Original Research

Matrix metalloproteinases are possible mediators for the development of alimentary tract mucositis in the dark agouti rat

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Abstract

Alimentary tract (AT) mucositis is a serious and debilitating side-effect of cancer therapy primarily characterized by damage of the mucous membranes throughout the AT. It is well established that this damage is a result of up-regulation of stress response genes and pro-inflammatory cytokines. Matrix metalloproteinases (MMPs) have been shown to function in several of the pathways known to be up-regulated in mucositis and play a key role in tissue injury and inflammation in many gastrointestinal disorders. This study aims to characterize the expression of multiple MMPs including MMP-1, -2, -3, -9 and -12 and their inhibitors, tissue inhibitor of metalloproteinase (TIMP)-1 and -2, in a rat model of irinotecan-induced mucositis. Dark agouti rats were administered a single 200 mg/kg intraperitoneal dose of irinotecan and killed at 1, 6, 24, 48, 72, 96 and 144 h following treatment. Hematoxylin and eosin staining, immunohistochemistry and realtime polymerase chain reaction were used to assess histopathological damage and MMP expression in the jejunum and colon. Marked histopathological evidence of mucositis was observed in the jejunum and colon as early as six hours following irinotecan treatment. A significant alteration in both gene expression and tissue levels of MMPs and TIMPs was noted following irinotecan. The increase in MMP-2, -3, -9 and -12 levels was associated with inflammatory infiltrate and maximum tissue damage. In contrast, MMP-1 expression correlated with tissue restitution. TIMP-1 and -2 levels were significantly altered in the jejunum following irinotecan. The augmentation in the expression profiles of MMPs and their inhibitors correlated with histopathological alterations observed in the tissue following irinotecan. This prompts the consideration of MMPs as possible mediators of chemotherapy-induced mucositis.

Keywords: mucositis, chemotherapy, alimentary tract, matrix metalloproteinases, tissue inhibitors of metalloproteinases

Experimental Biology and Medicine 2010; **235**: 1244–1256. DOI: 10.1258/ebm.2010.010082

Introduction

Irinotecan hydrochloride is a chemotherapeutic drug used for the treatment of a variety of solid tumours.[1] The mechanism of action of irinotecan is inhibition of DNA topoisomerase I, a key nuclear enzyme responsible for relaxing DNA during DNA replication.[1] Irinotecan acts on malignant as well as normal cells in the body and has been shown to cause severe alimentary tract (AT) mucositis in the clinic.[2,3] AT mucositis is characterized by clinical symptoms including ulceration, nausea and vomiting, abdominal pain and bloating and diarrhea.[4,5] This condition not only decreases patient quality of life, but also threatens the effectiveness of anti-cancer treatment as it is a dose-limiting toxicity.

Mucositis development is a multifactorial process and involves a cascade of events in multiple tissue regions.[5] It has been previously shown that irinotecan causes gross architectural disturbances in AT tissue, most notably the jejunum and colon, as a result of crypt cell damage, oxidative stress, inflammation and changes in gut flora to name a few.[2,6–8] Substantial research efforts have attempted to elucidate the signalling cascades responsible for this damage. Recent studies by Bowen and colleagues[9,10] illustrated that chemotherapy-induced mucositis is associated with the up-regulation of a range of stress response genes and activation of mitogen-activated protein kinase (MAPK) signalling, nuclear factor-κB (NF-κB) signalling, Fos/Jun signalling and Wnt signalling. Furthermore, the

downstream mediators of this damage include cytokines, ceramide and cyclooxygenase-2.[7,11] Matrix metalloproteinases (MMPs) have also been suggested to play a role in damage onset seen in the AT following chemotherapy administration.[5,12]

MMPs are zinc-dependent endopeptidases with a predominant role in extracellular matrix (ECM) homeostasis. Through regulation of ECM components, MMPs affect numerous biological phenomena, including cell growth, apoptosis, cell motility, immune responses and cytokine and chemokine bioactivity.[7,13–15] MMPs have been proposed to act as mediators of damage in mucositis development.[5] Although there is currently no sufficient data in the literature to explain all facets of MMPs' function and contribution to the development and amplification of chemotherapy-induced tissue injury, there is growing evidence for an important role of MMPs in a variety of tissue injury models. Furthermore, it has been suggested that MMPs are components of genetic programs such as the wound repair response where they are downstream targets of immediate-early response genes that are induced within minutes of cell stimulation.[14] Although MMPs have been shown to contribute to tissue injury and inflammation in many gastrointestinal diseases, no direct links have been established between MMPs and mucositis development.[15–17] Therefore, the current study aimed to characterize the expression of multiple MMPs, which have been shown to contribute to injury and inflammation including MMP-1, -2, -3, -9 and -12 as well as their inhibitors, tissue inhibitor of metalloproteinase (TIMP)-1 and -2, in a rat model of irinotecan-induced mucositis. This study also investigated the expression of the serine proteases urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) as well as plasminogen activator inhibitor-1 (PAI-1) as they have been known to play a role in MMP activation.

Materials and methods

Animals

Forty-eight female dark agouti rats weighing 150–170 g were used in this study. All experimental procedures were approved by the Animal Ethics Committees of the Institute of Medical and Veterinary Sciences and the University of Adelaide and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Training (2004). Due to the severe nature of irinotecan-induced diarrhea, animals were monitored four times daily. If animals showed any of the criteria specified by the Animal Ethics Committee, they were euthanized. These criteria included a dull ruffled coat with accompanying dull and sunken eyes, coolness to touch with no spontaneous movement and a hunched appearance. The criteria for animal monitoring and care are as described in previous studies.

Experimental plan

Rats were randomly allocated into control ($n = 6$) or experimental groups ($n = 42$). Experimental group rats received 0.01 mg/kg subcutaneous atropine in order to reduce cholinergic reaction to irinotecan immediately prior to the administration of a single intraperitoneal dose of 200 mg/kg irinotecan (previously shown to cause gastrointestinal mucositis). Irinotecan (kindly supplied by Pfizer, Kalamazoo, MI, USA) was administered in a sorbitol/lactic acid buffer (45 mg/mL sorbitol/0.9 mg/mL lactic acid, pH 3.4), required for the activation of the drug. Rats in the control group did not receive any treatment. Rats were assessed four times daily for mortality, body weights and diarrhea. Diarrhea was graded as follows: none; mild diarrhea (staining of anus); moderate diarrhea (staining top of legs and lower abdomen); and severe diarrhea (staining over legs and higher abdomen as well as continual anal leakage).

Rats were killed at various time points following irinotecan administration; 1, 6, 24, 48, 72, 96 and 144 h, by cardiac puncture and cervical dislocation under 3% halothane in 100% O_2 anesthesia. The gastrointestinal tract was dissected out from the pyloric sphincter to the rectum and flushed with chilled isotonic saline (0.9 w/v) to remove contents. A 1 cm sample of the small intestine and the colon were taken at approximately 25% and 50% of the lengths, respectively. For histological analysis, samples were fixed in 10% neutral-buffered formalin, processed and embedded in paraffin. Jejunal and colonic tissue was also frozen at -80°C until RNA extraction.

Histological assessment

Samples of jejunum and colon were collected, processed and stained as described in previous studies.[6,7,18] Sections were examined by light microscopy and analyzed by a professional veterinary pathologist.

RNA isolation and reverse transcription

Thirty micrograms of jejunal and colonic tissue was homogenized in TRIzol® Reagent (Invitrogen Life Technologies, Mulgrave, Australia). RNA was isolated using Nucleospin® RNA II kit (Macherey-Nagel, Duren, Germany). Total RNA was prepared according to the manufacturer's instructions. Briefly, cell lysis buffer was added to the ground tissue and filtered through silica membrane filter columns. Following a sequence of filtration steps, RNA binding conditions were adjusted and DNA digestion performed. A series of washing steps were carried out before highly pure RNA was eluted in RNase-free water. To determine RNA purity and concentration, each sample was analyzed using the Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA integrity was assessed by electrophoresis on 1.2% (w/v) agarose gels. RNA was converted to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). For each reaction, 1 μg of RNA was combined with iScript reaction mix, iScript reverse transcriptase and reaction volume made up to 20 μL using nuclease-free water. Samples were incubated for five minutes at 25°C, 30 min at 42°C and five minutes at 85°C. Total cDNA concentration was measured using the Nanodrop program and samples

diluted to 100 ng/μL with nuclease-free water (Promega, Madison, WI, USA).

Realtime polymerase chain reaction

Realtime polymerase chain reaction (PCR) was performed using the Rotor Gene 3000 (Corbett Research, Sydney, Australia). The expression of each candidate reference gene in each of the 42 experimental samples was determined relative to the mean expression of the same gene in tissue obtained from untreated animals' pooled cDNA. Amplification mixes contained 1 μL of cDNA sample, 5 μL of fluorescent dye SYBR green (Applied Biosystems, Foster City, CA, USA), 3 μL of nuclease-free water (Promega) and 0.5 μL of each forward and reverse primers (all primers were prediluted to 50 ρmol/μL) to make up a final volume of 10 μL. Thermal cycling conditions included a denaturation step at 95°C for 10 min, and then 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C (MMP-2 and MMP-9), 55°C (MMP-12, uPA, tPA, PAI-1), 56°C (TIMP-1 and TIMP-2) for 15 s and extension at 72°C for 20 s. Amplification was followed by melt-curve analysis to confirm product specificity. Primer pair sequences have been previously described in the literature[19–25] and are summarized in Table 1. Ubiquitin (UBC) was used as a housekeeping gene as it has shown stable expression throughout the time course of this irinotecan-induced model. For each set of primers, PCR cycling conditions were optimized to achieve similar amplification efficiency to the housekeeping gene UBC. Each sample was normalized by its UBC content by the RotorGene 6 program. Along with normalizing the expression of the gene of interest to the housekeeping genes, it performs the $2_{\mathrm{T}}^{-\Delta C}$ method to acquire the fold change in gene expression over the time course relative to the untreated control calibrator (with adjustment to amplification efficiency) according to the following formula:

$$\text{Ratio} = \frac{\text{Efficiency (target gene)}^{\Delta CT \text{ target (calibrator}-\text{test)}}}{\text{Efficiency (reference gene)}^{\Delta CT \text{ reference (calibrator}-\text{test)}}}$$

Immunohistochemistry

Control ($n = 6$) and experimental ($n = 42$) jejunum and colon sections were cut from paraffin blocks at 4 μm thickness and mounted onto silane-coated slides. Sections were dewaxed in xylene and rehydrated through a graded series of alcohols. Sections were immersed in 10 mmol/L citrate buffer (pH 6.0) and antigen retrieval performed by heating sections in a microwave on high until boiling and on low for 10 min. Sections were allowed to cool and endogenous peroxidase activity subsequently blocked with 3% H_2O_2 in methanol. Non-specific antibody binding was blocked with 20% normal goat or horse serum (NGS or NHS) (Sigma, St Louis, MO, USA) in phosphate-buffered saline for 30 min at room temperature (RT). Avidin–Biotin Blocking Kit (Vector Laboratories, Burlingame, CA, USA) was used to block endogenous avidin–biotin activity. Sections were incubated overnight with antibodies (diluted in 5% NGS or NHS) directed at MMP-1 at 1:800 (LifeSpan Biosciences, Seattle, WA, USA), MMP-2 at 1:10,000 (Abcam, Cambridge, UK), MMP-3 at 1:1000 (Epitomics, Burlingame, CA, USA), MMP-9 at 1:500 (Abcam), MMP-12 at 1:500 (Epitomics), TIMP-1 at 1:750 (Acris Antibodies, Herford, Germany) and TIMP-2 at 1:100 (Abcam) diluted in 5% NHS or NGS at 4°C. Tissue sections were incubated with the appropriate secondary antibody (20 min at RT), ABC labelling reagent (30 min at RT) and developed with DAB (3,3′-diaminobenzidine substrate). Sections were counterstained with Lillie-Mayer's hematoxylin, and dehydrated and cleared in xylene before being mounted. Qualitative immunohistochemistry was performed. Staining intensity was graded as follows: 0 – no staining; 1 – weak staining; 2 – moderate staining; 3 – strong staining; and 4 – intense staining. This qualitative staining assessment has been previously validated by published grading systems[7,11,26] and is routinely used within our laboratory. All assessments were carried out in a blinded manner by one investigator (NA).

Statistics

Results were statistically analyzed using a Kruskal–Wallis test. P value cutoffs for significance were adjusted to allow for multiple comparison according to the Bonferroni correction. Results were declared statistically significant at $P < 0.05$/group number.

Table 1 Primer pair sequences and characteristics

Gene	Primer sequence (5′–3′)	Nucleotide position	Amplicon length (bp)	T_m (°C)	Ref.
MMP-2	F: CTGATAACCTGGATGCAGTCGT R: CCAGCCAGTCCGATTTGA	2138–2272	135	55–50	19
MMP-9	F: AAGCCTTGGTGTGGCACGAC R: TGGAAATACGCAGGGTTTGC	760–876	117	56–52	20
MMP-12	F: CTGGGCAACTGGACACCT R: CTACATCCGCACGCTTCA	259–423	165	52–50	21
TIMP-1	F: TCCTGGTTCCCTGGCATAAT R: GGCAAAGTGATCGCTCTGGT	406–599	194	52–54	22
TIMP-2	F: CTACATCTCCTCCCCGGATGA R: GGTGCCCATTGATGCTCTTC	617–684	68	56–54	23
tPA	F: GTCAGATTCCAGTCAGTGTG R: GTTGCTCGTGATGGTTTTG	1340–1577	238	52–49	24
uPA	F: TCGGACAAGAGAGTGCCA R: TCACAATCCCGCTCAGAG	1018–1249	231	50–50	24
PAI-1	F: GACAATGGAAGAGCAACATG R: ACCTCGATCTTGACCTTTTG	966–1170	205	50–50	24
UBC	F: TCGTACCTTTCTCACCACAGTATCTAG R: GAAAACTAAGACACCTCCCCATCA	2406–2487	82	58–56	25

MMP, matrix metalloproteinases; TIMP, tissue inhibitor of metalloproteinase; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; PAI-1, plasminogen activator inhibitor-1; UBC, ubiquitin C

Results

Response to treatment and histological analysis

As reported previously,[7,27] rats treated with irinotecan began demonstrating clinical signs of mucositis from two hours following irinotecan administration where diarrhea was observed in 23% of rats. The prevalence of diarrhea peaked at 24 h where 39% of rats had mild diarrhea and 5% had moderate diarrhea. At 72 h, 33% of treated rats had mild diarrhea. All diarrhea was resolved at 144 h after irinotecan administration. None of the rats in the control groups developed diarrhea; irinotecan-induced death did not occur in this study.[7,27]

Marked histological evidence of mucositis was observed in the jejunum and colon following irinotecan treatment (Figure 1). These changes were evident as early as six hours following treatment and included the presence of degenerative enterocytes within the crypts. This was

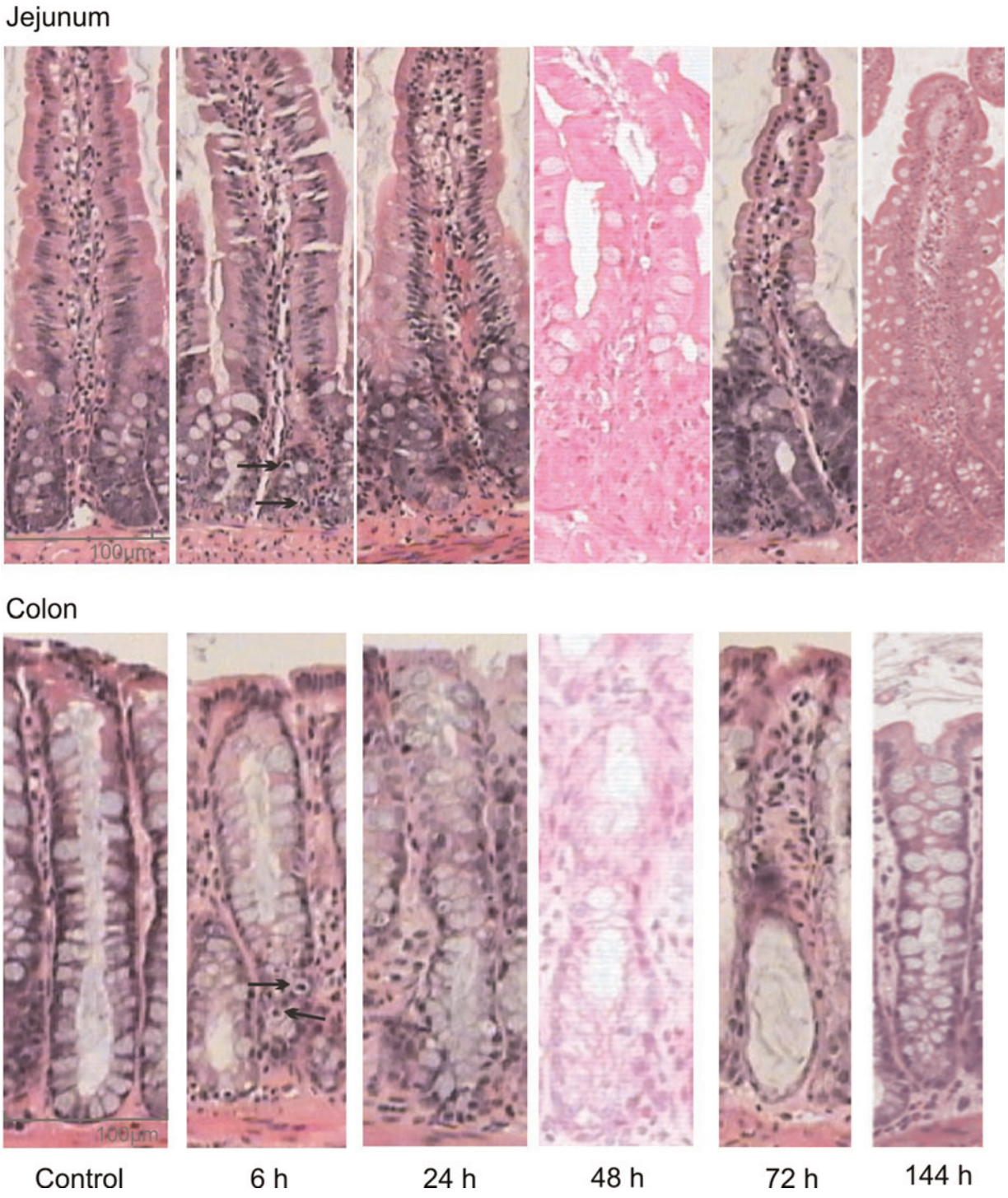

Figure 1 Hematoxylin and eosin stains illustrating histopathological damage at various time points following administration of a single 200 mg/kg intraperitoneal dose of irinotecan in the jejunum and colon. Early signs of damage are evident in the jejunum and colon at six hours (arrows). Villus blunting is noted in the jejunum at 48 h and complete crypt ablation occurs in the colon at 72 h. Tissue restitution is initiated at 144 h in both regions

followed by more gross architectural disturbances at later time points. In the jejunum, these changes included villus blunting, epithelial atrophy and increased intensity of inflammatory cell infiltrate through mucosal tissue. Similar damage was seen in the colon at later time points with focal complete crypt ablation observed at 72 h after treatment (Figure 1). Tissue restitution was initiated at 144 h.

MMP and TIMP gene expression following irinotecan treatment

Jejunum

A significant alteration in MMP-2, -9 and -12 gene expression was noted following irinotecan. MMP-2 levels were unchanged at the early time points, but increased at 48 h after treatment and peaked at an 8.8-fold change in gene expression at 72 h after treatment (Figure 2a). In comparison, MMP-9 expression was up-regulated 1.8-fold from baseline at six hours which was sustained until 96 h, after which a decrease in expression was noted (Figure 2a). The expression profile of MMP-12 was inconsistent with either MMP-2 or -9 as an initial decrease in expression was observed at six hours following treatment. A significant increase in MMP-12 was noted at 144 h ($P < 0.05$) where gene expression was 4.4-fold higher than controls (Figure 2a). TIMP-1 expression significantly decreased at one hour following treatment ($P < 0.05$) and this was followed by a peak 10.5-fold increase in gene expression

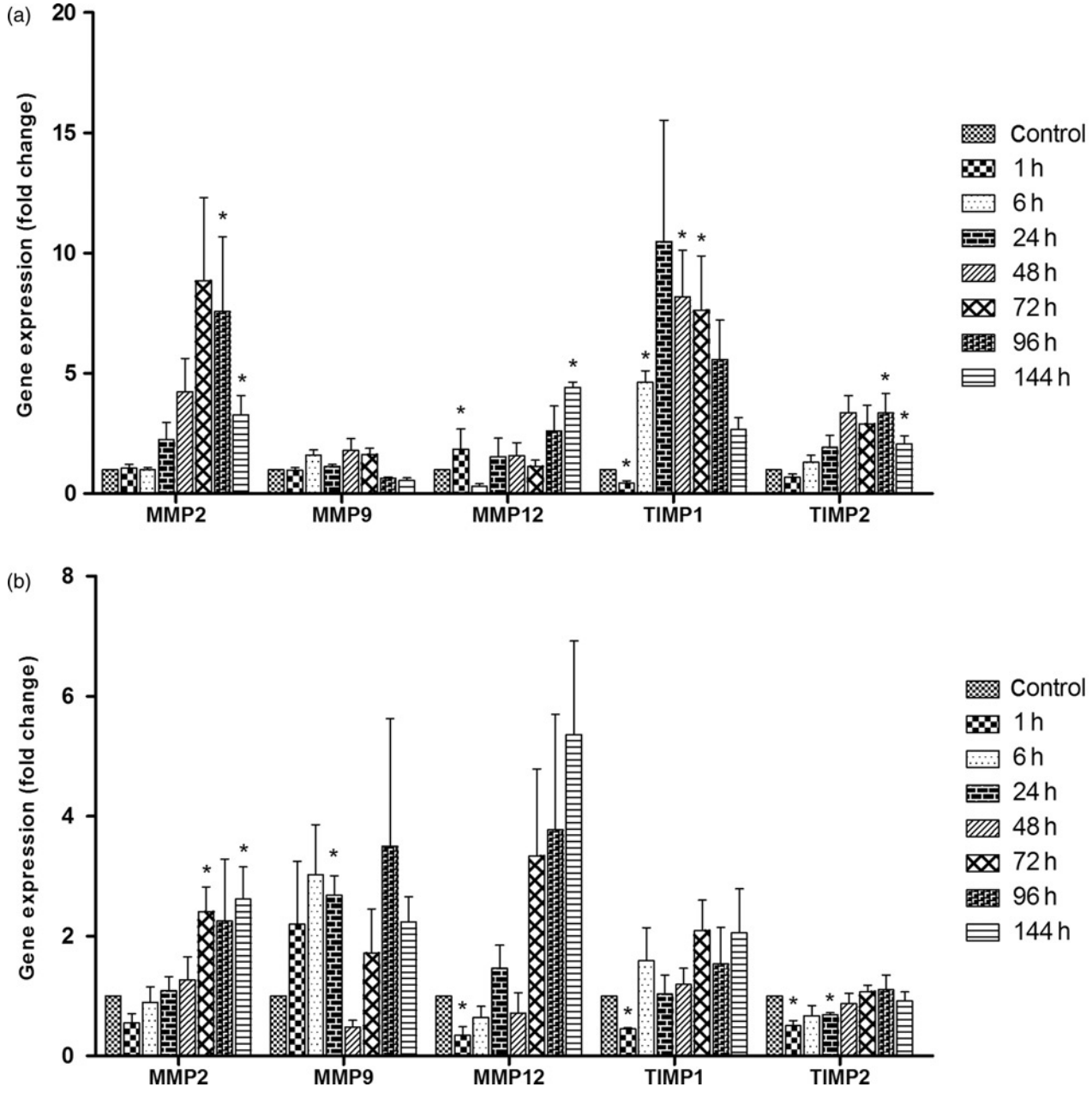

Figure 2 Effect of a single 200 mg/kg intraperitoneal dose of irinotecan on MMP and TIMP gene expression in the (a) jejunum and (b) colon at 1, 6, 24, 48, 72, 96 and 144 h after administration; the data are means ± standard error; $^{*}P < 0.05$: compared with pooled untreated controls. MMP, matrix metalloproteinases; TIMP, tissue inhibitor of metalloproteinase

at 24 h. TIMP-2 expression also increased to 3.4-fold at 96 h following treatment ($P < 0.05$) (Figure 2a).

Colon

MMP-2, -9 and -12 gene expressions were altered in the colon following irinotecan. MMP-2 expression decreased at one hour following irinotecan before gradually increasing at 72 h ($P < 0.05$). MMP-2 expression peaked at 2.6-fold change in gene expression at 144 h following treatment ($P < 0.05$) (Figure 2b). MMP-9 levels also increased following irinotecan treatment and peaked at 96 h with a 3.5-fold change in gene expression in comparison to controls (Figure 2b). There was a significant decrease in MMP-12 expression at one hour following irinotecan ($P < 0.05$). This was followed by an increase in MMP-12 at 72 h which peaked at 5.4-fold that of controls at 144 h (Figure 2b). TIMP-1 and -2 expression following irinotecan was consistent with that seen in the jejunum, but to a lesser extent (Figure 2a and b).

Tissue levels of MMPs following irinotecan

Jejunum

Tissue protease levels in this region were altered throughout the time course under investigation (Figure 3). In particular, MMP-1 levels decreased below that observed for untreated, control tissue at 1–24 h after irinotecan in both jejunal villi and crypts ($P < 0.05$) (Figure 4a and b). The decrease was followed by a statistically significant increase in staining levels for MMP-1 at 96 h following treatment in both regions ($P < 0.05$). MMP-2 levels were unchanged in the villi and decreased within the crypts early following irinotecan ($P < 0.05$). However, there was an increase in MMP-2 48 h after treatment in both regions ($P < 0.05$) (Figure 4a and b). MMP-3 was primarily expressed in the villus epithelial cells and absent in jejunal crypts in untreated control tissue (Figure 3). In both the crypts and villi, there was an increase in MMP-3 expression at one hour and 48 h, respectively ($P < 0.05$) (Figure 4a and b). This increase was sustained throughout the remainder of the time course (Figure 4a and b). MMP-9 levels also increased from moderate to intense staining in epithelial cells, basement membrane and underlying lamina propria at six hours onwards and in jejunal crypts at 24 h onwards after treatment ($P < 0.05$). There was a statistically significant increase in MMP-12 expression in the villi at 24, 96 and 144 h following treatment ($P < 0.05$). However, the most substantial increase in MMP-12 expression occurred in the crypts where intense staining was noted at 96 and 144 h following treatment ($P < 0.05$).

Colon

Similar changes in MMP expression were observed in the colon following irinotecan (Figure 5). In particular, an increase in MMP-1 levels was initiated at the basal level of the crypts 24 h after treatment ($P < 0.05$) and a corresponding subsequent increase in apical colon staining was noted at 72 h ($P < 0.05$) (Figure 6a and b). MMP-2 staining was weak in control tissue and increased significantly in the apical and basal colon at 96 h following treatment ($P < 0.05$) (Figure 6a and b). The expression of MMP-3 was minimal in the basal colon at all time points. In contrast, MMP-3 expression increased significantly in cells of the apical colon 24 h following irinotecan ($P < 0.05$) before subsequently significantly decreasing to levels below that seen in control tissue by 144 h ($P < 0.05$) (Figure 6a and b). MMP-9 levels were initially very low in untreated, control tissue. However, following irinotecan there was an increase in MMP-9 expression, which was intense at the late time points of 96 and 144 h ($P < 0.05$) (Figure 6a and b). An increase in MMP-12 from weak to moderate was noted in the apical and basal colon from 24 h following treatment ($P < 0.05$) (Figure 6a and b).

MMP expression changes throughout the time course following irinotecan administration is summarized in Table 2.

Tissue levels of TIMPs following irinotecan

TIMP-1 and -2 were expressed at low levels in the jejunum and colon of untreated, control animals (data not shown). TIMP-1 and -2 levels decreased significantly in the jejunal crypts and villi early on following irinotecan administration (1–6 h). There was a subsequent increase in TIMP-1 and -2 levels to moderate staining from 24 h onwards following irinotecan (data not shown). In contrast, no change in TIMP-1 and -2 were noted in the colon following irinotecan.

Plasminogens gene expression following irinotecan

tPA gene expression increased significantly in the jejunum from six hours following irinotecan onwards. tPA expression peaked in the jejunum at a 8.5-fold change 48 h following irinotecan ($P < 0.05$) (Figure 7a). uPA gene expression increased 2.6-fold at one hour in the jejunum, but a significant increase was only observed at 144 h following irinotecan ($P < 0.05$) (Figure 7a). PAI-1 increased significantly at six hours following treatment onwards ($P < 0.05$) (Figure 7a). There was no significant change in tPA and uPA in the colon (Figure 7b). A decrease in PAI-1 was noted at 48 h following irinotecan where gene expression was 0.39 (2.56-fold decrease) in comparison to control tissue ($P < 0.05$) (Figure 7b).

Discussion

The current study investigated the expression profiles of a range of MMPs, previously shown to act as mediators of tissue injury and inflammation,[17,28,29] in a time-course model of irinotecan-induced mucositis. Key findings from this study indicate a substantial augmentation in MMP-1, -2, -3, -9 and -12 as well as TIMP-1 and -2 expression following irinotecan. Furthermore, the expression profiles of these MMPs in correlation with the histopathological damage seen in the tissue suggest varying roles for the MMPs in the different stages of mucositis development within the AT. This study has also shown that MMP activators, tPA and uPA, are up-regulated in both the jejunum and colon. The expression of inhibitory PAI-1 increases slightly in the

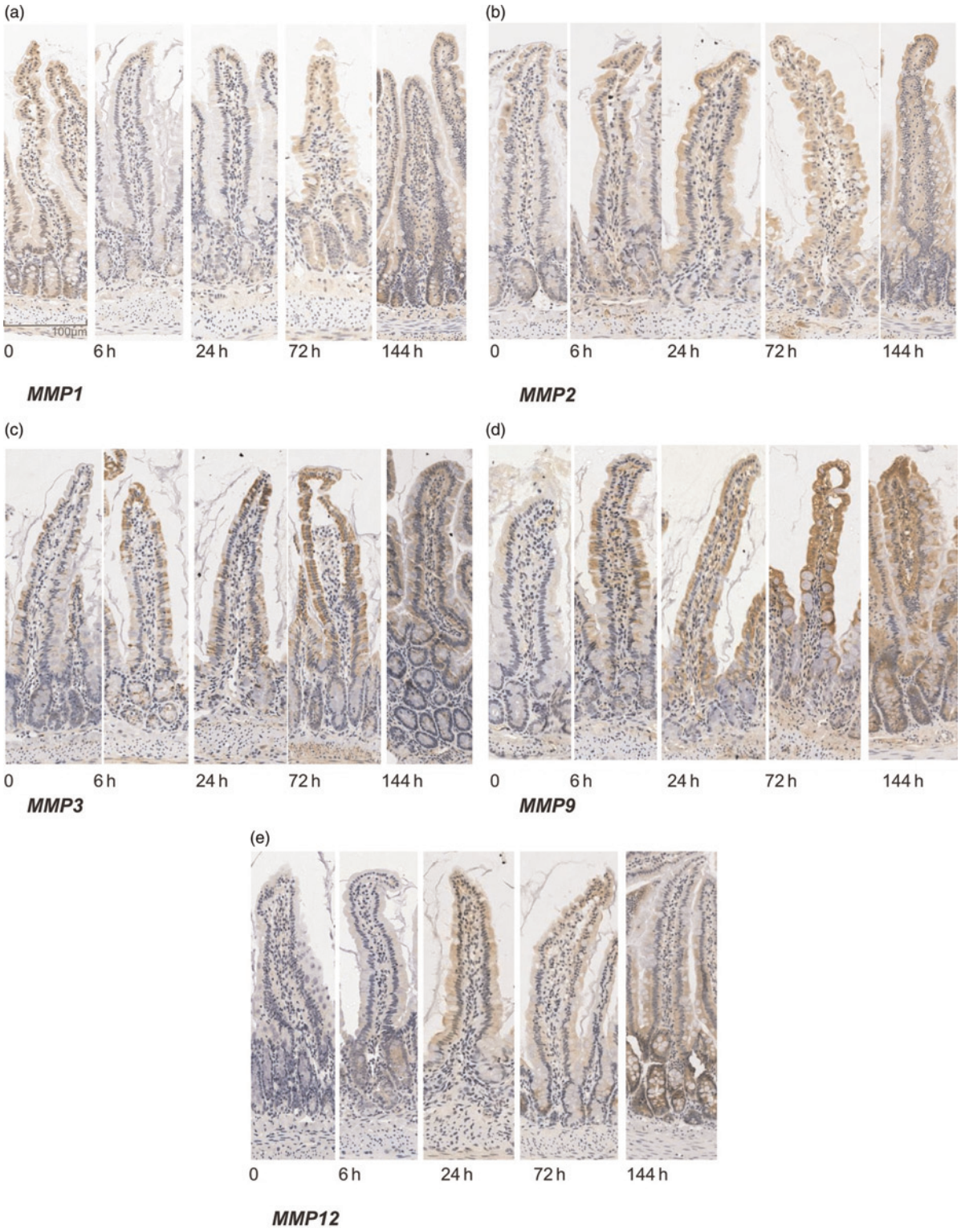

Figure 3 MMP immunostaining in the jejunum in control tissue and at selected time points following the administration of 200 mg/kg irinotecan. All photomicrographs taken at original magnification ×100. MMP, matrix metalloproteinases

jejunum, while it decreases in the colon following the administration of irinotecan.

From this study, it is now possible to elucidate the role that MMPs play in mucositis development by correlating their expression profiles with the underlying histopathology. The current study along with many past studies have described in detail the pathological alteration that occurs in the AT following irinotecan.[2,6–8,18] These have indicated the presence of degenerative enterocytes within jejunal and colonic crypts as early as six hours following irinotecan administration. Furthermore, intestinal morphometry studies have shown that irinotecan causes maximal villus

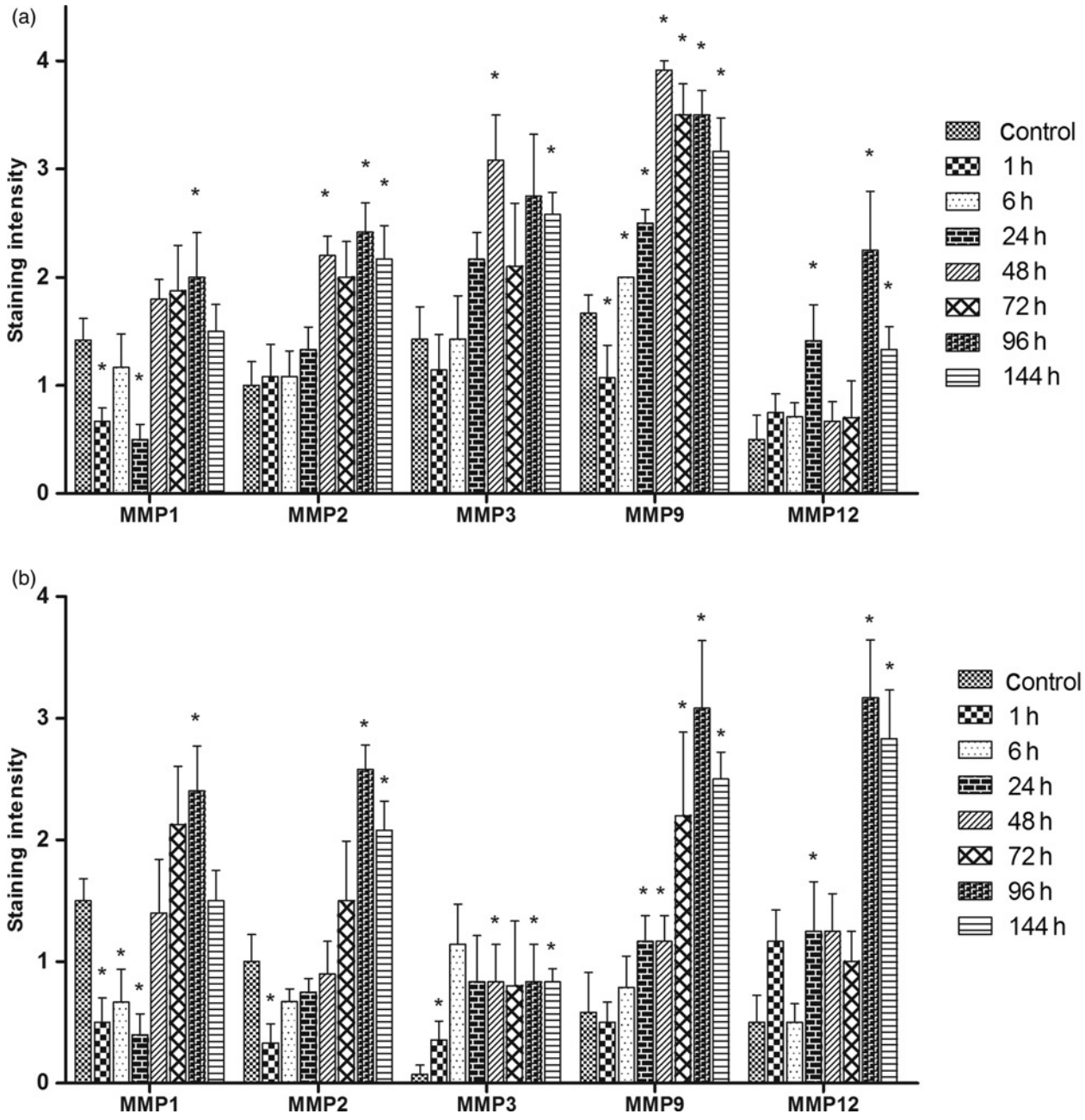

Figure 4 MMP staining in jejunal (a) villi and (b) crypts at 1, 6, 24, 48, 72, 96 and 144 h following the administration of 200 mg/kg irinotecan intraperitoneally. All control and experimental tissue were graded by a qualitative scale. The data are means ± standard error; $^{*}P < 0.05$: compared with untreated controls. MMP, matrix metalloproteinases

and crypt damage at 48–72 h following its administration, thus resulting in epithelial atrophy.[8] An increase in inflammatory infiltrate is also noted at those time points. In the colon, decreases in goblet cell numbers and mucus hypersecretion are also observed.[8] It has also been noted that in areas of crypt loss, collapse and condensation of the propria stroma occur.[2] In the jejunum and colon, tissue restitution is initiated generally by three days post-treatment, and as such hyperplasia was observed after 72 h and excess mitotic figures were present even at the later time points of 96–144 h following irinotecan administration.[6]

Although MMPs share similar structural homology and are all classed as ECM-degrading proteases, they have been shown to have distinct functional differences. For example, MMP-2 and -9 have been shown to aid in inflammatory cell infiltration into tissue following primary cell damage, while MMP-1 and -3 have been shown to be vital for inducing epithelial cell migration and tissue restitution following injury.[30] These differences could be attributed to differences in domain structure, activation mechanism and cellular location.[14] In our model of irinotecan-induced mucositis, MMP-1, -2, -3, -9 and -12 have shown differing expression profiles indicating possible differences in their roles in the pathogenesis of mucositis. The expression profiles of the MMPs of interest suggest they play the following roles: MMP-1, role in healing as it decreases early on then

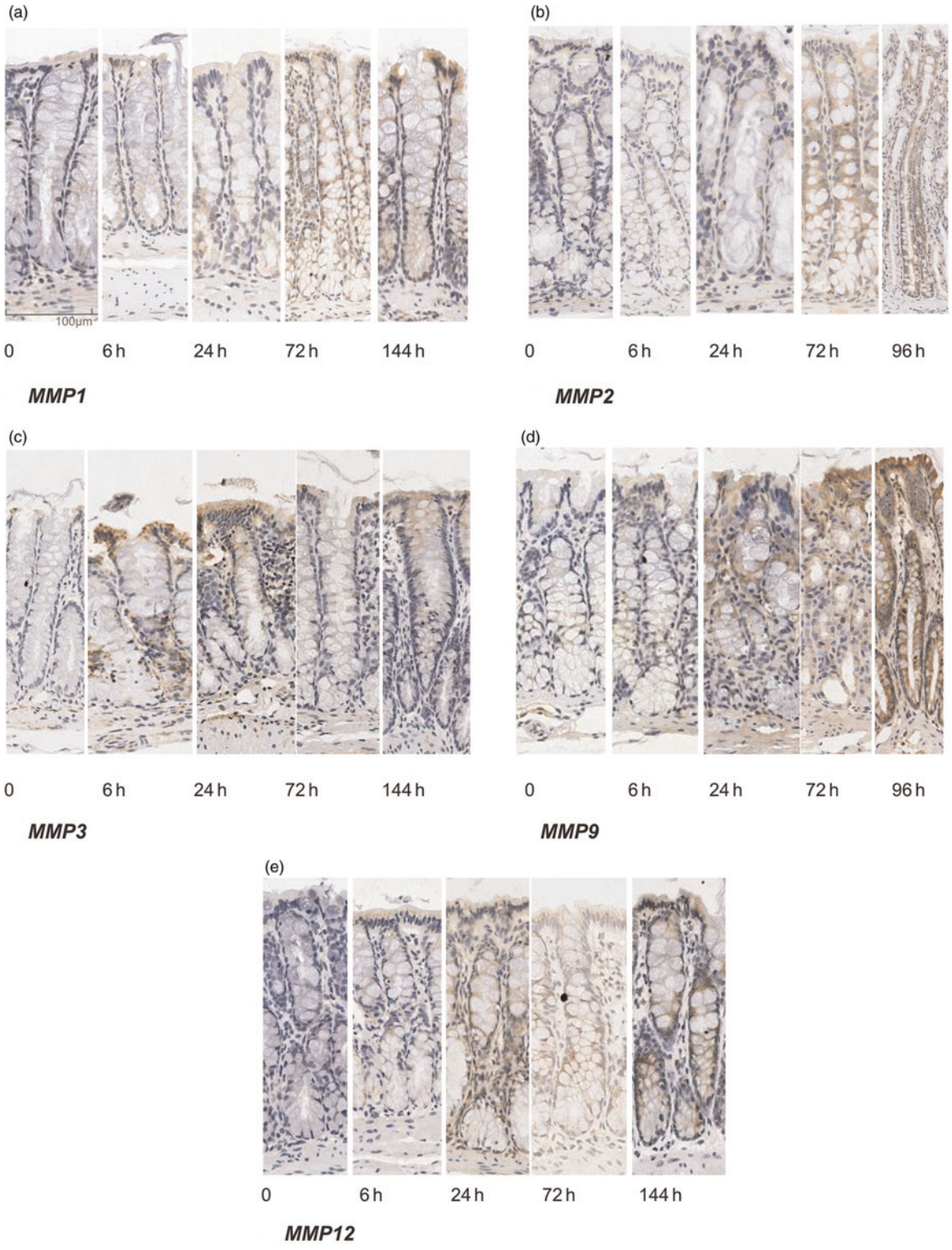

Figure 5 MMP immunostaining in the colon in control tissue and at selected time points following the administration of 200 mg/kg irinotecan. All photomicrographs taken at original magnification ×100. MMP, matrix metalloproteinases

increases at 96 and 144 h; MMP-2, MMP-9 and MMP-12, role in inflammation as expression decreases early on but increases at 48 h, consistent with inflammatory influx and maximal morphological damage; and MMP-3, possible role in the initiation of inflammation as well as hypoproliferation in jejunal and colonic crypts early following irinotecan, as peak expression was noted within crypts at very early time points before dissipating. However, it should be noted that causation has not been proven in this study.

MMP-1 showed a very distinct expression pattern relative to other MMPs under observation. MMP-1 (along with

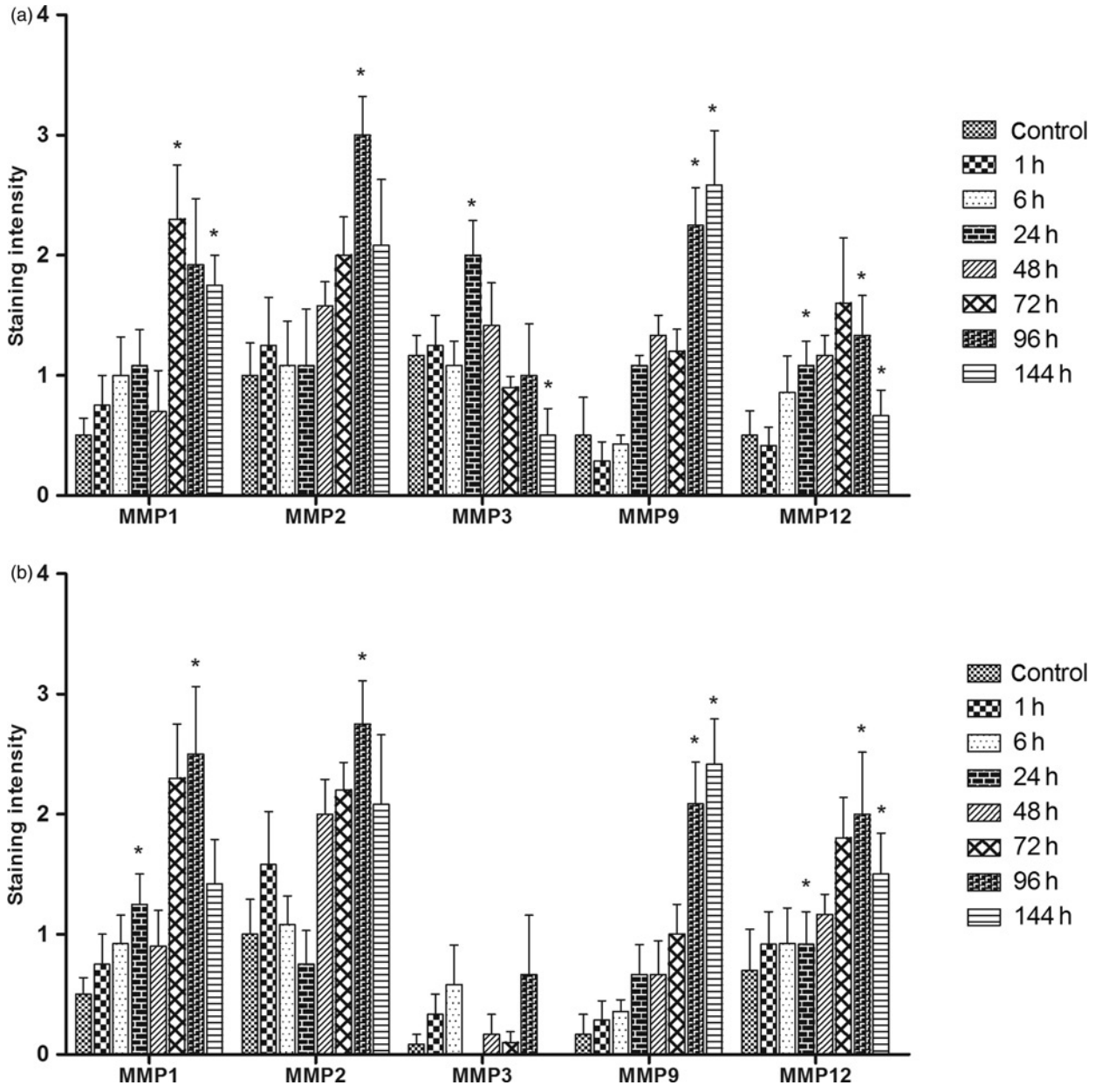

Figure 6 MMP staining in the (a) apical and (b) basal colon 1, 6, 24, 48, 72, 96 and 144 h following the administration of irinotecan 200 mg/kg irinotecan intraperitoneally. All control and experimental tissue were graded by a qualitative scale. The data are means ± standard error; $^{*}P < 0.05$: compared with untreated controls. MMP, matrix metalloproteinases

MMP-8, -13 and -14) is one of only four members of the MMPs, which have been shown to have fibrillar collagen degrading capacity.[31] Furthermore, MMP-1 promoter analysis studies have identified TATA box and activator protein 1 binding sites, as well as multiple consensus and composite sites.[14] The differential expression pattern for MMP-1 in this model may be attributed to its unique promoter conformation and hence the complexes by which it can be induced. A recent study by Salmela and colleagues[32] investigated the expression of MMP-1 in *in vivo* as well as *ex vivo* models of gut injury. They found MMP-1 to be expressed by migrating enterocytes and aid primarily in wound closure. Furthermore, they showed that MMP-1 expression was not up-regulated in response to different cytokine treatments, but rather increased in response to epidermal growth factor treatment; which is thought to have a vital role in the maintenance of mucosal layer integrity as well as in acute gut repair.[32] These studies support the suggestion that MMP-1 may play a vital role in tissue healing following mucosal injury caused by irinotecan. However, further studies are still required to fully understand the precise role of MMP-1 in tissue restitution and healing following mucositis development.

A hallmark of mucositis development following primary tissue injury is the initiation of an acute inflammatory response.[5,9,13] Previous studies have shown early up-regulation of MAPK signalling, NF-κB signalling, Fos/Jun signalling and Wnt signalling following irinotecan administration.[10,33] Furthermore, there is a peak in tissue levels of pro-inflammatory cytokines tumor necrosis factor,

Table 2 Summary of MMP tissue levels early (1–6 h) and late (24–144 h) following irinotecan administration

	Early	Late
Jejunum		
MMP-1	↓	↑
MMP-2	↓	↑
MMP-3	↑	↑
MMP-9	↓	↑
MMP-12	↔	↑
Colon		
MMP-1	↔	↑
MMP-2	↔	↑
MMP-3	↔	↓
MMP-9	↔	↑
MMP-12	↔	↑

MMP, matrix metalloproteinase

interleukin (IL)-1β and IL-6 at six hours following the administration of irinotecan.[7] *MMP* genes have been shown to possess functional elements which respond to cell injury and inflammatory pathways, such as those up-regulated in mucositis. Previous studies have implicated MMP-2, -3, -9 and -12 in inflammatory responses where they are produced by infiltrating leukocytes in order to facilitate their transendothelial migration into the mucosa.[34–36] In this study, inflammatory infiltrate was noted at 48–72 h following treatment and is consistent with the expression of MMP-2, -3, -9 and -12 in epithelial cells and the underlying blood vessel-rich lamina propria. Maximum tissue injury seen in AT mucositis is also consistently seen at these time points. This correlation between injurious signalling pathway activation, inflammatory infiltrate and the expression of MMPs implicates MMP-2, -3, -9

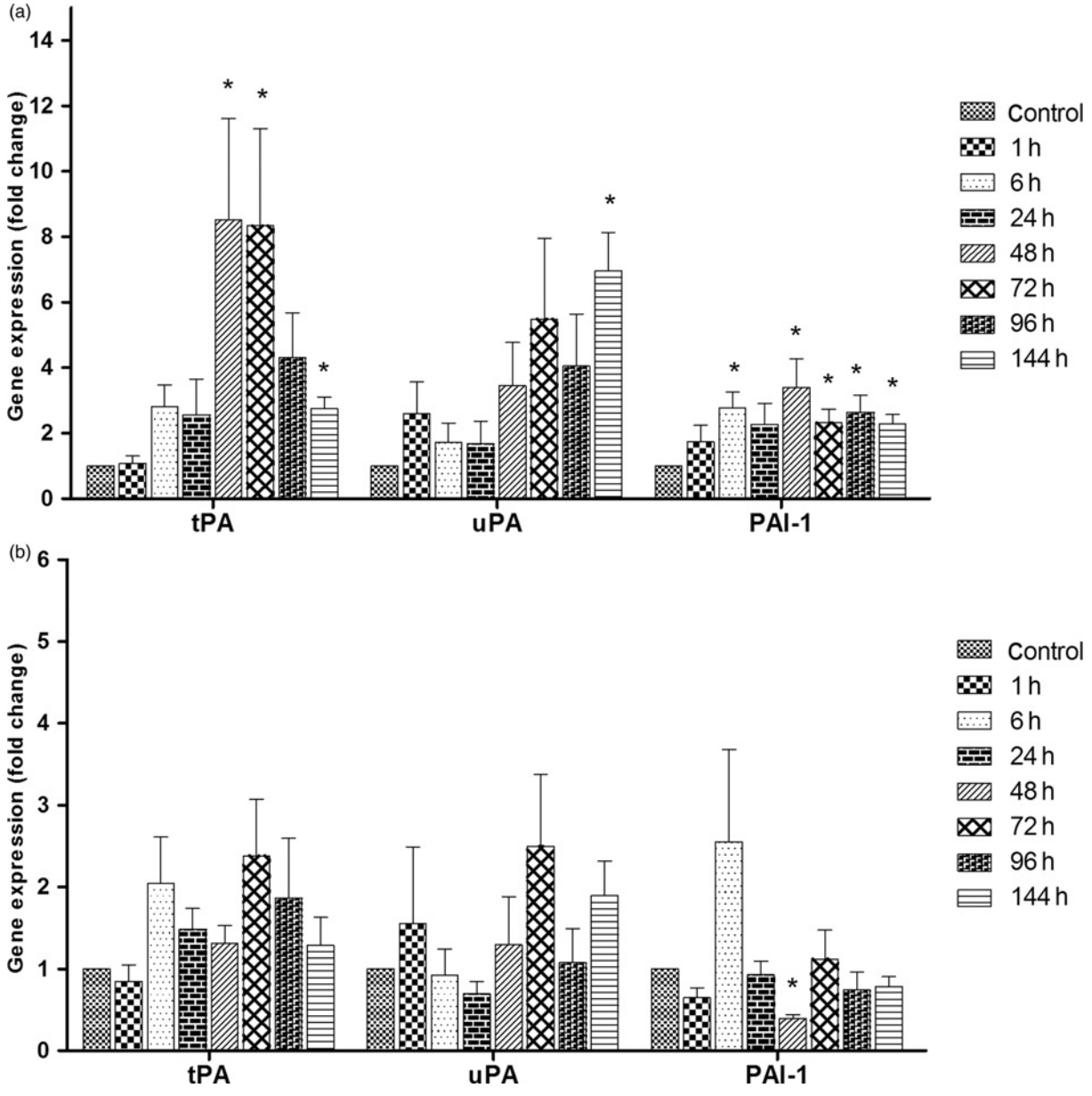

Figure 7 mRNA expression of MMP activators tPA and uPA and inhibitor PAI-1 in the (a) jejunum and (b) colon at 1, 6, 24, 48, 72, 96 and 144 h after administration; the data are means $\pm$ standard error; $^{*}P < 0.05$: compared with pooled untreated controls. MMP, matrix metalloproteinases; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; PAI-1, plasminogen activator inhibitor-1

and -12 in the acute inflammation seen in mucositis in our model. However, the role of MMPs in mucositis development is unlikely to be restricted to inflammation as they may also contribute to the profound changes in cellular kinetics, which are observed in AT mucositis.

MMPs are synthesized and secreted in their latent, inactive form. The regulatory mechanisms for MMP activation are complex and include allosteric activation, furin activation, serine proteinases and oxidation. This study investigated the expression of the plasminogen activators uPA and tPA following irinotecan administration. An early increase in uPA and tPA was noted in this study, possibly aiding in the activation of secreted MMPs. However, this study explored only one of many mechanisms for MMP activation. Further studies are now warranted to investigate the mechanisms responsible for MMP activation in tissue following irinotecan.

In conclusion, an augmentation in the expression profiles of MMP-1, -2, -3, -9 and -12 and their inhibitors TIMP-1 and -2 correlated with the histopathological alterations in the tissue following irinotecan treatment. This prompts the consideration of MMPs as possible mediators of damage in the AT following chemotherapy. Further studies are now required in order to elucidate the specific roles of each of these MMPs in mucositis. These could include intervention studies with specific MMP inhibitors in this model.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; RML, JMB, AMS and RJG carried out the animal trial; and NA-D conducted the experiments and wrote the manuscript.

ACKNOWLEDGEMENTS

NA-D was supported by an Australian Postgraduate Award and a GlaxoSmithKline Postgraduate Support Grant; RJG was supported by a Cancer Council Post-Doctoral Fellowship; JMB was supported by a Postdoctoral Research Fellowship from the National Health and Medical Research Council; AMS was supported by NHMRC Postgraduate Training Scholarship; and DMK is the Cancer Council SA Chair of Cancer Medicine.

All individuals who made contributions to this study are included either as authors or are acknowledged at the end of the paper. Postal Address for all authors: Royal Adelaide Hospital, Department of Medical Oncology, Level 4 East Wing, North Terrace, Adelaide 5000, SA, Australia.

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(Received March 3, 2010, Accepted May 21, 2010)