INFLAMMATORY CELLULAR RESPONSE AND CYTOKINES IL-1β, IL-6 AND TNFα IN RAT AND HUMAN SPINAL CORD INJURY

by

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ABSTRACT

The inflammatory response following spinal cord trauma plays an important role in the secondary SCI. The goal of this study was to characterize the posttraumatic inflammatory responses and localize cellular sources of IL-1β, IL-6 and TNF-α following SCI. Thus, we hypothesized that the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α may act as messengers to coordinate the inflammatory cascade in the secondary SCI and that the cytokine response should be greater in severe than in mild injury.

One hundred and twenty-six rats were used and rat spinal cord contusions were induced by the weight drop device. Mild and severe SCIs were respectively produced by dropping a 10-g weight from 3.0 and 12.0 cm. Inflammatory cellular responses were studied using immunohistochemistry and expressions of IL-1β, IL-6 and TNF-α mRNAs were analyzed by RT-PCR. Thirteen human spinal cords removed at autopsy were studied using immunohistochemistry and cellular sources of IL-1β, IL-6 and TNF-α were localized using double-label fluorescent confocal imaging.

In experimental SCI, neutrophils started to infiltrate primarily around blood vessels in the central gray matter at 6 hrs and peaked at 1 day. Macrophages were noted at 6 hrs and then progressively increased for the first 3 days postinjury. Activated microglia were found as early as 1 h after contusion and frequently wrapped around axonal swellings and healthy neurons. RT-PCR showed an early and robust up-regulation of IL-1β, IL-6 and TNF-α mRNAs in spinal cord after severe contusion injury, maximal at 6 h postinjury with return to control levels by 24 h postinjury, the changes being significantly less in mild injury. In human SCI, the inflammatory response paralleled the changes in experimental SCI, neurons and

microglia were identified as the cellular sources of IL-1β, IL-6 and TNF-α, and IL-1β co-existed with APP in the neurons and their axons.

RT-PCR analyses together with histological observations confirm that intrinsic CNS cells (neurons and microglia), not peripheral inflammatory cells, are the main source of cytokines because the peripheral inflammatory cells did not invade the injured spinal cord until 6 h postinjury, a time when cytokine mRNA levels had peaked and started to decline. Microglia around axons may have their possible beneficial effects on the injured axons by providing a trophic local environment to promote the regeneration of the injured axons and the co-existence of IL-1β and APP indicates a possible role of IL-1β in the production of APP. Furthermore, our comparative RT-PCR analyses, showing significantly increased expression of pro-inflammatory cytokine mRNAs in severe injury in contrast to mild injury, support the hypothesis that cytokine up-regulation is an important factor in the generation of the severity of the inflammatory response and thus a suitable target for pharmacological intervention to attenuate this response.

DECLARATION

This work contains no material that has been accepted for the award of any other degree or diploma in any university or 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.

I give consent for this copy of my thesis to be made available for loan and photocopying when this copy is placed in the Barr Smith Library.

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ABBREVIATIONS

AP1	activator protein-1
APP	amyloid precursor protein
ASD	acidic sphingomyelinase domain
BBB	blood brain barrier
BCIP	5-bromo-4-chloro-3-indolyl phosphate
cAMP	cyclic adenosine monophosphate
C-Fos	cytoplasmic fos
C-Jun	cytoplasmic jun
CNS	central nervous system
COX-2	cyclooxygenase-2
CRE	cAMP response element
CRP	c-reactive protein
CSF	colony stimulating factors
DNA	deoxy ribonucleic acid
EAA	excitatory amino acid
EGF	epidermal.growth factor
ERK	extracellular regulated kinases
FADD	Fas-associating protein with death domain
FAN	factor associated with neutral sphingomyelinase activation
FGF	fibroblast growth factor
GAP-43	growth-associated phosphoprotein-43
GFAP	glial fibrillary acidic protein
GRE	a glucocorticoid-responsive element

GS I-B_4	Griffonia simplicifolia I-B_4
H&E	haematoxylin and eosin
IFN	interferon
IκB	inhibitory κB
IL-1α	interleukin-1α
IL-1β	interleukin-1β
IL-1R-AcP	IL-1 receptor accessory protein
IL-6	interleukin-6
i-NOS	inducible-nitric oxide synthetase
JAK	janus activated kinase
JNK	c-Jun amino-terminal kinase
MAP	mitogen-activated protein
MEKK	MAP kinase/ERK kinase
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MRE	multiresponse element
mRNA	messenger ribonucleic acid
Mn-SOD	mitochondrial superoxide dismutases
NASCIS	national acute spinal cord injury study
NBT	nitro blue tetrazolium
NFAT	nuclear factor of activated T cells
NFβA	nuclear factor-βA
NF-IL6	nuclear factor-interleukin-6
NF-κB	nuclear factor-kappaB
NGF	nerve growth factor

NHS	normal horse serum
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NOS	nitric oxide synthase
NSD	neutral sphingomyelinase domain
Par-4	prostate apoptosis response-4
PBS	phosphate-buffered saline
PC-PLC	phosphatidylcholine-specific phospholipase c
PDGF	platelet-derived growth factor
PGI_2	prostaglandin I_2
PLA-2	phospholipase A-2
PNS	peripheral nervous system
RIP	receptor interacting protein
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SCI	spinal cord injury
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SHP-2	Src-homology phoshpatase-2
SRE	serum responsive element
STAT	signal transducer and activator of transcription
TGF-β	transforming growth factor-β
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TRADD	TNFR1-associated death domain protein
TRAF-2	TNF receptor-associated factor-2

TSCI	traumatic spinal cord injury
TXA_2	thromboxane A_2
VCAM	vascular cell adhesion molecule

PUBLICATIONS, PRESENTATIONS AND PRIZE

Publications

Yang L., Blumbergs PC, Jones NR, Manavis J, Sarvestani GT and Ghabriel MN. Early expression and cellular localization of pro-inflammatory cytokines in traumatic spinal cord injury in the human. Spine 29(9): 966-971; 2004

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Presentations at scientific meetings

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PRIZE

Rob Johnson Award 2001

For the best paper by a trainee

Spinal Society of Australia

Annual Scientific Meeting

INTRODUCTION

When you examine a man with a dislocation of a vertebra of his neck, and you find him unable to move his arms and his legs. His penis is erect; urine drips from his penis unknowingly. Then you have to say: A disease one cannot treat.

Edwin Smith Surgical Papyrus, about 2500 B.C.[312]

It has been more than forty-five centuries since an unknown Egyptian physician gave this above description of the clinical features of traumatic tetraplegia [218], but TSCI is still a prominent neurological health care issue for modern society. Until recently, the view of the irreversibility of spinal cord lesions was accepted almost as a "law of nature".

However, there has been a rapid expansion in this field of neuroscience during the last decades and we are now starting to understand the mechanisms of neuron survival and death, the crucial molecular aspects of neurite growth and the interaction of inflammatory cells with the CNS. Obviously, in such a large and old field, a full and total coverage of the existing literature is hardly possible. This review of the literature on TSCI in the following sections concentrates on the inflammatory response following TSCI and the underlying cellular and molecular mechanisms.

1. TRAUMATIC SPINAL CORD INJURY

1.1 EPIDEMIOLOGY

The annual incidence of TSCI has been suggested worldwide to range from 12.7 to 50 cases per million population [199]. The incidence of TSCI in Australia is about 300 new cases per year and is estimated to be 20-30 cases per million population per year [398].

Although the incidence of TSCI is increasing, the number of fatal cases has been decreasing each year in Australia since the early 1980s as a result of legislation related to safety measures, quicker retrieval from the accident site and improved emergency management [398].

The most common causes of TSCI are motor vehicle accidents (nearly 50%), followed by falls, penetrating injuries due to gunshot or knife wounds and sports accidents [312]. The majority of human TSCIs result from acute contusion due to displacement of bone or disk into the spinal cord during fracture dislocation or burst fracture of the spine [41,188]. The cervical spinal cord as well as the thoracolumbar junction are the regions that are most commonly affected [40,58]. Paralysis of the upper and lower extremities, so-called quadriplegia, accounts for 54% of the injured patients and the remaining 46% accounts for patients with paraplegia.

TSCI predominantly occurs in young men (61% have an age between 16 and 30 yr), and only 20-30% of the patients are women [199,312]. It is significantly uncommon for TSCI to occur in children under the age of 14 [56].

1.2 NATURE OF THE INJURY

The four vectors of forces applied to the spinal column in TSCI are flexion, extension, rotation and compression. In the majority of cases, a combination of forces acts on the spinal cord upon injury. The specific forces as well as the specific anatomic level to which the forces are applied results in a specific pattern of posttraumatic spinal cord pathology [36].

The spinal cord is the major conduit through which motor and sensory signals pass between brain and body. TSCI leads to a disruption of these pathways and a loss of motor and sensory

functions. It has been well established that much of the posttraumatic tissue damage and subsequent neurological deficits are due to secondary reactive processes [400,5,35,99,109,208]. The initial traumatic injury triggers a cascade of molecular and cellular changes that occur within minutes and continue for days or weeks. Therefore, the TSCI is a dynamic and complex process rather than an event.

1.3 SECONDARY SPINAL CORD INJURY

Allen first proposed the concept of secondary injury in 1911 [353], studying experimental acute spinal cord injury in dogs. Today, it is well known that loss of neural function following acute traumatic injury to the spinal cord only in part results from direct or immediate damage to spinal grey or white matter. The concept that secondary injury leads to much of the tissue damage after traumatic SCI has been suggested by the slow progress of histopathological changes [390] and the continuing death of neurons in the spinal cord after primary trauma to the cord [221]. Strong evidence has been provided by the demonstration that neurological impairment can be reduced by numerous postinjury treatments. Bracken et al. [47] in 1991 reported a large-scale clinical trial in patients with SCI, showing that high dose steroid methylprednisolone can reduce the secondary injury and functional deficits in patients with SCI by attenuating the biochemical and pathological changes in the cord after injury.

Now it is well established that damage to the spinal cord after SCI is produced by two distinct mechanisms: the primary mechanical injury and a secondary injury initiated by the primary injury [353]. Trauma to the spinal cord not only produces the primary mechanical damage but also causes progressive auto-destruction of tissue at the trauma site, the extent of which is dependent on the severity of the trauma [83]. Therefore, secondary injury processes play an important role in the overall functional deficit [104,392]. In the last 20 years, various putative

mechanisms of secondary injury have been proposed with the aim of finding methods to improve or restore neurological function (Table 1).

Table 1. *Mechanisms of secondary spinal cord injury*

1. Inflammatory response
   a. neutrophils
   b. macrophages
   c. microglia
   d. pro-inflammatory cytokines(IL-1β, IL-6 and TNF-α)
   e. autoimmune pathology (lymphocytes)
   f. free-radical production

2. Vascular changes
   a. loss of autoregulation
   b. systemic hypotension
   c. haemorrhage
   d. loss of microcirculation
   e. reduction in blood blow

3. Electrolyte changes
   a. increased intracellular calcium
   b. increased extracellular potassium
   c. increased sodium permeability

4. Biochemical changes
   a. neurotransmitter accumulation
      i) EAAs (e.g., glutamate)
      ii) biogenic amines (e.g., dopamine)
   b. arachidonic acid release
   c. lipid peroxidation
   d. endogenous opioids
   e. lysosome

5. Loss of energy metabolism
   (decreased adenosine triphosphate production)

1.3.1 INFLAMMATORY RESPONSE

Usually, an inflammatory response to traumatic injury brings about wound healing. However, the posttraumatic inflammatory response at CNS lesion sites has been suggested to contribute to secondary progressive tissue destruction [26,33,35,239], which precludes any meaningful tissue repair [406]. By causing damage to vascular, neuronal and glial cell populations, the primary traumatic injury is supposed to render these and adjacent structures susceptible to secondary injury by posttraumatic inflammation. The robust inflammatory response begins within hours and peaks within several days following TSCI [11,99]. This response mainly includes activation of microglia, release of cytokines, immigration of peripheral inflammatory cells, increase in vascular permeability and development of oedema [164,354].

1.3.1.1 MICROGLIA

It has been demonstrated in many studies that microglia are rapidly activated within hours after SCI [65,196,200,208,276,312,323,341,382]. Upon cellular activation, microglia, the resident cells of the CNS, can produce large quantities of a number of neurotoxic mediators, including EAAs, proteases, pro-inflammatory cytokines and NO, to further the secondary SCI. Details of biologic effects of microglia after SCI will be discussed in section 3.4.

1.3.1.2 CYTOKINES

Cytokines, such as IL-1β, IL-6 and TNFα, play a central role in the initiation, perpetuation and regulation of inflammatory response following SCI [26]. Details of the functions and significances of these pro-inflammatory cytokines will be discussed in section 2.

1.3.1.3 NEUTROPHIL AND MONOCYTE INFILTRATION

There are two main waves of cellular infiltration by leukocytes at the lesion site of the injured spinal cord [11,33,99,239,377]. The first wave consists of polymorphonuclear neutrophils and the second is dominated by phagocytic macrophages.

Polymorphonuclear neutrophils start to infiltrate the lesion site and the adjacent tissue in the first few hours after injury of the spinal cord in experimental animal models, reach a peak within 24 h and disappear by 3 days [65,99,239]. It has been observed that the number of polymorphonuclear neutrophils infiltrating the tissue is strongly correlated to the degree of haemorrhage occurring at the lesion site, suggesting that bleeding into tissue and subsequent degradation of haemoglobin products may be strong chemoattractant factors for polymorphonuclear neutrophils [113,239]. Activation of membrane phospholipases by certain clotting factors such as thrombin and bradykinin, which extravasate into the spinal cord tissue, may contribute to the initiation of the formation of chemoattractant factors for polymorphonuclear neutrophils [115]. Based on in vitro observations, neutrophils are potentially able to degrade connective tissue matrix and damage the tissue by their ability to generate oxygen radicals and secrete granule constituents such as lysosomal enzymes [9,358]. This view is supported by some findings that in vivo application of antibodies against the membrane glycoprotein complex CD18 (integrin β_2-subunit), which has been shown to be critically important for adhesion of leukocytes [328,347], induced a decrease in tissue damage of the spinal cord [72]. Furthermore, in a compression model of the spinal cord in the cat, Means and Anderson [239], observed signs of neuronophagia by polymorphonuclear neutrophils in the marginal zone of haemorrhagic necrosis, suggesting that neutrophils not only are general mediators of inflammatory events but also have specific cytotoxic effects on neuronal cell populations. In contrast, this view on neutrophils has been challenged by some studies showing that neutropenia induced by injection of vinblastine sulfate or methylprednisolone

has no effect on the extent of tissue damage following CNS injury [20,367]. Therefore, the detailed roles and functions of neutrophils in TSCI still remain controversial.

The second wave of immigration of peripheral inflammatory cells into the area of injury mainly consists of phagocytic macrophages [19,33]. It has to be stressed that it is technically difficult to distinguish between fully activated endogenous microglial cells and blood-derived extrinsic macrophages [25,85,101,272] and the roles and functions of microglia following TSCI will be discussed in detail in section 3. In recent years and mainly based on in vitro studies, macrophages have been found to not only participate in tissue destruction as phagocytes but also have strong secretory properties [136,271]. Macrophages can secrete a remarkable variety of macromolecules, including many cytokines (e.g., IL-1α, IL-1β, IL-6, TNF-α, interferons-α, -β, -γ, etc.), numerous growth factors (e.g., TGF-β, FGF, etc.) and various enzymes (e.g., lysozyme, elastase, collagenase etc.) and these bioactive molecules exert a wide range of biological effects, from mediating cytotoxicity to facilitation of tissue repair [212].

Some experimental evidence supports the involvement of macrophages in secondary tissue damage following TSCI. It was observed that demyelination of axons that survived the initial contusion injury is coincident in time with macrophage invasion [33,35]. In vitro studies showed that neutral proteases secreted by stimulated macrophages lead to demyelination [62]. In vivo studies in the guinea pig demonstrated that macrophage depletion by intraperitoneal application of silica dust at the time of spinal cord compression lesion produced a delay of up to 2 days in the onset of secondary functional loss and a reduction in the loss of myelinated fibres [34].

However, other experimental evidence has suggested that macrophages are essential for the regeneration of severed axons. Axonal transection in both the CNS and PNS results in degeneration of the distal nerve stump, so-called Wallerian degeneration. In the PNS, myelin is rapidly degraded over a period of a few days, but in the CNS, myelin and axonal debris can persist for months due to the poor recruitment of macrophages to areas of injury in the CNS compared with the massive recruitment in the PNS [30]. The persistence of myelin and axonal debris has been thought as an important factor in impeding the regeneration of injured axons in the CNS and thus the poor recruitment of macrophages has been proposed to be an important factor in the poor ability of the CNS to regenerate [54,271]. Furthermore, the presence of macrophages in the distal stump of a severed peripheral nerve is supposed to be not only necessary for removing myelin and axonal debris but also for initiating mitosis in Schwann cells and proliferation of fibroblasts [29]. Additionally, macrophages have a potential role in reconstruction of the injured tissue. The first evidence that macrophages are essential in tissue repair and remodelling was shown in 1975 by Leibovich and Ross, who found that macrophage depletion in the guinea pig was associated with impairment of skin wound healing [214]. Molecules produced by macrophages have been shown to have strong growth-permissive or proliferative properties for glial and endothelial cells in the CNS [312]. Thus, macrophages can enhance the production of NGF by non-neuronal cells [169,220] and modify glial cell surface properties to produce a substrate permissive for neurite elongation [78]. Molecules produced by macrophages (e.g., IL-1β, IL-6, TNF-α etc.) also have a direct protective and trophic effect on the injured CNS tissue [241,298]. On the other hand, these molecules are also known to have cytotoxic effects on the nervous system [194,354].

1.3.1.4 LYMPHOCYTE INFILTRATION

Relatively small numbers of different subpopulations of lymphocytes have been seen in spinal cord lesion sites [163,246,275]. Thus, the notion of an autoimmune process being initiated by TSCI was proposed by several research groups, but strong experimental evidence for this hypothesis is still lacking [63]. The majority of data indicate that a certain degree of bystander damage to surviving nerve fibres may be due to the inflammatory response rather than a targeted specific immune attack. However, it has to be ruled out by further experiments whether a specific immune response plays a role in TSCI.

1.3.1.5 FREE REDICALS

It has also been hypothesized that reperfusion injury of the endothelial cell after ischaemic changes may contribute some cytotoxic substances in the inflammatory response [312]. Ischaemia can transform xanthine dehydrogenase in the endothelial cell into a xanthine oxidase. Then, during reperfusion, oxygen activates the xanthine oxidase to oxidate xanthine, transferring its electrons to molecular oxygen to generate superoxide radicals. These radicals, which are toxic to the endothelial cell, increase vascular permeability. This vascular injury is exacerbated by neutrophils, which aggregate at sites of vascular damage, secreting additional reactive oxygen species [9,358]. In this way, the inflammatory response causes damage to the endothelial cell, disruption of vascular integrity, oedema, extravasation of erythrocytes, migration of neutrophils and monocytes into the injured region, and the subsequent release of cytotoxic substances [406]. Thus, the sustained release of cytotoxic substances in the inflammatory response has been believed to play a key role in progressive necrosis in spinal cord injury.

In summary, it has been suggested that the inflammatory response in spinal cord injury may initiate and maintain progressive secondary tissue damage, This notion has been supported by

numerous reports that the tissue necrosis can be significantly reduced by treatment with anti-inflammatory agents and that wound healing may be adversely affected by the presence of tissue necrosis [353,406]. However, the role of posttraumatic inflammatory response is still not very clear at present. The need to reduce the inflammation to limit the spread of damage may be in conflict with the need to permit the inflammation to promote tissue repair and regeneration. Furthermore, although the posttraumatic inflammatory response of TSCI is well documented, the functional significance of the inflammatory cells (e.g. neutrophils, macrophages, microglia) in traumatic spinal cord lesions still remain controversial and the posttraumatic inflammatory response has not been studied in quantitative detail.

1.3.2 LOCAL ISCHAEMIA

There is often a substantial decline in spinal cord blood flow after trauma; such ischemia is believed to contribute to secondary tissue injury [106]. Many studies have shown a major reduction in the microcirculation and a lack of perfusion at the injury site after experimental cord trauma [96,293,353]. Further, Wallace et al.[378] demonstrated that there was a lack of perfusion of the arterioles, capillaries and venules at the injury site and adjacent areas, considerably cephalad and caudad from the injury site, while the large vessels of the cord (the anterior spinal artery and the anterior sulcal arteries) always remained patent even after severe cord injury. The ischaemic areas included a large part of the grey matter and the surrounding white matter. Therefore, Tator et al.[353] postulated that secondary thrombosis or vasospasm of arterioles was due to the accumulation of noradrenaline at the injury site.

The normal cord can autoregulate blood flow to maintain a constant blood supply over a wide range of arterial pressure. However, autoregulation is markedly impaired after spinal cord trauma [149]. Moreover, neurogenic shock can be induced by acute cord trauma. It has been

shown in numerous studies that posttraumatic hypotension and decreased cardiac output are caused after experimental acute spinal cord injury. These declines result from a combination of decreased sympathetic tone and myocardial effects [148].

These results have suggested that multiple factors, including reduction of spinal cord microcirculation at and near the injury sites, impaired autoregulation of spinal cord blood flow, systemic hypotension, and diminished cardiac output, contribute to ischaemia after trauma in the spinal cord. Elevation of posttraumatic mean systemic arterial pressure to more than 160 mmHg failed to significantly improve spinal cord blood flow at the injury site and caused marked hyperaemia at adjacent sites [149]. A linear relationship was found to exist between the severity of cord injury and the reduction in blood flow at the injury site. Further, both the severity of cord injury and the degree of posttraumatic ischaemia were found to be significantly related to posttraumatic axonal dysfunction (motor and somatosensory tracts of the cord) [353].

Therefore, posttraumatic ischaemia is a direct and damaging reaction to trauma, and treatment of the ischaemia contributes to the restoration of cord function [112].

1.3.3 EAA

The EAA, glutamate, is widely distributed within the CNS and serves as an excitatory neurotransmitter [166]. EAAs, such as glutamate and NMDA, can destroy neurons in the mammalian CNS [249] and there are several types of EAA postsynaptic receptors, which mediate the neurotoxicity of EAAs [368]. It has been strongly suggested that EAAs are involved in secondary SCI following TSCI. There have been numerous studies that demonstrate that local levels of EAAs, especially glutamate, rise to toxic levels following TSCI [221,229,268,326]

and that support the involvement of EAAs in the pathologic changes after spinal cord injury. It was also observed that the extent of EAA increase was dependent on the severity of trauma [83,268]. By means of the microdialysis technique, Liu et al. [221] and other investigators [229,326] demonstrated that extracellular EAAs reached toxic levels upon impact injury to the spinal cord.

Further evidence was provided by reports of exacerbation of neuronal damage by EAA administration in the injured cord and attenuation of neuronal damage by some selective EAA antagonists (including antagonists of NMDA, KA and AMPA) following traumatic injury to the spinal cord [83,106,109,141,268,390,392].

Taken together, these results demonstrated that secondary damage to the spinal cord after traumatic injury can be partially attributed to delayed neurochemical change within CNS tissue, produced by increased extracellular EAAs. Significantly increased EAAs are thought to damage neurons by excessive activation mediated through specific membrane receptors mainly by two mechanisms: (a) an early chloride and sodium ion influx, leading to acute neuronal swelling and (b) a later calcium ion influx, leading to more delayed damage [221,229,299]. The later increased intracellular calcium is responsible for a cascade of cell events and further damage may occur in the absence of swelling [299,353]. Thus, excessive stimulation of membrane receptors by high extracellular concentration of EAAs may greatly increase Ca^{2+} influx to produce neuronal death in the CNS with accumulated intracellular Ca^{2+} [161,183]. The increased intracellular Ca^{2+} can activate a number of Ca^{2+}-stimulated enzymes, such as phospholipases, protein kinases, nitric oxide synthase, proteases and nucleases, which subsequently damage some organellae, including mitochondrion and nuclei. All these eventually result in cell damage [299]. These hypotheses have been supported by the

observations of a rapid decrease of extracellular calcium within the spinal cord following spinal injury and the restoration of neurological function after SCI in rat models by calcium-channel blockers [104,403]. Intracellular calcium influx and consequent accumulation is well known as the final common pathway of toxic cell death in the nervous system [306].

1.3.4 OTHER SECONDARY BIOCHEMICAL INJURY FACTORS

Biogenic amines (dopamine, noradrenaline, serotonin and histamine) act as neurotransmitters in the spinal cord. Some investigators [52,251,301,407] focused considerable attention on biogenic amines as possible mediators contributing to the secondary SCI. They reported that elevated levels of biogenic amines were observed within the spinal cord after trauma and paralleled a marked reduction in spinal cord blood flow in experimental trauma. However, conflicting results have been reported [359], which failed to confirm previous reports of amine alteration in spinal cord trauma. An impact injury to the thoracic cord did not lead to changes in noradrenaline, dopamine or serotonin despite the presence of severe haemorrhage and necrosis. Additionally, ∝-methyl-p-tyrosine, which reduces the levels of central dopamine and noradrenaline, did not demonstrate a protective effect after spinal cord trauma. Therefore, the role of biogenic amines in spinal cord trauma remains to be determined.

The lysosome mechanism was proposed by several authors [189]. They found that transection of the spinal cord was followed by massive accumulation of lysosomes and release of lysosomal hydrolases within spinal cord stumps. They suggested that release of lysosomal hydrolases resulted in secondary spinal cord damage.

The endogenous opioid mechanism was advanced by Faden et al.[107], who described the therapeutic effects of the opiate receptor antagonist naloxone in experimental models. They

suggested that endogenous opioids might play a pathophysiological role in the spinal cord following traumatic injury.

1.4 HISTOPATHOLOGICAL EVOLUTION

The sequential pathological changes in the cord following TSCI can be divided into acute, subacute and late phases. The acute phase is characterised by haemorrhage, rapid necrosis and glial activation, which is followed by reactive gliosis and recruitment of blood-born inflammatory cells in the subacute phase. The final appearance of the injured spinal cords some weeks after injury is surprisingly similar in the various lesion models: a cavity and scar tissue are formed and in white matter, different stages of Wallerian degeneration are observed.

1.4.1 ACUTE PHASE: HOURS AFTER INJURY

1.4.1.1 HAEMORRHAGE

An alteration in the microvasculature of central grey matter is the first detectable morphological change in the spinal cord [303]. Multifocal petechial haemorrhages at the site of compression are observed within minutes following TSCI with the light microscope. Postcapillary venules become distended with erythrocytes and red blood cells penetrate the injured vascular endothelium and are found within the perivascular spaces [8,91]. The haemorrhages at the very early time points are restricted to the central grey matter adjacent to the central canal [24]. The traumatic vascular lesions spread within the next few hours in a radial direction in the grey matter and to a small extent also into white matter [11,95]. Within the first 24 h, microthrombi containing degranulated platelets and abundant fibrin are frequently observed in capillary-sized vessels with structurally intact as well as necrotic walls [11]. The progression from comparatively normal vessels to vascular congestion and blood

extravasation can occur rapidly. As early as a few minutes following impact injury, separation of endothelial junctions and exposure of the microvascular basal lamina were observed [142]. Studies in cats using scanning electron microscopy showed craters in capillary endothelium and adherent noncellular material as early as 1 h posttrauma [279], suggesting that these endothelial abnormalities might be due to free radical-induced injury to membrane lipids, resulting in microcirculation failure. This view is supported by another finding that the endothelial abnormalities were prevented by pre-treatment with antioxidants [385]. Within hours postinjury, the hallmarks of ischaemic vascular injury (vacuolation and swelling) were present in endothelial cells of capillary and postcapillary venules throughout grey and white matter [91].

1.4.1.2 NECROSIS

Another early morphological abnormality in the acute phase is neuronal necrosis. Neurons with cytoplasmic eosinophilia, the first sign of necrotic alteration, appear in random distribution in grey matter within the first hour postinjury [378]. Other signs of necrotic alterations in neurons are also frequently observed, including ghost cells, cytoplasmic microvacuolation and shrunken neurons with indistinct nuclei, smudged cytoplasm, loss of Nissl bodies, irregular shape and hyperchromatization [341]. Ultrastructural observations revealed karyorrhexis and swelling of the rough endoplasmic reticulum in necrotic neuronal and glial cells [11]. The necrotic alterations quantitatively increase in the grey matter within the first several hours after injury [99], when many cell perikarya in the epicentre of the lesion have undergone homogeneous eosinophilic changes on H&E staining and karyolytic or clumped chromatin has been revealed in nuclei. Additionally, both neurons and glial cells are equally vulnerable and both cell populations undergo cell death in the same time interval [11,99].

1.4.1.3 AXONAL INJURY

Axonal injury in white matter is characterized by a finely granular appearance associated with a variable amount of swelling within the first several hours postinjury [11]. Characteristic early axonal abnormalities also include the development of a space between the axon and its myelin sheath, particularly in the central injury zone. This axonal swelling was mostly interpreted as due to oedema and was described as an enlarged periaxonal space [90,177,374]. However, electron microscopic studies [11,145] have showed that the majority of these spaces are intramyelinic vacuoles; the myelin sheaths are dilated or split. These focal changes of the myelin sheaths appear mainly in the central parts of white matter and account for the progressive spongy appearance of the white matter noted by light microscopy. By 4 h, numerous axons within the central white matter and to a lesser extent axons scattered throughout the peripheral white matter showed swelling and accumulation of organelles (mitochondria, neurofilaments, lysosomes and smooth endoplasmic reticulum) [11,202]. These axonal abnormalities continued in a centrifugal fashion toward the pial surface over the next several days [50]. Immunocytochemical technologies using antibodies against APP [39], synaptophysin, chromogranin A, cathepsin D [320], neurofilament protein subunits [277] and ubiquitin [314] have revealed a far greater occurrence of axonal injury than had been appreciated by use of classical silver staining techniques. Of these immunological markers, labelling for APP was found to be the most sensitive and specific in demonstrating axonal injury ranging from mildly damaged axons to axonal retraction bulbs, which represent severed axon cylinders [38,132].

1.4.1.4 MICROGLIAL ACTIVATION

As already described in section 1.3.1.1, rapid microglial activation was observed within hours after SCI [341,382]. Additionally, an in vitro study has demonstrated that microglia were activated

within a period as short as 5-10 minutes [356]. Details of microglial activation will be discussed in section 3.3.

1.4.2 SUBACUTE PHASE: DAYS AND WEEKS AFTER INJURY

This phase is characterized by the activation of astrocytes in the CNS as well as the recruitment of different peripheral cell populations into the area of lesion.

1.4.2.1 INFLUXES OF NEUTROPHILS AND MONOCYTES

As already described in section 1.3.1, two waves of infiltrating peripheral inflammatory cells, an early peak of neutrophils and a later peak of phagocytic macrophages, are seen following TSCI.

1.4.2.2 ASTROCYTE ACTIVATION

Astrocytes undergo remarkable hypertrophy and limited proliferation upon injury [259]. Reactive astrocytes have increased numbers of intermediate filaments comprised largely of GFAP and appear larger with more prominent and numerous processes than resting astrocytes in the normal CNS [291]. Reactive astrocytes also upmodulate the expression of a large number of molecules (e.g., enzymes, cytokines, neurotrophic factors) to exert some beneficial and injurious effects on the injured tissue. For example, upregulation of proteases and protease inhibitors help remodel the extracellular matrix, regulate the concentration of different proteins in the neutrophil and clear up debris from degenerating cells [260,291]. Cytokines are key mediators of immunity and inflammation and could play a critical role in the regulation of the blood-central nervous system interface [204,210,305]. Neurotrophic factors, transporter molecules and enzymes are involved in the metabolism of EAAs and in the antioxidant pathway [100,399]. Microscopic evaluation of spinal cord stab wound lesions at various postoperative time

intervals revealed a gradually increasing astrocytic reaction [260]. Between 1 and 2 days, mitotic activity of astrocytes increased markedly, indicating that the increased number of astrocytes at spinal cord lesion sites results in part from proliferation of the cell population [260]. Astrocyte reactivity upon injury extends in a radial direction into both adjacent grey and white matter, often over long distances [99,297]. The astrocytic response peaks at 14 days but is still present at 28 days [260,291].

1.4.2.3 INFILTRATION OF SCHWANN, MENINGEAL AND FIBROBLASTIC CELLS

Schwann cells, meningeal cells and fibroblasts are additional peripheral cell types that have been seen to invade spinal cord lesion sites [32]. However, the functional significance of these infiltrating cells is still not clear and remains controversial.

1.4.3 LATE PHASE: WEEKS TO MONTHS AFTER INJURY

1.4.3.1 POSTTRAUMATIC SYRINGOMYELIA

Resolution of the acute/subacute inflammatory response occurs several weeks after injury by the disappearance of the neutrophils and macrophages from the lesion area, leaving a fluid-filled cyst [397]. Weeks after injury, the cysts become better defined and extensive scar tissue forms around the cavity [397]. At very late stages in humans (months to years after the injury) secondary often very elongated syrinxes can appear preferentially rostral to the lesion [227]. The process leading to so-called syringomyelia is very poorly understood at present. It is an important late complication and can significantly contribute to further functional losses [332].

1.4.3.2 DEMYELINATION AND REMYELINATION

Another important aspect of the late phase is the continuous loss of myelin and axons in white matter. Apparent axonal loss and demyelination of a number of fibres were observed between 7 days and 3 months postinjury [33]. However, only a small proportion of the fibres were completely denuded of myelin and the majority showed abnormally thin sheaths surrounding axons of normal appearance [57,160]. Whether this specific appearance can also be due to demyelination is not completely clear. Interestingly, evidence of remyelination was also seen at 3 weeks postinjury [140,160]. Marked differences in the extent of remyelination between larger and smaller lesions were shown and remyelination was observed more extensively in smaller lesions [160]. At least a part of the remyelination event seems to be due to oligodendrocytes [139] and Schwann cells [57]. Schwann cell remyelination was predominantly observed in severe contusive injury associated with extensive Wallerian degeneration [36].

1.5 NEUROPROTECTIVE TREATMENT

The aim of neuroprotective therapies is to use pharmacologic agents to alter the detrimental neurochemical cascades initiated by mechanical deformation of neural tissue [22,240]. From the numerous substances tested in experimental spinal cord injury models for their neuroprotective efficacy, only one compound, methylprednisolone, was shown in well-controlled, multi-centre, large clinical trials to enhance functional outcome in the human. Although controversy exists about the benefits of its application, methylprednisolone is currently widely used in clinical practice and the only therapeutic agent approved by the Food and Drug Administration of U.S.A. for treating acute TSCI in humans. So far, the experimental and clinical trials of a large number of substances have shown very different and inconsistent results [108] and few have been evaluated in extensive dose-response studies, and rarely were the treatment schedules based on knowledge of the drug's pharmacokinetics [312]. In

addition, behavioral tests that are difficult to interpret were often used as the main outcome measurement, without careful and quantitative histology.

It is not proposed to undertake a comprehensive literature review of all these different therapies but to provide a selective overview as a background to the use of NF-κB-specific antisense oligodeoxynucleotides in our studies. In this section, we concentrate on the few groups of tested substances that showed interesting or promising results.

1.5.1 ANTI-INFLAMMATORY AGENTS

1.5.1.1 STEROIDS

Steroids have been tested extensively in animal models as well as in clinical trials [47,79,97]. Methylprednisolone megadoses, if applied early after lesion, were shown to exert beneficial effects in the various experimental CNS injury models, although the exact mechanism of action remained unclear [6,401,402]. Based on these results the NASCIS 1 study, the first multi-centre randomised clinical trial, was initiated to evaluate the steroid treatment of secondary damage following acute spinal cord trauma. A total 330 patients were enrolled in the study and were all treated within 48 h of injury. The study assessed the effects of high and low doses of methylprednisolone on recovery of neurological function in patients with spinal cord injury. Neurological evaluations performed at 6 month and 1 year postinjury did not show a significant difference in motor or sensory outcome between the high- and low-dose methylprednisolone groups [45]. A series of animal experiments conducted while NASCIS 1 was in progress suggested that higher doses than those used in NASCIS 1 might be needed to achieve a therapeutic response [48,152]. Thus, NASCIS 2 was started. A total 487 patients were randomised for treatments with methylprednisolone, naloxone, or placebo [47]. Based on

experimental studies, the administration of methylprednisolone was 30 mg/ kg followed by 5.4 mg/kg/hr for 23 h because these doses resulted in a positive effect on recovery in animals with TSCI. The maintenance dose was chosen according to theoretical calculations of the plasma half-life of the drug in humans and, to avoid complications of glucocorticoid administration, 24 h was chosen as the duration of drug application. Motor and sensory functions were assessed at 6 weeks, 6 months, and 1 year postinjury. The study found that the group of patients receiving methylprednisolone within 8 h after injury had significantly better sensory and motor function than those receiving placebo. Furthermore, the differences in motor improvement between the methylprednisolone and the placebo group were statistically significant at all three follow-up periods. Compared to the placebo group, no positive effect was shown in the group of patients who received naloxone or late methylprednisolone administration. A more detailed analysis of the data of NASCIS 2 also indicated that delayed treatment with methylprednisolone is associated with decreased neurological recovery [46].

Adverse effects of such high doses of methylprednisolone, such as urinary tract infections and pneumonia, were commonly found in NASCIS 2 [123]. The incidence of wound infection and gastrointestinal haemorrhage in methylprednisolone-treated patients in NASCIS2 was also slightly higher than in the control group.

An important issue, which was not addressed in the NASCIS 2 study, is the mechanism of action of methylprednisolone. It has been believed that the treatment of spinal cord injury with steroids is based on their anti-inflammatory actions [97], e.g., methylprednisolone has been recently found to reduce IL-1β and TNF-α expression by 80% and 55% respectively after SCI in rats via inhibiting NF-κB and AP-1 (two major proinflammatory transcription factors) [69,232,393,394]. Additionally, very high doses of the synthetic steroid methylprednisolone were

also found to exert non-specific radical scavenger effects [151] and reduce i-NOS expression by 70% after SCI in rats [69].

1.5.1.2 ANTISENSE OLIGODEOXYNUCLEOTIDES

Since the identification of the DNA double-stranded helix, the gene as a target of therapy and, moreover, the use of DNA as a drug have been possibilities. Antisense oligonucleotides, short strands of synthetic nucleic acids, are designed to bind to a target gene promoter through Watson-Crick base pairing (complementary base pairing) and the formation of this oligonucleotide/promoter heteroduplex results in downregulation of mRNA synthesis and consequent inhibition of synthesis of the protein product [174,376]. 'Antisense' is used by some living organisms, specifically viruses, to control gene replication [124]. Only recently has the use of antisense DNA as a mechanism to control human gene translation been appreciated. The first antisense-based drug Vitravene was successfully developed to inhibit human cytomegalovirus by Isis Pharmaceutical Inc., Carlsbad, California in 1998 [255].

Antisense oligodeoxynucleotides carry a high therapeutic potential for the treatment of human diseases and these molecules have been applied in several different fields, including oncology, haematology and cardiovascular and infectious diseases [124,162]. In recent years, some studies have been initiated and performed to evaluate the therapeutic usefulness of antisense technology in modulating physiological and pathological functions in the CNS. These include cytokines, neurotransmitter receptors, neuropeptides, trophic factors and other proteins [344]. Mayne et al. [237] demonstrated that administration of a TNF-α-specific antisense oligodeoxynucleotide into striatal parenchyma reduced cell death and improved neurobehavioral deficits in a rat model of intracerebral haemorrhage induced by disruption of the BBB integrity by collagenase, suggesting potential benefits through interference with

TNF-α production in the CNS injury. To date, few studies have been performed to evaluate the therapeutic usefulness of antisense oligodeoxynucleotides in modulating cytokine production to confer neuroprotection in TSCI. Thus, it would be of interest to examine such pharmacological agents in TSCI models in which cytokines may be a major mechanism leading to secondary tissue damage and functional deficits. For example, NF-κB-specific antisense oligodeoxynucleotides would be a potential pharmacological agent because NF-κB is a major transcriptional factor for the upregulation of IL-1β, IL-6 and TNFα [10,27,156,215] and a ubiquitous transcription factor involved in the proinflammatory response to cytokines such as IL-1β and TNFα [292] (for details, see sections 2.2, 2.3 and 2.4).

However, like any other treatments in TSCI, the antisense oligodeoxynucleotides have some pitfalls and limitations. First, non-specific functional neuronal alteration and detrimental effects on cellular morphology may be caused by the antisense oligodeoxynucleotides in the CNS [344] even though recent advances in antisense oligodeoxynucleotides chemistry have significantly decreased these side effects [376]. Second, while it is commonly believed that the CNS has limited capacity to launch immune reactions, it has not yet been established whether delivery of synthetic antisense oligodeoxynucleotides (a xenobiotic) into the CNS may ultimately stimulate immune competent cells to generate antibodies against RNA and/or DNA, triggering inflammation with severe CNS consequences. Finally, specificity of action is a key issue. Non-specific binding of the antisense oligodeoxynucleotides to mRNA species other than the intended target may cause unwanted interference with transcription and translation [174].

1.5.2 EAA ANTAGONISTS

Increased extracellular levels of EAAs (glutamate and aspartate) accumulate in the lesion area following CNS injury [65,105,221,240,245]. Numerous studies have demonstrated that glutamate and related excitatory amino acids can induce death of neurons [262,263,383] and it has been recognized for many years that excitatory amino acids may be implicated in the secondary tissue damage following CNS injury [70,222]. Development of selective NMDA receptor antagonists has shown that the NMDA receptor complex may be an important factor for the excitotoxic action of EAAs [299] and several groups have shown that treatment with competitive or noncompetitive NMDA antagonists improves neurological function after brain injury [105,106,165].

1.5.3 NORADRENERGIC AGONISTS

Osterholm et al. [265] proposed the monoamine theory of spinal cord injury, arguing that high levels of accumulation of norepinephrine at the lesion site crucially contributes to the pathological pattern of necrosis. The effect of catecholamine modulation using substances such as oz-methyltyrosine and clonidine was therefore studied [250,266]. Mixed results were obtained, and hence, the value of modulating noradrenergic transmission in the acute phase of spinal cord injury remains unclear [44]. However, these pharmacological studies attracted general interest about possible pharmacological interventions. Barbeau and co-workers [15] reported that modulation of the monoamine level by noradrenergic agonists might be important in the more chronic phase of spinal cord injury.

1.5.4 HYPOTHERMIA

Other strategies, i.e., hypothermia, have been tested in experimental spinal cord injury in addition to pharmacological means described above [231]. It has been hypothesized that low temperature would protect the CNS tissue against the effects of hypoxia or ischaemia because

the oxygen demand of the CNS tissue may drop due to the low metabolism induced by hypothermia [226]. Local spinal cord cooling was used in a series of experimental animal studies and the experimental data led to the trial of local spinal cord cooling in some cases of human spinal cord injury [158]. The data emerging from these studies have been difficult to interpret mainly due to the fact that low numbers of cases were reported and in none of the studies were appropriate controls included in the experimental design. Additionally, local spinal cord cooling is a technique fraught with technical difficulties, and the high rate of mortality reported in some of the trials [51] led to concerns about its clinical application.

1.6 ANIMAL MODELS

Interest in exploring spinal cord function using experimental models of SCI dates back to 177 A.D., when Galen performed spinal cord transections of dogs and other animals and recognized that resulting functional loss differed with the segmental level of the injury [389]. Numerous experimental animal models of TSCI have been developed during the last decades in order to find effective methods to manage spinal cord injury in humans. To obtain the most useful data, the experimental design should mimic as closely as possible the situation in the human and it should be as reproducible as possible. This task is difficult because human SCIs are multifactorial and include variables that may be difficult to mimic. For example, the majority of human SCIs occur in a closed vertebral system whereas most animal models use an open laminectomy for lesioning [312]. However, these models have provided the best and sometimes the only practical way to examine mechanisms of injury and to test potential treatments.

1.6.1 WEIGHT-DROP MODEL

The first well-controlled animal model was described by Allen [36] in 1911, who designed a weight-drop technique to deliver a quantifiable impact to the spinal cord of animals. The technique of weight-drop contusion has been refined and adapted over the years, but the central principle of using gravity to standardize the mechanical impact on the spinal cord has persisted in TSCI research.

A primary advantage of weight-drop models of SCI lies in their clinical relevance. First, the weight-drop method closely simulates some of the biomechanics of human SCI. For example, most human SCIs are believed to be due to rapid flexion-extension of the spinal column, resulting in a contusion of the cord when the vertebral bone hits the spinal cord [31,36,125,391]. Other models such as photochemistry, transection, hemisection, and static loading cannot model the clinical contusion injury [21]. Second, the similarities in histopathological appearance of the lesion site between rat weight-drop SCIs and human SCIs were observed by Balentine et al. [11] and other investigators [95,188,324]. The use of the weight-drop technique to produce traumatic injuries to the spinal cord in animals and the resulting stepwise sequential pathological changes in the cord were found to be similar to the configuration found in human spinal cord injuries. Spinal cord pathological changes, which were initially prominent in the centre of the cord, started as haemorrhages and oedema in the grey matter and progressed through central necrosis, adjacent white matter oedema, and demyelination, to finally involve the entire cord. The central fusiform zone of spinal cord necrosis later evolved into a cyst. Third, the weight-drop model has successfully predicted therapeutic potentials for steroids later tested in clinical trials [47].

It has been found in the weight-drop model that the reproducibility of the impact on the spinal cord at a given height is very good: the standard error is less than 2.5% of the mean force or

impulse [31,391]. Furthermore, the mean force or impulse of the impact is significantly correlated to the height from which the weight is dropped [31], functional deficits of the animals [125], somatosensory evoked potentials [288] and histopathology [31,389] seen at one month after SCI. Thus, the weight-drop model can be used to produce reproducible and graded spinal cord contusion injuries in the animal models [389,391].

1.6.2 PNEUMATIC/ELECTROMECHANICAL MODEL

The first of these devices, the constrained stroke pneumatic impactor was pioneered by the research group in the General Motors Research Laboratories in the 1980s [7,190]. An advantage of pneumatic or electromechanical production of spinal cord contusion is that injury biomechanics (e.g., contact velocity, amount of displacement of the surface of the tissue, force, etc.) can be controlled independently and therefore greater precision or actual determination of injury outcomes was allowed [335,336]. It has generally been used to injure the cervical spinal region with the vertebral column intact [335]. By maintaining the normal structure of the spinal column, certain artefacts associated with a laminectomy procedure (e.g., size of laminectomy site, etc.) are thus avoided and close approximation of the human injury process is achieved. However, such devices are complicated and very expensive, and therefore their use has not been widespread.

1.6.3 COMPRESSION MODEL

Tarlov and his colleagues [352] initiated the compression model and defined the gradation of neurological deficits with increasing compression of the spinal cord. A balloon catheter or a pneumatically driven mechanical plunger was used to compress the spinal cord in dogs. The clinical relevance of slow and maintained compression models is somewhat limited and is

distinct from that of the rapid contusion techniques, like the weight-drop or pneumatic/electromechanical techniques.

1.6.4 PHOTOCHEMICAL MODEL

Traumatic spinal cord injury results in degeneration of neural tissue by a number of pathophysiological mechanisms. Local spinal cord ischaemia is one of these mechanisms. It has been known that spinal cord blood flow is considerably reduced after traumatic injury [159].

Thus, an entirely different method was introduced by Watson et al. [384] on the basis of injuring the spinal cord vascular endothelium photochemically. After the injection a photosensitising organic dye (e.g., bengal rose) into the bloodstream, the spinal cord is irradiated with a light beam of appropriate wavelength. Damage to the vascular endothelium and subsequent thrombosis lead to ischaemic lesions and vasogenic oedema [61]. An advantage of the photochemical approach is that laminectomy is not required because the vertebral dorsal surfaces are sufficiently translucent.

Bunge et al. [57] and other researchers [159] used the photochemical technique to induce local spinal cord ischaemic injury in the rat, developing the photochemical lesion model with minimal variability to better understand the ischaemia mechanism of secondary SCI. Pathological changes after lesioning were studied by light and electron microscopy. The photochemical lesions resulted in extensive necrotic regions bordered by swollen axons, a massive influx of macrophages, an increase in the perivascular space in surrounding spared tissue, demyelination in the early stage and late myelination, by both oligodendrocytes and Schwann cells, in the tissue surrounding the necrotic lesion. These changes resembled those observed after SCI caused by contusion or compression.

2. CYTOKINES

In this review of the literature on cytokines, specific consideration has been paid to three main pro-inflammatory cytokines: IL-1β, IL-6 and TNF-α.

2.1 OVERVIEW

2.1.1 WHAT IS A CYTOKINE ?

Most cytokines are soluble simple polypeptides or glycoproteins with a molecular weight of 30 kDa or less, but some cytokines form higher molecular weight oligomers (e.g., TNF-α molecules form trimers) [60,256]. Cytokines are produced by leucocytes and a variety of other cells in the body, and act as chemical communicators between cells and have regulatory effects on haematopoietic and many other cell types that participate in host defence and repair processes [373]. Cytokines are secreted into the extracellular fluid or are membrane exposed by a cell. There they exert their effects on the same cells (autocrine activity) or on neighbouring cells (paracrine activity) by binding to specific high-affinity cell surface receptors which are coupled to intracellular signal transduction and second messenger pathways [60,256].

2.1.2 RESEARCH HISTORY

The field of cytokine research has evolved from four originally independent sources [103,373]. The first and most significant source is the field of lymphokine research. The origins of lymphokine research can be dated back to the mid-1960s when it was demonstrated that protein mediators secreted by lymphocytes regulated the growth and function of a variety of white blood cells. It soon became apparent that monocytes too are the source of these important proteins. The second source of cytokine research derives from the study of the interferons, which gradually became recognized as proteins exerting a broad range of actions on cell growth and differentiation. The third source of cytokine research is the field of

haematopoietic growth factors, or CSFs. The fourth source of cytokine research derives from the study of growth factors acting on non-haematopoietic cells, such as PDGF, EGF, TGF-β, FGF or NGF. Many of these factors, in addition to promoting cell growth, exert other 'cytokine-like' actions.

2.1.3 NOMENCLATURE

A unifying concept of cytokines was slow to emerge in the 1970s due to the fact that the cytokine field evolved from several separate sources. The term 'lymphokine', which originally denoted the product of activated lymphocytes [98], was also used less discriminately for secreted proteins from a variety of cell sources. To dispel the wrong notion that such proteins could be produced by lymphocytes alone, Cohen et al. [73] proposed the term 'cytokines'. After a long-standing reluctance, 'cytokine' has become the generally accepted name for this group of proteins. To designate individual cytokines, a group of participants at the Second International Lymphokine Workshop held in 1979 proposed the term 'interleukin' in order to develop a system of nomenclature [1] and 'interleukin' was proposed on the basis of the ability of these proteins to act as communication signals between different populations of leukocytes. The interleukin series has now reached 18 [373]. Although the name 'interleukin' implies that these agents function as communication signals between leukocytes, the term is not reserved for factors that can act only on leukocytes and a number of the proteins that have been labelled as interleukins not only are produced by a variety of non-haematopoietic cells but also affect the functions of many diverse somatic cells (e.g. IL-1 or IL-6) [1]. Many cytokines are now termed interleukins while others continue to be known by their older names (e.g. IFN-α/β/γ, TNF-α, TGF-β and many others).

2.1.4 CHARACTERISTIC FEATURES

Cytokines are the major orchestrators of host defence processes and are involved in responses to trauma, tumour and invading organisms, repair and restoration of homeostasis [373]. These regulatory proteins have a set of unique features outlined below [59,150,164,207,285,331,373].

- Constitutive production of cytokines is usually low or absent except for growth factors; production is tightly regulated at the level of transcription or translation. The production of growth factors tends to be constitutive and not as tightly regulated as that of other cytokines.
- Cytokines tend to be produced by less specialized cells and several unrelated cell types can produce the same cytokine (e.g., IL-1 is produced by neurons, microglia, monocytes-macrophages, fibroblasts, endothelial cells, etc.)
- Cytokines produce their actions by binding to specific high-affinity cell surface receptors. In addition to their cell surface (membrane-anchored) forms, many cytokine receptors (e.g., IL-1, IL-6 and TNF receptors) exist also as soluble cytokine receptors, lacking the residues that anchor the membrane, but retaining high-affinity binding properties. The soluble cytokine receptors play important roles as regulators of cytokine activity and can function as competitive inhibitors by sequestering cytokines away from their respective cell surface receptors.
- Most cytokine actions can be attributed to an altered pattern of gene expression in the target cells. Phenotypically, cytokine actions lead to an increase (or decrease) in the rate of cell proliferation, a change in cell differentiation state and/or a change in the expression of some differentiated functions.
- The range of actions displayed by individual cytokines can be broad and diverse, some actions targeting non-haematopoietic cells while other actions target haematopoietic cells.

- The 'redundancy' and 'pleiotropy' of cytokine actions, i.e. structurally dissimilar cytokines (e.g., TNF and IL-1) can have an overlapping spectrum of actions and individual cytokines tend to exert multiple actions on multiple target cells and tissues.
- Cytokine action is contextual. Actions of cytokines can be influenced profoundly by the milieu in which they act and especially by the presence of other biologically active agents (e.g., other cytokines). For example, a cytokine may increase (or decrease) the production of another cytokine in multiple target cells, may increase (or decrease) the expression of receptors in multiple target cells for another cytokine and may increase (or decrease) signalling by receptors in multiple target cells for another cytokine.

2.1.5 Sources of IL-1β, IL-6 and TNFα in SCI

Although the inflammatory response to TSCI has been extensively investigated in animal models, little is known about the cytokine response and the cellular sources of these cytokines (IL-1β, IL-6 and TNFα) in rat and human SCI.

2.2 IL-1β

There are two forms of IL-1, IL-1α and IL-1β, and in most studies their effects in terms of biologic activity are indistinguishable [88]. The sections below concentrate on IL-1β.

2.2.1 STRUCTURE

Human IL-1β is a single 153-residue polypeptide chain, whose structure is composed of 12 β-strands organized in a three-fold repeating motif (Figure 1). The core of the structure can best be described as a tetrahedron whose 4 faces are each made up of 3 β-strands, leaving only the end supporting the chain termini fully exposed. The exposed portions of the polypeptide chain are involved in the IL-1β receptor recognition [116,281,282,363].

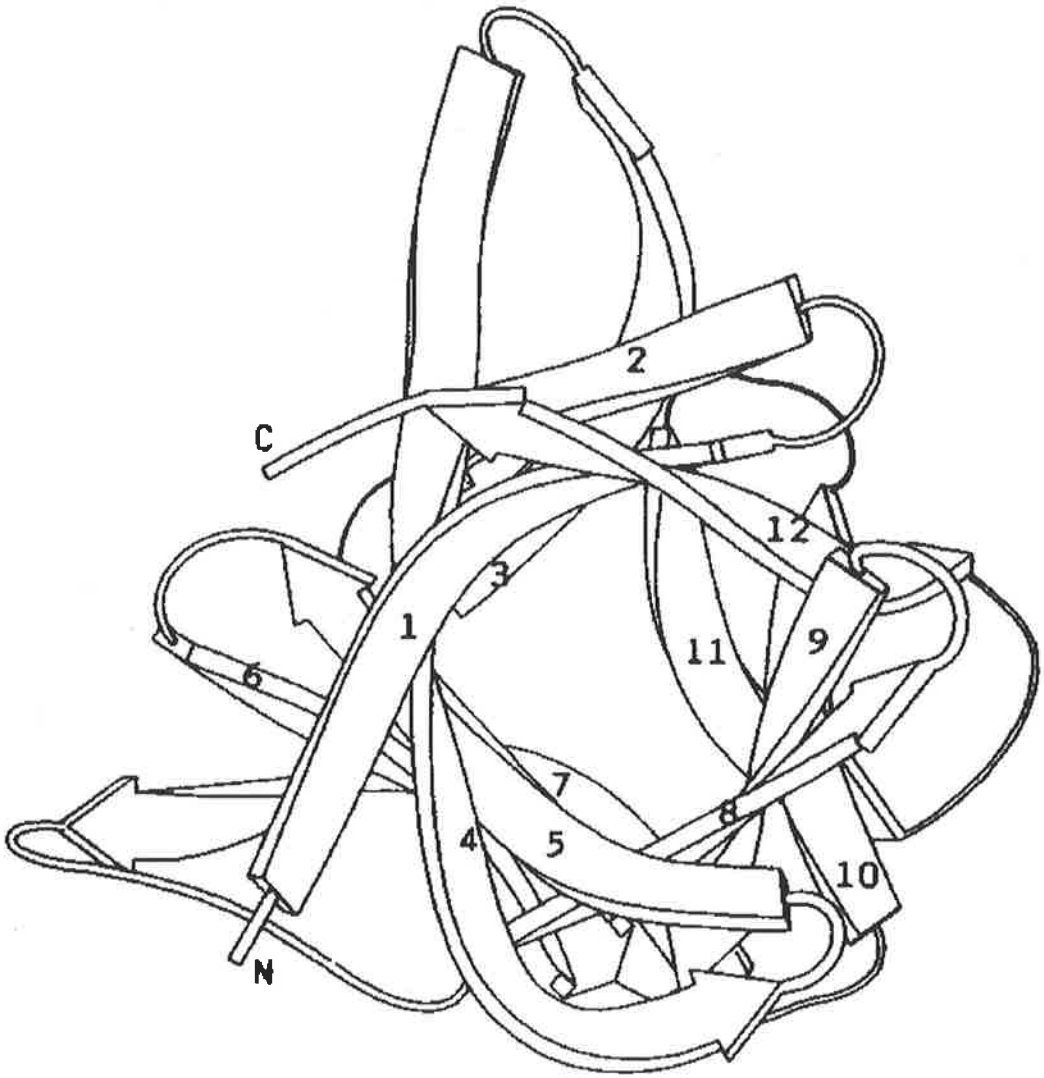


Figure 1. *Stereo structure of IL-1β. Two twisted arrows represent β-strands, who are numbered sequentially. N and C represent the termini of the single 153-residue polypeptide chain of IL-1β. The exposed portions of the polypeptide chain are involved in the IL-1β receptor recognition. (Adapted from Priestle JP, Schar HP and Grutter MG: Crystal structure of the cytokine interleukin-1β. The EMBO Journal 7(2):339-343, 1988.)*

2.2.2 IL-1β GENE AND IL-1β BIOSYNTHESIS

The human IL-1β gene consists of 7 exons on the long arm of chromosome 2 at the locus 2q13-2q21 [60,93].

Several potential transcriptional control elements, such as NFκB, AP1, CRE, NF-IL6 and NFβA, have been identified within the conserved region of the IL-1β promoter [195,198,321,342,364], as shown in Figure 2. These transcriptional regulators are up-regulated in response to many stimuli including injury, microbial products (e.g., bacterial lipopolysaccaride), cytokines (IL-1β, IL-2, TNF, etc.), T cell/antigen presenting cell interactions and immune complexes [88,89]. For example, IL-1β mRNA levels in human monocytes rise rapidly within 15 min after stimulation with bacterial lipopolysaccaride, but start to fall after 4 h [114]. The fall is caused by the synthesis of a transcriptional repressor and a decrease in mRNA half-life [184], suggesting that the production of IL-1β is tightly regulated. This is further supported by in vivo studies demonstrating a robust and transient increase of IL-1β mRNA expression that rapidly falls to a low and constant level within 6 hr after CNS injury in rats [193,341].

In addition to tightly regulated transcription, this regulation also extends to translation of IL-1β mRNA. Stimulants such as complement component C5a [308] or hyperosmolar NaCl [319] were found to induce the synthesis of large amounts of IL-1β mRNA in monocytic cells without significant translation into the IL-1β protein. This dissociation between transcription and translation suggests that the above stimuli are not sufficient to provide a signal for translation despite a vigorous signal for transcription [307].

Following synthesis, the 31-kDa precursor of IL-1β remains cytosolic and only marginally active until it is cleaved by IL-1β converting enzyme. The cleavage converts IL-1β precursor

to its 17 kDa mature form and then the mature IL-1β is secreted out of the cell [185]. Thus, the release of mature IL-1β depends on cleavage by IL-1β converting enzyme.

Constitutive production of IL-1β is usually low or absent and its expression is tightly regulated at the level of transcription, translation and secretion [88].

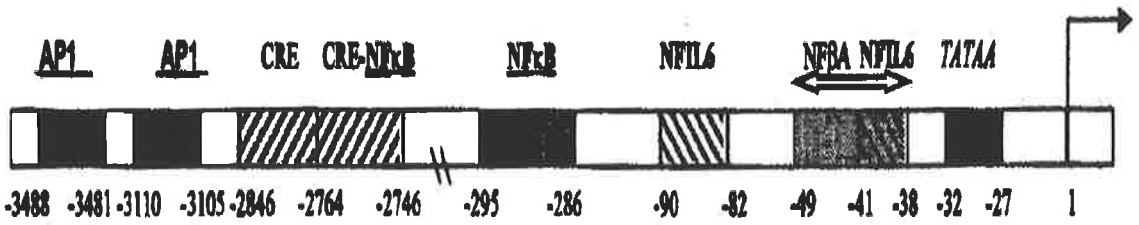


Figure 2. *Structure of the IL-1β gene promoter. The general structure of the IL-1β promoter is schematically illustrated. The approximate position of consensus sequences for known transcription factors are indicated (boxes), including NFκB, AP1, CRE, NF-IL6 and NFβA. The major transcription start site is located at position +1. (Adapted from Stylianou E and Saklatvala J: Interleukin-1. The International Journal of Biochemistry & Cell Biology 30:1075-1079,1988.)*

2.2.3 IL-1 RECEPTOR AND SIGNAL TRANSDUCTION

IL-1 receptors are expressed in most cells, including neurons, endothelial cells, neutrophils, T cells and B cells. [60,87]. There are two primary cell surface receptors for IL-1β. IL-1 type I receptor transduces a signal whereas the type II receptor binds IL-1β but does not transduce a signal. In fact, IL-1 type II receptor acts as a sink for IL-1β and has been termed a 'decoy' receptor [74]. A unique aspect of cytokine biology is a naturally occurring IL-1 receptor antagonist, which is structurally similar to IL-1β but lacking agonist activity [89].

When IL-1β binds to IL-1 type 1 receptor, a complex is formed which then binds to the IL-1R-AcP resulting in high-affinity binding [88]. Although it remains unclear how IL-1 type I receptor triggers the phosphorylation and activation, it has been found that, within a few minutes following binding to receptors, IL-1β induces intracellular multiple protein phosphorylations and activation of phosphatases via the receptor, resulting in cascades of activation of protein kinases [42,342].

One main cascade leads to phosphorylation of IκB (i.e. NF-κB inhibitory factor), which is rapidly degraded by the proteasome following its phosphorylation. Degradation of IκB activates NF-κB and releases it from the cytoplasmic NFκB-IκB complex [76]. Then, NF-κB translocates to the nucleus and binds to its DNA recognition site in the gene promoter to up-regulate the transcription of genes encoding pro-inflammatory cytokines (e.g., IL-1, 2, 6, 8, TNF, etc.), adhesion molecules (e.g., VCAM-1), chemokines, growth factors (e.g., CSF), inducible enzymes (e.g., COX-2, iNOS, etc.), anti-apoptotic enzymes (e.g., Mn-SOD) and growth proteins (e.g., c-myc) [10,156,215,236,357].

The other main cascade is the activation of three MAP kinases, which is a complicated pathway of phosphorylation of multiple protein kinases and is still poorly understood [342]. This signal transduction pathway mainly leads to the activation of cytoplasmic AP-1 (a dimer composed of various Fos and Jun family proteins), which translocates to the nucleus and binds to its DNA recognition site in the gene promoter to activate the transcription of many genes encoding proteolytic enzymes (e.g., MMPs), growth factors (e.g., NGF), growth protein (e.g., GAP-43), β-APP, galanin, neurofilament proteins and α-tubulin [53,167,178,236,309,394].

Thus, IL-1β exerts most of the biological effects on cells through the activation of NF-κB and AP-1, two major proinflammtory transcription factors common to many IL-1-induced genes [88].

An unusual discrepancy has been noted that low concentrations of IL-1β can induce a potent biologic response via a low number of receptors (fewer than 10-100 in some cells) [126,264], suggesting the efficiency of so few IL-1 type I receptors and the greatly amplified transduction of the post-receptor signals.

2.2.4 BIOLOGIC EFFECTS OF IL-1β IN SCI

IL-1β affects nearly every cell type, often in concert with other cytokines or small mediator molecules, e.g., TNF-α, as both IL-1β and TNF-α are potent activators of the transcription factor NF-κB [88,373]. IL-1β is a highly inflammatory cytokine and the margin between clinical benefit and unacceptable toxicity in humans is narrow [89]. It has been generally accepted that low concentrations of IL-1β protect neurons and are neurotrophic, whereas higher concentrations are neurotoxic [164,203].

Significantly increased IL-1β mRNA and protein expressions have been found in the cord after experimental SCI [19,164,192,216,341,357,380]. Some recent studies have demonstrated that within hours following SCI, activated microglia within the cord express IL-1β, which activates the endogenous glial cells [85] and stimulates the up-regulation of endothelial adhesion molecules to enhance leukocyte recruitment (e.g., ICAM-1 and VCAM-1) [19,164,179,341]. These findings suggesting that this early synthesis of IL-1β may be an important signal for initiating a progressive inflammatory response within the spinal cord.

The role that the increased IL-1β plays in mediating secondary SCI is still controversial. Many in vitro and vivo studies demonstrated that IL-1β may be neurotoxic. For example, it has been demonstrated by in vitro investigations that IL-1β is toxic to neurons [67]. Moreover, in vivo studies have suggested that IL-1β may contribute to tissue destruction, increased vascular permeability and oedema formation as it up-regulates MMP expression via the activation of AP-1, a major proinflammatory transcription factor [175,394,395]. MMP-1 plays a major role in tissue destruction in inflammation and MMP-9 degrades the extracellular matrix component of basement membrane leading to the loss of vascular integrity [394]. IL-1β is also implicated in the up-regulation of iNOS and COX-2 via the activation of NF-κB, another major proinflammatory transcription factor [10], resulting in an increase in NO and ROS production, and a TXA_2-PGI_2 imbalance, respectively [69,357]. An increased production of free radicals causes secondary tissue damage and an imbalanced production between TXA_2 and PGI_2 is involved in the microvascular thromboembolism and the secondary ischaemia following SCI. Additionally, IL-1β may stimulate formation of the astroglial scar [138,211], which is thought to be one element inhibiting the regeneration of injured axons through the lesion. Recently, Holmin and Mathiesen [175] found that intracerebral administration of IL-1β induces infiltration of inflammatory cells and apoptosis of intrinsic CNS cells. They also

found that IL-1β causes higher expression level of the pro-apoptotic Bax gene than the anti-apoptotic Bcl-2 gene. The most direct evidence implicating IL-1β in secondary SCI derives from experiments that employed a recombinant IL-1 receptor antagonist. Systemic injections of IL-1 receptor antagonist reduces neuronal damage, prevents apoptosis and improved cognitive function in rodents after CNS injury [122,223,257,302,360]. Furthermore, the administration of exogenous IL-1β markedly exacerbates ischaemic or excitotoxic CNS injury [206,300].

However, there are instances where IL-1β appears to act as a neurotrophic factor. In support of this, IL-1β attenuated the increased neuronal death, which was induced by adding an antiserum to IL-1α into dissociated mouse spinal cord cultures [49]. IL-1β could produce a neurotrophic effect indirectly via induction of some growth factors, such as NGF and FGF, in astrocytes [284,247]. Additionally, IL-1β can stimulate blood vessel growth [138], which is essential for the continued survival of tissue spared by the injury as well as any tissue repair that may contribute to functional recovery after SCI, which causes disruption of blood vessels and consequent ischaemia.

Thus, IL-1β is an important and modifiable component in the inflammatory response following SCI and has complex and bi-directional biological effects on the injured spinal cord.

2.3 IL-6

2.3.1 STRUCTURE

Human IL-6 is a glycoprotein with a molecular mass ranging from 21 to 28 kDa and consists of 212 amino acids including a hydrophobic signal sequence of 28 amino acids [60,345]. The structure is composed of four (A, B, C, D) helical bundles oriented in an 'up-up-down-down' configuration and three sites are involved in receptor interaction: site 1 is the binding site for IL-6 receptor α chain and sites 2 and 3 are those for gp130 (IL-6 receptor β chain) (Figure 3) [269].

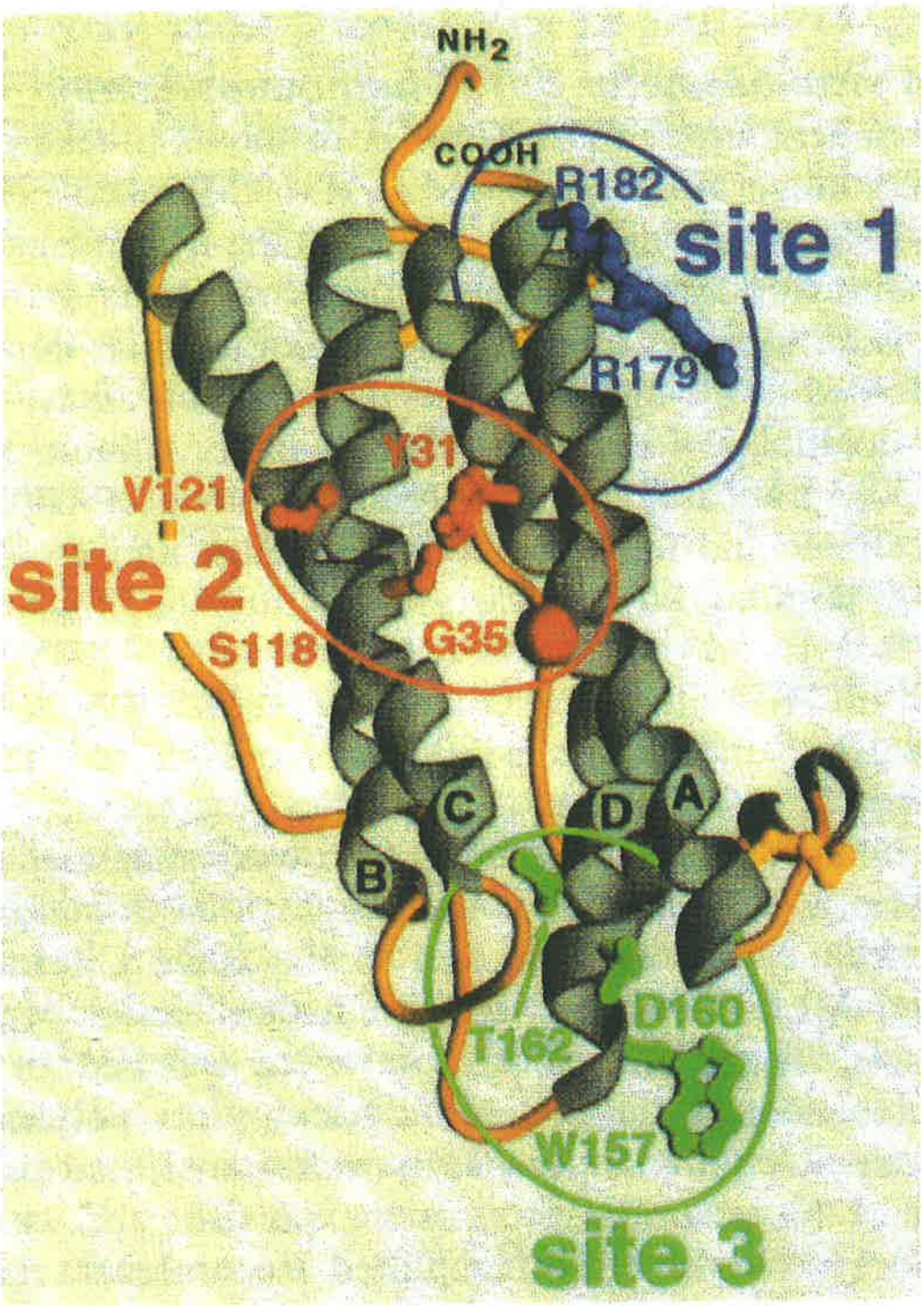


Figure 3. *Three-dimensional model of human IL-6. IL-6 is composed of four (A, B, C, D) helical bundles oriented in an 'up-up-down-down' configuration. Three sites are involved in receptor interaction: site 1(blue) is the binding site for IL-6 receptor α chain and sites 2 (red) and 3 (green) are those for gp130 (IL-6 receptor β chain). (Adapted from Paonessa G, Graziani R, Serio AD et al.: Two distinct and independent sites on IL-6 trigger gp130 dimer formation and signalling. The EMBO Journal 14(9): 1942-1951, 1995)*

2.3.2 IL-6 GENE AND IL-6 BIOSYNTHESIS

The human IL-6 gene is approximately 5 kilobases in length and consists of 5 exons and 4 introns on the short arm of chromosome 7 at the locus 7p14-7p21 [60,172,345].

Within the conserved region of the IL-6 promoter, there are several potential transcriptional factors such as an NF-κB binding site, a C-Fos SRE, a CRE, an AP-1 binding site and GRE [172,215,345]. Three DNA sequences in the IL-6 promoter are identified as the main functional transcriptional factors as shown in the figure: a 23-bp MRE, a 14-bp NF-IL6 and an NF-κB binding site (Figure 4). Additionally, the proposed stimuli to activate these three main functional transcriptional factors are also shown in the Figure 4, including IL-1, TNF, IL-6, LPS and serum.

The production of IL-6 is induced by a variety of stimuli, such as injury [157,197,204,315,354,365,388], pro-inflammatory cytokines (e.g., IL-1, TNF, IL-2, IFN-β etc.) [210,305,372], growth factors (e.g., PDGF) [172], LPS [210,369] and various viruses while its production is inhibited by glucocorticoids [316], IL-4 [133] and IL-13 [244].

Constitutive production of IL-6 is usually low or absent and its expression is tightly kept in check by a complex network, including sex steroids, e.g., estrogen and testosterone [372].

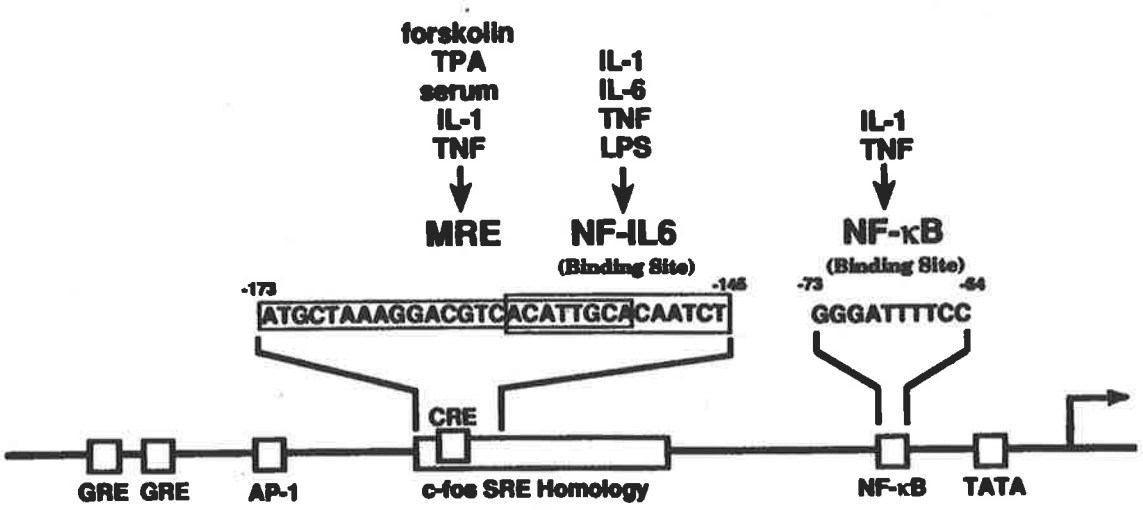


Figure 4. *A schematic structure of the IL-6 promoter region, where there are several potential transcriptional factors such as an NF-κB binding site, a C-Fos serum-responsive element (SRE), a cAMP-responsive element (CRE), an AP-1 binding site and a glucocorticoid-responsive element (GRE). Three DNA sequences in the IL-6 promoter are identified as functional transcriptional factors as shown in the figure: a 23-bp multiresponse element (MRE), a 14-bp sequence that is recognized by a nuclear factor, NF-IL6 and an NF-κB binding site. Additionally, the proposed stimuli to activate these three functional transcriptional factors are also shown. (Adapted from Aggarwal BB and Gutterman JU: IL-6, in Aggarwal BB and Gutterman JU (eds): Human cytokines: handbook for basic and clinical research. Boston, Blasckwell Scientific Publication:143-167,1992)*

2.3.3 IL-6 RECEPTOR AND SIGNAL TRANSDUCTION

IL-6 receptor is expressed on a wide variety of cells, including neurons, glial cells, monocytes, fibroblasts, T cells and activated B cells [60,345], IL-6 receptor consists of two molecules, one is an 80-kDa IL-6 binding protein (α chain) and the other a 130-kDa binding protein, gp130 (β chain) with 277-amino-acid residues in its cytoplasmic domain [172,269]. Thus, IL-6 receptor α chain and gp 130 (IL-6 receptor β chain) form a high-affinity IL-6 binding site. The cytoplasmic domain of IL-6 receptor α chain is not required for the IL-6-mediated signal transduction.

The binding of IL-6 to its receptor induces the formation of a hexamer composed of two each of IL-6, IL-6 receptor α chain and gp 130 [269], resulting in tyrosine phosphorylation of the cytoplasmic domain of gp130 [256]. Then, intracellular signalling molecules (e.g., STAT) specifically recognize and bind to the phosphorylated receptors (gp 130). Once bound to the phosphorylated receptors, these signalling molecules themselves become phosphorylated by receptor-associated kinases, enabling them to disengage from the receptors (gp 130) and interact with additional signalling molecules to form the intracellular IL-6 signal transduction pathway. The IL-6 signal transduction pathway mainly consists of the JAK-STAT and Ras-MAP kinase pathways [120,256].

JAK (named after Janus, the Roman god with two faces) are gp 130-associated kinases and become activated by cross-phosphorylation after IL-6-induced phosphorylation of the gp130. Although the JAK-STAT pathway is still not well understood, it is clear that JAK is critical for the activation of a specific signalling pathway, STAT [172,256]. Once phosphorylated by JAK, STAT molecules disengage from the receptor (gp 130) and translocate to the nucleus, where they bind to specific DNA motifs in the gene promoters and activate or inhibit the

transcription of these genes encoding anti-apoptotic proteins (e.g., bcl-2, pim-1, etc.), growth proteins (e.g., c-jun, c-myc, c-myb, etc.) and acute-phase proteins (e.g., CRP) [172,173,322].

In addition to the JAK-STAT signal transduction pathway, the other main pathway is the activation of Ras-MAP kinases through SHP-2. This cascade of kinase reactions lead to the phosphorylation and activation of NF-κB and ERKs [172,209,256]. Once activated, the NF-κB and ERKs translocate to the nucleus to phosphorylate their corresponding transcription factors, which up-regulate the expression of pro-inflammatory cytokines, adhesion molecules and growth proteins [10,156,256].

2.3.4 BIOLOGIC EFFECTS OF IL-6 IN SCI

IL-6, a multifunctional cytokine, is known to play an important role in a variety of CNS functions such as cell-to-cell signaling coordination of inflammatory response, protection of neurons from insult, as well as neuronal and glial differentiation, growth and survival [147,173,197,345], many of which overlap with those of IL-1β and TNF-α. It has been shown that a variety of stimuli, including injury, viruses and cytokines (e.g., IL-1β and TNF-α), can trigger IL-6 production within the CNS [381] and IL-6 receptors are present in neurons and oligodendrocytes [172,210,369,388].

Many experimental studies have demonstrated that IL-6 mRNA and protein are elevated in the cord following SCI [164,216,341]. It has been recently found that within hours following SCI, IL-6 protein is expressed by neurons and activated microglia within the cord [164].

The functional significance of IL-6 in the CNS is still hotly debated. It has been observed that IL-6 induces neurite outgrowth of neuronal cells in vitro [180], promotes oligodendrocyte

survival in vitro [18] and improves the survival of cultured rat cholinergic neurons with or without initiating the synthesis of neurotrophic factors [147,153,154,157,228]. Additionally, in vivo studies have demonstrated that local administration of IL-6 attenuates the neurotoxic effects of NMDA on rat striatal cholinergic neurons [361] and prevents degeneration of the spinal motoneurons of wobbler mice [181].

Conversely, it has been found in experimental SCI that anti-rat IL-6 antibody significantly inhibits the up-regulation of iNOS and reduces ultrastructural damages in the myelinated nerve fibres to attenuate secondary spinal cord damage [365]. Additionally, a severe neurologic syndrome with extensive neuropathologic changes (e.g., neuronal damage and reactive astrocytosis) was induced in transgenic mice with cerebral overexpression of IL-6 [64]. Moreover, the clinical features and neuropathology in the transgenic mice correlated with the level and spatial distribution of IL-6 overexpression.

Thus, IL-6 is both a neurotrophic and cytotoxic factor and is partially regulated by IL-1β and TNF-α [375].

2.4 TNF-α

2.4.1 STRUCTURE

TNF-α exists as a compact trimer composed of 3 identical subunits of 157 amino acids [3,150]. Each subunit consists almost entirely of antiparallel β-pleated sheets, organized into a 'jellyroll β sandwich' (Figure 5) [60,186]. The C-terminus of each subunit is embedded in the base of the trimer and the N-terminus is relatively free from the base of the trimer. Thus the N-terminus does not participate in trimer interaction and is not critical for TNF-α biologic activity. Additionally, mutations that destabilize the trimeric association of monomers result in the loss of TNF-α biologic activity, suggesting that the trimeric structure of TNF-α is important for its biologic functions [405].

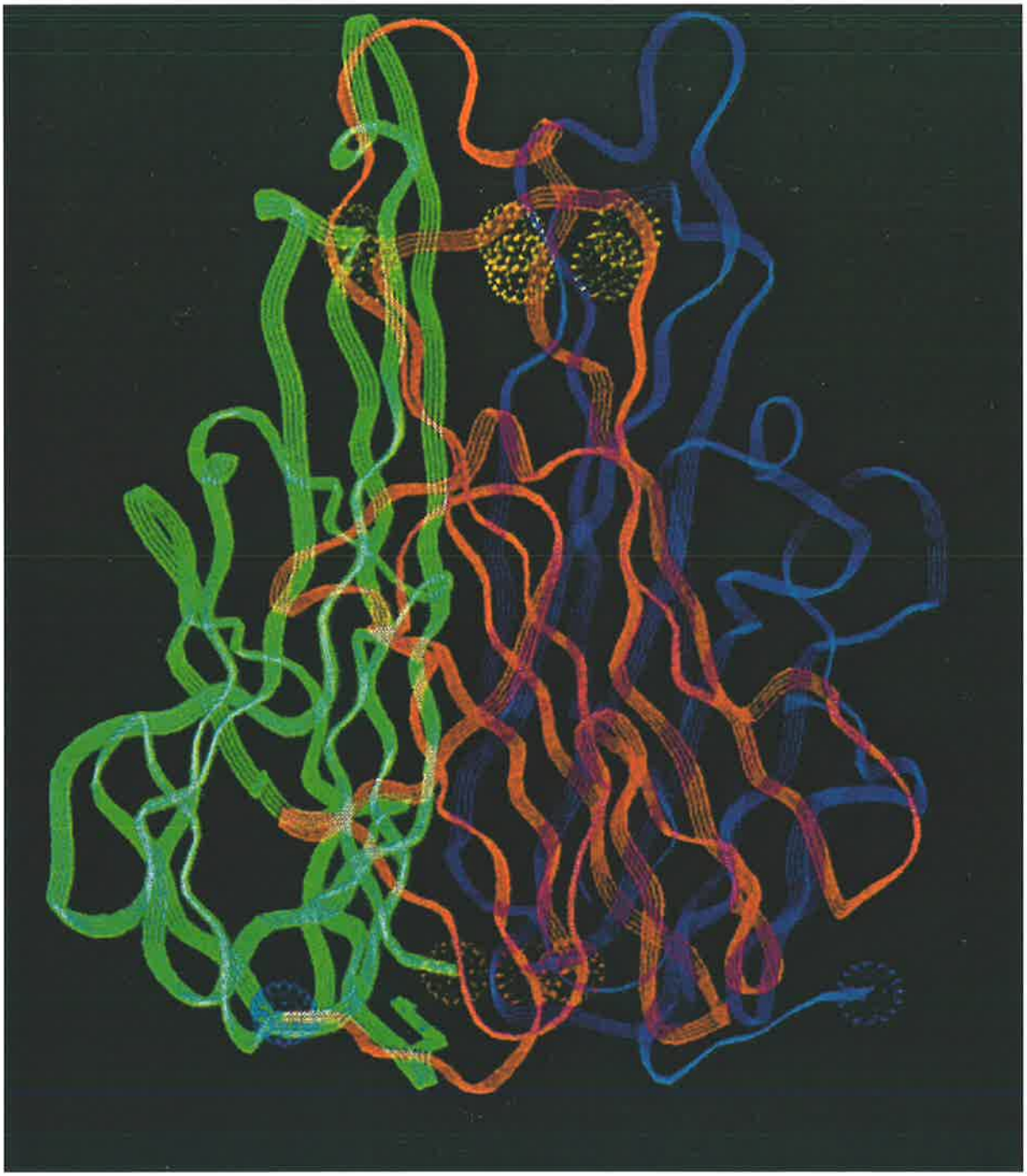


Figure 5. *Ribbon representation of the trimer with the 3-fold axis vertical. Individual subunits are colour-coded blue, green and red. N-termini are marked by blue spheres and C termini by red. Yellow spheres near the top of the molecule indicate the disulphide bridges. (Adapted from Jones EY, Stuart DI and Walker NPC et al.: Structure of tumour necrosis factor. Nature 338 (6212): 225-228, 1989)*

2.4.2 TNF-α GENE AND TNF-α BIOSYNTHESIS

The human TNF-α gene is approximately 3.6 kilobases in length and consists of 6 exons and 3 introns on the long arm of chromosome 6 at the locus 6q12-6q23 [3,60].

A number of regulatory transcription factors are found in the promoter of the TNF-α gene (Figure 6), including three NF-κB binding sites κ1, κ2 and κ3; three NFAT binding sites for NFAT-149, NFAT-117 and NFAT-76; one CRE for activation transcription factor-2/Jun, one AP-1 and one AP-2 binding site for Fos/Jun [405].

TNF-α is initially synthesized as a 233-amino-acid membrane-anchored prohormone, which is then cleaved by the TNF-α converting enzyme to yield the mature 157-amino-acid cytokine [60]. TNF-α has been shown to be produced by numerous cells, including immune cells (e.g., neutrophils, monocytic cells, B cells, T cells, natural killer cells, etc.) and non-immune cells (e.g., astrocytes, microglia, neurons, fibroblasts, osteoblasts, etc.) [405].

The biosynthesis of TNF-α is tightly controlled at many different levels to ensure the silence of the TNF-α gene under normal circumstances. Therefore, TNF-α is produced in vanishingly small quantities in quiescent cells, but is highly inducible by traumatic injury, ischaemia, cytokines (e.g., IL-1, IL-2, IFN, etc.), growth factors (e.g., CSF) and bacterial products (e.g., LPS) [208,210,237,258,379,396].

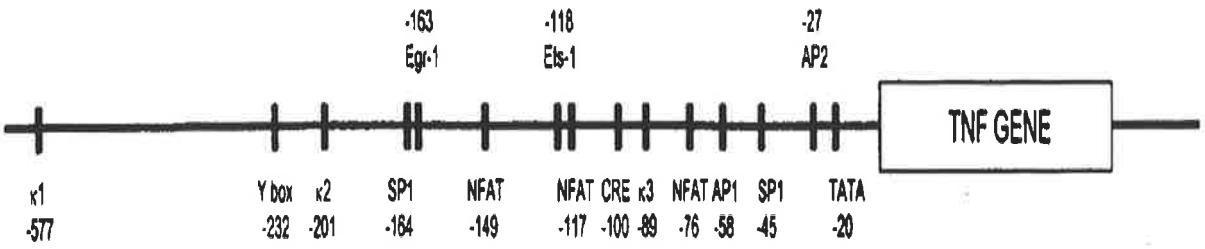


Figure 6. *Model of the TNF-α promoter. A number of transcription factor in the TNF-α promoter are found upstream of its gene, including three NF-κB sites κ1, κ2 and κ3; three NFAT binding sites, one CRE for activation transcription factor-2/Jun, one AP-1 and one AP-2 binding sites for Fos/Jun. (Adapted from Zhang M and Tracey KJ: Tumour necrosis factor, in Thomson A (eds): The cytokines handbook. San Diego, Academic Press:517-548,1998)*

2.4.3 TNF-α RECEPTOR AND SIGNAL TRANSDUCTION

TNF-α receptors are present on nearly all cell types with a few exceptions such as erythrocytes and resting T cells. There are two distinct but structurally homologous TNF-α receptors, type I and type II with molecular masses of 60 and 80 kDa, respectively. TNFR1 is found on most cell types whereas TNFR2 is more restricted to endothelial and haematopoietic cells [60,405].

It is generally believed that TNFR1 is responsible for the majority of biologic actions of TNF-α while signal transduction through TNFR2 occurs less extensively and is confined mainly to cells of the immune system. Thus, the section below concentrates on the signal transduction through TNFR1.

Three intracellular functional domains of TNFR1, i.e., death domain, NSD and ASD, are responsible for transferring signals from TNF-α to their corresponding intracellular adaptor proteins, TRADD, FAN, PC-PLC, via protein-protein interactions.

Once TRADD has bound to the TNFR1 death domain, it causes recruitment and activation of FADD and RIP through the C-terminal death domain and recruitment and activation of TRAF-2 through the N-terminal death domain. Activated FADD and RIP then bind and activate caspase-2 and caspase-8, respectively. These two caspases initiate a protease cascade, resulting in the cleaving of death substrates and activation of death protein (e.g., Par-4) to induce apoptosis [84,311,405]. Meanwhile, activated TRAF-2 triggered activations of MEKK and JNK, lead to the activation NF-κB and AP-1 to mediate the inflammatory response, by induction of pro-inflammatory cytokines (e.g., IL-1, 2, 6, 8, TNF, etc.), adhesion molecules (e.g., VCAM-1), inducible enzymes (e.g., COX-2, iNOS, etc.), chemokines [10,156,215,236,357], and

anti-apoptosis molecules (e.g., Mn-SOD, CSF, etc.) [171,209,370,405]. Thus, the TNFR1 death domain mediates both apoptotic and anti-apoptotic pathways.

FAN links the NSD of TNFR1 directly to neutral sphingomyelinase, which is then activated to mediate cell proliferation and the inflammatory response through MAP kinase ERK and PLA-2, respectively [370,405].

Once PC-PLC is activated, 1,2-diacylglycerol is produced by PC-PLC and activates acidic sphingomyelinase, which converts lipid sphingomyelin to ceramide. The rapidly raised intracellular level of ceramide acts as a secondary messenger, leading to NF-κB activation to mediate anti-apoptosis and the inflammatory response [370,405].

Thus, the downstream pathways of these three adaptor proteins result in various cell activities, including apoptosis, anti-apoptosis, inflammatory response and cell proliferation.

2.4.4 BIOLOGIC EFFECTS OF TNF-α IN SCI

The presence of TNF-α has been reported in various cells in the CNS, including microglia, astrocytes and neurons [68] and these cells also express high-affinity TNF-α receptors [329]. It is generally believed that high levels of TNF-α are injurious, but low levels can be beneficial [362,405].

TNF-α shares some similar properties with IL-1β in terms of pro-inflammatory effects, such as activation of endogenous glial cells [366], proliferation and hypertrophy of astrocytes [379], enhanced permeability of endothelial cells and enhanced recruitment of blood leucocytes [348].

This may be due to the fact that both TNF-α and IL-1β are potent activators of NF-κB, a major pro-inflammatory transcription factor [10].

Many experimental studies have demonstrated that TNF-α mRNA and protein are elevated in the cord following SCI [19,164,208,216,341,379,393]. It has been recently found that within hours following SCI, TNF-α protein is expressed by neurons and activated microglia within the cord [194,396].

The functional significance of TNF-α in the SCI is still not well understood. Some studies have demonstrated cytotoxic roles of TNF-α. It has been shown that TNF-α has a direct toxic effect on oligodendrocytes in vitro [94] and a single micro-injection of TNF-α caused demyelination in the spinal cord [325] and in the sciatic nerve [290]. Furthermore, in transgenic mice overexpressing TNF-α, a spontaneous inflammatory demyelination in the brain and spinal cord occurred and was partially prevented by exogenously delivered neutralizing antibodies to TNF-α [283]. In addition to the toxic effect on oligodendrocytes, TNF-α can induce apoptotic changes in cultured neurons and glial cells [238,346,370] and cause BBB injury with damage to endothelial cells [80]. In support of cytotoxic roles of TNF-α, some in vivo studies have also demonstrated that following CNS injury, inhibition of TNF-α by activated protein C, IL-10 or a neutralizing antibody significantly improved neurological outcome [28,205,349].

In contrast to its toxic actions, TNF-α has also been demonstrated to induce IL-6, NGF and other growth factors in astrocytes [305,399] and exert a regenerative effect on axons [313]. Additionally, TNF-α prevents glutamate-induced cell death and cytotoxic effects of amyloid β-peptides and iron by regulating intracellular calcium levels and suppressing the

accumulation of ROS in pure hippocampal cultures [16,68]. Another important neurotrophic effect of TNF-α is the induction of angiogenesis [213]. Additionally, improved recovery of motor function was observed in NOS-deficient, but not in TNF-α-deficient mice after SCI [111]. Furthermore, a study using TNFR knockout mice (both TNFR1 and TNFR2 deleted) has shown that neurons from the TNFR knockout mice exhibit reduced survival in culture compared with those from wild-type mice and doses of kainic acid that cause minimal damage in wild-type mice produce extensive neuronal loss in TNFR knockout mice [55].

It has been recently proposed that the neurotrophic and neuroprotective effects of TNF-α may depend on the ratio of TNFR1 to TNFR2 levels [26]. One possibility is that higher levels of TNFR2 may contribute to a cytoprotective response and increased levels of TNFR1 may induce a cytotoxic response [102].

In summary, TNF-α is a powerful inflammatory mediator with both cytotoxic and neurotrophic actions.

3. MICROGLIA

Microglia represent 5-12% of the cells in a given brain region [387] and these resident cells comprise about up to 20% of the total glial cell population [25]. It has been shown that microglia form an extremely stable cell population with little turnover and with less than 1% bone marrow precursor cells [85,170].

3.1 NOMENCLATURE

del Rio-Hortega in 1919 first used the term microglia to describe a type of glial cell that could be differentiated from neurons, astrocytes and oligodendrocytes by its distinctive morphology with silver impregnation techniques [81]. The nature and identity of microglial cells have triggered much debate and research since their discovery. However, it is now generally accepted that microglia are ubiquitous, mesodermally derived glial cells of characteristic morphology and phenotype which are evenly distributed throughout the central nervous system, and whose functions are primarily related to phagocytosis, antigen presentation, cytotoxicity and neuronal survival [338]. The term "macrophage" is used to refer to a monocyte-derived phagocyte in the parenchyma of CNS [92]. The distinction is complicated by the fact that macrophages are derived, at least in part, from intrinsic microglia in the CNS and that it is technically difficult to distinguish between phagocytic microglia and monocyte-derived macrophages [117,339]. Thus, microglia are also called as "resident macrophages of the CNS" [25].

3.2 CLASSIFICATION

In view of their morphological and functional characteristics in the mature CNS, three different types of microglia can be distinguished [25,339]:

1. the ramified (resting) microglia with long and branched processes, found predominantly in the white matter of normal mature CNS;

2. the ameboid (activated), but non-phagocytic microglia with cellular hypertrophy, and shortened and thickened processes, in response to sublethal injury without causing terminal degeneration and necrosis such as follows nerve transection; and
3. the foamy (phagocytic) microglia in areas of necrosis produced by varied mechanism including neurotrauma.

Figure 7 demonstrates the progressive transformation in the morphological appearance between ramified (resting) microglia and ameboid (activated) microglia.

Although neither the ameboid nor ramified shapes may be reliable indicators of the functional state of microglia, ramified microglia are often referred to as "resting" microglia and ameboid microglia as "activated" microglia in the mature CNS. However, there is no consensus as to what constitutes the best marker for microglial activation, especially in situ [85].

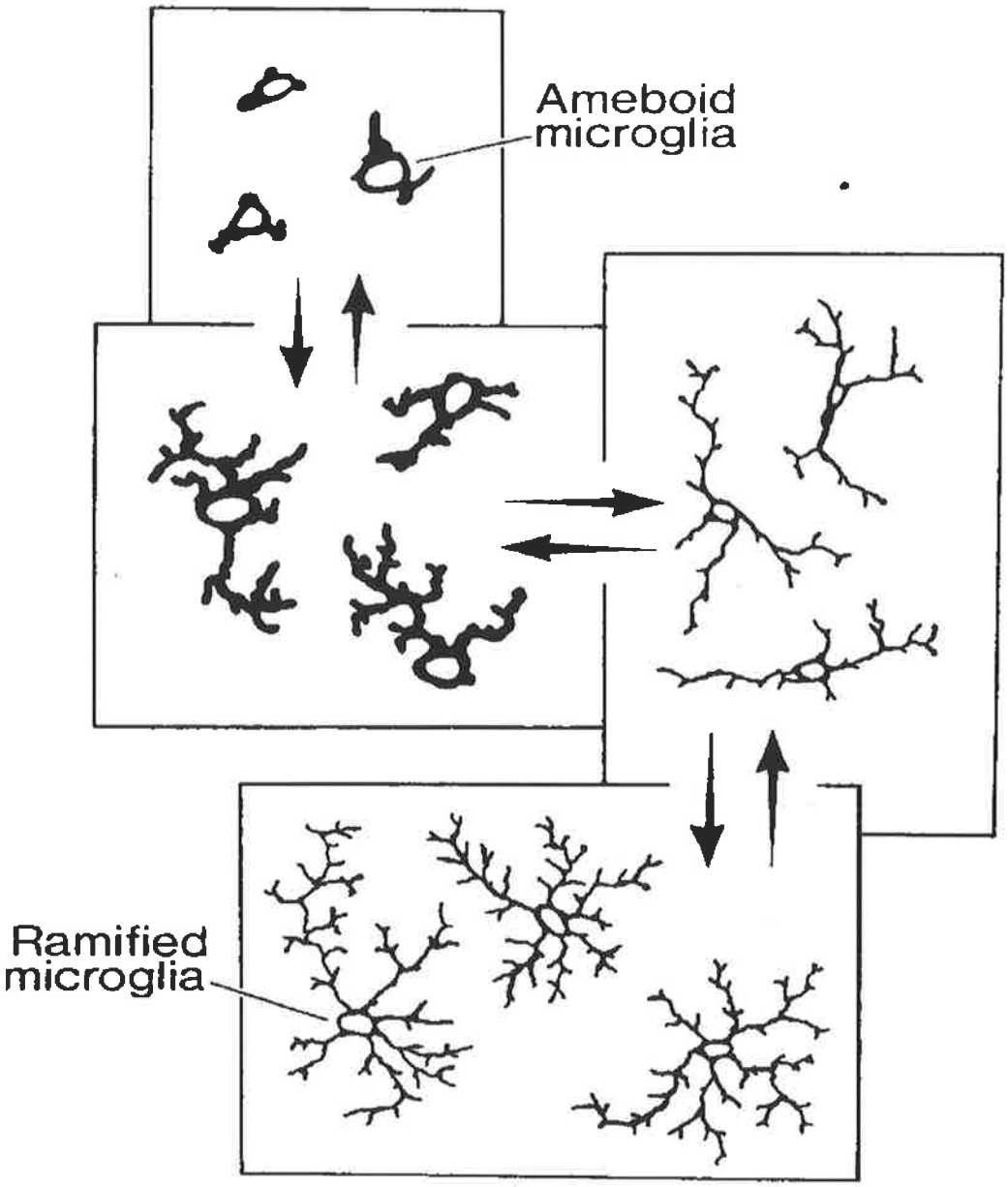


Figure 7. *Schematic diagram of interrelations between ramified and ameboid microglia. In disease states, the ramified microglia are gradually activated, showing progressive transformation in the morphological appearance. (Adapted from Dickson DW and Lee SC: Microglia, in Davis RL and Robertson DM (eds): Textbook of neuropathology. Boston, Williams & Wilkins: 165-205,1997)*

3.3 MICROGLIAL ACTIVATION

3.3.1 RESTING MICROGLIA

Resting microglia with numerous fine antler-like processes show a highly down-regulated immunophenotype and are in a functionally quiescent state [272,339]. The cell surface antigens on resting microglia in the physiologically quiescent state include complement receptor type 3 and Fc, but not MHC I, II and CD4 [382]. Microglia can most rapidly respond to injury in the CNS. They respond not only to changes in the structural integrity of CNS but also to very subtle alterations in their microenvironment, such as imbalances in ion homeostasis that precede pathological changes detectable histologically [129]. Thus, microglial activation often precedes reactions of any other cell type in the CNS [200].

3.3.2 ACTIVATED MICROGLIA

To define stages of microglia activation in vivo, the model of facial nerve axotomy, which leaves the BBB unimpaired, was used as it allows the study of the activation of microglia in the absence of infiltrating haematogenous macrophages [339]. In that study, the facial nerve was cut outside the brain and the reactions of facial motoneurons and their glial environment were studied in the brainstem. Following facial nerve transection, ramified microglia proliferated, became ameboid with cellular hypertrophy and shortened processes, and expressed several new surface antigens, including MHC I, II and CD4 [339]. It has also been noted that microglia in a perineuronal position started to ensheath the injured motor neurons and interpose processes between afferent synaptic terminals and the neuronal surface. The phenomenon, originally described by Blinzinger and Kreutzberg [37], is now known as "synaptic stripping", and its functional significance is not well understood. It has been suggested that detachment of synaptic terminals from the motor neuron surface may influence synaptic reorganization of injured motor neurons [25]. In addition, APP is newly expressed by activated perineuronal

microglia [12]. But, again, the significance of the presence of APP in activated microglia remains enigmatic.

It has also been observed that, upon cellular activation, microglia are capable of producing large quantities of a number of neurotoxic mediators, including EAAs, proteases, cytokines and NO [200,387]. Details will be discussed in section 3.4.

3.3.3 PHAGOCYTIC MICROGIA

Under the condition of facial nerve axotomy, microglia become activated but not phagocytic. However, if motor neuron death is induced by injection of toxic ricin into the facial nerve, microglia rapidly transform into phagocytic microglia [340], and gradually migrated into the injury site to remove the neuronal cell debris. In addition to complement receptor type 3, Fc, CD4, MHC I and II, these microglia-derived macrophages also express the ED1 epitope which is otherwise typical of peripheral macrophages [25]. Thus, it has to be stressed that it is technically difficult to distinguish between intrinsic phagocytic microglia and monocyte-derived macrophages [25,117,339,382].

3.3.4 SUMMARY

In summary, the microglial response to injury occurs in a rather stereotypic pattern, irrespective of the cause of the lesion [128,339]. Results obtained from the facial-nerve transection model show that activation of microglia displays a repertoire in terms of proliferation, migration to the site of injury, characteristic morphological, immunophenotypical and functional changes, and that microglial activation takes place in a graded fashion. In the first stage of activation, microglia become activated, but do not become phagocytic. In the second

stage of activation, following neuronal and/or axonal terminal degeneration, microglia transform into phagocytes, also known as microglia-derived brain macrophages.

Thus, the activation process is a graded event that is dependent on the severity of the insult and is most probably regulated by specific signals, e.g., cytokines, in a well-controlled manner [128].

3.4 FUNCTIONS OF MICROGLIA IN SCI

Functional significance of activated microglia in SCI is still poorly understood. Microglia have shown both cytotoxic and neurotrophic effects on the injured tissue, suggesting a possible dual-edged role [25].

3.4.1 CYTOTOXICITY

Many studies have demonstrated a swift activation of microglia following SCI [323,341,382]. There are two principal ways by which microglia may act as cytotoxic cells.

First, phagocytic microglia express Fc and complement receptors on their surfaces [85]. In particular, during antibody-mediated demyelination, activated microglia can lyse antibody-coated target cells via interaction of Fc and complement receptors on their surfaces with immune complexes and complement-opsonized antigens [404].

Second, activated microglia are capable of releasing several potentially cytotoxic substances in vitro such as cytokines (e.g., TNF-α, IL-1β and IL-6), free oxygen intermediates, NO, proteinases, arachidonic acid derivates and EAAs [12,75,242,323,355,387]. This is further supported by findings in animal models of CNS trauma that at the lesion site activated microglia have

been shown by microdialysis measurements and in situ hybridisation to release high levels of cytokines (e.g., IL-1) within 24 h following injury [388]. Shuman et al. [323] observed microglia with an activated morphology in the same regions as oligodendrocyte apoptosis and apparent contact between some of the apoptotic oligodendrocytes and microglial processes after spinal cord contusion in the rat, suggesting microglia-produced TNF-α may cause oligodendrocyte apoptosis. Microglia-produced TNF-α may also cause direct damage to myelin, furthering demyelination [317]. Additionally, Combs et al. [75] demonstrated in a tissue culture model that conditioned media from β-amyloid- and prion- stimulated microglia are neurotoxic to mouse neurons.

Interestingly, the cytotoxic properties of microglia can be modulated by cytokines, glucocorticoids and neurotransmitters [224]. Cytokines such as IFNγ prime microglia to become activated whereas other cytokines such as TGFβ1 or IL-4 downregulate microglial cytotoxicity [243]. Neuronal activity may also control microglial activation. β-adrenergic agonists like isoproterenol have been shown to reduce IL-1 and TNF-α production by microglia [200]. Additionally, glucocorticoids, such as dexamethasone and corticosterone, are also potent inhibitors of microglial activation [224]. Furthermore, potent inhibitors of macrophage/microglial activation have been shown to reduce the extent of tissue damage and improve the motor function after ischaemic injury in the rabbit spinal cord [137].

However, most of the information on the cytotoxic properties of activated microglia pertains to in vitro observations and still remains to be confirmed in vivo.

3.4.2 NEUROTROPHY

Although activated microglia are generally regarded as cytotoxic effector cells, they also exert neurotrophic effects on the injured tissue to promote tissue repair and regeneration after injury. For example, TGFβ1 produced by activated microglia after CNS trauma or after ischaemic injury may either promote tissue repair directly or indirectly by reducing astrocytic scar formation [219,386,388]. Microglia secrete both the urokinase-type plasminogen activator and plasminogen, which have been shown to promote neurite outgrowth [253,254]. Microglia-secreted plasminogen may also be involved in tissue remodelling after injury [25]. In fact, tissue remodelling by activated microglia precedes the regeneration of optic nerve of fish or amphibia which, unlike the mammalian CNS, has a high capacity for regeneration, [200], suggesting microglial activation may support regeneration. Furthermore, a recent study has demonstrated that transplantation of activated macrophage/mciroglial cells can significantly promote the regeneration of sensory axons into the injured spinal cord in the rat [280].

It is also reasonable to assume that following injury, immediately activated microglia release mediators such as IL-1, which may induce astrocyte activation [110], as IL-1 is capable of inducing hyperplasia and hypertrophy of astrocytes when injected intracerebrally [138]. Reactive astrocytes may, in turn, contribute to CNS regeneration by secreting neurotrophic factors such as neurotrophins or insulin-like growth factors, which promote neuronal and/or oligodendrocyte survival [130].

AIMS AND HYPOTHESES

HYPOTHESES

1. The pro-inflammatory cytokines IL-1β, IL-6 and TNF-α act as messengers to coordinate the inflammatory cascade in secondary SCI.
2. The cytokine response should be greater in severe than in mild SCI.
3. The NF-κB antisense should significantly inhibit the upregulation of IL-1β, IL-6 and TNF-α after SCI by blocking the activation of NF-κB.

AIMS

1. To investigate early posttraumatic inflammatory cellular response in rat and human SCIs.
2. To demonstrate and compare temporal mRNA expression patterns for IL-1ß, IL-6 and TNFα in mild and severe rat SCIs.
3. To demonstrate and compare temporal protein expression patterns for IL-1ß, IL-6 and TNFα in mild and severe rat SCIs.
4. To compare temporal mRNA and protein expression patterns for IL-1ß, IL-6 and TNFα in severe rat SCI with the patterns in rats treated with the NF-κB antisense after SCI.
5. To localize cellular sources of interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) in rat and human SCI.

RAT SCI EXPERIMENTS

1. MATERIALS AND GENERAL METHODS

1.1 ANIMALS USED AND ETHICS APPROVAL

All the experimental procedures were approved by the Animal Ethics Committees of the Institute of Medical and Veterinary Science and the University of Adelaide. One hundred and twenty-six male Sprague-Dawley rats were used in the experiments and were supplied by and housed in the Institute of Medical and Veterinary Science (Table 2). Prior to surgery, rats were kept in cages in groups of up to six. Rats were weighed at the first anaesthetic. Postoperatively, rats were housed in individual cages and given food and water ad libidum. Analgesia was administered as required.

Table 2. *Rats used to study traumatic spinal cord injury.*

Experiment	**Number of rats**	**Average weight ± SD**
Weight-drop model of SCI	33	412 ± 18 g
Inflammatory cellular response & cellular localization of IL-1β, IL-6 and TNFα after SCI	31*	420 ± 20 g
Studies of IL-1β, IL-6 & TNFα mRNAs and proteins after SCI	60	418 ± 22 g
NF-κB antisense treatment after SCI	14	422 ± 23 g

* Includes 12 rats in the 'weight-drop model of SCI'.

1.2 ANAESTHESIA

Anaesthesia was induced in each rat with 4% isoflurane in oxygen (3 L/min) in an anaesthetic chamber. Rats were positioned prone. Anaesthesia was maintained with the animal self ventilating 2.5% isoflurane in oxygen (1.5 L/min) through a nose cone. Core body temperature was continuously monitored and maintained at 38 ± 1 °C by a heating pad. Following the surgical procedure, anaesthesia was withdrawn and the rat supplied with 100% oxygen for 1-2 minutes before placing the rat in the postoperative recovery cage.

1.3 SURGICAL PROCEDURE

A 3 cm midline incision was made in the skin, followed by blunt dissection to expose the lower thoracic spine. Paraspinal muscles were stripped from the spinous processes of T10 to T13 under a dissecting microscope. A laminectomy of T12 was performed to expose a circular region of dura approximately 8 mm in diameter. The spinal column was stabilized by clamping the spinous processes of T11 and T13 with angled Allis clamps held in custom-machined holders. The clamps were then raised so that the lumbar region of the rat was suspended by the spinous processes. The prepared rat was positioned under a weight-drop device, which was used to induce the spinal cord contusions (details seen in 2.1.2). Mild and severe SCIs were respectively produced by dropping a 10-g weight from 3.0 or 12.0 cm onto the exposed dura of rat spinal cord at the T12 vertebral level. After injury, the wound was closed in layers using 3-0 synthetic absorbable sutures.

1.4 NEUROLOGICAL EXAMINATION

After rats recovered from surgery, neurological examinations were performed daily until sacrifice. Neurological function was evaluated with a grading system modified from those of Tarlov [351] and Delamarter et al. [82] (Table 3)

Table 3. *Motor score: grading of neurological function in experimental rats*

Grade	Function
0	No movement of hind limb; no weight bearing
1	Barely perceptible movement of hind limb; no weight bearing
2	Frequent movement of hind limb; no weight bearing
3	Can support weight on hind limb; may take one or two steps
4	Walks with only mild deficit
5	Normal walking

1.5 PERFUSION-SACRIFICE

Following a survival period of 1 h – 14 days, rats were induced and anaesthetized with isoflurane. The rat was positioned supine and the thoracic cage opened to expose the heart and aorta. A blunt 19 gauge needle was introduced through the apex of the heart into the ascending aorta. Two millilitres of 1000 IU/ml heparin was injected into the aorta. The heart was clamped transversely at the apex to secure the needle in place. The right atrium was opened. The rat was then perfused with either freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4, at room temperature) or normal saline for 10 minutes at a pressure of 120 mmHg. Perfusion pressures were generated with compressed air in a closed system and monitored with a sphygmomanometer [304].

1.6 TISSUE PROCESSING

1.6.1 REMOVAL OF SPINAL CORDS FROM RATS PERFUSED WITH 4% PARAFORMALDEHYDE

The entire spinal column was removed from each rat immediately after perfusion-fixation and postfixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight. The spinal cord was then dissected out and divided into nine 1-cm segments. Each segment was marked at the rostral end on the right side with a small vertical incision to allow orientation.

Tissue blocks were dehydrated in graded alcohol baths and cleared with chloroform, prior to processing into paraffin wax. Five micrometer serial sections were cut at (150 μm) levels with a microtome and mounted onto slides coated with APT (Sigma Chemical Corporation, US). The sections were dried at 37°C for at least 12 hours prior to use. Sections from each level were stained with H&E and immunohistochemically.

1.6.2 REMOVAL OF SPINAL CORDS FROM RATS PERFUSED WITH NORMAL SALINE

Spinal cord segments (2 cm in length and 190-210 mg in weight) encompassing the lesion epicentre were quickly removed from rats, snap-frozen in dry ice and placed in a sterile bag immediately after perfusion. Tissue samples were stored at -70°C until RT-PCR and Western blotting analyses.

1.7 PHOTOGRAPHY

Photomicrographs were taken with an Olympus PM20 camera system and an Olympus BX50 microscope. Film used for microscopic photography was Kodak EPY-36 Tungsten 64T Ektachrome.

2. WEIGHT-DROP MODEL

This experiment was performed to determine whether or not a graded (mild and severe) and consistent SCI could be produced in the rat with the weight-drop device.

2.1 METHODS

2.1.1 ANIMALS

Thirty-three adult male Sprague-Dawley rats were used and divided into three groups; laminectomy control, mild and severe SCI. The control group (N=3) received a T12 laminectomy only and were sacrificed at 14 d postinjury. The mild SCI group consisted of fifteen rats, which were injured by dropping the 10-g weight 3 cm onto the spinal cord and were sacrificed at 1 d, 7 d or 14 d postinjury (n=5 for each time point). In the severe SCI group, fifteen rats were injured by dropping the 10-g weight 12 cm onto the spinal cord and were sacrificed at 1 d, 7 d or 14 d postinjury (n=5 for each time point).

2.1.2 WEIGHT-DROP TECHNIQUE

The weight-drop device consisted of a Teflon impounder, 2 g weight, aligned but loosely fitted over the end of a brass rod inserted through a 10-g weight. The brass rod was attached to a stereotactic frame that allowed precise positioning along the X, Y, and Z coordinates. The spinal column was stabilized by clamping the spinous processes of T11 and T13 with angled Allis clamps held in custom-machined holders and the impounder was then centred over the laminectomy and lowered onto the dura. The 10-g weight was held in place by a pin which, when removed, allowed the weight to fall. The 10-g weight was then dropped 3.0 or 12.0 cm onto the impounder and 5 seconds later, the entire rod assembly was raised off the dura. For the laminectomy control group, the impounder was lowered onto the dura for 2 minutes but no weight was dropped.

2.1.3 MOTOR SCORE

Motor performances of hind limbs were analyzed with a grading system modified from those of Tarlov [351] and Delamarter et al. [82] (Table 3). A motor score was assigned to the left and right hind limb separately and the motor scores of the left and right hind limbs were averaged together to yield one score for a rat per test session.

2.1.4 HISTOPATHOLOGY

Tissue sections were stained with haematoxylin and eosin. The epicentre of the injury was identified histologically by reviewing serial transverse sections and selecting the section that reflected the maximal extent of the lesion with light microscopy. The area of the maximal lesion was measured with NIH image 1.62 software (NIH, Bethesda, MA) and the length of the spinal cord lesion was derived from the number of serial transverse sections showing histological abnormality.

2.1.5 STATISTICS

Wilcoxon Rank Sum Test and T-test were respectively used to compare the differences in Tarlov score and histopathology between mild and severe rat SCI. A P value less than 0.05 was considered significant.

2.2 RESULTS

2.2.1 MOTOR PERFORMANCE

As a motor score was assigned to the left and right hind limbs separately, asymmetry in the lesion was detected in some rats. However, for the most part, the deficits appeared

symmetrical with the same score given to both hind limbs. In no case did the score between hind limbs and vary by more than one point.

Table 4 shows the motor scores for laminectomy control, mild and severe SCI groups and statistical comparisons of pairs of the mild and severe SCI groups with respect to the motor score at 1, 7 and 14 days after injury (refer to Appendix 2).

The laminectomy control group did not show any motor function deficits while mild and severe SCI groups presented varying degrees of deficits. In no case did the score between rats in the same group vary by more than one point.

Motor functions of the hind limbs in the mild SCI group demonstrated mild and transient declines and then gradually and fully recovered to the normal level, whereas motor functions of rats with severe SCI were significantly impaired and improved marginally over a period of two weeks after injury. The mild SCI group had significantly better motor scores than the severe SCI group at 1, 7 and 14 days after injury.

Table 4. *Motor scores for laminectomy control, mild and severe SCI groups & comparisons of pairs of the mild and severe SCI groups with respect to the motor score*

Group	Weight-drop Height (cm)	Motor score (median)		
		1 d	7 d	14 d
Laminectomy control	0.0	5	5	5
Mild SCI	3.0	4	5	5
Severe SCI	12.0	0	0.5	1
Comparisons between mild and severe SCI		$P < 0.05$	$P < 0.05$	$P < 0.05$

2.2.2 HISTOPATHOLOGY

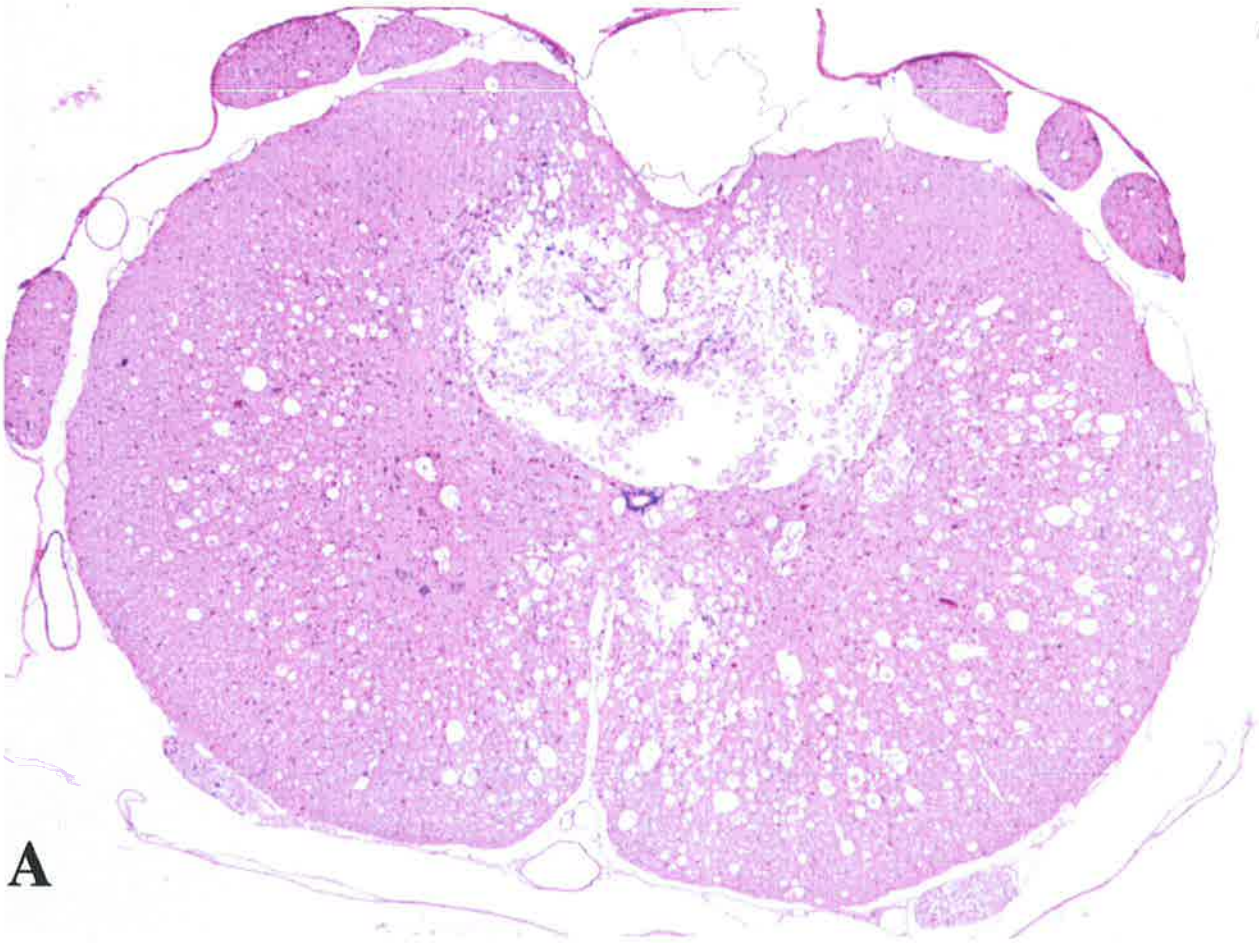
The 3-cm weight-drop contusions in the group of mild SCI produced central core lesions, which were characterized by cavities that replaced some of the grey and white matter. There were also multiple small microcysts in the white matter, some of which contained swollen axons or axonal debris. A representative transverse section at the lesion centre in mild SCI at 14 days after injury is shown in Figure 8A.

The 12-cm weight-drop contusions in the group of severe SCI created subtotal losses of grey and white matter and only tiny and isolated patches of white matter remained on peripheral edges of the ventral funiculus. A representative transverse sections in severe SCI at 14 days after injury is shown in Figure 8B.

No tissue loss or pathological changes were observed in the laminectomy control group (Figure 8C).

Statistical comparisons were done between the mild and severe SCI groups with respect to the area of maximal lesions and the length of lesions at 14 days after injury, showing significantly greater areas of maximal lesion (6.70 ± 0.37 mm^2) and lengths of lesion (13.23 ± 0.81 mm) in severe SCI than in mild SCI (area of maximal lesion: 1.21 ± 0.11 mm^2 & lesion length 2.88 ± 0.26 mm)(refer to Appendix 2). P values are both less than 0.05.

Figure 8. *Representative transverse sections at the epicentre of the lesion for a 3 cm (A) and 12 cm (B) weight-drop contusion SCI, 2 weeks after injury. C shows a transverse section of a laminectomy control rat spinal cord at T12 segment, 2 weeks after laminectomy. H&E, X10.*



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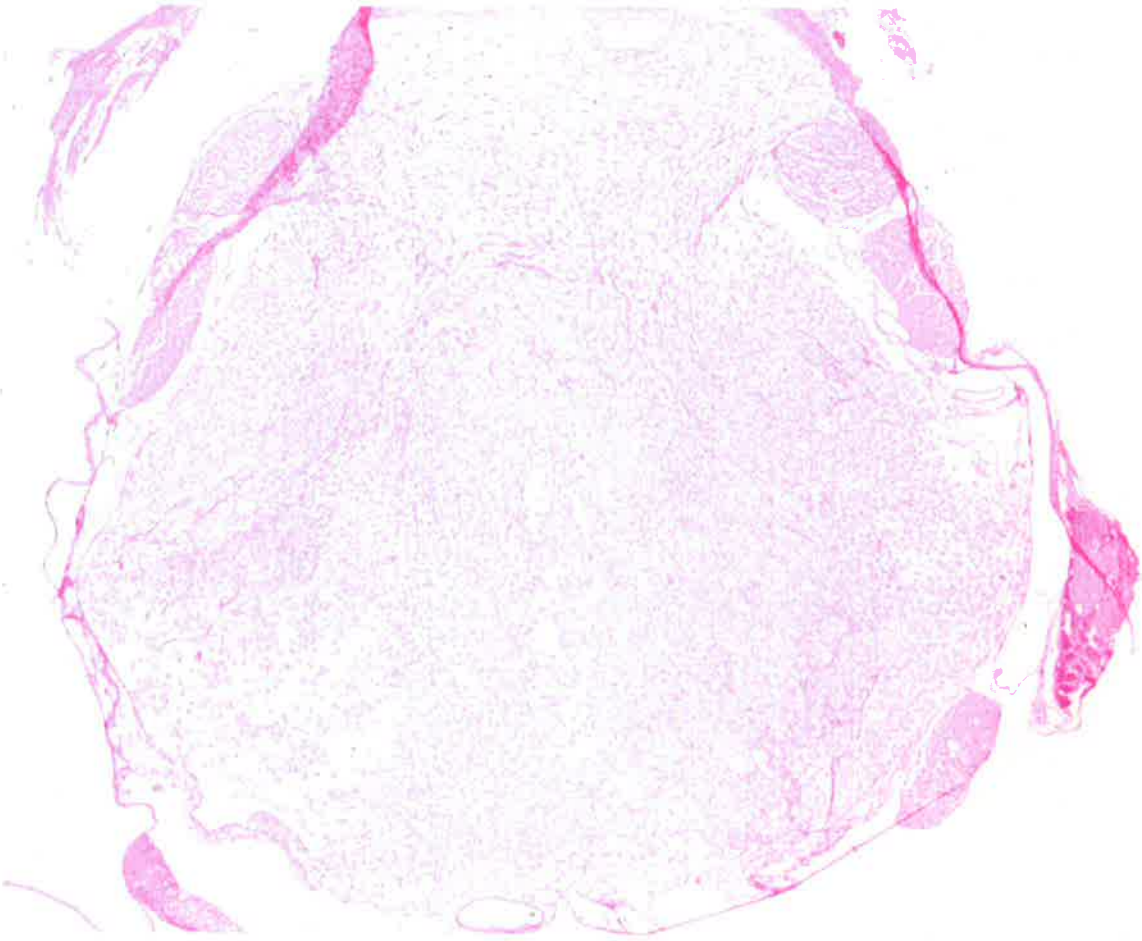

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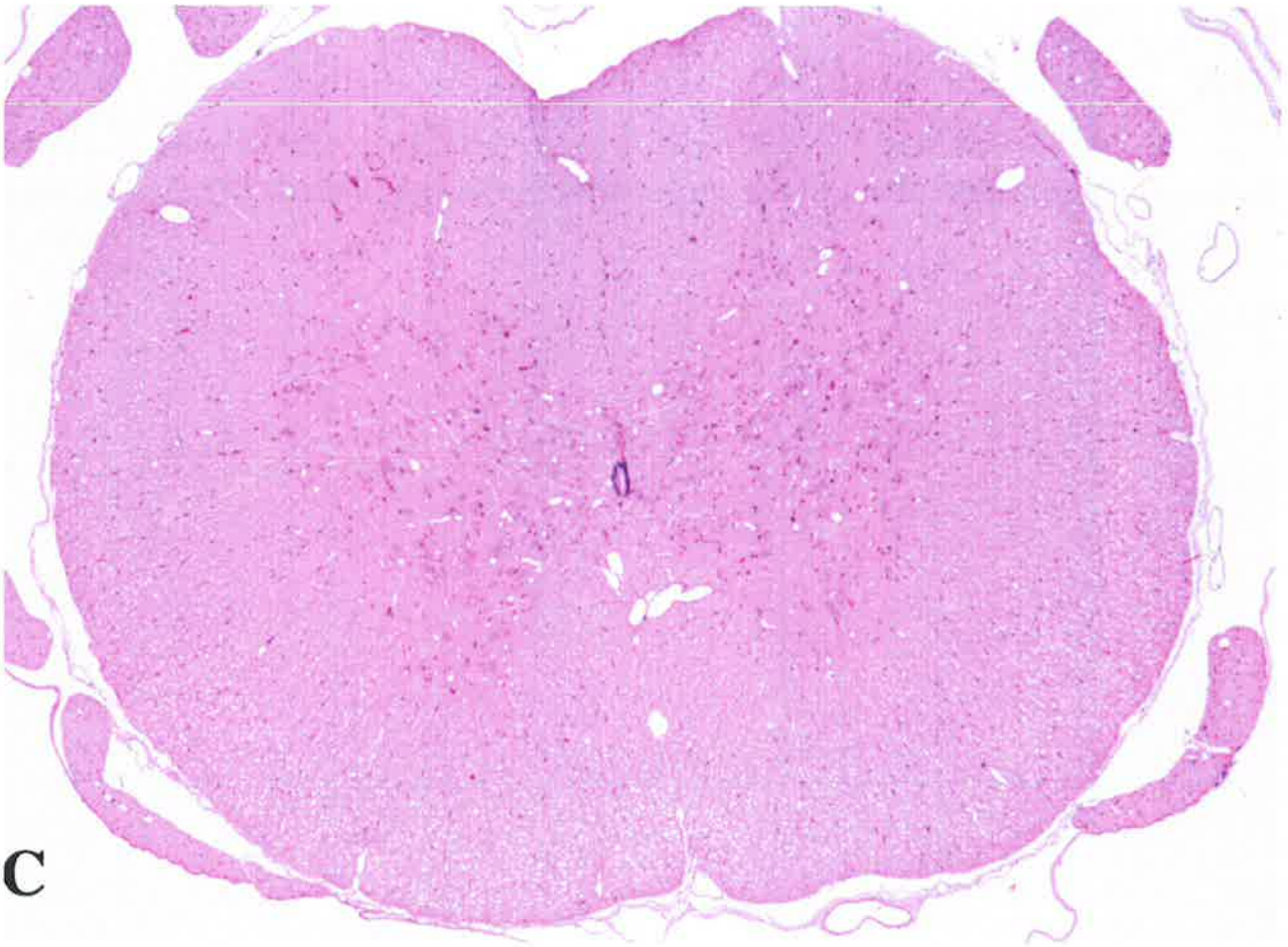

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2.3 DISCUSSION

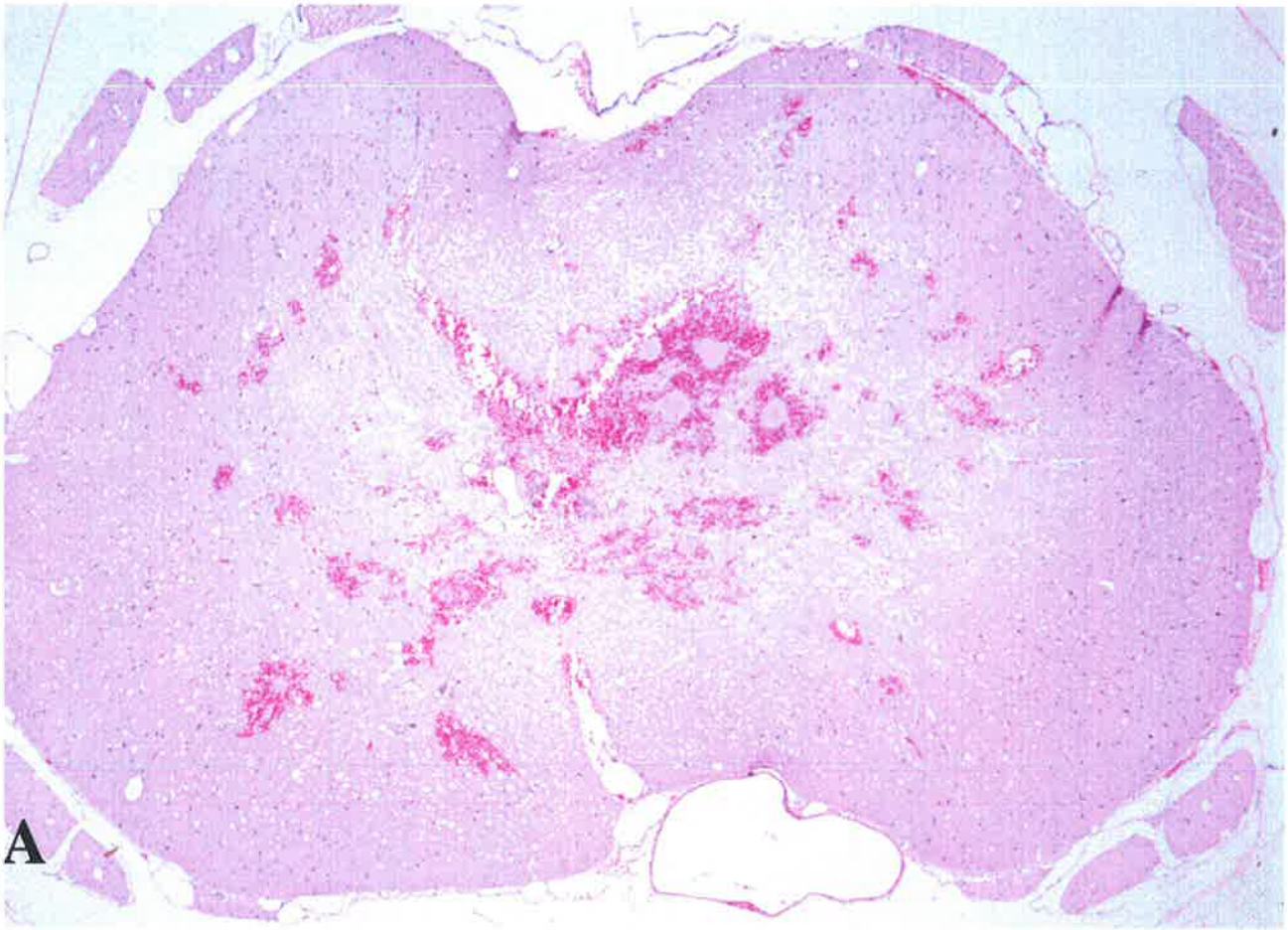
The results indicate that by using our weight-drop device, a graded SCI can be produced in rats with significant differences in outcomes of motor function and histopathology between mild and severe SCI groups. Furthermore, the spinal cord lesion induced by the weight-drop device is consistent: the standard error is less than 10 percent of the mean maximal lesion area or mean lesion length in the mild and severe SCI group.

Histopathology of spinal cords (area of maximal lesions and length of lesions) at 14 days after mild and severe SCI was chosen for statistical comparisons because it is more accurate to define the lesion area and intact area in the damaged spinal cord at 14 days postinjury (Figures 8A-B) than at earlier time points, such as 1 hr postinjury (Figures 9A-B).

There are two important differences in our weight-drop technique if compared to the classic Allen weight-drop technique [21,391]. The first is the extent of the laminectomy. We carried out a limited, one-level laminectomy just large enough to allow the impounder to contact the dura. An ideal experimental model of SCI would require minimal surgical preparation of the animal and multiple-level laminectomy itself is a major injury to the animal. The second is the extent to which the spinal column was stabilized prior to weight-drop injury. We stabilized the spinal column via clamps on the spinous processes of the vertebrae immediately rostral and caudal to the laminectomy. The rat was actually lifted by those clamps so that the weight-drop impact could not be cushioned by the body as a whole. Such stabilization also reduced the effects of respiratory movements that may alter the position of the spinal cord with respect to the impounder at the moment of impact and thus contribute to variability.

In conclusion, our weight-drop device produces graded and consistent contusion injuries of the spinal cord in the rat.

Figure 9. *Representative transverse sections at the epicentre of the lesion for a 3 cm (A) and 12 cm (B) weight-drop contusion SCI, 1 hour after injury. H&E, X10.*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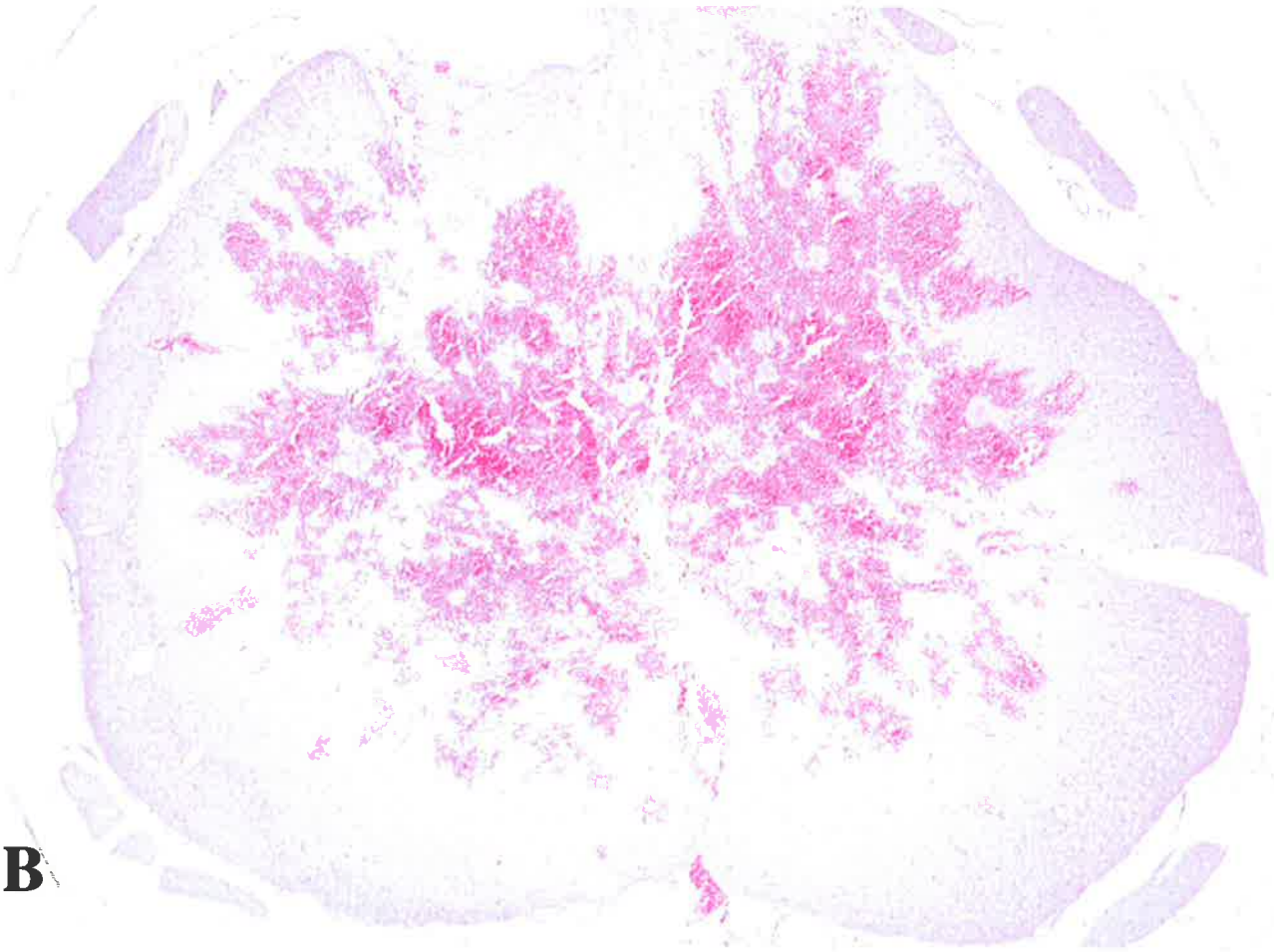
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3. INFLAMMATORY CELLULAR RESPONSE

This experiment was performed to investigate the posttraumatic inflammatory cellular response in the rat after SCI.

3.1 METHODS

3.1.1 ANIMALS

Thirty-one rats were used and divided into four groups: mild SCI, severe SCI, laminectomy control and naïve control. Fourteen rats with mild SCI were killed at 1 h (n=2), 3 h (n=2), 6 h (n=2), 1 d (n=2), 3 d (n=2), 7 d (n=2) or 14 d (n=2) postinjury. Severe SCI group also includes fourteen rats, which were sacrificed at 1 h (n=2), 3 h (n=2), 6 h (n=2), 1 d (n=2), 3 d (n=2), 7 d (n=2) or 14 d (n=2) postinjury. Two rats with laminectomy only were killed at 1 or 24 h (n=1 for each time point) after surgery and one normal rat was killed as a naïve control.

3.1.2 HISTOLOGICAL STAINING

Sections from each level were stained with haematoxylin and eosin to assess the extent and distribution of any pathologic abnormality of the cord with particular attention to haemorrhage, necrosis and migration of inflammatory cells (neutrophils and macrophages).

Immunocytochemical staining was carried out on 5-μm paraffin sections using standard immunoperoxidase staining protocols [334]. The sections were deparaffinized in xylene and cleared in graded ethanol baths. Endogenous peroxidase activity in the tissue was blocked by incubation with 3% H_2O_2 in PBS and non-specific binding was suppressed by incubation with blocking serum (15% NHS in PBS). Sections were then incubated overnight with the primary antibody diluted in NHS and stained with 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO) using avidin-biotin peroxidase (Vectastain ABC kit; Vector

Laboratories, Burlingame, CA) and counter-stained with haematoxylin. Negative control studies were performed by omitting the primary antibody step.

Macrophages were detected using the mouse monoclonal IgG antibody ED1 (Serotec Ltd., Oxford, UK) at a dilution of 1:400 [86]. Axonal injury was identified using a mouse anti-APP monoclonal antibody (DAKO, Carptinteria, CA) at a dilution of 1:10000 [132,217]. To identify microglia, the horseradish peroxidase conjugate of the B_4-isolectin from Griffonia simplicifolia (GS I-B_4-HRP, Sigma Chemical Co., CA) was used at a concentration of 20 μg/ml [337]. A rabbit anti-TNF-α polyclonal antibody (1:60, HyCult Biotechnology, Uden, Holland) was used to detect TNF-α expression. The antigen-specificity of the TNF-α antibody was checked by pre-absorbing the primary antibody with exogenous recombinant rat TNF-α (Pepro Tech, NJ).

Using double-label fluorescent immunohistochemistry, the cellular source of TNF-α was elucidated. The sections were incubated overnight with a combination of a rabbit anti-TNF-α antibody (as described before), and a human microglia marker demonstrating cross-reactivity with rat microglia (1:1000, a gift from Dr. Roggendorf W., University of Wurzburg, Germany). On the following day, the sections were incubated with a mixture of fluorescein-conjugated donkey anti-rabbit (FITC; 1:100; Jackson Immunoresearch Lab, West Grove, PA) and Cy™5-conjugated donkey anti-mouse (Cy5; 1:400; Jackson Immunoresearch Lab, West Grove, PA) antibodies. Sections were then mounted and examined under a fluorescence microscope (Biorad, LaserSharp 2100).

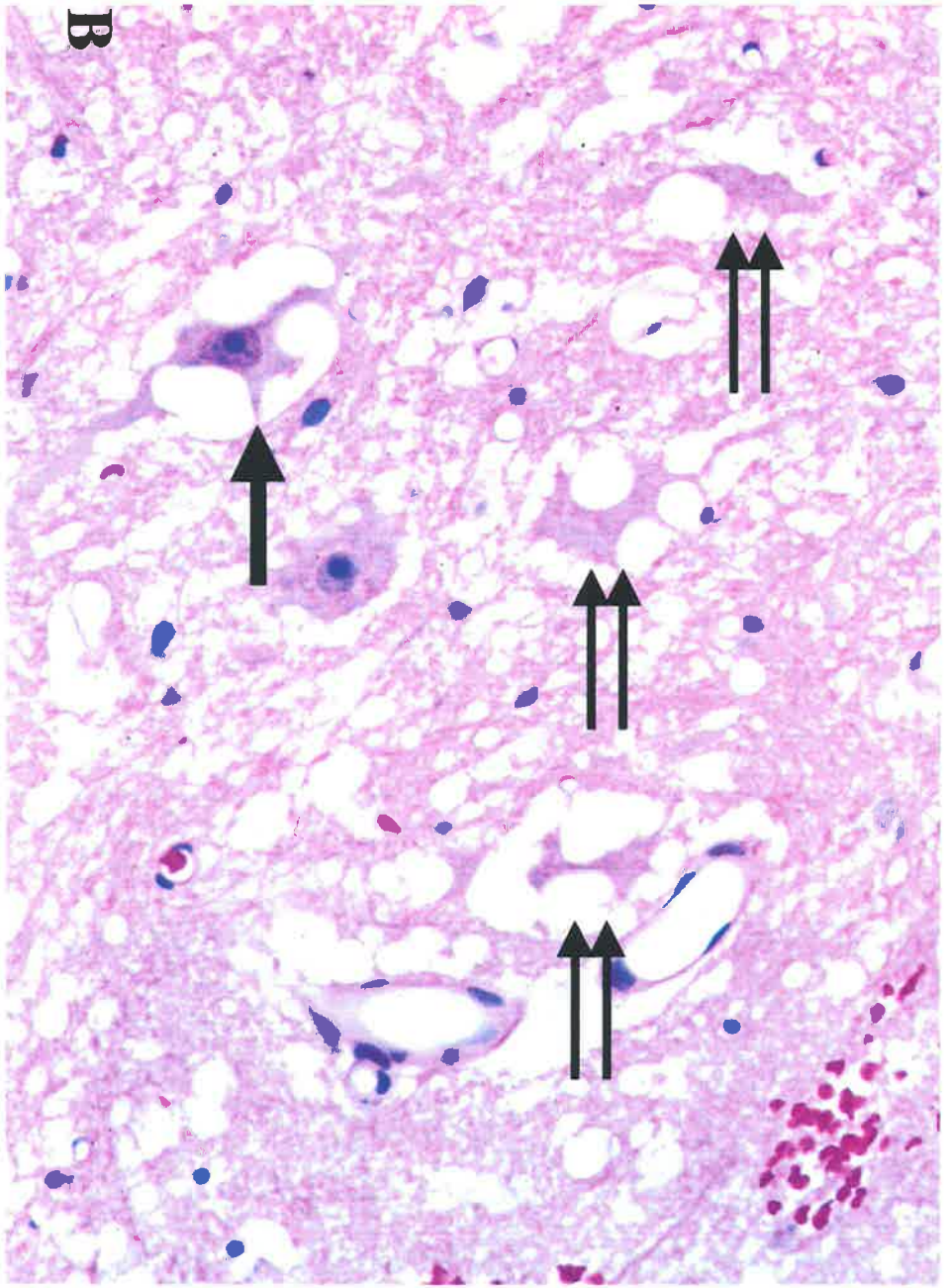
3.2 RESULTS

The nature and temporal development of the inflammatory response was the same in both mild and severe SCIs and differed only in degree, being much greater in severe injury. For example, transverse sections of lesion centres of mild and severe SCI at 1 h and 14 d postinjury are shown in Figures 9A-B and 8A-B, respectively.

3.2.1 ONE HOUR AFTER SCI

One hour following traumatic SCI, the lesion centre was marked by haemorrhage and necrosis (Figure 9), but no neutrophils or other leucocytes were seen in the areas of damaged tissue (Figures 10A-B). Some severely damaged neurons displayed extensive blebbing and shrinkage of their cytoplasms (Figures 10A-B). Signs of neuronal death, such as pyknosis and pale ghost-like cytoplasm were readily apparent in the grey matter (Figures 10A-B). At this early time point, the expression of TNF-α was found in many neurons (Figure 10C) and TNF-α positive neurons were found up to 0.6 cm from the lesion centre and there was no difference in the immunostaining pattern of TNF-α between the rostral and caudal parts of the lesion. However, more neurons expressed TNF-α after severe injury than mild injury. Furthermore, there were morphological signs of microglial activation, such as cellular hypertrophy and retraction of microglial processes (Figure 10D), and some activated (ameboid) microglia were found wrapping around some axonal swellings (Figure 10D). Meanwhile, early axonal injury was demonstrated by APP immunoreactivity in neurons and some axons (Figures 10E-F).

Figure 10. *Histological changes in the lesion at 1 h after SCI. (A-B) Marked haemorrhage without the infiltration of leucocytes in the lesion and some severely damaged neurons (single arrows) displaying extensive blebbing and shrinkage of their cytoplasms. There were signs of neuronal death, such as pyknosis (double-headed arrow) and pale ghost-like cytoplasm (double arrows) in the grey matter. H&E, X100. (C) TNF-α expression was detected in some neurons (arrows). TNF-α, X100. (D) Some activated (ameboid) microglia with morphological signs of cellular hypertrophy and retraction of processes wrapped around axons (arrows). B_4-isolectin, X100. (E-F) APP expression in some neurons and axons (arrows). APP, X100.*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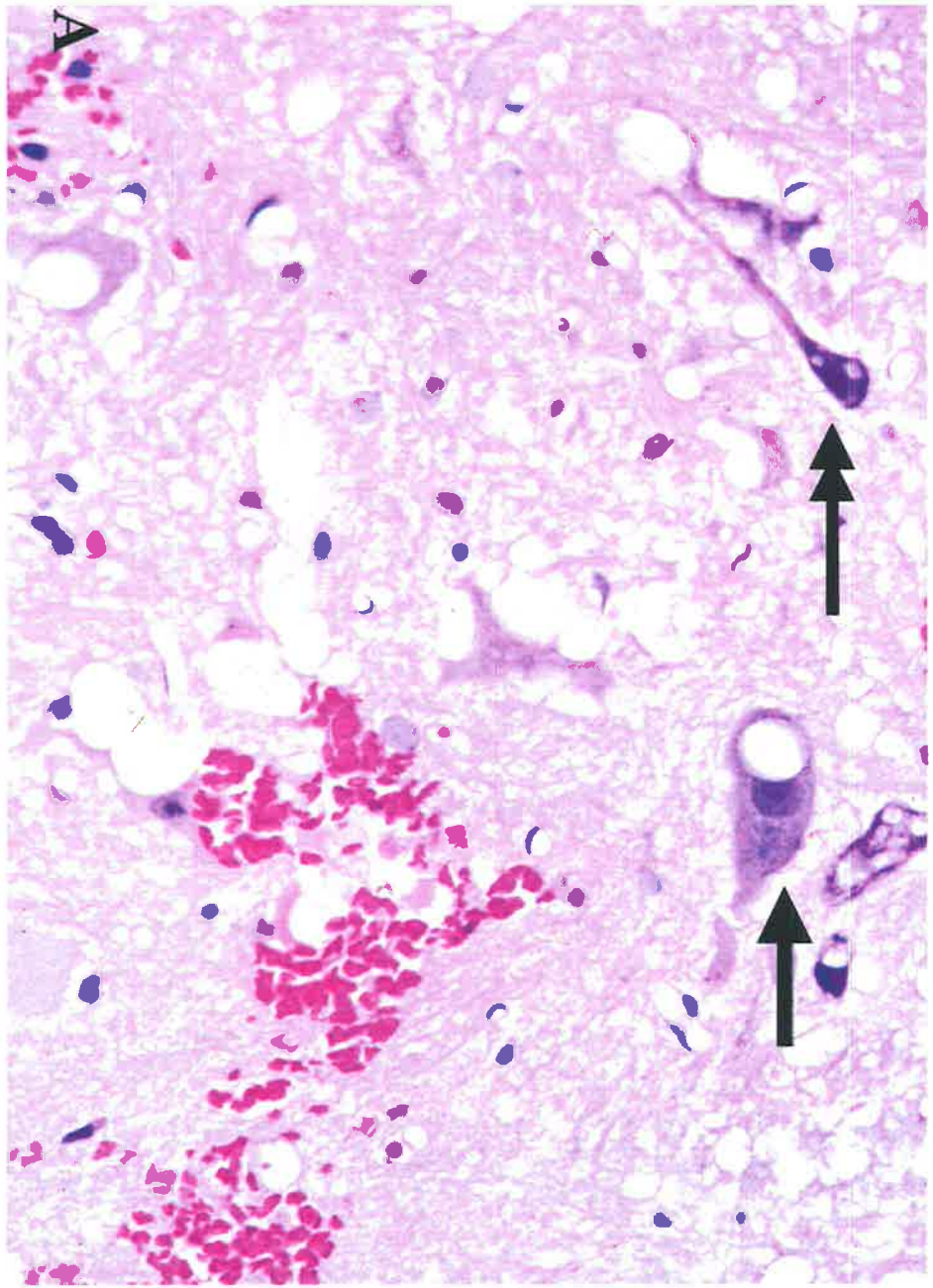
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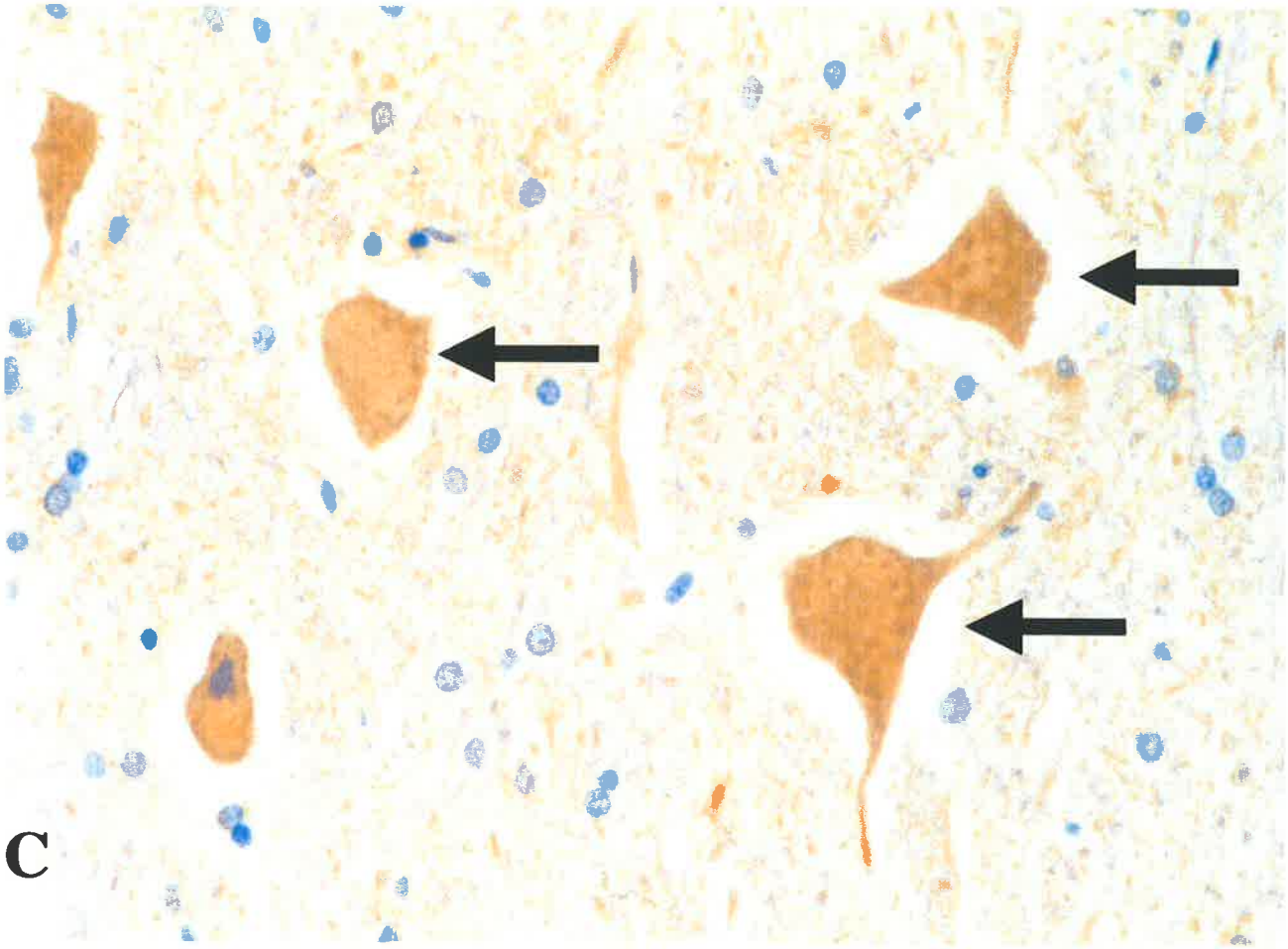
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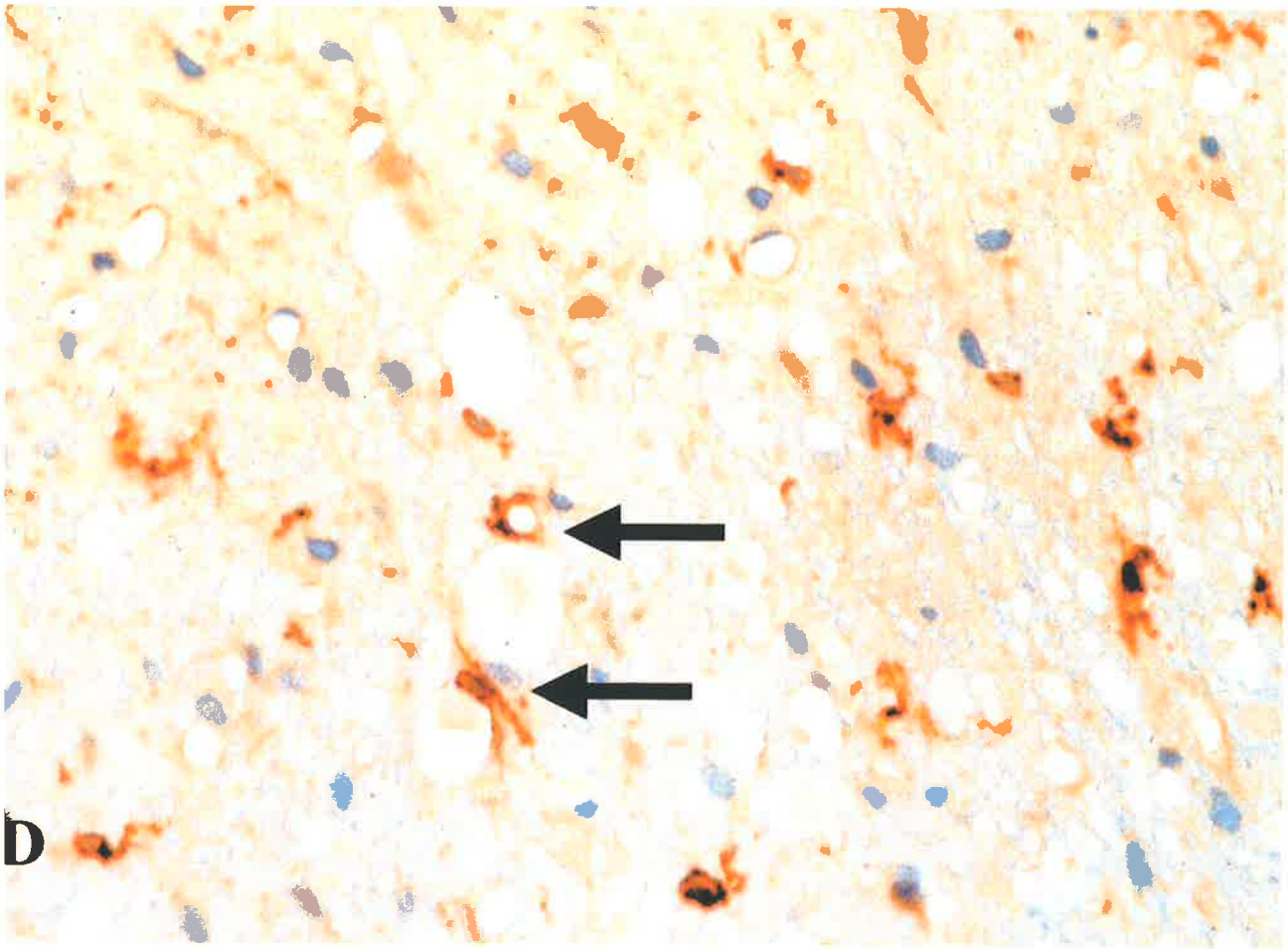
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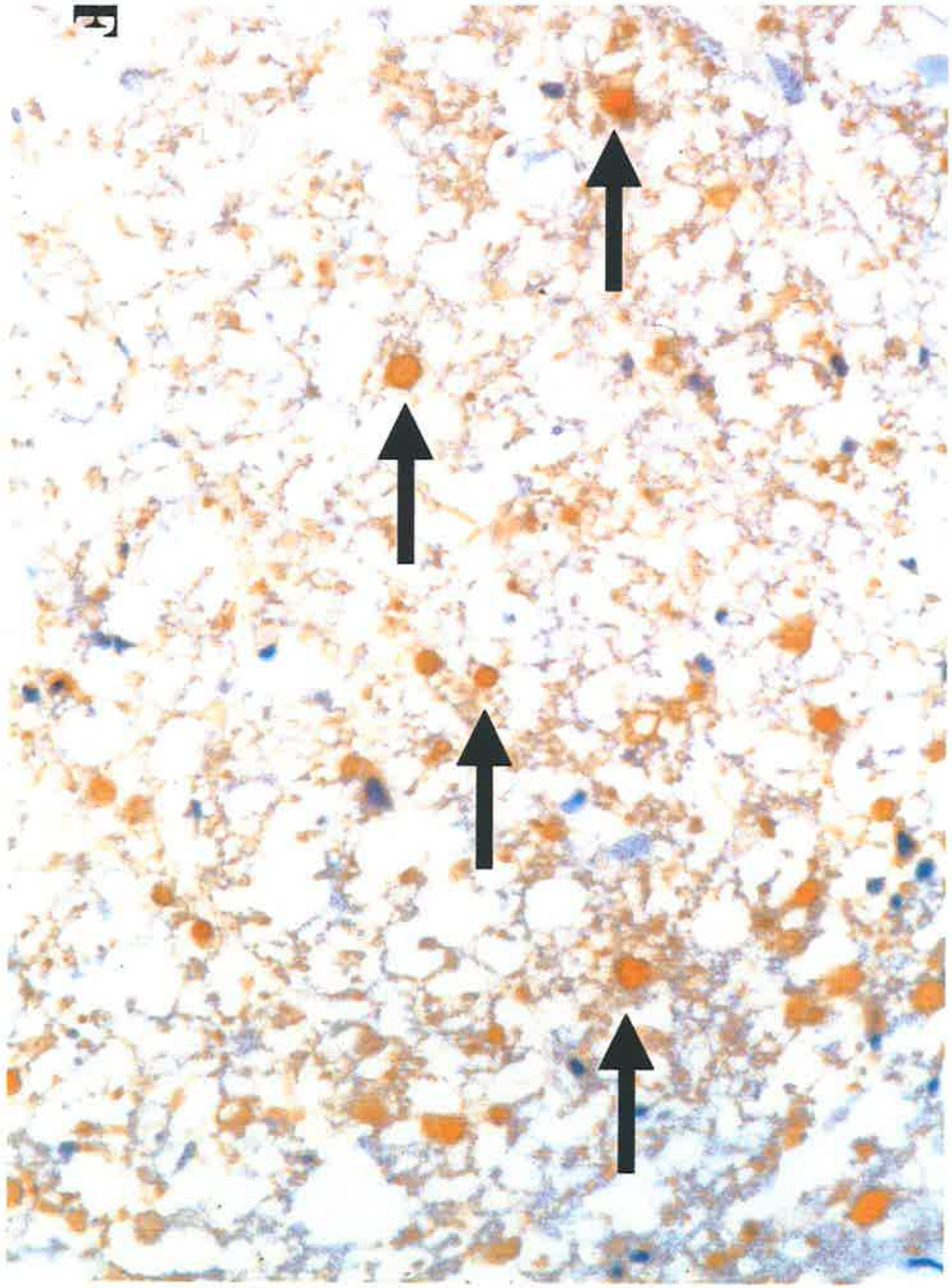
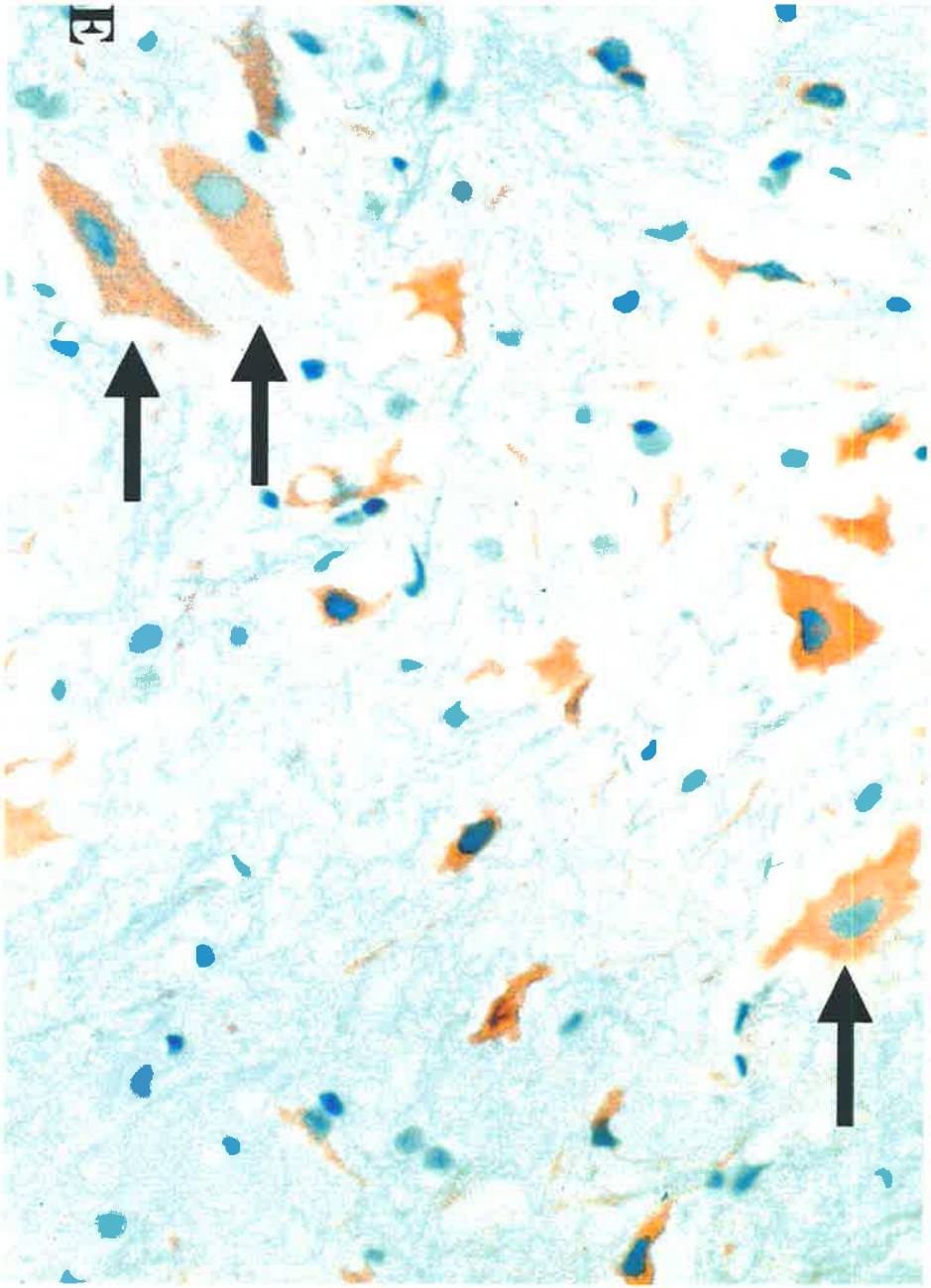


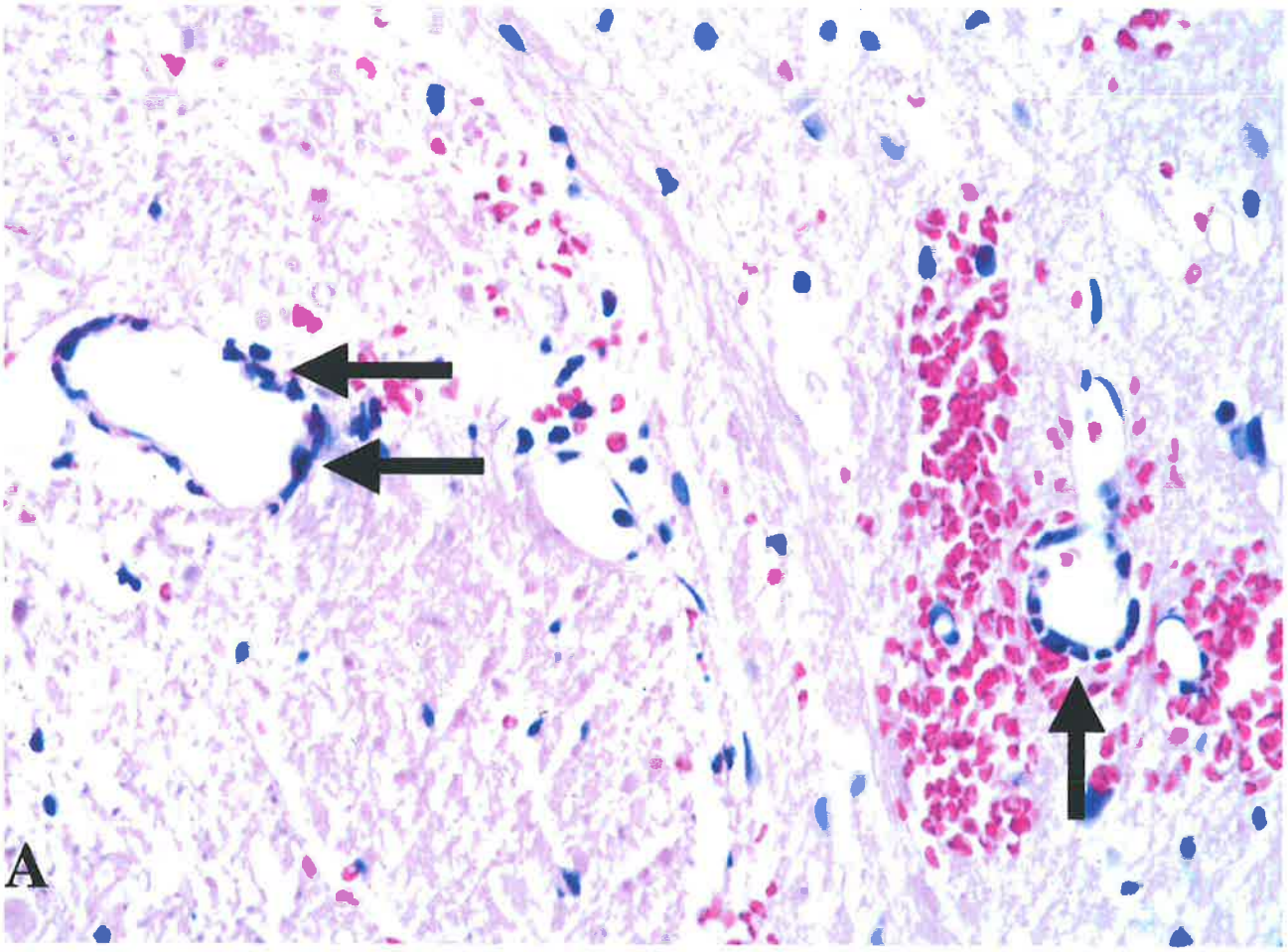


3.2.2 SIX HOURS AFTER SCI

Similar pathology was observed at 3 h after SCI compared to 1 h postinjury. However, at 6 h postinjury, a dramatic increase in the number of multinuclear neutrophils was found primarily in and around blood vessels (Figure 11A). Very occasional mononuclear cells in the walls of blood vessels were observed. TNF-α expression was detected not only in many neurons but also in some glial cells with microglial morphological appearance (Figure 11B). Some of these TNF-α positive glial cells were found surrounding axons (Figure 11B). TNF-α positive cells were found up to 1 cm from the lesion centre and there was no difference in the immunostaining pattern of TNF-α between the rostral and caudal parts of the lesion. There were few neurons and glial cells expressing TNF-α after mild injury compared with severe injury. More activated (ameboid) microglia were detected and some of them were found wrapping around axons as well as neurons (Figure 11C). Axonal injury was shown by APP immunoreactivity of many neurons and axons (Figure 11D).

To determine if TNF-α positive glial cells were microglia, double-label fluorescent immunohistochemistry was used, showing co-localization of TNF-α and the microglial marker KiM1P (Figure 12).

Figure 11. *Histological changes in the lesion at 6 h after SCI. (A) Neutrophil margination in the wall of a blood vessel (arrows). H&E, X100. (B) Glial cells expressed TNF-α and some of them surrounded axons (arrows). TNF-α, X1500. (C) More activated (ameboid) microglia were found and some of them wrapped around neurons (arrows). B_4-isolectin, X100. (D) APP positive neurons (double-headed arrow) and axons (arrows). APP, X100.*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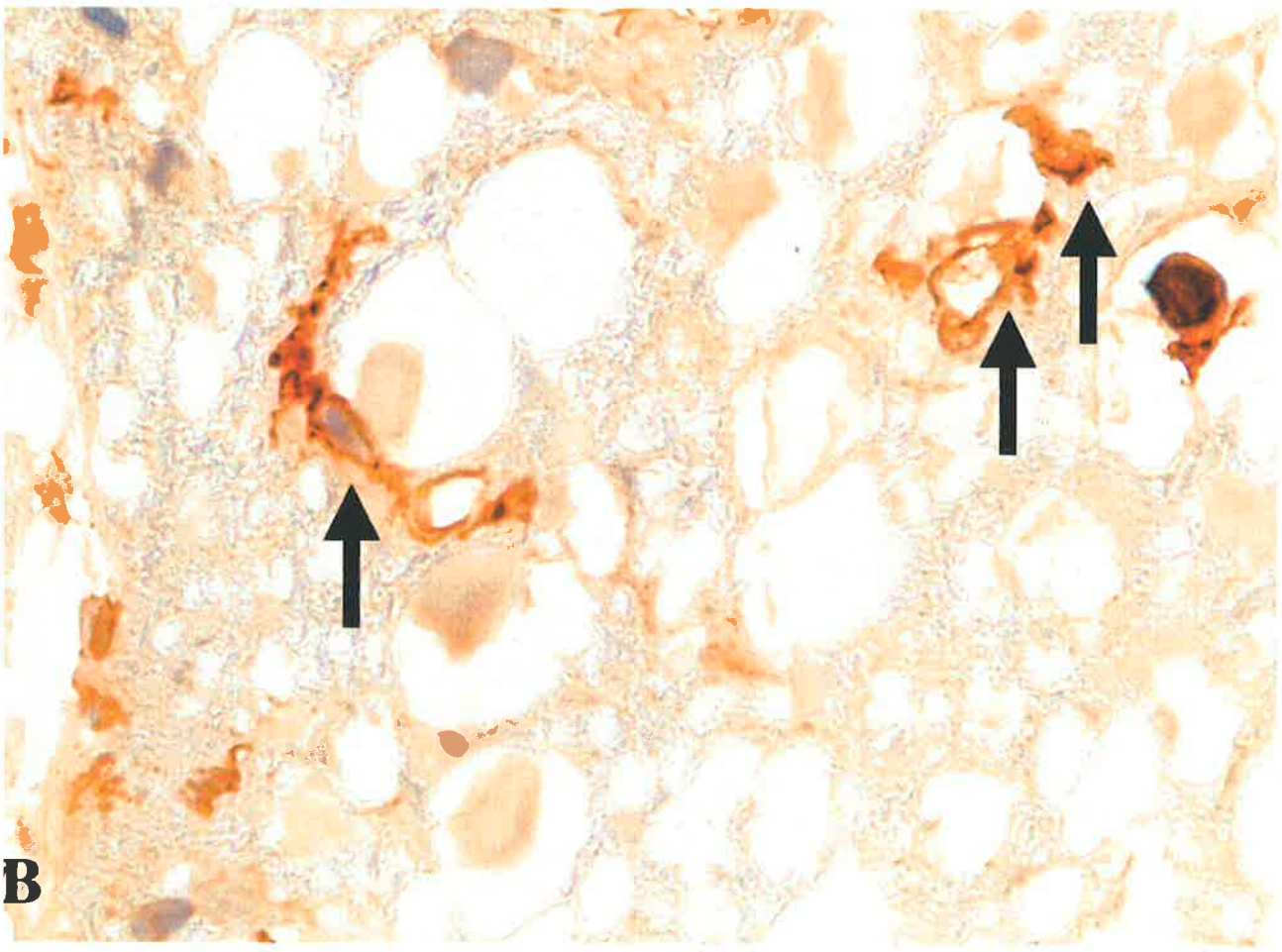
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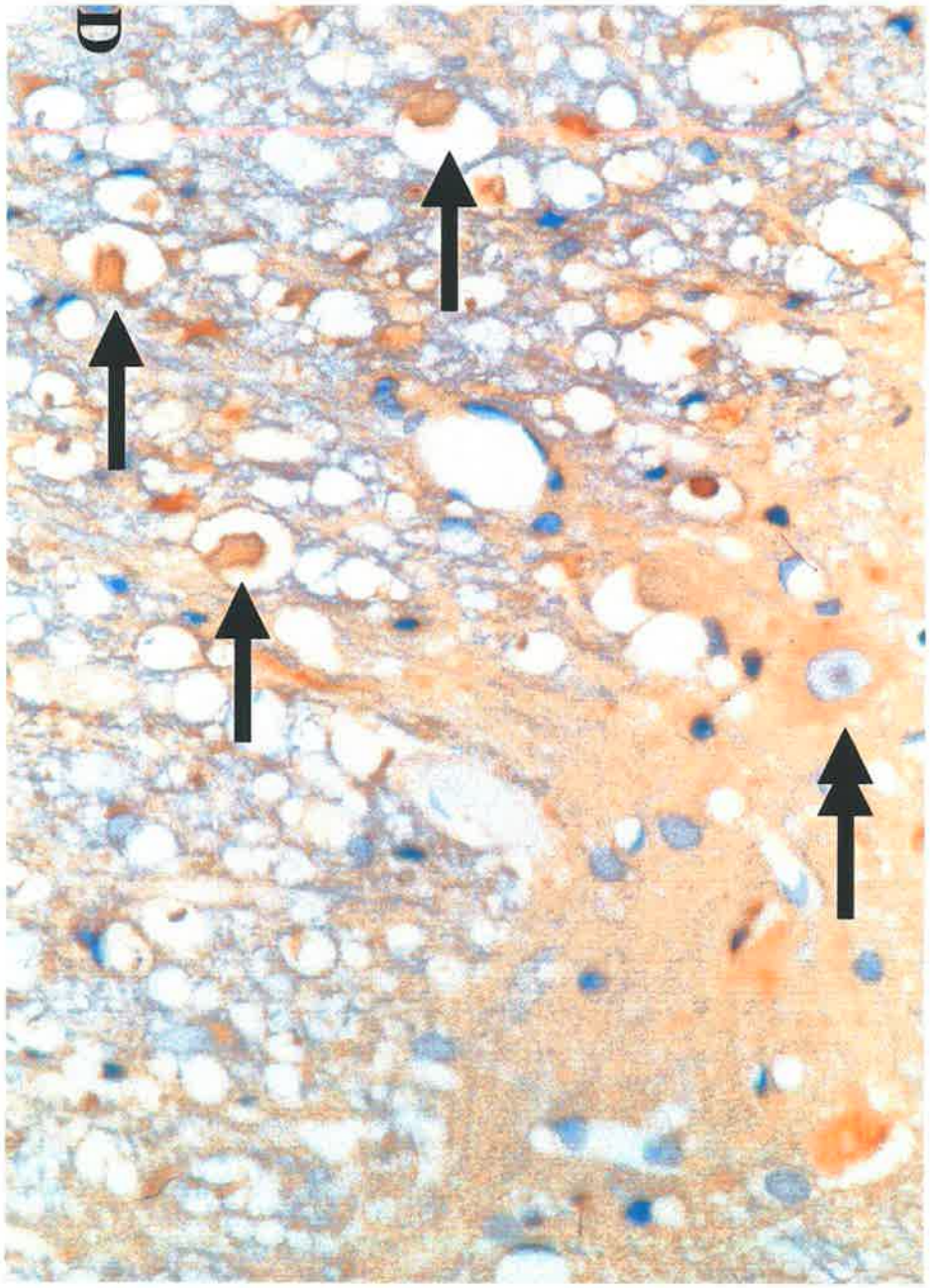
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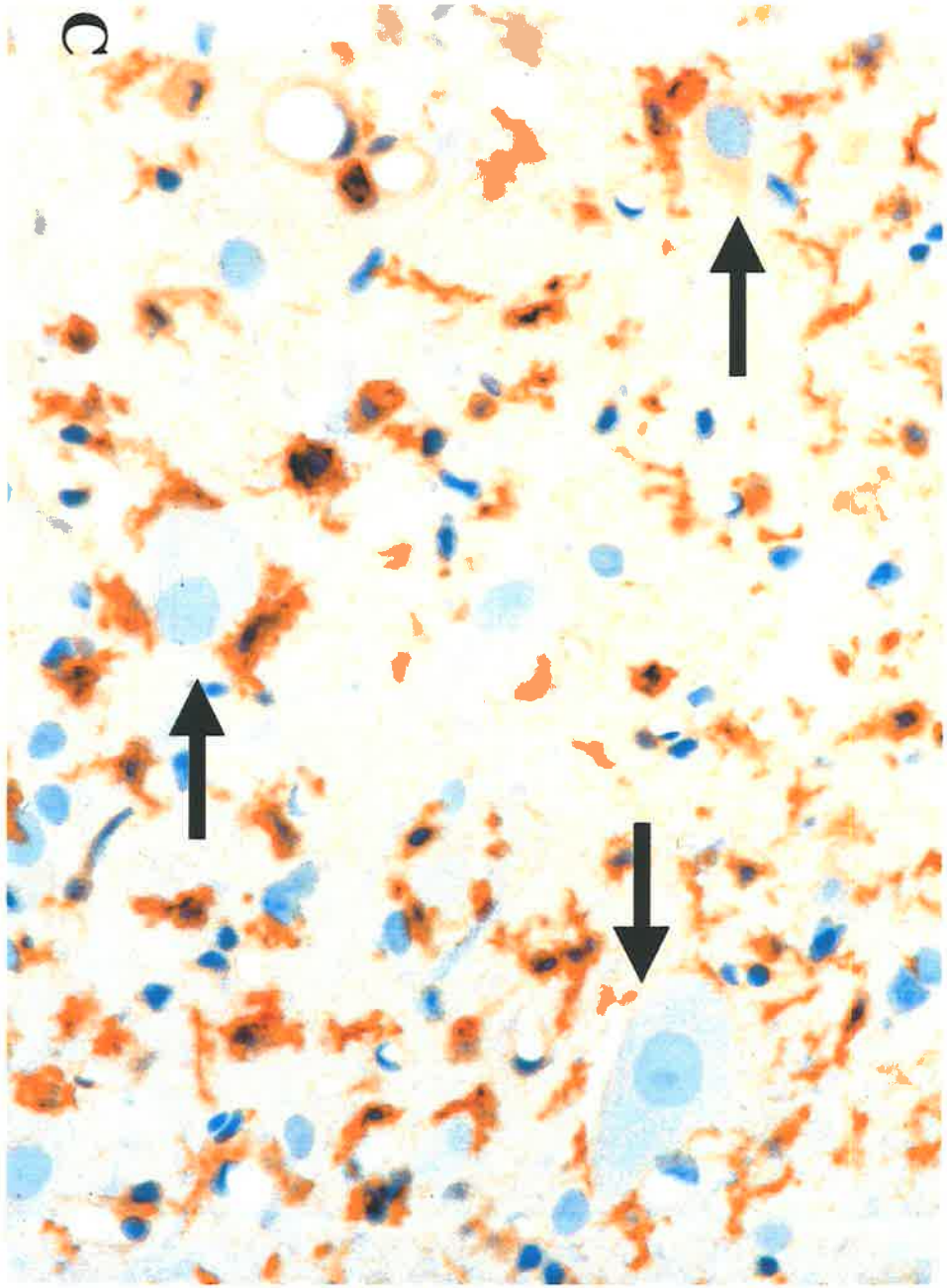
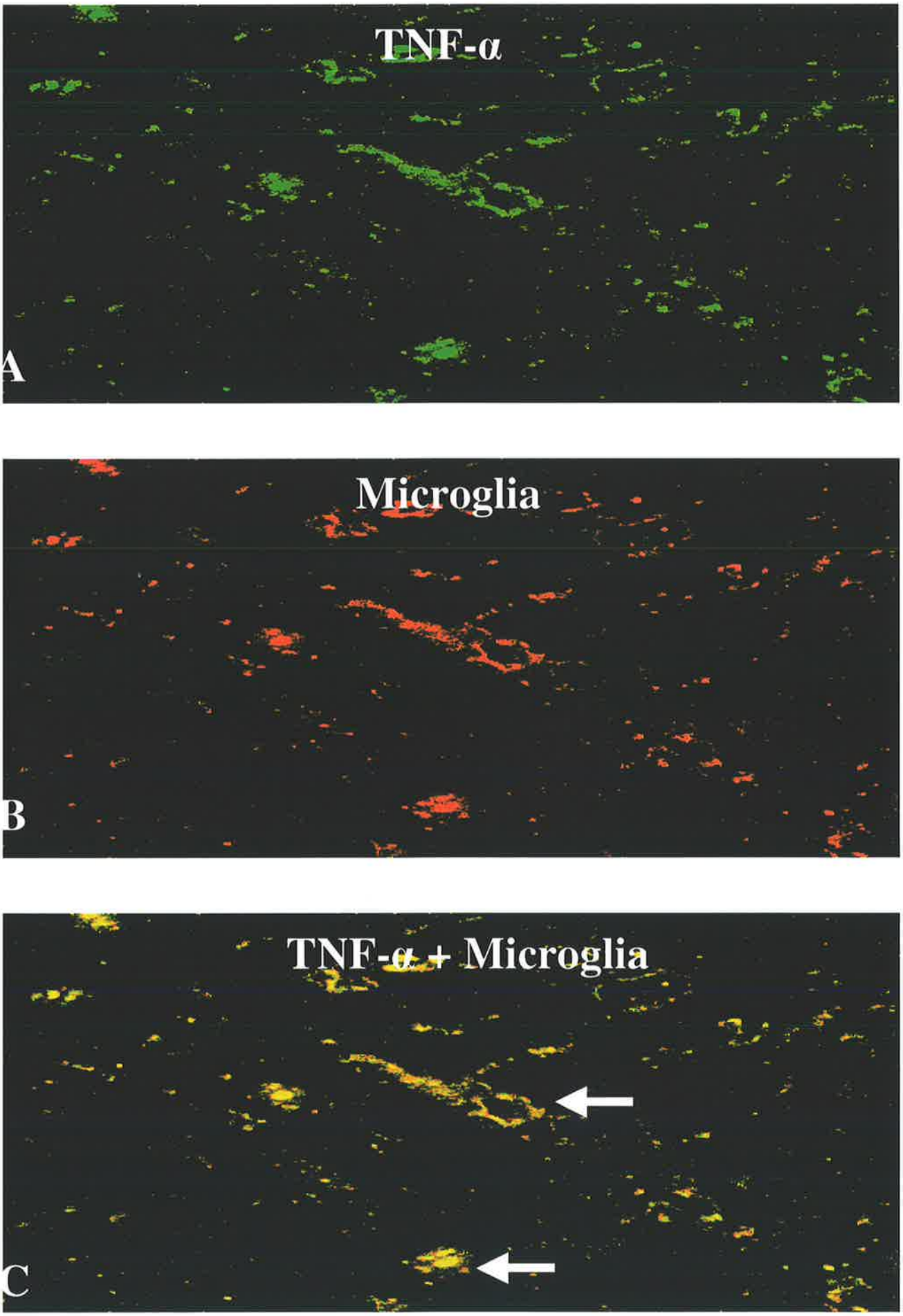

C

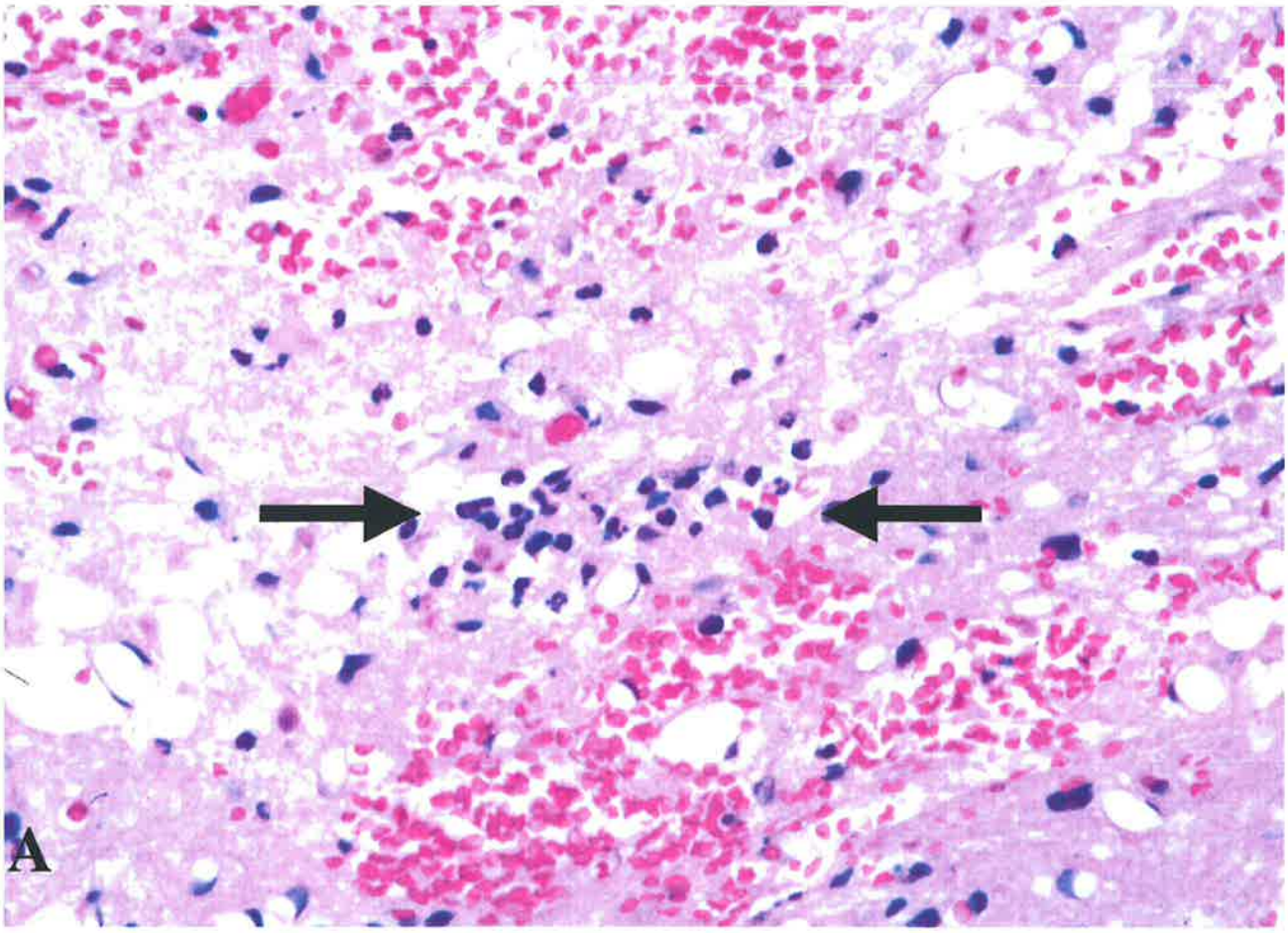
Figure 12. *Co-localization of the TNF-α (green) and the microglial marker KiM1P (red) in the lesion at 6 h after severe SCI. Cells stained with anti-TNF-α only and B_4-isolectin only were respectively shown in (A) and (B). Double-label immunofluorescent immunohistochemistry confirmed that TNF-α was co-localized in microglia (C). Arrows indicate cells co-localizing expression of the microglial marker (KiM1P) and TNF-α (A-C) (X600, zoom 2).*

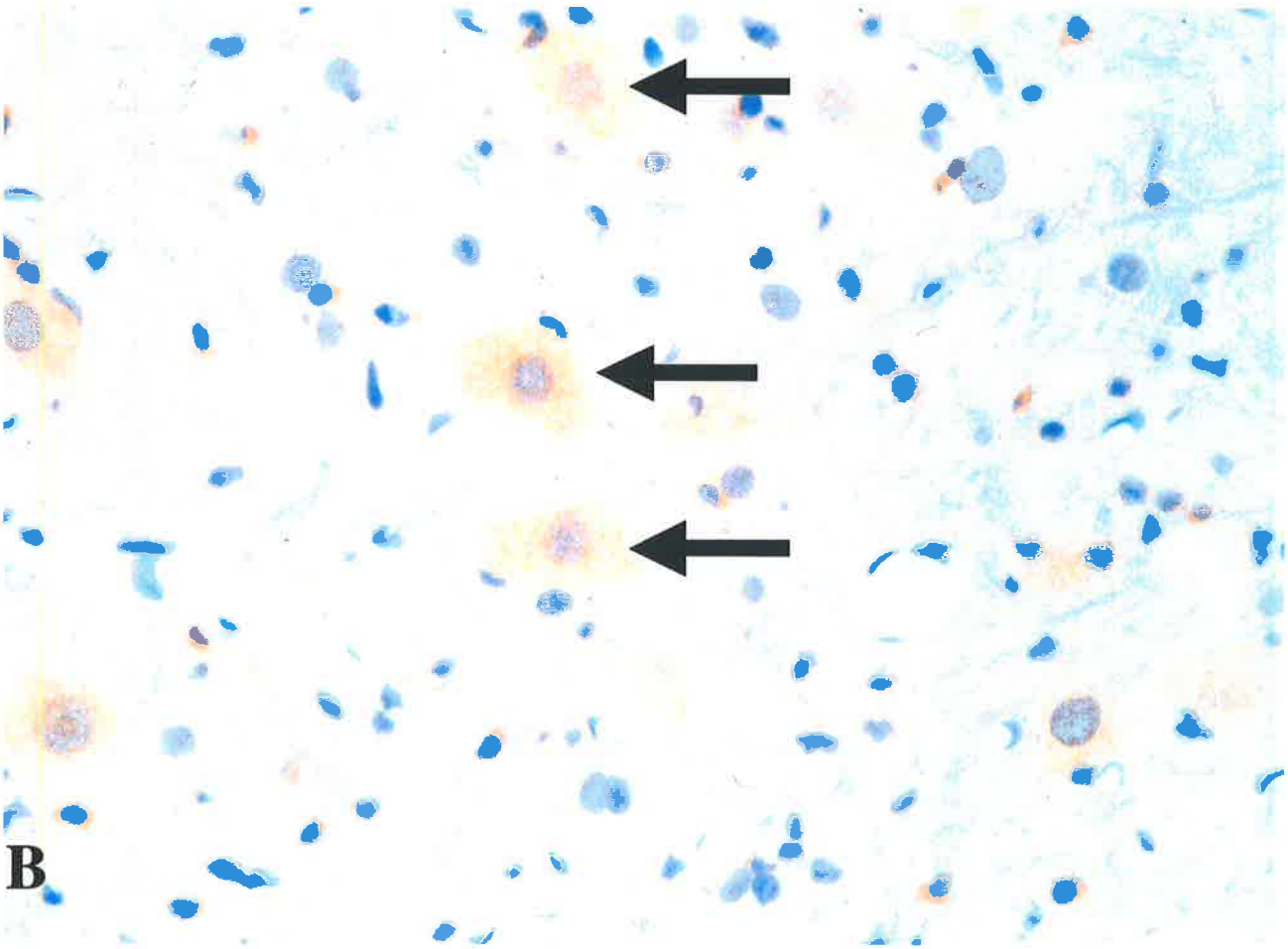

TNF-α
A
Microglia
B
TNF-α + Microglia
C

3.2.3 ONE DAY AFTER SCI

One day following SCI, numerous polymorphonuclear neutrophils were found in the damaged tissue (Figure 13A) and the number of neutrophils appeared to reach a peak at this time point. Weak expression of TNF-α was seen in the neurons (Figure 13B) while no expression was identified in the glial cells or neutrophils, suggesting that the level of TNF-α protein quickly declined. Some ED-1-stained macrophages started to appear in the injured tissue (Figure 13C) and many activated (ameboid) microglia were found wrapping around axonal swellings (Figure 13D). APP expression was also detected in neurons and axons.

Figure 13. *Histological changes in the lesion at 1 d after SCI. (A) Numerous neutrophils infiltrated the damaged spinal cord (arrow). H&E, X100. (B) Weak expression of TNF-α in neurons (arrows). TNF-α, X100. (C) Some macrophages started to appear in the lesion. ED-1, X1500. (D) Axons were surrounded by activated (ameboid) microglia (arrows). B_4-isolectin, X100.*


A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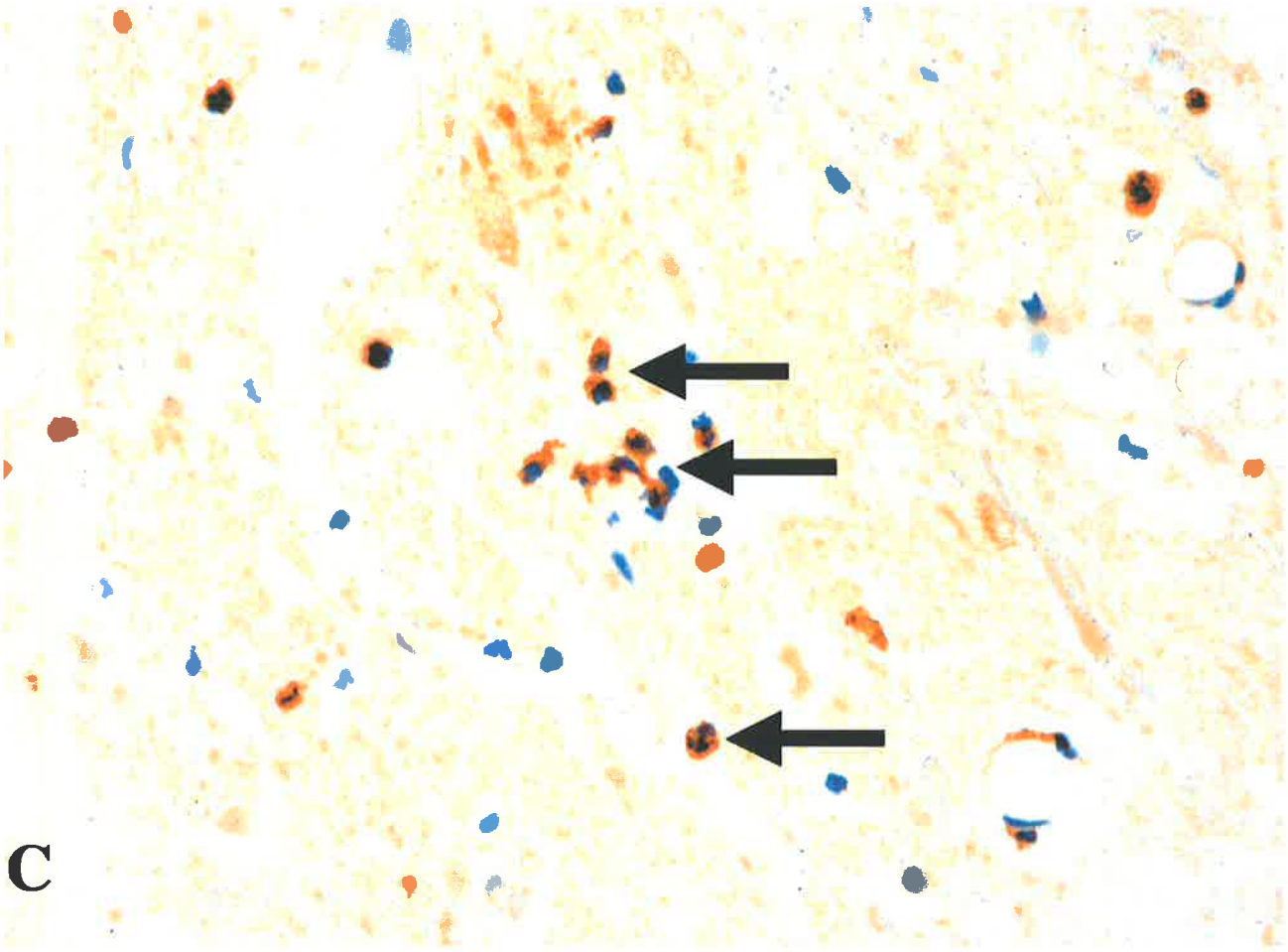

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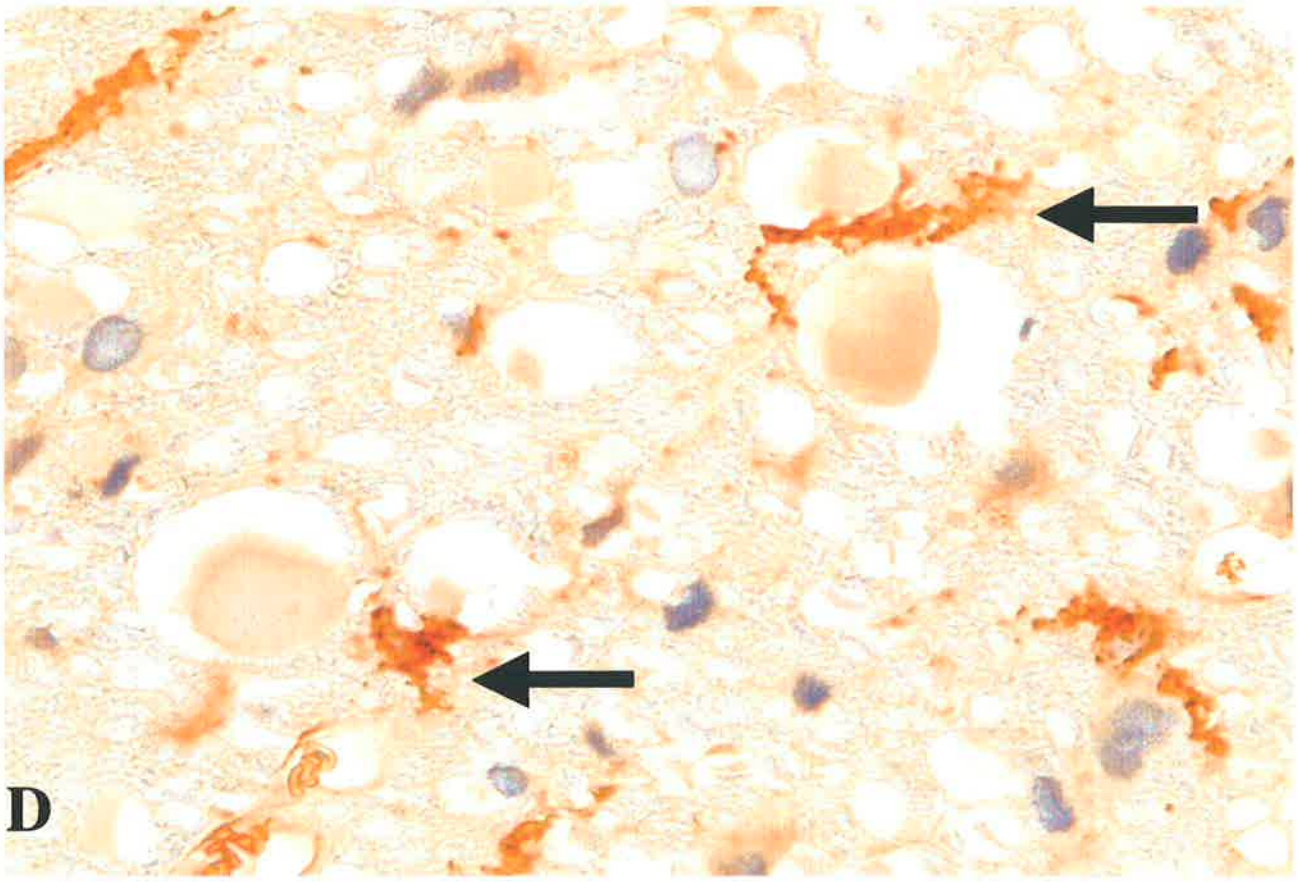


D

3.2.4 THREE DAYS AFTER SCI

At 3 d postinjury, the number of neutrophils dramatically declined while macrophages (Figures 14 A-B) and activated (ameboid) microglia (Figure 14 C) persisted in the spinal cord. Macrophages were found to be predominantly present in the necrotic areas (Figures 14 A-B) and largely absent from surviving areas. Instead, these regions were populated by activated (ameboid) microglia, some of which were found wrapping around axons (Figure 14 C). APP expression continued to be present in the neurons and axons (Figure 14 D), but no expression of TNF-α was observed.

3.2.5 SEVEN AND FOURTEEN DAYS AFTER SCI

Pathological changes at 7 and 14 days after SCI were very similar to those at 3 days postinjury. There were still many macrophages, predominantly in necrotic areas, and numerous activated (ameboid) microglia confined to the surviving areas. Some of activated microglia continued to surround axons and APP expression continued to be present in the neurons and axons.

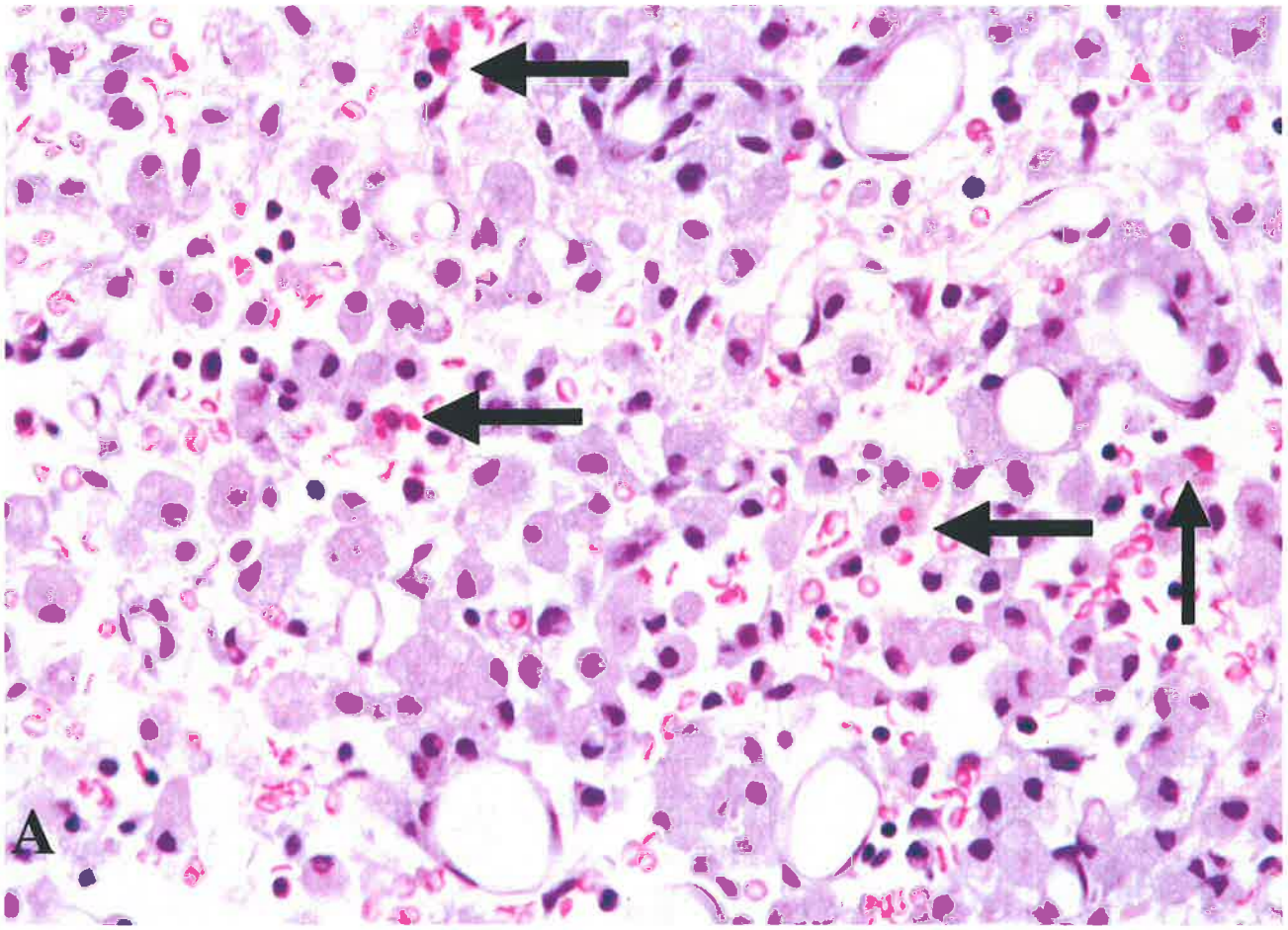
3.2.6 CONTROL STUDIES

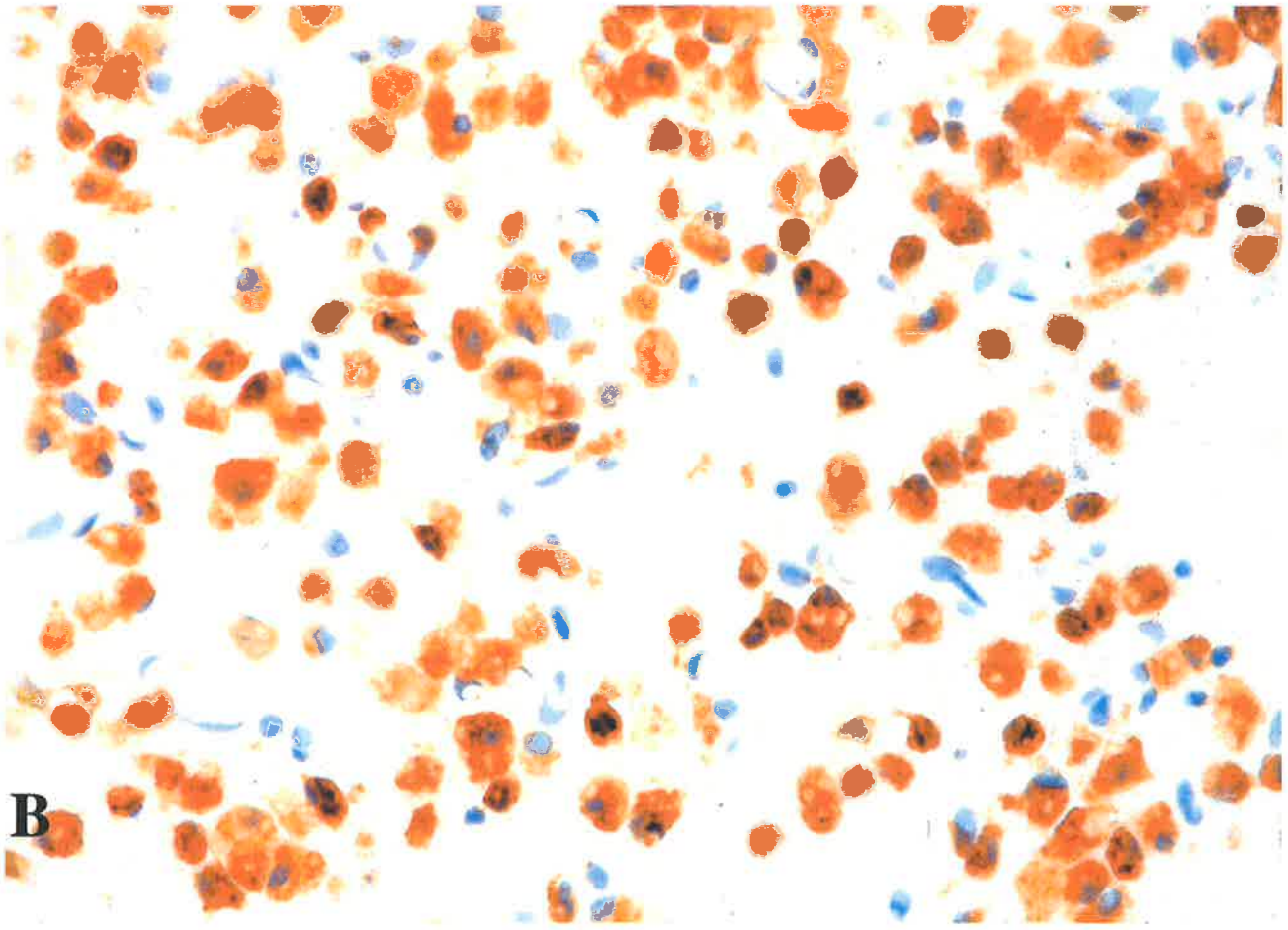
There was no expression of APP and TNF-α in two laminectomy controls and one normal control. Neither macrophage nor activated (ameboid) microglia were detected in the laminectomy controls and normal control.

No immunoreactivity of TNF-α was seen in the antigen-absorption controls.

No positive staining was observed in the negative immunostaining control studies, which were performed by omitting the primary antibody step.

Figure 14. *Histological changes in the lesion at 3 d after SCI. (A) Numerous macrophages were present in the damaged spinal cord, phagocytosing erythrocytes (arrows). H&E, X100. (B) Numerous ED-1 positive macrophages predominantly in the necrotic area. ED-1, X100. (C) Activated (ameboid) microglia mainly in the surviving tissue, surrounding around axons (arrow). B_4-isolectin, X100. (D) APP expression in the axons. APP, X100.*


A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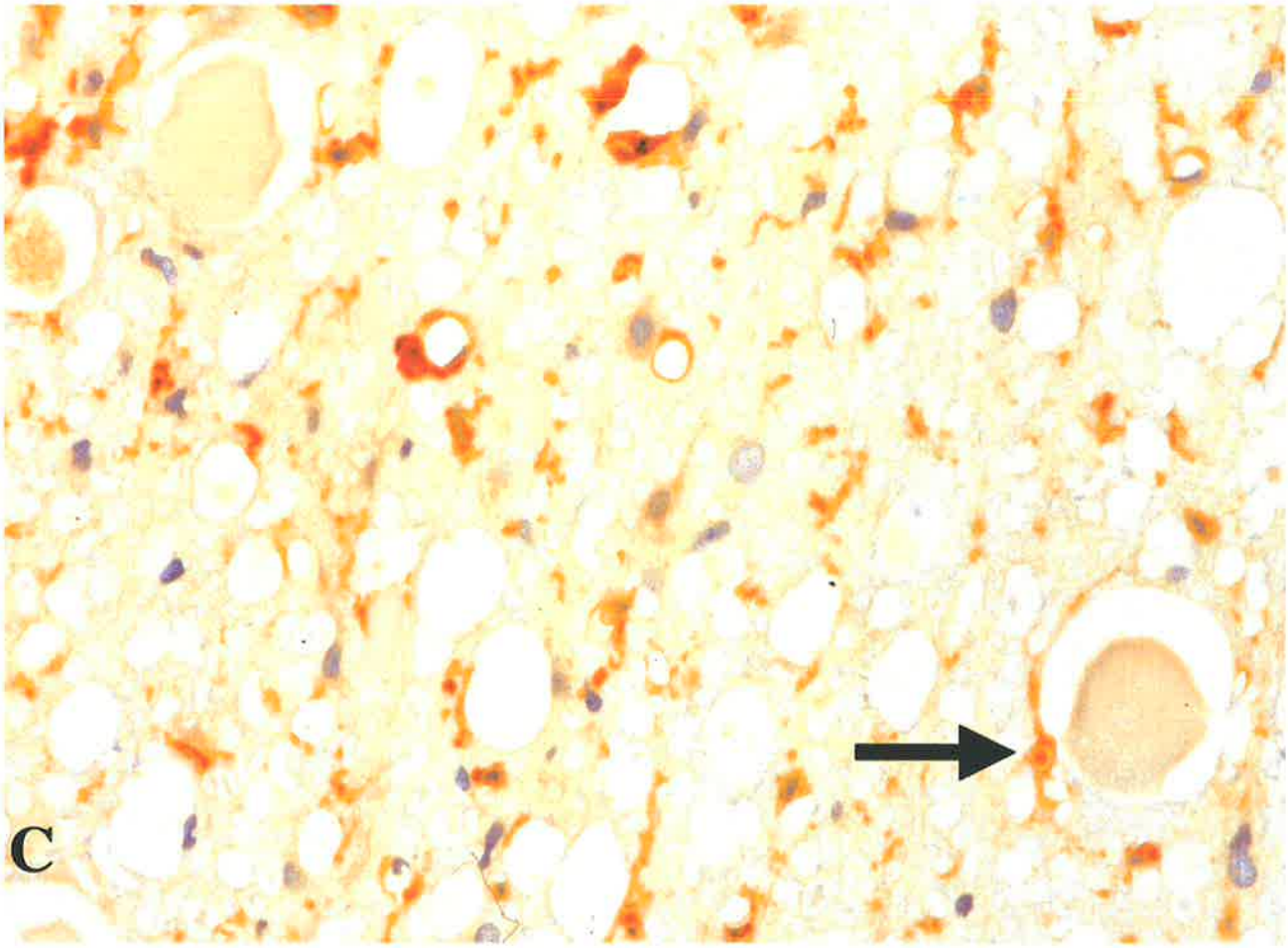
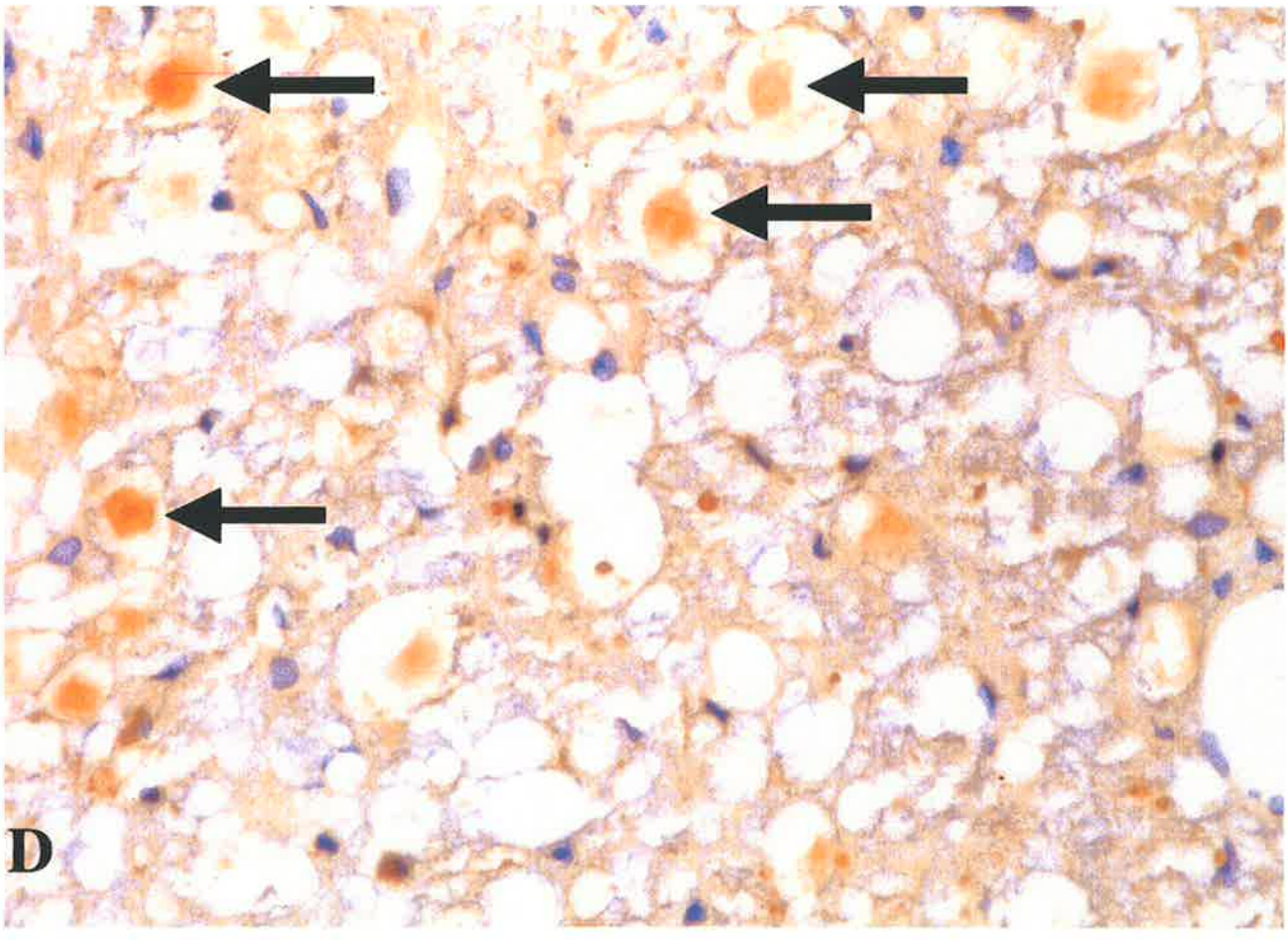

C


D

3.2.7 SUMMARY OF POSTTRAUMATIC INFLAMMATORY CELLULAR RESPONSE

The inflammatory cellular response after SCI is summarised in Table 5 below.

Table 5. *Inflammatory cellular response after SCI.*

Postinjury	Histopathology
1 & 3 hr	Haemorrhage and necrosis; no leukocyte infiltration. Neuronal pyknosis, vacuolation and pale ghost-like changes. Activated (ameboid) microglia, some of which surrounded axons. TNFα expression in neurons and APP expression in neurons & axons.
6 hr	Neutrophil margination. Activated (ameboid) microglia, some of which surrounded neurons. TNFα expression not only in neurons but also in some glial cells. APP expression in neurons and axons.
1 d	Peak of neutrophil number and ED-1+ macrophages started to appear. Activated (ameboid) microglia, some of which surrounded axons. A dramatic decline in TNFα expression. APP expression in neurons and axons.
3, 7 & 14 d	Few neutrophils in the damaged tissue. Numerous macrophages predominantly in necrotic areas. Activated (ameboid) microglia around axons mainly in surviving areas. APP expression in neurons and axons.

3.3 DISCUSSION

3.3.1 STAINING METHODS FOR RAT MICROGLIAL CELLS

The lack of a unique marker for microglia has made their study challenging. Many of the best markers for microglia are cell surface antigens that are unstable with routine histologic methods. Except for a few markers such as lectin, markers for microglia are either useless or suboptimal in paraformaldehyde-fixed or formalin-fixed paraffin sections [85,338].

Therefore, a simple method of using the lectin from GS I-B_4 for histochemical visualization of rat microglial cells was used in this study. In addition to providing good structural preservation, this method achieves complete labelling of all microglial cells and superior visualization of microglial processes. Furthermore, no other glial cell types aside from microglia demonstrate binding with the lectin from GS I-B_4 [337]. Therefore, this lectin is a very reliable microglial marker.

3.3.2 APP UPREGULATION

APP has been used extensively as a sensitive marker of axonal injury in human studies [39,131,320] and experimental studies [234,371], but the significance of APP upregulation in neurons and axons in response to TSCI is unknown. It has been suggested that induction of APP in the CNS is a normal "acute phase" response to neuronal stress [71,131,294,295] similar to the induction of heat shock proteins [225]. However, other studies have suggested that APP upregulation may have neuroprotective functions [143,235,330]. In the studies described here, it is not known whether APP expression increases early to perform some neuroprotective function. A possibility is that APP upregulation has no specific role in TSCI as alteration in APP expression could result from a number of factors common to local or diffuse areas of tissue damage such as oxidative stress and local inflammatory cytokines [71]. Thus, increased APP

immunoreactivity within neurons and axons following SCI may be an adaptive response to injury.

3.3.3 CHEMOATTRACTANTS OF INFILTRATING LEUKOCYTES

Haematogenous inflammatory cells (neutrophils and macrophages) did not start to infiltrate the injured spinal cord until 6 h postinjury, suggesting that these inflammatory cells were not spilled from damaged vessels, but recruited by some chemoattractants, including pro-inflammatory cytokines, haemorrhage and necrosis [88,113,239,347]. It has been reported that direct injection of TNFα or IL-1β into normal CNS causes widespread leukocyte margination [341]. This is further supported by our findings that a dramatic elevation of TNFα expression as early as 1 h after SCI, which may contribute to the recruitment of leukocytes into the damaged cord.

3.3.4 INFLAMMATORY CELLS

Three cell types are predominantly involved in the early aspects of an inflammatory response: microglia, neutrophils and macrophages. In contrast to some in vivo studies that failed to detect morphological signs of microglial activation by 1 d postinjury [261,341], activated (ameboid) microglia with cellular hypertrophy and retraction of processes were observed in this study as early as 1 h after TSCI and some of them were found surrounding axonal swellings. This novel finding challenged a current view that there is no direct topographical association between microglial cells and axons, and no association of axonal injury with activated microglia [127,261,278]. More studies are needed to elucidate the effect of activated microglia surrounding axons because activated microglia are able to produce pro-inflammatory cytokines (observed in this study), cytotoxic substances [25,387] and growth factors [341]. Activated microglia may provide a trophic local environment to promote axonal

regeneration as some studies have shown that activated microglia promoted regeneration of sensory axons in the injured rat spinal cord [248,280] and improved recovery of motor function in paraplegic rats [289].

The current study also found that activated (ameboid) microglia surrounded around neurons. This phenomenon originally found by the Blinzinger group [37] in the study of facial nerve axotomy, is now known as "synaptic stripping" [25]. Activated (ameboid) microglia are thought to interpose processes between afferent synaptic terminals and the neuronal surface and to be involved in limited phagocytosis of individual synaptic termini. It has been suggested that neuronal energy expenditure is minimised by cessation of the "transmission" function [252] and "synaptic stripping" may also have an impact on the synaptic reorganization of the injured neurons, but the effect of synaptic stripping on neurons is, as yet, largely unknown.

Neutrophils are the first peripheral inflammatory cells to migrate into the tissue. It has been suggested that neutrophils may be involved in neuronophagia [239], a view supported by in vitro literature about cytotoxic effects of neutrophils [9], but not by other in vivo data, which could not correlate secondary tissue damage with the presence of polymorphonuclear neutrophils [99,118]. In this study we have not found neutrophil-mediated neuronophagia and our data have shown that neutrophil influx in the contused spinal cord follows a time course of a detectable increase at 6 h and a rapid influx thereafter that peaks at 24 h. This is consistent with time courses described for other studies of SCI [65,99,341]. The fact that necrosis precedes the arrival of neutrophils at the injury site indicates that the tissue necrosis may not be caused by the invading leukocytes, but that it may, instead, provide a chemotactic stimulus for attracting neutrophils as the first line of recruited phagocytic cells to begin removal of cellular debris.

The second wave of peripheral inflammatory cells to enter damaged tissue in the CNS is monocyte-derived macrophages. It has been well-known that the population of macrophages is heterogeneous, consisting of microglia- and monocyte-derived [25,85,200,216,338]. Activated (ameboid) microglia can be distinguished from monocyte-derived macrophages by the morphology (microglial processes) before they further transform into round-shaped macrophages [338]. However, when activated (ameboid) microglia further transform into round-shaped macrophages, it is very difficult to determine their origin. This is because most techniques that label microglia will also label peripheral monocyte-derived macrophages [25,338]. Thus, the quantitative contributions of endogenous microglia-derived and monocyte-derived macrophages to the total macrophage pool are still not clear [25]. Macrophages arising from both sources are specifically labelled by the monoclonal antibody ED1, which does not label untransformed resident microglia, and is therefore the best label for macrophages [77].

Regardless of their origin, various reports have shown that macrophages can play both negative and positive roles in responding to injury. Macrophages (including microglia and monocytes) can be induced to produce several potentially cytotoxic substances in vitro, such as free oxygen intermediates, NO, proteases, EAAs and pro-inflammatory cytokines [13,200]. Thus, these cells may contribute to the secondary tissue destruction after TSCI. In support of this, depletion of macrophages has been shown to decrease tissue damage in the cord and delay functional loss after SCI [34,274]. In contrast, macrophages play a key role in the removal of cellular debris and erythrocytes, and produce the trophic cytokines, such as TGF-β1 and IL-4, to promote tissue repair and neuronal survival [14,78,271]. This is supported by some recent demonstrations that transplantation of cultured macrophage or microglia cause enhanced neurite outgrowth and better recovery of motor function [121,286,289]. Thus, the role of macrophages (including microglia and monocytes) is far from clear in studies of TSCI and

their function is likely to be dependent on the time point following injury and their location relative to the lesion.

3.3.5 CELLULAR SOURCES OF TNFα

It has been observed in our study that the rapid elevation of TNFα expression at 1, 3 and 6 h after SCI was followed by a dramatic decline of TNFα expressions prior to the peak appearance of neutrophils and macrophages, indicating that TNFα and other pro-inflammatory cytokines may be primarily produced by endogenous CNS cells rather than infiltrating blood-borne leukocytes. This is supported by our detection of TNFα expression in neurons and some microglial cells, using double-label fluorescent immunohistochemistry. Similar studies of other pro-inflammatory cytokines, such as IL-1β and IL-6, could not be done due to the unavailability of IL-1β and IL-6 specific antibodies for rat paraffin tissue.

4. UPREGULATION OF IL-1β, IL-6 AND TNFα mRNAs AND PROTEINS AFTER MILD AND SEVERE SCI

This experiment was performed to investigate the response of pro-inflammatory cytokines (IL-1β, IL-6 and TNFα) at mRNA and protein levels in the rat after SCI.

4.1 METHODS

4.1.1 ANIMALS

Sixty rats were used and divided into four groups: mild SCI, severe SCI, laminectomy control and naïve control. Twenty-one rats with mild SCI were killed at 1 h (n=4), 3 h (n=5), 6 h (n=4), 1 d (n=4) or 3 d (n=4) postinjury. The severe SCI group included twenty rats, which were sacrificed at 1 h (n=4), 3 h (n=4), 6 h (n=4), 1 d (n=4) or 3 d (n=4) postinjury. Sixteen rats with laminectomy only were killed at 1 h (n=3), 3 h (n=3), 6 h (n=3), 1 d (n=3) or 3 d (n=4) after surgery and three normal rats served as naïve controls.

4.1.2 RT-PCR

RT-PCR was used to analyse the mRNA expressions of IL-1β, IL-6 and TNFα in the rat spinal cord after TSCI.

4.1.2.1 MOLECULAR REAGENTS

One ml of Trizol® reagent was utilised in the RNA extraction process. Materials made use of in reverse transcription include 500ng random hexamers, 1x Reverse Transcription Buffer (50mM Tris-HCl, 50mM KCl, 10mM $MgCl_2$, 0.5mM spermidine, 10mM DTT), 1mM deooxynucleoside triphosphates (dNTPs) and 20U avian myclobastosis virus (AMV) (Promega, Madison, WI).

Reagents involved in PCR amplification comprise of 0.6μM each of sense and antisense oilgonuleotide primer, 1.7 mM $MgCl_2$, 200μM dNTPs, 1x PCR buffer (20mM Tris-HCl, 50mM KCl) and 1U of PlatinumTaq DNA polymerase and the internal control 18s primer mix.

Samples were prepared for visualisation with 6x Loading Buffer (50% glycerol, 0.25% bromophenol, 0.25% xylene cyanol) and run on 1.5% agarose in 1xTAE solution with DNA marker (20% DNA marker, 10% loading buffer, 70% water).

4.1.2.2 RNA EXTRACTION

For total RNA extraction, a 2cm segment of spinal cord encompassing the lesion epicentre was placed in 4 ml of TRIzol® Reagent (Life Technologies, Gaithersbug, MD). Tissue samples were completely homogenized and 800 μl of chloroform was added to the samples. Following a brief vortex, the samples were centrifuged at 12000 x g for 15 minutes at 4°C. The top layer (aqueous phase) containing total RNAs was carefully removed and transferred to sterile tubes while the bottom layer (organic phase) containing total DNAs and proteins was used for western blot analysis (refer to 4.1.3). An equal volume of isopropanol was added to the RNA samples, the tubes inverted to mix, and left at -20°C for 15 minutes to precipitate the RNA. RNA samples were centrifuged at 12000 x g for 15 minutes at 4°C. The supernatants were removed with care not to disturb the pellets and the pellets were washed in 70% ethanol. The RNA samples were centrifuged at 12000 x g for 10 minutes at 4°C. The supernatants were removed and the tubes briefly re-spun. Residual 70% ethanol was removed and RNA pellets allowed to air dry. RNA samples were re-suspended in 100μl of DEPC water and stored at -70°C until required.

Two ml of RNA sample was diluted 1:250 in DEPC water and the concentration determined using a UV-1601 Shimadzu Recording Spectrophotometer (Shimadzu Corporation, Kyoto, Japan), measuring the absorbance at a wavelength of 260nm. Purity of the RNA was analysed by comparing absorbance at 260nm with absorbance at 280nm.

4.1.2.3 REVERSE TRANSCRIPTION

The gene expression levels of IL-1β, IL-6, TNFα and cyclophilin control were determined by RT-PCR. One microgram of total RNA, 0.5 µl random hexamers (1 µg/µl) and DEPC water added to a volume of 12 µl was heated for 5 minutes at 70°C in a GeneAmp® PCR system 9700 thermal cycler to denature the RNA. Following the denaturation, 4 µl Reverse Transcription Buffer (5X), 2 µl dNTPs (10 mM) and 2 µl purified AMV Reverse Transcriptase (10 U/µl) was added to each sample to make a final volume of 20µl. Reverse transcription was performed at 30°C for 10 minutes and then 42°C for 45 minutes and this was followed by an incubation at 95°C for 5 minutes to inactivate the enzyme.

4.1.2.4 POLYMERASE CHAIN REACTION

The PCR amplification reaction was performed in a 20 µl volume containing 1 µl of the RT reaction solution, 0.8 mM each of sense and antisense primer (refer to Table 7), 50 mM $MgCl_2$, 2 µl dNTPs (2mM), 2 µl PCR buffer (10X), 1U of PlatinumTaq DNA polymerase (Life Technologies, Gaithersburg, MD) and DEPC water. Samples were amplified in GeneAmp® PCR system 9700 thermal cycler for initial denaturisation of 94°C for 3 minutes, followed by 35 cycles of denaturation (94 °C, 30 seconds), annealing (60 °C, 30 seconds) and extension (72 °C, 30 seconds) followed by a final extension of 72°C for 5 minutes to allow the completion of any partial extension products and the annealing of single-stranded complementary products. The PCR products were stored at -20°C until needed. Primers and

expected PCR product sizes for each target gene (IL-1β, IL-6 and TNFα) and cyclophilin control are listed in Table 7.

The PCR products were electrophorated on 2% agarose gels, stained with ethidium bromide and visualized by ultraviolet (UV) illumination. Digital images of the PCR products were produced under UV light and the intensity of PCR product staining was determined using NIH image 1.62 (NIH, Bethesda, MD) [343]. The amount of each PCR product of IL-1β, IL-6 and TNF-α was normalized to cyclophilin, a transcript whose levels do not change in contused spinal cord [318].

Table 6

PCR Primers and Amplification Product Sizes

Target	Sense primer & Antisense primer		Product size (base pair)
IL-1β	Sense (5'-3') Antisense (3'-5')	CAC CTT CTT TTC CTT CAT CTT TG GTC GTT GCT TGT CTC TCC TTG TA	241
IL-6	Sense (5'-3') Antisense (3'-5')	AAG TTT CTC TCC GCA AGA GAC TTC CAG AGG CAA ATT TCC TGG TTA TAT CCA GTT	299
TNF-α	Sense (5'-3') Antisense (3'-5')	GTA GCC CAC GTC GTA GCA AAC TGT GGG TGA GGA GCA CAT AGT C	196
Cyclophilin	Sense (5'-3') Antisense (3'-5')	GAC AAA GTT CCA AAG ACA GCA GAA A CTG AGC TAC AGA AGG AAT GGT TTG	470

4.1.3 WESTERN BLOT ANALYSIS

The protein levels of IL-1β, IL-6 and TNF-α were analysed by western blotting.

4.1.3.1 PROTEIN EXTRACTION AND PREPARATION

For total protein extraction, a 2 cm segment of spinal cord encompassing the lesion epicentre was placed in 4 ml of TRIzol® Reagent. Tissue samples were completely homogenized and 800 µl of chloroform was added to the samples. Following a brief vortex, the samples were centrifuged at 12000 x g for 15 minutes at 4°C. The top layer (aqueous phase) containing total RNAs was carefully removed and transferred to sterile tubes for RT-PCR while the bottom layer (organic phase) containing total DNAs and proteins was used for western blot assay. The bottom layer was mixed with 1.2 ml of 100% ethanol and the mixture was centrifuged at 2000 x g for 5 minutes at 4°C to sediment DNA. The supernatant containing total proteins was then mixed with isopropyl alcohol and the protein precipitate was sedimented at 12000 x g for 10 minutes at 4°C. The protein pellet was washed 3 times in a solution containing 0.3 M guanidine hydrochloride in 95% ethanol. The protein pellet was dissolved in 2% SDS by pipetting and complete dissolution of the protein pellet may require incubating the sample at 50°C. The protein concentration was determined by Bio-Rad (Hercules, CA, USA) protein assay. The sample was then mixed with laemlis buffer and stored at -20°C for use in western blot analysis.

Recombinant IL-1β, IL-6 and TNF-α (Peprotech, Rocky Hill, NJ) were also dissolved in laemlis buffer, serving as positive controls.

4.1.3.2 WESTERN BLOTTING

Samples and recombinant controls were heated in boiling water for 3 minutes and then cooled to room temperature. Twenty-five micrograms protein from each sample, recombinant positive controls and BenchMark™ protein marker (GIBCO BRL, Baithersburg, MD) were loaded onto 15% acrylamide gel [201]. The gel was run at 20 mA in 1 x SDS-PAGE running buffer for 1.5 hours. Following electrophoresis, the separating gel, a piece of nitrocellulose membrane (Millipore, Bedford, MA) of the same size, together with 4 pieces of blotting paper was soaked in Semi-Dry transfer buffer for approximately 10 minutes. The gel-membrane sandwich was then assembled in the Hoefer Semi-Dry transfer apparatus and the transfer carried out at 0.8 mA/cm^2 for 90 minutes. After the transfer, the membrane was blocked in 5% milk in Tris-buffered saline with 0.1% thimersoal (pH 7.5) at 4°C for overnight. The membrane was incubated for 2 hours with a primary antibody, which was rabbit anti-rat IL-1β, rabbit anti-rat IL-6 or rabbit anti-rat TNF-α antibodies (Peprotech, Rocky Hill, NJ) in1:500 dilution. Following the incubation with the primary antibody, the membrane was washed three times with Tris-buffered saline with 0.1% thimersoal (pH 7.5) and incubated for 1 hour with a secondary antibody (goat against rabbit IgG conjugated with alkaline phosphatase, 1: 10000) (Sigma, St. Louis, MO). The membrane was then incubated with substrate development solution (10 ml alkaline phosphatase substrate buffer, 100 µl NBT and 100 µl BCIP) for 5 minutes and rinsed in deionised water. The membrane was allowed to air dry on a paper towel and intensities of protein bands were quantified densitometrically by NIH image 1.62 (NIH, Bethesda, MD). In case where levels of IL-1β, IL-6 and TNF-α were not detectable, the data were recorded as 1 arbitrary densitometric unit. The amount of each product was normalized to the recombinant control for comparison.

4.1.3.3 STATISTICAL ANALYSES

Multiple samples were analysed by one-way analysis of variance (ANOVA). Differences between the mild and severe SCI groups were analysed by a post-hoc test of Student Neuman Keuls. A P value less than 0.05 was considered significant.

4.2 RESULTS

4.2.1 RT-PCR

The profile of cytokine transcription in contused spinal cord was marked by an early, robust but transient increase in IL-1β, IL-6 and TNF-α mRNAs.

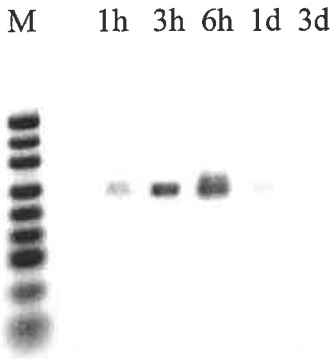
4.2.1.1 IL-1β mRNA

RT-PCR products representing IL-1β mRNA levels were shown in Figure 15 with a size of 241 base pairs. The staining intensity of RT-PCR products (amplified for 35 cycles) was directly proportional to the amount of DNA because pixel density units of these products were within the linear range of RT-PCR (refer to appendix 2).

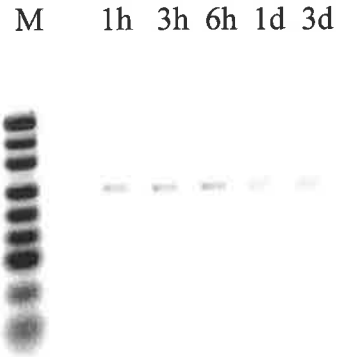
The levels of IL-1β mRNA in the group of severe SCI were elevated at 1 and 3 h after injury, peaked at 6 h and declined to near baseline levels by 1 d postinjury (Figures 15A and 16). Compared to the mild injury group (Figures 15B and 16), the mRNA levels of IL-1β in the group of severe SCI were significantly higher at 3 and 6 h postinjury ($P<0.05$). The maximum level of the IL-1β transcript in the severe SCI group at 6 h postinjury represented a 5-fold increase over the level of mild injury group. Additionally, the levels of the IL-1β mRNA in the mild SCI were significantly increased at 1 h, 3 h and 6 h postinjury ($P<0.05$) compared to laminectomy controls (Figures 15C and 16). In the group of laminectomy controls, fluctuations in IL-1β mRNA were hardly noted and their expression levels were quite similar to those in the naive control group ($P>0.05$) (refer to appendix 2).

Figure 15. *RT-PCR products for the IL-1β gene. Column M: molecular weight markers. Columns 1h, 3h, 6h, 1d and 3d: RT-PCR products of IL-1β at 1 hour, 3 hours, 6 hours, 1day and 3 days postinjury. A: IL-1β mRNA expressions after severe SCI; B: IL-1β mRNA expressions after mild SCI; and C: IL-1β mRNA expressions in laminectomy controls.*

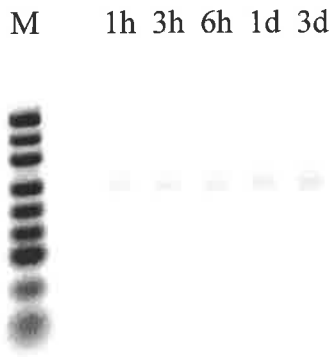
A: IL-1β mRNA after severe SCI



B: IL-1β mRNA after mild SCI



C: IL-1β mRNA in laminectomy controls



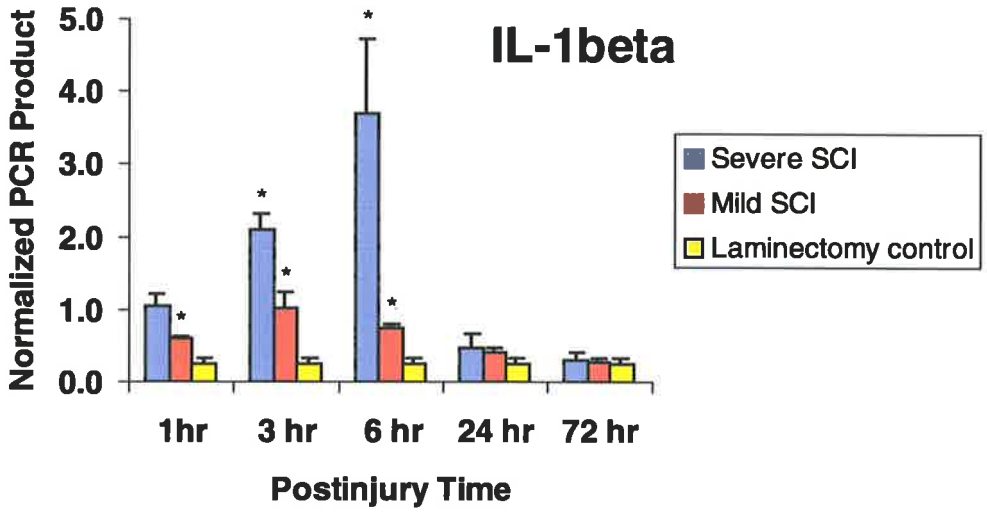


Figure 16. *Time courses of IL-1β mRNA expressions following SCI and in laminectomy controls. Levels of the IL-1β mRNA in the severe SCI were significantly greater than in the mild SCI at 3 hr and 6 hr postinjury. Compared to laminectomy controls, the levels of the IL-1β mRNA in the mild SCI were also significantly increased at 1 hr, 3 hr and 6 hr postinjury. Bars represent means ± SEM (standard error of the mean). (* $P < 0.05$).*

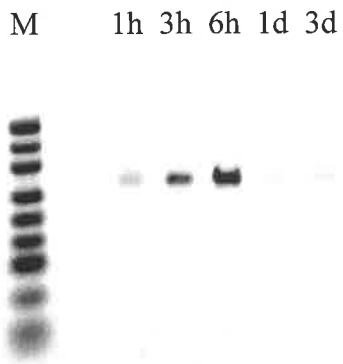
4.2.1.2 IL-6 mRNA

RT-PCR products representing IL-6 mRNA levels are shown in Figure 17 with a size of 299 base pairs. The staining intensity of RT-PCR products (amplified for 35 cycles) was directly proportional to the amount of DNA because pixel density units of these products were within the linear range of RT-PCR (refer to appendix 2).

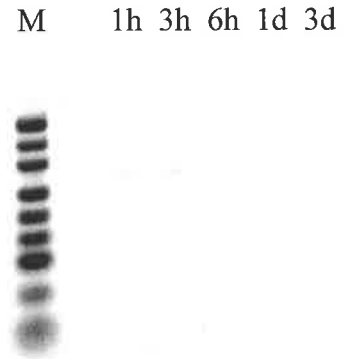
The expression profile of IL-6 mRNA in spinal cord was virtually identical to the IL-1β pattern, shown in Figures 15 and 16. The levels of IL-6 mRNA in the group of severe SCI were elevated at 1 and 3 h after injury, peaked at 6 h and declined to near baseline levels by 1 d postinjury (Figures 17A and 18). Compared to the mild injury group (Figures 17B and 18), the mRNA levels of IL-6 in the group of severe SCI were significantly higher at 3 and 6 h postinjury ($P<0.05$). The maximum level of the IL-6 transcript in the severe SCI group at 6 h postinjury represented a 7-fold increase over the level of mild injury group. Additionally, the levels of the IL-6 mRNA in the mild SCI were significantly increased at 1 h, 3 h and 6 h postinjury ($P<0.05$) compared to laminectomy controls (Figures 17C and 18). In the group of laminectomy controls, fluctuations in IL-6 mRNA were hardly noted and their expression levels were quite similar to those in the naive control group ($P>0.05$) (refer to appendix 2).

Figure 17. *RT-PCR products for the IL-6 gene. Column M: molecular weight markers. Columns 1h, 3h, 6h, 1d and 3d: RT-PCR products of IL-6 at 1 hour, 3 hours, 6 hours, 1day and 3 days postinjury. A: IL-6 mRNA expressions after severe SCI; B: IL-6 mRNA expressions after mild SCI; and C: IL-6 mRNA expressions in laminectomy controls.*

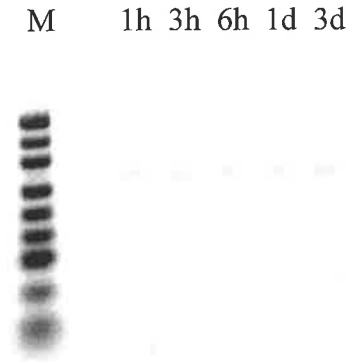
A: IL-6 mRNA after severe SCI



B: IL-6 mRNA after mild SCI



C: IL-6 mRNA in laminectomy controls



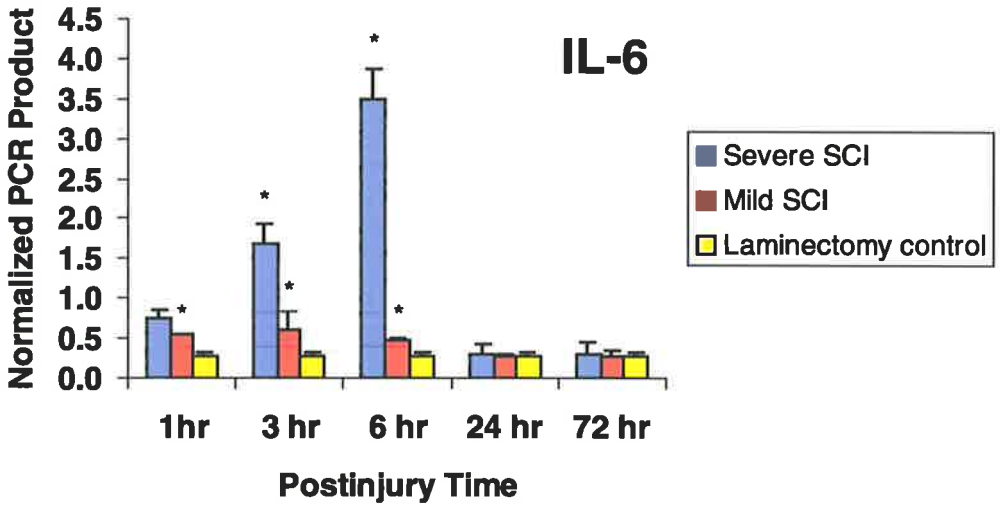


Figure 18. *Time courses of IL-6 mRNA expressions following SCI and in laminectomy controls. Levels of the IL-6 mRNA in the severe SCI were significantly greater than in the mild SCI at 3 hr and 6 hr postinjury. Compared to laminectomy controls, the levels of the IL-6 mRNA in the mild SCI were also significantly increased at 1 hr, 3 hr and 6 hr postinjury. Bars represent means ± SEM (standard error of the mean). (* $P < 0.05$).*

4.2.1.3 TNFα mRNA

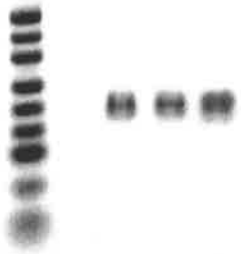
RT-PCR products representing TNF-α mRNA levels are shown in Figure 19 with a size of 196 base pairs. The staining intensity of RT-PCR products (amplified for 35 cycles) was directly proportional to the amount of DNA because pixel density units of these products were within the linear range of RT-PCR (refer to appendix 2).

The levels of TNF-α mRNA in the group of severe SCI were elevated at 1 and 3 h after injury, peaked at 6 h and declined to near baseline levels by 1 d postinjury (Figures 19A and 20). Compared to the mild injury group (Figures 19B and 20), the mRNA levels of TNF-α in the group of severe SCI were significantly higher at 1, 3 and 6 h postinjury ($P<0.05$). The maximum level of the TNF-α transcript in the severe SCI group at 6 h postinjury represented a 6-fold increase over the level of mild injury group. Additionally, the levels of the TNF-α mRNA in the mild SCI were significantly increased at 1 h, 3 h and 6 h postinjury ($P<0.05$) compared to laminectomy controls (Figures 19C and 20). In the laminectomy controls, no significant differences in TNF-α mRNA were seen when compared with naïve controls ($P>0.05$) (refer to appendix 2).

Figure 19. *RT-PCR products for the TNF-α gene. Column M: molecular weight markers. Columns 1h, 3h, 6h, 1d and 3d: RT-PCR products of TNF-α at 1 hour, 3 hours, 6 hours, 1 day and 3 days postinjury. A: TNF-α mRNA expressions after severe SCI; B: TNF-α mRNA expressions after mild SCI; and C: TNF-α mRNA expressions in laminectomy controls.*

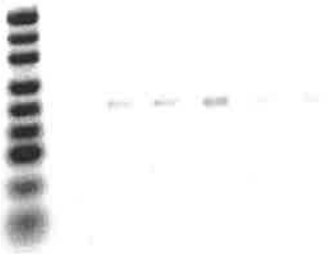
A: TNFα mRNA after severe SCI

M 1h 3h 6h 1d 3d



B: TNFα mRNA after mild SCI

M 1h 3h 6h 1d 3d



C: TNFα mRNA in laminectomy controls

M 1h 3h 6h 1d 3d

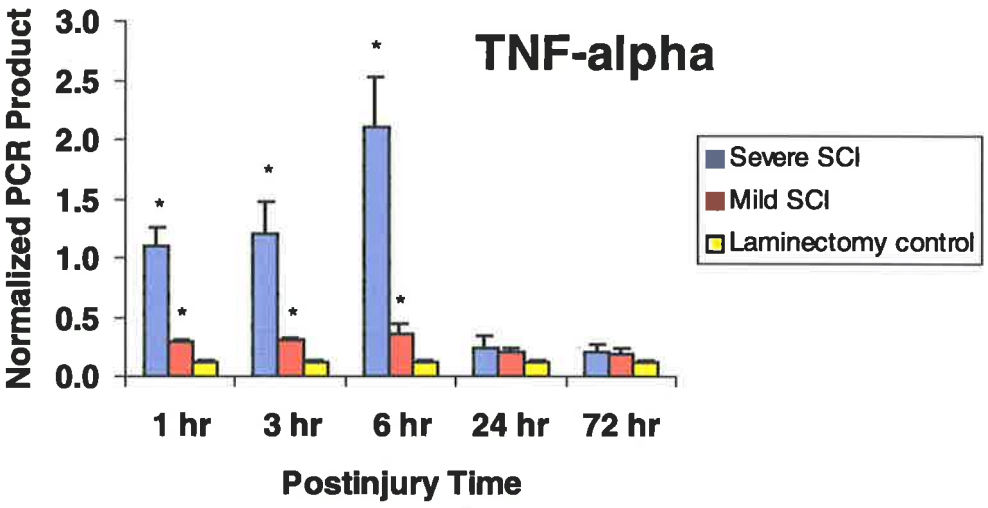


Figure 20. *Time courses of TNF-α mRNA expressions following SCI and in laminectomy controls. Levels of the TNF-α mRNA in the severe SCI were significantly greater than in the mild SCI at 1 hr, 3 hr and 6 hr postinjury. Compared to laminectomy controls, the levels of the TNF-α mRNA in the mild SCI were also significantly increased at 1 hr, 3 hr and 6 hr postinjury. Bars represent means ± SEM (standard error of the mean). (* $P < 0.05$).*

4.2.2 WESTERN BLOTTING

The profile of cytokine proteins in contused spinal cord was also marked by an early, robust but transient increase in IL-1β, IL-6 and TNF-α proteins. However, the increase in the pro-inflammatory cytokines was only observed in the group of severe SCI and no immunoreactive cytokine proteins were detected in the groups of mild SCI, laminectomy controls or naïve controls.

4.2.2.1 IL-1β PROTEIN

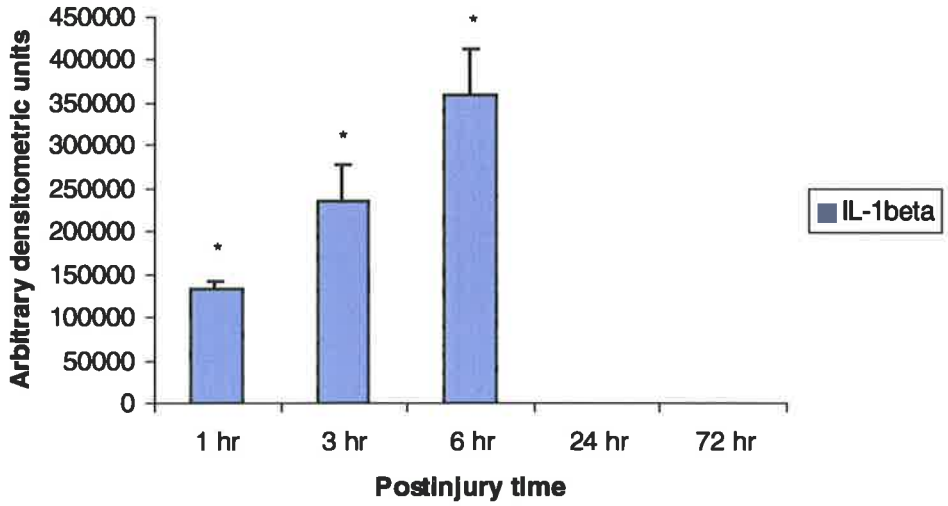
Western blotting showed labelling of single bands with a molecular weight of 17.3 kDa for IL-1β in Figure 21. The intensity of protein bands was directly proportional to the amount of protein because arbitrary densitometric units of these protein bands were within the linear range of western blot analysis (refer to appendix 2).

The levels of IL-1β protein in the group of severe SCI were elevated at 1 and 3 h after injury, peaked at 6 h and declined to undetectable levels by 1 d postinjury while no immunoreactive IL-1β was detectable in the spinal cord extracts from rats in the groups of mild SCI, laminectomy controls and naïve controls (Figures 21). Thus, compared to mild SCI, laminectomy controls and naïve controls, the levels of IL-1β protein at 1h, 3h and 6h following severe SCI were significantly higher ($P<0.05$) (refer to appendix 2).

Figure 21. *Temporal expressions of IL-1β at the protein level in the severe SCI demonstrated by western blot analysis. Levels of the IL-1β protein after severe SCI began to increase at 1 and 3 hr postinjury, peaked at 6 hr and quickly decline to non-detectable levels by 1 d. (A) IL-1β protein bands of the group of severe SCI. (B) Quantification of IL-1β protein bands of the group of severe SCI. Bars represent means ± SEM.*

A: IL-1β protein bands of the group of severe SCI.

B: Quantification of IL-1β protein bands in the group of severe SCI.



4.2.2.2 IL-6 PROTEIN

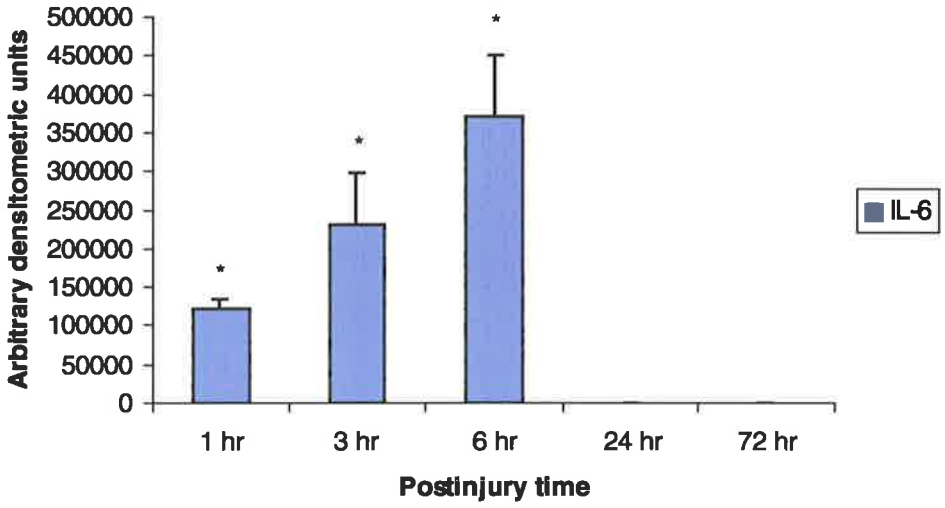
Western blotting showed labelling of single bands with a molecular weight of 23 kDa for IL-6 in Figure 22. The intensity of protein bands was directly proportional to the amount of protein because arbitrary densitometric units of these protein bands were within the linear range of western blot analysis (refer to appendix 2).

The levels of IL-6 protein in the group of severe SCI were elevated at 1 and 3 h after injury, peaked at 6 h and declined to undetectable levels by 1 d postinjury while no immunoreactive IL-6 was detectable in the spinal cord extracts from rats in the groups of mild SCI, laminectomy controls and naïve controls (Figures 22). Thus, compared to mild SCI, laminectomy controls and naïve controls, the levels of IL-6 protein at 1h, 3h and 6h following severe SCI were significantly higher ($P<0.05$) (refer to appendix 2).

Figure 22. *Temporal expressions of IL-6 at the protein level in the severe SCI demonstrated by western blot analysis. Levels of the IL-6 protein after severe SCI began to increase at 1 and 3 hr postinjury, peaked at 6 hr and quickly decline to non-detectable levels by 1 d. (A) IL-6 protein bands of the group of severe SCI. (B) Quantification of IL-6 protein bands of the group of severe SCI. Bars represent means ± SEM.*

A: IL-6 protein bands of the group of severe SCI.

B: Quantification of IL-6 protein bands in the group of severe SCI.



4.2.2.3 TNFα PROTEIN

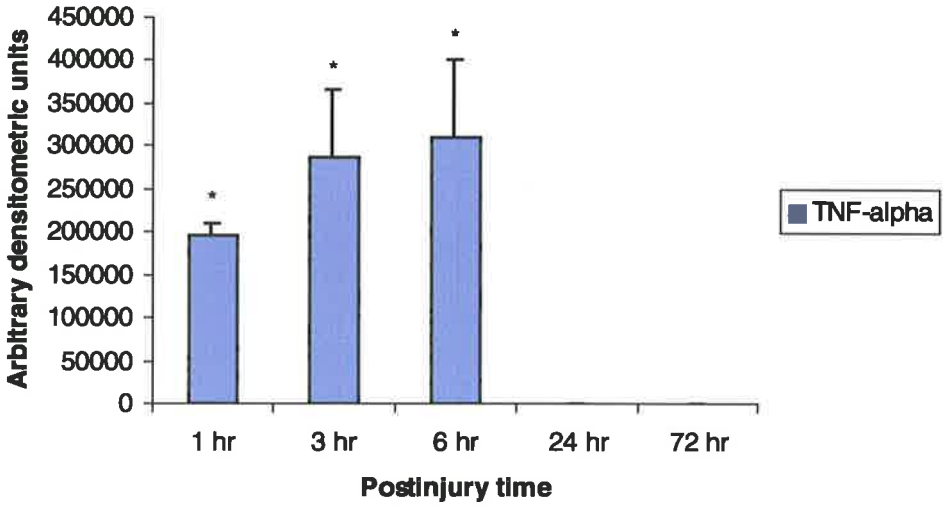
Western blotting showed labelling of single bands with a molecular weight of 17 kDa for TNFα in Figure 23. The intensity of protein bands was directly proportional to the amount of protein because arbitrary densitometric units of these protein bands were within the linear range of western blot analysis (refer to appendix 2).

The levels of TNFα protein in the group of severe SCI were elevated at 1 and 3 h after injury, peaked at 6 h and declined to undetectable levels by 1 d postinjury while no immunoreactive TNFα was detectable in the spinal cord extracts from rats in the groups of mild SCI, laminectomy controls and naïve controls (Figures 23). Thus, compared to mild SCI, laminectomy controls and naïve controls, the levels of TNFα protein at 1h, 3h and 6h following severe SCI were significantly higher ($P<0.05$) (refer to appendix 2).

Figure 23. *Temporal expressions of TNFα at the protein level in the severe SCI demonstrated by western blot analysis. Levels of the TNFα protein after severe SCI began to increase at 1 and 3 hr postinjury, peaked at 6 hr and quickly decline to non-detectable levels by 1 d. (A) TNFα protein bands of the group of severe SCI. (B) Quantification of TNFα protein bands of the group of severe SCI. Bars represent means ± SEM.*

A: TNFα protein bands of the group of severe SCI.

B: Quantification of TNFα protein bands in the group of severe SCI.



4.3 DISCUSSION

4.3.1 CELLULAR SOURCES OF IL-1β, IL-6 and TNF-α

The rapid elevation of IL-1β, IL-6 and TNF-α mRNA expressions at 1,3 and 6 h after SCI was followed by a dramatic decline of these expressions prior to the peak appearance of neutrophils and macrophages, indicating that these pro-inflammatory cytokines are primarily produced by endogenous CNS cells rather than infiltrating blood-borne leucocytes. This is supported by our detection of TNF-α expression in neurons and microglial cells after SCI (see section 3.2). These data suggest that neurons and microglial cells, not blood-born leucocytes proposed by many authors [135,347,354], may play a major role in the initiation and regulation of early posttraumatic inflammatory response of SCI by producing IL-1β, IL-6 and TNF-α.

4.3.2 DISPARITIES BETWEEN TRANSCRIPTION AND TRANSLATION OF IL-1β, IL-6 AND TNF-α

To our knowledge, this is the first study to address the disparities between syntheses of pro-inflammatory cytokine mRNAs and their bioactive proteins in SCI. Expressions of IL-1β, IL-6 and TNF-α at both mRNA and protein levels in severe SCI were dramatically elevated at 1, 3 h after injury, peaked at 6 h and declined to near baseline levels by 1 d postinjury. Thus, in the severe SCI, the temporal profiles of IL-1β, IL-6 and TNF-α protein expressions closely parallel their mRNA syntheses. Although IL-1β, IL-6 and TNF-α mRNA levels increased in mild injury, we were unable to detect the corresponding proteins histochemically.

The dissociation between mRNA and protein expression levels of these pro-inflammatory cytokines in the mild SCI may be attributed to a combination of two factors. First, different experimental techniques may cause the dissociation because two different techniques, RT-PCR and western blotting, were respectively used to detect the mRNA and protein

expressions of IL-1β, IL-6 and TNF-α. The RT-PCR process is exquisitely sensitive and a single copy of target nucleic acid can be detected by PCR because the number of target nucleic acid copies approximately doubles every cycle and 35 cycles of PCR in this study may amplify the target nucleic acid by about thirty billion times (2^{35}) [23,155]. Western blot analysis, when compared to RT-PCR, is relatively insensitive as the detection limits for IL-1β, IL-6 and TNF-α proteins are about 1.5-2.0 ng according to the antibody manufacturer's protocol (Peprotech, Rocky Hill, NJ). Second, mild SCI may not be sufficient to provide a signal for enough translation of the mRNAs into the bioactive cytokine proteins despite a vigorous signal for syntheses of the pro-inflammatory cytokine mRNAs. The syntheses of mature IL-1β, IL-6 and TNF-α proteins are tightly controlled at many different levels to ensure the silence of the pro-inflammatory cytokines in the CNS under normal circumstances and each one of the synthetic steps from sensing the presence of SCI to cytokine secretion is controlled by different molecular events regulating the cytokine gene, mRNA and protein [88,405]. Thus, in mild SCI, the syntheses of mature IL-1β, IL-6 and TNF-α proteins may not be able to overcome the apparently intrinsic inhibition to sufficiently translate the mRNAs. This is further confirmed by our finding that TNFα expression by immunohistochemistry occurs in fewer neurons and glial cells in the mildly injured spinal cord compared to severely injured. In summary, the syntheses of mature IL-1β, IL-6 and TNF-α proteins did not mirror their mRNA productions following mild SCI and were not sufficient for detection with western blot analysis.

4.3.3 SEVERITY-DEPENDENT EXPRESSIONS OF IL-1β, IL-6 AND TNF-α

Our results demonstrated significant elevations in IL-1β, IL-6 and TNF-α following severe SCI compared to mild SCI. To our knowledge, this is the first report of severity-dependent

expressions of IL-1β, IL-6 and TNF-α in SCI, suggesting that these cytokines may be suitable targets for pharmacological intervention.

These data also suggest a positive association between neurological impairment and excessive expressions of IL-1β, IL-6 and TNF-α as mild SCI did not cause either lasting functional deficits or substantially increased expression of pro-inflammatory cytokines. These results are consistent with similar changes observed previously in traumatic brain injury where no neurologic deficits were caused in a mild contusion injury with a small up-regulation of TNF-α expression [176,194]. Therefore, expression levels of the IL-1β, IL-6 and TNF-α may be associated with severity of SCI, especially with reference to resultant neurological deficits.

IL-1β, IL-6 and TNF-α are important and modifiable components of the inflammatory response to traumatic CNS injury. The production of IL-6 is regulated by a variety of stimuli, including IL-1β and TNF-α [172,369]. In experimental traumatic injury of the CNS, it has been shown that the IL-6 receptor was present in neurons and oligodendrocytes [381], and that IL-6 induced neurite outgrowth and improved neuronal survival by inducing the synthesis of neurotrophic factors [147,157,197]. Conversely, anti-rat IL-6 antibody was found to significantly attenuate the upregulation of inducible nitric oxide synthase and reduce secondary spinal cord damage [365]. Thus, IL-6 is thought as a kind of neurotrophic and cytotoxic factor and is partially regulated by IL-1β and TNF-α [375]. IL-1β is a highly inflammatory cytokine, which is implicated in neuronal necrosis and apoptosis, leukocyte infiltration, edema, activation of glial cells, induction of cytokines and synthesis of nitric oxide [88]. For example, IL-1β is able to induce IL-6 secretion from astrocytes [4], stimulate astrocytes to proliferate [135,211] and enhance the synthesis of nerve growth factor (NGF) and basic fibroblast growth factor (bFGF) in astrocytes [247,284]. It has been also reported that systemic injections of IL-1 receptor

antagonist reduced neuronal cell death and improved cognitive function after lateral fluid percussion head injury in the rat [302]. Thus, IL-1 has complex and bi-directional biological effects in the inflammatory response of CNS tissue to trauma. TNF-α shares some similar properties with IL-1β in terms of pro-inflammatory effects, such as the promotion of apoptosis in neurons [238,346], the activation of glial cells [366], the stimulation of astrocyte proliferation [379] and the induction of IL-6 and growth factors in astrocytes [305,399]. TNF-α is also believed to enhance the infiltration of blood leucocytes by modulating the expression of various surface antigens and adhesion molecules on endothelial cells [348]. Additionally, the cytotoxic effects of TNF-α on oligodendrocytes, leading to demyelination, have been shown in vitro [94] and in vivo [290,396]. In contrast to its toxic actions, TNF-α has also been demonstrated to have a regenerative effect on axons [313] and to have a neurotrophic effect by inducing synthesis of NGF in astrocytes [399]. Thus, TNF-α is a powerful inflammatory mediator with both cytotoxic and neurotrophic actions.

Taken together, IL-1β, IL-6 and TNF-α have been shown to produce tissue damage whereas they may also induce the production of neurotrophic molecules to rescue the injured tissue in the posttraumatic response. The explanation of these apparent contradictions requires a complete understanding of dynamics of the posttraumatic inflammatory response in SCI. The early and robust rise in IL-1β, IL-6 and TNF-α following SCI observed in this study may be detrimental because they may be overproduced beyond the level of neurotrophic effects and the margins between benefit and toxicity are quite narrow [405]. This is supported by the finding of spontaneous inflammatory demyelination in the CNS in transgenic mice overexpressing TNF-α [283]. In the present study no neurologic deficit was observed in mild SCI with a small upregulation of proinflammatory cytokine expression. The neurotrophic effects induced by

IL-1β, IL-6 and TNFα may not occur because the cells producing the neurotrophic molecules may be damaged or killed in CNS injury.

Therefore, these data suggest that there may be dual roles of IL-1β, IL-6 and TNF-α in SCI and the final effects of these pro-inflammatory cytokines may be greatly determined by many factors.

5. TREATMENT OF NF-κB-SPECIFIC ANTISENSE OLIGODEOXYNUCLEOTIDES AFTER SEVERE SCI

5.1 METHODS

5.1.1 NF-κB-SPECIFIC ANTISENSE OLIGODEOXYNUCLEOTIDES

The NF-κB-specific antisense oligodeoxynucleotides in albumin microencapsulation were a generous gift from Dr. C. Oettinger and Prof. M.J. D'Souza at Department of Pharmaceutical Sciences, College of Pharmacy, Mercer University, Atlanta, GA 30308, USA. The NF-κB-specific antisense oligodeoxynucleotides in their studies have been shown to significantly reduce the upregulation of TNFα, IL-1β and IL-6 in septic shock model in the rat, slashing 100% death rate after endotoxin injection to only 20%.

5.1.2 REFORMULATION OF NF-κB-SPECIFIC ANTISENSE OLIGODEOXYNUCLEOTIDES

Six hundred micrograms of oligodeoxynucleotides were weighed and re-suspended in 1 ml normal saline containing 2% Tween 80 as a suspending agent (pre-sterilized by autoclaving). The suspension sample was then sonicated for 30 seconds and ready for use.

5.1.3 INJECTION OF NF-κB-SPECIFIC ANTISENSE OLIOGODEOXYNUCLEOTIDES

Fourteen rats received a subarachnoid injection of 5 μl of suspension containing 3 μg NF-κB-specific antisense oligodeoxynucleotides and an intravenous injection of 955 μl of suspension containing 597 μg NF-κB-specific antisense oligodeoxynucleotides immediately after severe SCI produced by 12 cm weight-drop. Rats were sacrificed at 1 hr (N=4), 3 hr (N=5) and 6 hr (N=5) postinjury.

5.1.4 mRNA AND PROTEIN EXTRACTION

The mRNA and protein of IL-1β, IL-6 and TNFα were extracted from rat spinal cords according to the methods described in section 4.1.

5.2 RESULTS

The upregulation of IL-1β, IL-6 and TNFα at the levels of mRNA and protein after severe SCI was not significantly inhibited by the NF-κB-specific antisense oligodeoxynucleotides in the treatment group compared to non-treatment group. At 1, 3 and 6 hr postinjury, large increases in the production of IL-1β, IL-6 and TNFα mRNA and protein were still observed in spinal cord extracts from rats treated with antisense after severe SCI.

5.3 DISCUSSION

Although the NF-κB-specific antisense oligodeoxynucleotides have been shown to significantly reduce the upregulation of TNFα, IL-1β and IL-6 in a septic shock model in the rat, the sources of upregulated cytokines (IL-1β, IL-6 and TNFα) in SCI models are different from those in septic shock models. The upregulation of IL-1β, IL-6 and TNFα after CNS injury is mainly observed in the spinal cord and brain [168,267,354,379] whereas the increase in these cytokines is largely present in the systemic circulation after septic shock [230]. Thus, the NF-κB-specific antisense oligodeoxynucleotides administered intravenously in the septic shock model can easily get to the circulation to inhibit the cytokine upregulation. In contrast, most of the NF-κB-specific antisense oligodeoxynucleotides were administered via the femoral vein due to the limited subarachnoid space. Therefore, the main factor behind the failure of inhibition by the NF-κB-specific antisense oligodeoxynucleotides may be a lack of vehicle to deliver the antisense into the targeted cells (e.g., neurons and microglia), which are the main sources of upregulated cytokines, since our studies have shown endogenous cells

including neurons and microglia contribute substantially to the large increase in IL-1β, IL-6 and TNFα observed in the severely contused spinal cord.

Support from this comes from an unpublished study presented at the 6th International Neurotrauma Symposium in which haemagglutinating virus of Japan-liposome complex containing the NF-κB-specific antisense oligodeoxynucleotides was used in their study to significantly reduce the mRNA levels of IL-1β, IL-6 and TNFα in the rat spinal cord in the ischaemic SCI model. The use of NF-κB-specific antisense oligodeoxynucleotides for the treatment of traumatic SCI requires further study.

HUMAN SCI EXPERIMENTS

1. MATERIALS AND GENERAL METHODS

1.1 SELECTION OF MATERIAL

1.1.1 TRAUMA CASES

Eleven cases of human SCI resulting from acute trauma as a result of a motor vehicle accident (9 men, 2 women; age range, 18-83 years) were selected for the study (Table 4). The study samples were selected from a series of spinal cord injury cases that survived long enough to be admitted to the Spinal Unit at the Royal Adelaide Hospital. All subsequently died and underwent a full post-mortem examination during the period 1978-1997. All the spinal cords were delivered to the Neuropathology Department at the Institute of Medical and Veterinary Science for neuropathological examination.

Injuries that resulted from chronic compression of the spinal cord were excluded. Clinical information was obtained from hospital case notes and accident reports. Only those cases with adequate records of the circumstance of the injury, adequate patient clinical information and adequate neuropathologic data were included in the study. Details of a concomitant hypotension episode that may have compounded the traumatic event were also recorded (Table 4).

1.1.2 CONTROL CASES

Due to the difficulty of obtaining normal spinal cords from post-mortem, only two human spinal cords were available as controls (Table 4). These were from patients with traumatic brain injury only and had no evidence of pathological abnormality in the spinal cord.

1.2 TISSUE PREPARATION

All spinal cords were removed from the human body within 24 h after death and were then fixed in 40% formalin for a minimum of ten days. All the cords were then examined according to standard neuropathological techniques. Individual cord segment levels were determined by counting the segments above and below ventral nerve root of T2 (a landmark due to the marked anatomical disparity in size between T1 and T2 nerve roots).

Tissue blocks were dehydrated in graded alcohol baths and cleared with chloroform, prior to processing into paraffin wax. Five micrometer serial sections were cut at (150 μm) levels with a microtome and mounted onto slides coated with APT (Sigma Chemical Corporation, US). The sections were dried at 37°C for at least 12 hours prior to use. Sections from each level were stained with H&E and immunohistochemically.

Table 7. *Clinicopathologic features of traumatic SCI and control cases*

Case number	Sex	Age	Survival	Cause of SCT	Focal injury	Cause of Death	Hypotensive
1	M	83	30 minutes	MVA-pedestrian	HAEM T11	Multiple injuries, CA	Yes
2	M	22	5 hours	MVA-motorcyclist	HAEM C1-C2	Vertebral artery rupture	Yes
3	F	73	5 hours	MVA-cyclist	HAEM C1-C2	C1/C2 cord injury	Yes
4	M	18	1 day	MVA-driver	HAEM C1	Avulsion of left vertebral artery	Yes
5	M	48	1 day	MVA-rollover	HN C5-T2	Right vertebral artery dissection	No
6	F	77	37 hours	MVA-driver	HN C3-C6	Multiple injuries & CRA	Yes
7	M	52	38 hours	MVA-driver	HN C7-C8	CRA	Yes
8	M	18	2 days	MVA-driver	HN C4-C6	Right ASDH, raised ICP	Yes
9	M	63	2 days	MVA-driver	HAEM C7-T1	Bilateral bronchopneumonia, RA	Yes
10	M	30	88 hours	MVA-rollover	HN C5-C6	Closed head injury, VT, VF	Yes
11	M	32	5 days	MVA-driver	HN C2-T7	Bronchopneumonia	No
1	F	75	19 days	MVA	NIL	Severe head injuries	No
2	M	67	4 hours	MVA	NIL	Severe head injuries	No

ASDH = acute subdural haematoma; C = cervical; CA = cardiac arrest; CRA = cardiorespiratory arrest; HAEM = haemorrhage; HN = haemorrhagic necrosis; ICP = intracranial pressure; M = male; MVA = motor vehicle accident; RA = respiratory arrest; T = thoracic; VF = ventricular failure; VT = ventricular tachycardia.

2. INFLAMMATORY CELLULAR RESPONSE

This experiment was performed to investigate the posttraumatic inflammatory cellular response in the human after SCI.

2.1 METHODS

2.1.1 HISTOLOGIC STAINING

Haematoxylin and eosin (H&E) staining was used on 5-μm-thick sections to assess the extent and distribution of any pathologic abnormality of the cord with particular attention to haemorrhage, necrosis and migration of inflammatory cells (neutrophils and macrophages).

2.1.2 IMMUNOHISTOCHEMISTRY

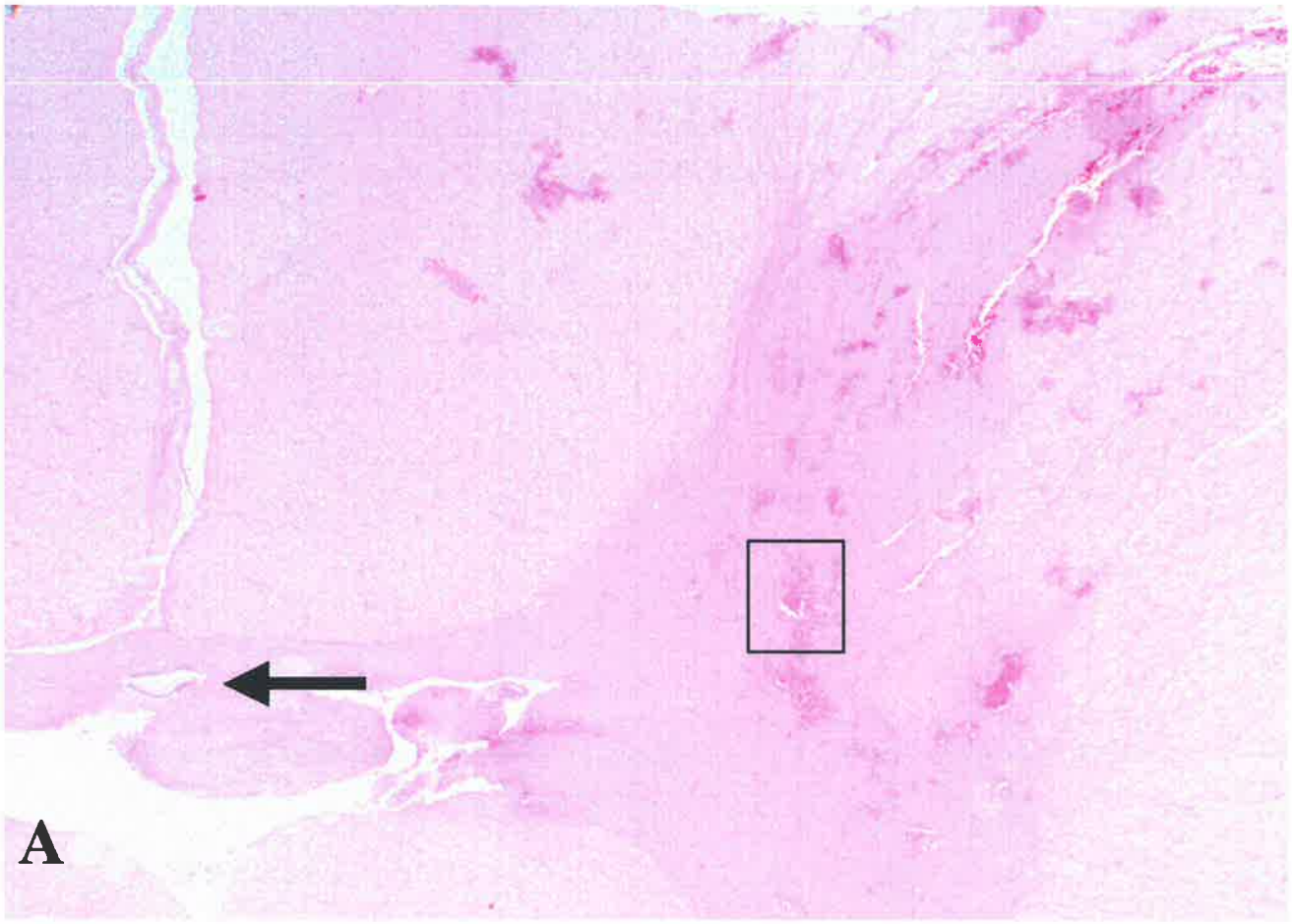
Five-micrometer-thick sections were incubated overnight with the primary antibody diluted in normal horse serum (NHS) and stained with 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO) using avidin-biotin peroxidase (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) and counter-stained with haematoxylin. Negative control studies were performed by omitting the primary antibody step. To identify activated microglia, a mouse anti-class II major histocompatibility (MHCII) antigen monoclonal antibody CR3/43 (DAKO, Carptinteria, CA) was used at a dilution of 1:200 [85,144]. Macrophages were visualized using a mouse monoclonal antibody CD68 (DAKO, Real Carptinteria, CA) at a dilution of 1:5000 [296]. Axonal injury was identified using a mouse anti-APP monoclonal antibody (DAKO, Real Carptinteria, CA) at a dilution of 1:10000 [132,217].

2.2 RESULTS

2.2.1 30 MINUTES POST SCI

At 30 minutes after traumatic SCI, the lesion centre was marked by haemorrhage mainly in the central grey matter, but no neutrophils or other leucocytes were seen in the areas of damaged tissue (Figures 24A-B). Meanwhile, early axonal injury was demonstrated by APP immunoreactivity in neurons and some axons (Figure 24C). At this early time point, some activated microglia were detected, wrapping around some axons (Figure 24D).

Figure 24. *Histological changes in the lesion at 0.5 h after SCI in the human. (A)Marked haemorrhage and disruption of normal spinal cord architecture. The central canal is marked by an arrow for orientation. H&E, X5. (B)Marked haemorrhage without the infiltration of leucocytes in the outlined region in (A) is magnified. H&E, X100. (C) APP expression in some neurons (arrows) and axons (double-headed arrows). APP, X100. (D) Some activated microglia stained by MHCII marker CR3/43 were found wrapping around axons (arrows). CR3/43, X100.*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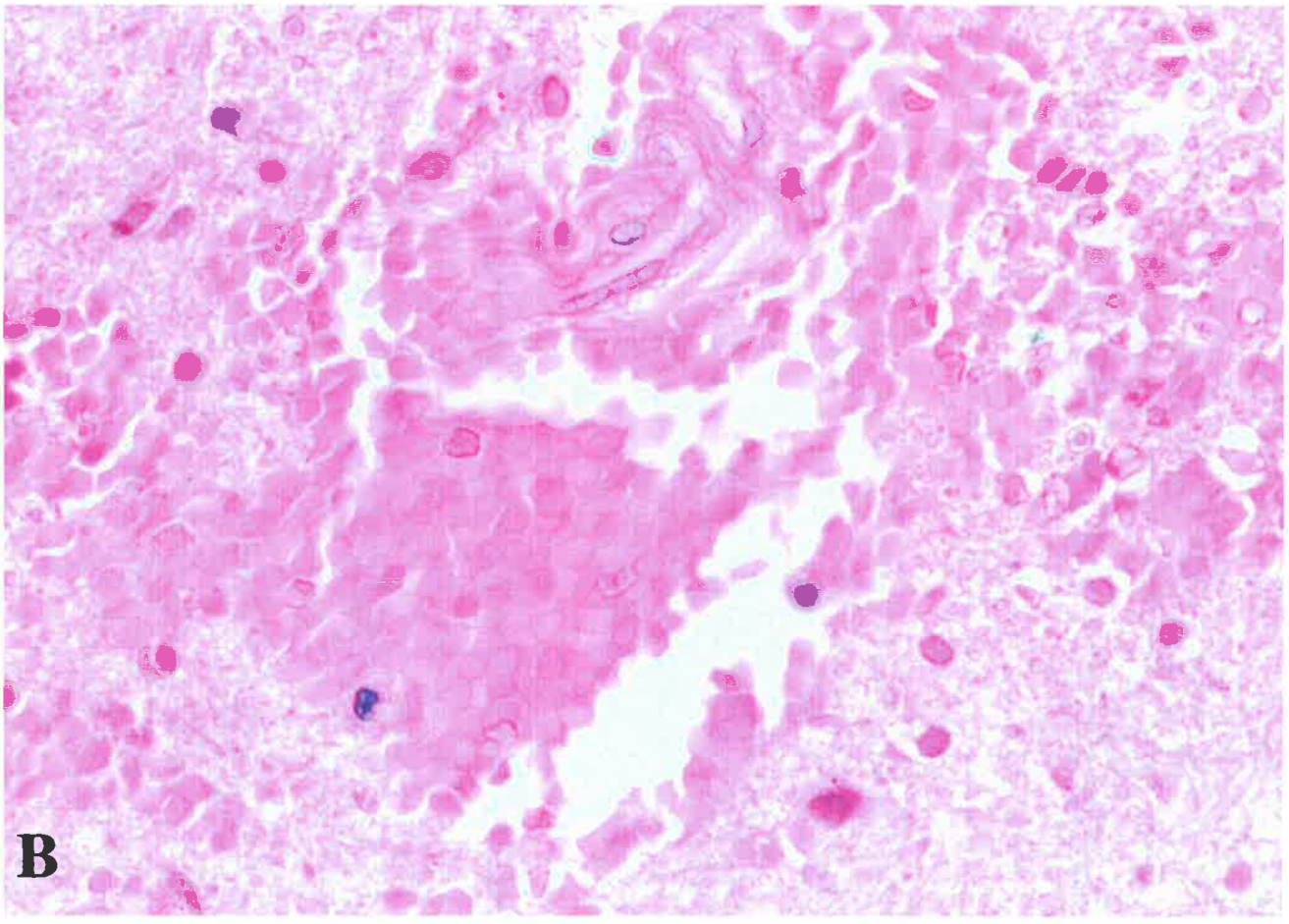
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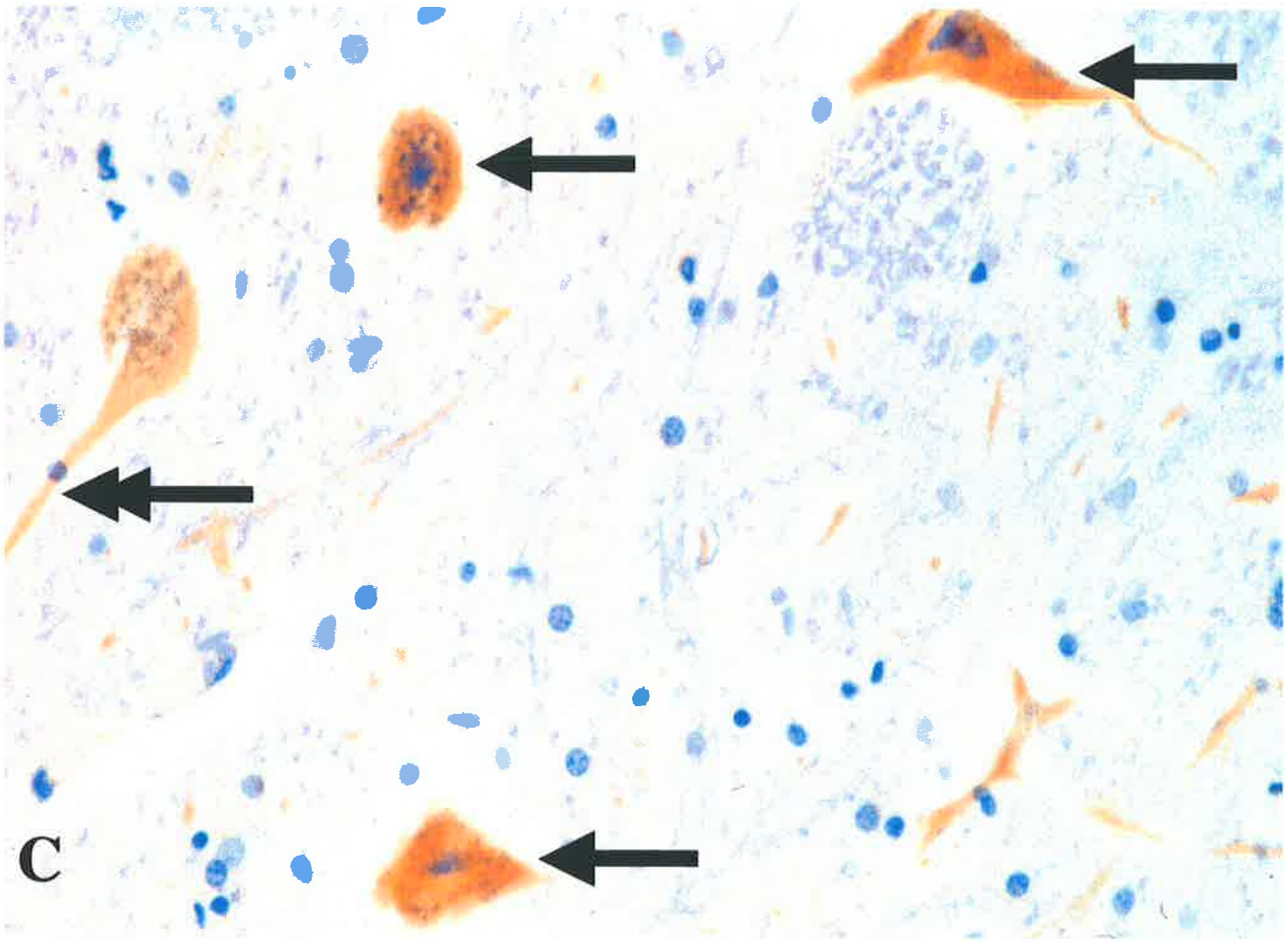
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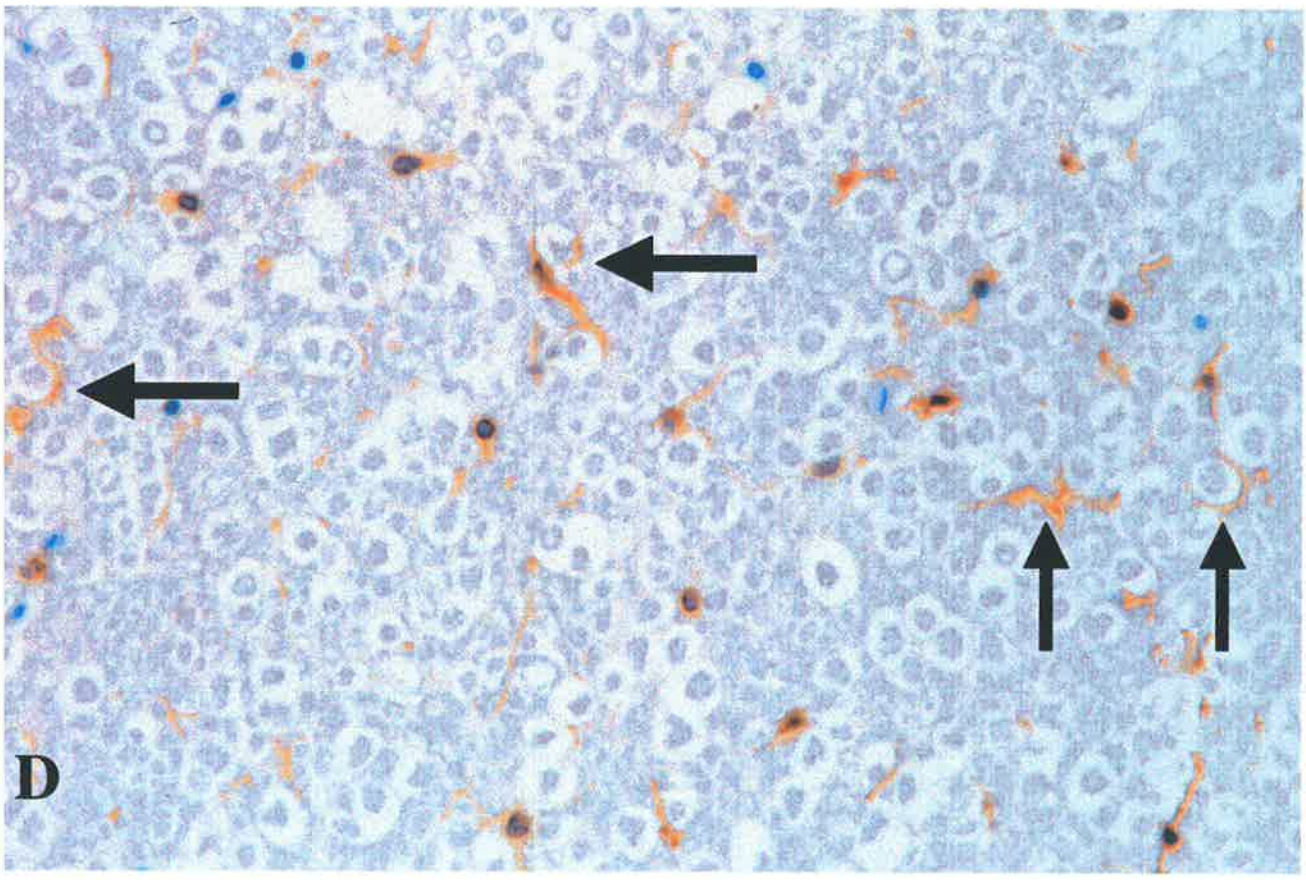
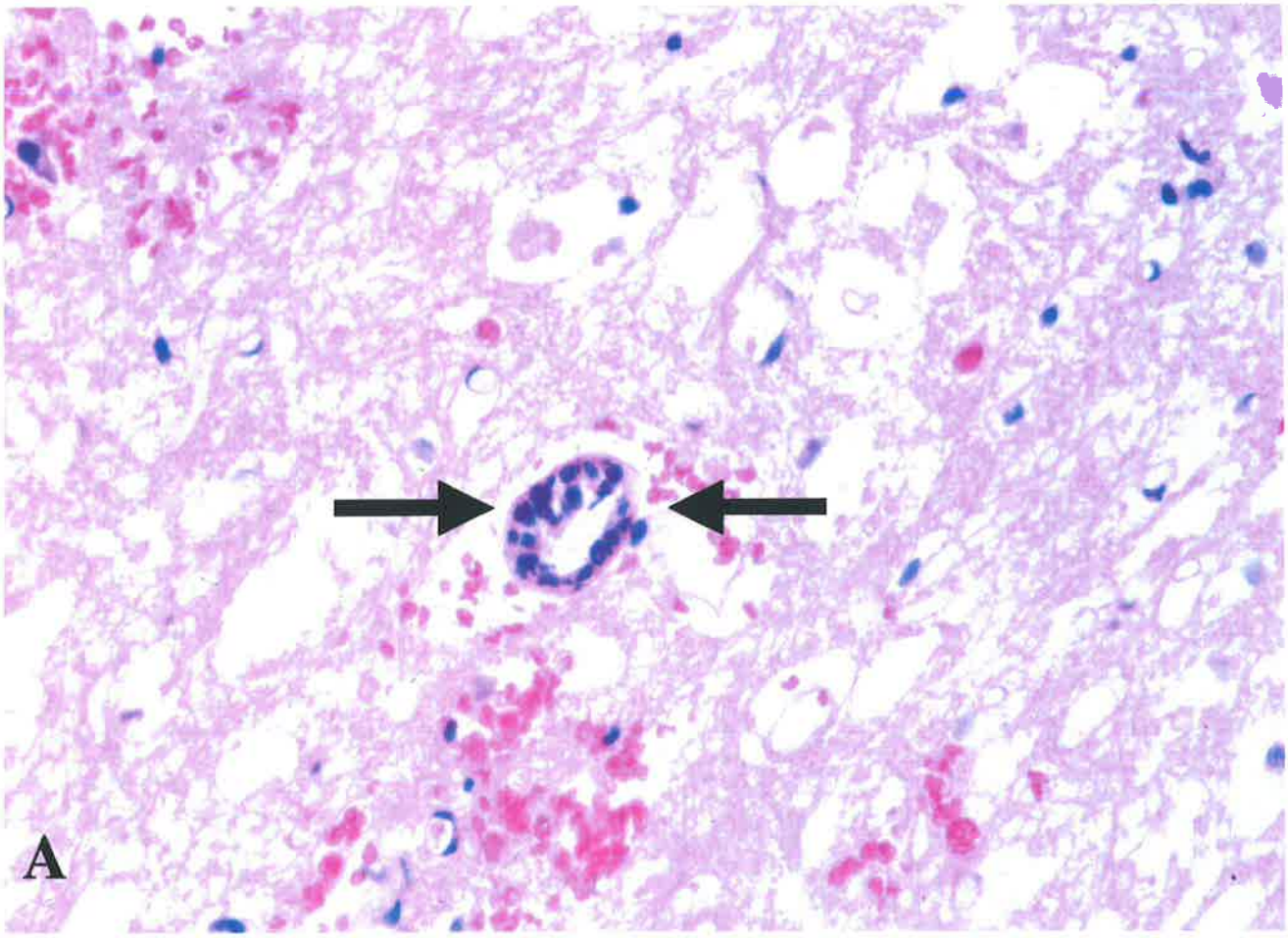

D

2.2.2 FIVE HOURS POST SCI

At 5 h postinjury, polymorphonuclear neutrophil margination was observed (Figure 25A). Very occasional mononuclear cells in the walls of blood vessels were observed. Axonal injury was shown by APP immunoreactivity in many neurons and axons (Figure 25B). Activated hypertrophied microglia were detected and some of their processes wrapped around swollen axons (Figure 25C).

Figure 25. *Histological changes in the lesion at 5 h after SCI in the human. (A) Neutrophil margination in the wall of a blood vessel (arrows). H&E, X100. (B) Expression of APP in many axons. APP, X100. (C) More activated microglia were found and some of them wrapped around swollen axons (arrows). CR3/43, X100.*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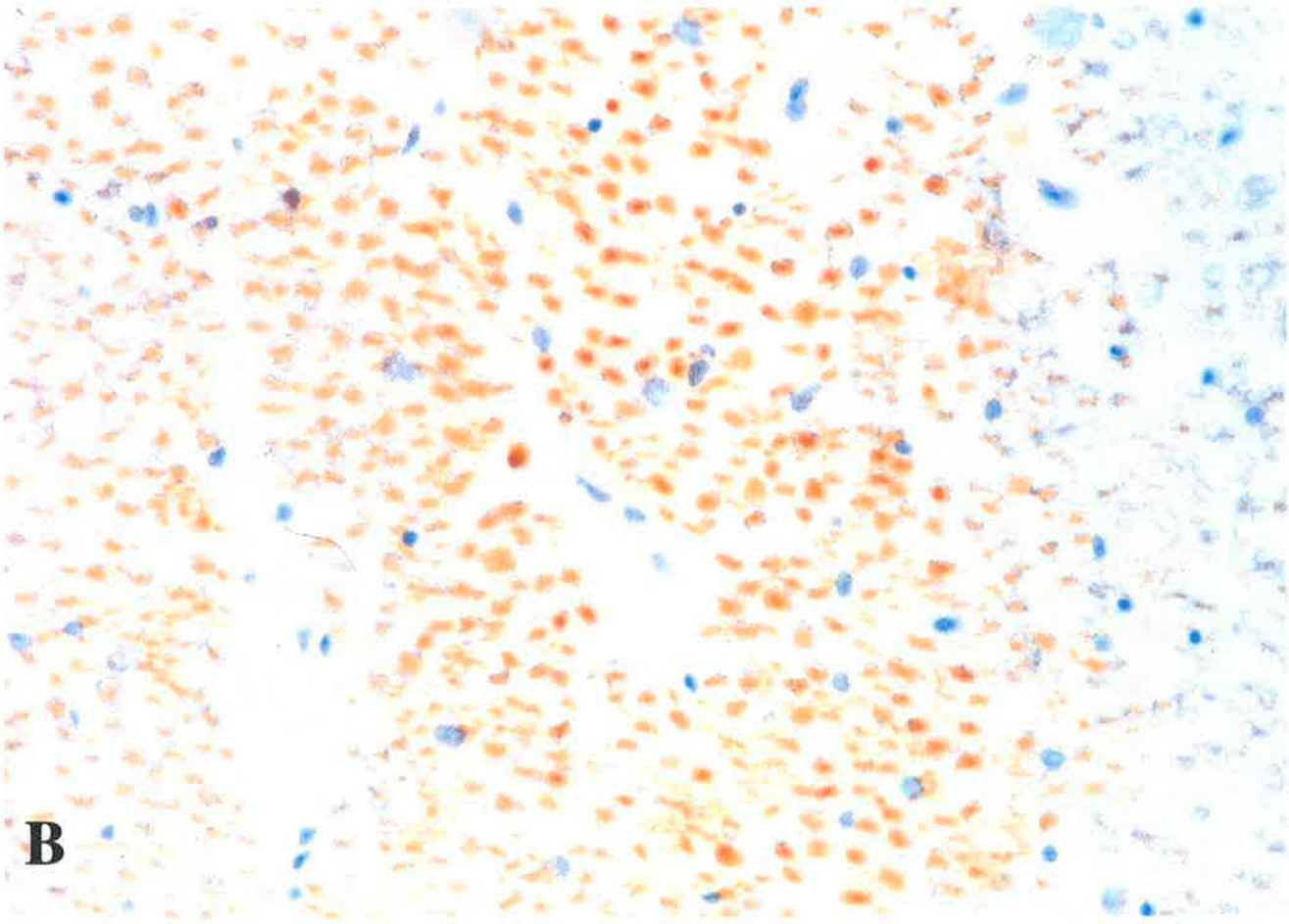
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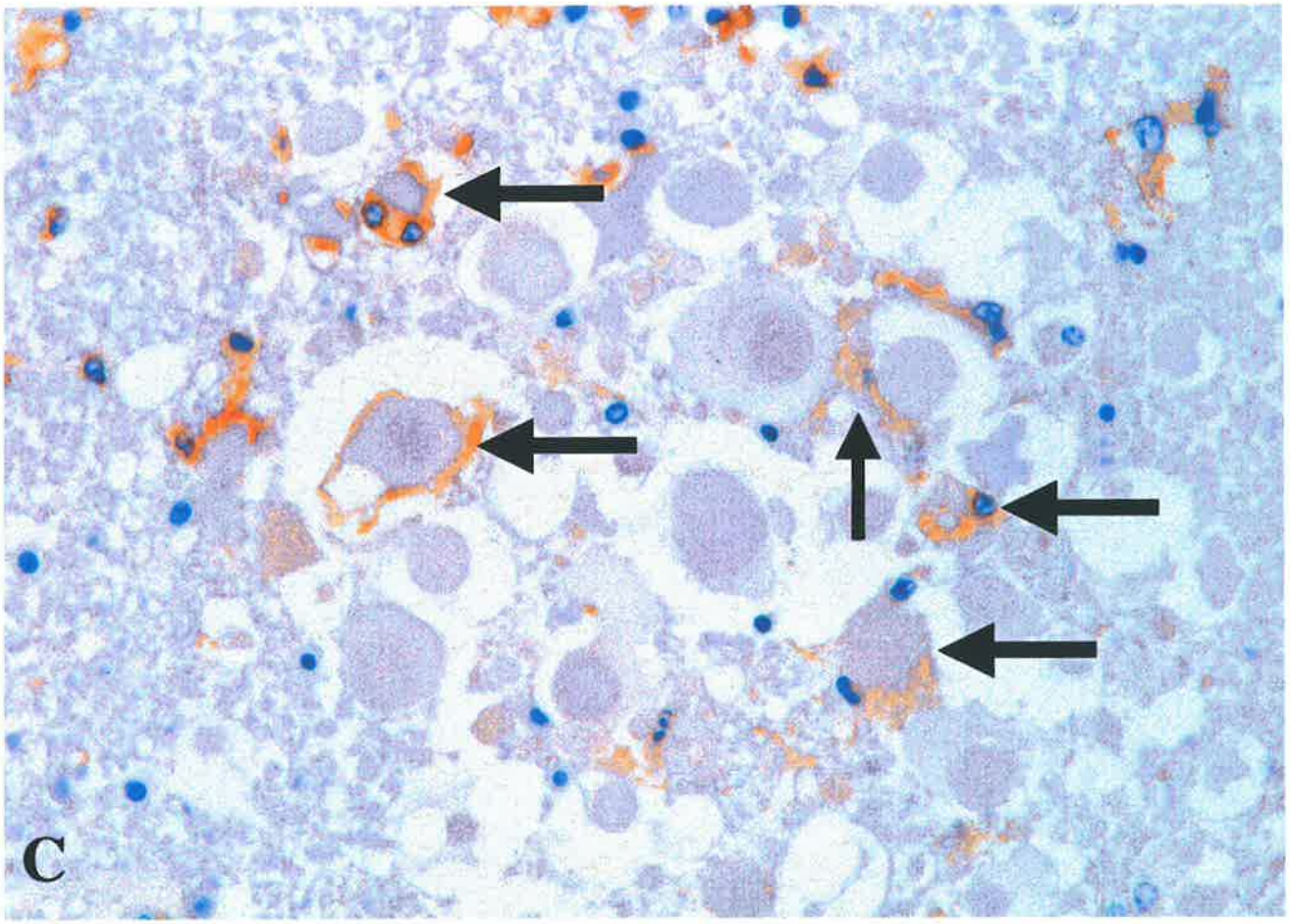
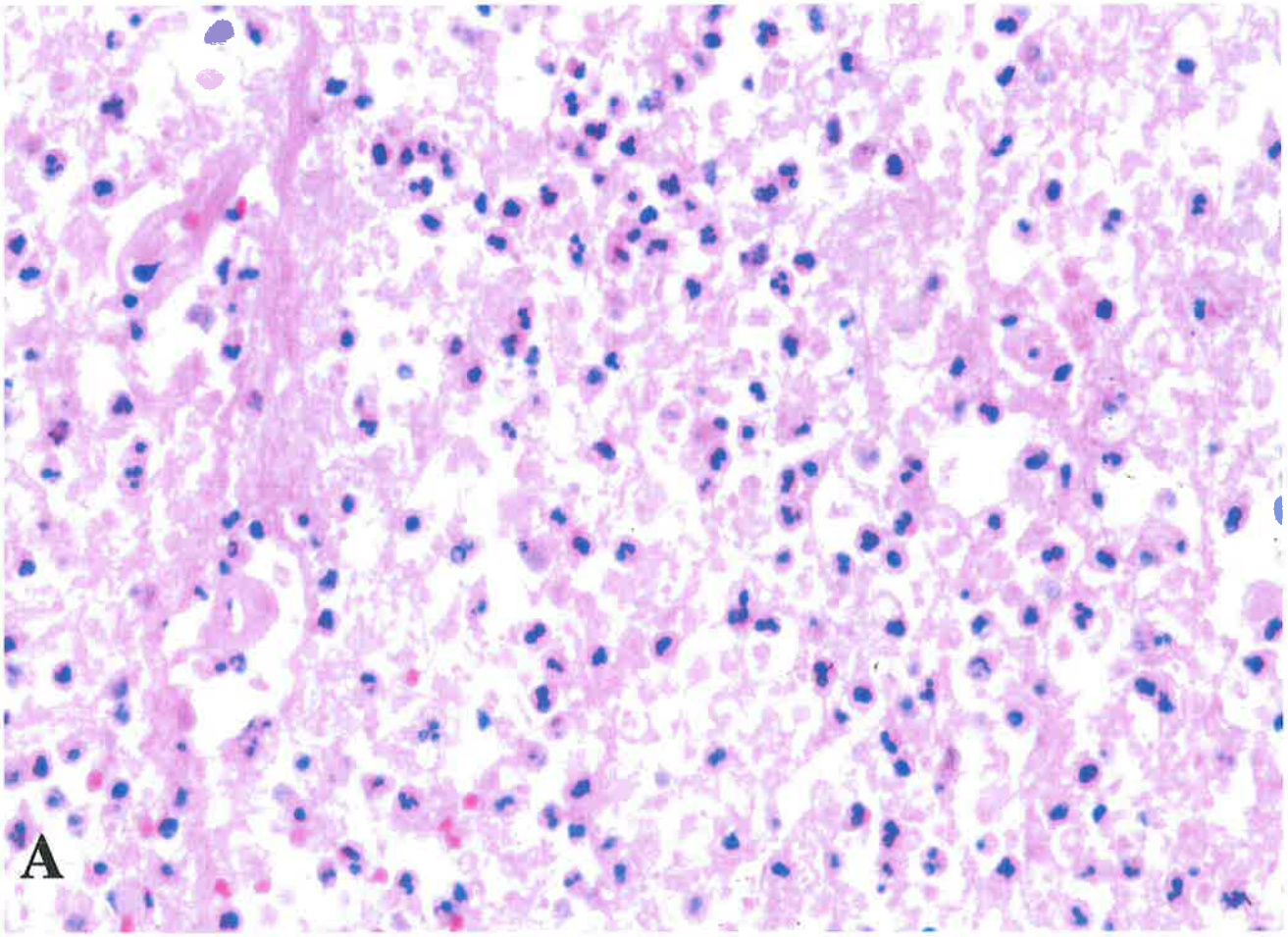

C

2.2.3 24 HOURS POST SCI

One day following SCI in the human, numerous polymorphonuclear neutrophils were widely spread throughout the damaged tissue (Figure 26A) and the number of neutrophils appeared to reach a peak at this time point. Many activated microglia were found wrapping around both axonal swellings and neuronal cell bodies (Figure 26B). Some CD68 positively-stained macrophages started to appear in the injured tissue (Figure 26C) and APP expression persisted in neurons and axons (not shown).

Figure 26. *Histological changes in the lesion at 1 d after SCI in the human. (A) Numerous polymorphonuclear neutrophils infiltrated the damaged spinal cord.. H&E, X100. (B) Neurons were surrounded by activated microglia (arrows). CR3/43, X100. (C) Some CD68 positively-stained macrophages started to appear in the lesion. CD68, X100.*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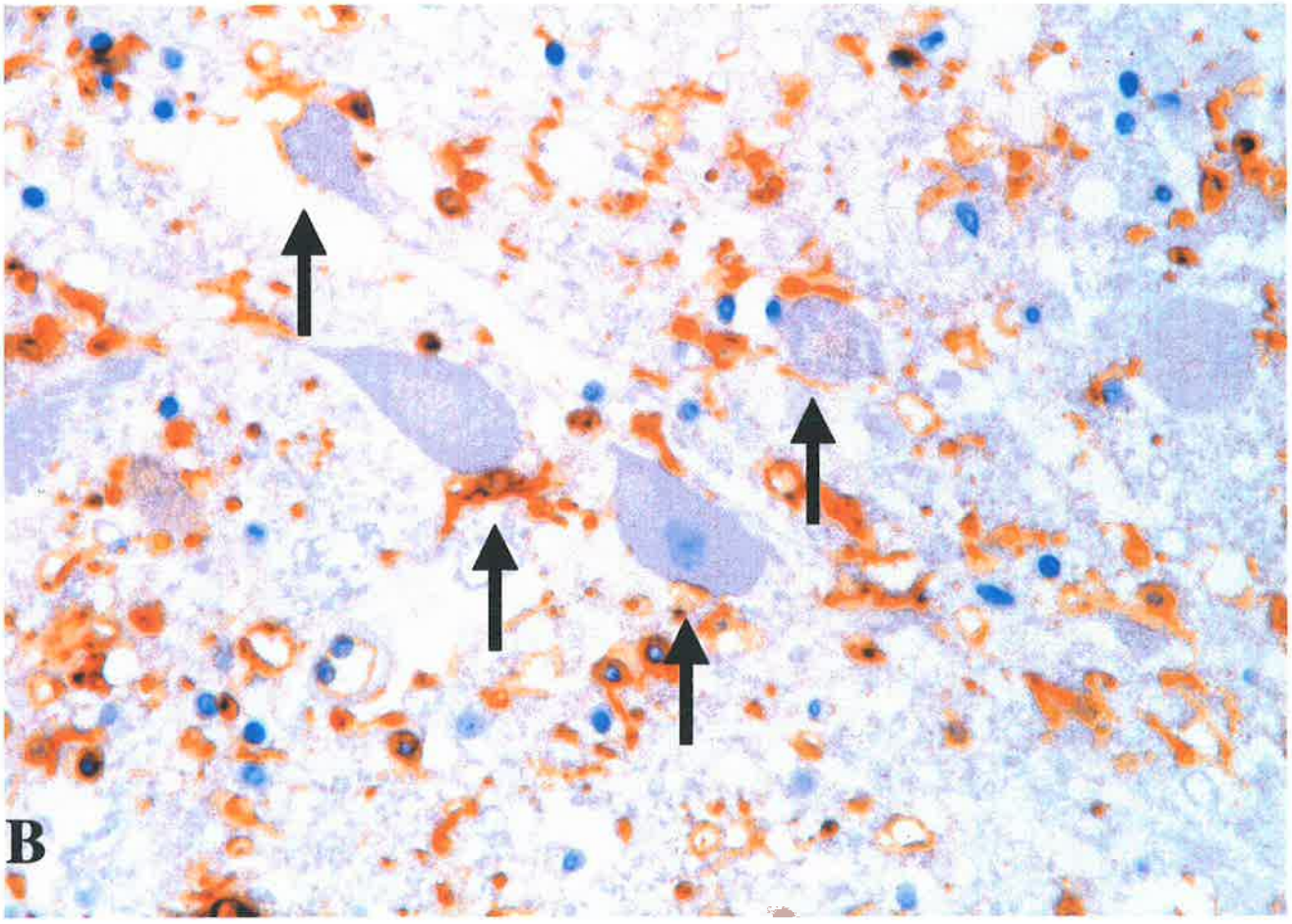
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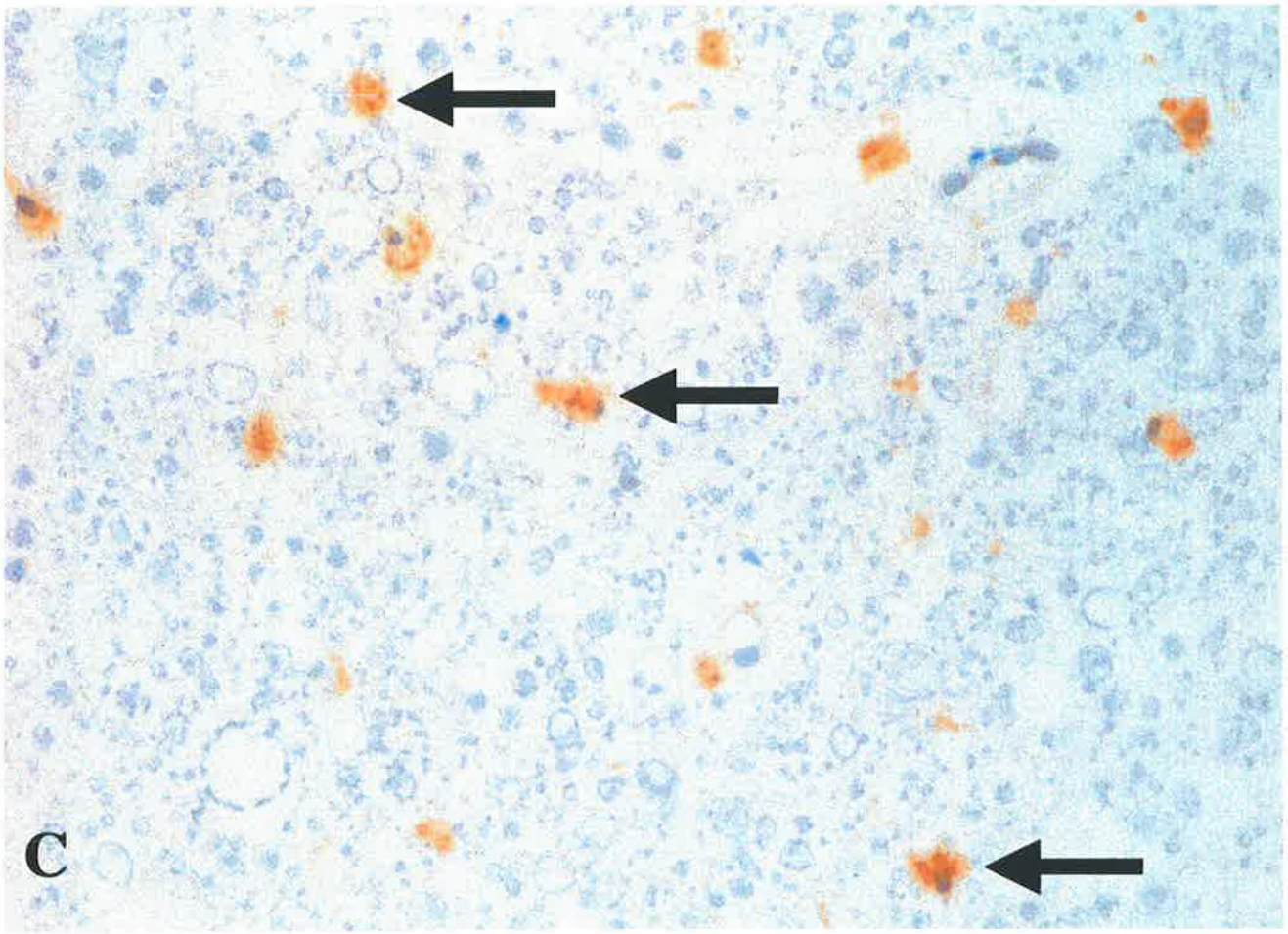

C

2.2.4 FROM TWO TO FIVE DAYS AFTER SCI

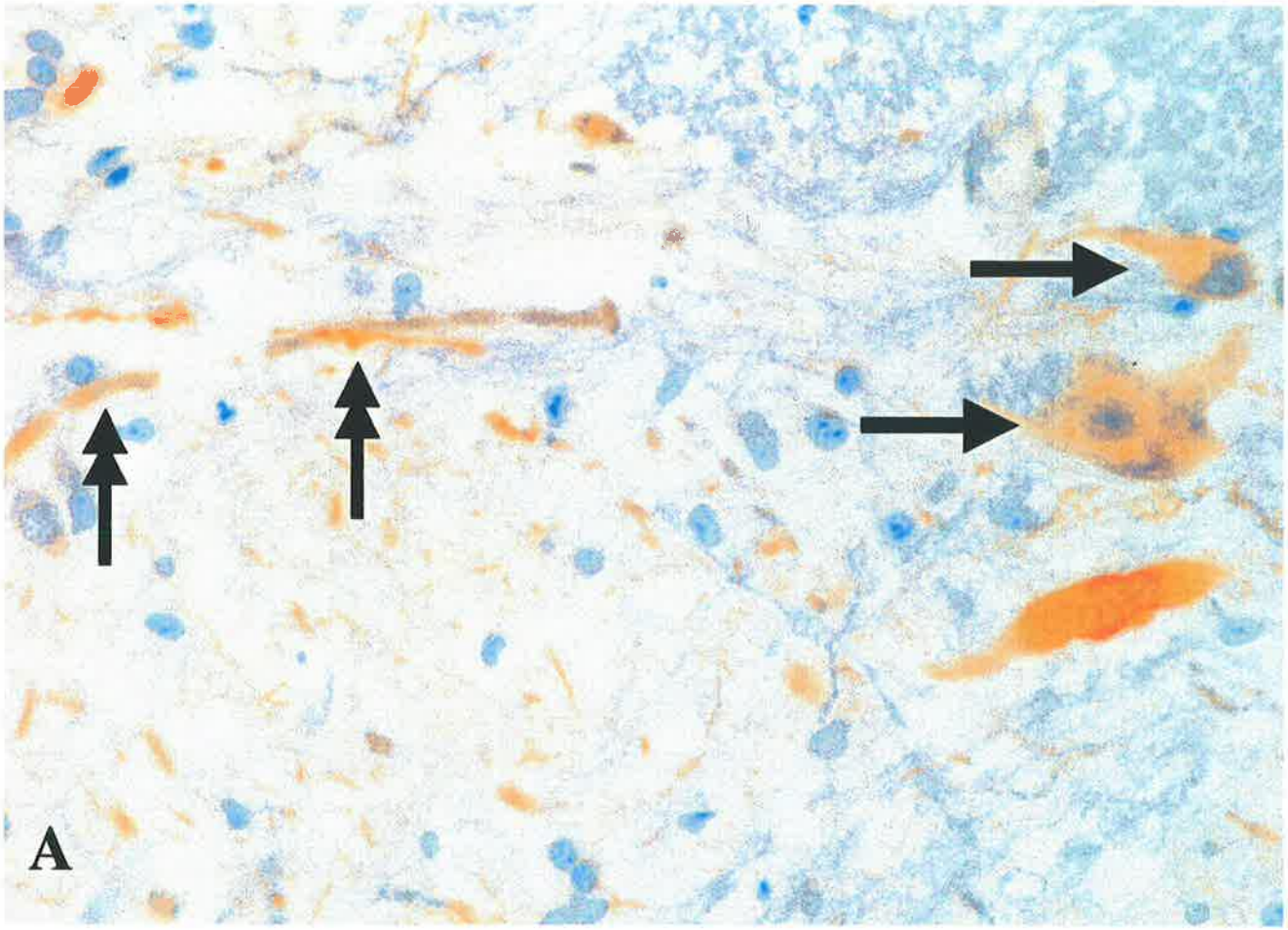
APP expression continued to be present in the neurons and axons (Figure 27A). From 2 d postinjury, the number of neutrophils dramatically declined while activated microglia (Figure 27B) and macrophages (Figure 27C) significantly increased their numbers and persisted in the spinal cord. Activated microglia were widely present throughout the damaged and surviving tissue (Figure 27B), but macrophages were found predominantly in the necrotic areas (Figure 27C).

2.2.5 CONTROL STUDIES

There was no expression of APP in the spinal cord of two control cases with head injury only. Neither macrophages nor activated microglia were detected in the controls.

No positive staining was observed in the negative immunostaining control studies, which were performed by omitting the primary antibody step.

Figure 27. *Histological changes in the lesion from 2 to 5 days after SCI in the human. (A) APP expression in the neurons (arrows) and axons (double-headed arrows). APP, X100. (B) Activated microglia mainly in the surviving tissue (arrows). CR3/43, X100. (C) Numerous foamy macorphages were largely present in the necrotic area (arrows). CD68, X100.*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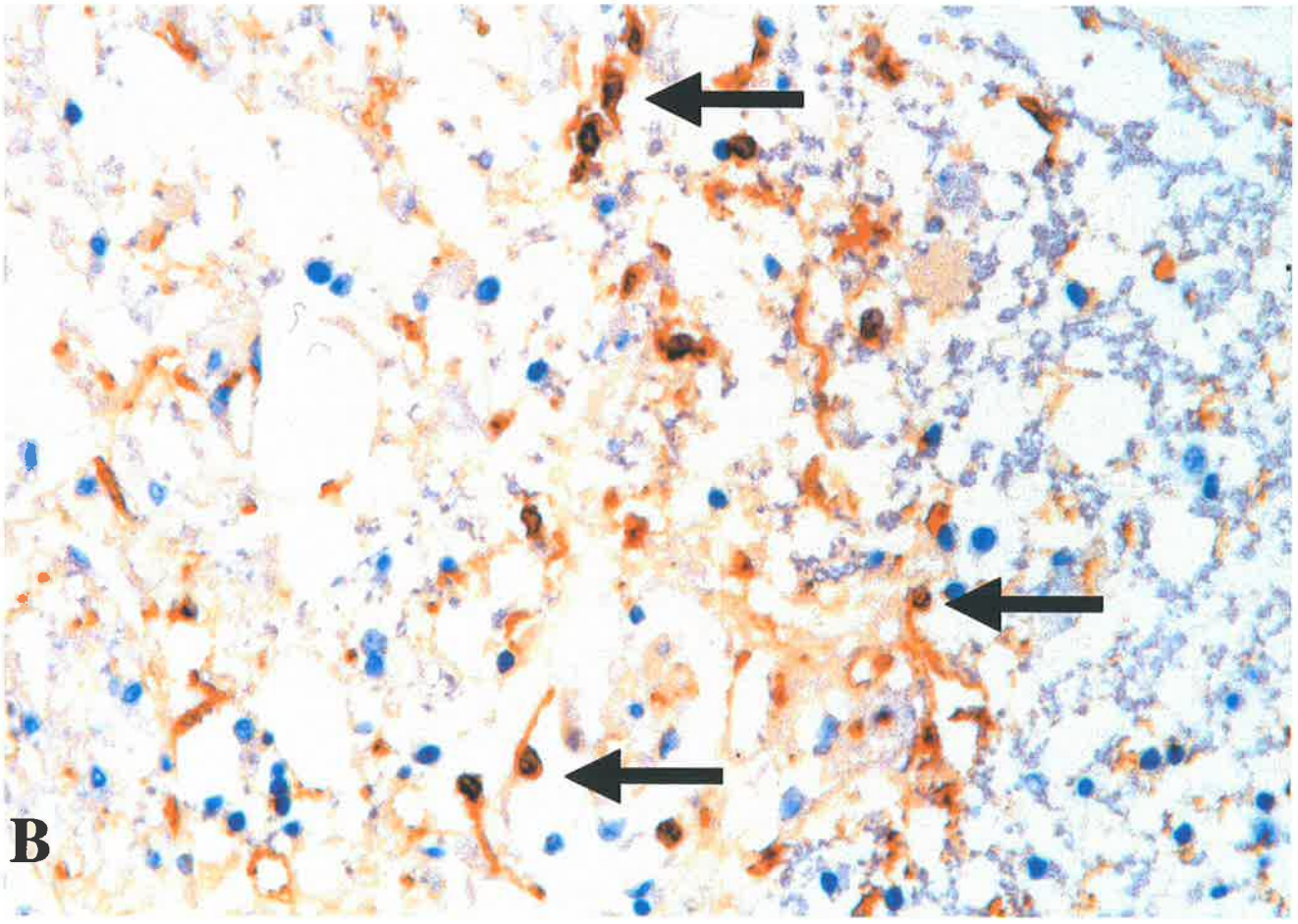
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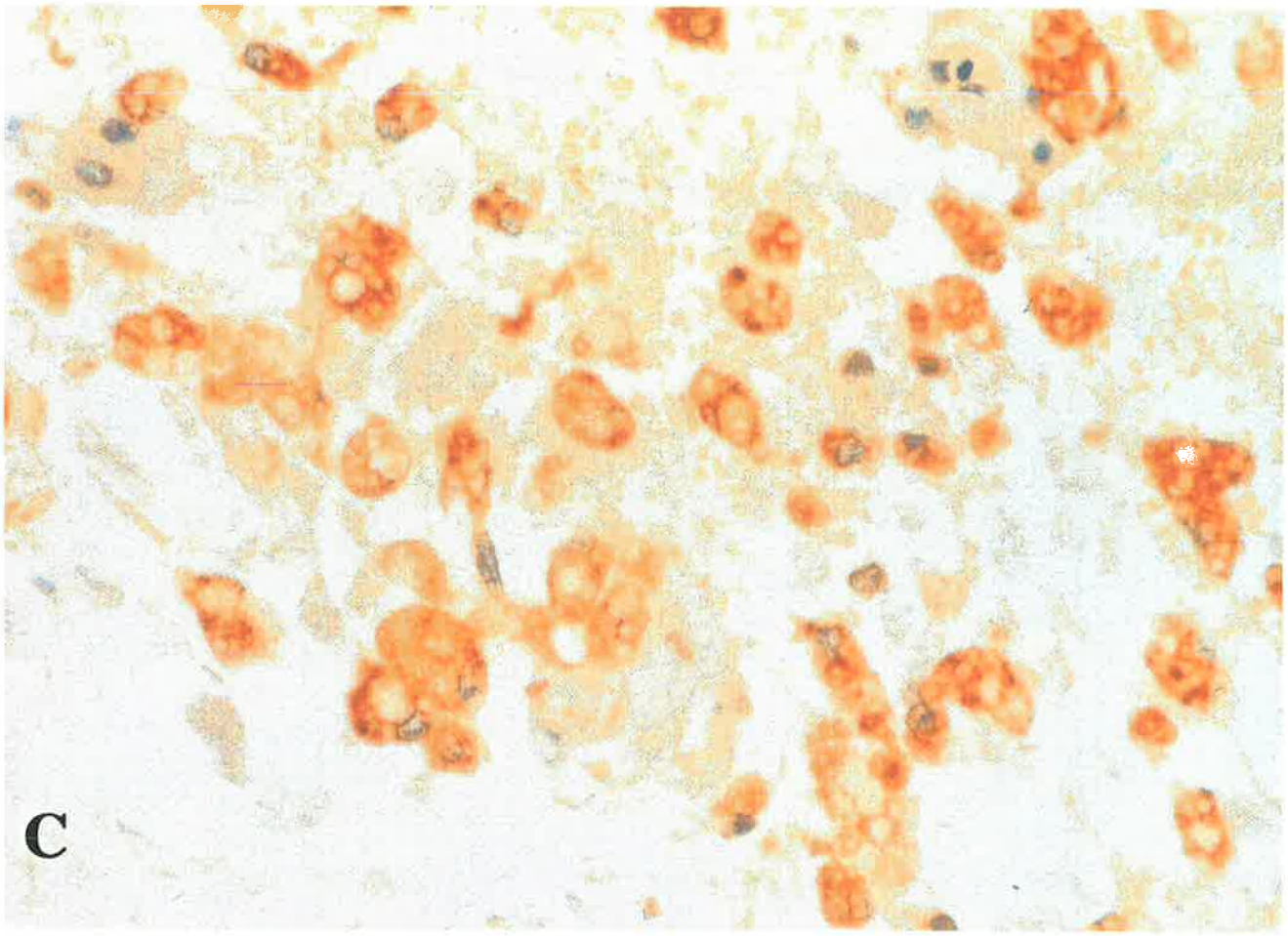

C

2.2.6 Summary of posttraumatic inflammatory cellular response

The inflammatory cellular response after SCI in the human is summarised in Table 5 below.

Table 8. *Inflammatory cellular response after SCI.*

Postinjury	Histopathology
0.5 hr	Haemorrhage and necrosis; No leukocyte infiltration; Activated microglia, some of which surrounded axons; APP expression in neurons & axons.
5 hr	Neutrophil margination. More activated microglia, some of which surrounded axons. APP expression in neurons and axons.
1 d	Peak of neutrophil number; CD68+ macrophages started to appear; Activated microglia, some of which surrounded neurons and axons; APP expression in neurons and axons.
2-5 d	Few neutrophils in the damaged tissue; Numerous macrophages predominantly in necrotic areas; Activated microglia mainly in surviving areas; APP expression in neurons and axons.

2.3 DISCUSSION

2.3.1 MARKERS FOR ACTIVATED HUMAN MICROGLIA

The MHC class II specific monoclonal antibody CR3/43 was used in this study to investigate activated microglia in routinely processed, paraffin-embedded human spinal cord. CR3/43 is directed against the β-chain of all products of the MHC class II gene subregions HLA-DR, HLA-DQ and HLA-DP [144].

CR3/43 labelling characteristics were systematically evaluated by Graeber and colleagues [144], using a panel of 50 autopsies and surgical cases. CR3/43 labelling was compared to the macrophage/microglia markers L-CA, KP1 and RCA-1. It has been found that resting microglia of histologically normal tissue were not stained by CR3/43 and CR3/43 was the most sensitive antibody to detect activated microglia in subtle tissue pathology. More importantly, astrocytes were never found to be labelled by CR3/43.

In summary, CR3/43 is an excellent marker for activated human microglia and it is specific for microglia in the sense that it does not label other glia. Visualization of microglial activation in this study was greatly aided by using this antibody.

2.3.2 APP UPREGULATION

This study demonstrated the presence of widespread APP immunoreactivity in the neurons and axons in all 11 human cases following SCI. APP positive staining was observed as early as half an hour after injury and persisted for at least 5 days. These observations parallel those observed in the rat SCI (described in Table 5, page 108). APP immunoreactivity occurs not only as a result of primary mechanical injury but also as a result of secondary complicating factors such as hypoxia, ischaemia and infarction [2,39,333], which are frequently found following

injury. Thus, APP is upregulated as an acute phase cell-stress response to injury. However, exactly where APP fits into the complex inflammatory cellular responses following SCI is unclear and requires further study.

2.3.3 INFLAMMATORY CELLULAR RESPONSE

The inflammatory cellular response following SCI in the human parallels the changes observed in rat SCI (described in Table 5, page 108). Like the response in rat SCI, there are mainly three cell types involved in the inflammatory response following SCI in the human: microglia, neutrophils and macrophages. The time course of inflammatory cellular response in human SCI is also very similar to that observed in rat SCI (described in Table 5, page 108).

In contrast to some in vivo studies that failed to detect morphological signs of microglial activation by 1 d postinjury [261,341], activated microglia in this study were detected as early as 0.5 h after injury and found surrounding axonal swellings and otherwise "normal" axons. This novel finding is in agreement with that seen in rat SCI in this study (described on page 110) and has further challenged the current view that there are no direct topographical association between microglial cells and axons, and no association of axonal injury with activated microglia [127,261,278]. More studies are needed to elucidate the effect of activated microglia surrounding axons because activated microglia are able to produce pro-inflammatory cytokines (observed in this study), cytotoxic substances [25,387] and growth factors [341]. Activated microglia may provide a trophic local environment to promote the axonal regeneration because some studies have shown that activated microglia promoted the regeneration of sensory axons in the injured rat spinal cord [248,280]. It has been also found in this study that activated microglia surrounded neurons. This is consistent with the finding in rat SCI (described on page 111) and is referred to as synaptic stripping. Microglia are thought to be

involved in limited phagocytosis of individual synaptic termini and to have an impact on the synaptic reorganization of the neurons, but the effect of synaptic stripping on neurons is, as yet, largely unknown.

Haematogenous inflammatory cells (neutrophils and macrophages) did not start to infiltrate the injured spinal cord until 6 h postinjury, suggesting that these peripheral inflammatory cells were not spilled from damaged vessels, but recruited by some chemoattractants including cytokines (e.g. IL-1β, IL-6 and TNFα) [88,348].

Neutrophils are the first peripheral inflammatory cells to migrate into the tissue. It has been suggested that infiltrating neutrophils may participate in tissue destruction and enlargement of the lesion because they can produce reactive oxygen species and other substances with potentially tissue-damaging effects [65,347,358]. Taoka and colleagues [347] recently found that inhibition of neutrophil activation alleviated the motor disturbances observed in a rat model of SCI. However, other studies reported that neutropenia had no effect on the extent of tissue damage following CNS injury [20,118,367]. The observation in this study that necrosis precedes the arrival of neutrophils at the injury site indicates that the tissue necrosis may not be caused by the invading leukocytes, but that it may, instead, provide a chemotactic stimulus for attracting neutrophils as the first line of recruited phagocytic cells to begin removal of cellular debris.

The second wave of peripheral inflammatory cells to enter damaged tissue in the spinal cord is monocyte-derived macrophages. Macrophages in the damaged spinal cord arise from two different sources; from the non-resident population of circulating blood monocytes recruited into injured cord and transformed into macrophages, and from the resident population of

ramified microglia in spinal cord activated and transformed into macrophages [25,85,200,216,338]. The quantitative contributions of endogenous microglia-derived and monocyte-derived macrophages to the total macrophage pool are still not clear [25].

Regardless of their origin, various reports have shown that macrophages can play both negative and positive roles in responding to injury. Macrophages (including microglia and monocytes) can be induced to produce several potentially cytotoxic substances in vitro, such as free oxygen intermediates, NO, proteases, EAAs and pro-inflammatory cytokines [13,200]. Thus, these cells may cause "bystander" damage to intact tissue and contribute to the secondary tissue destruction after TSCI. In support of this view, Blight [34] and Popovich [274] have observed a better anatomical and functional outcome in animals with CNS injuries when macrophage numbers are reduced experimentally. In addition, several studies have shown the damaging effects of pro-inflammatory cytokines produced by macrophages [17,298]. However, another view holds that macrophages play a key role in the removal of cellular debris which are essential for tissue remodelling, and produce the trophic cytokines, such as TGF-β1 and IL-4, to promote tissue repair and neuronal survival [14,78,271]. In support, several studies transplanting macrophages/microglia into an injured CNS showed increases in axonal regeneration [121,280]. However, this interpretation is flawed by a lack of quantitative, comparative cell counts conducted in the same animal. Therefore, the role of macrophages (including microglia and monocytes) is far from clear in studies of TSCI and their function is likely to be dependent on the time point following injury and their location relative to the lesion.

3. EARLY EXPRESSION AND CELLULAR LOCALIZATION IL-1β, IL-6 and TNFα

This experiment was performed to investigate the expression of IL-1β, IL-6 and TNFα in the human spinal cord after SCI.

3.1 METHODS

3.1.1 IMMUNOHISTOCHEMISTRY

For staining of IL-1β, IL-6 and TNF-α, a rabbit anti-IL-1β polyclonal antibody (1:200, Rockland, PA), a rabbit anti-IL-6 polyclonal antibody (1:25, HyCult Biotechnology, Uden, Holland) and a mouse anti-TNF-α monoclonal antibody (1:75, HyCult Biotechnology, Uden, Holland) were used to identify these pro-inflammatory cytokines. We also checked the antigen-specificity of the IL-1β, IL-6 and TNF-α antibodies by preabsorbing these primary antibodies with exogenous recombinant human IL-1β, IL-6 and TNF-α (Pepro Tech, NJ).

3.1.2 DOUBLE-LABEL FLUORESCENT CONFOCAL IMAGING

The cellular sources of IL-1β, IL-6 and TNF-α were elucidated using immunofluorescence double labelling confocal imaging. The sections were incubated overnight with combinations of anti-IL-1β, anti-IL-6 or anti-TNF-α antibody (as described before), and activated microglia-specific markers CR3/43 (as described on page 154) or anti-glucose transporter-5 (Glut-5) antibody (1:500), a gift from Dr. F. Maher, University of Melbourne) [270]. On the following day, the sections were incubated with a mixture of fluorescein-conjugated donkey anti-rabbit (FITC; 1:100; Jackson Immunoresearch Lab, West Grove, PA) and Cy™5-conjugated donkey anti-mouse (Cy5; 1:400; Jackson Immunoresearch Lab, West Grove, PA)

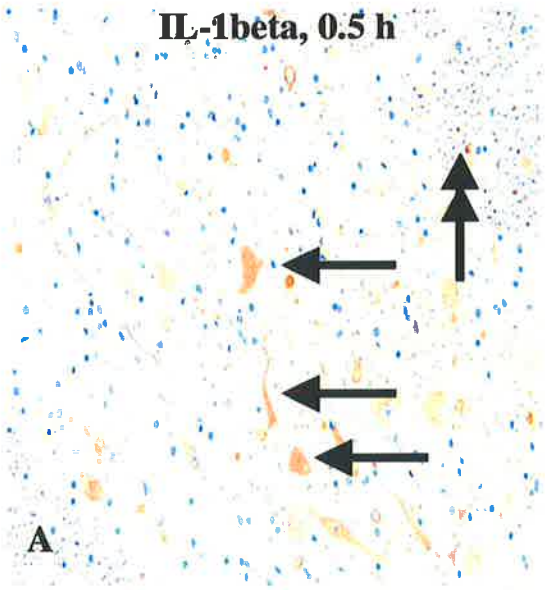
antibodies. Sections were then mounted and examined under a fluorescence microscope (Biorad, LaserSharp 2100).

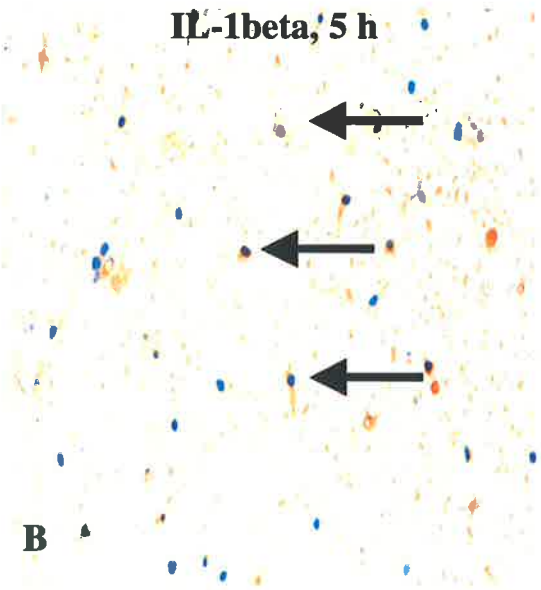
3.2 RESULTS

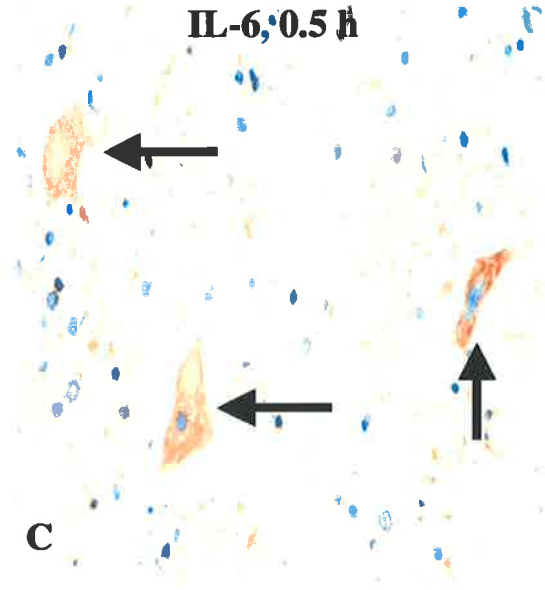
The staining intensity of IL-1β, IL-6 and TNF-α markedly increased at 0.5 and 5 h after traumatic SCI and quickly declined at 1 d postinjury (Figure 28). IL-1β, IL-6 or TNF-α positive cells were found up to 2 cord segments away from the lesion centre. There was no difference in the immunostaining pattern of IL-1β, IL-6 and TNF-α between the rostral and caudal parts of the lesion.

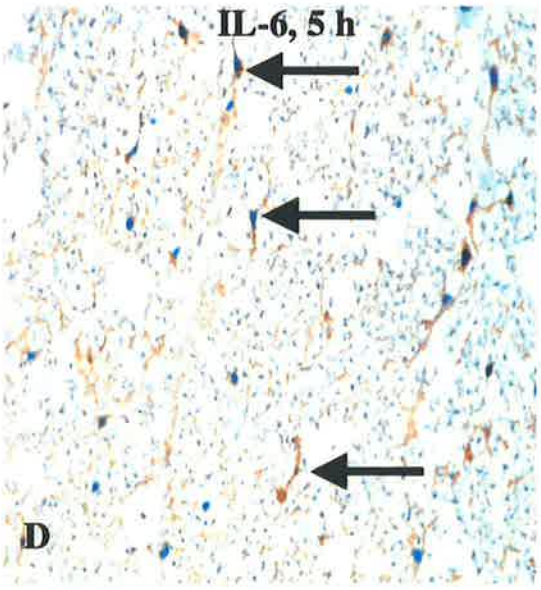
Half an hour after injury, the expression of IL-1β was found in some neurons and axons (Figure 28A), and IL-6 and TNF-α were mainly localized in the neurons (Figures 28D and 28G). At 5 h after injury, IL-1β, IL-6 and TNF-α were detected not only in many neurons but also in some microglial-like cells (Figures 28B, 28E and 28H). At 1 d after injury, very weak staining of IL-1β, IL-6 and TNF-α was seen in the neurons (Figures 28C, 28F and 28I) while no expression was identified in the glial cells. From 2 d postinjury, no staining of IL-1β, IL-6 and TNF-α was observed.

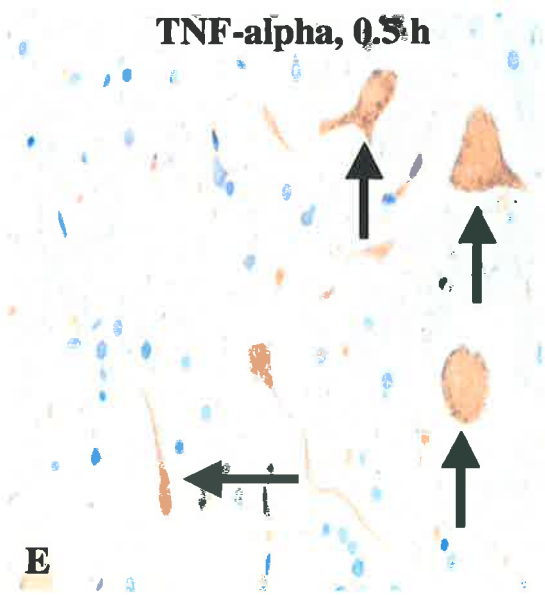
Figure 28. *Pro-inflammatory cytokine expression in the lesion after traumatic human SCI. At 0.5 h after spinal cord trauma, IL-1β expression (A) was found in neurons (arrows) and axons (double-headed arrow) while IL-6 (D) and TNF-α (G) was mainly localized in neurons. At 5 h after SCI, IL-1β (B), IL-6 (E) and TNF-α (H) was identified not only in neurons but also in some glial cells with microglial morphological appearance (arrows). At 1 d postinjury, the expression of IL-1β (C), IL-6 (F) and TNF-α (I) dramatically declined and weak staining was shown in neurons only (arrows). (A-C): IL-1β, X100; (D-F): IL-6, X100; (G-I): TNF-α, X100.*

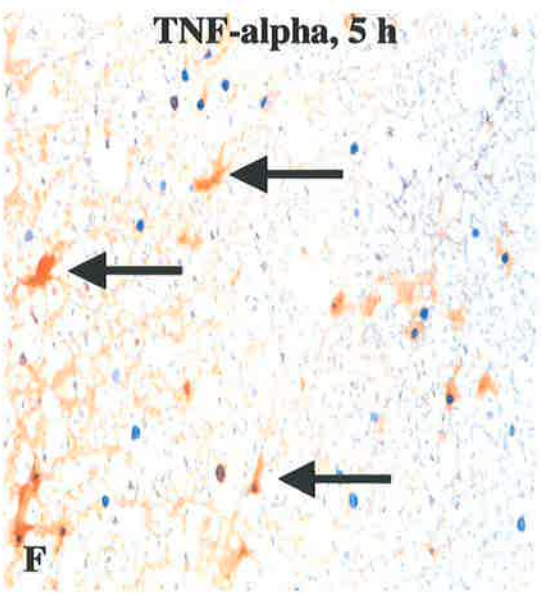

IL-1beta, 0.5 h
A


IL-1beta, 5 h
B


IL-6, 0.5 h
C


IL-6, 5 h
D


TNF-alpha, 0.5 h
E

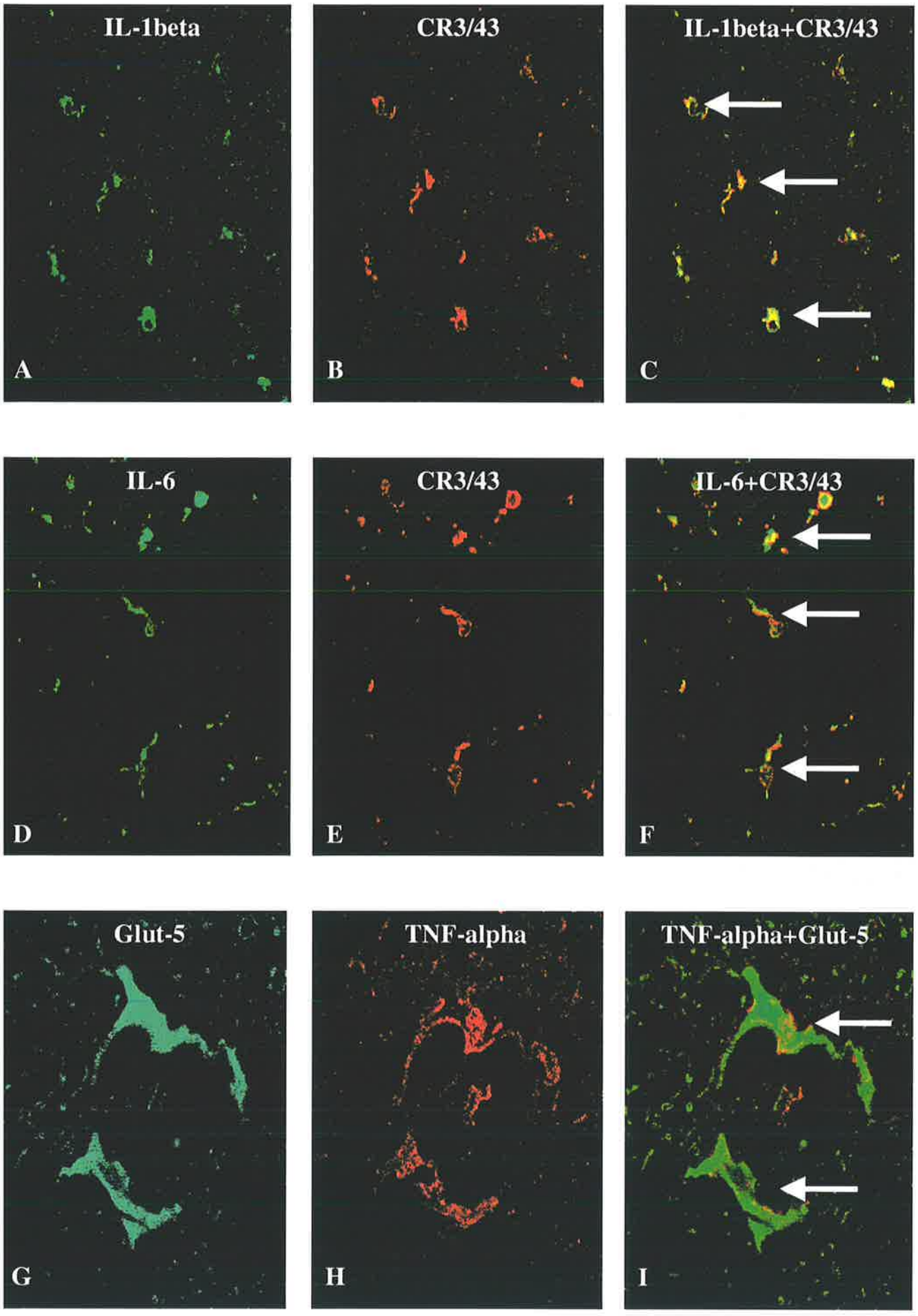

TNF-alpha, 5 h
F

Using double-label fluorescent immunohistochemistry, co-localization of IL-1β, IL-6 or TNF-α and microglial markers CR3/43 or glut-5 was found, confirming that IL-1β, IL-6 or TNF-α positive glial cells observed at 5 h postinjury were microglia (Figure 29).

In antigen-absorption experiments, the immunoreactivity of IL-1β, IL-6 and TNF-α was absent, showing the specificity of these antibodies in this study.

There was no expression of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) in the two normal control cases.

Figure 29. *Co-localization of IL-1β, IL-6 or TNF-α and microglial marker CR3/43 or glut-5 in the lesion at 5 h postinjury. Cells stained with anti-IL-1β, anti-IL-6 or anti-Glut-5 only (green) were shown in (A), (D) and (G) while cells stained with anti-CR/43 or anti-TNF-α only (red) in (B), (E) and (H). Double-label immunofluorescent immunohistochemistry confirmed that IL-1β, IL-6 or TNF-α was co-localized in microglia (yellow) in (C), (F) and (I). Arrows indicated cells that had the co-localized expression of the microglial markers (CR3/43 or Glut-5) and IL-1β, IL-6 or TNF-α. (A-F) (X600) and (G-I) (X1500)*


IL-1beta
A
CR3/43
B
IL-1beta+CR3/43
C
IL-6
D
CR3/43
E
IL-6+CR3/43
F
Glut-5
G
TNF-alpha
H
TNF-alpha+Glut-5
I

3.3 DISCUSSION

Our results have shown a rapid elevation of IL-1β, IL-6 and TNF-α expressions at 0.5 and 5 h after injury, and then a dramatic decline of these expressions prior to the appearance of neutrophils and macrophages at 1 d postinjury (described in Table 8 on page 171), suggesting that these pro-inflammatory cytokines are primarily produced by endogenous CNS cells rather than infiltrating blood-borne leucocytes in human SCI. These findings in human SCI have confirmed results observed in our weight-drop rat model of SCI and in other animal models of SCI [19,164,193] that neutrophils and macrophages are not the cellular sources of IL-1β, IL-6 and TNF-α.

Further, the present study demonstrates the production of IL-1β, IL-6 and TNF-α in neurons at 0.5 h after injury and in both neurons and microglia at 5 h after injury. To our knowledge, this study is the first to demonstrate the cellular sources of pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, in the injured human spinal cord. This novel finding is consistent with recent in vivo studies in rat models of SCI that identified TNF-α in neurons and microglia [194,396] and in vitro studies that showed the production of a variety of cytokines in neurons and microglia [134,210]. These data have confirmed that neurons and microglia (especially neurons), not blood-born leucocytes proposed by many authors [135,347,354], may play a major role in the initiation and regulation of early posttraumatic inflammatory response of the human SCI by producing IL-1β, IL-6 and TNF-α.

Thus, for the first time, human SCI has been shown in this study to induce the expression of the proinflammatory cytokines IL-1β, IL-6 and TNF-α in neurons within as little as 0.5 h following injury. These cytokines, a group of proteins that evoke remarkable responses in a variety of cell types, initiate a complex cascade leading to inflammatory responses, including

increased vascular permeability, leukocyte infiltration and induction of acute-phase response [19,164,208,341,379,380].

Inflammation may contribute to secondary tissue damage after SCI, for example by promoting tissue oedema and ischaemia. The proinflamatory cytokines induce several molecules capable of increasing vascular permeability as well as inducing cellular fluid loss [267]. These molecules include components of the complement cascade (C3a and C5a, which in turn may cause release of histamine, prostaglandins and leukotrienes from resident mast cells), proteases such as plasminogen activator, and bradykinin [267]. It has been shown that direct injection of IL-1β into spinal cord enhanced vascular permeability [310] and increased expression of pro-inflammatory cytokines in transgenic mice exacerbated neurological functional impairment after SCI [187]. Additionally, systemic administration of the anti-inflammatory cytokine IL-10 has been reported to limit neuronal damage and promote functional recovery following SCI in rats [28,273]. These results suggest that inflammation-promoted cellular damage exacerbates lesion volumes and SCI functional outcomes.

On the other hand, inflammatory responses have been found to promote beneficial response after SCI. It has been demonstrated that tissue loss after SCI was reduced by direct application of pro-inflammatory cytokines [192]. Furthermore, IL-1β or TNF-α receptor-knockout mice demonstrated reduced remyelination and diminished functional recovery [191,233].

Taken together, the role of pro-inflammatory cytokines after SCI is still controversial and it may be dependent on the balance of the injurious and beneficial effects of these cytokines.

4. CO-LOZALIZATION OF IL-1β AND APP

Because IL-1β immunoreactivity was localized in some neurons and axons (Figure 28A), we used immunofluorescence double labelling confocal imaging to examine whether IL-1β co-existed with amyloid precursor protein (APP) in the neurons and axons.

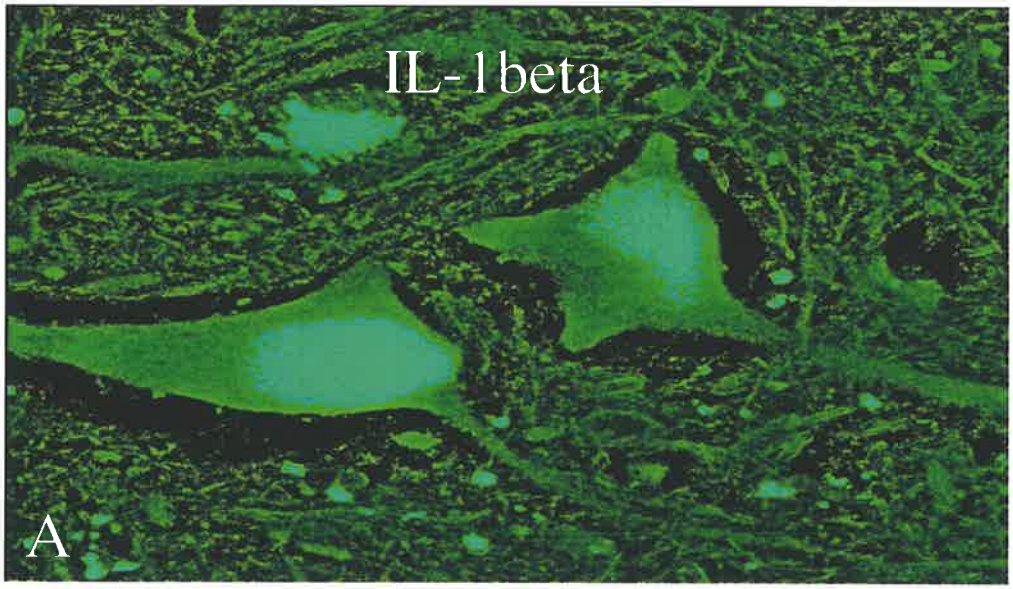
4.1 METHODS

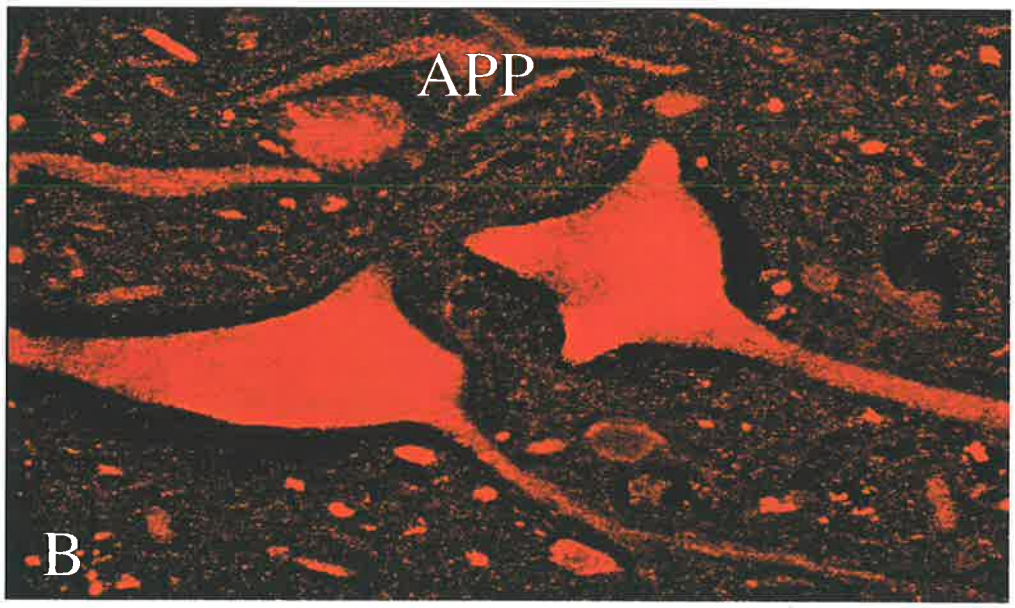
Co-existence of IL-1β with APP was demonstrated using immunofluorescence double labelling confocal imaging. The sections were incubated overnight with a combination of anti-IL-1β (as described before) and anti-APP antibodies (as described before). On the following day, the sections were incubated with a mixture of fluorescein-conjugated donkey anti-rabbit (FITC; 1:100; Jackson Immunoresearch Lab, West Grove, PA) and Cy™5-conjugated donkey anti-mouse (Cy5; 1:400; Jackson Immunoresearch Lab, West Grove, PA) antibodies. Sections were then mounted and examined under a fluorescence microscope (Biorad, LaserSharp 2100).

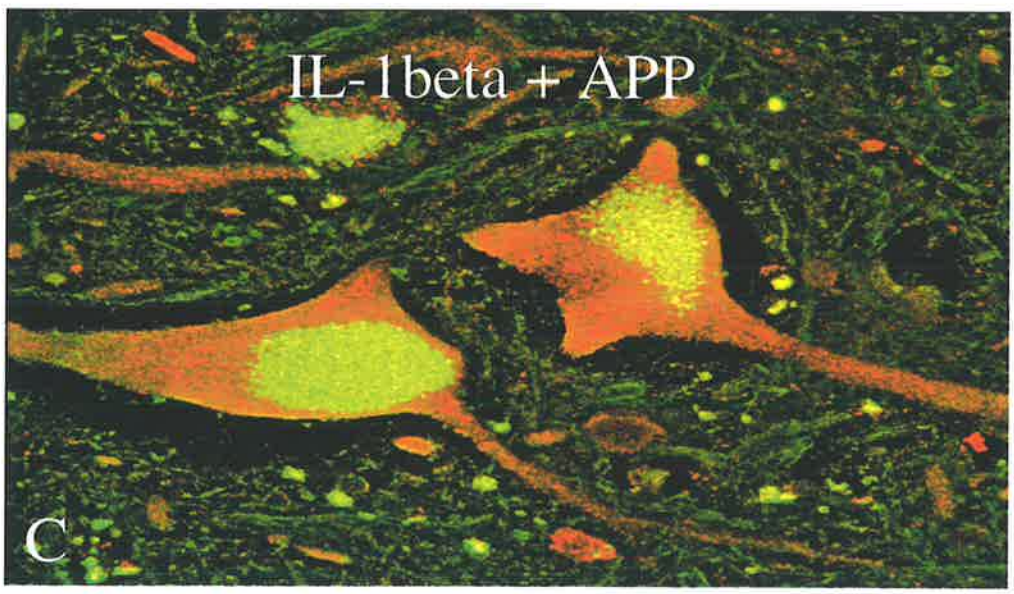
4.2 RESULTS

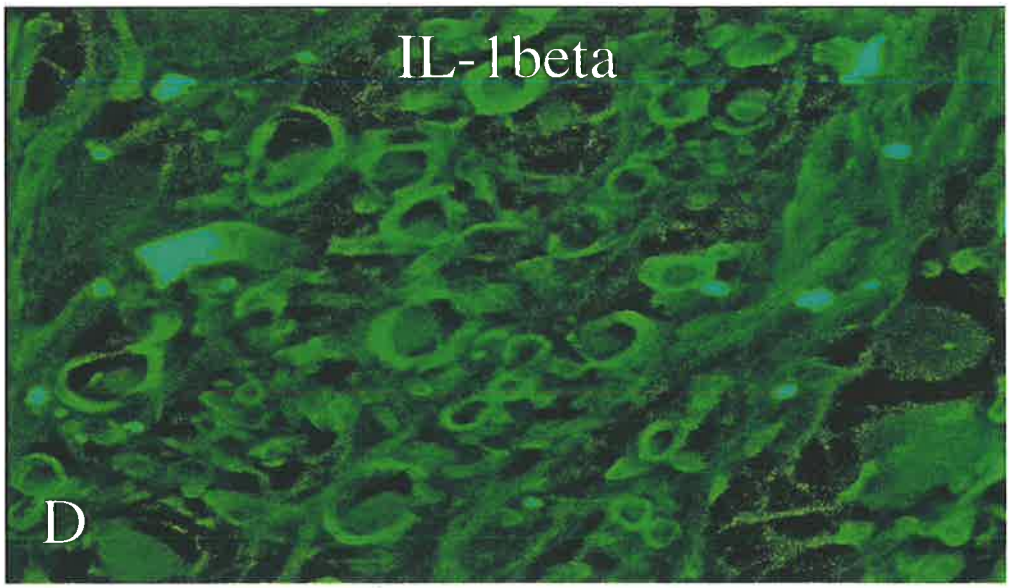
In the immunofluorescence experiments, IL-1β and APP were found co-localizing in the neurons and axons at 0.5 h and 5 h postinjury (Figure 30).

Figure 30. *Co-localization of IL-1β (green) and APP (red) in neurons and axons at 0.5 h postinjury. Neurons and axons stained with anti-IL-1β only were shown in (A) and (D) while neurons and axons stained with anti-APP only in (B) and (E). Double-label immunofluorescent immunohistochemistry revealed that neurons and axons expressed both IL-1β and APP in (C) and (F) (yellow). Arrows indicated that IL-1β positive neurons and axons were also stained positively with anti-APP. (A-F) (X600)*


IL-1beta
A


APP
B


IL-1beta + APP
C


IL-1beta
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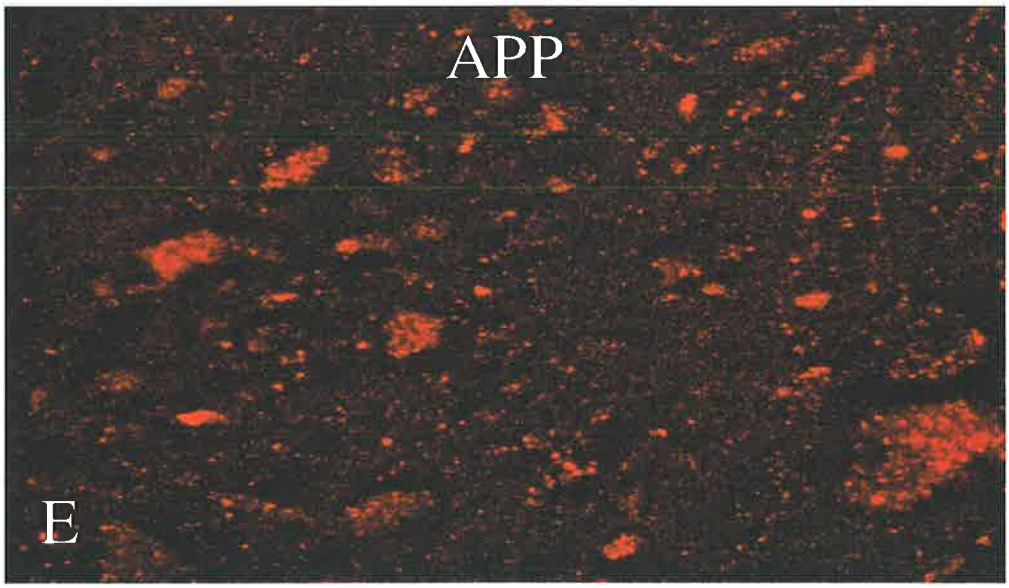
APP
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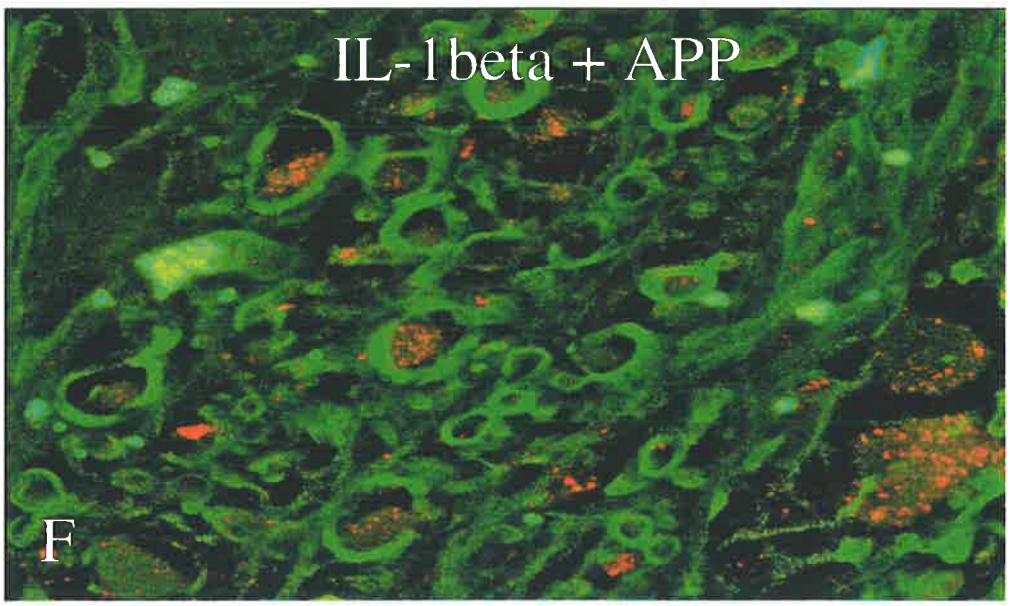

IL-1beta + APP
F

4.3 DISCUSSION

This study is the first to report the co-existence of APP and IL-1β in some neurons and axons as early as 0.5 h after human SCI. This observation suggests a possible link between IL-1β upregulation and APP expression. There is evidence that IL-1β can substantially up-regulate the expression of the APP gene by activating the nuclear factor kappa B (NF-κB) binding site of the regulatory region in primary neuronal cultures [119,146]. It has been demonstrated in vitro [143,235] and in vivo [330] that APP can protect neurons against excitotoxic, metabolic and oxidative insults. Thus, the link between APP and IL-1β may represent an important point of pharmacological intervention in the acute phase of SCI.

FINAL DISCUSSION

1. SUMMARY

Traumatic injury to the spinal cord initiates a cascade of secondary tissue damage beyond the original site of trauma, leading to a further loss of tissue and function [19,35,182,400]. The cascade of secondary tissue damage is accompanied by an inflammatory response marked by infiltration of neutrophils and macrophages, activation of glial cells and up-regulated expression of pro-inflammatory cytokines [99,164,354].

Neutrophils, macrophages and activated microglia may act as scavenger cells to remove cellular debris and release cytotoxic or neurotrophic molecules into the injured spinal cord [35,65,85,200,341,350], but the precise functions and effects of these cells remain unclear. Pro-inflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α), are involved in the regulation of the leukocyte recruitment and microglial activation [192,310], but their functional significances for secondary tissue damage following traumatic spinal cord injury (SCI) remain controversial [287]. IL-1β, IL-6 and TNF-α have been shown to have cytotoxic effects on the nervous system but also induce neurotrophic molecules involved in the repair of injured tissue [4,147,194,241,298,354]. Furthermore, the relationship between severity of SCI and expression levels of these pro-inflammatory cytokines has not been addressed and relationships between mRNA and protein levels of pro-inflammatory cytokines (e.g. IL-1β, IL-6 and TNF-α) in the central nervous system (CNS) have not been investigated. The cellular sources of IL-1β, IL-6 and TNF-α following traumatic SCI are controversial. Some authors believe that these cytokines are produced primarily by neutrophils and macrophages [239,347], but other studies have suggested that endogenous CNS cells including microglia may also produce pro-inflammatory cytokines

under various injury conditions [194,396]. Further studies of the posttraumatic inflammatory responses to SCI are required to resolve these controversies.

We hypothesized that the pro-inflammatory cytokines IL-1β, TNF-α and IL-6 produced by intrinsic cells in the spinal cord may act as messengers to coordinate the inflammatory cascade and the influx of neutrophils and monocytes to the site of damage and that the cytokine response should be greater in severe than in mild injury.

An in vivo model of SCI (the New York University Impactor model) was used in this study to induce acute SCI and inflammatory changes in rats. Analyses of the histological changes and inflammatory responses in rat SCI showed that activated microglia were found in mild and severe SCI as early as 1 h after contusion and increased dramatically at 1 d postinjury while neutrophils and macrophages were not noted in the injured spinal cord until 6 hrs postinjury. Increased immunoreactivity of TNF-α in mild and severe SCI was detected in neurons at 1 h postinjury, and in neurons and microglia at 6 h postinjury with much less increase in mild SCI, but the expression was short-lived and declined sharply by 1 d postinury. Furthermore, we quantified the expressions of IL-1β, IL-6 and TNF-α at both mRNA and protein levels over time following mild and severe SCI, utilizing RT-PCR and western blotting, and compared the expression levels of IL-1β, IL-6 and TNF-α between mild and severe traumatic SCI. Our results demonstrated an early significant up-regulation of IL-1β, IL-6 and TNF-α mRNAs, maximal at 6 h postinjury with return to control levels by 24 h postinjury, the changes being statistically significantly less in mild injury. The results also showed early transient increases of IL-1β, IL-6 and TNF-α proteins in severe SCI but not mild SCI.

Immunocytochemical, western blotting and RT-PCR analyses suggest that endogenous cells (neurons and microglia) in the rat spinal cord, not the blood-borne leucocytes, contribute to IL-1β, IL-6 and TNF-α production in the posttraumatic inflammatory response and that their up-regulation is greater in severe than mild SCI. This paper is the first to demonstrate significantly increased expression of IL-1β, IL-6 and TNF-α messenger ribonucleic acids (mRNAs) in severe SCI in contrast to mild SCI and that the temporal profiles of IL-1β, IL-6 and TNF-α protein expression in severe SCI paralleled their mRNA transcription whereas mild SCI resulted in increased synthesis of mRNAs but no detectable protein increase on western blotting. The disparities between transcription and translation of IL-1β, IL-6 and TNF-α in mild SCI could be due to the factors previously described (see Chapter RAT SCI EXPERIMENTS, section 4.3.2). Significantly increased expression of pro-inflammatory cytokines in severe injury in contrast to mild injury supports the hypothesis that cytokine up-regulation is an important factor in the generation of the severity of the inflammatory response. Thus, the significantly upregulated expression of cytokines IL-1ß, IL-6 and TNFα in severe SCI is a suitable target for pharmacological intervention to attenuate this response.

Almost all observations have been confined to animal models of CNS trauma and little is known of the cellular sources of IL-1β, IL-6 and TNF-α in the human. Therefore, we investigated the posttraumatic inflammatory response and the cellular sources of IL-1β, IL-6 and TNF-α in human spinal cord following contusion injury. This study is the first to demonstrate that, as early as 0.5 h after SCI, activated microglia were detected and their processes surrounded axonal swellings, suggesting that microglia are involved in the early response to traumatic axonal injury (see Chapter HUMAN SCI EXPERIMENTS, section 2.3.3).

Another novel finding in this study is that increased immunoreactivity of IL-1β, IL-6 and TNF-α was detected in neurons at 0.5 h postinjury, and in neurons and microglia at 5 h postinjury, but the expression of these pro-inflammatory cytokines was short-lived and declined sharply to baseline by 2 d postinjury. In human SCI as in our rat model, neutrophils and macrophages are not the cellular sources of IL-1β, IL-6 and TNF-α. We have found that endogenous cells in the human spinal cord, neurons and microglia, contribute to IL-1β, IL-6 and TNF-α production in the posttraumatic inflammatory response, suggesting that neurons and microglia, not "professional" blood-borne inflammatory cells, may play a major role in the initiation and regulation of the early post-traumatic inflammatory response of human SCI.

More studies are needed to elucidate the effect of activated microglia surrounding axons in both the rat and human SCI because activated microglia are able to produce pro-inflammatory cytokines (observed in this study), cytotoxic substances [25,387] and growth factors [341]. Activated microglia may provide a trophic local environment to promote axonal regeneration because some studies [248,280] have shown that activated microglia promoted the regeneration of sensory axons in the injured rat spinal cord. It has been also found in this study that activated microglia surrounded neurons. This phenomenon, originally described by the Blinzinger group [37], is now referred to as synaptic stripping. Microglia are thought to be involved in limited phagocytosis of individual synaptic termini and to have an impact on the synaptic reorganization of the neurons, but the effect of synaptic stripping on neurons is, as yet, largely unknown.

Finally, the combined results of rat and human SCI in this study indicate that expression of the pro-inflammatory cytokines IL-1ß, IL-6 and TNFα following SCI is robust, but short-lived, and that cytokine upregulation plays an important role in the initiation and regulation of

the early posttraumatic inflammatory response of SCI, where IL-1ß, IL-6 and TNFα are important molecules for the intercellular crosstalk between microglia, neurons, endothelial cells, oligodendrocytes and astrocytes. IL-1β activates microglia to express more IL-1ß, IL-6 and TNFα, stimulates astrocytes to form astroglial scars, upregulates expression of endothelial adhesion molecules to enhance leukocyte recuitment and produces MMP and free radicals to cause tissue ischaemia and destruction. IL-6 produces neuronal damage and reactive astrocytosis to inhibit the axonal regeneration. TNFα has a direct toxic effect on oligodendrocytes, damages vascular cells and activates the astrocytes and microglia. The production of IL-6 is regulated by a variety of stimuli, including IL-1β and TNFα (Figure 31) (see Chapter INTRODUCTION, sections 2.2.4, 2.3.4 & 2.4.4; Chapter HUMAN SCI EXPERIMENTS, section 3.3 and Chapter RAT SCI EXPERIMENTS, section 4.3.3).

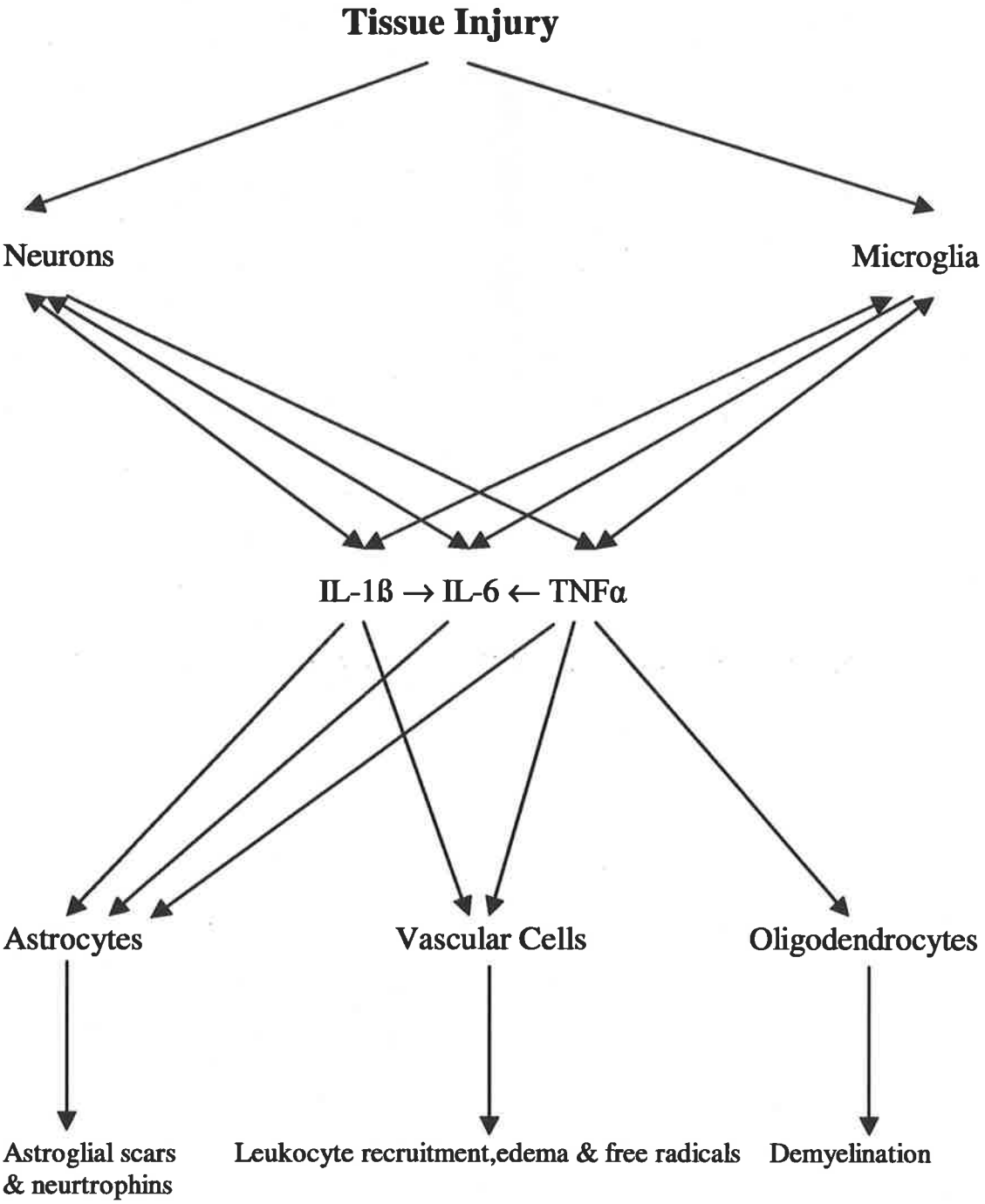


Figure 31. *Intercellular crosstalk between microglia, neurons, endothelial cells, astrocytes and oligodendrocytes in the posttraumatic inflammatory response to SCI. Tissue injury activates neuronal cells and microglia to express IL-1ß, IL-6 and TNFα, which are important molecules mediating the early posttraumatic inflammatory response. IL-1β activates astrocytes & microglia, upregulates expression of endothelial adhesion molecules and causes tissue ischaemia & destruction. IL-6 produces neuronal damage and reactive astrocytosis. TNFα has a direct toxic effect on oligodendrocytes, damages vascular cells and activates the astrocytes & microglia. The production of IL-6 is regulated by a variety of stimuli, including IL-1β and TNFα.*

IL-1ß, IL-6 and TNFα are important and modifiable components of the inflammatory response to traumatic CNS injury. The production of IL-1β, IL-6 and TNF-α is regulated by a variety of factors and these cytokines may function as both neurotrophic and cytotoxic factors (see Chapter RAT SCI EXPERIMENTS, section 4.3.3). IL-1β, IL-6 and TNF-α have been shown to produce tissue damage but may also rescue injured tissue in the posttraumatic response by inducing the production of neurotrophic molecules. There are several explanations for these apparent conflicting roles. First, the early and robust rise in IL-1β, IL-6 and TNF-α following SCI observed in this study may be detrimental if they are overproduced as the margins between neurotrophy and neurotoxicity are quite narrow [405]. This concept is supported by the finding of spontaneous inflammatory CNS demyelination in transgenic mice overexpressing TNF-α [283]. Second, the neurotrophic effects induced by IL-1β, IL-6 and TNFα may not occur in vivo because in vitro studies have shown that the production of neurotrophic molecules required intact cells. Third, cellular effects of cytokines are likely to be affected by other ongoing secondary injury events after SCI, e.g., APP expression can be substantially upregulated by IL-1β (see Chapter HUMAN SCI EXPERIMENTS, section 4.3) and the toxicity of excitatory amino acids, which are also upregulated early after CNS injury [105], can be enhanced by TNFα [66]. Fourth, the timing of the cytokine upregulation may influence their effects. Several studies [26,192] demonstrated that an injection of a cytokine cocktail of IL-1β, IL-6 and TNF-α into the spinal cord at 4 days postinjury resulted in a reduction in the recruitment of peripheral macrophages, microglial activation and tissue loss. However, the posttraumatic inflammatory response was enhanced and there was greater tissue loss when the cytokines were delivered 1 day after injury. Lastly, the cellular actions of cytokines are complex and subject to various regulation factors, including the number, subtype and proportion of activated receptors [43,327]. Also, the toxicity of excitatory amino acids may be enhanced by TNF-α [66].

Therefore, these data suggest that IL-1β, IL-6 and TNF-α are powerful inflammatory mediators in the posttraumatic inflammatory response of SCI and the final effects of these pro-inflammatory cytokines may be greatly determined by many factors, including the temporal and spatial relation of cytokines to their receptor-bearing target cells of the spinal cord.

2. LIMITATION OF THIS PROJECT AND FUTURE WORK

This study failed to demonstrate inhibition of the upregulation of cytokines IL-1β, IL-6 and TNFα in severe SCI by the NF-κB-specific antisense oligodeoxynucleotides. A lack of a suitable vehicle to deliver the antisense oligodeoxynucleotides into the targeted cells (e.g. neurons and microglia) is the main factor behind the failure. Further work is required to find an appropriate delivery mechanism for the antisense oligodeoxynucleotides in order to inhibit cytokine expression in severe SCI. This would provide information regarding the role of excessive cytokine expression.

The observation that activated microglia surrounded neurons and axons in both rat and human SCI requires further studies to determine exactly how microglia, neurons and axons interact with each other in SCI because information on intercellular communication is crucial for our understanding of the posttraumatic inflammatory response.

Other important future questions to be addressed are: (1) how and when cytokine receptors are expressed in the spinal cord following injury; (2) whether the expression of cytokine receptors is affected by the injury severity and (3) when, during the posttraumatic response to SCI, cytokines are either helpful or harmful.

CONCLUSIONS

There is an early, robust but transient upregulation of IL-1ß, IL-6 and TNFα mRNAs by 1 day after SCI in the rat. The cytokine mRNA responses are significantly greater in severe than in mild SCI in the rat. Protein levels of IL-1ß, IL-6 and TNFα in severe rat SCI were dramatically elevated in contrast to non-detectable levels in mild SCI. Furthermore, similar results in the expression of pro-inflammatory cytokines IL-1ß, IL-6 and TNFα were seen in human SCI. Our observations support the hypothesis that the cytokine upregulation is an important factor in the generation of the spinal cord injury, and is thus a suitable target for pharmacological intervention to attenuate this response.

Our studies of rat and human SCI have found that intrinsic CNS cells (neurons and microglia), not peripheral inflammatory cells, are the main source of pro-inflammatory cytokines. Thus, we believe that the inflammatory response in SCI is controlled by the central nervous system itself (neurons and microglia) rather than "professional" blood-borne inflammatory cells.

Our observations that microglial processes wrapped around axons in the rat and human SCI need further study.

APPENDIX

1. FIXATIVES AND BUFFERS

4% Paraformaldehyde in 0.1 M phosphate buffer (pH 7.4)

To make 5 L of fixative:
Solution 1

- Dissolve 200 g paraformaldehyde in 2500 ml distilled H_2O. Heat to 60° and stir.
- Add 1 mol/L NaOH by drops until clear.
- Filter.

Solution 2

- Dissolve 0.19 mole (26.20 g) of NaH_2PO_4 in 2500 ml distilled H_2O.
- Add 0.807 mole (114.56 g) of Na_2HPO_4.

Combine Solution 1 and Solution 2.
Cool down overnight.
Check pH and adjust pH to 7.4 with 5 M NaOH.

Tris buffer 0.05 M, pH 7.45

To make 1 L of buffer:

- Dissolve 6.06 g Tris in 1 L distilled H_2O.
- Add 8.77 g NaCl.
- Adjust pH to 7.45
- Allow at lease 10 minutes to fully dissolve and equilibrate, recheck pH.

Citric acid buffer 0.05 M, pH 6.0

To make 5 L of buffer:

- Dissolve 10.5 g citric acid monohydrate in 5 L distilled water.
- Adjust pH to 6.0.

PBS 0.01 M, pH 7.45

To make 20 L of buffer:

- Dissolve 1052 g NaCl into 3250 ml distilled water.
- Add 165.6 g $NaH_2PO_4 \cdot H_2O$.
- Adjust pH to 6.33.
  - Allow 2-3 minutes to ensure electrode stability, recheck pH of the stock solution.
- Dilute 660 ml of stock solution to 20 L with distilled water.
- Sample 200 ml, check pH and adjust pH to 7.45 using 5 M NaOH.
  - Adjust the 20 L buffer by adding 100 times of the NaOH added to 200 ml sample.

Ni DAB chromogen-substrate buffer 0.05 M, pH 7.45

To make 50 ml of buffer:

- Thaw 1 ml DAB and dilute it in 50 ml Tris buffer.
- Add 500 µl of 4% NiCl.
- Adjust pH to 7.45.
- Filter and add 50 µl of 30% hydrogen peroxide.

Laemlis buffer

To make 100 ml of buffer

- Glycerol 10 ml
- Stacking gel buffer 25 ml
- Urea-2M 12 g
- SDS 10% 20 ml
- PMSF 20 mg in ethanol
- Bromophenol Blue 5 ml

Add up to 100 ml with deionised water.

Stacking gel buffer

- Tris 3.03 g/100ml
- SDS 0.2 g/100 ml

PH to 6.8 with HCl.

Tris buffered saline

- 20 mM Tris: 4.84 g/2L
- 0.13 M NaCl: 15.18 g/2L
- Thimerosal: 100 mg/2L

PH to 7.4 with HCl.

Tris/Glycine Buffer

- 14.4 g glycine/4 L
- 3.02 g Tris/4 L

pH to 8.3 with NaOH.

Substrate Buffer

- 100mM Tris: 12.11 g/L
- 100mM NaCl: 5.844 g/L
- 50mM $MgCl_2$: 10.165 g/L

pH to 9.5 with HCl.

TAE buffer

- 40 mM Tris Aminomethane
- 20 mM glacial acetic acid
- 1 mM EDTA

pH to 8.0

2. STATISTICAL ANALYSES

Wilcoxon Rank Sum Test for comparisons of the Tarlov motor score

1. The Tarlov motor scores of mild SCI

1 d mild SCI	7 d mild SCI	14 d mild SCI
4	5	5
4.5	5	5
4	5	5
4	5	5
4	5	5
Median=4	Median=5	Median=5

2. The Tarlov motor scores of severe SCI

1 d severe SCI	7 d severe SCI	14 d severe SCI
0.5	1	1.5
0	0.5	1
0	1	1.5
0.5	0.5	1
0	0.5	1
Median=0	Median=0.5	Median=1

3. Wilcoxon Rank Sum Test

1 d mild SCI vs 1 d severe SCI

$n_1 = 5$, $n_2 = 5$, $T = 15$, $P_{0.05} = 17$

$T < 17$, $P < 0.05$

7 d mild SCI vs 7 d severe SCI

$n_1 = 5$, $n_2 = 5$, $T = 15$, $P_{0.05} = 17$

$T < 17$, $P < 0.05$

14 d mild SCIvs 14 d severe SCI

$n_1 = 5$, $n_2 = 5$, $T = 15$, $P_{0.05} = 17$

$T < 17$, $P < 0.05$

T-test for comparisons of histopathology

1. Areas of maximal lesions and lengths of lesions at 14 days after mild SCI

Areas of maximal lesions (mm^2)

0.97
1.56
1.23
0.98
1.32
Mean= 1.21
Standard error= 0.11

Lengths of lesions (mm)

2.60
3.60
2.75
2.15
3.30
Mean= 2.88
Standard error= 0.26

2. Areas of maximal lesions and lengths of lesions at 14 days after severe SCI

Areas of maximal lesions (mm^2)

7.88
6.93
6.86
5.73
6.12
Mean= 6.70
Standard error= 0.37

Lengths of lesions (mm)

15.60
13.15
12.90
10.60
13.90
Mean= 13.23
Standard error= 0.81

3. T-test

Comparisons of areas of maximal lesions between mild and severe SCI

$n_1 = 5$, $n_2 = 5$, $v = 8$, $S_c^2 = 0.0745$, $P_{0.05} = 2.306$, $t = 31.80$

$t > 2.306$, $P < 0.05$

Comparisons of lengths of lesions between mild and severe SCI

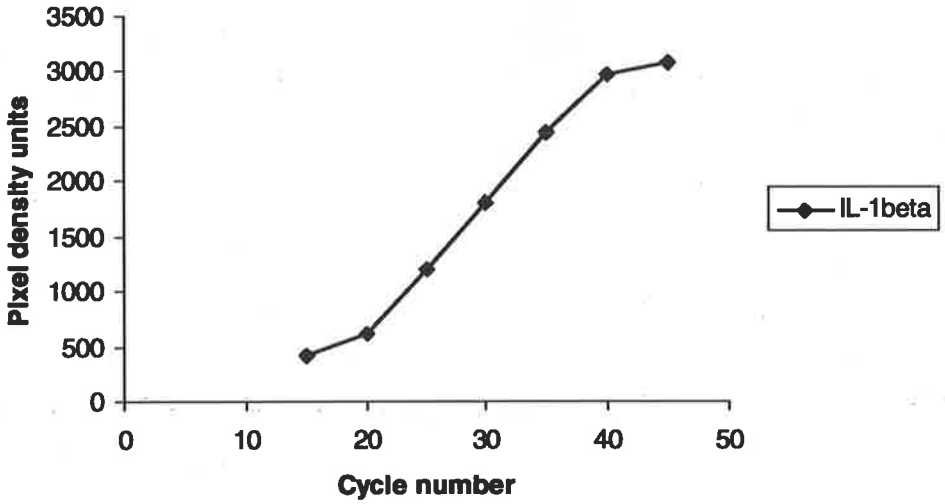
$n_1 = 5$, $n_2 = 5$, $v = 8$, $S_c^2 = 0.36185$, $P_{0.05} = 2.306$, $t = 27.24$

$t > 2.306$, $P < 0.05$

ANOVA test and Student-Neuman-Keuls multiple comparisons test for RT-PCR products

1. Determining linear range of RT-PCR products of IL-1β

15 cycles	20 cycles	25 cycles	30 cycles	35 cycles	40 cycles	45 cycles
419	623	1205	1798	2446	2973	3086



2. RT-PCR products of IL-1β after mild SCI

1 hr	3 hr	6 hr	1 d	3 d
0.654715	1.432875	0.824234	0.454498	0.251655
0.546999	0.897749	0.690003	0.350904	0.290935
0.632567	0.886983	0.709067	0.402909	0.350903
0.625609	0.968789	0.769082	0.426904	0.200065
	0.899099			
Mean= 0.61	Mean= 1.02	Mean= 0.75	Mean= 0.41	Mean= 0.27
SE= 0.02	SE= 0.10	SE= 0.03	SE= 0.02	SE= 0.03

SE: standard error

3. RT-PCR products of IL-1β after severe SCI

1 hr	3 hr	6 hr	1 d	3 d
1.238961	2.337077	4.987458	0.708855	0.275409
0.872524	1.843291	2.809917	0.468709	0.309043
1.090756	1.980989	3.678056	0.207809	0.429238
0.972678	2.198567	3.348796	0.455972	0.205989
Mean= 1.04	Mean= 2.09	Mean= 3.71	Mean= 0.46	Mean= 0.30
SE= 0.08	SE= 0.11	SE= 0.46	SE= 0.10	SE= 0.05

SE: standard error

4. RT-PCR products of IL-1β in laminectomy controls

1hr	3hr	6hr	1d	3d
0.399698	0.364675	0.395109	0.330689	0.399875
0.207609	0.179598	0.205412	0.243187	0.258796
0.154319	0.240985	0.231059	0.129678	0.128968
				0.209823
Mean= 0.25	Mean= 0.26	Mean= 0.28	Mean=0.26	Mean= 0.25
SE= 0.07	SE= 0.05	SE= 0.06	SE= 0.06	SE= 0.06

SE: standard error

5. RT-PCR products of IL-1β in naïve controls

0.376214
0.200815
0.211588
Mean= 0.26
SE= 0.06

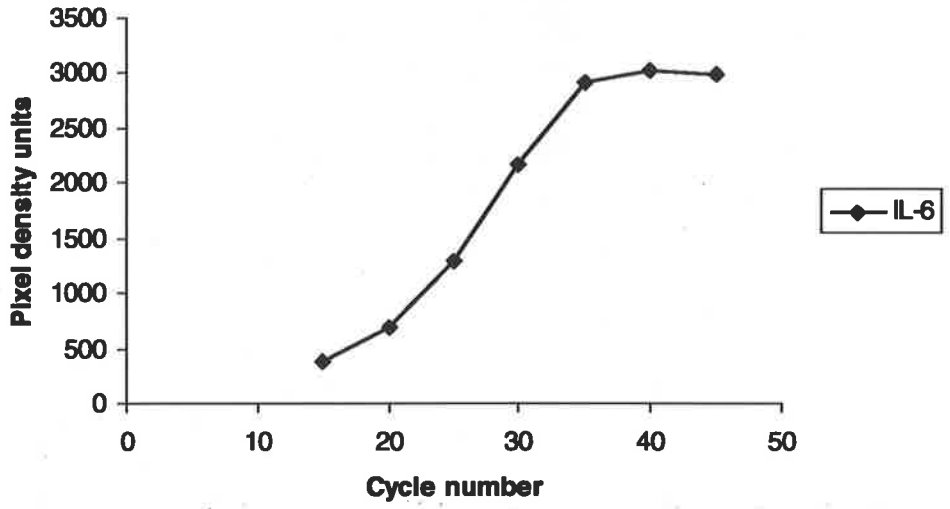
SE: standard error

6. ANOVA test and Student-Neuman-Keuls multiple comparisons test for IL-1β mRNA

Comparisons	P value
Severe 1 hr vs Mild 1 hr	P > 0.05
Severe 1 hr vs Naive control 1 hr	**P < 0.05**
Severe 1 hr vs Laminectomy control 1 hr	**P < 0.05**
Mild 1 hr vs Naïve control	**P < 0.05**
Mild 1 hr vs Laminectomy control 1 hr	**P < 0.05**
Laminectomy control 1 hr vs Naïve control	P > 0.05
Severe 3 hr vs Mild 3 hr	**P < 0.05**
Severe 3 hr vs Naive control 3 hr	**P < 0.05**
Severe 3 hr vs Laminectomy control 3 hr	**P < 0.05**
Mild 3 hr vs Naïve control	**P < 0.05**
Mild 3 hr vs Laminectomy control 3 hr	**P < 0.05**
Laminectomy control 3 hr vs Naïve control	P > 0.05
Severe 6 hr vs Mild 6 hr	**P < 0.05**
Severe 6 hr vs Naive control 6 hr	**P < 0.05**
Severe 6 hr vs Laminectomy control 6 hr	**P < 0.05**
Mild 6 hr vs Naïve control	**P < 0.05**
Mild 6 hr vs Laminectomy control 6 hr	**P < 0.05**
Laminectomy 6 hr vs Naïve control	P > 0.05
Severe 1 d vs Mild 1 d	P > 0.05
Severe 1 d vs Naive control 1 d	P > 0.05
Severe 1 d vs Laminectomy control 1 d	P > 0.05
Mild 1 d vs Naïve control	P > 0.05
Mild 1 d vs Laminectomy control 1 d	P > 0.05
Laminectomy control 1d vs Naïve control	P > 0.05
Severe 3 d vs Mild 3 d	P > 0.05
Severe 3 d vs Naive control 3 d	P > 0.05
Severe 3 d vs Laminectomy control 3 d	P > 0.05
Mild 3 d vs Naïve control	P > 0.05
Mild 3 d vs Laminectomy control 3 d	P > 0.05
Naïve control vs Laminectomy control 3 d	P > 0.05

7. Determining linear range of RT-PCR products of IL-6

15 cycles	20 cycles	25 cycles	30 cycles	35 cycles	40 cycles	45 cycles
378	698	1298	2167	2925	3027	2997



8. RT-PCR products of IL-6 after mild SCI

1 hr	3 hr	6 hr	1 d	3 d
0.608712	1.302448	0.542987	0.303511	0.207527
0.543551	0.995633	0.488938	0.289679	0.528671
0.527685	0.212294	0.402897	0.298876	0.144316
0.495829	0.238678	0.502261	0.200861	0.210903
	0.205892			
Mean= 0.54	Mean= 0.59	Mean= 0.48	Mean= 0.27	Mean= 0.27
SE= 0.02	SE= 0.23	SE= 0.03	SE= 0.02	SE= 0.09

SE: standard error

9. RT-PCR products of IL-6 after severe SCI

1 hr	3 hr	6 hr	1 d	3 d
0.762489	2.401069	4.100016	0.702878	0.707343
1.000991	1.428363	3.800702	0.199823	0.106897
0.509856	1.613268	3.647959	0.222696	0.227698
0.779621	1.278659	2.397682	0.105987	0.168565
Mean= 0.76	Mean= 1.68	Mean= 3.49	Mean= 0.31	Mean= 0.30
SE= 0.10	SE= 0.25	SE= 0.37	SE= 0.13	SE= 0.14

SE: standard error

10. RT-PCR products of IL-6 in laminectomy controls

1hr	3hr	6hr	1d	3d
0.378905	0.356981	0.378719	0.286523	0.409739
0.191326	0.322673	0.239765	0.422235	0.251387
0.171579	0.105982	0.134768	0.143276	0.160876
Mean= 0.25	Mean= 0.26	Mean= 0.25	Mean= 0.28	Mean= 0.27
SE= 0.07	SE= 0.08	SE= 0.07	SE= 0.08	SE= 0.06

SE: standard error

11. RT-PCR products of IL-6 in naïve controls

0.390919
0.164432
0.267892
Mean= 0.27
SE= 0.07

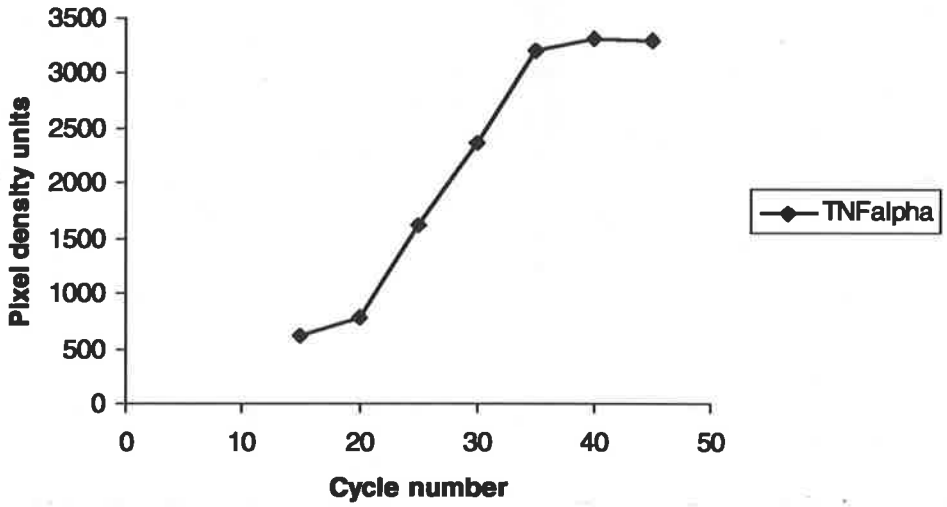
SE: standard error

12. ANOVA test and Student-Neuman-Keuls multiple comparisons test for IL-6 mRNA

Comparisons	P value
Severe 1 hr vs Mild 1 hr	P > 0.05
Severe 1 hr vs Naive control 1 hr	**P < 0.05**
Severe 1 hr vs Laminectomy control 1 hr	**P < 0.05**
Mild 1 hr vs Naïve control	**P < 0.05**
Mild 1 hr vs Laminectomy control 1 hr	**P < 0.05**
Laminectomy control 1 hr vs Naïve control	P > 0.05
Severe 3 hr vs Mild 3 hr	**P < 0.05**
Severe 3 hr vs Naive control 3 hr	**P < 0.05**
Severe 3 hr vs Laminectomy control 3 hr	**P < 0.05**
Mild 3 hr vs Naïve control	**P < 0.05**
Mild 3 hr vs Laminectomy control 3 hr	**P < 0.05**
Laminectomy control 3 hr vs Naïve control	P > 0.05
Severe 6 hr vs Mild 6 hr	**P < 0.05**
Severe 6 hr vs Naive control 6 hr	**P < 0.05**
Severe 6 hr vs Laminectomy control 6 hr	**P < 0.05**
Mild 6 hr vs Naïve control	**P < 0.05**
Mild 6 hr vs Laminectomy control 6 hr	**P < 0.05**
Laminectomy 6 hr vs Naïve control	P > 0.05
Severe 1 d vs Mild 1 d	P > 0.05
Severe 1 d vs Naive control 1 d	P > 0.05
Severe 1 d vs Laminectomy control 1 d	P > 0.05
Mild 1 d vs Naïve control	P > 0.05
Mild 1 d vs Laminectomy control 1 d	P > 0.05
Laminectomy control 1d vs Naïve control	P > 0.05
Severe 3 d vs Mild 3 d	P > 0.05
Severe 3 d vs Naive control 3 d	P > 0.05
Severe 3 d vs Laminectomy control 3 d	P > 0.05
Mild 3 d vs Naïve control	P > 0.05
Mild 3 d vs Laminectomy control 3 d	P > 0.05
Naïve control vs Laminectomy control 3 d	P > 0.05

13. Determining linear range of RT-PCR products of TNFα

15 cycles	20 cycles	25 cycles	30 cycles	35 cycles	40 cycles	45 cycles
622	783	1629	2370	3208	3321	3306



14. RT-PCR products of TNFα after mild SCI

1 hr	3 hr	6 hr	1 d	3 d
0.280207	0.291494	0.280489	0.252325	0.213612
0.320852	0.291557	0.411033	0.206957	0.229189
0.298907	0.343246	0.386758	0.193275	0.195687
0.250965	0.279016	0.376427	0.186936	0.126398
	0.300791			
Mean= 0.29	Mean= 0.30	Mean= 0.36	Mean= 0.21	Mean= 0.19
SE= 0.01	SE= 0.01	SE= 0.03	SE= 0.01	SE= 0.02

SE: standard error

15. RT-PCR products of TNFα after severe SCI

1 hr	3 hr	6 hr	1 d	3 d
1.203389	1.110673	2.659687	0.373112	0.294423
0.893555	1.395637	1.956812	0.124672	0.160087
1.169878	1.451376	2.036879	0.248287	0.168965
1.158216	0.902187	1.787953	0.209495	0.209497
Mean= 1.11	Mean= 1.21	Mean= 2.11	Mean= 0.24	Mean= 0.21
SE= 0.07	SE= 0.13	SE= 0.19	SE= 0.05	SE= 0.03

SE: standard error

16. RT-PCR products of TNFα in laminectomy controls

1hr	3hr	6hr	1d	3d
0.084126	0.163792	0.077589	0.119681	0.155902
0.159672	0.126509	0.132276	0.073985	0.098689
0.152556	0.120972	0.156592	0.128792	0.126721
Mean= 0.13	Mean= 0.14	Mean= 0.12	Mean= 0.11	Mean= 0.13
SE= 0.02	SE= 0.01	SE= 0.02	SE= 0.02	SE= 0.01

SE: standard error

17. RT-PCR products of TNFα in naive controls

0.113395
0.150865
0.102603
Mean= 0.12
SE= 0.01

SE: standard error

18. ANOVA test and Student-Neuman-Keuls multiple comparisons test for TNFα mRNA

Comparisons	P value
Severe 1 hr vs Mild 1 hr	**P < 0.05**
Severe 1 hr vs Naive control 1 hr	**P < 0.05**
Severe 1 hr vs Laminectomy control 1 hr	**P < 0.05**
Mild 1 hr vs Naïve control	**P < 0.05**
Mild 1 hr vs Laminectomy control 1 hr	**P < 0.05**
Laminectomy control 1 hr vs Naïve control	P > 0.05
Severe 3 hr vs Mild 3 hr	**P < 0.05**
Severe 3 hr vs Naive control 3 hr	**P < 0.05**
Severe 3 hr vs Laminectomy control 3 hr	**P < 0.05**
Mild 3 hr vs Naïve control	**P < 0.05**
Mild 3 hr vs Laminectomy control 3 hr	**P < 0.05**
Laminectomy control 3 hr vs Naïve control	P > 0.05
Severe 6 hr vs Mild 6 hr	**P < 0.05**
Severe 6 hr vs Naive control 6 hr	**P < 0.05**
Severe 6 hr vs Laminectomy control 6 hr	**P < 0.05**
Mild 6 hr vs Naïve control	**P < 0.05**
Mild 6 hr vs Laminectomy control 6 hr	**P < 0.05**
Laminectomy 6 hr vs Naïve control	P > 0.05
Severe 1 d vs Mild 1 d	P > 0.05
Severe 1 d vs Naive control 1 d	P > 0.05
Severe 1 d vs Laminectomy control 1 d	P > 0.05
Mild 1 d vs Naïve control	P > 0.05
Mild 1 d vs Laminectomy control 1 d	P > 0.05
Laminectomy control 1d vs Naïve control	P > 0.05
Severe 3 d vs Mild 3 d	P > 0.05
Severe 3 d vs Naive control 3 d	P > 0.05
Severe 3 d vs Laminectomy control 3 d	P > 0.05
Mild 3 d vs Naïve control	P > 0.05
Mild 3 d vs Laminectomy control 3 d	P > 0.05
Naïve control vs Laminectomy control 3 d	P > 0.05

ANOVA test and Student-Neuman-Keuls multiple comparisons test for western blot products

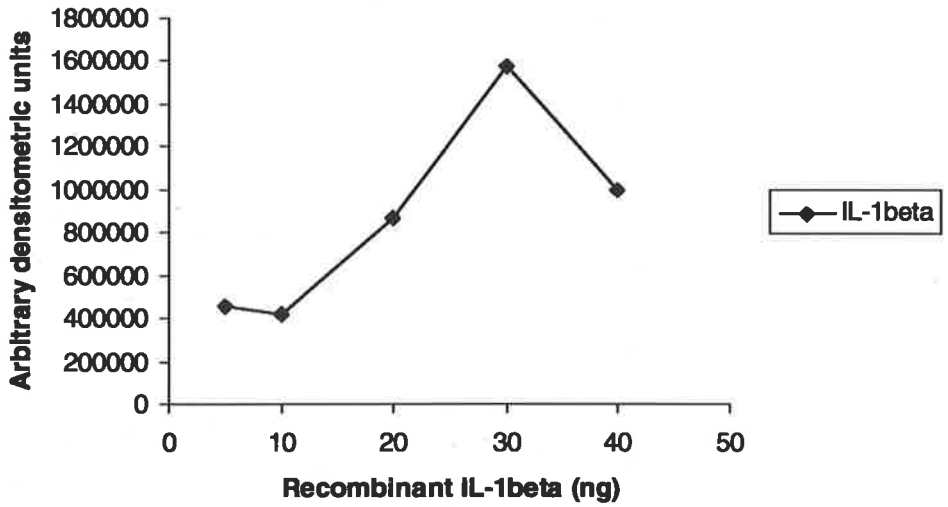
1. Western blot analysis for IL-1β after severe SCI

1 hr	3 hr	6 hr	24 hr	72 hr
138002	189435	504861	1	1
121299	321549	368982	1	1
156834	289939	319392	1	1
119074	137106	238958	1	1
Mean=133802	Mean=234507	Mean=358048	Mean=1	Mean=1
SE= 8762	SE= 42980	SE= 55790	SE= 0	SE= 0

SE: standard error

2. Western blot analysis for IL-1β standard recombinant controls

5 ng	10 ng	20 ng	30 ng	40 ng
457396.6	423950.7	869779.8	1579012	996399.8



3. ANOVA test and Student-Neuman-Keuls multiple comparisons test for IL-1β protein

Comparisons	P value
Severe 1 hr vs Mild 1 hr	**P < 0.05**
Severe 1 hr vs Naive control 1 hr	**P < 0.05**
Severe 1 hr vs Laminectomy control 1 hr	**P < 0.05**
Mild 1 hr vs Naïve control	P > 0.05
Mild 1 hr vs Laminectomy control 1 hr	P > 0.05
Laminectomy control 1 hr vs Naïve control	P > 0.05
Severe 3 hr vs Mild 3 hr	**P < 0.05**
Severe 3 hr vs Naive control 3 hr	**P < 0.05**
Severe 3 hr vs Laminectomy control 3 hr	**P < 0.05**
Mild 3 hr vs Naïve control	P > 0.05
Mild 3 hr vs Laminectomy control 3 hr	P > 0.05
Laminectomy control 3 hr vs Naïve control	P > 0.05
Severe 6 hr vs Mild 6 hr	**P < 0.05**
Severe 6 hr vs Naive control 6 hr	**P < 0.05**
Severe 6 hr vs Laminectomy control 6 hr	**P < 0.05**
Mild 6 hr vs Naïve control	P > 0.05
Mild 6 hr vs Laminectomy control 6 hr	P > 0.05
Laminectomy 6 hr vs Naïve control	P > 0.05
Severe 1 d vs Mild 1 d	P > 0.05
Severe 1 d vs Naive control 1 d	P > 0.05
Severe 1 d vs Laminectomy control 1 d	P > 0.05
Mild 1 d vs Naïve control	P > 0.05
Mild 1 d vs Laminectomy control 1 d	P > 0.05
Laminectomy control 1d vs Naïve control	P > 0.05
Severe 3 d vs Mild 3 d	P > 0.05
Severe 3 d vs Naive control 3 d	P > 0.05
Severe 3 d vs Laminectomy control 3 d	P > 0.05
Mild 3 d vs Naïve control	P > 0.05
Mild 3 d vs Laminectomy control 3 d	P > 0.05
Naïve control vs Laminectomy control 3 d	P > 0.05

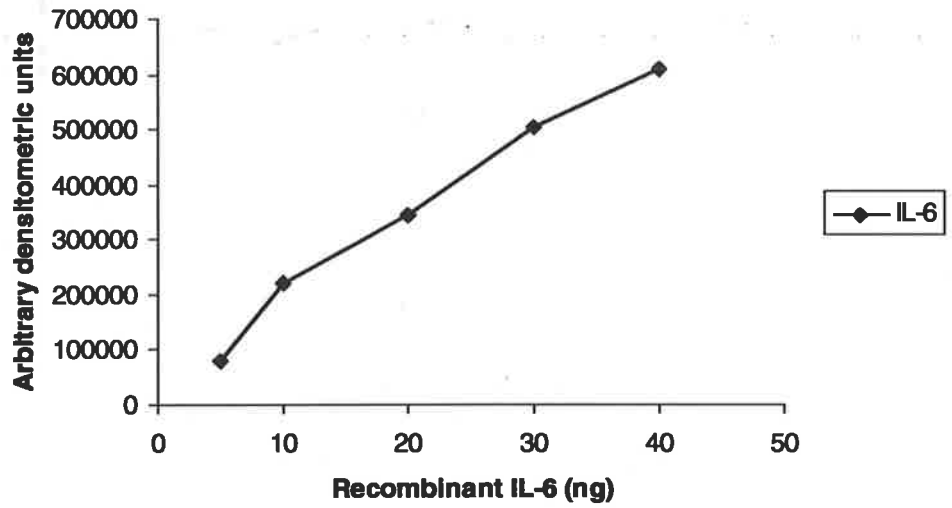
4. Western blot analysis for IL-6 after severe SCI

1 hr	3 hr	6 hr	24 hr	72 hr
161141	411546	364388	1	1
97102	221271	292769	1	1
112224	98234	230602	1	1
113532	192985	595845	1	1
Mean=121000	Mean=231009	Mean=370901	Mean=1	Mean=1
SE=13890	SE=65678	SE=79807	SE=0	SE=0

SE: standard error

5. Western blot analysis for IL-6 standard recombinant controls

5 ng	10 ng	20 ng	30 ng	40 ng
81413.75	219664.9	345559.4	504942.7	609273.1



6. ANOVA test and Student-Neuman-Keuls multiple comparisons test for IL-6 protein

Comparisons	P value
Severe 1 hr vs Mild 1 hr	**P < 0.05**
Severe 1 hr vs Naive control 1 hr	**P < 0.05**
Severe 1 hr vs Laminectomy control 1 hr	**P < 0.05**
Mild 1 hr vs Naïve control	P > 0.05
Mild 1 hr vs Laminectomy control 1 hr	P > 0.05
Laminectomy control 1 hr vs Naïve control	P > 0.05
Severe 3 hr vs Mild 3 hr	**P < 0.05**
Severe 3 hr vs Naive control 3 hr	**P < 0.05**
Severe 3 hr vs Laminectomy control 3 hr	**P < 0.05**
Mild 3 hr vs Naïve control	P > 0.05
Mild 3 hr vs Laminectomy control 3 hr	P > 0.05
Laminectomy control 3 hr vs Naïve control	P > 0.05
Severe 6 hr vs Mild 6 hr	**P < 0.05**
Severe 6 hr vs Naive control 6 hr	**P < 0.05**
Severe 6 hr vs Laminectomy control 6 hr	**P < 0.05**
Mild 6 hr vs Naïve control	P > 0.05
Mild 6 hr vs Laminectomy control 6 hr	P > 0.05
Laminectomy 6 hr vs Naïve control	P > 0.05
Severe 1 d vs Mild 1 d	P > 0.05
Severe 1 d vs Naive control 1 d	P > 0.05
Severe 1 d vs Laminectomy control 1 d	P > 0.05
Mild 1 d vs Naïve control	P > 0.05
Mild 1 d vs Laminectomy control 1 d	P > 0.05
Laminectomy control 1d vs Naïve control	P > 0.05
Severe 3 d vs Mild 3 d	P > 0.05
Severe 3 d vs Naive control 3 d	P > 0.05
Severe 3 d vs Laminectomy control 3 d	P > 0.05
Mild 3 d vs Naïve control	P > 0.05
Mild 3 d vs Laminectomy control 3 d	P > 0.05
Naïve control vs Laminectomy control 3 d	P > 0.05

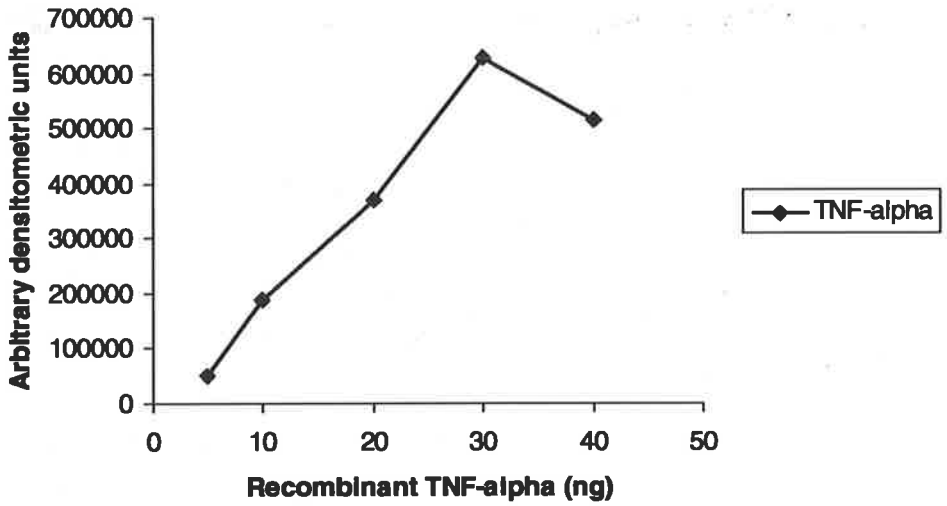
7. Western blot analysis for TNF-α after severe SCI

1 hr	3 hr	6 hr	24 hr	72 hr
237365	515193	576702	1	1
178526	277366	207789	1	1
195112	192888	247619	1	1
173028	162913	211526	1	1
Mean=196008	Mean=287090	Mean=310909	Mean=1	Mean=1
SE=14563	SE=79802	SE=89052	SE=0	SE=0

SE: standard error

8. Western blot analysis for TNF-α standard recombinant controls

5 ng	10 ng	20 ng	30 ng	40 ng
50450.4	187245.8	370216.6	628742.9	515664.2



9. ANOVA test and Student-Neuman-Keuls multiple comparisons test for TNFα protein

Comparisons	P value
Severe 1 hr vs Mild 1 hr	**P < 0.05**
Severe 1 hr vs Naive control 1 hr	**P < 0.05**
Severe 1 hr vs Laminectomy control 1 hr	**P < 0.05**
Mild 1 hr vs Naïve control	P > 0.05
Mild 1 hr vs Laminectomy control 1 hr	P > 0.05
Laminectomy control 1 hr vs Naïve control	P > 0.05
Severe 3 hr vs Mild 3 hr	**P < 0.05**
Severe 3 hr vs Naive control 3 hr	**P < 0.05**
Severe 3 hr vs Laminectomy control 3 hr	**P < 0.05**
Mild 3 hr vs Naïve control	P > 0.05
Mild 3 hr vs Laminectomy control 3 hr	P > 0.05
Laminectomy control 3 hr vs Naïve control	P > 0.05
Severe 6 hr vs Mild 6 hr	**P < 0.05**
Severe 6 hr vs Naive control 6 hr	**P < 0.05**
Severe 6 hr vs Laminectomy control 6 hr	**P < 0.05**
Mild 6 hr vs Naïve control	P > 0.05
Mild 6 hr vs Laminectomy control 6 hr	P > 0.05
Laminectomy 6 hr vs Naïve control	P > 0.05
Severe 1 d vs Mild 1 d	P > 0.05
Severe 1 d vs Naive control 1 d	P > 0.05
Severe 1 d vs Laminectomy control 1 d	P > 0.05
Mild 1 d vs Naïve control	P > 0.05
Mild 1 d vs Laminectomy control 1 d	P > 0.05
Laminectomy control 1d vs Naïve control	P > 0.05
Severe 3 d vs Mild 3 d	P > 0.05
Severe 3 d vs Naive control 3 d	P > 0.05
Severe 3 d vs Laminectomy control 3 d	P > 0.05
Mild 3 d vs Naïve control	P > 0.05
Mild 3 d vs Laminectomy control 3 d	P > 0.05
Naïve control vs Laminectomy control 3 d	P > 0.05

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