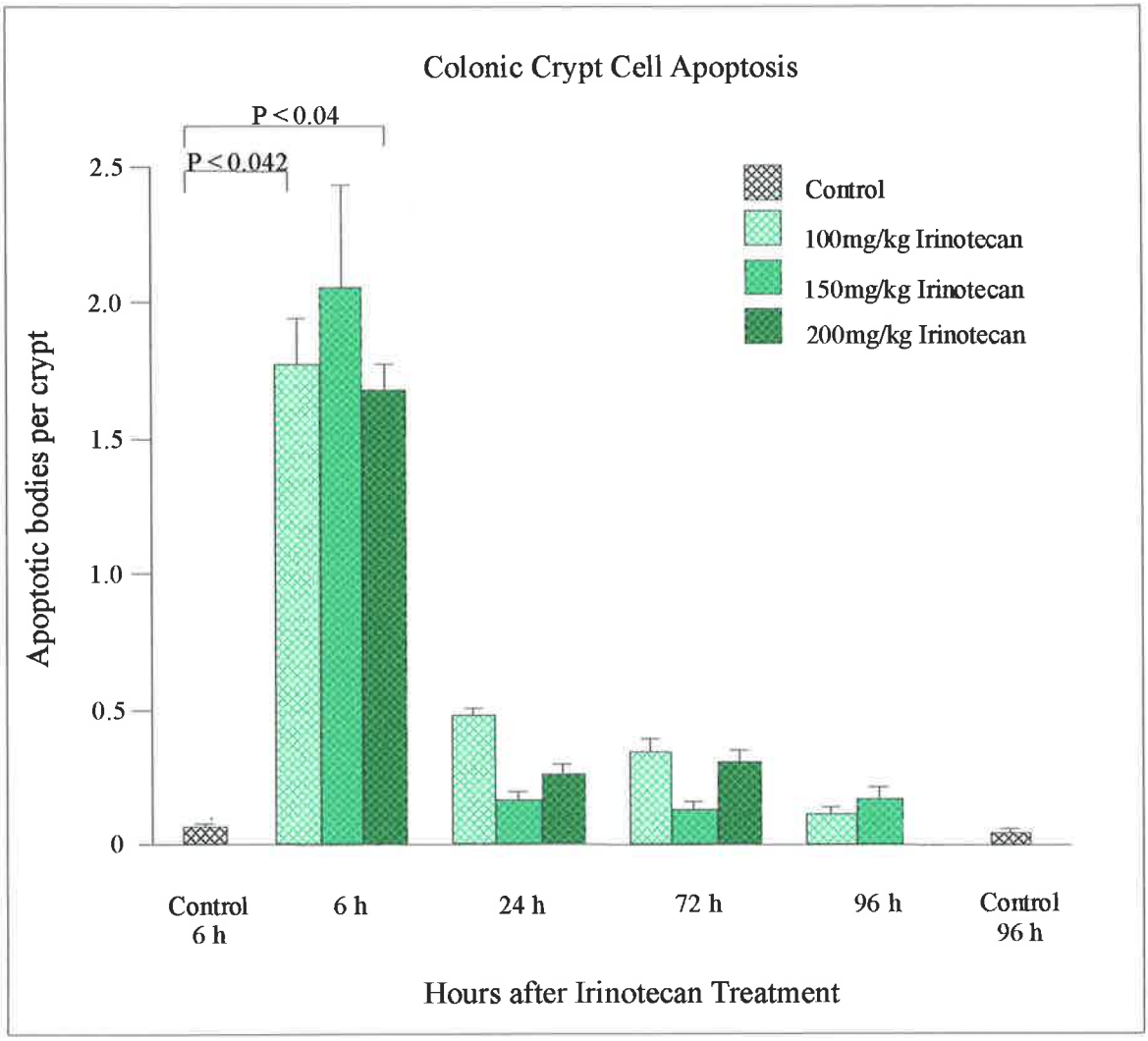
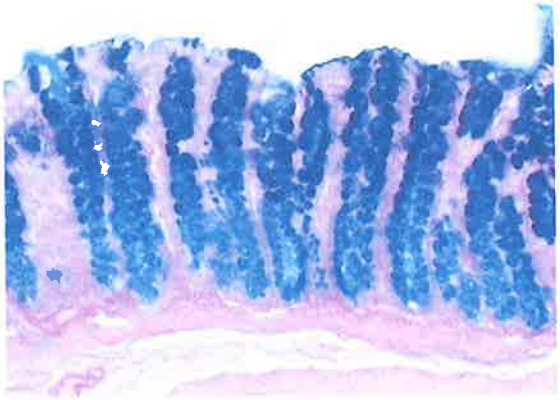
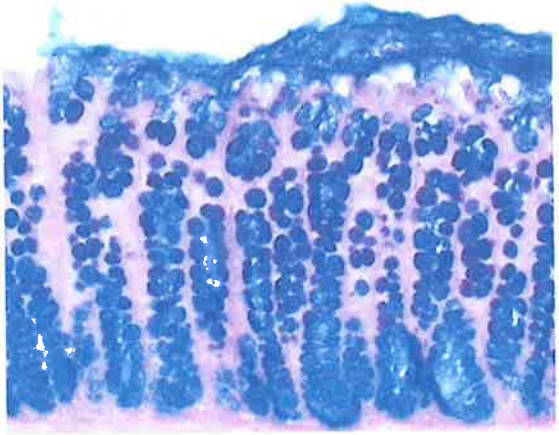


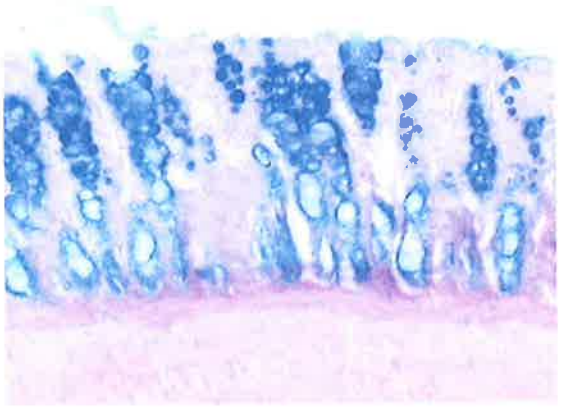
Figure 3.4 Effect of 100 mg, 150 mg and 200 mg/kg Irinotecan given i.p. over 2 days on colon crypt cell apoptosis in rats with breast cancer. Apoptosis was maximal in all doses 6 h following treatment (100 mg/kg $P < 0.042$; 200mg/kg $P < 0.04$).



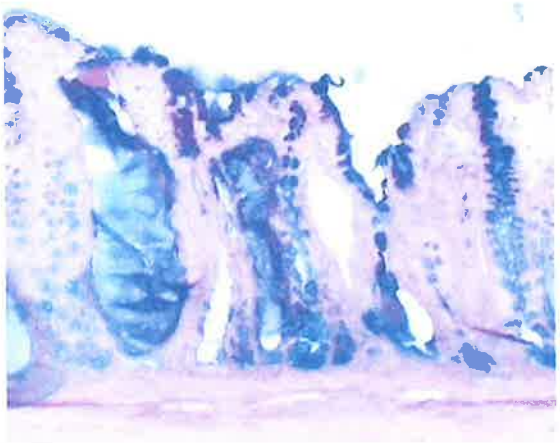
Control



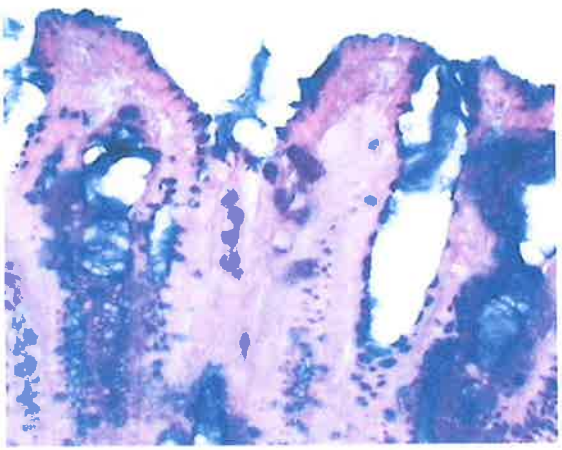
100 mg/kg 6 h



100 mg/kg 24 h



100 mg/kg 72 h



100 mg/kg 96 h

Figure 3.5 Photographs taken at 10 x magnification of colon at varying time points after treatment with 100 mg/kg irinotecan. There is increased mucin secretion at 6 h, followed by a gradual loss of normal architecture combined with increased mucin at all other time points.

3.4 Discussion

The DA rat breast carcinoma model is well-established for the study of gastrointestinal mucositis (52, 53), however previous studies have concentrated on methotrexate-induced damage and have focused on the small intestine (52, 53). In order to fully understand gastrointestinal mucositis it is important to study different chemotherapeutic agents, and to investigate the entire gastrointestinal tract as there may well be differences in drug action and hence in the development of mucositis.

Several possible mechanisms of irinotecan induced-delayed diarrhoea have been previously proposed, ranging from changes in architecture and large intestinal absorption rates (54, 56), to increases in intestinal β-Glucoronidase levels due to changes in intestinal bacteria (57). However, many of these studies were unable to assess the effect of irinotecan throughout the gastrointestinal tract. This study differs from these studies by quantitatively investigating apoptosis in both the small and large intestines, as well as investigating both histomorphological and histopathological changes. This study has shown that irinotecan caused apoptosis in crypts of the small and large intestines, but also uniquely caused colonic damage and excess mucus secretion in the large intestine. The degree of apoptosis observed was equivalent in both the small and large intestine, in contrast to the effect of methotrexate, which causes a 10-fold higher apoptosis in the small that in the large intestine (52).

Cytotoxic drugs are known to act by inducing apoptosis in cancer, but unfortunately the same apoptosis also occurs in intestinal crypts. This has been demonstrated in animals (52, 53) and in humans (2, 59). It is not surprising, therefore, that irinotecan caused apoptosis in crypts of the small and large intestines in the present study. This would explain the hypoplastic villous atrophy of the small intestine and colonic crypt damage, and this intestinal damage would contribute to diarrhoea. A previous study indicated that irinotecan caused enterocolitis of the ileum and caecum (56), but the present study extends the observation of damage to the jejunum and colon as well The observation of enterocolitis appears to be unique to irinotecan and was confirmed in our study with dilated crypts and areas of cellular necrotic debris and hyper-secreted mucus of the large intestine. Therefore the present study confirms that this is the likely mechanism behind irinotecan induced delayed diarrhoea. However, it has extended the above mentioned study by demonstrating the small intestine is also severely damaged by irinotecan and that this damage may exacerbate the delayed diarrhoea.

Changes in goblet cell numbers have been previously reported following irinotecan treatment in a number of studies (55, 56). However, these studies have reported conflicting results. Ikuno and colleagues reported that mice treated with irinotecan had increased numbers of mucin-secreting cells and a goblet cell hyperplasia in the caecum (55, 56). Conversely, Cao and colleagues reported that in their rat model irinotecan decreased goblet cell numbers in the colon (55, 56). The present study could not confirm increased numbers of goblet cells, but could confirm increased mucus secretion at all time points examined. Initially there was no discernible goblet cell hyperplasia. However, colonic hypersecretion of mucus was observed at all doses of irinotecan. This changed over time, and by 96 h, abnormal architecture, with few normal goblet cells was observed. However, there was increased colonic mucus secretion when compared to controls. Additionally, there were distinct changes in colonic mucin composition as detected by changes in staining patterns. This differs from that reported by Ikuno, and it is suggested that all of these changes taken together may play a role in the development of the delayed diarrhoea.

It is concluded that irinotecan is unusual for cytotoxic drugs in causing equivalent levels of colonic apoptosis to that usually predominantly seen in the small intestine and uniquely causes mucus secretion and colonic damage. The increased levels of cells undergoing apoptosis and tissue architectural changes within both the jejunal and colonic crypts, combined with changes in goblet cell numbers throughout the gastrointestinal tract, may lead to change in rates of absorption and possible diarrhoea.

The effect of interleukin-11 on ameliorating intestinal damage after methotrexate treatment of breast cancer in rats

4.1 Introduction

Mucositis is a clinical term used to describe the side-effects of cancer chemotherapy affecting mucosal surfaces such as that of the small intestine. Typical symptoms include bloating, abdominal pain and diarrhoea. Approximately 40–100% of cancer patients undergoing chemotherapy will develop mucositis (1, 3, 4, 55, 56, 139), and 50% of these will need reduction in the dose of chemotherapy or occasionally cessation of treatment. Mucositis can occasionally be fatal. Despite recent advances in the understanding of the underlying mechanism of small intestinal mucositis in experimental animals (1, 3, 4, 15, 55, 56, 139, 140) and humans (2), there is no effective treatment for established symptoms.

A study in patients with cancer following chemotherapy indicated that apoptosis precedes hypoproliferation of intestinal crypts (2). Previous animal studies indicate that radiotherapy and chemotherapy cause intestinal apoptosis and crypt hypoplasia, however they did not use animals with cancer (36, 37, 135). Any potential anti-mucotoxic agent should not be trophic to any tumour or prevent the induction of apoptosis in the tumour by chemotherapy treatment. This study utilised an animal model of small intestinal mucositis in the female DA rat with breast cancer to allow simultaneous investigation of the effect of chemotherapeutic and/or anti-mucotoxic agents on the small intestine and on the tumour.

Interleukin-11 (IL-11) is a pleiotrophic cytokine that stimulates proliferation of bone marrow stem cells and peripheral platelet counts (121, 141). IL-11 is secreted by bone marrow stromal cells and some mesenchymal cells (142). A previous study has shown that IL-11 increased survival of mice treated with combined radiation and chemotherapy, and aided the recovery of small intestinal villi and increased cell proliferation in crypts (143). Additionally, studies by Potten (123, 144) have shown that mice treated with IL-11 both prior to and during radiation therapy significantly increased intestinal crypt survival. However, these studies did not use tumour-bearing animals and were therefore unable to evaluate the effect of IL-11 on the tumour, nor the confounding effect of IL-11 and chemotherapy on the tumour.

The aims of this study were first to determine the time course of intestinal damage and repair in the DA rat and second, to determine the efficacy of IL-11 in preventing or ameliorating mucositis during chemotherapy treatment.

4.2 Materials and Methods

4.2.1 Passage of Tumour

The mammary adenocarcinoma used in this model arose spontaneously in the 1970s and was propagated by passage through female DA rats. This was kindly donated by Dr. A. Rofe (Institute of Medical and Veterinary Sciences, Adelaide, South Australia). Three female donor rats were injected s.c. on both flanks with 0.2 ml (2.0×10^7 cells per ml) tumour inoculum 11 days prior to the beginning of the study. These cells subsequently formed a tumour from which cells were harvested. The animals were culled by CO_2 asphyxiation and cervical dislocation, and subcutaneous tumours were dissected from the rats and placed into sterile Phosphate Buffered Saline (PBS - 16% NaCl; 0.4% KCl; 0.4% KH_2PO_4; 2.3% Na_2HPO_4 anhydr) for further passage. This study was approved by the Animal Ethics Committees of the North West Adelaide Health Services at The Queen Elizabeth Hospital, Adelaide, and of The Institute of Medical and Veterinary Sciences, Adelaide. The study complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Teaching (1997).

4.2.2 Preparation of Tumour Inoculum

Diced pieces of tumour were homogenised and filtered through sterile gauze. The tumour cell suspension was spun at 500 g for 3 mins each time, which was repeated four times. A viable cell count was carried out using 0.5% w/v trypan blue.

4.2.3 Experimental Animals

All experiments described below were conducted in female dark agouti (DA) rats, weighing approximately 150 g. All animals received breast cancer inoculum as described above. MTX was used as a known mucositis-inducing chemotherapy agent.

4.2.4 Biological Efficacy of IL-11

Sixteen female DA rats were randomly assigned into either control or experimental groups. Rats were treated with twice daily s.c. injections of IL-11 (100 μg/kg) or saline (0.9% w/v) for 5 consecutive days. Animals were exsanguinated, and a platelet count performed. The small and large intestines, liver, spleen and tumour were removed and weighed. Small samples of jejunum were fixed in Clarke's fixative for intestinal morphometry and in 10%

formalin for TUNEL assay. Small cross-sections of tumour were fixed in 10% formalin for TUNEL staining and histological examination.

4.2.5 Effect of IL-11 on Preventing Intestinal Mucositis

Sixty-eight female DA rats were randomly assigned to one of eight groups. Rats received either: IL-11; MTX; IL-11 and MTX, or saline. IL-11 (100 μg/kg) (as confirmed by the pilot study described in 4.2.4) or saline was administered s.c. twice a day for 5 days from days –3 to +1. Animals received two daily i.m. doses of 1.5 mg/kg MTX or saline (control) on days 0 and 1. Rats were killed on either day 1 (6 hours) or 3 following MTX treatment. The entire gastrointestinal tract from the pyloric sphincter to the rectum was removed, and the weight of the small intestine recorded. The tumours were removed and weighed. Samples of small intestine and tumour tissue were fixed in Clarke's fixative and 10% formalin for further analysis.

4.2.6 Dose-Response of IL-11

Forty-five female DA rats were randomly assigned to five groups: Control; MTX; 100 μg/kg IL-11/MTX; 200 μg/kg IL-11/MTX or 400 μg/kg IL-11/MTX. Rats received the appropriate dose of IL-11 or saline (control) as a s.c. injection twice a day for 3 days prior to treatment with MTX and for 2 days concurrently with MTX injections (ie day –3 to day +1). Animals were given 1.5 mg/kg MTX or saline (control) i.m. on days 0 and +1, and killed on day 3. The gastrointestinal tract was removed, and the weights of the small and large intestines recorded. The subcutaneous tumours were removed and weighed. Samples of small intestine and tumour tissue were fixed in Clarke's fixative and 10% formalin.

4.2.7 Intestinal Morphometry

Sections of jejunum were opened onto cardboard, fixed in Clarke's fixative for 24 h and stored in 70% ethanol. The stored tissue was rehydrated and hydrolysed in 1M HCl for 7 mins at 60°C. After two washes in double distilled water, the tissue was stained with Schiff's reagent (Sigma, St Louis, USA) for 30 mins before being microdissected using a cataract knife and stereomicroscope. The tissue was mounted in 45% (v/v) acetic acid, and, using a calibrated graticule, measurements were taken of the villus length and apical and basal widths of 15 villi and the lengths of 15 crypts (130). Villus area was calculated

as a trapezoid approximation. Villus area and crypt length correlate with villus and crypt epithelial cell populations, respectively.

4.2.8 Apoptosis Assessment of Small Intestine and Tumour

Sections of tumour and small intestine were fixed for 24 h in 10% formalin and stored in 70% ethanol. Tissue was dehydrated by passing through a graded series of ethanol and embedded in paraffin wax. 4 μm sections were cut using a microtome. The methodology for labelling the apoptotic cells was similar to that of Gavrieli (145).

4.2.9 Proliferative Assessment of Tumour

Cross-sections of tumour were fixed in 10% formalin and embedded in paraffin. Histological sections (4 μm) were cut and the numbers of mitotic figures per mm2 were counted after staining with haematoxylin and eosin. Counts were taken from the periphery of the tumour to avoid the central necrotic area of the tumour. Counts were expressed per mm^2 of tumour.

4.2.10 Statistical Analysis

All statistical analysis were conducted using the Peritz' F Test, which is a robust measure of differences of group means between multiple groups (138).

4.3 Results

4.3.1 Biological Activity of IL-11

IL-11 increased the mean (SE) platelet count 9823.8 (24.3) vs 723.4 (24.6), x 10^9/L $p < 0.050$, indicating that this dose was biologically active in this model. There were no other significant differences between IL-11 and control. IL-11 had no effect on tumour weight, or on proliferation, as measured by tumour mitotic count. IL-11 did not alter villus area, crypt length or crypt mitotic count, nor did it increase jejunal apoptosis (data not shown). In addition, IL-11 did not affect bone, spleen, small and large intestine or tumour weights (data not shown).

4.3.2 Effect of IL-11 on Ameliorating Small Intestinal Mucositis

The effect of 100 μg/kg IL-11 twice daily s.c. on weights of the small intestine and breast cancer at 6 h and 48 h after MTX are given in Table 4.1. There was no difference in the weight of the small intestine or tumour at day 1 following MTX treatment. However by day 3, IL-11 had a beneficial effect on minimising small intestinal weight loss after MTX (IL-11/MTX vs saline/MTX $p<0.025$). Tumour weights were reduced after MTX treatment.

The effect of MTX and IL-11 on intestinal morphometry is given in Fig. 4.1. MTX did not reduce villus area or crypt length at day 1 after treatment, but reduced both measures at 3 days after treatment. IL-11 treatment reduced this damage so that villus area ($p < 0.025$) was greater and intestinal crypts were longer ($p < 0.025$) than in animals receiving MTX alone. The effect of IL-11 and MTX on apoptosis in small intestinal crypts is given in Fig 4.2. MTX induced apoptosis in small intestinal crypts at day 1 after treatment that improved by day 3 (Fig 4.2). IL-11 treatment increased apoptosis in the crypts of the small intestine at day 1 after MTX. The effect of MTX and IL-11 on tumour apoptosis and proliferation is given in Fig 4.3. MTX reduced mitotic count in tumours at day 1 which started to return to pre-treatment values by day 3. There were no significant differences seen in tumour apoptotic levels between any groups.

4.3.3 Dose Response of IL-11

IL-11 at 200 μg/kg ($p < 0.0203$) and 400 μg/kg ($p < 0.0203$) improved small intestinal weight at day 3 after treatment. Colonic weight was not altered. IL-11 significantly

Time after MTX	Group	Small Intestine (g)	Tumour (g)
6 h	IL-11/MTX	5.20 (0.35)	12.05 (2.43)
	Saline/MTX	5.58 (1.01)	11.35 (5.76)
	IL-11/Saline	6.13 (0.72)	15.33 (5.89)
	Saline/Saline	5.88 (0.76)	18.94 (4.61)
48 h	IL-11/MTX	4.44 (0.31)[a]	7.67 (2.83)
	Saline/MTX	3.95 (0.30)[a]	6.37 (2.99)
	IL-11/Saline	6.24 (0.77)[b]	30.24 (4.64)
	Saline/Saline	5.62 (0.37)[b]	28.84 (7.90)

Table 4.1 Weight of small intestine and tumour in rats that were pre-treated with IL-11 or left untreated (control) for 3 days prior and 2 days during treatment with MTX. Rats were killed at 6 and 48 h after the second MTX injection. Data are expressed as mean weight (SE) g in groups of 8 rats.

a. $P < 0.025$

b. $P = NS$

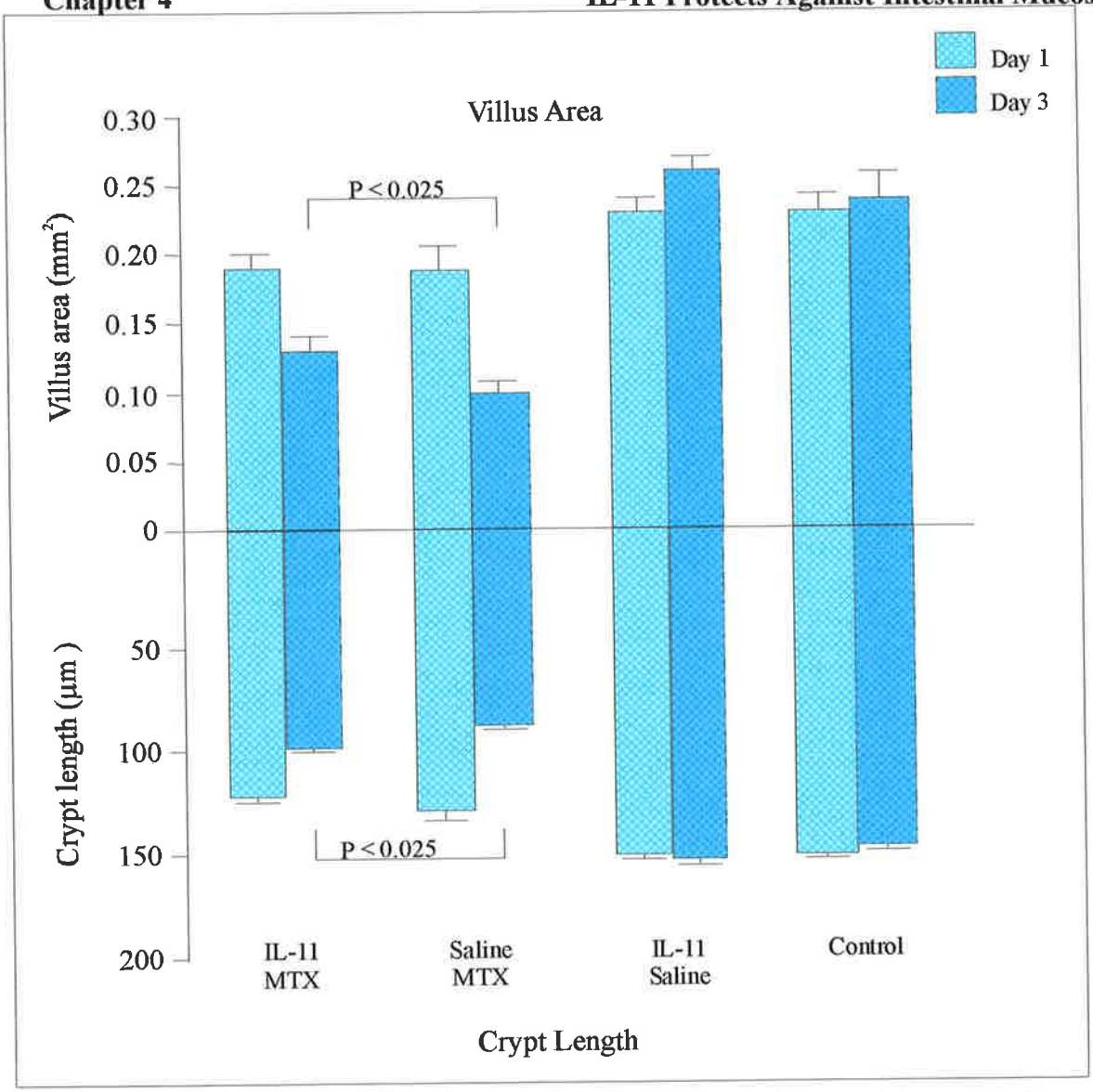


Figure 4.1 Effect of 100 µg/kg twice a day s.c. IL-11 for 5 days at 1 and 3 days after MTX on intestinal morphometry in rats with breast cancer. $P < 0.025$

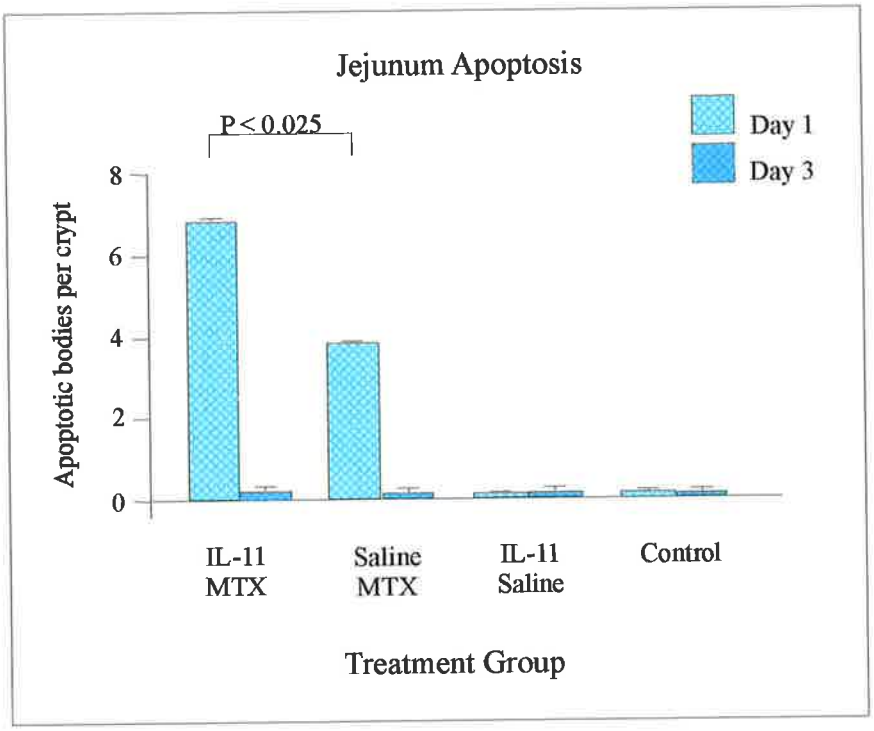


Figure 4.2 Effect of 100 μg/kg/twice a day s.c for 5 days on jejunal apoptosis at 1 and 3 days after MTX in rats with breast cancer. $P < 0.025$

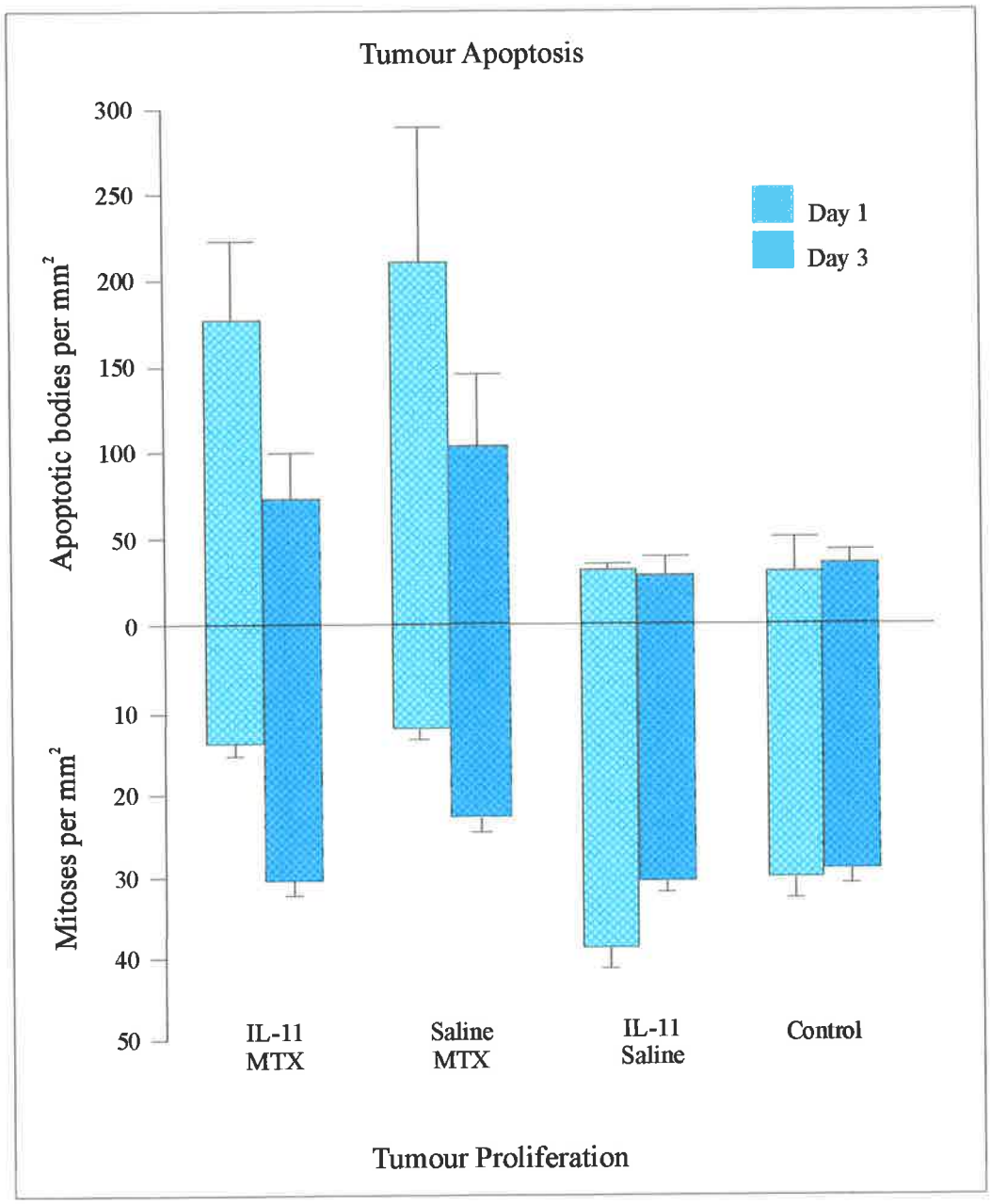


Figure 4.3 Effect of 100 μg/kg/twice a day s.c. for 5 days on tumour apoptosis and proliferation at 1 and 3 days after MTX in rats with breast cancer. There is no significant differences between any of the groups at any time point.

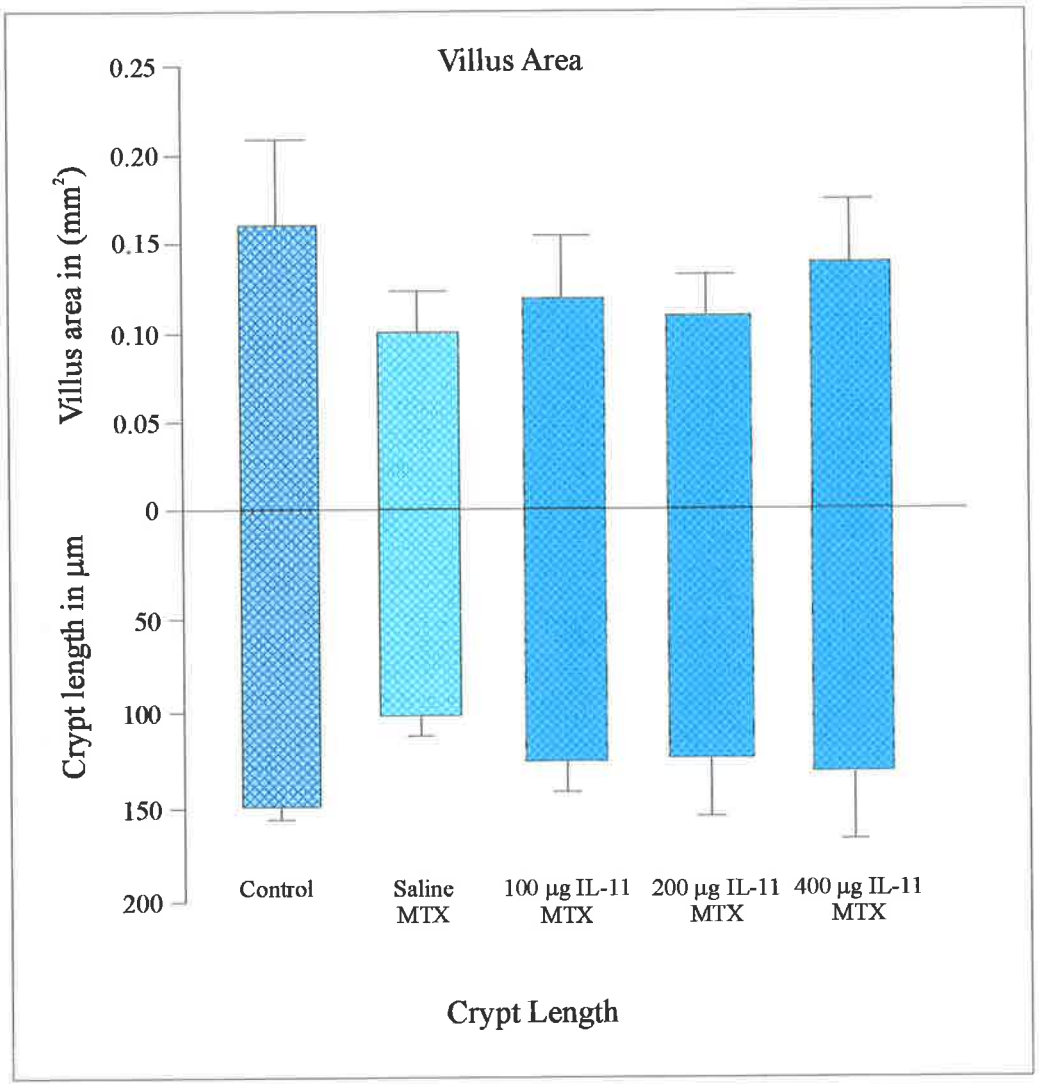


Figure 4.4 Effect of 100 µg, 200 µg and 400 µg/kg/twice a day s.c. IL-11 for 5 days on intestinal morphometry at day 3 after MTX treatment in rats with breast cancer.

reduced the severity of change in villus area (400 μg/kg $p < 0.0402$), crypt length (400 μg/kg $p < 0.0402$; 200 μg/kg $p < 0.0203$; 100 μg/kg $p < 0.0203$) (Fig 4.4) and crypt cell proliferation (400 μg/kg $p < 0.0303$; 200 μg/kg $p < 0.0402$; 100 μg/kg $p < 0.0203$) (Fig 4.5). MTX increased apoptosis in crypts of all groups compared to the control group, regardless of IL-11 treatment or dose of IL-11 (Fig 4.5).

IL-11 did not have a dose response in tumour proliferation. There were no significant differences in apoptosis between the groups, nor was there an increase in tumour proliferation (data not shown).

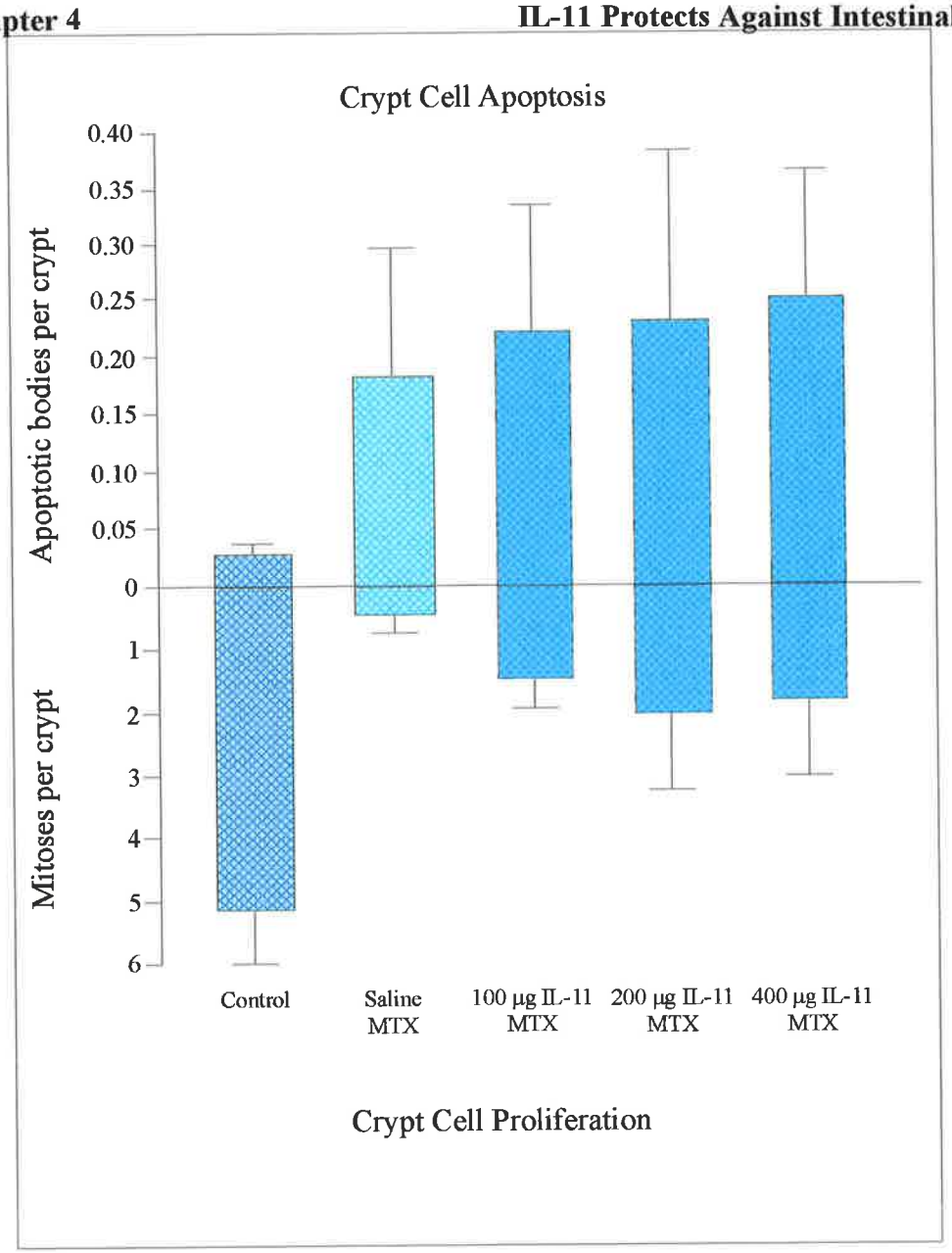


Figure 4.5 Effect of 100 µg, 200 µg and 400 µg/kg/twice a day s.c. IL-11 for 5 days on crypt cell proliferation and apoptosis at day 3 after MTX treatment in rats with breast cancer.

4.4 Discussion

In this study the laboratory rat with syngeneic breast cancer to study the time-course of mucositis and the effect of IL-11 on preventing intestinal damage was used. The advantages of this model in comparison to previous studies were that it controlled for any systemic effect of cancer on intestinal response, and allowed evaluation of any effect of IL-11 in preventing the anti-tumour effect of MTX.

The efficacy of IL-11 in ameliorating intestinal mucositis after MTX was investigated. Firstly the effect of IL-11 on untreated animals was evaluated followed by the effect of IL-11 on improving mucositis after MTX. Biological efficacy of IL-11 was confirmed by an increased peripheral platelet count using a dose of 100 μg/kg administered twice daily. This initial study also showed that administration of IL-11 caused no change in small intestine weight indicating IL-11 did not have any direct trophic effect on the small intestine. This is not surprising given that IL-11 is a cytokine and not a growth factor. Additionally, tumour growth was not adversely induced.

The effect of IL-11 on preventing or ameliorating small intestinal damage (mucositis) was investigated at two different time-points. Mucositis was assessed by apoptosis and by intestinal morphometry. IL-11 did not reduce apoptosis after MTX (Fig 4.2) as previously reported after radiotherapy or combined radiotherapy/chemotherapy (122, 143). Indeed, IL-11 in this study increased apoptosis after MTX in crypts of the small intestine (Fig 4.2). This did not result in increased intestinal damage, but villus atrophy and crypt length and proliferation were relatively less severe, i.e. IL-11 ameliorated intestinal damage (Fig 4.1). Thus, apoptosis itself does not inevitably lead onto mucositis. Pritchard and colleagues (39) found that apoptosis was not directly associated with intestinal damage after chemotherapy. Their study found that chemotherapy caused both apoptosis and hypoproliferation and both factors contributed to intestinal damage. They showed chemotherapy activated p53/p21 that induced cytostasis of crypt cells and this effect was abrogated in p53 knockout mice. Possibly IL-11 could down-regulate p53 and prevent cytostasis. This could explain the finding of the present study that IL-11 allowed relative crypt proliferation to proceed even though MTX caused hypo-proliferation. This would have ameliorated villus atrophy as was observed.

IL-11 did not protect the breast cancer from MTX (Fig 4.3). IL-11 did not alter the MTX-induced reduction in tumour weight, proliferation or apoptosis. Moreover, IL-11 had no

trophic effect on growth of breast cancer. Thus, IL-11 does not compromise effect of MTX on the tumour.

Current literature suggests that IL-11 belongs to a family of cytokines, which act through a common glycoprotein, gp130 (146, 147). The expression of the IL-11 receptor within the gastrointestinal tract has not been fully elucidated; however this may well have implications for the mechanisms of action of IL-11. Within corticotrophic and non-functioning tumours the IL-11 receptor has been shown to be present, however functional studies using normal and tumoural cells have yet to be carried out (148).

In conclusion, IL-11 ameliorates mucositis as measured by intestinal weight and intestinal morphometry, even though it did not prevent apoptosis. Although MTX induced hypoproliferation of crypt cells, IL-11 improved this reduction and presumably this helped to maintain villus area.

Investigation of pro- and anti-apoptotic gene expression in the normal human gastrointestinal tract.

5.1 Introduction

Gastrointestinal apoptosis has long been known to be a side effect of administration of chemotherapeutic agents for cancer. Initial studies of the effect of methotrexate (MTX), a commonly used chemotherapeutic agent, on the human small intestine identified pyknotic bodies in cells lining the crypts, now known to be apoptotic cells (5, 6). These cells were observed within 6 hours of MTX administration and were still present at 48 hours. Further studies conducted by Keefe and colleagues (2) confirmed the presence of these apoptotic cells in the human small intestine following treatment with chemotherapy. Their study showed small intestinal apoptosis increased seven-fold at 1 day after chemotherapy and was followed by a reduction in intestinal morphometry (villus area, crypt length mitotic count and enterocyte height) 3 days later, and coincided with patient symptoms of mucositis. The authors concluded that small intestinal apoptosis leads to crypt hypoproliferation and that the two are directly related to mucositis. This group went on to show that these subjects had regained normal structure and function by day 16 following chemotherapy (1).

The apoptosis that occurs in the crypts of the small intestine shortly after the administration of chemotherapy has been hypothesised to relate to the damage that is subsequently seen (2). There are two distinct apoptotic pathways, an extrinsic pathway from activation of death receptors (eg Fas/CD95 ligand, TNF receptor, TRAIL) on the cellular membrane, and an intrinsic pathway that induces mitochondrial release of cytochrome C and subsequent activation of caspases in the cytoplasm. Both pathways lead to fragmentation of DNA and apoptosis. Bcl-2 and other family members are either anti-apoptotic (Bcl-2, Bcl-xL, Bcl-w, Mcl-1) or pro-apoptotic (Bax, Bak, Bad, BIM) (149, 150). Although there is still some uncertainty, the ratio of anti-apoptotic to pro-apoptotic proteins (eg Bcl-2:Bax) is believed to regulate apoptosis (151-153). The relative expression of both the pro- and anti-apoptotic proteins remains unknown especially in the gastrointestinal tract of humans.

Previous research conducted in our laboratory has shown that the anti-apoptotic proteins Bcl-2 and Bcl-xL are relatively deficient in small intestinal crypts of the rat (66% and 66% respectively), when compared to large intestinal crypts (154, 155). This finding could provide an explanation as to why the small intestine is more vulnerable to chemotherapy.

Furthermore it has been shown in the laboratory rat, that the small intestine displayed a 3-fold increase in Bax expression after methotrexate treatment. Bcl-2 protein also decreased whereas Bcl-xL had a more variable response after treatment. However, expression of both of these anti-apoptotic proteins was unable to compensate for the heightened Bax expression. These preliminary data are compatible with activation of the intrinsic apoptotic pathway (154, 155).

p53 and p21 are two common transcription factors which are also known to play a role in the development of apoptosis and therefore may be important in mucositis. The p53 gene is commonly mutated in cancer (15). Normally expressed in low levels, p53 becomes upregulated when DNA damage occurs, moving to the nucleus where it acts to regulate several other genes, including $p21^{waf1/cip1}$ (15, 40, 41). Upregulation of p53 can have a number of consequences for the cell following DNA damage. It can either initiate G1 arrest, allowing the cell time to repair itself or undergo apoptosis, or it can directly initiate apoptosis (15, 42). It has also been suggested that low levels of p53 can induce differentiation (41). When p53 is upregulated it activates the transcription of $p21^{waf1/cip1}$, a cyclin dependent kinase (cdk) inhibitor (42). $p21^{waf1/cip1}$ binds with other cellular components to form a quaternary complex which is necessary for normal functioning of DNA polymerase (42), thereby inducing cell arrest in the G1 phase. $p21^{waf1/cip1}$ is necessary to obtain cell cycle arrest that is p53-dependent in response to DNA damage (43).

Current evidence suggests that the Bcl-2 family of proteins, and the two transcription factors, p53 and p21, play a critical role in the development of apoptosis and hence in the development of mucositis. Despite this, the expression of both remains unknown in the 'normal' human gastrointestinal tract. Therefore, the aim of this study was to characterise the expression of pro- and anti-apoptotic genes, as well as p53 and p21 within the oesophagus, stomach, duodenum, ileum, caecum, colon and rectum of humans, in order to provide a baseline for future comparison with subjects who have had cytotoxic chemotherapy for cancer.

5.2 Subjects and Methods

5.2.1 Subjects

Subjects were recruited from those undergoing routine endoscopies and colonoscopies within the Department of Gastroenterology and Hepatology at The Queen Elizabeth Hospital. There were 50 subjects recruited to this study; 24 males and 26 females with a median age of 54.7 years (range 16-84). Most subjects had multiple biopsies taken, depending on the type of procedure they were undergoing. Subjects were excluded from the study if they were undergoing endoscopies and/or colonoscopies for cancer or if they had a known bleeding disorder. This study was approved by the Ethics of Human Research Committee at The Queen Elizabeth Hospital and was carried out in accordance with the Declaration of Helsinki. Informed consent was obtained from each patient prior to enrolment in the study.

5.2.2 Endoscopy and Colonoscopy Biopsies

Subjects were prepared in the normal manner for endoscopy and colonoscopy. For endoscopy, subjects fasted overnight (i.e. minimum 6 hours), before the upper gastrointestinal tract was inspected and biopsies taken from the oesophagus, antrum and upper duodenum. Biopsies were immediately fixed in 10% neutral buffered formalin. For colonoscopy, subjects were also fasted overnight; however subjects were required to drink Fleet (sodium phosphate) the night prior to, and the morning of, their procedure. The lower gastrointestinal tract was inspected and biopsies taken from the ileum, caecum, midcolon and rectum. Biopsies were immediately fixed in 10% neutral buffered formalin.

5.2.3 Number of Samples

There were 16 oesophageal biopsies, 15 gastric antral biopsies, 16 duodenal biopsies, 14 ileal biopsies, 18 caecal biopsies, 13 mid colonic biopsies, and 20 rectal biopsies taken from 50 subjects.

5.2.4 Light Microscopy

All biopsies were fixed in 10% neutral buffered formalin before being dehydrated through graded ethanols and infiltrated with, and embedded in paraffin wax. Using a rotary microtome, 4 μm sections were cut and stained with haematoxylin and eosin to ascertain correct orientation.

5.2.5 Immunohistochemistry for Detection of Transcription Factors p53 and p21 and Pro- and Anti-Apoptotic Family Members

Prior to beginning immunohistochemistry, all antibodies were optimised for human gastrointestinal tissue. The method used for optimisation and all subsequent immunohistochemistry is detailed below. For antibodies that were not raised in mouse (BIM and Bcl-w) a slightly modified protocol was used (20% horse serum and an anti-goat secondary antibody). All remaining steps were the same.

Two consecutive, 4 μm paraffin sections of the various regions of the gastrointestinal tract were cut using a rotary microtome, dewaxed in xylene and rehydrated through a graded series of alcohols. One section was designated as the negative control, the other as the experimental section. Slides were washed for 2 x 5 mins in PBS prior to being subjected to heat mediated antigen retrieval in citrate buffer (pH 6.5). Slides were quenched in 3% hydrogen peroxide in methanol for 1 min to deactivate endogenous peroxidase activity, before non-specific antigens were blocked by normal serum for 20 mins. Finally, endogenous avidin and biotin was eliminated using a commercial avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA USA). Slides were incubated overnight at 4°C with the primary antibody (Table 5.1 for antibodies and appropriate concentrations) on the experimental section, and PBS only on the negative section. The following morning, slides were incubated with a universal linking antibody, before the reaction was amplified with Ultra-Streptavidin conjugated to horseradish peroxidase and visualised with diaminobenzidine chromogen. Slides were counterstained with Lillie-Mayer's Haematoxylin, dehydrated through a graded series of alcohols and mounted with PIX mounting medium. Both positive and negative control sections were included in each run (Table 5.1 for positive tissue which was used for each antibody).

5.2.6 Quantitative Immunohistochemistry

In order to quantify staining, an algorithm which calculated the cumulative signal strength or energy of a digital file of the image was utilised (132, 133). It was assumed that all staining in both the experimental and negative control images was important (132, 133). Digital photographs were taken of identical intestinal crypt sections of experimental and control sections (Fig 5.1) using a Nikon 800 research microscope (x63 dry lens) and SPOT-RT camera (Diagnostic Instruments Inc, Michigan, Detroit). A 33x33 pixel area over the nucleus (p53 and p21) or cytoplasm (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Bax, Bak,

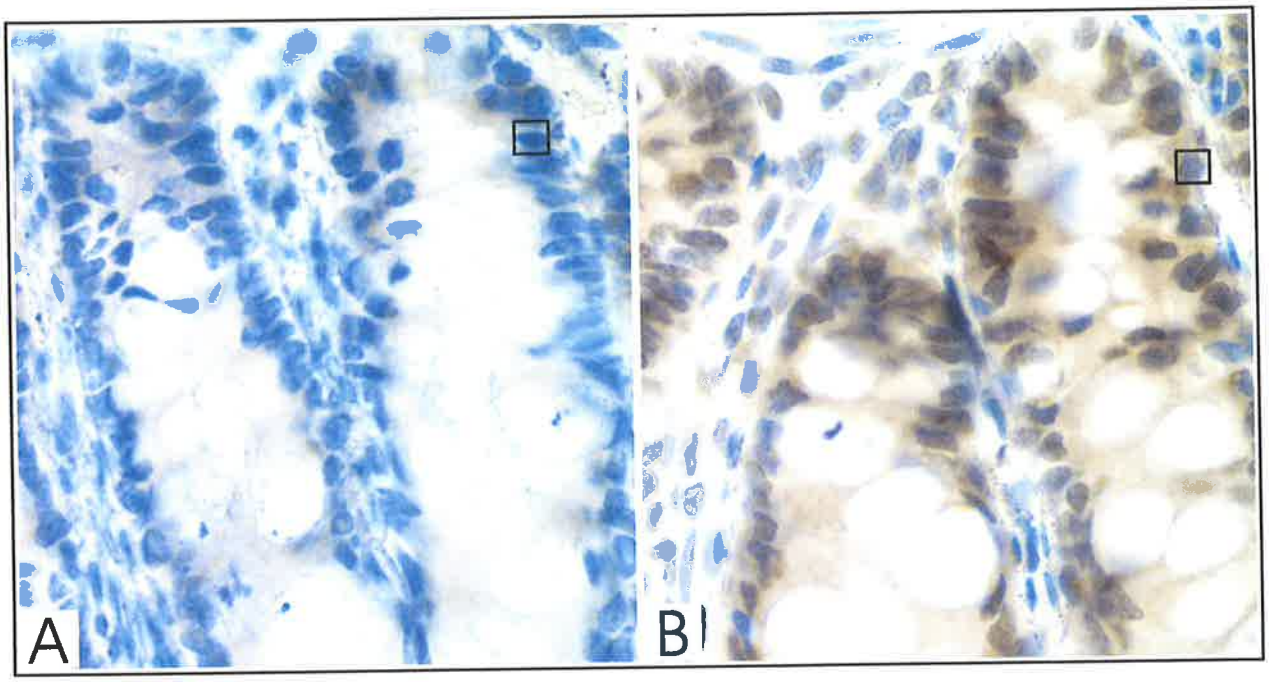


Figure 5.1 Photomicrographs of a serial A) unstained control and B) stained experimental (mouse monoclonal p53 antibody) section of human duodenum taken at 600 x magnification overlayed with a 33 x 33 area of interest box.

Antibody	Clone, Source and Type	Dilution	Positive Control
p53	NCL-p53-240 Novacastra, Mouse Monoclonal	1:50	Breast Tumour
p21	C19 sc-397 Santa Cruz, Rabbit Polyclonal	1:1200	Liver
Bak	sc-832 Santa Cruz, Rabbit Polyclonal	1:1200	Stomach
Bcl-xl	H-62 sc-7195 Santa Cruz, Rabbit Polyclonal	1:850	Thymus
Bcl-2	N-19 sc-492 Santa Cruz, Rabbit Polyclonal	1:1500	Spleen
Bax	P19 sc-526 Santa Cruz, Rabbit Polyclonal	1:1300	Thymus
BID	FL195 sc-11243 Santa Cruz, Rabbit Polyclonal	1:4200	Spleen
Mcl-1	S19 sc-819 Santa Cruz, Rabbit Polyclonal	1:7000	Spleen
Bcl-w	N19 sc-6172 Santa Cruz, Goat Polyclonal	1:500	Spleen/Testis
BIM	N20 sc-8265 Santa Cruz, Goat Polyclonal	1:1500	Spleen/Testis

Table 5.1: The source, clone, concentration and appropriate positive control tissue used for immunohistochemical analysis throughout the normal human gastrointestinal tract.

BIM, BID) of individual cells were selected in both experimental and control sections (excluding the goblet cells), using Image Pro Plus (Media Cybernetics, Silver Spring MD) (Fig 5.1). These were saved as TIFF files, which allowed for LZW compression without the loss of any data (132, 133), before being converted to image matrices, and normalised using functions in Matlab (Mathworks Inc. Natick, MA USA) (132, 133). The intensity of staining was expressed as the pixel energy difference in cumulative signal between the control and experimental image matrices (Figs 5.1, 5.2. 5.3).

To simplify results, data were normalised for each protein. To do this, maximal expression along the gastrointestinal tract was determined for each protein and given an arbitrary value of 1. All other data for that protein was then expressed relative to that value. In this way, trends could be more easily observed.

5.2.7 Statistical Analysis

All statistical analysis were conducted using the Peritz' F Test, which is a robust measure of differences of group means between multiple groups (138).

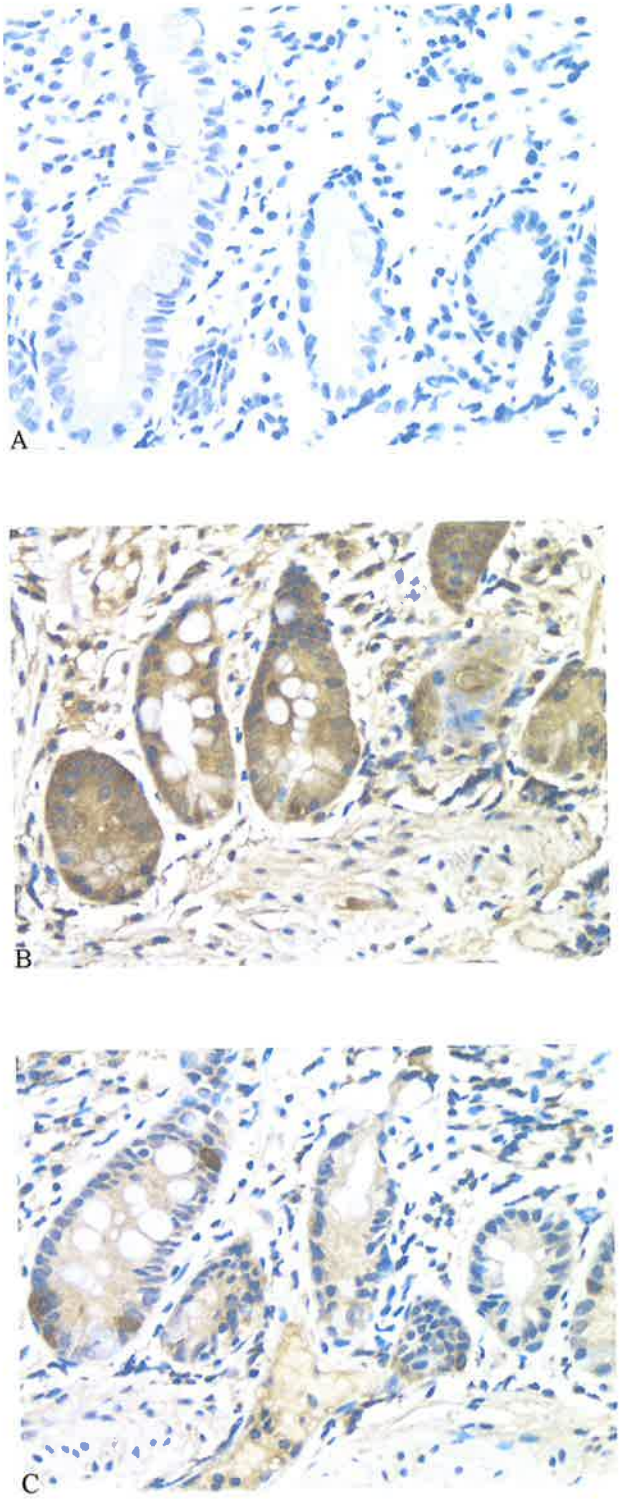


Figure 5.2 Photomicrographs at 400 x magnification of A) unstained control B) Pro-apoptotic Bim, and C) Anti-apoptotic Bcl-w within the normal human duodenum.

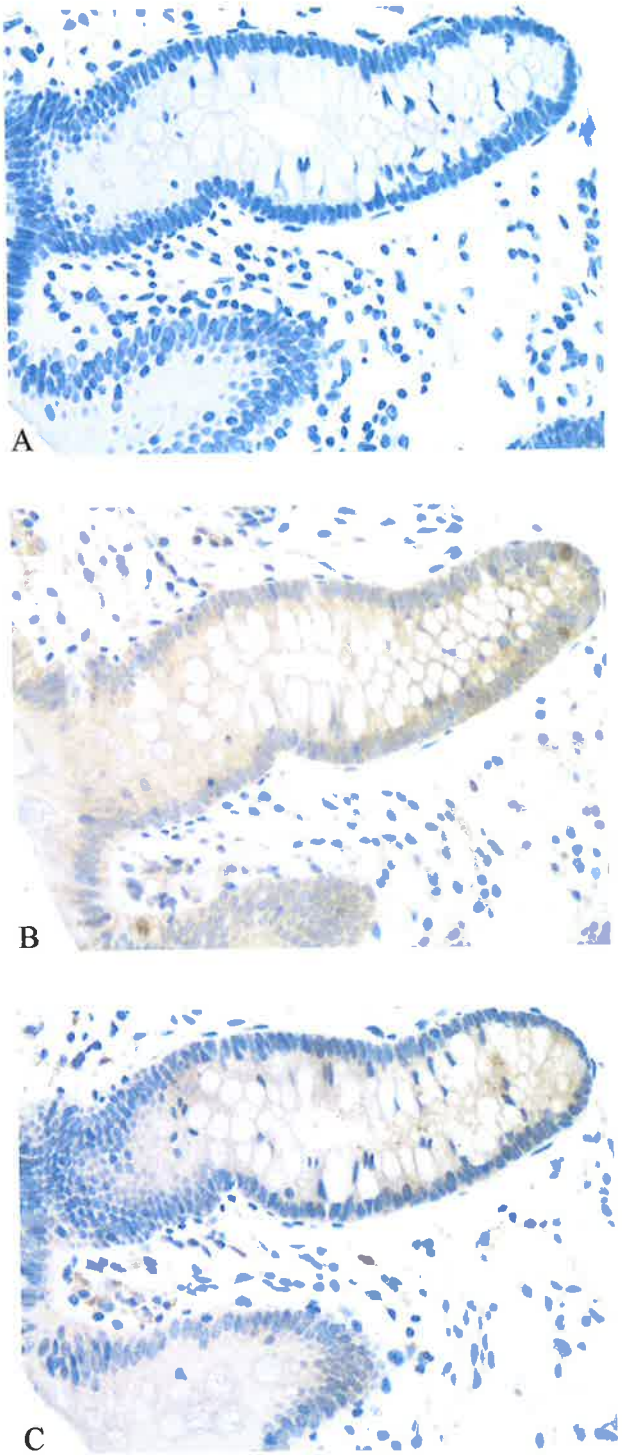


Figure 5.3 Photomicrograph at 400 x magnification of A) unstained control, B) Pro-apoptotic Bim, and C) Anti-apoptotic Bcl-w within the normal human caecum.

5.3 Results

5.3.1 Pro-Apoptotic Expression Throughout the Gastrointestinal Tract

Four pro-apoptotic proteins, Bax, Bak, BIM and BID, were investigated for levels of expression throughout seven regions of the normal human gastrointestinal tract. All regions of the tract showed expression of pro-apoptotic proteins, with positive staining occurring in the cytoplasm of the cells under investigation. Qualitatively, there appeared to be little difference in staining intensity in all four pro-apoptotic proteins between the crypts and villi, with staining appearing consistent in both parts (Figs 5.2, 5.3). Additionally, there was a consistent level of staining across all the tissue sections, which allowed for any region of the tissue to be examined and recorded as an accurate representation of expression.

Bax

Bax was expressed through all regions of the gastrointestinal tract. The expression was 1.6 fold higher in the duodenum (110.1) than in the caecum (70.2) ($p < 0.05$). All remaining regions investigated showed relatively consistent expression of Bax with no significant differences recorded (Fig 5.4).

Bak

The expression of Bak was lowest in the oesophagus (81.5) and highest in the antrum (158.5). There was a 1.9 fold change in expression in the antrum (81.5 vs 158.5 $p < 0.05$), and rectum (81.5 vs 157.8; $p < 0.043$), a 1.2 fold change in the duodenum (81.5 vs 146.7; $p < 0.029$) and ileum (81.5 vs 147.8; $p < 0.036$), and a 1.8 fold change in the colon (81.5 vs 145.6; $p < 0.22$). There was no significant difference between the oesophagus and caecum (Fig 5.4).

BIM

The pattern of expression of BIM was similar to Bak. The oesophagus had the lowest (37.7) and ileum had the highest (134.9) expression. Fold changes ranged from 2.7 in the antrum (37.7 vs 101.2; $p < 0.015$) to 3.6 in the ileum (37.7 vs 134.9; $p < 0.05$) (Fig 5.4).

BID

The expression of BID was the most variable of all pro-apoptotic proteins examined. Like Bak and BIM, the oesophagus had the lowest expression of BID (21). Highest expression was found in the duodenum (70), these values being significantly different < 0.05) (Fig 5.4).

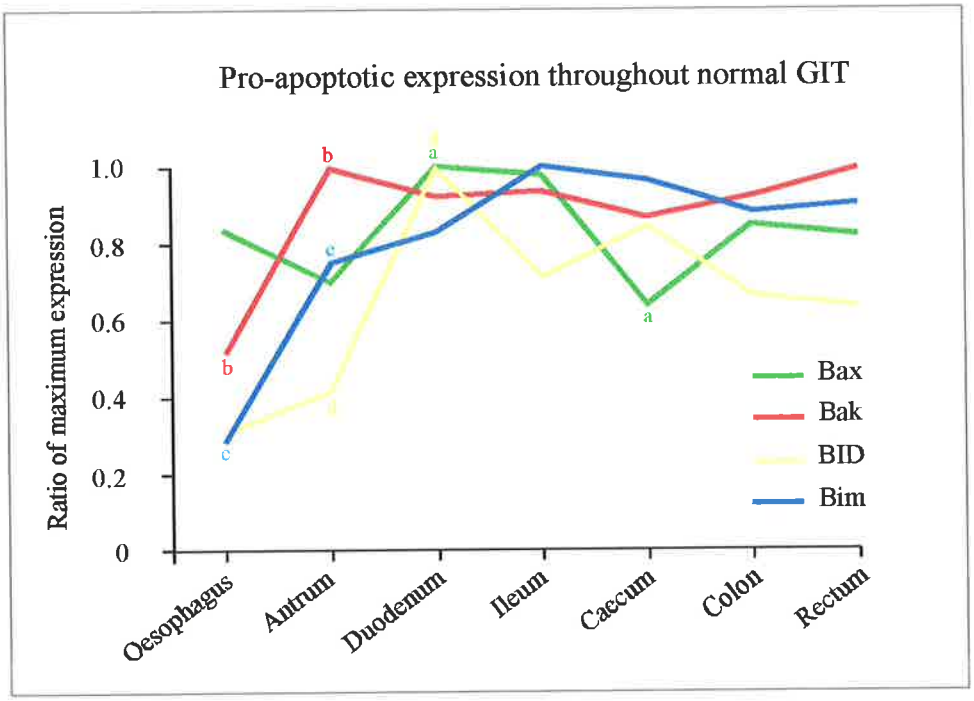


Figure 5.4 Expression of 4 pro-apoptotic proteins throughout the normal gastrointestinal tract of humans. With the exception of Bax, the oesophagus always has a lower level of expression.
A = $p < 0.05$; b = $p < 0.05$; c = $p < 0.015$; d = $p < 0.043$.

5.3.2 Anti-Apoptotic Expression Throughout the Gastrointestinal Tract

Four anti-apoptotic proteins, Bcl-2, Bcl-w Mcl-1 and Bcl-xL, were investigated to determine their expression levels in seven regions of the normal human gastrointestinal tract. All regions of the tract showed expression of anti-apoptotic proteins, and like pro-apoptotic proteins, positive staining occurred in the cytoplasm of the cells. When examined qualitatively, there appeared to be minimal differences in staining intensity between the crypts and villi, with staining appearing consistent in both parts. Additionally, there was a consistent level of staining across all the tissue sections, which allowed for any region of the tissue to be examined and recorded as an accurate representation of expression (Figs 5.2, 5.3).

Bcl-2

Mean Bcl-2 expression was significantly lower in the oesophagus when compared to all other regions. Expression increased in duodenal crypts compared to the crypts of the colon with a 1.3 fold increase (161.7 vs120.3). There were no significant differences detected between any other regions of the tract (Fig 5.5).

Bcl-w

Bcl-w protein expression was also low in the oesophagus, but showed a 2.5 fold increase in the rectum (35.1 vs 122; $p < 0.05$). There was also a 1.9 fold change between caecum and rectum (65 vs 122; $p < 0.029$) (Fig 5.5).

Mcl-1

Mcl-1 expression was also consistently lower in the oesophagus than in any other region examined with the biggest change, a 2.2 fold increase seen between the oesophagus and the caecum (51.9 vs 112; $p < 0.05$). All other regions of the gastrointestinal tract that were investigated were relatively constant with no significant differences detected (Fig 5.5).

Bcl-xL

Bcl-xL had relatively constant expression along the gastrointestinal tract, with no significant differences observed between any regions (Fig 5.5).

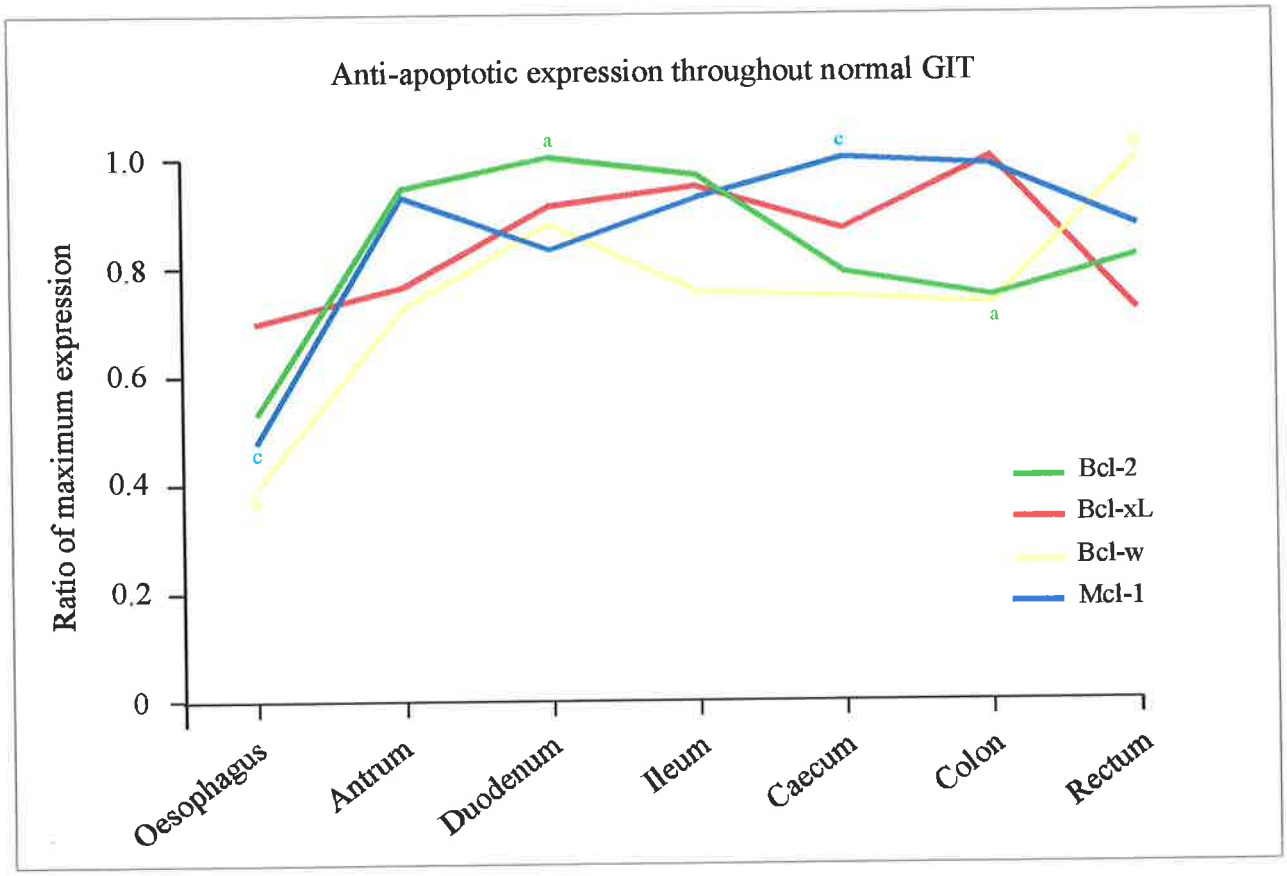


Figure 5.5 Expression of 4 anti-apoptotic proteins throughout the normal gastrointestinal tract of humans. With the exception of Bcl-xL, the oesophagus always has a lower level of expression. Bcl-xL expression shows no significant difference throughout the tract.
a = $p < 0.05$; b = $p < 0.05$; c = $p < 0.05$.

5.3.3 Expression of p53 and p21 Through the Gastrointestinal Tract.

Two transcription factors, p53 and p21 were also investigated to determine their expression levels in seven regions of the normal human gastrointestinal tract. All regions of the tract showed expression of these transcription factors, however, positive staining occurred in the nucleus, of the cells. When examined qualitatively, there appeared to be minimal differences in staining intensity between the crypts and villi, with staining appearing consistent in both parts. Additionally, there was a consistent level of staining across all the tissue sections, which allowed for any region of the tissue to be examined and recorded as an accurate representation of expression.

p53

p53 had highly variable expression throughout the gastrointestinal tract and was significantly lower in the oesophagus and the colon compared to all other regions. There was a 2.1 fold change in expression from oesophagus to antrum (39.9 vs 82.2; $p < 0.043$) and a 2.4 fold change in expression from antrum to colon (82.2 vs 34.6; $p < 0.050$). Maximum protein expression occurred in the jejunum, with a 2.0 fold change in expression from the oesophagus and a 2.3 fold change in expression from the colon (79.2 vs 34.6; $p < 0.04$) (Fig 5.6).

p21

Mean expression of p21 showed a general decrease from the upper regions of the tract to the lower regions of the tract. Highest expression was detected in the antrum, with lowest expression was detected in the rectum ($p < 0.050$) (Fig 5.6).

p53 and p21

As p53 is reported to regulated expression of p21 (156, 157), the significance of the relationship between these two transcription factors was investigated. The results from this analysis showed that there was a significant increase in p21 when compared to p53 in the oesophagus; antrum, ileum and colon ($p < 0.05$ respectively) and no significance reported in the duodenum, caecum and rectum (Fig 5.6).

5.3.4 Correlation of Results with Subjects' H. pylori Status

Helicobacter pylori was present in 8 of 50 subjects. Expression of pro- and anti-apoptotic proteins as well as p53 and p21 were compared to *H. pylori* negative, and it was found that there was no significant difference in expression for any protein, over any region (data not shown).

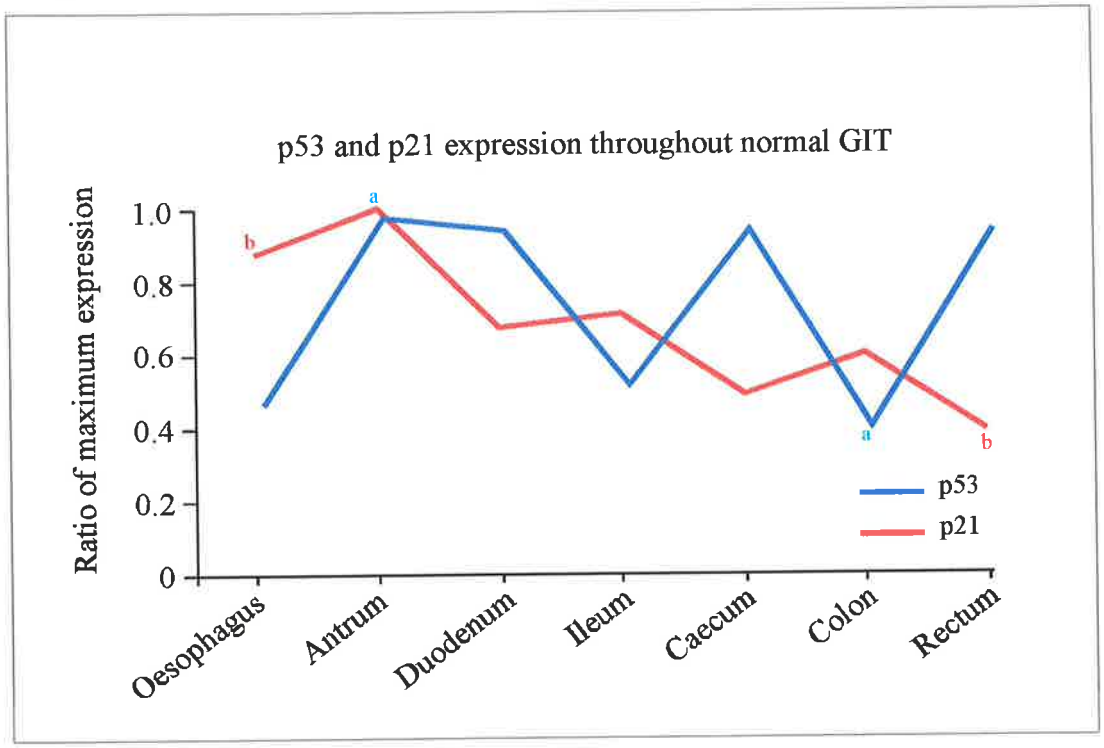


Figure 5.6 Expression of transcription factors p53 and p21 throughout the normal human gastrointestinal tract. p53 expression is highly variable, whereas p21 expression decreases along the tract, with highest expression seen in the upper regions and lowest levels seen in the rectum.
A = $p < 0.05$; b = $p < 0.05$.

5.4 Discussion

Bcl-2 is the founding member of a family of genes that currently have at least 20 known members (158, 159). Members can be either pro- or anti-apoptotic, and contain several conserved regions of amino acids known as Bcl-2 homologies or BH domains (149). The function of these domains is to allow interactions to occur between family members thus promoting or inhibiting cell death (149). These interactions are called heterodimerisation when a pro- and an anti-apoptotic protein interact, and homodimerisation when two of the same proteins (ie pro and pro or anti and anti) interact. Previous research has shown that the ratio of pro- to anti- apoptotic proteins within a cell is vital for allowing interactions between members and to potential apoptotic stimuli (152, 153). There are two hypotheses relating to how the ratio of proteins found within a cell will ultimately determine its fate. The first hypothesis is that an excess of the anti-apoptotic Bcl-2 and Bcl-xL proteins heterodimerises with the pro-apoptotic Bax protein and prevents Bax from forming "toxic" homodimers with itself. The second hypothesis is that Bcl-2 and Bcl-xL are both active repressors of cell death and heterodimerise with Bax to prevent cell death (149, 160). Apoptosis has been shown to be an important mechanism in the development of gastrointestinal mucositis, therefore understanding the role that the Bcl-2 family members play is vital to further our understanding of mucositis.

Quantitative immunohistochemistry is an emerging technique that was used in the present study to demonstrate the expression of pro-and anti-apoptotic proteins as well as two transcription factors throughout the normal human gastrointestinal tract. The technique described has been previously validated when chromagen exposure times, counterstain times, or day-day variations were altered (133). Despite previous immunohistochemistry research on determining the relative expression of many of these proteins throughout the body (161-165), these studies have mainly relied on tissue from autopsy cases, as well as from biopsies. Results from these studies have shown that there is variation in staining between the small and large intestine, with a greater ratio of pro-apoptotic proteins expressed in the small intestine and more anti-apoptotic proteins expressed in the colon (48, 166). Indeed these results have been able to be successfully correlated with damage in the tissues, with the suggestion that this pattern of expression can explain why there is a preponderance of colon cancer as opposed to small intestinal cancer (48, 154, 155, 166).

The present study differs from previous studies in that it utilises tissue from living subjects. Subjects that had a malignancy or had previously undergone chemotherapy were

specifically excluded, to ensure "normal" healthy tissue was used. Results showed for most of the proteins that were analysed that the oesophagus had the lowest expression. It is hypothesised that this could be due to the function of this tissue as a transit tube for a variety of dietary substances. In its capacity as a transit tube, the oesophagus needs to be relatively robust, able to handle a variety of potential damaging conditions. Having a relative lower expression of proteins may enable this to occur.

The remainder of the tract had relatively consistent levels of both pro- and anti-apoptotic protein expression. It may be that in tissue from "normal" subjects, there is no difference between pro- and anti-apoptotic protein expression, with levels of both remaining consistent. This suggests that in the absence of a stress, such as chemotherapy, levels of these proteins remained balanced. When there is a "stress" placed on the system, imbalances as previously shown, (48, 154, 155, 166) may occur. This may be a genetic mutation, the presence of *H. pylori,* or when a cytotoxic agent is administered.

Previous studies have suggested that *H. pylori* may alter expression of the transcription factors, p53 and p21 (167-169). In order to determine this, the expressions of these two proteins were compared between subjects positive and negative for *H. pylori.* For both p53 and p21, in all regions of the tract, no significant difference was detected, suggesting that *H. pylori* does not cause expressional changes for these two proteins. It is known that *H. pylori* is the main cause of chronic gastritis (167, 168), with a change in expression of p53 and p21 reported to be related to *H. pylori* infection. As only 16% of subjects that were recruited were *H. pylori* positive, it may be that the sample size was too small. In addition, it may be that the subjects recruited did not have chronic gastritis.

In conclusion, relative expression of pro- and anti-apoptotic proteins, and p53 and p21 were determined for the first time throughout the normal gastrointestinal tract. This will allow reference to be made for future studies determining the change in expression following damage to the intestine.

Ultrastructural and immunohistochemical changes within the oral mucosa following cancer chemotherapy

6.1 Introduction

Mucositis is a major oncological problem, causing pain and ulceration in the mouth, and abdominal bloating, vomiting and diarrhoea throughout the gastrointestinal tract (2-4). It affects mainly the mouth and small intestine, and occurs in 100% of subjects undergoing high dose chemotherapy and stem cell or bone marrow transplantation (2-4). Mucositis increases morbidity of subjects undergoing cancer treatment, prolongs hospital stay, increases re-admission rates (4), and is occasionally fatal. Currently there is no known treatment, with management being limited to pain relief and maintenance of good oral hygiene. Alleviating mucositis would lead to increased maximum tolerated doses of chemotherapy, and improved quality of life for cancer subjects. This increased dose of tolerable chemotherapy by subjects may also increase the likelihood for cancer cure. In order to gain a complete understanding of gastrointestinal mucositis, oral mucositis must be evaluated. Therefore, the primary purpose of this study was to increase the current understanding of the mechanisms of human oral mucositis.

Little research has been reported on the mechanism behind oral mucositis. However, there has been research reported on treatment and prevention options of oral mucositis after chemotherapy (62, 66) and on possible preventative agents such as GM-CSF (64, 77) and sucralfate (79). All analyses of oral mucositis have been based on a purely observational basis, and whilst there have been a number of hypotheses as to the mechanisms behind this, the actual pathophysiology remains undefined (4). Several studies suggest four distinct phases of damage: (1): inflammatory/vascular phase; (2): epithelial phase; (3): ulcerative/bacteriological phase and (4): healing phase (63, 67, 68), however, these have not examined the time-course of damage following chemotherapy or whether apoptosis is a component of the mechanism. Oral mucositis has been studied in the cheek-pouch of the hamster (73, 74), although this model is limited by the lack of associated tumour and spontaneous chemotherapy-induced mucositis. Mucositis in this model is induced by mechanical scratching of the mucosal surface following chemotherapy administration and may therefore introduce the confounding factor of wound healing (73, 74). Another confounding factor is that rodents have an increased thickness of the oral mucosa, with the doses of chemotherapy required to cause mucositis being lethal (D.Wilson, pers. com.

2001). There are no data on the morphological changes in the human oral mucosa following chemotherapy. Clinical studies have shown that oral mucositis peaks at days 10-14 whereas intestinal mucositis peaks at days 3-7 after chemotherapy (2). The reason for this dissociation is unknown. Past studies conducted by our research group have shown intestinal mucositis is due to apoptosis of crypt epithelial cells, but it is unknown if apoptosis also occurs in the mouth or whether the repair is delayed. Therefore the aim of this part of my PhD was to determine the time-course of damage that occurs in the oral mucosa following cytotoxic cancer chemotherapy.

6.2 Subjects and Methods

6.2.1 Subjects

Subjects were recruited from those being treated with chemotherapy and being seen in the Department of Medical Oncology at the Royal Adelaide Hospital. There were 20 subjects recruited to this study; 4 males and 16 females with a median age of 52.4 years (range 32-86). Subjects were excluded from the study if they were undergoing concurrent radiotherapy to the head and neck, or if they had pre-existing mucosal damage. Standard dose chemotherapy was used in all subjects, with multiple drug regimes used and most were administered over 1-4 hours. The malignancies (number of subjects) were: breast cancer (eleven), colorectal cancer (three), small cell lung cancer (one), non small cell lung carcinoma (one), neuroendocrine pancreatic carcinoma (one), B-cell lymphoma (two), and Hodgkin's lymphoma (one). There were not enough subjects to each group to perform a subset analysis based on the chemotherapeutic agents that were administered (1, 2). However, the number of cycles of chemotherapy that each patient previously had was recorded to determine if this played a role in determining the ultrastructural changes within the oral mucosa. In addition to the subjects, four normal volunteers (1 male and 3 females) were recruited to the study. These volunteers were healthy individuals who had no previous history of cancer, had not undergone previous chemotherapy and were asked to give one biopsy to act as a true control. This study was approved by the Ethics of Human Research Committee of the Royal Adelaide Hospital and was carried out in accordance with the Declaration of Helsinki. Informed consent was obtained from each patient prior to enrolment in the study.

6.2.2 Oral Biopsies

Subjects were asked to have two buccal biopsies, one prior to the commencement of chemotherapy and one at varying timepoints after chemotherapy (up to 11 days post chemotherapy). Two of the twenty subjects withdrew from the study after the first biopsy, one due to patient request and another due to withdrawing from chemotherapy treatment. The remaining eighteen subjects underwent two buccal punch biopsies performed by Dr. Richard Logan within the Special Needs Unit located in the Adelaide Dental Hospital. Pre chemotherapy biopsies were taken on one side of the mouth and post chemotherapy biopsies were taken on the opposite side. The surrounding buccal mucosa was numbed

with local anaesthetic, and a small punch biopsy was taken. A single stich was placed at the site of the biopsy.

6.2.3 Histological and Ultrastructural Preparation

Following removal, the buccal mucosa was immediately divided into two smaller pieces, one of which was fixed in 10% neutral buffed formalin for routine pathological assessment and the other was fixed in 1.25% gluteraldehyde/4% paraformaldehyde in 4% sucrose in phosphate buffered saline (pH 7.2) at 4°C for electron microscopical examination. The tissue was then post fixed in 2% osmium tetroxide, dehydrated through graded ethanols, before being placed in propylene oxide. The tissue was then infiltrated with resin (Procure-Araldite Embedding Kit, ProSciTech, Queensland, Australia). Following embedding in epoxy resin, 1μm sections were cut using an ultramicrotome and stained with toluidine blue. These were examined under a light microscope and an area of underlying mucosa and epithelium were selected for electron microscopical examination. Ultrathin sections were cut using a diamond knife and ultramicrotome at a thickness of approximately 100 nm and were mounted on copper grids. (All processing and subsequent staining for electron microscopical analysis was conducted by Ms. Lyn Waterhouse from Adelaide Microscopy, University of Adelaide). Following staining with uranyl acetate and lead citrate, grids were analysed using a Philips CM-100 transmission electron microscope (Adelaide Microscopy, University of Adelaide). The entire epithelium was examined (including the basal layer, prickle cell layer, intermediate layer and the superficial layer).

6.2.4 Immunohistochemical Analysis of p53, p21, Pro- and Anti-Apoptotic Protein Expression in the Oral Mucosa

Prior to beginning immunohistochemistry, all antibodies were optimised for human gastrointestinal tissue. The method used for optimisation and all subsequent immunohistochemistry is detailed below. For antibodies that were not raised in mouse (BIM and Bcl-w) a slightly modified protocol was used (20% horse serum and anti-goat secondary antibody). All remaining steps are the same.

Two consecutive, 4 μm paraffin sections of the oral mucosa were cut using a rotary microtome, dewaxed in xylene and rehydrated through a graded series of alcohols. One section was designated as the negative control, the other as the experimental section. Slides were washed for 2 x 5mins in PBS prior to being subjected to heat mediated antigen retrieval in citrate buffer (pH 6.5). Slides were quenched in 3% hydrogen peroxide in

methanol for 1 min to deactivate endogenous peroxidase activity, before non-specific antigens were blocked by normal serum for 20 mins. Finally, endogenous avidin and biotin was eliminated using a commercial avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA USA). Slides were incubated overnight at 4°C with the primary antibody (refer to Table 6.1 for antibodies and appropriate concentrations) on the experimental section, and PBS only on the negative section. The following morning, slides were incubated with a universal linking antibody, before the reaction was amplified with Ultra-Streptavidin conjugated to horseradish peroxidase and visualised with diaminobenzidine chromogen. Slides were counterstained with Lillie-Mayer's Haematoxylin, dehydrated through a graded series of alcohols and mounted with PIX mounting medium. Both positive and negative control sections were included in each run (refer to Table 6.1 for positive tissue which was used for each antibody)

6.2.5 Quantitative Immunohistochemistry

In order to quantify staining, an algorithm which calculated the cumulative signal strength or energy of a digital file of the image in question was used (132, 133). It was assumed that all staining in both the experimental and negative control image was important (132, 133). Digital photographs were taken of identical regions of experimental and control sections (Fig 6.1) using a Nikon 800 research microscope (x43 dry lens) and SPOT-RT camera (Diagnostic Instruments Inc, Michigan, Detroit). A 99 x 99 pixel area over the nucleus (p53 and p21) or cytoplasm (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Bax, Bak, BIM, BID) of individual cells were selected in both experimental and control sections, using Image Pro Plus (Media Cybernetics, Silver Spring MD)(Fig 6.1). These were saved as TIFF files, which allowed for LZW compression without the loss of any data (132, 133), before being converted to image matrices, and normalised using functions in Matlab (Mathworks Inc. Natick, MA USA) (132, 133). The intensity of staining was expressed as the pixel energy difference in cumulative signal between the control and experimental image matrices.

6.2.6 Detection of Apoptosis

Sections of oral buccal mucosa were fixed for 24 h in 10% formalin and stored in 70% ethanol. Tissue was dehydrated, embedded in paraffin wax and histological 4 μm sections were cut. The method for labelling the apoptotic cells was performed by using *In Situ Cell Death Detection Kit AP* (Roche, Mannheim, Germany). After dewaxing, the slides were rehydrated, prior to immersion in a 0.1% TX-100 in 0.1% (w/v) sodium citrate buffer for 8

mins at room temperature. Following two rinses in PBS, slides were placed in a TUNEL buffer solution (150mM Tris; 0.7M NaCaCo; 10mM $CoCL_2$; 10% BSA and sterile H_20) for a further 10 mins at room temperature. Slides were immediately placed into the reaction mixture and incubated in a humidified chamber for 3 h at 37°C. Following three rinses in PBS slides were incubated with Converter-AP in a humidified chamber for 60 mins at 37°C, after which they were rinsed a further 2 changes of PBS. Fast red chromogen (Roche, Germany) was applied for 15 minutes. Slides were rinsed and counterstained with haematoxylin. Apoptotic bodies were counted per mm^2 in the basal layer of oral tissue per 4 μm section.

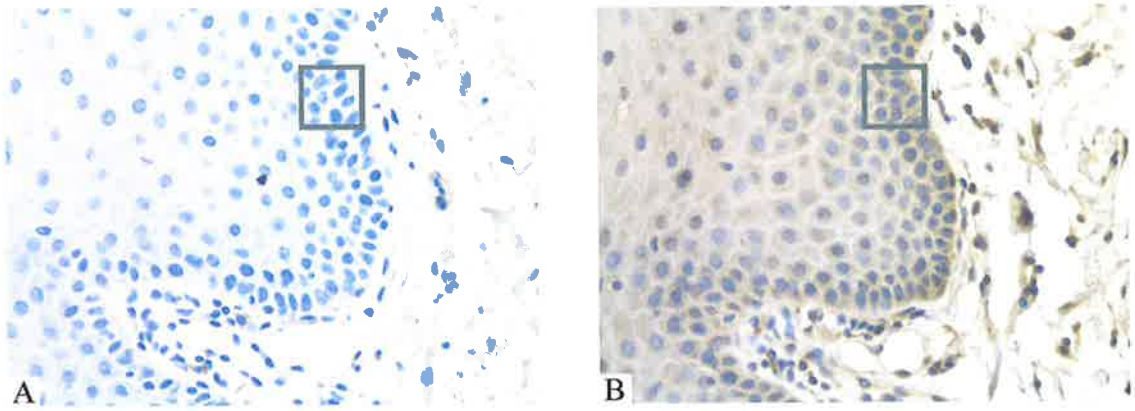


Figure 6.1 Photomicrographs of a serial A) unstained control and B) stained experimental section taken at 600x magnification overlayed with a 99 x 99 area of interest box.

Antibody	Clone and Source	Dilution	Positive Control
p53	NCL-p53-240 Novacastra, Mouse Monoclonal	1:50	Breast Tumour
p21	C19 sc-397 Santa Cruz, Rabbit Polyclonal	1:1200	Liver
Bak	sc-832 Santa Cruz, Rabbit Polyclonal	1:1200	Stomach
Bcl-xl	H-62 sc-7195 Santa Cruz, Rabbit Polyclonal	1:850	Thymus
Bcl-2	N-19 sc-492 Santa Cruz, Rabbit Polyclonal	1:1500	Spleen
Bax	P19 sc-526 Santa Cruz, Rabbit Polyclonal	1:1300	Thymus
BID	FL195 sc-11243 Santa Cruz, Rabbit Polyclonal	1:4200	Spleen
Mcl-1	S19 sc-819 Santa Cruz, Rabbit Polyclonal	1:7000	Spleen
Bcl-w	N19 sc-6172 Santa Cruz, Goat Polyclonal	1:500	Spleen/Testis
BIM	N20 sc-8265 Santa Cruz, Goat Polyclonal	1:1500	Spleen/Testis

Table 6.1: The source, clone, concentration and appropriate positive control tissue used for immunohistochemical analysis throughout the normal and post chemotherapy treated human oral mucosa.

6.3 Results

6.3.1 Subjects

One patient complained of soreness at the biopsy site and subsequently withdrew from the study. The remaining nineteen subjects, plus the four normal controls, showed no untoward complication. Four normals acted as untreated controls, and twenty subjects each had pre chemotherapy biopsies. Post chemotherapy biopsies were taken at days 1 (n = 4), 2(1), 3(4), 5(3), 6(2), 7(1), 8(2) and 11(1).

13 of the 20 subjects had their pre and post chemotherapy biopsies assessed by pathologists at The Institute of Medical and Veterinary Sciences, Adelaide, South Australia. Of these, six subjects had normal pathological reports for both pre and post chemotherapy biopsies and one subject had a normal report for their pre chemotherapy biopsy only. The remaining six subjects were reported to have a mild to moderate chronic inflammation in both pre and post chemotherapy biopsies. In addition some of these subjects had mild keratinisation.

6.3.2 Electron Microscopy

6.3.2.1 Normal Subjects

Basal Cell Layer

All four normal subjects had similar basal layers in their oral epithelium. Cells lined the basement membrane and were elongated in shape with an uneven basal surface. These cells contained large irregular shaped nuclei sometimes containing one or more nucleoli (Fig 6.2). Many desmosomes were present. The majority of the cells were electron dense but occasional cells were electron lucent. At higher magnification, these cells had scarce cytoplasm with few organelles (Fig 6.2).

Prickle-Cell Layer:

The prickle cell layer had membranes with many desmosomes in an irregular arrangement. Some cells were in mitosis. Lymphocytes were also present in this region. Prickle cells had cytoplasm with few organelles, the nuclei were not as large as those that were observed in the basal layer, and some contained one or more nucleoli (Fig 6.2). Overall, the sizes of the cells in this layer were larger, often with an irregular shape. Tonofilaments were present (Fig 6.2).

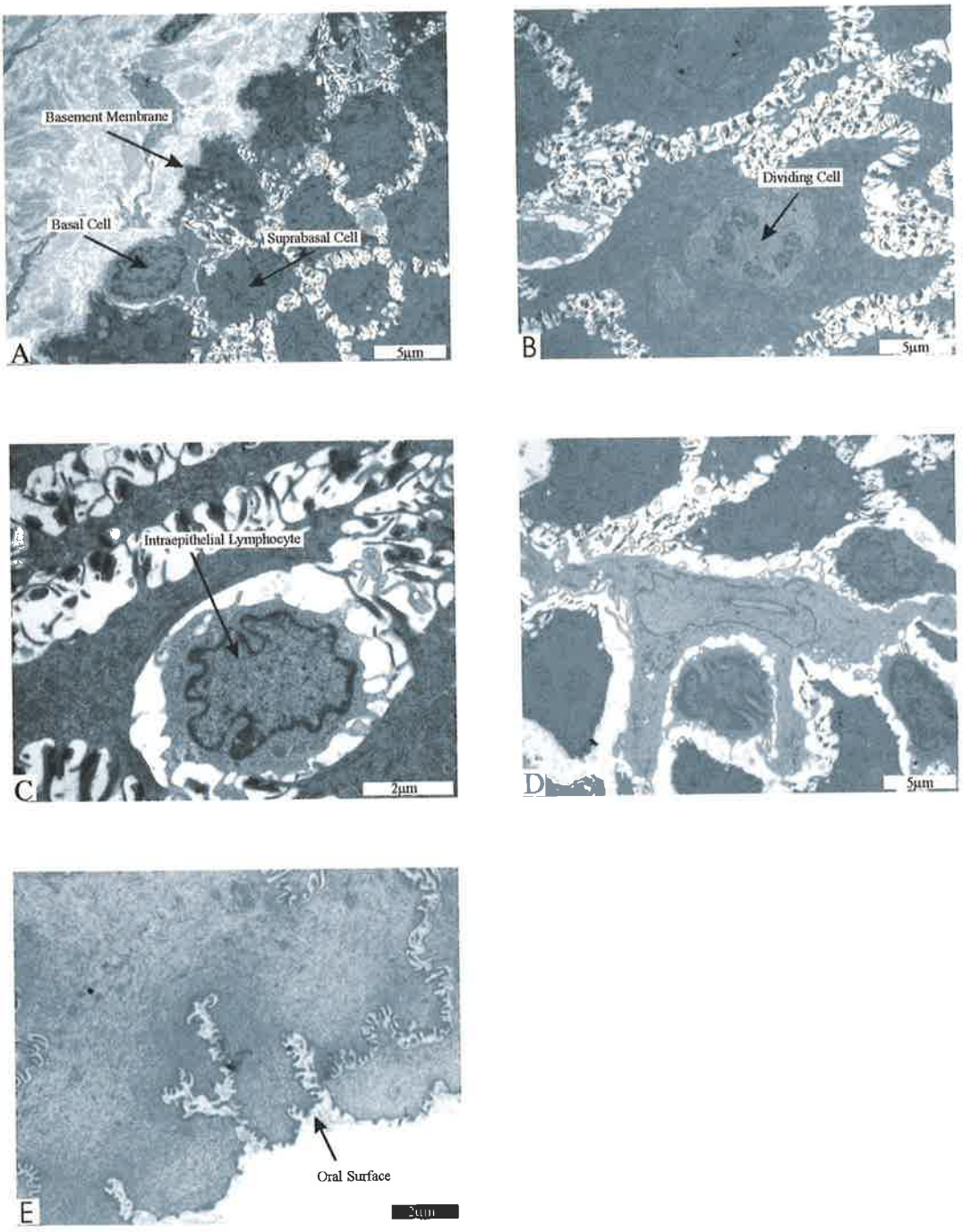


Figure 6.2 Electron micrographs of normal human oral mucosa. In A) The basal cells are elongated in shape and have an uneven basal surface. B) A cell of the prickle-cell layer dividing. C) The presence of intraepithelial lymphocytes were observed within the prickle-cell layer. D) E) The superficial layer of cells.

Granular Layer

These cells were elongated with elongated nuclei. They were not as electron dense as the previous two layers. Some of the cells contained cytoplasmic granules (Fig 6.2).

Superficial Layer

In all normal subjects examined these cells often were highly irregular in shape and varied in electron density. Some contained no nuclei (Fig 6.2).

6.3.2.2 Subject Biopsies

Pre-Chemotherapy Biopsies

Biopsies of subjects before chemotherapy, had changes in ultrastructure compared to biopsies from healthy controls. Cells in the basal layer had enlarged intercellular spaces, increased cytoplasmic filaments, and contained cytoplasmic vacuoles. Wide intercellular gaps were also present in the other cell layers. Their nuclei were darkly stained and some cytoplasmic vacuoles were present (Fig 6.3).

Post-Chemotherapy Biopsies

At day 1 after chemotherapy, biopsies showed ultrastructural changes throughout the layers of the epithelium. The basal cell layer had increased intercellular filaments arranged circumferentially around the cytoplasm, and increased space between the nuclear membrane and cytoplasm. Cells in the basal and suprabasal layers had more vacuoles (Fig 6.4). The prickle, granular and superficial cell layers had cells with general degeneration, void cytoplasmic space and electron density variation (Fig 6.4).

At day 2, the basal and suprabasal cells had increased thickened filaments arranged in a haphazard fashion throughout the cytoplasm, or arranged around the circumference of the cytoplasm. These cells were multinucleated, containing many vacuoles in their cytoplasm (Fig 6.4). The only discernible organelle was the mitochondria. Basal cells developed enlarged intercellular spaces between the basement membrane and often contained vacuoles. Apoptosis was evident with occasional very dark cells observed along the basement membrane, containing pyknotic nuclei (Fig 6.5). Cells in the suprabasal region often had a loss of obvious shape, degrading cytoplasm and loss of organelles. Cells in the higher regions of the epithelium displayed similar changes to those seen at day 1, with cells containing vacuoles and general cytoplasmic degradation (Fig 6.4).

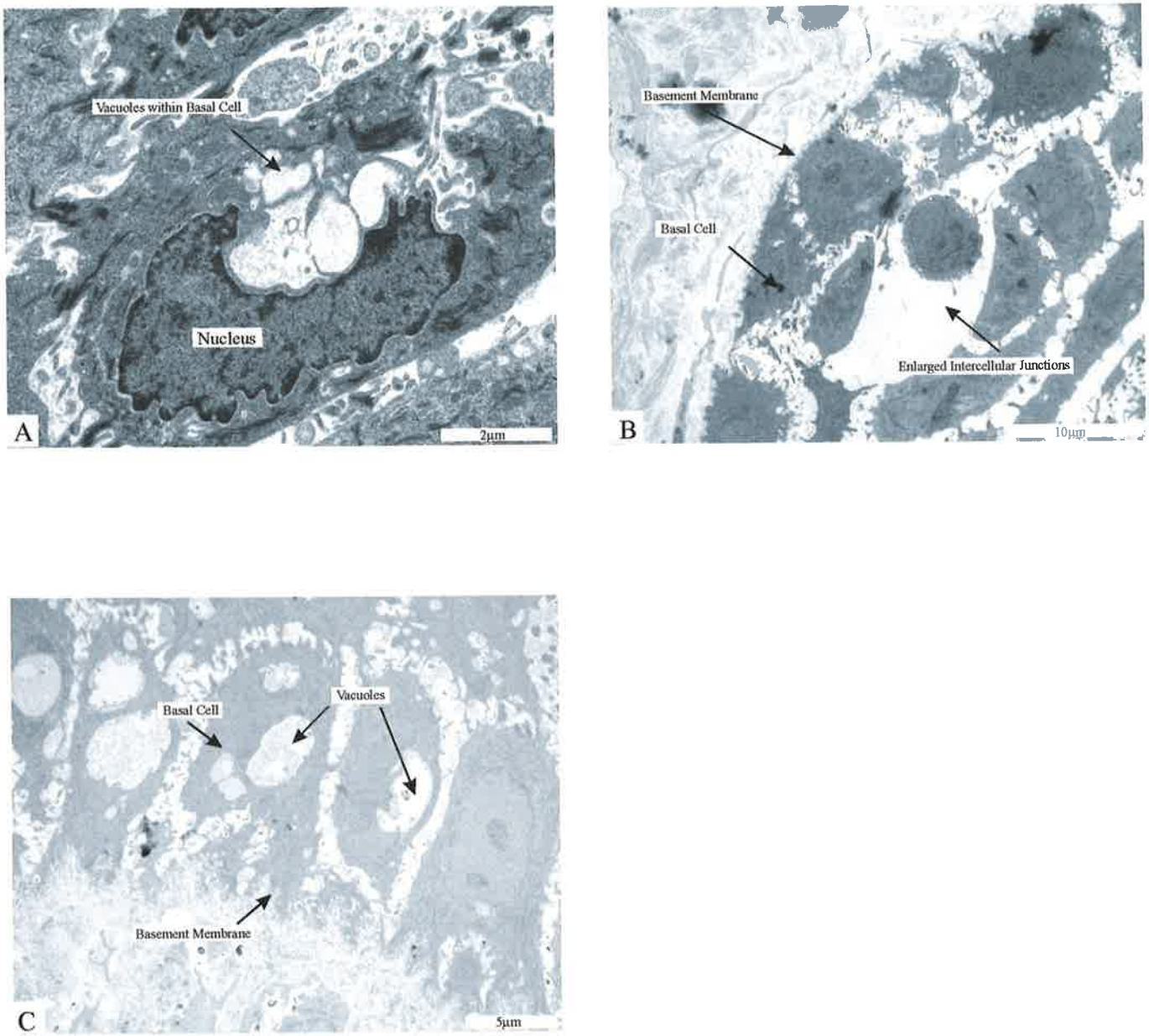


Figure 6.3 Electron micrographs from pre chemotherapy biopsies. A) Note the vacuoles present within the cytoplasm of basal cells. B) There are enlarged intercellular spaces between the cells of the basal layer. C) The basal cells contain many vacuoles.

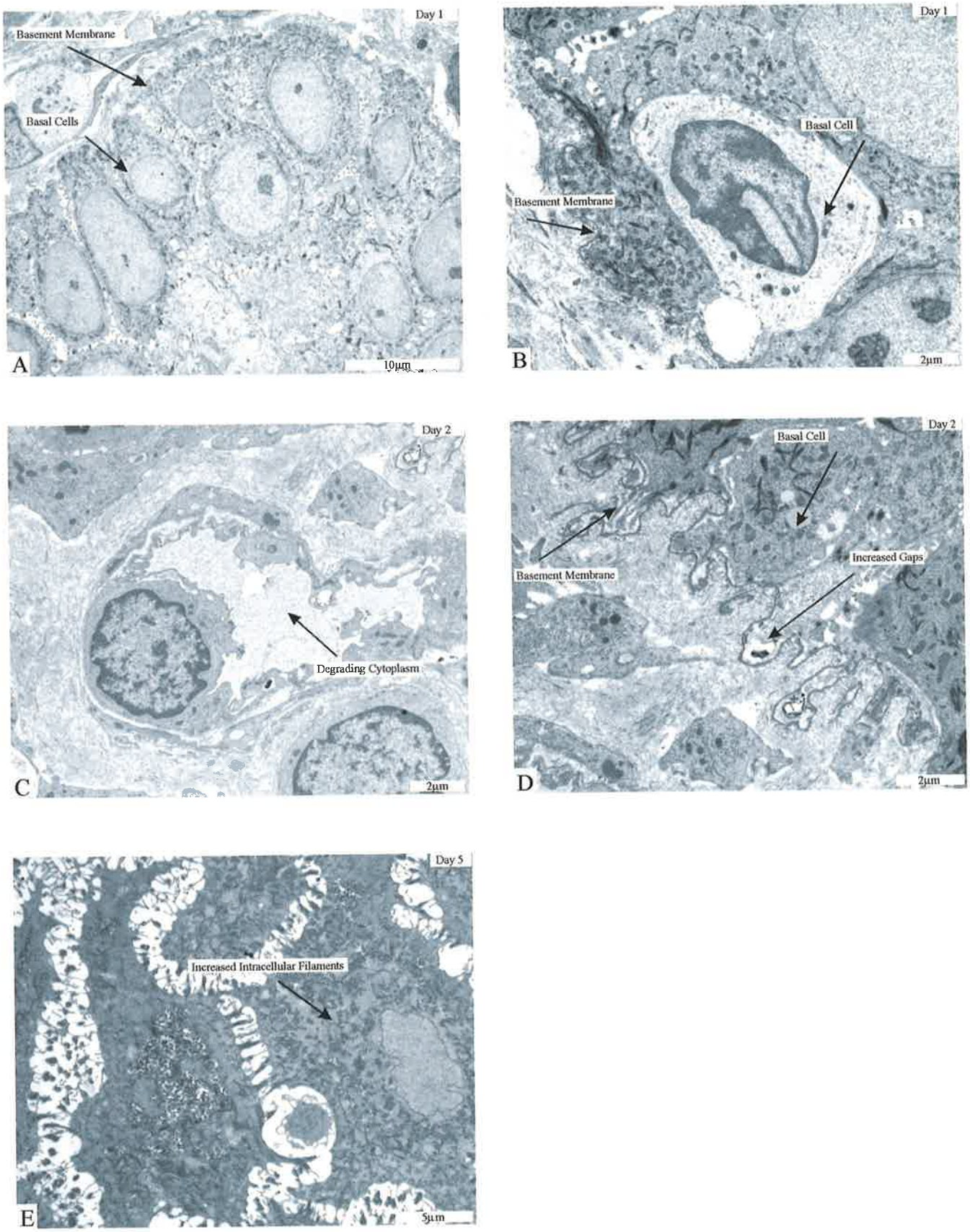


Figure 6.4 Electron micrographs of human oral mucosa following treatment with chemotherapy. A) Note the increased intracellular filaments arranged around the circumference of the basal cells. There are also enlarged intercellular spaces. B) Again, note the increase intracellular filaments, and the vacuole. C) Basal cell, where the cytoplasm is void of organelles and is beginning to degrade. D) There are increased gaps between the basement membrane and the basal cells. E) Cells within the prickle layer have increased filaments within the cytoplasm.

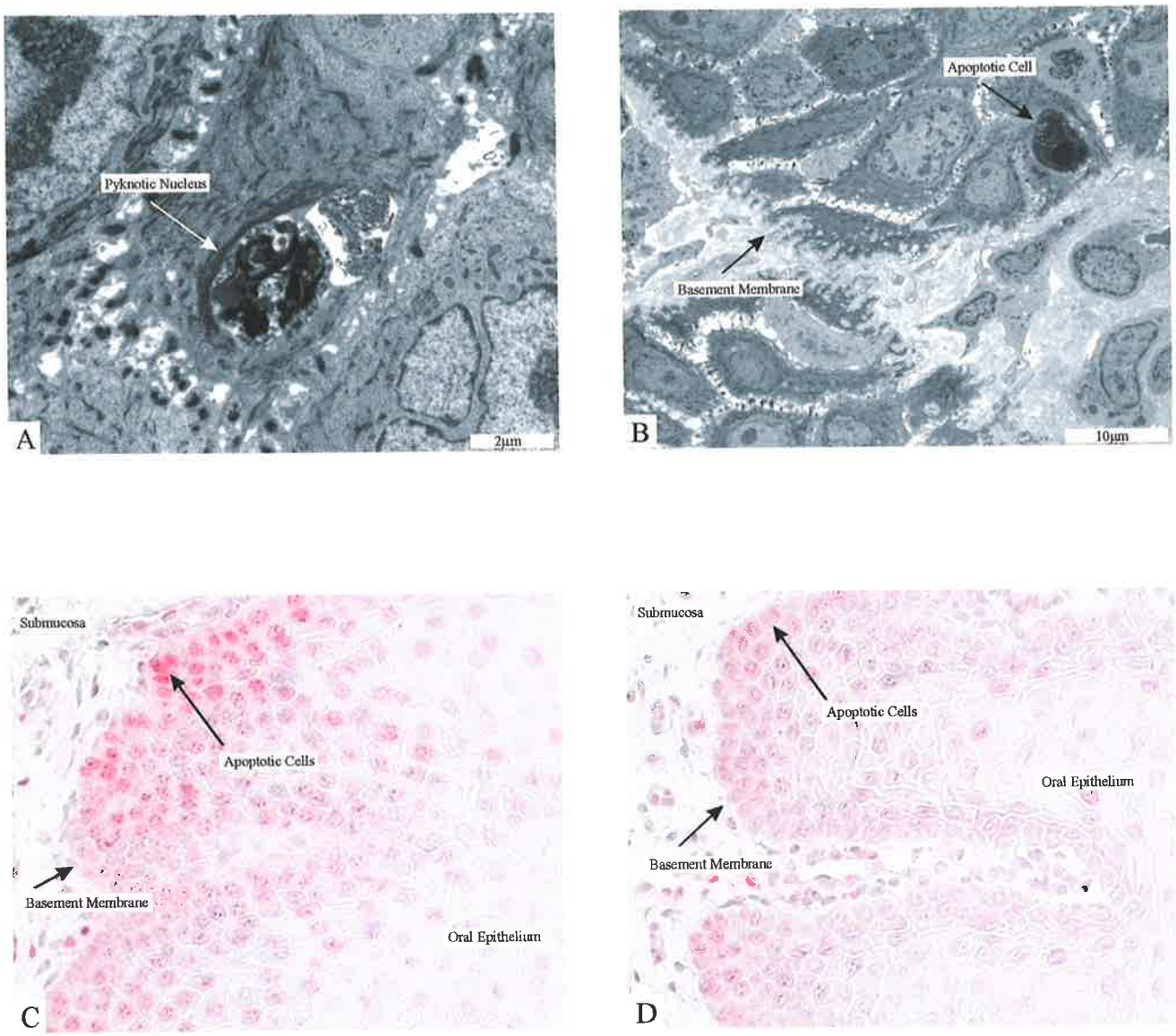


Figure 6.5 A) and B) Electron micrographs demonstrating the presence of apoptotic cells. Note the dark pyknotic nuclei. C) and D) Photomicrographs of apoptotic cells detected using the TUNEL assay (Biopsies are from the same patient).

By day 3 electron lucent cells containing few mitochondria, cytoplasmic vacuoles and multiple nucleoli were visible along the basement membrane. In the suprabasal region a small number of cells were observed to have electron dense nuclei, in addition to round dense vesicles around the periphery of the cytoplasm with no other organelles (Fig 6.4). Cells in this region had increased cytoplasmic filaments, although these had decreased from day 2.

At day 5 basal cells had large intercellular spaces with cytoplasmic vesicles, and increased cytoplasmic filaments arranged haphazardly throughout the cytoplasm. Intracellular lymphocytes were present.

At day 6 basal cells contained multiple nucleoli and large clear cytoplasmic vesicles. Pronounced intercellular spaces were still observed. Cytoplasmic filaments had decreased from earlier timepoints.

At days 7 and 8, the ultrastructural changes were similar to those described for day 6.

At day 11 cells of the oral mucosa contained large cytoplasmic vacuoles with large intercellular spaces observed. These changes were evident throughout the entire epithelium.

6.3.3 Pro-Apoptotic Protein Expression

Four pro-apoptotic proteins, Bax, Bak, BIM and BID, were examined for expression in the oral buccal mucosa. The oral mucosa showed expression of pro-apoptotic proteins, with positive staining occurring in the cytoplasm of the cells under investigation. Qualitatively, there appeared to be little difference in staining intensity in all four pro-apoptotic proteins within the basal layer, with staining appearing consistent. There was more variable staining observed in the prickle-cell, granular and superficial layers; however these were not examined quantitatively. Additionally, there was a consistent level of staining across all the tissue sections, which allowed for any region of the tissue to be examined and recorded as an accurate representation of expression.

Expression of the four pro-apoptotic proteins examined was relatively constant in the normal controls. Pre-chemotherapy biopsies however, showed variation from these normal controls, with BIM, Bak and Bax showing an increase and BID showing a decrease. When post chemotherapy biopsies were examined, there was a general elevation in expression

(from normal controls) over time (Fig 6.6). Pro-apoptotic Bax, Bak and BIM were elevated and BID was depressed. At day 1 following chemotherapy, there was a 1.06 fold decrease in Bak expression (170.7 vs 159.6) and a 3.03 fold decrease in BID expression (164.4 vs 54.2). In contrast there was a 1.52 and 1.74 fold increase in Bax and BIM respectively (111.1 vs 168.2; 126 vs 219.2). By day 2, there was an increase in expression of all proteins from day 1, but Bid was decreased 0.76 fold from normals. At day 5 after chemotherapy all pro-apoptotic proteins showed similar expression levels and these were all increased from normal controls. By day 11 post chemotherapy, expression had not returned to levels of normal controls (Fig 6.6).

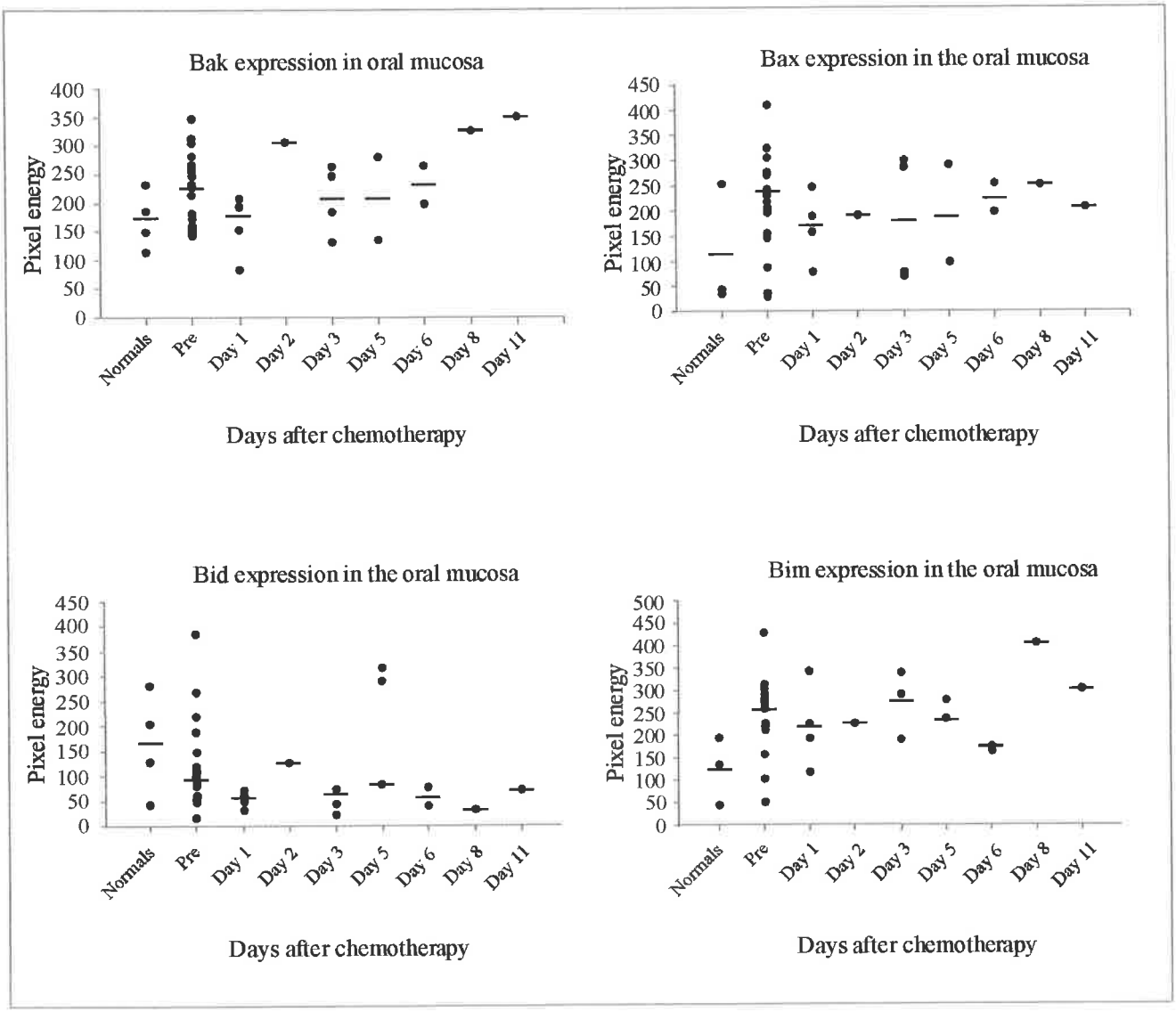


Figure 6.6 Expression of four pro-apoptotic proteins were examined in human oral mucosa before and after chemotherapy treatment. Expression of all were highly variable, and no levels had returned to normal by day 11 following treatment.

6.3.4 Anti-Apoptotic Protein Expression

Four anti-apoptotic proteins, Bcl-2, Bcl-w, Mcl-1 and Bcl-xL, were examined for expression in the oral buccal mucosa. The oral mucosa showed expression of anti-apoptotic proteins, with positive staining occurring in the cytoplasm of the cells under investigation. Qualitatively, there appeared to be little difference in staining intensity in all four anti-apoptotic proteins within the basal layer, with staining appearing consistent. There was more variable staining observed in the prickle-cell, granular and superficial layers; however these were not examined quantitatively. Additionally, there was a consistent level of staining across all the tissue sections, which allowed for any region of the tissue to be examined and recorded as an accurate representation of expression.

Expression of the four anti-apoptotic proteins examined was also relatively consistent in the normal controls (Fig 6.7). However when the pre-chemotherapy biopsies were examined there were changes in two of these anti-apoptotic proteins from the normal controls. Bcl-2 and Bcl-w showed elevated expression from normals whereas Mcl-1 and Bcl-xL showed no change. Post chemotherapy biopsies again showed a general elevation over the times examined for Bcl-2 and Mcl-1 whereas a more varied response was observed with Bcl-w and Bcl-2. Day 2 and Day 6 after chemotherapy seemed to be the days in which the most change in these proteins was observed. At day 2 there was an increase in expression of Bcl-2, Bcl-xL and Mcl-1 (1.89; 1.20 and 1.88 fold increase respectively from controls) and a 1.45 fold decrease in Bcl-w expression. At day 6 after chemotherapy there was a 1.70 fold increase in Bcl-2 expression, but a decrease in Bcl-xL and Mcl-1 expression (1.27 and 1.87 respectively) with Bcl-w returning to relatively normal levels. By day 11, only Bcl-w had returned to levels of normal controls (Fig 6.7).

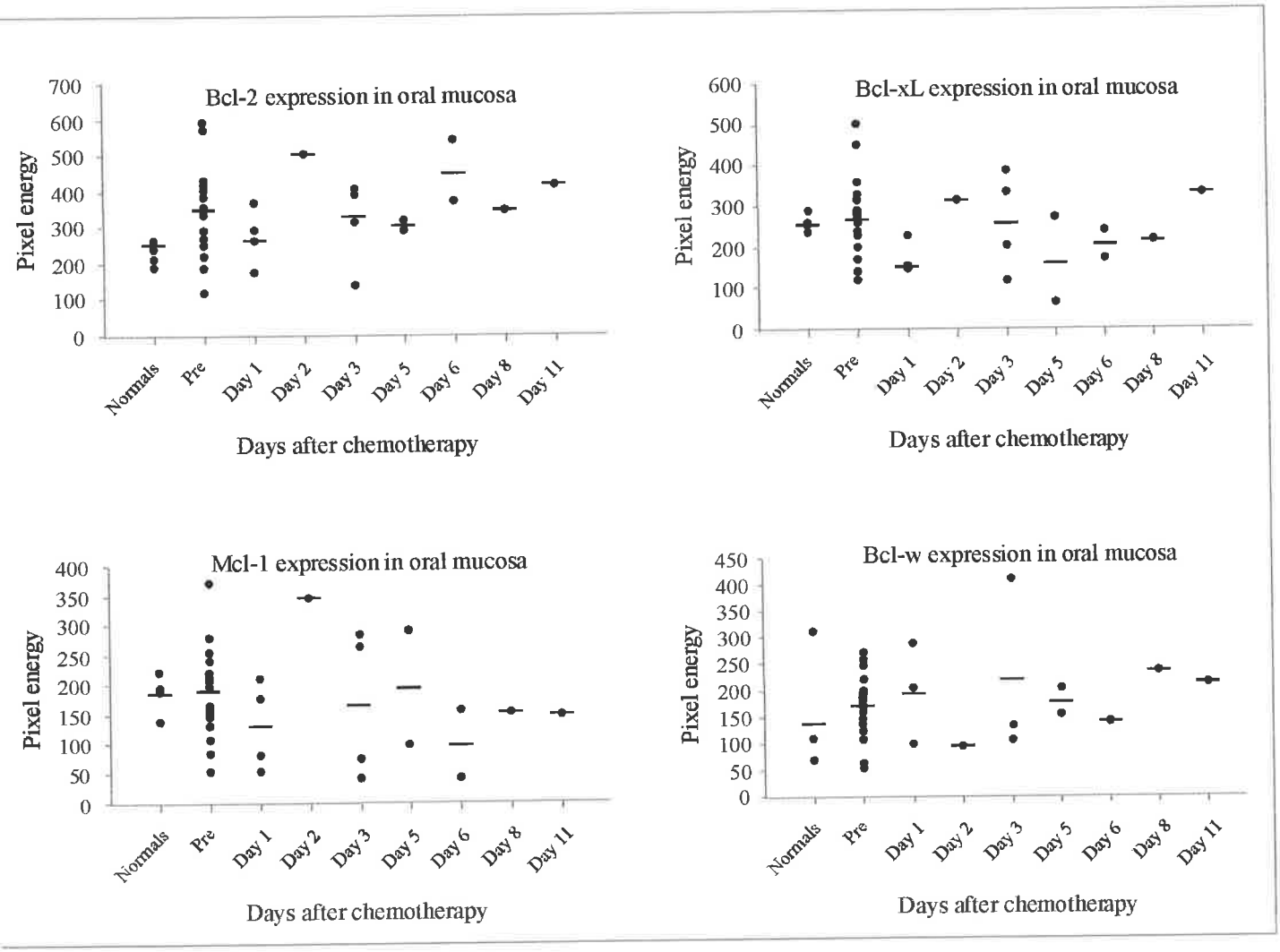


Figure 6.7 Expression of four anti-apoptotic proteins were examined in human oral mucosa before and after chemotherapy treatment. Expression of all four was highly variable, with only Bcl-w returning to normal levels by day 11 following treatment.

6.3.5 Transcription Factor Expression

The expression of two transcription factors, p53 and p21, were examined. In both normal subjects and pre chemotherapy biopsies, there were no difference between the two (p53 = 229.3; p21 = 220.8 and p53 = 253.54; p21 = 220.08 respectively) (Fig 6.8). However, following chemotherapy, p53 seemed to have biphasic expression with a sharp increase in p53 expression at day 1 and 2, before decreases at day 3 and 5, before expression levels rose again at day 6 and remained elevated over the remainder of the times investigated. p21 expression followed a similar pattern of expression changes, although they were not as marked as for p53. Both had not returned to levels of controls by day 11 following chemotherapy (Fig 6.8).

6.3.6 Apoptosis

Apoptosis was minimal (0.06 apoptotic bodies mm^2 of tissue), in normal control subjects but increased in subjects prior to their next cycle of chemotherapy ie, at least 21 days after previous chemotherapy (20.67 apoptotic bodies per mm^2 of tissue) (Figs 6.5; 6.9). After chemotherapy, apoptosis had a biphasic pattern with an early peak at day 3 and a later peak at day 6 with a long tail.

In normal control tissue there was very little apoptosis observed (0.06 apoptotic bodies per mm^2 of tissue), however when pre chemotherapy biopsies were examined there was found to be an average of 20.67 apoptotic bodies per mm^2 of tissue (Figs 6.5, 6.9). Post chemotherapy biopsies revealed that there was still elevated levels of apoptosis at day 1 (3.1 apoptotic bodies per mm^2 of tissue), when compared to normal controls, but decreased levels when compared to pre chemotherapy biopsies. This increased to 14.8 at day 2 and 51.9 at day 3. Apoptosis was able to be confirmed ultrastructurally at these time points (Fig 6.5). At Day 5 after chemotherapy 11.3 apoptotic bodies per mm^2 of tissue were identified 33.5 at day 6, 29.7 at day 7 and 14.3 at day 8 (Fig 6.9).

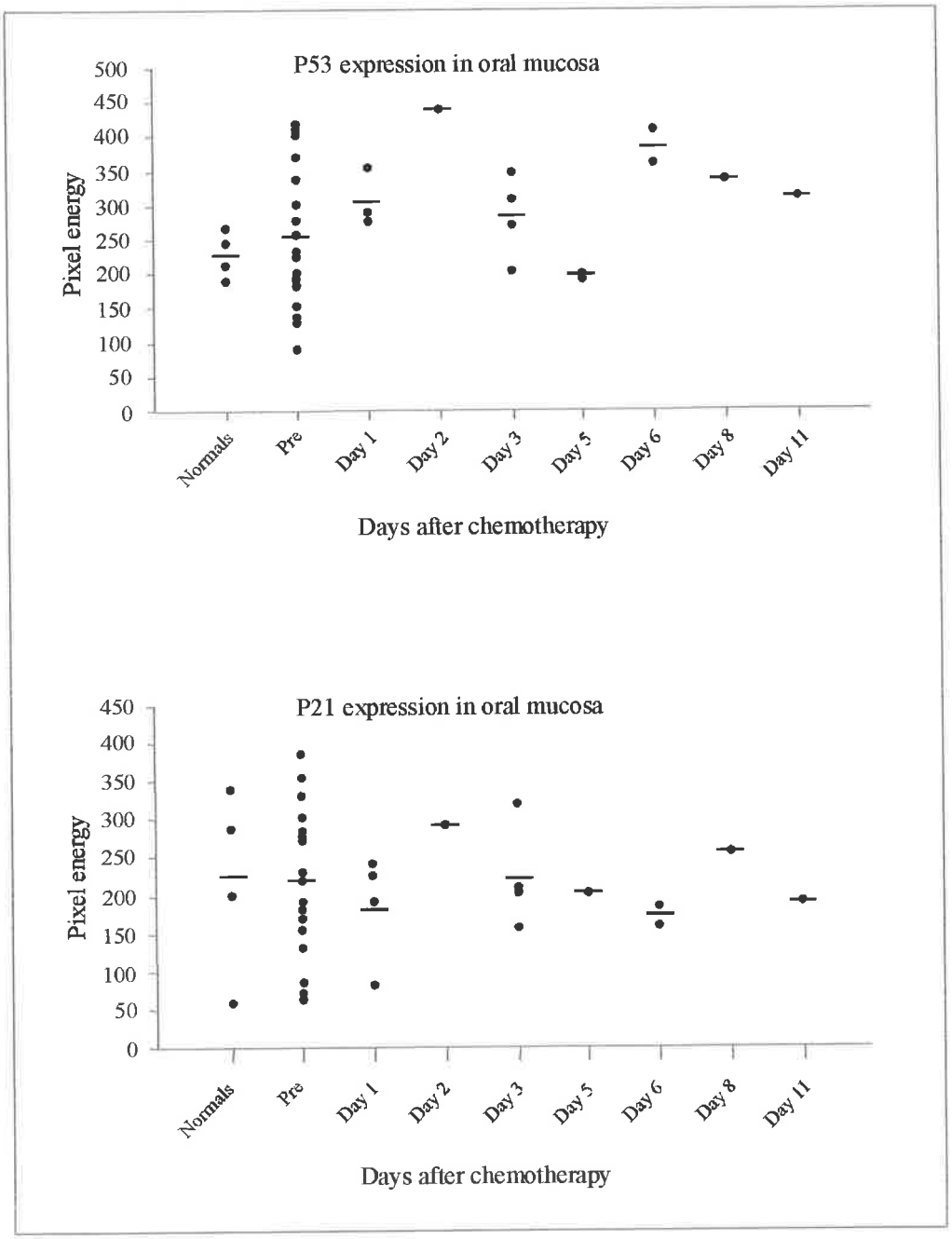


Figure 6.8 Expression of two transcription factors, p53 and p21, in human oral mucosa before and after chemotherapy treatment. There is no difference between normal controls and pre-chemotherapy biopsies. However, after treatment both show changes in expression that had not returned to normal levels by day 11 following treatment.

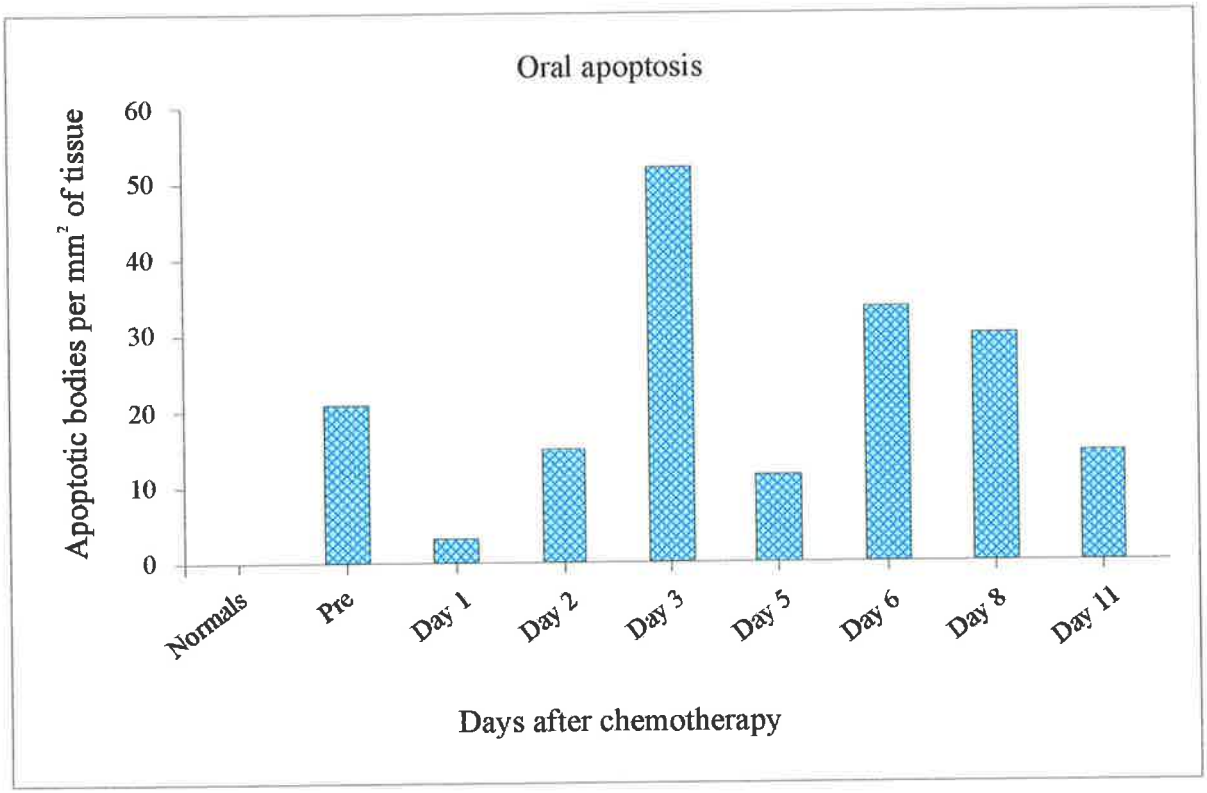


Figure 6.9 Levels of apoptosis in the oral mucosa were examined pre and post chemotherapy. Normal controls showed very little apoptosis (0.125 per mm2 of tissue - too small to see on graph), whereas all patients treated with chemotherapy had elevated levels, which had not returned to normal by day 11 following treatment.

6.4 Discussion

Oral mucositis as a condition was first described approximately 18 years ago and was used to describe the oral mucosal damage that was caused by both chemotherapy and radiotherapy (64). Like the remainder of the gastrointestinal tract, the oral mucosa has epithelial cells which rapidly divide and mature, causing it to be highly sensitive to the cytotoxic effects of the chemotherapy (64). Whilst there has been research reported on the treatment and prevention options of oral mucositis after chemotherapy (62, 66) and on possible preventative agents, very little has been reported on the mechanisms behind oral mucositis. This is the first time that anyone has looked at the histological and ultrastructural changes that occur in the oral mucosa at early time points following chemotherapy.

This study examined both the histological and ultrastructural changes that occurred post chemotherapy with a view to better understanding the mechanisms behind oral mucositis. The histopathology reports showed there was no change to the mucosa reported, other than a mild inflammatory change in some but not all subjects. This was not expected, as it has been shown in the gastrointestinal tract that architectural changes are observed at the light microscopical level (2, 3). However when the oral mucosa was examined ultrastructurally it was evident that there were cellular changes that were not seen at the light microscopical level.

The present study has demonstrated that, following chemotherapy, ultrastructural changes occur early and persist for at least 11 days following treatment. However it is more than likely that they persist for much longer than this. The earliest change observed was an increase in the number of intercellular filaments within the cells of the basal and suprabasal region. These increases in filaments were observed over all times examined; however they began to decrease after day 5 post treatment. It is known that intercellular filaments, in particular tonofilaments, provide structural support to the cells. Their increase following cytotoxic injury may suggest that the cells are trying to 'protect' themselves against damage by increasing the amount of structural support. This would explain why they remained increased over all times examined. The basal and suprabasal cells started to have vacuoles present within their cytoplasm at day 1 and these increased over time. This observation is highly suggestive of a role of endocytosis, which would be expected with increased apoptosis levels.

An unexpected finding from this study was the observation that all pre-chemotherapy biopsies showed ultrastructural changes different to those seen in the normal controls. All but one of our recruited subjects to this study had undergone previous cycles of chemotherapy meaning that they were not chemotherapy naïve. The ultrastructural changes that we observed in these pre chemotherapy biopsies indicate that the oral mucosa takes longer than the 21 days between cycles of chemotherapy to heal. Therefore following the initial cycle of chemotherapy, all subsequent cycles are administered to tissue that may not have fully recovered, initiating further cellular changes.

The present study also confirmed that like the small intestine, apoptosis occurs as a result of chemotherapy in the oral mucosa. Using transmission electron microscopy, early stages of apoptosis were observed at both day 1 (with nuclear separation) and day 2 (with pyknotic nuclei) but these were not observed at later times. The finding of apoptosis in the oral mucosa was able to be confirmed using the TUNEL assay. Apoptosis increased following administration of cytotoxic chemotherapy, reaching a maximum at day 3 post treatment. In order to determine if the apoptosis that was observed was p53-dependent results from p53 and TUNEL were correlated. This correlation showed a similar pattern for both, with increasing expression from day 1, peaking at day three before decreasing again, suggesting the apoptosis observed in this study is related to the expression of p53. These findings support previous research into the mechanisms behind the development of small intestinal mucositis (2, 3). Therefore the results from the present study confirm the hypothesis that, as the entire gastrointestinal tract has the same embryological route of development, with differences only being due to specialised functions, the development of mucositis will follow a similar pattern (7).

Changes in pro- and anti-apoptotic protein expression were identified both pre and post chemotherapy, when compared to normal controls. Normal expression was relatively consistent between the four normal volunteers for both pro- and anti-apoptotic expression as well as the two transcription factors and apoptosis investigated in the present study. However, an unexpected result from this study was the change from this normal expression in pre chemotherapy biopsies. For the majority of proteins investigated, there was an increase in expression; the only exception to this was BID where a decrease was observed. As all but one of the subjects that were enrolled for this study had undergone previous cycles of chemotherapy 21 days prior to this pre chemotherapy biopsy being taken, it may be that these changes represent a late healing phase of oral mucosal damage. Post chemotherapy biopsies also showed a change in expression of all proteins examined from

normal controls, and these levels had not returned to normal levels by day 11. As previous reports have suggested that gastrointestinal damage (mucositis) is mediated by a change in ratio of pro- to anti-apoptotic proteins (154, 155), it was hoped that the same change would occur in human oral mucositis. However, due to the highly variable response between subjects this was unable to be confirmed in the present study. It is planned for this study to continue, and with more subjects enrolled, will give a better understanding of the changes that occur in the pro- and anti-apoptotic protein ratio post chemotherapy.

In conclusion, this study has shown that, following chemotherapy, changes occur early and that these changes are similar to those seen in the small intestine.

General Discussion

7.1 Introduction

Mucositis is recognised as a major clinical problem; however the underlying mechanisms behind the condition remain poorly defined. Several studies over the last decade have made important contributions (1, 2, 67), however much of the detailed histology, ultrastructure and protein changes which lead to the development of mucositis have yet to be described. The studies contained within this thesis utilised an established animal model for the study of mucositis, as well as the human clinical setting, to provide detailed descriptions of the underlying mechanisms behind gastrointestinal mucositis. This was achieved using a myriad of techniques including routine histology, transmission electron microscopy, intestinal morphometry, TUNEL detection of apoptosis and quantitative immunohistochemistry.

7.2 The Gastrointestinal Tract and Cytotoxic Agents

The gastrointestinal tract extends from the mouth through to the anus and contains some of the fastest proliferating cells in the body (9, 10) with each region of the tract being highly specialised for specific functions. All regions of the gastrointestinal tract can be affected by cytotoxic agents such as chemotherapy and radiotherapy, leading to mucositis. Gastrointestinal damage as a result of radiotherapy was first described over 100 years ago (170), and since then has been well characterised with the mechanisms underlying the condition better understood than those underlying chemotherapy-induced mucositis. For this thesis, gastrointestinal side effects caused by chemotherapy were the focus, and more detail on the side effects caused by radiotherapy are available in review articles (171-173).

Studies to date have focused on oral mucositis (62, 66, 67), with the remainder of the tract, from the oesophagus to the colon, receiving little attention (1). The main reason proposed for this imbalance is the difficulty in accessing these sites, combined with the differences in tissue types between the varying regions (8). The little research that has been conducted on the remainder of the tract, combined with the more extensive knowledge of the oral cavity, has provided evidence of similarities in the development of mucositis. This has led to the recent development of an hypothesis of mucositis which proposes the development will be the same throughout the gastrointestinal tract (7, 8). The results presented in this thesis have added further evidence to support this hypothesis.

7.3 Oral Cavity

There have been many reports on possible treatment and prevention options for oral mucositis after chemotherapy (62, 66, 69, 77, 78, 174-178), however very little research exists on the underlying mechanisms. One hypothesis for mucositis advocated by Sonis and co-workers states that mucositis occurs in four phases: inflammatory/vascular, epithelial, ulcerative/microbiological and healing phases, with each phase assumed to be independent of the others (4, 65, 67, 69, 70). The present study in this thesis found that 30% of subjects enrolled, had mild inflammatory changes occurring in the oral mucosa early after chemotherapy. This observation agrees in part with the hypothesis proposed by Sonis (1998), which states the first phase in the development of mucositis is inflammatory (4, 67). However, the present study demonstrated the remaining 70% of subjects, had relatively normal histopathology reports, providing evidence that not all phases of the proposed hypothesis occur in all subjects. Previous studies has suggested that changes in pro-inflammatory cytokines, such as tumour necrosis factor α, are involved in this initial inflammatory response (4, 67, 179), however this study did not investigate these cytokines and so was unable to confirm if this occurs. Given that there was evidence of inflammatory change in 30% of subjects in this study, the next logical step would be to examine pro-inflammatory cytokines in order to prove or disprove the hypothesis.

This present study also differs from the work of Sonis in finding early induction of apoptosis and ultrastructural changes following chemotherapy despite 70% of subjects appearing to have relatively normal tissue histologically. These changes were observed from day 1 to day 11 following chemotherapy, and it is likely that these changes would have continued to be evident at later time points. In the first two days following chemotherapy, which correspond to the proposed inflammatory phase (4, 67, 179), the present study reported cell-cell junction disruption, in accordance with previous literature (4, 67, 179). However this study differed from previous literature (4, 67, 179) by demonstrating ultrastructural apoptosis at days 1 and 2, which was confirmed using the TUNEL assay. Early apoptosis at day 1 after chemotherapy has been previously reported in the small intestine (1, 2), and when considered in conjunction with the current results, provide evidence that the development of mucositis follows a similar pattern in all regions of the gastrointestinal tract (7, 8).

This study confirmed the presence of apoptosis in the oral mucosa following chemotherapy. As there have been previous reports which suggest that changes in pro- and

anti-apoptotic protein expression may be responsible for the development of apoptosis (31, 149-153), expression of these proteins were examined in this study. Changes in both pro- and anti-apoptotic proteins were identified in biopsies taken both prior to, and following chemotherapy, when compared to healthy volunteers. This part, like the ultrastructural study, highlighted changes in protein expression in pre chemotherapy biopsies and it is proposed that these changes are due to previous exposure to chemotherapy. Post chemotherapy biopsies also showed a change in expression of all proteins examined from healthy volunteers, and these levels had not returned to normal by day 11. Previous reports have suggested that gastrointestinal mucositis is mediated by a change in ratio of pro- to anti-apoptotic proteins (154, 155). It was anticipated that the same changes would occur in oral mucositis, adding further weight to the hypothesis that mucositis throughout the gastrointestinal tract has a similar development (7, 8). However, due to the highly variable response between subjects this was unable to be confirmed in the present study. It is hoped that this study will continue, and with more subjects enrolled, may give a better understanding of the changes that occur in the pro- and anti-apoptotic protein ratio post chemotherapy.

Current literature suggests that the final phase of oral mucositis is the healing phase whereby the oral mucosa returns to normal (4, 67, 179), however the results from the present study suggest that the duration of the healing phase is poorly defined. Patients in the current study that had undergone previous cycles of chemotherapy 21 days prior to participation, continued to exhibit ultrastructural damage to the mucosa when compared to healthy volunteers. This suggests that the oral mucosa takes longer than the 21 days between cycles of chemotherapy to heal. Further studies are now warranted to determine when, or indeed if, the oral mucosa ever completely heals, with biopsies being taken at varying time points following completion of treatment.

7.4 Oesophagus

Anatomically, the oesophagus extends from the upper oesophageal sphincter to the lower oesophageal sphincter, these sphincters delineating it from the oropharynx and the stomach respectively (92, 93). Very little information exists on oesophageal mucositis (induced by chemotherapy) other than reports that many subjects who undergo chemotherapy develop some form of oesophagitis (8). Symptoms are often misdiagnosed as gastro-oesophageal reflux disease, *Candida* oesophagitis, cytomegalovirus oesophagitis, or oesophagitis induced by herpes virus (98). The present study aimed to further the understanding of

mucositis in this part of the gastrointestinal tract, by providing a baseline of normal expression of pro- and anti-apoptotic proteins. These results will allow further studies to be conducted in subjects undergoing chemotherapy and will allow the change in expression in these proteins to be determined. Although previous studies have investigated pro- and anti-apoptotic proteins within the oesophagus (164), they have relied heavily on autopsy samples which may have different expression profiles from live patients. The results from this study have shown that expression of the majority of pro- and anti-apoptotic protein were significantly (and consistently) lower in the oesophagus than all other regions. Further studies can now be conducted in cancer subjects to see if these levels change following chemotherapy treatment.

7.5 Stomach

Like the oesophagus, very little research has been published on the mechanisms and development of mucositis in the stomach (7, 8). As in the oesophagus, this thesis aimed to determine normal levels of expression of pro- and anti-apoptotic proteins in the human, thus allowing future studies to determine the expressional changes following chemotherapy. In addition, this study aimed to determine whether *H. pylori* affected expression levels. Despite previous studies having suggested that *H. pylori* may alter expression of the transcription factors, p53 and p21 (167-169), the present study did not show this. Of the 50 subjects that were recruited for this study only 16% tested positive for *H. pylori.*, however approximately 30% of the general population carry this bacteria and it may be that the sample size of the present study was too small to detect any significant change. In addition, it is known that *H. pylori* is the main cause of chronic gastritis (167, 168), and it may be that the subjects recruited to this study did not have chronic gastritis. Nevertheless this study was able to construct a baseline of expression for many pro- and anti-apoptotic proteins and transcription factors, and further studies in subjects with chemotherapy can now be conducted.

7.6 Small Intestine

The small intestine has been the focus of many previous studies on chemotherapy-induced mucositis (1, 2, 19, 36, 38, 99, 180). These studies have demonstrated that apoptosis is an early response to chemotherapy (1, 2, 5, 6), with some studies confirming that apoptosis leads to crypt hypoproliferation and the conclusion that the two are directly related to mucositis (1, 2). This thesis aimed to build on this theory and investigated the changes that

occur in the rat small intestine following administration of two different cytotoxic agents. Furthermore, this thesis investigated the expression of normal levels of pro- and anti-apoptotic protein expression in the clinical setting, in order to provide a baseline data for comparison with future research in cancer subjects.

Following on from previous research using MTX (5, 6, 51), results presented in this thesis defined the role of apoptosis in the development of mucositis in the rat small intestine. This study confirmed, that unlike the human intestine, where apoptosis precedes crypt hypoproliferation and subsequent mucositis (1, 2), the relationship in the rat is not always as clear and is dependent upon the dose of chemotherapy. It was found that low dose MTX (0.5 mg/kg) causes a high level of apoptosis, minimal crypt cell hypoproliferation and no loss of tissue architecture; these results are similar to those described by Pritchard and colleagues (39). Furthermore, the present study attempted to determine whether changes in transcription factor expression were responsible for this apparent discrepancy. It was found that a change in p53 expression did not precede the high levels of apoptosis at 6 hours, but an increase in p53 expression at 24 hours may be associated with apoptosis. However, this study could not conclusively determine whether this latter increase in apoptosis was dependent on expression of p53, due to the dual dose of chemotherapy administered. Further studies are warranted using a single dose of chemotherapy to determine if this can detect changes in transcription factor expression before relationships between p53 expression and apoptosis can be confirmed in this model.

This thesis also clearly demonstrated that different chemotherapy agents act in the same way in causing mucositis in the small intestine. Irinotecan is a chemotherapeutic agent which is known affect the large intestine as it causes severe diarrhoea (54, 56, 57, 60, 181). However, the effect of this cytotoxic agent on the small intestine was previously unknown. It was found that apoptosis and crypt cell hypoproliferation in the small intestine developed in a similar fashion to MTX.

Expressions of pro- and anti-apoptotic proteins in the normal human small intestine were also the subject of investigation in this thesis. The majority of previous work on these proteins have either been investigated in animal models (154, 155) or human autopsy samples (161-165), with few studies investigating expression from fresh samples (182). This thesis aimed to determine levels in normal subjects in order to provide a baseline for future research. Unlike other studies (154, 155, 161-165), results presented here suggest that in tissue from "normal" subjects, there is no difference between pro- and anti-

apoptotic protein expression, with levels of both remaining consistent. This suggests that in the absence of a stress, such as chemotherapy, levels of these proteins remained balanced. When there is "stress" placed on the system, an imbalance may be created thus enabling either apoptosis or repair.

7.7 Large Intestine

The large intestine has often been overlooked, despite diarrhoea being a major side effect of cancer chemotherapy. Much of the research has focused on the small intestine due to the higher rates of apoptosis (17, 24). Results presented in this thesis in the rat colon showed irinotecan induced severe damage and a high level of apoptosis, which resulted in severe loss of tissue integrity. This differed from other studies (chapter 2) in this thesis using methotrexate, where apoptosis was seen following chemotherapy, however there was no accompanying loss of tissue integrity.

When normal human colon was examined for expression of pro- and anti-apoptotic proteins, like the other parts of the gastrointestinal tract, no significant differences were seen between the majority of proteins examined. Unlike other literature (154, 155, 161-165), results presented here suggest that in tissue from "normal" subjects, there is no difference between pro- and anti-apoptotic protein expression, with levels of both remaining consistent. This suggests that in the absence of a stress, such as chemotherapy, levels of these proteins remained balanced. When there is "stress" placed on the system, an imbalance may be created thus enabling either apoptosis or repair.

7.8 Rectum

Like in the oesophagus and stomach, very little research has been conducted on the effect of cancer chemotherapy on the rectum. This thesis assessed the expression of pro- and anti-apoptotic proteins in normal human subjects and in keeping with the majority of all other regions of the tract, found no significant imbalance between them. Further clinical investigations into the protein changes following chemotherapy can now be conducted in cancer subjects.

7.9 Potential Anti-Mucotoxics

IL-11 is a potential anti-mucotoxic cytokine that has already been the focus of much research into the protection and recovery of stem cells found within the haematopoietic system (117, 124, 126, 141, 183). Previous animal studies investigating the effects of IL-

11 have found that this potential anti-mucotoxic cytokine aided in gut recovery, caused increased gut proliferation and reduced the incidence of apoptosis (75, 123, 143), however none of these studies utilised an animal model with tumours. The present study extended this research by using the laboratory rat with syngeneic breast cancer to study the effect of IL-11 on preventing intestinal damage. The advantages of this model in comparison to previous studies were that it controlled for any systemic effect of cancer on intestinal response, and allowed evaluation of any effect of IL-11 in preventing the anti-tumour effect of MTX. Results from this study clearly showed that IL-11 did not protect the breast cancer from MTX, nor alter the MTX-induced reduction in tumour weight, proliferation or apoptosis. Moreover, IL-11 had no trophic effect on growth of breast cancer and therefore does not compromise effect of MTX on the tumour.

In this thesis, mucositis was assessed in the small intestine and it was shown that unlike in previous studies (123, 143), there was no reduction in apoptosis, rather there was an increase. However, like in other studies, the increased apoptosis did not cause increased intestinal damage, rather these measures were improved. Thus, this research adds further evidence to results presented in Chapter 2 whereby apoptosis itself, does not inevitably lead onto mucositis. Pritchard and colleagues found that apoptosis was not directly associated with intestinal damage after chemotherapy. It is therefore suggested that IL-11 may down-regulate the transcription factor p53 and prevent cytostasis, which could explain the finding of the present study that IL-11 allowed relative crypt proliferation to proceed even though MTX caused hypo-proliferation. This would have ameliorated the villus atrophy observed. Preliminary studies (Bowen, *et al.*, unpublished results) conducted by others in our research laboratory have begun to investigate the role that p53 may play in response to IL-11 administration.

Despite literature and this study providing clear evidence which suggests that IL-11 is beneficial in ameliorating the side effects of cancer chemotherapy (75, 117, 123, 142, 143), the exact mechanism by which it acts remains unclear. Current literature suggests that IL-11 belongs to a family of cytokines, which act through a common glycoprotein, gp130 (146, 147). The expression of the IL-11 receptor within the gastrointestinal tract has not been fully elucidated; however this may have implications for understanding the mechanisms of action of IL-11. Further studies need to be conducted in order to fully determine the role that this receptor plays.

7.10 Future Directions

Chemotherapy-induced gastrointestinal mucositis is an emerging field of supportive care in cancer research. The research presented in this thesis has aimed to fill some gaps in the knowledge. There are many potential anti-mucotoxics that are under investigation, including transforming growth factor-β (184), epidermal growth factor (74), nuclear factor kappa B (185), whey growth factor (186), and glutamine (187, 188) just to name a few. Currently in our laboratory we are investigating the role that another potential anti-mucotoxic, keratinocyte growth factor, plays in ameliorating MTX (53) and irinotecan induced mucositis. In addition to potential anti-mucotoxics, further work utilising microarray technology to investigate gene changes following chemotherapy is an emerging and exciting technique, and will hopefully provide a clearer understanding of the development of gastrointestinal mucositis

7.11 Conclusions

This thesis has provided clear evidence that the entire gastrointestinal tract follows a similar pattern of development of mucositis. Results presented show that different chemotherapeutic agents act in a similar manner in causing small intestinal mucositis. This is an important finding for the development and targeting of appropriate preventative strategies, such as IL-11 which may play an important role in ameliorating the symptoms of mucositis. Apoptosis was also found to have a key role in mucositis and was detected in the oral cavity, small intestine and large intestine following chemotherapy. Although apoptosis was present following administration of chemotherapy, there was not always histological evidence of mucositis. In conclusion, mucositis is a complex side effect of cancer treatment, and this research has increased our understanding of its mechanisms of development.

References

1. Keefe, DMK (1998). The effect of cytotoxic chemotherapy on the mucosa of the small intestine. Department of Medicine, University of Adelaide.

2. Keefe, DM, Brealey, J, Goland, GJ and Cummins, AG (2000). Chemotherapy for cancer causes apoptosis that precedes hypoplasia in crypts of the small intestine in humans. *Gut*, 47: 632-7.

3. Keefe, DM, Cummins, AG, Dale, BM, Kotasek, D, Robb, TA and Sage, RE (1997). Effect of high-dose chemotherapy on intestinal permeability in humans. *Clinical Science*, 92: 385-9.

4. Pico, J-L, Avila-Garavito, A and Naccache, P (1998). Mucositis: its occurrence, consequences, and treatment in the oncology setting. *Oncologist*, 3: 446-451.

5. Trier, JS (1962). Morphological alterations induced by methotrexate in the mucosa of human proximal intestine I. Serial observations by light microscopy. *Gastroenterology*, 42: 295-305.

6. Trier, JS (1962). Morphologic alterations induced by methotrexate in the mucosa of human proximal intestine II. Electron microscopic observations. *Gastroenterology*, 43: 407-424.

7. Keefe, DM (2003). Gastrointestinal mucositis: a new biological model. *Support Care Cancer*, .

8. Keefe, DM, Gibson, RJ and Hauer-Jenson, M (2004). Gastrointestinal Mucositis. *Seminars in Oncology Nursing*, 20: 38-47.

9. Potten, CS (1990). A comprehensive study of the radiobiological response of the murine (BDF1) small intestine. *International Journal of Radiation Biology*, 58: 925-973.

10. Tortora, GJ and Grabowski, SR (1993). Principles of Anatomy and Physiology. HarperCollins, New York.

11. Burkitt, HG, Young, B and Heath, JW (1993). Oral Tissues. Burkitt, HG, Young, B and Heath, JW, Wheater's Functional Histology A text and colour atlas, Churchill Livingstone, New York, 235-246.

12. Squier, CA and Hill, MW (1994). Oral Mucosa. Ten Cate, AR, Oral Histology Development, Structure and Function, Mosby-Year Book Inc, St Louis, 389-431.

13. Wright, NA (1997). Stem cell repertoire in the intestine. Potten, CS, Stem Cells, Academic Press, London, 315-330.

14. Bach, SP, Renehan, AG and Potten, CS (2000). Stem cells: the intestinal stem cell as a paradigm. *Carcinogenesis*, 21: 469.

15. Potten, CS, Wilson, JW and Booth, C (1997). Regulation and significance of apoptosis in the stem cells of the gastrointestinal epithelium. *Stem Cells*, 15: 82-93.

16. Potten, CS and Loeffler, M (1990). Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. *Development - Supplement*, 110: 1001-20.

17. Potten, CS, Booth, C and Pritchard, DM (1997). The intestinal epithelial stem cell: the mucosal governor. *International Journal of Experimental Pathology*, 78: 219-43.

18. Paulus, U, Potten, CS and Loeffler, M (1992). A model for the control of cellular regeneration in the intestinal crypt after perturbation based solely in local stem cell regulation. *Cell Proliferation*, 25: 559-578.

19. Potten, CS and Loeffler, M (1987). A comprehensive model of the crypts of the small intestine of the mouse provides insight into the mechanisms of cell migration and the proliferation hierarchy. *Journal of Theoretical Biology*, 127: 381-91.

20. Loeffler, M, Stein, R, Wichmann, HE, Potten, CS, Kaur, P and Chwalinski, S (1986). Intestinal cell proliferation. I. A comprehensive model of steady-state proliferation in the crypt. *Cell & Tissue Kinetics*, 19: 627-45.

21. Potten, CS, Taylor, Y and Hendry, JH (1988). The doubling time of regenerating clonogenic cells in the crypts of the irradiated mouse small intestine. *International Journal of Radiation Biology*, 54: 1041-51.

22. Hendry, JH, Potten, CS, Ghafoor, A, Moore, JV, Roberts, SA and Williams, PC (1989). The response of murine intestinal crypts to short-range promethium-147 beta irradiation: deductions concerning clonogenic cell numbers and positions. *Radiation Research*, 118: 364-74.

23. Potten, CS, Owen, G, Hewitt, D, Chadwick, CA, Hendry, H, Lord, BI and Woolford, LB (1995). Stimulation and inhibition of proliferation in the small intestinal crypts of the mouse after in vivo administration of growth factors. *Gut*, 36: 864-873.

24. Potten, CS (1998). Stem cells in gastrointestinal epithelium: numbers, characteristics and death. *Philosophical Transactions of the Royal Society of London - Series B: Biological Sciences*, 353: 821-830.

25. De Vita Jr, V (1997). Principals of cancer management: Chemotherapy. De Vita Jr, V, Cancer: Principles and Practice of Oncology, Lippincott-Raven, Philadelphia, 333-347.

26. Kaufmann, SH (1989). Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: a cautionary note. *Cancer Research*, 49: 5870-8.

27. Walker, PR, Smith, C, Youdale, T, Leblanc, J, Whitfield, JF and Sikorska, M (1991). Topoisomerase II-reactive chemotherapeutic drugs induce apoptosis in thymocytes. *Cancer Research*, 51: 1078-1085.

28. Havrilesky, LJ, Elbendary, A, Hurteau, JA, Whitaker, RS, Rodriguez, GC and Berchuck, A (1995). Chemotherapy-induced apoptosis in epithelial ovarian cancers. *Obstetrics and Gynecology*, 85: 1007-1010.

29. Huschtscha, LI, Bartier, WA, Andersson Ross, CE and Tattersall, MHN (1996). Characteristics of cancer cell death after exposure to cytotoxic drugs *in vitro*. *British Journal of Cancer*, 73: 54-60.

30. Hannum, YA (1997). Apoptosis and the dilemma of cancer chemotherapy. *Blood*, 89: 1845-4853.

31. Kerr, JFR, Winterford, CM and Harmon, BV (1994). Apoptosis: its significance in cancer and cancer therapy. *Cancer*, 73: 2013-2026.

32. D'Amico, AV and McKenna, WG (1994). Apoptosis and a re-investigations of the biologic basis for cancer therapy. *Radiotherapy and Oncology*, 33: 3-10.

33. Schmitt, CA and Lowe, SW (1999). Apoptosis and therapy. *Journal of Pathology*, 187: 127-137.

34. Reed, JC (2000). Mechanisms of apoptosis. *American Journal of Pathology*, 157: 1415-1430.

35. Potten, CS (1997). Epithelial cell growth and differentiation II. Intestinal apoptosis. *American Journal of Physiology*, G253-G257.

36. Ijiri, K and Potten, CS (1983). Response of intestinal cells of differing topographical and hierarchical status to ten cytotoxic drugs and five sources of radiation. *British Journal of Cancer*, 47: 175-85.

37. Ijiri, K and Potten, CS (1987). Further studies on the response of intestinal crypt cells of different hierarchical status to eighteen different cytotoxic agents. *British Journal of Cancer*, 55: 113-23.

38. Ijiri, K and Potten, CS (1990). The circadian rhythm for the number and sensitivity of radiation-induced apoptosis in the crypts of mouse small intestine. *International Journal of Radiation Biology*, 58: 165-75.

39. Pritchard, DM, Potten, CS and Hickman, JA (1998). The relationships between p53-dependent apoptosis, inhibition of proliferation, and 5-fluorouracil-induced histopathology in murine intestinal epithelia. *Cancer Research*, 58: 5453-65.

40. el-Deiry, WS, Tokino, T, Velculescu, VE, Levy, DB, Parsons, R, Trent, JM, Lin, D, Mercer, WE, Kinzler, KW and Vogelstein, B (1993). WAF1, a potential mediator of p53 tumor suppression. *Cell*, 75: 817-25.

41. Wilson, JW, Pritchard, DM, Hickman, JA and Potten, CS (1998). Radiation-induced p53 and $^{p21WAF-1CIP-1}$ expression in the murine intestinal epithelium. *American Journal of Pathology*, 153: 899-909.

42. Bilim, V, Kawasaki, T, Takahashi, K and Tomita, Y (2000). Adriamycin induced G2/M cell cycle arrest in transitional cell cancer cells with wt p53 and $p21^{WAF1/CIP1}$ genes. *Journal of Experimental and Clinical Cancer Research*, 19: 483-488.

43. Deng, C, Zhang, P, Harper, JW, Elledge, SJ and Leder, P (1995). Mice lacing $p21^{cip1/waf1}$ undergo normal development but are defective in G1 checkpoint control. *Cell*, 82: 675-684.

44. Clarke, AR, Howard, LA, Harrison, DJ and Winton, DJ (1997). p53, mutation frequency and apoptosis in the murine small intestine. *Oncogene*, 14: 2015-2018.

45. Merritt, AJ, Potten, CS, Kemp, CJ, Hickman, JA, Balmain, A, Lane, DP and Hall, PA (1994). The role of p53 in spontaneous and radiation-induced apoptosis in the gastrointestinal tract of normal and p53-deficient mice. *Cancer Research*, 54: 614-7.

46. Donehower, LA, Harvey, M, Slagle, BL, McArthur, MJ, Montogomery Jr, CA, Butel, JS and Bradley, A (1992). Mice deficient fo p53 are developmentally normal but susceptible to spontaneous tumours. *Nature*, 356: 215-221.

47. Potten, CS (2001). Apoptosis in oral mucosa: lessons from the crypt. A commentary. *Oral Diseases*, 7: 81-85.

48. Merritt, AJ, Potten, CS, Watson, AJ, Loh, DY, Nakayama, K and Hickman, JA (1995). Differential expression of bcl-2 in intestinal epithelia. Correlation with attenuation of apoptosis in colonic crypts and the incidence of colonic neoplasia. *Journal of Cell Science*, 108: 2261-71.

49. Huennekens, FM (1994). The methotrexate story: a paradigm for development of cancer chemotherapeutic agents. *Adv Enzyme Regul*, 34: 397-419.

50. Eichholtz, H and Trott, KR (1980). Effect of methotrexate concentration and exposure time on mammalian cell survival in vitro. *Br J Cancer*, 41: 277-84.

51. Taminiau, JAJM, Gall, DG and Hamilton, JR (1980). Response of the rat small-intestine epithelium to methotrexate. *Gut*, 21: 486-492.

52. Gibson, RJ, Keefe, DMK, Thompson, FM, Clarke, JM, Goland, GJ and Cummins, AG (2002). Effect of interleukin-11 on ameliorating intestinal damage after methotrexate treatment of breast cancer in rats. *Digestive Diseases & Sciences*, 47: 2751-2757.

53. Gibson, RJ, Keefe, DMK, Clarke, JM, Regester, GO, Thompson, FM, Goland, GJ, Edwards, BE and Cummins, AG (2002). The effect of keratinocyte growth factor on tumour growth and small intestinal mucositis after chemotherapy in the rat with breast cancer. *Cancer Chemotherapy & Pharmacology*, 50: 53-58.

54. Araki, E, Ishikawa, M, Iigo, M, Koide, T, Itabashi, M and Hoshi, A (1993). Relationship between development of diarrhea and the concentration of SN-38, and active metabolite of CPT-11, in the intestine and the blood plasma of athymic mice following intraperitoneal administration of CPT-11. *Japanese Journal of Cancer Research*, 84: 697-702.

55. Cao, S, Black, JD, Troutt, AB and Rustum, YM (1998). Interleukin 15 offers selective protection from Irinotecan-induced intestinal toxicity in a preclinical animal model. *Cancer Research*, 58: 3270-3274.

56. Ikuno, N, Soda, H, Watanabe, M and Oka, M (1995). Irinotecan (CPT-11) and characteristic changes in the mouse ileum and cecum. *Journal of the National Cancer Institute*, 87: 1876-1883.

57. Takasuna, K, T., H, Hirohashi, M, Kato, M, Nomura, M, Nagai, E, Yokoi, T and Kamataki, T (1996). Involvement of B-Glucuronidase in intestinal microflora in the intestinal toxicity of the antitumor camptothecin derivative irinotecan hydrochloride (CPT-11) in rats. *Cancer Research*, 56: 3752-3757.

58. Abigerges, D, Armand, JP, Chabot, GG, DaCosta, L, Fadel, E, Cote, C, Heraot, P and Gandia, D (1994). Irinotecan (CPT-11) high-dose escalation using intensive high-dose loperamide to control diarrhoea. *Journal of the National Cancer Institute*, 86: 446-449.

59. Masuda, N, Fukuoka, M, Kudoh, S, Kusunoki, Y, Matsui, K, Nakagawa, K, Hirashima, T, Tamanoi, M, Nitta, T, Yana, T, Negoro, S, Takifuji, N and Takada, M (1994). Phase I study of irinotecan and cisplatin with granulocyte colony-stimulating factor support for advanced non-small-cell lung cancer. *Journal of Clinical Oncology*, 12: 90-96.

60. Cao, S, Troutt, AB and Rustum, YM (1998). Interleukin 15 protects against toxicity and potentiates against antitumor activity of 5-flououracil alone and in combination with leucovorin in rats bearing colorectal cancer. *Cancer Research*, 58: 1695-1699.

61. Reinecker, HC, MacDermott, RP, Mirau, S, Dignass, A and Podolsky, DK (1996). Intestinal epithelial cells both express and respond to interleukin 15. *Gastroenterology*, 111: 1706-1713.

62. Kostler, WJ, Hejna, M, Wenzel, C and Zielinski, CC (2001). Oral mucositis complicating chemotherapy and/or radiotherapy: options for prevention and treatment. *Ca: a Cancer Journal for Clinicians*, 51: 290-315.

63. Peterson, DE (1999). Research advances in oral mucositis. *Current Opinion in Oncology*, 11: 261-266.

64. Serdar Turhal, N, Erdal, S and Karacay, S (2000). Efficacy of treatment to relieve mucositis-induced discomfort. *Support Care Cancer*, 8: .

65. Biron, P, Sebban, C, Gourmet, R, Chvetzoof, G, Philip, I and Blay, JY (2000). Research controversies in management of oral mucositis. *Support Care Cancer*, 8: 68-71.

66. Wilkes, JD (1998). Prevention and treatment of oral mucositis following cancer chemotherapy. *Seminars in Oncology*, 25: 538-551.

67. Sonis, ST (1998). Mucositis as a biological process: a new hypothesis for the development of chemotherapy-induced stomatotoxicity. *Oral Oncology*, 34: 39-43.

68. Stiff, P (2001). Mucositis associated with stem cell transplantation: current status and innovative approaches to management. *Bone Marrow Transplantation*, 27: S3-S11.

69. Sonis, ST, Lindquist, L, Van Vugt, A, Stewart, AA, Stam, K, Qu, GY, Iwata, KK and Haley, JD (1994). Prevention of chemotherapy-induced ulcerative mucositis by transforming growth factor beta 3. *Cancer Research*, 54: 1135-8.

70. Cope, PO, DeMeo, M and Stiff, P (1997). Prophylactic enteral support to BMT patients reduces length of hospital stay, improves GI integrity and nutritional status, and reduces intake requirements required for positive outcome. *Proceedings of ASCO*, 16: 218a.

71. Sonis, A and Sonis, S (1979). Oral complications of cancer chemotherapy in pediatric patients. *Journal of Pedodontics*, 3: 122-8.

72. Herrstedt, J (2000). Prevention and management of mucositis in patients with cancer. *International Journal of Antimicrobial Agents*, 16: 161-163.

73. Sonis, ST, Tracey, C, Shklar, G, Jenson, J and Florine, D (1990). An animal model for mucositis induced by cancer chemotherapy. *Oral Surgery, Oral Medicine, Oral Pathology*, 69: 437-43.

74. Sonis, ST, Costa, JW, Jr., Evitts, SM, Lindquist, LE and Nicolson, M (1992). Effect of epidermal growth factor on ulcerative mucositis in hamsters that receive cancer chemotherapy. *Oral Surgery, Oral Medicine, Oral Pathology*, 74: 749-55.

75. Sonis, ST, Van Vugt, AG, McDonald, J, Dotoli, E, Schwertschlag, U, Szklut, P and Keith, J (1997). Mitigating effects of interleukin 11 on consecutive courses of 5-fluorouracil-induced ulcerative mucositis in hamsters. *Cytokine*, 9: 605-12.

76. Sonis, ST, Peterson, RL, Edwards, LJ, Lucey, CA, Wang, L, Mason, L, Login, G, Ymamkawa, M, Moses, G, Bouchard, P, Hayes, LL, Bedrosian, C and Dorner, AJ (2000). Defining mechanisms of action of interleukin-11 on the progression of radiation-induced oral mucositis in hamsters. *Oral Oncology*, 36: 373-81.

77. Sprinzl, GM, Galvan, O, de Vries, A, Ulmer, H, Gunkel, AR, Lukas, P and Thumfart, WF (2001). Local application of granulocyte-macrophage colony stimulating factor (GM-CSF) for the treatment of oral mucositis. *European Journal of Cancer*, 37: 2003-2009.

78. Berger, A, Henderson, M, Nadoolman, W, Duffy, V, Cooper, D, Saberski, L and Bartoshuk, L (1995). Oral capsaicin provides temporary relief for oral mucositis pain secondary to chemotherapy/radiation therapy. *Journal of Pain and Symptom Management*, 10: 243-248.

79. Shenep, JL, Kalwinsky, DK, Hutson, PR, George, SL, Dodge, RK, Blankenship, KR and Thornton, D (1988). Efficacy of oral sucralfate suspension in prevention and treatment of chemotherapy-induced mucositis. *Journal of Pediatrics*, 113: 758-763.

80. Allison, RR, Vongtama, V, Vaughan, J and Shin, KH (1995). Symptomatic acute mucositis can be minimized or prophykaxed by the combination of sucralfate and fluconazole. *Cancer Investigation*, 13: 16-22.

81. Duenas-Gonzalea, A, Sobrevilla-Calvo, P, Frias-Mendivil, M, Gallardo-Rindon, D, Lara-Medina, F, Aguilar-Ponce, L, Miranda-Lopez, E, Zinser-Sierra, J and Reynosa-Gomez, E (1996). Misoprostol prophylaxis for high-dose chemotherapy-induced mucositis: a randomized double-blind study. *Bone Marrow Transplantation*, 17: 809-812.

82. Dorr, W and Weber-Frisch, M (1995). Effect of changing weekly dose on accelerated repopulation during fractionated irradiation of mouse tongue mucosa. *International Journal of Radiation Biology*, 67: 577-85.

83. Dorr, W, Jacubek, A, Kummermehr, J, Herrmann, T, Dolling-Jochem, I and Eckelt, U (1995). Effects of stimulated repopulation on oral mucositis during conventional radiotherapy. *Radiotherapy & Oncology*, 37: 100-7.

84. Dorr, W and Weber-Frisch, M (1995). Repopulation response of mouse oral mucosa during unconventional radiotherapy protocols. *Radiotherapy & Oncology*, 37: 230-6.

85. Rocke, LK, Loprinzi, CL, Lee, JK, Kunselman, SJ, Iverson, RK, Finck, G, Lifsey, D, Glaw, KC, Stevens, BA and Hatfield, AK (1993). A randomized clinical trial of two different durations of oral cryotherapy for prevention of 5-fluorouracil-related stomatitis. *Cancer*, 72: 2234-8.

86. Mahood, DJ, Dose, AM, Loprinzi, CL, Veeder, MH, Athmann, LM, Therneau, TM, Sorensen, JM, Gainey, DK, Mailliard, JA, Gusa, NL, Finck, GK, Johnson, C and Goldberg, RM (1991). Inhibition of fluorouracil-induced stomatitis by oral cryotherapy. *Journal of Clinical Oncology*, 9: 449-452.

87. Loprinzi, CL and Dose, AM (1990). Studies on prevention of 5-fluorouracil-induced oral mucositis. *NCI Monographs*, 9: 93-94.

88. Garewal, HS, Meyskens Jr, FL, Killen, D, Reeves, D, Kiersch, TA, Elletson, H, Strosberg, A, King, D and Steinbronn, K (1990). Response of oral leukoplakia to beta-carotene. *Journal of Clinical Oncology*, 8: 1715-1720.

89. Mills, EED (1988). The modifying effect of beta-carotene on radiation and chemotherapy induced oral mucositis. *British Journal of Cancer*, 57: 416-417.

90. Sonis, ST, Van Vugt, AG, Brien, JP, Muska, AD, Bruskin, AM, Rose, A and Haley, JD (1997). Transforming growth factor-beta 3 mediated modulation of cell cycling and attenuation of 5-fluorouracil induced oral mucositis. *Oral Oncology*, 33: 47-54.

91. Wymenga, AN, van der Graaf, WT, Hofstra, LS, Spijkervet, FK, Timens, W, Timmer-Bosscha, H, Sluiter, WJ, van Buuren, AH, Mulder, NH and de Vries, EG (1999). Phase I study of transforming growth factor-beta3 mouthwashes for prevention of chemotherapy-induced mucositis. *Clinical Cancer Research*, 5: 1363-8.

92. Gavaghan, M (1999). Anatomy and physiology of the esophagus. *AORN J*, 69: 372-86.

93. Squier, CA and Kremer, MJ (2001). Biology of oral mucosa and esophagus. *Journal of the National Cancer Institute Monographs*, 29: 7-15.

94. Long, JD and Orlando, RC (1999). Esophageal submucosal glands: structure and function. *American Journal of Gastroenterology*, 94: 2818-2824.

95. Baker, DG (1982). The radiobiological basis for tissue reactions in the oral cavity following therapeutic x-irradiation. *Archives of Otolaryngology*, 108: 21-24.

96. Sonis, ST (1983). Epidemiology, frequency, distribution, mechanisms, and histopathology. Peterson, DE and Sonis, ST, Oral complications of cancer chemotherapy, M. Nijhof, The Hague, 1-12.

97. Squier, CA (1990). Mucosal alterations. *NCI Monographs*, 9: 169-172.

98. Maule, WF (1997). Gastrointestinal toxicity of chemotherapeutic agents. Perry, MC, The Chemotherapy Source Book, Williams and Wilkins, Baltimore, 697-707.

99. Slavin, RE, Dias, MA and Saral, R (1978). Cytosine arabinoside induced gastrointestinal toxic alterations in sequential chemotherapeutic protocols. *Cancer*, 42: 1747-1759.

100. Rofe, AM, Bourgeois, CS, Washington, JM, Philcox, JC and Coyle, P (1994). Metabolic consequences of methotrexate therapy in tumour-bearing rats. *Immunology and Cell Biology*, 72: 43-48.

101. Tomas, FM, Chandler, CS, Coyle, P, Bourgeois, CS, Burgoyne, JL and Rofe, AM (1994). Effects of insulin and insulin-like growth factors on protein and energy metabolism in tumour-bearing rats. *Journal of Biochemistry*, 301: 769-775.

102. Boyle, FM, Wheeler, HR and Shenfield, GM (1996). Glutamate ameliorates experimental vincristine neuropathy. *Journal of Pharmacology and Experimental Therapeutics*, 279: .

103. Booth, D and Potten, CS (2001). Protection against mucosal injury by growth factors and cytokines. *Journal of the National Cancer Institute Monographs*, 29: 16-20.

104. Paul, SR, Bennet, F, Calvetti, JA, Kelleher, KG, Wood, CR, R.M., OHJ, Leary, AC, Sibley, B, Clark, SC, Williams, DA and Yang, Y-C (1990). Molecular cloning of a cDNA encoding interleukin 11, a stromal cell-derived lymphopoietic and haematopoietic cytokine. *Proceedings of the National Academy of Sciences*, 87: 7512-7516.

105. Soda, H, Raymond, E, Sharma, S, Lawrence, R, Cerna, C, Gomez, L, Schaub, R, Von Hoff, DD and Izbicka, E (1999). Recombinant human interleukin-11 is unlikely to stimulate the growth of the most common solid tumors. 10: 97-101.

106. Yang, Y-C (1993). Interleukin-11: an overview. *Stem Cells*, 11: 474-86.

107. Tepler, I, Elias, L, Smith, JW, 2nd, Hussein, M, Rosen, G, Chang, AY, Moore, JO, Gordon, MS, Kuca, B, Beach, KJ, Loewy, JW, Garnick, MB and Kaye, JA (1996). A randomized placebo-controlled trial of recombinant human interleukin-11 in cancer patients with severe thrombocytopenia due to chemotherapy. *Blood*, 87: 3607-14.

108. Gordon, MS (1996). Thrombopoietic activity of recombinant human interleukin 11 in cancer patients receiving chemotherapy. *Cancer Chemotherapy & Pharmacology*, 38 Suppl: S96-8.

109. Gordon, MS, McCaskill-Stevens, WJ, Battiato, LA, Loewy, J, Loesch, D, Breeden, E, Hoffman, R, Beach, KJ, Kuca, B, Kaye, J and Sledge, GW, Jr. (1996). A phase I trial of recombinant human interleukin-11 (neumega rhIL-11 growth factor) in women with breast cancer receiving chemotherapy. *Blood*, 87: 3615-24.

110. Walmsley, M, Butler, DM, Marinova-Mutafchieva, L and Feldmann, M (1998). An anti-inflammatory role for interleukin-11 in an established murine collagen-induced arthritis. *Immunology*, 95: 31-37.

111. Sands, BE, Bank, S, Sninsky, CA, Robinson, M, Katz, S, Singleton, JW, Miner, PB, Safdi, MA, Galandiuk, S, Hanauer, SB, Varilek, GW, Buchman, AL, Rodgers, VD, Salzberg, B, Cai, B, Loewy, J, DeBruin, MF, Rogge, H, Shapiro, M and Schwertschlag, US (1999). Preliminary evaluation of safety and activity of recombinant human interleukin 11 in patients with active Crohn's disease. *Gastroenterology*, 117: 58-64.

112. Greenwood-Van Meerveld, B, Tyler, K and Keith Jr, JC (2000). Recombinant human interleukin-11 modulates ion transport and mucosal inflammation in the small intestine and colon. *Laboratory Investigation*, 80: 1269-1280.

113. Liu, Q, Du, XX, Schindel, DT, Yang, ZX, Rescorla, FJ, Williams, DA and Grosfield, JL (1996). Trophic effects of interleukin-11 in rats with experimental short bowel syndrome. *Journal of Pediatric Surgery*, 31: 1047-1051.

114. Alavi, K, Prasad, R, Lundgren, K and Schwartz, MZ (2000). Interleukin-11 enhances small intestinal absorptive function and mucosal mass after intestinal adaptation. *Journal of Pediatric Surgery*, 35: 371-374.

115. Musashi, M, Yang, Y-C, Paul, SR, Clark, SC, Sudo, T and Ogawa, M (1991). Direct and synergistic effects of interleukin-11 on murine hemopoiesis in culture. *Proceedings of the National Academy of Sciences*, 88: 765-769.

116. Leonard, JP, Quinto, CM, Kozitza, MK, Neben, TY and Goldman, SJ (1994). Recombinant human interleukin-11 stimulates multilineage hematopoietic recovery in mice after a myelopsuppressive regimen of sublethal irradiation and carboplatin. *Blood*, 83: 1499-1506.

117. Du, XX, Neben, DT, Goldman, S and Williams, DA (1993). Effects of recombinant human interleukin-11 on hematopoietic reconstitution in transplant mice: acceleration of recovery of peripheral blood neutrophils and platelets. *Blood*, 81: 27-34.

118. Tohyama, K, Yoshida, Y, Ohashi, K, Sano, E, Kobayashi, H, Endo, K, Naruto, M and Nakamura, T (1992). Production of multiple growth factors by a newly established human thyroid carcinoma cell line. *Japanese Journal of Cancer Research*, 83: 153-8.

119. Maier, R, Ganu, V and Lotz, M (1993). Interleukin-11, and inducible cytokine in human articular chondrocytes and synoviocytes, stimulateds the production of the tissue inhibitor of metalloproteinases. *Journal of Biological Chemistry*, 268: 21527-21532.

120. Elias, JA, Zheng, T, Einarsson, O, Landry, M, Trow, T, Rebert, N and Panuska, J (1994). Epithelial interleukin-11 regulation by cytokines, respiratory syncytial virus and retinoic acid. *Journal of Biological Chemistry*, 269: 22261-22268.

121. Keith Jr, JC, Albert, A, Sonis, ST, Pfeiffer, CJ and Schaub, RG (1994). IL-11, a pleiotrophic cytokine:exciting new effects of IL-11 on gastrointestinal mucosal biology. *Stem Cells*, 12: 79-90.

122. Booth, C and Potten, CS (1995). Effects of IL-11 on the growth of intestinal epithelial cells *in vivo*. *Cell Proliferation*, 28: 581-594.

123. Potten, CS (1995). interleukin-11 protects the clonogenic stem cells in murine small-intestinal crypts from impairment of their reproductive capacity by radiation. *International Journal of Cancer*, 62: 356-361.

124. Du, XX, Doerschuk, CM, Orazi, A and Williams, DA (1994). A bone marrow stromal-derived growth factor, interleukin-11, stimulates recovery of small intestinal mucosal cells after cytoablative therapy. *Blood*, 83: 33-7.

125. Peterson, RL, Trepicchio, WL, Bozza, MM, Wang, L and Dorner, AJ (1994). G1 growth arrest and reduced proliferation of intestinal epithelial cells induced by rhIL-11 may mediate protection against mucositis. *Blood*, 86: 311a.

126. Du, X and Williams, DA (1997). Interleukin-11: review of molecular, cell biology, and clinical use. *Blood*, 89: 3897-3908.

127. Peterson, RL, Bozza, MM and Dorner, AJ (1996). Interleukin-11 induces intestinal epithelial cell growth arrest through effects on retinoblastoma protein phosphorylation. *American Journal of Pathology*, 149: 895-902.

128. Lacroix, M, Siwek, B, Marie, PJ and Body, JJ (1998). Production and regulation of interleukin-11 by breast cancer cells. *Cancer Letters*, 127: 29-35.

129. Morelli, D, Menard, S, Colnaghi, MI and Balsari, A (1996). Oral administration of anti-doxorubicin monoclonal antibody prevents chemotherapy-induced gastrointestinal toxicity in mice. *Cancer Research*, 56: 2082-2085.

130. Cummins, AG, LaBrooy, JT, Stanley, DP, Rowland, R and Shearman, DJC (1990). A quantitative histological study of enteropathy associated with HIV infection. *Gut*, 31: 317-321.

131. Hasan, M and Ferguson, A (1981). Measurements of intestinal villi non-specific and ulcer-associated duodenitis-correlation between area of microdissected villus and villus epithelial cell count. *Journal of Clinical Pathology*, 34: 1181-6.

132. Matkowskyj, KA, Cox, R, Jensen, RT and Benya, RV (2003). Quantitative immunohistochemistry by measuring cumulative signal strength accurately measures receptor number. *Journal of Histochemistry & Cytochemistry*, 51: 205-214.

133. Matkowskyj, KAS and Benya, RV (2000). Quantitative immunohistochemistry by measuring cumulative signal strength using commerically available software and matlab. *Journal of Histochemistry & Cytochemistry*, 48: 303-311.

134. Harper, JW, Adami, GR, Wei, N, Keyomarsi, K and Elledge, SJ (1993). The p21 cdk-interacting protein cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell*, 75: 805-816.

135. Ijiri, K and Potten, CS (1986). Radiation-hypersensitive cells in small intestinal crypts; their relationships to clonogenic cells. *British Journal of Cancer Supplement*, 7: 20-22.

136. Martin, K, Potten, CS and Kirkwood, TB (2000). Age-related changes in irradiation-induced apoptosis and expression of p21 and p53 in crypt stem cells of murine intestine. *Ann N Y Acad Sci*, 908: 315-8.

137. Ishizuka, S, Martin, K, Booth, C, Potten, CS, de Murcia, G, Burkle, A and Kirkwood, TB (2003). Poly(ADP-ribose) polymerase-1 is a survival factor for radiation-exposed intestinal epithelial stem cells in vivo. *Nucleic Acids Res*, 31: 6198-205.

138. Harper, JF (1994). Peritz' F test: basic programme of a robust multiple comparison test for statistical analysis of all differences among group means. *Comput Biol Med*, 14: 437-445.

139. Keefe, DMK, Cummins, AG, Kotasek, D, Dale, BM and Sage, RE (1996). Small intestinal side effects of chemotherapy: a study comparing symptoms, functino and morphology. *Journal of Gastroenterology and Hepatology*, 11: A121.

140. Potten, CS (1992). The significance of spontaneous and induced apoptosis in the gastrointestinal tract of mice. *Cancer and Metastasis Reviews*, 11: 179-195.

141. Du, XX and Williams, DA (1994). Interleukin-11: a multifunctional growth factor derived from the hematopoietic microenvironment. *Blood*, 83: 2023-30.

142. Orazi, A, Cooper, RJ, Tong, J, Gordon, MS, Battiato, L, Sledge, GW, Jr., Kaye, JA, Kahsai, M and Hoffman, R (1996). Effects of recombinant human interleukin-11 (Neumega rhIL-11 growth factor) on megakaryocytopoiesis in human bone marrow. *Experimental Hematology*, 24: 1289-97.

143. Orazi, A, Du, X, Yang, Z, Kashai, M and Williams, DA (1996). Interleukin-11 prevents apoptosis and accelerates recovery of small intestinal mucosa in mice treated with combined chemotherapy and radiation. *Laboratory Investigation*, 75: 33-42.

144. Potten, CS (1996). Protection of the small intestinal clonogenic stem cells from radiation-induced damage by pretreatment with interleukin 11 also increases murine survival time. *Stem Cells*, 14: 452-459.

145. Gavrieli, Y, Sherman, Y and Ben-Sasson, SA (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *Journal of Cell Biology*, 119: 493-501.

146. Nandurkar, HH, Hilton, DJ, Nathan, P, Willson, T, Nichola, N and Begley, GC (1996). The human IL-11 receptor requires gp130 for signalling: demonstration by molecular cloning of the receptor. *Oncogene*, 12: 585-593.

147. Hilton, DJ, Hilton, AA, Raicevic, A, Rakar, S, Harrison-Smith, M, Gough, NM, Begley, GC, Metcalf, D, Nicola, NA and Willson, TA (1994). Cloning of a murine IL-11 receptor-a-chain: requirement for gp130 for high affinity binding and signal transduction. *EMBO Journal*, 13: 4765-4775.

148. Perez Castro, C, Carbia Nagashima, A, Paez Pereda, M, Goldberg, V, Chervin, A, Carrizo, G, Molina, H, Renner, U, Stalla, GK and Arzt, E (2001). Effects of the gp130 cytokines ciliary neurotropic factor (CNTF) and interleukin-11 on pituitary cells: CNTF receptors on human pituitary adenomas and stimulation of prolactin GH secretion in normal rat anterior pituitary aggregate cultures. *Journal of Endocrinology*, 169: 539-547.

149. Kelekar, A and Thompson, CB (1998). Bcl-2 family proteins: the role of the BH3 domain in apoptosis. *Trends in Cell Biology*, 8: 324-330.

150. Adams, JM and Cory, S (1998). The Bcl-2 protein family: arbiters of cell survival. *Science*, 281: 1322-1326.

151. Gross, A, McDonnell, JM and Korsmeyer, SJ (1999). Bcl-2 family members and the mitochondria in apoptosis. *Genes & Development*, 13: 1899-1911.

152. Oltvai, ZN, Milliman, CL and Korsmeyer, SJ (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell*, 609-619: .

153. Raisova, M, Hossini, AM, Eberle, J, Riebeling, C, Wieder, T, Sturm, I, Daniel, PT, Orfanos, CE and Geilen, CC (2001). The Bax/Bcl-2 ratio determined the susceptibility of human melanoma cells to CD95/Fas-mediated apoptosis. *Journal of Investigative Dermatology*, 117: 333-340.

154. Bowen, JM, Gibson, RJ, Keefe, DM and Cummins, AG (2003). Cytotoxic chemotherapy increases p53 and pro apoptotic Bax and Bak expression in crypts of the rat and human small intestine. *Gut*, Submitted: .

155. Bowen, JM (2001). The Expression of the Bcl-2 family members in the small and large intestine. Department of Medicine, University of Adelaide.

156. el-Deiry, WS (1998). Regulation of p53 downstream genes. *Seminars in Cancer Biology*, 8: 345-357.

157. el-Deiry, WS, Tokino, T, Waldman, T, Oliner, JD, Velculescu, VE, Burrell, M, Hill, DE, Healy, E, Rees, JL and Hamilton, SR (1995). Topological control of p21WAF1/CIP1 expression in normal and neoplastic tissues. *Cancer Research*, 55: 2910-9.

158. Reed, JC (1998). Bcl-2 family proteins. *Oncogene*, 17: 3225-3236.

159. Herr, I and Debatin, KM (2001). Cellular stress response and apoptosis in cancer therapy. *Blood*, 98: 2603-2614.

160. Konopleva, M, Zhao, S, Xie, Z, Segall, H, Younes, A, Claxton, DF, Estrov, Z, Kornblau, SM and Andreeff, M (1999). Apoptosis molecules and mechansims. Kaspers, ea, Drug Resistance in Leukemia and Lymphoma III, Kluwer Academic/Plenum Publishers, New York, 217-234.

161. Krajewski, M, Fenoglio-Preiser, CM, Krajewski, S, Song, K, Macdonald, JS, Stemmerman, G and Reed, JC (1996). Immunohistochemical analysis of Bcl-2 family proteins in adenocarcinomas of the stomach. *American Journal of Pathology*, 149: 1449-1457.

162. Krajewski, S, Bodrug, S, Krajewski, M, Shabaik, A, Gascoyne, R, Berean, K and Reed, JC (1995). Immunohistochemical analysis if Mcl-1 protein in human tissues. Differential regulation of Mcl-1 and Bcl-2 protein production suggests a unique role for Mcl-1 in control of programmed cell death in vivo. *American Journal of Pathology*, 146: 1309-1319.

163. Krajewski, S, Krajewski, M and Reed, JC (1996). Immunohistochemical analysis of *in vivo* patterns of *Bak* expression, a proapoptotic member of the Bcl-2 protein family. *Cancer Research*, 56: 2849-2855.

164. Krajewski, S, Krajewski, M, Shabaik, A, Miyasaka, T, Wang, H-G and Reed, JC (1994). Immunohistochemical determination of *in vivo* distribution of Bax, a dominant inhibitor of Bcl-2. *American Journal of Pathology*, 145: 1323-1336.

165. Krajewski, S, Krajewski, M, Shabaik, A, Wang, H-G, Irie, S, Fong, L and Reed, JC (1994). Immunohistochemical analysis of *in vivo* patterns of Bcl-X expression. *Cancer Research*, 54: 5501-5507.

166. Pritchard, DM, Potten, CS, Korsmeyer, SJ, Roberts, S and Hickman, JA (1999). Damage-induced apoptosis in intestinal epithelia from bcl-2-null and bax-null mice: investigations of the mechanistic determinants of epithelial apoptosis in vivo. *Oncogene*, 18: 7287-93.

167. Unger, Z, Molnar, B, Pronai, L, Szaleczky, E, Zagoni, T and Tulassay, Z (2003). Mutant p53 expression and apoptotic activity of *Helicobacter pylori* positive and negative gastritis in correlation with the presence of intestinal metaplasia. *European Journal of Gastroenterology and Hepatology*, 15: 389-393.

168. Petersson, F, Borch, K and Franzen, LE (2002). Gastric epithelial proliferation and p53 and p21 exprression in a general population sample: relations to age, sex and mucosal changes associated with *H. pylori* infection. *Digestive Diseases & Sciences*, 47: 1558-1566.

169. Teh, M, Tan, KB, Seet, BL and Yeoh, KG (2002). Study of p53 immunostaining in the gastric epithelium of CagA-positive and CagA-negative *Helicobacter pylori* gastritis. *Cancer*, 95: 499-505.

170. Walsh, D (1897). Deep traumatism from roentgen ray exposure. *Br Med J*, July: 273-374.

171. Carr, KE (2001). Effects of radiation damage on intestinal morphometry. *Int Rev Cytol*, 208: 1-119.

172. Fajardo, LF, Berthrong, M and Anderson, RE (2001). Alimentary Tract. Fajardo, LF, Berthrong, M and Anderson, RE, Radiation Pathology, New York, 209-247.

173. Hauer-Jenson, M and Wang, J (2002). Mechanisms and modification of the radiation response of gastrointestinal organs. Milas, L, Ang, KK and Nieder, C, Biological modification of radiation response, Heidelberg, in press.

174. Borowski, B, Benhamou, E, Pico, J-L, Laplanche, A, Margainaud, JP and Hayat, M (1994). Prevention of oral mucositis in patients treated with high-dose chemotherapy and bone marrow transplantation: a randomised controlled trial comparing two protocols of dental care. *Oral Oncol Eur J Cancer*, 30B: 93-97.

175. Chi, K-H, Chen, C-H, Chan, W-K, Chow, K-C, Chen, S-Y, Yen, S-H, Chao, JY, Chang, C-Y and Chen, KY (1995). Effect of granulcyte-macrophage colony-stimulating factor on oral mucositis in head and neck cancer patients after cisplatin, fluouracil and leucovorin chemotherapy. *Journal of Clinical Oncology*, 13: 2620-2628.

176. Katano, M, Nakamura, M, Matsuo, T, Iyama, A and Hisatsuga, T (1995). Effect of granulocyte colony-stimulating factor (G-CSF) on chemotherapy-induced oral mucositis. *Japanese Journal of Surgery*, 25: 202-206.

177. Hejna, M, Kostler, WJ, Raderer, M, Steger, GG, Brodowicz, T, Scheithauer, W, Wiltschke, C and Zielinski, CC (2001). Decrease of duration and symptoms in

chemotherapy-induced oral mucositis by topical GM-CSF: results of a prospective randomised trial. *European Journal of Cancer*, 37: 1994-2002.

178. Verdi, CJ, Garewal, HS, Koenig, LM, Vaughn, B and Burkhead, T (1995). A double-blind, randomized, placebo-controlled, crossover trial of pentoxifylline for the prevention of chemotherapy-induced oral mucositis. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology & Endodontics*, 80: 36-42.

179. Duncan, M and Grant, G (2003). Oral and intestinal mucositis - causes and possible treatments. *Alimentary Pharmacology & Therapeutics*, 18: 853-874.

180. Klimberg, VS, Salloum, RM, Kasper, M, Plumley, DA, Dolson, DJ, Hautamaki, RD, Mendenhall, WR, Bova, FC, Bland, KI and Copeland, EM, 3rd (1990). Oral glutamine accelerates healing of the small intestine and improves outcome after whole abdominal radiation. *Archives of Surgery*, 125: 1040-5.

181. Cao, S and Rustum, YM (2000). Synergistic antitumor activity of irinotecan in combination with 5-fluorouracil in rats bearing advanced colorectal cancer: role of drug sequence and dose. *Cancer Research*, 60: 3717-3721.

182. Moss, SF, Agarwal, B, Arber, N, Guan, RJ, Krajewski, M, Krajewski, S, Reed, JC and Holt, PR (1996). Increased intestinal Bak expression results in apoptosis. *Biochemical & Biophysical Research Communications*, 223: 199-203.

183. Du, XX, Liu, Q, Yang, Z, Orazi, A, Rescorla, FJ, Grosfield, JL and Williams, DA (1997). Protective effects of interleukin-11 in a murine model of ischemic bowel necrosis. *American Journal of Physiology*, 272: G545-G552.

184. Booth, C, Haley, JD, Bruskin, AM and Potten, CS (2000). Transforming growth factor-β3 protects murine small intestinal crypt stem cells and animal survival after irradiation, possibly by reducing stem-cell cycling. *International Journal of Cancer*, 86: 53-59.

185. Sonis, ST (2002). The biologic role for nuclear factor-KappaB in disease and its potential involvement in mucosal injury associated with anti-neoplastic therapy. *Crit Rev Oral Biol Med*, 13: 380-389.

186. Howarth, GS, Francis, GL, Cool, JC, Xu, X, Byard, RW and Read, LC (1996). Milk growth factors enriched from cheese whey ameliorate intestinal damage by methotrexate when administered orally to rats. *Journal of Nutrition*, 126: 2519-30.

187. Savarese, DMF, Savy, G, Vahdat, L, Wischmeyer, PE and Corey, B (2003). Prevention of chemotherapy and radiation toxicity with glutamine. *Cancer Treatment Reviews*, 29: 501-213.

188. Anderson, PM, Schroeder, G and Skubitz, KM (1998). Oral glutamine reduces the duration and severity of stomatitis after cytotoxic cancer chemotherapy. *Cancer Investigation*, 83: 1433-1439.

R.J. Gibson, J.M. Bowen, M.R.B. Inglis, A.G. Cummins and D.M.K. Keefe (2003) Irinotecan causes severe small intestinal damage, as well as colonic damage, in the rat with implanted breast cancer.
Journal of Gastroenterology and Hepatology, v. 18 (9), pp. 1095–1100, September 2003

R.J. Gibson, D.M.K. Keefe, F.M. Thompson, J.M. Clarke, G.J. Goland and A.G. Cummins (2002) Effect of Interleukin-11 on Ameliorating Intestinal Damage After Methotrexate Treatment of Breast Cancer in Rats.
Digestive Diseases and Sciences, v. 47 (12), pp. 2751-2757, December 2002

Keefe, D.M.K., Gibson, R.J. and Hauer-Jensen, M. (2004) Gastrointestinal mucositis. *Seminars in Oncology Nursing, v. 20 (1), pp. 38-47, February 2004*