Alimentary Tract Mucositis: NF-κB and Pro-Inflammatory Cytokines in the Tissues and Serum Following Chemotherapy

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Declaration

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Abstract

Mucositis refers to the widespread damage of mucosal surfaces throughout the length of the alimentary tract (AT) that can occur during cancer treatment. Its development is an important clinical problem that complicates and limits treatment options as well as adversely affecting the quality of life and treatment outcomes for patients. Recent studies directed at determining the pathobiology of mucositis have indicated increasing evidence for the role of transcription factors, such as nuclear factor-κB (NF-κB), and certain pro-inflammatory cytokines, for example tumour necrosis factor (TNF), interleukin-1β (IL-1β) and interleukin-6 (IL-6), in its development. This thesis developed from an initial clinical investigation in which the expression of NF-κB and COX-2 in oral mucosa was investigated in patients undergoing chemotherapy. Increased levels of NF-κB were demonstrated in the buccal mucosa following chemotherapy.

It is well established that mucositis occurs in different sites of the AT. The aims of this research, therefore, were to compare and contrast the changes that do occur at different sites of the AT following chemotherapy in an established animal model (Dark Agouti (DA) rat). Furthermore, the studies were conducted to determine whether changes in tissue and serum levels of NF-κB and pro-inflammatory cytokines occurred following chemotherapy and, with respect to tissue levels, identify whether there were differences in expression at different sites throughout the AT. The final aim was to examine whether the histological changes and changes in pro-inflammatory cytokines were affected by the type of chemotherapy drug used.

The effects of three chemotherapy drugs with different mechanisms of action (irinotecan, methotrexate and 5-fluorouracil) were investigated, all of which can cause mucositis in the clinical setting.

The thesis is divided into a *Literature Review* (Chapter 1) followed by 4 research papers: Chapter 2 – "*Nuclear factor- κB (NF- κB) and cyclooxygenase-2 (COX-2) expression in the oral mucosa following cancer chemotherapy*" Chapter 3 – "*Characterisation of mucosal changes in the alimentary tract following administration of irinotecan: Implications for the pathobiology of mucositis*" Chapter 4 – "*Is the pathobiology of chemotherapy-induced alimentary tract mucositis influenced by the type of mucotoxic drug administered*?", Chapter 5 – "*Serum levels of NF-κB and pro-inflammatory cytokines following administration of mucotoxic drugs*". Chapter 6 provides an overall summary and discussion of the results.

Previous research has indicated that following administration of chemotherapeutic agents there may be subclinical changes occurring in the mucosa prior to obvious clinical manifestations. The results presented in this thesis also demonstrate this in both humans and animals following administration of chemotherapy. Immunohistochemical analysis of tissue taken from the oral cavity, jejunum and colon from the DA rats following chemotherapy demonstrated that changes in NF-κB and the pro-inflammatory cytokines, TNF, IL-1β and IL-6, occurred at all sites over a 72 hour time period. This was evident before severe histological evidence of mucositis were observed such as epithelial atrophy in the oral mucosa, atrophy, blunting and fusion of the villi in the jejunum and crypt ablation in the jejunum and colon. Furthermore, each of the three drugs caused different patterns of NF-κB and pro-inflammatory cytokine expression in the tissues; in spite of this, however, histological features of damage were similar. With respect to serum levels of NF-κB and pro-inflammatory cytokines, differences were observed between the serum and tissue levels. Generally, serum changes followed initial histological changes in the tissues, or occurred simultaneously with histological changes. The mechanisms behind this are unclear; however it may be that elevated cytokines in the tissues "overflow" into the serum as tissue damage

increases. Furthermore, the use of serum cytokine level measurement to predict mucosal damage is limited because of the differences in timing and short time intervals between changes in the serum and tissues.

This thesis has provided additional important information on mucositis pathobiology and highlights its complexity. In particular, it has provided new evidence supporting the notion that mucositis is not restricted to the oral cavity and that other sites of the AT are also affected. Furthermore, these results confirm previous data indicating that subclinical changes occur in the mucosa prior to the development of obvious histological damage or clinical manifestations of mucositis. Contrary to previous reports, these studies have indicated that, although the clinical and histological changes may be similar, the alterations in NF-κB and pro-inflammatory cytokines in the tissues are affected by the type of drug used. This has important implications in the management and prevention of mucositis in the clinical setting particularly when multi-drug or chemotherapy-radiotherapy regimens are used. A common pathway that leads to mucosal damage is yet to be determined. The fact that serum levels appear to reflect the "global" nature of the effects of chemotherapy, highlights the fact that ongoing research needs to be directed, not necessarily at specific side effects, but rather how side effects of chemotherapy are interrelated so that better patient management can be achieved and ultimately provide optimum treatment and better survival for patients with cancer.

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Co-author Contributions

Professor Dorothy M. K. Keefe

Professor Keefe was my principal supervisor and therefore listed as a co-author on all publications arising from this thesis. She assisted in the development of my original research proposal and provided funding for the work that was completed during my candidature. In addition she read through many drafts of the individual papers as well as this thesis.

Professor Stephen T. Sonis

Professor Sonis was my co-supervisor who, in spite of being on the opposite side of the world, was crucial in helping to develop my research project. He read drafts of each paper and this thesis.

Dr Rachel J. Gibson

Dr Gibson is a member of the Mucositis Research Group. She helped plan and provided valuable assistance with the initial irinotecan animal experiment and provided advice on laboratory techniques. Dr Gibson also read numerous drafts of the individual papers that make up this thesis.

Dr Joanne M. Bowen

Dr Bowen is a member of the Mucositis Research Group. She assisted with all of the animal experiments undertaken in this study and provided advice on laboratory techniques. She also read numerous drafts of the individual papers that make up this thesis.

Andrea M. Stringer

Ms Stringer is a fellow PhD student and member of the Mucositis Research Group. She assisted with all of the animal experiments and provided advice on laboratory techniques. She also read numerous drafts of the individual papers that make up this thesis.

Ann S-J. Yeoh

Ms Yeoh is a fellow PhD student and member of the Mucositis Research Group. She read and commented on numerous drafts of the literature review for which she is listed as a co-author.

Explanation of the Thesis

This thesis is composed of 6 chapters: a literature review, four distinct research papers and finally, summary and conclusions. During the course of my candidature the literature review and 3 research papers were published or accepted for publication (Appendix II); Chapter 5 has been submitted for publication. Accordingly, each research chapter is written as a publication complete with introduction, materials and methods, results and discussion. Some minor editing of the chapters has been made to avoid significant repetition; the chapters have also been updated since publication and, in some instances, additional figures have been added. Unavoidable repetition has occurred only as necessary due to the format of the papers.

Animal Ethics

The animal studies (Chapters 3-5) were approved by the Animal Ethics Committees of The Institute of Medical and Veterinary Sciences and of The University of Adelaide. They complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Training (2004). Due to the potentially severe nature of the diarrhoea that can be induced by irinotecan, animals were monitored four times daily and if any animal showed certain criteria (as defined by the Animal Ethics Committee) they were euthanised. These criteria included a dull ruffled coat with accompanying dull and sunken eyes, coolness to touch with no spontaneous movement, and a hunched appearance.

Chapter 1

Literature Review

The role of pro-inflammatory cytokines in cancer treatment-induced alimentary tract mucositis: Pathobiology, animal models and cytotoxic drugs.

This chapter has been published as:

Logan RM, Stringer AM, Bowen JM, Yeoh A S-J, Gibson RJ, Sonis ST and Keefe DMK, "The role of pro-inflammatory cytokines in cancer treatment-induced alimentary tract mucositis: Pathobiology, animal models and cytotoxic drugs" Cancer Treat Rev (2007), 33(5): 448-460

1.1 Introduction

Mucositis is a major problem for patients undergoing treatments such as chemotherapy and radiotherapy (Figure 1.1) It is a complication that occurs throughout the alimentary tract (AT) [77, 119] and causes a spectrum of clinical signs and symptoms which range from intractable and debilitating oral pain as a result of ulceration through to, and including, gastrointestinal symptoms such as abdominal bloating, vomiting and diarrhoea [79, 120]. Recently it has also been suggested that other mucosal surfaces throughout the body, such as the genitourinary and respiratory mucosae, may also be affected [73].

Other side effects of cancer treatment, such as severe nausea or potentially life threatening events, such as neutropenia, are now relatively well managed [13]. Mucositis however, remains an important dose-limiting factor in a patient's cancer treatment [13, 118]. The debilitating effects of mucositis can result in unplanned interruptions or even premature cessation of treatment. The risk of systemic infections and even death is increased in patients with mucositis. For patients who are undergoing head and neck radiotherapy, the dose of treatment is limited by the proximity of the mouth to critical anatomical structures such as the brain and spinal cord and therefore increases in dose may not be feasible. However with respect to chemotherapy, the effective treatment or prevention of mucositis may allow an increase in the maximum tolerated doses of treatment and improved quality of life for cancer patients during and after treatment. Conceivably, this would also translate to an increased likelihood for cancer remission.

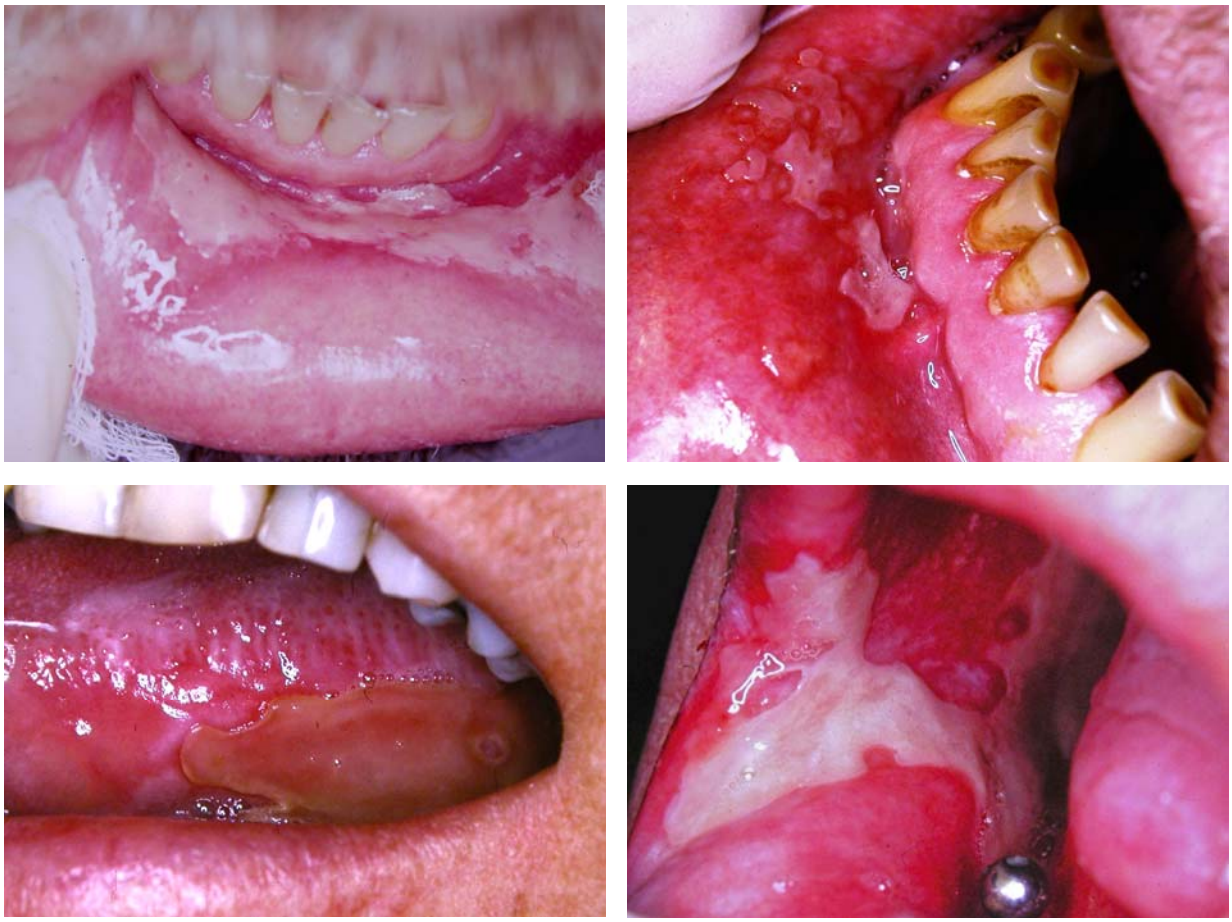

Figure 1.1

Clinical photographs demonstrating the clinical appearance of oral mucositis during cancer treatment which presents as widespread, irregular ulceration of the oral mucosa with surrounding erythema.

Mucositis increases the morbidity of patients undergoing cancer treatment, results in extended hospital stays and, in addition, re-admission rates are also increased [42, 120]. Hospitalisation of patients for supportive care and pain management because of mucositis has significant economic consequences [42, 108]. As well as being important from a clinical and economical point of view, patient perceptions of mucositis and the impact that it has on their treatment and quality of life are equally, if not more, important. A study that investigated patient reported complications of bone marrow transplantation clearly identified mucositis as the single most debilitating side effect of treatment [13]. Furthermore, opioid analgesics used the management of mucositis had secondary effects on the quality of life of patients because of adverse drug reactions such as decreased mental acuity and hallucinations [13].

The prevalence of mucositis is variable and appears to be dependent on the type of treatment given as well as the disease that is being treated. For example, mucositis occurs in 80-100% of patients undergoing so called "high-risk" regimens such as radiotherapy to the head and neck or high dose chemotherapy and stem cell (or bone marrow) transplantation [79, 120]. Furthermore, specific cytotoxic chemotherapy agents, such as 5-fluorouracil (5-FU), are associated with more severe mucositis [128]. In regimens considered to be "low-risk" for the development of mucosal toxicity, the prevalence of mucositis may be as low as 10-15%, however given the numbers of people receiving chemotherapy, this is still represents a significant number of patients that are affected by mucositis [128].

It is important that the pathobiology of mucositis is determined and fully described so that effective targeted treatment strategies can be developed. The most recent hypothesis for the pathogenesis of mucositis has implicated an important role for transcription factors and pro-

inflammatory cytokines in its development [130, 134]. The focus of this review is to examine the literature relating to the role that pro-inflammatory cytokines might play in the pathobiology of mucositis, particularly in the context of chemotherapy-induced mucositis, as well as examining animal models currently used to study mucositis pathobiology. Finally, the review will discuss the role of specific cytotoxic drugs in the pathobiology of mucositis.

1.2 Alimentary tract

The AT extends from the oral cavity to the anus, encompassing the oesophagus, stomach, small and large intestines and rectum (Figure 1.2). Its primary role is in the digestion and absorption of food as well as elimination of waste and water absorption. In addition, the AT has an important role in protection, particularly with respect to barriers to infection [69].

Differences are apparent in the structure of the mucosa along the AT. This is due to the different specialised roles that are played by the different regions in the AT (Figure 1.2). The oral mucosa, for example, has a predominantly protective function forming a barrier between the oral cavity and the underlying tissues, whilst the mucosa of the small intestine has an absorptive function which is reflected in its architecture where it is arranged in villi and crypts. Disruption of the normal structure of the AT tissues that occurs as a result of mucosal damage manifests clinically as the symptoms of mucositis which, as previously outlined, include mouth pain and abdominal symptoms. In addition, the risk of local and systemic infections is increased because natural defences are compromised.

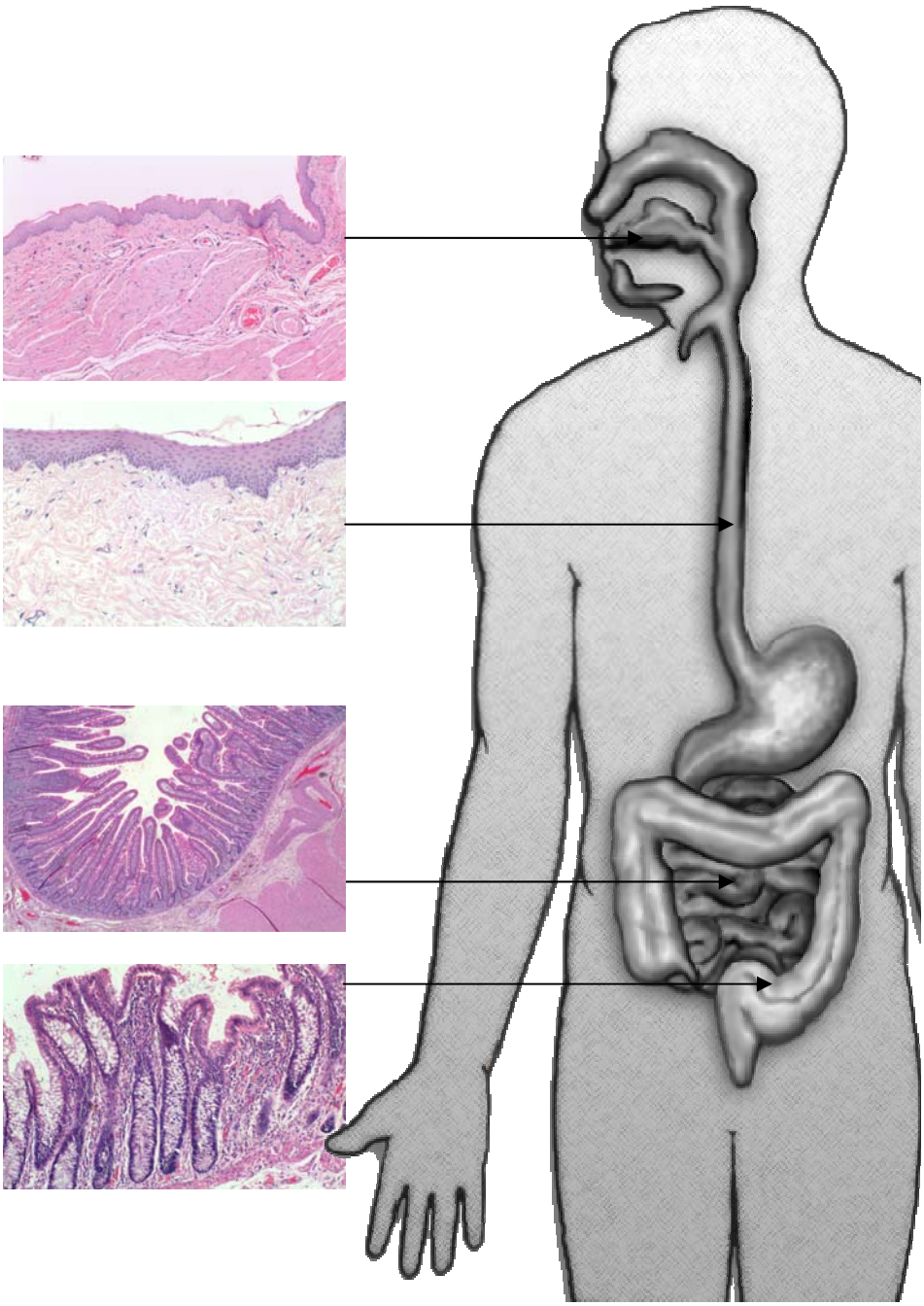

Figure 1.2

Photomicrographs illustrating the differences in histology of the mucosa at different sites of the alimentary tract.

1.3 Mucositis pathobiology

Historically, the primary mechanism through which mucositis developed was considered to be the result of direct cytotoxic effects of chemotherapy or radiotherapy on the basal cells of the epithelium which line the AT [147, 148]. It was thought that the epithelial cells were particularly vulnerable to the effects of treatment because of their high cell turnover rate. Following the development of an appropriate animal model to study mucositis [143], it became clear that the pathobiology was more complex and involved an interplay between all compartments of the mucosa including the connective tissue elements as well as the epithelium. For instance, subsequent researchers investigating intestinal damage that occurred following radiation, found that the primary damage response occurred in endothelial cells [49, 99, 103, 117]. Such damage has also been shown to occur in the oral mucosa following exposure to radiation [142]. Sonis *et al* demonstrated that this primary damage to endothelial cells occurred well before any detectable changes were apparent in the epithelium [142].

In 1998 Sonis proposed a four stage model that described the stages involved in the development of oral mucositis [134]. This model was subsequently modified to comprise five continuous overlapping phases [130, 134, 139] (Figure 1.3). The first of these phases is described as an *initiation phase*. This occurs immediately following exposure to cytotoxic agents and results in direct tissue damage to mucosal components as a result of the production of reactive oxygen species. This is followed by an *upregulation and message generation phase*, an important element of which is the activation of transcription factors, in particular nuclear factor-κB (NF-κB). This transcription factor is activated in response to chemotherapy and radiotherapy and is responsible for the upregulation of up to 200 genes that have an effect on mucosal integrity by inducing clonogenic cell death, apoptosis and

tissue injury throughout the mucosa, not limited to the epithelium [137]. NF-κB activation results in the production of pro-inflammatory cytokines, including tumour necrosis factor (TNF, formerly referred to as TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) [135]. In the context of mucositis, these cytokines have all been demonstrated in the mucosa as well as in the peripheral blood of patients undergoing cancer treatment [46, 59]. The third phase involves *signal amplification* and occurs as a consequence of the pro-inflammatory cytokines acting via positive feedback mechanisms causing further activation of NF-κB and subsequent increased production of cytokines. Other biologically active proteins or pro-inflammatory mediators, such as cyclo-oxygenase-2 (COX-2) [135], are upregulated and initiate an inflammatory cascade leading to activation of matrix metalloproteinases whose production elicit further tissue damage [152]. The *ulcerative phase* develops where there is a loss of the epithelial layer of the mucosa accompanied by bacterial colonisation. It is not until this stage that mucositis becomes clinically evident. The patient will experience significant pain and possibly abdominal symptoms; the risk of systemic infection is increased, particularly if the patients are immunosuppressed. Furthermore, bacterial products can stimulate further amplification of cytokine production leading to further potentiation of tissue injury [134]. Following cessation of the cancer treatment, the final *healing phase* occurs. This phase results in the restoration of normal mucosal appearance at the clinical level. Re-epithelialisation of the mucosa initiated by signals from the extracellular matrix is observed histologically. The healing phase is probably the least well understood and studied with respect to mucositis pathobiology.

It is important to reiterate that the clinical manifestations of mucositis are not apparent until the ulcerative phase develops. A normal clinical appearance prior to this phase belies the fact that a complex myriad of biological events is thought to take place subclinically. Elucidation

of the exact mechanisms involved could potentially reveal therapeutic targets to enable the cessation of mucositis development before clinical signs and symptoms, such as ulceration, occur. Furthermore, it has also been highlighted that although healing does occur, ultrastructural and histological evidence indicates that the structure of the mucosa is altered for an extended period following the completion of treatment [55, 95]. The ability of the mucosa to withstand further trauma or insult may therefore be compromised.

A review by Anthony *et al* highlighted the fact that there are various factors that impact on the risk or likelihood that a patient will develop mucositis [5]. That review divided these "drivers" of mucotoxicity into either global factors or tissue specific factors, including those at a cellular or molecular level. It was postulated that it was the presence and interaction of these factors which led to an increased risk of mucositis and may explain the increased propensity for mucositis that is apparent in some individuals compared to others who undergo comparable treatment regimens. Other risk factors for oral mucositis have recently been reported in the literature. Cheng *et al* examined risk factors in children undergoing chemotherapy and found that, in this cohort, low body weight, neutropenia and elevation in creatine and transaminase levels increased the risk of developing oral mucositis [27]. Furthermore, a recent paper by Aprile *et al*, investigating associations between acute toxicities in colorectal patients suggested that many "co-occurring toxicities" may share common pathobiology [8]. These recent papers highlight the fact that additional work needs to be completed to determine mucosal susceptibility to injury subsequent to cancer treatment and its role in the context of other toxicities as this may lead to better outcomes for patients.

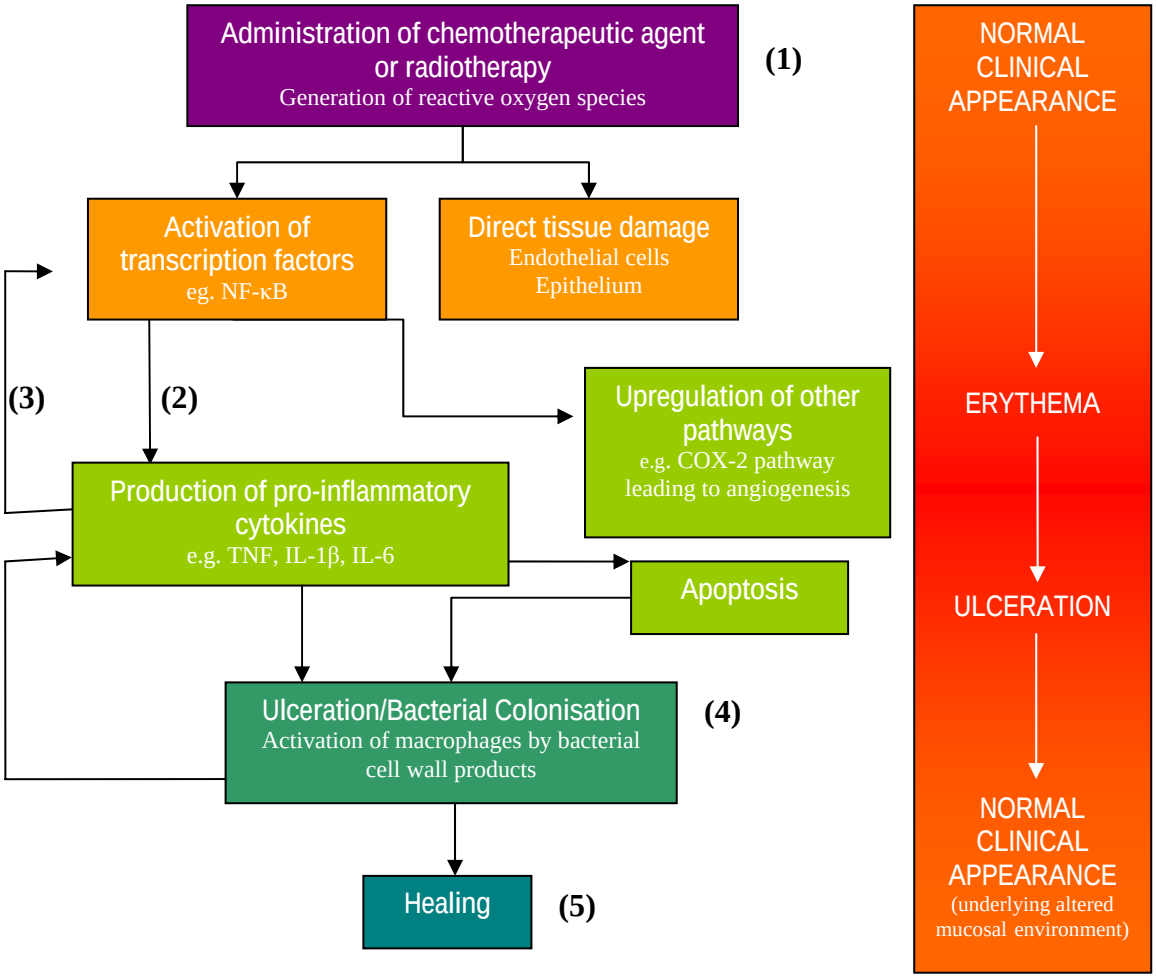

Figure 1.3

Diagram illustrating the mucosal and clinical changes that occur following chemotherapy or radiotherapy which, according to the current hypothesis, lead to mucositis (Sonis 1998; Scully et al, 2003). The 5 overlapping stages are demonstrated (1) Initiation; (2) Upregulation and message generation; (3) Signalling and amplification; (4) Ulceration; (5) Healing.

1.4 Evidence for the role of nuclear factor-κB and pro-inflammatory cytokines in mucositis

Cytokines are inducible chemical messengers which are produced by a variety of cells throughout the body. They are low molecular weight glycoproteins and are involved in both the inflammatory and immune responses [36]. The role of cytokines in the pathogenesis of mucositis has been investigated by various studies. Sonis *et al* found that cytokines that targeted epithelial proliferation such as epidermal growth factor [138] and transforming growth factor-β3 [144] were able to modify the course of mucositis. Antin and Ferrara [6] described dysregulation of TNF and IL-1 production following conditioning regimens that included radiation.

The recent advent of more targeted therapy for mucositis in specific patient populations using Palifermin (recombinant keratinocyte growth factor-1), has provided further support for the role cytokines play in the development of mucosal toxicity [123, 146]. One of the effects of Palifermin is to cause alteration of cytokine profiles resulting, amongst other things, in downregulation of TNF [123].

As indicated previously, NF-κB is thought to play an important role in the pathobiology of mucositis, particularly with respect to the upregulation and subsequent expression of the pro-inflammatory cytokines TNF, IL-1β and IL-6.

1.4.1 Nuclear factor-κB

A thorough review of the role of NF-κB in diseases and its potential involvement in the pathology of mucositis has been previously published [135]. Collectively the NF-κB family comprises a group of 5 different members (NF-κB 1(p50/p105), NF-κB 2(p52/p100), p65(Rel

A), Rel 3 and cRel) which have a diverse range of biological effects [50, 135]. This is indicated by the large number of target genes that are influenced by NF-κB activation. These target genes include various cytokines (including, as already mentioned, TNF, IL-1β and IL-6), immunoreceptors, cell adhesion molecules, acute phase proteins, stress response genes and cell-surface receptors [135]. Generally NF-κB plays important roles in inflammatory and immune responses as well as in the development of haematopoietic cells, keratinocytes and lymphoid structures. With respect to inflammation, NF-κB can have both pro- and anti-inflammatory effects depending at which stage during the inflammatory process the pathway is stimulated [60]. The activation of NF-κB can be facilitated by various factors including both radiation and chemotherapy [123] as well as infectious agents, physiological stress and inflammatory cytokines [135].

The evidence for NF-κB's role in mucositis is based on various observations as outlined by Sonis [135]. Cell death that results from cytotoxic chemotherapy is attributed to programmed cell death (PCD) or apoptosis – this occurs in both normal cell populations and in neoplastic cells. NF-κB plays an important role in the process of apoptosis [135]. A recent study provided data on morphological and ultrastructural changes in the oral mucosa following cytotoxic chemotherapy [55]. This study demonstrated that the level of apoptosis occurring in the oral mucosa peaked at 3 days following chemotherapy at 400 times the level seen in the healthy controls. A further study, conducted within the same patient group, demonstrated that tissue levels of NF-κB were also elevated following chemotherapy [95]. It has also been postulated that reactive oxygen species produced in tissues by ionising radiation may cause activation of NF-κB in normal alimentary mucosa and cause increased apoptosis, manifesting clinically as mucosal damage [161]. Further circumstantial evidence for the role of NF-κB in the pathogenesis of mucositis is that the onset and severity of mucositis are associated with

its activation and that inhibition of pro-inflammatory cytokines and reduction in bacterial load leads to decreased levels of NF-κB, which clinically translates to a reduction in mucositis [98, 142].

As well as having pro-apoptotic properties NF-κB can, in some situations, demonstrate anti-apoptotic properties [16, 135]. Studies have demonstrated that generally NF-κB has a pro-apoptotic effect on normal cells and an anti-apoptotic or cytoprotective effect on tumour or neoplastic cells [135]. The reason for this dichotomy of action is unclear. Sonis suggested various explanations for this. For example, stimulation of different Bcl-2 genes occurs via NF-κB activation [135]. The Bcl-2 family of genes can have either pro-apoptotic or anti-apoptotic actions. In the rat and in humans, increased expression of the Bcl-2 members Bax and Bak has been demonstrated in the small intestine following cytotoxic chemotherapy [20]. These Bcl-2 family members have been demonstrated to promote apoptosis [149]. Alternative explanations for the duality of NF-κB's action include alternative pathway mediation between neoplastic and normal cells resulting in amplified tissue damage in normal cell populations compared to neoplastic populations. Other factors such as the oral environment and specific features such as epithelial type, local microbial flora and underlying pathology may also be important influences [5, 135].

1.4.2 Tumor necrosis factor

TNF is a pleiotropic protein that was initially isolated from mouse serum following exposure to bacterial endotoxin [25]. It was demonstrated that TNF replicated endotoxin's ability to cause haemorrhagic necrosis in methylcolantrene-induced sarcomas [25]. Subsequent to this discovery, it became evident that this protein belonged to a larger group, or family, of proteins that have both beneficial, as well as potentially damaging, effects throughout the

body [62]. The beneficial roles played by members of the TNF family include inflammatory and protective immune responses as well as being important factors in organogenesis of secondary lymphoid organs and lymphoid structure maintenance [62]. Conversely, TNF has also been shown to have a host damaging role in the context of sepsis and autoimmune diseases such as rheumatoid arthritis and inflammatory bowel disease (IBD) [62, 97].

TNF is predominantly produced by activated macrophages, NK cells and T lymphocytes [106]. The two receptors for TNF are expressed either on all cell types (TNF-R1) or only on immune or endothelial cells (TNF-R2) [106]. TNF through the interaction with TNF-R1 causes various cellular events including activation of the caspase cascade which leads to apoptosis. TNF interaction with TNF-R1 also leads to activation of NF-κB. TNF-R2 signalling is less well characterised, however it is known that this receptor does not possess a death domain and can therefore not directly precipitate apoptosis. The role of NF-κB activation leading to apoptosis via TNF-R2 signalling is unclear [106]. In addition to causing the "classical" caspase-dependent form of apoptosis or PCD, TNF has also been demonstrated to induce necrosis-like caspase-independent PCD [106].

Clinically it has been shown that increased serum levels of TNF occur in patients who have undergone bone marrow transplantation and that this event precedes the development of major transplant related complications [15, 63]. Other researchers have demonstrated elevated TNF levels occurring in association with non-haematological toxicities [46, 59, 125, 131]. Inhibition of TNF using agents such as pentoxifylline reduced these non-haematological toxicities [15]. With respect to mucositis, various animal and human studies have shown a decrease in the occurrence or severity of mucositis following administration of TNF inhibitors [15, 47, 92]. Interestingly, Orlicek *et al* demonstrated that isolates from viridans streptococci were able to induce TNF production by murine macrophages [115]. These

organisms are normal commensal flora in the mouth and respiratory tract, the induction of TNF by these bacteria therefore may be important in the context of mucositis development. This is particularly so in the ulcerative phase of the tissue damage process resulting in further amplification of pro-inflammatory cytokine production and subsequent further tissue damage. It has been demonstrated, using a hamster model of 5-FU induced mucositis, that administration of pentoxifylline and thalidomide, both of which inhibit cytokine synthesis, had a protective effect [92]. These authors concluded that this indicated an important role for TNF in the pathobiology of 5-FU induced oral mucositis.

1.4.3 Interleukin-1β

IL-1β is a multifunctional cytokine that has an affect on a wide variety of cell types and also interacts with many other cytokines [35]. IL-1β is part of a family of cytokines which also include IL-1α and IL-1 receptor antagonist (IL-1Ra). The latter molecule binds to each of the two IL-1 receptors.

IL-1β has multiple biologic effects which have been demonstrated in *in vitro* and *in vivo* including systemic reactions such as fever and increased gene expression of a range of genes including pro-inflammatory cytokines and pro-inflammatory mediators. IL-1β production can be stimulated by both microbiological and non-microbiological factors. The latter includes, among many things, other cytokines and irradiation [35]. Along with TNF, IL-1β is an important cytokine that is involved in the activation of the NF-κB pathway. In fact IL-1β and TNF have been reported to have a synergistic effect, for example causing induction of endothelial adhesion molecules essential for the initial phases of the inflammatory response [36].

Local tissue levels of IL-1β and TNF have been demonstrated to markedly increase in animal models of radiation-induced oral mucositis concurrently with the development of mucositis [142]. IL-1β may also have a role to play in the healing phase of mucositis development [17]. There is, however, a paucity of data in the literature about the exact role that IL-1β plays in the context of mucositis pathobiology.

1.4.4 Interleukin-6

Like TNF and IL-1β, IL-6 has a broad range of biological activities which affect a range of target cells. It is particularly involved in the immune response and in the pathogenesis of inflammatory diseases such as rheumatoid arthritis, Castleman's disease and Crohn's disease [114, 155]. Initially, it was identified as a factor that induced B-cells to produce immunoglobulins [112, 114]. It is produced by a variety of cell types including T and B lymphocytes, fibroblasts, keratinocytes, endothelial cells and some tumour cells [82, 112].

IL-6 production is induced by TNF. Conversely, TNF is strongly inhibited by IL-6 thereby forming an effective negative feedback loop which inhibits the activation or hyperactivation of the pro-inflammatory cytokine cascade [46]. IL-6 therefore, can have anti-inflammatory as well as pro-inflammatory effects [107].

In the context of intestinal damage, elevation of IL-6 levels have been observed in both the serum and tissues of patients with IBD and, in addition, the levels of IL-6 correspond with the severity of the disease [157]. In this situation, epithelial cells and mononuclear cells in the lamina propria are the major source of this cytokine [157]. IBD appears to result from interactions between antigen presenting cells and local microflora leading to activation of

mucosal T cells with subsequent release of pro-inflammatory cytokines [111]. It has been demonstrated that anti-IL-6 receptor antibodies can block these inflammatory processes in IBD [111]. Furthermore, IL-6 can induce activation of NF-κB in the intestinal epithelia [157] and it is postulated that it is via this NF-κB activation that IL-6 exerts its biological effects leading to inflammatory diseases such as IBD and rheumatoid arthritis [157]. Again, as is the case with IL-1β, there is little data on the specific role of IL-6 in the pathobiology of mucositis.

1.5 Animal models

Various animal models have been developed to investigate oral mucositis with respect to the changes that occur in mucosae subsequent to the administration of cytotoxic agents and to determine the effects of antimucotoxic medication. The choice of animal model depends on various factors such as accessibility to the animal, cost and, of course, what is being investigated. In addition, many animal models have been used to study radiation-induced mucositis; these, however, are beyond the scope of this review.

1.5.1 Hamsters

The first animal model to investigate chemotherapy-induced mucositis involved the use of Golden Syrian hamsters following administration of 5-FU [143]. Clinical and histological evaluation indicated that the changes that occurred in the mucosa were similar to that seen in humans with mucositis. In addition, the changes were also influenced by the degree of myelosuppression experienced by the animals. Subsequent studies using this model, or modified versions of it, have been used to investigate various aspects of mucositis including pathobiology and treatment as well as mucotoxicity of cytotoxic medication [1, 4, 28-30, 80, 92, 98, 105, 110, 132, 133, 140-142, 145].

Some of the first studies using the hamster model investigated the roles of epidermal growth factor (EGF) [138], transforming growth factor-β3 (TGF-β3) [140, 144] and recombinant human interleukin-11 (rhIL-11) [80, 133, 145] in the pathobiology of mucositis.

Early studies using the hamster as a model for mucositis investigated features relating to the biology of mucositis and the susceptibility of mucosa to injury. EGF is a protein that

stimulates the growth and differentiation of epithelial cells [44]. Early on it was considered that susceptibility to mucosal damage may be related to the rate of epithelial cell replication. Sonis *et al*, demonstrated that, by stimulating epithelial basal cell turnover rate by administration of EGF, mucosal injury was increased, supporting the concept that the epithelial turnover affects the susceptibility of the mucosa to chemotherapy [138].

TGF-β is a regulatory growth factor that has the ability to reversibly arrest proliferating cells in the G1 phase of the cell cycle. Sonis *et al*, demonstrated that topical application of TGF-β3 to the oral mucosa of hamsters prior to administration of 5-FU reduced various parameters related to mucositis including incidence, severity and duration as well as other factors such as weight-loss attributed to chemotherapy [140]. Conversely, survival in this animal model was increased. Again, this study demonstrated that mucosal susceptibility to chemotherapeutic agent may be affected by epithelial cell turnover. A subsequent study that demonstrated the proliferation of basal cells in the epithelium by measuring proliferating cell nuclear antigen (PCNA) confirmed that topical application of TGF-β3 significantly reduced basal cell replication in the oral epithelium of the hamster [144].

IL-11 is a pleiotropic cytokine that causes stimulation of bone marrow proliferation and has been demonstrated to ameliorate mucosal injury following 5-FU administration in a hamster model of mucositis [80]. Subsequent studies further demonstrated that the administration of rhIL-11 had beneficial effects with respect to frequency, severity and duration of mucositis as well as weight loss [133, 145]. The animal model developed by Sonis *et al* [143] was modified to investigate radiation-induced mucositis, specifically relating to the mechanism behind the action of IL-11 on the progression of mucosal damage [142]. rhIL-11 was demonstrated to attenuate mucositis resulting from radiation in the hamster model. This was attributed to the

effect of IL-11 in maintaining mucosal integrity as well as causing a reduction in pro-inflammatory cytokine expression by inflammatory cells within the mucosa. This study provided further evidence for the interplay that occurs between epithelial and connective tissue compartments of the mucosa in the development of mucosal injury.

Other studies using the hamster model to investigate various aspects of mucositis pathology have included Sonis *et al*, who investigated COX-2 expression in experimental radiation-induced mucositis [141] and Leitão *et al* who investigated the role of nitric oxide (NO) on the pathogenesis of 5-FU-induced oral mucositis [90]. Both of these studies demonstrated the complexities of mucositis pathogenesis and the multiple pathways that may be involved in the development of mucosal injury.

Studies have also employed the hamster model to describe potential treatment strategies for mucositis. The use of fibroblast growth factor-20 (FGF-20) or Velafermin has been shown to be effective in helping to maintain mucosal integrity in the context of experimental radiation- and combined chemotherapy/radiation-induced mucositis [4]. The TNF-inhibiting effects of pentoxifylline and thalidomide were investigated for the management of experimental oral mucositis using the hamster model [92]. As well as demonstrating that these drugs had a protective effect for the development of mucositis in the animals, this study also demonstrated the potentially important role for TNF in the pathogenesis of mucositis as already described in this review. The use of topical granulocyte-macrophage colony stimulating factor (GM-CSF) has also been evaluated using the hamster model of oral mucositis [28]. As well as having a beneficial effect on mucositis, this study provided additional support for the role of pro-inflammatory cytokines in the development of mucositis. GM-CSF administration appeared to cause a decrease in the expression of the pro-

inflammatory cytokines TGF-β, IL-2, TNF, IL-1β and β-actin in the oral mucosa of the hamsters.

Patients undergoing cancer treatment are susceptible to increased oral infections, the most common of which are due to *Candida albicans*, a commensal organism in the mouth. Various studies have demonstrated that infections may exacerbate the severity of mucositis, although the role of antimicrobial and antifungal agents in the treatment of mucositis is debatable [11]. Regardless of this, the use of a suitable delivery mechanism to effectively administer antifungal agents may improve oral health in some patients who develop secondary infections. Aksungur *et al* used a hamster model, based on that developed by Sonis *et al*, to develop a medication delivery system for nystatin for the treatment and prophylaxis of oral mucositis [1]. This study demonstrated a beneficial effect of the use of topical nystatin with respect to mucositis scores and survival.

The hamster model developed by Sonis *et al* has also been used in drug development [26]. Protegrins are molecules with antimicrobial activity against gram-positive and negative bacteria as well as yeasts. Chen *et al* described the development and selection of an appropriate protegrin for the potential treatment of mucositis using the hamster model [26].

1.5.2 Mice

The murine model represents another commonly used animal model to investigate aspects of mucositis. Again the diversity of studies is wide and encompasses investigation into the treatment of mucositis [10, 18, 37, 38, 45, 66, 109, 126] and pathobiology [12, 33, 70, 158].

Murine models have been used to test treatment strategies related to toxicities associated with specific drugs. Administration of doxorubicin has been shown to cause apoptosis in the intestinal epithelium of rats resulting in manifestations of gastrointestinal toxicity [151]. Using mice, Morelli *et al* and Balsari *et al* investigated the effect of systemic and topical applications of an anti-doxorubicin monoclonal antibody for the treatment and prevention of mucositis [10, 109]. They demonstrated reduced signs of gastrointestinal toxicity in mice following the oral administration of the anti-doxorubicin antibody [109]. In the oral mucosa, using the murine model, epithelial apoptosis due to doxorubicin was eliminated by the topical application of the anti-doxorubicin antibody [10].

The development of Palifermin or keratinocyte growth factor (KGF) for clinical use was preceded by numerous pre-clinical studies, many of which involved murine models to determine any adverse effects of KGF as well as to determine the effectiveness of KGF in preventing mucosal toxicity. Early investigations using murine models of chemotherapy-induced injury where KGF was administered prior to chemotherapy using 5-FU or methotrexate (MTX) demonstrated that survival was improved as well as histological parameters in the small intestine such as increased villus height and crypt depth [45]. Subsequent studies confirmed this and looked at other parameters such as cell proliferation markers as well as radiation-induced and radiochemotherapy-induced mucositis [18, 37-41, 122].

Other studies that have investigated treatment strategies for mucositis using murine models have included the addition of short-chain fatty acids to the diet of mice treated with Ara-C (cytarabine) [126]. This study demonstrated that histological features of the small intestinal mucosa such as villus height were increased and that inflammation and necrosis was decreased. Woo *et al*, demonstrated in a mouse model that mucositis due to cyclophosphamide could be reduced by the administration of clarithromycin [158]. Huang *et al* investigated the role of EGF administration in the treatment of chemotherapy-induced intestinal injury in mice [66]. Transgenic mice, that over-express EGF, were administered 5-FU. These mice did not demonstrate any reduced effects of mucosal damage compared to control mice, for example, features such as loss of mucosal histological architecture and weight loss were no different between the groups. Likewise mice treated with exogenous EGF after receiving 5-FU did not demonstrate a beneficial effect. These results appear to concur with those of the early studies done in hamsters as outlined previously [138].

Mice have also been used in studies investigating aspects of mucositis pathobiology. Kang *et al* investigated the role of caspase activation, particularly caspase-11, in the context of mucosal injury subsequent to melphalan administration in mice [70]. Caspase-11 is a murine caspase which is 60 per cent homologous with human caspase-4 [71]. Caspase-11 is an important regulator of apoptosis in various pathological conditions [71]. In the context of mucositis however, gastrointestinal damage due to melphalan appeared to be independent of caspase-11 and therefore this study indicated that other pathways leading to intestinal apoptosis might be involved [70].

Beck *et al* investigated the role of trefoil factors in chemotherapy- and radiotherapy-induced mucositis in mice [12]. They found that mice deficient in intestinal trefoil factor were more susceptible to mucosal damage following chemotherapy or radiotherapy compared to their non-deficient counterparts. In addition, supplementary intestinal trefoil factor included in the diets of mice reduced the severity of intestinal mucositis following chemotherapy or radiotherapy. This study demonstrated the important role of protective factors of the AT in the maintenance of mucosal integrity.

1.5.3 Rats

Rats have also been used widely in studies investigating mucositis pathobiology [12, 19, 20, 53, 94] and treatment [32, 54, 56, 64, 156]. In addition, there have been studies which have been designed to determine non-invasive methods for the detection of intestinal mucositis [31, 65].

One of the most extensively used models to investigate chemotherapy-induced mucositis is that that employs the use of the female Dark Agouti rat [19, 20, 53, 56, 57, 94]. This model has been demonstrated to effectively parallel the development of mucositis that occurs in humans. This model has the added benefit of the rats bearing tumours; the "tumour effect" can be studied as well as mucosal damage. Various cytotoxic chemotherapy agents have been investigated with this model including irinotecan, MTX and 5-FU.

Gibson *et al* investigated the effect of KGF administration on small intestinal mucositis and tumour growth following administration of MTX [56]. The study found that although KGF administration increased intestinal growth prior to chemotherapy, it provided no benefit

with respect to mucositis. KGF administration appeared to reduce tumour growth; this is an important factor when an agent is considered as a potential therapeutic option. A subsequent study by Gibson *et al* investigated another potential therapeutic agent, IL-11 [57]. The administration of this pleiotropic cytokine to rats with breast carcinomas resulted in attenuation of MTX-induced mucositis measured by maintenance of intestinal weight and morphometrical features. This appeared to occur via the induction of compensatory crypt cell proliferation rather than by inhibiting apoptosis. Furthermore, the administration of IL-11 did not increase the growth of the tumours in the rats [57].

The DA rat model has also been used to characterise the small intestinal damage that occurs following the administration of specific drugs [53]. Irinotecan (see 1.6.1) causes severe diarrhoea, the mechanism by which this occurs is poorly characterised. Gibson *et al* demonstrated that irinotecan administration in tumour-bearing rats resulted in apoptosis and hypoproliferative changes in the small and large intestines. In addition, goblet cell number and mucus secretion were affected in the large intestine. The diarrhoea that is induced by irinotecan was attributed to these factors collectively. The effect of Palifermin, or KGF, on diarrhoea and survival following irinotecan administration was also investigated by Gibson *et al* [54]. It was found that in tumour-bearing rats, diarrhoea was reduced and survival increased following KGF administration without promoting tumour growth [54].

Bowen *et al* further characterised the effect of irinotecan administration in tumour-bearing DA rats by investigating changes in gene expression in the small intestine [19]. It was found that irinotecan resulted in differential regulation of various genes associated with the mitogen-activated protein kinase (MAPK) signalling pathway which is involved in the caspase-cascade, the activation of which ultimately results in apoptosis.

Obviously animal models do not always accurately replicate what happens in humans in a true clinical situation; however these studies are important in determining events that occur in the mucosa following to administration of chemotherapy or radiation. Another important difference between these animal models and humans are the histological differences between the mucosa of animals and human subjects, particularly with respect to oral mucosa. In humans, the oral mucosa is largely non-keratinised with the exception of specific sites such as the dorsal aspect of the tongue and masticatory mucosae. In the rat, however, the oral mucosal epithelium is generally a keratinised phenotype giving it increased resilience to physical damage. With respect to oral mucositis, many animal studies employ mechanical irritation to induce ulceration of the mucosa [1, 28-30, 90, 92, 105, 110, 132, 143, 145]. This potentially adds a further complicating issue to these studies in that they appear to be based on the historical paradigm that considers mucositis to be a predominantly epithelial phenomenon and can only be said to occur when there is clinical evidence of ulceration, that is, loss of epithelium. If mucositis is considered to be a true *mucosal* phenomenon, then clinical evidence of ulceration shouldn't be strictly necessary. Whilst different types of epithelium may be more resilient and resistant to injury, the events occurring in the underlying connective tissue should conceivably be consistent regardless of whether or not they lead to frank ulceration. It is conceivable that induction of ulceration by mechanical irritation of the mucosa may initiate biological events which might complicate or even mask the histological and molecular changes induced by chemotherapeutic agents or radiation.

To date, all animal models have focussed predominantly on one area of the AT, particularly the oral cavity. Until recently, there have been no studies that have compared the changes that occur in different sites of the AT. In Chapters 3 and 4 of this thesis, results of studies are

presented which investigate the changes that occur along the AT comparing the effects of different cytotoxic agents using the female DA rat model.

1.6 Specific cytotoxic chemotherapeutic agents

In the literature, the development of mucositis has largely been assumed to be independent of the agent causing it. This idea, however, is simplistic in that different cytotoxic agents obviously have different modes of action and, in themselves, affect different molecular pathways in both neoplastic and normal cells. Also, many chemotherapeutic agents are given in combination; a fact that further complicates the characterisation of the pathways affected by these agents. It is therefore conceivable that, although the clinical outcomes and the histological evidence of tissue damage may be similar, the process of mucositis development may also differ according to cytotoxic agent administered. If this is the case, there would therefore be important implications for the effective treatment of mucositis.

1.6.1 Irinotecan

Irinotecan hydrochloride is a chemotherapy agent that has been used to treat various types of solid tumours (Figure 1.4A). It exerts its cytotoxic effect by inhibiting DNA topoisomerase 1; this requires conversion of irinotecan to its active metabolite SN-38 by carboxylesterase [51]. Irinotecan causes severe side effects which limit the dose of drug that can safely be delivered to patients. These side effects include myelosuppression and severe diarrhoea [2, 72]. The features of irinotecan-induced damage to the small intestine are well documented and include an increase in apoptosis in the crypts as well as effacement of the villi in the small intestine [53]. Increased apoptosis within the crypts of the colon mucosa have also been described [53]. Clinically this damage manifests as diarrhoea, abdominal bloating and pain [76, 77, 79, 120].

The diarrhoea that results following irinotecan administration occurs in two phases. The first occurs within 24 hours of administration of the drug and is considered to be a result of its cholinergic effects. This diarrhoea is treated with atropine and, in most cases, is not severe [2]. The second phase of diarrhoea develops 5 to 11 days following administration of irinotecan [2]. This phase of diarrhoea is more prolonged and can be potentially life-threatening due to severe dehydration and resulting electrolyte imbalance [2, 153].With respect to the late onset diarrhoea, the toxic effects of irinotecan are thought to be potentiated by factors related to the intestinal microflora [2]. Following conversion by carboxylesterase to its active metabolite SN-38 in the liver, detoxification occurs which involves glucuronidation to form the inactive SN-38G. SN-38G is secreted into the bile and, from there, is emptied into the small intestine. Within the lumen of the small intestine, it has been demonstrated that bacterial β-glucuronidase can convert SN-38G back to the active SN-38, where it causes direct damage to the epithelial cells of the intestinal mucosa. Antimicrobial strategies to alter the intestinal microflora have been trialled in order to reduced the levels of bacterial β-glucuronidase in the intestine and therefore reduce the degree of mucosal damage.

The cytotoxic effects of irinotecan are also potentiated by the fact that TNF production is increased by the drug [58]. The DNA damage that is caused by irinotecan, in addition to elevated TNF, activates NF-κB thereby effecting tumour cell death via pro-apoptotic pathways. This also has important implications in the pathobiology of mucositis. However, as already mentioned, NF-κB activation can have anti-apoptotic effects with respect to neoplastic cells. It is thought that tumour resistance to irinotecan may be enhanced by pathways promoting cell survival and also counteract the effect of TNF activation [160].

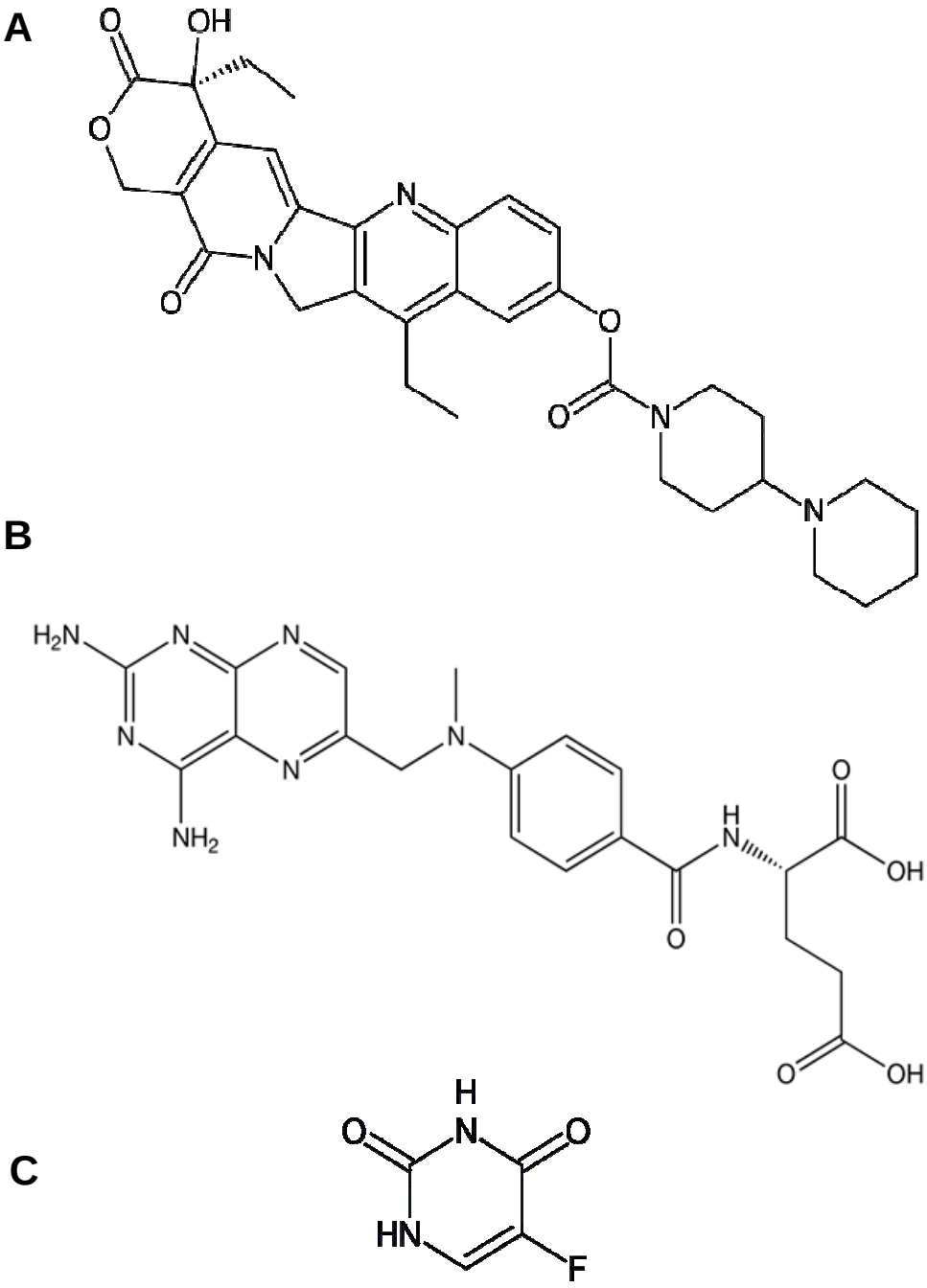

Figure 1.4

Chemical structure of (A) irinotecan, (B) methotrexate, (C) 5-fluorouracil

1.6.2 Methotrexate

MTX is a commonly used drug for the treatment of various neoplastic diseases [23] as well as some inflammatory diseases (for example, rheumatoid arthritis) (Figure 1.4B). It belongs to a class of drug that acts by antagonising folate, although its exact mode of action is not fully understood [100]. MTX has anti-inflammatory effects that are a result of various molecular events including decreased gene expression of pro-inflammatory cytokines IL-1, 2, 6 and interferon-γ [86]. It also inhibits COX-2 synthesis and chemotaxis of neutrophils [86]. In addition, Majumdar and Aggarwal demonstrated that, in Jurkat cells, MTX caused suppression of NF-κB that was induced by inflammatory factors such as TNF [100]. All of this evidence helps to explain the beneficial effect of MTX in treatment of inflammatory diseases, however does not indicate how the development of mucositis might occur in the AT associated with MTX administration. It is well documented that MTX causes oral mucositis [23] and damage to the small intestinal mucosa [57].

In spite of its reported anti-inflammatory effects, MTX has been shown to have other actions that may explain its associated toxicity, particularly in the gastrointestinal mucosa. For example, MTX administration causes apoptosis of intestinal epithelial cells in rat models of mucositis [52]. MTX-associated apoptosis specifically occurs in the intestinal crypts and causes villous atrophy [57]. In addition, damage to goblet cells in the gastrointestinal epithelium as a result of MTX administration may reduce the natural, non-specific defences of the epithelium by reducing mucin secretion and other proteins such as the trefoil factors that have important protective roles in the gut [33, 155]. Also, in the context of mucositis following MTX administration, production of TNF by mucosal T cells and macrophages is increased in response to lipopolysaccharide derived from commensal gut flora [34]. This

indicates that the immune cells within the mucosa may, in themselves, contribute to mucositis development [34].

1.6.3 5-Fluorouracil

5-FU is a commonly used chemotherapeutic agent and is well documented to cause oral mucositis. This drug is a uracil analogue (Figure 1.4C) and its major effect is to inhibit nucleotide metabolism. 5-FU has been demonstrated to inhibit NF-κB activation [7, 9]. It is therefore likely that the pathways through which mucosal damage is mediated is different from the other, previously described cytotoxic drugs. Pritchard *et al* demonstrated in a murine model that the intestinal toxicity of 5-FU occurs as a results of the combined effects of apoptosis and inhibition of cell proliferation [124]. These events led to reduced cellularity in both the intestinal crypts and villi. Furthermore, the same authors demonstrated that the changes that occurred in the intestinal mucosa in mice subsequent to 5-FU administration were p53 dependent [124]. Leitão *et al* investigated the role of nitric oxide (NO) in the pathogenesis of 5-FU-induced experimental oral mucositis using a hamster model [90]. NO is known to induce apoptosis in various cell types and is associated with p53 and changes in the expression of members of the Bcl-2 family [91]. Therefore, whilst NF-κB may not be a significant factor in 5-FU associated mucositis, alternative pathways leading to apoptosis appear to be involved in causing mucosal damage. The exact mechanism, however, behind the development of 5-FU induced mucositis remains to be fully determined.

1.6.4 Combined chemotherapy-radiotherapy regimens

The treatment of haematological diseases has used radiation, in the form of total body irradiation, combined with chemotherapy for myeloablation prior to bone marrow or stem

cell transplantation. More recently, combined modality treatments have been investigated for other forms of neoplastic disease. In the case of head and neck squamous cell carcinoma, for example, standard treatment of surgery plus or minus radiotherapy and/or chemotherapy has an overall 4 year survival rate of 30 to 40 per cent [127]. In the specific case of tongue cancer for example, a recent study which looked at data collected between 1987 to 2004 found that 5 year survival rates were between 30 and 50 per cent [89]. Moreover, this survival rate had not improved during the 18 year period studied. Such statistics have prompted the investigation of other methods or combinations of treatment to attempt to improve survival rates [127]. Furthermore, surgery can be disfiguring (particularly in the head and neck region), affect function and consequently result in significant problems in the quality of life for patents following treatment. Treatment strategies that preserve organ function and minimise the need for invasive and disfiguring surgery have led to the use of combined modality treatments employing both chemotherapy and radiotherapy [61]. Whilst treatment outcomes may be improved by more aggressive treatment strategies, this benefit comes at a cost of increased risk of toxicity.

As mentioned elsewhere in this review, both radiotherapy and chemotherapy can cause mucositis and, when combined, the prevalence of mucositis is increased. Woo *et al* demonstrated that, in the context of bone marrow transplantation, oral ulceration occurred in over 75 per cent of patients [159]. Numerous studies reporting various combinations of combined chemoradiotherapy or induction chemotherapy followed by different schedules of radiotherapy have been reported in the literature [3, 48, 67, 127]. Comparison between these various studies is complicated because of the number of variables involved. In addition many single-institution studies have limited numbers of patients enrolled in the trials which makes the ability to form conclusive judgements about the value of the results difficult. The

prevalence of mucositis in the studies however is consistently high. One study, investigated the treatment of 127 patients with advanced squamous cell carcinoma of the head and neck cancer, Hanna *et al* reported that 64 per cent of patients developed mucositis, and 33 per cent had grade 3-4 mucositis [61]. All of the patients in the study received standard fractionation radiotherapy concurrently with at least 2 cycles of cisplatin and 5-FU. Other gastrointestinal symptoms reported in the study that could be attributed to AT mucositis included severe nausea, dehydration and electrolyte imbalance which required intravenous or enteral fluid replacement [61]. Other studies have reported similar results [3, 48, 67, 127]. The benefit in survival as a result of these new treatment regimens is variable.

1.7 Conclusions

Whilst there has been a great deal of work conducted into the pathogenesis of mucositis, the role(s) of pro-inflammatory cytokines needs to be further defined. Furthermore there have been no studies that have investigated the role of pro-inflammatory cytokines and the development of mucositis along the entire AT. The complex interplay between the cytokines themselves and the cytokines and treatment modality needs to be elucidated. Certainly it is clear that different cytotoxic drugs activate different molecular pathways and, whilst the clinical outcomes may be similar, the routes leading to those outcomes may be vastly different. This has important implications for the development of targeted treatment for AT mucositis. Further characterisation of the biological events occurring in the context of mucositis will inevitably lead to improved treatment outcomes and quality of life for patients undergoing cancer treatment.

Chapter 2

Nuclear factor-κB (NF-κB) and cyclooxygenase-2 (Cox-2) expression in the oral mucosa following cancer chemotherapy.

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2.1 Introduction

As indicated in the preceding chapter, there has been a significant amount of research into mucositis involving the use of animal models and there is a distinct paucity of research in the literature relating to mucositis pathobiology in the clinical setting. A study by Gibson *et al* provided data on morphological and ultrastructural changes in the oral mucosa following cytotoxic chemotherapy [55]. This study demonstrated that the level of apoptosis occurring in the oral mucosa peaked at 3 days following chemotherapy and was 400 times the level that was observed in the in the healthy controls. Likewise, ultrastructural changes were demonstrated in the mucosa after chemotherapy and persisted up to 11 days following treatment. Gibson's data support the 5 stage hypothesis of mucositis pathogenesis [139] in which transcription factors activated by chemotherapy, radiation therapy or reactive oxygen species play a major role in the biological cascade that leads to mucosal injury.

In the context of mucositis, the transcription factor NF-κB is considered to be one of the main "drivers" of the condition. Its activation results in the upregulation of a variety of genes and subsequent production of pro-inflammatory cytokines including tumor necrosis factor, interleukin-1β and interleukin-6, which results in increased tissue injury in all compartments of the mucosa, not exclusively the epithelium. In addition, NF-κB also causes upregulation of COX-2 which has also been implicated in increasing levels of matrix metalloproteinases, a likely mediator of tissue damage [81]. It is thought that the subsequent amplification of these biological events via positive feedback loops, as well as stimulation by bacterial cell wall products, result in the widespread tissue damage seen in the clinical setting as ulceration. The finding that NF-κB expression and COX-2 levels were elevated in the irradiated colorectum in association with histopathological changes consistent with mucositis [162] provides further support for a role for NF-κB in regimen-related mucosal barrier injury. Given

that mucositis is considered a "whole gut" phenomenon, it is suggested that the underlying mechanisms of mucosal tissue injury in the oral and colorectal region are similar. Accordingly, the purpose of the current study was to characterise the expression of NF-κB and COX-2 in the human oral mucosa following cytotoxic chemotherapy.

2.2 Materials and methods

2.2.1 Subjects

Twenty adult patients scheduled to receive cytotoxic chemotherapy for the treatment of non-head and neck solid tumours were enrolled in this study. Standard dose chemotherapy using multiple drug regimens known to be associated with mucositis was administered to all patients over a 1-4 hour time period. Four healthy adults (1 male and 3 females) with no history of cancer or previous chemotherapy, served as controls. The study was approved by the Ethics of Human Research Committee of the Royal Adelaide Hospital and was carried out in accordance with the Declaration of Helsinki. Informed consent was obtained from each subject prior to enrolment in the study.

2.2.2 Experimental design

Each patient underwent 2 punch biopsies of their buccal mucosa. One biopsy was completed prior to the commencement of a cycle of chemotherapy and the second biopsy up to 11 days after chemotherapy. Two of the 20 patients withdrew from the study following the first biopsy, one was due to subject request and another was due to withdrawal from chemotherapy treatment. Briefly, pre-chemotherapy biopsies were taken on the right side of the mouth and post-chemotherapy biopsies were taken on the left side, ensuring that the post-chemotherapy biopsies were obtained from non-ulcerated sites. The surrounding buccal mucosa was injected with local anaesthetic, and a small (4 mm) punch biopsy was taken. A single stitch was placed at the site of the biopsy if necessary. The sample of buccal mucosa was immediately fixed in 10% neutral buffered formalin.

2.2.3 Sample preparation for haematoxylin and eosin staining

Tissue samples were embedded in paraffin wax, cut at 4 μm sections and mounted on glass slides. The sections were dewaxed, rehydrated and stained in Lillie-Mayer's haematoxylin for 10 minutes. Sections were then differentiated in 1% acid alcohol and blued in Scott's tap water before being counterstained in eosin. The sections were then dehydrated and mounted prior to histological examination.

2.2.4 Immunohistochemistry

NF-κB and COX-2 expression in the buccal mucosa were detected using standard immunohistochemical techniques [162].

Serial 4 μm sections of the fixed paraffin tissues were cut and mounted on silane-coated microscope slides and dried on a hot-plate for approximately 2 hours. The sections were deparaffinised in xylene before being rehydrated through a series of alcohols and distilled water, followed by two rinses in phosphate buffered saline (PBS, pH 7.5). Antigen retrieval was carried out by microwaving the slides whilst immersed in citrate buffer (pH 6.0) for 3 minutes on high power and 15 minutes on low power. The slides were allowed to cool to room temperature (20 minutes). They were dehydrated and endogenous peroxidase was blocked by immersion in 3% H_2O_2 in methanol for 1 minute. Following this the slides were rehydrated and treated for 8 minutes with a Triton-X100 block (0.1% TX-100 in citrate buffer) before incubation. The slides were covered with 50% horse serum in PBS for 30 minutes, rinsed with PBS, and then incubated using the Avidin Biotin Blocking Kit (Vector Laboratories, Burlingame, CA) to block endogenous avidin-biotin activity. NF-κB p65 (F-6) (Santa Cruz Biotechnology, Santa Cruz, CA; sc-8008) is a affinity purified mouse monoclonal IgG1 antibody was used at a dilution of 1:2,300 and COX-2 (M-19) (Santa Cruz

Biotechnology; sc-1747) , a goat affinity purified polyclonal antibody, was used at a dilution of 1:1,700.

NF-κB or COX-2 antibody was then applied with 5% horse serum (Sigma) in PBS and the slides were left overnight (16 hours) at 4°C in a humidified chamber. Following incubation with the primary antibody, sections were washed with 2 changes of PBS and incubated with a secondary antibody (Biotinylated anti-mouse immunoglobulin G purified antibody (Vector Laboratories)) at a dilution of 1:200 with 5% horse serum for slides treated with NF-κB and biotinylated anti-goat immunoglobulin G purified antibody (Vector laboratories) of 1:200 dilution with 5% horse serum for slides treated with COX-2 antibody for 20 minutes at room temperature. Subsequent to this, the labelling reagent ultrastreptavidin peroxidise (Signet Pathology Systems Inc., Dedham, MA) was applied for 20 minutes at room temperature. Antibody binding was visualised with 3,3'diaminobenzidine tetrachloride at room temperature. The slides were then washed in two changes of distilled water and counterstained with Lillie-Mayer's haematoxylin for 5 minutes. The slides were dehydrated, cleared to xylene and mounted. Positive and negative controls were used. Positive controls for each antibody were rat breast adenocarcinoma tumour tissue, whilst the negative control was rat spleen and human tonsil for NF-κB and COX-2 respectively.

Qualitative immunohistochemistry was performed. Staining was observed using a light microscope. The intensity of NF-κB and COX-2 staining was scored as follows: 0, no staining, 1, weak staining, 2, moderate staining, 3, moderate to intense staining, 4, intense staining. This qualitative staining assessment has been previously validated by published grading systems [83-85] and is routinely used in our laboratory [20, 162]. All analyses were done in a blinded fashion by one investigator (RML).

2.2.5 Statistical analysis

Statistical significance between mean intensity scores comparing pre- and post-chemotherapy specimens was assessed using the Wilcoxon signed rank test (SAS Institute Inc).

2.3 Results

2.3.1 Subjects

Four male and sixteen female subjects with a median age of 52.4 years were enrolled for study. Patients received a range of chemotherapy regimens for a variety of tumour diagnoses (Table 2.1).

With the exception of one subject who noted post-biopsy soreness and withdrew from the study, study subjects tolerated the procedure well. One subject did not receive their anticipated cancer therapy and was consequently withdrawn from the study after their pre-treatment biopsy. Post-chemotherapy biopsies were taken at days 1 (n = 4), 2 (n = 1), 3 (n = 4), 5 (n = 3), 6 (n = 2), 7 (n = 1), 8 (n = 2) and 11 (n = 1) following infusion. The decision as to which sampling time point was assigned to a subject was made randomly.

Eleven of the 18 patients developed symptoms of mucositis. Nine patients had clinically evident mucositis as well as other oral symptoms including loss of taste, mouth dryness, a "thick" feeling over the tongue and cheek area and split tongues. Two patients had symptoms of small intestinal mucositis including nausea and vomiting (Table 2.1). None of the patients developed mucositis to a level that warranted reduction in cytotoxic treatment during their participation in this study.

Sex	Age (yrs)	Tumour type	Chemotherapy regimen	Cycle	Mucositis
Female	48	Breast	AC and CMF	3 (CMF)	No
Female	47	Breast	DOX;Docetaxel;CMF	2 (CMF)	Yes
Female	60	Breast	CMF	6	Yes
Female	55	Breast	CMF	3	Yes
Female	52	Breast	CMF	5	Yes
Female	60	Breast	CMF	2	No
Female	48	Breast	CMF	2	No
Female	55	Breast	AC	3	No
Female	63	Breast	Docetaxel	6	Yes
Female	32	Breast	AC and CMF (Withdrew)		Yes
Male	62	NHL	CHOP	1	No
Female	86	NHL	CHOP	6	No
Female	52	HL	ABVD	3	Yes*
Female	56	Colorectal	5FU/ Folinic acid	3	Yes
Female	68	Colorectal	5FU/ Leucovorin	3	Yes*
Female	62	Colorectal	5FU/ Folinic acid	2	Yes
Female	64	Colorectal	5FU/ Folinic acid	1	Yes
Male	66	Lung	CAV	3	No
Male	56	Lung	Withdrew		
Male	56	Pancreas	Streptozocin/5FU	3	Unknown

*These patients demonstrated signs of small intestinal mucositis such as nausea

Table 2.1

Characteristics of patients that participated in the study.

Drug abbreviations: AC = Doxorubicin/Cyclophosphamide; CMF = Cyclophosphasmide/Methotrexate/5-Fluorouracil; DOX = Doxorubicin; CHOP = Cyclophosphamide/Doxorubicin/Vincristine/Prednisolone; 5FU = 5-Fluorouracil
ABVD = Adriamycin/Bleomycin/Vincristine/Dacarbazine; CAV = Cyclophosphamide/Doxorubicin/Vincristine

2.3.2 Histological findings

All specimens were examined by a specialist oral pathologist (RML). Post-chemotherapy specimens were compared to the pre-treatment specimen for the same patient. In 6 of the 18 subjects no abnormal features in either the pre or post-chemotherapy biopsies were seen. Generally the histological features seen in the remaining patients were non specific and included mild to moderate chronic inflammation and mild epithelial keratinisation.

The degree of changes seen within with mucosa did not appear to be related to the timing of the biopsy post-chemotherapy, nor were any atrophic changes apparent in the specimens. The biopsies taken from the buccal mucosa of the 4 normal volunteers did not demonstrate any atypical histological features (Figures 2.1 and 2.2)

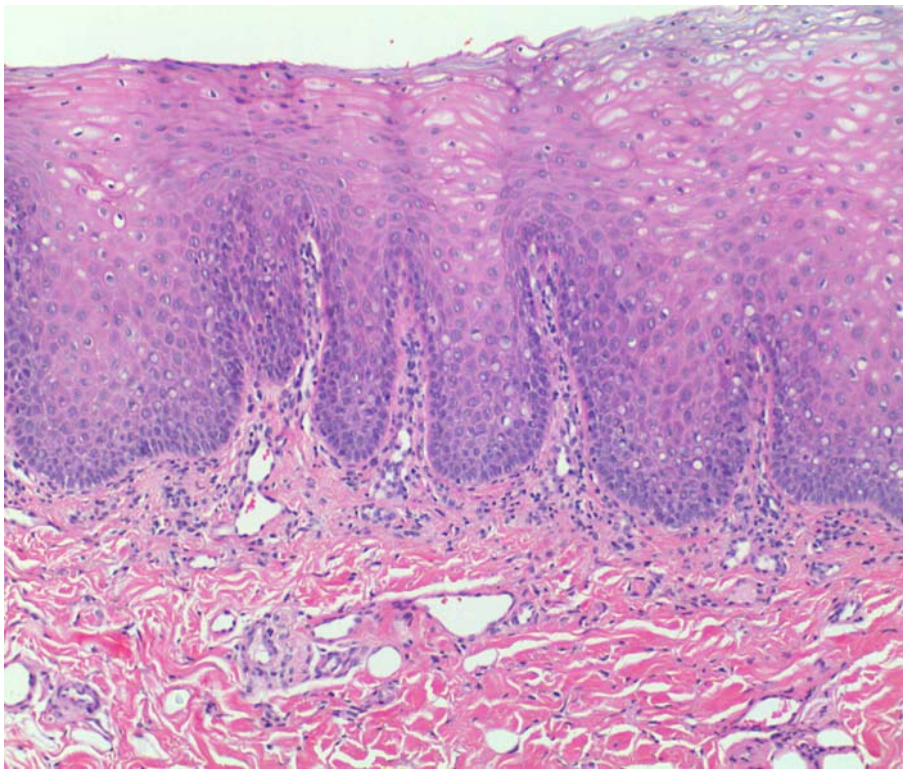

Figure 2.1

An example of the histological appearance of the buccal oral mucosa prior to chemotherapy. (Original magnification x10)

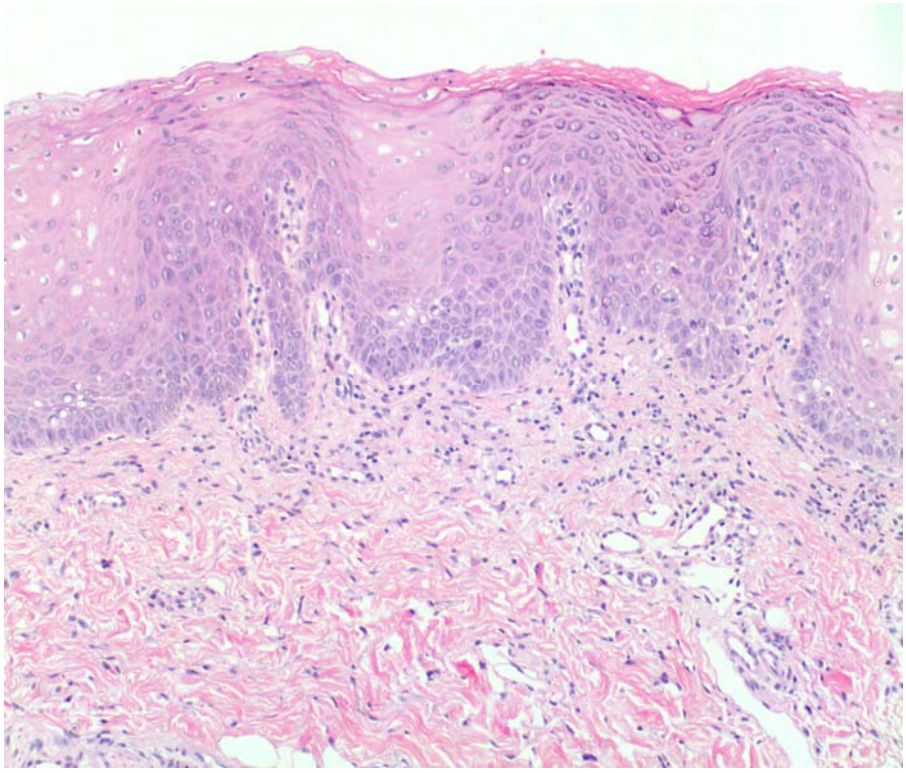

Figure 2.2

An example of the histological appearance of the buccal oral mucosa following chemotherapy. (Original magnification x10)

2.3.3 Immunohistochemistry

Overall, the staining intensity of both NF-κB and COX-2 was increased in the post-chemotherapy biopsies compared to the pre-chemotherapy biopsies and normal controls (Figures 2.3 and 2.4).

COX-2

The chemotherapy naïve specimens did not demonstrate significant staining for COX-2 (data not shown), whilst some of the pre-chemotherapy specimens did demonstrate some COX-2 immunoreactivity (Figure 2.3).

Increased staining for COX-2 was observed in the post-chemotherapy specimens. Positively stained areas particularly included the nuclei of cells in basal layer of the epithelium (Figure 2.5). In some cases scattered positively stained nuclei were also seen in cells within the upper layers of the prickle cell layer of the epithelium. Fibroblasts and endothelial cells were also stained with COX-2 and again this was mainly confined to the cell nuclei (Figure 2.5). These results were statistically significant using the Wilcoxon paired rank test ($p<0.05$).

NF-κB

In the chemotherapy naïve specimens no significant staining with NF-κB was observed (data not shown), whilst some staining was evident in the pre-chemotherapy specimens. Post-chemotherapy increased numbers of specimens demonstrated staining for NF-κB (Figure 2.4).

NF-κB staining was observed within the basal and prickle cell layers of the epithelium, however it appeared to involve the cytoplasm of the cells (Figure 2.6) rather than having prominent nuclear staining as was apparent with COX-2. Staining of fibroblasts was also evident with NF-κB. In addition there was some NF-κB staining of inflammatory cells within the connective tissue (Figure 2.6). These results were statistically significant using the Wilcoxon paired rank test ($p<0.05$).

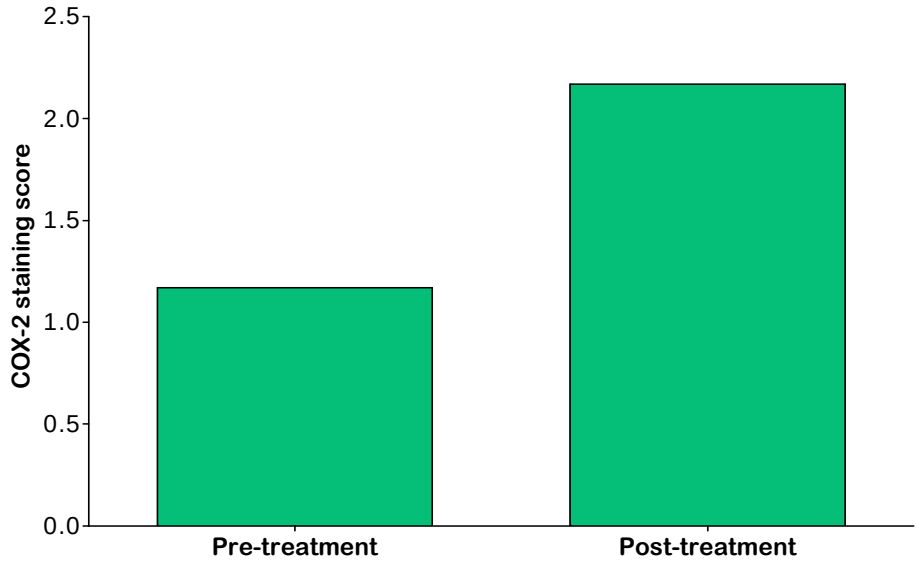

Figure 2.3

Intensity of staining for COX-2 in the buccal mucosa pre-treatment and post-treatment (Wilcoxon signed rank test, $p<0.05$).

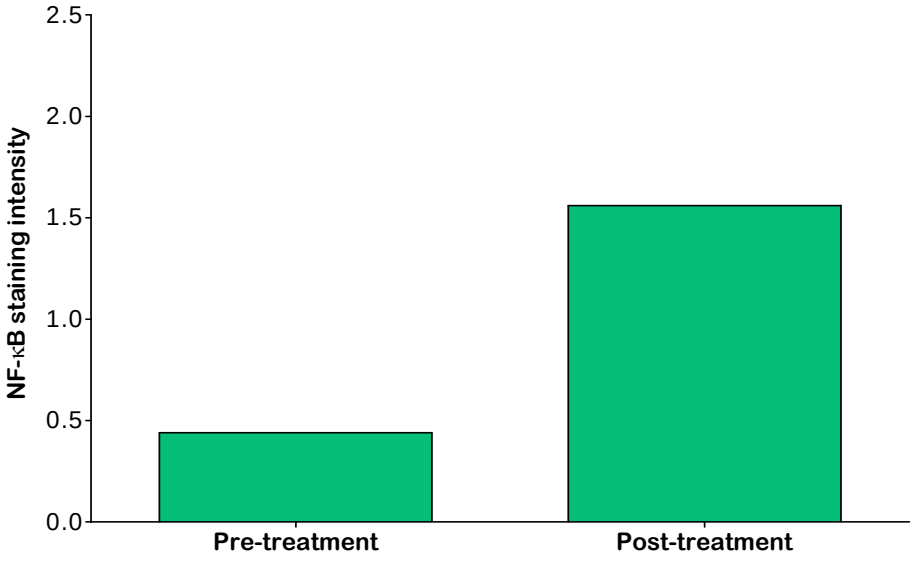

Figure 2.4

Intensity of staining for NF-κB in the buccal mucosa pre-treatment and post-treatment (Wilcoxon signed rank test, $p<0.05$).

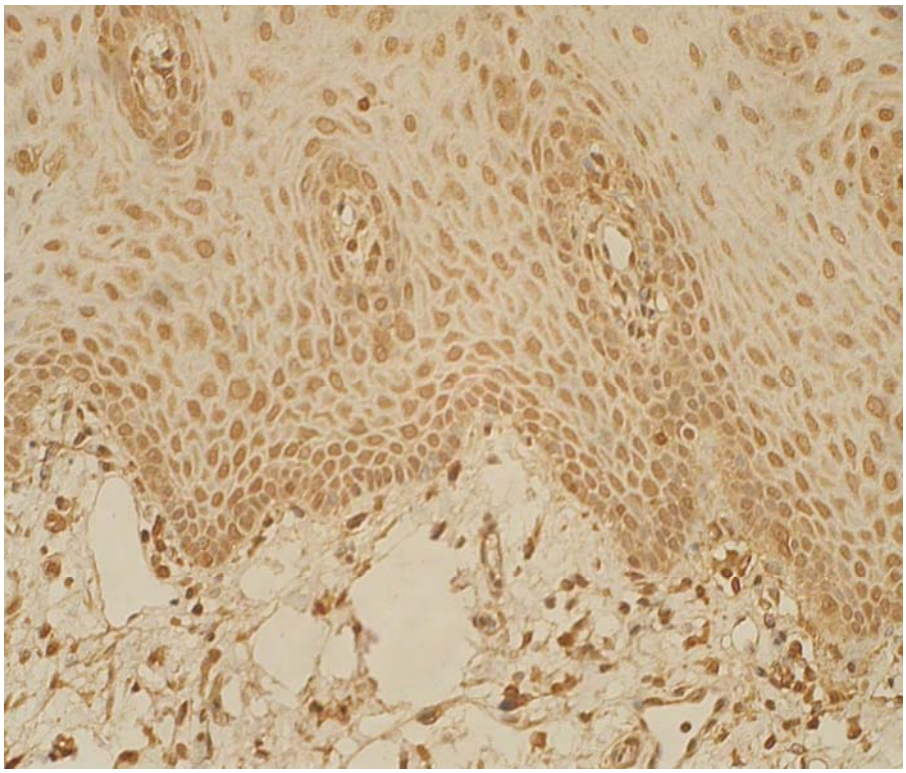

Figure 2.5

Immunohistochemical staining patterns of COX-2 in the oral mucosa following chemotherapy. (Original magnification x10)

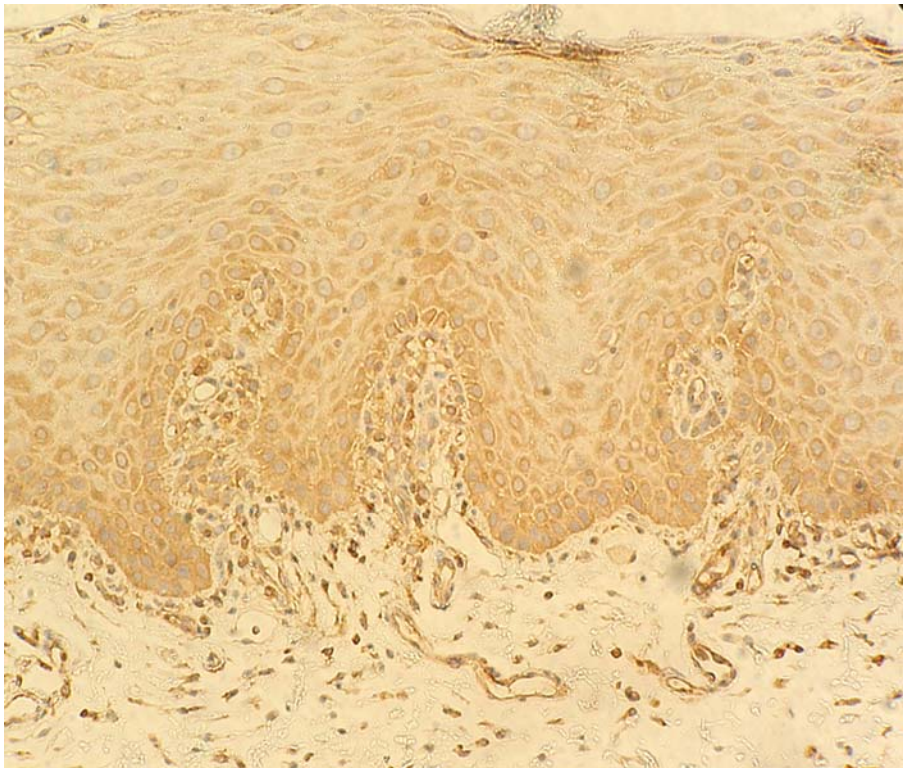

Figure 2.6

Immunohistochemical staining patterns of NF-κB in the oral mucosa following chemotherapy. (Original magnification x10)

2.4 Discussion

There has been substantial literature published on various management strategies to treat oral mucositis [11, 14, 22, 78, 88, 101, 104]. However there has been a paucity of data reporting the mechanistic aspects of the development or pathobiology of oral mucositis, particularly in the clinical setting. The present study examined the expression of NF-κB and COX-2 in the oral mucosa of patients following cytotoxic chemotherapy. Although preliminary, this study demonstrated that NF-κB and COX-2 were elevated following chemotherapy even when histologically, there appeared to be little difference between the pre- and post-chemotherapy appearance of the tissue.

The role of NF-κB in the context of radiation-induced oral mucositis has been demonstrated previously [141]. In the context of cytotoxic chemotherapy however there are also confounding factors due to the action of drugs in combination and their pharmacology. For example, it has been shown that 5-FU can suppress the transcription factor NF-κB [7, 9]. This raises questions about how factors such as treatment modality and other specific patient factors affect the development of side effects that they experience. Unfortunately because of the low numbers of patients in this study it was not possible to draw any specific conclusions from this data relating to this question and this is the main limitation of the current study. Certainly however, as demonstrated by the current study, patients who received 5-FU still experienced mucositis and it is conceivable that this is a result of other "mucotoxic" pathways being activated. In order to accurately define the time course of histological changes that occur subsequent to treatment, further large scale studies with increased patient numbers are required. Additionally, increased patient numbers would enable more detailed analysis of the effects of specific drugs or drug combination on the tissue changes as well as defining the roles of NF-κB and COX-2 in clinical lesion development and resolution.

Studies using a validated animal model may be the next step in determining any difference that specific drugs might have in the upregulation and expression of factors such as NF-κB and COX-2 as well as other pro-inflammatory mediators and the development of mucositis.

Previous studies in humans and in those using animal models have demonstrated positive staining of endothelial cells for COX-2 following radiation [141, 162]. The present study demonstrated that endothelial cell staining for COX-2 is also increased following chemotherapy regimens. The significance of this is not clear, however in the context of radiation, endothelial cell damage is considered to be one of the first changes that occur before the ulcerative phase of mucositis develops [117]. The expression of COX-2 in these cells may be the initial signs of the inflammatory cascade which leads to the production of prostaglandins and further tissue damage. Certainly COX-2 is upregulated by NF-κB and as well as playing an important role in inflammatory pathways, it has also been demonstrated to contribute to tumour development by stimulating angiogenesis [87].

The changes that were observed in NF-κB and COX-2 staining in the oral mucosa of patients undergoing chemotherapy were interesting in that clinically and, for many of the patients, histologically, the mucosa appeared normal. This was in keeping with a previous study that demonstrated ultrastructural changes occurring in the oral mucosa in the absence of changes at the histological level [55]. The significance of this is two-fold. Firstly these changes may represent the early changes that occur in the mucosa subsequent to either radiation or, as in these patients, cytotoxic chemotherapy. This would be consistent with the 5 phase model of mucositis pathobiology as proposed by Sonis [134, 139]. Alternatively, as suggested by Gibson *et al*, because these patients were not chemotherapy naïve (apart from one patient) the changes in the tissue might represent residual damage in the mucosa [55]. This indicates that clinical appearances are deceiving and the time taken for the mucosa to heal following a

mucotoxic insult is extended, lasting longer than the intervening time periods between cycles of chemotherapy. This is also consistent with current thinking on mucositis pathobiology in that following healing there is significant alteration of the mucosal environment [139]. Residual angiogenesis is one such alteration and, as previously mentioned, COX-2 plays a role in promoting angiogenesis. The clinical significance of this is not clear, although it has been suggested that affected patients would be at increased risk of oral mucositis during subsequent treatment for their cancer [139].

In conclusion, the findings from this pilot study have confirmed the presence of NF-κB and COX-2 in oral mucosa following cytotoxic chemotherapy and provided further evidence for their involvement in the pathobiology of oral mucositis. It appears from the data from this study that the early changes that occur in the oral mucosa are similar to that seen as a result of radiation. However, further work needs to be done to determine the effects of individual chemotherapeutic agents on the expression of these factors in patients undergoing chemotherapy and their subsequent role in the pathobiology of mucositis. In order to begin this process, experiments using validated animal models have been undertaken to further investigate the effect of individual chemotherapy agents on different sites of the AT. The chapters that follow comprise the results of these animal experiments.

Chapter 3

Characterisation of mucosal changes in the alimentary tract following administration of irinotecan: Implications for the pathobiology of mucositis.

This chapter has been published as:

Logan RM, Gibson RJ, Bowen JM, Stringer AM, Sonis ST and Keefe DMK, "Characterisation of mucosal changes in the alimentary tract of the Dark Agouti rat following administration of irinotecan: Implications for the pathobiology of mucositis" Cancer Chemotherapy and Pharmacology (2007), doi: 10.1007/s00280-007-0570-0

3.1 Introduction

The role of the transcription factor NF-κB has been postulated as a key element in the development of mucositis [96, 135]. This has been established using animal models as well as clinical studies in patients who have undergone radiotherapy or, as described in the previous chapter, chemotherapy [95, 162]. NF-κB is responsible for the upregulation of approximately 200 genes. In the context of mucositis however, it is the subsequent upregulation of TNF, IL-1β and IL-6 and their amplification through positive feedback loops which are currently considered responsible for the tissue damage that occurs in a patient with mucositis [5]. The result of the upregulation of these pro-inflammatory cytokines is widespread tissue damage and this occurs within the various epithelial and connective tissue compartments that make up the mucosal tissue.

Irinotecan hydrochloride is a chemotherapy agent that has been used to treat a variety of solid tumours. It has its cytotoxic effect by inhibiting DNA topoisomerase 1, which occurs following conversion of irinotecan to its active metabolite SN-38 by carboxylesterase [51]. The dose of irinotecan that can be safely administered is limited by severe side effects such as leukopaenia and severe diarrhoea [2, 72]. In the clinical setting, the occurrence of toxicities varies between patients [68]. Glucuronidation of SN-38 is protective of against irinotecan toxicity. Hepatic and intestinal uridine diphosphate glucuronoslytransferase (UGT), particularly the UGT1A1 isoform, are involved in the glucuronidation of the active metabolite [68, 154]. It has been suggested that patients with low activity of UGT1A1 may have a genetic predisposition for increased irinotecan toxicity. Furthermore, the intestinal microflora may also have an important role to play in the toxicity of this drug. Many resident bacteria in the gut have β-glucuronidase activity and are able to convert the glucuronidated SN-38 back to the active metabolite within the intestine potentiating the toxicity of the drug resulting in an

increase in side effects such as diarrhoea [150]. Furthermore, bacteria in damaged mucosa may directly stimulate NF-κB and pro-inflammatory cytokine expression thereby potentiating tissue damage [139]. The effect of irinotecan on the structure of the small intestine mucosa is well documented and includes increased apoptosis in the crypts of the jejunum and colon leading to loss of normal histology of the tissue as well as effacement of the normal structure of the crypts and villi [53]. These histological features of intestinal mucositis manifest clinically as diarrhoea, abdominal bloating and pain [76, 77, 79, 120].

Previous studies have postulated that, allowing for regional differences in the mucosa, the mechanism of mucositis development is similar regardless of the site of the AT [162]. The aims of the current study were:

1. To determine a time course for histological changes at different sites of the AT following administration of irinotecan;

2. To determine whether NF-κB and the pro-inflammatory cytokines TNF, IL-1β and IL-6 are expressed in different regions of the AT following irinotecan administration;

3. To determine whether there is a difference in the timing of changes in tissue levels of NF-κB and pro-inflammatory cytokines in different regions of the AT.

3.2 Materials and Methods

3.2.1 Experimental design

Eighty-one rats were randomly divided into 9 groups of 9 rats that corresponded to a specific time point, that is, 30, 60, 90 minutes, 2, 6, 12, 24, 48 and 72 hours. Each group comprised 3 control and 6 experimental animals. Immediately prior to the administration of a single intraperitoneal dose of 200 mg/kg of irinotecan, all rats in the experimental groups received 0.01 mg/kg subcutaneous atropine (to reduce any cholinergic reaction to irinotecan). Irinotecan (supplied by Pfizer) was administered in a sorbitol/lactic acid buffer (45 mg/ml sorbitol/0.9 mg/ml lactic acid pH 3.4), required for activation of the drug. Rats in the control groups did not receive any drug. Subsequent to administration of irinotecan the following endpoints were assessed four times per 24 hour period: mortality, diarrhoea, and general clinical condition. Diarrhoea was classified according to the following criteria: mild diarrhoea (staining of anus); moderate diarrhoea (staining top of legs and lower abdomen; severe diarrhoea staining over legs and higher abdomen as well as continual anal leakage).

Rats were killed at the specific time points following administration of irinotecan as indicated above by exsanguination and cervical dislocation. A section of the AT extending from the pyloric sphincter to the rectum was dissected out and flushed with chilled isotonic saline (0.9 w/v) to remove contents. Samples (1 cm in length) of the small intestine (taken at 25% of the length of the small intestine from the pylorus) and the colon (taken at midcolon position) were dissected and removed for further analysis. In addition, samples of oral mucosa were also removed. All tissue samples were immediately fixed in 10% neutral buffered formalin before processing and embedding in paraffin wax.

3.2.2 Sample preparation for haematoxylin and eosin staining

Sections were cut at 4 μm and mounted on glass slides. The sections were dewaxed, rehydrated and stained in Lillie-Mayer's haematoxylin for 10 minutes. Sections were then differentiated in 1% acid alcohol and blued in Scott's tap water before being counterstained in eosin. The sections were dehydrated and mounted prior to histological examination. Photographs of the specimens were taken using an Olympus Altra 20 camera. Measurement of epithelial thickness in the oral mucosa, villus length (in the jejunum) crypt length (in the jejunum and colon) was undertaken using analySIS® FIVE software.

3.2.3 Immunohistochemistry

The expression of NF-κB, TNF, IL-1β and IL-6 in the oral mucosa, jejunum and colon was detected using standard immunohistochemical techniques [162]. Briefly, serial 4 μm sections of the fixed paraffin tissues were cut and mounted on silane-coated microscope slides and dried on a hot-plate for approximately 2 hours. The sections were deparaffinised in xylene before being rehydrated through a series of alcohols and distilled water, followed by two rinses in phosphate buffered saline (PBS, pH 7.5). Antigen retrieval was carried out by microwaving the slides whilst immersed in citrate buffer (pH 6.0) for 3 minutes on high power and 15 minutes on low power. The slides were allowed to cool to room temperature (20 minutes). They were dehydrated and endogenous peroxidase was blocked by immersion in 3% H_2O_2 in methanol for 1 minute. The slides were covered with 50% horse serum in PBS for 30 minutes, rinsed with PBS, and incubated using the Avidin Biotin Blocking Kit (Vector Laboratories, Burlingame, CA) to block endogenous avidin-biotin activity. NF-κB p65 (F-6) (Santa Cruz Biotechnology, Santa Cruz, CA; sc-8008) is an affinity purified mouse monoclonal IgG1 antibody and was used at a dilution of 1:2,300; TNF (HP8001) (HyCult Biotechnology b.v.), a rabbit affinity purified polyclonal antibody, was used at a dilution of 1:1000; IL-1β (H-153) (Santa Cruz Biotechnology, Santa Cruz, CA; sc-7884), a rabbit affinity

polyclonal antibody, was used at a dilution of 1:1000; IL-6 (M-19) (Santa Cruz Biotechnology, Santa Cruz, CA; sc-1265), a goat affinity polyclonal antibody, was used at a dilution of 1:1000.

NF-κB, TNF, IL-1β or IL-6 antibody was then applied with 5% horse serum (Sigma) in PBS and the slides were left overnight (16 hours) at 4°C in a humidified chamber. Following incubation with the primary antibody, sections were washed with 2 changes of PBS and incubated with a secondary antibody (Biotinylated anti-mouse immunoglobulin G purified antibody (Vector Laboratories)) at a dilution of 1:200 with 5% horse serum for slides treated with NF-κB, TNF, Il-1β and biotinylated anti-goat immunoglobulin G purified antibody (Vector laboratories) at 1:200 dilution with 5% horse serum for slides treated with IL-6 antibody, for 20 minutes at room temperature. Subsequent to this, the labelling reagent ultrastreptavidin peroxidase (Signet Pathology Systems Inc., Dedham, MA) was applied for 20 minutes at room temperature. Antibody binding was visualised with 3,3'diaminobenzidine tetrachloride at room temperature. The slides were washed in two changes of distilled water and counterstained with Lillie-Mayer's haematoxylin for 5 minutes. The slides were dehydrated, cleared to xylene and mounted. Positive controls for NF-κB were rat breast adenocarcinoma tumour tissue and, for TNF, IL-1β and IL-6, normal rat lung tissue.

Qualitative immunohistochemistry was performed. Staining was observed using a light microscope. The intensity of staining for was scored as follows: 0, no staining, 1, weak staining, 2, moderate staining, 3, moderate to intense staining, 4, intense staining. This qualitative staining assessment has been previously validated by published grading systems [83-85] and is routinely used in our laboratory [20, 162]. All assessments were done in a blinded fashion by one investigator (RML).

3.2.4 Statistical analysis

Statistical analysis of the morphological features of the specimens was carried out using an unpaired t-test (Graphpad Prism 5).

Due to the ordinal nature of the outcome data, the effect of irinotecan on the various outcome measures (i.e. tissue expression of NF-κB and pro-inflammatory cytokines) was assessed using ordinal logistic regression models. Statistical significance was set at p=0.05. All calculations were performed using SAS Version 9.1 (SAS Institute Inc., Cary, NC, USA).

3.3 Results

3.3.1 Response to treatment

Treated rats began to demonstrate "clinical" signs and symptoms of gastrointestinal mucositis at 2 hours following the administration of irinotecan. The main manifestation of this was diarrhoea. Mild diarrhoea was observed in 23% of treated rats 2 hours following the administration of irinotecan. Twelve hours following administration of the drug, 30% of rats had mild diarrhoea and 5% were classed as having moderate diarrhoea. Diarrhoea was most prevalent at 24 hours where 39% of rats had mild diarrhoea and 5% had moderate diarrhoea. At 72 hours, 33% of the rats in the treatment groups had mild diarrhoea. None of the rats in the control groups had diarrhoea. Chemotherapy-induced death did not occur in this study.

3.3.2 Histological findings

Oral mucosa

In the oral mucosa, changes were noted in the thickness of the epithelium over the 72 hour period. However, no obvious areas of ulceration were identified (Figure 3.1). The appearance of the oral mucosal epithelium at 48 hours is shown in Figure 3.1. Rats in the treatment groups demonstrated marked epithelial atrophy of the oral epithelium compared with controls at 60 minutes ($p<0.0005$), 12 hours ($p=0.035$), 24 hours ($p=0.035$) and at 48 hours ($p=0.012$) (Figure 3.2).

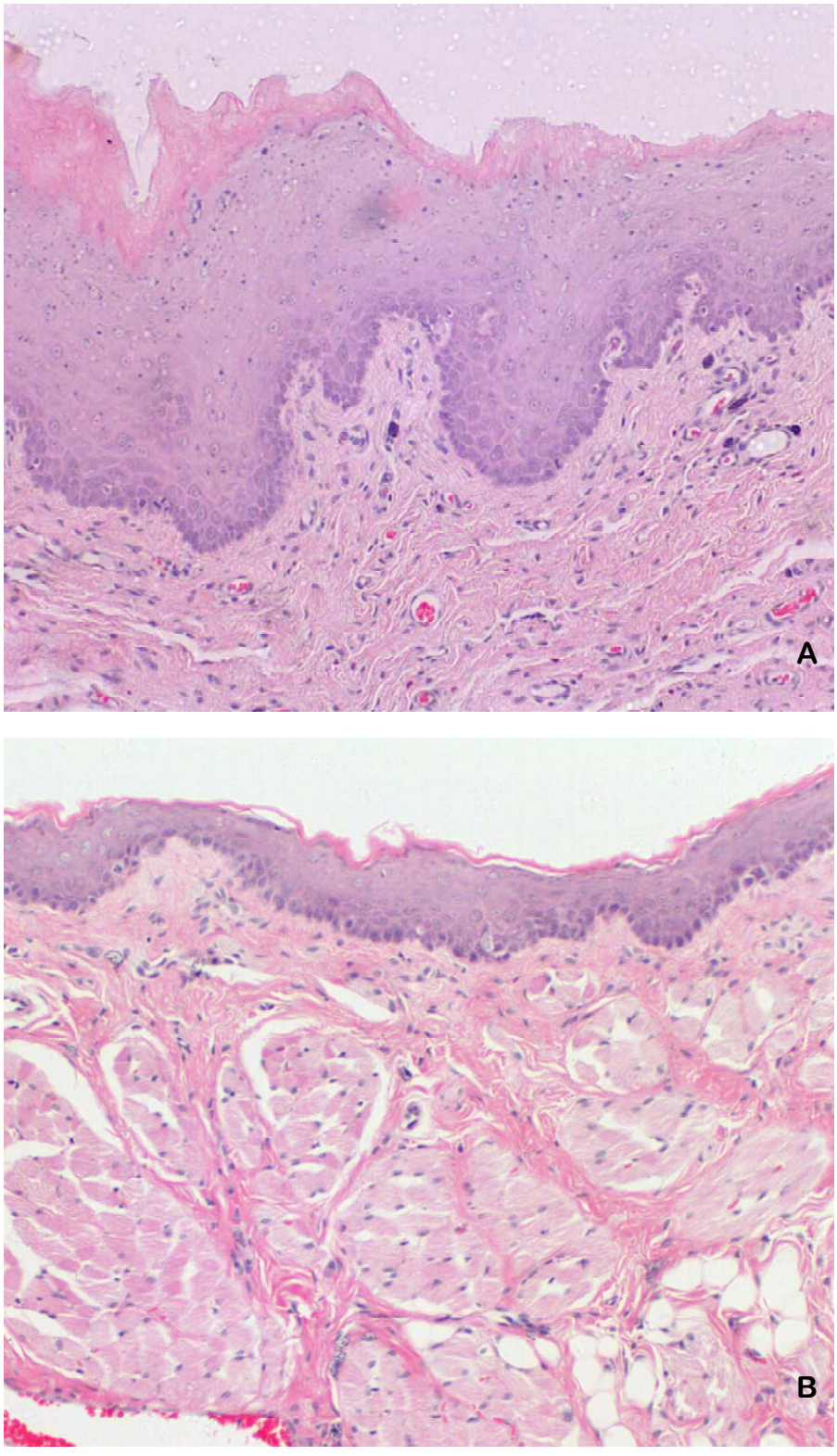

Figure 3.1

Histological features of the oral mucosa at baseline (A) and at 48 hours (B) following irinotecan administration. (Original magnification x20)

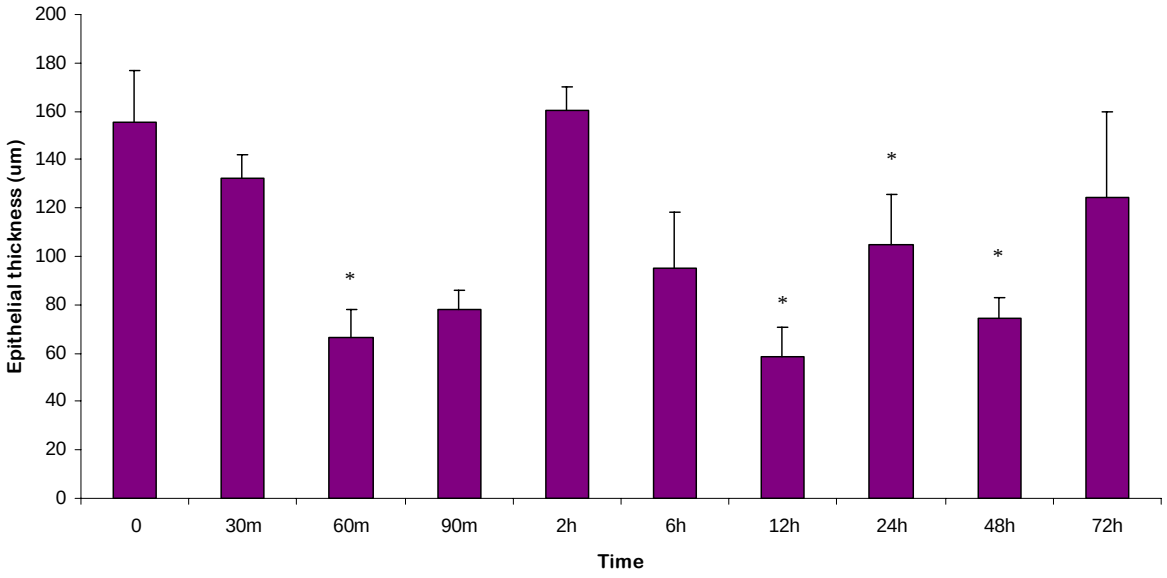

Figure 3.2

Changes in epithelial thickness of the oral mucosa over the 72 hour time period following the administration of irinotecan.

Jejunum

Marked histological evidence of mucositis was observed in the jejunum following treatment with irinotecan. This was evident from 6 hours following administration of irinotecan (Figure 3.3). These changes included the presence of degenerative enterocytes within the crypts followed by more gross architectural disturbances which were seen at the later time points where changes such as villus blunting, epithelial atrophy and increased intensity of inflammatory cell infiltration throughout the mucosal tissue were noted (Figure 3.3). Changes in crypt length (Figure 3.4) were similar to that observed in the oral mucosa demonstrating a decrease in length at 30 minutes ($p=0.017$) respectively to the control group. This was followed by a resolution at 2 hours followed by another significant reduction compared to controls at 12 hours ($p=0.0022$) before returning to lengths comparable to controls by 24 hours.

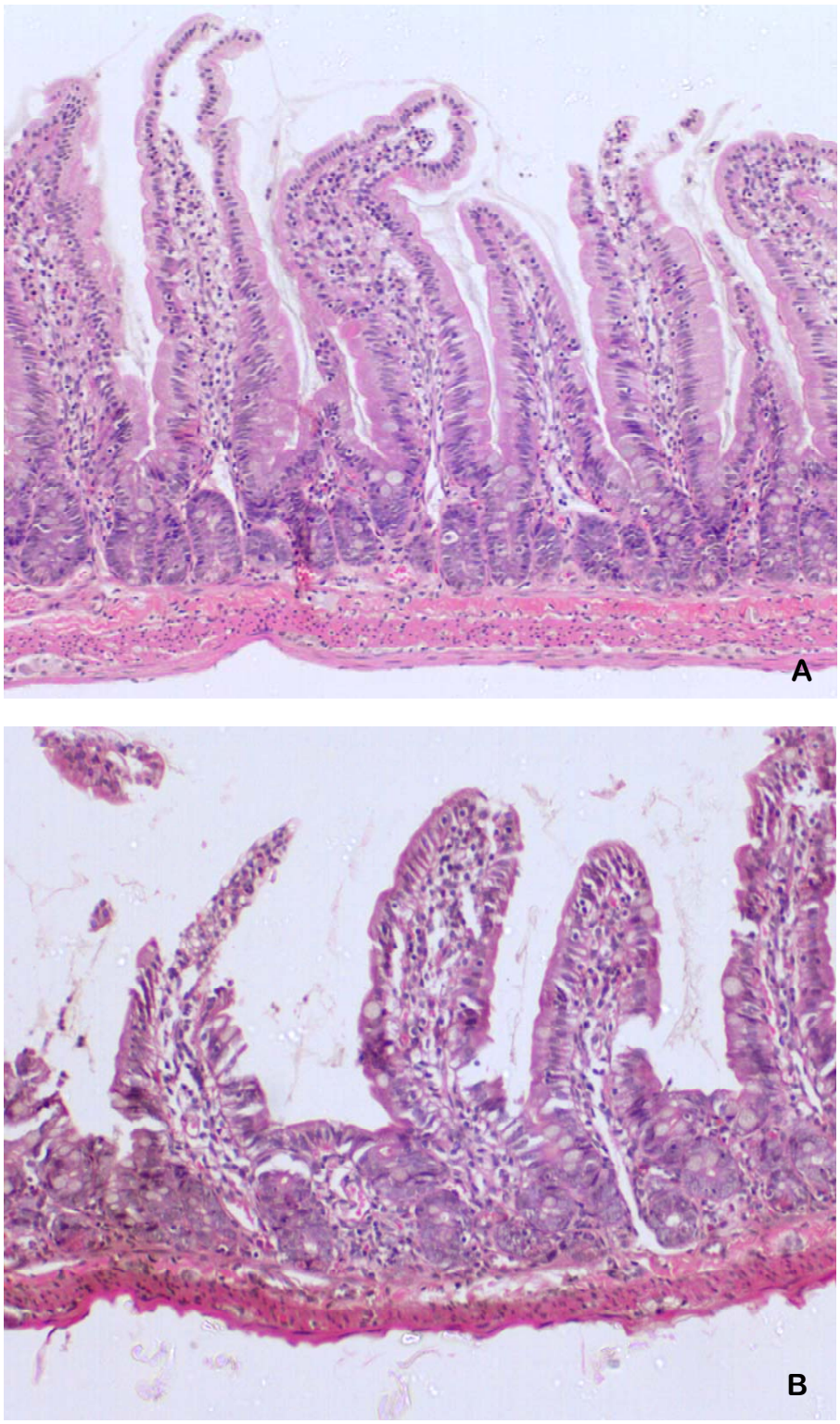

Figure 3.3

Histological features of the mucosa of the jejunum at baseline (A) and at 48 hours (B) following irinotecan administration. (Original magnification x20)

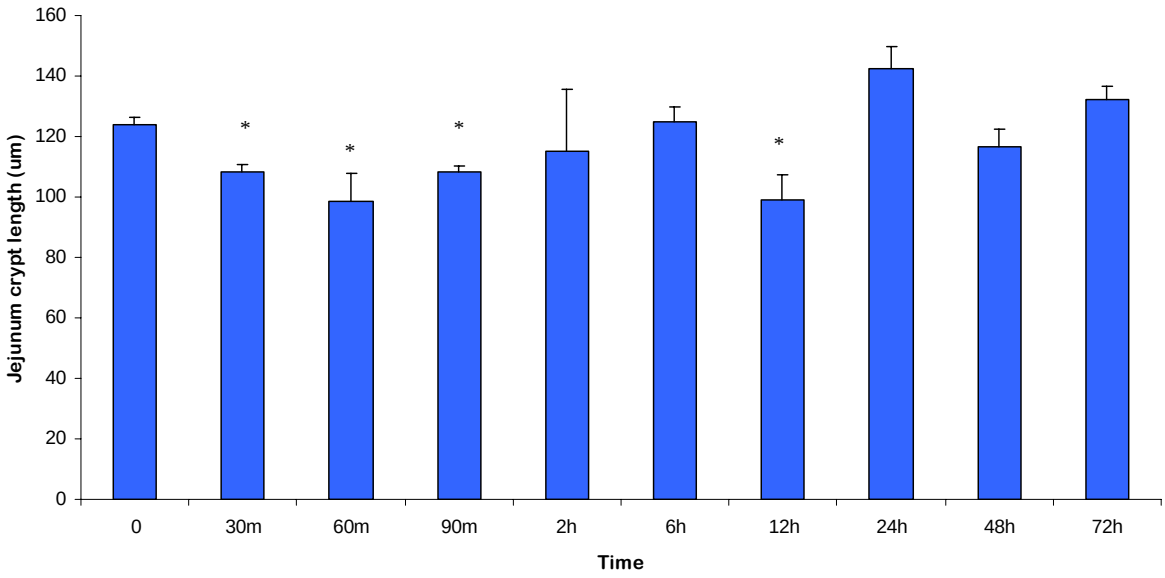

Figure 3.4

Changes in crypt length of the jejunum over the 72 hour time period following the administration of irinotecan.

Colon

Likewise, there was also marked histological evidence of mucositis in the colon following administration of irinotecan. Initial histological changes seen included the presence of individual degenerative enterocytes within the crypts. Whilst at later time points complete ablation of the crypts was observed (Figure 3.5). Crypt length in the colon increased at 30 and 60 minutes ($p=0.0006$ and 0.0036 respectively) returned to normal levels at 2 hours and then increased again at 24 hours ($p<0.0001$) (Figure 3.6).

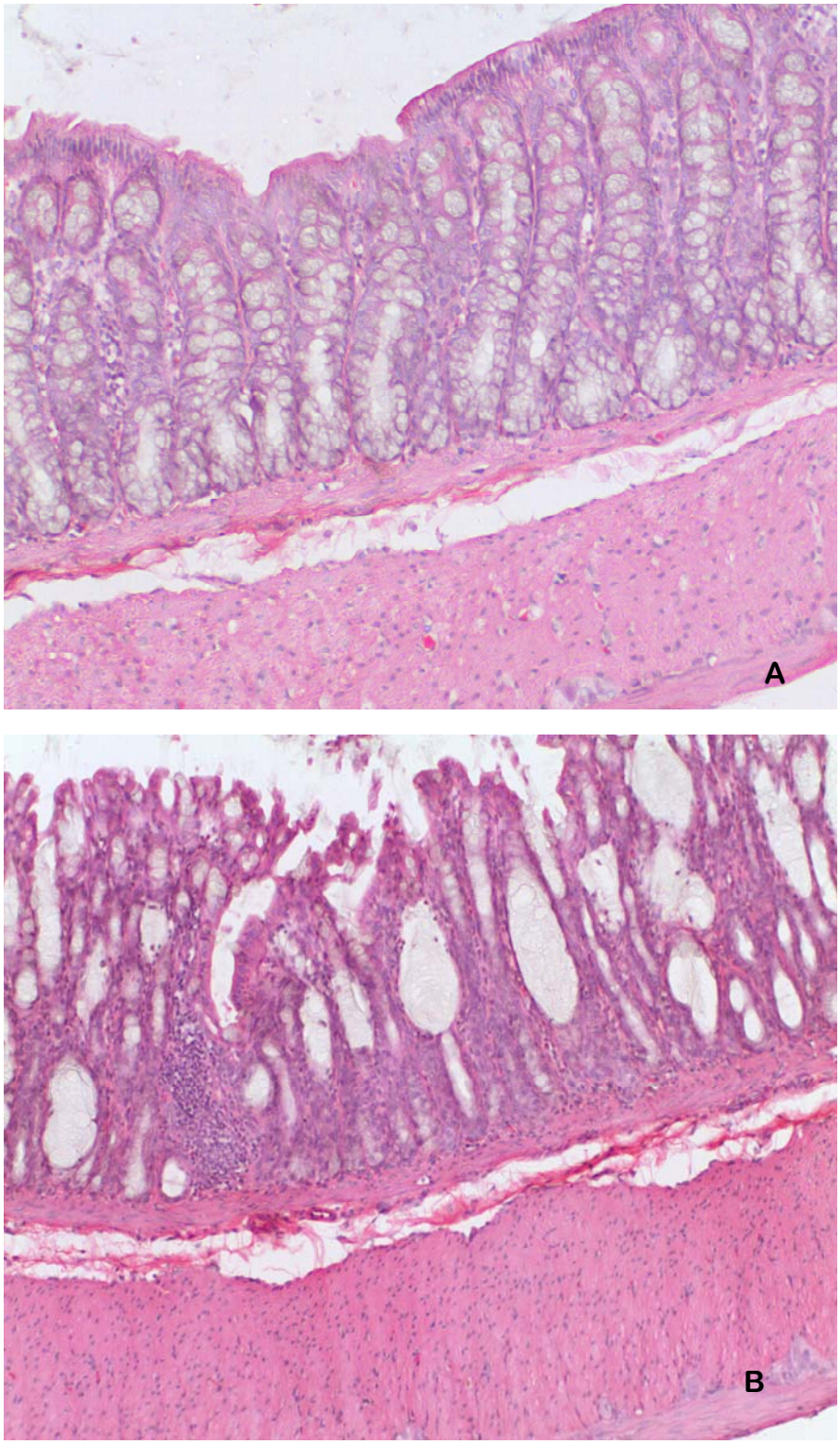

Figure 3.5

Histological features of the mucosa of the colon at baseline (A) and at 48 hours (B) following irinotecan administration. (Original magnification x20)

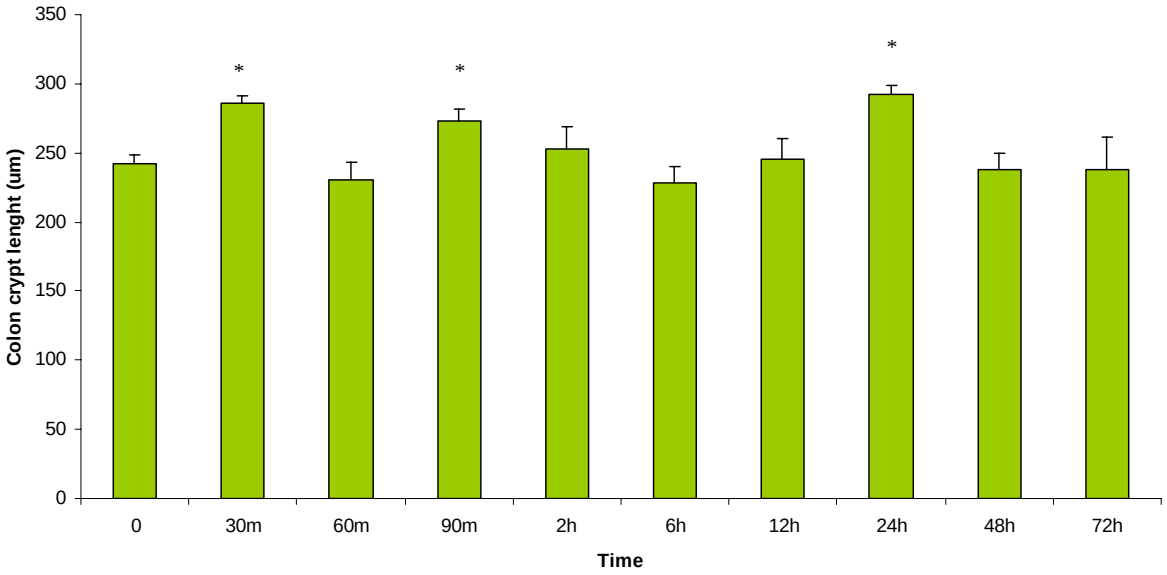

Figure 3.6

Changes in crypt length of the colon over the 72 hour time period following the administration of irinotecan.

3.3.3 Immunohistochemistry

NF-κB

Significantly elevated staining of NF-κB was observed in the oral mucosa, jejunum and colon. Levels peaked in the oral mucosa at 2 hours following irinotecan administration (Figure 3.7) and were consistently higher throughout the experimental period ($p=0.0002$). In the jejunum, staining of NF-κB peaked at 12 hours before subsiding and then slowly increasing over the later time points ($p=0.0033$) (Figure 3.7). In the colon, staining of NF-κB were also elevated at all time points in the mucosa and the difference between the experimental and control groups was statistically significant ($p<0.0001$) (Figure 3.7).

Positive staining for NF-κB was observed in the epithelium of all tissues as well as occasional fibroblasts and inflammatory cells in the submucosa.

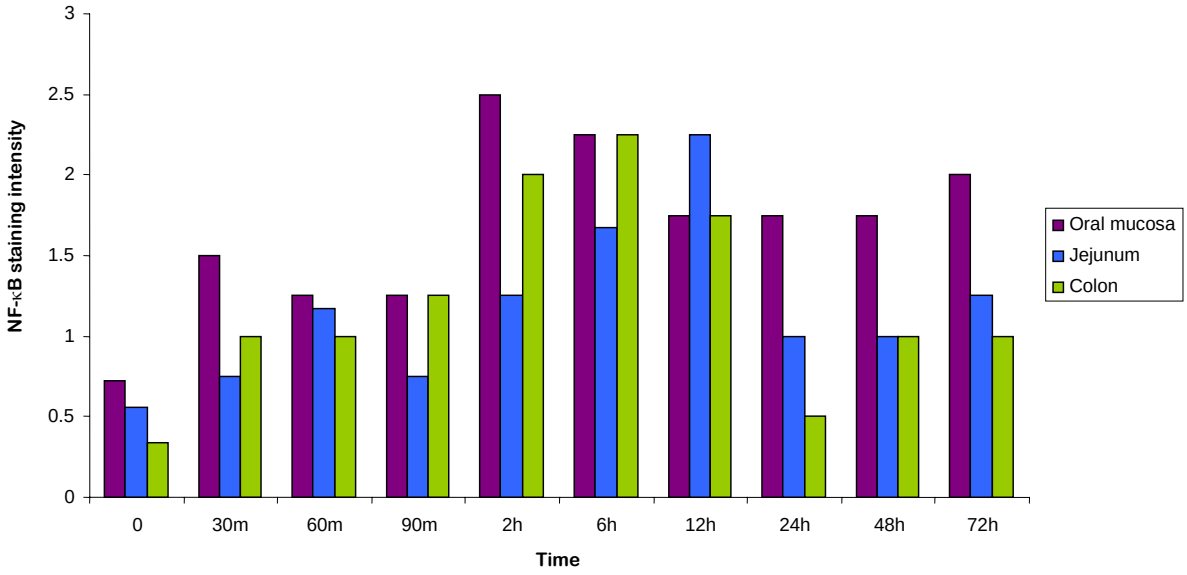

Figure 3.7

NF-κB staining in the oral mucosa, jejunum and colon over the 72 hour time period following the administration of irinotecan.

TNF

The immunohistochemical staining for TNF was more intense in the experimental groups compared with the control groups in all of the sites of the rat AT that were examined (Figure 3.10). When compared with the control group of rats significantly elevated tissue staining for TNF was observed in the oral mucosa peaking at 6 hours (p=0.0101) (Figure 3.8) . In the jejunum, the tissue levels of TNF were also elevated and peaked at 6 hours although this was not significant (p=0.1235) (Figure 3.8). Significantly elevated tissue staining for TNF was observed in the colon compared to the control groups, peaking early at 2 hours (p=0.0254) (Figure 3.8). The trend observed in TNF staining tended to parallel the expression of NF-κB in the tissues of the rat AT.

Positive staining for TNF was also evident in the fibroblast population as well as inflammatory cells in all the tissues examined.

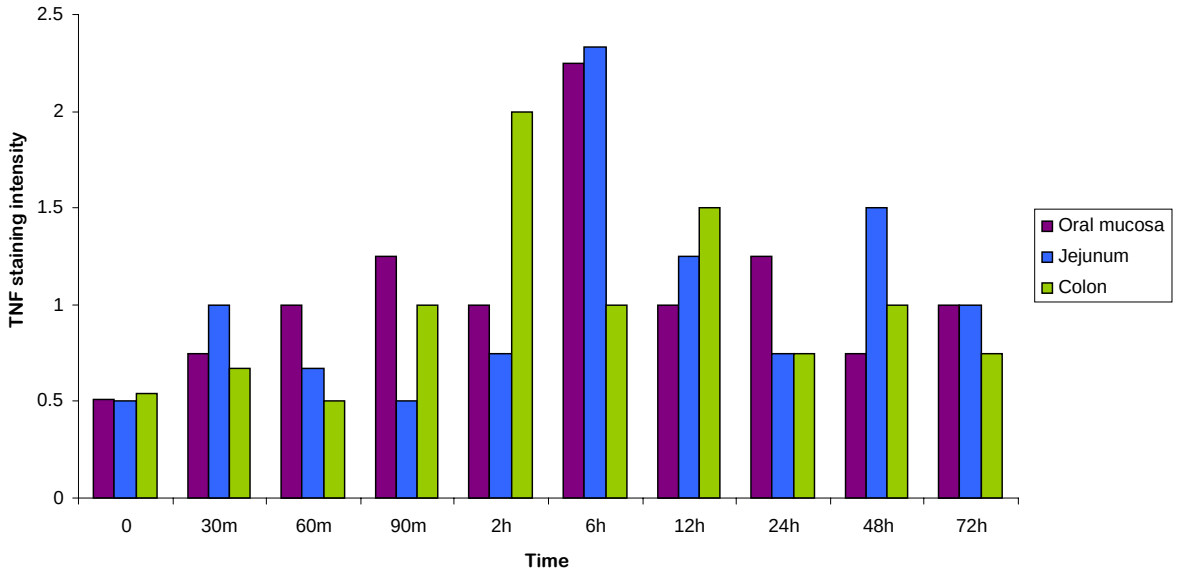

Figure 3.8

TNF staining in the oral mucosa, jejunum and colon over the 72 hour time period following the administration of irinotecan.

IL-1β

Elevated staining for IL-1β was observed in the oral mucosa at the later time points investigated in the study however this was not significant (p=0.0733) (Figure 3.9). Whilst in the jejunum, significantly elevated tissue staining in was observed compared to controls peaking at 6 hours (p=0.017) (Figure 3.9). Following the peak, staining intensity then returned to the levels observed in the control rats. In the colon, the tissue levels of IL-1β peaked at 12 hours (Figure 3.9). As in the jejunum this result was statistically significant when compared with the control group of rats (p=0.0038).

Occasional endothelial cell staining was noted in the oral mucosa and jejunum.

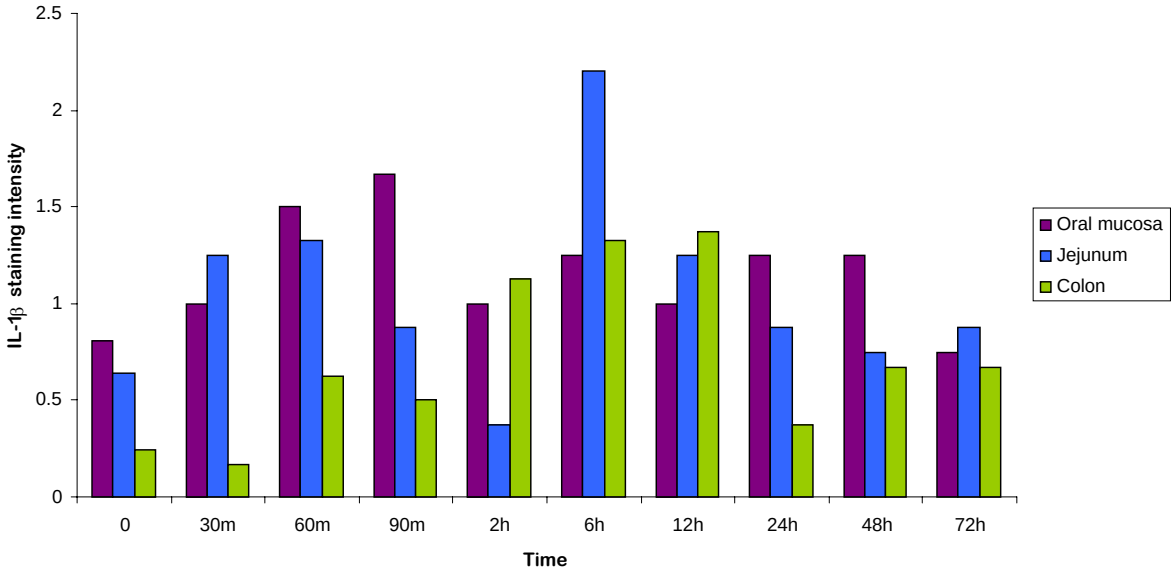

Figure 3.9

IL-1β staining in the oral mucosa, jejunum and colon over the 72 hour time period following the administration of irinotecan.

IL-6

As for IL-1β, tissue levels of IL-6 in the oral mucosa were significantly elevated at the later time points compared with control groups (p=0.0006) (Figure 3.10). There was a peak in tissue levels of IL-6 in the jejunum and colon at 6 hours (Figure 3.10), these results however were not significant compared to controls (p=0.4230 and p=0.0976 respectively). The trend observed in the tissues examined with respect to changes in tissue levels of IL-6 over time was consistent with the other results.

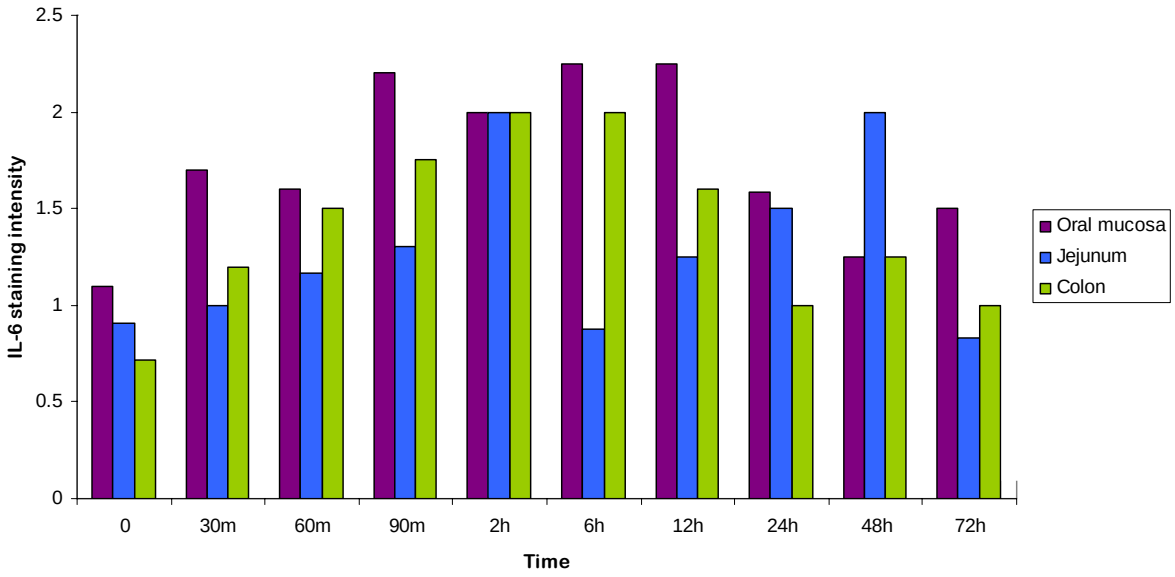

Figure 3.10

IL-6 staining in the oral mucosa, jejunum and colon over the 72 hour time period following the administration of irinotecan.

3.4 Discussion

It has been suggested previously that the pathogenesis of mucositis is consistent throughout the AT with differences occurring as a result of anatomical and histological variation [77]. The results of this study support this notion. The current study demonstrated that NF-κB, thought to be a key driver of mucositis, and pro-inflammatory cytokines (TNF, IL-1β and IL-6) were expressed in different sites along the AT following the administration of irinotecan in the DA rat and that this coincided with histological evidence of tissue damage at early time points. Obviously there are limitations in this study in that it is not longitudinal; however, a true longitudinal study using serial biopsies from the same rat taken at different time points is not practical and would pose increased risk of mortality from procedures and compromise results. This study does have a large sample size and, as such, changes in cytokine expression over time can be implied.

The results from this study demonstrated that the staining for NF-κB, TNF, IL-1β and IL-6 in the oral mucosa, jejunum and colon was similar following the administration of irinotecan (Figures 3.9-3.12). Tissue staining was generally elevated between 2 and 12 hours following chemotherapy. The peak in tissue staining of NF-κB and pro-inflammatory cytokines coincided with the initial histological signs of tissues changes and the initial occurrence of diarrhoea. However, the epithelial atrophy in the oral mucosa and reduction in villus and crypt lengths in the intestine resolved by 12 hours. Gross architectural disturbances such as increased apoptotic bodies, inflammation, villus blunting and crypt ablation were not observed until the later time points. Likewise the early diarrhoea could be attributed to cholinergic effects of irinotecan in spite of the administration of atropine. More severe diarrhoea occurred at the later time points, coinciding with the more severe changes in mucosal histology seen in the intestine. This indicates that changes occur in the mucosa prior to the development of symptoms of mucositis. As well as demonstrating subclinical changes,

the present study also demonstrates that these changes are similar regardless of anatomical site. This provides further evidence for a common pathway for mucositis development which is modified as a consequence of local structural differences in mucosae. There are obvious histological differences between the mucosae of the oral cavity and the intestinal mucosa. In the rat, the epithelial compartment of the oral mucosa is ortho-keratinised unlike in humans where the oral epithelium is generally non-keratinised or para-keratinised. The small and large intestinal mucosae in the rat have similar histology to that seen in humans in that it has structural features which are in keeping with its absorptive, digestive and protective functions [69]. These structural differences in the mucosa appear to have implications for the resilience that different mucosae may exhibit in response to the effects of chemotherapeutic drugs.

The occurrence of subclinical changes in levels of staining for pro-inflammatory cytokines in the AT demonstrated in this study provides support for the hypothesis proposed by Sonis *et al* [130, 134] whereby the onset on clinical symptoms of mucositis is preceded by a complex cascade of molecular events triggered by the production of reactive oxygen species within the mucosa including the activation of transcription factors such as NF-κB leading to upregulation of pro-inflammatory cytokines. These pro-inflammatory cytokines (such as TNF- IL-1β and IL-6) are responsible for the tissue damage that occurs as a result of promoting apoptosis within the mucosa. Recent studies in clinical settings have demonstrated the activation of NF-κB in the oral mucosa and small intestinal mucosa of patients that had undergone clinical chemotherapy and radiotherapy [95, 162]. In other clinical settings, for example inflammatory bowel disease (IBD), the role of cytokines is well established [113]. TNF has been demonstrated to have a host damaging role in this context [106]. It has also been suggested that NF-κB activation by IL-6 may also play a role in IBD [157]. The role of pro-inflammatory cytokines in IBD has led to the use of novel treatments

that target and ultimately inhibit components of the inflammatory cascade, for example the anti-TNF monoclonal antibody, infliximab has a beneficial effect in the management of Crohn's disease [113, 121]. There do not appear to be any similar studies which have investigated targeted treatments in the context of mucositis. A recent publication by Melo *et al* indicated that irinotecan-induced diarrhoea in a mouse model may be modified by administering pentoxifylline and thalidomide [102]. Both of these agents inhibit cytokine production. Pentoxifylline administration delayed the onset of diarrhoea as well as reducing the intestinal changes induced by irinotecan, including tissue levels of TNF and IL-1β [102]. Thalidomide, whilst not affecting diarrhoea onset also attenuated tissue changes in the intestine [102]. The authors of that study concluded that this provided evidence for the role of TNF and IL-1β in mucositis pathobiology and indicated that further investigations were necessary.

As well as the subclinical changes that occur in the intestinal mucosa before the development of diarrhoea, this study also suggests that ulceration in the oral mucosa is preceded by subclinical mucosal changes. Following irinotecan administration, alterations in NF-κB and pro-inflammatory cytokines occurred in the oral mucosa in the absence of ulceration. With respect to oral mucositis, many studies using animal models "induce" mucositis by traumatizing the mucosa by mechanical irritation following chemotherapy in order to create clinical evidence of ulceration [96]. This study is one of the first to demonstrate histological, as well as molecular changes occurring in the oral mucosa subsequent to chemotherapy in a rat model of mucositis *without* the need for trauma. This is important because, mechanical irritation of the mucosa may cause the induction of biological events which might complicate the histological and molecular changes induced by chemotherapy. The need to actually observe epithelial loss (that is, ulceration) is based on a dated paradigm that mucositis is solely an epithelial phenomenon and purely relates to the development of ulceration, more

recent ideas suggest that many subclinical events occur in the mucosa well before ulceration manifests clinically. The current study confirms this hypothesis. Other studies have also reported immunohistochemical, as well as ultrastructural, evidence of tissue damage irrespective of histological changes as well as an absence of clinical changes [55, 95]. We have previously demonstrated that elevated tissue levels of NF-κB occurred in the oral mucosa subsequent to chemotherapy. In that study, however no histological evidence of structural alteration of the mucosa was apparent. Likewise, Gibson and colleagues demonstrated ultrastructural changes occurring in the oral mucosa in the absence of changes at the histological level. In the current study, the mucosa, particularly the epithelial barrier, although altered, remained intact providing additional support for the current pathobiology model of mucositis whereby many of the molecular events leading to ulceration occur before clinical evidence of mucositis is apparent [139]. In the rat, the distinct structural or phenotypic differences between the oral and intestinal mucosae, particularly in terms of keratinisation, may provide additional resilience of the oral mucosa to the effects of irinotecan.

In conclusion, the results of the study provide further evidence for the role of NF-κB and associated pro-inflammatory cytokines in the pathobiology of AT mucositis and addressed the three stated aims of the study. This is the first study to demonstrate changes in the tissue levels of these factors throughout the AT following administration of irinotecan. This study also indicates that there appears to be a common pathway for mucosal damage subsequent to irinotecan administration, irrespective of differences in structural variation between different regions of the AT. In the clinical setting, other commonly used chemotherapy drugs cause mucositis (for example MTX and 5-FU) and have also been demonstrated to cause histological damage to tissues [32, 57]. Further complicating the clinical setting is that patients are often administered combinations of drugs each of which may potentially cause

mucositis. The results of this study raise the question as to whether different drugs, which clinically also cause mucositis, also cause similar changes in pro-inflammatory cytokine expression at sites throughout the AT; this question is addressed in the following chapters.

Chapter 4

Is the pathobiology of chemotherapy-induced alimentary tract mucositis influenced by the type of mucotoxic drug administered?

This chapter has been published as:

Logan RM, Gibson RJ, Bowen JM, Stringer AM, Sonis ST and Keefe DMK, "Is the pathobiology of chemotherapy-induced alimentary tract mucositis influenced by the type of mucotoxic drug administered?" Cancer Chemotherapy and Pharmacology (2008), doi: 10.1007/s00280-008-0732-8

4.1 Introduction

In the preceding chapter it was demonstrated that histological and immunohistochemical changes occur concurrently at different sites of the AT subsequent to the administration of irinotecan in the DA rat [94]. Irinotecan, a topoisomerase-1 inhibitor is well known to cause mucositis, particularly small intestinal mucositis and causes significant clinical problems for patients undergoing treatment protocols that include this drug. Following administration of irinotecan in the DA rat, histological evidence of tissue damage was observed in the oral mucosa, jejunum and colon. This coincided with changes in the tissue expression of NF-κB as well as TNF, IL-1β and IL-6 [94]. In order to determine whether different drugs caused similar changes in the AT to that observed previously, the study using irinotecan was repeated, substituting irinotecan with either MTX or 5-FU both of which are commonly used drugs in clinical practice.

Although both of these drugs ultimately kill tumour cells through inhibiting DNA synthesis, they do this through different mechanisms and have different pharmacological profiles. MTX is a folate antagonist and inhibits the enzyme dihydrofolate reductase which is required for the production of thymidine; as a result, DNA synthesis is blocked. 5-FU is a pyrimidine analogue and interferes with DNA synthesis by blocking thymidylate synthetase; in addition it is also incorporated into RNA thereby affecting RNA function and protein synthesis. MTX also has anti-inflammatory actions which are exploited in the management of diseases such as rheumatoid arthritis. It is well established that both of these drugs cause mucositis both in the oral mucosa and in the small intestine [23, 57, 116, 124]. MTX causes damage to the small intestine characterised by increased crypt apoptosis and villus atrophy (Gibson *et al*, 2002) 5-FU administration also results in increased apoptosis and decreased cellularity in the small intestine [124]. However, the mechanisms by which MTX and 5-FU cause mucosal toxicity, particularly in the oral mucosa are not well understood. There appear to be some paradoxes

in the current hypothesis regarding mucositis pathobiology with respect to these drugs. For example, in the context of its antiinflammatory action, MTX causes a reduction in pro-inflammatory cytokine gene expression, particularly IL-1, 2 and 6 as well as interferon-γ [86]. Furthermore, MTX has been demonstrated to suppress NF-κB in Jurkatt cells [100] as well as pro-inflammatory cytokines such as IL-6 [86]. Likewise, 5-FU has also been shown to decrease NF-κB activation [7, 9]. As NF-κB activation and subsequent pro-inflammatory cytokine production have been implicated to play an important role in mucositis pathobiology, given these actions, do MTX and 5-FU cause mucosal damage in the alimentary tract through alternative pathways?

The aims of this study therefore were to:

1. Determine the histological changes that occur in the oral mucosa, jejunum and colon in rats following administration of either MTX or 5-FU;

2. Determine whether there is immunohistochemical evidence of changes in tissue expression of NF-κB and pro-inflammatory cytokines (TNF, IL-1β and IL-6) at different sites of the AT following the administration of either MTX or 5-FU;

3. Determine whether histological changes and the tissue expression of NF-κB and pro-inflammatory cytokines varies according to the type of drug administered.

Based on the fact that irinotecan, MTX and 5-FU have different mechanisms of action, it was hypothesised that the changes observed in the AT of the DA rat following chemotherapy would be influenced by the type of drug administered.

4.2 Materials and methods

4.2.1 Experimental design

The design of this experiment is identical to that described in Chapter 3, except that two groups of 81 rats were used substituting irinotecan for either MTX or 5-FU. Again each group of 81 rats was divided randomly into 9 groups which corresponded to a specific time point – 30, 60 and 90 minutes, 2, 6, 12, 24, 48 and 72 hours. Each group included 3 control rats and 6 experimental rats. All rats in the experimental groups received a single intramuscular dose of 1.5 mg/kg of MTX or a single intraperitoneal dose of 150mg/kg of 5-FU. As in the previous chapter, rats in the control groups did not receive any drug. Subsequent to administration of the drug the following endpoints were assessed four times per 24 hour period: mortality, diarrhoea, and general clinical condition.

Again, as described in Chapter 3, rats were killed by exsanguination and cervical dislocation at the specific time points following administration of the drug according to their group.

4.2.2 Sample preparation for haematoxylin and eosin staining

Tissue samples were taken from the oral mucosa and small and large intestine and prepared for histological examination as described previously in Chapter 3.

4.2.3 Immunohistochemistry

Immunohistochemical staining of the tissue for NF-κB, TNF, IL-1β and IL-6 was carried out as described previously in Chapter 3.

4.2.4 Statistical analysis

Statistical analysis of morphological changes in different site of the AT was carried out using an unpaired t-test (Graphpad Prism 5).

With respect to the tissue levels of NF-κB and pro-inflammatory cytokines, due to the ordinal nature of the outcome data, the effect of treatment on these outcome measures was assessed using ordinal logistic regression models. Statistical significance was set at p=0.05. All calculations were performed using SAS Version 9.1 (SAS Institute Inc., Cary, NC, USA).

In order to compare epithelial thickness in the oral mucosa and crypt length in the jejunum and colon over time and between the different drugs, a general linear model was fitted to the data. In the model a generalised estimating equation was used to account for the dependence in observations from the same rat. The analysis was done using SAS version 9.1 (SAS Institute Inc. Cary, NC, USA).

4.3 Results

4.3.1 Response to treatment

The rats tolerated both MTX and 5-FU well. Clinical indications of deterioration were not observed in the experimental groups of rats with respect to either drug. Furthermore, significant diarrhoea was not observed in either group of rats.

4.3.2 Histological findings

Oral mucosa

Methotrexate

MTX administration caused a decreased in epithelial thickness in the oral mucosa however ulceration was not a feature (Figure 4.1). This was evident at 90 minutes following drug administration ($p=0.0059$) followed by brief return to normal thickness then a gradual decline over the rest of the time period (12 hours, $p=0.004$; 24 hours, $p=0.001$; 48 hours, $p=0.0004$; 72 hours, $p=0.0019$) (Figure 4.2).

5-Fluorouracil

Likewise, 5-FU also caused a decrease in the thickness of the epithelium without resulting in ulceration (Figure 4.3). This was evident at 2 hours ($p=0.0002$) followed by a brief return to normal levels between 6 and 24 hours followed by a gradual decline over the rest of the 72 hour period (48hours, $p<0.0001$; 72 hours, $p<0.0001$) (Figure 4.4).

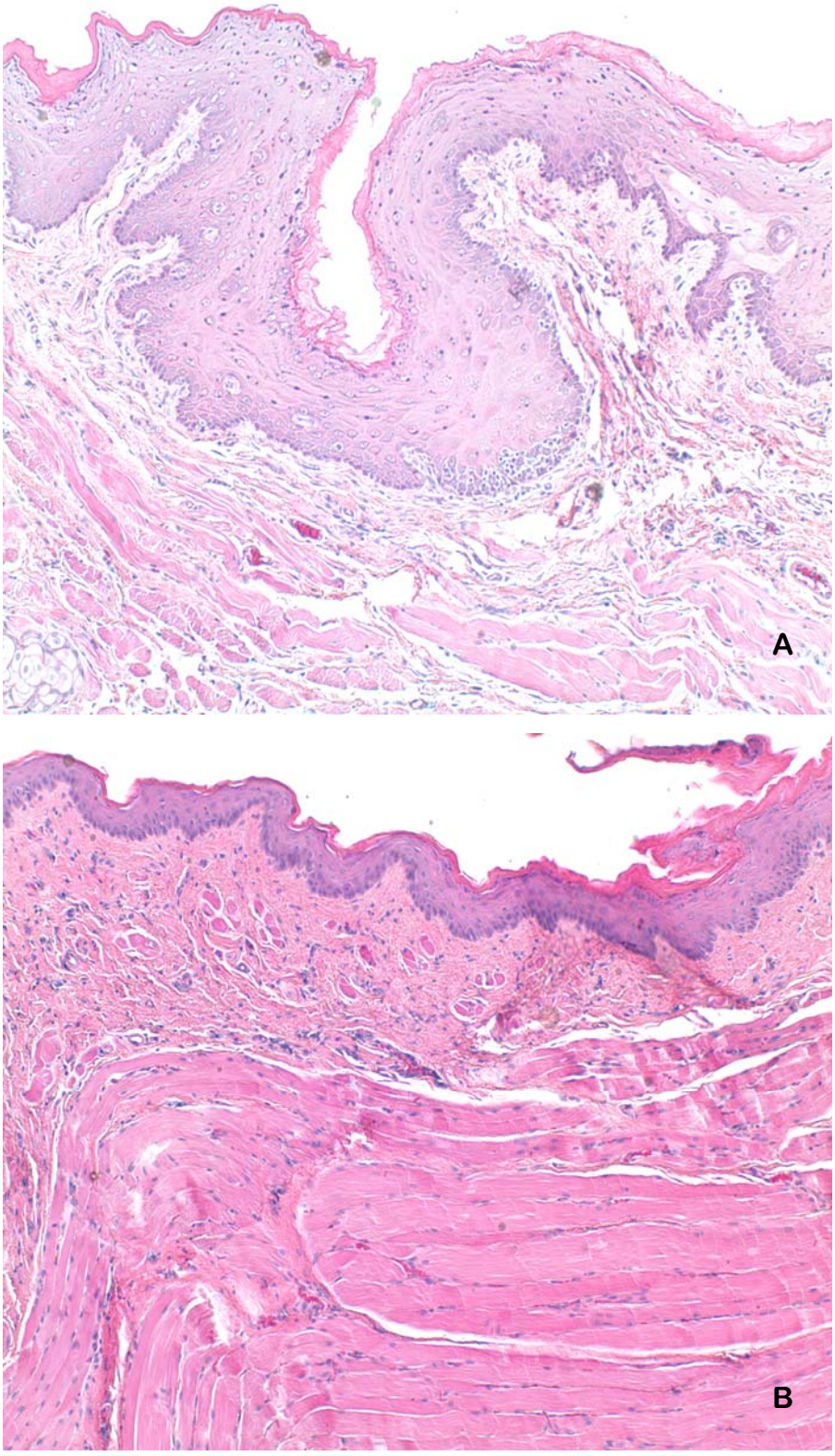

Figure 4.1

Histological features of the oral mucosa at baseline (A) and at 12 hours (B) following methotrexate administration. (Original magnification x20)

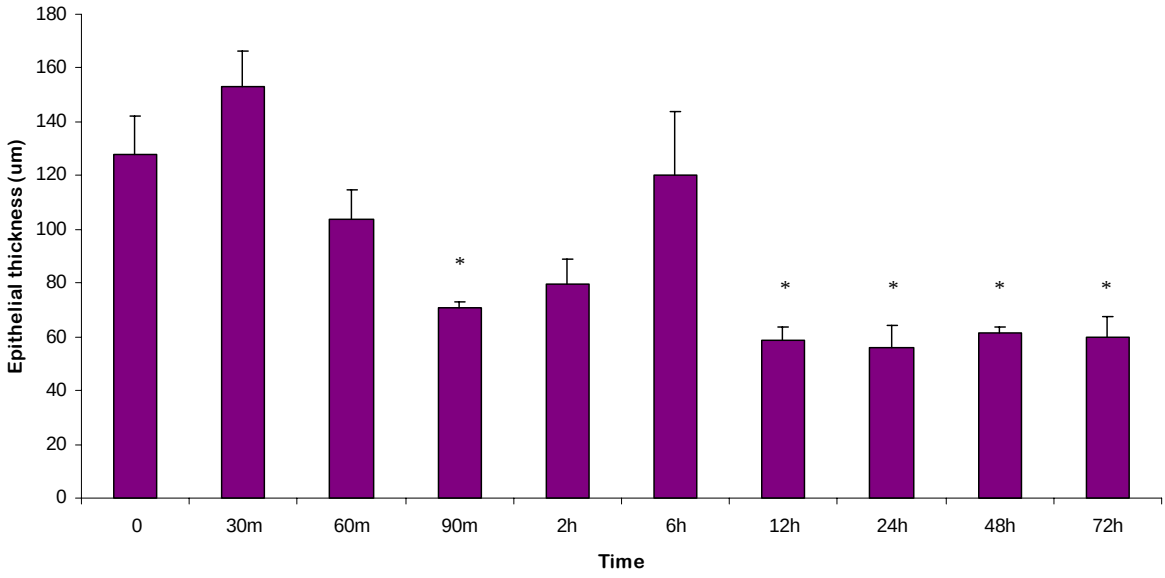

Figure 4.2

Changes in epithelial thickness of the oral mucosa over the 72 hour time period following the administration of methotrexate.

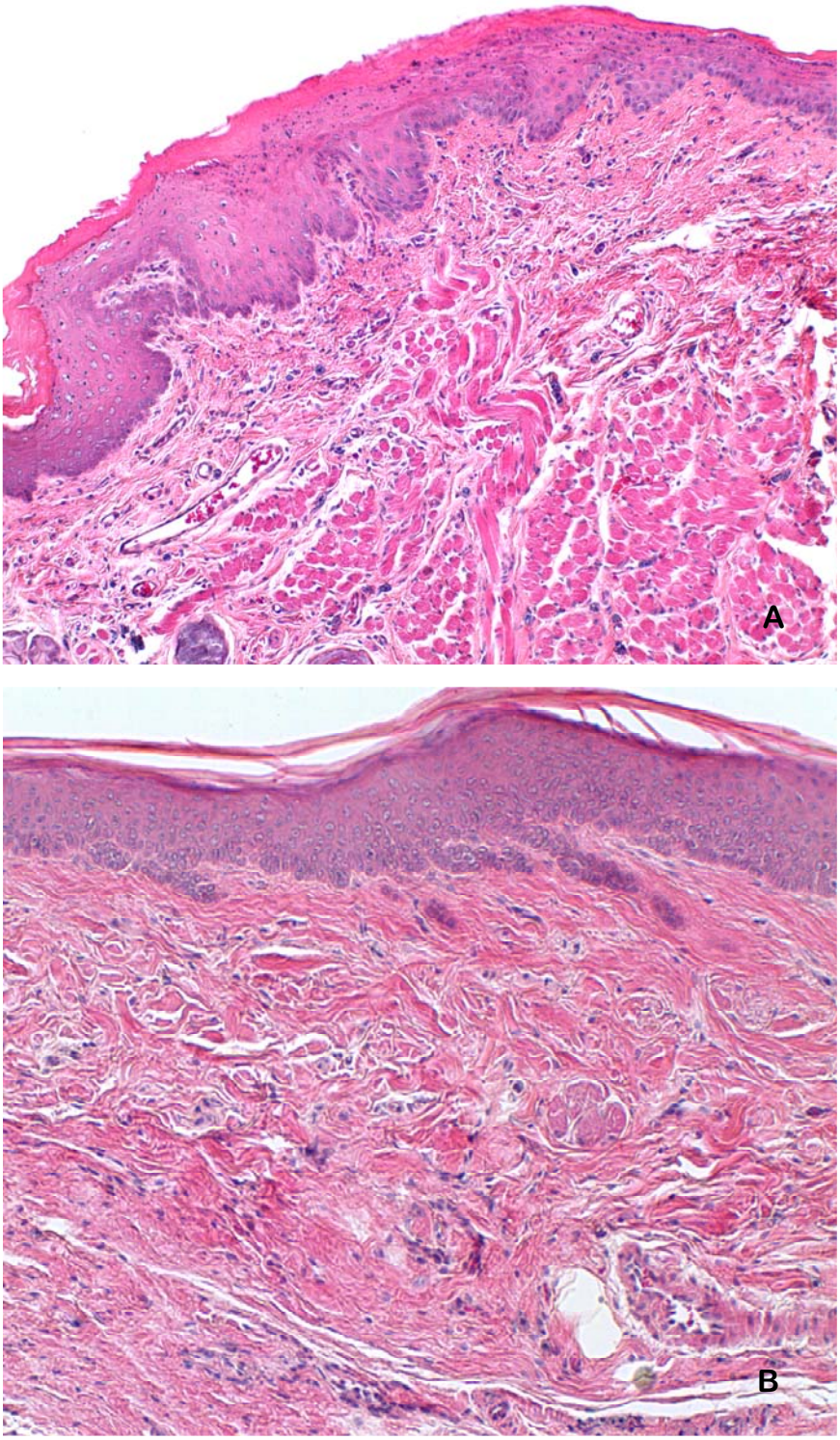

Figure 4.3

Histological features of the oral mucosa at baseline (A) and at 48 hours (B) following 5-fluorouracil administration. (Original magnification x20)

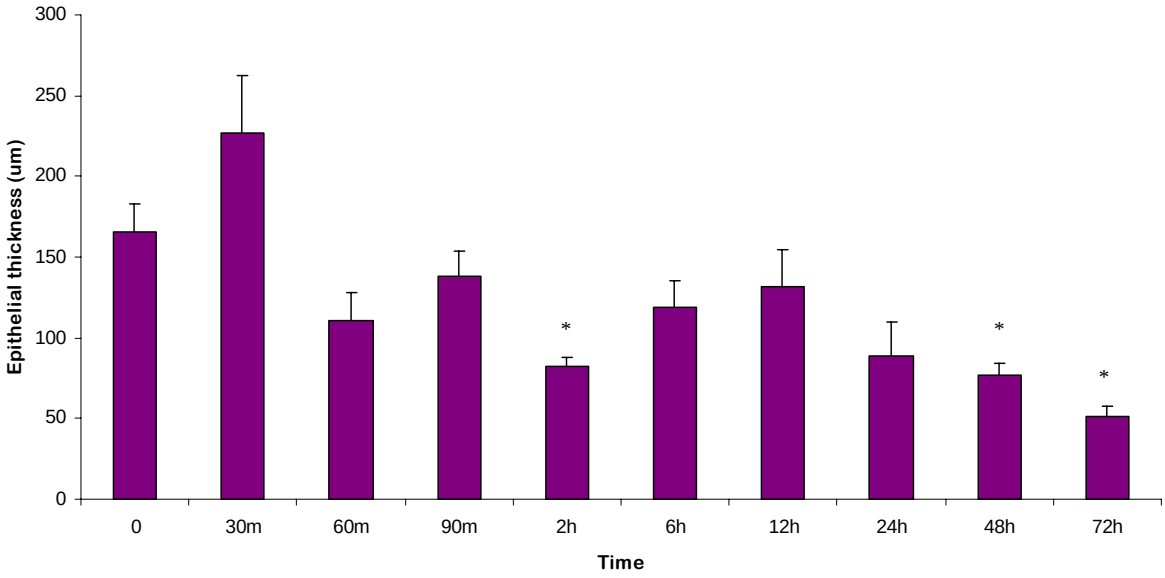

Figure 4.4

Changes in epithelial thickness of the oral mucosa over the 72 hour time period following the administration of 5-fluorouracil.

Jejunum

Methotrexate

In the jejunum, a reduction in crypt length was observed (Figure 4.5) following MTX administration from 6 hours ($p<0.0001$) and throughout the remaining time period (12 hours, $p<0.0001$; 24 hours, $p<0.0001$; 48 hours, $p<0.0001$; 72 hours, $p=0.0061$(Figure 4.6). Other signs of mucosal damage included blunting and fusion of the villi and obliteration of the crypts.

5-Fluorouracil

5-FU caused blunting and fusion of the villi (Figure 4.7). 5-FU administration also caused changes in jejunal crypt length. After remaining stable for the first 12 hours a reduction in crypt length occurred from 12 hours ($p=0.0038$) extending over the remainder of the 72 hour time period (24 hours, $p<0.0001$; 48 hours $p<0.0001$; 72 hours, $p=0.0109$) (Figure 4.8).

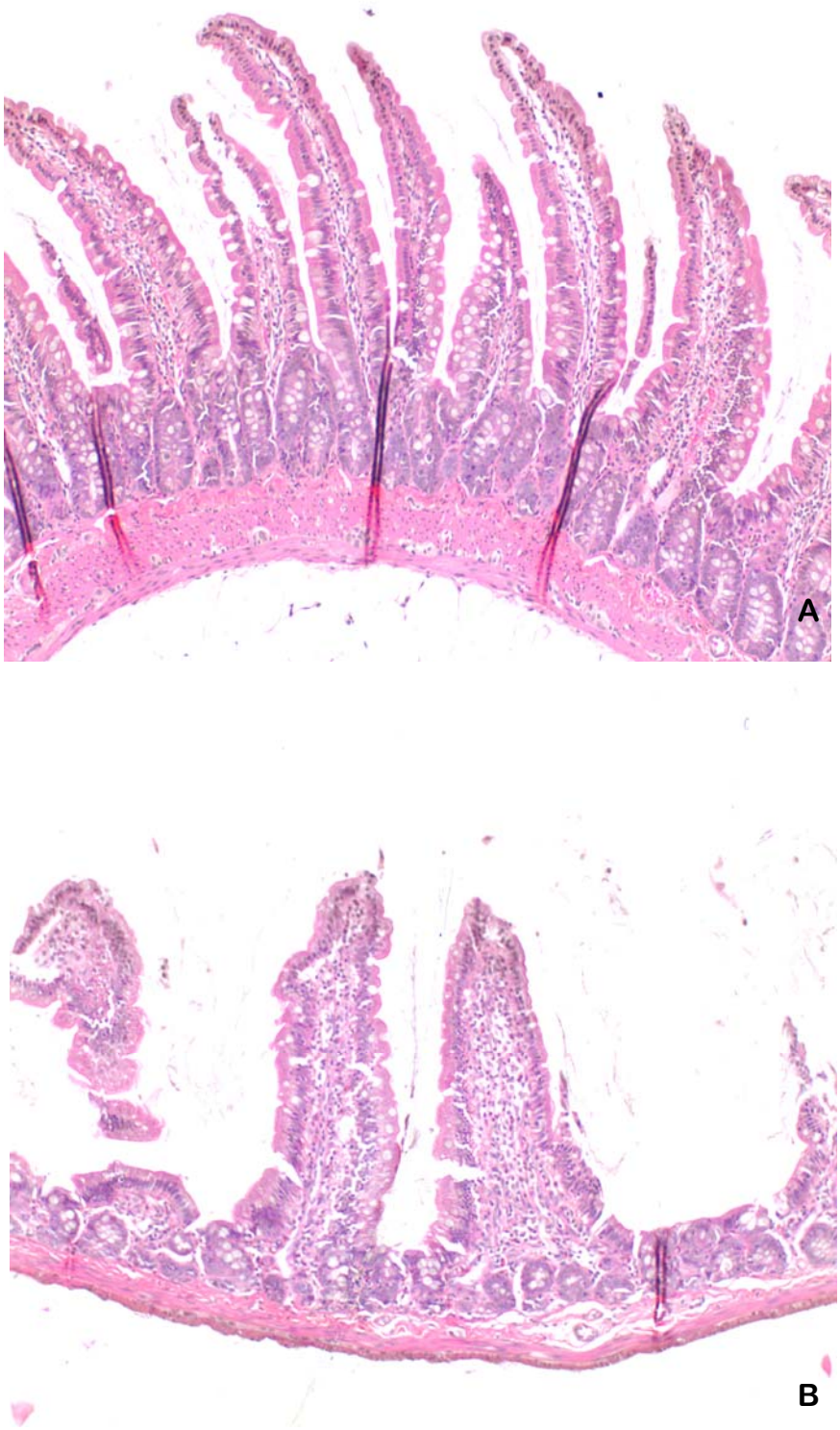

Figure 4.5

Histological features of the jejunum at baseline (A) and at 12 hours (B) following methotrexate administration. (Original magnification x20)

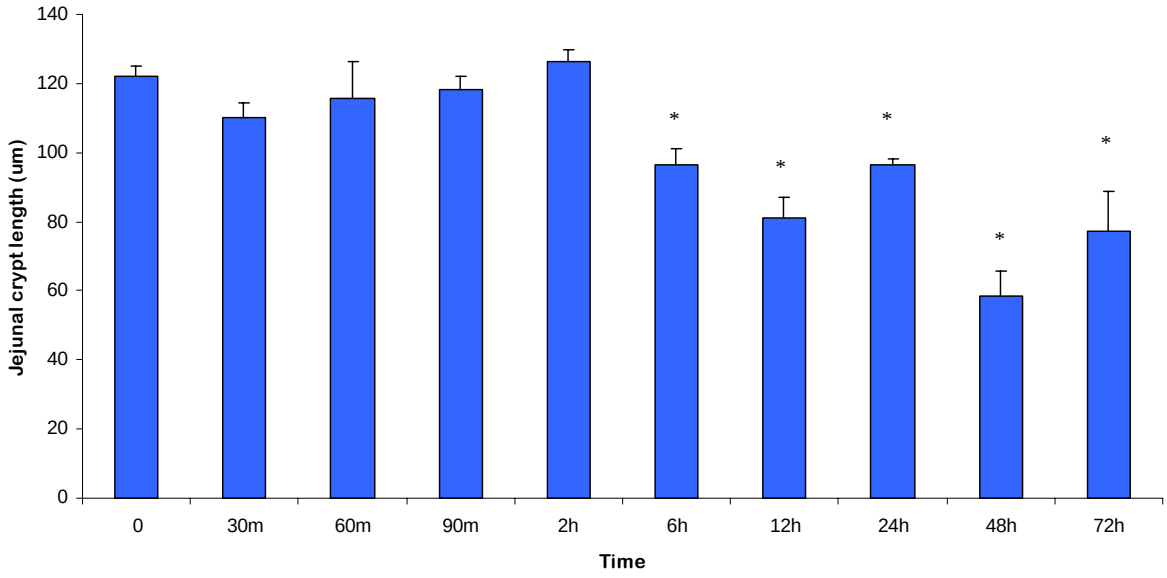

Figure 4.6

Changes in the crypt length of the jejunum mucosa over the 72 hour time period following the administration of methotrexate.

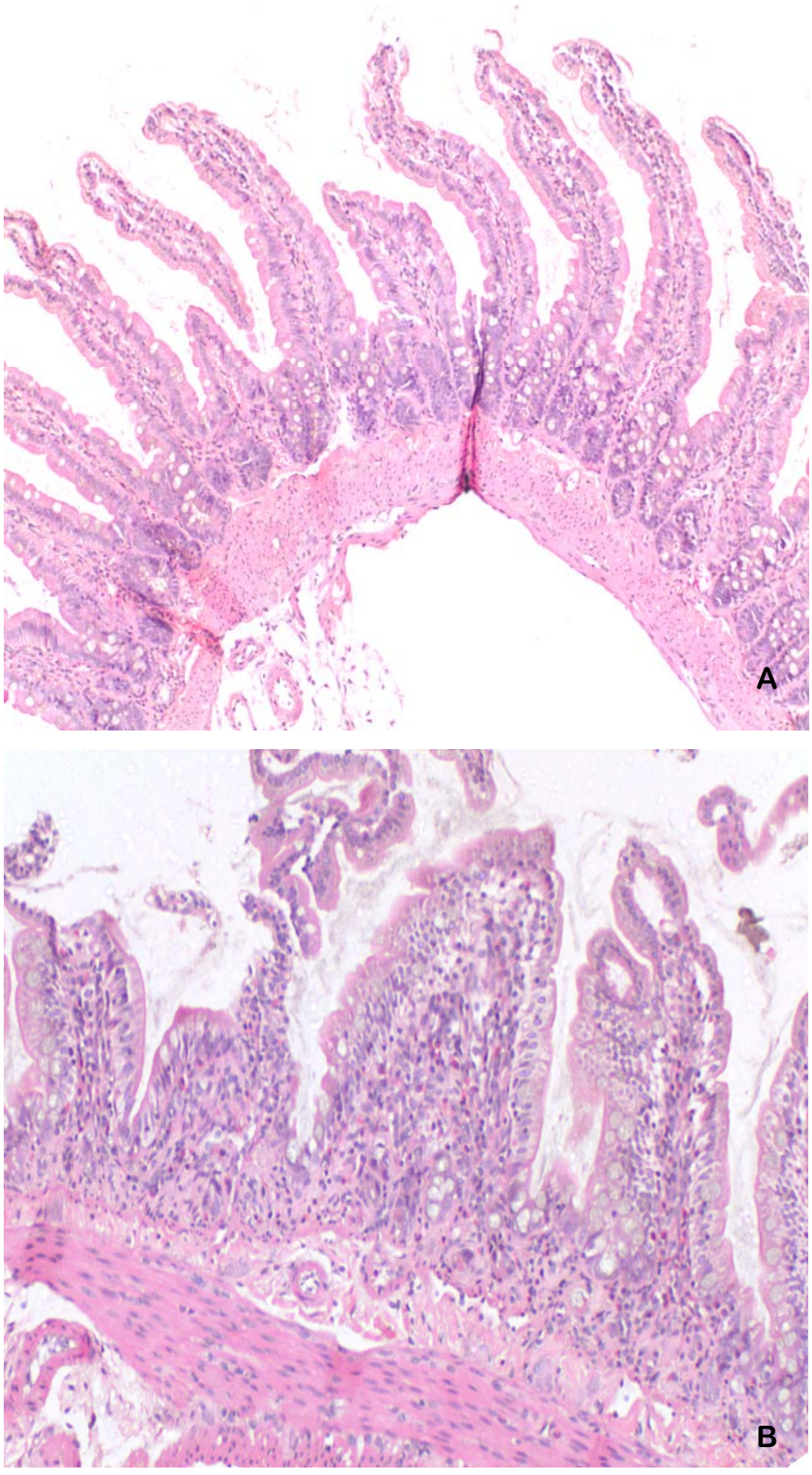

Figure 4.7

Histological features of the jejunum at baseline (A) and at 48 hours (B) following 5-fluorouracil administration. (Original magnification x20)

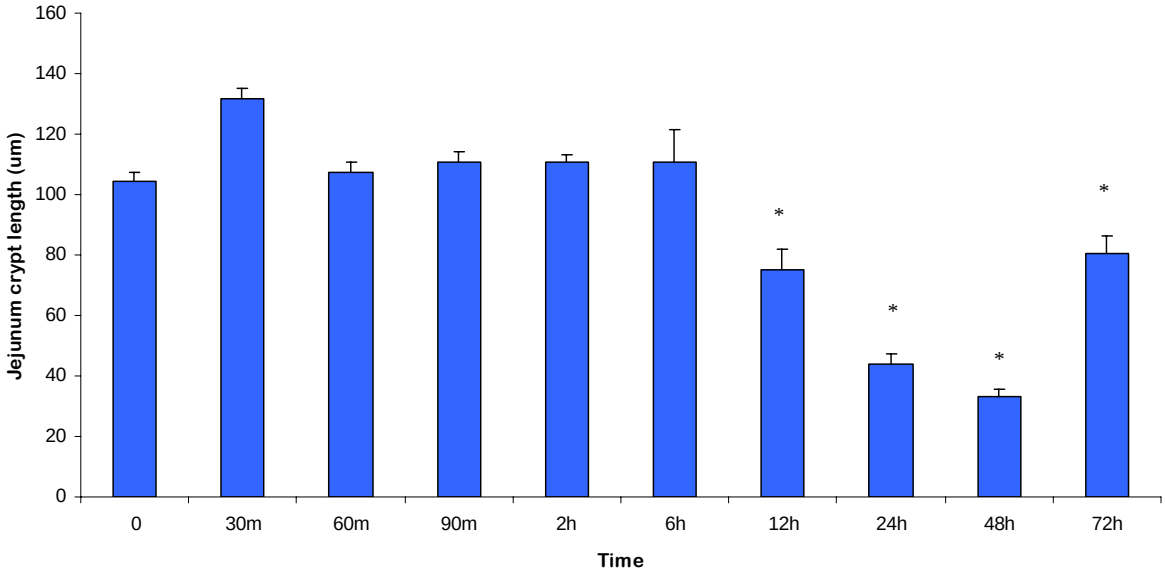

Figure 4.8

Changes in the crypt length in the jejunum mucosa over the 72 hour time period following the administration of 5-fluorouracil.

Colon

Methotrexate

Histological examination revealed minimal changes in the colon in rats given MTX (Figure 4.9). For MTX treated rats an increase in crypt length was observed between 2 and 6 hours ($p=0.0207$ and $p<0.0001$ respectively) followed by a return to normal levels (Figure 4.10).

5-Fluorouracil

Likewise, 5-FU also caused minimal histological change in the colon (Figure 4.11). 5-FU administration resulted in increased numbers of apoptotic bodies within the deep aspects of the crypts. With respect to crypt length, increased length was noted 90 minutes ($p=0.0007$) followed by a brief reduction at 6 hours ($p=0.0036$) a further reduction occurred by 72 hours ($p=0.00145$) (Figure 4.12).

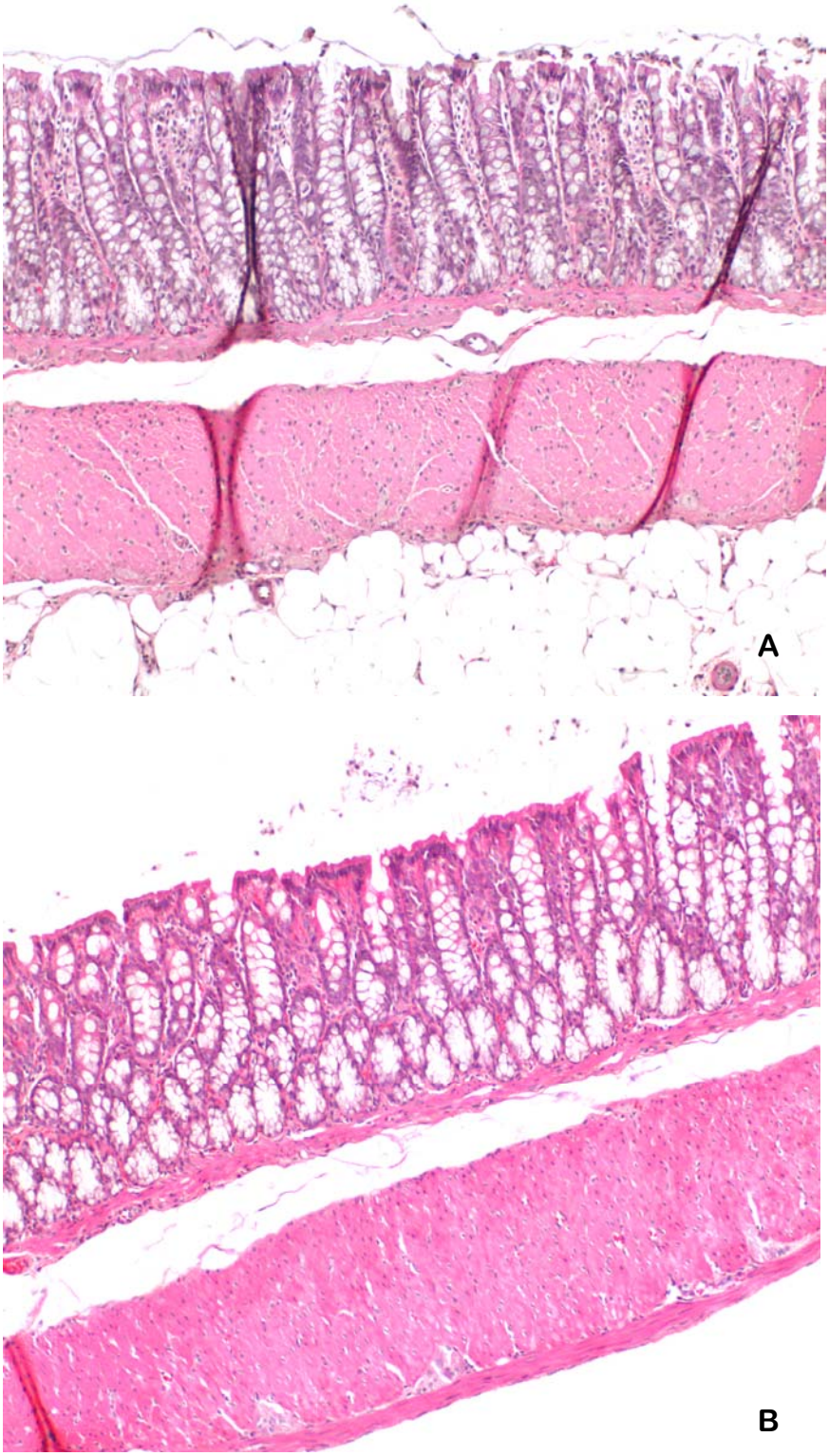

Figure 4.9

Histological features of the colon at baseline (A) and at 24 hours (B) following methotrexate administration. (Original magnification x20)

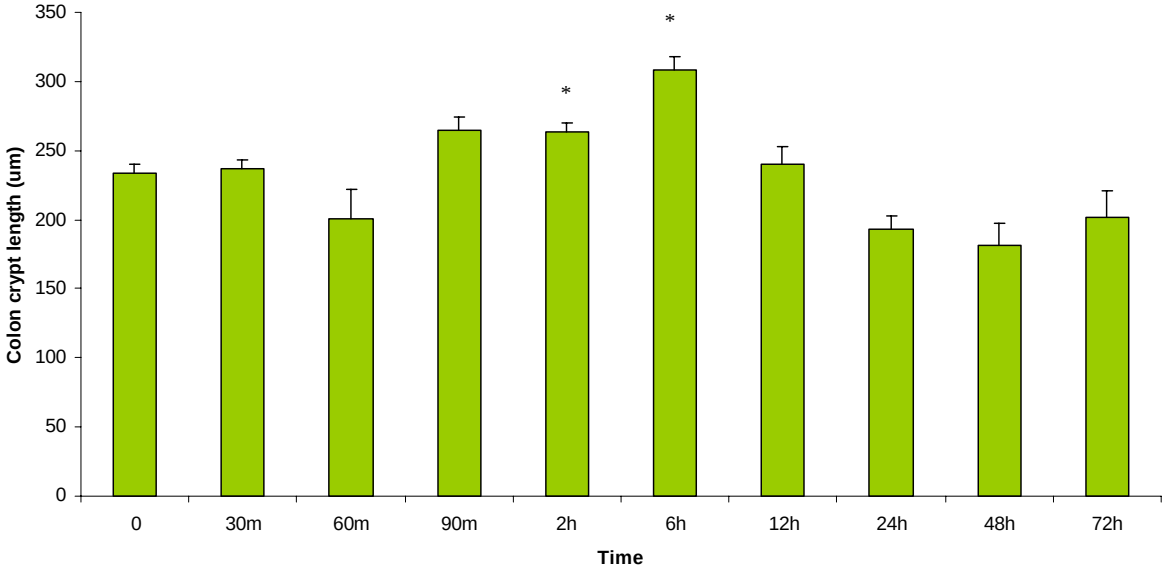

Figure 4.10

Changes in the crypt length of the colon mucosa over the 72 hour time period following the administration of methotrexate.

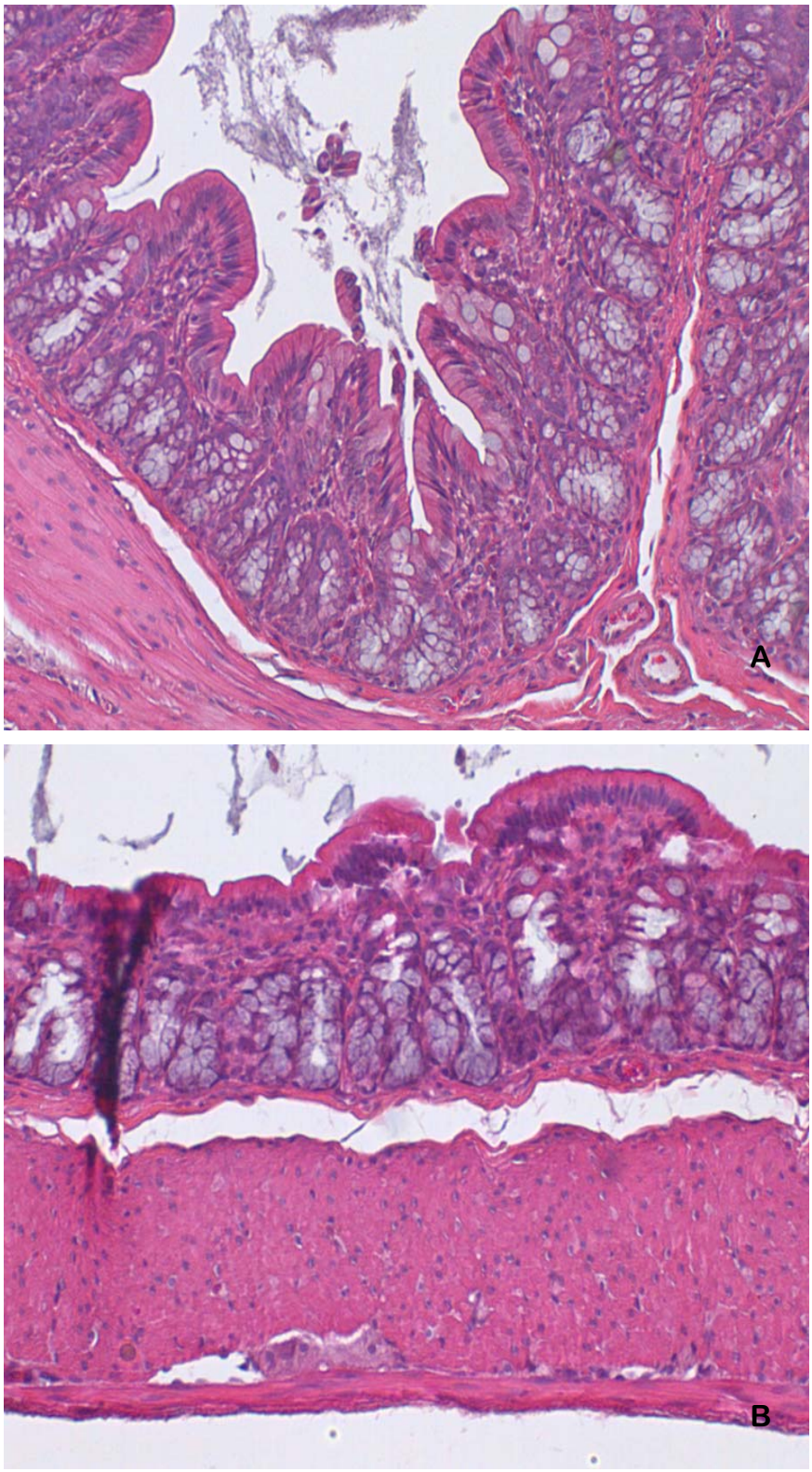

Figure 4.11

Histological features of the colon at baseline (A) and at 48 hours (B) following 5-fluorouracil administration. (Original magnification x20)

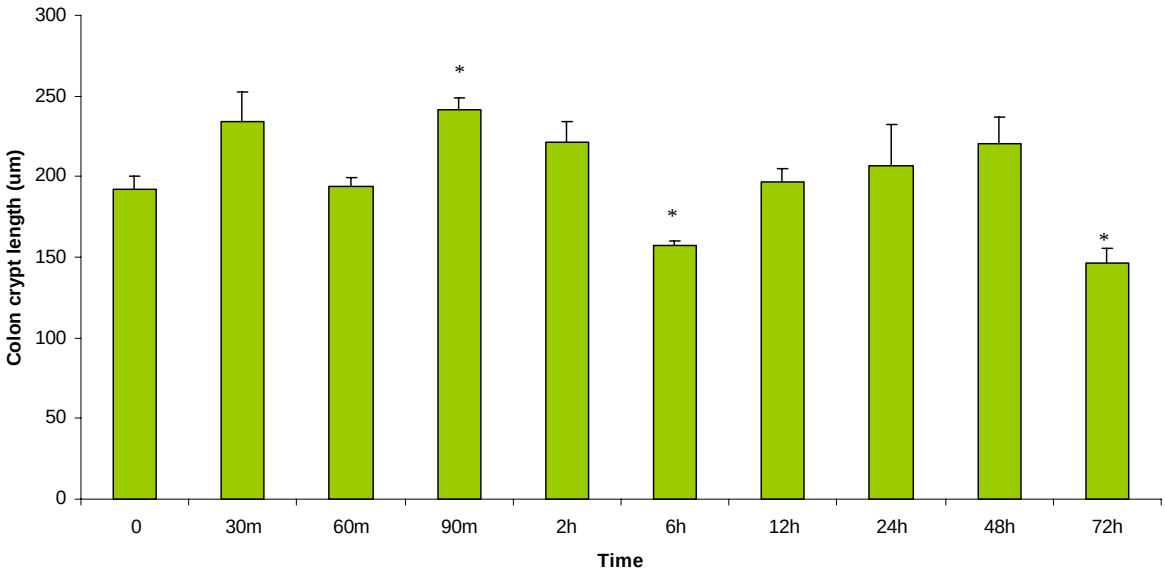

Figure 4.12

Changes in the crypt length of the colon mucosa over the 72 hour time period following the administration of 5-fluorouracil.

4.3.3 Comparison between changes caused by different drugs

Post hoc comparison of data (including irinotecan data from Chapter 3) demonstrated that there was a significant difference in the changes in epithelial thickness of the oral mucosa caused by the different drugs ($p=0.0002$). This difference was dependent on time ($p<0.0001$) and group ($p=0.0044$). With respect to specific time points, post hoc comparison demonstrated that irinotecan and MTX caused earlier changes in epithelial thickness compared to 5-FU at 90 minutes ($p=0.0197$ and $p=0.0005$ respectively). A difference was also observed between changes caused by irinotecan and the other drugs at 2 hours ($p<0.0001$). No difference was observed between MTX and 5-FU at later time points (Figure 4.13).

Likewise a significant difference was observed in the jejunum with respect to changes in crypt length caused by the 3 different drugs ($p<0.0001$), and again it was dependent on time ($p<0.0001$) and group ($p<0.0001$). Differences were observed at 30 minutes between rats who received irinotecan compared to 5-FU ($p<0.0001$) with an earlier reduction in crypt length observed in the rats who received irinotecan. A significant difference between the irinotecan group and MTX group was observed at 6 hours ($p=0.0008$). Again earlier resolution of damage in the irinotecan group was observed with differences at later time points (24 hours, $p<0.0001$; 48 hours, $p<0.0001$ and 72 hours, MTX, $p=0.0004$ and 5-FU, $p<0.0001$). Comparisons between MTX and 5-FU demonstrated that 5-FU caused a greater reduction in crypt length compared with MTX at 24 and 48 hours ($p<0.0001$ and $p=0.0598$ respectively) (Figure 4.14).

In the colon, a significant difference was also observed in changes caused by all three drugs ($p<0.0001$) with dependence on time ($p<0.0001$) and group ($p=0.0041$). Differences were observed at 30 minutes between rats who received irinotecan compared to 5-FU ($p<0.0001$) with an early increase in crypt length observed in the rats who received irinotecan. At 6 hours, MTX caused an increase in crypt length with resolution noted in rats who received

irinotecan ($p<0.0001$). Conversely 5-FU administration resulted in a decrease in crypt length at 6 hours which was significant compared to irinotecan and MTX ($p<0.0001$). At 24 hours a significant difference was observed between irinotecan which caused an increase in crypt length compared to MTX and 5-FU ($p<0.0001$ and $p=0.0434$ respectively) (Figure 4.15).

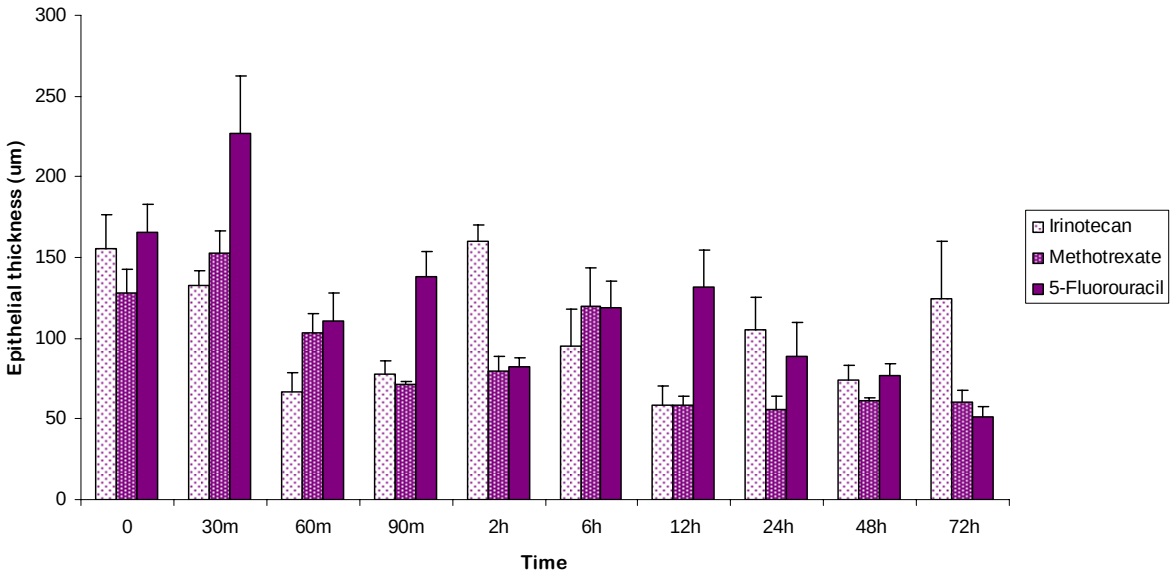

Figure 4.13

Comparison between changes that occurred in the thickness of the oral epithelium over the 72 hour time period following the administration of irinotecan, methotrexate or 5-fluorouracil.

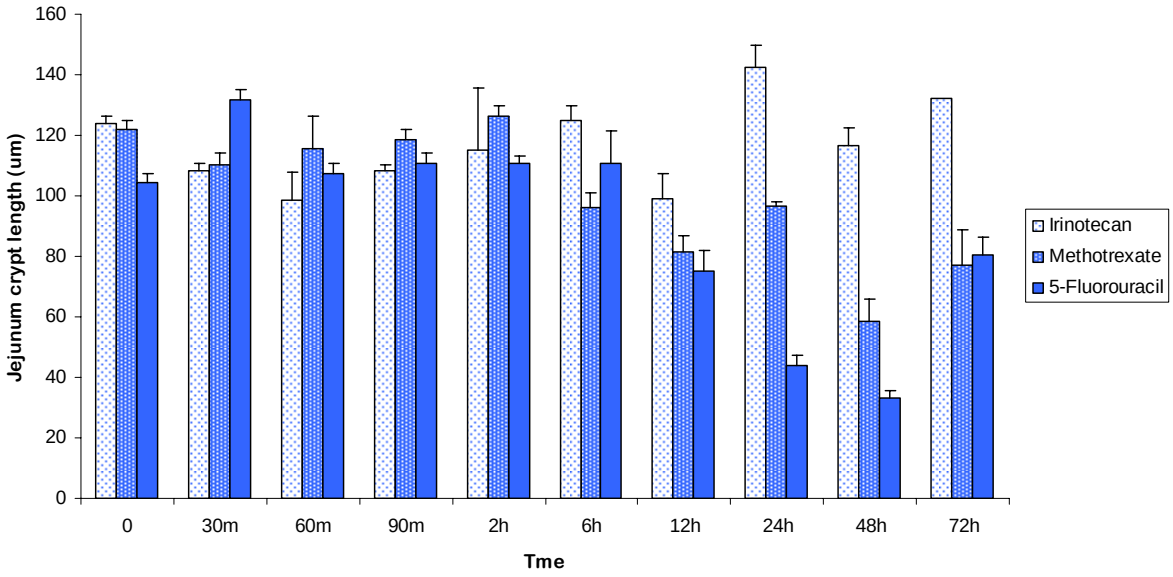

Figure 4.14

Comparison between changes that occurred in the crypt length in the jejunum mucosa over the 72 hour time period following the administration of irinotecan, methotrexate or 5-fluorouracil.

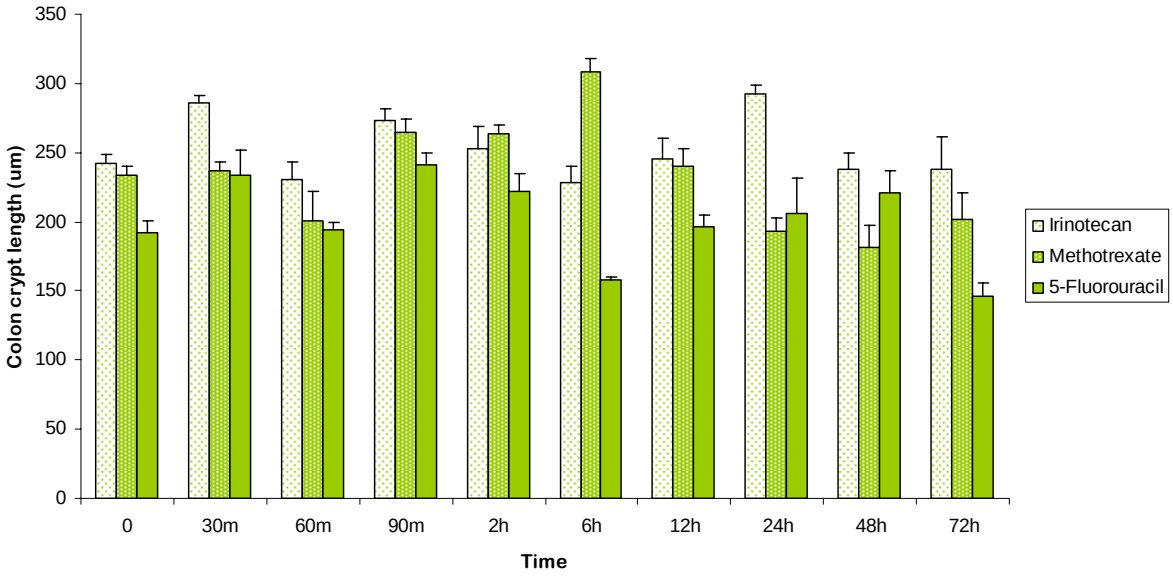

Figure 4.15

Comparison between changes that occurred crypt length in the colon mucosa over the 72 hour time period following the administration of irinotecan, methotrexate or 5-fluorouracil.

4.3.4 Immunohistochemistry

NF-κB

The level of staining for NF-κB within the oral mucosa was elevated at the majority of time points subsequent to MTX administration (Figure 4.16). Although approaching significance, the results for the tissue levels of NF-κB expression were not significant (p=0.078). In the jejunum and colon statistically significant levels of tissue staining for NF-κB were observed peaking at 6 hours in the jejunum (p=0.008) and at 12 hours in the colon (p=0.005) (Fig 4.16).

Positive staining for NF-κB was observed in the epithelium of all of the tissues examined as well as occasional fibroblasts and inflammatory cells within the submucosa.

5-FU administration did not cause significantly elevated tissue staining for NF-κB within the oral mucosa, jejunum or the colon (Figure 4.17). Again, the epithelial cells stained positively for NF-κB as well as fibroblasts and inflammatory cells within the underlying submucosal tissue.

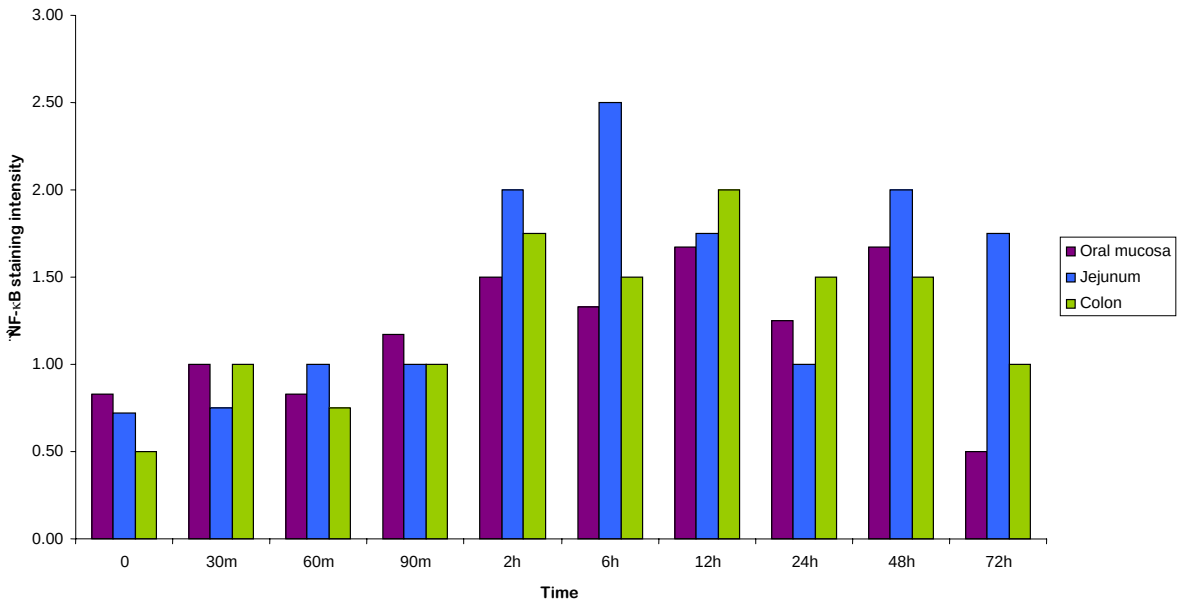

Figure 4.16

NF-κB staining in the oral mucosa, jejunum and colon over the 72 hour time period following the administration of methotrexate.

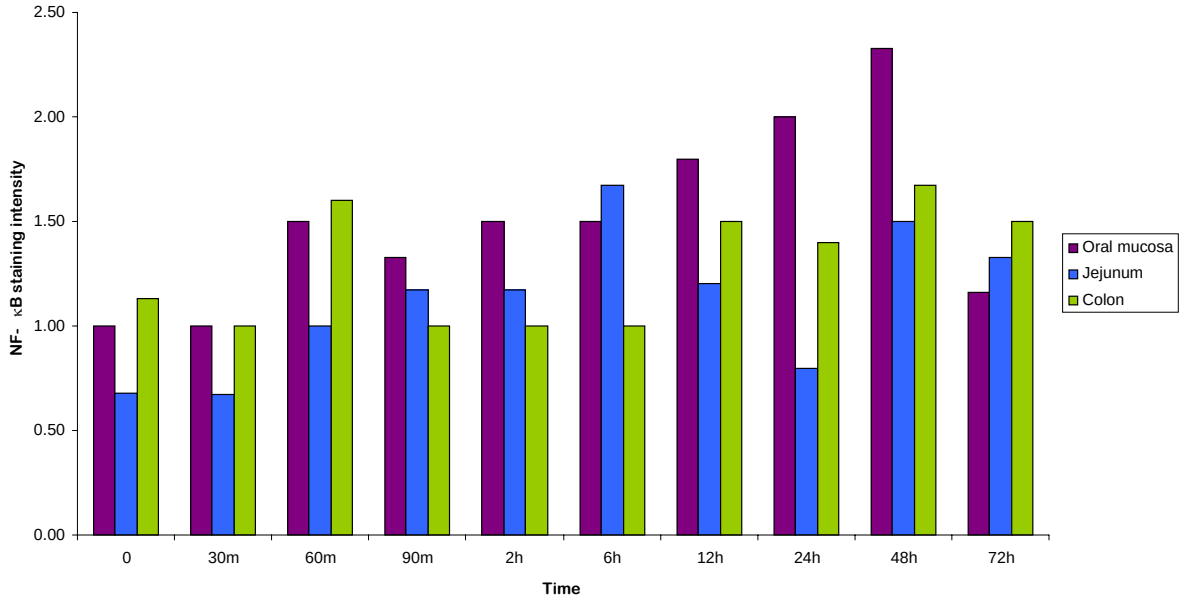

Figure 4.17

NF-κB staining in the oral mucosa, jejunum and colon over the 72 hour time period following the administration of 5-fluorouracil.

TNF

Significantly elevated levels of TNF were observed in the oral mucosa, jejunum and colon subsequent to MTX administration ($p=0.0005$, $p=0.0007$ and $p=0.002$ respectively) (Figure 4.18). In the oral mucosa the tissue staining for TNF peaked at between 2 and 6 hours, in the jejunum at 6 hours, whilst in the colon the elevation of TNF was more prolonged and peaked at 24 hours.

TNF staining was significantly elevated in the oral mucosa and colon following administration of 5-FU ($p=0.008$, oral mucosa; $p=0.001$, jejunum; $p=0.0002$, colon). As for MTX, TNF levels peaked earlier in the oral mucosa and jejunum, whilst in the colon the peak levels occurred later and were more prolonged (Figure 4.19).

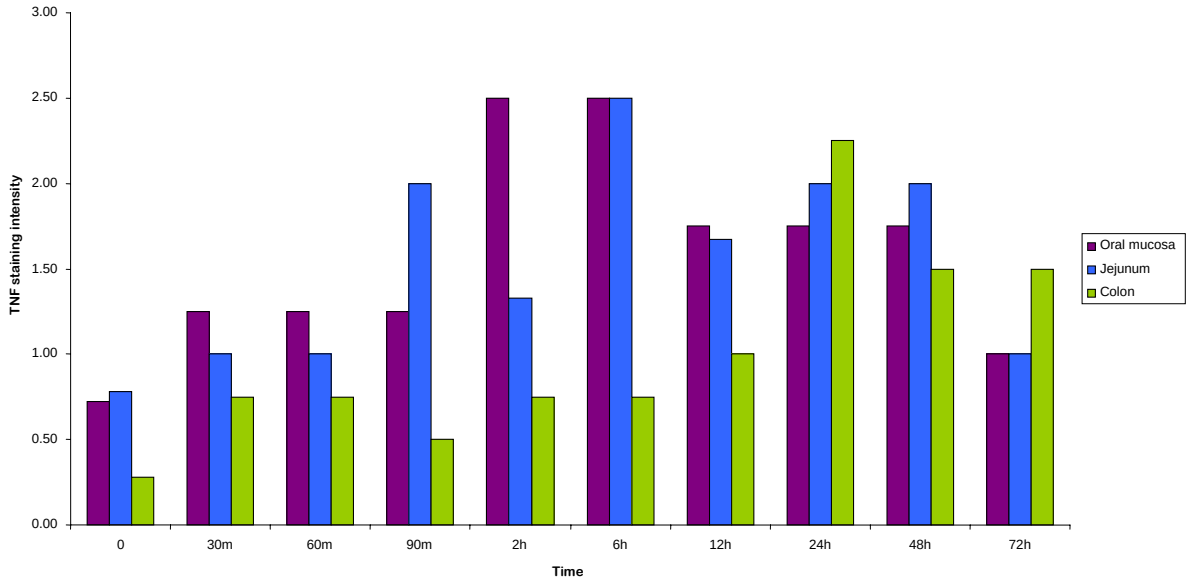

Figure 4.18

TNF staining in the oral mucosa, jejunum and colon over the 72 hour time period following the administration of methotrexate.

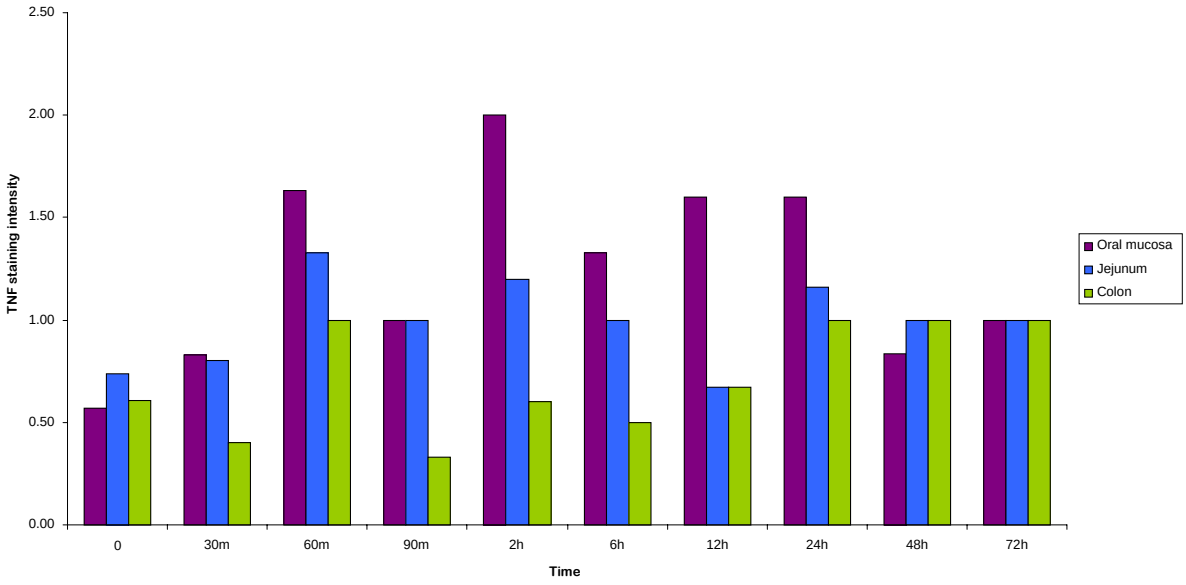

Figure 4.19

TNF staining in the oral mucosa, jejunum and colon over the 72 hour time period following the administration of 5-fluorouracil.

IL-1β

Following MTX administration, significantly elevated levels of IL-1β were observed in the oral mucosa, jejunum and colon ($p=0.0016$, $p<0.0001$ and $p=0.0064$ respectively). In each of the three AT sites examined there appeared to be two peaks of IL-1β, at 6 and 48 hours in the oral mucosa, at 2 and 24 hours in the jejunum and colon (Figure 4.20).

With respect to IL-1β levels following 5-FU administration, only oral mucosa demonstrated significant results ($p=0.0013$) with a peak in staining intensity observed at 6 hours (Figure 4.21). Although not significant, peaks of IL-1β were observed in the jejunum and colon at 90 minutes (Figure 4.21).

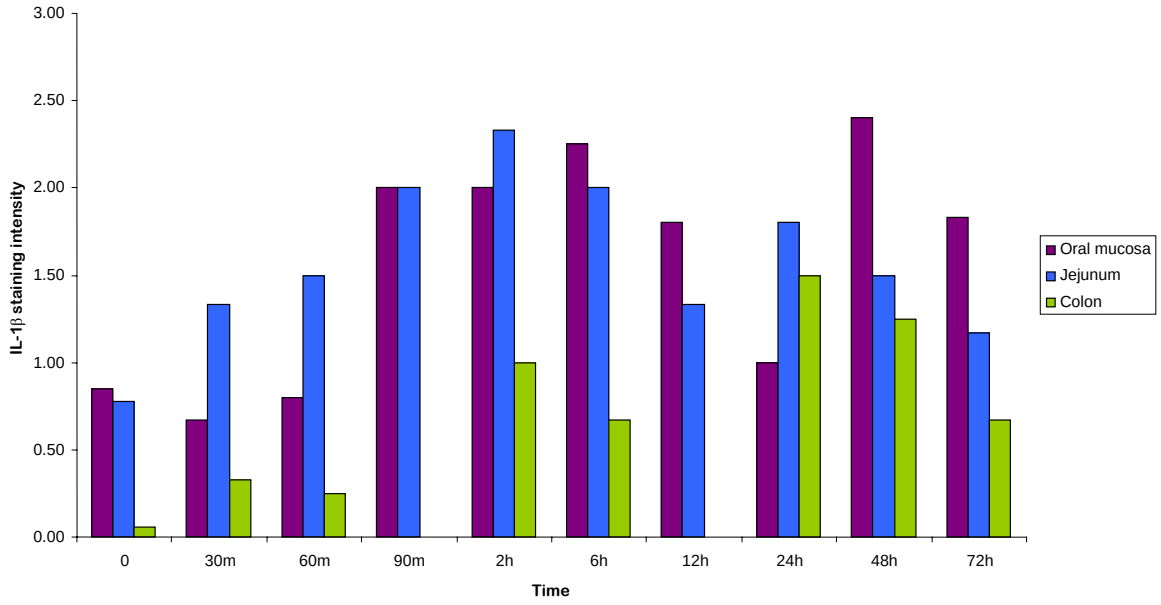

Figure 4.20

IL-1β staining in the oral mucosa, jejunum and colon over the 72 hour time period following the administration of methotrexate.

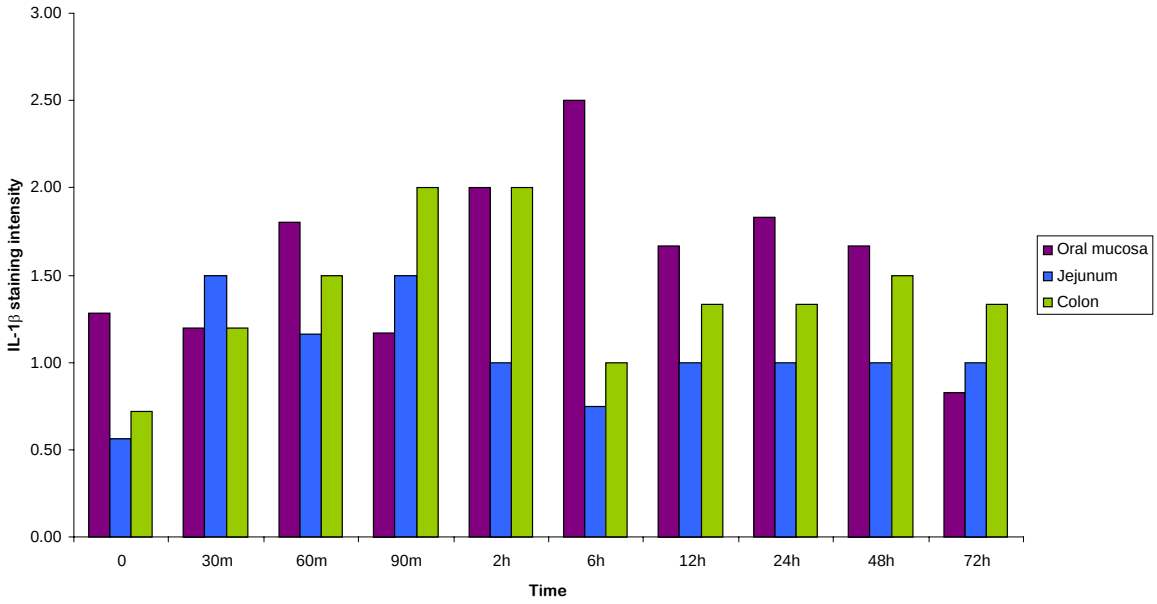

Figure 4.21

IL-1β staining in the oral mucosa, jejunum and colon over the 72 hour time period following the administration of 5-fluorouracil.

IL-6

The level of IL-6 expressed in the tissues following MTX was significantly elevated in the oral mucosa, jejunum and colon ($p=0.0004$, $p=0.0083$ and $p=0.0013$ respectively). Early peaks in IL-6 staining were observed in the oral mucosa and colon at 6 hours (Figure 4.22), whilst in the jejunum, IL-6 staining peaked at 2 hours and again at 48 hours (Figure 4.22)

Following the administration of 5-FU, no significant staining for IL-6 was observed in any of the tissues examined (Figure 4.23).

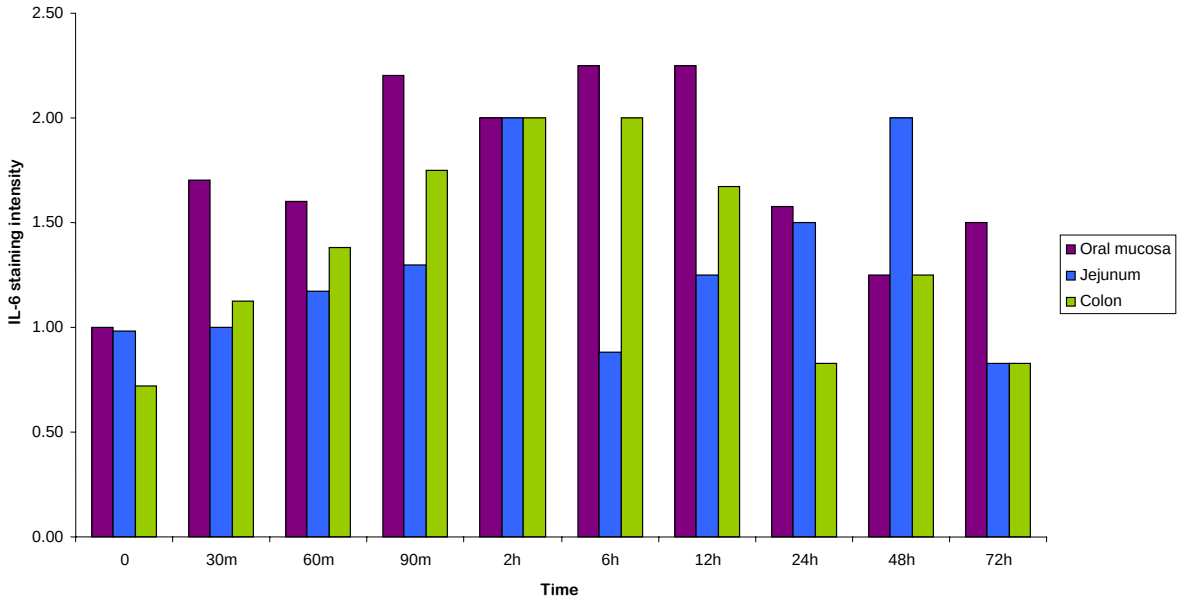

Figure 4.22

IL-6 staining in the oral mucosa, jejunum and colon over the 72 hour time period following the administration of methotrexate.

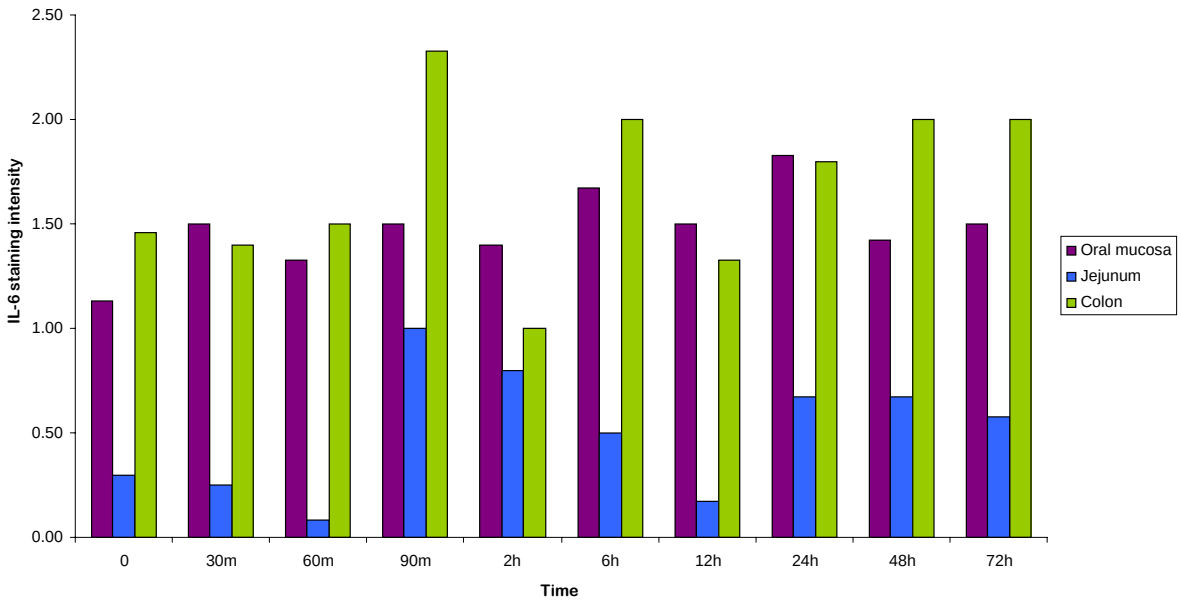

Figure 4.23

IL-6 staining in the oral mucosa, jejunum and colon over the 72 hour time period following the administration of 5-fluorouracil.

4.4 Discussion

In Chapter 3 it was demonstrated that, in the DA rat, the administration of irinotecan caused histological changes consistent with AT mucositis in separate anatomical sites as well as altering the tissue expression of proinflammatory cytokines [94]. This observation supported the hypothesised role of NF-κB activation and subsequent elevation of tissue levels of TNF, IL-1β and IL-6 [94]. The main objective of this part of the study was to determine whether different drugs with different modes of action caused similar changes in the AT of the DA rat.

Following MTX or 5-FU administration, the *type* and *pattern* of histological change that was observed at different sites of the AT was similar over the 72 hours following administration of the drug. However when the two experimental groups were compared, differences were observed with respect to the *timing* of changes. MTX administration resulted in an initial decrease in epithelial thickness of the oral mucosa followed by a brief increase to normal levels and then a subsequent decrease in epithelial thickness over the 72 hour time period. Similar changes were observed in the jejunum and colon with respect to crypt length; however the initial decrease was not significant. 5-FU administration, on the other hand, although not significant in the oral mucosa and colon caused an initial increase in oral epithelial thickness and intestinal crypt length followed by a gradual reduction over the remainder of the 72 hour period.

An interesting feature observed in this study was the apparent increased resilience of the colon compared to other sites of the AT. In this site, an increase in crypt length occurred following administration of MTX and 5-FU. A decrease in crypt length was either brief (in the case of MTX) or apparent only at the 72 hour time point. This "hyperplastic" response in the colon was also observed following irinotecan administration [94]. Does this difference between the colon and the other mucosae reflect a true increased resilience of the colon? If so,

what protective mechanisms are present in the colon that promote this hyperplastic response? It has previously been suggested that the location of stem cells within the colon renders them more resistant to damage, so that although apoptosis may be increased this does not necessarily lead to tissue damage occurring [52].

In Chapter 3 the histological changes in the AT caused by irinotecan are similar to those that resulted from MTX and 5-FU administration. It has been suggested that the mucosae may have a relatively limited "repertoire" in its response to the insult caused by drug administration [75]. These studies support this notion. Irinotecan administration, however appeared to cause histological changes that were more severe than those resulting from MTX and 5-FU [94]. The timing of changes observed following irinotecan administration were also different and indicated a shorter, more intense insult with evidence of resolution by 72 hours [94] unlike in the current study where oral epithelial thickness and jejunum crypt length remained reduced compared to baseline levels at 72 hours. It is unclear why irinotecan caused this difference in timing and intensity, however it is postulated that the cholinergic side effects of irinotecan may potentiate the damage that occurs following its administration. As mentioned the same *type* of damage appears to be caused by MTX and 5-FU, however this is protracted compared to irinotecan; it would be interesting to observe ongoing changes at time points beyond 72 hours to determine whether the pattern of damage is similar. As this damage appears to occur over a more extended time period following MTX and 5-FU administration it is likely that this results in reduced severity of the histological changes observed.

With respect to tissue levels of NF-κB and pro-inflammatory cytokines, further differences were observed between the drugs. Similar staining to that seen previously with irinotecan was observed following the administration of MTX whereby an early peak in tissue staining

for NF-κB, TNF, IL-1β and IL-6 occurred [94]. 5-FU however showed no significant increase in tissue staining for NF-κB or IL-6 in the experimental groups compared with controls.

Why was there a difference in the changes caused by different drugs? It is well documented that MTX, 5-FU and irinotecan all cause mucositis [24, 57, 90, 94] and the histological examination of the tissues in this study demonstrated damage particularly in the jejunum and colon. MTX has been shown to cause suppression of NF-κB in Jurkatt cells [100] as well as suppression of pro-inflammatory cytokines such as IL-6 [86]. This, however, does not appear to have occurred subsequent to the administration of this drug in the DA rats in the current study. Apart from direct effects of MTX it is possible that the increased expression of NF-κB and pro-inflammatory cytokines may be a secondary phenomenon that occurs subsequent to MTX-induced tissue damage via other unidentified pathways as there are many other factors that can activate NF-κB apart from chemotherapy [135]. Furthermore, protective factors in the gut, for example trefoil factors, have also been shown to be affected by MTX, possibly rendering the AT more susceptible to tissue damage [33, 155]. Tissue damage would expose the tissues to other factors that may promote NF-κB activation such as cell wall products from resident bacteria within the gut [136].

Like MTX, 5-FU administration has been shown to inhibit NF-κB activation [7, 9]. The results of this study support this in that there was no observed difference between experimental and control groups in NF-κB following 5-FU administration. A murine model describing 5-FU-induced intestinal damage demonstrated that intestinal toxicity arose due to the combined effects of apoptosis and inhibition of cell proliferation. These events lead to reduced cellularity in the crypts and villi of the intestine [124]. The same authors suggest that the mechanisms that lead to such damage were dependant on p53. It has been suggested that nitric oxide (NO) may a play a role in 5-FU induced mucositis through NO-dependent

activation of p53 as well as members of the Bcl-2 family [90]. Further investigation into the mechanisms through which 5-FU causes mucosal toxicity is required.

The results from this study confirmed our hypothesis that the expression of NF-κB and pro-inflammatory cytokines in the AT of the DA rat following chemotherapy appears to be influenced by the type of drug administered. Furthermore, the pattern of histological changes that occurred at different sites of the AT also appeared to be influenced to some degree by the type of drug. Of course, the type of drug is unlikely to be the only variable that determines whether or not patients will develop mucositis. Other factors may also impact on a patient's risk for developing mucositis including hormonal factors, underlying systemic illness and genetic factors, not to mention factors in the local tissue environment [5]. In addition, many treatment protocols involve the combination of different drugs which adds further complexity to the mechanisms through which AT toxicity occurs. Further investigation is required so that more effective, or even targeted, strategies can be developed for the treatment of mucositis.

Chapter 5

Serum levels of NF-κB and pro-inflammatory cytokines following administration of mucotoxic drugs.

5.1 Introduction

In the previous chapters, immunohistochemical evidence of tissue changes in levels of NF-κB, TNF, IL-1β and IL-6 in the AT of the DA rat following the administration of irinotecan, MTX or 5-FU. These changes in NF-κB and pro-inflammatory cytokines were apparent prior to *marked* histological evidence of damage to the AT [93, 94]. Clinical evidence from patients undergoing chemotherapy, described in Chapter 2, also suggests that changes occur in the mucosa prior to the development of clinical indications of damage, such as ulceration [55, 95]. The ability to detect these subclinical changes would potentially be a useful tool in the management of mucositis. However, routinely taking tissue samples from different sites of the AT, particularly the small and large intestine is not a practical option for all patients undergoing cancer treatment as a means to determine their likelihood for developing mucositis because of the increased risk of complications associated with invasive procedures in these patients.

Previous studies have demonstrated the association between changes in serum levels of proinflammatory cytokines, for example TNF, and non-haematological side effects of bone marrow transplantation [15, 63]. These studies found that the changes in serum levels preceded the development of major transplant related complications. Changes in serum levels of pro-inflammatory cytokines have not *specifically* been investigated in the context of mucositis. It is hypothesized that this may be a useful clinical tool in evaluating a patient's risk of developing mucositis. Conceivably, the identification of these sub-clinical events may facilitate better management of mucositis enabling the institution of more appropriate treatment for mucositis or, more importantly, prevent mucositis occurring completely.

The aims of this part of the study were to:

1. Determine whether changes in serum levels of NF-κB, TNF, IL-1β and IL-6 occurred following chemotherapy and whether these were dependent on the type of drug administered;

2. Compare changes in serum levels with histological evidence of tissue damage at different sites of the AT.

5.2 Materials and Methods

Detailed descriptions of the experimental design have been included in the preceding chapters. Briefly, 3 groups of 81 female DA rats were administered irinotecan, MTX or 5-FU. For each drug, rats were randomly assigned to one of nine groups according to a specified time point (30, 60, 90 minutes, 2, 6, 12, 24, 48 and 72 hours) each group included 3 controls and 6 experimental animals. All rats in the experimental groups received a single intraperitoneal dose of either 200mg/kg of irinotecan, a single intramuscular dose of 1.5 mg/kg of MTX or a single intraperitoneal dose of 150mg/kg of 5-FU. Rats in the control groups did not receive any drug. Subsequent to the administration of the drug the following endpoints were assessed four times per 24 hour period: mortality, diarrhoea, and general clinical condition. At the appropriate time point, the rats were killed by exsanguination and cervical dislocation.

Specimens from the oral mucosa, jejunum and colon were dissected and fixed for analysis as described previously.

5.2.1 Sample preparation for ELISA detection of serum NF-κB and pro-inflammatory cytokines

Blood samples were collected from all rats via cardiac puncture and centrifuged at 5000rpm to collect serum. Serum levels of NF-κB, TNF, IL-1β and IL-6 were measured using NF-κB/p65 ActivELISA (Imgenex Corporation), Rat TNF-α ELISA Ready-SET-Go! (Bioscience Inc.), Rat IL-1β and Rat IL-6 Immunoassay (Biosource International Inc) ELISA kits respectively according to each manufacturer's instructions. The standards for NF-κB, TNF, IL-1β and IL-6 ranged from 0-100ng/mL, 0-100pg/mL, 0-2000pg/mL and 0-2000pg/mL respectively. All samples were tested in duplicate.

5.2.2 Statistical analysis

Statistical analysis of the data was carried out using an unpaired t-test (Graphpad Prism 5).

5.3 Results

5.3.1 Response to treatment

The rats' response to drug administration has been described in previous chapters.

5.3.2 Serum levels of NF-κB and pro-inflammatory cytokines

NF-κB

A peak in serum concentration of NF-κB was detected at 6 hours following the administration of irinotecan ($p=0.006$). Levels remained significantly elevated compared to baseline at 12 hours ($p=0.046$). It was observed that serum NF-κB levels peaked earlier following MTX administration at 2 hours ($0<0.0001$). Conversely, 5-FU administration resulted in an early decrease in serum levels of NF-κB at 60 minutes ($p=0.02$). Serum levels then increased, peaking at 12 hours ($p<0.01$) before returning to baseline levels by 48 hours (Figure 5.1).

TNF

Administration of irinotecan resulted in two peaks in serum levels of TNF at 60 minutes ($p<0.0001$) and 24 hours ($p<0.0001$). In rats administered MTX, an early decreased in serum TNF was observed at 30 minutes ($p=0.01$) and later, at 72 hours ($p=0.01$). Increased levels of serum TNF were observed at 90 minutes ($p=0.02$) and 2 hours ($p=0.01$). Following 5-FU administration, a peak in serum TNF was observed at 90 minutes ($p=0.0001$) (Figure 5.2).

IL-1β

Serum IL-1β levels were decreased at 60 minutes ($p=0.002$) and were then increased at 2 hours ($p=0.04$) following irinotecan administration. A decrease in serum IL-1β was also observed in rats administered MTX at 6 hours ($p=0.01$). Following 5-FU administration IL-1β serum levels peaked at 60 minutes ($p<0.0001$) and at 12 hours ($p<0.0001$) (Figure 5.3).

IL-6

Following irinotecan administration, IL-6 demonstrated an increase in concentration peaking at 12 hours ($p<0.001$) and then rapidly reducing to baseline levels. In rats given MTX, IL-6 serum concentration was elevated compared to baseline levels between 90 minutes and 6 hours, peaking at 2 hours ($p<0.0001$). 5-FU administration did not result in any significant change in serum concentration of IL-6 over the 72 hour time period (Figure 5.4).

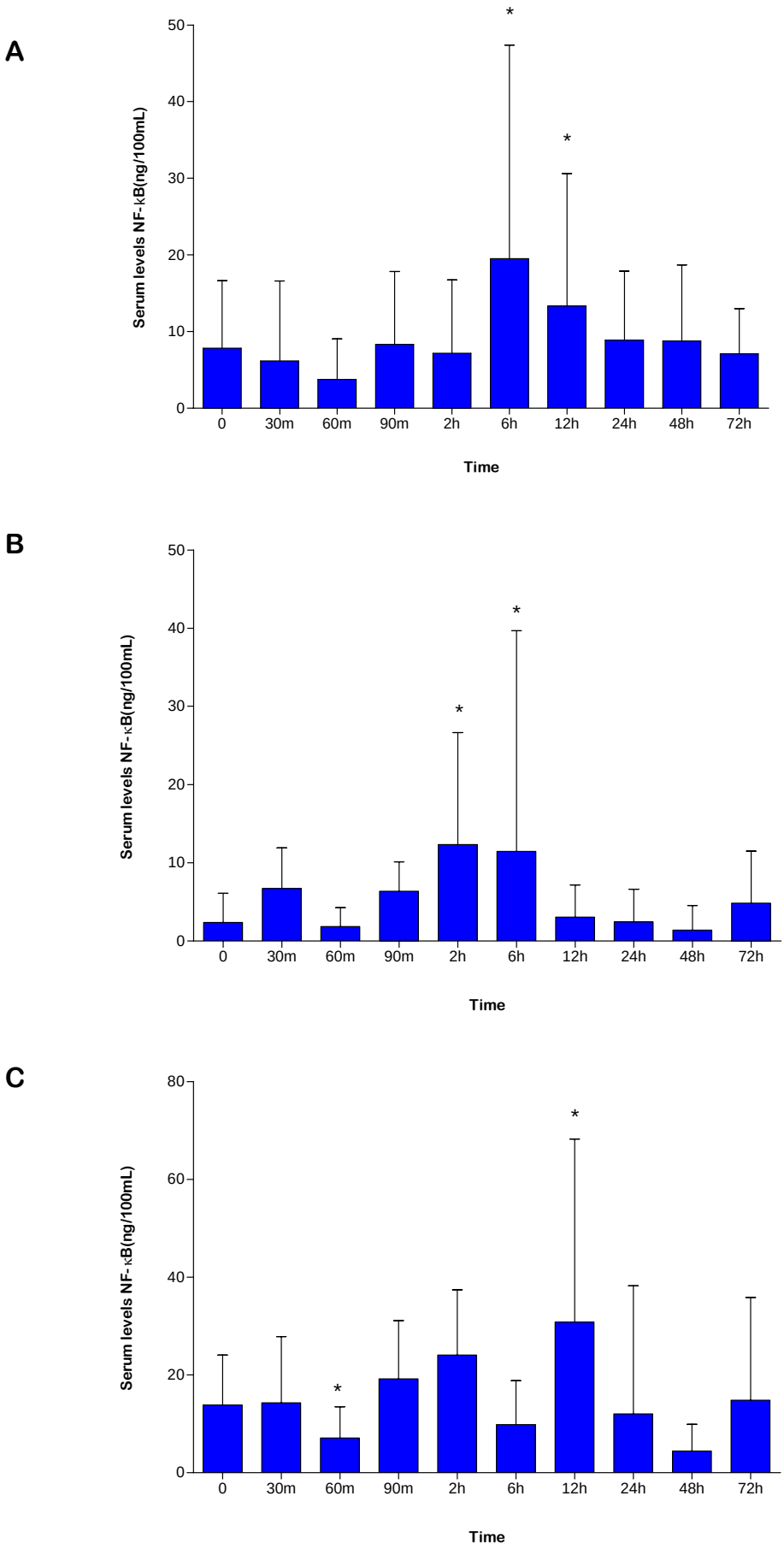

Figure 5.1

Serum concentration of NF-κB over the 72 hour time period following the administration of irinotecan (A), methotrexate (B) and 5-fluorouracil (C).

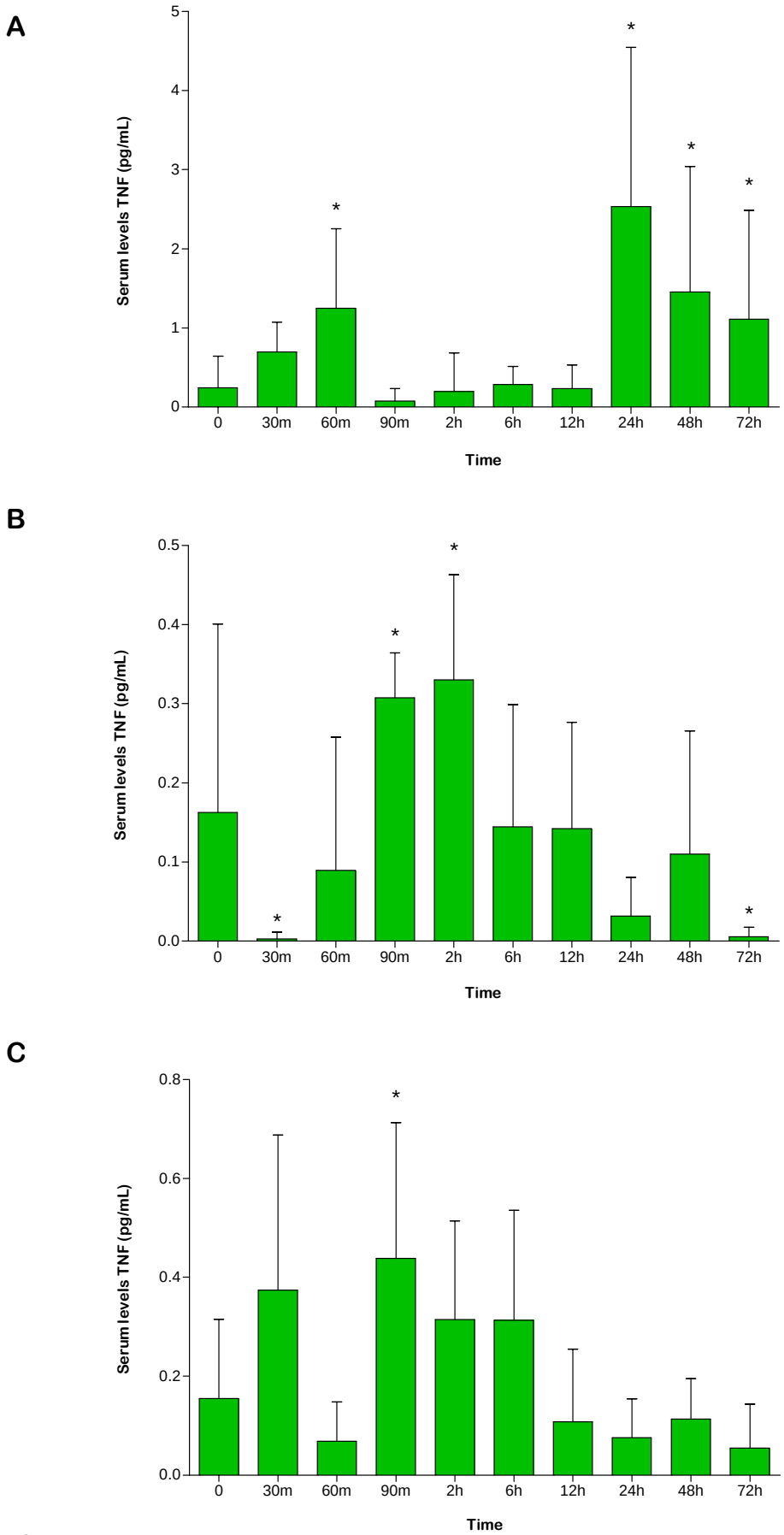

Figure 5.2

Serum concentration of TNF over the 72 hour time period following the administration of irinotecan (A), methotrexate (B) and 5-fluorouracil (C).

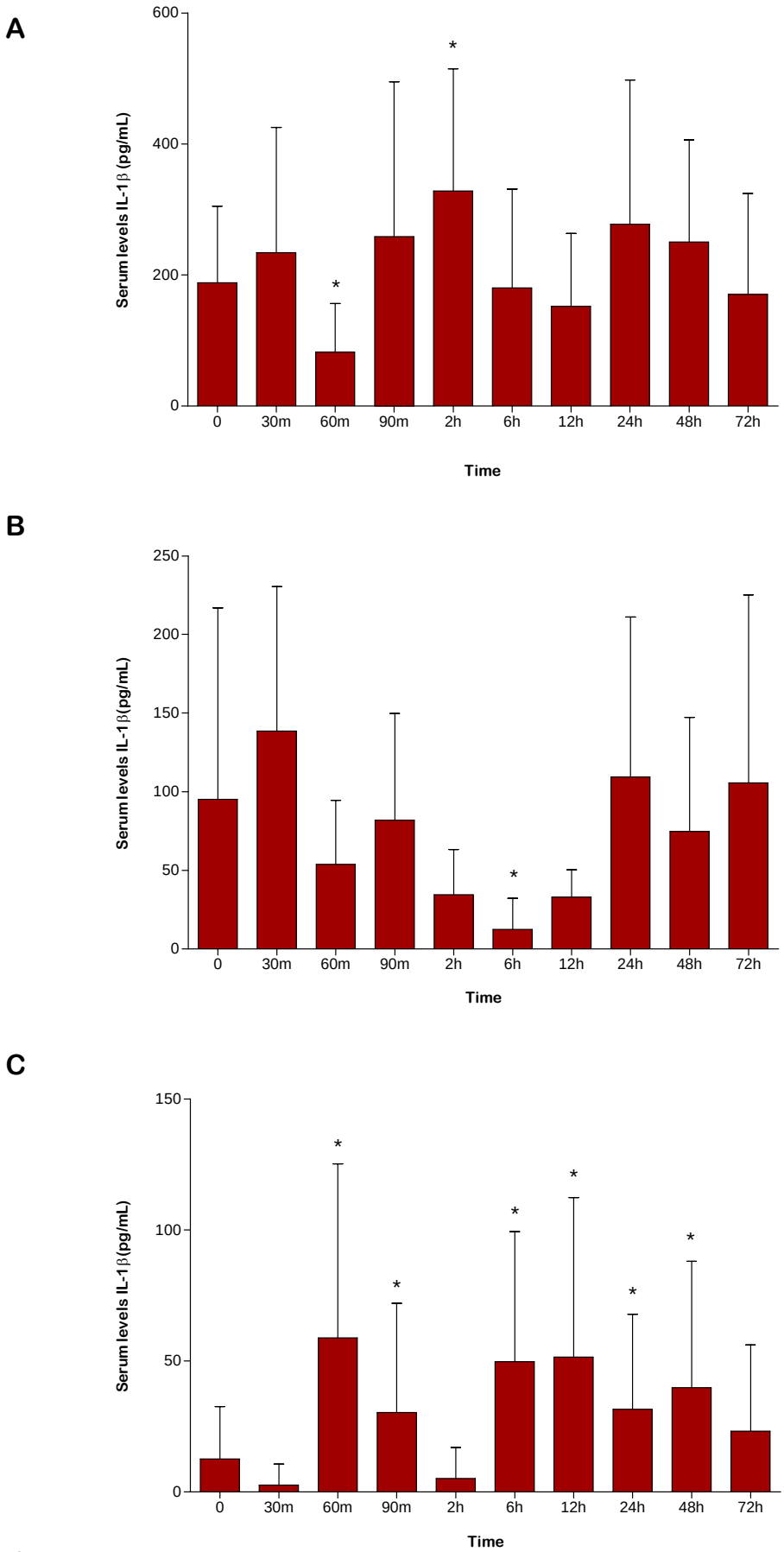

Figure 5.3

Serum concentration of IL-1β over the 72 hour time period following the administration of irinotecan (A), methotrexate (B) and 5-fluoruracil (C).

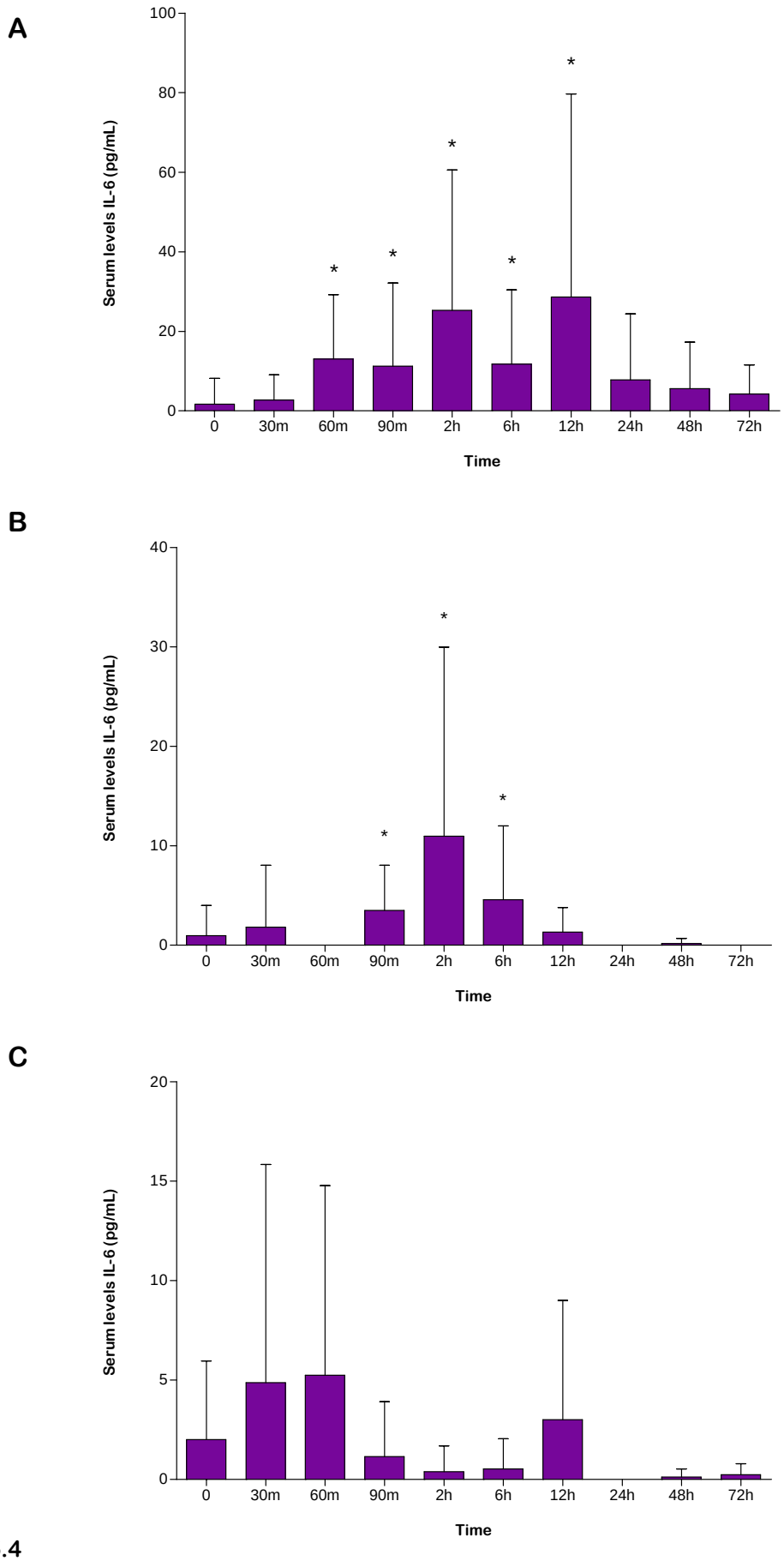

Figure 5.4

Serum concentration of IL-6 over the 72 hour time period following the administration of irinotecan (A), methotrexate (B) and 5-fluorouracil (C).

5.3.3 Comparison of serum levels with histological changes in different sites of the AT

The changes that occur in the mucosal histology at different sites of the AT following administration of irinotecan, MTX or 5-FU have been described in the previous chapters [93, 94]. These data, in comparison to the changes in serum levels, are summarised in figures 5.5 - 5.16. Following the administration of irinotecan and MTX, the peaks that occurred in serum concentrations occurred after initial histological changes in the tissues were observed. Exceptions to this included serum TNF following irinotecan administration, in which case increasing serum TNF coincided with histological changes (Figure 5.6). Peak serum IL-1β occurred prior to histological changes in the tissues following MTX administration (Figure 5.11). With respect to 5-FU administration, peaks in serum NF-κB, TNF, IL-1β and IL-6 were all observed prior to initial histological evidence of tissue damage (Figure 5.13 - 5.16).

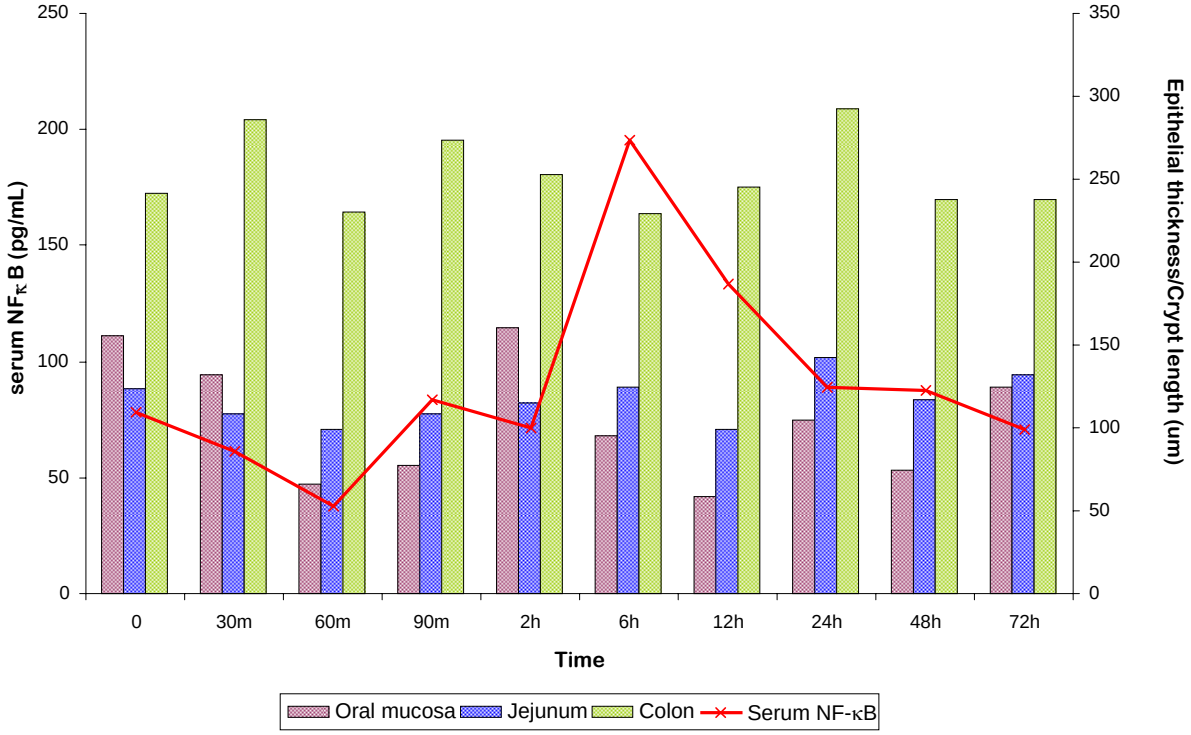

Figure 5.5

Comparison between serum NF-κB and histological changes in the AT following the administration of irinotecan.

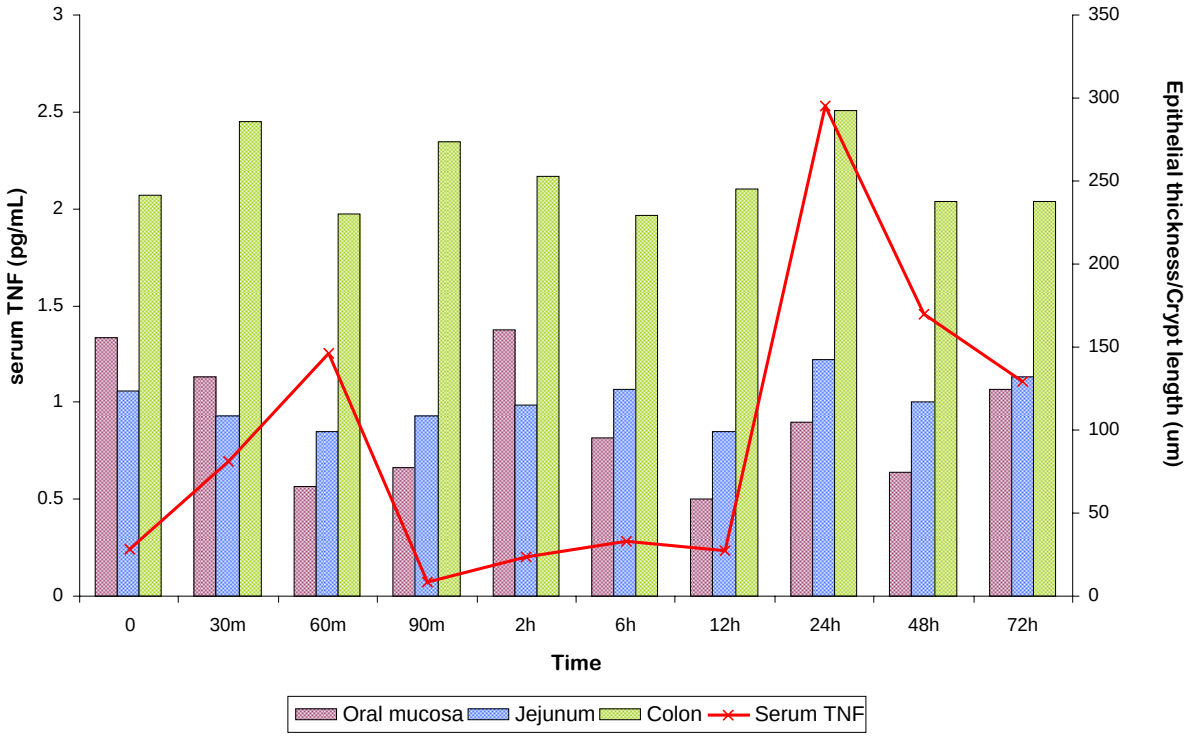

Figure 5.6

Comparison between serum TNF and histological changes in the AT following the administration of irinotecan.

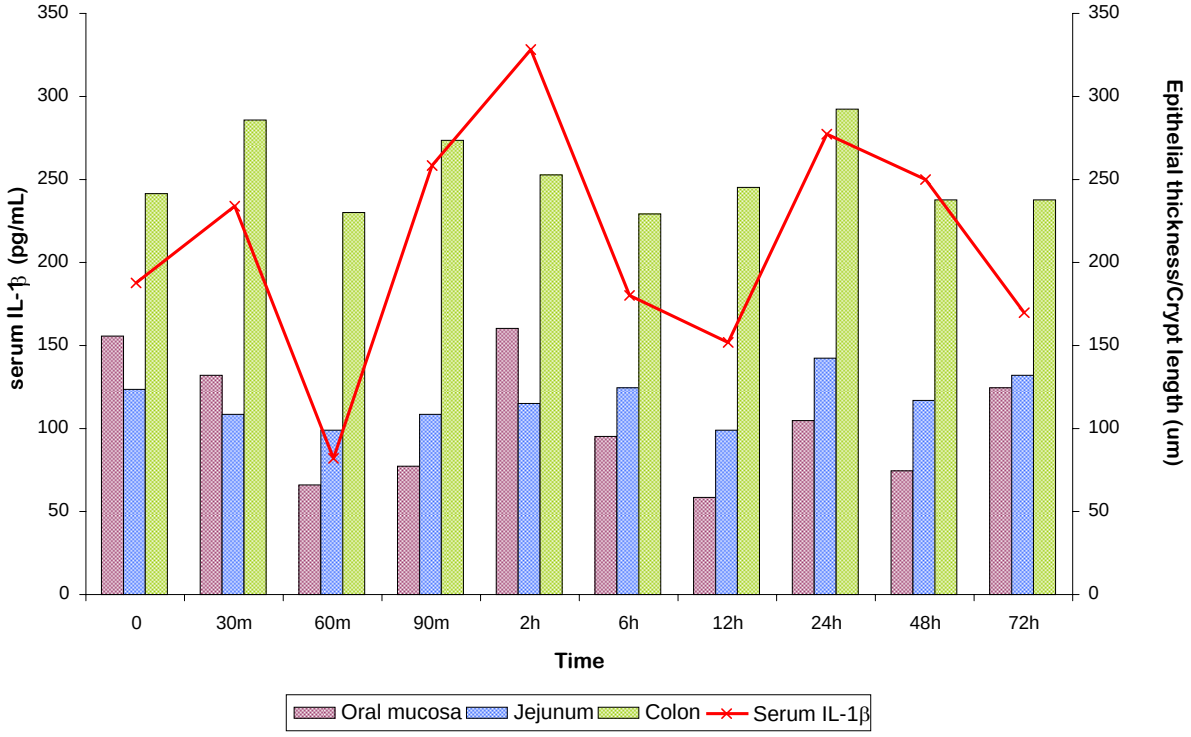

Figure 5.7

Comparison between serum IL-1β and histological changes in the AT following the administration of irinotecan.

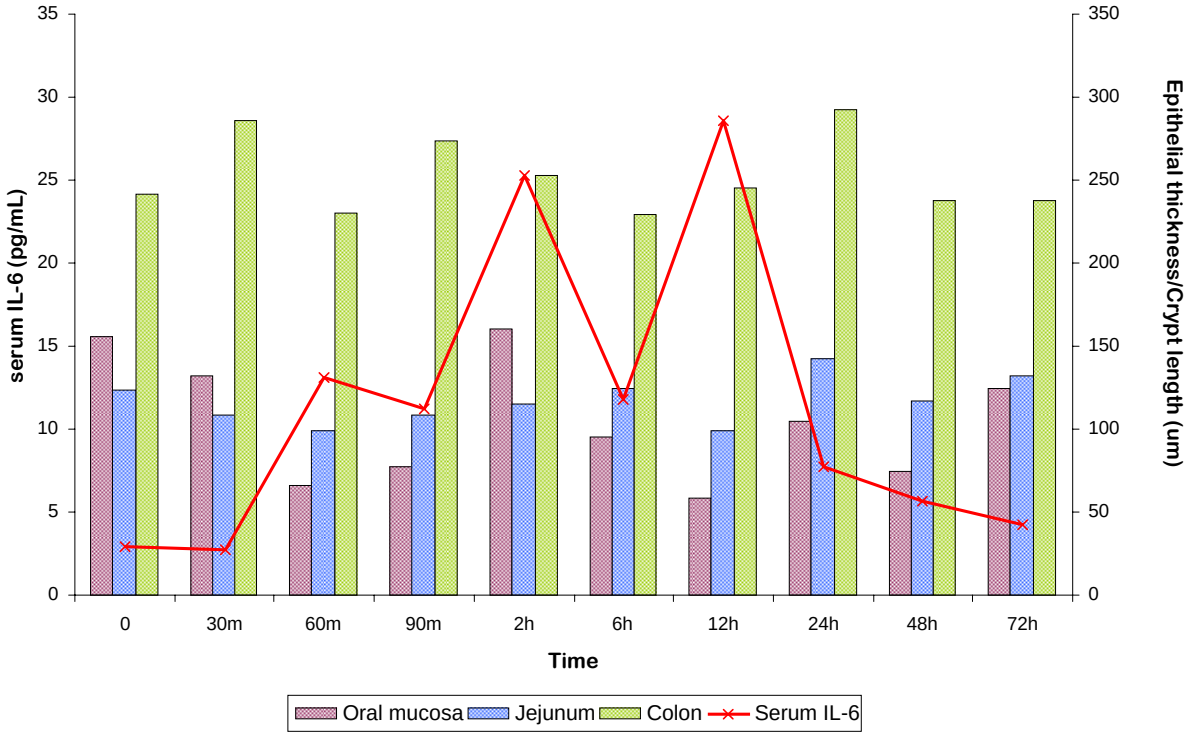

Figure 5.8

Comparison between serum IL-6 and histological changes in the AT following the administration of irinotecan.

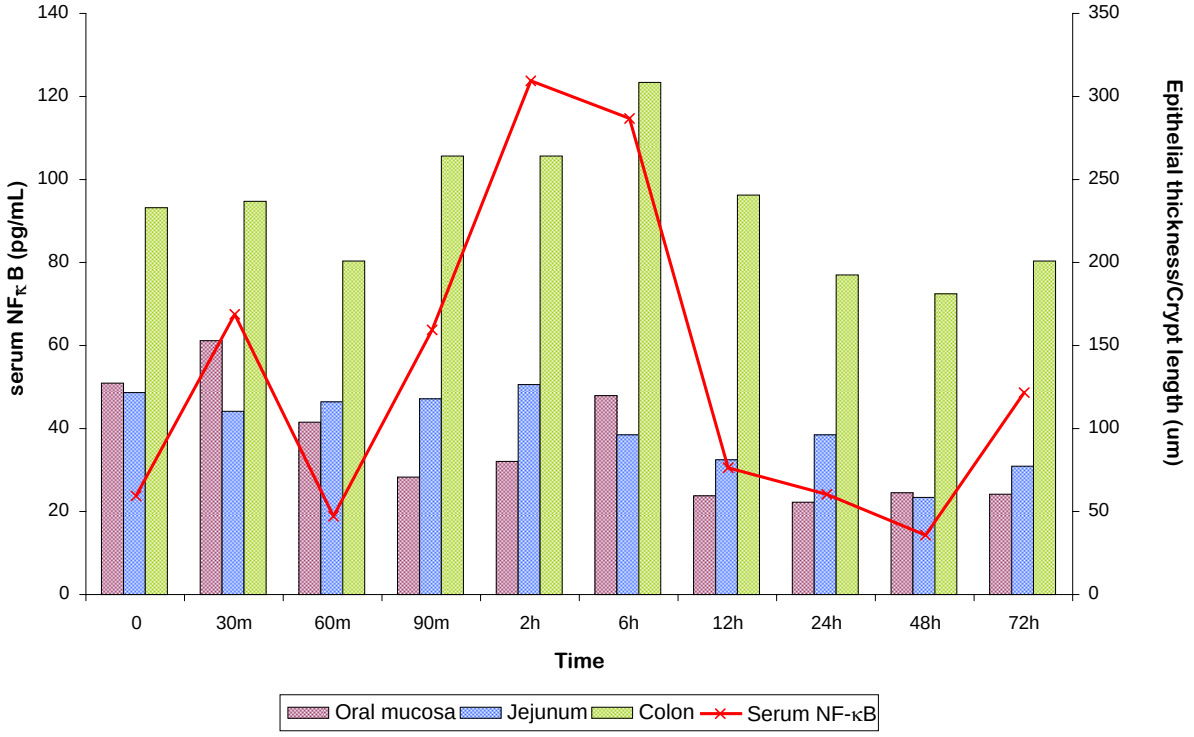

Figure 5.9

Comparison between serum NF-κB and histological changes in the AT following the administration of methotrexate.

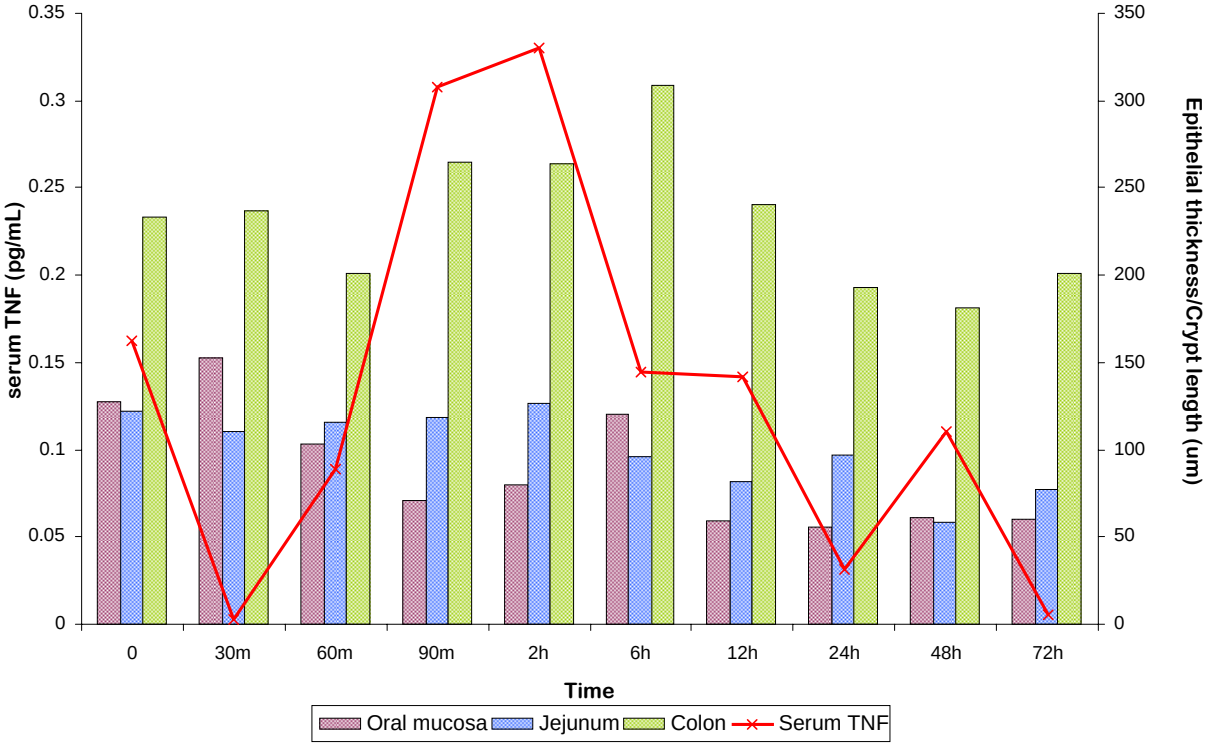

Figure 5.10

Comparison between serum TNF and histological changes in the AT following the administration of methotrexate.

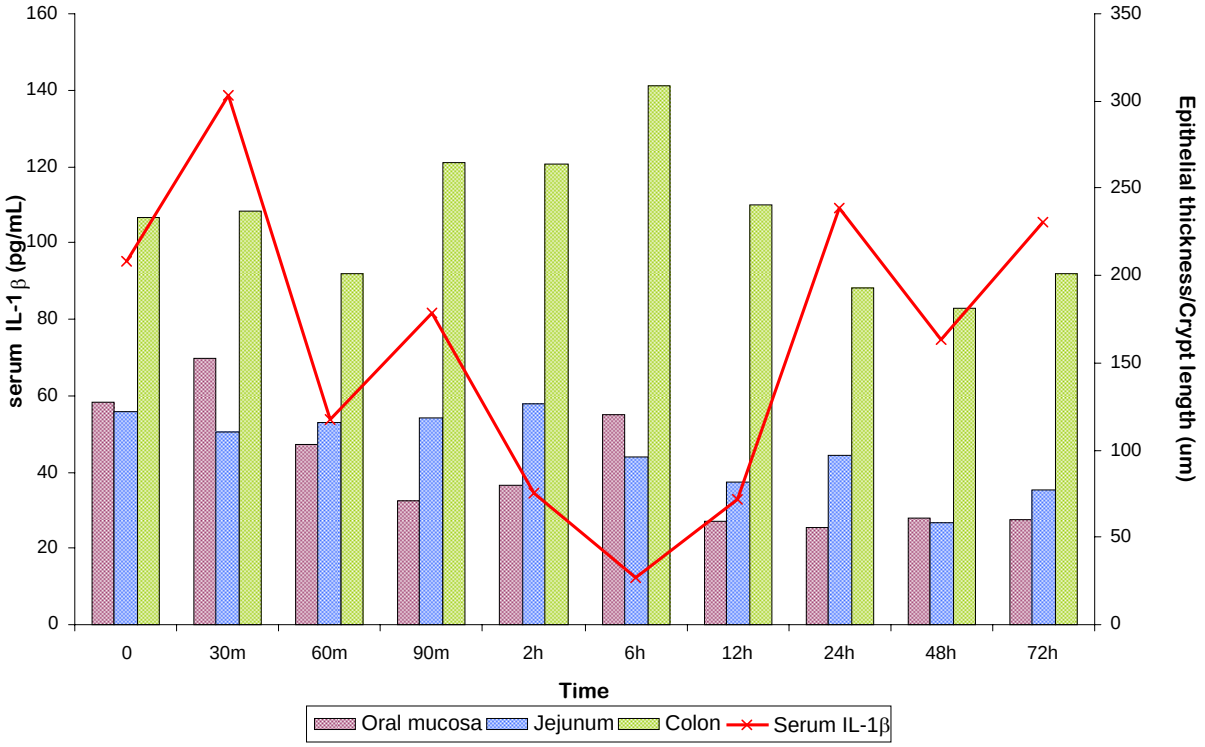

Figure 5.11

Comparison between serum IL-1β and histological changes in the AT following the administration of methotrexate.

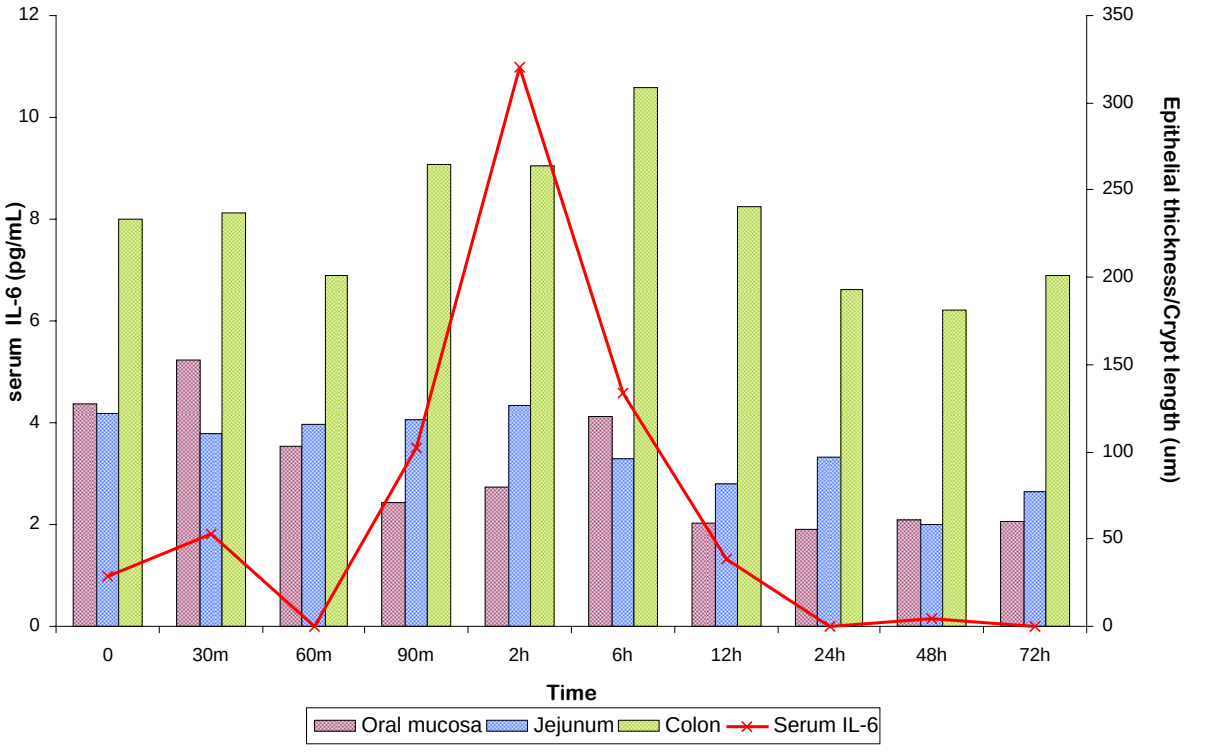

Figure 5.12

Comparison between serum IL-6 and histological changes in the AT following the administration of methotrexate.

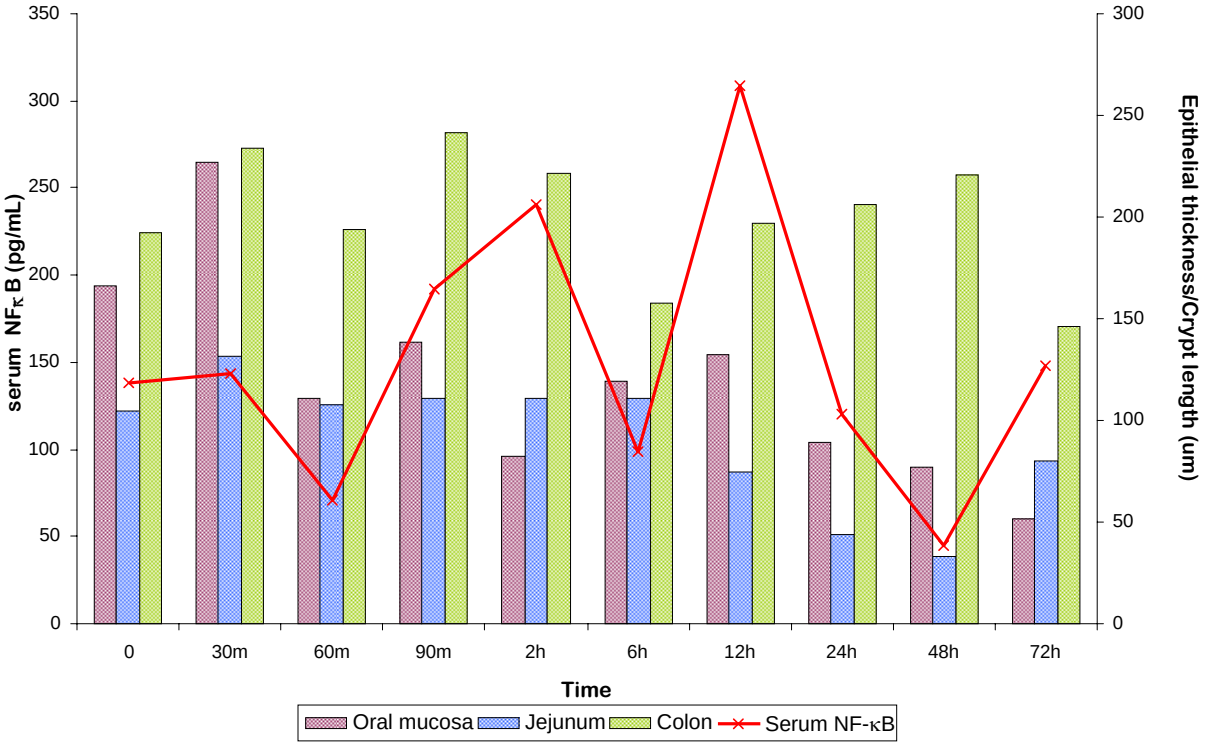

Figure 5.13

Comparison between serum NF-κB and histological changes in the AT following the administration of 5-fluorouracil.

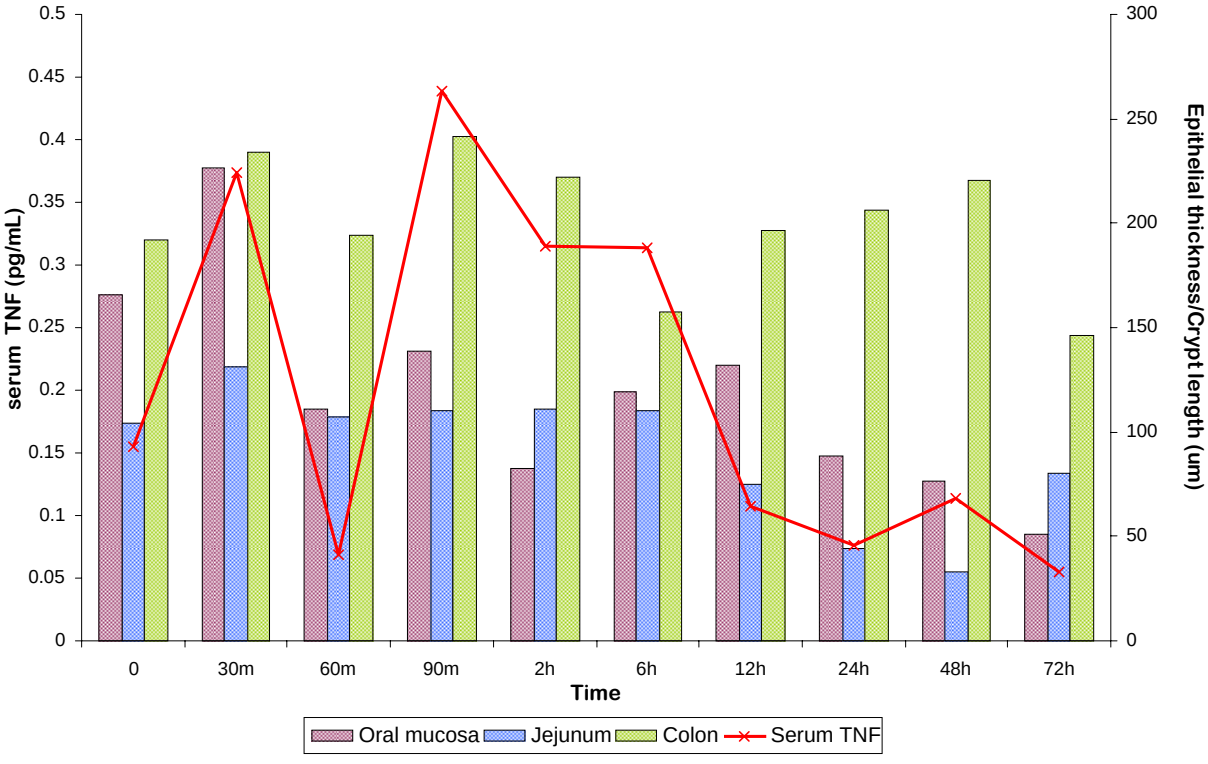

Figure 5.14

Comparison between serum TNF and histological changes in the AT following the administration of 5-fluorouracil.

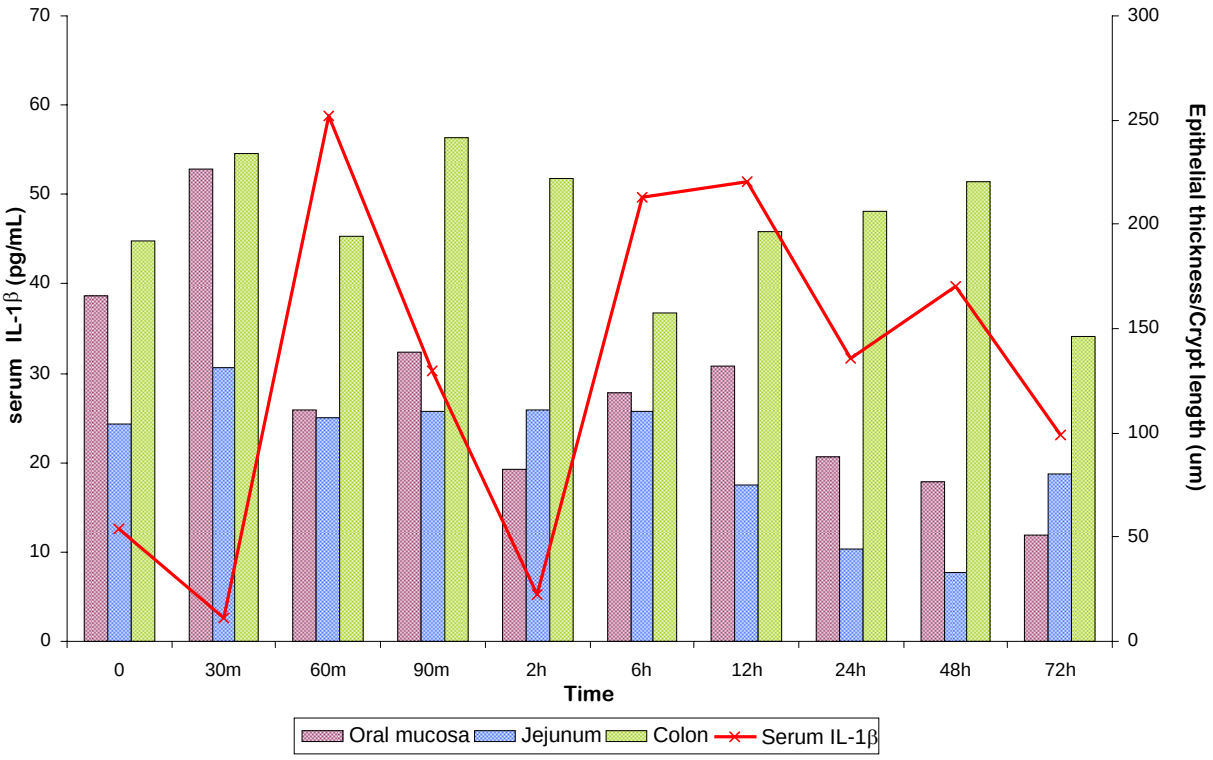

Figure 5.15

Comparison between serum IL-1β and histological changes in the AT following the administration of 5-fluorouracil.

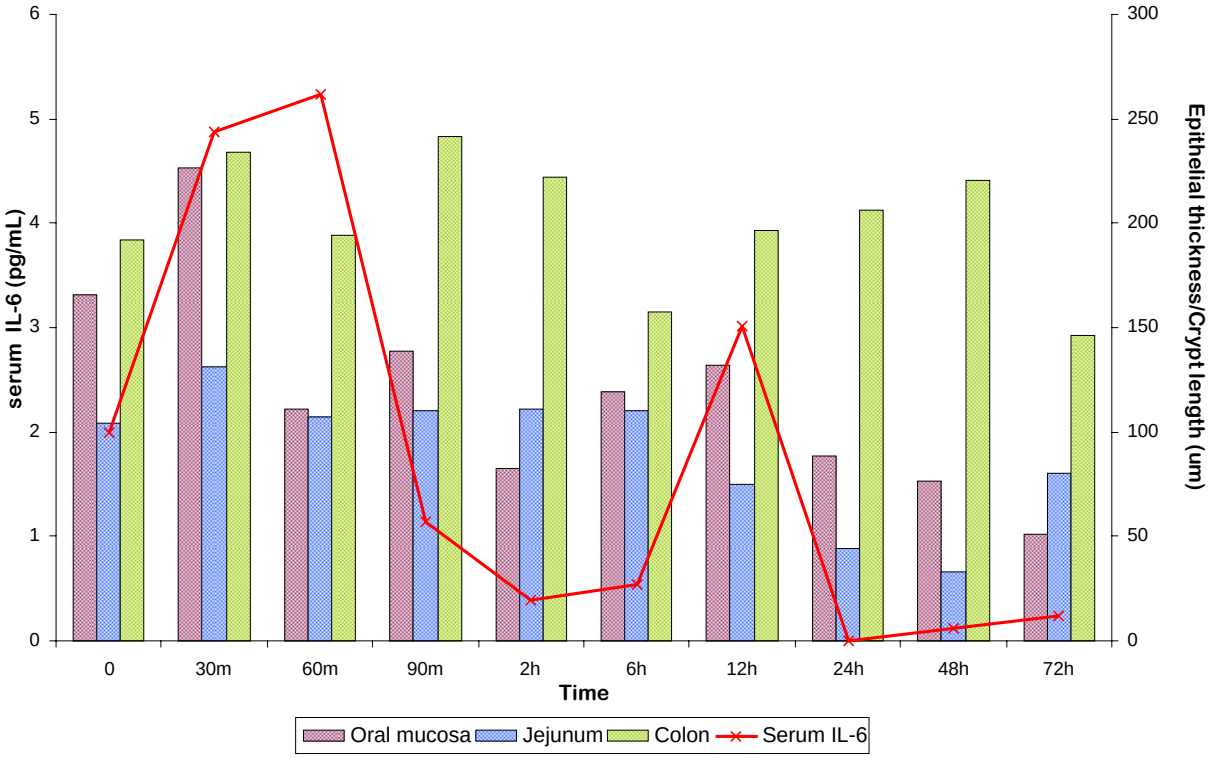

Figure 5.16

Comparison between serum IL-6 and histological changes in the AT following the administration of 5-fluorouracil.

5.4 Discussion

The roles of NF-κB, TNF, IL-1β and IL-6 in the pathobiology of mucositis have increasingly been reported in the literature [93, 95, 96, 162]. It is also well recognised that there may be subclinical events occurring in the affected tissues prior to clinical evidence of mucositis manifesting. In the preceding chapters it was shown that *tissue* levels of NF-κB, TNF, IL-1β and IL-6 are altered in different sites of the AT tract prior to marked histological evidence of damage following chemotherapy [93, 94]. The main objective of the current study was to determine, following administration of different drugs that are known to cause mucositis, whether changes in *serum* concentrations of NF-κB and the selected pro-inflammatory cytokines as previously studied [93, 94] occurred and secondly, whether these changes corresponded to, or indeed predicted, histological evidence of tissue damage.

This study demonstrated that serum concentrations of NF-κB, TNF, IL-1β and IL-6 did change following the administration of irinotecan, MTX or 5-FU. However, just as these drugs cause different timing of histological changes in the AT (Figures 5.5 - 5.16) [93, 94], each drug also resulted in differences in changes in serum cytokine concentrations over the 72 hour time period. In some situations, serum changes followed tissue changes. The exact reasons for this are unclear, however it is postulated that this may reflect an "overflow" effect of cumulative damage in the tissues eventually manifesting in the serum. With respect to *tissue* levels of pro-inflammatory cytokines reported previously, these were generally elevated prior to histological evidence of damage and remained elevated compared to baseline levels in the tissue [93, 94]. Serum levels, on the other hand, tended to return to baseline levels by 72 hours after drug administration. This difference between serum and tissue levels may be explained by the fact that the serum levels are more likely to reflect the global or systemic effects of drugs which may have the result of moderating the general levels in the serum

whilst ongoing tissue damage as well as other factors such as alterations in local environment may encourage tissue levels of NF-κB, TNF, IL-1β and IL-6 to remain elevated. Further investigation of systemic factors and their interaction with serum pro-inflammatory cytokine changes are indicated.

The fact that differences were observed in serum changes in rats that received different drugs is not unexpected as it would be relatively simplistic to believe that different drugs with different mechanisms of action would cause similar changes in pro-inflammatory cytokine expression either in the tissues or serum. For example, in the case of irinotecan, this drug has causes marked cholinergic side effects due to the inhibition of acetylcholinesterase. Whilst the drug may have direct effects on the mucosa of the AT, it may also act indirectly as a result of its cholinergic activity which may cause changes in the microenvironment of the AT. Changes in the intestinal luminal environment have been suggested to play a role in the development of chemotherapy-induced mucositis [5, 129, 150]. Different drugs may also have direct pharmacological actions which could impact on mucositis pathobiology according to the current hypothesis. 5-FU, for example, has been demonstrated to inhibit NF-κB activation [7, 9]. Following 5-FU administration in the DA rat, serum levels of NF-κB did decrease early on, however this was followed by an increase between 2 and 12 hours. This discrepancy can be accounted for by the fact that, as previously highlighted, serum concentration is more likely a measure of systemic changes rather than purely a reflection of the changes occurring in the AT. Furthermore, whilst the initial drop in NF-κB is consistent with the effects of 5-FU administration, the later increase may arise due to other activating factors once 5-FU has been metabolised and removed from the tissues and circulation as this drug has a half life of only 8-22 minutes, depending on dose. NF-κB may be activated through a wide variety of mechanisms such as via IL-1β- and TNF-related pathways – both IL-1β and TNF were elevated at early time points following 5-FU administration.

As previously mentioned, one of the reasons for examining serum levels of NF-κB, TNF, IL-1β and IL-6 was to determine whether there was any value in this as a means to predict or determine mucosal changes before manifestations of tissue damage become evident. The clinical utility of measuring serum NF-κB, TNF, IL-1β and IL-6, however, is limited by the fact that in order for this to be of any benefit to patients, the timing is critical. In this animal model, the time difference between peak serum levels and initial histological changes in the tissues was, in some instances, as little as 30 minutes. Measuring serum levels and instituting appropriate preventive measures before there is obvious histological change in the tissues therefore would essentially be impossible. Furthermore, in the clinical setting, chemotherapy regimens often involve multiple drugs combinations with potentially synergistic or, alternatively, antagonistic effects which may complicate the evaluation of serum changes if they occur.

In conclusion, whilst the results of this study demonstrated that, following drug administration, changes were evident in the serum concentrations of NF-κB, TNF, IL-1β and IL-6, these changes differed according to the type of drug administered. In some instances changes in serum levels coincided or preceded histological changes in tissues at different sites of the AT. In spite of this, it is concluded that the utility of serological testing to predict AT damage is not warranted based on the results of this study. A more useful strategy would be to determine a patient's individual risk for developing toxicities to cancer treatment. Along those lines, these results do pose further questions about the nature of drug toxicities. In the future it may be more realistic and of more benefit to consider global effects of drugs or "clusters of toxicities" rather than limiting the focus of research to a specific site or organ [8]. Further studies are required to determine the possible relationships between different toxicities and determine how, once these links are known, patient management can be improved.

Chapter 6

Summary and Conclusions

6.1 Introduction

Mucositis is a major clinical problem caused by different modalities of cancer treatment. In recent years there has been increased understanding of the histology and kinetics of mucositis The molecular changes that occur in the context of mucositis, for example the role of NF-κB and pro-inflammatory cytokines, still require further investigation. The initial investigation looking at NF-κB expression in oral mucosa as outlined in Chapter 2 [95] led to the development of the further studies that comprise this thesis using an established animal model to provide further information about the pathobiology of AT mucositis following chemotherapy. These subsequent studies employed various techniques including routine histology, immunohistochemistry and enzyme-linked immunosorbent assay (ELISA).

6.2 Histological evidence of mucositis

The results presented in this thesis support previous work indicating that mucosal damage occurs throughout the length of the AT, that is, it is not restricted to a specific site such as the oral cavity or small intestine. Differences were observed between the different regions of the AT which related to the normal architectural differences in the mucosa as a result of each area's specialised functions.

6.2.1 Oral mucositis

The easily accessible nature of the oral cavity and obvious clinical manifestations of oral mucositis that occur following chemotherapy and radiotherapy have led to numerous studies into the pathobiology of oral mucositis. In Chapters 3 and 4, the administration of irinotecan, MTX or 5-FU caused atrophy of the epithelium, occasionally with inflammation within the underlying connective tissue. An important fact is that rat oral mucosa has a resilient keratinised phenotype unlike the human oral mucosa which is largely non-keratinised and

more susceptible to damage. Past studies using rats as the animal models of mucositis have "initiated" mucosal damage by traumatising the mucosa in order to cause ulceration [1, 28-30, 90, 92, 105, 110, 132, 143, 145]. Previous clinical investigations (Chapter 1) have demonstrated that changes occur in the mucosa such as increased apoptosis following chemotherapy in the absence of ulceration [55, 95]. For this thesis it was hypothesised that these subclinical events would also occur in the rat mucosa following drug administration and, more importantly, that minor trauma to "initiate" changes may complicate or even mask potential events occurring in the mucosa. As expected, ulceration was not observed in the rats; however it was considered that ulceration per se was not required to confirm whether or not the mucosa was affected by the drugs. As mentioned, generalised epithelial atrophy was observed over the 72 hour time period examined. Therefore, to the best of our knowledge, these are the first studies to demonstrate histological changes in the mucosa in the absence of traumatic initiating events.

6.2.2 Small intestine mucositis

The results from these studies demonstrate that the histological changes that occur following administration of irinotecan, MTX or 5-FU are similar. These changes include a reduction in villous height and crypt length. The intensity of damage was greater in irinotecan treated rats and this was consistent with their clinical symptoms of diarrhoea which were less marked in the rats that were administered MTX or 5-FU. Associated with the epithelial changes was a concomitant increase in the degree of inflammation in the underlying connective tissue. These changes were consistent with those reported previously in the literature [52, 53, 56, 57, 79]. Histological evidence of severe damage was seen at the later time points including blunting and fusion of villi and crypt ablation.

6.2.3 Large intestine mucositis

Mucositis involving the large intestine is less well characterised [53]. Slight differences were observed between the drugs with respect to timing of changes, however each caused crypt hyperplasia. This was evident at early time points for irinotecan (30-60 minutes) and at later time points for MTX and 5-FU (2–6 hours and 90 minutes respectively). Degeneration of enterocytes within the crypts and eventually crypt ablation was observed at the later time points.

These differences in histological features of damage that occur between the small intestine and colon are in keeping with the notion that the features of mucositis differ according to the individual specialised functions of each area of the AT. In the colon it has been suggested that the different location of stem cells within crypts compared to the small intestine renders them more resistant to damage [52].

6.3 NF-κB and pro-inflammatory cytokine expression

In recent years the role of transcription factors such as NF-κB has increasingly been considered to be important in the pathobiology of mucositis [135]. NF-κB is an important regulator of pro-inflammatory cytokines that have been implicated in the development of mucosal damage; these include TNF, IL-1β and IL-6. The results included in this thesis support the role of NF-κB in the pathobiology of mucositis as well as the involvement of these pro-inflammatory cytokines.

The involvement of NF-κB, TNF, IL-1β and IL-6 was investigated in three separate studies in which, in each, a different drug was used. The three drugs, irinotecan, MTX and 5-FU are all potentially mucotoxic drugs in their own right in the clinical setting. As hypothesised, the papers that make up this thesis indicated that:

1. The expression of NF-κB, TNF, IL-1β and IL-6 change in the mucosa and in the serum following drug administration;

2. The pattern and timing of changes of NF-κB, TNF, IL-1β and IL-6 differs in the tissue and serum depending on the drug administered;

3. Differences were observed in the pattern and timing of changes of NF-κB, TNF, IL-1β and IL-6 between the tissue and serum.

The results from the three experiments are summarised in Tables 6.1 – 6.2. These results all indicate that NF-κB and proinflammatory cytokines are altered in the mucosa and serum following chemotherapy; however the exact role that they play requires more detailed

investigation. The fact that NF-κB expression is increased in the mucosa does provide further evidence for its hypothesised role. Complicating this however, is the observation in 5-FU treated rats that NF-κB decreased in the early time points. This suggests that other pathways or specific factors may lead to mucosal damage.

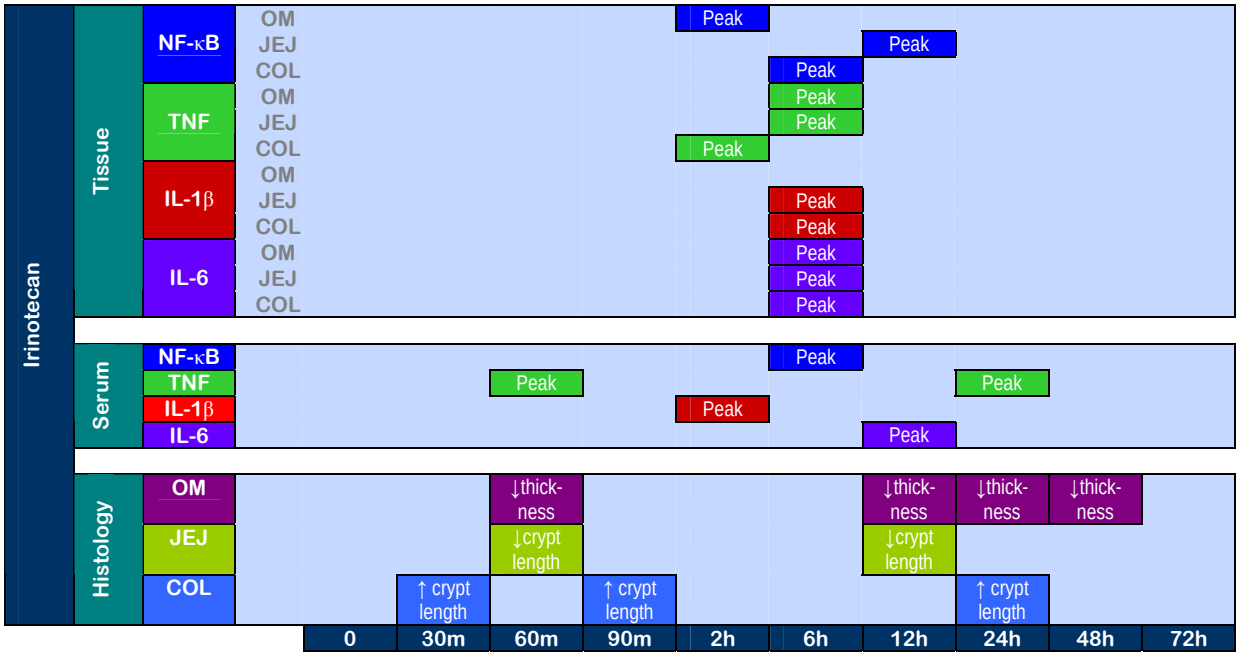

Table 6.1

Summary of peak changes in tissue and serum levels of NF-κB and pro-inflammatory cytokines and the histological changes that occur in different sites of the AT following irinotecan administration.

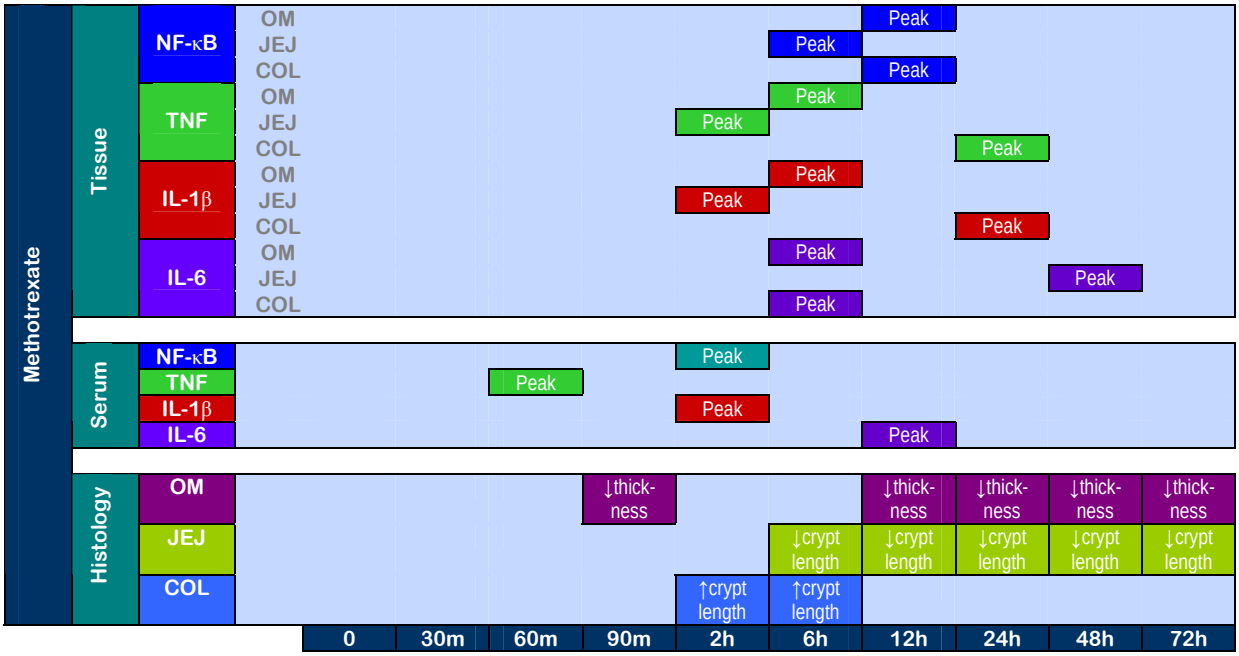

Table 6.2

Summary of peak changes in tissue and serum levels of NF-κB and pro-inflammatory cytokines and the histological changes that occur in different sites of the AT following methotrexate administration.

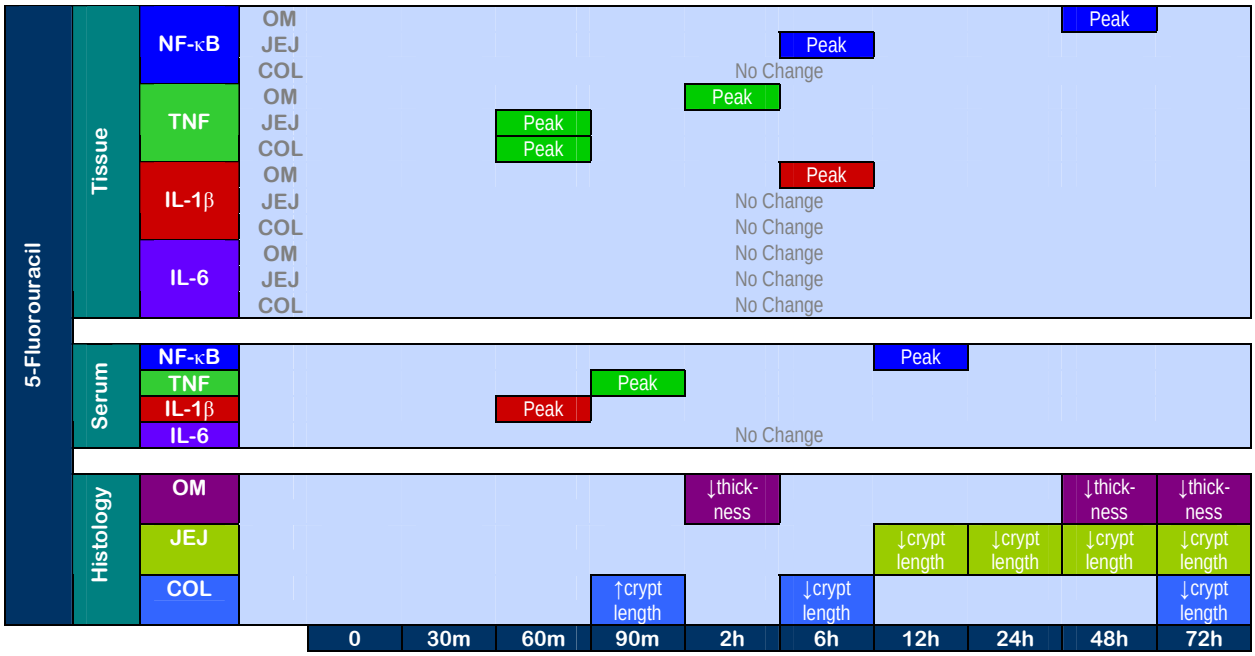

Table 6.3

Summary of peak changes in tissue and serum levels of NF-κB and pro-inflammatory cytokines and the histological changes that occur in different sites of the AT following 5-fluorouracil administration.

6.4 Future research

Based on the results of this thesis it is clear that increased understanding of the molecular basis of mucositis will be an important factor in the future management of mucositis. An example of more targeted management of mucositis is that using Palifermin (or keratinocyte growth factor-1) which has been approved for the management of mucositis and its use is recommended in specific clinical settings [17, 74]. This is the first drug which has a basis in molecular targeting of the processes involved in the pathobiology of mucositis. In addition to having a measurable clinical benefit, Elting *et al* demonstrated an economical benefit in the use of Palifermin in patients with haematological malignancies who received total body irradiation. In this group of patients, the expense of Palifermin was offset by a reduction in hospital costs associated with patient care [43]. A potential complicating factor in mucositis management is that there are numerous pathways that can potentially lead to the tissue damage that occurs following chemotherapy. The differences that were observed in the pattern of pro-inflammatory cytokines expression in this thesis attest to this. In spite of these differences, however, the *histological* features observed were similar at 72 hours. This would indicate that, in general, the tissues have a limited repertoire with respect to different biological insults. It may be that there whilst there are different pathways activated initially, these then converge to a common final pathway that leads to the histological damage and ultimately the clinical manifestations of mucositis. Newer technologies, for example, microarray analysis, may assist in further characterising the genetic changes that occur following cancer treatment and identifying a common pathway [19, 21].

The other important factor highlighted by the results of this research is the fact that cancer treatment, particularly chemotherapy, has systemic effects. This was evident in the disparity observed between the tissue levels of NF-κB and proinflammatory cytokines following chemotherapy and those levels in the serum. These results do, however, pose further

questions about the nature of drug toxicities. In the future it may be more realistic and of more benefit to consider global effects of drugs or "clusters of toxicities" rather than limiting the focus of research to a specific site or organ [8]. Further studies are required to determine the possible relationships between different toxicities and determine how, once these links are known, patient management can be improved.

6.5 Conclusions

In conclusion, this thesis has provided further information on mucositis pathobiology and highlights its complexity. In particular, it has provided additional evidence that mucositis is not just restricted to the oral cavity and that other sites of the AT are affected. Furthermore, these results support previous data indicating that subclinical changes occur in the mucosa prior to the development of obvious histological damage or clinical manifestations of mucositis. Contrary to previous reports, these studies have indicated that, although the clinical and histological changes may be similar, the alterations in NF-κB and pro-inflammatory cytokines in the tissues are affected by the type of drug used. The use of measuring serum levels of factors thought to be involved in the pathobiology of mucositis is limited because of the systemic effects of the drugs. This, however, highlights the fact that further research needs to be directed, not at specific side effects, but rather how side effects of chemotherapy are interrelated so that better patient management can be achieved and ultimately provide optimum treatment and better survival for patients with cancer.

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Appendices

Appendix I

Other work completed during candidature.

Conference presentations

October 2004

Colgate Australian Clinical Dental Research Centre, Seminar Series
Oral presentation:
"Oral side effects of oncology treatment"

February 2006

The 2006 Amgen Clinical Symposium, Haematology: today, tomorrow and beyond
Invited Speaker – Oral presentations
"Oral Mucositis" – Advanced Trainee Master Class
"Update on the MASCC Guidelines for Oral Mucositis" – Plenary Session

June 2006

Multinational Association for Supportive Care in Cancer/International Society for Oral Oncology, 18th International Symposium.
Poster presentations:
"Cancer chemotherapy causes gene expression changes throughout the gastrointestinal tract of rats"
Tyskin A., Bowen J., Gibson R., Stringer A., Logan R., Keefe D.
"Chemotherapy-induced diarrhoea is associated with changes in the luminal environment in the DA rat"
Stringer A., Gibson R., Logan R., Bowen J., Yeoh A., Keefe D.
"Chemotherapy-induced changes in mucin composition and secretion"
Stringer A., Gibson R., Logan R., Bowen J., Yeoh A., Keefe D.
"Serum levels of nuclear factor kappa B and tumour necrosis factor following irinotecan administration in the DA rat"
Logan R., Gibson R., Stringer A., Bowen J., Sonis S., Keefe D.
"Nuclear factor kappa B and cyclooxygenase-2 expression in the oral mucosa following cancer chemotherapy"
Logan R., Gibson R., Stringer A., Bowen J., Sonis S., Keefe D.

August 2006

Colgate Australian Clinical Dental Research Centre, Research Day
Oral presentation:
"Nuclear factor-κB and cyclooxygenase-2 expression in the oral mucosa following cancer chemotherapy"
Poster presentation:
"Serum levels of nuclear factor kappa B and tumour necrosis factor following irinotecan administration in the DA rat"
Logan R., Gibson R., Stringer A., Bowen J., Sonis S., Keefe D.

May 2007

1st World Congress of the International Academy of Oral Oncology, Amsterdam, The Netherlands
Poster presentations:
"Nuclear factor-κB and cyclooxygenase-2 expression in the oral mucosa following cancer chemotherapy"
Logan R., Gibson R., Sonis S., Keefe D.
"Serum levels of nuclear factor kappa-B and tumour necrosis factor following irinotecan administration in the DA rat"
Logan R., Gibson R., Stringer A., Bowen J., Sonis S., Keefe D

June 2007

Multinational Association for Supportive Care in Cancer/International Society for Oral Oncology, 20th International Symposium. St Gallen, Switzerland

Mucositis Research Day
Oral presentation:
"Expression of NF-κB and pro-inflammatory cytokines along the alimentary tract of the Dark Agouti rat following chemotherapy"

Poster presentations:
"Irinotecan causes elevated expression of NF-kB and pro-inflammatory cytokines along the alimentary tract of the DA rat"
Logan R., Gibson R., Stringer A., Bowen J., Sonis S., Keefe D.
"The protective mechanism of palifermin in alimentary mucositis"
Chan T., Gibson R., Bowen J., Stringer A., Yeoh, A., Logan R., Keefe D.
"Irinotecan-induced diarrhoea is associated with changes in MUC gene expression"
Stringer A., Gibson R., Logan R., Bowen J., Yeoh A., Keefe D.
"Chemotherapy-induced diarrhoea and changes in the faecal flora of cancer patients"
Stringer A., Yeoh A., Gibson R., Logan R., Bowen J., Keefe D.

Other publications during candidature

Von Bültzingslöwen I., Brennan, M.T., Spijkervet, F., **Logan R.**, Stringer A., Raber, J., Keefe, D., "Growth factors and cytokines in the prevention and treatment of oral and gastrointestinal mucositis" *Supportive Care in Cancer*, 2006; 14(6):519-527

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Stringer A.M., Gibson R.J., **Logan R.M.**, Bowen J.M., Yeoh A.S-J, Burns, J., Finnie J.W., Keefe D.M.K. "Chemotherapy-induced diarrhoea is associated with changes in the luminal environment in the DA rat" *Exp Biol Med*, 2007; 232: 96-106

Gibson R.J., Stringer A.M., Bowen J.M., **Logan R.M.,** Burns J., Alvarez E., Keefe D.M., "Velafermin is effective in improving gastrointestinal mucositis following Irinotecan treatment in tumour-bearing DA rats" *Cancer Biology and Therapy*, 2007; 6(4)

Stringer A.M., Gibson R.J., **Logan R.M.,** Bowen J.M., Yeoh A.S.J., Keefe, D.M.K., "Chemotherapy-induced mucositis: the role or gastrointestinal microflora and mucins in the luminal environment", *Journal of Supportive Oncology*, 2007; 5(6): 259-267

Logan R.M., "First World Congress of the International Academy of Oral Oncology (IAOO) - Oral Cancer: A Global Challenge" Conference Report. *Expert Reviews in Anticancer Therapy*, 2007, 7(7); 975-976

Bowen J.M., Gibson R.J., Tsykin A., Stringer A.M., **Logan R.M.**, Keefe D.M.K., "Gene expression analysis of multiple gastrointestinal regions reveals activation of common cell regulatory pathways following cytotoxic chemotherapy" *Int J Cancer*, 2007, in press

Appendix II

Publications arising from this Thesis.

1. Logan RM, Stringer AM, Bowen JM, Yeoh A S-J, Gibson RJ, Sonis ST and Keefe DMK, "The role of pro-inflammatory cytokines in cancer treatment-induced alimentary tract mucositis: Pathobiology, animal models and cytotoxic drugs" Cancer Treatment Reviews (2007), 33(5): 448-460

2. Logan RM, Gibson RJ, Sonis ST and Keefe DMK, "Nuclear factor-κB (NF-κB) and cyclooxygenase-2 (COX-2) expression in the oral mucosa following cancer chemotherapy", Oral Oncology (2007), 43; 395-401

3. Logan RM, Gibson RJ, Bowen JM, Stringer AM, Sonis ST and Keefe DMK, "Characterisation of mucosal changes in the alimentary tract of the Dark Agouti rat following administration of irinotecan: Implications for the pathobiology of mucositis" Cancer Chemotherapy and Pharmacology (2007), doi: 10.1007/s00280-007-0570-0

4. Logan RM, Gibson RJ, Bowen JM, Stringer AM, Sonis ST and Keefe DMK, "Is the pathobiology of chemotherapy-induced alimentary tract mucositis influenced by the type of mucotoxic drug administered?" Cancer Chemotherapy and Pharmacology (2008), doi: 10.1007/s00280-008-0732-8

Logan, R.M., Stringer, A.M., Bowen, J.M., Yeoh, A., Gibson, R.J., Sonis, S.T. and Keefe, D.M.K. (2007) The role of pro-inflammatory cytokines in cancer treatment-induced alimentary tract mucositis: Pathobiology, animal models and cytotoxic drugs. *Cancer Treatment Reviews, vol 33 (5), pp. 448-460*

Logan, R.M., Gibson, R.J., Sonis, S.T. and Keefe, D.M.K. (2007) Nuclear factor-κB (NF-κB) and cyclooxygenase-2 (COX-2) expression in the oral mucosa following cancer chemotherapy.
Oral Oncology, vol. 43 (4), pp. 395-401

Logan, R.M., Gibson, R.J., Bowen, J.M., Stringer, A.M., Sonis, S.T. and Keefe, D.M.K. (2008) Characterisation of mucosal changes in the alimentary tract of the Dark Agouti rat following administration of irinotecan: Implications for the pathobiology of mucositis.
Cancer Chemotherapy and Pharmacology, vol. 62 (1), pp. 33-41

Logan, R.M., Gibson, R.J., Bowen, J.M., Stringer, A.M., Sonis, S.T. and Keefe, D.M.K. (2008) Is the pathobiology of chemotherapy-induced alimentary tract mucositis influenced by the type of mucotoxic drug administered?.
Cancer Chemotherapy and Pharmacology, in press