Intestinal microvascular changes and matrix metalloproteinases in radiotherapy-induced gastrointestinal toxicity

A thesis submitted in fulfilment for the degree of

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by

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Table of Contents

Abstract

Radiotherapy-induced gastrointestinal toxicity (RIGT) involves damage to the gastrointestinal mucosa and is associated with symptoms including but not limited to, diarrhoea, pain, and rectal bleeding. Members of the matrix metalloproteinase (MMP) family have recently been identified as being upregulated in RIGT. Furthermore, the microvasculature has long been implicated in the development of toxicities following radiotherapy, however, the mechanisms behind this are yet to be explored. This thesis aimed to assess the microvascular response to irradiation, to further elucidate the role of MMPs in RIGT, and to assess the effect of MMP inhibition on microvascular endothelium following irradiation.

This thesis consists of a general introduction, published literature review, three research chapters, one published and two submitted, and a general discussion. In chapter 1, the topic of this thesis is introduced, discussing the epidemiology and underlying pathobiology of RIGT. Chapter 2, a published critical review of the literature, consolidates literature on the role of MMPs, intestinal microvasculature, and vascular mediators in RIGT. This literature review surmised MMPs to be key regulators of endothelial mediators, and to play a key role in inducing damage to intestinal microvasculature following radiotherapy.

The third chapter, published in Supportive Care in Cancer, utilized a Dark Agouti (DA) rat model of fractionated abdominal irradiation to assess changes to the intestinal microvasculature. A significant increase in apoptosis of microvascular cells 6 and 15 weeks from the first dose of irradiation was found, corresponding with histopathological damage and apoptosis in the jejunal and colonic crypts. This study suggested regional and timing-specific changes in the intestinal microvasculature to occur in response to fractionated radiotherapy.

Chapter four assessed levels of MMPs in the jejunum and colon in the same DA rat model of RIGT. Whilst mRNA expression MMP-1, -2, and -14 significantly increased in the jejunum, only MMP-2 expression increased in the colon. MMP-2 immunostaining was also observed to be increased in both the jejunum and colon, a finding supported by western blotting, showing significantly increased MMP-2 protein levels in both the jejunum and colon at week 6. This supported a role for MMP-2 in the pathobiology of RIGT.

Chapter five, the final research chapter, assessed vascular mediator expression in the DA rat model of RIGT, as well as the effects of irradiation and MMP inhibition on tumour-associated microvascular endothelial cells derived from DA rat mammary adenocarcinoma. This study confirmed an *in vivo* increase in the vascular mediators, VEGF, TGFβ, angiostatin, and endostatin. Cell culture results confirmed an increase in both MMP-2 and -9 following irradiation, significantly attenuated by MMP inhibition, however this attenuation did not alter the expression of vascular mediators or the toxicity profile of irradiation.

In summary, this thesis contributed to the field of supportive care in cancer by elucidating a role for the intestinal microvasculature, MMPs, and vascular mediators, in RIGT. The findings of this thesis suggest these factors are likely part of a complex pathway involving many other mediators and intestinal components. Further research is now warranted to assess efficacy of treatments for RIGT targeting the intestinal microvasculature and MMPs.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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Publications arising from thesis

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Stansborough R.L., Al-Dasooqi N., Bateman E.H., Bowen J.M., Keefe D.M, Logan R.M., Yeoh A.S.J., Yeoh E.E.K., Gibson R.J. (2018) Matrix metalloproteinase-2 expression is increased in the small and large intestine following fractionated radiation *in vivo. Supportive Care in Cancer* (published online ahead of print 12 May). Available at: https://doi.org/10.1007/s00520-018-4255-5.

Stansborough R.L., Bateman E.H., Al-Dasooqi N., Bowen J.M., Keefe D.M.K., Yeoh A.S.J., Logan R., Yeoh E.E.K., Stringer A.M., Gibson R.J. (2017) Fractionated abdominal irradiation induces intestinal microvascular changes in an *in vivo* model of radiotherapy-induced gut toxicity. *Supportive Care in Cancer*. 25(6): 1973-1983.

Stansborough R.L., Al-Dasooqi N., Bateman E.H., Keefe D.M., Gibson R.J. (2016) Radiotherapy-induced gut toxicity: Involvement of matrix metalloproteinases and the intestinal microvasculature. *International Journal of Radiation Biology*. 92(5): 241-248.

Additional Studies and Publications

Stansborough R.L, Gibson R.J. (2017) Proteasome inhibitor-induced gastrointestinal toxicity. *Current Opinion in Supportive and Palliative Care*. 11(2): 133-137.

Williams B., Tsangari H., **Stansborough R.L.**, Marino V., Cantley M.D., Dharmapatni A., Gibson R.J., Perilli E., Crotti T.N. (2016) Mixed effects of caffeic acid phenethyl ester (CAPE) on joint inflammation, bone loss and gastrointestinal inflammation in a murine model of collagen antibody-induced arthritis. *Inflammopharmacology*. 25(1): 55-68.

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Zawawi M.S.F., Perilli E., **Stansborough R.L.**, Marino V., Cantley M.D., Xu J., Dharmapatni A.A.S.S.K., Haynes D.R., Gibson R.J., Crotti T.N. (2015) Caffeic acid phenethyl ester abrogates bone resorption in a murine calvarial model of polyethylene particle-induced osteolysis. *Calcified Tissue International and Musculoskeletal Research.* 96(6): 565-574.

Wardill H.R., Bowen J.M., Al-Dasooqi N., Sultani M., Bateman E., **Stansborough R.**, Shirren J., Gibson R.J. (2014). Irinotecan disrupts tight junction proteins within the gut: implications for chemotherapy-induced gut toxicity. *Cancer Biology and Therapy***.** 15(2): 236-244.

Chapter 1 General introduction

Radiotherapy is one of the most commonly prescribed therapies for cancer, with an optimal radiotherapy utilisation rate of 48% in Australia (Barton et al. 2014). In 2014 alone, over 60,000 people receiving medicine-subsidised radiotherapy services in Australia (Barton et al. 2014; Welfare 2017). Although developments have been made in increasing the safety profile of radiotherapeutic techniques, the prevalence of radiotherapy-induced toxicities remains high (Shadad et al. 2013a). Radiotherapy may induce many different toxicities, dependent on targeted area and treatment schedule. However one of the most common toxicities, particularly following abdominal and pelvic irradiation, is radiotherapy-induced gastrointestinal toxicity (RIGT) (Shadad et al. 2013a). RIGT is a broad term, encompassing clinical and histopathological manifestations of toxicity occurring throughout the gastrointestinal tract following irradiation. RIGT can present as both acute (occurring within 3 months of treatment) or chronic (3 months – several years following treatment) (Theis et al. 2010). This introductory chapter will further explore the epidemiology and pathobiology of RIGT, focussing on two key components, matrix metalloproteinases (MMPs) and the intestinal microvasculature.

1.1 Clinical and histopathological manifestation of RIGT

RIGT can manifest as both acute and chronic toxicity, with distinct side effects and histopathological features. Although the two forms of RIGT were once thought to be unrelated (Denham & Hauer-Jensen 2002), it is now understood they are continuations of the same pathology, with acute RIGT contributing to the development of chronic RIGT (Hauer-Jensen, Denham & Andreyev 2014).

1.1.1 Acute RIGT

Acute RIGT is a term encompassing acute radiation enteritis, radiation-induced enteropathy, and radiation-induced proctitis. It may occur during, or within days of, radiotherapy, with clinical symptoms including, but not limited to, diarrhoea, abdominal pain, and nausea and vomiting (Theis et al. 2010; Berbee & Hauer-Jensen 2012). Whilst acute RIGT may develop into chronic RIGT, these two conditions have distinct histopathological manifestations (Theis et al. 2010). This is likely due to differences in pathobiology, with acute RIGT driven by the direct effect of radiation, inducing reactive oxygen species (ROS), oxidative damage, activating transcription factors and pro-inflammatory mediators, and inducing an inflammatory response (Sonis, S. T. 2004; Liu et al. 2016).

1.1.2 Chronic RIGT

Chronic RIGT, also known as chronic radiation enteritis or chronic radiation enteropathy, typically occurs anywhere from 3 months to 6 years following radiotherapy, however cases have been reported up to 15 years following treatment (Theis et al. 2010). The clinical presentation of chronic RIGT includes a diverse set of symptoms such as diarrhoea, constipation, abdominal pain, rectal bleeding, and distention (Qin et al. 2013). Several complications are also associated with chronic RIGT, including faecal incontinence, bowel obstruction, fistula formation, transfusion-dependent bleeding, and secondary malignancy (Andreyev 2007; Qin et al. 2013). The pathobiology of acute and chronic RIGT will be explored in greater depth in both this introductory chapter and in the review of the literature in chapter 2.

1.2 Epidemiology

The general prevalence of both acute and chronic RIGT ranges from 33-72% depending on treatment modality, dose schedule, and target area (Roszak et al. 2012; Shadad et al. 2013b). However is difficult to accurately determine, largely due to discrepancies in the definition and grading of clinical RIGT (Roszak et al. 2012; Shadad et al. 2013a). Despite this, several clinical studies have assessed the prevalence of radiation toxicity in specific cohorts. Roszak and colleagues (2012) assessed acute RIGT in 263 patients with cervical and endometrial cancer who had received definitive (radiation as sole local treatment) or adjuvant (treatment to lower risk of recurrence) radiotherapy. Acute RIGT occurred in 33.1% of patients, with definitive radiotherapy resulting in a 65.5% incidence of RIGT of any grade (grade 3-4 =24.1%) (Roszak et al. 2012). Further, acute diarrhoea and abdominal pain have been shown in a systematic review to occur in anywhere from 20% to 70% of patients following abdominal or pelvic radiotherapy (Shadad et al. 2013a, 2013b).

Rectal bleeding is one of the most common symptoms of chronic RIGT (Andreyev 2007; Kim, Huh & Park 2013). A recent study by Kim and colleagues (2013) assessed 32 patients who had received definitive or postoperative radiotherapy for uterine cervical cancer and were subsequently diagnosed with rectal mucosal damage. The median interval was 19.5 months (2 - 114 month range) from last radiotherapy treatment and rectal bleeding was the most frequent symptom, occurring in 72.4% of patients (Kim, Huh & Park 2013). The most common endoscopic findings were congested mucosa (62.5%), telangiectasia (56.3%), and ulceration (15.6%) (Kim, Huh & Park 2013). In another study assessing 100 patients who had received radiotherapy for pelvic malignancies 5 years prior, 20% of patients presented with chronic RIGT (grade 1-2 – 75%, grade 3-4 – 25%) (Hernandez-Moreno et al. 2015). In addition to

this, 28.3% of patients had a change in daily number of stools, 35% had changes to the consistency of stools, and 12.1% had blood or mucus in stools (Hernandez-Moreno et al. 2015). Thus, the prevalence of chronic RIGT continues to be high, and places a significant burden on patients, often long after the cessation of treatment.

1.3 Pathobiology

1.3.1 5-phase model

The pathobiology of RIGT is complex and not yet fully understood. Much of the current understanding of the development of RIGT can be summarized by the 5-phase model developed by Sonis in 2004. In the first phase, initiation, radiation directly damages DNA, as well as generates reactive oxygen species (ROS) (Sonis, S. T. 2004; Liu et al. 2016). The second phase, message generation, involves the up-regulation of transcription factors, particularly NFκB (Sonis, S. T. 2004). This then results in the activation of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, and many other downstream mediators, including MAPK, and cyclooxygenase-2 (COX-2) (Sonis, S. T. 2004; Yeoh et al. 2007; Al-Dasooqi et al. 2013; Liu et al. 2016). Following this, the signalling and amplification stage occurs, in which these cytokines and mediators cause tissue damage, as well creating a positive feedback loop to further upregulate transcription factors and cytokines (Sonis, S. T. 2004; Al-Dasooqi et al. 2013). The fourth phase, ulceration, encompasses the accumulation of this tissue damage, resulting in ulceration, bacterial penetration, and immune cell infiltration. The final phase is healing, in which resolution of ulcers, remodelling of the extracellular matrix (ECM), and epithelial proliferation and differentiation occurs (Sonis, S. T. 2009). Despite this, the intestinal tissue often never returns to its ‘normal’ state, and fibrotic thickening and

long term changes in mediator expression can lead to chronic RIGT (Hauer-Jensen, Denham & Andreyev 2014).

1.3.2 Recent updates in the pathobiology of RIGT

The study of the pathobiology of RIGT continues to be a focus in research surrounding cancer therapy-induced toxicities, due to the limited number of currently available treatments (Lalla et al. 2014). Matrix metalloproteinases (MMPs) have now been implicated in the pathobiology of RIGT (Strup-Perrot et al. 2006; Angenete et al. 2009). Strup-Perrot and colleagues (2006) found a significant increase in mRNA expression of MMP -2, -3, -9, and -14, and protein levels of MMP-2 and -14, associated with increased diarrhoea and histopathological damage, in the colon of rats irradiated with a single abdominal dose of 10 Gy. Further, Mangoni and colleagues (2015) reported MMP-2 and -13 to be significantly increased in both the jejunum and colon following 12 Gy total body irradiation in mice (Mangoni et al. 2015). In the same study, inhibition of MMPs, reduced radiotherapy-induced jejunal and colonic damage (Mangoni et al. 2015). One of the proposed mechanisms by which MMPs contribute to the development of RIGT is through the degradation of the extracellular matrix (ECM), leading to microvascular tube formation and the release of vascular mediators.

The intestinal endothelium is also intricately involved in the development of RIGT, with several pre-clinical studies, utilising rat and human endothelial cell culture models, implicating vascular mediators in its pathobiology (Wang, J et al. 2002; Oh et al. 2014). Several of these mediators, including vascular endothelial growth factor (VEGF), and the anti-angiogenic mediators, angiostatin and endostatin, are potentially involved in pathological angiogenesis, telangiectasia, and rectal bleeding in chronic RIGT (Park et al. 2012; Laterza et al. 2013; Stansborough et al. 2016). Transforming growth factor β (TGFβ), a multifunctional

growth factor involved in fibrosis, has also been suggested to play a key role in the development of intestinal fibrosis and vascular sclerosis characteristic of chronic RIGT (Wang, J, Zheng & Hauer-Jensen 2001; Boerma et al. 2013). Further, TGFβ has been shown to be increased in both pre-clinical and clinical studies, supporting its role in the development of chronic RIGT (Wang, J, Zheng & Hauer-Jensen 2001; Milliat et al. 2006; Kruse et al. 2009; Al-Dasooqi et al. 2013). It is clear from these studies the pathobiology of RIGT is truly complex, involving relationships between transcription factors and many downstream mediators, thus further studies are required to identify targets for potential novel treatments of both acute and chronic RIGT.

1.4 Treatment of RIGT

The recommendation of preventative and therapeutic measures for both acute and chronic RIGT remain limited (Lalla et al. 2014). Preventative agents recommended, or suggested, by the Multinational Association for Supportive Care in Cancer (MASCC) in the most recent mucositis guidelines include intravenous amifostine for radiation proctitis, and systemic sulfasalazine for pelvic radiation-induced enteropathy (Lalla et al. 2014). These agents scavenge free radicals, preventing oxidative damage, and inhibit pro-inflammatory mediators, respectively (Uzal et al. 2012; Fuccio, Frazzoni & Guido 2015). Currently, the only suggested treatments for RIGT include hyperbaric oxygen for radiation-induced proctitis, and sucrulfate enemas for chronic radiation-induced proctitis in patients with rectal bleeding (Laterza et al. 2013; Lalla et al. 2014; Bansal et al. 2016). These treatments act by promoting neovascularisation and decreasing tissue hypoxia, and forming a protective barrier over damaged mucosa and stimulating epithelial healing, respectively (Laterza et al. 2013; Lalla et al. 2014; Bansal et al. 2016). Whilst these agents may reduce the severity, or promote healing,

of RIGT in some patients, a lack of understanding of the pathobiology of RIGT hinders the development of more targeted treatments. Better understanding the role of factors contributing to RIGT pathobiology, such as those explored in this thesis, MMPs and the intestinal microvasculature, can contribute to the development of targeted and efficacious preventative and therapeutic agents.

1.5 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a group of enzymes, consisting of 24 different members in humans, which primarily are responsible for the remodelling of the ECM (Biancheri et al. 2013). However, the roles of MMPs in normal and pathological states are diverse. They can upregulate many mediators, including many of the pro-inflammatory cytokines known to be involving in RIGT pathobiology, and activating vascular mediators such as VEGF, angiostatin, and endostatin, via the sequestering of ECM components, expanded upon in Chapter 2 (Bergers et al. 2000; Strup-Perrot et al. 2006; Mittal et al. 2016). MMPs are predominantly regulated at the transcription level and are released as inactive zymogens by various cell types, including epithelial cells, endothelial cells, macrophages, neutrophils, and T cells (Biancheri et al. 2013). They can then be activated via cleavage of the pro-domain (Yan & Boyd 2007). Several MMPs have been implicated in the development of RIGT, however the MMP -1, -2, -9, -12, and -14 will be explored in more depth, both in this introductory chapter, and throughout the thesis, due to their possible roles and relationships with other mediators and the intestinal microvasculature in the development of acute and chronic RIGT (Strup-Perrot et al. 2006; Angenete et al. 2009; Al-Dasooqi et al. 2010).

1.5.1 MMP-1

MMP-1 is classified as a collagenase due to its ability to degrade collagen and gelatin. However, it is also capable of activating pro-MMP-9 (Mittal et al. 2016). Transcription of MMP-1 can be increased in response to pro-inflammatory cytokines, epidermal growth factor, as well as following activation of p38 mitogen-activated protein kinases (MAPK) (Salmela et al. 2004; Mittal et al. 2016). Both of these mediators have previously been shown to be increased in pre-clinical studies of RIGT and are thought to be involved in regulating apoptosis (Sonis, S. T. 2002; Salmela et al. 2004; Mittal et al. 2016).

1.5.2 MMP-2

MMP-2, also known as gelatinase A, is capable of degrading several ECM components, including collagen IV and VXII, gelatin, elastin, plasminogen, and decorin, to activate downstream products such as VEGF, TGFβ, and form angiostatin, endostatin, and tumstatin (Sternlicht & Werb 2001; Manicone & McGuire 2008). MMP-2 is uniquely regulated by MMP-14 (MT1-MMP), and once activated able to shed and activate pro-inflammatory cytokines such as TNF-α and IL-1β (Chien et al. 2013). MMP-2 has been consistently shown to be upregulated following irradiation in both pre-clinical and clinical studies, occurring prior to, or concurrently with, intestinal histopathological damage in acute RIGT (Strup-Perrot et al. 2006; Angenete et al. 2009; Mangoni et al. 2015).

1.5.3 MMP-9

MMP-9 is also a member of the gelatinase group of MMPs, classified as gelatinase B (Yan & Boyd 2007). Similarly to MMP-2, MMP-9 is capable of degrading type IV collagen, gelatin and elastin, among several other ECM components (Visse & Nagase 2003). Several pre-

clinical studies have suggested a disruptive effect of MMP-9 in pathological states. MMP-9 is predominantly secreted by neutrophils and can impair re-epithelialization (Castaneda et al. 2005; Biancheri et al. 2013). MMP-9 is also known to alter pro-inflammatory cytokine expression, capable of both processing IL-1β from its precursor and degrading it (Visse & Nagase 2003). Whilst MMP-9 has been shown to be increased in the large intestine following irradiation in some models, its role is slightly less clear than that of MMP-2, and the literature is inconsistent in its findings (Strup-Perrot et al. 2006; Angenete et al. 2009).

1.5.5 MMP-14

MMP-14 has been shown to cleave the cell adhesion receptor CD44, promoting cell adhesion and migration (Yan & Boyd 2007). Additionally, MMP-14 is intricately involved with the activation of pro-MMP-2. Active MMP-14 expressed on the cell surface, when bound to tissue inhibitor of metalloproteinase 2 (TIMP2),acts as a receptor for pro-MMP-2 after which a free MMP-14 molecule is able to cleave and activate pro-MMP-2 (Chien et al. 2013). Whilst MMP-14 has remained largely unexplored in RIGT, its involvement with MMP-2 regulation suggests it may play an important role (Strup-Perrot et al. 2006).

1.6 Intestinal microvasculature

The intestinal microvasculature plays an important role in the maintenance and regulation of the intestinal tract. It not only regulates blood delivery and perfusion, but also acts as a barrier controlling the exchange of fluids and nutrients, while preventing the systemic circulation of pathogenic bacteria from the intestinal lumen (Spadoni et al. 2015). Endothelial cells are a primary component of the microvasculature, and can regulate mediator expression and immune cell infiltration in response to pathological conditions (Cromer et al. 2011). Cell culture studies comparing different endothelial cell culture human intestinal microvascular

endothelial cells (HIMECs) have been compared with human umbilical vein endothelial cells (HUVECs) and have been found to have different inflammatory responses (Nilsen et al. 1998; Cromer et al. 2011). HIMECs, unlike HUVECs, when stimulated with lipopolysaccharide (LPS), found in the intestine as a major component of gram-negative bacteria, produce IL-1β, IL-3, and IL-6 (Nilsen et al. 1998; Cromer et al. 2011). The identification of an intestine-vascular barrier, similar to the blood-brain barrier, has also recently been described by Spadoni and colleagues (2015). In the gastrointestinal tract, permeability of intestinal endothelial cells is closely regulated by the presence of intercellular tight junctions, such as occludin, zonula occludens-1, and adherens junctions, such as vascular endothelial cadherin and β-catenin (Spadoni et al. 2015). Whilst outside the scope of this thesis, the role of tight junctions in the development of cancer-treatment related toxicities has been explored in more detail by Wardill and colleagues (2014; 2015; 2016; 2016). The intestinal microvasculature is known to be involved in RIGT. Clinically, telangiectasis, or the formation of pathological, vasodilated microvessels, has been documented in both acute and chronic RIGT (Kruse et al. 2004; Yeoh et al. 2005). Pre-clinically, endothelial apoptosis, as well as a shift of the intestinal microvasculature to a pro-coagulant and pro-thrombotic phenotype, have been shown to occur following irradiation (Paris et al. 2001; Wang, J et al. 2002). Despite these changes, the pathobiology of microvascular changes during RIGT, particularly the relationship between the intestinal microvasculature and MMPs, require further exploration to identify targets for novel treatments. Previous literature surrounding the relationship of these aspects of RIGT will be explored in chapter 2, forming the basis for the aims of the research presented in this thesis.

1.7 Aims of Thesis

Whilst the pathobiology of acute and chronic RIGT continues to be explored, there remains a lack of targets for the development of novel treatments. In addition, the relationship between some of the newer aspects of RIGT development, the intestinal microvasculature, MMPs, and vascular mediators, need to be explored further so that their potential in RIGT treatment can be evaluated. Thus, to further elucidate the role of these components in RIGT, the aims and hypotheses of this thesis are as below;

1. I hypothesise that microvascular alteration occurs during both during both acute and chronic phases in a rat model of RIGT, and that these changes are associated with increases in the vascular mediators VEGF, TGFβ, von willebrand factor (VWF), angiostatin, and endostatin. I aim to assess the microvascular response to irradiation, and the alteration of vascular mediators, VEGF, TGFβ, VWF, angiostatin, and endostatin, in an *in vivo* model of fractionated radiation-induced RIGT.
2. An increase in MMPs -2, -9, and -14, and a decreased in MMP-1, is hypothesised to occur alongside microvascular alterations and intestinal histopathological changes. Thus, I aim to further elucidate the roles of MMPs, particularly MMP-1, -2, -9, and -14, in the same model of fractionated radiation-induced RIGT.
3. MMP expression is hypothesised to increase following irradiation of endothelial, and the attenuation of these MMPs via specific inhibition is hypothesised to alter endothelial viability and regulate VEGF and TGFβ expression. Thus, I aim to assess the effect of MMP inhibition using the selective MMP-2 and -9 inhibitor, SB-3CT, on tumour-associated microvascular endothelial cells (TAMECs) derived from

spontaneous Dark Agouti rat mammary adenocarcinoma, with and without irradiation *in vitro*.

Statement of Authorship

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Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Chapter 2 Radiotherapy-induced gut toxicity: Involvement of matrix metalloproteinases and the intestinal microvasculature

[Stansborough et al., (2016) *International Journal of Radiation Biology.* 92(5): 241-248]

Referencing has been altered from original publication for consistency between chapters.

2.1 Abstract

Purpose: To review the literature surrounding the involvement of the endothelium and matrix metalloproteinases (MMP) in radiotherapy-induced gut toxicity (RIGT) and further elucidate its complex pathobiology.

Results: RIGT involves damage to the gastrointestinal mucosa and is associated with diarrhoea, pain, and rectal bleeding depending on the area of exposure. The mechanisms underpinning RIGT are complex and have not yet been elucidated. Members of the MMP family, particularly MMP -2 and -9, have recently been identified as being key markers in RIGT and chemotherapy-induced gut toxicity (CIGT). Furthermore, the microvasculature has long been implicated in the development of toxicities following both chemotherapy and radiotherapy, however, the mechanisms behind this are yet to be explored.

Conclusions: It is proposed that matrix metalloproteinases are key regulators of endothelial mediators, and may play a key role in inducing damage to intestinal microvasculature following radiotherapy.

2.2 Introduction

Radiotherapy-induced gut toxicity (RIGT) is a dose-limiting pathology involving damage to the gastrointestinal mucosa caused by radiotherapy for cancer. Its symptoms including, but not limited to pain, diarrhoea, nausea, and rectal bleeding, are debilitating and significantly reduce the patients' quality of life (Sonis, Stephen T. & Keefe 2013). The overall incidence of RIGT is not well understood, due to discrepancies in the definition of gastrointestinal toxicity following radiotherapy, and the classification of symptoms. Acute diarrhea and abdominal pain occur in 20-70% of patients receiving radiotherapy for abdominal or pelvic malignancies, and acute RIGT affecting quality of life has been shown to occur in 47% of women receiving radiotherapy for cervical or endometrial cancer (Classen et al. 1998; Abayomi, Kirwan & Hackett 2009; Shadad et al. 2013b).

2.3 Gastrointestinal response to radiotherapy

Gastrointestinal toxicities from radiotherapy are classified as being either acute (occurring hours after treatment) or delayed (months after treatment) (Al-Dasooqi et al. 2013). Acute RIGT occurs shortly after radiation and can continue for up to 3 months following treatment (Theis et al. 2010). Symptoms of acute toxicity include, but are not limited to, mucositis, nausea and vomiting, pain, and diarrhoea (Al-Dasooqi et al. 2013; Elting et al. 2013; Sonis, Stephen T. & Keefe 2013). Further, acute RIGT is characterised by a plethora of histopathological alterations including mucosal atrophy, inflammation, and increased intestinal permeability (Theis et al. 2010). Several mediators have been identified as being key regulators in the development of acute RIGT, particularly the transcription factor nuclear factor-κB (NF-κB); which acts to up-regulate over 200 genes involved in this pathology (Bowen et al. 2007). Key downstream mediators of NF-κB include pro-inflammatory

cytokines, mitogen-activated protein kinase (MAPK), and matrix metalloproteinases (MMP) (Sonis, S. T. 2004; Logan, Gibson, et al. 2008; Logan, Stringer, et al. 2008; Al-Dasooqi et al. 2010). These mediators act together to cause significant levels of apoptosis, decrease proliferation, and degrade the extracellular matrix (Sonis, S. T. 2004).

Delayed toxicity, also known as chronic radiation enteropathy, occurs between 18 months and 6 years following irradiation and is reported in up to 20% of patients receiving pelvic radiotherapy (Theis et al. 2010). Commonly reported symptoms include changes in stool frequency and rectal bleeding (in ~70% of cases); however, lesser reported complications may also occur, including small intestinal obstruction and fistulation (occurring in 0.6 and 13% of cases, respectively) (Henson 2010; Theis et al. 2010). Histopathologically, delayed RIGT is characterised by tissue ischaemia and necrosis, ulceration, telangiectasia, fibrosis of both the submucosa and the microvasculature, and mesenchymal cell activation (Richter et al. 1998; Theis et al. 2010; Al-Dasooqi et al. 2013). It has been shown that several mediators up-regulated in the delayed phase of RIGT, such as transforming growth factor-β1 (TGF-β1) and thrombomodulin, interact with the extracellular matrix (ECM) to cause deposition of ECM components in the intestinal smooth muscle wall and perhaps induce long-term injury to the epithelium and endothelium of the gastrointestinal tract (Andreyev 2007; Gervaz, Morel & Vozenin-Brotons 2009; Wang, FZ et al. 2012; Al-Dasooqi et al. 2013). Remodelling of the ECM has many downstream effects, including the release of sequestered growth factors resulting in alterations to the microvasculature, possibly linking back to related symptoms such as rectal bleeding and telangiectasis (Imai et al. 1997; Bergers et al. 2000). These studies suggest a complex mediator pathway associated with delayed toxicity following radiation.

Both acute and delayed toxicities result in a significant reduction of quality of life of patients receiving radiotherapy for cancer (Bentzen 2006; Lalla, Saunders & Peterson 2014). Despite the clinical significance of RIGT, its pathogenesis remains poorly understood. This lack of understanding of the mechanisms by which RIGT occurs hinders the development of effective treatments. Previous studies have suggested a complex pathway of mediator release, inflammation, and apoptosis, of not only the epithelial mucosa, but the underlying submucosa, including microvascular endothelium (Paris et al. 2001; Kruse et al. 2004).

2.4 Matrix metalloproteinases (MMP)

Matrix metalloproteinases, a class of zinc-dependent endopeptidases, have been previously shown to be altered in RIGT and chemotherapy-induced gut toxicity (CIGT) (Strup-Perrot et al. 2006; Al-Dasooqi et al. 2010). MMP subtypes are classified either functionally or structurally, with 24 members of the MMP family currently identified in humans, all of which share a catalytic domain coordinated by zinc (Yan & Boyd 2007). Their gene expression is predominantly regulated at the transcriptional level, with MMP members being regulated by transcription factors such as beta-catenin and NF-κB (Yan & Boyd 2007). However, post-transcriptional regulation of MMP does occur, with TGF-β, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and various pro-inflammatory cytokines being capable of activating MMP promoters, particularly in disease states (Yan & Boyd 2007). MMP family members vary structurally and functionally, with differences also occurring in the transcriptional and post-transcriptional regulation (Strongin et al. 1995). Whilst the general role of the MMP family is primarily in homeostasis of the extracellular matrix, specific MMP members may have different roles and have been identified as altering cell growth, immune responses, chemokine and cytokine expression, as well as apoptosis, a key histopathological feature of RIGT (Castaneda et al. 2005; Manicone & McGuire 2008). The recent implication of the MMP family in RIGT and CIGT suggests a complex and intricate involvement with associated mediators and tissue types in RIGT, as summarised in previous reviews (Sternlicht & Werb 2001; Al-Dasooqi et al. 2009).

2.5 Matrix metalloproteinases may be key mediators in RIGT

Studies by Strup-Perrot et al. (2005; 2006) identified matrix metalloproteinases as regulating RIGT. Male Wistar rats received a single dose of radiation (10 Gy) to the abdomen, and gene

expression of MMP -2, -3, -9, and -14 in the colon was assessed. MMP -2 was significantly increased at 1, 3, and 7 days following irradiation, MMP -3 was significantly increased at 3 days, MMP -9 at 3 and 9 days, and MMP -14 at 1 and 3 days (Strup-Perrot et al. 2006). Strup-Perrot et al. (2004) also assessed intestinal gene expression of MMP -2, -9, and -14, as well as tissue inhibitor of matrix metalloproteinase (TIMP) -1, and -2, in patients undergoing surgery for intestinal occlusion caused by delayed radiation-induced enteritis. MMP -2, -9, and -14, and TIMP -1, and -2 mRNA expression were significantly increased compared to healthy controls (Strup-Perrot et al. 2004). Conflicting results have been found in later studies. Angenete et al. (2009) studied levels of various matrix metalloproteinases in normal and tumour tissue from the rectum of rectal cancer patients scheduled for open surgery with or without pre-operative radiation. The study recruited 32 patients, 20 of which received short-term pre-operative fractionated radiotherapy (5 x 5 Gy) with a median time of tissue collection following completion of radiotherapy of 3 days. The remaining 12 patients received no pre-operative radiation. Patients receiving chemoradiotherapy were excluded and groups were matched for age and gender. Whilst MMP -2 levels were significantly increased in the rectal mucosa following radiotherapy, no significant changes in MMP -1, MMP -9, or TIMP -1 levels were observed following radiotherapy in these patients (Angenete et al. 2009). These discrepancies may be due to the many differences between the studies, including species difference in preclinical vs clinical studies, differences between acute and delayed RIGT, and single-dose vs fractionated irradiation. This is particularly relevant in the context of acute vs delayed RIGT, in which many differences can be observed at the clinical, histopathological, and molecular levels (Theis et al. 2010). It is likely that MMP expression would differ depending on time from the last radiotherapy course. Differences in normal tissue response in single vs fractionated dose radiotherapy have also been widely observed, with the short breaks

between doses in fractionated irradiation thought to lead to an accumulative affect, although not always linear (Yeoh et al. 2007; Hauer-Jensen, Denham & Andreyev 2014).

Recent studies characterizing MMP expression in the development of CIGT have suggested particular roles for different MMP members depending on their patterns of expression, and their coincidence with stages of CIGT development (Al-Dasooqi et al. 2010). MMP -2, -9, and -12 levels have been shown to peak 48 hours following chemotherapy, coinciding with maximal mucosal damage in the jejunum of Dark Agouti rats (Al-Dasooqi et al. 2010). This implicates these specific MMP subtypes in the progression of inflammation in CIGT. In contrast, MMP -1 has been found to be down-regulated early in the development of CIGT, however, is later up-regulated at 96 and 144 hours following chemotherapy administration, suggesting it may play a role in the healing stages of the pathology (Al-Dasooqi et al. 2010). This is supported by the ability of MMP -1 to cleave type-1 collagen to regulate cell migration in re-epithelialization (Pilcher et al. 1997; Al-Dasooqi et al. 2010). Alterations in MMP -1 expression may therefore be involved in the development of delayed RIGT, in which collagen deposition and reactive fibrosis commonly occur, also this has not yet been explored (Hauer-Jensen, Denham & Andreyev 2014). Such studies are yet to be conducted in RIGT models, particularly in regards to the differences between single dose *in vivo* RIGT studies and the fractionated radiation doses commonly used clinically.

MMP -2 and -9 in particular, have many downstream mediators linked to the pathobiology of RIGT. MMP -2 and -9 are capable of up-regulating pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), as well as forming a positive feedback loop with the key transcriptional factor NF-κB, (Sengupta & MacDonald 2007). Certain MMP members, MMP -2, -7, and -9 in particular, have also been linked with the disruption of endothelial and

epithelial tight junctions (Al-Dasooqi, Wardill & Gibson 2014; Wardill et al. 2014). No studies to date have assessed changes in endothelial tight junction protein levels in the intestinal microvasculature following radiation. However, studies have assessed intestinal epithelial tight junctions and blood-brain barrier tight junctions following radiation (Fauquette et al. 2012; Shukla et al. 2016). A recent study by Garg et al. (2016) assessed changes in tight junction expression in the jejunum, ileum, and colon of non-human primates following total-body irradiation. Claudin -1 and -10 expression was significantly increased at day 4, and claudin -4 and -7 expression was repressed at days 4, 7, and 12. These changes were also shown to be segment-specific, with increased expression of claudin-1 occurring only in the colon, and repression of claudin-7 expression occurring only in the ileum (Garg et al. 2016). Fauquette et al. (2012) assessed permeability and changes to tight junction protein levels following irradiation of a bovine brain capillary endothelial cell (BBCEC) monolayer co-cultured with glial cells. Permeability to fluorescein isothiocyanate (FITC)-dextran (70kDa) was significantly increased 8 days following 25 Gy irradiation, and immunoflourescent staining of the tight junction protein zonula occludens-1 (ZO-1) was decreased 8 days following both 12 Gy and 25 Gy irradiation. Whilst endothelial cells of the blood-brain barrier are structurally distinct to those of the intestinal microvascular, alterations to endothelial tight junction integrity following irradiation may still lead to increased permeability in such vessels. Similarly, a significant loss of junctional distribution of tight junction proteins occludin, claudin-3, and ZO-1 was shown to occur in the colon epithelium 2 hours following a single dose of 4 Gy whole body irradiation in female C57BL6 mice (Shukla et al. 2016)**.** Further exploration of tight junction alterations in the intestinal endothelium following radiation is warranted, particularly with increasing evidence of the importance of the gut-brain axis in many pathologies, including CIGT (Tillisch 2014; Wardill et al. 2015).

Despite these implications of the MMP family in RIGT and CIGT, a causative role has not yet been confirmed in previous literature, and several aspects of MMP functionality in RIGT remain entirely unexplored.

2.6 Response of intestinal microvasculature to radiotherapy

The microvasculature, and specifically the endothelium, plays a large role in toxicity following radiotherapy. Despite this, the extent of the involvement of the intestinal microvasculature, and the mechanisms behind this beyond their direct clinical manifestation in RIGT are unknown. Kruse et al. (2004) assessed changes to the microvasculature of the rectum of female C3H/HenAF-nu^{+} mice following a single dose of 20 Gy irradiation. Extensive vascular changes in the rectum, particularly tortuous telangiectatic vessels, were observed from 10 weeks following irradiation, with increasing severity, and were not resolved at 30 weeks post-irradiation (Kruse et al. 2004). Telangiectatic vessels are defined as 'thin-walled, tortuous vascular channels that often occur in groups and are accompanied by an area of leukocytic infiltration' (Kruse et al. 2004; Yeoh et al. 2005). Whilst the involvement of these telangiectatic vessels in the development of RIGT is unclear, gastrointestinal bleeding is one of the primary symptoms in patients with hereditary haemorrhagic telangiectasia presenting with intestinal telangiectatic lesions (Kjeldsen & Kjeldsen 2000). Mucosal telangiectasia resulting in rectal bleeding is one of the primary dose-limiting factors for irradiation of prostate cancer (Kruse et al. 2004). Further understanding of how intestinal telangiectasis occurs in response to radiation may lead to potential targets for the treatment or prevention of rectal bleeding in RIGT.

Although few preclinical studies have assessed changes to the intestinal microvasculature following irradiation, these changes have been observed clinically in several studies following

radiotherapy for cancer (Yeoh et al. 2005; Takeuchi et al. 2012). Takeuchi et al. (2012) assessed histopathological features of radiation toxicity of the lower rectum. The study included patients with a history of radiation therapy for an intrapelvic malignancy, characteristic colonoscopic findings of radiation proctitis, and histopathological exclusion of other diseases. Six male patients with radiation proctitis received 70 Gy external irradiation for prostate cancer, and 2 female patients received 50 Gy external irradiation and 20 or 27 Gy internal irradiation for uterine and vaginal cancers, respectively. Endoscopies and rectal biopsies were performed 15.4 ± 10.2 months from the completion of irradiation. Telangiectasia was observed in the superficial layer of the rectal mucosa upon endoscopy, and was confirmed in the upper layer of the lamina propria upon histological examination of rectal biopsies (Takeuchi et al. 2012). These findings are consistent with those of Yeoh et al. (2005) who demonstrated telangiectatic, fibrosed, and sclerosed blood vessels in the lamina propria, submucosa and fat layer of the gastrointestinal tract following short-term fractionated pelvic irradiation (25 Gy total/5 fractions/1 week), or long-term neoadjuvant chemoradiotherapy (45 Gy total/25 fractions/5 weeks, with concurrent infusions of 5-fluorouracil), in patients with colorectal carcinoma. Although these studies have highlighted a key role of the intestinal microvasculature during RIGT they have not described the effect these changes have on the underlying pathobiology. The formation of telangiectatic vessels in the rectal mucosa following radiation has been associated with increased expression of jagged 1 and Kruppel-like factor 5 (KLF5), both of which are involved in vascular remodeling and have been linked to the development of interstitial fibrosis (Kruse et al. 2004). KLF5 in particular is capable of upregulating growth factors such as PDGF and TGF-β (Kruse et al. 2004). The upregulation of these mediators would suggest a greater involvement of telangiectatic vessels in RIGT development than currently understood.

Endothelial apoptosis has also been suggested to be an initiating feature in the pathology of RIGT (Paris et al. 2001). Several *in vivo* and *in vitro* studies have found that endothelial apoptosis occurs hours following various radiation doses in several different tissue types, including the intestinal, lung, and central nervous system (CNS) microvasculature (Pena, Fuks & Kolesnick 2000; Paris et al. 2001; Zhang, Y et al. 2012; Li, G et al. 2014). Li, G et al. (2014) assessed apoptosis in the spinal cord of female Fisher 344 rats following a single dose of 50 Gy radiation to the spinal cord. Apoptosis was analysed via histology and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and 3.2 ± 10% of apoptotic cells in the spinal cord 10 hours following irradiation were identified as endothelial (cells positively stained for factor-VIII-related antigen). A similar study conducted by Pena, Fuks and Kolesnick (2000) found 16-20% of endothelial cells in the spinal cord of male mice underwent apoptosis at 12 hours following exposure to 50 Gy of irradiation. Zhang, Y et al. (2012) observed positive apoptotic staining co-localised to the lung endothelium of C57BL/6J mice following a single dose of 15 Gy irradiation to the thorax. It was reported in the study that 11% of apoptotic cells in the irradiated lung were endothelial (Zhang, Y et al. 2012). Changes to endothelium following irradiation cause significant long-term effects, with irradiated endothelium lacking the ability to regulate thrombogenic, inflammatory and coagulation processes months to years following irradiation (Boerma et al. 2004; Otterson et al. 2012). Many of these effects of radiation on the endothelium are related to changes in angiogenic signalling.

Angiogenic changes have been described *in vitro*, with the angiogenesis pathway being over-expressed in primary human endothelial cells from 12 hours to 21 days following a single irradiation dose of 2Gy (Heinonen et al. 2015). The angiogenic mediator TGF-β1 has been found to be up-regulated preclinically in both early and delayed RIGT and is an upstream

mediator of thrombomodulin, capable of reducing thrombomodulin immunoreactivity in the endothelium. Thrombomodulin is a transmembrane glycoprotein present on endothelial cells which is capable of forming a complex with thrombin, preventing the formation of fibrin and the activation of protease activated receptor-1 (PAR-1). This leads to the activation of protein C, an anticoagulant and anti-inflammatory protein (Wang, J et al. 2002). When thrombomodulin reactivity is reduced following irradiation PAR-1 is up-regulated, decreasing thromboresistance and inducing endothelial dysfunction (Wang, J et al. 2002; Wang, FZ et al. 2012; Boerma et al. 2013). TGF-β1 has also been shown to enhance radiation-induced fibrotic thickening of the intestinal wall and subserosa in a rat model of early and delayed RIGT following fractionated irradiation (Boerma et al. 2013). Although these microvascular changes have been described in several studies, the causal relationship of these changes to RIGT pathobiology are yet to be explored, as are the opportunities for novel therapies that may arise from this.

2.7 Matrix metalloproteinases and the intestinal microvasculature following radiotherapy

The MMP family has been strongly linked to endothelial regulation and angiogenesis, and could play a direct role in pathological angiogenesis associated with RIGT (Bergers et al. 2000; Al-Dasooqi et al. 2010). Much of what is known regarding the involvement of MMP in the regulation of the endothelium, particularly MMP -2 and -9, is from their involvement in tumour invasion and tumour angiogenesis. MMP -9 has been shown to be integral in the progression of some cancer types based on its ability to regulate angiogenesis (Bergers et al. 2000; Bhoopathi et al. 2010). MMP members degrade the basement membrane allowing for endothelial proliferation and migration, and remodeling of the extracellular matrix, inducing

tube formation (Chidlow et al. 2007; Al-Dasooqi et al. 2011). Members of the MMP family also indirectly induce pathological angiogenesis via regulation of angiogenic mediators such as vascular endothelial-cadherin (VE-cadherin), TGF-β, chemokines, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), angiostatin, and endostatin (Ichikawa et al. 2006; Xu, Yu & Duh 2006; Bendrik et al. 2008; Manicone & McGuire 2008; Tolstanova et al. 2011). These mediators contribute to many facets of pathological angiogenesis including increased vascular permeability, vasodilation, endothelial proliferation, and inhibition of vessel maturation and pericyte stabilization (Kalluri 2003; Bendrik et al. 2008; Tolstanova et al. 2011). Due to this cumulative evidence of a role for the MMP family in endothelial regulation, MMP inhibitors have been explored for their efficacy in the reduction of tumour burden and metastasis. Whilst broad-acting MMP inhibitors such as marimastat significantly inhibited tumour angiogenesis and reduced tumour burden preclinically, phase II and III clinical trials have been unsuccessful (Zucker, Cao & Chen 2000; Zucker & Cao 2009). This may be due to the vastly different, and even opposing affects, of various MMP types, as well as the exclusion of patients with early stage cancer (Zucker & Cao 2009). Specific inhibition of MMP members known to be involved in tumour angiogenesis, such as MMP -2 and -9, may be more efficacious in ongoing research, and provide insights in to how matrix metalloproteinases may be regulating these endothelial changes.

Few studies have been conducted to assess the role of matrix metalloproteinases in changes to the intestinal microvasculature in RIGT, and most have been performed *in vitro*. Park et al. (2012) found MMP -2 and vascular endothelial growth factor receptor (VEGFR)-2 to be significantly increased 24 hours following a single radiation dose of 4 Gy in human brain microvascular endothelial cells (HBMECs) and human dermal microvascular endothelial cells

(HDMECs). In a similar *in vitro* model, capillary endothelial cells were irradiated with a single dose of 10 Gy and displayed significantly increased apoptosis, as well as an increase in active MMP -1 and -2 (Vorotnikova, Tries & Braunhut 2004). MMP -2 expression was also increased at 24 hours, as was MMP -2 enzyme activity at 24 and 48 hours, following a single dose of 4 Gy irradiation in endothelial cells cultured from normal human breast tissue (Oh et al. 2014). In contrast, MMP -9 expression decreased in these cells at 24 and 48 hours following irradiation (Oh et al. 2014). Whilst these studies suggest MMP, particularly the gelatinase MMP -2, are directly involved in the endothelial response to radiation, further *in vivo* studies are warranted.

2.8 MMP signalling regulates endothelial mediators of RIGT

2.8.1 MMP and Vascular Endothelial Growth Factor (VEGF)

VEGF plays a variety of roles in the regulation of microvasculature. It is capable of increasing vascular permeability, causing transient vasodilation, and promoting endothelial proliferation, migration, and differentiation, all of which stimulate angiogenesis (Ferrara, Gerber & LeCouter 2003; Chidlow et al. 2007). The primary ability of the MMP family, particularly MMP -2 and -9, to increase VEGF, lies in their ability to cleave collagen IV to release sequestered VEGF (Bergers et al. 2000; Kalluri 2003). Once VEGF has been released it is able to bind to VEGF receptors on endothelial cells and induce angiogenesis via a tyrosine kinase pathway (Lee et al. 2015). MMP -2, and 9 have also been suggested to change the structure of VEGF, cleaving larger VEGF isoforms and increasing bioavailability, however, Hawinkels et al. (2008) found MMP -9 to have no effect in cleaving $VEGF_{165}$ to the smaller, soluble isoform $VEGF_{121}$ in HT29 colon carcinoma and fibroblast spheroids (Belotti et al. 2003; Kalluri 2003; Hawinkels et al. 2008). Hawinkels and colleagues (2008) showed a

decrease in extracellular localization of heparan sulphate proteoglycans (HSPG), suggesting a release of VEGF from the ECM via cleavage of HSPG by MMP -9. Bergers et al. (2000) cultured normal, non-transgenic pancreatic islets from C57/B16 mice with MMP -9. Cells were then washed and embedded in collagen gels containing endothelial cells. Whilst control islets, with no added MMP -9, failed to elicit an angiogenic response from the endothelial cells, islets treated with MMP -9 promoted the endothelial cells to form sprouts towards the islet. VEGF levels were 2-fold higher in the medium of MMP -9 treated islets, and MMP -9 treated islets showed no angiogenic response of endothelial cells when an anti-VEGF antibody was added. This suggests that the MMP -9 mediated endothelial response is regulated by VEGF (Bergers et al. 2000). A study by Bhoopathi and colleagues (2010) found VEGF expression following secreted protein acidic and rich in cysteine (SPARC) overexpression to be MMP -9 dependent in human medulloblastoma cell lines. These studies highlight a complex involvement of MMP, particularly MMP -9, with VEGF and it is likely that differences in the pathway occur based on tissue types and the tissue environment (pathological vs normal tissue).

2.8.2 MMP and Transforming Growth Factor-β

The effect of TGF-β on the microvasculature is complex, with TGF-β having several different effects on the endothelium, depending on the signalling pathway (Goumans et al. 2003). Ionizing radiation has been shown to alter the signalling pathways of TGF-β in endothelium, reducing the activation of the activin receptor-like kinase 1 (ALK1) pathway and enhancing the ALK5 pathway activation (Kruse et al. 2009; Scharpfenecker et al. 2009). This change in signalling pathways is thought to alter the way in which endothelial cells respond to TGF-β, with ALK5 inhibiting and ALK1 stimulating endothelial cell proliferation and migration

(Goumans et al. 2003). The degradation of the small collagen-associated proteoglycan, decorin, by several MMP members has been shown in several studies to sequester latent TGF-β, converting it to its active state (Imai et al. 1997). TGF-β1 and MMP -9 mRNA levels have been shown to be significantly increased at 3 and 7 days, as was TGF-β2 from day 1, following 10 Gy abdominal X-irradiation (Strup-Perrot et al. 2005). Despite this link between MMP -9 and TGF-β, associated changes to the microvasculature are yet to be explored.

2.8.3 MMP and anti-angiogenic mediators

Several MMP subtypes have been associated with the anti-angiogenic mediators, endostatin, tumstatin, and angiostatin (Kalluri 2003; Tolstanova et al. 2011). Endostatin and tumstatin are both anti-angiogenic mediators formed from the c-terminal portions of collagens XVIII and IV respectively (Sudhakar et al. 2003). Both collagen IV and XVII, found in vascular basement membranes, can be broken down by active forms of MMP -2, cleaving endostatin from collagen XVII, and increasing thrombospondin-1, which has been shown to be up-regulated early and late in an animal model of CIGT (Bowen et al. 2007). MMP -2, -7, -9, and -12 are also capable of cleaving plasminogen to form angiostatin (Sternlicht & Werb 2001). MMP -9, endostatin, VEGF, and platelet-derived growth factor receptor (PDGFR) were increased at different time points in an animal model of ulcerative colitis, with increased endostatin and VEGF being correlated with increased size of colonic lesions (Tolstanova et al. 2011). However, the effects of endostatin and angiostatin on endothelial cells are yet to be clarified, with transfection of the MMP -9 gene *in vivo* inducing endostatin release and significantly reducing tumour growth rate and microvessel area in mammary adenocarcinomas in mice (Bendrik et al. 2008). This could be due to biphasic effects of endostatin, and differential effects depending on cell type (Guan et al. 2003; Folkman 2006).

Despite this, COL-3 (Metastat; Collagenex Pharmaceuticals, Newtown, PA, USA)**,** a specific inhibitor of MMP -2 and MMP -9, did not increase tumour growth rate of advanced solid malignancies in clinical trials and three patients with disease progression prior to treatment had prolonged stable disease (>6 months) (Syed et al. 2004). Further studies are now warranted to characterise MMP -9 and associated downstream angiogenic mediators in RIGT.

2.8.4 MMP and endothelial permeability

MMP -2, -7, and -9 have also been shown to play extensive roles in endothelial permeability due to their ability to shed VE-cadherin and cleave occludin; two tight junction proteins with essential roles in maintaining tissue permeability. MMP -7 sheds epithelial-cadherin from adherens tight junctions to remodel cell-cell contacts and facilitate cell migration (McGuire, Li & Parks 2003). VEGF-A, a possible downstream mediator of several MMP subtypes, is also capable of increasing junction adhesion molecule -C expression, stimulating occludin and VE-cadherin phosphorylation and decreasing occludin and VE-cadherin at endothelial tight junctions (Antonetti et al. 1998). This link between matrix metalloproteinases, which have been shown to be up-regulated in RIGT, and the phosphorylation of endothelial tight junctions, highlights a potential new involvement for the MMP family in intestinal endothelial dysfunction and increased permeability in RIGT.

2.9 Take home messages

RIGT is one of the most prevalent dose-limiting toxicities manifesting following radiotherapy (Sonis, Stephen T. & Keefe 2013; Lalla, Saunders & Peterson 2014). It involves debilitating pain and increases the risk of infection in patients receiving radiotherapy. Due to many of the severe side effects of RIGT, it is often necessary to reduce the dosage, or halt anti-neoplastic therapies, leading to decreased remission and increased mortality (Lalla, Saunders & Peterson

2014). Despite the prevalence and burden of this pathology, there are currently no effective treatments for RIGT and its pathobiology remains to be clearly understood. Various MMP members have been shown to be altered in the intestine following irradiation, in the settings of both acute and delayed RIGT (Strup-Perrot et al. 2004; Strup-Perrot et al. 2006; Angenete et al. 2009). The gastrointestinal microvasculature has also been implicated in RIGT development, with endothelial apoptosis occurring early in the pathology, and pathological angiogenesis being observed in several models of RIGT (Paris et al. 2001; Kruse et al. 2004; Yeoh et al. 2005). Several MMP subtypes are capable of regulating many angiogenic and anti-angiogenic endothelial mediators, such as VEGF, TGF-β, endostatin, and angiostatin, highlighting a strong link for their role in RIGT-associated microvascular changes. Thus the present review proposes a vascular mediator pathway, regulated by altered MMP expression or activation following irradiation, as being involved in intestinal microvascular changes seen in both acute and delayed RIGT. Although further study is necessary, the MMP family presents a unique target in the development of novel therapies for RIGT. Due to the complex nature of members of the MMP family, and differential effects based on the type of MMP, tissue type, and pathology, extensive studies are required. As RIGT is a prevalent and debilitating adverse effect of anti-neoplastic therapies, any future studies focusing on the biological basis of this pathology will aid in the development of anti-mucotoxic therapies of much clinical value.

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Overall percentage (%)	75%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Chapter 3 Fractionated abdominal irradiation induces intestinal microvascular changes in an *in vivo* model of radiotherapy-induced gut toxicity

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Referencing has been altered from original publication for consistency between chapters.

3.1 Abstract

Purpose: Radiotherapy-induced gut toxicity (RIGT) is associated with diarrhoea, pain and rectal bleeding and can occur as an acute or chronic toxicity. The microvasculature has been shown to be altered in the development of RIGT; however, the features are not yet characterized. We hypothesized that apoptosis of microvascular cells would occur early in the gastrointestinal tract following fractionated irradiation, followed by late microvascular changes, including sclerosis and telangiectasis.

Methods: Female Dark Agouti rats were treated with a 6-week fractionated radiation schedule of 3 × 2.5 Gy doses per week localized to the abdomen. At 3, 6 and 15 weeks, the intestines were assessed for markers of acute and chronic injury including morphological changes, collagen deposition, apoptosis and proliferation.

Results: Apoptosis of microvascular cells significantly increased at 6 and 15 weeks in the jejunum ($p = 0.0026$ and $p = 0.0062$, respectively) and at 6 and 15 weeks in the colon ($p < 0.0001$ and $p = 0.0005$, respectively) in rats receiving fractionated radiation to the abdomen. Histopathological changes of the colon microvasculature were also seen from week 3, including thickening of the lamina propria and dilated, thickened, telangiectatic vessels.

Conclusions: Findings of this study provide evidence of regional and timing-specific changes in the intestinal microvasculature in response to fractionated radiotherapy which may play a role in development of both acute and chronic RIGT.

3.2 Introduction

Radiotherapy-induced gut toxicity (RIGT) is an adverse effect of radiotherapy for cancer, which may occur as an acute toxicity (also referred to as gastrointestinal mucositis or acute radiation enteropathy) or chronic toxicity (also chronic radiation enteropathy) (Hauer-Jensen, Denham & Andreyev 2014; Stansborough et al. 2016). In patients receiving radiotherapy for abdominal or pelvic tumours, approximately 60–80% develop symptoms of acute gut toxicity, including, but not limited to, nausea, diarrhoea, pain and fatigue (Hauer-Jensen, Denham & Andreyev 2014). Chronic gut toxicity may also occur following radiotherapy and includes symptoms such as altered intestinal transit, gut dysmotility and less commonly intestinal obstruction or fistulae formation (Hauer-Jensen, Denham & Andreyev 2014). Despite these symptoms, there remain few treatment options for RIGT, largely hindered by a lack of understanding of the underlying pathobiology (Hauer-Jensen, Denham & Andreyev 2014; Lalla et al. 2014). This is particularly true for the endothelium. Late rectal bleeding is one of the most common symptoms of RIGT following high-dose radiotherapy for prostate cancer, occurring in up to 70% patients (Akimoto et al. 2004; Kruse et al. 2004). This points to a likely impact of radiation on the normal intestinal microvasculature. Whilst intestinal epithelial cell apoptosis (Potten et al. 1994; Qiu et al. 2010), activation of transcription factors such as NF-κB (Yeoh et al. 2005) and a cascade of inflammatory mediators including pro-inflammatory cytokines and matrix metalloproteinases (Strup-Perrot et al. 2005; Strup-Perrot et al. 2006) are well known to be involved in RIGT, these pathways are yet to be characterized in the endothelium.

Paris and colleagues (2001) assessed changes to endothelial apoptosis in the jejunum of C57BL/6 mice who received 8–15 Gy whole body irradiation. Whilst this study showed that

endothelial apoptosis occurred early (4 h) following irradiation, the model was largely based on GI syndrome, in which doses are not clinically translatable in the context of radiotherapy for cancer (Paris et al. 2001). Additionally, later preclinical studies showed conflicting results, in which endothelial apoptosis did not to occur early during GI syndrome and did not contribute to the severity of toxicity (Schuller et al. 2007; Kirsch et al. 2010). Abderrahmani and colleagues (2012) found a significant increase in endothelial apoptotic cells in the villus lamina propria 4 and 5 h following 19 Gy intestinal irradiation in mice (Abderrahmani et al. 2012). This was significantly lower in plasminogen activator inhibitor-1 (PAI-1) knockout mice, which had significantly reduced acute and late radiation-induced intestinal injury when compared to wild-type mice (Abderrahmani et al., 2012). Whilst this again suggests a link between early endothelial apoptosis and intestinal injury, epithelial apoptosis was also significantly lower in PAI-1 knockout mice at the 5-h time point, making it difficult to suggest which may be contributing to the reduced toxicity (Abderrahmani et al. 2012).

Despite the lack of consensus of the literature on endothelial apoptosis in RIGT, changes to intestinal microvasculature have been identified, both clinically and pre-clinically (Kruse et al. 2004; Yeoh et al. 2005). Yeoh and colleagues (2005) assessed histological changes of archived colorectal tissue samples from 28 patients with colorectal carcinoma who had received either preoperative short term pelvic irradiation or long-term neoadjuvant chemoradiotherapy. Short-term radiation involved a total dose of 25 Gy using the 4 field box technique, over 5 fractions in 1 week. Long-term neoadjuvant chemoradiotherapy involved concurrent 5-FU (300 mg/m2/day) and pelvic irradiation with 45 Gy in 25 fractions over 5 weeks. Yeoh et al. (2005) demonstrated telangiectatic, fibrosed and sclerosed blood vessels in the lamina propria, submucosa and fat layer of the gastrointestinal tract following abdominal radiation. The study did not assess these changes in direct relation to the occurrence of RIGT

in the patients; however, 16 out of 28 of recruited patients did experience symptoms of gastrointestinal toxicity during treatment (Yeoh et al. 2005). In pre-clinical studies, tortuous telangiectatic vessels have been seen in the rectum of mice irradiated with a single dose of 20 Gy. These did not resolve at the completion of the study (30 weeks following irradiation); however, these changes were not investigated in relation to clinical parameters of gastrointestinal toxicity in these mice (Kruse et al. 2004).

Despite the observation of pathological blood vessels in RIGT, few studies have investigated specific vascular changes in an in vivo model of fractionated irradiation. Several studies, however, have assessed thrombogenic changes to the microvasculature in an attempt to characterize mediator pathways underpinning vascular-related symptoms such as rectal bleeding, including changes to levels of vascular mediators such as thrombomodulin, protease-activated receptor-1 (PAR-1) and von Willebrand factor (vWF) (Jahroudi, Ardekani & Greenberger 1996; Wang, J et al. 2002; Hauer-Jensen, Fink & Wang 2004). Wang and colleagues (2002) found a statistically significant decrease in the number of thrombomodulin-positive blood vessels in the intestines of Sprague-Dawley rats who had received fractionated radiation (33.6 Gy in 8 daily fractions or 67.2 Gy in 16 daily fractions) to a surgically induced scrotal hernia. Irradiated rats also displayed mucosal ulceration and inflammation during early RIGT (1 day and 2 weeks following cessation of radiation), vascular sclerosis and reactive fibrosis of the subserosa and submucosa in delayed RIGT (6 and 26 weeks following radiation).

Decreases in thrombomodulin are known to increase PAR-1 and decrease protein C activation, leading to upregulation of inflammatory mediators and endothelial dysfunction (Wang, J et al. 2002; Hauer-Jensen, Fink & Wang 2004). These changes in vascular mediator

upregulation following radiotherapy suggest a change in pathophysiological state of the intestinal microvasculature; however, further studies are needed to determine how this is affecting microvascular cell kinetics.

Thus, the present study aimed to determine changes to microvascular apoptosis and proliferation following fractionated abdominal radiation in an animal model of RIGT. The study also aimed to assess clinical and histopathological parameters of RIGT including changes to body weight and epithelial apoptosis to assess the timeline of these changes in the context of microvascular damage. In the present study, we demonstrate for the first time significantly increased intestinal microvascular apoptosis and significantly reduced microvascular proliferation following fractionated radiation in rats.

3.3 Materials and Methods

3.3.1 Ethics

This study was conducted in accordance with ethics approved by the Animal Ethics Committees at both The University of Adelaide (M-041-2006) and the Institute of Medical and Veterinary Science (IMVS) (78/05) and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Teaching (2007).

3.3.2 Animals and Experimental Design

Seventy female Dark Agouti (DA) rats, weighing 155 to 170 g on arrival, were housed under controlled conditions with 12 h dark and 12 h light cycles. Rats had ad libitum access to water and standard rat chow. The experimental design was based on our previously published pilot study (Yeoh et al. 2007). Briefly, rats (n = 5 per time point) were randomly assigned into three groups receiving radiation and three groups receiving sham irradiation. Prior to irradiation, rats in both treatment and control groups were anaesthetized with 4% isofluorane and placed into a custom-built container (Yeoh et al. 2007). Rats in the treatment groups were irradiated, limited to the abdomen by lead shielding, at a dose of 2.5 Grey (Gy)/fraction three times a week to a total of 45 Gy/18 fractions/6 weeks prescribed to depth of 3.3 cm using a Varian Clinac Linear Accelerator (Varian Medical Systems) with focus-skin distance of 130 cm. Control rats received sham irradiation in the same course as treated rats. Groups of rats were killed at the ends of weeks 1 to 6, representing acute toxicities from short- and long-course radiation, and at 15 weeks, representing a delayed, or chronic, toxicity. Animals were closely monitored in the immediate recovery period (30 min from radiation) and were then

monitored daily for weight change, diarrhoea, dull/ ruffled coat, change in temperament, reluctancy to move and skin reaction to radiation, throughout the experimental time course.

3.3.3 Gastrointestinal Tissue collection

Gastrointestinal tissue was collected as previously described by Yeoh and colleagues (2007). Following flushing of the small and large intestine with cold isotonic saline, sections of jejunum (at 25% length of small intestine from the pylorus) and colon (at 50% length of large intestine) were resected into 2-cm sections, fixed in 10% neutral formalin and embedded in paraffin for further analysis. In preparation for staining, sections were cut using a rotary microtome (Leica) to 4 μm and were mounted on uncoated slides for haemotoxylin & eosin (H&E) and Picro sirius red staining or FLEX immunohistochemistry (IHC) microscope slides (Dako) for IHC.

3.3.4 Histology

H&E staining was conducted to determine morphological changes to the jejunum and colon following irradiation as described previously (Yeoh et al. 2007), including villous blunting or fusion, crypt loss, inflammation, vessel dilation and oedema. Picro Sirius red staining was used to determine total tissue collagen levels as described previously (Al-Dasooqi et al. 2011). Briefly, sections were dewaxed in xylene and hydrated in a series of graded ethanol. Picro Sirius red solution (0.5 g Sirius red F3B in 500-ml saturated aqueous picric acid solution) was applied to sections and left at room temperature for 1 h. Sections were then washed using acid water (0.5% acetic acid in H2O), dehydrated, cleared and coverslipped. Images of H&E and Picro Sirius red-stained tissue were taken using NanoZoomer scanning equipment (Hamamatsu, Japan), and images were analysed using NanoZoomer Digital Pathology software (NDP.view2, Hamamatsu). H&E-stained tissue was assessed using a

previously validated damage scoring method in which sections were given a score of either 0 or 1 for the presence of the following factors: disruption of the brush border, crypt loss/architectural disruption, disruption of crypt cells, infiltration of polymorphonuclear cells and lymphocytes, dilation of lymphatics and capillaries, oedema and villous fusion and atrophy in the jejunum. Picro Sirius red-stained tissue was assessed qualitatively. All assessments were conducted in a blinded fashion by one assessor (RLS).

3.3.5 Immunohistochemistry (IHC)

Activated caspase 3 and Ki67 proteins were visualized by IHC staining in intestinal tissue from rats killed at the end of weeks 3, 6 and 15 to determine levels of apoptosis, proliferation and microvascular cell dysfunction, respectively. Caspase 3 and Ki67 primary antibodies were utilized as previously published (Marshman et al. 2001; Al-Dasooqi et al. 2011). A Dako-automated staining system (Autostainer link 48 and PT Link, Dako) was used to conduct IHC for caspase 3 (Abcam #ab44976, 0.200 mg/ml), Ki67 (Abcam #ab16667, concentration not provided) at dilutions of 1:1000 and 1:1200, respectively. IHC was conducted according to manufacturer's instructions using the EnVision™ FLEX kit for Dako autostainer link systems (Dako) and as previously described (Wardill et al. 2015). Briefly, 4-μm sections of jejunum and colon mounted on IHC microscope slides (Dako) were dewaxed in histolene and rehydrated through a series of graded ethanols. Antigen retrieval was conducted using a Tris/EDTA antigen retrieval buffer (0.37 g/l EDTA, 1.21 g/l Tris; pH 9.0) in the PT link antigen retrieval system (Dako) in which the buffer was preheated to 65 °C following which slides were added then heated to 97 °C for 20 min. Slides were placed in the autostainer, and sections were blocked for 5 min using the FLEX 0.3% hydrogen peroxide block (Dako). A protein block (0.25% casein in PBS, containing stabilizing protein and 0.015

mol/l sodium azide, Dako) was then applied for 30 min to reduce non-specific background staining of tissue, followed by application of the primary antibody for 60 min. Rabbit HRP-labelled polymer conjugated to goat anti-rabbit or goat anti-mouse (Dako), depending on the specifications of the antibody, was then applied for 30 min as a labelling and secondary antibody system. Sections were visualized using DAB (EnVision™ FLEX kit, Dako) and counterstained with Harris' haematoxylin for 20 s, dehydrated in 100% ethanol, cleared with histolene and coverslipped. Images were taken using NanoZoomer scanning equipment (Hamamatsu) and DAB staining of villi (jejunum only), crypts, and microvasculature was analysed on NanoZoomer Digital Pathology software (NDP.view2, Hamamatsu). Cell counts per crypt (average of 15 crypts per section) were taken for caspase 3 and Ki67 staining in the jejunum and colon mucosa as well as in the villi of the jejunum. Cell counts of caspase 3 and Ki67-immunostained cells were also performed on the microvasculature in the lamina propria and submucosa in which stained cells were counted in 15 microvessels per section and were then averaged to give stained cells per vessel. All sections were assessed in a blinded fashion by one assessor (RLS).

3.3.6 Statistical Analysis

Group means for histological damage scoring and apoptotic and proliferative cell counts were compared between sham irradiated and irradiated rats per time point. All statistical analysis was conducted using the program Graphpad Prism (version 6). Differences between study group means were analysed using a two-way ANOVA with Sidak's multiple comparison. Asterisks denote significance compared to control at the time point, where *<0.05, **<0.01, ***<0.001 and ****<0.0001. Bars on graphs represent standard error of the mean.

3.4 Results

3.4.1 Response of animals to fractionated radiation

Animals receiving fractionated irradiation to the abdomen three times a week displayed significantly lower weight gain (represented as a % from baseline) at weeks 3 ($p = 0.0055$) and from 6 weeks onwards ($p < 0.05$) when compared to animals receiving sham irradiation (Fig. 1). Whilst some rats were observed to have a dull/ruffled coat raised around the neck following irradiation, no significant differences were observed in any other clinical parameters including diarrhoea, change in temperament, reluctancy to move or skin reaction to radiation (data not shown). Small intestinal weight was significantly higher in irradiated rats at 15 weeks ($p < 0.0001$) (Table 1). There were no significant differences in large intestinal weight between irradiated and control rats (Table 1).

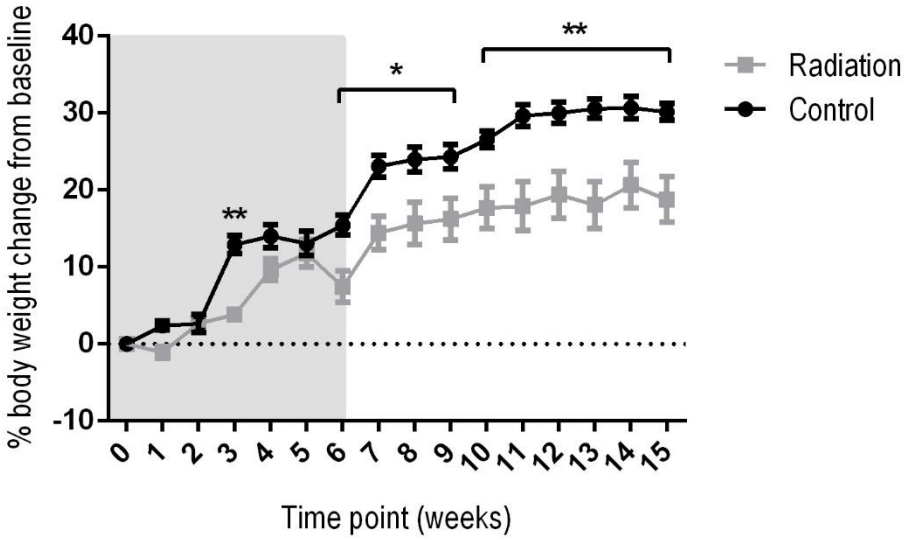


Figure 1. *Percentage body weight change from baseline (day 0) of DA rats following fractionated abdominal irradiation.* Percentage body weight gain was significantly lower in irradiated rats when compared to controls at week 3 ($p < 0.0001$) and every week from week 6 until study completion in week 15 (6–9 weeks, $p < 0.05$; 10–15 weeks, $p < 0.01$). Shaded area of graph represents course of fractionated or sham irradiation. Asterisk denotes significances compared to control where *$p < 0.05$, **$p < 0.01$

3.4.2 Fractionated radiation induced microvascular changes to the jejunum and colon

Jejunum

Damage scoring was not significantly altered in the jejunum at any time point (Suppl. 1). Despite this, thickening of the lamina propria and dilated, thickened, telangiectatic vessels were seen at week 6 in H&E-stained sections (Fig. 2). This thickening of the intestinal microvasculature was confirmed using Picro Sirius red in which fibrotic thickening of the microvasculature was also seen, extending from weeks 3 to 15 (Fig. 2). No significant difference was seen in the villus height or crypt depth (data not shown).

Colon

Damage scoring of the colon was significantly increased compared to controls at week 3 ($p = 0.043$) (Suppl. 1). Histopathological changes of the colon microvasculature were seen in rats receiving irradiation from week 3. Thickening of the lamina propria and dilated, thickened, telangiectatic vessels were seen from 3 weeks after the first radiation dose and had resolved by week 6 (Fig. 2). This thickening of the intestinal microvasculature was confirmed using Picro Sirius red staining and vasodilation, and fibrosis of the microvasculature was observed 6 weeks from the first radiation dose (Fig. 2). Crypt depth was significantly higher in the colon of rats receiving radiation when compared to control rats at 5 weeks ($p = 0.022$). No significant differences were noted at any other time point (data not shown).

Table. 1 Small intestinal weight and large intestinal weight of DA rats following fractionated irradiation to the abdomen.

Time Point	**Treatment Group**	**Small Intestinal Weight (g)**	**Large Intestinal Weight (g)**
Week 3	*Control*	4.96±0.70	1.10±0.12
	Radiation	4.64±0.29	1.04±0.06
Week 6	*Control*	5.00±0.25	1.24±0.12
	Radiation	5.76±0.33	1.42±0.10
Week 15	*Control*	4.91±0.36	1.31±0.08
	Radiation	6.43±0.94********	1.51±0.11

Small intestinal weight was significantly higher in irradiated rats at week 15 ($p < 0.0001$) when compared to time matched controls. Large intestinal weight did not significantly differ between groups at any time point. Data presented as mean ± standard deviation. * denotes significance compared to time matched control where ****$p < 0.0001$

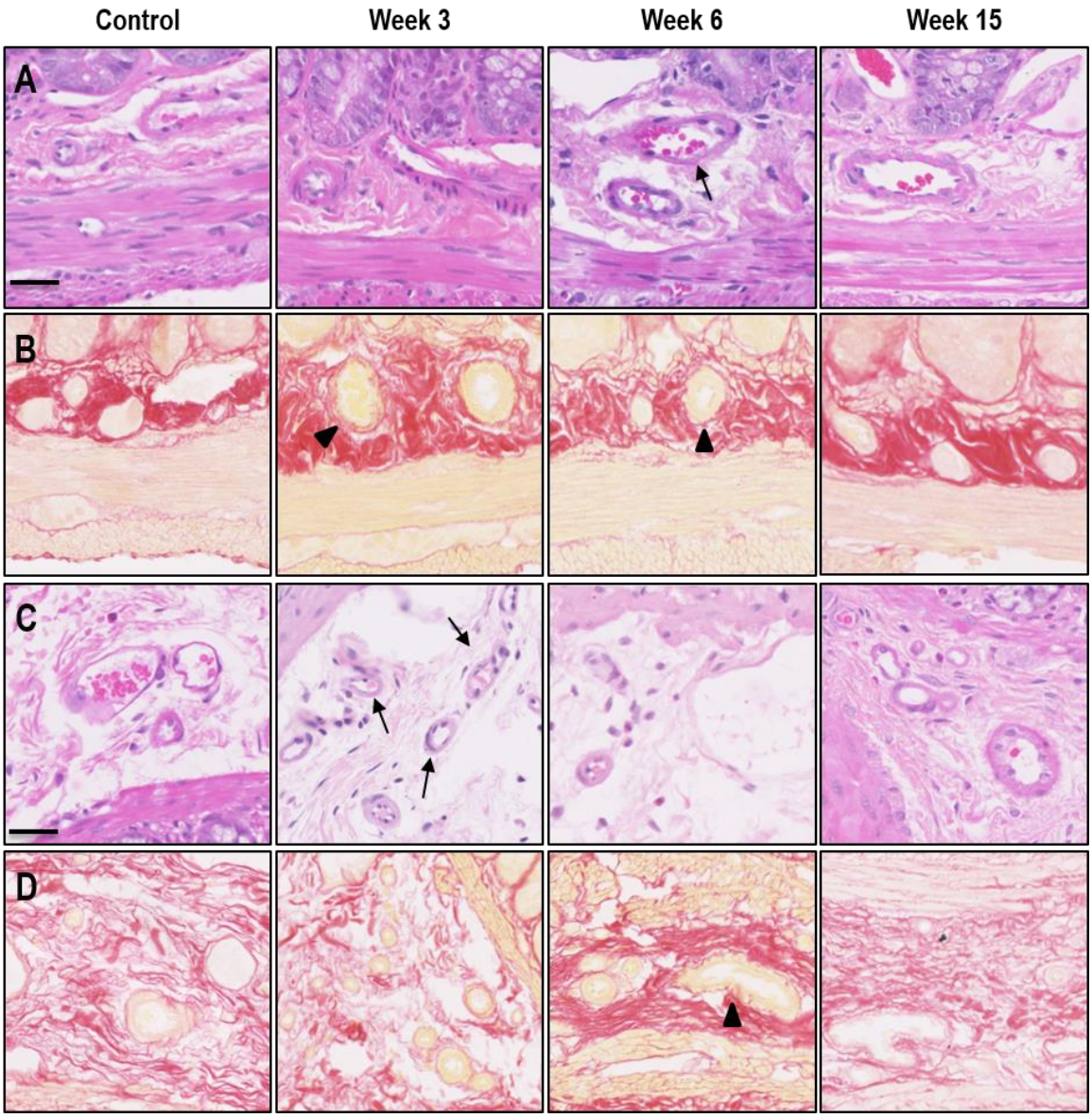


Figure 2 *Morphological changes to jejunum and colon microvasculature following fractionated irradiation*. **A** H&E-stained jejunal microvasculature with dilated, telangiectatic vessels indicated by arrows. **B** Picro Sirius red-stained jejunal microvasculature in which fibrotic thickening of vessels can be seen from week 3 following irradiation (indicated by arrowheads). **C** H&E-stained colonic microvasculature displaying formation of telangiectatic vessels (indicated by arrows) following fractionated irradiation. **D** Picro Sirius red-stained colonic microvasculature with a thickened vessel indicated by arrowhead. ×40 magnification. Scale bar = 30 μm

3.4.3 Fractionated radiation induced microvascular cell apoptosis and reduced microvascular proliferation in the jejunum and colon

Jejunum

Apoptosis of microvascular cells significantly increased at 6 and 15 weeks in the jejunum ($p = 0.0026$ and $p = 0.0062$, respectively) in rats receiving fractionated radiation to the abdomen (Fig. 3). Cell proliferation, measured by Ki67 immunostaining, was not significantly altered at any time point in the jejunal microvasculature (Fig. 4).

Colon

Colonic microvascular cell apoptosis was significantly increased at 6 weeks ($p < 0.0001$) and 15 weeks ($p = 0.0005$) in rats receiving fractionated radiation when compared to control (Fig. 3). No significant changes in microvascular apoptosis were seen between groups at 3 weeks. Microvascular cell proliferation was significantly decreased 3 weeks from first dose in the colon ($p = 0.0072$) and was not significantly altered at week 6 (Fig. 4). In contrast, proliferation significantly increased at 15 weeks in the colon microvasculature ($p = 0.0041$).

3.4.4 Fractionated radiation induced apoptosis in the epithelial cells of the crypts of the jejunum and colon

Jejunum

Apoptosis, as determined by caspase 3 immunostaining, was significantly higher in the epithelial cells of the jejunum crypts of irradiated rats at 3 and 6 weeks ($p = 0022$ and $p < 0.0001$, respectively) (Fig. 5). Jejunal crypt apoptosis was not significantly altered at the 15-week time point. There were also no significant changes in apoptosis in the villi of the jejunum at any time point assessed.

Colon

Apoptosis was also significantly increased in the epithelial cells lining the colonic crypts at 6 weeks when compared to control ($p = 0.014$) (Fig. 5). Again, no significant changes were seen at any other time point.

3.4.5 Fractionated radiation induced proliferation in the epithelial cells of the jejunum and colon

Jejunum

There were no significant changes seen to proliferation indices in the jejunal crypts or villi at any time point after irradiation (data not shown).

Colon

Proliferative cells in the colon crypts, as determined by Ki67 immunostaining, were significantly higher in the radiation group at 6 weeks when compared to control ($p = 0.026$) (Supp. 2). No further significant changes were seen in the proliferative indices at any other time point.

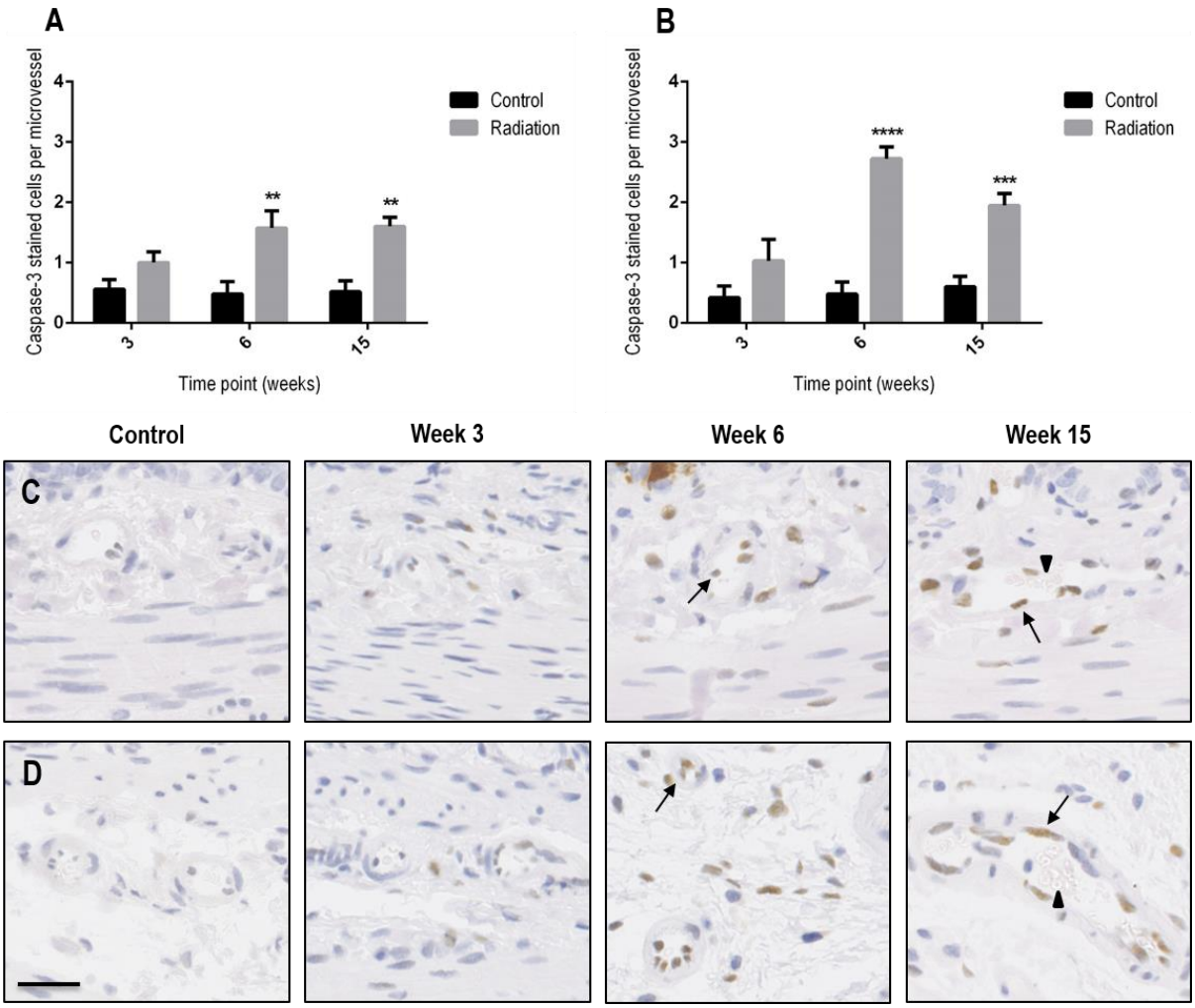


Figure 3. *Caspase 3 immunostaining of the jejunum and colon microvasculature*. **A** Caspase 3 significantly increased in the micro vessel of the jejunum at 6 and 15 weeks. **B** Caspase 3 staining increased in the colon microvasculature at 6 and 15 weeks. **C** Caspase 3 staining of the jejunal microvasculature. Arrow indicates apoptotic microvascular cells, with arrowhead indicating unstained red blood cells. **D** Caspase 3 staining of the colonic microvasculature with arrows indicating apoptotic microvascular cells at 6 and 15 weeks. Scale bar = 30 μm, ×40 magnification. * Significance compared to control where **p < 0.01, ***p < 0.001, ****p < 0.0001

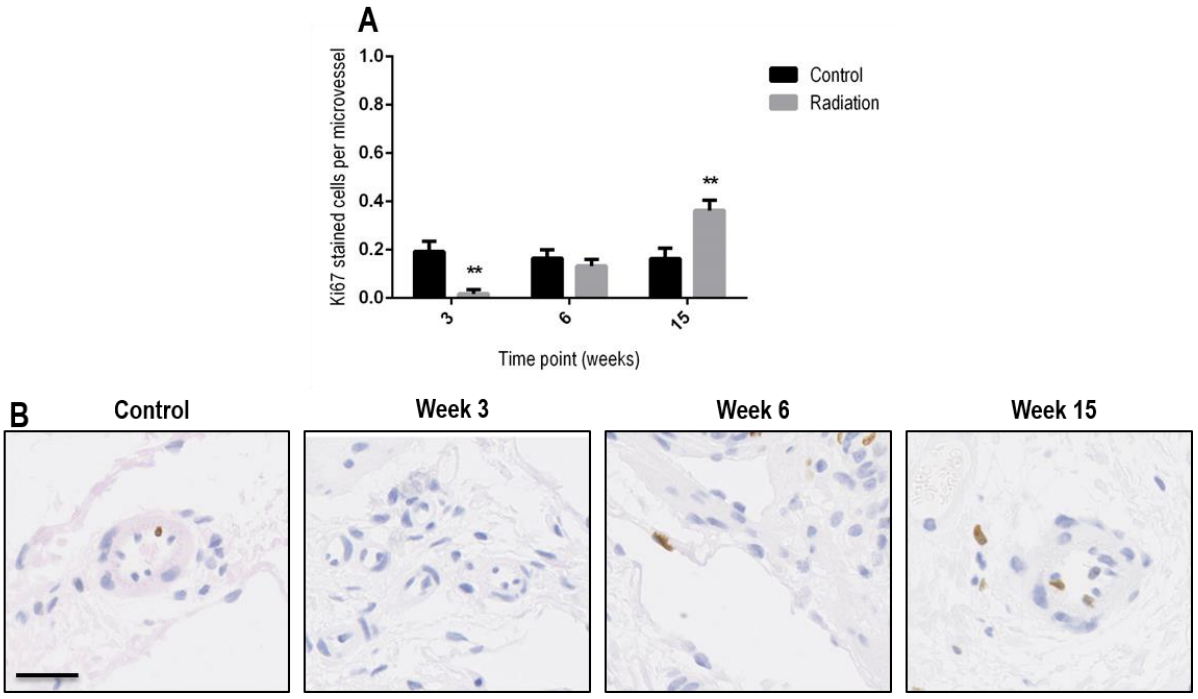


Figure 4. *Ki67 immunostaining of the colon microvasculature*. **A** Ki67-stained microvascular cells significantly decreased in the colon at week 3 and significantly increased at week 15. **B** Ki67 staining of the colon microvasculature. Scale bar = 30 μm, ×40 magnification. * Significance compared to control where **p < 0.01

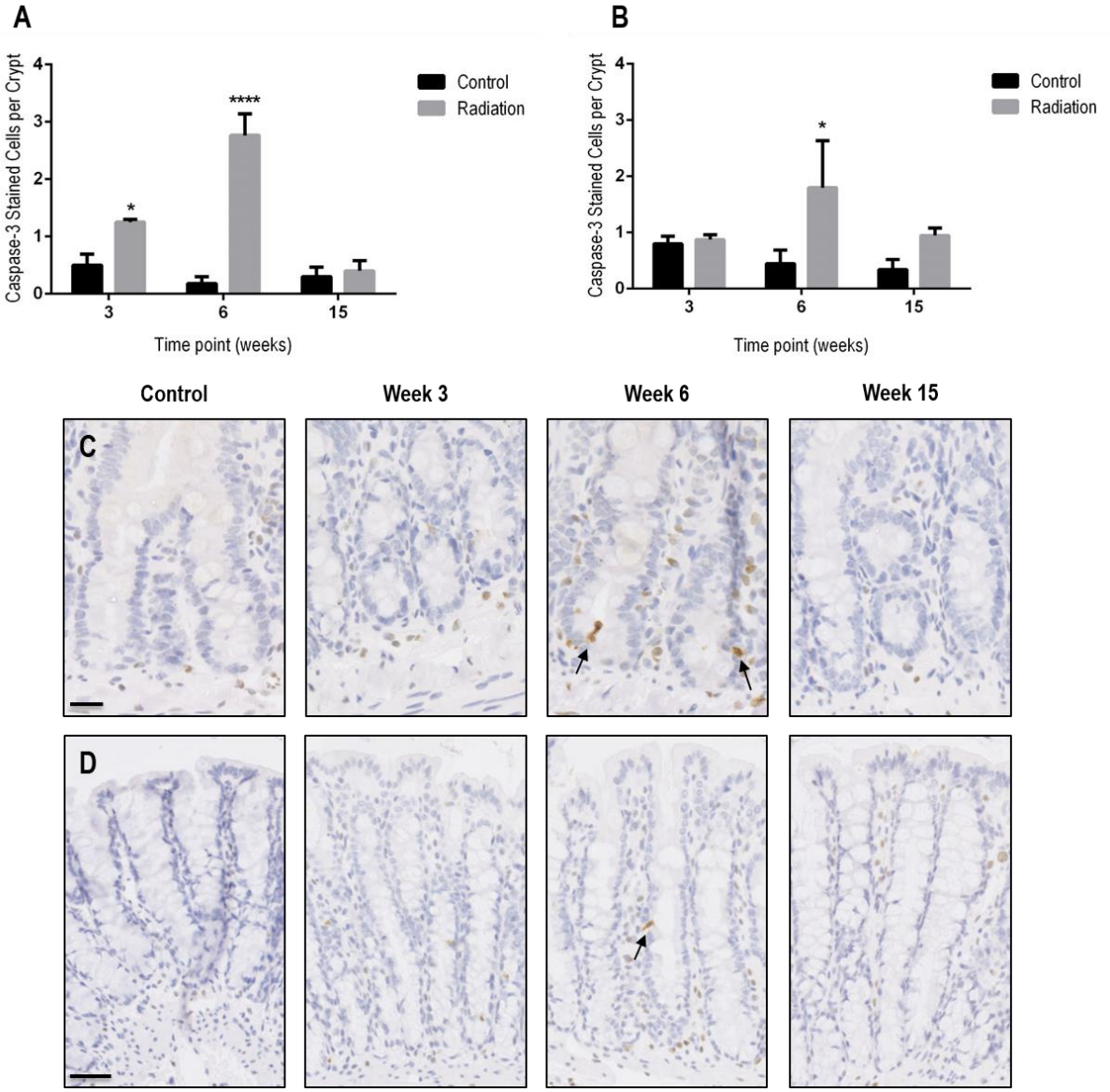


Figure 5. *Apoptosis of jejunal and colonic crypts following fractionated irradiation*. **A** Caspase 3-immunostained cells significantly increased in the jejunal crypts at 3 ($p = 0.0029$) and 6 weeks ($p < 0.0001$) when compared to control. **B** Caspase 3-immunostained cells were significantly increased in the colon crypts at 6 weeks in the irradiated group ($p = 0.0145$). **C** Caspase 3 immunostaining in the jejunal crypts with apoptotic cells indicated by arrows. **D** Caspase 3 immunostaining in the colonic crypts with apoptotic cells visible at week 6. Scale bars = 40 μm. Asterisk denotes significance compared to controls where *$p < 0.05$, ****$p < 0.0001$

3.5 Discussion

RIGT is a debilitating adverse effect of radiotherapy for cancer; however, RIGT pathobiology is not entirely understood, hindering the development of novel treatments. Key findings from this study demonstrate an increase in apoptotic cells of the microvasculature following fractionated abdominal radiation in rats. Of particular interest, apoptosis of the colon microvasculature was shown to occur 15 weeks following initial irradiation in the colon, 9 weeks following the last dose of radiation. Although previous studies have assessed changes in vascular mediator expression and histopathological features of endothelial dysfunction in RIGT (Richter et al. 1998; Wang, J et al. 2002), this is the first study showing microvascular apoptosis to be occurring in the delayed, or chronic, phase of RIGT, as displayed in the 15-week time point of the current study. This late microvascular apoptosis in the colon may be linked to mediators previously known to contribute to intestinal endothelial dysfunction following irradiation such as transforming growth factor beta (TGFβ), PAR-1 and von willebrand factor (Jahroudi, Ardekani & Greenberger 1996; Wang, J et al. 2002).

RIGT was shown to occur in the present study, with irradiated rats gaining significantly less weight compared to sham irradiated controls and increased apoptosis in crypts of both the jejunum and colon. This is in support of previous studies assessing RIGT following fractionated radiation in vivo (Al-Dasooqi et al. 2011). Yeoh and colleagues (2007) described significantly increased apoptosis in crypts of both the jejunum and colon from weeks 1–6 in DA rats administered a 6-week course of fractionated radiation to the abdomen (3 × 2.5 Gy/week). The studies did, however, differ with regard to proliferation of cells in the crypts. The present study showed an increase in proliferation at 6 weeks in the colonic crypts, as

opposed to a significant decrease in proliferating cells throughout the course of radiation in the aforementioned study (Al-Dasooqi et al. 2011). This could be explained in part by the differences between assessments of proliferating cells, with the present study using immunostaining of Ki67, a protein expressed in all active phases of the cell cycle, as opposed to mitotic cell count. Apoptosis was also shown to be highest at 6 weeks in both the jejunum and colon, indicating an accumulative effect of fractionated radiation on damage to the gastrointestinal mucosa. This is consistent with what has previously been seen in models of fractionated irradiation, in which it is proposed that the epithelial cells are able to regenerate due to breaks in the course of radiation (Al-Dasooqi et al. 2011). This is reflected in the relatively unchanged levels of proliferation in the jejunum and colon throughout the time course of the study.

The significant increase in wet weight of the small intestine is most likely explained by changes associated with chronic, or delayed, RIGT. In this late phase, reactive fibrosis often occurs, leading to fibrotic thickening of the muscle layer, submucosa and vascular walls (Wang, J et al. 2002). Several studies have assessed this in in vivo models of delayed radiation-induced enteropathy, and increases in collagen deposition and upregulation of fibrotic mediators including TGFβ have been shown (Jahroudi, Ardekani & Greenberger 1996; Wang, J et al. 2002). These changes, coupled with oedema and inflammatory infiltrate, could be leading to this increased wet weight of the small intestine, although parameters of histopathological damage, including oedema and presence of neutrophils, were not significantly increased in the jejunum at any time point in the current study.

This study is the first to show changes to microvascular apoptosis and proliferation in an in vivo fractionated radiation model. Of particular interest is the sustained microvascular cell

apoptosis occurring 9 weeks following the cessation of irradiation in the colon (week 15 of the study). Despite this, numbers of apoptotic cells in the crypts of the colon were not significantly higher than controls at the 15-week time point. Whilst this long-term microvascular apoptosis has not previously been shown, changes to the intestinal microvasculature have been observed many months following chemotherapy, and the biology of these cells has shown to be altered (Richter et al. 1998; Wang, J et al. 2002).

Wang and colleagues (2002) have shown an increase in thrombomodulin, an endothelial transmembrane glycoprotein and a decrease in protein C, an anticoagulant and anti-inflammatory protein, in a rat model of chronic radiation enteropathy. This increase in thrombomodulin leads to endothelial dysfunction, involving decreased thromboresistance and the activation of protease-activated receptor 1 (PAR-1) (Wang, J et al. 2002; Hauer-Jensen, Fink & Wang 2004). These mediator changes, particularly thrombomodulin and activated protein C, could play a role in the microvascular cell apoptosis seen in this delayed toxicity phase in the present study. Both thrombomodulin and protein C have been shown to be protective in models of endothelial apoptosis, thought to be due to the ability of protein C to decrease the expression and activation of transcription factor NF-kB and downregulate pro-apoptotic proteins p53 and Bax (Cheng et al. 2003; Mosnier, Zlokovic & Griffin 2007; Li, Y-H et al. 2012), however, this link has not yet been explored in the context of RIGT.

The current study provides a link between the development of RIGT, particularly late RIGT, and intestinal microvascular changes, including microvascular cell apoptosis, early senescence and late proliferation. Whilst mucosal damage was significantly increased in the colon 3 and 6 weeks into the fractionated radiation schedule, as shown by various indices including damage scoring, apoptosis and decreased proliferation, apoptosis and proliferation

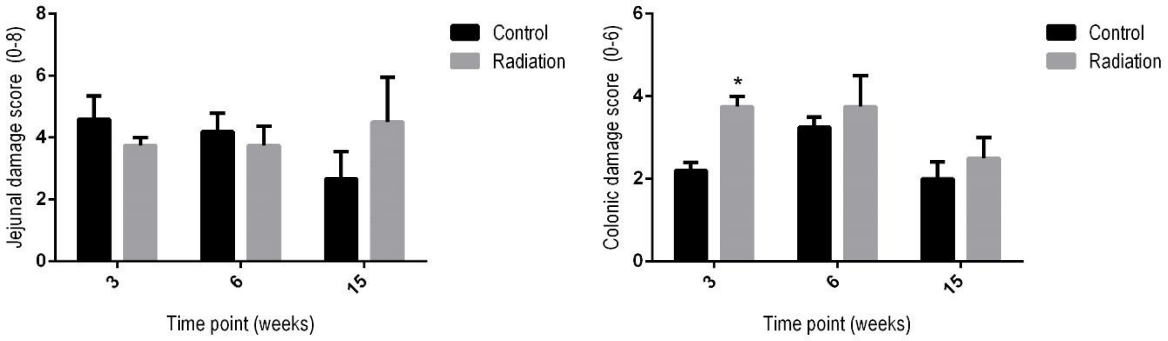
were observed in the microvascular cells in the delayed RIGT time point of 15 weeks, 9 weeks following the last dose of fractionated irradiation. Despite this, weight gain of irradiated rats was significantly lower than control rats from 6 weeks onwards. These results indicate that changes to the intestinal microvasculature may be driving toxicity observed in the later time points, indicative of chronic, or delayed, RIGT. In order to determine whether these factors are causally related, the effects of endothelial damage on the intestinal response to RIGT need to be explored in more depth. Preliminary, in vitro studies have indicated that the endothelial cell response to radiation may induce epithelial dysfunction, independent of the BAX-mediated apoptosis of epithelial cells following direct irradiation (Gaugler, MH et al. 2007). If this pathway could be attenuated, it may lessen gastrointestinal toxicity, thus reducing the severity of RIGT. However, this distinct mechanism needs to be further explored to determine possible mediators of this response, thus providing targets for the development of novel treatments.

Apoptosis of endothelial cells following radiation has also been shown to occur in endothelial cells of other tissues, including lung, brain, spinal cord and kidney (Pena, Fuks & Kolesnick 2000; Yeoh et al. 2007; Li, G et al. 2014). Whilst few studies have assessed changes to intestinal endothelium following irradiation, endothelial apoptosis of the intestinal microvasculature has been shown to occur shortly following single-dose irradiation in vivo (Sung et al. 2006; Abderrahmani et al. 2012). Endothelial apoptosis in vascular endothelial cells in the lamina propria of intestinal crypts was observed 4 h following 14 Gy whole-body irradiation of C57BL/6 J mice (Sung et al. 2006). In a similar study by Abderrahmani and colleagues (2012), C57BL/6 J mice received a single dose of 19 Gy gamma irradiation to a segment of exposed intestine. Endothelial apoptosis in the lamina propria of the villi was shown to be significantly increased at 4 and 5 h following irradiation when compared to

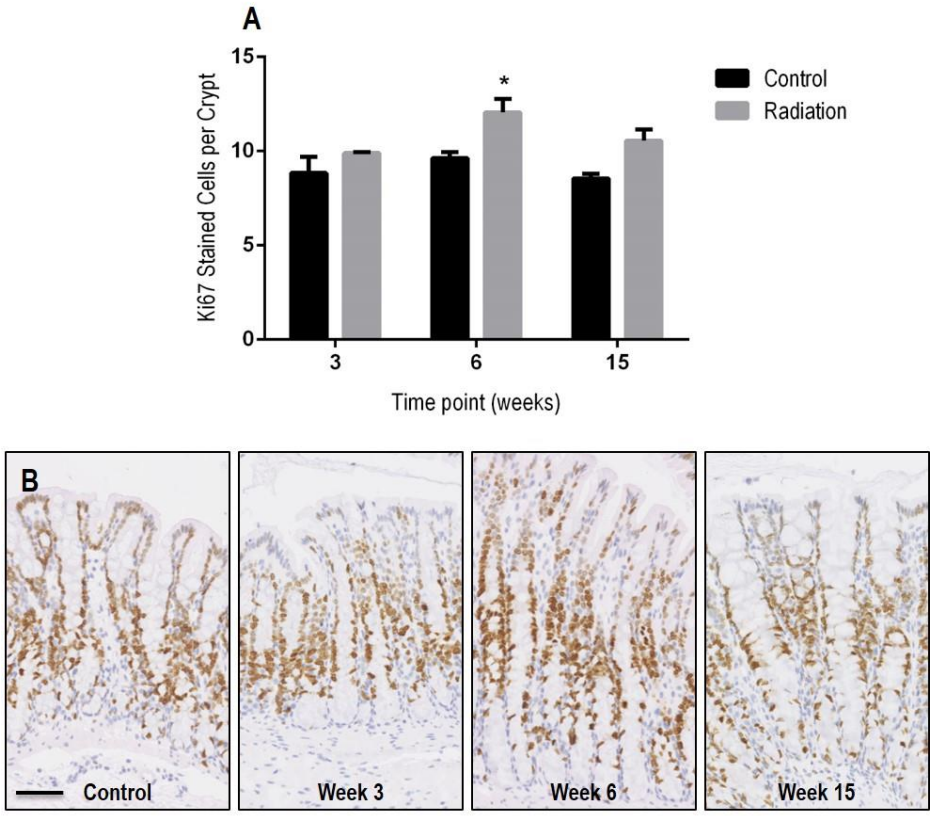
control (Abderrahmani et al. 2012). Whilst microvascular apoptosis was not observed in the earliest time point of the present study (3 weeks), this may be due to the differences between fractionated and single-dose radiation. These studies, together with common clinical symptoms including rectal bleeding and late reactive fibrosis, and results of the present study, highlight a role of the intestinal microvasculature in the pathophysiology of RIGT.

3.6 Conclusion

Changes in cell kinetics, specifically apoptosis and proliferation, were observed in the intestinal microvasculature throughout the entire time course of fractionated irradiation in vivo. Findings of this study highlight a role for intestinal microvasculature in both acute and chronic RIGT and implicate intestinal endothelium as being a potential target for the development of novel treatments.



Supplementary Figure 1 *Damage scoring of the colon and jejunum of irradiated rats*. * denotes significances compared to control where * ($p < 0.05$).



Supplementary figure 2 *Ki67 immunostaining of the colon crypts*. **A** Proliferation was significantly higher in the colon crypts at 6 weeks in the irradiated group ($p = 0.0260$) **B** Ki67 immunostaining of the colon crypts. * = $p < 0.05$.

Statement of Authorship

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Name of Principal Author (Candidate)	Romany Laura Stansborough		
Contribution to the Paper	I was responsible for all experimental work using archival tissue from the original animal study (DAR28). This included histopathology, immunohistochemistry, PCR, and western blotting. I prepared the manuscript with editorial assistance from co-authors.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

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Chapter 4 Matrix metalloproteinase expression is altered in the small and large intestine following fractionated radiation *in vivo*

[Stansborough R.L. et al., (2018). *Supportive Care in Cancer* (Available at: https://doi.org/10.1007/s00520-018-4255-5)]

4.1 Abstract

Purpose: Radiotherapy-induced gut toxicity (RIGT) is associated with significant diarrhoea, pain, and rectal bleeding. Matrix metalloproteinases (MMPs) have been reported to be involved in chemotherapy-induced gut toxicity and RIGT following single-dose irradiation *in vivo*. We therefore proposed MMPs would be involved in the pathobiology of RIGT following fractionated irradiation.

Methods: Dark Agouti rats were treated with fractionated radiation (3 x 2.5 Gy/week for 6 weeks). Rats were killed at 3, 6 and 15 weeks to represent acute and chronic toxicities. Section of jejunum and colon were immunostained for MMP-1, -2, -9, and -14. Relative mRNA expression in jejunum and colon was quantified by RT-PCR for MMP-1, -2, -9, and -14. Western blotting was also conducted on jejunum and colon tissue collected at week 6 to determine protein levels of pro- and active- MMP-2.

Results: MMP-2 total protein levels, determined by western blotting, significantly increased in both the jejunum ($p = 0.0359$), and the colon ($p = 0.0134$) 6 weeks into the fractionated radiation schedule. MMP-1, -2, and -14 mRNA expression significantly increased in the jejunum. MMP-2 mRNA expression was also significantly increased in the colon. Immunostaining of MMP-2 was observed to be increased in both crypt enterocytes and the lamina propria.

Conclusions: MMP-2 plays a role in the pathobiology of gastrointestinal toxicities following fractionated irradiation. Whilst MMP-1 and -14 mRNA expression was increased, this occurred

only in the jejunum, suggesting MMPs are differentially involved in RIGT depending on the intestinal region. Further studies are needed to elucidate the role these mediators play in the development and potentiation of RIGT.

4.2 Introduction

Radiotherapy-induced gut toxicity (RIGT) is a debilitating side effect of radiotherapy for cancer, estimated to affect over 80% of patients depending on target area and radiation schedule (Theis et al. 2010; Shadad et al. 2013b). Symptoms differ depending on time from last dose, and can be generally divided into acute and chronic toxicities, with acute toxicities involving diarrhoea, pain, rectal bleeding, and histopathological changes to the gut, including villous blunting, crypt disruption, and telangiectasia (Yeoh et al. 2007; Stansborough et al. 2016). Chronic symptoms include incontinence, rectal bleeding, and ulceration, often due to reactive fibrotic thickening of the muscle layer and microvascular walls in the small and large intestines (Theis et al. 2010). Both acute and chronic RIGT lead to significant decreases in quality of life, and can lead to radiotherapy dose reductions or cessation of treatment (Sonis, Stephen T. & Keefe 2013). Despite this, limited treatment options remain, and most target individual symptoms alone (Lalla et al. 2014).

Matrix metalloproteinases (MMPs), a 24-member family of zinc-dependent endopeptidases, have recently been identified in the development of RIGT (Strup-Perrot et al. 2005; Strup-Perrot et al. 2006; Angenete et al. 2009). Expression of MMPs can be increased at the transcriptional level by mediators known to contribute to the pathogenesis of RIGT, including pro-inflammatory cytokines and the activation of p53 by NF-κB (Yeoh et al. 2005; Nagase, Visse & Murphy 2006; Strup-Perrot et al. 2006; Yue et al. 2015). MMPs are synthesized as pro-zymogens and can either be secreted, or in the case of membrane type-MMPs (MT-MMPs), expressed on the cell surface (Sternlicht & Werb 2001; Yan & Boyd 2007). Pro-MMPs require proteolytic activation to allow for degradation of the extracellular matrix. Activation of pro-MMPs can be induced via the plasminogen-plasmin cascade, or in the case of MMP-2

specifically, by MT1-MMP (also known as MMP-14) (Strup-Perrot et al. 2004). MMP activation is also regulated by tissue inhibitors of matrix metalloproteinases (TIMPs), which are capable of inhibiting active MMPs (Strup-Perrot et al. 2004; Al-Dasooqi et al. 2010).

Following activation, MMPs are capable of altering cell growth, inflammation, cell death, or angiogenesis via the upregulation of downstream mediators such as pro-inflammatory cytokines, growth factors, and chemokines (Castaneda et al. 2005; Manicone & McGuire 2008; Stansborough et al. 2016). MMP-2, -3, and -9, for example, are capable of activating the pro-inflammatory cytokine interleukin-1β (Schonbeck, Mach & Libby 1998). Many of these mediators are known to be involved in the development of RIGT, with pro-inflammatory cytokines such as tumour necrosis factor alpha (TNFα), and interleukin-1beta (IL-1β) being involved in the propagation of inflammation and eventually ulceration, implicating MMPs in this pathobiology (Strup-Perrot et al. 2006; Logan, Stringer, et al. 2008).

Due to these widespread effects and possible link to RIGT, Strup-Perrot and colleagues (2006) assessed levels of MMP-2, -3, -9, and -14 in the colon of rats irradiated with a single abdominal dose of 10 Gy. mRNA expression of all assessed MMPs were significantly increased in the colon, and protein levels of both pro- and active MMP-2 were also significantly increased 1, 3, and 7 days following irradiation (Strup-Perrot et al. 2006). Few studies have assessed changes in MMP expression in RIGT clinically, however protein expression of MMP-2, but not -1 or -9, has been shown to be significantly increased in the irradiated normal rectal tissue of patients who had received short-term preoperative fractionated radiotherapy for rectal cancer (Angenete et al. 2009). These studies clearly highlight possible differences in MMP expression dependent on intestinal region, time since

last dose, and single dose vs fractionated radiation schedules. Thus further research investigating MMPs in different intestinal regions is required in order to better clarify our understanding of the role of the MMP pathway in RIGT.

Whilst studies have linked increases in MMPs to RIGT, no study to date has assessed MMPs in multiple regions of the gut following fractionated irradiation in a clinically relevant model of RIGT. As it is clear from the aforementioned studies that MMP expression is dependent on many factors, including intestinal region and radiation schedule, the present study aimed to assess expression and localization of MMPs -1, -2, -9, and -14, in both the jejunum and colon in a rat model of fractionated RIGT. The Dark Agouti rat model of RIGT has been utilized in this study due to prior characterisation of the histopathological response to RIGT in this model, the fractionated radiation schedule reflecting clinical schedules, and the inclusion of both acute and chronic time points (Yeoh et al. 2007; Stansborough et al. 2017).

4.3 Materials and Methods

4.3.1 Ethics

This study was conducted in accordance with ethics approved by the Animal Ethics Committees at the University of Adelaide (M-041-2006) and the Institute of Medical and Veterinary Science (78/05) and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Teaching at the time of the study.

4.3.2 Animals and Experimental Design

Experimental design was as previously described (Yeoh et al. 2007; Stansborough et al. 2017). Briefly, thirty female Dark Agouti (DA) rats were randomly assigned into groups receiving radiation, and groups receiving sham irradiation). Prior to irradiation, rats in treatment and control groups were anaesthetised and placed into a custom-built container, limiting radiation to the abdomen (Yeoh et al. 2007; Stansborough et al. 2017). Rats in the radiation group were irradiated at a dose of 2.5 Gray (Gy)/fraction three times a week for 6 weeks prescribed to a depth of 3.3 cm using a Varian Clinac Linear Accelerator (Varian Medical Systems, USA) with focus-skin distance of 130 cm. Rats in both sham irradiated and irradiated groups were killed at the ends of weeks 3, 6, and 15 (n=5 per treatment group at each time point). These time points corresponded to total accumulated doses of 0 Gy (sham irradiated controls), 22.5 Gy (3 week time point), or 45 Gy (6 and 15 week time points). These time points and doses were calculated to represent acute toxicity (< 12 weeks from start of abdominal irradiation) and delayed toxicity (≥12 weeks from start of abdominal irradiation). Gastrointestinal tissue was collected following flushing, with two 2 cm sections of jejunum (at 25 % length of small intestine from the pylorus) and colon (at 50 % length of

large intestine) resected. One section of tissue was formalin-fixed and paraffin-embedded, and the other was snap frozen in liquid nitrogen and stored at -80 °C.

4.3.3 RNA isolation and reverse transcription

RNA isolation was performed using the Nucleospin® RNA II kit (Macherey-Nagel, Duren, Germany) as previously described (Stansborough et al. 2017). Briefly, ~15 mg of tissue was homogenized in cell lysis buffer and beta-mercaptoethanol using the TissueLyser LT (Qiagen) with stainless steel beads (Qiagen). The lysate was filtered through silica membrane filter columns, 70 % ethanol was added, the silica membrane was desalted, and DNA was digested using the DNAse reaction mixture. Following washing and drying of the silica membrane RNA was eluted in 60 µl of RNase-free water. RNA concentration and purity was determined using the TAKE3 plate and Synergy My Reader (BioTek). RNA was converted to cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to manufacturers' protocol. Total cDNA concentration and purity was quantified using the TAKE3 plate and Synergy My Reader. Stock cDNA was diluted to 100 ng/µl with nuclease-free water.

4.3.4 Real-time polymerase chain reaction (qRT-PCR)

Quantitative RT-PCR was performed using the Rotor Gene 3000 (Corbett Research, Sydney, Australia). Reaction mixtures contained 1 µl of cDNA, 5 µl of SYBR green PCR Master Mix (Applied Biosystems), 5 µl of nuclease-free water, and 0.5 µl each of 50 pmol/µl forward and reverse primers (table 1). Thermal cycling conditions were optimised for each target gene and melt curve analysis was performed to determine presence of any dimers. Conditions included a denaturation step at 95 °C for 10 minutes, and 40 cycles at 95 °C (15 seconds), annealing 55 °C (MMP-1 and -9), 56°C (MMP-14), or 60 °C (MMP-2) (15 seconds), and acquiring at 72

°C (20 seconds). The housekeeping gene used was 18S, which was determined to be stable throughout treatment groups. Primer efficiency was between 90-110 % for each target gene against 18S, and thus the $2^{-\Delta\Delta CT}$ method was used to determine fold change in gene expression relative to 18S and untreated controls from each time point.

Table 1. Primer sequences for housekeeping and target genes.

Gene	Primer sequence (5'-3')	Nucleotide Position	Amplicon length (bp)	Tm (°C)	Ref
18S	F; CAT TCG AAC GTC TGC CCT AT R; GTT TCT CAG GCT CCC TCT CC	344-452	109	52 56	(Uchida et al. 2010)
MMP-1	F; CCT TCC TTT GCT GTT GCT TC R; CTG AAA CAC GGG GAA ACT GT	51-108	58	52 52	Designed in Primer3
MMP-2	F; CTG ATA ACC TGG ATG CAG TGCT R; CCA GCC AGT CCG ATT TGA	2138-2272	135	55 50	(Al-Dasooqi et al. 2010)
MMP-9	F; AAG CCT TGG TGT GGC ACG AC R; TGG AAA TAC GCA GGG TTT GC	760-876	117	56 52	(Vikman, Ansar & Edvinsson 2007)
MMP-14	F; GTG GAT GGA CAC CGA GAA CT R; CTT TGT GGG TGA CCC TGA CT	949-1039	91	54 54	Designed in Primer3

4.3.5 Immunohistochemistry (IHC)

MMP proteins were visualised using immunohistochemical (IHC) staining in jejunal and colonic tissue from rats killed at the end of weeks 3, 6, and 15 to determine protein localization. An automated staining system (Autostainer link 48 and PT Link, Dako) was used to conduct IHC for MMP-1 (#PAB12708, Abnova), MMP-2 (#ab37150, Abcam), MMP-9 (#ab58803, Abcam), and MMP-14 (#ab53712, Abcam), at concentrations of 2 µg/ml (MMP-1), 1.25 µg/ml (MMP-2 and MMP-9), 2.5 µg/ml (MMP-14). The EnVision™ FLEX kit for Dako autostainer link systems (Dako) was used as previously described (Wardill et al. 2015). Slides were scanned using the Nanozoomer (Hamamatsu Photonics) and protein localization was qualitatively assessed for each slide and stain.

4.3.6 Western blot

Protein Extraction

Protein was extracted from 30 mg sections of snap frozen jejunum and colon. Sections were homogenized in cooled Eppendorf tubes with homogenizing beads at 50 GHz for 5 minutes with 250 µl of 1:10 protease inhibitor cocktail (10 µg/ml in sterile PBS, EDTA free; #P8340, Sigma) in RIPA buffer (#R0278, Sigma) using the TissueLyser LT (Qiagen). Beads were removed and tubes were centrifuged at 10,000 x g for 15 minutes at 4 ºC. Supernatant was collected and protein was quantified using the BCA protein quantification assay (#23227, Thermo Scientific) according to manufacturer's protocol, after which it was stored at -80 ºC.

Western blotting

Western blotting was performed to quantify levels of pro- and active forms of MMP-2 protein in the jejunum and colon following fractionated irradiation. The iBlot™ 2 dry blotting system

(Invitrogen) was used to perform the western blot according to manufacturer's protocol. Samples were prepared with 30 µg of total protein, 10 µl Bolt LDS Sample Buffer (#B0008, ThermoFisher), 4 µl of Bolt Reducing Agent (#B0009, ThermoFisher) and dH_2O to yield a total sample volume of 40 µl. Samples were vortexed, heated at 70 ºC for 10 minutes, and pipetted into lanes of a 4-12 % Bis-Tris plus 12-well gel (#NW04122, ThermoFisher) in the iBlot™ mini gel tank with 1x SDS Running buffer (20x Bolt MES Running Buffer in dH2O, #B0002, ThermoFisher). 5 µl BioRad Precision Plus Protein™ dual colour standard (#1610374, BioRad) was used as a standard ladder. Electrophoresis was run at a constant 150V for 45 minutes. The iBlot transfer system was used with copper transfer stacks to transfer from gel to a PVDF membrane (#IB24002, ThermoFisher). The membrane was washed with TBST, re-activated with methanol, rinsed with dH_20 and immersed in 1x iBind fluorescent detection (FD) solution (iBindTM FD Solution Kit, #SLF1019, ThermoFisher). Primary antibodies for MMP-2 (#ab37150, Abcam) and beta-actin (#ab8224, Abcam) were diluted in iBind FD solution at 5 µg/ml and 0.167 µg/ml, respectively. The secondary antibody (IRDye 800CW goat anti-Rabbit, #925-32211, Li-Cor) was diluted in iBind FD solution at a concentration of 0.33 µg/ml. The iBind system (#SLF1010, ThermoFisher) was used, with iBind FD solution applied to the card, to add the primary and secondary antibodies to the membrane. The membrane was left overnight and scanned using the LI-COR Odyssey CLx imager at 800 nM with an intensity of 3.0. MMP-2 bands were quantified against the beta-actin housekeeping band using Image Studio™ software (Li-Cor).

Statistical Analysis

Means for each treatment group (sham irradiated and irradiated) were compared at each time point. Following normality testing, differences between treatment group means were analysed

using either a two-way ANOVA with Sidak's multiple comparison or a Kruskal-Wallis test with multiple comparison. Asterisks denote significance compared to control at the time point, where * <0.05, ** <0.01, *** <0.001, and **** <0.0001.

4.4 Results

4.4.1 Fractionated irradiation induced intestinal upregulation of MMP -1, -2, and -14 at the transcriptional level

Jejunum

RT-qPCR was performed to determine mRNA expression of MMP-1, -2, -9, and -14 following fractionated irradiation. MMP-1 mRNA expression was significantly increased in the jejunum 3 weeks into the fractionated radiation schedule ($p=0.0086$), but not at the 6- or 15-week time points (figure 1A). MMP-2 mRNA expression increased 172 fold at week 3 in the jejunum ($p<0.0001$) but was not sigificantly different at other timepoints (figure 1B). MMP-9 mRNA expression was not significantly altered at any time point under investigation (figure 1C). MMP-14 mRNA expression was significantly increased in the jejunum at week 3, by 26-fold, and at week 6, by 24-fold ($p=0.0002$, and $p=0.0065$, respectively). There was no significant difference seen at 15 weeks following radiation (Figure 1E).

Colon

There were no significant changes to MMP-1 mRNA expression in the colon (figure 2A) at any time point under investigation. MMP-2 mRNA expression significantly increased by 14- and 24-fold at 3 and 6 weeks in the colon ($p=0.0313$, and $p=0.0001$, respectively) (Figure 2B). However, no significant change was seen at 15 weeks after radiation. MMP-9 mRNA expression was not significantly altered at any of the time points investigated (Figure 2C). In contrast to what was observed in the jejunum, MMP-14 was not significantly altered at any time point in the colon (Figure 2E).

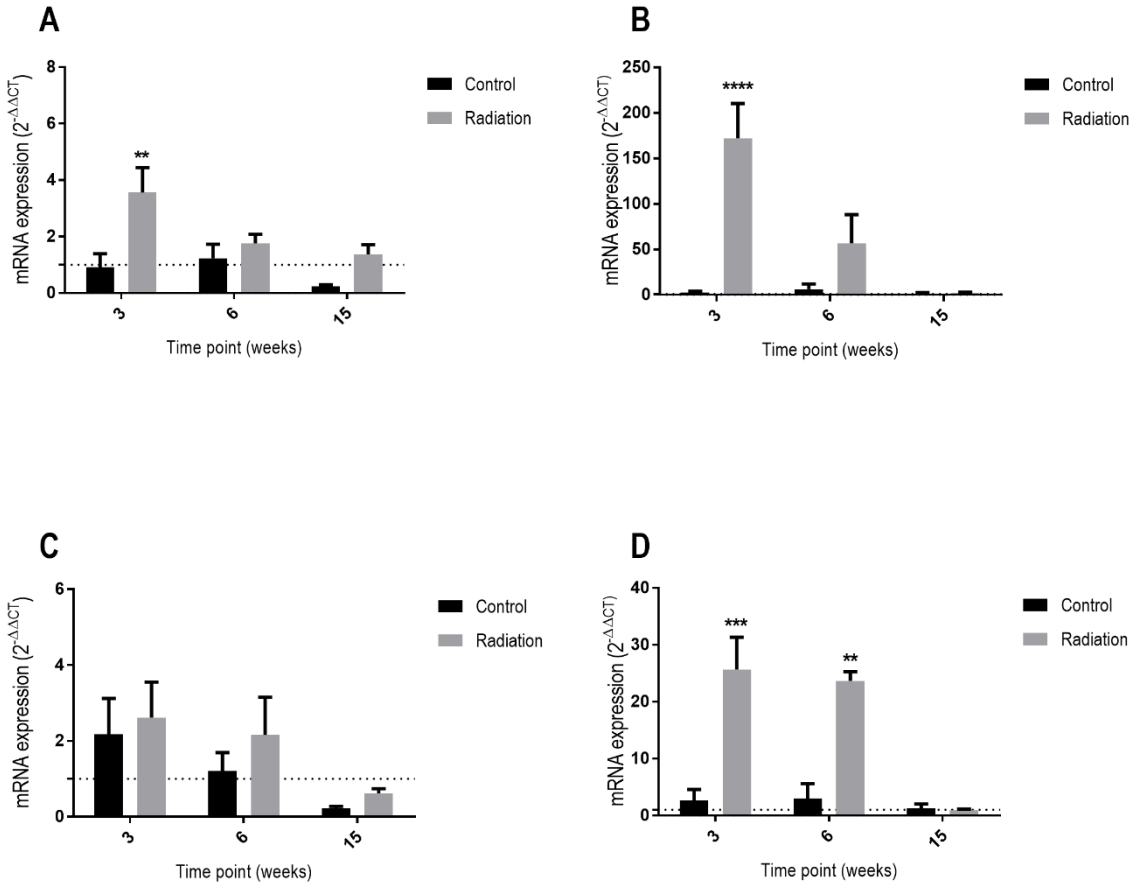


Figure 1. *Expression of MMP -1, -2, -9, and -14 in the jejunum following abdominal irradiation.* **A** mRNA expression of MMP-1 was significantly increased by 3.5-fold at week 3 (p=0.0086). **B** MMP-2 was significantly increased by 172-fold 3 weeks into the fractionated radiation course (p<0.0001). **C** MMP-9 mRNA expression was not significantly altered at any time point in the jejunum. **D** MMP-14 expression was significantly increased by 26-fold and 24-fold at the 3- and 6-week time points, respectively (p=0.0002; p=0.0065). Dotted line represents baseline fold change. Data shown is mean + standard error of the mean (SEM). Asterisks denote significance compared to controls at each time point, where **<0.01, ***<0.001, and ****<0.0001, n=5.

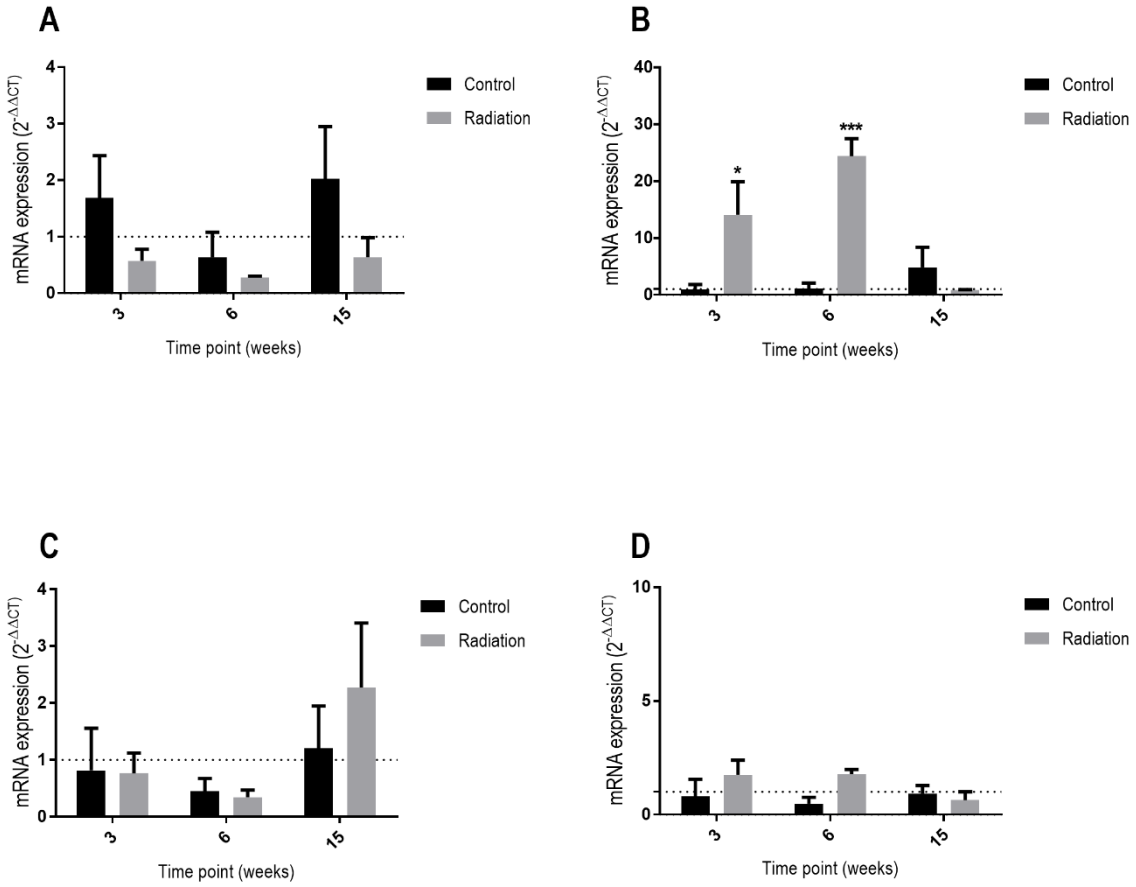


Figure 2. *Expression of MMP-1, -2, -9, and -14 in the colon following abdominal irradiation.* **A** Colonic MMP-1 mRNA expression was not significantly altered at any time point ($p>0.05$). **B** Colonic MMP-2 mRNA expression was significantly increased at week 3, with a 14--fold increase ($p=0.0313$). MMP-2 expression was also significantly increased at week 6 by 24-fold ($p=0.0001$). Colonic mRNA expression of MMP-9 (**C**) and -14 (**D**) were not significantly altered at any time point. Dotted line indicates baseline fold change. Data shown is mean + SEM. Asterisks denote significance compared to controls at each time point, where *<0.05, and ***<0.001, n=5.

4.4.2 Protein expression and localization of MMP-2 was altered in the colon following fractionated irradiation

As changes in mRNA expression of MMP-1, -2, and -14 were observed, immunohistochemistry was qualitatively assessed to initially determine any changes in protein localization or staining intensity prior to conducting western blotting. MMP-2 had an increase in total immunostaining represented by an increase in cytoplasmic staining in crypt enterocytes observed at weeks 3 and 6 (figure 3). There was also a slight increase in localization of MMP-2 to the lamina propria, particularly of the microvasculature, at weeks 3 and 6 (Supplementary figure 1). Staining intensity of the other MMPs assessed, -1, -9, and -14, was not altered in the jejunum or colon at any time point under investigation.

4.4.3 Pro- and active forms of MMP-2 protein were significantly increased in the jejunum following fractionated irradiation

As increases in immunostaining of MMP-2 were seen at 6 weeks, but unable to be quantified, western blotting was undertaken for MMP-2 in both the jejunum and colon at the 6-week time point. The 6-week time point was chosen due to a maximum radiation dose at this time point (45 Gy), as well as significant histopathological damage, including apoptosis of both the jejunal and colonic crypts and microvascular cells as previously reported [17]. Levels of both pro- and active- MMP-2 were able to be quantified via western blotting, as these forms have different molecular weights and thus present as separate bands. Pro-MMP-2 was significantly increased in both the jejunum ($p<0.0001$) and colon ($p<0.0001$) when compared against housekeeping band intensity (β-actin) and average intensity of controls (figure 4). Active MMP-2 was significantly increased following irradiation in the jejunum ($p=0.0011$) but not in the colon (figure 4).

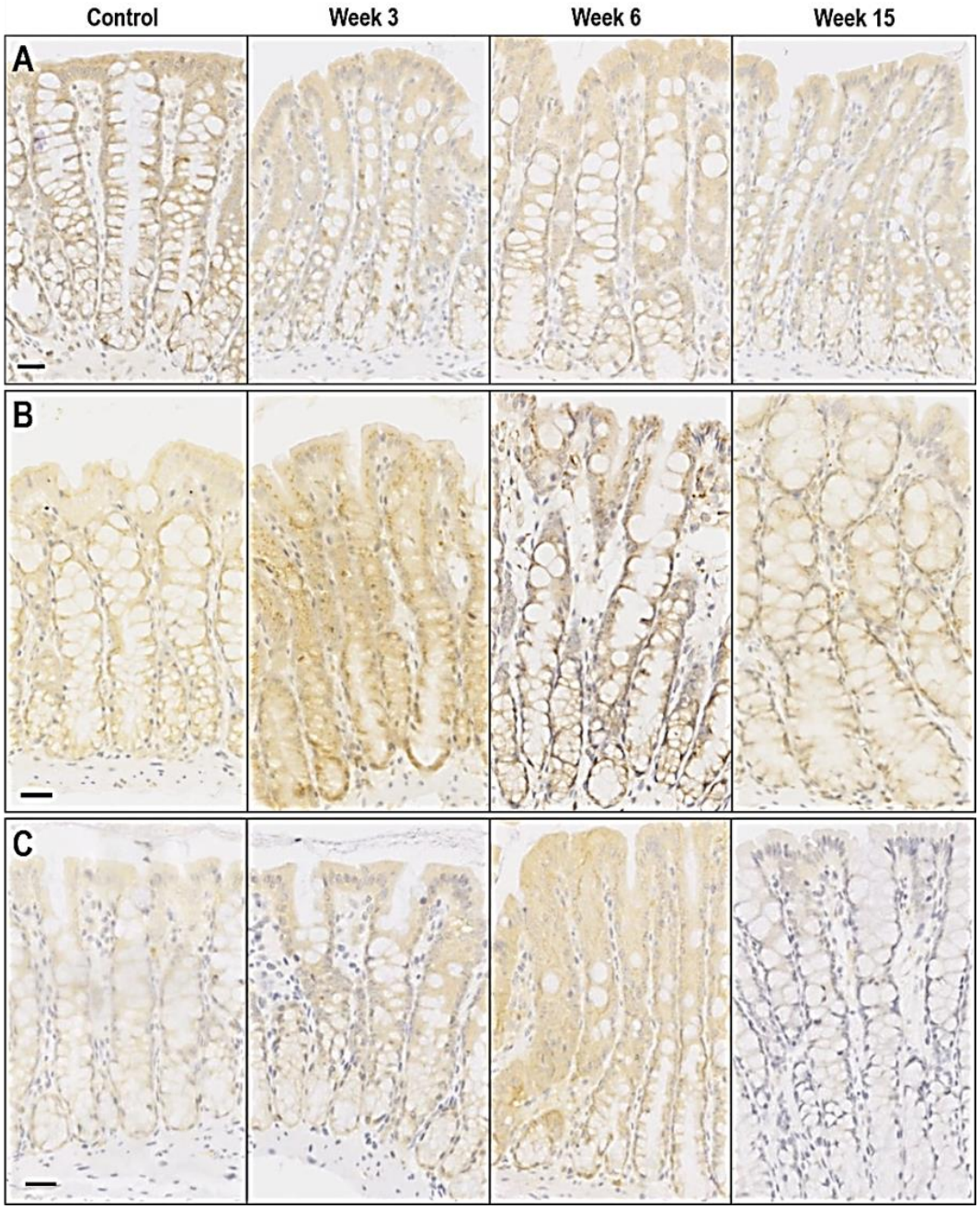


Figure 3. *MMP -1, -2, and -9 immunostaining in the colonic crypts following fractionated irradiation.* **A** MMP-1 staining intensity in the colonic crypts was not altered at any time point following fractionated irradiation **B** MMP-2 immunostaining was increased in the colonic crypts following 22.5 Gy and 45 Gy fractionated irradiation (weeks 3 and 6, respectively) **C** MMP-9 immunostaining was not altered in the colonic crypts 10x magnification, scale bar = 30 µm (applies to all images in same row), n=5.

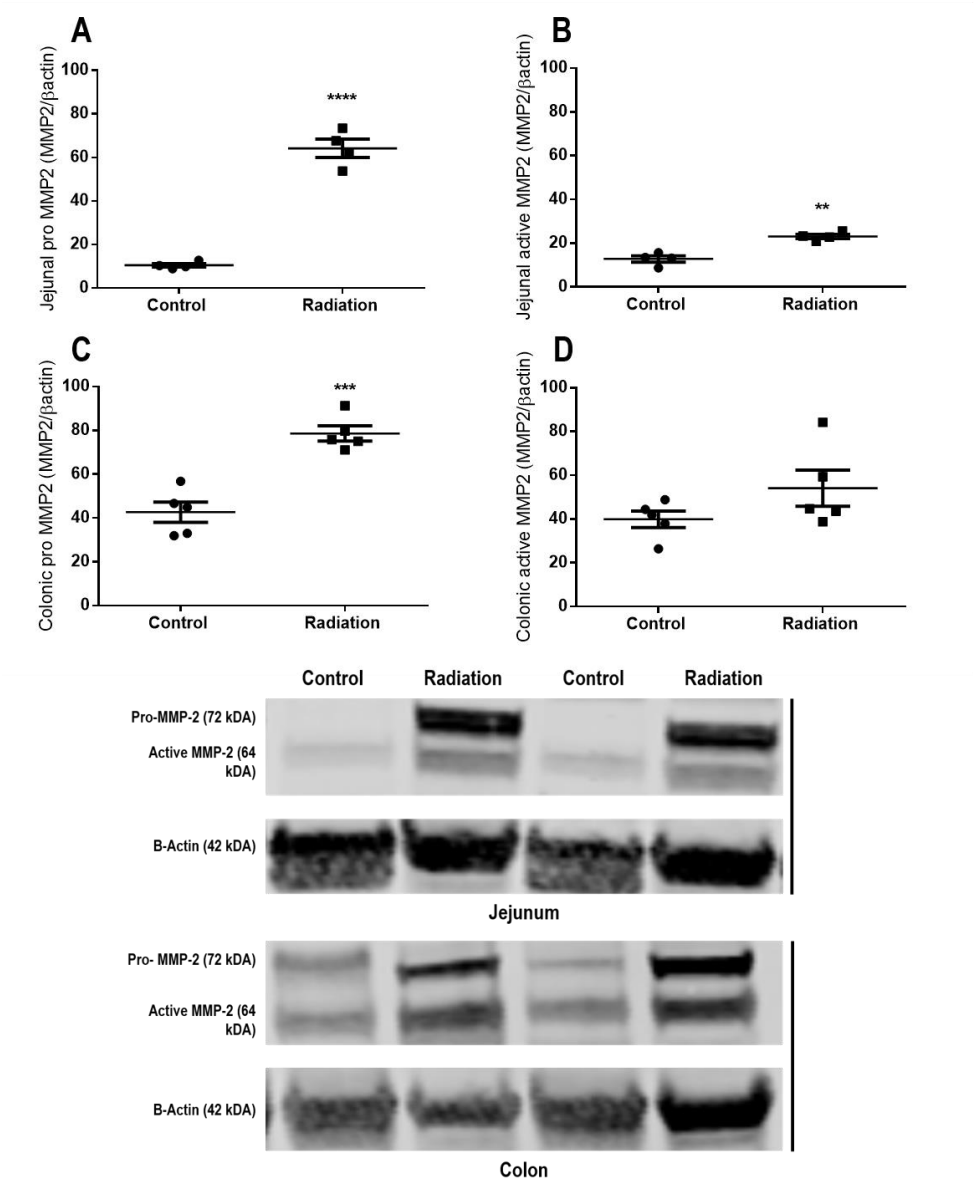


Figure 4. *MMP-2 protein expression was significantly increased in the jejunum and colon 6 weeks into the fractionated radiation schedule.* **A** Jejunal levels of pro-MMP-2 were significantly increased at 6 weeks ($p<0.0001$) **B** Jejunal levels of active MMP-2 were significantly increased at 6 weeks ($p=0.0011$) **C** Colonic levels of pro-MMP-2 were significantly increased at week 6 ($p<0.0001$) **D** Colonic levels of active MMP-2 were not significantly altered at 6 weeks ($p>0.05$). Data shown is mean + SEM. Asterisks denote significance compared to controls at each time point, where **<0.01, ***<0.001, and ****<0.0001, n=5.

4.5 Discussion

The current study assessed mRNA and protein expression of several MMPs thought to be involved in the development of RIGT in a model of fractionated irradiation. A key finding of this study suggests MMP -2 mRNA expression is upregulated in both the small and large intestine in response to abdominal fractionated irradiation. MMP-1 and -14 mRNA expression was also significantly increased in the jejunum only. The increases in these MMPs occurred alongside loss of body weight, increases in histopathological damage and microvascular changes previously shown in the same model (Stansborough et al. 2017).

The key finding of a significant increase in MMP-2 mRNA expression in both the jejunum and colon early in the fractionated radiation schedule accords with the consistent upregulation of MMP-2 reported in both clinical and preclinical studies of RIGT (Strup-Perrot et al. 2006; Angenete et al. 2007). MMP-2 expression was increased 172-fold at the 3-week time point in the jejunum, but not significantly altered at 6-weeks, despite an increase in cumulative dose. It is possible that the fractionated schedule of radiation is a contributor to this, with repeated healing occurring between doses over the 6-week radiation schedule, possibly leading to a decrease in MMP-2 expression. As MMP-2 immunohistochemical staining was also increased in the colonic crypts at the 3 and 6-week time points, protein levels were further quantified using western blotting to ascertain differences in pro and active forms. Whilst MMPs are expressed in their latent forms, activation is essential to many of the downstream effects (Hofmann 2000). Both pro- and active- MMP -2 were significantly increased in the jejunum following irradiation, however, only pro-MMP-2 was significantly increased in the colon. Rats receiving fractionated irradiation had significantly lower weight gain, significantly increased histopathological damage scoring in the colon, telangiectatic vessel formation, and apoptosis in

the crypts of the jejunum and colon occurring at the same time points as significant increases in MMP-2 mRNA and protein expression (Stansborough et al. 2017).

Strup-Perrot and colleagues (2006) showed protein levels of pro- and active MMP-2 to be increased 1, 3, and 7 days in the colon following 10 Gy abdominal irradiation in rats. However, the jejunum was not assessed in the aforementioned study. MMP-2 protein expression has been correlated with decreased mean bursting pressure and breaking strength during anastomic healing 3 and 7 days following 40 Gy fractionated irradiation in Wistar rats (Bedirli et al. 2007). Clinically, active MMP-2 levels have correlated to poor outcomes in RIGT and radiation enteritis (Angenete et al. 2007). Angenete and colleagues (2007) found increased levels of MMP-2 in both tumour tissue and tumour-adjacent normal rectal tissue within the radiation field to correlate with fistula formation in patients who had received pre-operative fractionated irradiation (5x 5 Gy) for rectal cancer. This increase of MMP-2 following radiation, in both pre-clinical and clinical studies, supports the findings of the present study. Correlation of this increase with poor outcomes such as impaired anastomic healing, and fistula formation, suggests MMP-2 may be a clinical target for the treatment of RIGT.

Following transcriptional upregulation and activation of secreted pro-MMP-2, MMP-2 is capable of degrading several ECM components, such as collagen IV and VXII, plasminogen, and decorin, to active downstream products such as vascular endothelial growth factor (VEGF), transforming growth factor beta (TGFβ), and form angiostatin, endostatin, and tumstatin (Stansborough et al. 2016). Active MMP-2 is also able to shed and activate pro-inflammatory cytokines such as TNF-α and IL-1β (Chien et al. 2013). Active MMP-2 is thus able to indirectly alter cell signalling, altering cell growth, migration, and angiogenesis, and in the present study is upregulated at the same time point as apoptosis in both the jejunum and colon (Yan & Boyd

2007; Stansborough et al. 2017). Previous studies have confirmed significantly increased expression of proinflammatory mediators, such as TNFα and IL-1β, in animal models of RIGT (Ong et al. 2010; Gerassy-Vainberg et al. 2018). This supports a possible pathway in which MMP-2 may be contributing to the exacerbation of acute RIGT through the activation of these proinflammatory mediators. Future studies assessing specific inhibition of MMPs, particularly MMP-2, as a possible treatment for RIGT should investigate the potential involvement of proinflammatory cytokines in this pathway. From the results of the present study, when considered in the context of previous literature, it is likely that the upregulation and activation of intestinal MMP-2 following irradiation plays an important role in the pathogenesis of RIGT (Strup-Perrot et al. 2006; Angenete et al. 2009; Chien et al. 2013).

MMP-14 was also significantly upregulated in the jejunum following irradiation in the present study. As previously mentioned, MMP-14 is intricately involved with the activation of pro-MMP-2 (Chien et al. 2013). Active MMP-14 expressed on the cell surface binds to TIMP-2 via the N-terminal domain. This complex then acts as a receptor for pro-MMP-2 after which a free MMP-14 molecule is able to cleave and activate pro-MMP-2 (Chien et al. 2013). This mechanism of MMP-2 activation by MMP-14 may be reflected in the present study, in which levels of both pro- and active MMP-2 were concurrently increased with MMP-14 expression in the jejunum. However, pro-, but not active MMP-2 was increased in the colon, in which MMP-14 mRNA expression was not significantly altered. Differences between the response of the jejunum and colon to radiation have been characterized in previous studies (Cameron et al. 2012; Stansborough et al. 2017). Loss of epithelial cell lining and villi have been noted 6-24 hours following single-dose irradiation in rats (Cameron et al. 2012). In contrast, damage to the structure of the colonic crypts occurs from 24 hours following irradiation (Cameron et al. 2012). The upregulation of MMP-14 at acute time points in the jejunum corresponds to significantly

lower weight gain, and apoptosis in both epithelial and endothelial apoptosis in the same model (Stansborough et al. 2017). Apoptosis has also been shown to occur earlier in the jejunum than in the colon in the model of fractionated irradiation used in the present study (Stansborough et al. 2017). Whilst the timing of these events is likely altered in a fractionated radiation schedule, this reflects a differing profile of RIGT in the different intestinal regions. Additionally, the increase in MMP-14 expression in the present study does not appear to correspond to increased cumulative doses, with relative expression similar at both 3 and 6 week time points. However, the direction of the relationship between the increase in MMP expression and the histopathological damage in the gut remains unclear. The pattern of MMP-2 and MMP-14 expression following radiation potentially supports the role of MMP-14 in the activation of MMP-2, which should be considered when exploring MMP inhibition as a treatment for RIGT.

In the present study MMP-1 mRNA expression was found to be significantly increased in the jejunum following 22.5 Gy fractionated abdominal irradiation. This was not reflected in protein localization, with no changes in MMP-1 immunostaining in the jejunum or colon. MMP-1 is classified as a collagenase due to its ability to degrade collagen and gelatin, however it is also capable of activating pro-MMP-9 (Mittal et al. 2016). Transcription of MMP-1 can be increased in response to proinflammatory cytokines, epidermal growth factor, as well as following activation of p38 mitogen-activated protein kinases (MAPK), which has previously been shown to be increased in RIGT (Sonis, S. T. 2002; Salmela et al. 2004; Mittal et al. 2016). Al-Dasooqi and colleagues (2010) found MMP-1 protein expression to be down-regulated early, and upregulated late during chemotherapy-induced gut toxicity (CIGT), suggesting a role for MMP-1 in healing. MMP-1 could be playing a similar role in the present study as it is known to regulate cell migration and re-epithelialization, however MMP-1 was not altered in the chronic RIGT time point of the present study, 6 weeks following the cessation of radiation

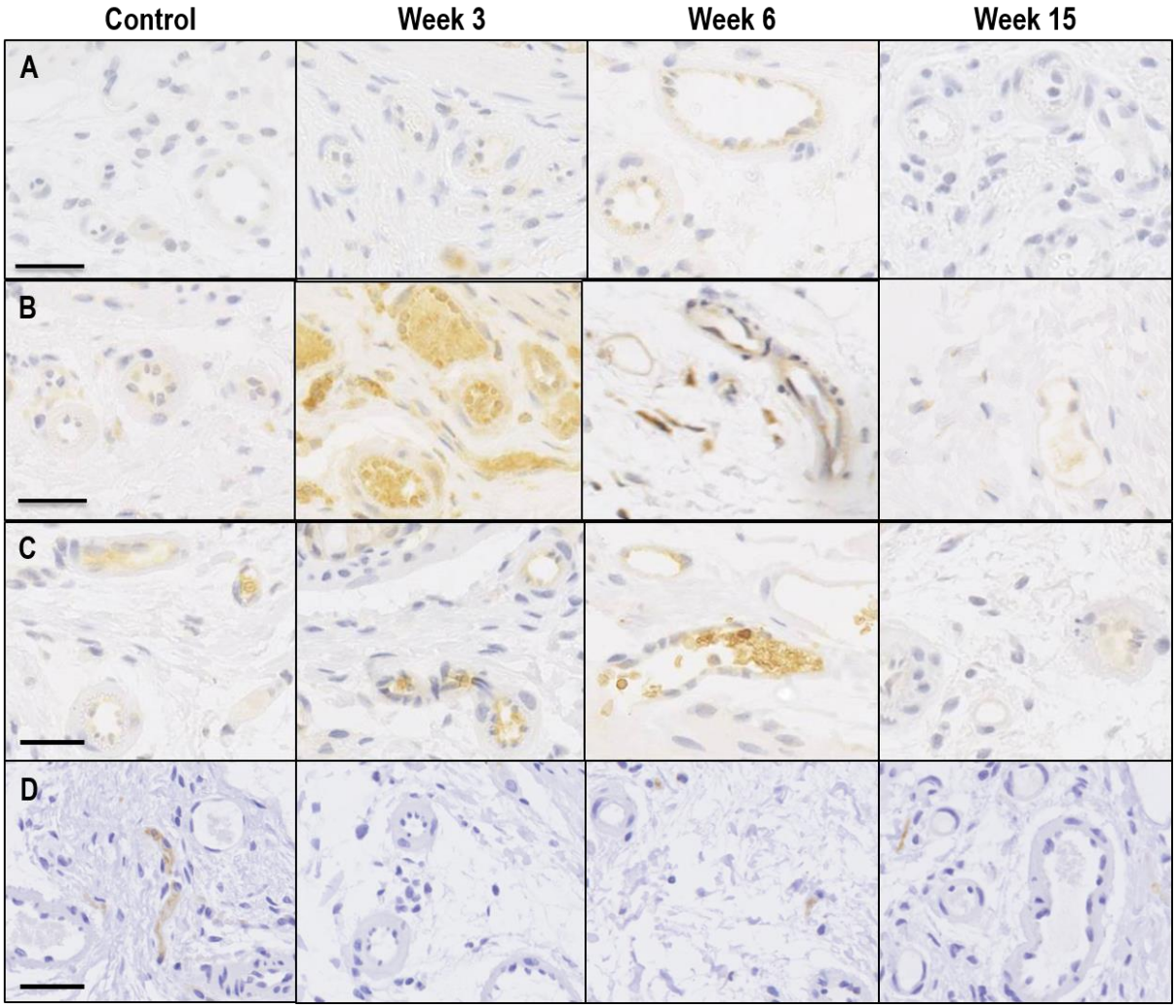
(Stansborough et al. 2016). It is possible that any increase in MMP-1 could have returned to baseline at this point.

In contrast with the Strup-Perrot and colleagues (2006) study assessing MMP levels following irradiation in rats we found no significant difference in MMP-9 mRNA expression. MMP-1 and -14 mRNA expression, like MMP-2, were significantly increased in the jejunum. Despite the significantly increased mRNA expression of these MMPs, no changes in immunostaining intensity or localisation were observed. This lack of post-translational modification of the remaining MMPs may be due to the time points of the present study in which all tissue was collected 24 hours following irradiation, despite different cumulative radiation doses at the 3- and 6-week time points. MMP-9 has been shown to be increased 3-7 days following irradiation in rats, however this has been assessed in models of single-dose irradiation (Strup-Perrot et al. 2006; Angenete et al. 2009). Changes in mRNA expression do not always correlate strongly with protein expression so it is also possible that these changes had no molecular manifestation (de Sousa Abreu et al. 2009). Additional studies are required to further assess the role of these MMPs in RIGT in different regions, models and with different treatment schedules.

4.6 Conclusion

Whilst this study is consistent with previous studies assessing MMP expression following radiation, this is the first study to assess the MMP expression in multiple regions of the gut in a fractionated radiation model of RIGT. Key findings of this study suggest MMP-2 to be involved in the development of RIGT in both the small and large intestine following abdominal fractionated radiation. MMP-2 is widely understood to be overexpressed in many human cancer and levels of active MMP-2 have been shown to increase risk of metastasis of head and neck cancer (Chien et al. 2013). This study presents a unique possibility for specific

MMP inhibitors to improve radiotherapy-induced gastrointestinal toxicities whilst not reducing efficacy of cancer treatment. Further studies are now warranted to assess these inhibitors for their therapeutic potential in RIGT.



Supplementary Figure 1. *MMP -1, -2, -9, and -14 immunostaining in the colonic lamina propria following fractionated irradiation.* **A** MMP-1 staining intensity in the colonic lamina propria was not altered at any time point following fractionated irradiation **B** MMP-2 immunostaining was increased in the colonic lamina propria at weeks 3 and 6 **C** MMP-9 immunostaining was not altered in the colonic lamina propria, although staining of red blood cells was increased at weeks 3 and 6 **D** MMP-14 immunostaining was not increased in the colonic lamina propria at any time point. 20x magnification, scale bar = 20 μm (applies to all images in same row).

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Romany Laura Stansborough
Contribution to the Paper	I was responsible for all experimental work using archival tissue from the original animal study (DAR28). This included immunohistochemistry, and PCR. I conducted isolation of primary endothelial cells, with assistance, and conducted all associated experiments. I prepared the manuscript with editorial assistance from co-authors.
Overall percentage (%)	85%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 8/12/2017

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Chapter 5 Vascular endothelial growth factor (VEGF), transforming growth factor beta (TGFβ), angiostatin, and endostatin are increased in radiotherapy-induced gastrointestinal toxicity

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5.1 Abstract

Purpose: Radiotherapy-induced gut toxicity (RIGT) is a debilitating effect of radiotherapy for cancer, often resulting in significant diarrhoea and pain. Previous studies have highlighted roles of the intestinal microvasculature and matrix metalloproteinases (MMPs) in the development of RIGT. We hypothesized vascular mediators would be significantly altered in a dark agouti (DA) rat model of RIGT. Additionally, we aimed to assess the effect of MMP-2 and -9 inhibition on the response of tumour-associated microvascular endothelial cells (TAMECs) to radiation.

Methods: Dark Agouti (DA) rats were administered 2.5 Gy abdominal irradiation (3 times/week over 6 weeks). Vascular endothelial growth factor (VEGF), transforming growth factor beta (TGFβ), von Willebrand factor (VWF), angiostatin, and endostatin expression was assessed at 3, 6 and 15 weeks. Additionally, DA rat mammary adenocarcinoma tumour-associated microvascular endothelial cells (TAMECs) were used to assess the effects of radiation (12 Gy) and the MMP inhibitor SB-3CT on MMP, VEGF, and TGFβ expression, and cell viability.

Results: VEGF mRNA expression was significantly increased in the colon at week 15 ($p = 0.0012$), and TGFβ mRNA expression was significantly increased in both the jejunum and colon at week 3 ($p = 0.0280$, and $p = 0.0310$, respectively). Endostatin immunostaining was

significantly increased at week 3 ($p = 0.0046$), and angiostatin at 3 and 6 weeks ($p = 0.0022$, and $p = 0.0135$, respectively). MMP-2 and -9 mRNA and total protein levels were significantly increased following irradiation of TAMECs. Although this increase was significantly attenuated by SB-3CT, it did not significantly alter endothelial cell viability or VEGF and TGFβ mRNA expression.

Conclusions: Findings of this study support the involvement of VEGF, TGFβ, angiostatin, endostatin, and MMP-2 in the pathobiology of RIGT. However, the relationship between these mediators is complex and needs further investigation to improve understanding of their therapeutic potential in RIGT.

5.2 Introduction

Radiotherapy-induced gut toxicity (RIGT), estimated to affect 60-80% of patients receiving radiotherapy for abdominal or pelvic tumours, induces debilitating symptoms including, but not limited to, diarrhoea, pain, rectal bleeding, and incontinence (Theis et al. 2010; Shadad et al. 2013a; Hauer-Jensen, Denham & Andreyev 2014). RIGT is often divided into acute and chronic toxicity, with acute changes involving histopathological damage to villi and crypts, and chronic involving reactive fibrotic thickening (Yeoh et al. 2007; Theis et al. 2010). Previous research has suggested the involvement of the intestinal microvasculature in the development of RIGT, and several mediators have been investigated (Kruse et al. 2004; Yeoh et al. 2007; Stansborough et al. 2017). NFκB, proinflammatory cytokines, and matrix metalloproteinases (MMPs) are all upregulated in the intestine following radiation, and have downstream effects on vascular mediators such as vascular endothelial growth factor (VEGF), transforming growth factor beta (TGFβ), von Willebrand factor (VWF), and the antiangiogenic mediators endostatin and angiostatin (Stansborough et al. 2016). Whilst a link has been proposed in RIGT (Stansborough et al. 2016) there is limited research which has investigated changes in these mediators in the intestinal microvasculature during a fractionated radiation schedule.

Our previous studies have shown the intestinal microvasculature to be involved in RIGT, with apoptosis, cytostasis, telangiectasis, and fibrotic thickening occurring early and late following fractionated irradiation in dark agouti (DA) rats (Yeoh et al. 2005; Stansborough et al. 2017). In addition, several regulators of angiogenesis and vascular physiology including, but not limited to, TGFβ, VEGF, and VWF, are known to be altered in the intestine following irradiation (Wang, J, Zheng & Hauer-Jensen 2001; Wang, J et al. 2002; Milliat et al. 2006; Lenting et al. 2012; Boerma et al. 2013). VEGF, bound to heparin in the extracellular matrix,

is able to be released by matrix metalloproteinases (MMPs) such as MMP-2, and MMP-9 (Carmeliet 2005). The anti-angiogenic mediators angiostatin and endostatin have also been shown to increase in response to irradiation of cancer endothelial cells derived from human breast cancer tissue (Oh et al. 2014). A reduction in angiostatin via knockdown of plasminogen restored tube formation of these cells, suggesting angiostatin is involved in endothelial regulation following irradiation (Oh et al. 2014). Although it appears angiostatin and endostatin are involved in endothelial response to irradiation this connection is yet to be explored in RIGT. With the increasing investigation of the possible use of anti-angiogenic agents as adjuvant cancer therapies alongside chemotherapy or radiotherapy, targeting mediators such as vascular endothelial growth factor (VEGF) and endostatin, the role of these mediators in RIGT needs to be further explored.

Previous research has clearly shown MMPs are involved in RIGT, with MMP-2 in particular shown to be significantly increased during RIGT (Strup-Perrot et al. 2006). It has also been hypothesised MMPs are involved in microvascular changes and alteration to vascular mediators such as VEGF, TGFβ, and VWF in RIGT (Stansborough et al. 2016). MMPs are able to regulate the expression of several of these mediators via the degradation of the extracellular matrix (ECM) leading to the release of latent VEGF and TGFβ, as well as forming angiostatin and endostatin by cleaving plasminogen and collagen XVIII respectively (Sternlicht & Werb 2001; Kalluri 2003; Carmeliet 2005). In degrading the ECM, MMPs also promote endothelial migration, proliferation and tube formation (Kalluri 2003). Despite previous literature, a direct link has not been established between MMPs and vascular mediator expression following radiation, and the effect of these changes on endothelial cells. Thus the present study aimed to first confirm the alteration of vascular mediators VEGF, TGFβ, VWF, angiostatin, and endostatin in the jejunum and colon following fractionated

irradiation in DA rats. We then aimed to determine whether inhibition of MMP-2 and -9 with SB-3CT altered endothelial viability and expression of these vascular mediators in primary tumour-associated microvascular endothelial cells (TAMECs) derived from DA rat mammary adenocarcinomas. TAMECs have been successfully cultured for previous, similar experiments, have been phenotyped, and their cultivation in DA rats allows for consistency between both components of the study (Bateman et al. 2013). In utilising this primary *in vitro* model we aimed to assess the effects of SB-3CT on tumour endothelial response to irradiation as an initial investigation into the response of tumour endothelium to MMP-2 and -9 inhibition. As the cell type used in the *in vitro* component of the study does not directly translate to RIGT, further studies are required to assess the effects of SB-3CT on intestinal endothelium following irradiation. However, results of the present study form a foundation for further investigation into the possible use of SB-3CT following irradiation.

5.3 Materials and Methods

5.3.1 Ethics

Both studies were conducted in accordance with ethics approved by the Animal Ethics Committees at the University of Adelaide (M-041-2006; M-2015-117) and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Teaching (2013).

5.3.2 Animals and Experimental Design

Archival tissue from a prior study was utilised in the *in vivo* component of the present study, and experimental design has been previously described (Yeoh et al. 2007; Stansborough et al. 2017)**.** Briefly, 30 female Dark Agouti (DA) rats were randomly assigned into two groups, one group receiving sham irradiation, and one group receiving a schedule of fractionated irradiation. Both groups of rats were anaesthetised using 3% halothane in 100% oxygen and placed into a custom-built container, with rats in the treatment group receiving fractionated abdominal irradiation using a Varian Clinac Linear Accelerator (Varian Medical Systems, USA) at a dose of 2.5 Gray (Gy) (depth = 3.3 cm, focus-skin distance = 130 cm) 3x per week for a total dose of 22.5 Gy in 9 fractions and 45 Gy in 18 fractions over 3 and 6 weeks, respectively (Yeoh et al. 2007). Rats were killed at 3, 6, or 15 weeks from first irradiation dose (n = 5 per time point). Animals were monitored daily and clinical record sheets kept. Sections of jejunum (30% of small intestinal length when measured from the pylorus) and colon (midlength of the large intestine) were collected as previously described and either formalin-fixed and paraffin-embedded, or snap frozen and stored at -80º (Yeoh et al. 2007; Stansborough et al. 2017).

5.3.3 RNA isolation and reverse transcription

RNA isolation was performed using the Nucleospin® RNA II kit (Macherey-Nagel, Germany) according to manufacturers' protocol. Briefly, 15-20 mg of jejunal and colonic tissue was homogenized using the TissueLyser LT (Qiagen) and Nucleospin® RNA II kit reagents (Macherey-Nagel, Germany). The lysate was filtered through silica membrane filter columns, desalted, DNA was digested using the DNAse reaction mixture, and the silica membrane was washed and dried. RNA was eluted in 60 µl RNase-free water. The TAKE3 plate and Synergy MyReader (BioTek) was used to determine RNA concentration and purity. RNA (1 µg) was converted to cDNA using the iScript cDNA synthesis (Bio-Rad, USA) according to manufacturers' protocol. The TAKE3 plate and Synergy MyReader was again used to determine total cDNA concentration and purity and stock cDNA was diluted to 100 ng/µl with nuclease-free water.

5.3.4 Real-time polymerase chain reaction (RT-PCR)

RT-PCR was performed using the Rotor Gene 3000 (Corbett Research, Sydney, Australia) according to manufacturer's protocol. Reaction mixtures contained 1 µl of cDNA, 5 µl of SYBR green (Applied Biosystems, Foster City, CA), 5 µl of nuclease-free water, and 0.5 µl each of 50 pmol/µl forward and reverse primers (Table 1). Thermal cycling conditions were as described; denaturation step at 95°C for 10 min, and included 40 cycles at 95°C for 10 s, annealing at 60ºC (VWF, TGFβ, and MMP-2), or 55ºC (VEGF and MMP-9), for 15 s, and extension and acquiring at 72°C for 20 s. 18S was used as the housekeeping gene. Primer efficiency was calculated to be between 90-110% for each target gene against 18S, and thus the $2^{-\Delta\Delta CT}$ method was used to determine fold change in gene expression relative to the housekeeping gene and sham irradiated controls from each time point.

5.3.5 Immunohistochemistry (IHC)

Angiostatin and endostatin proteins were visualised by IHC staining in the colon at 3, 6, and 15 week time points. A Dako automated staining system (Autostainer link 48 and PT Link, Dako, Denmark) was used with the EnVision™ FLEX kit for Dako autostainer link systems (Dako, Denmark) as previously described (Stansborough et al. 2017). The anti-angiostatin primary antibody (#ab2904, Abcam), and anti-endostatin primary antibody (#GTX37706, GeneTex), were used at 6.66 µl/ml and 20 µl/ml, respectively. Slides were scanned using the Nanozoomer (Hamamatsu Photonics, Japan) at 40x magnification. ImageScope imaging software (Leica Biosystems, Germany) was used to analyse angiostatin and endostatin in which percentage of positively stained cells was calculated in the lamina propria of each section (averaged from ten randomly selected 40x sections per slide).

5.3.6 Isolation of tumour-associated microvascular endothelial cells (TAMECs)

Tumour-associated microvascular endothelial cells were isolated and cultured as previously described (Bateman et al. 2013). Briefly, Dark Agouti (DA) rats were injected subcutaneously with DA mammary adenocarcinoma cells, and 8 days following, tumours were removed and processed under sterile conditions. Surrounding connective tissue was removed and tissue was mechanically disrupted into 1 mm fragments. The tissue was enzymatically digested in collagenase/dispase (Sigma), and incubated with 0.25% trypsin/EDTA (Bateman et al. 2013). Following this, the tissue was centrifuged and resuspended in complete growth medium, consisting of supplemented Dulbecco's Modified Eagle Media (DMEM) (28% HEPES buffer, 10% foetal bovine serum (FBS), and 1% L-glutamine, penicillin, streptomycin) and filtered through a 100 µm nylon cell strainer (Corning). The resultant suspension was added to gelatinised culture flasks and incubated at 37ºC. Tumour-conditioned medium (TCM) was

added to the TAMECs every 24-48 h to maintain the endothelial cell culture. Endothelial culture was confirmed using immunostaining for endothelial cell markers, as previously described (Bateman et al. 2013).

Table 1. 18S, VEGF, TGFβ, VWF, MMP-2, and MMP-9 primer sequences

Gene	Primer sequence (5'-3')	Nucleotide Position	Amplicon length (bp)	Tm (°C)	Ref
18S	**F** CATTCGAACGTCTGCCCTAT **R** GTTTCTCAGGCTCCCTCTCC	344-452	109	60 60	(Uchida et al. 2010)
VEGF	**F** AGGCGAGGCAGCTTGAGTTA **R** CTGTCGACGGTGACGATGGT	1601-1766	166	62 64	(Zhang, M et al. 2014)
TGFβ	**F** ATGACATGAACCGACCCTTC **R** ACTTCCAACCCAGGTCCTTC	897-1073	177	60 60	(Close, Gumuscu & Reh 2005)
VWF	**F** GCCTCTACCAGTGAGGTTTTGAAG **R** ATCTCATCTCTTCTCTGCTCCAGC	4292-4587	296	63 63	(Boerma et al. 2004)
MMP-2	**F** CTG ATA ACC TGG ATG CAG TGCT **R** CCA GCC AGT CCG ATT TGA	2138-2272	135	55 50	(Al-Dasooqi et al. 2010)
MMP-9	**F** AAG CCT TGG TGT GGC ACG AC **R** TGG AAA TAC GCA GGG TTT GC	760-876	117	56 52	(Vikman, Ansar & Edvinsson 2007)

5.3.7 Treatment of TAMECs

TAMECs at passages 1-2 were detached with trypsin, pelleted, counted, diluted to 1 x 10^9 cells/mL, and split evenly across 4 x 50 ml falcon tubes to represent the four treatment groups (vehicle control, SB-3CT alone, irradiation alone, and irradiation and SB-3CT combined). Cells were then treated for 24 hours with either 0.01% DMSO in H20 (vehicle control), or 1 µm SB-3CT. SB-3CT concentration was determined by dose response assays (data not shown) and was determined to be within the K_i range of inhibiting MMP-2 and MMP-9 (K_i = 14 nM and K_i = 600 nM, respectively), without inhibiting MMP-1, -3, or -7 (K_i = 206 µM, K_i = 15 µM, K_i = 96 µM, respectively) (Brown et al. 2000). Following this, falcon tubes containing the cells were either sham irradiated, or received 12 Gy gamma irradiation using a Cs^{137} source (IBL-437 Blood Irradiator). Cells were immediately plated according to individual experimental conditions. An XTT assay was used to conduct a radiation dose response assay and was based on an initial dose range of 0-12 Gy. 12 Gy resulted in a significant decrease in cell viability without causing excessive cell death (data not shown). Dosimetry was calculated prior to radiation and based on dose rate calculations, calibrated monthly based on degradation constant.

5.3.8 Protein and RNA extraction from TAMECs

Following irradiation and/or SB-3CT pre-treatment, TAMECs were seeded into 6-well plates at a cell density of 1 x 10^5 cells/well, and incubated for 24 h. Following incubation, the media was collected and immediately placed on ice. Cells were pelleted and supernatant collected and stored at -80ºC. Lysis buffer (Macherey-Nagel RNA kit) was added to each well. Wells were scraped and cells and buffer were collected and disrupted using a 26-gauge needle. β-

mercaptoethanol (3.5 µl) was added to each tube, vortexed, and further RNA extraction and cDNA conversion was performed according to kit protocols, as described earlier.

5.3.9 MMP 2 and 9 activity assays

MMP-2 and -9 activity was assessed in supernatant collected from treatment TAMECs, using MMP-2 and -9 activity assay kits (QuickZyme). Experiments were conducted according to kit protocol. Briefly, duplicate diluted samples (1:2 dilution with assay buffer), serially diluted standards, and assay buffer controls, were pipetted into the assay plate. The plate was covered and incubated at 4ºC overnight. The wells were aspirated and washed with assay buffer, and 50 µl of P-Aminophenyl mercuric acetate (APMA) solution (0.5 mM APMA in 0.05% DMSO/assay buffer) was added to wells containing standards, and to one of the two duplicate sample wells. Assay buffer was added to the remaining sample wells, and detection reagent was added to all wells. The plate was shaken for 20 s and read at 405 nm to obtain the baseline value, repeated at 6 and 22 h. Data was analysed by subtracting the average blank value from each sample value, creating a standard curve based on the 6 h data and plotting the standard concentration against the blank subtracted standard values, applying a best-fit curve, and calculating the sample concentrations graphically.

5.3.10 XTT Assay

In preparation for XTT assay, $1x10^5$ treated TAMECs were passaged intro 96-well flasks and were left to adhere for 24 h. Following adhering, XTT was performed according to kit protocol (Cell Proliferation Kit II (XTT, Sigma), with labelling and electron coupling reagents being prepared immediately before removing media from wells, replacing with 100 µl fresh media, and adding 50 µl per well of the prepared XTT reagent. The plates were incubated for 6 h and the plate was read at 490 nm using the TAKE3 plate and Synergy MyReader.

5.3.11 Immunocytochemistry (ICC)

Prior to experimentation of TAMECs, expression of VEGF, VWF, TGFβ, MMP-2, and MMP-9 under normal conditions was confirmed using immunocytochemistry (Supplementary Figure 1). Briefly, treated TAMECs were passaged into 8-well chamber slides at $2x10^5$ cells/well and left to adhere for 24 h. Following this, cells were fixed with 4% paraformaldehyde for 30 minutes at 4ºC, washed twice with PBS, incubated with 3% H2O2 in PBS for 5 min to reduce non-specific staining, washed, and incubated with Triton-X block (100 µl TX-100 and 0.1 g sodium citrate in 100 mL H20) for 8 min to permeate the cell membrane. The cells were washed and blocked using the normal serum blocking reagent (Level 2 USA™ Ultra Streptavidin Detection System kit, Signet Laboratories). Primary antibodies (MMP-2; #ab37150, Abcam, MMP-9; #ab58803, Abcam, VEGF; #ab46154, Abcam, VWF; #ab6994, Abcam, TGFβ; #ab92486, Abcam) were prepared at previously optimised concentrations of 2 µl/ml (VWF & MMP-2), 4 µl/ml (VEGF & MMP-9), and 10 µl/ml (TGFβ), and added to each well for 60 min. Cells were washed and a linking reagent (Level 2 USA™ Ultra Streptavidin Detection System kit, Signet Laboratories) was applied for 20 min. A peroxidase-labelled ultra-streptavidin labelling reagent (Signet Laboratories) was applied for 20 min, followed by a 1 min incubation with diaminobenzidine (DAB) chromogen in 0.03% hydrogen peroxidase (Signet Laboratories) for visualization. Cells were washed, chambers were removed, and slides were counterstained with Lillie Meyers haematoxylin (1:10), washed, dehydrated, and coverslipped.

5.3.12 Statistical Analysis

Graphpad Prism (version 6) software was used to perform statistical analysis. Normality was determined and differences between study group means were analysed using either a one-way

or two-way ANOVA with Sidak's multiple comparison, or a Kruskal-Wallis test with multiple comparison. Asterisks denote significance compared to control at the time point, where * <0.05, ** <0.01, *** <0.001, and **** <0.0001. Bars on graphs represent standard error of the mean.

5.4 Results

5.4.1 Fractionated irradiation induced changes in weight gain and histopathological damage

Clinical and histological response to fractionated irradiation for this study has been previously published (Stansborough et al. 2017). Briefly, rats receiving fractionated irradiation had significantly lower weight gain at week 3 ($p = 0.0055$), and from week 6 until the completion of the study ($p < 0.05$), when compared to sham irradiated controls (Stansborough et al. 2017). Histopathological damage scoring was significantly increased in the colon, but not in the jejunum, at week 3 ($p = 0.043$). However, thickening of the lamina propria, telangiectatic vessel formation, and apoptosis were observed in both the jejunum and colon following irradiation (Stansborough et al. 2017).

5.4.2 VEGF and TGFβ were significantly increased in the colon following irradiation in DA rats

Despite no significance effect in the jejunum, VEGF mRNA expression was significantly increased in the colon at the 15-week time point ($p = 0.0012$) (Figure 1A+B). TGFβ mRNA expression significantly increased in both the jejunum and colon 3 weeks into the radiation schedule ($p = 0.0280$ and $p = 0.0310$, respectively), however was not significantly increased at 6- or 15-weeks in either region (Figure 1C+D). VWF mRNA expression was not significantly altered at any time point following fractionated irradiation in jejunum or colon (Figure 1E + 1F).

5.4.3 Angiostatin and endostatin immunostaining was significantly increased in the colon following fractionated irradiation

Endostatin was immunostaining significantly increased in the lamina propria of the colon at the 3-week time point only ($p = 0.0046$) (Figure 2A + 2C). Angiostatin immunostaining was significantly increased at 3- and 6-weeks in the colon lamina propria ($p = 0.0022$, and $p = 0.0135$, respectively) (Figure 2B + 2C). There were no significant alterations at week 6 or 15.

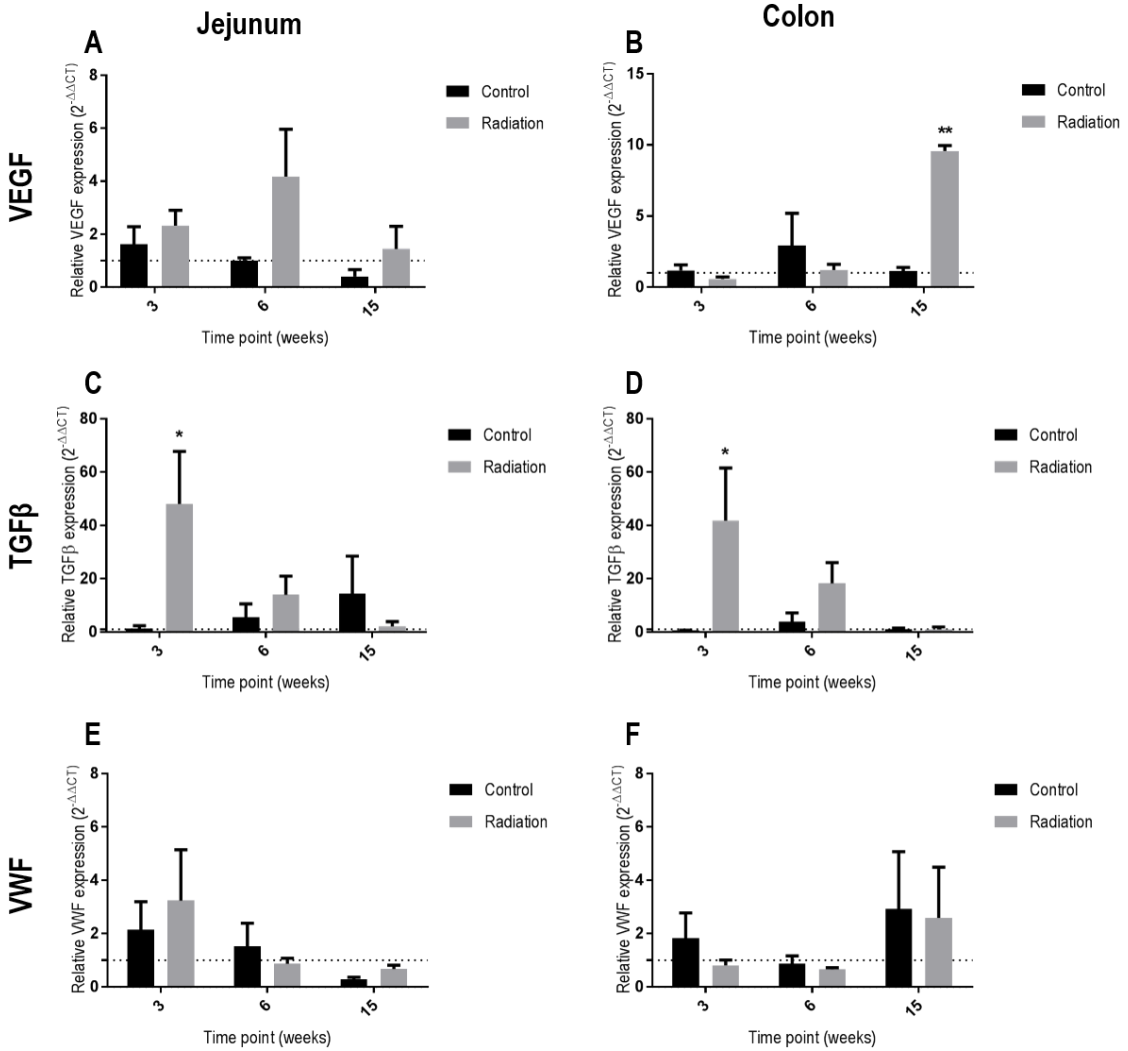


Figure 1. *VEGF, TGFβ, and VWF expression in the jejunum and colon following fractionated radiation*. **A** VEGF mRNA expression in the jejunum following fractionated irradiation **B** VEGF mRNA expression in the colon following fractionated irradiation **C** TGFβ mRNA expression in the jejunum fractionated irradiation. **D** TGFβ expression in the colon following fractionated irradiation. **E** Relative mRNA expression of VWF in the jejunum following fractionated irradiation **F** VWF mRNA expression in the colon. Dotted line represents baseline fold change. * = significance compared to control. * <0.05, ** <0.01, n=5. Data = mean + standard error of the mean (SEM).

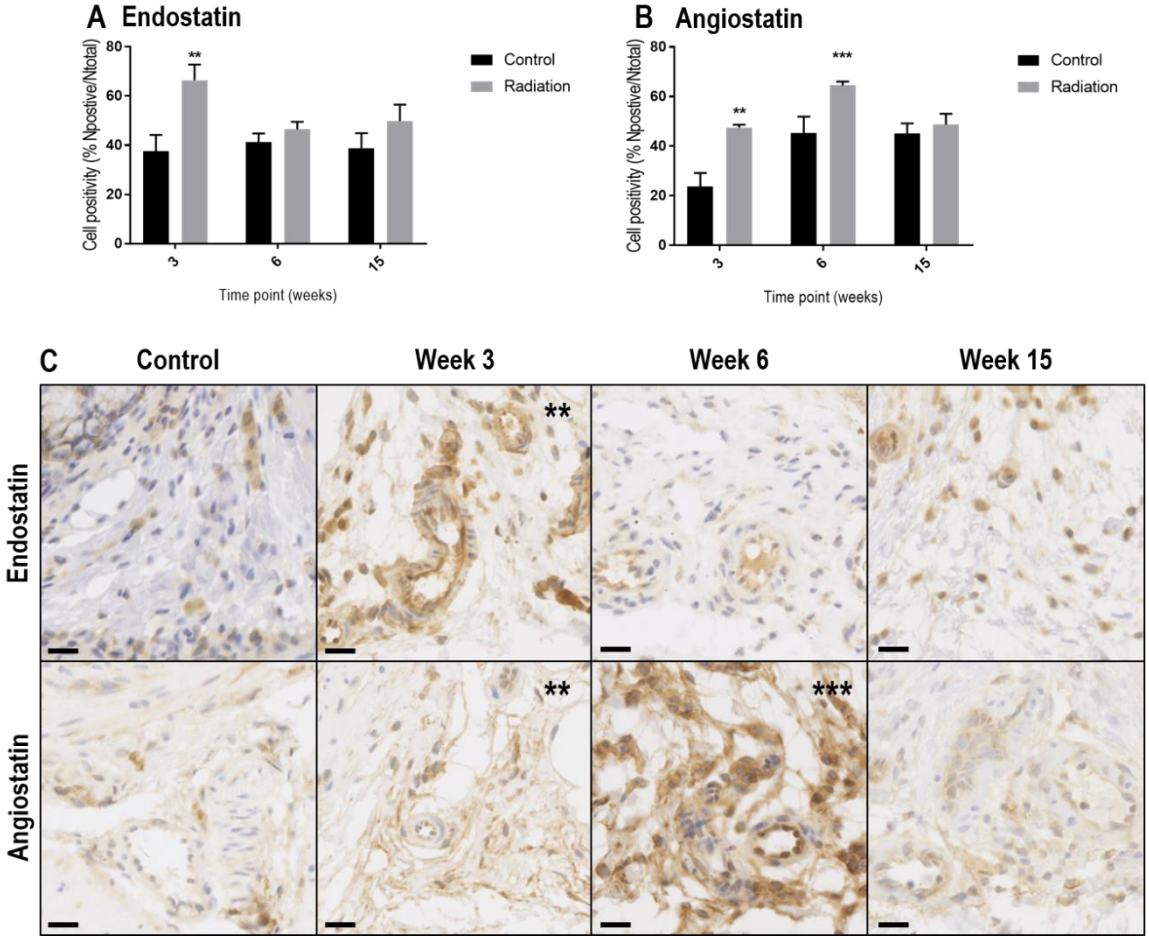


Figure 2. *Endostatin (**A**) and angiostatin (**B**) immunostaining in the lamina propria of the colon following fractionated irradiation.* Cell positivity was calculated using ImageScope software and is determined as number of positively stained cells/number of total cells. * denotes significance compared to controls at the same time-points, ** <0.01, *** <0.001. Data = mean + SEM, n=5. Scale bars = 20 µm, 40x original magnification.

5.4.4 SB-3CT significantly attenuated the expression of MMP-9 following irradiation of TAMECs

Relative mRNA expression of MMP-2 was not significantly altered following irradiation of TAMECs when compared to controls (p = ns; Figure 3A). However, TAMEC MMP-2 expression following irradiation was significantly reduced when pre-treated with SB-3CT, as compared to the radiation only group (p = 0.0034) (Figure 3A). MMP-9 mRNA expression was significantly increased following irradiation of TAMECs (0.0022). This was significantly attenuated by SB-3CT (p < 0.0001). Despite this attenuation of MMP-2 and -9, SB-3CT did not significantly alter cell viability following irradiation, compared to radiation alone (p = ns; figure 5).

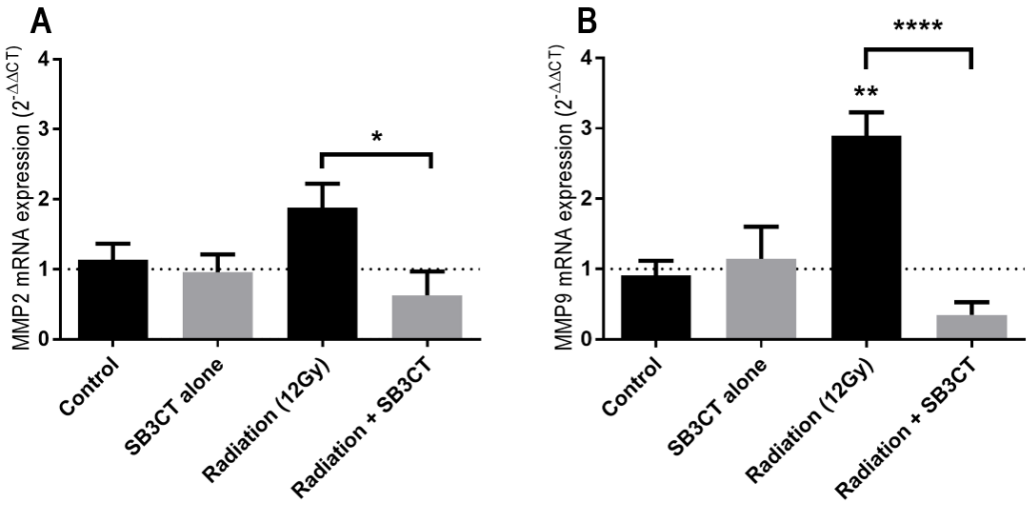


Figure 3. *MMP-2 (**A**) and -9 (**B**) mRNA expression following irradiation of TAMECs, with/without SB-3CT*. Dotted line represents baseline fold change. * = significance compared to control. * <0.05, ** <0.01, **** <0.0001. Data = mean + SEM. n=12

5.4.5 SB-3CT significantly attenuated MMP-2 and -9 protein expression following irradiation

Total protein levels of both MMP-2 and MMP-9 were significantly increased in TAMEC supernatant following 12 Gy irradiation ($p = 0.046$, and $p = 0.0401$, respectively) (Figure 4A and 4C). This increase in total MMP-2 and -9 protein levels was significantly attenuated by the administration of SB-3CT ($p = 0.0119$, and $p = 0.0209$, respectively). The ratio of active: total MMP-2 and -9 were not significantly altered following irradiation of TAMECs. However the ratio of active: total MMP-2 was significantly increased in the radiation + SB-3CT group, compared to control ($p = 0.0379$). The ratio of active: total MMP-9 was not significantly altered in the radiation + SB-3CT group when compared to all other groups ($p =$

ns; Figure 4D).

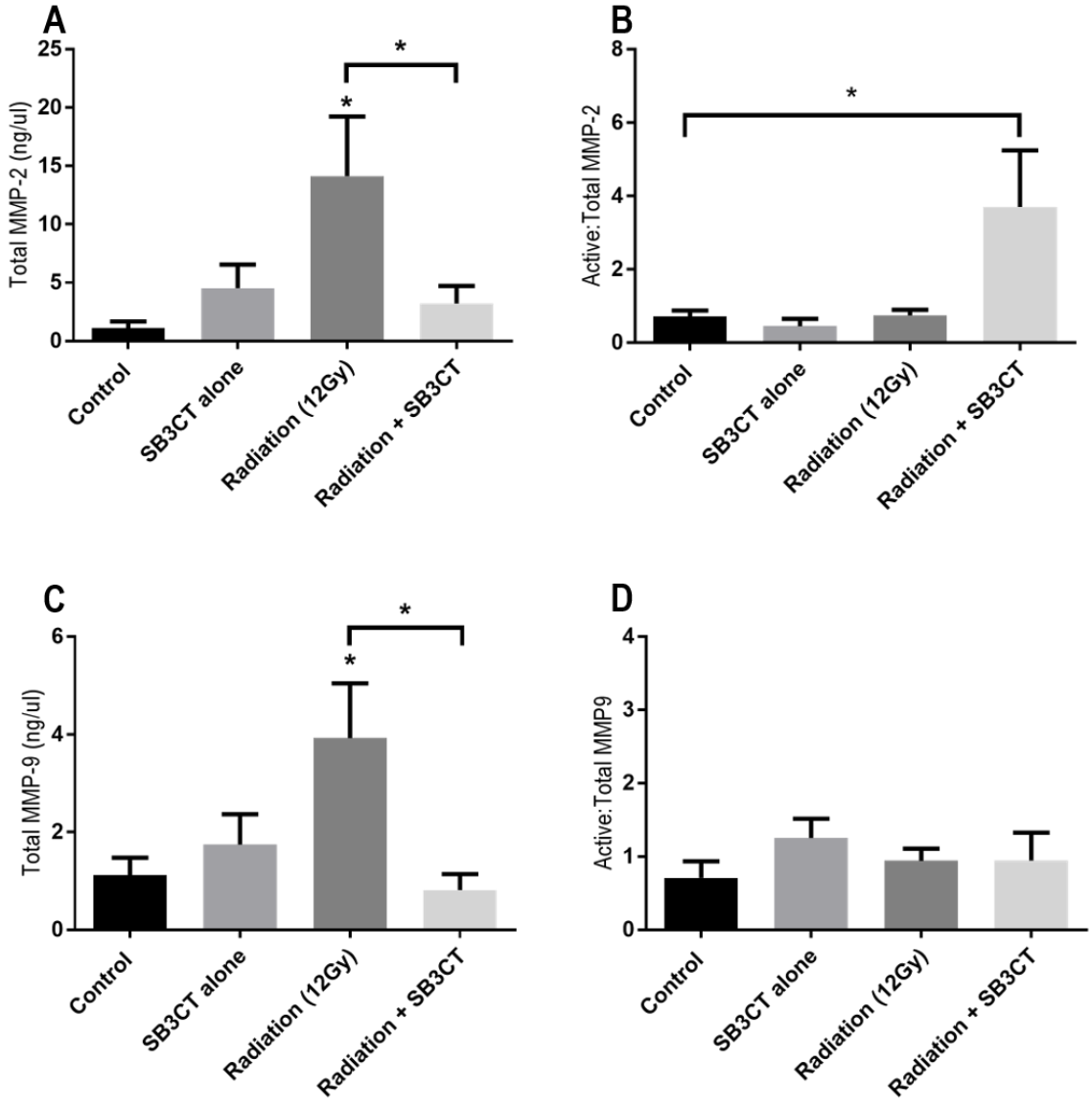


Figure 4. *Total and active: total ratio of secreted MMP-2 and -9 protein concentration following irradiation and MMP inhibition in TAMECs.* **A** Total MMP-2 protein concentration **B** Ratio of active: total MMP-2 protein levels **C** Total MMP-9 protein concentration **D** Ratio of active: total MMP-9 protein levels. * = significance compared to control. * <0.05. Data = mean + SEM. n = 12

5.4.6 SB-3CT did not alter tumour-associated microvascular endothelial cell viability following irradiation

SB-3CT alone did not significantly alter cell viability; as measured via XTT assay; both irradiation (12 Gy), and irradiation + SB-3CT significantly reduced cell viability of TAMECs ($p = 0.0236$, and $p = 0.0003$, respectively) (Figure 5). There was no significant difference between the radiation alone, and radiation + SB-3CT groups ($p = ns$; Figure 5).

5.4.7 VEGF and TGFβ mRNA expression were not significantly altered following irradiation of TAMECs

RT-PCR was used to assess relative mRNA expression of VEGF and TGFβ following irradiation and MMP inhibition in TAMECs. VEGF and TGFβ mRNA expression were unaltered by both irradiation and SB-3CT treatment (Figure 6).

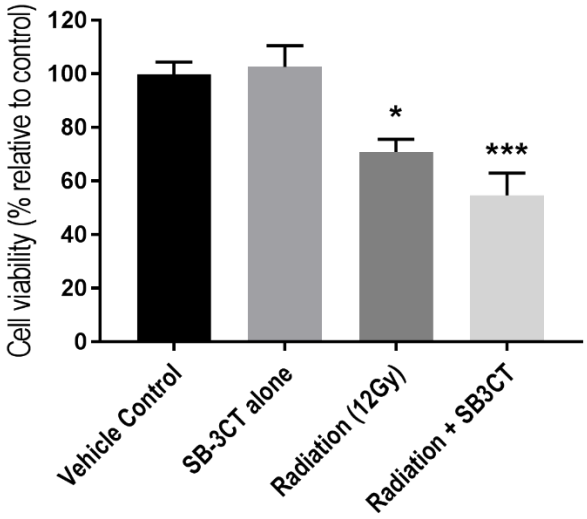


Figure 5. *Cell viability of TAMECs following irradiation and MMP inhibition.* * = significance compared to control. * <0.05, *** <0.001. Data = mean + SEM. *n = 8*

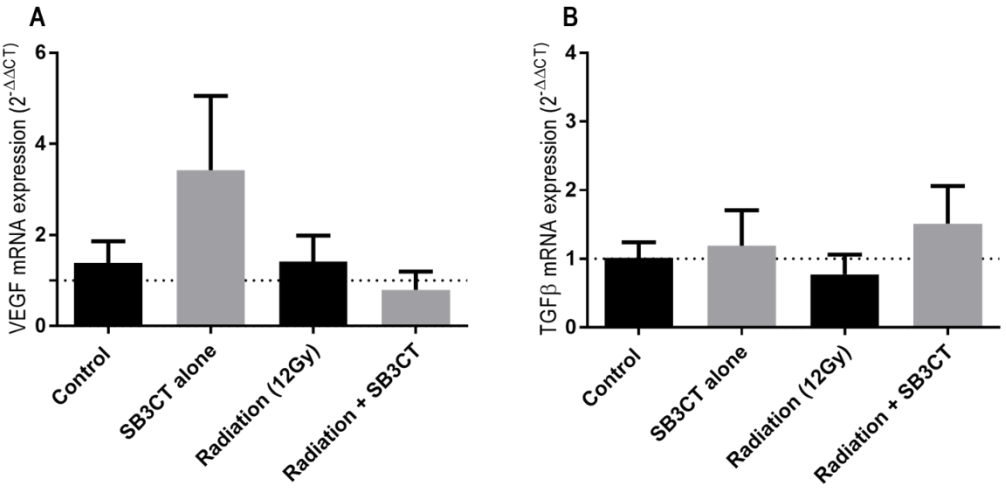


Figure 6. *VEGF and TGFβ mRNA expression following irradiation of TAMECs with or without SB-3CT.* VEGF (**A**) and TGFβ (**B**) mRNA expression were not significantly altered following irradiation of TAMECs, with or without the presence of SB-3CT. Dotted line represents baseline fold change. Data = mean + SEM. n = 12

5.5 Discussion

Although the pathobiology of RIGT is complex, MMPs, the intestinal microvasculature, and associated vascular mediators have been shown to be involved (Stansborough et al. 2016). Key findings from this study show mRNA expression of VEGF and TGFβ mRNA expression to be significantly increased in the intestines following RIGT, and is the first study to show an increase in angiostatin and endostatin during RIGT. However, the pattern of upregulation of these mediators was different, occurring at different time points and in different intestinal regions. Further, this study assessed the effect of the MMP-2 and -9 inhibitor, SB-3CT, on tumour-associated microvascular endothelial cells (TAMECs) following irradiation. Both mRNA and protein expression of MMP-2 and -9 were significantly upregulated in TAMECs following irradiation, however irradiation did not significantly alter VEGF or TGFβ expression in these cells. SB-3CTsignificantly attenuated MMP-2 and -9 mRNA and protein expression, however, it did not alter cell viability of TAMECs following irradiation.

VEGF mRNA expression was significantly increased in the colon at the 15 week time point of the DA rat RIGT model. VEGF may have a protective role in gastrointestinal injury, with higher levels of VEGF expression correlating with less severe gastric ulcers in murine models of stress-induced gastric ulceration (Malara et al. 2005). VEGF has also been shown to regulate normal vasculature in the small intestinal villi, with excessive inhibition resulting in regression of normal microvasculature, and reduced vascular density (Howdieshell et al. 2001; Saif, Elfiky & Salem 2007; Pollom et al. 2015). Clinically, the inhibition of VEGF has been shown to delay healing of gastric erosions, reduce vascular density in the small intestinal villi, and induce epithelial ulceration, supporting a role for VEGF in healing (Mangoni et al. 2012; Pollom et al. 2015). This regulatory role could be consistent with the timing of VEGF

upregulation seen in the present study, occurring at the 15 week time-point in the colon only. At this time point, 9 weeks following the last radiation dose, we have clearly documented and reported on a transition from acute to chronic RIGT phenotype which is able to be seen histologically (Stansborough et al. 2017). This effect is likely due to alteration of the wound healing process, as well as vascular changes resulting in impaired delivery of healing agents to the damaged tissue (Mangoni et al. 2012). This study, in combination with findings of previous literature, suggests a role for VEGF in the progression to chronic RIGT, possibly playing a part in wound healing following acute RIGT. This potential role of VEGF in RIGT needs to be further explored, particularly with the increased study of anti-VEGF as adjuvant cancer therapies (Barney et al. 2013; Pollom et al. 2015). When VEGF inhibitors (VEGFIs) are combined with radiotherapy, gastrointestinal toxicity has been shown to significantly increase (Barney et al. 2013; Pollom et al. 2015). This potential for increased intestinal toxicity needs to be considered when determining the clinical potential of these agents.

Whilst TGFβ expression was significantly increased 3 weeks into the fractionated radiation schedule in our rat model of RIGT, consistent with previous literature, it was not significantly upregulated at 6 and 15 weeks, nor was it altered following irradiation of TAMECs (Richter et al. 1998; Wang, J, Zheng & Hauer-Jensen 2001). The lack of increase in TGFβ at the chronic RIGT time point was somewhat unexpected and in contrast with previous literature (Kleifeld et al. 2001). This may be explained by the different models used in the study by Wang and colleagues (2001, in which a scrotal hernia is surgically induced and the fractionated schedule uses higher doses over a shorter period of time (Wang, J, Zheng & Hauer-Jensen 2001). Our previous studies have shown fibrotic changes to the lamina propria and microvasculature at both the 6 and 15 week time points (Stansborough et al. 2017). With the complex interactions of TGFβ it is possible that the early upregulation of TGFβ, or

alterations in the receptors of TGFβ are involved in these fibrotic changes. It is also unclear what the potential consequences of TGFβ involvement in RIGT may be, with TGFβ being capable of opposing angiogenic effects, highly dependent on conditions and tightly regulated in the endothelium by the receptors ALK1 and ALK5 (Goumans et al. 2003). Whilst TGFβ binding with ALK5 has an anti-angiogenic effect, inhibiting endothelial cell migration and proliferation, ALK1 binding has the opposite effect (Goumans et al. 2003). Thus the ratio of ALK5 to ALK1 seems to be key to the functional role of TGFβ in pathological conditions. Further studies, both *in vitro* and *in vivo*, are needed to investigate the ALK5 to ALK1 ratio and determine where this balance lies in endothelial response to irradiation, and during RIGT, in order to determine the role of TGFβ in its pathobiology.

Angiostatin and endostatin immunostaining was shown to be significantly elevated in the lamina propria of both the jejunum and colon. This occurred only during the acute time points, with angiostatin increasing at 3 and 6 weeks, and endostatin at 3 weeks. The anti-angiogenic mediators angiostatin and endostatin, fragments of plasmin and type XVIII collagen, respectively, are responsible for inhibiting endothelial cell proliferation and migration, as well as inducing apoptosis in the case of endostatin (Ribatti 2009). Endostatin is also known to directly inhibit MMP-2 (Abdollahi et al. 2004). Although this current study is the first to assess angiostatin and endostatin in an animal model of RIGT, response of angiostatin to radiation has previously been assessed *in vitro* (14). Oh and colleagues (2014) finding angiostatin protein levels to be significantly increased in human breast tissue derived cancer endothelial cells following 4 Gy irradiation (Oh et al. 2014). Whilst endostatin has not yet been investigated in RIGT, Endostar, a recombinant human endostatin, has been shown to significantly decrease MMP-2, -9, -14, and VEGF immunostaining in nasopharyngeal carcinoma xenografts in mice following 6 Gy irradiation (Peng et al. 2012). Although the

roles of angiostatin and endostatin likely differ in normal vs tumour tissue response to radiation, these studies, in combination with the present study, suggest a role of these anti-angiogenic mediators in radiation response, likely linked to the MMP pathway.

MMP-2 and -9 mRNA and protein expression was significantly increased following irradiation of TAMECs. The significant increase in MMP-2 expression is consistent with previous studies finding an increase in MMP-2 24 hours following 4 Gy irradiation of human normal endothelial cells and TAMECS (NECs and CECs, respectively) derived from breast tissue (Oh et al. 2014). However, the same study found MMP-9 not to be altered by irradiation of TAMECs in contrast to the findings of our current study (Oh et al. 2014). This is not entirely unexpected, due to species and tissue differences between the two studies, with MMP-9 regulation known to depend on cell type (Vincenti & Brinckerhoff 2007). Despite these conflicting results, the similarities between the studies highlight a role for MMP-2, in particular, in endothelial regulation following irradiation.

The increase in MMP-2 and -9 expression was also significantly attenuated, both at the mRNA and protein level, by the pre-treatment of TAMECs with SB-3CT. SB-3CT is the first mechanism-based MMP inhibitor, potently and selectively inhibiting MMP-2 and -9. The effect of SB-3CT is unique in its selectivity, as it forms a reactive species within the active sites of MMP-2 and -9 (Kruger et al. 2005) and was developed in response to a lack of efficacy of broad-spectrum, synthetic MMP inhibitors when used as anti-cancer therapies in clinical trials.

An unexpected finding in the present study was the significant decrease in mRNA expression of MMP-2 and MMP-9 following pre-treatment with SB-3CT in TAMECs following irradiation, as the mechanism of action of SB-3CT is in inhibiting activation. Additionally,

SB-3CT significantly increased MMP-2 activity in TAMECs when combined with irradiation, having no effect on activity of MMP-9. The significant decrease in proMMP expression by SB-3CT is not unprecedented, with SB-3CT significantly decreasing proMMP-9 levels in a mouse model of transient focal cerebral ischemia (37). The study hypothesized this decrease was likely due to a positive feedback mechanism between MMP activity and gene transcription (Gu et al. 2005).

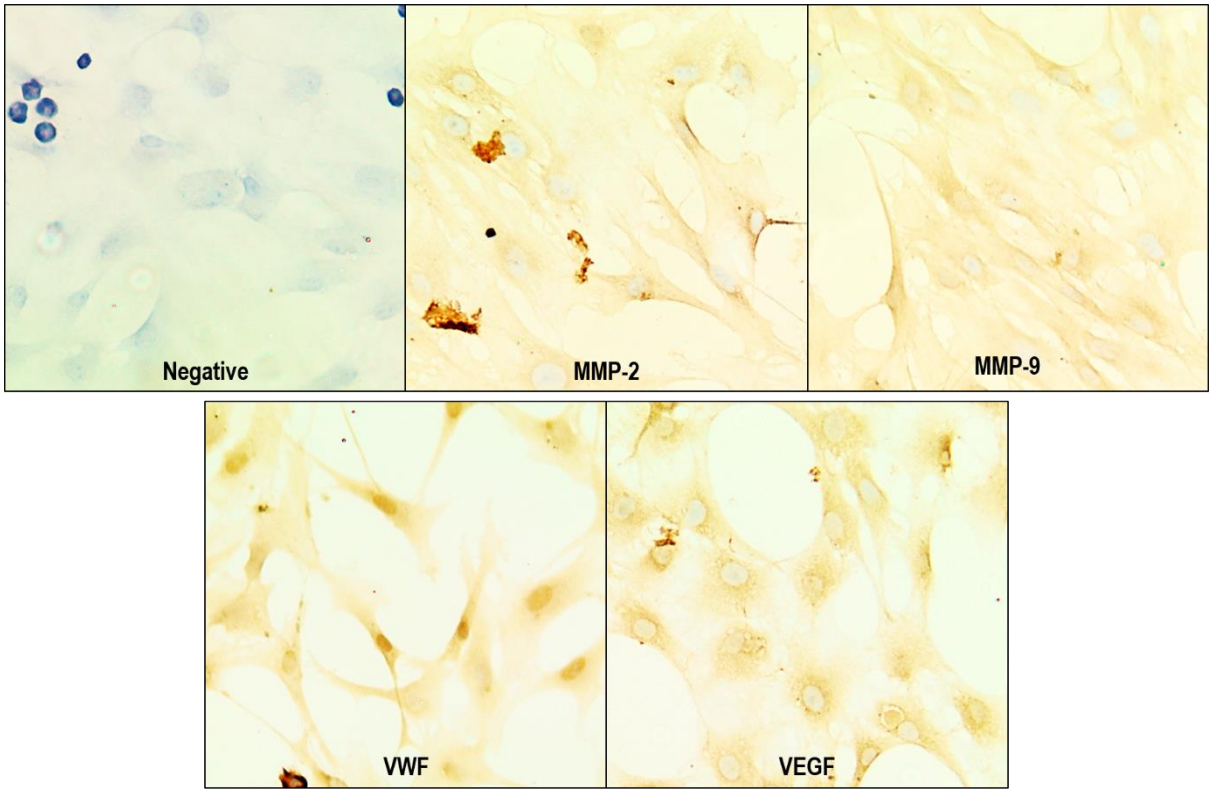
To our knowledge, this is the first study to assess mRNA expression of MMP-2 and -9 following SB-3CT administration. There are endogenous mediators that can be regulated by active MMP-2 and -9, such as TGFβ, and pharmacological reagents, such as doxycycline, that have been shown to regulate the mRNA expression of MMP-2 and -9 by altering mRNA stability (Yan & Boyd 2007). Whether SB-3CT is regulating transcription via a similar process, by affecting the promotor regions of these MMPs, or through disruption of a positive feedback mechanism, is unclear. The regulation of mRNA expression of both MMP-2 and MMP-9 by SB-3CT should be investigated further and current studies are underway in our laboratory.

The use of a single cell type in this study is a limitation as normal and tumour endothelial cells are phenotypically different and subsequently respond differently to irradiation; therefore it is likely that the response of normal gut endothelial to SB-3CT would differ to the findings of the present study (Oh et al. 2014). Thus, in order to further assess the possible effects of MMP inhibition in RIGT, future studies assessing SB-3CT in an *in vivo* model of RIGT are warranted. However, the findings of this study highlight a relationship between radiation, MMP expression and tumour endothelium. Due to the potential use of specific MMP inhibitors as cancer therapies, and with the involvement of both MMP's and the

endothelium in the development and progression of RIGT, the findings of this study highlight the need for further research and consideration as to possible dual effects of MMP inhibitors in this setting (Strup-Perrot et al. 2006; Angenete et al. 2009; Stansborough et al. 2017).

5.6 Conclusion

The findings of this study support a role for the vascular mediators VEGF, TGFβ, angiostatin, and endostatin in RIGT following fractionated irradiation. Additionally, MMP-2 and -9 were shown to be involved in endothelial response to radiation *in vitro,* however inhibition by SB-3CT did not alter cell viability. The findings of the *in vitro* component of the present study elucidate the effects of SB-3CT on the endothelium, with unexpected findings of significantly inhibited proMMP expression, but not active MMP expression, and confirm the role of MMP-2 in particular in the endothelial response to irradiation. These results highlight the need for further pre-clinical studies to assess the effect of MMP inhibitors and anti-angiogenic drugs in RIGT, particularly as these drugs are currently being investigated as adjuvant cancer therapies.



Supplementary Figure 1. Conformational staining of MMP-2, MMP-9, VWF, and VEGF in tumour-associated microvascular endothelial cells (TAMECs). 100x magnification

Chapter 6 General Discussion

6.1 Introduction

RIGT encompasses both acute, and chronic, intestinal toxicity following radiotherapy for cancer, with symptoms developing in 33-72% of patients during, or following radiotherapy, depending on target area and treatment regime (Roszak et al. 2012; Kim, Huh & Park 2013; Shadad et al. 2013b). RIGT incorporates the previously defined acute radiation enteropathy, chronic radiation enteropathy, radiation enteritis, radiation proctitis, and radiation-induced gastrointestinal mucositis (Theis et al. 2010). This thesis explored new facets of the pathobiology of RIGT, clarifying and expanding on the existing understanding of the role of intestinal microvasculature, vascular mediators, and matrix metalloproteinases (MMPs) (Paris et al. 2001; Wang, J et al. 2002; Strup-Perrot et al. 2006; Stansborough et al. 2016).

Throughout this thesis I aimed to assess each of these components, beginning with changes to the intestinal microvasculature (chapter 3), expression of MMPs (chapter 4), and expression of vascular mediators (chapter 5) in a well-established *in vivo* model of fractionated radiation-induced gastrointestinal toxicity. My studies were the first to show apoptosis and cytostasis in the intestinal microvasculature in a fractionated radiation model of RIGT, which preceded peak epithelial damage, and telangiectasis and fibrotic thickening of the microvasculature (Stansborough et al. 2017). I then assessed MMPs and found MMP-2 was associated with these changes, with increases in both mRNA and protein expression occurring during RIGT. Consistent with my hypothesised link between MMPs, vascular mediators, and subsequent vascular modification in RIGT (chapter 2), I also found VEGF, TGFβ, angiostatin, and endostatin, were significantly increased in the same model (chapter 5). Finally, I developed a novel *in vitro* model of endothelial response to irradiation to assess the effect of MMP

inhibition on endothelial cell viability, MMP expression, and the subsequent expression of VEGF and TGFβ. Using this model I demonstrated irradiation of tumour-associated microvascular endothelial cells (TAMECs) significantly upregulated both MMP-2 and MMP-9, suggesting a direct effect of radiation on endothelial regulation of MMPs. Whilst SB-3CT did significantly attenuate their upregulation and translation of MMP-2 and -9, it did not significantly alter TAMEC viability. Additionally, VEGF and TGFβ were not significantly upregulated following irradiation in the *in vitro* model, possibly due to the required involvement of other tissue components, not present in the single cell model.

6.2 Intestinal Microvasculature in RIGT

Previous research has shown an involvement of the intestinal microvasculature in the development of RIGT, however, to date the interpretation of the findings of these studies has been controversial (Richter et al. 1998; Paris et al. 2001; Gaugler, M-H et al. 2007). Paris et al. (2001) reported endothelial apoptosis occurs hours following irradiation in a mouse model of GI syndrome, in which extensive intestinal damage is caused by high-dose of radiation. Despite this finding, endothelial response to the large doses of irradiation in GI syndrome may not be consistent with that during RIGT, in which radiotherapy is typically administered in a fractionated schedule consisting of smaller doses over time. Since the Paris (2001) study, findings of endothelial involvement in RIGT have been conflicting, with some studies finding no endothelial apoptosis in GI syndrome (Schuller et al. 2007; Kirsch et al. 2010), and another finding endothelial apoptosis to occur within hours of intestinal irradiation in mice (Abdderahmani et al 2012). Key findings in this thesis (chapter 3) support the role of endothelial apoptosis in RIGT, with apoptosis of small and large intestinal microvascular cells occurring in the acute (24 hours following last radiation dose of a 3 or 6 week fractionated

radiation schedule) and chronic (9 weeks following last radiation dose of a 6 week fractionated radiation schedule) RIGT stages, in an *in vivo* rat model of fractionated radiation-induced gastrointestinal toxicity (Stansborough et al. 2017). Cytostasis was shown to precede apoptosis of these cells in the colon and was significantly increased compared to control at the chronic point. My findings coincided with increased damage scoring in the colon, epithelial apoptosis in both the jejunum and colon, significantly reduced weight gain, and histopathological changes to the lamina propria and microvasculature, including fibrotic thickening and telangiectasis (Stansborough et al. 2017). These results reflect the clinical presentation of RIGT, with telangiectatic, fibrosed vasculature occurring in colorectal tissue following short-term fractionated pelvic irradiation or long-term neoadjuvant chemoradiotherapy for colorectal cancer (Yeoh et al. 2005). These findings of similarities between the rat model used in this study and clinical presentation of RIGT support the validity of this RIGT model for further experimental use. Taken together, my suggest changes to the intestinal microvasculature may be key drivers in the progression of acute RIGT.

Although the microvascular changes I demonstrated in chapter 3 coincided with significantly lower weight gain, the clinical implications of endothelial apoptosis and telangiectasis in RIGT require further study (Stansborough et al. 2017). Determining the transition from acute to chronic RIGT is key in developing treatments and preventative measures. This transition has previously been explained in a model of consequential late effects, in which acute toxicity directly induces chronic toxicities (Peters, Ang & Thames 1988). Although findings of chapter 3 appear to support a relationship between intestinal microvascular alteration in acute RIGT and microvascular sclerosis and telangiectasis in chronic RIGT, this link is difficult to confirm. It is possible apoptosis and cytostasis of the microvasculature in acute RIGT, as shown in chapter 3, with the altered expression of tissue factors and vascular mediators in

chapter 5, may create a pro-coagulant endothelial surface and drive the development of chronic RIGT (Denham & Hauer-Jensen 2002). The role of intestinal telangiectasis in this shift, however, is particularly unclear. Previously telangiectasis has been shown to be more common after acute radiation toxicities (Turesson et al. 1996); it seems acute inflammatory damage to microvasculature is important in the development of telangiectasia, but not in the induction of post-irradiation fibrosis (Denham & Hauer-Jensen 2002). Although the findings of chapter 3 suggest intestinal microvasculature alteration plays some role in the shift from acute to chronic RIGT, it is likely to involve several other factors. Recent literature finding mast cell deficiency, sensory-nerve ablation, and TGFβ modulation in rats produce dissociations between acute and chronic toxicity (Hauer-Jensen, Denham & Andreyev 2014). Further studies are required, to build on these findings, assessing a possible link in processes inducing microvascular changes and driving the shift from acute to chronic RIGT. In particular, *in vivo* models of RIGT assessing the inhibition of MMPs or vascular mediators such as VEGF, angiostatin and endostatin should also include assessment of intestinal microvascular changes to determine any affect of inhibition of these mediators on the shift from acute to chronic RIGT.

6.3 MMPs in RIGT

Utilizing the same model as chapter 3, in which histopathological characteristics of RIGT, particularly the response of the microvasculature, were explored, allowed me to investigate possible links between MMP alteration and RIGT development. Results of my study highlighted an involvement of MMP-2 in RIGT, having been upregulated in the jejunum and colon, both at the protein and mRNA levels following 6 weeks of fractionated irradiation. When considering the results of chapters 3 and 4, with telangiectasis, microvascular apoptosis

and cytostasis, and epithelial damage, occurring alongside an increase in MMP-2 expression, a relationship between these factors is possible. MMPs are regulated at the transcriptional level by several transcription factors, primarily including β-catenin, and NFκB. MMPs are also regulated post-transcriptionally via transforming growth factor-β (TGFβ), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and pro-inflammatory cytokines (Yan & Boyd 2007; Stansborough et al. 2016). These mediators are capable of increasing the half-life of MMP mRNAs by binding to the coding regions of the transcript and regulating mRNA stability, and possibly regulating translation efficiency via the recruitment of mRNA to membrane-bound ribosomes (Yan & Boyd 2007). It is likely this increase in expression of MMP-2 is an indirect result of the effect of radiation on transcriptional regulators, such as NFκB, and pro-inflammatory cytokines, including TNF and IL-1β (Yeoh et al. 2007). Whilst these mediators were not assessed in this thesis, they are well known to be involved in the pathogenesis of RIGT, including the same *in vivo* model of RIGT as in chapters 3-5 of this thesis (Yeoh et al. 2005; Boerma et al. 2013; Yue et al. 2015). Additionally, MMP-2 degrades the capillary basement membrane, primarily via the degradation of type IV collagen (Kalluri 2003). This allows for endothelial migration and proliferation; this function possibly contributes to the telangiectasis seen in chapter 3, particularly at the chronic time point, week 15, in which endothelial proliferation was significantly increased. Alternatively, it is possible MMP-2 may have dual functions in regulating the intestinal microvascular changes in RIGT. This may be through sequestering the anti-angiogenic mediators angiostatin and endostatin (Kalluri 2003; Tolstanova et al. 2011), increased in the same model (chapter 5), via the digestion of plasminogen and type XVIII collagen, which could contribute to endothelial cytostasis (Cauwe et al., 2007).

The demonstrated increase in both MMP-2 and MMP-14 at the transcriptional level in this thesis (chapter 4) is one which should be considered when investigating the role of MMP-2, or the inhibition of MMP-2, in future models of RIGT. This increase in both MMP-2 and -14 expression is consistent with previous findings of intestinal upregulation of these same MMPs following abdominal irradiation in rats (Strup-Perrot et al. 2005). As MMP-2 is uniquely controlled by a mechanism of enzyme activation in which MMP-2 and MMP-14 are co-regulated (Sternlicht & Werb 2001), combined with the findings of chapter 4 that both MMP-2 and -14 are upregulated in RIGT, development of specific inhibitors of MMPs for the treatment of RIGT should consider MMP-14 as a possible target.

6.4 Vascular mediators in RIGT

A link between MMP-2, shown to be significantly increased in chapter 4, and microvascular alteration, seen in chapter 3, was hypothesised in chapter 2 (Stansborough et al. 2017). Chapter 5 aimed to explore this hypothesised link, assessing the vascular mediators VEGF, TGFβ, VWF, angiostatin, and endostatin. As discussed in chapter 2, all of these vascular mediators are able to be sequestered from the ECM by MMP-2, shown to be increased in chapter 4 (Yan & Boyd 2007; Stansborough et al. 2016). mRNA expression of VEGF, TGFβ, angiostatin, and endostatin were found to be increased; primarily in the acute phases of the fractionated radiation schedule. The exception was VEGF, which was significantly increased during the chronic phase, in the colon only. These findings support the hypothesised pathway of MMP inducing microvascular alteration in RIGT via the regulation of vascular mediators (Stansborough et al. 2016). Additionally, my study was the first pre-clinical study to assess intestinal VEGF expression following fractionated irradiation. An increase during the chronic RIGT time point may suggest a role in healing, supported by evidence from the literature

which show the inhibition of VEGF to significantly enhance intestinal damage when combined with total body irradiation in mice (Mangoni et al. 2012). VEGF acts to maintain immature vasculature and enhance angiogenesis, thus it is likely an increase in VEGF weeks following the last radiotherapy dose is also contributing to telangiectasis in chronic RIGT (Carmeliet 2005). This is supported by a study by Trzcinski and colleagues (2017) in which VEGF expression significantly correlated with chronic radiation proctitis in patients who had undergone radiotherapy for prostate, cervical, or uterine cancers. Clarity is needed in the implications of VEGF inhibition on acute and chronic RIGT, and intestinal toxicity should be investigated further in future clinical trials in which VEGF inhibitors are combined with abdominal irradiation.

My study also demonstrated TGFβ was significantly increased in both the jejunum and colon early in the DA rat model of RIGT (chapter 5). TGFβ has previously been shown to increase in the small intestine following irradiation in both pre-clinical and clinical studies (Wang, J, Zheng & Hauer-Jensen 2001; Milliat et al. 2006). This increase has largely been associated with fibrotic changes occurring during chronic RIGT, rather than acute RIGT (Wang, J, Zheng & Hauer-Jensen 2001). However, this early increase is not entirely unexpected as it has been suggested ionizing radiation may directly induce TGFβ1 release via reactive oxygen species (Boerma et al. 2013). Additionally, MMP-2, coinciding with TGFβ upregulation, increases the bioavailability of TGFβ (Egeblad & Werb 2002). Both MMP-2 and -9 have been shown to proteolytically cleave latent TGFβ, sequestering its inactive extracellular complex and thus leading to the activation of TGFβ (Yu & Stamenkovic 2000). Clinically, enhanced rectal expression of TGFβ has been shown to correlate with radiation injury score, highlighting TGFβ as a possible target for the treatment of acute RIGT, and even the prevention of the development of chronic RIGT (Milliat et al. 2006). The findings of this

thesis suggest a link between MMP-2 expression and acute TGFβ, and further investigations are now necessary to determine whether the inhibition of MMP-2 alters TGFβ expression and to elucidate the role of TGFβ during acute RIGT.

This is the first study to assess intestinal angiostatin and endostatin protein expression using immunostaining during RIGT. Both angiostatin and endostatin were found to be significantly increased in the lamina propria of the colon 3 weeks into the fractionated radiation schedule, with angiostatin also significantly increased at week 6. Angiostatin and endostatin, when increased in early RIGT, may contribute to the endothelial apoptosis and cytostasis observed in chapter 3 (Itasaka et al. 2007). Angiostatin and endostatin are both sequestered by MMP-2, from plasmin and collagen type XVIII respectively (Ribatti 2009). When the findings of chapter 5 are considered with the increase in MMP-2 in chapter 4, the role of MMP-2 in endothelial apoptosis and cytostasis in RIGT is further supported (Stansborough et al. 2017). With this implication of anti-angiogenic mediators in the development of RIGT, particular consideration should be given to intestinal toxicity when these angiogenic mediators are used in combination with radiation as anti-cancer therapies.

6.5 Modulation of microvascular changes in RIGT via MMP inhibition

As a link could be seen between microvascular changes, MMP expression, and vascular mediator expression in chapters 3-5, primary tumour-associated microvascular endothelial cells (TAMECs) isolated from DA rat mammary adenocarcinoma were used to assess the direct effects of radiation and MMP inhibition on endothelial cells. I found MMP-2 and -9 expression was significantly increased in these endothelial cells following 12 Gy irradiation. Further, MMP-2 and -9 were significantly inhibited by the addition of the inhibitor SB-3CT. The increase in MMP-9 in the *in vitro* model, not seen in the *in vivo* model of RIGT, is likely

due to the differences between tumour and normal endothelium. Although MMP-9 plays an important role in tumour response to irradiation, it does not seem to be involved in gastrointestinal damage from fractionated irradiation (Bergers et al. 2000; Angenete et al. 2009). This highlights a limitation in the model used in this study, in that MMPs are highly tissue-specific in their upregulation and downstream effects, thus changes observed in tumour endothelium may not reflect those seen in normal endothelium following irradiation.

Radiation significantly reduced cell viability of TAMECs. However, despite the significantly inhibited expression of MMP-2 and -9 by SB-3CT in irradiated TAMECs, this inhibition did not alter cell viability of TAMECs. Whilst the presence of TGFβ and VEGF expression was confirmed in the present study, they were not significantly altered following irradiation of TAMECs as seen in both my *in vivo* studies and also supported by previous literature (Wang, J, Zheng & Hauer-Jensen 2001). Regulation of endothelium by MMPs is largely due to the sequestering of ECM components, and subsequent release and activation of growth factors such as TGFβ and VEGF (Hawinkels et al. 2008; Manicone & McGuire 2008; Stansborough et al. 2016). Thus, it is likely the lack of ECM components in the TAMEC model which would normally be present in the intestinal submucosa may have hindered the effect of MMP inhibition on endothelial cell regulation. However, the lack of alteration of VEGF expression is consistent with findings of Oh and colleagues (2014) in which VEGF and VEGFR2 expression was not altered in human breast tissue-derived endothelial cells following 4 Gy irradiation.

Despite some limitations, my study confirmed a direct effect of radiation on MMP-2 and -9 in endothelial cells, supporting a link between the significant increase in MMP-2 observed in chapter 4, and the microvascular changes observed in chapter 3. Future *in vivo* studies should

now be conducted to determine the effect of MMP-2 and -9 inhibition, or even knockout, in RIGT, with a particular focus on the intestinal microvasculature. This is increasingly important with the continued development of MMP inhibitors as anti-cancer agents, particularly when combined with radiation, as the implications on intestinal toxicity are currently unknown.

6.6 Therapeutic implications

The findings of this thesis, when taken together, support a role for MMPs and the vascular mediators VEGF, TGFβ, angiostatin, and endostatin, in the development of RIGT. These mediators are likely part of a larger pathway and contribute to the microvascular changes seen in chapter 3 (Stansborough et al. 2017). Due to these findings, both MMP-2 and the aforementioned vascular mediators appear to be attractive targets for the development of novel RIGT treatments. This section of the general discussion provides context, and discusses possible considerations, for the development of these treatments, particularly as similar agents have additional implications in cancer treatment.

6.6.1 MMP inhibition

Whilst MMP inhibitors are yet to be investigated for the treatment of RIGT, they have a long, and tumultuous path in their use as anti-cancer therapies (Pavlaki & Zucker 2003; Syed et al. 2004; Chu et al. 2007). Although MMP inhibitors have shown a great deal of promise in reducing tumour burden and invasion, and preventing metastases in pre-clinical studies, the majority of these inhibitors failed to produce objective anti-tumour responses in early clinical trials and their development has been halted (Syed et al. 2004; Chu et al. 2007). This is largely due to two factors; 1. the use of broad-spectrum MMP inhibitors, and 2. their use in patients with treatment-resistant, advanced malignancies (Syed et al. 2004). With increased

specificity of MMP inhibitors, such as that used in chapter 5, SB-3CT, their use in early stages of tumour progression, or in the prevention of further metastases, warrants further investigation. MMP-2, and -14, upregulated in RIGT (chapter 4), and MMP-9, upregulated in endothelial cells following irradiation (chapter 5), have been shown to be involved in tumour angiogenesis, with specific inhibition of these MMPs reducing endothelial migration and invasion, and impairing tumour angiogenesis in pre-clinical studies (Egeblad & Werb 2002). If clinical development of specific inhibitors of these MMPs shows therapeutic potential, it is possible their combination with radiotherapy may not only enhance the anti-tumour effects of irradiation, but reduce associated intestinal toxicity. Thus, the investigation of the role of MMPs in RIGT, and the effects of their inhibition on its pathobiology is increasingly important.

Li and colleagues (2017) conducted the first study to assess the effect to MMP inhibition on radiation toxicity, determining the effects of the broad-spectrum MMP inhibitor, Ilomastat, on radiation-induced pulmonary toxicity in mice. Despite differences in pathology, increases in MMPs, cytokines, and reactive fibrosis are all characteristic features of both RIGT and radiation-induced pulmonary toxicity (Li, X et al. 2017). Ilomastat significantly reduced MMP-2 and -9 expression, attenuated pulmonary toxicity, fibrosis, inflammation, and apoptosis, and significantly increased survival time (Li, X et al. 2017). This very recent study, combined with the findings of this thesis, suggesting the involvement of MMP-2 in RIGT, highlight the need to further investigation and development of specific MMP inhibitors in the treatment of RIGT.

6.6.2 Vascular mediator inhibition

Due to the role of VEGF in the 'angiogenic switch' during tumorigenesis, inhibitors of VEGF (VEGFIs) are currently being investigated as cancer therapies. Despite promising results of VEGFIs in clinical trials, adverse effects, including hypertension, haemorrhage, thromboembolism, and GI perforation, have severely limited their use, leading to them primarily being investigated as adjuvant therapies (Hapani, Chu & Wu 2009; Pollom et al. 2015). When VEGFIs are combined with radiotherapy, gastrointestinal toxicity has been shown to significantly increase (Barney et al. 2013; Pollom et al. 2015). The inhibition of VEGF has been shown to delay healing of gastric erosions, reduce vascular density in the small intestinal villi, induce epithelial ulceration. Specifically, in the case of inhibition using bevacizumab, a monoclonal antibody against VEGF, induces platelet aggregation and degranulation, possibly resulting in ischemia and GI perforation (Mangoni et al. 2012; Pollom et al. 2015). Barney and colleagues (2013) retrospectively assessed patients with primary or metastatic abdominal tumours who had undergone stereotactic body radiation therapy (SBRT), in which highly precise, high-dose fractionations of radiotherapy are used, and found the rate of serious bowel injury (SBI) in patients who had received post-SBRT VEGFI to be 35%, with no SBI reported in patients undergoing SBRT alone (Barney et al. 2013). These SBI included gastric/duodenal ulceration, gastric ulceration, stomach perforation, small bowel perforation, and duodenal perforation leading to sepsis and death (Barney et al. 2013). The findings of chapter 5, in which VEGF was significantly increased during chronic RIGT in DA rats, support the findings of a protective role for VEGF during RIGT. Although the involvement of VEGF in tumorigenesis and tumour angiogenesis prevents the targeting of this mediator for the treatment of chronic RIGT, it is important to better understand its role in the

pathology in order to better understand the enhancement of RIGT caused by VEGFI's clinically.

6.7 Conclusions and future directions

The studies conducted in this thesis aimed to elucidate the role of the intestinal microvasculature, and the potential mediators of these microvascular changes, matrix metalloproteinases, and associated vascular mediators, in the development of radiotherapy-induced gastrointestinal toxicity. Chapter 3 is the first study to assess intestinal endothelial apoptosis and cytostasis following fractionated irradiation. The findings of this thesis help clarify the role of the microvasculature in acute RIGT, and support the role of telangiectasis and microvasculature fibrosis in chronic RIGT. MMP-2 was found to be significantly increased in the same model of RIGT in chapter 4, and was associated with these microvascular changes and intestinal damage, supporting the hypothesis developed in the review of the literature (chapter 2). MMP-2 and -9 were also shown to be involved in endothelial response to radiation *in vitro* (chapter 5). Additionally, the importance of MMP-2 in tumorigenesis, when combined with the findings of a significant increase in RIGT, encourage the use of MMP-2 inhibitors in the treatment of RIGT, with the possibility of enhancing the efficacy of radiotherapy.

The additional findings of increased expression of VEGF, TGFβ, angiostatin, and endostatin during RIGT present a possible link between the increased MMP-2 observed in chapter 4, and the microvascular changes observed in chapter 3, with MMP-2 acting to sequester ECM components, thus releasing these mediators. When taken together, the findings of this thesis support the proposed mechanism of MMP and microvascular involvement in RIGT. Although further studies are now required to elucidate this pathway, these results have greatly

contributed to the knowledge of the pathobiology of RIGT, and have highlighted MMPs, and the microvasculature, as targets for novel RIGT treatments.

Chapter 7 References

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Chapter 8 Appendices

The following section includes pdf copies of the published manuscripts included as chapters 2-5 of this thesis.

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REVIEW

Radiotherapy-induced gut toxicity: Involvement of matrix metalloproteinases and the intestinal microvasculature

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ABSTRACT

Purpose To review the literature surrounding the involvement of the endothelium and matrix metalloproteinases (MMP) in radiotherapy-induced gut toxicity (RIGT) and further elucidate its complex pathobiology.

Results RIGT involves damage to the gastrointestinal mucosa and is associated with diarrhoea, pain, and rectal bleeding depending on the area of exposure. The mechanisms underpinning RIGT are complex and have not yet been elucidated. Members of the MMP family, particularly MMP-2 and -9, have recently been identified as being key markers in RIGT and chemotherapy-induced gut toxicity (CIGT). Furthermore, the microvasculature has long been implicated in the development of toxicities following both chemotherapy and radiotherapy, however, the mechanisms behind this are yet to be explored.

Conclusions It is proposed that matrix metalloproteinases are key regulators of endothelial mediators, and may play a key role in inducing damage to intestinal microvasculature following radiotherapy.

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Introduction

Radiotherapy-induced gut toxicity (RIGT) is a dose-limiting pathology involving damage to the gastrointestinal mucosa caused by radiotherapy for cancer. Its symptoms including, but not limited to, pain, diarrhoea, nausea, and rectal bleeding, are debilitating and significantly reduce the patients' quality of life (Sonis and Keefe 2013). The overall incidence of RIGT is not well understood, due to discrepancies in the definition of gastrointestinal toxicity following radiotherapy, and the classification of symptoms. Acute diarrhea and abdominal pain occur in 20–70% of patients receiving radiotherapy for abdominal or pelvic malignancies, and acute RIGT affecting quality of life has been shown to occur in 47% of women receiving radiotherapy for cervical or endometrial cancer (Classen et al. 1998, Abayomi et al. 2009, Shadad et al. 2013).

Gastrointestinal response to radiotherapy

Gastrointestinal toxicities from radiotherapy are classified as being either acute (occurring hours after treatment) or delayed (months after treatment) (Al-Dasooqi et al. 2013). Acute RIGT occurs shortly after radiation and can continue for up to 3 months following treatment (Theis et al. 2010). Symptoms of acute toxicity include, but are not limited to, mucositis, nausea and vomiting, pain, and diarrhoea (Al-Dasooqi et al. 2013, Elting et al. 2013, Sonis and Keefe 2013). Further, acute RIGT is characterized by a plethora of histopathological alterations including mucosal atrophy, inflammation, and increased intestinal permeability (Theis et al. 2010). Several mediators have been identified as being key regulators in the development of acute RIGT, particularly the transcription factor nuclear factor-κB (NF-κB); which acts to upregulate over 200 genes involved in this pathology (Bowen et al. 2007). Key downstream mediators of NF-κB include pro-inflammatory cytokines, mitogen-activated protein kinase (MAPK), and matrix metalloproteinases (MMP) (Sonis 2004, Logan et al. 2008a, 2008b, Al-Dasooqi et al. 2010). These mediators act together to cause significant levels of apoptosis, decrease proliferation, and degrade the extracellular matrix (Sonis 2004).

Delayed toxicity, also known as chronic radiation enteropathy, occurs between 18 months and 6 years following irradiation and is reported in up to 20% of patients receiving pelvic radiotherapy (Theis et al. 2010). Commonly reported symptoms include changes in stool frequency and rectal bleeding (in ~70% of cases); however, lesser reported complications may also occur, including small intestinal obstruction and fistulation (occurring in 0.6 and 13% of cases, respectively) (Henson 2010, Theis et al. 2010). Histopathologically, delayed RIGT is characterized by tissue ischaemia and necrosis, ulceration, telangiectasia, fibrosis of both the submucosa and the microvasculature, and mesenchymal cell activation (Richter et al. 1998, Theis et al. 2010, Al-Dasooqi et al. 2013). It has been shown that several mediators upregulated in the delayed phase of RIGT, such as transforming growth factor-β1 (TGF-β1) and thrombomodulin, interact with the extracellular matrix (ECM) to cause deposition of ECM components in the intestinal smooth muscle wall and perhaps induce long-term injury to the epithelium and endothelium of the gastrointestinal tract (Andreyev 2007, Gervaz et al. 2009, Wang et al. 2012, Al-Dasooqi et al. 2013). Remodelling of the ECM has

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many downstream effects, including the release of sequestered growth factors resulting in alterations to the microvasculature, possibly linking back to related symptoms such as rectal bleeding and telangiectasis (Imai et al. 1997, Bergers et al. 2000). These studies suggest a complex mediator pathway associated with delayed toxicity following radiation.

Both acute and delayed toxicities result in a significant reduction of quality of life of patients receiving radiotherapy for cancer (Bentzen 2006, Lalla et al. 2014). Despite the clinical significance of RIGT, its pathogenesis remains poorly understood. This lack of understanding of the mechanisms by which RIGT occurs hinders the development of effective treatments. Previous studies have suggested a complex pathway of mediator release, inflammation, and apoptosis, of not only the epithelial mucosa, but the underlying submucosa, including microvascular endothelium (Paris et al. 2001, Kruse et al. 2004).

Matrix metalloproteinases (MMP)

Matrix metalloproteinases, a class of zinc-dependent endopeptidases, have been previously shown to be altered in RIGT and chemotherapy-induced gut toxicity (CIGT) (Strup-Perrot et al. 2006, Al-Dasooqi et al. 2010). MMP subtypes are classified either functionally or structurally, with 24 members of the MMP family currently identified in humans, all of which share a catalytic domain coordinated by zinc (Yan and Boyd 2007). Their gene expression is predominantly regulated at the transcriptional level, with MMP members being regulated by transcription factors such as beta-catenin and NF-κB (Yan and Boyd 2007). However, post-transcriptional regulation of MMP does occur, with TGF-β, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and various pro-inflammatory cytokines being capable of activating MMP promoters, particularly in disease states (Yan and Boyd 2007). MMP family members vary structurally and functionally, with differences also occurring in the transcriptional and post-transcriptional regulation (Strongin et al. 1995). Whilst the general role of the MMP family is primarily in homeostasis of the extracellular matrix, specific MMP members may have different roles and have been identified as altering cell growth, immune responses, chemokine and cytokine expression, as well as apoptosis, a key histopathological feature of RIGT (Castaneda et al. 2005, Manicone and McGuire 2008). The recent implication of the MMP family in RIGT and CIGT suggests a complex and intricate involvement with associated mediators and tissue types in RIGT, as summarized in previous reviews (Sternlicht and Werb 2001, Al-Dasooqi et al. 2009).

Matrix metalloproteinases may be key mediators in RIGT

Studies by Strup-Perrot et al. (2005,2006) identified matrix metalloproteinases as regulating RIGT. Male Wistar rats received a single dose of radiation (10 Gy) to the abdomen, and gene expression of MMP-2, -3, -9, and -14 in the colon was assessed. MMP-2 was significantly increased at 1, 3, and 7 days following irradiation, MMP-3 was significantly increased at 3 days, MMP-9 at 3 and 9 days, and MMP-14 at 1 and 3 days (Strup-Perrot et al. 2006). Strup-Perrot et al. (2004) also assessed intestinal gene expression of MMP-2, -9, and -14, as well as tissue inhibitor of matrix metalloproteinase (TIMP)-1, and -2, in patients undergoing surgery for intestinal occlusion caused by delayed radiation-induced enteritis. MMP-2, -9, and -14, and TIMP-1, and -2 mRNA expression were significantly increased compared to healthy controls (Strup-Perrot et al. 2004). Conflicting results have been found in later studies. Angenete et al. (2009) studied levels of various matrix metalloproteinases in normal and tumour tissue from the rectum of rectal cancer patients scheduled for open surgery with or without pre-operative radiation. The study recruited 32 patients, 20 of which received short-term pre-operative fractionated radiotherapy (5×5 Gy) with a median time of tissue collection following completion of radiotherapy of 3 days. The remaining 12 patients received no pre-operative radiation. Patients receiving chemoradiotherapy were excluded and groups were matched for age and gender. Whilst MMP-2 levels were significantly increased in the rectal mucosa following radiotherapy, no significant changes in MMP-1, MMP-9, or TIMP-1 levels were observed following radiotherapy in these patients (Angenete et al. 2009). These discrepancies may be due to the many differences between the studies, including species difference in preclinical vs. clinical studies, differences between acute and delayed RIGT, and single-dose vs. fractionated irradiation. This is particularly relevant in the context of acute vs. delayed RIGT, in which many differences can be observed at the clinical, histopathological, and molecular levels (Theis et al. 2010). It is likely that MMP expression would differ depending on time from the last radiotherapy course. Differences in normal tissue response in single vs. fractionated dose radiotherapy have also been widely observed, with the short breaks between doses in fractionated irradiation thought to lead to an accumulative affect, although not always linear (Yeoh et al. 2007, Hauer-Jensen et al. 2014).

Recent studies characterizing MMP expression in the development of CIGT have suggested particular roles for different MMP members depending on their patterns of expression, and their coincidence with stages of CIGT development (Al-Dasooqi et al. 2010). MMP-2, -9, and -12 levels have been shown to peak 48 h following chemotherapy, coinciding with maximal mucosal damage in the jejunum of Dark Agouti rats (Al-Dasooqi et al. 2010). This implicates these specific MMP subtypes in the progression of inflammation in CIGT. In contrast, MMP-1 has been found to be down-regulated early in the development of CIGT, however, is later upregulated at 96 and 144 h following chemotherapy administration, suggesting it may play a role in the healing stages of the pathology (Al-Dasooqi et al. 2010). This is supported by the ability of MMP-1 to cleave type-1 collagen to regulate cell migration in re-epithelialization (Pilcher et al. 1997, Al-Dasooqi et al. 2010). Alterations in MMP-1 expression may therefore be involved in the development of delayed RIGT, in which collagen deposition and reactive fibrosis commonly occur, also this has not yet been explored (Hauer-Jensen et al. 2014). Such studies are yet to be conducted in RIGT models, particularly in regards to the differences between single dose in vivo RIGT studies and the fractionated radiation doses commonly used clinically.

MMP-2 and -9 in particular, have many downstream mediators linked to the pathobiology of RIGT. MMP-2 and -9 are capable of upregulating pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), as well as forming a positive feedback loop with the key transcriptional factor NF-κB, (Sengupta and MacDonald 2007). Certain MMP members, MMP-2, -7, and -9 in particular, have also been linked with the disruption of endothelial and epithelial tight junctions (Al-Dasooqi et al. 2014, Wardill et al. 2014). No studies to date have assessed changes in endothelial tight junction protein levels in the intestinal microvasculature following radiation. However, studies have assessed intestinal epithelial tight junctions and blood-brain barrier tight junctions following radiation (Fauquette et al. 2012, Shukla et al. 2015). A recent study by Garg et al. (2016) assessed changes in tight junction expression in the jejunum, ileum, and colon of non-human primates following total-body irradiation. Claudin-1 and -10 expression was significantly increased at day 4, and claudin-4 and -7 expression was repressed at days 4, 7, and 12. These changes were also shown to be segment-specific, with increased expression of claudin-1 occurring only in the colon, and repression of claudin-7 expression occurring only in the ileum (Garg et al. 2016). Fauquette et al. (2012) assessed permeability and changes to tight junction protein levels following irradiation of a bovine brain capillary endothelial cell (BBCEC) monolayer co-cultured with glial cells. Permeability to fluorescein isothiocyanate (FITC)-dextran (70 kDa) was significantly increased 8 days following 25 Gy irradiation, and immunoflourescent staining of the tight junction protein zonula occludens-1 (ZO-1) was decreased 8 days following both 12 Gy and 25 Gy irradiation. Whilst endothelial cells of the blood-brain barrier are structurally distinct to those of the intestinal microvascular, alterations to endothelial tight junction integrity following irradiation may still lead to increased permeability in such vessels. Similarly, a significant loss of junctional distribution of tight junction proteins occludin, claudin-3, and ZO-1 was shown to occur in the colon epithelium 2 h following a single dose of 4 Gy whole body irradiation in female C57BL6 mice (Shukla et al. 2015). Further exploration of tight junction alterations in the intestinal endothelium following radiation is warranted, particularly with increasing evidence of the importance of the gut-brain axis in many pathologies, including CIGT (Tillisch 2014, Wardill et al. 2015). Despite these implications of the MMP family in RIGT and CIGT, a causative role has not yet been confirmed in previous literature, and several aspects of MMP functionality in RIGT remain entirely unexplored.

Response of intestinal microvasculature to radiotherapy

The microvasculature, and specifically the endothelium, plays a large role in toxicity following radiotherapy. Despite this, the extent of the involvement of the intestinal microvasculature, and the mechanisms behind this beyond their direct clinical manifestation in RIGT are unknown. Kruse et al. (2004) assessed changes to the microvasculature of the rectum of female C3H/HenAF-nu^{+} mice following a single dose of 20 Gy irradiation. Extensive vascular changes in the rectum, particularly tortuous telangiectatic vessels, were observed from 10 weeks following irradiation, with increasing severity, and were not resolved at 30 weeks post-irradiation (Kruse et al. 2004). Telangiectatic vessels are defined as 'thin-walled, tortuous vascular channels that often occur in groups and are accompanied by an area of leukocytic infiltration' (Kruse et al. 2004, Yeoh et al. 2005). Whilst the involvement of these telangiectatic vessels in the development of RIGT is unclear, gastrointestinal bleeding is one of the primary symptoms in patients with hereditary haemorrhagic telangiectasia presenting with intestinal telangiectatic lesions (Kjeldsen and Kjeldsen 2000). Mucosal telangiectasia resulting in rectal bleeding is one of the primary dose-limiting factors for irradiation of prostate cancer (Kruse et al. 2004). Further understanding of how intestinal telangectasis occurs in response to radiation may lead to potential targets for the treatment or prevention of rectal bleeding in RIGT.

Although few preclinical studies have assessed changes to the intestinal microvasculature following irradiation, these changes have been observed clinically in several studies following radiotherapy for cancer (Yeoh et al. 2005, Takeuchi et al. 2012). Takeuchi et al. (2012) assessed histopathological features of radiation toxicity of the lower rectum. The study included patients with a history of radiation therapy for an intrapelvic malignancy, characteristic colonoscopic findings of radiation proctitis, and histopathological exclusion of other diseases. Six male patients with radiation proctitis received 70 Gy external irradiation for prostate cancer, and two female patients received 50 Gy external irradiation and 20 or 27 Gy internal irradiation for uterine and vaginal cancers, respectively. Endoscopies and rectal biopsies were performed 15.4 ± 10.2 months from the completion of irradiation. Telangiectasia was observed in the superficial layer of the rectal mucosa upon endoscopy, and was confirmed in the upper layer of the lamina propria upon histological examination of rectal biopsies (Takeuchi et al. 2012). These findings are consistent with those of Yeoh et al. (2005) who demonstrated telangiectatic, fibrosed, and sclerosed blood vessels in the lamina propria, submucosa and fat layer of the gastrointestinal tract following short-term fractionated pelvic irradiation (25 Gy total/5 fractions/1 week), or long-term neoadjuvant chemoradiotherapy (45 Gy total/25 fractions/5 weeks, with concurrent infusions of 5-fluorouracil), in patients with colorectal carcinoma. Although these studies have highlighted a key role of the intestinal microvasculature during RIGT they have not described the effect these changes have on the underlying pathobiology. The formation of telangiectatic vessels in the rectal mucosa following radiation has been associated with increased expression of jagged 1 and Kruppel-like factor 5 (KLF5), both of which are involved in vascular remodeling and have been linked to the development of interstitial fibrosis (Kruse et al. 2004). KLF5 in particular is capable of upregulating growth factors such as PDGF and TGF-β (Kruse et al. 2004). The upregulation of these mediators would suggest a greater involvement of telangiectatic vessels in RIGT development than currently understood.

Endothelial apoptosis has also been suggested to be an initiating feature in the pathology of RIGT (Paris et al. 2001). Several in vivo and in vitro studies have found that endothelial

apoptosis occurs hours following various radiation doses in several different tissue types, including the intestinal, lung, and central nervous system (CNS) microvasculature (Pena et al. 2000, Paris et al. 2001; Zhang et al. 2012, Li et al. 2004). Li et al. (2004) assessed apoptosis in the spinal cord of female Fisher 344 rats following a single dose of 50 Gy radiation to the spinal cord. Apoptosis was analysed via histology and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and 3.2 ± 10% of apoptotic cells in the spinal cord 10 h following irradiation were identified as endothelial (cells positively stained for factor-VIII-related antigen). A similar study conducted by Pena et al. (2000) found 16–20% of endothelial cells in the spinal cord of male mice underwent apoptosis at 12 h following exposure to 50 Gy of irradiation. Zhang et al. (2012) observed positive apoptotic staining co-localized to the lung endothelium of C57BL/6J mice following a single dose of 15 Gy irradiation to the thorax. It was reported in the study that 11% of apoptotic cells in the irradiated lung were endothelial (Zhang et al. 2012). Changes to endothelium following irradiation cause significant long-term effects, with irradiated endothelium lacking the ability to regulate thrombogenic, inflammatory and coagulation processes months to years following irradiation (Boerma et al. 2004, Otterson et al. 2012). Many of these effects of radiation on the endothelium are related to changes in angiogenic signalling.

Angiogenic changes have been described in vitro, with the angiogenesis pathway being over-expressed in primary human endothelial cells from 12 h to 21 days following a single irradiation dose of 2Gy (Heinonen et al. 2015). The angiogenic mediator TGF-β1 has been found to be upregulated preclinically in both early and delayed RIGT and is an upstream mediator of thrombomodulin, capable of reducing thrombomodulin immunoreactivity in the endothelium. Thrombomodulin is a transmembrane glycoprotein present on endothelial cells which is capable of forming a complex with thrombin, preventing the formation of fibrin and the activation of protease activated receptor-1 (PAR-1). This leads to the activation of protein C, an anticoagulant and anti-inflammatory protein (Wang et al. 2002). When thrombomodulin reactivity is reduced following irradiation PAR-1 is upregulated, decreasing thromboresistance and inducing endothelial dysfunction (Wang et al. 2002, 2012, Boerma et al. 2013). TGF-β1 has also been shown to enhance radiation-induced fibrotic thickening of the intestinal wall and subserosa in a rat model of early and delayed RIGT following fractionated irradiation (Boerma et al. 2013). Although these microvascular changes have been described in several studies, the causal relationship of these changes to RIGT pathobiology are yet to be explored, as are the opportunities for novel therapies that may arise from this.

Matrix metalloproteinases and the intestinal microvasculature following radiotherapy

The MMP family has been strongly linked to endothelial regulation and angiogenesis, and could play a direct role in pathological angiogenesis associated with RIGT (Bergers et al. 2000, Al-Dasooqi et al. 2010). Much of what is known regarding the involvement of MMP in the regulation of the endothelium, particularly MMP-2 and -9, is from their involvement in tumour invasion and tumour angiogenesis. MMP-9 has been shown to be integral in the progression of some cancer types based on its ability to regulate angiogenesis (Bergers et al. 2000, Bhoopathi et al. 2010). MMP members degrade the basement membrane allowing for endothelial proliferation and migration, and remodeling of the extracellular matrix, inducing tube formation (Chidlow et al. 2007, Al-Dasooqi et al. 2011). Members of the MMP family also indirectly induce pathological angiogenesis via regulation of angiogenic mediators such as vascular endothelial-cadherin (VE-cadherin), TGF-β, chemokines, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), angiostatin, and endostatin (Ichikawa et al. 2006, Xu et al. 2006, Bendrik et al. 2008, Manicone and McGuire 2008, Tolstanova et al. 2011). These mediators contribute to many facets of pathological angiogenesis including increased vascular permeability, vasodilation, endothelial proliferation, and inhibition of vessel maturation and pericyte stabilization (Kalluri 2003, Bendrik et al. 2008, Tolstanova et al. 2011). Due to this cumulative evidence of a role for the MMP family in endothelial regulation, MMP inhibitors have been explored for their efficacy in the reduction of tumour burden and metastasis. Whilst broad-acting MMP inhibitors such as marimastat significantly inhibited tumour angiogenesis and reduced tumour burden preclinically, phase II and III clinical trials have been unsuccessful (Zucker et al. 2000, Zucker and Cao 2009). This may be due to the vastly different and even opposing effects of various MMP types, as well as the exclusion of patients with early stage cancer (Zucker and Cao 2009). Specific inhibition of MMP members known to be involved in tumour angiogenesis, such as MMP-2 and -9, may be more efficacious in ongoing research, and provide insights in to how matrix metalloproteinases may be regulating these endothelial changes.

Few studies have been conducted to assess the role of matrix metalloproteinases in changes to the intestinal microvasculature in RIGT, and most have been performed in vitro. Park et al. (2012) found MMP-2 and vascular endothelial growth factor receptor (VEGFR)-2 to be significantly increased 24 h following a single radiation dose of 4 Gy in human brain microvascular endothelial cells (HBMEC) and human dermal microvascular endothelial cells (HDMEC). In a similar in vitro model, capillary endothelial cells were irradiated with a single dose of 10 Gy and displayed significantly increased apoptosis, as well as an increase in active MMP-1 and -2 (Vorotnikova et al. 2004). MMP-2 expression was also increased at 24 h, as was MMP-2 enzyme activity at 24 and 48 h, following a single dose of 4 Gy irradiation in endothelial cells cultured from normal human breast tissue (Oh et al. 2014). In contrast, MMP-9 expression decreased in these cells at 24 and 48 h following irradiation (Oh et al. 2014). Whilst these studies suggest MMP, particularly the gelatinase MMP-2, are directly involved in the endothelial response to radiation, further in vivo studies are warranted.

MMP signalling regulates endothelial mediators of RIGT

MMP and Vascular Endothelial Growth Factor (VEGF)

VEGF plays a variety of roles in the regulation of microvasculature. It is capable of increasing vascular permeability, causing transient vasodilation, and promoting endothelial proliferation, migration, and differentiation, all of which stimulate angiogenesis (Ferrara et al. 2003, Chidlow et al. 2007). The primary ability of the MMP family, particularly MMP-2 and -9, to increase VEGF, lies in their ability to cleave collagen IV to release sequestered VEGF (Bergers et al. 2000, Kalluri 2003). Once VEGF has been released it is able to bind to VEGF receptors on endothelial cells and induce angiogenesis via a tyrosine kinase pathway (Lee et al. 2015). MMP-2, and 9 have also been suggested to change the structure of VEGF, cleaving larger VEGF isoforms and increasing bioavailability; however, Hawinkels found MMP-9 to have no effect in cleaving $VEGF_{165}$ to the smaller, soluble isoform $VEGF_{121}$ in HT29 colon carcinoma and fibroblast spheroids (Belotti et al. 2003, Kalluri 2003, Hawinkels et al. 2008). Hawinkels and colleagues (2008) showed a decrease in extracellular localization of heparan sulphate proteoglycans (HSPG), suggesting a release of VEGF from the ECM via cleavage of HSPG by MMP-9. Bergers et al. (2000) cultured normal, non-transgenic pancreatic islets from C57/B16 mice with MMP-9. Cells were then washed and embedded in collagen gels containing endothelial cells. Whilst control islets, with no added MMP-9, failed to elicit an angiogenic response from the endothelial cells, islets treated with MMP-9 promoted the endothelial cells to form sprouts towards the islet. VEGF levels were 2-fold higher in the medium of MMP-9-treated islets, and MMP-9-treated islets showed no angiogenic response of endothelial cells when an anti-VEGF antibody was added. This suggests that the MMP-9 mediated endothelial response is regulated by VEGF (Bergers et al. 2000). A study by Bhoopathi and colleagues (2010) found VEGF expression following secreted protein acidic and rich in cysteine (SPARC) overexpression to be MMP-9-dependent in human medulloblastoma cell lines. These studies highlight a complex involvement of MMP, particularly MMP-9, with VEGF and it is likely that differences in the pathway occur based on tissue types and the tissue environment (pathological vs. normal tissue).

MMP and Transforming Growth Factor-β

The effect of TGF-β on the microvasculature is complex, with TGF-β having several different effects on the endothelium, depending on the signalling pathway (Goumans et al. 2003). Ionizing radiation has been shown to alter the signalling pathways of TGF-β in endothelium, reducing the activation of the activin receptor-like kinase 1 (ALK1) pathway and enhancing the ALK5 pathway activation (Kruse et al. 2009, Scharpfenecker et al. 2009). This change in signalling pathways is thought to alter the way in which endothelial cells respond to TGF-β, with ALK5 inhibiting and ALK1 stimulating endothelial cell proliferation and migration (Goumans et al. 2003). The degradation of the small collagen-associated proteoglycan, decorin, by several MMP members has been shown in several studies to sequester latent TGF-β, converting it to its active state (Imai et al. 1997). TGF-β1 and MMP-9 mRNA levels have been shown to be significantly increased at 3 and 7 days, as was TGF-β2 from day 1, following 10 Gy abdominal X-irradiation (Strup-Perrot et al. 2005). Despite this link between MMP-9 and TGF-β, associated changes to the microvasculature are yet to be explored.

MMP and anti-angiogenic mediators

Several MMP subtypes have been associated with the anti-angiogenic mediators, endostatin, tumstatin, and angiostatin (Kalluri 2003, Tolstanova et al. 2011). Endostatin and tumstatin are both anti-angiogenic mediators formed from the c-terminal portions of collagens XVIII and IV, respectively (Sudhakar et al. 2003). Both collagen IV and XVII, found in vascular basement membranes, can be broken down by active forms of MMP-2, cleaving endostatin from collagen XVII, and increasing thrombospondin-1, which has been shown to be upregulated early and late in an animal model of CIGT (Bowen et al. 2007). MMP-2, -7, -9, and -12 are also capable of cleaving plasminogen to form angiostatin (Sternlicht and Werb 2001). MMP-9, endostatin, VEGF, and platelet-derived growth factor receptor (PDGFR) were increased at different time-points in an animal model of ulcerative colitis, with increased endostatin and VEGF being correlated with increased size of colonic lesions (Tolstanova et al. 2011). However, the effects of endostatin and angiostatin on endothelial cells are yet to be clarified, with transfection of the MMP-9 gene in vivo inducing endostatin release and significantly reducing tumour growth rate and microvessel area in mammary adenocarcinomas in mice (Bendrik et al. 2008). This could be due to biphasic effects of endostatin, and differential effects depending on cell type (Guan et al. 2003, Folkman 2006). Despite this, COL-3 (Metastat; Collagenex Pharmaceuticals, Newtown, PA, USA), a specific inhibitor of MMP-2 and MMP-9, did not increase tumour growth rate of advanced solid malignancies in clinical trials and three patients with disease progression prior to treatment had prolonged stable disease (>6 months) (Syed et al. 2004). Further studies are now warranted to characterize MMP-9 and associated downstream angiogenic mediators in RIGT.

MMP and endothelial permeability

MMP-2, -7, and -9 have also been shown to play extensive roles in endothelial permeability due to their ability to shed VE-cadherin and cleave occludin; two tight junction proteins with essential roles in maintaining tissue permeability. MMP-7 sheds epithelial-cadherin from adherens tight junctions to remodel cell-cell contacts and facilitate cell migration (McGuire et al. 2003). VEGF-A, a possible downstream mediator of several MMP subtypes, is also capable of increasing junction adhesion molecule -C expression, stimulating occludin and VE-cadherin phosphorylation and decreasing occludin and VE-cadherin at endothelial tight junctions (Antonetti et al.

1998). This link between matrix metalloproteinases, which have been shown to be upregulated in RIGT, and the phosphorylation of endothelial tight junctions, highlights a potential new involvement for the MMP family in intestinal endothelial dysfunction and increased permeability in RIGT.

Take home messages

RIGT is one of the most prevalent dose-limiting toxicities manifesting following radiotherapy (Sonis and Keefe 2013, Lalla et al. 2014). It involves debilitating pain and increases the risk of infection in patients receiving radiotherapy. Due to many of the severe side-effects of RIGT, it is often necessary to reduce the dosage, or halt anti-neoplastic therapies, leading to decreased remission and increased mortality (Lalla et al. 2014). Despite the prevalence and burden of this pathology, there are currently no effective treatments for RIGT and its pathobiology remains to be clearly understood. Various MMP members have been shown to be altered in the intestine following irradiation, in the settings of both acute and delayed RIGT (Strup-Perrot et al. 2004, 2006, Angenete et al. 2009). The gastrointestinal microvasculature has also been implicated in RIGT development, with endothelial apoptosis occurring early in the pathology, and pathological angiogenesis being observed in several models of RIGT (Paris et al. 2001, Kruse et al. 2004, Yeoh et al. 2005). Several MMP subtypes are capable of regulating many angiogenic and anti-angiogenic endothelial mediators, such as VEGF, TGF-β, endostatin, and angiostatin, highlighting a strong link for their role in RIGT-associated microvascular changes. Thus the present review proposes a vascular mediator pathway, regulated by altered MMP expression or activation following irradiation, as being involved in intestinal microvascular changes seen in both acute and delayed RIGT. Although further study is necessary, the MMP family presents a unique target in the development of novel therapies for RIGT. Due to the complex nature of members of the MMP family, and differential effects based on the type of MMP, tissue type, and pathology, extensive studies are required. As RIGT is a prevalent and debilitating adverse effect of anti-neoplastic therapies, any future studies focusing on the biological basis of this pathology will aid in the development of anti-mucotoxic therapies of much clinical value.

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ORIGINAL ARTICLE

Fractionated abdominal irradiation induces intestinal microvascular changes in an in vivo model of radiotherapy-induced gut toxicity

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Abstract
Purpose Radiotherapy-induced gut toxicity (RIGT) is associated with diarrhoea, pain and rectal bleeding and can occur as an acute or chronic toxicity. The microvasculature has been shown to be altered in the development of RIGT; however, the features are not yet characterized. We hypothesized that apoptosis of microvascular cells would occur early in the gastrointestinal tract following fractionated irradiation, followed by late microvascular changes, including sclerosis and telangiectasis.
Methods Female Dark Agouti rats were treated with a 6-week fractionated radiation schedule of 3 × 2.5 Gy doses per week localized to the abdomen. At 3, 6 and 15 weeks, the intestines were assessed for markers of acute and chronic injury including morphological changes, collagen deposition, apoptosis and proliferation.
Results Apoptosis of microvascular cells significantly increased at 6 and 15 weeks in the jejunum ($p = 0.0026$ and $p = 0.0062$, respectively) and at 6 and 15 weeks in the colon ($p < 0.0001$ and $p = 0.0005$, respectively) in rats receiving fractionated radiation to the abdomen. Histopathological changes of the colon microvasculature were also seen from week 3, including thickening of the lamina propria and dilated, thickened, telangiectatic vessels.
Conclusions Findings of this study provide evidence of regional and timing-specific changes in the intestinal microvasculature in response to fractionated radiotherapy which may play a role in development of both acute and chronic RIGT.

Keywords Mucositis · Radiotherapy · Microvasculature · Gastrointestinal · Apoptosis

Introduction

Radiotherapy-induced gut toxicity (RIGT) is an adverse effect of radiotherapy for cancer, which may occur as an acute toxicity (also referred to as gastrointestinal mucositis or acute radiation enteropathy) or chronic toxicity (also chronic radiation enteropathy) [1, 2]. In patients receiving radiotherapy for abdominal or pelvic tumours, approximately 60–80% develop symptoms of acute gut toxicity, including, but not limited to, nausea, diarrhoea, pain and fatigue [1]. Chronic gut toxicity may also occur following radiotherapy and includes symptoms such as altered intestinal transit, gut dysmotility and less commonly intestinal obstruction or fistulae formation [1]. Despite these symptoms, there remain few treatment options for RIGT, largely hindered by a lack of understanding of the underlying pathobiology [1, 3]. This is particularly true for the endothelium. Late rectal bleeding is one of the most common symptoms of RIGT following high-dose radiotherapy for prostate cancer, occurring in up to 70% patients [4, 5]. This points to a likely impact of radiation on the normal intestinal microvasculature. Whilst intestinal epithelial cell apoptosis [6, 7], activation of transcription factors such as NF-κB [8] and a cascade of inflammatory mediators including pro-

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inflammatory cytokines and matrix metalloproteinases [9, 10] are well known to be involved in RIGT, these pathways are yet to be characterized in the endothelium.

Paris and colleagues (2001) assessed changes to endothelial apoptosis in the jejunum of C57BL/6 mice who received 8–15 Gy whole body irradiation. Whilst this study showed that endothelial apoptosis occurred early (4 h) following irradiation, the model was largely based on GI syndrome, in which doses are not clinically translatable in the context of radiotherapy for cancer [11]. Additionally, later preclinical studies showed conflicting results, in which endothelial apoptosis did not to occur early during GI syndrome and did not contribute to the severity of toxicity [12, 13]. Abderrahmani and colleagues (2012) found a significant increase in endothelial apoptotic cells in the villus lamina propria 4 and 5 h following 19 Gy intestinal irradiation in mice [14]. This was significantly lower in plasminogen activator inhibitor-1 (PAI-1) knockout mice, which had significantly reduced acute and ate radiation-induced intestinal injury when compared to wild-type mice [14]. Whilst this again suggests a link between early endothelial apoptosis and intestinal injury, epithelial apoptosis was also significantly lower in PAI-1 knockout mice at the 5-h time point, making it difficult to suggest which may be contributing to the reduced toxicity [14].

Despite the lack of consensus of the literature on endothelial apoptosis in RIGT, changes to intestinal microvasculature have been identified, both clinically and pre-clinically [4, 8]. Yeoh and colleagues (2005) assessed histological changes of archived colorectal tissue samples from 28 patients with colorectal carcinoma who had received either preoperative short-term pelvic irradiation or long-term neoadjuvant chemoradiotherapy. Short-term radiation involved a total dose of 25 Gy using the 4 field box technique, over 5 fractions in 1 week. Long-term neoadjuvant chemoradiotherapy involved concurrent 5-FU (300 mg/m^2/day) and pelvic irradiation with 45 Gy in 25 fractions over 5 weeks. Yeoh et al. (2005) demonstrated telangiectatic, fibrosed and sclerosed blood vessels in the lamina propria, submucosa and fat layer of the gastrointestinal tract following abdominal radiation. The study did not assess these changes in direct relation to the occurrence of RIGT in the patients; however, 16 out of 28 of recruited patients did experience symptoms of gastrointestinal toxicity during treatment [8]. In pre-clinical studies, tortuous telangiectatic vessels have been seen in the rectum of mice irradiated with a single dose of 20 Gy. These did not resolve at the completion of the study (30 weeks following irradiation); however, these changes were not investigated in relation to clinical parameters of gastrointestinal toxicity in these mice [4].

Despite the observation of pathological blood vessels in RIGT, few studies have investigated specific vascular changes in an in vivo model of fractionated irradiation. Several studies, however, have assessed thrombogenic changes to the microvasculature in an attempt to characterize mediator pathways underpinning vascular-related symptoms such as rectal bleeding, including changes to levels of vascular mediators such as thrombomodulin, protease-activated receptor-1 (PAR-1) and von Willebrand factor (vWF) [15–17]. Wang and colleagues (2002) found a statistically significant decrease in the number of thrombomodulin-positive blood vessels in the intestines of Sprague-Dawley rats who had received fractionated radiation (33.6 Gy in 8 daily fractions or 67.2 Gy in 16 daily fractions) to a surgically induced scrotal hernia. Irradiated rats also displayed mucosal ulceration and inflammation during early RIGT (1 day and 2 weeks following cessation of radiation), vascular sclerosis and reactive fibrosis of the subserosa and submucosa in delayed RIGT (6 and 26 weeks following radiation). Decreases in thrombomodulin are known to increase PAR-1 and decrease protein C activation, leading to upregulation of inflammatory mediators and endothelial dysfunction [15, 17]. These changes in vascular mediator upregulation following radiotherapy suggest a change in pathophysiological state of the intestinal microvasculature; however, further studies are needed to determine how this is affecting microvascular cell kinetics.

Thus, the present study aimed to determine changes to microvascular apoptosis and proliferation following fractionated abdominal radiation in an animal model of RIGT. The study also aimed to assess clinical and histopathological parameters of RIGT including changes to body weight and epithelial apoptosis to assess the timeline of these changes in the context of microvascular damage. In the present study, we demonstrate for the first time significantly increased intestinal microvascular apoptosis and significantly reduced microvascular proliferation following fractionated radiation in rats.

Materials and methods

Ethics

This study was conducted in accordance with ethics approved by the Animal Ethics Committees at both The University of Adelaide (M-041-2006) and the Institute of Medical and Veterinary Science (IMVS) (78/05) and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Teaching (2007).

Animals and experimental design

Seventy female Dark Agouti (DA) rats, weighing 155 to 170 g on arrival, were housed under controlled conditions with 12 h dark and 12 h light cycles. Rats had ad libitum access to water and standard rat chow. The experimental design was based on our previously published pilot study [18]. Briefly, rats (n = 5 per time point) were randomly assigned into three groups

receiving radiation and three groups receiving sham irradiation. Prior to irradiation, rats in both treatment and control groups were anaesthetized with 4% isofluorane and placed into a custom-built container [18]. Rats in the treatment groups were irradiated, limited to the abdomen by lead shielding, at a dose of 2.5 Grey (Gy)/fraction three times a week to a total of 45 Gy/18 fractions/6 weeks prescribed to depth of 3.3 cm using a Varian Clinac Linear Accelerator (Varian Medical Systems) with focus-skin distance of 130 cm. Control rats received sham irradiation in the same course as treated rats. Groups of rats were killed at the ends of weeks 1 to 6, representing acute toxicities from short- and long-course radiation, and at 15 weeks, representing a delayed, or chronic, toxicity. Animals were closely monitored in the immediate recovery period (30 min from radiation) and were then monitored daily for weight change, diarrhoea, dull/ruffled coat, change in temperament, reluctancy to move and skin reaction to radiation, throughout the experimental time course.

Gastrointestinal tissue collection

Gastrointestinal tissue was collected as previously described by Yeoh and colleagues (2007). Following flushing of the small and large intestine with cold isotonic saline, sections of jejunum (at 25% length of small intestine from the pylorus) and colon (at 50% length of large intestine) were resected into 2-cm sections, fixed in 10% neutral formalin and embedded in paraffin for further analysis. In preparation for staining, sections were cut using a rotary microtome (Leica) to 4 μm and were mounted on uncoated slides for haemotoxylin & eosin (H&E) and Picro sirius red staining or FLEX immunohistochemistry (IHC) microscope slides (Dako) for IHC.

Histology

H&E staining was conducted to determine morphological changes to the jejunum and colon following irradiation as described previously [18], including villous blunting or fusion, crypt loss, inflammation, vessel dilation and oedema.

Picro Sirius red staining was used to determine total tissue collagen levels as described previously [19]. Briefly, sections were dewaxed in xylene and hydrated in a series of graded ethanol. Picro Sirius red solution (0.5 g Sirius red F3B in 500-ml saturated aqueous picric acid solution) was applied to sections and left at room temperature for 1 h. Sections were then washed using acid water (0.5% acetic acid in H_2O), dehydrated, cleared and coverslipped.

Images of H&E and Picro Sirius red-stained tissue were taken using NanoZoomer scanning equipment (Hamamatsu,Japan), and images were analysed using NanoZoomer Digital Pathology software (NDP.view2, Hamamatsu). H&E-stained tissue was assessed using a previously validated damage scoring method in which sections were given a score of either 0 or 1 for the presence of the following factors: disruption of the brush border, crypt loss/architectural disruption, disruption of crypt cells, infiltration of polymorphonuclear cells and lymphocytes, dilation of lymphatics and capillaries, oedema and villous fusion and atrophy in the jejunum. Picro Sirius red-stained tissue was assessed qualitatively. All assessments were conducted in a blinded fashion by one assessor (RLS).

Immunohistochemistry

Activated caspase 3 and Ki67 proteins were visualized by IHC staining in intestinal tissue from rats killed at the end of weeks 3, 6 and 15 to determine levels of apoptosis, proliferation and microvascular cell dysfunction, respectively. Caspase 3 and Ki67 primary antibodies were utilized as previously published [19, 20]. A Dako-automated staining system (Autostainer link 48 and PT Link, Dako) was used to conduct IHC for caspase 3 (Abcam #ab44976, 0.200 mg/ml), Ki67 (Abcam #ab16667, concentration not provided) at dilutions of 1:1000 and 1:1200, respectively. IHC was conducted according to manufacturer's instructions using the EnVision™ FLEX kit for Dako autostainer link systems (Dako) and as previously described [21]. Briefly, 4-μm sections of jejunum and colon mounted on IHC microscope slides (Dako) were dewaxed in histolene and rehydrated through a series of graded ethanols. Antigen retrieval was conducted using a Tris/EDTA antigen retrieval buffer (0.37 g/l EDTA, 1.21 g/l Tris; pH 9.0) in the PT link antigen retrieval system (Dako) in which the buffer was preheated to 65 °C following which slides were added then heated to 97 °C for 20 min. Slides were placed in the autostainer, and sections were blocked for 5 min using the FLEX 0.3% hydrogen peroxide block (Dako). A protein block (0.25% casein in PBS, containing stabilizing protein and 0.015 mol/l sodium azide, Dako) was then applied for 30 min to reduce non-specific background staining of tissue, followed by application of the primary antibody for 60 min. Rabbit HRP-labelled polymer conjugated to goat anti-rabbit or goat anti-mouse (Dako), depending on the specifications of the antibody, was then applied for 30 min as a labelling and secondary antibody system. Sections were visualized using DAB (EnVision™ FLEX kit, Dako) and counterstained with Harris' haematoxylin for 20 s, dehydrated in 100% ethanol, cleared with histolene and coverslipped. Images were taken using NanoZoomer scanning equipment (Hamamatsu) and DAB staining of villi (jejunum only), crypts, and microvasculature was analysed on NanoZoomer Digital Pathology software (NDP.view2, Hamamatsu). Cell counts per crypt (average of 15 crypts per section) were taken for caspase 3 and Ki67 staining in the jejunum and colon mucosa as well as in the villi of the jejunum. Cell counts of caspase 3 and Ki67-immunostained cells were also performed on the

microvasculature in the lamina propria and submucosa in which stained cells were counted in 15 microvessels per section and were then averaged to give stained cells per vessel. All sections were assessed in a blinded fashion by one assessor (RLS).

Statistical analysis

Group means for histological damage scoring and apoptotic and proliferative cell counts were compared between sham-irradiated and irradiated rats per time point. All statistical analysis was conducted using the program Graphpad Prism (version 6). Differences between study group means were analysed using a two-way ANOVA with Sidak's multiple comparison. Asterisks denote significance compared to control at the time point, where *<0.05, **<0.01, ***<0.001 and ****<0.0001. Bars on graphs represent standard error of the mean

Results

Response of animals to fractionated radiation

Animals receiving fractionated irradiation to the abdomen three times a week displayed significantly lower weight gain (represented as a % from baseline) at weeks 3 ($p = 0.0055$) and from 6 weeks onwards ($p < 0.05$) when compared to animals receiving sham irradiation (Fig. 1). Whilst some rats were observed to have a dull/ruffled coat raised around the neck following irradiation, no significant differences were observed in any other clinical parameters including diarrhoea, change in temperament, reluctancy to move or skin reaction to radiation (data not shown). Small intestinal weight was significantly higher in irradiated rats at 15 weeks ($p < 0.0001$) (Table 1). There were no significant differences in large intestinal weight between irradiated and control rats (Table 1).

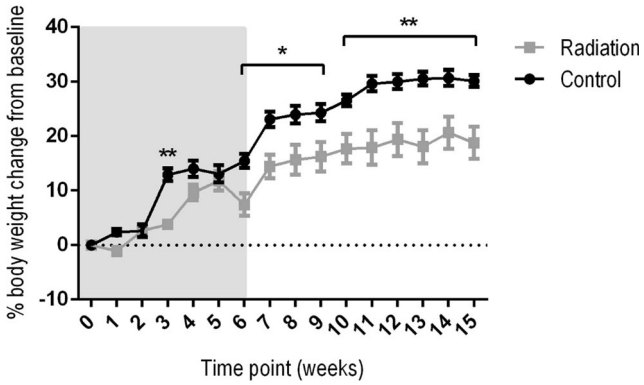

Fig. 1 Percentage body weight change from baseline (day 0) of DA rats following fractionated abdominal irradiation. Percentage body weight gain was significantly lower in irradiated rats when compared to controls at week 3 ($p < 0.0001$) and every week from week 6 until study completion in week 15 (6–9 weeks, $p < 0.05$; 10–15 weeks, $p < 0.01$). *Shaded area of graph* represents course of fractionated or sham irradiation. *Asterisk* denotes significances compared to control where *$p < 0.05$, **$p < 0.01$

Fractionated radiation-induced microvascular changes to the jejunum and colon

Jejunum

Damage scoring was not significantly altered in the jejunum at any time point (Suppl. 1). Despite this, thickening of the lamina propria and dilated, thickened, telangiectatic vessels were seen at week 6 in H&E-stained sections (Fig. 2). This thickening of the intestinal microvasculature was confirmed using Picro Sirius red in which fibrotic thickening of the microvasculature was also seen, extending from weeks 3 to 15 (Fig. 2). No significant difference was seen in the villus height or crypt depth (data not shown).

Colon

Damage scoring of the colon was significantly increased compared to controls at week 3 ($p = 0.043$) (Suppl. 1). Histopathological changes of the colon microvasculature were seen in rats receiving irradiation from week 3. Thickening of the lamina propria and dilated, thickened, telangiectatic vessels were seen from 3 weeks after the first radiation dose and had resolved by week 6 (Fig. 2). This thickening of the intestinal microvasculature was confirmed using Picro Sirius red staining and vasodilation, and fibrosis of the microvasculature was observed 6 weeks from the first radiation dose (Fig. 2). Crypt depth was significantly higher in the colon of rats receiving radiation when compared to control rats at 5 weeks ($p = 0.022$). No significant differences were noted at any other time point (data not shown).

Small intestinal weight was significantly higher in irradiated rats at week 15 ($p < 0.0001$) when compared to time matched controls. Large intestinal weight did not significantly differ between groups at any time point. Data presented as mean ± standard deviation. * denotes significance compared to time matched control where ****$p < 0.0001$

Fractionated radiation-induced microvascular cell apoptosis and reduced microvascular proliferation in the jejunum and colon

Jejunum

Apoptosis of microvascular cells significantly increased at 6 and 15 weeks in the jejunum ($p = 0.0026$ and

Table 1 Small intestinal weight and large intestinal weight of DA rats following fractionated irradiation to the abdomen

Time point	Treatment group	Small intestinal weight (g)	Large intestinal weight (g)
Week 3	*Control*	4.96 ± 0.70	1.10 ± 0.12
	Radiation	4.64 ± 0.29	1.04 ± 0.06
Week 6	*Control*	5.00 ± 0.25	1.24 ± 0.12
	Radiation	5.76 ± 0.33	1.42 ± 0.10
Week 15	*Control*	4.91 ± 0.36	1.31 ± 0.08
	Radiation	6.43 ± 0.94****	1.51 ± 0.11

p = 0.0062, respectively) in rats receiving fractionated radiation to the abdomen (Fig. 3). Cell proliferation, measured by Ki67 immunostaining, was not significantly altered at any time point in the jejunal microvasculature (Fig. 4).

Colon

Colonic microvascular cell apoptosis was significantly increased at 6 weeks ($p < 0.0001$) and 15 weeks ($p = 0.0005$) in rats receiving fractionated radiation when compared to control (Fig. 3). No significant changes in microvascular apoptosis were seen between groups at 3 weeks. Microvascular cell proliferation was significantly decreased 3 weeks from first dose in the colon ($p = 0.0072$) and was not significantly altered at week 6 (Fig. 4). In contrast, proliferation significantly increased at 15 weeks in the colon microvasculature ($p = 0.0041$).

Fractionated radiation-induced apoptosis in the epithelial cells of the crypts of the jejunum and colon

Jejunum

Apoptosis, as determined by caspase 3 immunostaining, was significantly higher in the epithelial cells of the jejunum crypts of irradiated rats at 3 and 6 weeks (p = 0022 and $p < 0.0001$, respectively) (Fig. 5). Jejunal crypt apoptosis was not significantly altered at the 15-week time point. There were also no significant changes in apoptosis in the villi of the jejunum at any time point assessed.

Colon

Apoptosis was also significantly increased in the epithelial cells lining the colonic crypts at 6 weeks when compared to control ($p = 0.014$) (Fig. 5). Again, no significant changes were seen at any other time point.

Fractionated radiation-induced proliferation in the epithelial cells of the jejunum and colon

Jejunum

There were no significant changes seen to proliferation indices in the jejunal crypts or villi at any time point after irradiation (data not shown).

Colon

Proliferative cells in the colon crypts, as determined by Ki67 immunostaining, were significantly higher in the radiation group at 6 weeks when compared to control ($p = 0.026$) (Supp. 2). No further significant changes were seen in the proliferative indices at any other time point.

Discussion

RIGT is a debilitating adverse effect of radiotherapy for cancer; however, RIGT pathobiology is not entirely understood, hindering the development of novel treatments. Key findings from this study demonstrate an increase in apoptotic cells of the microvasculature following fractionated abdominal radiation in rats. Of particular interest, apoptosis of the colon microvasculature was shown to occur 15 weeks following initial irradiation in the colon, 9 weeks following the last dose of radiation. Although previous studies have assessed changes in vascular mediator expression and histopathological features of endothelial dysfunction in RIGT [15, 22], this is the first study showing microvascular apoptosis to be occurring in the delayed, or chronic, phase of RIGT, as displayed in the 15-week time point of the current study. This late microvascular apoptosis in the colon may be linked to mediators previously known to contribute to intestinal endothelial dysfunction following irradiation such as transforming growth factor beta (TGFβ), PAR-1 and von willebrand factor [15, 16].

RIGT was shown to occur in the present study, with irradiated rats gaining significantly less weight compared to sham-irradiated controls and increased apoptosis in crypts of both the jejunum and colon. This is in support of previous studies

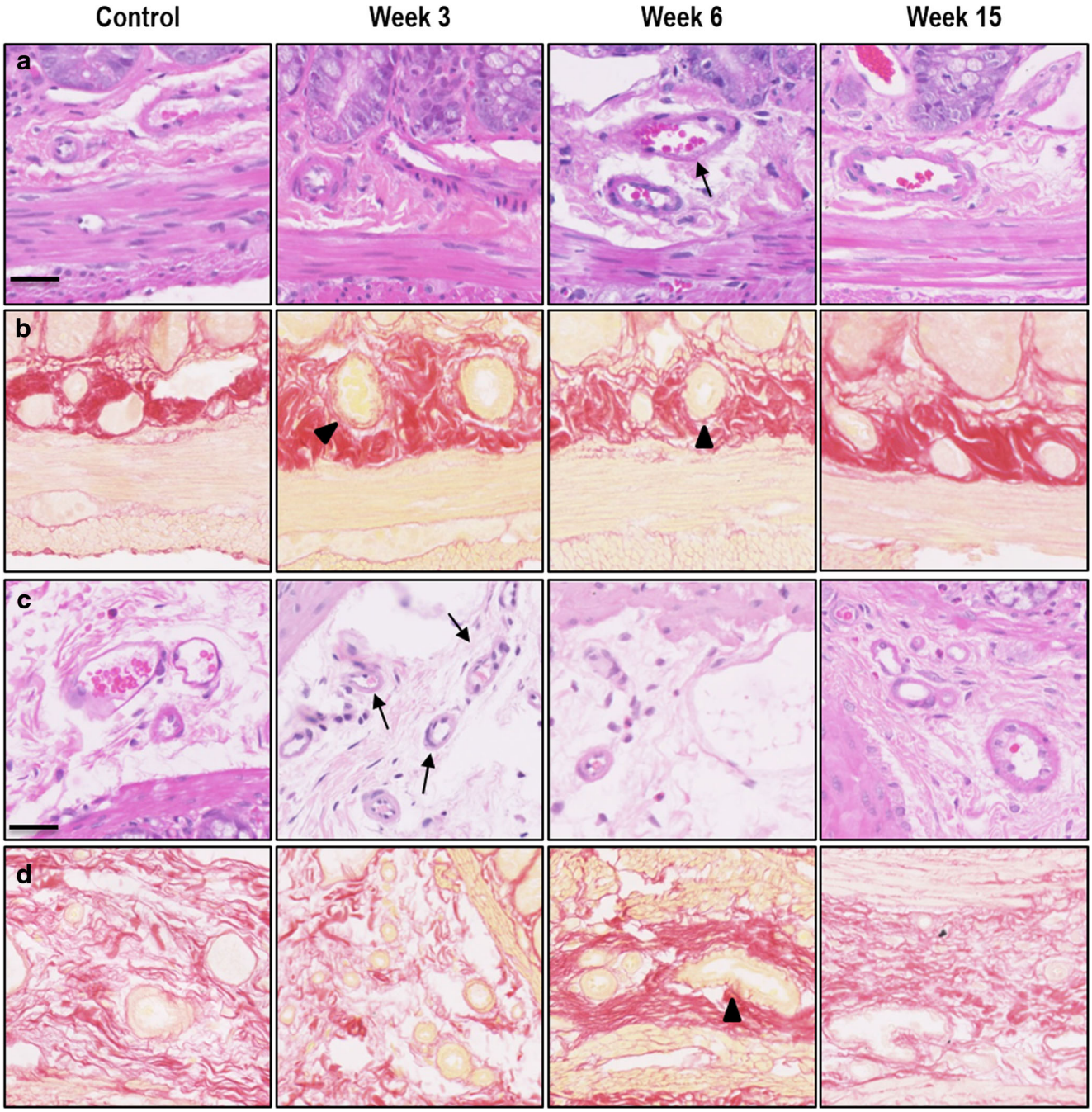

Fig. 2 Morphological changes to jejunum and colon microvasculature following fractionated irradiation. **a** H&E-stained jejunal microvasculature with dilated, telangiectatic vessels indicated by *arrows*. **b** Picro Sirius red-stained jejunal microvasculature in which fibrotic thickening of vessels can be seen from week 3 following irradiation (indicated by *arrowheads*). **c** H&E-stained colonic microvasculature displaying formation of telangiectatic vessels (indicated by *arrows*) following fractionated irradiation. **d** Picro Sirius red-stained colonic microvasculature with a thickened vessel indicated by *arrowhead*. ×40 magnification. *Scale bar* = 30 μm

assessing RIGT following fractionated radiation in vivo [19]. Yeoh and colleagues (2007) described significantly increased apoptosis in crypts of both the jejunum and colon from weeks 1–6 in DA rats administered a 6-week course of fractionated radiation to the abdomen (3 × 2.5 Gy/week). The studies did, however, differ with regard to proliferation of cells in the crypts. The present study showed an increase in proliferation at 6 weeks in the colonic crypts, as opposed to a significant decrease in proliferating cells throughout the course of radiation in the aforementioned study [19]. This could be explained in part by the differences between assessments of proliferating cells, with the present study using immunostaining of Ki67, a protein expressed in all active phases of the cell cycle, as opposed to mitotic cell count. Apoptosis was also shown to

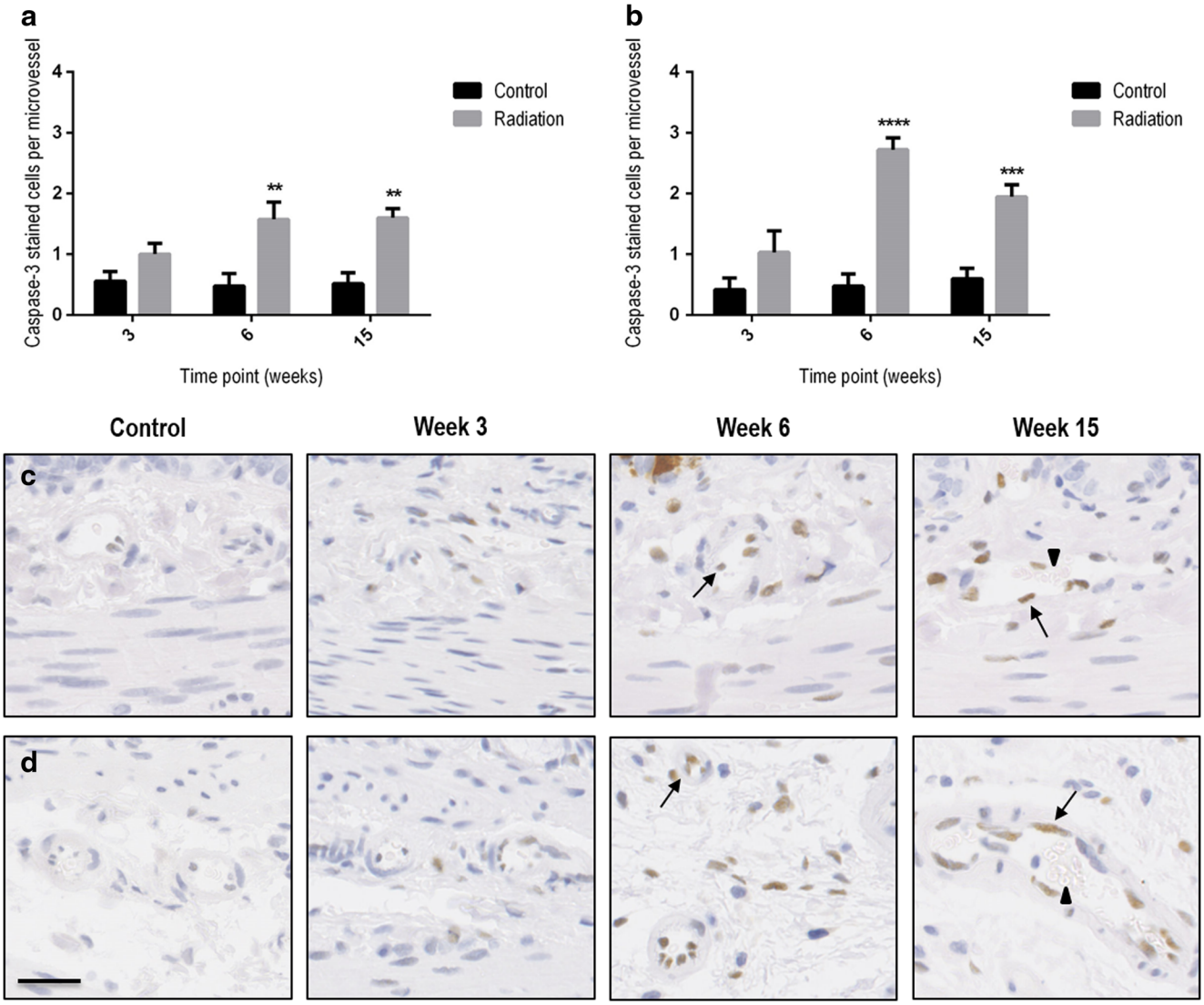

Fig. 3 Caspase 3 immunostaining of the jejunum and colon microvasculature. **a** Caspase 3 significantly increased in the microvessel of the jejunum at 6 and 15 weeks. **b** Caspase 3 staining increased in the colon microvasculature at 6 and 15 weeks. **c** Caspase 3 staining of the jejunal microvasculature. *Arrow* indicates apoptotic microvascular cells, with *arrowhead* indicating unstained red blood cells. **d** Caspase 3 staining of the colonic microvasculature with *arrows* indicating apoptotic microvascular cells at 6 and 15 weeks. *Scale bar* = 30 μm, ×40 magnification. * Significance compared to control where $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$

be highest at 6 weeks in both the jejunum and colon, indicating an accumulative effect of fractionated radiation on damage to the gastrointestinal mucosa. This is consistent with what has previously been seen in models of fractionated irradiation, in which it is proposed that the epithelial cells are able to regenerate due to breaks in the course of radiation [19]. This is reflected in the relatively unchanged levels of proliferation in the jejunum and colon throughout the time course of the study.

The significant increase in wet weight of the small intestine is most likely explained by changes associated with chronic, or delayed, RIGT. In this late phase, reactive fibrosis often occurs, leading to fibrotic thickening of the muscle layer, submucosa and vascular walls [15]. Several studies have assessed this in in vivo models of delayed radiation-induced enteropathy, and increases in collagen deposition and upregulation of fibrotic mediators including TGFβ have been shown [15, 16]. These changes, coupled with oedema and inflammatory infiltrate, could be leading to this increased wet weight of the small intestine, although parameters of histopathological damage, including oedema and presence of neutrophils, were not significantly increased in the jejunum at any time point in the current study.

This study is the first to show changes to microvascular apoptosis and proliferation in an in vivo fractionated radiation model. Of particular interest is the sustained microvascular cell apoptosis occurring 9 weeks following the cessation of irradiation in the colon (week 15 of the study). Despite this,

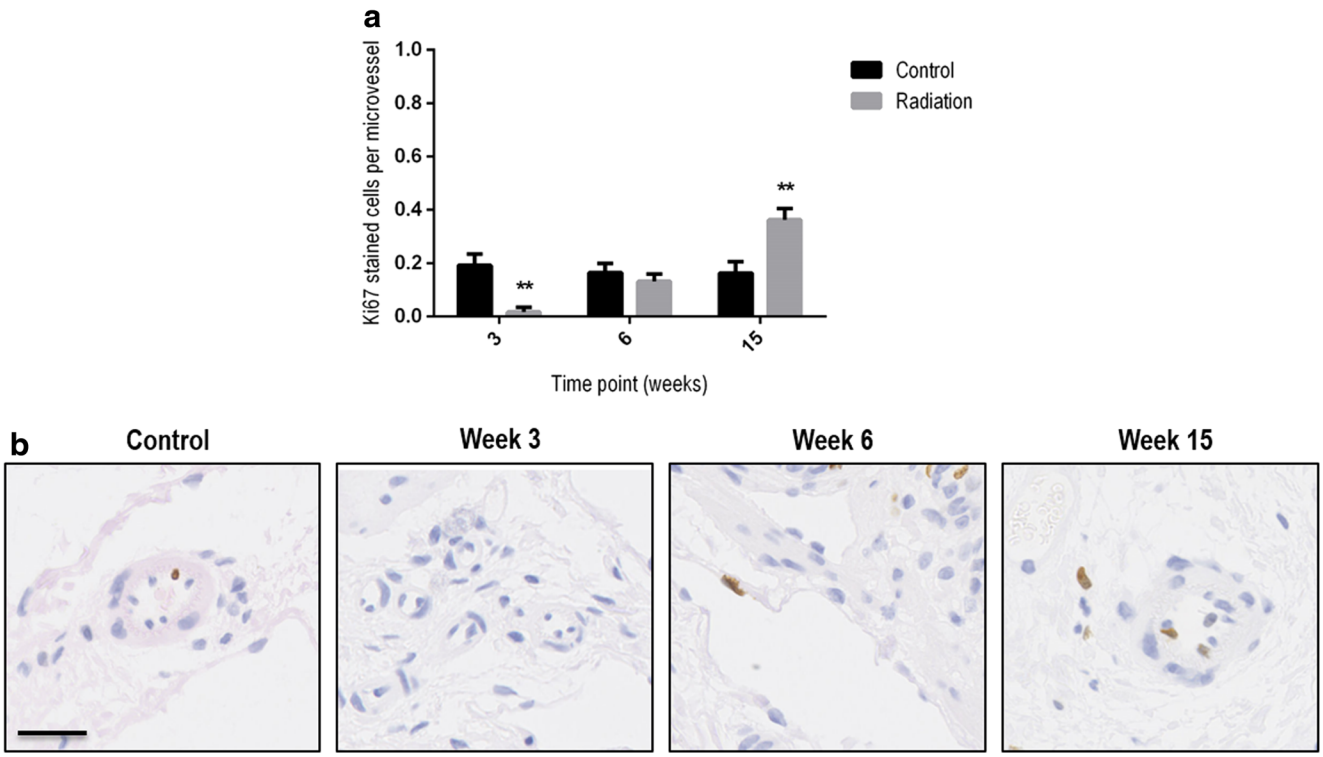

Fig. 4 Ki67 immunostaining of the jejunum and colon microvasculature. **a** Ki67-stained microvascular cells significantly decreased in the colon at week 3 and significantly increased at week 15. **b** Ki67 staining of the colon microvasculature. *Scale bar* = 30 μm, ×40 magnification. * Significance compared to control where $^{**}p < 0.01$

numbers of apoptotic cells in the crypts of the colon were not significantly higher than controls at the 15-week time point. Whilst this long-term microvascular apoptosis has not previously been shown, changes to the intestinal microvasculature have been observed many months following chemotherapy, and the biology of these cells has shown to be altered [15, 22]. Wang and colleagues (2002) have shown an increase in thrombomodulin, an endothelial transmembrane glycoprotein and a decrease in protein C, an anticoagulant and anti-inflammatory protein, in a rat model of chronic radiation enteropathy. This increase in thrombomodulin leads to endothelial dysfunction, involving decreased thromboresistance and the activation of protease-activated receptor 1 (PAR-1) [15, 17]. These mediator changes, particularly thrombomodulin and activated protein C, could play a role in the microvascular cell apoptosis seen in this delayed toxicity phase in the present study. Both thrombomodulin and protein C have been shown to be protective in models of endothelial apoptosis, thought to be due to the ability of protein C to decrease the expression and activation of transcription factor NF-kB and downregulate pro-apoptotic proteins p53 and Bax [23–25]; however, this link has not yet been explored in the context of RIGT.

The current study provides a link between the development of RIGT, particularly late RIGT, and intestinal microvascular changes, including microvascular cell apoptosis, early senescence and late proliferation. Whilst mucosal damage was significantly increased in the colon 3 and 6 weeks into the fractionated radiation schedule, as shown by various indices including damage scoring, apoptosis and decreased proliferation, apoptosis and proliferation were observed in the microvascular cells in the delayed RIGT time point of 15 weeks, 9 weeks following the last dose of fractionated irradiation. Despite this, weight gain of irradiated rats was significantly lower than control rats from 6 weeks onwards. These results indicate that changes to the intestinal microvasculature may be driving toxicity observed in the later time points, indicative of chronic, or delayed, RIGT. In order to determine whether these factors are causally related, the effects of endothelial damage on the intestinal response to RIGT need to be explored in more depth. Preliminary, in vitro studies have indicated that the endothelial cell response to radiation may induce epithelial dysfunction, independent of the BAX-mediated apoptosis of epithelial cells following direct irradiation [26]. If this pathway could be attenuated, it may lessen gastrointestinal toxicity, thus reducing the severity of RIGT. However, this distinct mechanism needs to be further explored to determine possible mediators of this response, thus providing targets for the development of novel treatments.

Apoptosis of endothelial cells following radiation has also been shown to occur in endothelial cells of other tissues, including lung, brain, spinal cord and kidney [18, 27, 28]. Whilst few studies have assessed changes to intestinal

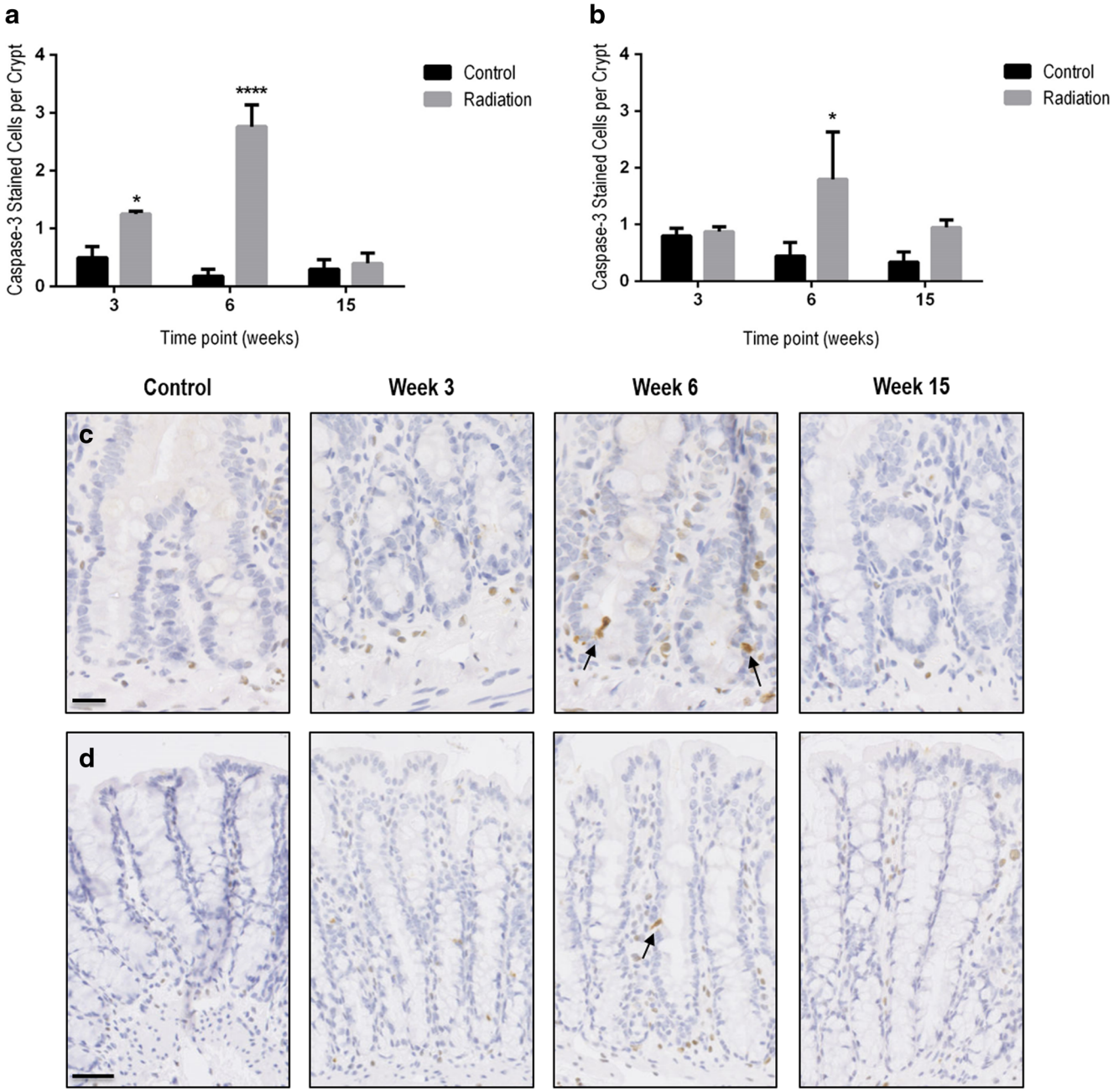

Fig. 5 Apoptosis of jejunal and colonic crypts following fractionated irradiation. **a** Caspase 3-immunostained cells significantly increased at 3 ($p = 0.0029$) and 6 weeks ($p < 0.0001$) when compared to control. **b** Caspase 3-immunostained cells were significantly increased in the colon crypts at 6 weeks in the irradiated group ($p = 0.0145$). **c** Caspase 3 immunostaining in the jejunal crypts with apoptotic cells indicated by *arrows*. **d** Caspase 3 immunostaining in the colonic crypts with apoptotic cells visible at week 6. *Scale bars* = 40 μm. *Asterisk* denotes significance compared to controls where $*p < 0.05$, $****p < 0.0001$

endothelium following irradiation, endothelial apoptosis of the intestinal microvasculature has been shown to occur shortly following single-dose irradiation in vivo [14, 29]. Endothelial apoptosis in vascular endothelial cells in the lamina propria of intestinal crypts was observed 4 h following 14 Gy whole-body irradiation of C57BL/6 J mice [29]. In a similar study by Abderrahmani and colleagues (2012), C57BL/6 J mice received a single dose of 19 Gy gamma irradiation to a segment of exposed intestine. Endothelial apoptosis in the lamina propria of the villi was shown to be significantly increased at 4 and 5 h following irradiation when compared to control [14]. Whilst microvascular apoptosis was not observed in the earliest time point of the present study (3 weeks), this may be due to the differences between fractionated and single-dose radiation. These studies, together with common clinical symptoms including rectal

bleeding and late reactive fibrosis, and results of the present study, highlight a role of the intestinal microvasculature in the pathophysiology of RIGT.

Conclusion

Changes in cell kinetics, specifically apoptosis and proliferation, were observed in the intestinal microvasculature throughout the entire time course of fractionated irradiation in vivo. Findings of this study highlight a role for intestinal microvasculature in both acute and chronic RIGT and implicate intestinal endothelium as being a potential target for the development of novel treatments.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE

Matrix metalloproteinase expression is altered in the small and large intestine following fractionated radiation in vivo

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Abstract

Purpose Radiotherapy-induced gut toxicity (RIGT) is associated with significant diarrhoea, pain and rectal bleeding. Matrix metalloproteinases (MMPs) have been reported to be involved in chemotherapy-induced gut toxicity and RIGT following single-dose irradiation in vivo. We therefore proposed MMPs would be involved in the pathobiology of RIGT following fractionated irradiation.

Methods Dark Agouti rats were treated with fractionated radiation (3 × 2.5 Gy/week for 6 weeks). Rats were killed at 3, 6 and 15 weeks to represent acute and chronic toxicities. Sections of jejunum and colon were immunostained for MMP-1, MMP-2, MMP-9 and MMP-14. Relative mRNA expression in jejunum and colon was quantified by RT-PCR for MMP-1, MMP-2, MMP-9 and MMP-14. Western blotting was also conducted on jejunum and colon tissue collected at week 6 to determine protein levels of pro- and active MMP-2.

Results MMP-2 total protein levels, determined by western blotting, significantly increased in both the jejunum ($p = 0.0359$) and the colon ($p = 0.0134$) 6 weeks into the fractionated radiation schedule. MMP-1, MMP-2, and MMP-14 mRNA expression significantly increased in the jejunum. MMP-2 mRNA expression was also significantly increased in the colon. Immunostaining of MMP-2 was observed to be increased in both crypt enterocytes and the lamina propria.

Conclusions MMP-2 plays a role in the pathobiology of gastrointestinal toxicities following fractionated irradiation. Whilst MMP-1 and MMP-14 mRNA expression was increased, this occurred only in the jejunum, suggesting MMPs are differentially involved in RIGT depending on the intestinal region. Further studies are needed to elucidate the role these mediators play in the development and potentiation of RIGT.

Keywords Mucositis · Radiotherapy · Endothelium · Gastrointestinal · Matrix metalloproteinase

Introduction

Radiotherapy-induced gut toxicity (RIGT) is a debilitating side effect of radiotherapy for cancer, estimated to affect over 80% of patients depending on target area and radiation schedule [18, 27]. Symptoms differ depending on time from last dose and can be generally divided into acute and chronic toxicities, with acute toxicities involving diarrhoea, pain, rectal bleeding and histopathological changes to the gut, including villous blunting, crypt disruption and telangiectasia [21, 33]. Chronic symptoms include incontinence, rectal bleeding and ulceration, often due to reactive fibrotic thickening of the muscle layer and microvascular walls in the small and large intestines [27]. Both acute and chronic RIGT lead to significant decreases in quality of life and can lead to radiotherapy dose reductions or cessation of treatment [20]. Despite this, limited treatment options remain, and most target individual symptoms alone [10].

Matrix metalloproteinases (MMPs), a 24-member family of zinc-dependent endopeptidases, have recently been

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identified in the development of RIGT [3, 25, 26]. Expression of MMPs can be increased at the transcriptional level by mediators known to contribute to the pathogenesis of RIGT, including pro-inflammatory cytokines and the activation of p53 by NF-κB [14, 26, 32, 34]. MMPs are synthesised as pro-zymogens and can either be secreted or, in the case of membrane type-MMPs (MT-MMPs), expressed on the cell surface [23, 31]. Pro-MMPs require proteolytic activation to allow for degradation of the extracellular matrix. Activation of pro-MMPs can be induced via the plasminogen-plasmin cascade or, in the case of MMP-2 specifically, by MT1-MMP (also known as MMP-14) [24]. MMP activation is also regulated by tissue inhibitors of matrix metalloproteinases (TIMPs), which are capable of inhibiting active MMPs [1, 24].

Following activation, MMPs are capable of altering cell growth, inflammation, cell death or angiogenesis via the up-regulation of downstream mediators such as pro-inflammatory cytokines, growth factors and chemokines [6, 12, 21]. MMP-2, MMP-3 and MMP-9, for example, are capable of activating the pro-inflammatory cytokine interleukin-1β [17]. Many of these mediators are known to be involved in the development of RIGT, with pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α), and interleukin-1beta (IL-1β) being involved in the propagation of inflammation and eventually ulceration, implicating MMPs in this pathobiology [11, 26].

Due to these widespread effects and possible link to RIGT, Strup-Perrot and colleagues (2006) assessed levels of MMP-2, MMP-3, MMP-9 and MMP-14 in the colon of rats irradiated with a single abdominal dose of 10 Gy. mRNA expression of all assessed MMPs was significantly increased in the colon, and protein levels of both pro- and active MMP-2 were also significantly increased 1, 3 and 7 days following irradiation [26]. Few studies have assessed changes in MMP expression in RIGT clinically; however, protein expression of MMP-2, but not MMP-1 or MMP-9, has been shown to be significantly increased in the irradiated normal rectal tissue of patients who had received short-term preoperative fractionated radiotherapy for rectal cancer [3]. These studies clearly highlight possible differences in MMP expression dependent on intestinal region, time since last dose and single-dose vs fractionated radiation schedules. Thus, further research investigating MMPs in different intestinal regions is required in order to better clarify our understanding of the role of the MMP pathway in RIGT.

Whilst studies have linked increases in MMPs to RIGT, no study to date has assessed MMPs in multiple regions of the gut following fractionated irradiation in a clinically relevant model of RIGT. As it is clear from the aforementioned studies that MMP expression is dependent on many factors, including intestinal region and radiation schedule, the present study aimed to assess expression and localisation of MMP-1, MMP-2, MMP-9 and MMP-14, in both the jejunum and colon in a rat model of fractionated RIGT. The Dark Agouti rat model of RIGT has been utilised in this study due to prior characterisation of the histopathological response to RIGT in this model, the fractionated radiation schedule reflecting clinical schedules and the inclusion of both acute and chronic time points [22, 33].

Materials and methods

Ethics

This study was conducted in accordance with ethics approved by the Animal Ethics Committees at the University of Adelaide (M-041-2006) and the Institute of Medical and Veterinary Science (78/05) and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Teaching at the time of the study.

Animals and experimental design

Experimental design was as previously described [22, 33]. Briefly, 30 female Dark Agouti (DA) rats were randomly assigned into groups receiving radiation and groups receiving sham irradiation). Prior to irradiation, rats in treatment and control groups were anaesthetised and placed into a custom-built container, limiting radiation to the abdomen [22, 33]. Rats in the radiation group were irradiated at a dose of 2.5 Gy/fraction three times a week for 6 weeks prescribed to a depth of 3.3 cm using a Varian Clinac Linear Accelerator (Varian Medical Systems, USA) with focus-skin distance of 130 cm. Rats in both sham irradiated and irradiated groups were killed at the ends of weeks 3, 6 and 15 ($n = 5$ per treatment group at each time point). These time points corresponded to total accumulated doses of 0 Gy (sham irradiated controls), 22.5 Gy (3-week time point), or 45 Gy (6- and 15-week time points). These time points and doses were calculated to represent acute toxicity (< 12 weeks from start of abdominal irradiation) and delayed toxicity (≥ 12 weeks from start of abdominal irradiation). Gastrointestinal tissue was collected following flushing, with two 2-cm sections of jejunum (at 25% length of small intestine from the pylorus) and colon (at 50% length of large intestine) resected. One section of tissue was formalin-fixed and paraffin-embedded, and the other was snap frozen in liquid nitrogen and stored at − 80 °C.

RNA isolation and reverse transcription

RNA isolation was performed using the Nucleospin® RNA II kit (Macherey-Nagel, Duren, Germany) as previously described [22]. Briefly, ~ 15 mg of tissue was homogenised in

cell lysis buffer and beta-mercaptoethanol using the TissueLyser LT (Qiagen) with stainless steel beads (Qiagen). The lysate was filtered through silica membrane filter columns, 70% ethanol was added, the silica membrane was desalted and DNA was digested using the DNAse reaction mixture. Following washing and drying of the silica membrane, RNA was eluted in 60 μl of RNase-free water. RNA concentration and purity were determined using the TAKE3 plate and Synergy My Reader (BioTek). RNA was converted to cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's protocol. Total cDNA concentration and purity were quantified using the TAKE3 plate and Synergy My Reader. Stock cDNA was diluted to 100 ng/μl with nuclease-free water.

Real-time polymerase chain reaction

Quantitative RT-PCR was performed using the Rotor Gene 3000 (Corbett Research, Sydney, Australia). Reaction mixtures contained 1 μl of cDNA, 5 μl of SYBR green PCR Master Mix (Applied Biosystems), 5 μl of nuclease-free water and 0.5 μl each of 50 pmol/μl forward and reverse primers (Table 1). Thermal cycling conditions were optimised for each target gene and melt curve analysis was performed to determine the presence of any dimers. Conditions included a denaturation step at 95 °C for 10 min and 40 cycles at 95 °C (15 s), annealing at 55 °C (MMP-1 and MMP-9), 56 °C (MMP-14) or 60 °C (MMP-2) (15 s) and acquiring at 72 °C (20 s). The housekeeping gene used was 18S, which was determined to be stable throughout treatment groups. Primer efficiency was between 90 and 110% for each target gene against 18S, and thus the $2^{-\Delta\Delta CT}$ method was used to determine fold change in gene expression relative to 18S and untreated controls from each time point.

Immunohistochemistry

MMP proteins were visualised using immunohistochemical (IHC) staining in jejunal and colonic tissue from rats killed at the end of weeks 3, 6 and 15 to determine protein localisation. An automated staining system (Autostainer link 48 and PT Link, Dako) was used to conduct IHC for MMP-1 (no. PAB12708, Abnova), MMP-2 (no. ab37150, Abcam), MMP-9 (no. ab58803, Abcam) and MMP-14 (no. ab53712, Abcam), at concentrations of 2 μg/ml (MMP-1), 1.25 μg/ml (MMP-2 and MMP-9) and 2.5 μg/ml (MMP-14). The EnVision™ FLEX kit for Dako autostainer link systems (Dako) was used as previously described [30]. Slides were scanned using the Nanozoomer (Hamamatsu Photonics) and protein localisation was qualitatively assessed for each slide and stain.

Western blot

Protein extraction

Protein was extracted from 30-mg sections of snap frozen jejunum and colon. Sections were homogenised in cooled Eppendorf tubes with homogenising beads at 50 GHz for 5 min with 250 μl of 1:10 protease inhibitor cocktail (10 μg/ml in sterile PBS, EDTA free; no. P8340, Sigma) in RIPA buffer (no. R0278, Sigma) using the TissueLyser LT (Qiagen) with stainless steel beads (Qiagen). Beads were removed and tubes were centrifuged at 10,000×*g* for 15 min at 4 °C. Supernatant was collected and protein was quantified using the BCA protein quantification assay (no. 23227, Thermo Scientific) according to manufacturer's protocol; after which, it was stored at − 80 °C.

Western blotting

Western blotting was performed to quantify levels of pro- and active forms of MMP-2 protein in the jejunum and colon following fractionated irradiation. The iBlot™ 2 dry blotting system (Invitrogen) was used to perform the western blot according to manufacturer's protocol. Samples were prepared with 30 μg of total protein, 10 μl Bolt LDS Sample Buffer (no. B0008, ThermoFisher), 4 μl of Bolt Reducing Agent (no. B0009, ThermoFisher) and dH_2O to yield a total sample

Table 1 Primer sequences for housekeeping and target genes

Gene	Primer sequence (5′-3′)	Nucleotide position	Amplicon length (bp)	Tm (°C)	Ref
18S	F: CAT TCG AAC GTC TGC CCT AT	344-452	109	52	[28]
	R: GTT TCT CAG GCT CCC TCT CC			56	
MMP-1	F: CCT TCC TTT GCT GTT GCT TC	51-108	58	52	Designed in Primer3
	R: CTG AAA CAC GGG GAA ACT GT			52	
MMP-2	F: CTG ATA ACC TGG ATG CAG TGCT	2138-2272	135	55	[1]
	R: CCA GCC AGT CCG ATT TGA			50	
MMP-9	F: AAG CCT TGG TGT GGC ACG AC	760-876	117	56	[29]
	R: TGG AAA TAC GCA GGG TTT GC			52	
MMP-14	F: GTG GAT GGA CAC CGA GAA CT	949-1039	91	54	Designed in Primer3
	R: CTT TGT GGG TGA CCC TGA CT			54	

volume of 40 μl. Samples were vortexed, heated at 70 °C for 10 min and pipetted into lanes of a 4–12% Bis-Tris plus 12-well gel (no. NW04122, ThermoFisher) in the iBlot™ mini gel tank with 1× SDS Running buffer (20× Bolt MES Running Buffer in dH2O, no. B0002, ThermoFisher). Five-microliter Bio-Rad Precision Plus Protein™ dual colour standard (no. 1610374, Bio-Rad) was used as a standard ladder. Electrophoresis was run at a constant 150 V for 45 min. The iBlot transfer system was used with copper transfer stacks to transfer from gel to a PVDF membrane (no. IB24002, ThermoFisher). The membrane was washed with TBST, reactivated with methanol, rinsed with dH_20 and immersed in 1× iBind fluorescent detection (FD) solution (iBindTM FD Solution Kit, no. SLF1019, ThermoFisher). Primary antibodies for MMP-2 (no. ab37150, Abcam) and beta-actin (no. ab8224, Abcam) were diluted in iBind FD solution at 5 and 0.167 μg/ml, respectively. The secondary antibody (IRDye 800CW goat anti-Rabbit, no. 925–32,211, Li-Cor) was diluted in iBind FD solution at a concentration of 0.33 μg/ml. The iBind system (no. SLF1010, ThermoFisher) was used, with iBind FD solution applied to the card, to add the primary and secondary antibodies to the membrane. The membrane was left overnight and scanned using the LI-COR Odyssey CLx imager at 800 nM with an intensity of 3.0. MMP-2 bands were quantified against the beta-actin housekeeping band using Image Studio™ software (Li-Cor).

Statistical analysis

Means for each treatment group (sham irradiated and irradiated) were compared at each time point. Following normality testing, differences between treatment group means were analysed using either a two-way ANOVA with Sidak's multiple comparison or a Kruskal-Wallis test with multiple comparison. Asterisks denote significance compared to control at the time point, where *< 0.05, **< 0.01, ***< 0.001 and ****< 0.0001.

Results

Fractionated irradiation induced intestinal upregulation of MMP-1, MMP-2 and MMP-14 at the transcriptional level

Jejunum

RT-qPCR was performed to determine mRNA expression of MMP-1, MMP-2, MMP-9 and MMP-14 following fractionated irradiation. MMP-1 mRNA expression was significantly increased in the jejunum 3 weeks into the fractionated radiation schedule ($p = 0.0086$) but not at the 6- or 15-week time points (Fig. 1a). MMP-2 mRNA expression increased 172-fold at week 3 in the jejunum ($p < 0.0001$) but was not significantly different at other timepoints (Fig. 1b). MMP-9 mRNA expression was not significantly altered at any time point under investigation (Fig. 1c). MMP-14 mRNA expression was significantly increased in the jejunum at week 3, by 26-fold, and at week 6, by 24-fold ($p = 0.0002$ and $p = 0.0065$, respectively). There was no significant difference seen at 15 weeks following radiation (Fig. 1e).

Colon

There were no significant changes to MMP-1 mRNA expression in the colon (Fig. 2a) at any time point under investigation. MMP-2 mRNA expression significantly increased by 14- and 24-fold at 3 and 6 weeks in the colon ($p = 0.0313$ and $p = 0.0001$, respectively) (Fig. 2b). However, no significant change was seen at 15 weeks after radiation. MMP-9 mRNA expression was not significantly altered at any of the time points investigated (Fig. 2c). In contrast to what was observed in the jejunum, MMP-14 was not significantly altered at any time point in the colon (Fig. 2e).

Protein expression and localisation of MMP-2 was altered in the colon following fractionated irradiation

As changes in mRNA expression of MMP-1, MMP-2 and MMP-14 were observed, immunohistochemistry was *qualitatively assessed* to *initially* determine any changes in protein localisation or staining intensity *prior to conducting western blotting*. MMP-2 immunostaining appeared to increase following irradiation, represented by an increase in cytoplasmic staining in crypt enterocytes observed at weeks 3 and 6 (Fig. 3). There was also a slight increase in localisation of MMP-2 to the lamina propria, particularly of the microvasculature, at weeks 3 and 6 (Supplementary Fig. 1). Staining intensity of the other MMPs assessed, MMP-1, MMP-9 and MMP-14, did not appear to be altered in the jejunum or colon at any time point under investigation.

Pro- and active forms of MMP-2 protein were significantly increased in the jejunum following fractionated irradiation

As increases in immunostaining of MMP-2 were seen at 6 weeks, but unable to be quantified, western blotting was undertaken for MMP-2 in both the jejunum and colon at the 6-week time point. The 6-week time point was chosen due to a maximum radiation dose at this time point (45 Gy), as well as significant histopathological damage, including apoptosis of both the jejunal and colonic crypts and microvascular cells as previously reported [17]. Levels of both pro- and active MMP-2 were able to be quantified via western blotting, as these forms have different molecular weights and thus present

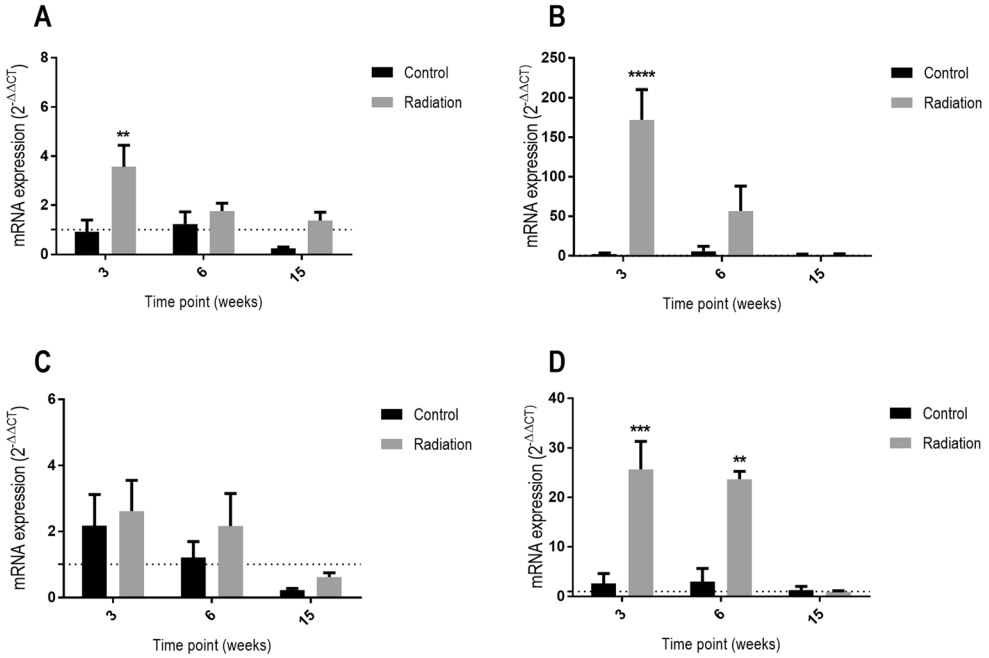

Fig. 1 Expression of MMP-1, MMP-2, MMP-9 and MMP-14 in the jejunum following abdominal irradiation. **a** mRNA expression of MMP-1 was significantly increased by 3.5-fold at week 3 ($p = 0.0086$). **b** MMP-2 was significantly increased by 172-fold 3 weeks into the fractionated radiation course ($p < 0.0001$). **c** MMP-9 mRNA expression was not significantly altered at any time point in the jejunum. **d** MMP-14 expression was significantly increased by 26-fold and 24-fold at the 3- and 6-week time points, respectively ($p = 0.0002$; $p = 0.0065$). Dotted line represents baseline fold change. Data shown is mean + standard error of the mean (SEM). Asterisks denote significance compared to controls at each time point, where **< 0.01, ***< 0.001 and ****< 0.0001, $n = 5$

as separate bands. Pro-MMP-2 was significantly increased in both the jejunum ($p < 0.0001$) and colon ($p < 0.0001$) when compared against housekeeping band intensity (β-actin) and average intensity of controls (Fig. 4). Active MMP-2 was significantly increased following irradiation in the jejunum ($p = 0.0011$) but not in the colon (Fig. 4).

Discussion

The current study assessed mRNA and protein expression of several MMPs thought to be involved in the development of RIGT in a model of fractionated irradiation. A key finding of this study suggests MMP-2 mRNA expression is upregulated

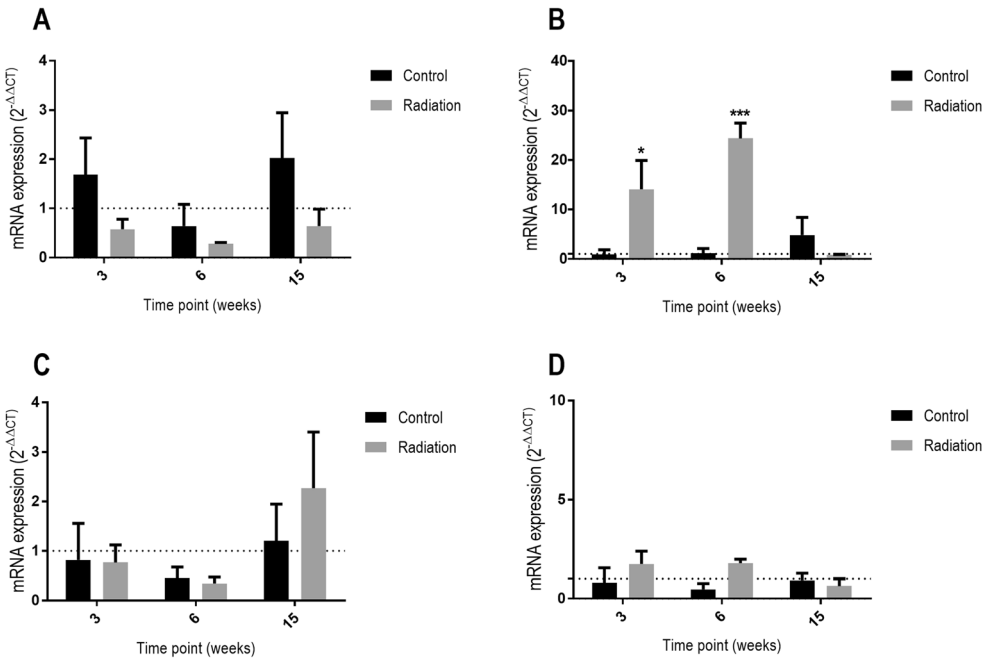

Fig. 2 Expression of MMP-1, MMP-2, MMP-9 and MMP-14 in the colon following abdominal irradiation. **a** Colonic MMP-1 mRNA expression was not significantly altered at any time point ($p > 0.05$). **b** Colonic MMP-2 mRNA expression was significantly increased at week 3, with a 14-fold increase ($p = 0.0313$). MMP-2 expression was also significantly increased at week 6 by 24-fold ($p = 0.0001$). Colonic mRNA expression of MMP-9 (**c**) and MMP-14 (**d**) were not significantly altered at any time point. Dotted line indicates baseline fold change. Data shown is mean + SEM. Asterisks denote significance compared to controls at each time point, where *< 0.05 and ***< 0.001, $n = 5$

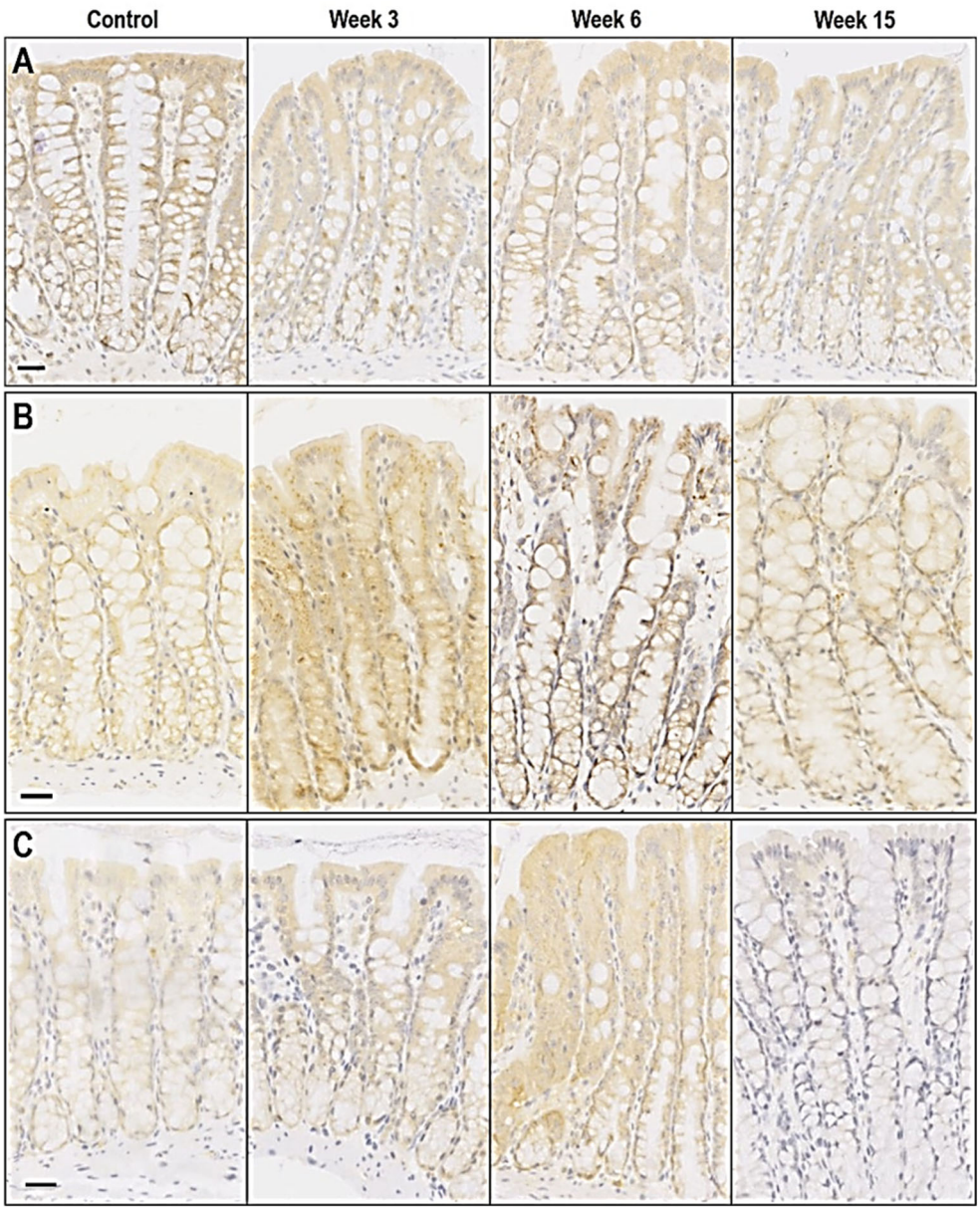

Fig. 3 MMP-1, MMP-2 and MMP-9 immunostaining in the colonic crypts following fractionated irradiation. **a** MMP-1 staining intensity in the colonic crypts was not altered at any time point following fractionated irradiation. **b** MMP-2 immunostaining was increased in the colonic crypts following 22.5 and 45 Gy fractionated irradiation (weeks 3 and 6, respectively). **c** MMP-9 immunostaining was not altered in the colonic crypts. ×10 magnification, scale bar = 30 μm (applies to all images in same row), $n = 5$

in both the small and large intestine in response to abdominal fractionated irradiation. MMP-1 and MMP-14 mRNA expression was also significantly increased in the jejunum only. The increases in these MMPs occurred alongside loss of body weight, increases in histopathological damage and microvascular changes previously shown in the same model [22].

The key finding of a significant increase in MMP-2 mRNA expression in both the jejunum and colon early in the fractionated radiation schedule accords with the consistent upregulation of MMP-2 reported in both clinical and preclinical studies of RIGT [2, 26]. MMP-2 expression was increased 172-fold at the 3-week time point in the jejunum, but not significantly altered at 6 weeks, despite an increase in cumulative dose. It is possible that the fractionated schedule of radiation is a contributor to this, with repeated healing occurring between doses over the 6-week radiation schedule, possibly leading to a decrease in MMP-2 expression. As MMP-2 immunohistochemical staining was also increased in the colonic crypts at the 3- and 6-week time points, protein levels were further quantified using western blotting to ascertain differences in pro and active forms. Whilst MMPs are expressed in their latent forms, activation is essential to many of the downstream effects (Hofmann 2000). Both pro- and active MMP-2 were significantly increased in the jejunum following irradiation; however, only pro-MMP-2 was significantly increased in the colon. Rats receiving fractionated irradiation had significantly lower weight gain, significantly increased histopathological damage scoring in the colon, telangiectatic vessel formation and apoptosis in the crypts of the jejunum and colon occurring at the

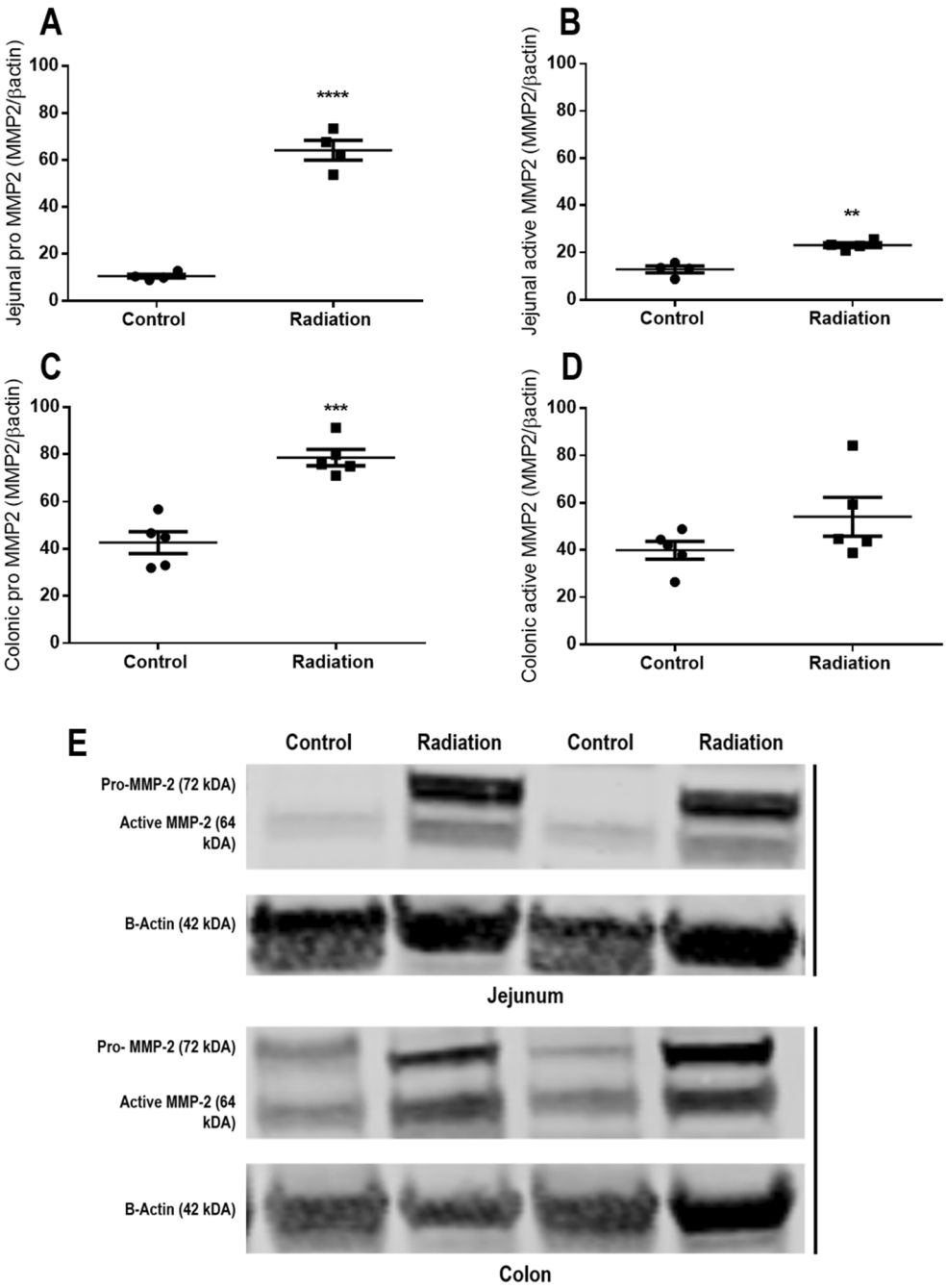

Fig. 4 MMP-2 protein expression was significantly increased in the jejunum and colon 6 weeks into the fractionated radiation schedule. **a** Jejunal levels of relative pro-MMP-2 were significantly increased at 6 weeks ($p < 0.0001$). **b** Jejunal levels of active MMP-2 were significantly increased at 6 weeks ($p = 0.0011$). **c** Colonic levels of pro-MMP-2 were significantly increased at week 6 ($p < 0.0001$). **d** Colonic levels of active MMP-2 were not significantly altered at 6 weeks ($p > 0.05$). **e** Representative MMP-2 bands at 72 kDA (pro-MMP-2) and 64 kDA (Active MMP-2) and B-actin housekeeping band at 42 kDa in both the jejunum and colon. MMP-2 bands were quantified against the beta-actin housekeeping band using Image Studio™ software. Data shown is mean + SEM. Asterisks denote significance compared to controls at each time point, where **< 0.01, ***< 0.001 and ****< 0.0001, $n = 5$

same time points as significant increases in MMP-2 mRNA and protein expression [22].

Strup-Perrot and colleagues (2006) showed protein levels of pro- and active MMP-2 to be increased 1, 3 and 7 days in the colon following 10-Gy abdominal irradiation in rats. However, the jejunum was not assessed in the aforementioned study. MMP-2 protein expression has been correlated with decreased mean bursting pressure and breaking strength during anastomic healing 3 and 7 days following 40-Gy fractionated irradiation in Wistar rats [4]. Clinically, active MMP-2 levels have correlated to poor outcomes in RIGT and radiation enteritis [2]. Angenete and colleagues (2007) found increased levels of MMP-2 in both tumour tissue and tumour-adjacent normal rectal tissue within the radiation field to correlate with fistula formation in patients who had received preoperative fractionated irradiation (5 × 5 Gy) for rectal cancer. This increase of MMP-2 following radiation, in both preclinical and clinical studies, supports the findings of the present study. Correlation of this increase with poor outcomes such as impaired anastomic healing, and fistula formation, suggests MMP-2 may be a clinical target for the treatment of RIGT.

Following transcriptional upregulation and activation of secreted pro-MMP-2, MMP-2 is capable of degrading several ECM components, such as collagen IV and VXII, plasminogen and decorin, to active downstream products such as vascular endothelial growth factor (VEGF), transforming growth factor beta (TGFβ) and form angiostatin, endostatin and tumstatin [21]. Active MMP-2 is also able to shed and activate pro-inflammatory cytokines such as TNF-α and IL-1β [7]. Active MMP-2 is thus able to indirectly alter cell signalling, altering cell growth, migration and angiogenesis and in the present study is upregulated at the same time point as apoptosis in both the jejunum and colon [22, 31]. Previous studies have confirmed significantly increased expression of pro-inflammatory mediators, such as TNF-α and IL-1β, in animal models of RIGT [9, 15]. This supports a possible pathway in which MMP-2 may be contributing to the exacerbation of acute RIGT through the activation of these pro-inflammatory mediators. Future studies assessing specific inhibition of MMPs, particularly MMP-2, as a possible treatment for RIGT should investigate the potential involvement of pro-inflammatory cytokines in this pathway. From the results of the present study, when considered in the context of previous literature, it is likely that the upregulation and activation of intestinal MMP-2 following irradiation play an important role in the pathogenesis of RIGT [3, 7, 26].

MMP-14 was also significantly upregulated in the jejunum following irradiation in the present study. As previously mentioned, MMP-14 is intricately involved with the activation of pro-MMP-2 [7]. Active MMP-14 expressed on the cell surface binds to TIMP-2 via the N-terminal domain. This complex then acts as a receptor for pro-MMP-2 after which a free MMP-14 molecule is able to cleave and activate pro-MMP-2 [7]. This mechanism of MMP-2 activation by MMP-14 may be reflected in the present study, in which levels of both pro- and active MMP-2 were concurrently increased with MMP-14 expression in the jejunum. However, pro- but not active MMP-2 was increased in the colon, in which MMP-14 mRNA expression was not significantly altered. Differences between the response of the jejunum and colon to radiation have been characterised in previous studies [5, 22]. Loss of epithelial cell lining and villi has been noted 6–24 h following single-dose irradiation in rats [5]. In contrast, damage to the structure of the colonic crypts occurs from 24 h following irradiation [5]. The upregulation of MMP-14 at acute time points in the jejunum corresponds to significantly lower weight gain and apoptosis in both epithelial and endothelial apoptosis in the same model [22]. Apoptosis has also been shown to occur earlier in the jejunum than in the colon in the model of fractionated irradiation used in the present study [22]. Whilst the timing of these events is likely altered in a fractionated radiation schedule, this reflects a differing profile of RIGT in the different intestinal regions. Additionally, the increase in MMP-14 expression in the present study does not appear to correspond to increased cumulative doses, with relative expression similar at both 3- and 6-week time points. However, the direction of the relationship between the increase in MMP expression and the histopathological damage in the gut remains unclear. The pattern of MMP-2 and MMP-14 expression following radiation potentially supports the role of MMP-14 in the activation of MMP-2, which should be considered when exploring MMP inhibition as a treatment for RIGT.

In the present study, MMP-1 mRNA expression was found to be significantly increased in the jejunum following 22.5-Gy fractionated abdominal irradiation. This was not reflected in protein localisation, with no changes in MMP-1 immunostaining in the jejunum or colon. MMP-1 is classified as a collagenase due to its ability to degrade collagen and gelatin; however, it is also capable of activating pro-MMP-9 [13]. Transcription of MMP-1 can be increased in response to pro-inflammatory cytokines, epidermal growth factor as well as following activation of p38 mitogen-activated protein kinases (MAPK), which has previously been shown to be increased in RIGT [13, 16, 19]. Al-Dasooqi and colleagues (2010) found MMP-1 protein expression to be downregulated early and upregulated late during chemotherapy-induced gut toxicity (CIGT), suggesting a role for MMP-1 in healing. MMP-1 could be playing a similar role in the present study as it is known to regulate cell migration and reepithelialisation; however, MMP-1 was not altered in the chronic RIGT time point of the present study, 6 weeks following the cessation of radiation [21]. It is possible that any increase in MMP-1 could have returned to baseline at this point.

In contrast with the Strup-Perrot and colleagues (2006) study, assessing MMP levels following irradiation in rats we found no significant difference in MMP-9 mRNA expression. MMP-1 and MMP-14 mRNA expressions, like MMP-2, were significantly increased in the jejunum. Despite the significantly increased mRNA expression of these MMPs, no changes in immunostaining intensity or localisation were observed. This lack of post-translational modification of the remaining MMPs may be due to the time points of the present study in which all tissue was collected 24 h following irradiation, despite different cumulative radiation doses at the 3- and 6-week time points. MMP-9 has been shown to be increased 3–7 days following irradiation in rats; however, this has been assessed in models of single-dose irradiation [3, 26]. Changes in mRNA expression do not always correlate strongly with protein expression so it is also possible that these changes had no molecular manifestation [8]. Additional studies are required to further assess the role of these MMPs in RIGT in different regions, models and with different treatment schedules.

Conclusion

Whilst this study is consistent with previous studies assessing MMP expression following radiation, this is the first study to

assess the MMP expression in multiple regions of the gut in a fractionated radiation model of RIGT. Key findings of this study suggest MMP-2 to be involved in the development of RIGT in both the small and large intestine following abdominal fractionated radiation. MMP-2 is widely understood to be overexpressed in many human cancer and levels of active MMP-2 have been shown to increase risk of metastasis of head and neck cancer [7]. This study presents a unique possibility for specific MMP inhibitors to improve radiotherapy-induced gastrointestinal toxicities whilst not reducing efficacy of cancer treatment. Further studies are now warranted to assess these inhibitors for their therapeutic potential in RIGT.

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Compliance with ethical standards

Conflict of interest The authors report no declarations of interest.

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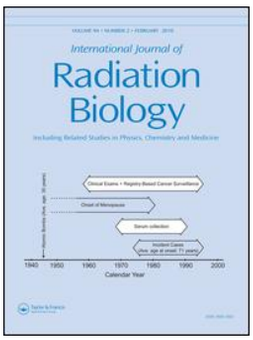

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Vascular endothelial growth factor (VEGF), transforming growth factor beta (TGFβ), angiostatin, and endostatin are increased in radiotherapy-induced gastrointestinal toxicity

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Abstract

Purpose: Radiotherapy-induced gut toxicity (RIGT) is a debilitating effect of radiotherapy for cancer, often resulting in significant diarrhoea and pain. Previous studies have highlighted roles of the intestinal microvasculature and matrix metalloproteinases (MMPs) in the development of RIGT. We hypothesized vascular mediators would be significantly altered in a dark agouti (DA) rat model of RIGT. Additionally, we aimed to assess the effect of MMP-2 and -9 inhibition on the response of tumour-associated microvascular endothelial cells (TAMECs) to radiation.

Methods: Dark Agouti (DA) rats were administered 2.5 Gy abdominal irradiation (3 times/week over 6 weeks). Vascular endothelial growth factor (VEGF), transforming growth factor beta (TGFβ), von Willebrand factor (VWF), angiostatin, and endostatin expression was assessed at 3, 6 and 15 weeks. Additionally, DA rat mammary adenocarcinoma tumour-associated microvascular endothelial cells (TAMECs) were used to assess the effects of radiation (12 Gy) and the MMP inhibitor SB-3CT on MMP, VEGF, and TGFβ expression, and cell viability.

Results: VEGF mRNA expression was significantly increased in the colon at week 15 ($p = 0.0012$), and TGFβ mRNA expression was significantly increased in both the jejunum and colon at week 3 ($p = 0.0280$, and $p = 0.0310$, respectively). Endostatin immunostaining was significantly increased at week 3 ($p = 0.0046$), and angiostatin at 3 and 6 weeks ($p = 0.0022$, and $p = 0.0135$, respectively). MMP-2 and -9 mRNA and total protein levels were significantly increased following irradiation of TAMECs. Although this increase was significantly attenuated by SB-3CT, it did not significantly alter endothelial cell viability or VEGF and TGFβ mRNA expression.

Conclusions: Findings of this study support the involvement of VEGF, TGFβ, angiostatin, endostatin, and MMP-2 in the pathobiology of RIGT. However, the relationship between these mediators is complex and needs further investigation to improve understanding of their therapeutic potential in RIGT.

Introduction

Radiotherapy-induced gut toxicity (RIGT), estimated to affect 60-80% of patients receiving radiotherapy for abdominal or pelvic tumours, induces debilitating symptoms including, but not limited to, diarrhoea, pain, rectal bleeding, and incontinence (Theis et al. 2010; Shadad et al. 2013; Hauer-Jensen et al. 2014). RIGT is often divided into acute and chronic toxicity, with acute changes involving histopathological damage to villi and crypts, and chronic involving reactive fibrotic thickening (Yeoh et al. 2007; Theis et al. 2010). Previous research has suggested the involvement of the intestinal microvasculature in the development of RIGT, and several mediators have been investigated (Kruse et al. 2004; Yeoh et al. 2007; Stansborough et al. 2017). NFκB, proinflammatory cytokines, and matrix metalloproteinases (MMPs) are all upregulated in the intestine following radiation, and have downstream effects on vascular mediators such as vascular endothelial growth factor (VEGF), transforming growth factor beta (TGFβ), von Willebrand factor (VWF), and the antiangiogenic mediators endostatin and angiostatin (Stansborough et al. 2016). Whilst a link has been proposed in RIGT (Stansborough et al. 2016) there is limited research which has investigated changes in these mediators in the intestinal microvasculature during a fractionated radiation schedule.

Our previous studies have shown the intestinal microvasculature to be involved in RIGT, with apoptosis, cytostasis, telangiectasis, and fibrotic thickening occurring early and late following fractionated irradiation in dark agouti (DA) rats (Yeoh et al. 2005; Stansborough et al. 2017). In addition, several regulators of angiogenesis and vascular physiology including, but not limited to, TGFβ, VEGF, and VWF, are known to be altered in the intestine following irradiation (Wang et al. 2001; Wang et al. 2002; Milliat et al. 2006; Lenting et al. 2012; Boerma et al. 2013). VEGF, bound to heparin in the extracellular matrix, is able to be released by matrix metalloproteinases (MMPs) such as MMP-2, and MMP-9 (Carmeliet 2005). The anti-angiogenic mediators angiostatin and endostatin have also been shown to increase in response to irradiation of cancer endothelial cells derived from human breast cancer tissue (Oh et al. 2014). A reduction in angiostatin via knockdown of plasminogen restored tube

formation of these cells, suggesting angiostatin is involved in endothelial regulation following irradiation (Oh et al. 2014). Although it appears angiostatin and endostatin are involved in endothelial response to irradiation this connection is yet to be explored in RIGT. With the increasing investigation of the possible use of anti-angiogenic agents as adjuvant cancer therapies alongside chemotherapy or radiotherapy, targeting mediators such as vascular endothelial growth factor (VEGF) and endostatin, the role of these mediators in RIGT needs to be further explored.

Previous research has clearly shown MMPs are involved in RIGT, with MMP-2 in particular shown to be significantly increased during RIGT (Strup-Perrot et al. 2006). It has also been hypothesised MMPs are involved in microvascular changes and alteration to vascular mediators such as VEGF, TGFβ, and VWF in RIGT (Stansborough et al. 2016). MMPs are able to regulate the expression of several of these mediators via the degradation of the extracellular matrix (ECM) leading to the release of latent VEGF and TGFβ, as well as forming angiostatin and endostatin by cleaving plasminogen and collagen XVIII respectively (Sternlicht and Werb 2001; Kalluri 2003; Carmeliet 2005). In degrading the ECM, MMPs also promote endothelial migration, proliferation and tube formation (Kalluri 2003). Despite previous literature, a direct link has not been established between MMPs and vascular mediator expression following radiation, and the effect of these changes on endothelial cells. Thus the present study aimed to first confirm the alteration of vascular mediators VEGF, TGFβ, VWF, angiostatin, and endostatin in the jejunum and colon following fractionated irradiation in DA rats. We then aimed to determine whether inhibition of MMP-2 and -9 with SB-3CT altered endothelial viability and expression of these vascular mediators in primary tumour-associated microvascular endothelial cells (TAMECs) derived from DA rat mammary adenocarcinomas. TAMECs have been successfully cultured for previous, similar experiments, have been phenotyped, and their cultivation in DA rats allows for consistency between both components of the study (Bateman et al. 2013). In utilising this primary *in vitro* model we aimed to assess the effects of SB-3CT on tumour endothelial response to irradiation as an initial investigation into the response of tumour endothelium to MMP-2 and -9 inhibition. As the cell type used in the *in vitro*

component of the study does not directly translate to RIGT, further studies are required to assess the effects of SB-3CT on intestinal endothelium following irradiation. However, results of the present study form a foundation for further investigation into the possible use of SB-3CT following irradiation.

Materials and Methods

Ethics

Both studies were conducted in accordance with ethics approved by the Animal Ethics Committees at the University of Adelaide (M-041-2006; M-2015-117) and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Teaching (2013).

Animals and Experimental Design

Archival tissue from a prior study was utilised in the *in vivo* component of the present study, and experimental design has been previously described (Yeoh et al. 2007; Stansborough et al. 2017). Briefly, 30 female Dark Agouti (DA) rats were randomly assigned into two groups, one group receiving sham irradiation, and one group receiving a schedule of fractionated irradiation. Both groups of rats were anaesthetised using 3% halothane in 100% oxygen and placed into a custom-built container, with rats in the treatment group receiving fractionated abdominal irradiation using a Varian Clinac Linear Accelerator (Varian Medical Systems, USA) at a dose of 2.5 Gray (Gy) (depth = 3.3 cm, focus-skin distance = 130 cm) 3x per week for a total dose of 22.5 Gy in 9 fractions and 45 Gy in 18 fractions over 3 and 6 weeks, respectively (Yeoh et al. 2007). Rats were killed at 3, 6, or 15 weeks from first irradiation dose (n = 5 per time point). Animals were monitored daily and clinical record sheets kept. Sections of jejunum (30% of small intestinal length when measured from the pylorus) and colon (midlength of the large intestine) were collected as previously described and either formalin-fixed and paraffin-embedded, or snap frozen and stored at -80º (Yeoh et al. 2007; Stansborough et al. 2017).

RNA isolation and reverse transcription

RNA isolation was performed using the Nucleospin® RNA II kit (Macherey-Nagel, Germany) according to manufacturers' protocol. Briefly, 15-20 mg of jejunal and colonic tissue was homogenized using the TissueLyser LT (Qiagen) and Nucleospin® RNA II kit reagents (Macherey-Nagel, Germany). The lysate was filtered through silica membrane filter columns, desalted, DNA was digested using the DNAse reaction mixture, and the silica membrane was washed and dried. RNA was eluted in 60 µl RNase-free water. The TAKE3 plate and Synergy MyReader (BioTek) was used to determine RNA concentration and purity. RNA (1 µg) was converted to cDNA using the iScript cDNA synthesis (Bio-Rad, USA) according to manufacturer's protocol. The TAKE3 plate and Synergy MyReader was again used to determine total cDNA concentration and purity and stock cDNA was diluted to 100 ng/µl with nuclease-free water.

Real-time polymerase chain reaction (RT-PCR)

RT-PCR was performed using the Rotor Gene 3000 (Corbett Research, Sydney, Australia) according to manufacturer's protocol. Reaction mixtures contained 1 µl of cDNA, 5 µl of SYBR green (Applied Biosystems, Foster City, CA), 5 µl of nuclease-free water, and 0.5 µl each of 50 pmol/µl forward and reverse primers (Table 1). Thermal cycling conditions were as described; denaturation step at 95°C for 10 minutes, and included 40 cycles at 95°C for 10 s, annealing at 60ºC (VWF, TGFβ, and MMP-2), or 55ºC (VEGF and MMP-9), for 15 s, and extension and acquiring at 72°C for 20 seconds. 18S was used as the housekeeping gene. Primer efficiency was calculated to be between 90-110% for each target gene against 18S, and thus the $2^{-\Delta\Delta CT}$ method was used to determine fold change in gene expression relative to the housekeeping gene and sham irradiated controls from each time point.

Immunohistochemistry (IHC)

Angiostatin and endostatin proteins were visualised by IHC staining in the colon at 3, 6, and 15 week time points. A Dako automated staining system (Autostainer link 48 and PT Link, Dako, Denmark) was

used with the EnVision™ FLEX kit for Dako autostainer link systems (Dako, Denmark) as previously described (Stansborough et al. 2017). The anti-angiostatin primary antibody (#ab2904, Abcam), and anti-endostatin primary antibody (#GTX37706, GeneTex), were used at 6.66 µl/ml and 20 µl/ml, respectively. Slides were scanned using the Nanozoomer (Hamamatsu Photonics, Japan) at 40x magnification. ImageScope imaging software (Leica Biosystems, Germany) was used to analyse angiostatin and endostatin in which percentage of positively stained cells was calculated in the lamina propria of each section (averaged from ten randomly selected 40x sections per slide).

Isolation of tumour-associated microvascular endothelial cells (TAMECs)

Tumour-associated microvascular endothelial cells were isolated and cultured as previously described (Bateman et al. 2013). Briefly, Dark Agouti (DA) rats were injected subcutaneously with DA mammary adenocarcinoma cells, and 8 days following, tumours were removed and processed under sterile conditions. Surrounding connective tissue was removed and tissue was mechanically disrupted into 1 mm fragments. The tissue was enzymatically digested in collagenase/dispase (Sigma), and incubated with 0.25% trypsin/EDTA (Bateman et al. 2013). Following this, the tissue was centrifuged and resuspended in complete growth medium, consisting of supplemented Dulbecco's Modified Eagle Media (DMEM) (28% HEPES buffer, 10% foetal bovine serum (FBS), and 1% L-glutamine, penicillin, streptomycin) and filtered through a 100 µm nylon cell strainer (Corning). The resultant suspension was added to gelatinised culture flasks and incubated at 37ºC. Tumour-conditioned medium (TCM) was added to the TAMECs every 24-48 hours to maintain the endothelial cell culture. Endothelial culture was confirmed using immunostaining for endothelial cell markers, as previously described (Bateman et al. 2013).

Table 1. 18S, VEGF, TGFβ, VWF, MMP-2, and MMP-9 primer sequences

Treatment of TAMECs

TAMECs at passages 1-2 were detached with trypsin, pelleted, counted, diluted to 1 x 10^9 cells/mL, and split evenly across 4 x 50 ml falcon tubes to represent the four treatment groups (vehicle control, SB-3CT alone, irradiation alone, and irradiation and SB-3CT combined). Cells were then treated for 24 hours with either 0.01% DMSO in H20 (vehicle control), or 1 µm SB-3CT. SB-3CT concentration was determined by dose response assays (data not shown) and was determined to be within the K_i range of inhibiting MMP-2 and MMP-9 (K_i = 14 nM and K_i = 600 nM, respectively), without inhibiting MMP-1, -3, or -7 (K_i = 206 µM, K_i = 15 µM, K_i = 96 µM, respectively) (Brown et al. 2000). Following this, falcon tubes containing the cells were either sham irradiated, or received 12 Gy gamma irradiation using a Cs^{137} source (IBL-437 Blood Irradiator). Cells were immediately plated according to individual experimental conditions. An XTT assay was used to conduct a radiation dose response assay and was based on an initial dose range of 0-12 Gy. 12 Gy resulted in a significant decrease in cell viability without causing excessive cell death (data not shown). Dosimetry was calculated prior to radiation and based on dose rate calculations, calibrated monthly based on degradation constant.

Protein and RNA extraction from TAMECs

Following irradiation and/or SB-3CT pre-treatment, TAMECs were seeded into 6-well plates at a cell density of 1 x 10^5 cells/well, and incubated for 24 hours. Following incubation, the media was collected and immediately placed on ice. Cells were pelleted and supernatant was collected and stored at -80ºC. Lysis buffer (Macherey-Nagel RNA kit) was added to each well. Wells were scraped and cells and buffer were collected and disrupted using a 26-gauge needle. β-mercaptoethanol (3.5 µl) was added to each tube, vortexed, and further RNA extraction and cDNA conversion was performed according to kit protocols, as described earlier.

MMP 2 and 9 activity assays

MMP-2 and -9 activity was assessed in supernatant collected from treatment TAMECs, using MMP-2 and -9 activity assay kits (QuickZyme). Experiments were conducted according to kit protocol. Briefly, duplicate diluted samples (1:2 dilution with assay buffer), serially diluted standards, and assay buffer controls, were pipetted into the assay plate. The plate was covered and incubated at 4ºC overnight. The wells were aspirated and washed with assay buffer, and 50 µl of P-Aminophenyl mercuric acetate (APMA) solution (0.5 mM APMA in 0.05% DMSO/assay buffer) was added to wells containing standards, and to one of the two duplicate sample wells. Assay buffer was added to the remaining sample wells, and detection reagent was added to all wells. The plate was shaken for 20 seconds and read at 405 nm to obtain the baseline value, repeated at 6 and 22 hours. Data was analysed by subtracting the average blank value from each sample value, creating a standard curve based on the 6 hour data and plotting the standard concentration against the blank subtracted standard values, applying a best-fit curve, and calculating the sample concentrations graphically.

XTT Assay

In preparation for XTT assay, $1x10^5$ treated TAMECs were passaged intro 96-well flasks and were left to adhere for 24 hours. Following adhering, XTT was performed according to kit protocol (Cell Proliferation Kit II (XTT, Sigma), with labelling and electron coupling reagents being prepared immediately before removing media from wells, replacing with 100 µl fresh media, and adding 50 µl per well of the prepared XTT reagent. The plates were incubated for 6 hours and the plate was read at 490 nm using the TAKE3 plate and Synergy MyReader.

Immunocytochemistry (ICC)

Prior to experimentation of TAMECs, expression of VEGF, VWF, TGFβ, MMP-2, and MMP-9 under normal conditions was confirmed using immunocytochemistry (Supplementary Figure 1). Briefly, treated TAMECs were passaged into 8-well chamber slides at $2x10^5$ cells/well and left to adhere for 24 hours. Following this, cells were fixed with 4% paraformaldehyde for 30 minutes at 4ºC, washed twice with

PBS, incubated with 3% H2O2 in PBS for 5 minutes to reduce non-specific staining, washed, and incubated with Triton-X block (100 µl TX-100 and 0.1 g sodium citrate in 100 mL H20) for 8 minutes to permeate the cell membrane. The cells were washed and blocked using the normal serum blocking reagent (Level 2 USA™ Ultra Streptavidin Detection System kit, Signet Laboratories). Primary antibodies (MMP-2; #ab37150, Abcam, MMP-9; #ab58803, Abcam, VEGF; #ab46154, Abcam, VWF; #ab6994, Abcam, TGFβ; #ab92486, Abcam) were prepared at previously optimised concentrations of 2 µl/ml (VWF & MMP-2), 4 µl/ml (VEGF & MMP-9), and 10 µl/ml (TGFβ), and added to each well for 60 minutes. Cells were washed and a linking reagent (Level 2 USA™ Ultra Streptavidin Detection System kit, Signet Laboratories) was applied for 20 minutes. A peroxidase-labelled ultra-streptavidin labelling reagent (Signet Laboratories) was applied for 20 minutes, followed by a 1 minute incubation with diaminobenzidine (DAB) chromogen in 0.03% hydrogen peroxidase (Signet Laboratories) for visualization. Cells were washed, chambers were removed, and slides were counterstained with Lillie Meyers haematoxylin (1:10), washed, dehydrated, and coverslipped.

Statistical Analysis

Graphpad Prism (version 6) software was used to perform statistical analysis. Normality was determined and differences between study group means were analysed using either a one-way or two-way ANOVA with Sidak's multiple comparison, or a Kruskal-Wallis test with multiple comparison. Asterisks denote significance compared to control at the time point, where * <0.05, ** <0.01, *** <0.001, and **** <0.0001. Bars on graphs represent standard error of the mean.

Results

Fractionated irradiation induced changes in weight gain and histopathological damage

Clinical and histological response to fractionated irradiation for this study has been previously published (Stansborough et al. 2017). Briefly, rats receiving fractionated irradiation had significantly lower weight gain at week 3 ($p = 0.0055$), and from week 6 until the completion of the study ($p < 0.05$), when compared to sham irradiated controls (Stansborough et al. 2017). Histopathological damage scoring was significantly increased in the colon, but not in the jejunum, at week 3 ($p = 0.043$). However, thickening of the lamina propria, telangiectatic vessel formation, and apoptosis were observed in both the jejunum and colon following irradiation (Stansborough et al. 2017).

VEGF and TGFβ were significantly increased in the colon following irradiation in DA rats

Despite no significance effect in the jejunum, VEGF mRNA expression was significantly increased in the colon at the 15-week time point ($p = 0.0012$) (Figure 1A + B). TGFβ mRNA expression significantly increased in both the jejunum and colon 3 weeks into the radiation schedule ($p = 0.0280$ and $p = 0.0310$, respectively), however was not significantly increased at 6- or 15-weeks in either region (Figure 1C + D). VWF mRNA expression was not significantly altered at any time point following fractionated irradiation in jejunum or colon (Figure 1E + 1F).

Figure 1. VEGF, TGFβ, and VWF expression in the jejunum and colon following fractionated radiation. **A** VEGF mRNA expression in the jejunum following fractionated irradiation **B** VEGF mRNA expression in the colon following fractionated irradiation **C** TGFβ mRNA expression in the jejunum fractionated irradiation. **D** TGFβ expression in the colon following fractionated irradiation. **E** Relative mRNA expression of VWF in the jejunum following fractionated irradiation **F** VWF mRNA expression in the

colon. Dotted line represents baseline fold change. * = significance compared to control. * <0.05, ** <0.01, n=5. Data = mean + standard error of the mean (SEM).

Angiostatin and endostatin immunostaining was significantly increased in the colon following fractionated irradiation

Endostatin was immunostaining significantly increased in the lamina propria of the colon at the 3-week time point only (p = 0.0046) (Figure 2A + 2C). Angiostatin immunostaining was significantly increased at 3- and 6-weeks in the colon lamina propria (p = 0.0022, and p = 0.0135, respectively) (Figure 2B + 2C). There were no significant alterations at week 6 or 15.

Figure 2. *Endostatin (**A**) and angiostatin (**B**) immunostaining in the lamina propria of the colon following fractionated irradiation.* Cell positivity was calculated using ImageScope software and is determined as number of positively stained cells/number of total cells. * denotes significance compared to controls at the same time-points, ** <0.01, *** <0.001. Data = mean + SEM, n=5. Scale bars = 20 μm, 40x original magnification.

SB-3CT significantly attenuated the expression of MMP-9 following irradiation of TAMECs

Relative mRNA expression of MMP-2 was not significantly altered following irradiation of TAMECs when compared to controls (p = ns; Figure 3A). However, TAMEC MMP-2 expression following irradiation was significantly reduced when pre-treated with SB-3CT, as compared to the radiation only group (p = 0.0034) (Figure 3A). MMP-9 mRNA expression was significantly increased following irradiation of TAMECs (0.0022). This was significantly attenuated by SB-3CT (p < 0.0001). Despite this attenuation of MMP-2 and -9, SB-3CT did not significantly alter cell viability following irradiation, compared to radiation alone (p = ns; figure 5).

Figure 3. *MMP-2 (**A**) and -9 (**B**) mRNA expression following irradiation of TAMECs, with/without SB-3CT*. Dotted line represents baseline fold change. * = significance compared to control. * <0.05, ** <0.01, **** <0.0001. Data = mean + SEM. n=12

SB-3CT significantly attenuated MMP-2 and -9 protein expression following irradiation

Total protein levels of both MMP-2 and MMP-9 were significantly increased in TAMEC supernatant following 12 Gy irradiation ($p = 0.046$, and $p = 0.0401$, respectively) (Figure 4A and 4C). This increase in total MMP-2 and -9 protein levels was significantly attenuated by the administration of SB-3CT ($p = 0.0119$, and $p = 0.0209$, respectively). The ratio of active: total MMP-2 and -9 were not significantly altered following irradiation of TAMECs. However the ratio of active: total MMP-2 was significantly increased in the radiation + SB-3CT group, compared to control ($p = 0.0379$). The ratio of active: total MMP-9 was not significantly altered in the radiation + SB-3CT group when compared to all other groups (p = ns; Figure 4D).

Figure 4. *Total and active: total ratio of secreted MMP-2 and -9 protein concentration following irradiation and MMP inhibition in TAMECs.* **A** Total MMP-2 protein concentration **B** Ratio of active: total MMP-2 protein levels **C** Total MMP-9 protein concentration **D** Ratio of active: total MMP-9 protein levels. * = significance compared to control. * <0.05. Data = mean + SEM. n = 12

SB-3CT did not alter tumour-associated microvascular endothelial cell viability following irradiation

SB-3CT alone did not significantly alter cell viability; as measured via XTT assay; both irradiation (12 Gy), and irradiation + SB-3CT significantly reduced cell viability of TAMECs ($p = 0.0236$, and $p = 0.0003$, respectively) (Figure 5). There was no significant difference between the radiation alone, and radiation + SB-3CT groups (p = ns; Figure 5).

Figure 5. *Cell viability of TAMECs following irradiation and MMP inhibition.* * = significance compared to control. * <0.05, *** <0.001. Data = mean + SEM. *n = 8*

VEGF and TGFβ mRNA expression were not significantly altered following irradiation of TAMECs

RT-PCR was used to assess relative mRNA expression of VEGF and TGFβ following irradiation and MMP inhibition in TAMECs. VEGF and TGFβ mRNA expression were unaltered by both irradiation and SB-3CT treatment (Figure 6).

Figure 6. *VEGF and TGFβ mRNA expression following irradiation of TAMECs with or without SB-3CT.* VEGF (**A**) and TGFβ (**B**) mRNA expression were not significantly altered following irradiation of TAMECs, with or without the presence of SB-3CT. Dotted line represents baseline fold change. Data = mean + SEM. n = 12

Discussion

Although the pathobiology of RIGT is complex, MMPs, the intestinal microvasculature, and associated vascular mediators have been shown to be involved (Stansborough et al. 2016). Key findings from this study show mRNA expression of VEGF and TGFβ mRNA expression to be significantly increased in the intestines following RIGT, and is the first study to show an increase in angiostatin and endostatin during RIGT. However, the pattern of upregulation of these mediators was different, occurring at different time points and in different intestinal regions. Further, this study assessed the effect of the MMP-2 and -9 inhibitor, SB-3CT, on tumour-associated microvascular endothelial cells (TAMECs) following irradiation. Both mRNA and protein expression of MMP-2 and -9 were significantly upregulated in TAMECs following irradiation, however irradiation did not significantly alter VEGF or TGFβ expression in these cells. SB-3CTsignificantly attenuated MMP-2 and -9 mRNA and protein expression, however, it did not alter cell viability of TAMECs following irradiation.

VEGF mRNA expression was significantly increased in the colon at the 15 week time point of the DA rat RIGT model. VEGF may have a protective role in gastrointestinal injury, with higher levels of VEGF expression correlating with less severe gastric ulcers in murine models of stress-induced gastric ulceration (Malara et al. 2005). VEGF has also been shown to regulate normal vasculature in the small intestinal villi, with excessive inhibition resulting in regression of normal microvasculature, and reduced vascular density (Howdieshell et al. 2001; Saif et al. 2007; Pollom et al. 2015). Clinically, the inhibition of VEGF has been shown to delay healing of gastric erosions, reduce vascular density in the small

intestinal villi, and induce epithelial ulceration, supporting a role for VEGF in healing (Mangoni et al. 2012; Pollom et al. 2015). This regulatory role could be consistent with the timing of VEGF upregulation seen in the present study, occurring at the 15 week time-point in the colon only. At this time point, 9 weeks following the last radiation dose, we have clearly documented and reported on a transition from acute to chronic RIGT phenotype which is able to be seen histologically (Stansborough et al. 2017). This effect is likely due to alteration of the wound healing process, as well as vascular changes resulting in impaired delivery of healing agents to the damaged tissue (Mangoni et al. 2012). This study, in combination with findings of previous literature, suggests a role for VEGF in the progression to chronic RIGT, possibly playing a part in wound healing following acute RIGT. This potential role of VEGF in RIGT needs to be further explored, particularly with the increased study of anti-VEGF as adjuvant cancer therapies (Barney et al. 2013; Pollom et al. 2015). When VEGF inhibitors (VEGFIs) are combined with radiotherapy, gastrointestinal toxicity has been shown to significantly increase (Barney et al. 2013; Pollom et al. 2015). This potential for increased intestinal toxicity needs to be considered when determining the clinical potential of these agents.

Whilst TGFβ expression was significantly increased 3 weeks into the fractionated radiation schedule in our rat model of RIGT, consistent with previous literature, it was not significantly upregulated at 6 and 15 weeks, nor was it altered following irradiation of TAMECs (Richter et al. 1998; Wang et al. 2001). The lack of increase in TGFβ at the chronic RIGT time point was somewhat unexpected and in contrast with previous literature (Kleifeld et al. 2001). This may be explained by the different models used in the study by Wang and colleagues (2001, in which a scrotal hernia is surgically induced and the fractionated schedule uses higher doses over a shorter period of time (Wang et al. 2001). Our previous studies have shown fibrotic changes to the lamina propria and microvasculature at both the 6 and 15 week time points (Stansborough et al. 2017). With the complex interactions of TGFβ it is possible that the early upregulation of TGFβ, or alterations in the receptors of TGFβ are involved in these fibrotic changes. It is also unclear what the potential consequences of TGFβ involvement in RIGT may be, with

TGFβ being capable of opposing angiogenic effects, highly dependent on conditions and tightly regulated in the endothelium by the receptors ALK1 and ALK5 (Goumans et al. 2003). Whilst TGFβ binding with ALK5 has an anti-angiogenic effect, inhibiting endothelial cell migration and proliferation, ALK1 binding has the opposite effect (Goumans et al. 2003). Thus the ratio of ALK5 to ALK1 seems to be key to the functional role of TGFβ in pathological conditions. Further studies, both *in vitro* and *in vivo,* are needed to investigate the ALK5 to ALK1 ratio and determine where this balance lies in endothelial response to irradiation, and during RIGT, in order to determine the role of TGFβ in its pathobiology.

Angiostatin and endostatin immunostaining was shown to be significantly elevated in the lamina propria of both the jejunum and colon. This occurred only during the acute time points, with angiostatin increasing at 3 and 6 weeks, and endostatin at 3 weeks. The anti-angiogenic mediators angiostatin and endostatin, fragments of plasmin and type XVIII collagen, respectively, are responsible for inhibiting endothelial cell proliferation and migration, as well as inducing apoptosis in the case of endostatin (Ribatti 2009). Endostatin is also known to directly inhibit MMP-2 (Abdollahi et al. 2004). Although this current study is the first to assess angiostatin and endostatin in an animal model of RIGT, response of angiostatin to radiation has previously been assessed *in vitro* (14). Oh and colleagues (2014) finding angiostatin protein levels to be significantly increased in human breast tissue derived cancer endothelial cells following 4 Gy irradiation (Oh et al. 2014). Whilst endostatin has not yet been investigated in RIGT, Endostar, a recombinant human endostatin, has been shown to significantly decrease MMP-2, -9, -14, and VEGF immunostaining in nasopharyngeal carcinoma xenografts in mice following 6 Gy irradiation (Peng et al. 2012). Although the roles of angiostatin and endostatin likely differ in normal vs tumour tissue response to radiation, these studies, in combination with the present study, suggest a role of these anti-angiogenic mediators in radiation response, likely linked to the MMP pathway.

MMP-2 and -9 mRNA and protein expression was significantly increased following irradiation of TAMECs. The significant increase in MMP-2 expression is consistent with previous studies finding an increase in MMP-2 24 hours following 4 Gy irradiation of human normal endothelial cells and TAMECS (NECs and CECs, respectively) derived from breast tissue (Oh et al. 2014). However, the same study found MMP-9 not to be altered by irradiation of TAMECs in contrast to the findings of our current study (Oh et al. 2014). This is not entirely unexpected, due to species and tissue differences between the two studies, with MMP-9 regulation known to depend on cell type (Vincenti and Brinckerhoff 2007). Despite these conflicting results, the similarities between the studies highlight a role for MMP-2, in particular, in endothelial regulation following irradiation.

The increase in MMP-2 and -9 expression was also significantly attenuated, both at the mRNA and protein level, by the pre-treatment of TAMECs with SB-3CT. SB-3CT is the first mechanism-based MMP inhibitor, potently and selectively inhibiting MMP-2 and -9. The effect of SB-3CT is unique in its selectivity, as it forms a reactive species within the active sites of MMP-2 and -9 (Kruger et al. 2005) and was developed in response to a lack of efficacy of broad-spectrum, synthetic MMP inhibitors when used as anti-cancer therapies in clinical trials.

An unexpected finding in the present study was the significant decrease in mRNA expression of MMP-2 and MMP-9 following pre-treatment with SB-3CT in TAMECs following irradiation, as the mechanism of action of SB-3CT is in inhibiting activation. Additionally, SB-3CT significantly increased MMP-2 activity in TAMECs when combined with irradiation, having no effect on activity of MMP-9. The significant decrease in proMMP expression by SB-3CT is not unprecedented, with SB-3CT significantly decreasing proMMP-9 levels in a mouse model of transient focal cerebral ischemia (37). The study hypothesized this decrease was likely due to a positive feedback mechanism between MMP activity and gene transcription (Gu et al. 2005).

To our knowledge, this is the first study to assess mRNA expression of MMP-2 and -9 following SB-3CT administration. There are endogenous mediators that can be regulated by active MMP-2 and -9, such as TGFβ, and pharmacological reagents, such as doxycycline, that have been shown to regulate the mRNA expression of MMP-2 and -9 by altering mRNA stability (Yan and Boyd 2007). Whether SB-3CT is regulating transcription via a similar process, by affecting the promotor regions of these MMPs, or through disruption of a positive feedback mechanism, is unclear. The regulation of mRNA expression of both MMP-2 and MMP-9 by SB-3CT should be investigated further and current studies are underway in our laboratory.

The use of a single cell type in this study is a limitation as normal and tumour endothelial cells are phenotypically different and subsequently respond differently to irradiation; therefore it is likely that the response of normal gut endothelial to SB-3CT would differ to the findings of the present study (Oh et al. 2014). Thus, in order to further assess the possible effects of MMP inhibition in RIGT, future studies assessing SB-3CT in an *in vivo* model of RIGT are warranted. However, the findings of this study highlight a relationship between radiation, MMP expression and tumour endothelium. Due to the potential use of specific MMP inhibitors as cancer therapies, and with the involvement of both MMP's and the endothelium in the development and progression of RIGT, the findings of this study highlight the need for further research and consideration as to possible dual effects of MMP inhibitors in this setting (Strup-Perrot et al. 2006; Angenete et al. 2009; Stansborough et al. 2017).

Conclusion

The findings of this study support a role for the vascular mediators VEGF, TGFβ, angiostatin, and endostatin in RIGT following fractionated irradiation. Additionally, MMP-2 and -9 were shown to be involved in endothelial response to radiation *in vitro,* however inhibition by SB-3CT did not alter cell viability. The findings of the *in vitro* component of the present study elucidate the effects of SB-3CT on the endothelium, with unexpected findings of significantly inhibited proMMP expression, but not active

MMP expression, and confirm the role of MMP-2 in particular in the endothelial response to irradiation. These results highlight the need for further pre-clinical studies to assess the effect of MMP inhibitors and anti-angiogenic drugs in RIGT, particularly as these drugs are currently being investigated as adjuvant cancer therapies.

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Declarations of Interest

The authors report no declarations of interest.

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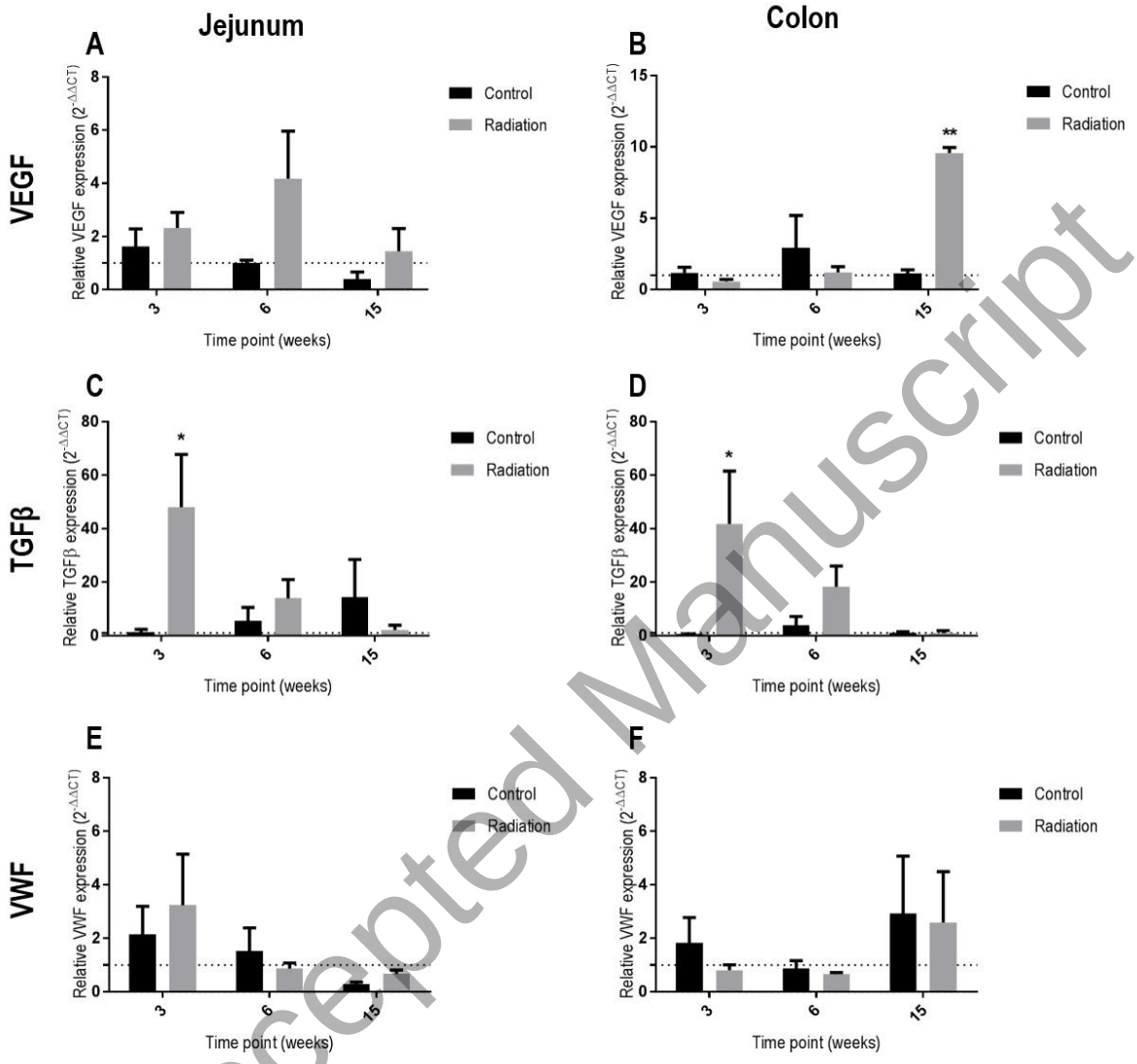
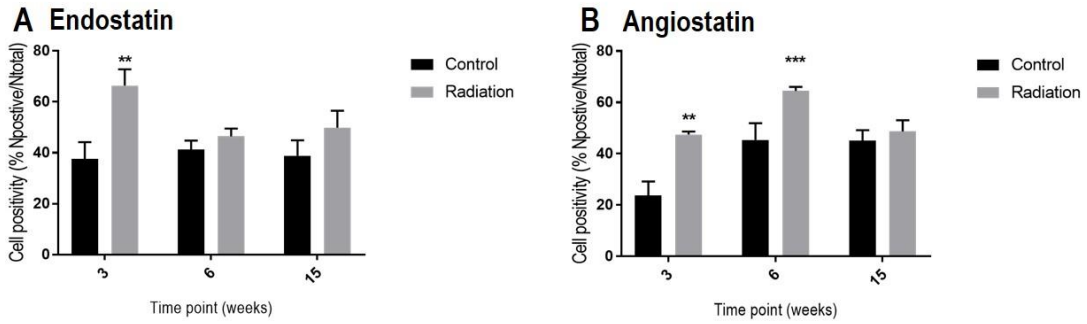
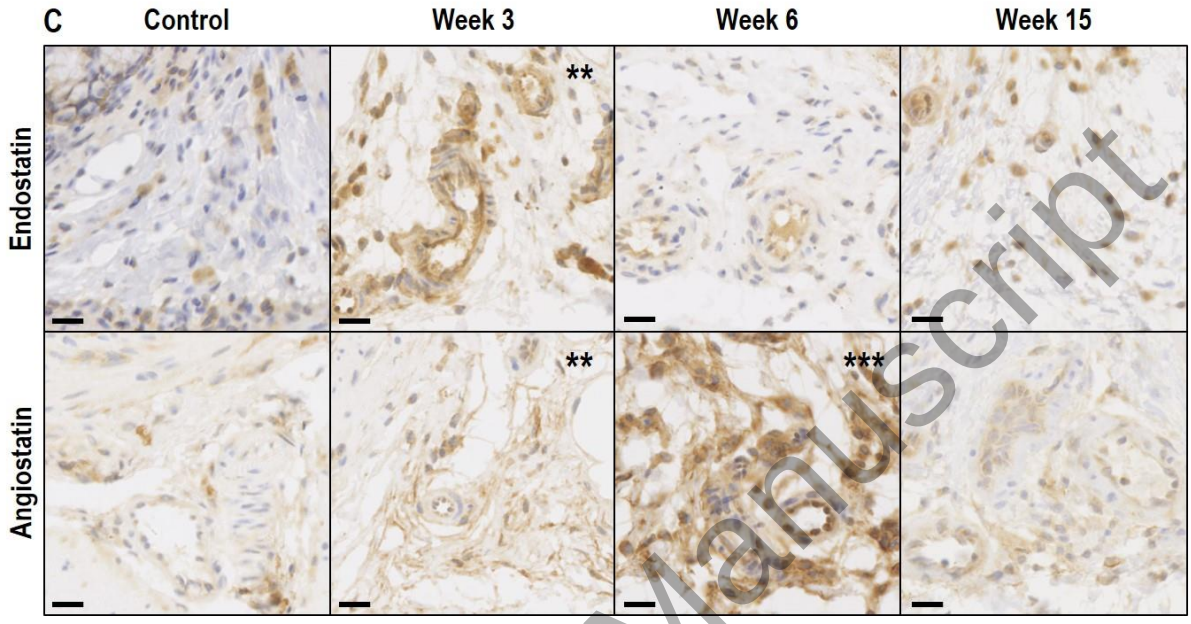
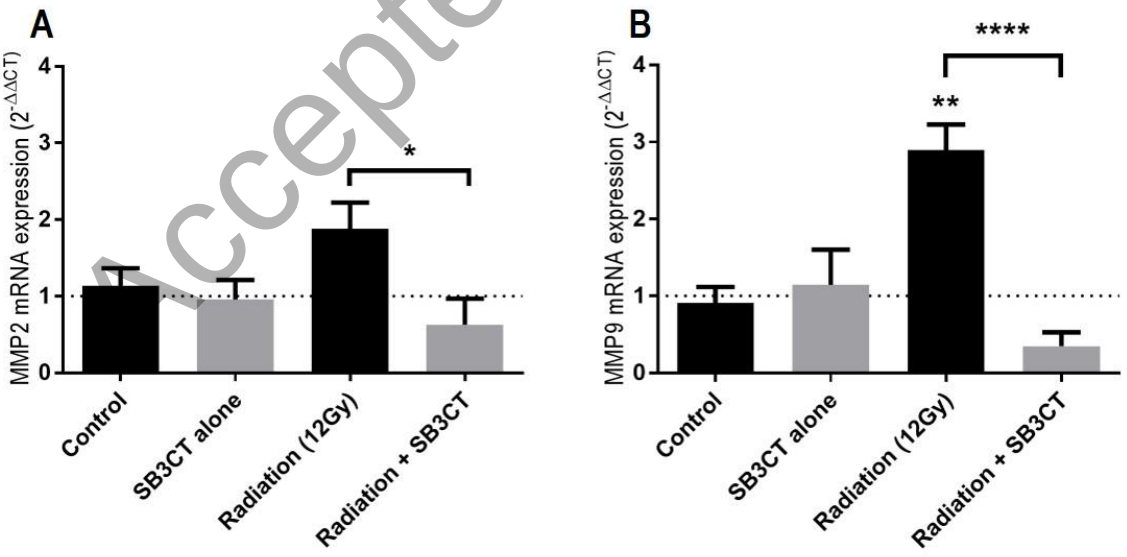
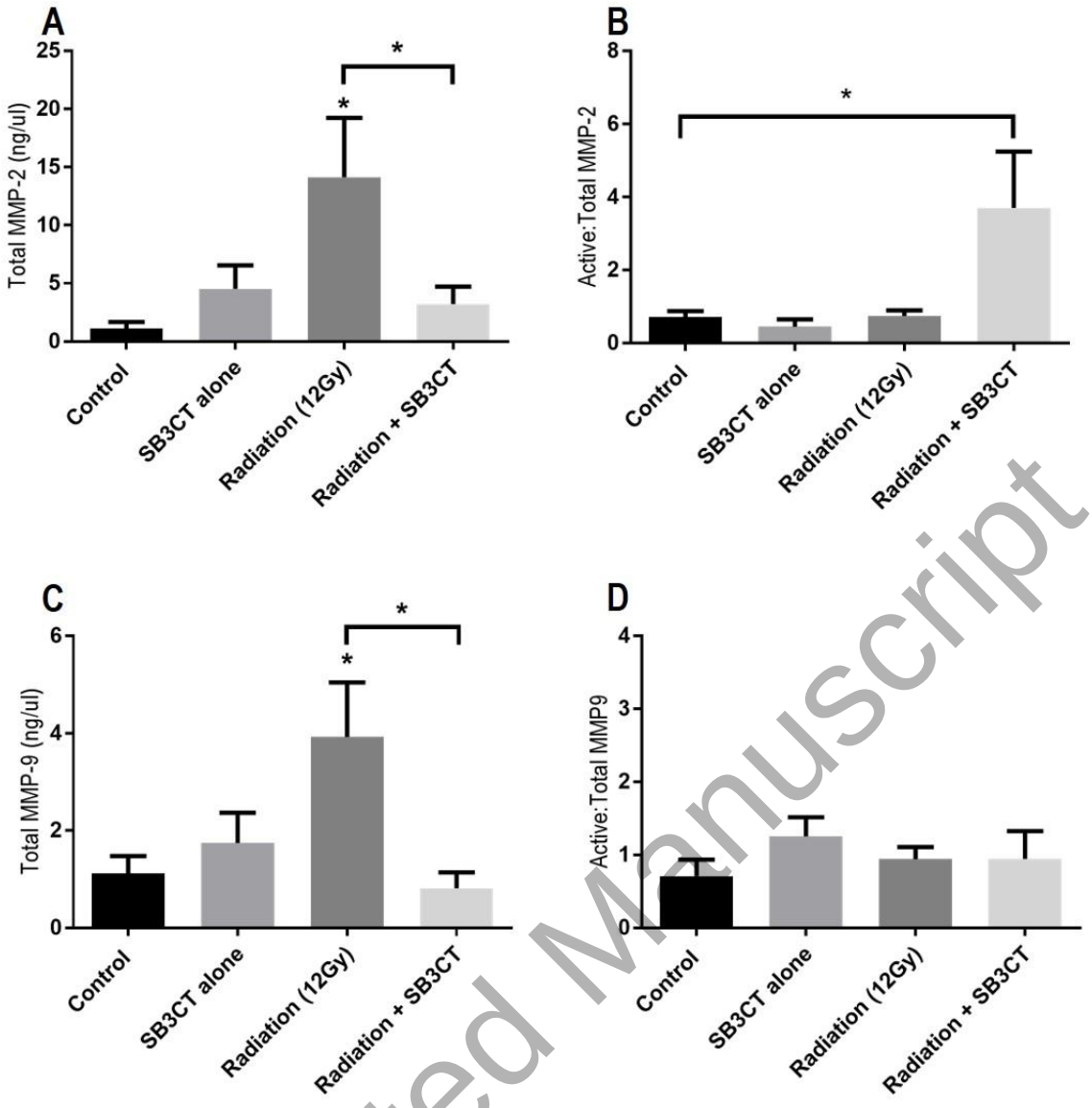
Supplementary Figure 1. Conformational staining of MMP-2, MMP-9, VWF, and VEGF in tumour-associated microvascular endothelial cells (TAMECs). 100x magnification

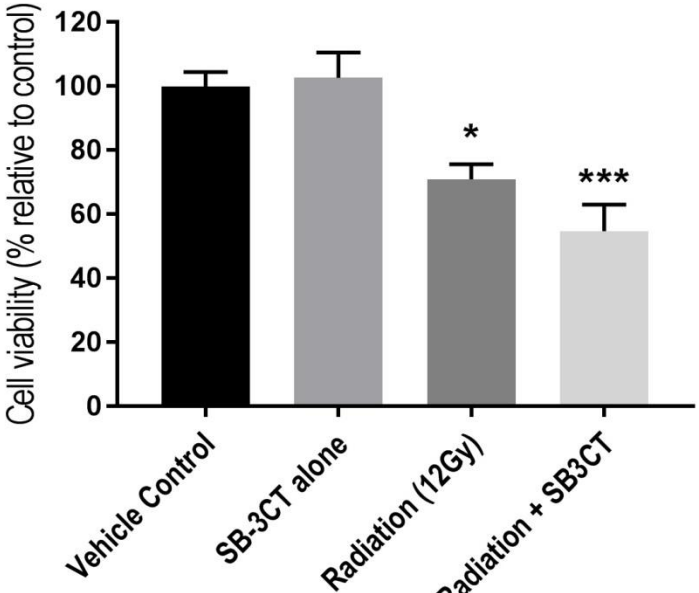












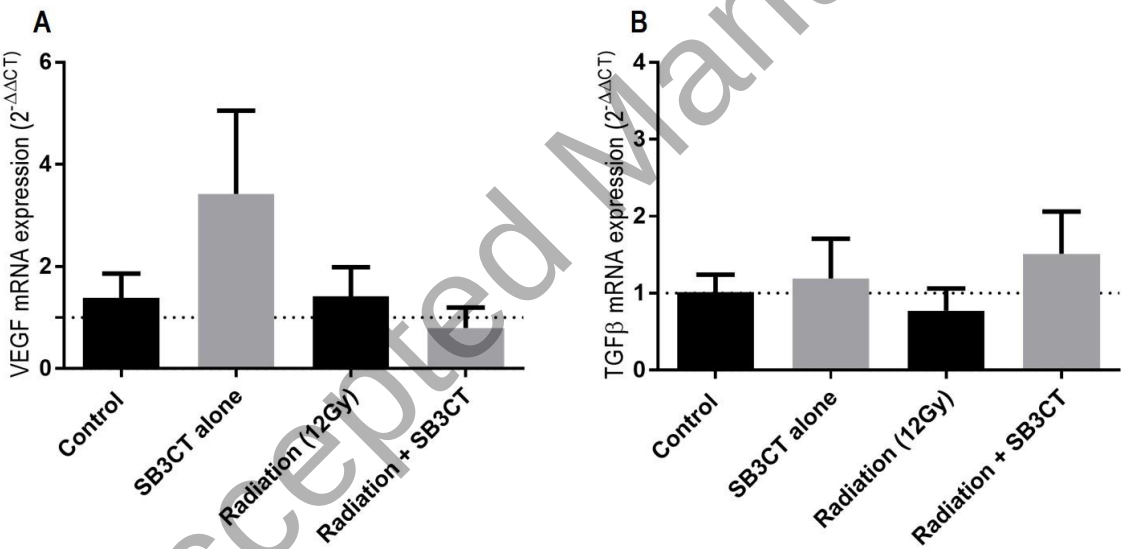


Table 1. 18S, VEGF, TGFβ, VWF, MMP-2, and MMP-9 primer sequences

Gene	Primer sequence (5'-3')	Nucleotide Position	Amplicon length (bp)	Tm (°C)	Ref
18S	**F** CATTCGAACGTCTGCCCTAT	344-452	109	60	(Uchida et al. 2010)
	R GTTTCTCAGGCTCCCTCTCC			60	
VEGF	**F** AGGCGAGGCAGCTTGAGTTA	1601-1766	166	62	(Zhang et al. 2014)
	R CTGTCGACGGTGACGATGGT			64	
TGFβ	**F** ATGACATGAACCGACCCTTC	897-1073	177	60	(Close et al. 2005)
	R ACTTCCAACCCAGGTCCTTC			60	
VWF	**F** GCCTCTACCAGTGAGGTTTTGAAG	4292-4587	296	63	(Boerma et al. 2004)
	R ATCTCATCTCTTCTCTGCTCCAGC			63	
MMP-2	**F** CTG ATA ACC TGG ATG CAG TGCT	2138-2272	135	55	(Al-Dasooqi et al. 2010)
	R CCA GCC AGT CCG ATT TGA			50	
MMP-9	**F** AAG CCT TGG TGT GGC ACG AC	760-876	117	56	(Vikman et al. 2007)
	R TGG AAA TAC GCA GGG TTT GC			52	