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Bowel irradiation: the effects on inflammation and matrix-metalloproteinases

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Abstract

Background and aims: Radiotherapy is a valid treatment in cancer multimodal management but it is affected by severe systemic and local side effects. A murine small bowel model was used for the investigation of inflammatory features and apoptosis of local radiotherapy. Similarly the same model was used to analyze the combined effects of radiotherapy and antibiotics in the expression of matrilysin, MMP involved in murine bowel, in the production of antimicrobical peptides when it is exposed to bacteria. Matrilysin is furthermore expressed in human colorectal cancer and can be overxpressed by radiation exposure. Clinical studies of radiated and non radiated colorectal patients were used to correlate matrilysin expression with different tumor stages and different radiotherapy protocols.

Results: Features of radiation injury observed in murine small bowel may be mainly summarized as early increases of inflammatory proteins as MIP-2, a decrease in leucocyte CD 45 positive cells over time and increased dose dependent cellular death. Not only radiotherapy or antibiotics alone, but the combination of both preoperative radiotherapy and antibiotics leads to marked increases in matrilysin expression in the rodent intestine. Higher levels of matrilysin in stage I/II adenocarcinoma compared to stage III/IV and varying grades of dysplasia were observed in non radiated colo-rectal cancers. Radiotherapy affects MMP-7 expression in different grade depending on the radiation dose delivered with long course therapy showing limited effect on MMP-7 expression.

Conclusions: a murine model of local irradiation is effective in studying the early radiation-induced tissue injury of small bowel. Radiotherapy tends to override the effect of antibiotics and leads to an up-regulation of MMP-7 and TGF-β and MIP-2 expression in the murine intestine. Significantly increased concentrations of MMP-7 in tumor tissues, lymph nodes and in serum is associated with increasing grade of dysplasia and adenocarcinoma infiltration. Immunohistochemistry and ELISA are simple cost-benefit methods when examining tumor behaviour in resected specimens. High dose (50 Gy) radiotherapy administered in preoperatively long course induces significantly less MMP-7 over-expression compared to short term 25 Gy irradiation at surgery and which itself has an overriding effect of up regulation of MMP-7.

Key words: Radiotherapy, Inflammation, Colorectal Cancer, MMP-7, Matrilysin, Bacteria

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Bowel irradiation: the effects on inflammation and matrix-metalloproteinases

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To: all who supported me

Ὁ βίος βραχύς, ἡ δὲ τέχνη μακρή, ὁ δὲ καιρὸς ὀξύς, ἡ δὲ πεῖρα σφαλερή, ἡ δὲ κρίσις χαλεπή

Vita brevis, ars longa, occasio praeceps, experimentum periculosum, iudicium difficile

Life is short, art long, opportunity evanescent, experiment insidious, judgement difficult

Hippocrates

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Abstract

Background and aims: Radiotherapy is a validated treatment in multimodal management of cancer but leads to severe systemic and local side effects. A murine small bowel model was used for the investigation of inflammatory features and apoptosis of local radiotherapy. Similarly the same model was used to analyze the combined effects of radiotherapy and antibiotics in the expression of matrilysin /MMP-7 involved in murine bowel, in the production of antimicrobical peptides when exposed to bacteria. Matrilysin is furthermore expressed in human colorectal cancer and can be overxpressed by radiation exposure. Clinical studies of irradiated and non irradiated colorectal cancer patients were used to correlate matrilysin expression with different tumor stages and different radiotherapy protocols.

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List of papers

This thesis is based upon the following original articles. In the text of the following chapters these papers and the manuscript will be referred to according to their sequential numbers.

Paper I

Polistena A, Johnson LB, Ohiami-Masseron S, Wittgren L, Back S, Thornberg C,Gadaleanu V, Adawi D, Jeppsson B. **Local radiotherapy of exposed murine small bowel: Apoptosis and inflammation.BMC Surg. 2008;8(1):1. ***

Paper II

Polistena A, Johnson LB, Röme A, Wittgren L, Bäck S, Osman N, Molin G, Adawi D, Jeppsson B. **Matrilysin Expression Related to Radiation and Microflora Changes in Murine Bowel. J Surg Res. 2011;167:137-43. ***

Paper III

Polistena A, Cucina A, Dinicola S, Stene C, Cavallaro G, Ciardi A, Orlando G, Arena R, D'Ermo G, Cavallaro A, Johnson LB, De Toma G. **MMP7 expression in colorectal tumours of different stages. In Vivo. 2014;28:105-10. ***

Paper IV

Polistena A, Stene C, Gaber A, Nodin B, Ottochian B, Adawi D, Avenia N, Jirström K, Johnson LB. **MMP-7 modulation by short and long term radiotherapy in rectal cancer patients.** *Manuscript*

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Abbreviations

RI	radiation injury
ECM	extracellular matrix
MMPs	matrix-metalloproteinases
PDGF	platelet derived growth factor
TGF-β	transforming growth factor beta
PMNs	polymorphonuclear leucocytes
IL	interleukin
TNF-α	tumor necrosis factor
MIP-2	macrophage inflammatory protein-2
BCL2	B-cell lymphoma 2
ROS	Radical oxygen species
BAD	associated agonist of cell death
MMP-7	matrilysin
TIMPs	matrix-metalloproteinases tissue inhibitors
Gy	gray
TME	total mesorectal excision
RT-PCR	Reverse transcriptase polymerase chain reaction

General Background

Radiotherapy

In medicine human ionizing radiation exposure occurs either in diagnostic procedures or in radiotherapy. Radiotherapy is a therapeutic approach used in multimodal treatment against several neoplastic diseases including cancer of the breast, prostate, rectum, lung and head and neck. The maximum applied dosage in clinical use is strictly limited by potential side effects occurring to normal surrounding organs despite the use of proper shields (Molla et al. 2001). The risk of injury to healthy surrounding tissues limits the radiation dose that can be safely delivered to a tumor, and thereby its curability (Zheng et al. 2000).

The difficulty of planning a correct radiation schedule is therefore regulated by the difficulty in balancing dose and exposition time for a maximum tumoricidal effect on one side and the maximum protection of surrounding tissue from the side effects on the other. This issue has still not been totally overcome by current standard protocols used in radiotherapy.

The small bowel is highly radiosensitive and very mobile and is thus an important dose limiting organ during radiation therapy for abdominal and pelvic cancer (Zheng et al. 2000). Radiation induces an inflammatory response in target and surrounding tissues which is characterised by tissue accumulation of plasma proteins and leucocytes.

Radiation injury on surrounding organs

The wide use of radiotherapy in abdominopelvic cancer, although offers important advantages, represented mainly by tumor downstaging in neoadjuvant therapy and in controlling recurrent disease after surgical excision in both neoadjuvant and adjuvant form, nevertheless presents several and severe side effects. Radiation induces injury to rapidly dividing tissues. In the abdomino-pelvic field, small bowel is the surrounding organ mainly involved. It is in fact highly radiosensitive and extremely mobile and represents an important dose-limiting organ during abdomino-pelvic irradiation. Intestinal radiation toxicity is characterized by mucosal barrier breakdown and inflammation, followed by development of progressive vascular sclerosis and intestinal wall fibrosis. The process is accompanied by sustained over-expression of inflammatory and fibrogenic cytokines (Zheng et al. 2000). Radiation induced damage on the intestinal mucosa leads to a severe clinical syndrome associated to radiotherapy, characterized by diarrohoea, nausea, abdominal pain, and bleeding known as radiation enteropathy (Denham et al. 2002).

Radiation injury (RI) is characterized by the death of basal and suprabasal layers in the epithelial surfaces and by the inflammatory responses in the underlying tissue. It is associated to a precocious re-epithelization after an initial period of growth arrest. The severity of mucosal damage depends on the balance between epithelial denudation and new proliferation. When the rate at which surviving epithelial cells are killed exceeds the maximum rate at which new cells are replaced the injury is more evident. The more deep is the lesion the slower is the re-epithelization since the main damage occurs on the epithelial stem cells and on the basement membrane zone which otherwise determines loss of the barrier function of the intestinal mucosa (Somozy et al. 2002). Whereas acute normal tissue toxicity is generally a result of death of rapidly proliferating cells, delayed toxicity in many organs is characterized by progressive fibrosis and vascular sclerosis. The RI can therefore be defined by primary direct cytocidal effect or direct dysfunctional cellular effect and by indirect secondary phenomena including inflammation, reparative processes with collagen and extracellular matrix (ECM) deposition regulated by matrix-metalloproteinases (MMPs) involvement and following fibrosis. All the factors in the radiotherapeutic fractioning undergo a repeated new induction, leading at least to delay in wound healing and to chronic lesion formation. For these reasons RI can be defined as an uncommon kind of injury (Denham et al. 2002). Wound healing therefore presents four classical phase which might be summarized as follows (Johnson 2005):

- *Haemostasis:* in the wound, bleeding capillaries release blood products and initiate the coagulation cascade, similarly radiotherapy activates the same cascade in intact cells. Platelets secrete cytokines such as platelet derived growth factor (PDGF) and transforming growth factor beta (TGF-β).

- *Inflammation*: within 6 to 8 hours of the trauma vasodilation of blood vessels occurs with transmigration of polymorphonuclear leucocytes (PMNs) including macrophages, monocytes, neutrophils and lymphocytes. These cells are recruited to "clean" the wound of debris as necrotic material like bacteria. Collagen matrix undergoes degradation by metalloproteinases during this phase.

- *Granulation/Fibroplasia/Proliferation*: starts three days after wound healing and lasts for about three to four week. After five days collagen degradation ends and its deposition starts mediated by TGF-β. Angiogenesis and collagen deposition from fibroblast is stimulated by growth factors and cytokines. Collagen deposition continues after its peak by the third week for other 2-4 weeks. Oxygen is fundamental for collagen synthesis since its involvement in the hydroxylation of lysine and proline. Capillary formation continues followed by ECM deposition which is regulated by MMPs. This is followed by progressive re-epithelization which occurs from the periphery of the wound which will be successively contracted by the action of myofibroblast containing actine and myosin.

- *Remodelling/maturation*: After the third week the wound presents continuous remodeling which lasts for about 2 years. This is based on the equilibrium of collagen deposition and degradation. The maximal wound strength is reached after 12 weeks to 2 years.

In irradiated tissues this sequence of events is delayed and affected by cellular dysfunction and loss of mesenchymal cell support. Usually delayed radiation injury is characterized by fibrosis which is the result of continuous signaling for connective tissue deposition and or failure in the down-regulatory processes which terminates fibrogenesis (Denham et al. 2002).

RI models

In order to define common criteria for defining RI, different scoring systems of histological damage have been used in animal models (Richter et al. 1997, Wang et al. 1999, Langberg et al. 1996, Wachter et al. 2000, Olofse-van Acth et al. 2001, Hauer-Jensen et al. 1983, Hauer-Jensen et al. 1983). Basically two major models were described: total or topic abdomino-pelvic radiation (Freeman et al. 2001, Molla' et al. 2001, Picard et al.2001) and selective radiation (Zheng et al. 2000, Delaney et al. 1994) with the surgical exposition of a short segment of small bowel. In our opinion this second procedure offers several advantages from an experimental point of view including the possibility of delivering high dose radiation to a limited target, limiting in the meantime the exposition of the surrounding organs by the use of proper shield. This setting represents an excellent model to investigate radiation induced damage in comparison to normal non radiated tissue. Furthermore this animal model reduces the mortality rate cost by systemic damage.

Inflammatory mediators

Cytokines are small secreted proteins released by cells that have a specific effect on the interactions and communications between cells (Zhang et al. 2007). Cytokine is a general name; other names include lymphokine (cytokines made by lymphocytes), monokine (cytokines made by monocytes), chemokine (cytokines with chemotactic activities), and interleukin (cytokines made by one leukocyte and acting on other leukocytes). They are often produced in a cascade, as one cytokine stimulates its target cells to make additional cytokines. Cytokines can also act synergistically or antagonistically. Cytokines are made by many cell populations, but the predominant producers are lymphocytes T helper and macrophages.

Pro-inflammatory cytokines: are produced predominantly by activated macrophages and are involved in the up-regulation of inflammatory reactions. There is abundant evidence that certain pro-inflammatory cytokines such as interleukins (IL) mainly IL-6 and tumor necrosis factor-α (TNF-α). IL-6 is released primarily by monocytes and macrophages as well as by nonimmune cells, such as fibroblasts and endothelial cells, during cell injury, infection, invasion, and inflammation.

Chemokines: includes a variety of cytokines which are known to induce chemotaxis. One particular subgroup of structurally related cytokines is known as chemokines. The term chemotactic cytokines (CHEMOtactic CytoKINES) usually refers to this. These factors represent a family of low molecular weight secreted

proteins that primarily function in the activation and migration of leukocytes although some of them also possess a variety of other functions. Various chemokines including macrophage inflammatory protein-2 (MIP-2) are up-regulated not only in models of inflammatory disease but also in various forms of trauma and in injury.

Anti-inflammatory cytokines: are a series of immunoregulatory molecules that control the pro-inflammatory cytokine response. Cytokines act in concert with specific cytokine inhibitors and soluble cytokine receptors to regulate the human immune response. Their physiologic role in inflammation and pathologic role in systemic inflammatory states are increasingly recognized. Major anti-inflammatory cytokines include interleukin (IL)-1 receptor antagonist, IL-4, IL-10, IL-11, and IL-13. Leukemia inhibitory factor, interferon-alpha, IL-6, and transforming growth factor (TGF)-β are categorized as either anti-inflammatory or proinflammatory cytokines, under various circumstances. Specific cytokine receptors for IL-1, TNF-α, and IL-18 also function as inhibitors for pro-inflammatory cytokines. Among all the anti-inflammatory cytokines, IL-10 is a cytokine with potent anti-inflammatory properties, repressing the expression of inflammatory cytokines such as TNF-α, IL-6 and IL-1 by activated macrophages. In addition, IL-10 can up-regulate endogenous anti-cytokines and down-regulate pro-inflammatory cytokine receptors. Thus, it can counter-regulate production and function of pro-inflammatory cytokines at multiple levels. The family of TGF-β comprises 5 different isoforms (TGF-β1 to -β5).

Radiation and inflammation

The immune system has the power to modulate the expression of radiation-induced normal and tumor tissue damage. (Schaue et al. 2015) On the one hand it can contribute to cancer cure, on the other it can influence acute and late radiation side effects, which in many ways resemble acute and chronic inflammatory disease states.

Radiation-damaged tissues often show cardinal signs of inflammation. In fact, rubor, tumor, calor, dolor and functio laesa may be reasons for the Radiation Oncologist to limit treatment dose. While the physicochemical and free radical events are over within a microsecond of radiation exposure, the inflammatory response that ensues perpetuates the response by generating recurring waves of cytokines, chemokines and growth factors with associated inflammatory infiltrates.

An early inflammatory response, beginning few hours after irradiatiton is characterized by leucocyte rolling and adhesion and following tissue infiltration, mediated by cell adhesion molecules (selectins, integrins and immunoglobulines super-genes families) into the irradiated organs, phenomena constituting one of the main determinants of radiation-induced damage (Johnson et al. 2004). This local effect is combined to a systemic haematopoetic effect which is characterized by fall in haematological count, with lymphocytes being the most sensitive leucocyte since they most easily undergo apoptosis (van der Kogel 1993) and while the monocytes are the most refractory to change (Plowman et al. 1983). These phenomena in the early period lead to increased susceptibility to infection. As a result, radiation-induced damage in normal tissues evolves with time as it is impacted by the various

regulatory immune mechanisms associated with wound healing. Importantly, the persistent crosstalk between the radiation-damaged lesion and the immune system in turn initiates inflammatory loops that feed back to the bone marrow to mobilize inflammatory cells, with further systemic consequences. One such consequence of radiation-induced inflammation is that it can lead to out-of-field effects (Kim et al. 2010).

That the damage response can extend beyond the locally irradiated area was shown for example by bilateral pneumonitis that can occur after unilateral irradiation (Morgan et al. 1995).

Indirect support for a self-perpetuating pro-oxidant and pro-inflammatory scenario stems from the fact that non-steroidal anti-inflammatory drugs and antioxidants can alleviate some of that latent damage, at least in vivo, as well as reduce inflammation-induced mutations (Mukherjee et al. 2014). Multiple features determine the host tissue response. There is a genetic component that dictates how different tissues deal with external and internal threats such as pathogens (van de Vosse et al. 2009) and injury, and the acute and late effects that are manifested following irradiation reflect this link.

In most cases, by the time radiation therapy is initiated the cancer already exists in a relatively stable state of equilibrium with inflammatory host cells and hypoxia already present. The impact of irradiation, and probably the outcome of treatment, will therefore depend upon the preexisting status quo. One contribution to the preexisting condition is the oxidative stress that tumors are under. This is often poorly defined but its consequences can be seen in the abnormalities in anti-oxidant genes. General principles of immune regulation in normal and tumor tissues have been derived from animal models. Irradiated tissues generally interact with the innate immune system in a manner similar to those damaged by pathogens (McBride et al. 2004). One common resulting paradigm is stimulation of granulocyte-macrophage colony formation with entry of early myeloid progenitors into the circulation and into the inflamed site within hours of irradiation (Ahn et al. 2010). In irradiated tumors, granulocytes gravitate towards areas of necrosis, while tumor-associated macrophages infiltrate areas of hypoxia amplified by radiation-induced vascular damage.28 These myeloid cells may differentiate further and join pre-existing tumor-associated macrophages throughout the tumor mass. In normal and tumor tissues, these infiltrating macrophages should be considered as being distinct from resident tissue macrophages and pre-existing tumor-associated macrophages as they have obvious inflammatory functions (Chen et al. 2009). Inflammatory macrophages are fascinating cells with a myriad of tools at their disposal including the ability to produce high levels of pro-inflammatory or anti-inflammatory cytokines and high pro-/anti-oxidants. They may even differentiate into antigen presenting cells and attract lymphocytic immune cell infiltrates so as to allow immunologically adaptive responses or, alternatively, they may drive angiogenesis and wound healing. They also appear to be required for stimulating growth of primary tumors and metastases. These divergent functions reflect a basic principle of the immune system, namely a network of finely tuned but opposing cellular and soluble forces that appear functionally linked to classes of cytokines and to redox status.

Radiation and apoptosis

Radiation induces mostly the ***intrinsic apoptotic pathway*** (mitochondrial release of cytochrome c and subsequent apoptosome formation), but depending on dose and cell type, the ***extrinsic apoptotic pathway*** (death receptor-mediated caspase activation) or the ***membrane stress pathway*** (ceramide production and subsequent second messenger signaling) might be the consequence of irradiation (Maier et al. 2016).

The ***intrinsic apoptotic pathway*** is initiated by signaling following single or a double-strand break if DNA repair is not successful (Gudkov et al. 2003). The stronger and longer the activation of p53 as a key determinant in DNA damage response, the higher the chances for apoptosis instead of growth arrest. p53 can contribute to both the intrinsic mitochondria-mediated and the extrinsic death-receptor-mediate. Apoptosis is mostly induced by irradiation in hematopoietic cells, or in cells of the mucosa in the gastro-intestinal tract, or in p53 wildtype tumor cells. Double-strand break -dependent activation of p53 results in increased expression pro-apoptotic genes, inducing the intrinsic apoptotic pathway. In contrast, the cell fate in most normal tissues is senescence induced by the p53/p21 and the p16/RB1pathways, which result in cell-cycle arrest in the G1 phase and subsequent senescence, in the intrinsic apoptotic pathway the control and regulation of apoptotic mitochondrial events occurs through members of the B-cell lymphoma 2 (BCL2) family of proteins, which govern mitochondrial membrane permeability and can be either pro-apoptotic or anti-apoptotic. Nuclear accumulation of p53 activates the expression of the pro-apoptotic BCL2 genes leading to cell death by permeabilization of the outer mitochondrial membrane and subsequent release of cytochrome c (Cory et al. 2002).

Furthermore, ionizing radiation can directly enhance the production of O2 by mitochondria triggering the release of cytochrome c. O2, but also other ROS, like H2O2 or OH radicals, can cause the release of calcium from mitochondria, provoking various possible pro-apoptotic consequences: loss of the mitochondrial membrane potential, release of proapoptotic mitochondrial proteins, which is coupled to stress response, known as the inner mitochondrial membrane permeability transition, production of ROS due to binding of calcium to cardiolipin which results in the oxidation of membrane phospholipids and proteins and, thus, in increased membrane permeability, dephosphorylation of pro-apoptotic BCL2-associated agonist of cell death (BAD) by the calcium/Calmodulin-dependent protein phosphatise calcineurin causing translocation of BAD from the cytoplasm to the mitochondria followed by release of cytochrome c from mitochondria which leads to caspase-9 stimulation that initiates the cascade and then activates the effectors caspases-3 and -7, thus inducing the post-mitochondrial-mediated caspase cascade (Cain et al. 1999).

Radiation-induced apoptosis is also executed through the canonical ***extrinsic apoptotic pathway*** by signaling through death receptors, which belong to the TNF receptor super family. Activation of p53 by radiation causes downstream transactivation of the receptor CD95 the CD95 ligand whose interaction results in trimerization of CD95 and clustering of its intracellular death domain.

Subsequently, procaspase-8 interacts with the death effector domain determining activation of the initiator caspase-8 results again in activation of procaspase-3 and procaspase-7. In addition, this activation of caspases can also proceed through the intrinsic mitochondria-dependent mechanisms.

In contrast to DNA damage-dependent apoptotic processes, DNA damage-independent apoptotic processes do not require p53. Radiation-induced ROS inflict lipid oxidative damage in the plasma membrane, which results in activation of sphingomyelinase followed by rapid hydrolysis of sphingomyelin in the plasma membrane, releasing the second messenger ceramide which is the activator of the membrane ***stress apoptotic pathway***. The most important effect of ceramide is the activation of the apoptotic effector caspases-1, -3 and -6, as well as the autocrine stimulation of the death receptor pathway.

Radiation and bowel microflora

When high dose radiation is delivered on the bowel a well defined RI occurs as above decribed. Furthermore the ionizing radiations act on the residential bowel microflora which presents quantitative and qualitative changes in the different symbiotic bacterial strains resident in the bowel. After radiation exposure changes occur to the homeostatic role of bacteria in the function of intestinal membrane concerning especially inflammation and innate defensive system. Radiation induces the break of the balance among symbiotic bacteria and can lead to the selection of opportunistic pathogens and further to infection. A clear link can be observed between injury and infection, since radiation (as injury) can open the mucosal barrier to infection, and infection itself with the related inflammatory pathway can lead to injury increasing the damage of the RI. Several observations (Ouwehand et al. 2000, Korschunov et al. 1996, Benova et al. 2002, Pinegin et al. 1977) showed how bowel radiation leads to a general decrease in intestinal microflora with the possibility of opportunistic germs selection and subsequent pathogenic effect on a damaged mucosa. Microflora changes are involved in pathogenesis both directly and indirectly. The modifications of the physiological action of symbiotic bacteria in fermentation of nutrients especially of unabsorbed carbohydrate, with the production of short chain fatty acids, is considered involved in the membrane dysfunction typical of the post radiation syndrome. Based on the consideration above, several studies were performed to investigate the effect of probiotic administration as radioprotectors which have shown a general benefit to the gastrointestinal side effects of radiotherapy (Delia et al. 2002, Gopal et al. 2001, Urbanecsek et al. 2001, Smirnova et al. 2000, Pikina et al. 2000, Korschunov et al. 1996, Liu et al. 2001, Tsuneoka et al. 1994). Similarly the prophylactic and/or therapeutic effect of antibiotics in modulating microflora during radiotherapy has been proposed as a potential radiomodulatory therapy (Wijers et al. 2001).

MMPs and bowel microflora

MMPs are a family of a Zn +2-dependent proteolytic enzyme (Verma et al. 2007), involved in physiological and pathological remodeling of extracellular matrix in proliferation, angiogenesis and wound healing. MMPs are divided in different groups named by substrate specificity in :

- **collagenases:** expressively involved in matrix turnover and remodeling **(MMP-1, MMP-8, MMP-13)**

- **streomelysins***:* (including matrilysin otherwise known as **MMP-7**) involved in the degradation of proteoglycans, laminin, fibronectin, elastin and degradeted collagens (**MMP-3, MMP-10, MMP-11**)

- **gelatinases:** specific for collagen type IV,V, elastin, fibronectin and denaturated collagens (**MMP-2, MMP-9**)

- **membrane type proteinases** (MT-MMPs): while most of the MMPs are secreted, the MT-MMPs are membrane associated and a number of these have cytoplasmic domains which may be important in cellular signaling part of. MT-MMPS play a central role in tumor invasion process (**MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, MMP-25**)

As proteolytic enzymes MMPs undergo to a catalytical activation from proenzymatic zymogenic forms and can be inhibited by endogenous specific tissue inhibitors (**TIMPs**), which engage the enzymatic site blocking their proteolytic activity. TIMPs family is grouped in four types (TIMP-1, TIMP-2, TIMP-3, TIMP-4) *(Louis et al. 2000).*

Matrilysin is the smallest metalloproteinase known (28kd) and presents a wide spectrum of different substrates including collagen type IV and X, but also MMP-9. Previous studies demonstrated its peculiarity among MMPs, based on the constitutive expression in normal, not inflamed, not injured epithelia in several organs (Parks et al. 2001, Wilson et al. 1995, Lopez-Boado et al. 2000, Wilson et al. 1999), while MMPs typically are not expressed or seldom expressed nearly undetectable levels in normal tissues. MMPs expression usually follows specific immunological stimuli in physiological and pathological process respectively as proliferation and remodeling in tissue growth and differentiation, and as tissue repairing and wound healing after inflammation or different types of injury.

Several experimental studies in murine models support this observation, suggesting an important role of Matrilysin in the differentiated function of epithelia especially in the mucosal barrier *(Parks et al. 2001, Wilson et al. 1995, Wilson et al. 1999).*

This homeostatic function has been clearly demonstrated in small bowel, with murine in vivo and in vitro model, with the evidence of indirect bactericidal action of matilysin in a complex and widely regulated system of innate immunity. Important role in this innate immunity is played by bactericidal proteins produced

in the epithelial cells as lysozima, phospholipase A2 or a e b defensins, which are produced and secreted when the epithelial barrier is exposed to bacteria or to bacterial antigens.

For their nature, in pathological conditions, MMPs are devoted to matrix remodeling after different kind of injury, indeed injury modifies the mucosal barrier and can favor infection and bacterial transmigration, on the other side severe infection and the related inflammation can lead to mucosal injury, therefore infection and injury are linked and MMPs, especially matrilysin in murine small bowel, can play a key role in controlling not only wound healing process but also in regulating this complex innate immunological system (Lopez-Boado et al. 2000).

In mice matrilysin is constitutively expressed in the Paneth cells in the small bowel crypts. Several studies using mice wild type and deficient for matrilysin gene (MAT+/+ vs MAT-/- mices) showed the clear role of matrilisn in the activation of the precursor of a-defensins peptides, named cryptidins since involved in crypt defense against bacteria (Parks et al. 2001, Ogle et al. 2002, Ayabe et al. 2000, Ganz 2000, Ogawa et al. 2000, Oulette et al. 2000, Harwing et al. 1995, Eisenhauer et al. 1992, Selsted et al. 1992, Oulette et al. 1999).

Lopez-Boado et al. (Lopez-Boado et al. 2000) showed the role of bacterial exposition for the induction of matrilysin expression and for its activation in human mucosal epithelial cells. Although in this setting cells from human carcinoma lines of colon, lung and bladder and normal tracheal tissue were used and not of normal small bowel, the experimental results of this investigation clearly demonstrated several key points of the subject:

- The specific bacterial stimulation (strains of E. Coli and S. thyphimurium) of the cell culture for exposition times ranging from 60 to 90 minutes, showed an increased expression of matrilysin detectable on m-RNA level, from 2 hours after infection maintaining an high value up to 24 hours allowing the detection of increased level of secreted enzyme up to 48 hours.

- Matrilysin is produced as inactive zymogene at the beginning but proceeds to activate itself by proteolysis. Furthermore shows a capacity of degrading itself in a concentration dependent manner, which is supported by the presence of both activated and degraded matrilysin in the immunoblot assay performed after stimulation with adherent bacteria.

- Adherent bacteria induce more expression of MMP-7 in comparison to not adherent. LPS seems not to have a central role in this pathway since its inhibition with polymyxin did not block matrilysin expression in response to bacterial broth filtrate, suggesting the role for an undetected inductive factor not LPS related.

- In comparison to bacterial stimulation no induction of MMP-7 was detected using different type of cytokines and other stimuli, confirming a specific bacterial involvement in matrilysin activation. Furthermore not all the bacterial strains tested showed their effect on MMP-7 over expression.

- Not using epithelial cells as substrate it is evident that matrilysin shows a cell specificity, being expressed only in the epithelial cell lines although other lines can produce other MMPs but not MMP-7.

- Bacterial exposition in epithelial cells does not induce the production of other MMPs.

- Other authors (Putsep et al. 2000) have observed that germ-free mice are able to produce cryptidins as the colonized wild type despite a lack in MMP-7 expression, minimizing the importance of bacterial exposition in this pathway suggesting the involvement of other independent factors. Lopez-Boado et al. (2000), in an in vivo section of their investigation, showed that in GF/mice MMP-7 expression could be induced by bacterial exposition. This evidence confirms that also in vivo model (in mice, not in human in the present paper) bacteria have a key role in Matrilysin expression. Numerous publications focused on the pathways activated by luminal microflora and all the changes that in it occur, to induce modifications in gastrointestinal inflammation. This is due to direct antigenic stimulation but also to food fermentation with production of short-chain fatty acids as butyrate (fermentation of no absorbed carbohydrates). This evidence suports the link between nutrition and flora and in a in vitro human model butyrate has been shown responsible for the transcriptional regulation of stromelysin-1, explaining one of the possible mechanism for the induction of inflammation in the bowel, concerning especially the field of chronic bowel inflammatory disease (Pender et al. 2000).

According to the available literature, humans differ from mice because they seem not to produce matrilysin in the small bowel. Consequently the human system of intestinal defensins, named HD5 and HD6, requires another activating enzyme which experimental results seem identify mainly in Trypsin, maybe with the possible involvement of others undetected enteropeptidase (Zaslof 2002, Ghosh et al. 2002). Numerous publications on Matrilysin in human bowel have focused on its role in cancer growth and proliferation (Kumar et al. 2002, Hodvenak et al. 2002).

MMPs and irradiated colorectal cancer

Previous studies showed a general over expression radiation induced of MMPs genes (especially MMP-7, MMP-2 and MMP-9) suggesting a possible role of over expressed MMPs in the abnormal tissue remodeling (Kumar et al. 2002, Hodvenak et al. 2002). Kumar et al. (2002) investigated the effect of radiotherapy on matrilysin gene in human rectum showing the specific over expression of MMP-7 in irradiated rectal cancer tissue compared to surrounding normal irradiated rectal tissue. This observation highlights the complex implications in progression and metastatic spreading (phenomena related to matrix remodeling MMP mediated) of colorectal cancer treated with neoadjuvant and/or adjuvant radiotherapy since the well demonstrated role of MMP-7 and its radiation induced over expression on one side and the possibility of a preventive or therapeutic usage of specific TIMP to radiotherapy in rectal cancer on the other.

Matrilysin and colorectal cancer

A significant genetic matrilisyn over expression in advanced colo-rectal cancer has been shown and also associated with increased metastatic spreading (Kumar et al. 2002, Declerck et al. 1992, Mori et al. 1995). The expression of MMP-7 m-RNA in humans has a high specificity in colorectal cancer, being expressed exclusively in malignant epithelial cells whereas only few observations seldom showed weak expression in normal colorectal mucosa with a progressive trend from normal mucosa to cancer with different quantitative expressions in different grades of dysplasia (Kumar et al. 2002, Ishikawa et al. 1996, Newell et al. 2002, Polistena et al. 2014). In contrast, most of the other members of MMP/TIMP family (MMP-2, MT1-MMP, TIMP 2-3) are also expressed also in normal colonic mucosa and in tumoral tissue located in both epithelial and surrounding stromal cells (Kumar et al. 2002). A lot of evidence confirms that there is a clear role of MMP-7 and of other MMPs in tumor growth and invasion (Ishikawa et al. 1996, Declerck et al. 1992). Increased MMP-7 expression in tumour tissue, in serum, lymph nodes and in peritoneal liquid generally correlates with worse prognosis, tendency for metastatic disease and reduced overall survival and based on this evidence a role of sensitive tumor marker for MMP-7 has been proposed (Ishikawa et al. 1998, Ishida et al. 2003, Lloyd et al. 2006, Martinez-Fernandez et al. 2009, Polistena et al. 2014, Fuksiewicz et al. 2015, Sun et al. 2015, Sica et al. 2015).

Cancer and ECM remodeling

Metastasis, a major cause of mortality and morbidity in cancer patients, is a complex multistep process, during which tumor cells locally invade the surrounding tissues, penetrate blood or lymphatic vessels (intravasation), and exit vessels at distant sites (extravasation) to form secondary tumors. Proteolytic degradation of the ECM is an important part of this process and several classes of enzymes, including MMPs, serine proteinases (plasminogen activators), and cathepsins, all abundantly secreted by a variety of tumor cells, have been implicated. A second mechanism regulating the extracellular activity of these enzymes is provided by TIMP (Declerck et al. 1992).

Under pathological conditions associated with excessive degradation of the ECM such as tumor invasion and metastasis, there is an imbalance between MMPs and TIMPs. The observation of a direct correlation between the secretion of MMP by tumor cells and their invasive and metastatic potential suggests that such imbalance can be achieved by increased production of MMP by tumor cells. Alternatively, it could be created by a decreased production of inhibitors, as suggested by the observation of an inverse correlation between levels of TIMP production and the invasive potential of tumor cells. Thus, down-regulation of metalloproteinase activity has a striking effect on local invasion and partially suppresses hematogenous metastasis (Declerck et al. 1992). It is suggested that genetic manipulation in tumor cells by gene therapy may provide an alternative to conventional approaches for the treatment of cancer. Thus, in this aspect, protease inhibitor genes may represent attractive candidates in a gene therapy approach to cancer because it may not be necessary

to deliver and express these genes in every single tumor cell as long as the level of expression in a limited number of transduced cells is sufficient to prevent the excessive breakdown of the ECM. The data indicate that retroviral-mediated transduction of TIMP-2 cDNA into a limited population of tumor cells in vivo is sufficient to increase the accumulation of connective tissue proteins in tumor tissue, to inhibit growth and to prevent local invasion (Imren et al. 1996).

Current clinical application of radiotherapy in rectal cancer

The first successful treatment of rectal cancer employing radiotherapy was reported by Symonds in 1914. Advancements in this field have led to the development of several techniques of administration including external beam radiation, endocavitary radiation, intraoperative therapy and brachytherapy and combinations of both. Adjuvant radiotherapy aims to improve survival and reduce local recurrence by treating any residual microscopic disease, (Lidder et al. 2005). Only trials using a dose greater than 20 Gy for preoperative radiotherapy have demonstrated lower recurrence rates compared to surgery alone (Wheeler et al. 1999) Radiotherapy has in the last decades been shown to be beneficial in the management of resectable rectal cancer disease adding more to the already observed benefits of total mesorectal excision (TME) surgery described by Heald in 1986 (Heald et al 1986). In most trials a higher morbidity in the early postoperative period has been observed in the radiotherapy groups (Swedish Rectal Cancer Trial 1993). Nevertheless preoperative short course radiotherapy with 5 Gy for 5 days in one week before curative surgery for rectal cancer was shown to reduce local recurrence and improve survival in the Swedish Trials (Swedish Rectal Cancer Trial 1993, 1997) compared to surgery alone. The following Dutch trial using short course radiotherapy combined with surgery with TME for resectable rectal cancer disease showed reduction of the risk of local recurrence at 2 years (2.4% vs 8.2%) and at 5 years (6% vs 11%) compared to optimal surgery alone by TME although no benefits in terms of improved survival were observed (Kapiteijn et al. 1999, 2001). The main related problems observed in the short term course were perineal wound infections cardiovascular and thromboembolic complications, disturbed anorectal dysfunction and physiology have. Downstaging and downsizing advanced rectal tumors emerged as important clinical issue after the positive results obtained by short term neoadjuvant radiotherapy. Downstaging radiotherapy is used to downstage a primarily unresectable tumour to facilitate resection in case of a tumour invading adjacent organs such as the base of the bladder or bony pelvis which are not readily resectable. Only neoadjuvant radiotherapy can produce this response. For this purpose the conventional therapy uses 50 Gy in 2 Gy daily fractions administered over a 5 week period. Usually chemotherapy is added to radiotherapy mainly to increase radiosentisitivity (Lidder et al. 2005). The effect of reducing the tumor mass inducing cellular necrosis is defined as downsizing and allows together with the downstaging effects on the local stage of the tumor a better control of local disease and improves resecability especially of lower rectal tumors with sphincter saving procedures since originally these tumors

were approached by abdominoperineal resections with permanent stomas. According to current protocols, surgery follows the long term radiotherapy course within 4-6 weeks to achieve better results with adequate shrinkage of the tumoral mass and adequate recovery from the acute radiotherapy injuries.

Palliative radiotherapy is otherwise indicated a certain group of patients with rectal cancer not amendable to surgery because of comorbidity or for advanced extra-pelvic disease. It may also be effective in reducing pain or bleeding (Glimelius et al. 2003, Lidder et al. 2005).

Briefly the *Current radiotherapeutic regimens* can be summarized as follows*:*

- **Short course preoperative radiotherapy: 25 Gy/week (5Gy x 5 days)** with the intent of reducing the impact of local recurrence and improve survival after standard optimal surgery with TME in already resectable rectal cancer without evidence of nodal mesorectal disease and for tumor lesser than T3

- **Long course preoperative radiotherapy ± chemotherapy: 50 Gy/ 5 weeks (2Gy x 25 days)** with the intent of downstaging and downsize tumor at least T3 with or without evidence of nodal mesorectal disease allowing better control of local disease and improving resecability especially of lower rectal tumors with sphincter saving procedures, otherwise receiving abdominoperineal resections.

- **Long course postoperative radiotherapy ± chemotherapy** indicated for advanced tumors which could not undergo neoadjuvant regimens or alone as palliative regimen in patients not suitable for surgery or with advanced disease without potential benefits from intervention.

Complications associated with radiotherapy and radiation protective agents

The severity of postradiation symptoms is dependent on the type of radiotherapy regimen, pre- or postoperative administration, field, port and fraction sizes and eventually concomitant chemotherapy. Short- term complication within the first three months are usually transient but can be prolonged. These include lethargy, nausea (4-17%), diarrhoea (7-30%), skin erythema and desquamation, cystitis (11-28%), bleeding, perineal wound infections, sepsis lumbosacral plexopathy, cardiopulmonary and thromboembolic (7-13%) (Lidder et al. 2005). In the Dutch trial the acute postoperative complication rate was 48% in the radiated group vs 41% in the not radiated one. Long term complications include intestinal obstruction (5-11%), ileus (5%), intestinal perforation, anorectal and genitourinary dysfunction, delayed wound healing, fistulae, intestinal obstruction, bleeding, enteritis, malabsorption, neuropathy, cardiovascular, thromboembolic, femoral neck and pelvic fractures (Wheeler et al. 1999). The preventive measures include several different approaches which were used clinically to reduce side effects of radiotherapy in healthy intestines. Local administration of sucralphate or mesalasine showed some effect but their use is not standardized. Clinically relevant results were observed with the administration of short-chain fatty acids (products of bacterial fermentation) based

on the evidence that radiotherapy reduces the mucosa associated lactobacilli (Vernia et. al. 2000). More recently radiation protective agents are classified on the basis of their mechanism of action into 3 groups (Vasin 2014):

- Radiation protective agents, with the implementation of radiation protective action taking place at the cellular level in the course of rapidly proceeding radiation-chemical reactions. At the same time, when the ionizing radiation energy is absorbed, these agents partially neutralize the "oxygen effect" as a radiobiological phenomenon, especially in the radiolysis of DNA (β-mercaptoethylamines, aminoalkyl thiosulphates, aminoalkyl dithiophosphates, aminoalkyl isothiouronium, and thiazolidine and thiazoline derivatives)

- Radiation protective agents that exert their effect at the system level by accelerating the post-radiation recovery of radiosensitive tissues through activation of a number of pro-inflammatory signaling pathways *(immunologic adjuvants and high-molecular structures of microbe, plant and animal sources like vaccines, endotoxins, polysaccharides, glucans, polynucleotides, cytokines such as IL-1beta, IL-8, IL-12,IL-18,TNF, hemopoietic growth factors, soluble cytokines, interferons, and immune regulatory peptides)* and an increase in the secretion of hematopoietic growth factors, including their use as mitigators in the early period after irradiation prior to the clinical development of acute radiation syndrome

- Radiomodulators including drugs and nutritional supplements (vitamins A, C, and E, polyphenols, anthocyanins, flavonoids, isothiocyanates, and other natural antioxidants) that can elevate the resistance of the organism to adverse environmental factors, including exposure to ionization by means of modulating the gene expression through a hormetic effect of small doses of stressors and a "substrate" maintenance of adaptive changes, resulting in an increased antioxidant protection of the organism. Radiation protective agents having polyvalence in implementation of their action may simultaneously induce radioprotective effect by various routes with a prevalence of basis mechanisms of the action.

Aims of the study

I. To validate a murine small bowel model for the investigation of inflammatory features and apoptosis as local effects of radiotherapy

II. To correlate, in the same murine model, the radiation induced microflora changes in the small bowel to the expression of MMP-7, matrilysin with modulation of the resident bacteria by antibiotic pretreatment.

III. To confirm in an in vivo human model the different grade of MMP7 expression in different stages of not radiated colorectal tumours.

IV. To analyze the potential differences of MMP-7 modulation by short and long term radiotherapy in rectal cancer patients.

Methods

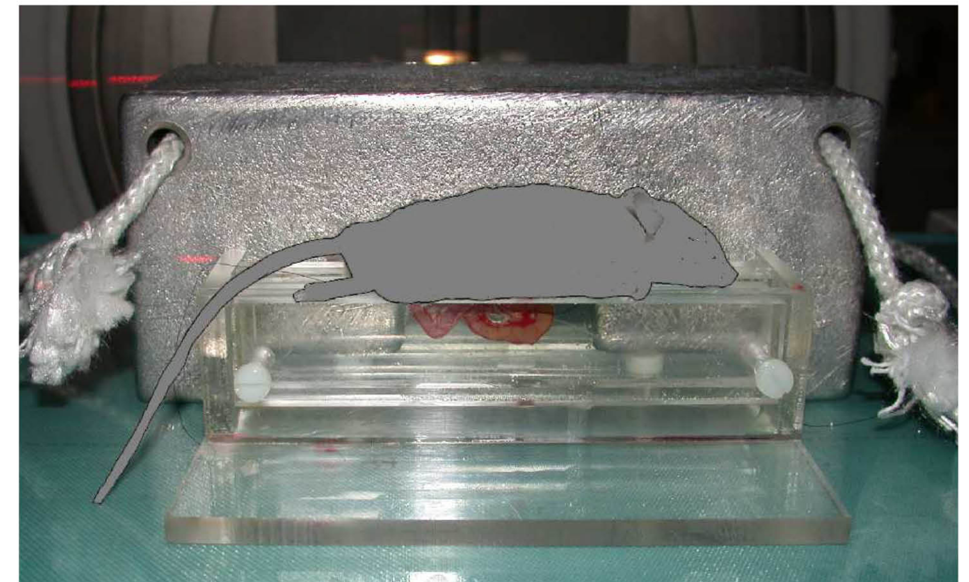
All studies were approved by the Ethics Committee of the Faculty of Medicine, Lund University, Sweden (I,II,IV) and of Rome University, La Sapienza, Italy (III) and the experimental studies (I,II) were performed in accordance with legislation on the protection of animals and were reviewed and approved by the Animal Ethics Committee at Lund University, Sweden.

Experimental Models

Radiation Chamber and Lead Shield (Paper I and II)

A special chamber designed by the authors group (**Figure 1**) for segmental irradiation of rodent small bowel was constructed. It consists of a plexiglass structure with an enclosure encompassing only the chosen intestinal segment with its mesentery. The lead shield has an aperture of 3cm x 4cm square allowing x-ray passage to the exposed intestine whilst pro- tecting the mouse from direct irradiation.

Figure 1



The chamber for segmental intestinal radiation exposure, reproduced from: Johnson LB et al. Radiation enteropathy and leucocyte-endothelial cell reactions in a refined small bowel model. BMC Surgery 2004, 4:10

Experimental Studies *(Paper I and II)*

Animals

Male C57B1/6J mice (weight 22–26 g, supplied by Taconic, Denmark) were kept under standard laboratory conditions with a 12 hour light and 12 hour dark cycle, and were allowed free access to animal food (Lactamin,Sweden) and tap water ad libitum.

Anesthesia

Mice were anesthesized with 7.5 mg Ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg Xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100 g body weight by intraperitoneal (i.p.) injection.

Surgical procedures

Animals were placed in supine position on a heating pad (37°C) for maintenance of body temperature. A short midline abdominal incision (1.0–1.5 cm) was performed and a 5 cm segment of ileum located 5 cm from the ileocaecal valve was exteriorized and marked with 5-0 nonabsorbable sutures. Any other visible prolapsed abdominal content was replaced back into the abdomen and the animal was placed on a specially designed frame with the loop of intestine fixed between two perspex sheets. The adjustable spacing between the slabs was just sufficient to support the exteriorized mouse intestine (maximum 0.5 cm). After irradiation the intestine was replaced in the abdomen and the incision closed with a polypropylene suture. To reduce water losses exposed bowels were covered with moist swabs, before and after radiotherapy.

Irradiation protocol

Paper I

54 animals were divided in two main groups: radiated mice (n = 36) and sham radiated (n = 18). The animals were further divided into smaller groups (6 animals each) according to the dose of radiation (19 or 38 Gy) or sham radiation.

Paper II

108 animals were divided into two main groups; radiated mice (n = 72 animals) and sham radiated (n = 36), The animals were further divided into smaller groups (6 animals each) according to the dose of radiation (19 or 38 Gy) or sham radiation. During the 7 days before radiotherapy, a broad spectrum antibiotic (ampicillin 500 mg/kg/d) was administered intramuscularly (antibiotic groups).

Paper I and Paper II

All animals underwent laparotomy for exposure of the intestinal segment that was analysed. The exposed ileum was subjected to a single high absorbed dose radiation of either 19 or 38 Gy and the mouse protected by an 8 cm thick lead metal shield. The irradiations were undertaken using a clinical linear accelerator (Varian Clinac 2100C) and in normal room temperature. The method has been described elsewhere. The absorbed dose was verified with independent measurements and was found to be within 5% throughout the intended volume using this technique. The use of an asymmetrically half blocked 6 MV beam and extra lead shielding assured that the treatment field perfectly fitted the exteriorized intestine while the remaining mouse body was kept outside the radiation beam. The absorbed dose rate was 3.2 Gy/minute and consequently the irradiation time for each animal was approximately 6 minutes for 19 Gy (n = 18) and 12 minutes for 38 Gy (n = 18). An absorbed dose of 19 Gy delivered to the intestine is known to cause consistent structural, cellular, and molecular changes. The sham operated mice underwent the same surgical procedure except irradiation. Exposure time from surgery, through irradiation to wound closure was kept at a minimum in order to reduce stress and trauma levels, the whole procedure taking approximately 15 minutes.

Collection of samples (Paper I and Paper II)

Specimens were collected for the 3 different time points (2, 24 and 48 hours). At each timepoint 3 groups (19 Gy,38 Gy and Sham) consisting of 6 animals per group were studied. A second laparotomy was performed; the marked bowel segment was exteriorized and excised. It was then divided into segments of 0.5–1.0 cm lengths which were weighed and stored for histology, immunohistochemistry, and cytokine analysis (Paper I) and for microflora analysis, western blotting for MMP-7, cytokine analysis (Paper II).Blood samples were collected from each animal (Paper I and paper II).

Analysis of samples

Paper I

Histological study

Samples were collected at different time points after irradiation. The marked irradiated bowel segment was excised and rinsed with normal saline. The samples were fixed in 4% buffered paraformaldehyde, dehydrated in gradedethanol and embedded in paraffin. The sections were cut on a microtome and mounted on glass slides. The slides were stained with hematoxylin and eosin for histological evaluation under light microscopy, which was done by the pathologist in a blinded fashion.

Cytokines

Tissue samples were washed in PBS containing penicillin, streptomicin and fungizon (100 U/ml) and kept cool in cold serum-free medium (DMEM). Bowel was incubated in 1 ml of DMEM solution 10% FCS and penicillin, streptomycin for 24 hours (37°C) in a 12-wells plate. The cultured medium was harvested and stored in -20°C until analysis of MIP-2 (Macrophage inflammatory protein-2), TNF-α (Tumour necrosis factor-α) and IL-10 using a colorimetric sandwich ELISA kit with recombinant murine proteins as standard (Quantikine, R&D Systems, Europe). The minimal detectable protein concentration in these kits is <1.5 pg/ml for MIP-2, <5.1 pg/ml for TNF-α and < 4.0 pg/ml for IL-10.

Immunohistochemistry

For immunohistochemistry, standard avidin-biotin procedures for mouse CD45 (defined as leucocyte common antigen) and for active caspase-3 were used. After deparaffinization and washing in phosphate buffered solution (PBS), endogenous peroxidase activity was blocked by incubating the sliced sections in 3% hydrogen peroxide in PBS for 10 minutes. For CD45 analysis the slides were fixed with BD Retrievagen fixative A (BD Biosciences, Europe) and then incubated overnight at 60°C. Slides for Caspase-3 were microwave-treated. The sections were blocked in 5% fetal calf serum with PBS for 30 minutes and then incubated for 1 hour at room temperature with 1:100 anti mouse CD45 monoclonal antibody (RnD Systems Inc., Minneapolis, USA, cat n° MAB114) and with 1:750 anti-active human-mouse caspase-3 affinity-purified rabbit antibody (RnD Systems Inc., Minneapolis, USA, cat n° AF835) respectively. Biotin-conjugated secondary antibody and streptavidin-conjugated horseradish peroxidase (DAKO, USA) were applied to sections for 45 minutes at room temperature, and developed using the 3,3'-diaminobenzidine (DAB) as substrate. Finally counterstaining with haematoxylin for both CD45 and caspase was performed. Sections were mounted and leucocyte and apoptotic cell counts were determined on randomly selected areas using the point counting technique by a blinded observer. Each slide contains 3 sections and from each section we choose randomly 3 areas under light microscopy with a high power field ×100. We then take the mean value of all the counts in each slide.

Paper II

Intestinal Microflora

Tissue samples from the irradiated small intestine were first placed in 5 mL of sterile transport medium [17]. Samples were then placed in an ultrasonic bath (Millipore, Sweden) for 5 min and then rotated on Chiltern (Terma-Glas, Gothenberg, Sweden) for 2 min. After a conven- tional dilution procedure, viable counts were obtained from brain heart infusion (BHI) that was incubated aerobically and anaerobically at 37o C for 72 h (aerobic and anaerobic bacterial count, respectively), and from Rogosa agar (Oxoid, Hampshire, England) that was incubated anaerobically at 37o C for 72 h (lactobacilli counts). Viable counts were also obtained from violet red-bile-glucose agar (VRBD) (Oxoid, Hampshire, England) that was incubated aerobically at 37o C for 24 h (enterobacteriaceae counts).

Western Blotting for MMP-7

Western blotting was conducted using equivalent amounts of super- natant from tissue homogenates, which were loaded onto each lane of the 4% to 12% Nu-PAGE Bis-TrisGel (Invitrogen, Lidingö, Sweden) and were run under reducing conditions using an electrophoretic ma-chine (XCell II mini-cell; NOVEX, Frankfurt am Main, Germany) powered by a 1000/500 units power supply (Bio-Rad, Sundbyberg, Sweden). Molecular multicolored standard (MultiMark, Invitrogen) and human recombinant MMP-7 (cat. no. 444270; Calbiochem) were included as double controls in MMP-7 detection. Proteins from the gel were then transferred to nitrocellulose membranes (Trans-Blot, Bio-Rad). Blots were blocked in blocking solution and then incubated respectively with a 1:500 dilution of rabbit anti-murine matrilysin polyclonal antibody (Ab) (cat. no. PC492; Calbiochem-Oncogene, via Merck, Darmstadt, Germany) and all kept in Ab buffer for 4 h at room temperature. Anti-MMP-7 antibody detects both human and murine protein in both the precursor (28 kD) and active (19 kD) forms of the protein. Blots were subsequently incubated after washing in tris(hydroxymethyl)methylamine with a 1:3000 dilution of a phospha- tase conjugated goat anti-rabbit IgG (cat. no. 170-6518; Bio-Rad) in Ab buffer for 1 h. Blots were finally developed with a substrate reaction by adding a solution of 0.19 mg/mL NBT/BCIP stock solution (Roche, Diagnostics GmbH, Mannheim, Germany) in ultra pure water. After 10 to 20 min. the reaction was stopped by washing in H20. Semiquan- titative measurement of blots was performed by densitometry analy- sis (GS-710 calibrate imaging densitometer; Bio-Rad) and software data evaluation (QUANTITIVE ONE: quantitation software; Bio- Rad). The densitometry values are expressed as percentage (%) of the control antigen.

Cytokines

Samples of small bowel were weighed and homogenized for 1 min in phosphate buffer. The homogenates were centrifuged at 10,000 g at 4o C for 5 min. MIP-2 and TGF-b concentrations in the supernatants were determined by ELISA using the commercially available Quanti- kine kit (R&D Systems, Minneapolis, MN). Optical densities were measured on an ELISA reader at a wavelength of 450 nm. Data were analyzed against the linear portion of the generated standard curve.

Paper I and II

Systemic Leucocyte Counts

20 mL blood was mixed with Turk's solution (0.2 mg gentian violet in 1 mL glacial acetic acid, 6.25% v/v) in a 1:10 dilution. Leucocytes were counted and differentiated as polymorphonuclear (PMNL) or mono- nuclear (MNL) cells in a Burker chamber.

Clinical Studies *(Paper III and IV)*

Paper III

A prospective study over a period of three years (September 2005- September 2008) was undertaken of 8 patients with benign and 20 patients with malignant not radiated colorectal tumours with indication for elective colorectal surgery after acceptance of informed consent. The study was carried-out. Staging was performed after colonoscopy and biopsy, with abdominal computed tomography (CT) and chest radiography. Clinical variables were included in a database. Intraoperative blood samples were collected to determine baseline parameters, specific oncomarkers (carcinoembryogenic antigen, CEA; cancer antigen 19- 9, CA19-9, cancer antigen 50, CA 50) and MMP7.

Surgical procedure

The intervention carried out were: 8 right hemicolectomy, 6 left hemicolectomy, 4 sigmoidectomy, 2 subtotal colectomy, 6 anterior rectal resection, 2 abdominoperineal rectal excision.

Collection of samples

Serum samples were taken from all the surgical patients and from 10 healthy volunteers after acceptance of informed consent.

Intraoperative specimens were collected from cancer tissues and from normal surrounding mucosa (about 2-2.5 cm from the tumour edge). Lymph nodes from the colonic mesentery close to the tumour were collected. The specimens were fixed in 4% formaldehyde before histopathological examination.

Samples and serum preparation

Tissue samples from tumoural and mucosal tissue, as well as from lymph nodes, were kept in sterile tubes at –80°C. They were then cut obtaining aliquots weighing between 50 and 100 mg. These were treated with 300 µl of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% NP-40, 1% CHAPS, 2 mM EDTA dissolved in tetra-distilled water). A mixture of protease inhibitors (Complete-Mini Protease Inhibitor Cocktail Tablets; Roche, Mannheim, Germany) was added just before use. Samples were first homogenized by Ultra-Turrax® (T10 basic; IKA®, Staufen, Germany), then sonicated for 20 sec and centrifuged at 14,000 rpm for 20 min (19). The supernatants were then collected. Venous blood samples were drawn into sterile vacuum tubes and left at room temperature for 30 min, then centrifuged at 4,000 rpm for 15 min, to divide serum from pellet, as standard laboratory protocol. Serum was immediately aliquoted and stored at –80°C until assayed. The protein content of supernatants and serum samples was determined by using the Bradford assay.

Analysis of samples

Histology

Samples were fixed in 4% phosphate-buffered formaldehyde and later embedded in paraffin. Sliced specimens stained with hematoxylin and eosin were analyzed under light microscopy. At least three slides were studied from each specimen by a blinded observer. Stage definition was stated according to 2002 UICC classification.

Immunohistochemistry

For immunohistochemistry, standard avidin- biotin procedures for human MMP7 were used. After de- paraffinization and washing in phosphate-buffered solution (PBS), endogenous peroxidase activity was blocked by incubating the sliced sections in 3% hydrogen peroxide in PBS for 10 min. Analysis for MMP7 was performed using anti-MMP7 (MAB-10756; Immunological Sciences, Rome, Italy) following the manufacturer's instructions. Biotin-conjugated secondary antibody and streptavidin- conjugated horseradish peroxidase (Dako North America, Inc., CA, USA) was applied to sections for 45 min at room temperature, and developed using 3,3'-diaminobenzidine (DAB) as substrate. Finally, counterstaining with haematoxylin was performed. Sections were mounted and the grade of staining was determined on randomly selected areas counter-checked for intensity by a blinded observer.

MMP7 determination

In supernatants and serum samples, total human MMP7 levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Quantikine®, R&D Systems, Minneapolis/USA). Diluted samples (150 µl) were added to a 96- well microtiter plate, pre-coated with a monoclonal antibody to human MMP7 and incubated at room temperature for a further 2 h on a microplate shaker. After washing, 200 µl of the secondary antibody solution were added, and the plate was incubated for 2 h at room temperature on the shaker. After washing, the substrate solution was added and incubated at room temperature in the dark. A 50 µl stop solution was added after 30 min and the optical density was measured using a microtiter plate reader (Opsys MR™; Dinex Technologies, Inc.; Chantilly, VA, USA) at 450 nm, with correction wavelength set at 570 nm.

Western blot analysis

For western blot analysis, supernatants obtained from lymph node specimens were separated on a sodium dodecyl sulphate-polyacrylamide electrophoresis gel with a concentration of acrylamide specific for MMP7 and β-actin. Proteins were blotted onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA) and probed with the following antibodies: anti-MMP7 (MAB-10756 Immunological Sciences) and anti-β-actin (A 5060; Sigma Chemical Co., St. Louis, MO, USA). Antigens were detected with an enhanced chemoluminescence (ECL) kit from

Amersham (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). All western blotting images were acquired and analyzed through an Imaging Fluor S densitometer (Bio-Rad Laboratories, Hercules). The optical density (O.D.) of each condition was correlated to the signal of the β-actin internal control.

Paper IV

A case-controlled study comprising 77 patients with rectal cancer treated at the University Hospital of Malmö between 2003 and 2007 was undertaken. The study cohort consisted of 53 patients. Three treatment groups were defined: one group receiving short term preoperative RT of 25 Gray (5 X 5 Gy), 20 patients; another group treated with long term preoperative RT of 50 Gy (25 X 2 Gy), 21 patients; and a control group undergoing surgery alone without RT, 12 patients.

Surgical procedures

Were performed either anterior resection or abdominoperineal resection, by the total mesorectal excision technique.

Preoperative and perioperative endoscopy

Two endoscopic biopsies were taken: at inclusion before RT prior to start of surgery to eliminate possible effects of surgical trauma and ischaemia on MMP expression; at surgery and from the excised specimen. Two millimeter punch biopsies were obtained from tumour tissue as well as from normal mucosa not less than 2 centimeters from the tumour edge within the irradiated field.

Collection of samples

Intraoperative specimens were collected from cancer tissues and from normal surrounding mucosa (about 2 cm from the tumour edge). All tissue samples were instantly formalin fixed and paraffin embedded.

Analysis of samples

Tissue microarrays

Tissue microarray (TMA) were performed in two series; one biopsy TMA with 1 x 1 mm cores were taken out of biopsies from normal tissue as well as from tumour tissue, and one TMA from the complete surgical specimens through which 2 x 1 mm cores were drawn from areas comprising viable, non-necrotic tumour, and nearby microscopically benign rectal mucosa, respectively. This was performed by using a manual arraying device (MTA-1; Beecher Inc., Sun Prairie, WI, USA) and mounted in a recipient block.

Immunohistochemistry and staining evaluation

The TMAs were sectioned into 4 micrometer (μm) samples pretreated in the DAKO PT-link module using a standard protocol and buffer supplied by the manufacturer. Thereafter slides were stained in a DAKO Autostainer-plus using the EnVision™ FLEX including Peroxidise-Blocking Reagent (DAKO, Glostrup, Denmark) with monoclonal antibodies MMP-7 Santa Cruz mouse (clone MM0022-4C21) sc-101566 dilution 1:50 and TGF-beta Abcam rabbit poly (ab6603) dilution 1:200. Immunohistochemistry was performed by an automated staining machine (Ventana Medical Systems, Inc., Tucson, AZ, USA). Three research scientists jointly annotated cytoplasmic expression of inflammatory markers for each core, in tumour tissue and mucosa. Annotation of absolute percent of positive cells was multiplied with annotated intensity (0-3) of stained cells, and a mean expression score was subsequently calculated for each patient at each time point. Discrepant cores were discussed until consensus was reached.

Statistics

Paper I

Sham and radiated groups were statistically analysed by one-way analysis of variance (ANOVA) with a post hoc Turkey test for all pairwise multiple comparison procedures. Non-parametrically distributed groups were studied by the Kruskal-Wallis analysis of variance on ranks. A probability (P) value ≤ 0.05 was accepted as significant. Differences were expressed as mean values ± SEM.

Paper II

Statistical evaluations were performed using the Kruskal-Wallis one way analysis of variance on ranks for unpaired samples (Dunn's post hoc test was used). For bacterial microflora in comparing two groups we used Mann-Whitney rank sum test, and for the comparison of the different time points within the radiated groups we used oneway analysis of variance (ANOVA) followed by multiple comparisons versus control group (Dunnett's method). For MIP-2 and TGF-b, we used for multiple comparison Kruskal-Wallis one-way ANOVA on ranks followed by all pairwise multiple comparison procedures (Student-Newman-Keuls Method). In comparing two groups, we used

Mann-Whitney rank sum test. The results are presented as mean values 6 SEM or median (25th–75th percentile) as appropriate. Differences were considered to be significant at $P < 0.05$.

Paper III

Data are expressed as the mean±standard deviation (SD). The statistical comparisons between groups were performed by using the analysis of variance (ANOVA) followed by the Bonferroni post hoc test. Differences were considered significant at $p<0.05$. Analysis was performed by using a statistical software (GraphPad Software, Inc., San Diego, CA, USA).

Paper IV

Spearman's Rho and χ^2 tests were used to investigate RT groups and patient characteristics. Mann-Whitneys U-test and Wilcoxon Z-test (Z) were used to investigate MMP7 expression differences between tissue from baseline before RT, after RT prior to surgery, and after resection, in the RT subgroups. All statistics were performed using SPSS version 21.0 (SPSS Inc, Chicago, IL, USA). P-values over 0.05 was considered significant.

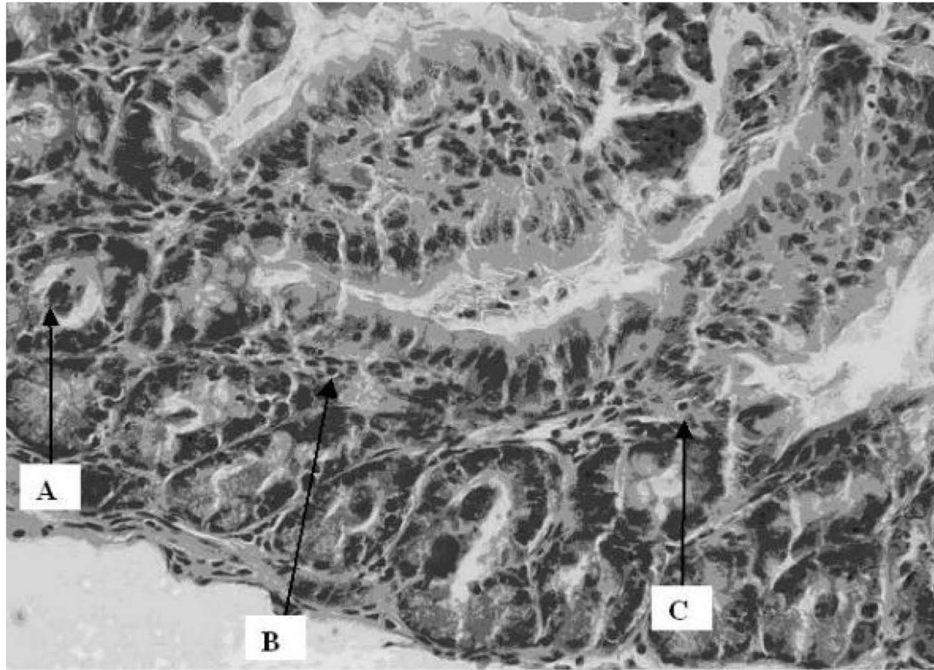
Study design and results

Apoptosis and Inflammation in irradiated murine small bowel *(Paper I)*

Our model is a refinement of the latter model previously used in our group (Johnson et al. 2004) where a murine small bowel segments can be subjected to irradiation while minimizing secondary radiation effects to other organs, lymphoid tissue and bone marrow. Local effects of subsequent radiation injury with limited effects of immunological involvement can be more accurately assessed. The aim of this study was to define the features of early, local radiation-induced damage to small bowel, focusing on inflammatory changes and apoptosis. As presented in methods 54 mice were divided into two groups 36 irradiated (subjected to a single absorbed dose of 19 or 38 Gy) and 18 sham irradiated. Specimens were collected for histology, immunohistochemistry (IHC) and ELISA analysis (for IL-10, MIP-2, TNF-α and Caspase-3) after 2, 24 and 48 hours.

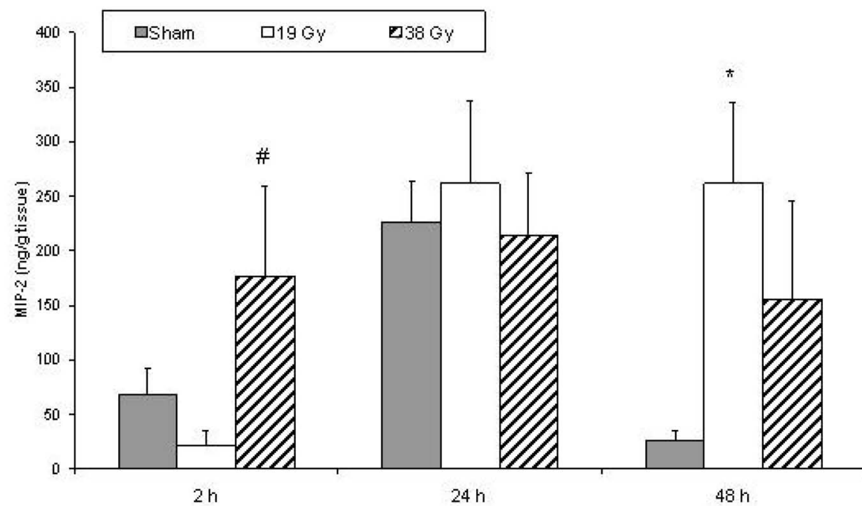
Histology demonstrated progressive infiltration of inflammatory cells with cryptitis and increased apoptosis (**Figure 2**). MIP-2 concentration was significantly increased in irradiated animals up to 48 hours (**Figure 3**). No significant differences were observed in IL-10 and TNF-α levels. Immunoistochemistry with CD45 (**Figure 4**) showed a significant increase at 2 hours of infiltrating leucocytes and lymphocytes after irradiation followed by progressive decrease with time. Caspase-3 expression (**Figure 5**) increased significantly in a dose dependent trend in both irradiated groups up to 48 hours. The total leucocyte count in this study did not show any significant changes in the different groups.

Figure 2



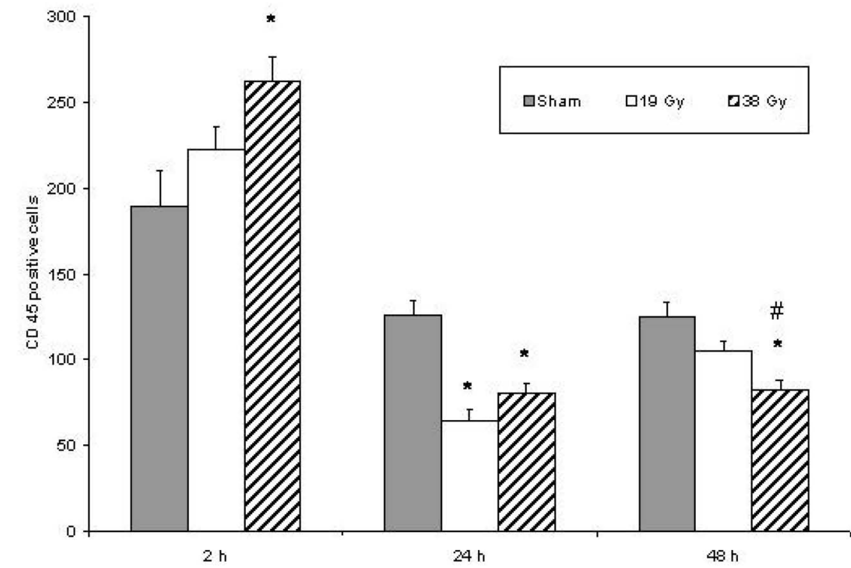
Cross section of intestinal wall 48 hrs after 38 Gy irradiation. An increase in apoptosis (A), intraepithelial granulocytes (B) and lymphocytes (C) was observed with degenerative epithelium and granulocyte exudate in the lumen (between the villi and even in the crypts, suggesting cryptitis). The slides were stained with hematoxylin and eosin for histological evaluation under light microscopy, which was done by the pathologist in a blinded fashion.

Figure 3



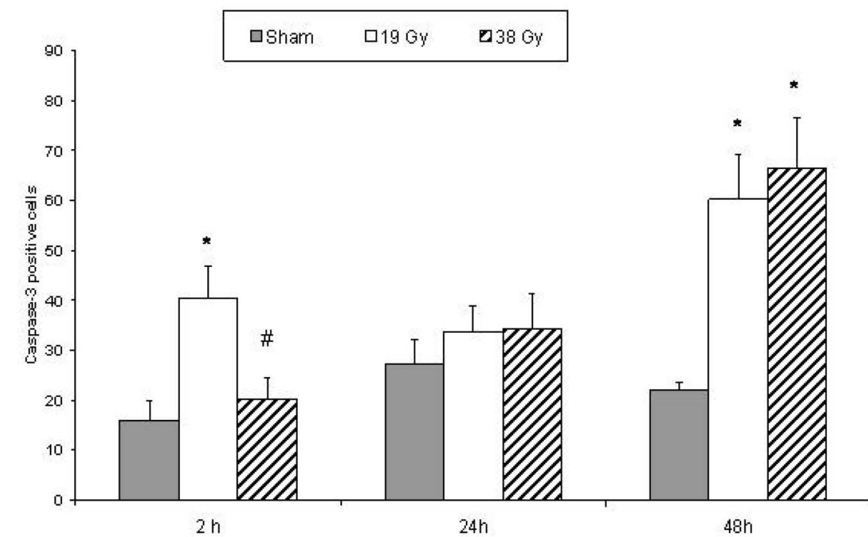
*MIP-2 concentration in small bowel tissue comparing sham and different doses of radiation within each time point. * denotes p < 0.05 compared to sham group, # denotes p <0.05 compared to 19 Gy group.*

Figure 4



*CD45 positive stained cells in small bowel tissue comparing sham and different doses of radiation within each time point. * denotes p < 0.05 compared to sham group, # denotes p <0.05 compared to 19 Gy group.*

Figure 5



*Caspase-3 positive stained cells in small bowel tissue comparing sham and different doses of radiation within each time point. * denotes p < 0.05 compared to sham group, # denotes p < 0.05 compared to 19 Gy group.*

Matrilysin expression related to radiation and microflora changes in murine bowel *(Paper II)*

Since matrilysin is prominently upgraded in tissues with heavy bacterial load with indirect bactericidal action in the innate immunity by regulation of the level of antimicrobial peptides (α and β defensins) and in the mean time its overexpression has been observed after irradiation, our aim was the study of how the modification of gut flora by administration of antibiotics and the local bowel irradiation could modulate the expression of matrilysin. As presented in methods

Animals were divided into two different groups a 72 animals (divided into smaller groups according to radiation dose 19 or 38 Gy) and 36 animals sham radiated. After 7 days antibiotic administration an exteriorized segment of ileum was subjected to single high dose radiation and samples were collected 2, 24, and 48 h and analyzed for microflora, MIP-2, TGF-b, and MMP-7.

The combination of antibiotics and irradiation leads to an early significant reduction of bacteria, down-regulates MIP-2 (**Table 1**), up-regulates TGF-b (**Table 2**) and elevation of MMP-7 (**Figure 6**) to levels achieved by antibiotics or irradiation alone. Lactobacilli were reduced to non-existent levels after antibiotics.

Table 1.

	Sham radiation	19 Gy radiation	38 Gy radiation
Preradiation antibiotics			
2 h	6133 (3406–7676)[§]	1040 (809–1252)[*,§]	339 (268–408)[*,†]
24 h	27435 (8286–47931)	1708 (1689–2312)[*]	1378 (829–1601)[*,‡]
48 h	3002 (1661–4400)[§]	1866 (1413–2139)	2099 (1264–3457)[‡]

Values presented as median (25th–75th percentile).
[*] $P < .05$ compared with sham radiation.
[†] $P < 0.05$ compared with 19 Gy.
[‡] $P < 0.05$ compared with 2 h.
[§] $P < 0.05$ compared with 24 h.

MIP-2 Values of the Small Bowel (pg/g tissue) after Different Doses of Radiation and Antibiotic Treatment

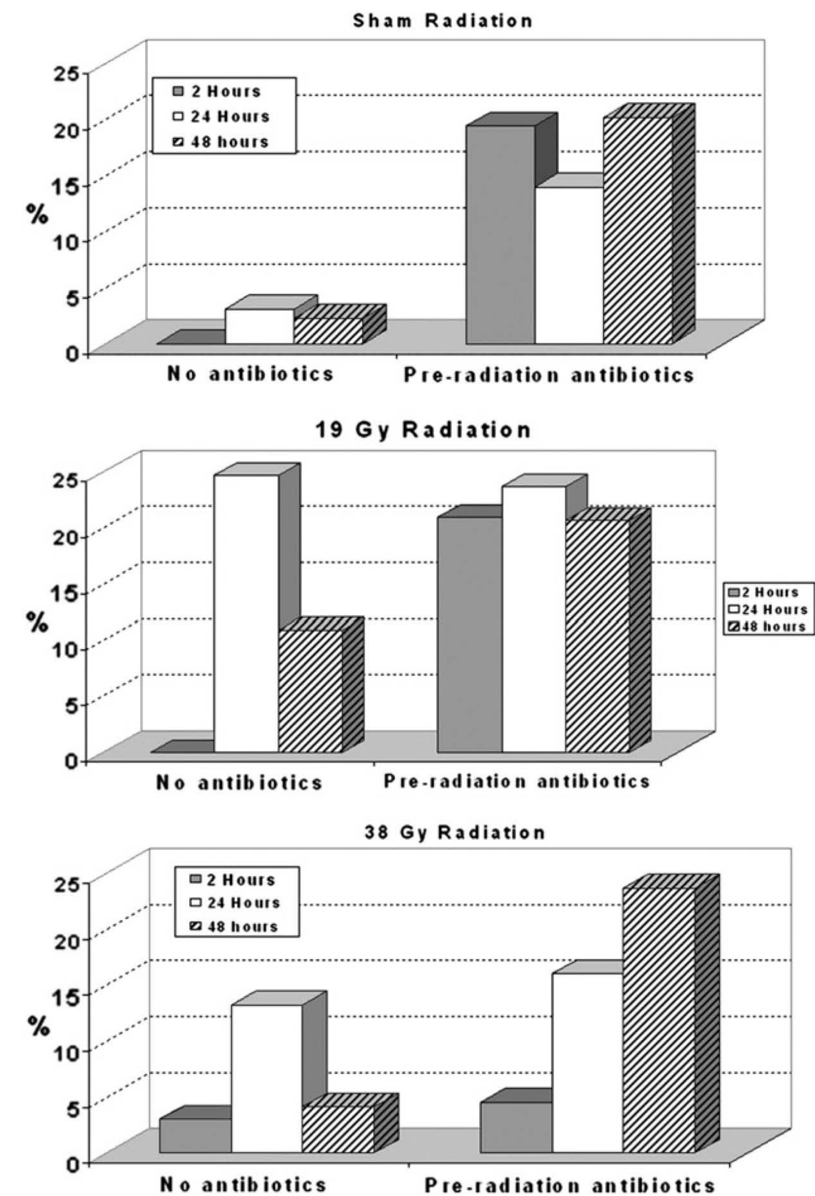
Table 2.

	Sham radiation	19 Gy radiation	38 Gy radiation
Preradiation antibiotics			
2 h	8900 (7659–9710)	10591 (8464–13601)[†]	8013 (6222–9111)
24 h	4144 (3904–4496)	5800 (5116–6016)[*]	6668 (5226–9843) [*]
48 h	3911 (3241–5370)	10109 (9400–10771)[*,†]	7348 (4971–10373)[*]

Values presented as median (25th-75th percentile).
[*] denotes $P < 0.05$ compared with sham radiation.
[†] denotes $P < 0.05$ compared with 24 h.

TGF-b Values of the Small Bowel (pg/g Tissue) After Different Doses of Radiation and Antibiotic Treatment

Figure 6.



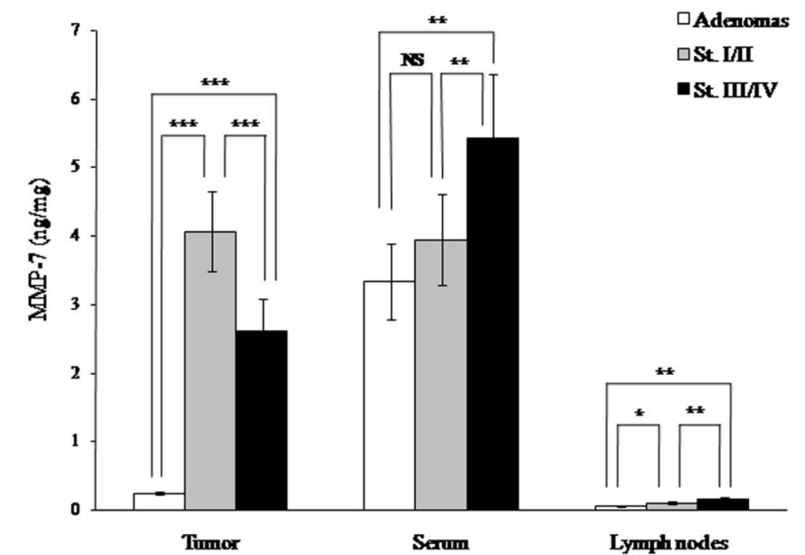
MMP-7 expression %, 2, 24, and 48 h after sham radiation, 19 Gy and 38 Gy radiations 6 antibiotics pretreatment.

MMP7 Expression in Colorectal Tumours of Different Stages *(Paper III)*

The expression of MMP-7 mRNA in humans has a high specificity in colorectal cancer, especially in malignant epithelial cells, but with weaker expression also in normal colorectal mucosa, as well as in different grades of dysplasia to cancer. The purpose of the study was to evaluate the correlation between matrilysin expression at different stages of tumour progression from dysplasia to cancer with levels expressed in adjacent lymph nodes in the resected specimen and if this would be useful for the assessment of colorectal cancer prognosis. For this purpose specimen of colorectal tumor (dysplastic polyps and cancers), normal mucosa, lymph nodes and serum were collected from 28 patients and 20 healthy volunteers and analyzed for MMP-7 expression by immunohistochemistry, ELISA and western blotting.

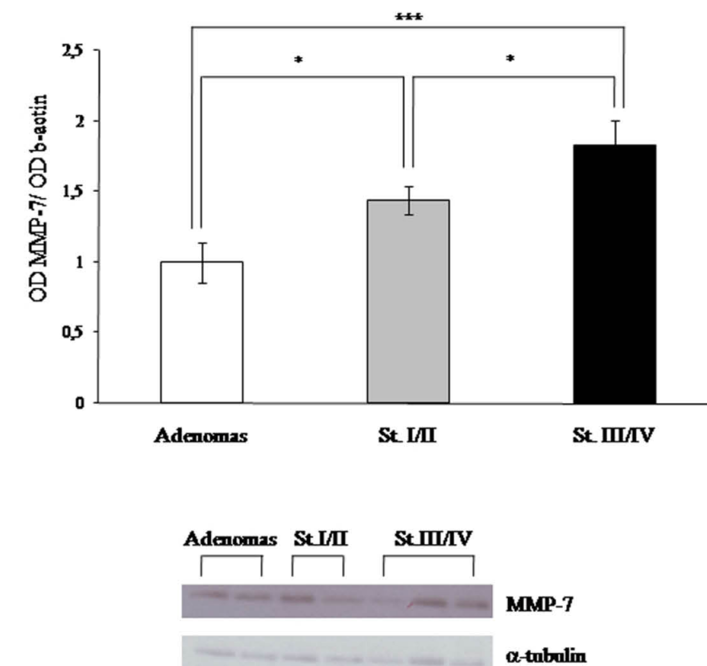
Histology demonstrated no MMP-7 expression was in normal mucosa, but progressive increased expression from benign to malignant tumor among which well differentiated carcinoma had the most evident staining. With ELISA MMP-7 expression was significantly ($p<0.01$) higher in stage I and II cancer tissues compared to adenomas (low and moderate dysplasia) and significantly lower compared to stage III and IV cancers **(Figure 7)**. Levels in adenomas were also significantly lower ($p<0.001$) compared to those in stage III and IV disease. Normal mucosa did not show any measurable levels of MMP-7. No significant difference was observed in serum levels of MMP-7 comparing patients with benign adenomas to those with stage I and II cancers. A significant increase ($p<0.01$) was evident in serum comparing patients with adenomas to those with stage III and IV cancer. However, no significant differences were observed for MMP-7 expression within the groups comparing stage I to II and III to IV in both tumour and serum. Serum obtained from healthy controls showed very low or undetectable levels of MMP-7. Lymph nodes presented lower levels of MMP-7 compared to serum and tumoural tissue. Significant differences in expression in lymph nodes were noticed among groups, adenoma vs. stages I and II ($p<0.05$) and stages III and IV vs. both stages I and II and adenomas ($p<0.001$) **(Figure 7)**. Western blot analysis. Further analysis of lymph nodes by western blotting, with a semi-quantitative measurement of MMP-7 expression, confirmed the ELISA results. We observed a significant positive trend in the expression of MMP-7 from adenoma to increasing cancer stage. MMP-7 was evaluated by comparative detection of β-actin. Lymph nodes of patients with stage I and II tumours had significant higher expression than those in patients with adenomas ($p<0.05$) and those in patients with stage III and IV tumors had significantly higher levels of MMP-7 ($p<0.05$) compared to those with stage I and II adenocarcinomas and those with adenomas ($p<0.001$) **(Figure 8)**.

Figure 7.



*Matrix metalloproteinase-7 expression in tumoral tissue,serum and lymph nodes. Significantly different at *p<0.05, **p<0.01, ***p<0.001; NS, p>0.005.*

Figure 8.

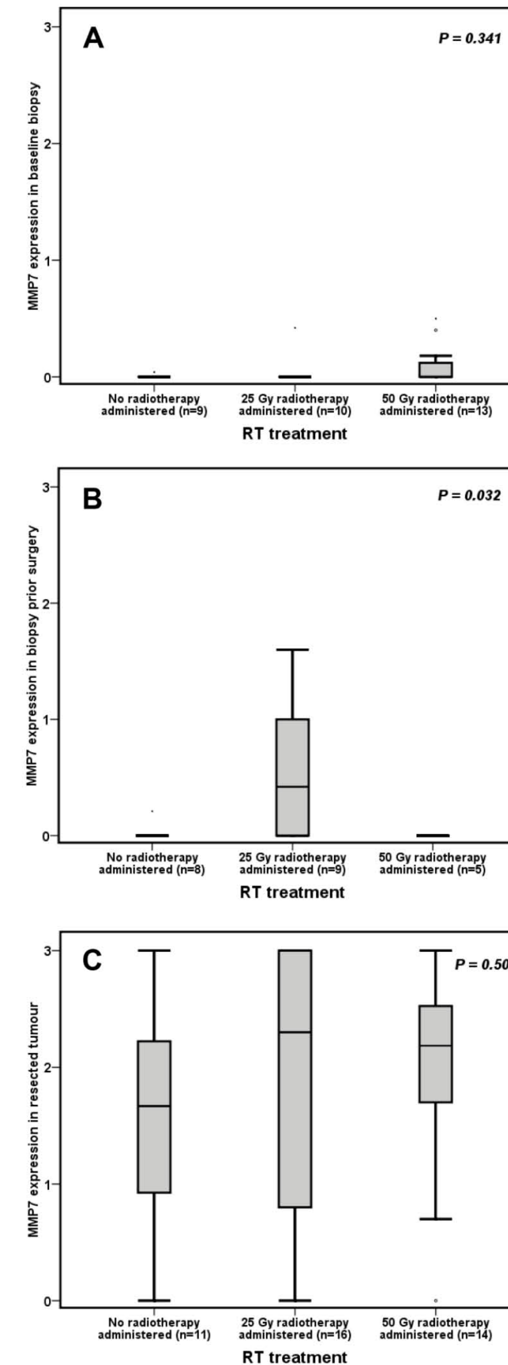


*Western blot analysis showing the expression of matrix metalloproteinase-7 in lymph nodes. Significantly different at *p<0.05, *** p<0.001.*

Modulation of MMP-7 by short and long term radiotherapy in rectal cancer patients *(Paper IV)*

The aim of the study is to analyse the effects of irradiation on inflammation and MMP-7 in patients with rectal cancer undergoing different regimens of neoadjuvant radiotherapy. Properative and post-irradiation (in radiated groups), perioperative endoscopic biopsy and biopsy of excised specimens from tumor burden and surrounding mucosa from 53 patients divided into three treatment groups receiving short term (25 Gy) and long term (50 Gy) preoperative RT and controls receiving only surgery, underwent analysis by immunohistochemistry on tissue microarray for MMP-7 and TGF-beta. We observed that surgery up-regulated MMP-7 in all groups. Short term 25 Gy radiotherapy induces over-expression of MMP-7 before and at the time of surgery but it is not observed following 50 Gy radiotherapy **(Figure 9)**. In all three groups no significant increase of TGF-beta was observed before surgery. TGF-beta showed significant 2- to 3-fold increase only after rectal resection.

Figure 9



MMP7 tumour expression at baseline, in preoperative biopsy and in the specimen.

Discussion

The effects of radiation on small bowel which is the principal healthy surrounding organ in the abdomino-pelvic field is the result of inflammatory damage and consequent repairing process which induces re-epithelization of intestinal mucosa with a dose depending relation of radiation on damage. MMP-7 has been shown to be expressed in human colo-rectal cancer and overexpressed after radiation exposure and also increased in murine small bowel after different kind of stimuli including radiation. MMP-7 has been proposed as a biomarker in colo-rectal cancer due to its modulation by irradiation.

The present research project was planned to analyze the secondary effects of RI on small bowel during therapeutic radiotherapy. The first objective of the research was to clearly define the radiation-induced damage on small bowel, using a murine model built by our research group. Features focused on were inflammation and apoptosis. Further analyses focused on radiation induced changes on intestinal microflora correlated to MMP-7 expression. Based on the results of the experimental phase of the project we approached to the clinical phase, to verify the potential role of matrilysin as biomarker of cancer growth and dissemination in relation to different tumor stages and further to analyze the expression of matrilysin and of inflammatory proteins in human colorectal cancer patients undergoing different radiotherapeutic regimen in the short and long term protocols.

Inflammation and apoptosis as markers of RI in murine small bowel

A major problem in planning radiotherapy is achieving an optimal radiation schedule, balancing dose, fractionation and exposure time for maximum tumoricidal effect, limiting at the same time the injury to healthy surrounding tissues. In radiotherapy of the abdominopelvic region the small bowel is frequently injured and the severity of mucosal damage depends on the balance between epithelial denudation and new cellular proliferation. When the rate at which surviving epithelial cells killed exceeds the maximum rate at which new cells are replaced the injury is more evident. The deeper the lesion the slower the re-epithelization because the epithelial stem cells at the basement membrane zone are mainly affected. This event reflects also on the potential loss of barrier function with risk of consequent bacterial penetration and infection.

The current paper confirms the validity of our designed model of murine small bowel local irradiation, to study the acute features of RI. Features observed here may be mainly summarized as early increases in MIP-2 levels, a decrease in CD 45 positive cells over time and increased dose dependent apoptosis. There is evidence

that MIP-2 is one of the major chemokines that lead to neutrophil recruitment and infiltration in several animal models of inflammation (Han et al. 2004, Uchimura et al. 2000). Among pro-inflammatory proteins MIP-2 and TNF-α are over-expressed after whole body rodent irradiation (Kyrkanides et al. 2002, Weichselbaum et al. 2002). MIP-2 secretion is enhanced by inflammatory stimuli and radiation leads to an inflammatory damage with consequent effects in leucocyte recruitment and consequent inflammatory patways activation. Main inflammatory pathways include the ingestion of apoptotic cells by macrophages which lead to the production of MIP-2 which is a potent chemoattractants for neutrophils. In our model there was a successive elevation of MIP-2 levels up to 24 hours after which the irradiated groups generally maintained their levels whilst the levels in sham, following only surgical trauma, dropped at 48 hours. Previous investigations focused on the increase of leucocyte intravascular rolling and adhesion after radiation with resultant inflammatory infiltrate composed mainly of lymphocytes has been observed in the lamina propria of intestinal villi (Richter et al. 1997). The CD45 antigen family is expressed on the membranes of all leucocytes (haematopoetic cells). Its role in membrane signal transduction and lymphocyte activation is well defined and allows a specific function as marker of B and T cells which is suitable for immunohistochemical study as done in our analysis. CD 45 values show a sharp increase of in all the groups at 2 hours, in the early phase of acute RI, in a dose dependent manner, showed by the significant increase compared to sham in the 38 Gy group. The values of CD45 then fell again at 24 hours, maintaining the same level of staining up to 48 hours, with the irradiated groups showing a reduction to less than half of their values at 2 hours, probably in relation to the concomitant increase of apoptosis which probably affect leucocytes. There is evidence in our data that radiation therefore tends to reduce the number of active CD45 bearing leucocytes/lymphocytes locally in the inflammatory infiltrate of the irradiated tissue. On the other hand the total leucocyte count in this study did not show any significant changes in the different groups. This may be explained by the fact that radiation was properly locally administered without bone marrow or systemic involvement, leaving systemic leucocyte production mainly unaffected.

The other fundamental scar of the acute RI is the increase in apoptosis, with a dose related trend which is associated to the granulocyte exudative inflammation mostly seen in the crypts. Some of the changes observed could most possibly be the pre-stadium to crypt abscess formation. Active caspase-3 immunostaining, specific for the late phase of the apoptotic process, in our observation was functional to describe this aspect of RI as previously experienced by other authors in an in vivo γ-radiated animal model at the crypt level (Marsham et al. 2001). This phenomenon is clearly observed in our experiment and is explained by the fact that proliferating crypt cells are more susceptible to radiation-induced apoptosis as opposed to already differentiated villi epithelial cells (Ramachandran et al. 2000). Our experimental results showed caspase expression from the 2 hour time point increasing to maximum expression at 48 hours after both 19 and 38 Gy irradiation. A significant expression compared to sham at this time point. This trend corresponds to results of earlier in vivo studies (Labejof et al. 2002). This increase in the levels of apoptosis is not associated in our setting to high level of TNF-α which is often found after

general inflammatory stimulus and radiation and it has been shown to be one of the most important inducers of the apoptotic process (Ramachandran et al. 2000). This suggests that either the local effect of radiotherapy probably was not enough to affect a noticable stimulus for TNF-α production or that probably wrong timing and/or incorporation of a less sensitive method of analysis could be an explanation of this finding. No significant difference was either observed in IL-10 expression as antinflammatory cytokine within our groups. This study defined some of the inflammatory features of the acute RI in a in vivo model and confirmed the validity of our model for such type of study.

The relation between radiation and antibiotics to microbiological and matrilysin changes in the murine small bowel

Radiation has been shown to both change the bacterial environment (Johnson et al. 2004) as well as up-regulate matrilysin in rectal cancer (Kumar et al. 2002). Matrilysin has an important role in the differentiated function of epithelia especially as mucosal barrier (Wilson et al. 1999). The epithelial barrier function in the gut is disturbed following radiotherapy (Nejdfors et al. 2000). Matrilysin is also involved in the secretion of the precursors of α-defensins, antimicrobial peptides named cryptidins (Parks et al. 2001, Lopez-Boado et al. 2000, Ayabe et al. 2000). Not only radiotherapy or antibiotics alone, but the combination of both preoperative radiotherapy and antibiotics leads to marked increases in MMP-7 expression in the rodent intestine. The up-regulation of MMP-7 observed after antibiotic prophylaxis might probably be due to stimulus trigged off by the imbalance of bacterial flora in favour of more potentially pathogenic flora. The fluctuations in bacterial microflora after irradiation alone in our study compare with results obtained in an earlier study by our group using a similar 19 Gy dose (Johnson et al. 2004). As here, there is a rise and fall in enterobacteriaceae bacterial counts with time. Further, similar results are observed in this study with a tendency to increasing bacterial counts with time, with regards to aerobes and anaerobes just as no significant changes are observed in levels of the lactobacilli 24 h after irradiation. We also find that as in the previous study that most bacterial counts peak within 24 h. This study confirms this by showing a clear fall in counts 48 h after irradiation. The preoperative prophylactic antibiotic regimen given in this study resulted in general suppression of lactobacilli after laparotomy devoid of radiotherapy. Lactobacilli seem thus to be more resistant to radiotherapy than to antibiotic therapy. However, at the late time point, 48 h after irradiation, all bacterial counts are markedly reduced after having been previously exposed to antibiotics. This could imply an over- riding effect of the antibiotics in the late stage over radiotherapy.

Enterobacteriaceae, were the only flora to rise at the early time points. The effect being further bolstered after increases in anaerobes and aerobes at later time points. We observed an increased expression of matrilysin at 24 h after radiation, with detectable levels up to 48 h which corresponds (at protein level, regarding time-points) to that previously observed in a human in vitro model after bacterial exposition (Lopez-Boado et al. 2000).

Our study is unique as it examines the direct effect of antibiotics (ampicillin) on matrilysin expression. MMP expression may be due to different modes of regulation and activation of the different MMP groups that, as previously showed for the mouse gelatinases (MMP-2 and 9), react differently to antibiotics exposure (Ueberham et al. 2003).Matrilysin is quite sensitive to microbial changes. In this case, we suspect a stimulation of matrilysin by the presence of the potentially pathogenic enterobacteriaceae. The imbalance of the intestinal microflora affected by antibiotic therapy thereby leading to a stimulation of matrlysin production to similar levels observed after radiotherapy.

TGF-β is a fibrogenic cytokine that induces collagen synthesis and linked to radiation fibrosis. TGF-β levels in our study show trends similar to the results of Ueberham et al. (2003). Mourelle et al. (1998), and Saha et al. (2007) show a down-regulation of TGF-β after antibiotic/antimicrobial therapy only. We also found decreasing levels of TGF- β with time, in small bowel tissue after antibiotic treatment alone. However, the combination of both radiotherapy regimes and antibiotics led to a short dip in both radiotherapy treatment regimens at 24 h, thereafter significant increases reaching initial levels noted at 48 h compared with antibiotics alone. Earlier studies by our group (Johnson et al. 2006) and of other authors (Fukuchi et al. 1999) show that a combination of two therapies (radiotherapy and surgery, and chemotherapy and surgery, respectively) leads to depression of TGF- β in the first postoperative week followed by an up-regulation in the late period. Here antibiotics and surgery lead to a continued dip in TGF-β at 48 h, however, the three therapies here (antibiotics and radio- therapy and surgery) all lead to a short lived depression of TGF-β. This phenomenon will have to be studied more closely. Studies have showed areas of active histopathologic RI as in radiation enteritis with increased immunoreactivity of TGF-β and collagen in the intestinal wall.

Antibiotics alone in our study led to MIP-2 peaking at 24 h corresponding to what has been earlier reported (Armstrong et al. 2004) after surgical injury. Antibiotics and radiotherapy together lead to a successive increase in MIP-2 levels. In our model, preoperative antibiotics seem to enhance an early inflammatory response of MIP-2 to surgical injury within the first 24 hours, whilst the addition of radiotherapy leads to an up-regulation of MIP-2.

Our results indicate that inhibition of MMP-7 or reduction in radiosensitivity in radiotherapeutic regimes can not be achieved by regulation of the microflora with the antibiotic treatment. In light of the above results an interesting correlation between an antibiotic and MMP-7 is observed but further studies are needed to validate this. It will be of clinical importance to study the regulation of the remodelling process of the ECM with radiation associated fibrosis and tumour progression.

Different expression of MMP-7 is related to different stages of colorectal cancer

The significance of MMP-7 elevation in resected specimens and in serum for the definition of oncological risk and prognosis for patients is still not clearly defined. In cancer immunology, a clear role of MMP-7 and other MMPs has been shown for tumour growth, invasion and spread (Declerck et al. 1992, Mori et al. 1995). Tumour specimens in our study showed significantly higher levels of MMP-7 in adenocarcinoma compared to varying grades of dysplasia ($p<0.001$). Even though the level of MMP-7 in those with disseminated disease was less than in those with stage I and II cancer, it was nevertheless still significantly higher than that in adenomas ($p<0.001$). The explaination of this non progressive trend of matrilysin expression in tumoral tissue could be related to the evidence that neoplastic infiltration is associated with degradation of elastin, laminin, proteoglycans, osteopontin, fibronectin and type IV collagen which is mediated by MMP-7 and this patway could be more active in stage I and II compared to already invasive tumors.Furthermore MMP-7 was shown to be overexpressed after radiation compared to preoperative levels in patients with rectal cancer (Kumar et al. 2002) and it was generally confirmed by a previous study from our group showing, in an in vivo animal model, the correlation of MMP-7 expression and irradiation and that this radiological trauma was not modulated by microfloral regulation when antibiotics were administred (Polistena et al. 2011). Similarly Western blot showed a significant progressive increase in lymph node MMP-7 with increasing dysplasia and infiltrative disease stage. MMP-7 levels were lower in lymph nodes compared to tumour tissue, but here again, we found successive significant increases compared to adenoma through stage I and II disease to locally advanced cancer. Interesting results presented in a study by Ichikawa et al. who studied MMP-7 expression by RT-PCR in lymph nodes from patients with colon cancer showed that its expression increased accuracy in diagnosis compared to ordinary histology (1996). MMP-7 detected in adenocarcinoma (by RT-PCR) was associated with over 90% of histologically-positive cancer, whereas 30% of lymph nodes primarily defined as negative at histology were found to be positive for MMP-7 RNA (Ohlsson et al. 2006).

This MMP-7 analysis again suggests its importance as a potential clinical biomarker of MMP-7 and this is based on the evidence that increased MMP-7 expression in serum in multivariate analysis generally correlates with worse prognosis, local invasiveness, a tendency for metastatic disease and with reduced overall survival (Waas et al. 2002, Polistena et al. 2011, Maurel et al. 2007). In colorectal cancer, levels of MMP-9 (which is activated by MMP-7) investigated in peripheral and portal blood showed increased levels and correlated with advanced and metastatic disease stage (Ishida et al. 2003, Ichikawa et al. 1996). Our serum ELISA results showed a clearly significant increase of MMP-7 in locally advanced disease over adenoma and stage I and II cancer. Serum concentration of MMP-7 showed no significant differences between adenoma and non-invasive cancer, but within the two cancer groups, there was again a significant increase in MMP-7 from non-metastatic to metastatic disease. Our study, did not use the more sensitive analysis

of RT-PCR, but nevertheless shows that with simpler cost-benefit methods, similar results or trends can be observed when examining tumor behaviour with resected specimens. The complexity of cancer warrants combinations of different analyses in order to achieve better prognostic goals. Therefore in the analyses of lymph nodes we can confirm that application of ELISA and western blotting is a good alternative and reliable compared to RT-PCR in staging for advanced cancer. Furthermore, immunohistochemistry seems to be a good complement.

Matrilysin expression following different radiotherapeutic regimens in radiated rectal cancer patients

Due to the strict correlation between surgery and radiotherapy in current multimodal management of rectal cancer, the effect and the consequent possible modulation of radiation induced MMP-7 over-expression, remains a current field of investigation which is still far from explaning these mechanisms due to the complex features and the several molecules and pathways involved. The effect of radiotherapy on matrilysin gene in human rectal cancer in vivo was first investigated by Kumar et al. (2002) who observed an over expression of MMP-7 in radiated rectal cancer tissue compared to normal irradiated rectal tissue. The over-expression of MMP-7 after radiotherapy in neoadjuvant and/or adjuvant radiotherapy, given the role of matrilysin in abnormal tissue remodelling after radiotherapeutic injury (Kumar et al. 2002, Waas et al. 2002, Martinez-Fernandez et al. 2009) and its relation to the progression and metastatic spreading of colorectal cancer leads to the rationale of investigating potential preventive or therapeutically use of specific tissue inhibitors to MMP`s (TIMP) in rectal cancer treatment with the intent of containing the increased morbidity mediated by metalloproteinases (Kumar et al. 2002). Although there is evidence of the effects of short term radiotherapy on MMP-7 expression, to our knowledge there are no previous publications comparing in rectal cancer patients the effects of short and long course RT on MMP-7 expression.

For this reason the present study must be considered as a pilot investigation on the modulation of different radiotherapeutic treatments in this setting. Due to the low number of patients enrolled in the different groups of examined patients further studies are needed to validate our observations. Radiotherapy alone, surgery alone or both in combination, clearly affect MMP-7 expression, acting as a consequence on ECM remodelling. Significantly higher values of MMP-7 were evident after surgery even in non irradiated patients. Radiotherapy instead affects MMP-7 expression in different grade depending on the radiation dose delivered. In the 50 Gy irradiated cases the effect of RT itself seemed not to influence MMP-7 expression, analyzed before operation compared to level of protein observed at baseline, being evident a tendency to decrease its concentration. This effect might be ascribed to the severe effects of elevated cumulative dosage. Only after surgery did these 50 Gy irradiated patients present with significantly higher values of MMP-7 comparable to the 25 Gy group which had already, after radiotherapy alone, a significant increase in matrilysin expression before surgery. These values showed a further significant increase

after the operation, showing the combined effects of both surgery and radiotherapy. The explanation of this picture after higher doses of radiotherapy (50 Gy) might be ascribed to the waiting time after sample collection since patients, according to the neoadjuvant protocol, undergo surgery four to six weeks after the end of the radiotherapy which is a sufficient time for the effects of the over-expression induced to be phased out. Another possible explanation might be the severe effects of high cumulative dosage radiotherapy with persistent oedema diluting the concentration of MMP-7 or probably due to necrosis with loss of tumoral cells and consequently of their protein products including matrilysin. Surgery itself determinates MMP-7 increase which might affect the tumor growth and spreading, but it is not possible to eliminate the surgical trauma in the treatment of rectal cancer. The more relevant experimental information we can consider on a clinical point of view is that according to our results, radiotherapy instead affects differently MMP-7 expression in relation to the long or short course radiation treatment used.

This preliminary observation might support a more favourable use of long course therapy, for its limited effect on MMP-7 expression, which might act in containing tumour progression during radiotherapy. The limited effect on normal mucosa of both surgery and radiotherapy in our setting confirms that previously shown by other authors and in a previous publication from our group attesting a progressive expression of MMP-7 from normal mucosa to cancer through different grades of dysplasia. TGF-beta participates in the remodelling of the ECM, but has many other functions such as suppression of the immune system and regulation of cell growth. It was shown also to act both as an inhibitor of tumour growth and as a promoter of tumour progression. The activation of TGF-beta can be elicited by endogenous agents, for example plasmin or MMP-9 but also exogenous factors such as irradiation and radiation-activated TGF-beta may be involved in the mechanisms behind fibrosis. However some studies have found a decrease or no effect on the TGF-β levels after radiotherapy.

An homogenous trend was observed in the expression of TGF-β, as a marker of inflammation and fibrosis, in tumour before and after treatment compared to baseline. In the three groups no significant increase of TGF-β was observed after 25 and 50 Gy radiotherapy just before surgery. TGF-β showed however significant 2 to 3 fold increases only after surgery, having its maximum increase in the 25 Gy radiated group. A previous investigation from our group showed that radiotherapy and surgery induce depression of TGF-β in rats in the first postoperative week followed by an up-regulation in the late period (35, 37). Similarly other authors showed lower active TGF-β levels in rectal cancer irradiated patients both in tumour tissue and rectal mucosa (35). Evidence points toward a radiation induced activation of latent TGF-β but this activation may only be seen in a limited window time, most probably in the later effect of TGF-β induced fibrosis which may explain why in our study as previously similarly observed (35) it was not seen at the time of the biopsy after termination of radiotherapy.

Conclusions

- Early local radiation-induced tissue injury of small bowel in this refined rodent model shows a progressive, infiltration of inflammatory cells into tissues probably mediated by the release of MIP-2
- A radiation dose related increase of apoptosis is observed.
- Pretreatment with ampicillin in combination with radiotherapy leads to an over-expression of MMP-7 similar to that achieved by either radiation or antibiotics alone.
- Radiotherapy tends to override the effect of antibiotics and leads to an up-regulation of MMP-7 and TGF-β and MIP-2 expression between 24 h and 48 hours.
- Antibiotics alone have a suppressive effect on TGF-β.
- Inhibition of MMP-7 can not be achieved by regulation of the microflora with antibiotic regimen.
- Significantly increased concentrations of MMP-7 in tumor tissues, lymph nodes and in serum is associated with increasing grade of dysplasia and adenocarcinoma infiltration.
- Increased MMP-7 expression in tumour tissue, in serum, lymph nodes is observed in advanced colorectal cancer disease.
- There is a correlation between MMP-7 expression and the risk of lymph nodal involvement.
- High dose (50 Gy) radiotherapy administered preoperatively induces significantly less MMP-7 over-expression compared to short term 25 Gy irradiation at surgery.
- Surgery itself has an overriding effect of up regulation of MMP-7.
- TGF- β showed however significant 2 to 3 fold increases only after surgery, having its maximum increase in the 25 Gy radiated group.

Future prospectives

- Further studies regarding collagen deposition and ECM remodelling of MMPs mediated in human rectum undergoing radiotherapy need to be investigated.
- Clinical use of matrilysin inhibitors as a useful preventive or therapeutic adjunct to radiotherapy in rectal cancer has to be investigated.
- The expression of MMP-7 in chronic inflammatory diseases as well as the expression in dysplatic polips has to be further investigated.

Swedish Summary

Bakgrund

Strålbehandling

Strålning används för behandling av bl a ändtarmstarmcancer för att minska tumörens storlek och göra den tillgänglig för kirurgi samt för att minska risken för återfall. Strålning påverkar dock kringliggande organ och vävnader, t ex tunntarm som är strålkänslig, på ett ogynnsamt sätt p g a en inflammatorisk reaktion. Denna inflammation leder till en utsvämning av inflammatoriska signalproteiner vilken är reglerad av bl a en familj av speciella proteiner, MMP (matrixmetalloproteinaser), i vilken matrilysin/MMP-7 är den minsta medlemmen. Den har visats ha en bakteriedödande effekt och deltar i immunförsvaret samt spelar en roll vid sårläkningen. Tidigare studier har visat förhöjda nivåer av MMP-7 vid inflammatoriska tillstånd samt hos korttids strålbehandlade patienter med ändtarmscancer. Samtidigt har man beskrivit sämre sjukdomsprognos hos patienter som har en högre MMP-7-nivå i blod, lymfkörtlar och tumörvävnad.

Målsättning

I. Att utvärdera den lokala effekten av strålbehandling på tunntarm i en musmodell.

II. Att undersöka påverkan av strålning på bakterieflora och MMP-7 i tunntarm hos mus efter förebyggande antibiotikabehandling.

III. Att undersöka MMP-7-nivåer hos icke strålbehandlade patienter med tjock- och ändtarmscancer i olika stadier.

IV. Att studera MMP-7-nivåer hos korttids- respektive långtidsstrålbehandlade patienter med ändtarmscancer.

Delarbete I: Inflammation och celldöd som markörer för strålskada på tunntarm i en musmodell

Denna studie visar att strålpåverkan på tarm leder till förhöjda nivåer av inflammatoriska proteiner såsom MIP-2 (Macrophage Inflammatory Protein 2) och ökande celldöd med ökande stråldos vilket är i överensstämmelse med tidigare studier som visar ökad produktion av MIP-2 , ett protein som drar till sig vita blodkroppar för

att bekämpa inflammationen. Studien påvisar också en ökad celldöd i senare skeden efter 19 Gy respektive 38 Gy strålning.

Delarbete II: Samspelet mellan strålbehandling, antibiotikabehandling och matrilysinnivåer i tunntarm i en musmodell

Kombinationen av strålbehandling och antibiotikabehandling leder till en ökad produktion av matrilysin. Lactobacilli var mer motståndskraftiga mot strålning än mot antibiotika. Bakterienivåerna i tarmen hos mus som erhållit både antibiotika och strålning var kvarstående låga i senare stadier. Nivåerna av TGF-β, ett signalprotein som är förknippat med bindvävsutveckling, var låga efter enbart antibiotikabehandling. Våra resultat visar att även om bakterietillväxten hämmas så påverkas inte matrilysinnivåerna.

Delarbete III: MMP-7-nivåer varierar med olika stadier av tjock- och ändtarmscancer

Denna studie visar högre nivåer av matrilysin i cancertumörer jämfört med i polyper med enbart cellförändringar av varierande grad. Man såg också ökande matrilysinnivåer med ökande grad cellförändringar och ännu högre nivåer vid spridd cancersjukdom. Dock förelåg inga signifikanta skillnader i blod mellan polyper och tidiga cancerformer.

Delarbete IV: Varierande matrilysin-/MMP-7-nivåer hos korttids- respektive långtidsstrålade patienter med rektalcancer

Denna jämförelse av matrilysinnivåer vid två olika strålbehandlingar är unik. I den korttidsstrålade gruppen ökade MMP-7 redan vid avslutad strålning och kvarstod signifikant förhöjd t o m efter att kirurgin hade utförts. I den korttidsstrålade gruppen genomfördes kirurgin veckan efter avslutad strålbehandling medan i den långtidsstrålade gruppen genomfördes det kirurgiska ingreppet först 4-6 veckor efter avslutad strålbehandling. I den långtidsstrålade gruppen var MMP-7 förhöjd enbart efter kirurgin. Denna studie talar för att en långtidsstrålbehandling skulle kunna vara mera gynnsam för patienten, sett till nivåer av MMP-7, där lägre nivåer tyder på mindre tendens till spridning.

Sammanfattning

- Ökad nivå av MIP-2 leder till ökad förekomst av inflammatoriska celler efter strålskada på tunntarm hos mus.
- Ökande stråldos leder till ökad celldöd.
- Förebyggande antibiotikabehandling i kombination med strålning leder till ökad MMP-7-produktion på likartat sätt som uppnås efter enbart endera strålning eller antibiotikabehandling.

- Hämning av MMP-7 kan inte uppnås genom reglering av bakteriefloran via antibiotikabehandling.
- Vid avancerad tjocktarms- och ändtarmscancersjukdom ses ökade nivåer av MMP-7 i blod, lymfkörtlar och tumörvävnad.
- Det föreligger ett samband mellan MMP-7-nivåer och risken för lymfkörtelengagemang.
- Långtidsstrålbehandling (50 Gy) leder till mindre uttalad höjning av MMP-7-nivåer vid kirurgi än korttidsstrålbehandling (25 Gy) gör.
- Kirurgi enbart leder till ökad produktion av MMP-7.

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Local radiotherapy of exposed murine small bowel: Apoptosis and inflammation

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Abstract

Background: Preoperative radiotherapy of the pelvic abdomen presents with complications mostly affecting the small bowel. The aim of this study was to define the features of early radiation-induced injury on small bowel.

Methods: 54 mice were divided into two groups (36 irradiated and 18 sham irradiated). Animals were placed on a special frame and (in the radiated group) the exteriorized segment of ileum was subjected to a single absorbed dose of 19 or 38 Gy radiation using 6 MV high energy photons. Specimens were collected for histology, immunohistochemistry (IHC) and ELISA analysis after 2, 24 and 48 hours. Venous blood was collected for systemic leucocyte count in a Burker chamber.

Results: Histology demonstrated progressive infiltration of inflammatory cells with cryptitis and increased apoptosis. MIP-2 (macrophage inflammatory protein) concentration was significantly increased in irradiated animals up to 48 hours. No significant differences were observed in IL-10 (interleukin) and TNF-α (tumour necrosis factor) levels. IHC with CD45 showed a significant increase at 2 hours of infiltrating leucocytes and lymphocytes after irradiation followed by progressive decrease with time. Caspase-3 expression increased significantly in a dose dependent trend in both irradiated groups up to 48 hours.

Conclusion: Acute small bowel injury caused by local irradiation is characterised by increased apoptosis of crypt epithelial cells and by lymphocyte infiltration of the underlying tissue. The severity of histological changes tends to be dose dependent and may affect the course of tissue damage.

Background

Radiotherapy is used in the multimodal treatment for neoplastic diseases in the pelvic abdomen [1-4]. Radiation induces injury to rapidly dividing tissues and in the pelvic region small bowel is usually affected. Acute complications consist of nausea, vomiting, abdominal pain, diarrhea, gastro-intestinal haemorrhage and bacterial infection. Chronic damage is presented as radiation enteritis, ulcerations, fibrosis, stricture formation, fistulae, malabsorption and dysmotility [5-8]. Features of radiation injury at early time points can be detected mainly by histological assesment although more sensitive evaluation can be assessed by immunohistochemistry [9].

Several animal models have been used to investigate radiation-induced damage of the small bowel. Two major murine models have been described: total body or topic abdomino-pelvic irradiation [5,10,11] and selective segmental irradiation [12,13] of an exposed portion of the small bowel. Our model is a refinement of the latter model where small bowel segments can be subjected to irradiation while minimizing secondary radiation effects to other organs, lymphoid tissue and bone marrow. Local effects of subsequent radiation injury with limited effects of immunological involvement can be more accurately assessed. The aim of this study was to define the features of early, local radiation-induced damage to small bowel, focusing on inflammatory changes and apoptosis.

Methods

Animals

Male C57B1/6J mice (weight 22–26 g, supplied by Taconic, Denmark) were kept under standard laboratory conditions with a 12 hour light and 12 hour dark cycle, and were allowed free access to animal food (Lactamin, Sweden) and tap water *ad libitum*. All experiments were performed in accordance with legislation on the protection of animals and were approved by the Regional Ethic's Committee for Animal Experimentation at Lund University, Sweden.

Anesthetic and surgical procedures

The mice were anesthesized with 7.5 mg Ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg Xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100 g body weight by intraperitoneal (*i.p.*) injection. The animals were placed in supine position on a heating pad (37°C) for maintenance of body temperature. A short midline abdominal incision (1.0–1.5 cm) was performed and a 5 cm segment of ileum located 5 cm from the ileocaecal valve was exteriorized and marked with 5-0 non-absorbable sutures. Any other visible prolapsed abdominal content was replaced back into the abdomen and the animal was placed on a specially designed frame [14] with the loop of intestine fixed between two perspex sheets. After irradiation the intestine was replaced in the abdomen and the incision closed with a polypropylene suture. To reduce water losses exposed bowels were covered with moist swabs, before and after radiotherapy.

Irradiation protocol

54 animals were divided in two main groups: radiated mice (n = 36) and sham radiated (n = 18). All animals underwent laparotomy for exposure of the intestinal segment that was analysed. The exposed ileum was subjected to a single high absorbed dose radiation of either 19 or 38 Gy and the mouse protected by an 8 cm thick lead metal shield. The irradiations were undertaken using a clinical linear accelerator (Varian Clinac 2100C) and in normal room temperature. The method has been described elsewhere [14]. Briefly, the exteriorized mouse intestine was positioned between 1.5 cm thick perspex slabs (10 × 15 cm^2) to sufficiently reduce secondary radiation scatter and thereby accomplish a reproducible and homogenous dose distribution. The (adjustable) spacing between the slabs was just sufficient to support the exteriorized mouse intestine (maximum 0.5 cm). The absorbed dose was verified with independent measurements and was found to be within 5% throughout the intended volume using this technique. The use of an asymmetrically half blocked 6 MV beam and extra lead shielding assured that the treatment field perfectly fitted the exteriorized intestine while the remaining mouse body was kept outside the radiation beam. The absorbed dose rate was 3.2 Gy/minute and consequently the irradiation time for each animal was approximately 6 minutes for 19 Gy (n = 18) and 12 minutes for 38 Gy (n = 18). An absorbed dose of 19 Gy delivered to the intestine is known to cause consistent structural, cellular, and molecular changes [15]. The sham operated mice underwent the same surgical procedure except irradiation. Exposure time from surgery, through irradiation to wound closure was kept at a minimum in order to reduce stress and trauma levels, the whole procedure taking approximately 15 minutes.

Collection of samples

Specimens were collected for the 3 different time points (2, 24 and 48 hours). At each timepoint 3 groups (19 Gy, 38 Gy and Sham) consisting of 6 animals per group were studied. A second laparotomy was performed; the marked bowel segment was exteriorized and excised. It was then divided into segments of 0.5–1.0 cm lengths which were weighed and stored for histology, immunohistochemistry and cytokine analysis.

Histological study

Samples were collected at different time points after irradiation. The marked irradiated bowel segment was excised and rinsed with normal saline. The samples were fixed in 4% buffered paraformaldehyde, dehydrated in graded

ethanol and embedded in paraffin. The sections were cut on a microtome and mounted on glass slides. The slides were stained with hematoxylin and eosin for histological evaluation under light microscopy, which was done by the pathologist in a blinded fashion.

Cytokines

Tissue samples were washed in PBS containing penicillin, streptomicin and fungizon (100 U/ml) and kept cool in cold serum-free medium (DMEM). Bowel was incubated in 1 ml of DMEM solution 10% FCS and penicillin, streptomycin for 24 hours (37°C) in a 12-wells plate [16]. The cultured medium was harvested and stored in -20°C until analysis of MIP-2 (Macrophage inflammatory protein-2), TNF-α (Tumour necrosis factor-α) and IL-10 (Interleukin-10) using a colorimetric sandwich ELISA kit with recombinant murine proteins as standard (Quantikine, R&D Systems, Europe). The minimal detectable protein concentration in these kits is < 1.5 pg/ml for MIP-2, <5.1 pg/ml for TNF-α and < 4.0 pg/ml for IL-10.

Immunohistochemistry

For immunohistochemistry, standard avidin-biotin procedures for mouse CD45 (defined as leucocyte common antigen) [17-20] and for active caspase-3 were used. After deparaffinization and washing in phosphate buffered solution (PBS), endogenous peroxidase activity was blocked by incubating the sliced sections in 3% hydrogen peroxide in PBS for 10 minutes. For CD45 analysis the slides were fixed with BD Retrievagen fixative A (BD Biosciences, Europe) and then incubated overnight at 60°C. Slides for Caspase-3 were microwave-treated [21]. The sections were blocked in 5% fetal calf serum with PBS for 30 minutes and then incubated for 1 hour at room temperature with 1:100 anti mouse CD45 monoclonal antibody (RnD Systems Inc., Minneapolis, USA, cat n° MAB114) and with 1:750 anti-active human-mouse caspase-3 affinity-purified rabbit antibody (RnD Systems Inc., Minneapolis, USA, cat n° AF835) respectively. Biotin-conjugated secondary antibody and streptavidin-conjugated horseradish peroxidase (DAKO, USA) were applied to sections for 45 minutes at room temperature, and developed using the 3,3'-diaminobenzidine (DAB) as substrate. Finally counterstaining with haematoxylin for both CD45 and caspase was performed. Sections were mounted and leucocyte and apoptotic cell counts were determined on randomly selected areas using the point counting technique by a blinded observer. Each slide contains 3 sections and from each section we choose randomly 3 areas under light microscopy with a high power field ×100. We then take the mean value of all the counts in each slide.

Statistical analysis

Sham and radiated groups were statistically analysed by one-way analysis of variance (ANOVA) with a post hoc Turkey test for all pairwise multiple comparison procedures. Non-parametrically distributed groups were studied by the Kruskal-Wallis analysis of variance on ranks. A probability (P) value ≤ 0.05 was accepted as significant. Differences were expressed as mean values ± SEM.

Results

All animals survived the operation and radiotherapy sessions

Histology

At the normal controls: No inflammatory changes were observed 2 hours after surgery. There was similarly no major difference at 24 and 48 hours except for the presence of some granulocytes and a few apoptotic cells in the crypts. At 19 Gy: normal cell structure was kept at 2 hours, apart from some dilated peripheral vessels, indicating hyperaemia. At 24 hours some lymphocytes, dilated vessels and some apoptotic cells were present in the crypts. At 48 hours, signs of hyperaemia, a further increase in apoptosis and mitosis (active cells) as well as an increase in granulocytes in the epithelium (Figure 1), lamina propria and subserosa was observed. At 38 Gy: an increase in the inflammatory infiltrate was observed. At 2 hours some inflammatory cells (granulocytes) and few apoptotic cells together with other degerative epithelial cells were observed. At 24 hours we found an increase in granulocytes, massive apoptosis and increased exudative inflammation in the lumen (between the villi and even in the crypts, suggesting cryptitis). The changes after 48 hrs were similar to those found at 24 hours but to a much lesser degree (Figure 2).

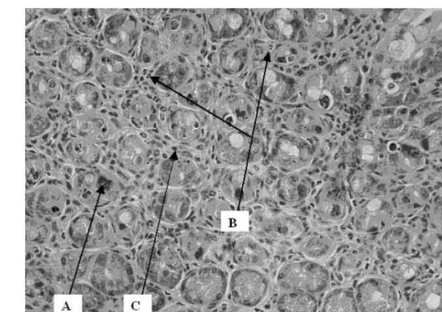

Figure 1
Cross section of intestinal wall 48 hrs after 19 Gy irradiation. An increase in apoptosis (A), intraepithelial granulocytes (B) and lymphocytes (C) was observed. The slides were stained with hematoxylin and eosin for histological evaluation under light microscopy, which was done by the pathologist in a blinded fashion.

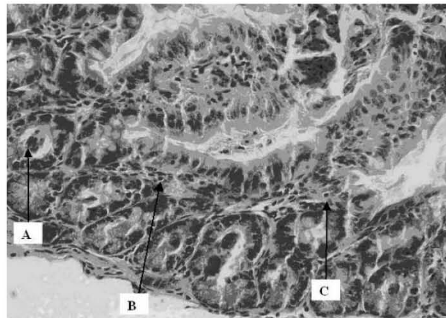

Figure 2
Cross section of intestinal wall 48 hrs after 38 Gy irradiation. An increase in apoptosis (A), intraepithelial granulocytes (B) and lymphocytes (C) was observed with degenerative epithelium and granulocyte exudate in the lumen (between the villi and even in the crypts, suggesting cryptitis). The slides were stained with hematoxylin and eosin for histological evaluation under light microscopy, which was done by the pathologist in a blinded fashion.

Caspase-3 Immunohistochemistry staining

Two hours after radiation there was a significant increase in caspase-3 positive cells in the 19 Gy group compared to sham and 38 Gy irradiated groups (Figure 3). 24 Hours after radiation there was no difference between the groups (Figure 3). At 48 hours there was a significant increase in both irradiated groups compared to the sham irradiated group (Figure 3).

CD 45 Immunohistochemistry staining

Two hours after irradiation there was an increase in CD45 positive cells in the irradiated group exposed to 38 Gy compared to the sham irradiated group (Figure 4). 24 hours after irradiation there was a significant decrease in both irradiated groups compared to sham irradiation (Figure 4). 48 hours after irradiation, there was a decrease in both irradiated groups with a significant difference in the 38 Gy group compared to both sham and 19 Gy irradiated groups.

Cytokines

Two hours after irradiation there was a significant increase in MIP-2 in 38 Gy group compared to 19 Gy group (Figure 5). 24 hours after irradiation there was no difference between the groups. 48 hours after irradiation, there was an increase in both irradiated groups with a significant difference in the 19 Gy group compared to sham irradiated group. When comparing the different time points in each treatment group, we found difference in the sham and 19 Gy groups while there was no difference within the 38 Gy groups. MIP-2 activity showed the significantly highest values in the sham group at 24 hours followed by a significant decrease after 48 hours. In the 19 Gy groups higher values were observed at 24 and 48 hours compared to 2 hours values. TNF-α and IL-10 levels did not exhibit any significant changes in neither sham nor irradiated groups.

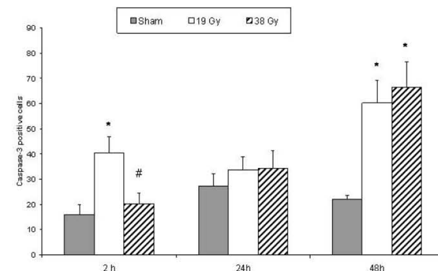

Figure 3
Caspase-3 positive stained cells in small bowel tissue comparing sham and different doses of radiation within each time point. * denotes $p < 0.05$ compared to sham group, # denotes $p < 0.05$ compared to 19 Gy group.

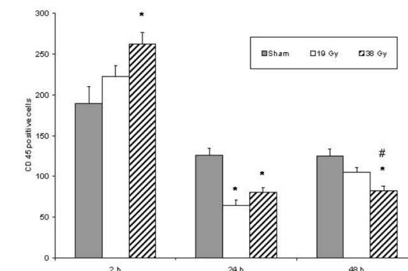

Figure 4
CD45 positive stained cells in small bowel tissue comparing sham and different doses of radiation within each time point. * denotes $p < 0.05$ compared to sham group, # denotes $p < 0.05$ compared to 19 Gy group.

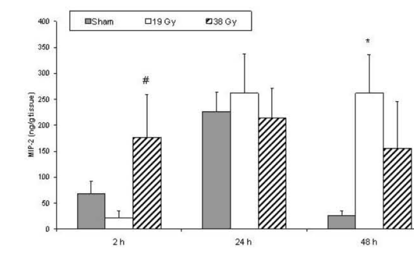

Figure 5
MIP-2 concentration in small bowel tissue comparing sham and different doses of radiation within each time point. * denotes $p < 0.05$ compared to sham group, # denotes $p < 0.05$ compared to 19 Gy group.

Discussion

The local effect of radiotherapy on an exposed segment of small bowel includes early increases in MIP-2 levels, a decrease in CD 45 positive cells over time and increased dose dependent apoptosis. Many studies show a general over-expression of pro-inflammatory proteins such as MIP-2 and TNF-α after whole body rodent irradiation [22,23]. Macrophage inflammatory protein-2 (MIP-2) is a CXC chemokine and its secretion enhanced by inflammatory stimuli. There is evidence that MIP-2 is one of the major chemokines that lead to neutrophil recruitment and infiltration in several animal models of inflammation [24,25]. Some of the main pathways include the ingestion of apoptotic cells by macrophages which lead to the production of MIP-2 which is a potent chemoattractants for neutrophils. Recent reports indicate that MIP-2 is also regulated by oxygen radicals [21]. In our model there was a successive elevation of all MIP-2 levels up to 24 hours after which the irradiated groups generally maintained their levels whilst the sham levels dropped at 48 hours. 19 Gy irradiation being significantly raised compared to sham treatment at this time point.

A major problem in planning radiotherapy is acheiving an optimal radiation schedule, balancing dose, fractionation and exposure time for maximum tumoricidal effect. At the same time there must be protection against injury to healthy surrounding tissues. In radiotherapy of the abdominopelvic region the small bowel is frequently injured and the severity of mucosal damage depends on the balance between epithelial denudation and proliferation. When the rate at which surviving epithelial cells killed exceeds the maximum rate at which new cells are replaced the injury is more evident. The deeper the lesion is the slower the re-epithelization with loss of barrier function since the main damage is to the epithelial stem cells at the basement membrane zone.

An increased leucocyte intravascular rolling and adhesion after radiation with resultant inflammatory infiltrate, composed mainly of lymphocytes has been observed in the lamina propria of intestinal villi [9]. The CD45 antigen family is a group of high molecular weight glycoproteins expressed on the membranes of all leucocytes (haematopoetic cells). Its role in membrane signal transduction and lymphocyte activation is well defined and allows a specific function as marker of B and T cells. Our CD 45 values show a sharp increase of in all the groups at 2 hours but with the 38 Gy group significantly increased compared to sham. The values then fell again at 24 hours, with a reduction of the irradiated groups to less than half of their values at 2 hours. All levels continued to be maintained in generally the same way from 24 to 48 hours post-irradiation. Radiation thus tends to reduce the number of active CD45 bearing leucocytes/lymphocytes locally in the inflammatory infiltrate of the irradiated tissue. The total leucocyte count in this study did not show any significant changes in the different groups. This may be explained by the fact that radiation was locally administered without bone marrow or systemic involvement, leaving systemic leucocyte production mainly unaffected.

In the acute phase, increase in radiation dosage leads to increase in apoptosis and granulocyte exudative inflammation mostly seen in the crypts. Some of the changes observed could most possibly be the pre-stadium to crypt abscess formation. Active caspase-3 immunostaining is specific for the late phase of the apoptotic process. Immunostaining technique in an in vivo irradiated animal model showed a significant expression of caspase-3 activity in γ-radiated mice at the crypt level although no difference was noticed at the villus tip [26]. This phenomenon is clearly observed in our experiment and is explained by the fact that proliferating crypt cells are more susceptible to radiation-induced apoptosis as opposed to already differentiated villi epithelial cells [27]. Our experimental results showed caspase expression from the 2 hour time point increasing to maximum expression at 48 hours after both 19 and 38 Gy irradiation. A significant expression compared to sham at this time point. This trend corresponds to results of earlier in vivo studies [28].

A high level of TNF-α is often found after general inflammatory stimulus and radiation and it has been shown to be one of the most important inducers of the apoptotic process [27]. Nevertheless in our setting no detectable level of TNF-α could be measured, suggesting that either the local effect of radiotherapy was not enough to affect a noticable stimulus for TNF-α production or that probably

wrong timing and/or incorporation of a less sensitive method of analysis could be an explanation of this finding. No significant difference was either observed in IL-10 expression within our groups.

Conclusion

Early local radiation-induced tissue injury of small bowel in this refined rodent model shows a progressive, infiltration of inflammatory cells into tissues probably mediated by the release of MIP-2 and a dose related increase of apoptosis. High irradiation may lead to a shift from crypt apoptosis to cryptitis (the advanced forms being most probably a pre-stage to crypt abscess formation) and inevitably necrosis. This model has proven to be good at studying the early local effects of radiotherapy on the bowel and has the potential of even being a useful model for long term effect study.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

AP: Participated in the design of the study and performed experimental studies and drafted the manuscript.

LBJ: Designed the study and participated in construction of the chamber. Performed experimental studies and drafted the manuscript.

SOM: Performed experimental studies.

LW: Participated in the radiological design of the study, construction of the chamber and the implementation of radiotherapy.

SB: Participated in the radiological design of the study, chamber and the implementation of radiotherapy.

CT: Participated in the implementation of radiotherapy.

VC: Performed the histological analysis.

DA: Participated in the design of the study, performance of experimental studies, drafting the manuscript.

BJ: Conceived the design and participated in construction of the chamber. Co-ordination of the study as well as supervision and draft of the manuscript.

All authors have read and approved the final version of the manuscript.

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Matrilysin Expression Related to Radiation and Microflora Changes in Murine Bowel

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Background. **Matrilysin (MMP-7) elevation after radiotherapy is shown in humans. Matrilysin regulates certain cytokines and the production of bactericidal proteins when the mucosa is exposed to bacterial antigens. We investigate the effect of irradiation on matrilysin and microflora in murine bowel, after modulation with antibiotics.**

Methods. **Animals were divided into two different groups a radiation group (72 animals) and sham radiation group (36 animals). Animals were divided into smaller groups of six according to radiation dose (19 or 38 Gy or sham). Seven days before radiotherapy ampicillin 500 mg/kg/d was administered intramuscularly, in the antibiotic groups. An exteriorized segment of ileum was subjected to single high dose radiation (19 or 38 Gy). Samples were collected 2, 24, and 48 h and analyzed for microflora, MIP-2, TGF-β, and MMP-7.**

Results. **The combination of antibiotics and irradiation leads to an early significant reduction of bacteria, down-regulates MIP-2, up-regulates TGF-β and elevation of MMP-7 to levels achieved by antibiotics or irradiation alone. Lactobacilli were reduced to non-existent levels after antibiotics.**

Conclusions. **Pretreatment with Ampicillin before irradiation and laparotomy in a murine model leads to Matrilysin over-expression as achieved by radiotherapy alone. Microfloral regulation does not affect MMP-7 stimulation after surgical or radiological trauma. Radiotherapy overrides the effect of antibiotics leading to an up-regulation of MMP-7, TGF-β and MIP-2 expression between 24 h and 48 h.** © 2011 Elsevier Inc. All rights reserved.

Key Words: **MMP-7; Matrilysin; antibiotics; radiation; microflora; TGF-β; MIP-2.**

INTRODUCTION

Pelvic radiation therapy is almost invariably accompanied by acute intestinal inflammation and often followed by a progressive fibrosis months to years later. Since radiotherapy is increasingly used in the therapy of gynecologic cancer as well as cancer in the urinary bladder, prostate, and rectum, an increase in problems with adverse reactions from intestines can be anticipated. Up to 50% of patients are left with long-term chronic gastrointestinal side effects affecting quality of life [1]. Early response to radiation includes microvascular injury, endothelial and epithelial cell apoptosis as well as leukocyte and platelet recruitment. This is followed by an increased connective tissue deposition in the deeper layers of the bowel wall. These fibrotic changes will persist and cause problems with stricture formation and intestinal obstruction [2]. The degree of initial inflammation may influence later development of fibrosis.

Matrix metalloproteinases (MMPs) are a family of a Zn^{+2}-dependent proteolytic enzymes, involved in physiologic and pathologic remodeling of extracellular matrix in proliferation, angiogenesis, and wound healing [3]. Matrilysin (MMP-7) is expressed in blood monocytes, tissue macrophages of atherosclerotic lesions, and in normal glandular epithelial cells

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of different organs [4] where it plays an important role in mucosal barrier function. It is often expressed in epithelial tumor cells of various origins [4] and is believed to play a significant role in cancer invasion and spread [5]. Matrilysin has a broad substrate specificity including collagen, elastin, fibronectin, and proteoglycans [4]. Matrilysin is prominently upgraded in tissues with heavy bacterial load (eg, lungs in cystic fibrosis) [6]. Several experimental studies in murine models have shown the indirect bactericidal action of matrilysin in innate immunity by regulation of the level of antimicrobial peptides such as α and β defensins, produced by several cell types including phagocytes, Paneth cells, and other epithelial cells of the small intestine when the mucosal barrier is exposed to bacterial antigens [6–9].

In humans an overexpression of MMP-7 has been observed in radiated rectal cancer tissue [5] and in dermal connective tissue during long-term exposure to sunlight [4]. Several observations [10–13] indicate that bowel irradiation leads initially to a general decrease in intestinal microflora with the possibility of opportunistic germ selection and subsequent pathogenic effect on damaged mucosa, favoring infection. α-defensins (mouse cryptidins produced by Paneth cells) are broad-spectrum antimicrobial peptides that are activated intracellularly by matrilysin and are active against both Gram-negative (G-) and Gram positive (G+) bacteria [14]. α-defensins play a major role in the maintenance of intestinal immune homeostasis, first, by acting as innate antibacterial agents and, second, by regulating inflammatory cytokines. Gut microbes affect the radiosensitivity of endothelial cells and lymphocytes populating the mesenchyme of small intestinal villi. Mice with a normal microbiota have been shown to exhibit enhanced intestinal radiosensitivity with marked features of radiation enteritis and increased mortality after irradiation compared with germ-free mice [15].

Our hypothesis is that suppression of gut flora by administration of antibiotics could modulate the expression of matrilysin following radiotherapy thereby making the gut more resistant to radiation induced injury.

MATERIALS AND METHODS

Animals

Male C57Bl/6J mice weighing 22–26 g were kept under standard laboratory conditions maintained on a 12 h light and 12 h dark cycle and were allowed free access to animal chow and tap water *ad libitum*. All experimental procedures were performed in accordance with legislation on the protection of animals and were reviewed and approved by the Lund University Ethic's Committee for Animal Experimentation.

Anesthetic and surgical preparation

The mice were anesthetized with 7.5 mg ketamine hydrochloride (Hoffman-La Roche, Basel Switzerland) and 2.5 mg xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100 g body weight by intraperitoneal (i.p.) injection. The animals were placed in supine position on a heating pad (37°C) for maintenance of body temperature. A small midline incision (1.0–1.5cm) was performed and a 5 cm segment of ileum located 5 cm from the ileocaecal valve was exteriorized and marked with 5-0 nonabsorbable sutures. Any other visible prolapsed abdominal content was replaced back into the abdomen and the animal was placed on the specially designed frame/chamber, with the loop of intestine fixed between two perspex sheets. The exposed ileum was subjected to a single dose of 19 Gy or 38 Gy and thereafter replaced in the abdomen and the incision closed with a polypropylene suture.

Experimental Protocol

Animals were divided into two main groups; (R+) radiation and surgery group (72 animals) and (R-) sham radiation and surgery group (36 animals), which served as controls. The animals were further divided into smaller groups (six animals each) according to the dose of radiation (19 or 38 Gy) or sham radiation. During the 7 d before radiotherapy, a broad spectrum antibiotic (ampicillin 500 mg/kg/d) was administered intramuscularly (antibiotic groups). A 5 cm long exteriorized segment of ileum was subjected to single radiation dose (19 or 38 Gy). Samples were collected 2, 24, and 48 h after radiation or sham exposure for microfloral culture, ELISA analysis for MIP-2 and TGF-β, leucocyte counts, and MMP-7 expression *via* Western blotting. MMP levels were further quantified by densitometry.

Radiation

We standardized our method for surgery and irradiation so this was the same for all groups. Conditions and time factors during surgery/irradiation and sample collection were the same for all animals. The irradiations were undertaken using a linear accelerator for clinical use (Varian Clinac 2100C, Varian Medical Systems, Palo Alto, CA, USA). The exteriorized intestine was positioned between perspex slabs to accomplish sufficient secondary radiation scatter and thereby a reproducible and homogenous dose distribution. The absorbed dose was verified with independent measurements and was found to be within 5% throughout the intended volume using this technique. Using an asymmetrically half blocked 6 MV beam and extra lead shielding, the treatment field perfectly fitted the exteriorized intestine while the remaining body was kept outside the radiation beam. An absorbed dose of 19 or 38 Gy was delivered to the intestine as this dose causes consistent structural, cellular, and molecular changes [16]. The absorbed dose rate was 3.2 Gy/min, and consequently, the irradiation time for each animal was approximately 6 and 8 min for 19 and 38 Gy groups, respectively. During irradiation, the intestine in the chamber was protected from large temperature variations and trauma by perspex sheets. The exposure time from surgery, through irradiation to wound closure is kept at a minimum, taking approximately 15–17 min, thus keeping stress and trauma levels low.

Intestinal Microflora

Tissue samples from the irradiated small intestine were first placed in 5 mL of sterile transport medium [17]. Samples were then placed in an ultrasonic bath (Millipore, Sweden) for 5 min and then rotated on Chiltern (Terma-Glas, Gothenberg, Sweden) for 2 min. After a conventional dilution procedure, viable counts were obtained from brain heart infusion (BHI) that was incubated aerobically and anaerobically at 37°C for 72 h (aerobic and anaerobic bacterial count, respectively), and from Rogosa agar (Oxoid, Hampshire, England) that was

incubated anaerobically at 37°C for 72 h (lactobacilli counts). Viable counts were also obtained from violet red-bile-glucose agar (VRBD) (Oxoid, Hampshire, England) that was incubated aerobically at 37°C for 24 h (enterobacteriaceae counts).

Western Blotting for MMP-7

Western blotting was conducted using equivalent amounts of supernatant from tissue homogenates, which were loaded onto each lane of the 4% to 12% NuPAGE Bis-TrisGel (Invitrogen, Lidingö, Sweden) and were run under reducing conditions using an electrophoretic machine (XCell II mini-cell; NOVEX, Frankfurt am Main, Germany) powered by a 1000/500 units power supply (Bio-Rad, Sundbyberg, Sweden). Molecular multicolored standard (MultiMark, Invitrogen) and human recombinant MMP-7 (cat. no. 444270; Calbiochem) were included as double controls in MMP-7 detection. Proteins from the gel were then transferred to nitrocellulose membranes (Trans-Blot, Bio-Rad). Blots were blocked in blocking solution and then incubated respectively with a 1:500 dilution of rabbit anti-murine matrilysin polyclonal antibody (Ab) (cat. no. PC492; Calbiochem-Oncogene, via Merck, Darmstadt, Germany) and all kept in Ab buffer for 4 h at room temperature. Anti-MMP-7 antibody detects both human and murine protein in both the precursor (28 kD) and active (19 kD) forms of the protein. Blots were subsequently incubated after washing in tris(hydroxymethyl)methylamine with a 1:3000 dilution of a phosphatase conjugated goat anti-rabbit IgG (cat. no. 170-6518; Bio-Rad) in Ab buffer for 1 h. Blots were finally developed with a substrate reaction by adding a solution of 0.19 mg/mL NBT/BCIP stock solution (Roche, Diagnostics GmbH, Mannheim, Germany) in ultra pure water. After 10 to 20 min. the reaction was stopped by washing in H20. Semiquantitative measurement of blots was performed by densitometry analysis (GS-710 calibrate imaging densitometer; Bio-Rad) and software data evaluation (QUANTITIVE ONE: quantitation software; Bio-Rad). The densitometry values are expressed as percentage (%) of the control antigen.

Cytokines

Samples of small bowel were weighed and homogenized for 1 min in phosphate buffer. The homogenates were centrifuged at 10,000 g at 4°C for 5 min. MIP-2 and TGF-β concentrations in the supernatants were determined by ELISA using the commercially available Quantikine kit (R&D Systems, Minneapolis, MN). Optical densities were measured on an ELISA reader at a wavelength of 450 nm. Data were analyzed against the linear portion of the generated standard curve.

Systemic Leucocyte Counts

20 μL blood was mixed with Turk's solution (0.2 mg gentian violet in 1 mL glacial acetic acid, 6.25% v/v) in a 1:10 dilution. Leucocytes were counted and differentiated as polymorphonuclear (PMNL) or mononuclear (MNL) cells in a Burker chamber.

Statistical Analysis

Statistical evaluations were performed using the Kruskal-Wallis one way analysis of variance on ranks for unpaired samples (Dunn's *post hoc* test was used). For bacterial microflora in comparing two groups we used Mann-Whitney rank sum test, and for the comparison of the different time points within the radiated groups we used one-way analysis of variance (ANOVA) followed by multiple comparisons *versus* control group (Dunnett's method). For MIP-2 and TGF-β, we used for multiple comparison Kruskal-Wallis one-way ANOVA on ranks followed by all pairwise multiple comparison procedures (Student-Newman-Keuls Method). In comparing two groups, we used Mann-Whitney rank sum test. The results are presented as mean values $\pm$ SEM or median (25th–75th percentile) as appropriate. Differences were considered to be significant at $P < 0.05$.

RESULTS

There was no mortality among the experimental groups.

Intestinal Microflora

After both 19 Gy and 38 Gy irradiation, the enterobacteriaceae bacterial count was significantly reduced at 2 h, rising and peaking at 24 h. The aerobic and anaerobic flora followed a similar trend only when 38 Gy irradiation was given; otherwise levels were unchanged at the different time points after 19 Gy irradiation. There were no significant variations in the lactobacilli group with time with radiation alone (Table 1).

After pretreatment with antibiotics, there was an initial suppression of all microbes, followed by a successive rise in numbers with the exception of lactobacilli. Lactobacilli remained significantly suppressed at all time points monitored (ie, until 48 h) (Table 1). The combination of antibiotics and 19 Gy or 38 Gy irradiation led to a general decrease in microflora levels at the 2 h point. By 24 h, all groups had recovered and peak levels were recorded here for all groups. At 48 h postirradiation, microbial levels were decreasing; 38 Gy irradiation led, however, to further decreases in microbial levels compared with the same groups without antibiotic treatment (Table 1).

Western Blotting for MMP 7

Radiation alone led to more than a 5-fold increase in matrilysin at 24 h for 19 Gy irradiation and a 3-fold increase at 38 Gy, dropping drastically to less than half of their peak values at 48 h (Fig. 1). Antibiotics alone led to increasing MMP levels with a 5–6-fold increase at 48 h. The combination of irradiation and antibiotics led to similar increased levels of MMPs at 24 h and 48 h compared with radiation only. The MMP levels at 48 h were similar in all groups pretreated with antibiotics with or without irradiation (Fig. 1). 38 Gy irradiation in combination with antibiotics led to a successive increase in MMP-7 with time. Sham irradiation without antibiotics gave low levels of MMP-7 at all time points.

Cytokines

MIP-2

Pretreatment with antibiotics led to a significant reduction of MIP-2 values 2 h and 24 h after surgery in the irradiated groups compared to sham irradiation. There was a successive increase with time in the irradiated groups with all the groups having similar levels at 48 h. The sham irradiated group had, however, significantly higher initial values at 2 h peaking later with

TABLE 1

Ileum Bacterial Microflora Differences (log CHU/g Tissue) Between Groups With and Without Antibiotic Treatment at Different Time Points of Radiation

		Aerobic	Anaerobic	Enterobacteriaceae	Lactobacilli
2 h after radiation					
Sham	− Antibiotic	7.35 ± 035	7.06±0.41	3.24±1.47	5.23±1.10
	+ Antibiotic	5.03 ± 1.59	4.87 ± 1.55	4.90 ± 1.56	0.00 ± 0.00*
19 Gy	− Antibiotic	6.38 ± 0.26	7.01 ± 0.40	0.00 ± 0.00	4.30 ± 1.38
	+ Antibiotic	1.23 ± 1.23*	2.38 ± 1.50*	1.23 ± 1.23	0.00 ± 0.00*
38 Gy	− Antibiotic	5.92 ± 0.22	5.99 ± 0.30	1.86 ± 1.17	4.62 ± 0.99
	+ Antibiotic	3.34 ± 1.49	0.00 ± 0.00*	0.00 ± 0.00	0.00 ± 0.00*
24 h after radiation					
Sham	− Antibiotic	7.97 ± 0.26	8.06 ± 0.24	6.34 ± 1.27	5.76 ± 1.19
	+ Antibiotic	7.35 ± 0.34	7.49 ± 0.35	7.24 ± 0.36	0.00 ± 0.00*
19 Gy	− Antibiotic	8.63 ± 0.40	8.49 ± 0.43	6.96 ± 1.42	4.01 ± 1.79
	+ Antibiotic	9.66 ± 0.27	9.73 ± 0.27*	9.71 ± 0.25 *	0.00 ± 0.00*
38 Gy	− Antibiotic	8.28 ± 0.29	8.36 ± 0.25	7.80 ± 0.33	6.40 ± 1.08
	+ Antibiotic	8.45 ± 0.54	8.59 ± 0.54	8.21 ± 0.65	0.00 ± 0.00*
48 h after radiation					
Sham	− Antibiotic	8.15 ± 0.17	8.31 ± 0.21	3.65 ± 1.63	8.03 ± 0.20
	+ Antibiotic	8.72 ± 0.56	8.70 ± 0.58	8.44 ± 0.62*	0.00 ± 0.00*
19 Gy	− Antibiotic	6.79 ± 1.38	7.04 ± 1.44	5.20 ± 1.65	4.35 ± 1.38
	+ Antibiotic	8.56 ± 0.47	8.67 ± 0.44	7.07 ± 1.51	2.56 ± 1.62
38 Gy	− Antibiotic	7.86 ± 0.26	7.89 ± 0.31	7.30 ± 0.34	5.65 ± 1.24
	+ Antibiotic	5.07 ± 1.69	3.72 ±1.79*	2.92 ± 1.84*	0.00 ± 0.00*

Data presented as mean ± SEM.
*$P < 0.05$ compared with sham (–) antibiotics.

a 4-fold increase at 24 h with levels and then dropping sharply to less than half of the initial 2 h values at 48 h, where all experimental groups had similar values (Table 2). The higher radiation dosage of 38 Gy produced the least of MIP-2 levels at 2 h and 24 h.

TGF-β

Pretreatment with antibiotics led to a successive decrease in TGF-β levels with increasing time in the sham irradiated group. All irradiated groups showed a dip in TGF-β levels at 24 h, rising again at 24 h (Table 3). Irradiated groups had, however, significantly higher TGF-β levels compared to sham irradiation at 24 h and 48 h. (Table 3).

DISCUSSION

The fluctuations in bacterial microflora after irradiation alone in our study compare with results obtained in an earlier study by our group using a similar 19 Gy dose [13]. As here, there is a rise and fall in enterobacteriaceae bacterial counts with time. Further, similar results are observed in this study with a tendency to increasing bacterial counts with time, with regards to aerobes and anaerobes just as no significant changes are observed in levels of the lactobacilli 24 h after irradiation.

We also find that as in the previous study that most bacterial counts peak within 24 h. This study confirms this by showing a clear fall in counts 48 h after irradiation. The preoperative prophylactic antibiotic regimen given in this study resulted in general suppression of lactobacilli after laparotomy devoid of radiotherapy. Lactobacilli seem thus to be more resistant to radiotherapy than to antibiotic therapy. However, at the late time point 48 h after irradiation, all bacterial counts are markedly reduced after having been previously exposed to antibiotics. This could imply an overriding effect of the antibiotics in the late stage over radiotherapy.

Radiation has been shown to both change the bacterial environment [13] as well as up-regulate matrilysin in rectal cancer [5]. Matrilysin has an important role in the differentiated function of epithelia especially as mucosal barrier [18, 19]. The epithelial barrier function in the gut is disturbed following radiotherapy [20]. Matrilysin is also involved in the secretion of the precursors of α-defensins, antimicrobial peptides named cryptidins [6, 7, 21–24].

Not only radiotherapy or antibiotics alone, but the combination of both preoperative radiotherapy and antibiotics leads to marked increases in MMP-7 expression in the rodent intestine. The up-regulation of MMP-7 observed after antibiotic prophylaxis might probably be due to stimulus trigged off by the imbalance of bacterial flora in favor of more potentially pathogenic

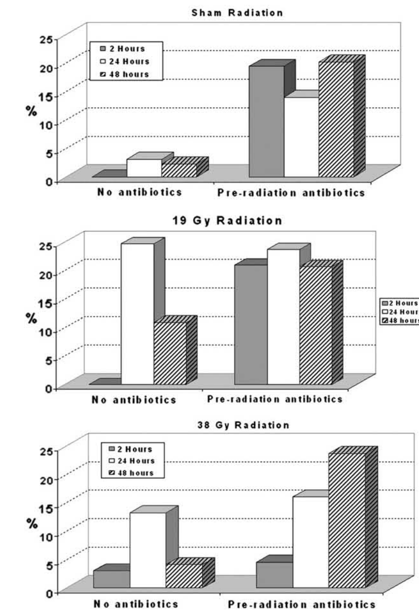

FIG. 1. MMP-7 expression %, 2, 24, and 48 h after sham radiation, 19 Gy and 38 Gy radiations ± antibiotics pretreatment.

enterobacteriaceae, which were the only flora to rise at the early time points. The effect being further bolstered after increases in anaerobes and aerobes at later time points. We observed an increased expression of matrilysin at 24 h after radiation, with detectable levels up to 48 h which corresponds (at protein level, regarding time-points) to that previously observed in a human *in vitro* model after bacterial exposition [7].

Our study is unique as it examines the direct effect of antibiotics (ampicillin) on matrilysin expression. The closest study available to us [25] is based on a tetracycline-regulated gene expression system in a mouse model where fibrosis induced by TGF-β 1 is reversed by the introduction of the tetracycline derivative doxycycline. Here MMP-2 mRNA and TIMP-1 mRNA, which were up-regulated whilst MMP-9 mRNA levels were down-regulated under TGF-β 1 stimulation, were all returned to control levels when doxycycline was introduced. The discrepancy between our two studies with regards to MMP expression may be due to different modes of regulation and activation of the different MMP groups, where the mouse gelatinases (MMP-2 and 9) react differently to antibiotics exposure. Matrilysin is quite sensitive to microbial changes. In this case, we suspect a stimulation of matrilysin by the presence of the potentially pathogenic enterobacteriaceae. The imbalance of the intestinal microflora affected by antibiotic therapy thereby leading to a stimulation of matrilysin production to similar levels observed after radiotherapy.

TGF-β is a fibrogenic cytokine that induces collagen synthesis and linked to radiation fibrosis [13]. TGF-β levels in our study show trends similar to the results of Ueberham *et al* [25], Mourelle *et al.* [26], and Saha *et al.* [27] where a down-regulation of TGF-β is observed after antibiotic/antimicrobial therapy only. We also found decreasing levels of TGF-β with time, in small bowel tissue after antibiotic treatment alone. However, the combination of both radiotherapy regimes and antibiotics led to a short dip in both radiotherapy treatment regimens at 24 h, thereafter significant increases reaching initial levels noted at 48 h compared with antibiotics alone. Earlier studies by our group [28] and Fukuchi *et al.* [29] show that a combination of two therapies (radiotherapy and surgery, and chemotherapy and surgery, respectively) leads to depression of TGF-β in the first postoperative week followed by an up-regulation in the late period. Here antibiotics and

TABLE 2

MIP-2 Values of the Small Bowel (pg/g tissue) after Different Doses of Radiation and Antibiotic Treatment

	Sham radiation	19 Gy radiation	38 Gy radiation
Preradiation antibiotics			
2 h	6133 (3406–7676)§	1040 (809–1252)*,§	339 (268–408)*,†
24 h	27435 (8286–47931)	1708 (1689–2312)*	1378 (829–1601)*,‡
48 h	3002 (1661–4400)§	1866 (1413–2139)	2099 (1264–3457)‡

Values presented as median (25th–75th percentile).
*$P < .05$ compared with sham radiation.
†$P < 0.05$ compared with 19 Gy.
‡$P < 0.05$ compared with 2 h.
§$P < 0.05$ compared with 24 h.

TABLE 3

TGF-β Values of the Small Bowel (pg/g Tissue) After Different Doses of Radiation and Antibiotic Treatment

	Sham radiation	19 Gy radiation	38 Gy radiation
Preradiation antibiotics			
2 h	8900 (7659–9710)	10591 (8464–13601)[†]	8013 (6222–9111)
24 h	4144 (3904–4496)	5800 (5116–6016)[*]	6668 (5226–9843)[*]
48 h	3911 (3241–5370)	10109 (9400–10771)[*,†]	7348 (4971–10373)[*]

Values presented as median (25th-75th percentile).
[*]denotes $P < 0.05$ compared with sham radiation.
[†]denotes $P < 0.05$ compared with 24 h.

surgery lead to a continued dip in TGF-β at 48 h, however, the three therapies here (antibiotics and radiotherapy and surgery) lead to a short lived depression of TGF-β. This phenomenon will have to be studied more closely. Studies have showed areas of active histopathologic radiation injury as in radiation enteritis having increased immunoreactivity of TGF-β and collagen in the intestinal wall.

Macrophage-inflammatory protein-2 (MIP-2) is a major CXC chemokine expressed by macrophages at sites of tissue inflammation following injury and/or infection. MIP-2 production appears to be restricted to infiltrating inflammatory leucocytes including neutrophils and monocytes, with a relative late response, peaking between 16 h and 24 h after surgical injury to the skin of rodents [30].

MIP-2 levels have been found to decrease during antifungal treatment of infected rats with amphotericin B, but no influence on MIP-2 levels was seen in the treatment of uninfected rodents compared with untreated uninfected rodents suggesting a decreased cytokine response as a result of the reduction of fungal load caused by the treatment. Antibiotics alone in our study led to MIP-2 peaking at 24 h corresponding to what has been earlier reported [30] after surgical injury. Antibiotics and radiotherapy together lead to a successive increase in MIP-2 levels (Table 2). In this model, preoperative antibiotics seem to enhance an early inflammatory response of MIP-2 to surgical injury within the first 24 h, whilst the addition of radiotherapy leads to a later up-regulation of MIP-2.

The results of this study indicate that pretreatment with the antibiotic ampicillin in combination with radiotherapy leads to an overexpression of MMP-7 similar to that achieved by either radiation or antibiotics alone. This may be the result of both microbial/mucosal changes and inflammation from the radiotherapy. Radiotherapy tends to override the effect of antibiotics and leads to an up-regulation of MMP-7 and TGF-β and MIP-2 expression between 24 h and 48 h. Antibiotics alone have a suppressive effect on TGF-β. Our results indicate that inhibition of MMP-7 or reduction in radiosensitivity in radiotherapeutic regimes can not be achieved by regulation of the microflora with the antibiotic regimen used. Our data thus did not support fully the hypothesis but has rather shown some interesting correlation between an antibiotic and MMP-7. Further studies are needed to understand the activation and regulation of matrilysin. Information, which will be of clinical importance in the quest for regulating the remodellingprocess of the extracellular matrix associated with radiation associated fibrosis and tumour progression.

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paper III

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MMP7 Expression in Colorectal Tumours of Different Stages

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Abstract. *Background/Aim: Matrix metalloproteinases (MMPs) are involved in cancer biology. Expression of MMP7 (matrilysin) in colorectal cancer is associated with metastatic disease even though it is expressed in most tumour states. In the present study, our purpose was to analyze MMP7 in bowel and lymph nodes of different tumour stages and to evaluate its expression as a cancer biomarker. Patients and Methods: 28 patients surgically-treated for benign and malignant colorectal tumours were recruited and analyzed for MMP7 in tumoural tissue, lymph nodes and serum by histology, immunohistochemistry, ELISA and western blotting. Results: Immunohistochemistry showed prevalent expression of MMP7 in advanced cancer. A significant increase ($p<0.001$) was evident in serum of stage III/IV cancers compared to both adenomas and non-metastatic disease. MMP7 was increased in cancer tissues with prevalence in stage I/II. Lymph nodes presented a significant increase of MMP7 ($p<0.05$ adenoma vs. stage I/II and $p<0.001$ vs. stage III/IV). Conclusion: MMP7 increases with dysplasia and cancer disease stage in tumour tissue as well as in the regional lymph nodes. It may be used as a complement in investigating suspected locally advanced cancer.*

Matrix metalloproteinases (MMPs) are a family of a Zn^{2+}-dependent proteolytic enzymes involved in physiological and pathological remodeling of extracellular matrix in proliferation, angiogenesis and wound healing. MMP7 matrilysin is the smallest metalloproteinase and is known to degrade collagen type IV and X, but also MMP2 and MMP9 (1). Previous studies demonstrated its peculiarity among MMPs, based on its expression in normal, non-inflamed, non-injured epithelia in several organs, as opposed to other MMPs which are normally or seldom not expressed at those sites (2, 3). MMP7 is produced as an inactive zymogene (28 kD). Experimental data suggest it is transformed into an active form (19 kD) by proteolysis and is capable of degrading itself in a concentration-dependent manner (1-5).

An overexpression of MMP7 in advanced stages of colorectal cancer has been linked to increased metastatic disease (6, 7). MMP7 can be found in cancer tissue, as well as in serum and in peritoneal fluid during peritoneal carcinosis (8-12). MMP7 was found to be a requirement for tumour formation but not related to depth of tumour invasion nor to surrounding stromal fibrosis (13). A rodent model for colorectal carcinoma using mice deficient for matrilysin with an *MMP7*-knockout mutation, presented a reduced number and size of tumours. The mutation did not block invasion since that seems to be related to activation of other MMPs, such as MMP2 and MMP9 which are produced in stromal and not in epithelial cells, where MMP7 is produced (13).

Previous studies also showed an increase in MMPs (especially MMP2, MMP7 and MMP9) after neoadjuvant radiotherapy for rectal cancer, suggesting their possible role in abnormal tissue remodeling after radiotherapeutic injury (14-16). The expression of MMP7 mRNA in humans has a high specificity in colorectal cancer, especially in malignant epithelial cells, but some studies have shown its expression in normal colorectal mucosa, as well as in different grades of dysplasia to cancer (17, 18).

The purpose of the present study was to determine whether matrilysin expression at different stages of tumour progression to cancer within the bowel can be correlated to levels in adjacent lymph nodes in the resected specimen as this would be useful for the assessment of colorectal cancer prognosis.

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Key Words: Matrilysin, MMP7, colorectal cancer, adenoma, lymph nodes.

Patients and Methods

Patients. A prospective study was undertaken of patients referred for elective colorectal cancer treatment to the Pietro Valdoni Department of Surgery, Sapienza University of Rome. Exclusion criteria were neoadjuvant radiotherapy, chemoradiotherapy, language problems and consent withdrawal. Twenty-eight patients were recruited prospectively according to guidelines for treatment of colorectal disease after routine clinical assessment. The study was approved by the Human Ethics Committee at the Sapienza University of Rome and registered at Clinical Trials, ID NCT 01570452. The study was carried-out over a period of three years (September 2005- September 2008). Twenty-eight patients completed the study (eight with benign and 20 with malignant colorectal tumours) after acceptance of informed consent. Among benign tumours, only polyps not suitable for endoscopic resection were included. Serum controls were taken from 10 healthy volunteers after acceptance of informed consent. Staging was performed after colonoscopy and biopsy, with abdominal computed tomography (CT) and chest radiography. Clinical variables were included in a database. Patients with severe dysplasia or large symptomatic low-to-moderate dysplastic adenomas and adenocarcinoma underwent surgery. Intraoperative blood samples were collected to determine baseline parameters, specific oncomarkers (carcinoembryogenic antigen, CEA; cancer antigen 19-9, CA19-9, cancer antigen 50, CA 50) and MMP7. Intraoperative specimens were collected from cancer tissues and from normal surrounding mucosa (about 2-2.5 cm from the tumour edge). Lymph nodes from the colonic mesentery close to the tumour were collected. The specimens were fixed in 4% formaldehyde before histopathological examination.

Samples and serum preparation. Tissue samples from tumoural and mucosal tissue, as well as from lymph nodes, were kept in sterile tubes at –80˚C. They were then cut obtaining aliquots weighing between 50 and 100 mg. These were treated with 300 μl of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% NP-40, 1% CHAPS, 2 mM EDTA dissolved in tetra-distilled water). A mixture of protease inhibitors (Complete-Mini Protease Inhibitor Cocktail Tablets; Roche, Mannheim, Germany) was added just before use. Samples were first homogenized by Ultra-Turrax® (T10 basic; IKA®, Staufen, Germany), then sonicated for 20 sec and centrifuged at 14,000 rpm for 20 min (19). The supernatants were then collected. Venous blood samples were drawn into sterile vacuum tubes and left at room temperature for 30 min, then centrifuged at 4,000 rpm for 15 min, to divide serum from pellet, as standard laboratory protocol. Serum was immediately aliquoted and stored at –80˚C until assayed. The protein content of supernatants and serum samples was determined by using the Bradford assay.

Histology. Samples were fixed in 4% phosphate-buffered formaldehyde and later embedded in paraffin. Sliced specimens stained with hematoxylin and eosin were analyzed under light microscopy. At least three slides were studied from each specimen by a blinded observer. Stage definition was stated according to 2002 UICC classification (20).

Immunohistochemistry. For immunohistochemistry, standard avidin-biotin procedures for human MMP7 were used. After de-paraffinization and washing in phosphate-buffered solution (PBS), endogenous peroxidase activity was blocked by incubating the sliced sections in 3% hydrogen peroxide in PBS for 10 min. Analysis for MMP7 was performed using anti-MMP7 (MAB-10756; Immunological Sciences, Rome, Italy) following the manufacturer's instructions. Biotin-conjugated secondary antibody and streptavidin-conjugated horseradish peroxidase (Dako North America, Inc., CA, USA) was applied to sections for 45 min at room temperature, and developed using 3,3'-diaminobenzidine (DAB) as substrate. Finally, counterstaining with haematoxylin was performed. Sections were mounted and the grade of staining was determined on randomly selected areas counter-checked for intensity by a blinded observer.

MMP7 determination. In supernatants and serum samples, total human MMP7 levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Quantikine®, R&D Systems, Minneapolis/USA). Diluted samples (150 μl) were added to a 96-well microtiter plate, pre-coated with a monoclonal antibody to human MMP7 and incubated at room temperature for a further 2 h on a microplate shaker. After washing, 200 μl of the secondary antibody solution were added, and the plate was incubated for 2 h at room temperature on the shaker. After washing, the substrate solution was added and incubated at room temperature in the dark. A 50 μl stop solution was added after 30 min and the optical density was measured using a microtiter plate reader (Opsys MR™; Dinex Technologies, Inc.; Chantilly, VA, USA) at 450 nm, with correction wavelength set at 570 nm.

Western blot analysis. For western blot analysis, supernatants obtained from lymph node specimens were separated on a sodium dodecyl sulphate-polyacrylamide electrophoresis gel with a concentration of acrylamide specific for MMP7 and β-actin. Proteins were blotted onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA) and probed with the following antibodies: anti-MMP7 (MAB-10756 Immunological Sciences) and anti-β-actin (A 5060; Sigma Chemical Co., St. Louis, MO, USA). Antigens were detected with an enhanced chemoluminescence (ECL) kit from Amersham (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). All western blotting images were acquired and analyzed through an Imaging Fluor S densitometer (Bio-Rad Laboratories, Hercules). The optical density (O.D.) of each condition was correlated to the signal of the β-actin internal control.

Statistical methods. Data are expressed as the mean±standard deviation (SD). The statistical comparisons between groups were performed by using the analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test. Differences were considered significant at p<0.05. Analysis was performed by using a statistical software (GraphPad Software, Inc., San Diego, CA, USA).

Results

Patients' characteristics. The study group consisted of 28 patients (16 males, 12 females) with mean age±SD (standard deviation) of 74±6 years and a median of 72 years (range=55-88 years). Age was equally distributed between males and females. Tumour sites were: right colon in eight, left colon in twelve, rectum in eight. The surgical techniques used were: right hemicolectomy in eight, left hemicolectomy in six, sigmoidectomy in four, subtotal colectomy in two,

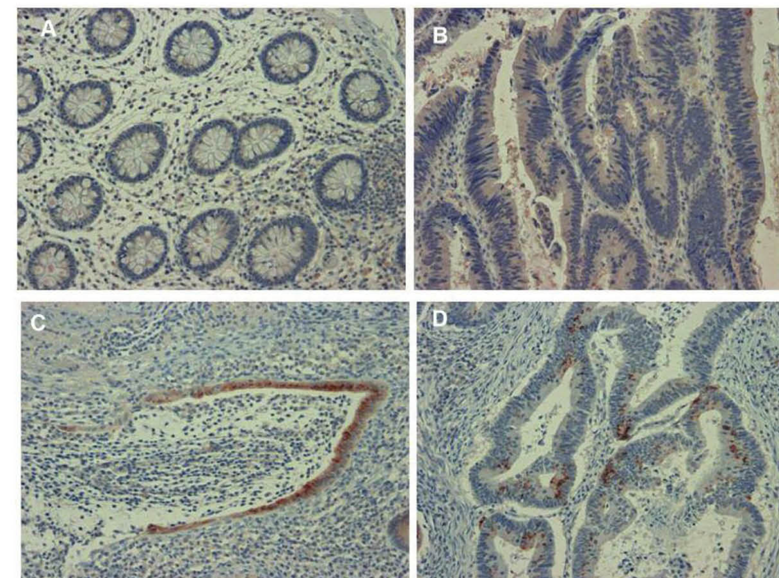

Figure 1. *Immunohistochemistry for matrix metalloproteinase-7 (MMP7). Negative staining in healthy mucosa (A). MMP7 expression in low-grade dysplastic adenoma (B), well-differentiated carcinoma T2N0M0 (C) and in poorly-differentiated carcinoma T3N2 M0 (D), original magnification, ×20.*

anterior rectal resection in six, abdominoperineal rectal excision in two. Among eight patients with rectal cancer, none underwent neoadjuvant radiotherapy or chemotherapy. No major complications were observed after surgery. No mortality was registered within 30 days after operation. Specimens were divided into four groups: A, serum controls of healthy volunteers; B, benign tumors (dysplastic adenomas); C, stage I and II disease (adenocarcinomas); and D, stage III and IV disease (adenocarcinomas).

CEA was increased in seven patients (>5 ng/ml) belonging to group D. CA19-9, CA50 and the clinical variables recorded pre-operatively did not show any significant differences.

Histology. Stained specimens of the tissues were examined and staged according to three groups as dysplastic adenomas (n=8); no disseminated disease (n=10), including stage I (n=7) and II (n=3); and disseminated disease (n=10), including stage III (n=8) and IV (n=2).

Immunohistochemistry. No MMP7 expression was observed in normal mucosa (Figure 1A). MMP7 was expressed in benign tumours (Figure 1B). A tendency for more evident expression in well-differentiated (Figure 1C) compared to non-differentiated carcinomas (Figure 1D) was observed.

ELISA. MMP7 expression was significantly (p<0.01) higher in stage I and II cancer tissues compared to adenomas (low and moderate dysplasia) and significantly lower compared to stage III and IV cancers (Figure 2). Levels in adenomas were also significantly lower (p<0.001) compared to those in stage III and IV disease. Normal mucosa (negative tissue control) did not show any measurable levels of MMP7 in any of the samples (data not shown). No significant difference was observed in serum levels of MMP7 comparing patients with benign adenomas to those with stage I and II cancers.

A significant increase (p<0.01) was evident in serum comparing patients with adenomas to those with stage III and IV cancer. However, no significant differences were observed for MMP7 expression within the groups comparing stage I to II and III to IV in both tumour and serum. Serum obtained from healthy controls showed very low or undetectable levels of MMP7. Lymph nodes presented lower levels of MMP7 compared to serum and tumoural tissue. Significant differences in in expression in lymph nodes were noticed

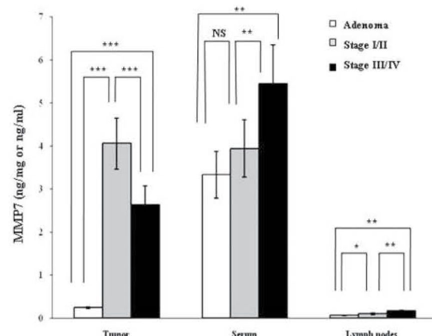

Figure 2. *Matrix metalloproteinase-7 expression in tumoral tissue, serum and lymph nodes. Significantly different at *p<0.05, **p<0.01, ***p<0.001; NS, p>0.005.*

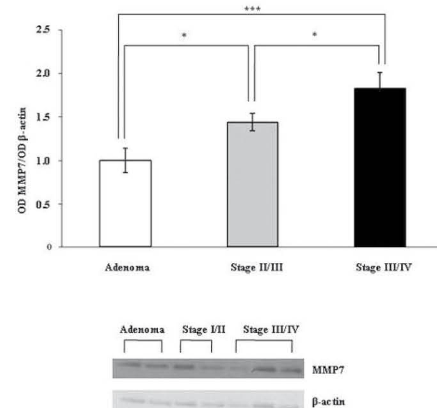

Figure 3. *Western blot analysis showing the expression of matrix metalloproteinase-7 in lymph nodes. Significantly different at *p<0.05, *** p<0.001.*

among groups, adenoma *vs*. stages I and II (p<0.05) and stages III and IV *vs*. both stages I and II and adenomas (p<0.001) (Figure 2).

Western blot analysis. Further analysis of lymph nodes by western blotting, with a semi-quantitative measurement of MMP7 expression, confirmed the ELISA results. We observed a significant positive trend in the expression of MMP7 from adenoma to increasing cancer stage. MMP7 was evaluated by comparative detection of β-actin. Lymph nodes of patients with stage I and II tumours had significant higher expression than those in patients with adenomas (p<0.05) and those in patients with stage III and IV tumors had significantly higher levels of MMP7 (p<0.05) compared to those with stage I and II adenocarcinomas and those with adenomas (p<0.001) (Figure 3).

Discussion

The prognostic significance of MMP7 and its role in tumour biology has been widely investigated. Nevertheless the specific mechanism through which it promotes tumour invasion and spread is still unclear. The significance of MMP7 increase in resected specimens and in serum for the definition of oncological risk and prognosis for patients is still not clearly defined. In cancer immunology, a clear role of MMP7 and other MMPs has been shown for tumour growth, invasion and spread (6, 7, 17). Tumour specimens in our study had significantly higher levels of MMP7 in adenocarcinoma compared to varying grades of dysplasia (p<0.001). Even though the level of MMP7 in those with disseminated disease was less than in those with stage I and II cancer, it was nevertheless still significantly higher than that in adenomas (p<0.001). Immunohistochemistry showed a progressive increase with increasing dysplasia and cancer disease stage. Western blot showed a significant progressive increase in lymph node MMP7 with increasing dysplasia and infiltrative disease stage. MMP7 levels were lower in lymph nodes compared to tumour tissue, but here again, we found successive significant increases compared to adenoma through stage I and II disease to locally advanced cancer. Interesting results presented in a study by Ichikawa *et al*. who studied MMP7 expression by RT-PCR in lymph nodes from patients with colon cancer and showed that its expression increased accuracy in diagnosis compared to ordinary histology (17). MMP7 detected in adenocarcinoma (by RT-PCR) was associated with over 90% of histologically-positive cancer, whereas 30% of lymph nodes primarily defined as negative at histology were found to be positive for MMP7 RNA (21).

Neoplastic infiltration is related to degradation of elastin, laminin, proteoglycans, osteopontin, fibronectin and type IV collagen which is mediated by MMP7. MMP7 is even overexpressed after radiation compared to preoperative levels in patients with rectal cancer (14). In a previous study, our group confirmed the correlation of MMP7 expression and immune system status, where levels increased significantly whenever the intra-luminal microflora was suppressed with antibiotics. We found that microfloral regulation does not affect MMP7 stimulation after surgical or radiological trauma (22).

Increased MMP7 expression in serum in multivariate analysis generally correlates with worse prognosis, local invasiveness, a tendency for metastatic disease and with reduced overall survival (16, 22, 23). In colorectal cancer, levels of MMP9 (which is activated by MMP7) investigated in peripheral and portal blood showed increased levels and correlated with advanced and metastatic disease stage (8, 17). Our serum ELISA results showed a clearly significant increase of MMP7 in locally advanced disease over adenoma and stage I and II cancer. No significant differences were observed in MMP7 between adenoma and non-invasive cancer, but within the two cancer groups, there was again a significant increase in MMP7 from non-metastatic to metastatic disease.

Our study, although lacking the sensitivity of RT-PCR analyses, shows that with simpler cost-benefit methods, similar results or trends can be observed when examining tumor behaviour with resected specimens. The complexity of cancer warrants combinations of different analyses in order to achieve better prognostic goals.

The results of the analyses of lymph nodes suggest a reliable application of ELISA and western blotting as an alternative to RT-PCR in staging for advanced local cancer in order to avoid down-staging in cases with histologically-negative nodes. Furthermore, immunohistochemistry seems to be a good complement.

MMPs, including MMP7, continue to be an interesting group in the quest for choosing biomarkers that can help us in the clinical setting. Evidence that T-cells generated *in vitro* may target MMP7-derived antigen expressed on the cellular surface of antigen-presenting cells might represent a potential option in immunotherapy against cancer (24).

Conclusion

Significantly increased concentrations of MMP7 were observed in tumor tissues, lymph nodes and in serum in and were associated with increasing grade of dysplasia and adenocarcinoma infiltration. We showed a correlation between MMP7 expression and the risk of lymph nodal involvement. This suggests a possible role for MMP7 in the determination of locally advanced cancer in resected specimens, in staging and in planning for eventual adjuvant therapy. Further investigations are, however, required to determine the exact role the complex molecule MMP7 may play in cancer treatment.

Competing Interests

None of the Authors of the study have any conflicts of interest with regards to funding or support of any kind of the study.

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paper IV

MMP-7 modulation by short and long term radiotherapy in rectal cancer patients.

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Abstract

Background: MMP-7 in humans has a high specificity for colorectal cancer cells and modulates tumour growth and invasion correlating with worse prognosis, tendency for metastatic disease and reduced overall survival. A radiation induced over-expression of MMP-7 has been observed in rectal cancer.

Purpose: The aim of the study is to analyse the effects of irradiation on inflammation and MMP-7 in patients with rectal cancer undergoing different regimens of neoadjuvant radiotherapy.

Methods: 53 patients were divided into three treatment groups receiving short term 25 Gray (Gy) preoperative radiotherapy (RT), 50 Gy long term preoperative RT and controls receiving only surgery. Three biopsies were taken from each patient at inclusion before RT / at diagnosis, prior to surgery and from the excised specimen. All tissue samples were formalin fixed and paraffin embedded and analysis was performed by immunohistochemistry on tissue microarray for MMP-7 and TGF-beta. Mann-Whitneys U-test and Wilcoxon Z-test (Z) were used for statistical validation.

Results: Surgery up-regulated MMP-7 in all groups. Short term 25 Gy RT induces over-expression of MMP-7 at/before surgery but not 50 Gy RT. In all three groups no significant increase of TGF-beta was observed before surgery. TGF-beta showed significant 2- to 3-fold increase only after rectal resection.

Conclusions: 50 Gy preoperative radiotherapy induces significantly less MMP-7 over-expression compared to short term 25 Gy irradiation at surgery. Surgery has an overriding effect over up regulation of MMP-7.

Key Words: matrilysin; matrix metalloproteinase; rectal cancer; radiotherapy; inflammation

Introduction

Colorectal cancer is the third most common malignancy in the world, thus constituting the most frequent reason for gastrointestinal resections (1). In Sweden 5755 persons were diagnosed with colorectal cancer of which 2144 persons had rectal cancer in 2009. Approximately 70% of rectal cancer patients underwent a surgical resection (2).

Neoadjuvant radiotherapy (RT) is used in more than 60% of Swedish patients with the intent to either reduce local recurrences by ameliorated local control or to downsize tumours that primarily are not resectable. Preoperative short-term RT 5 x 5 Gray (Gy) for one week followed by surgery within three days has been increasingly used as a treatment for patients with resectable rectal cancer. Long-term RT 5 x 1.8-2 Gy for five weeks followed by surgery after six to eight weeks, is used preoperatively to downsize tumours that are originally diagnosed as locally advanced.

The use of preoperative RT in rectal cancer treatment has been resulting in decreased rate of local recurrences and an increased survival rate has also been shown (*3*). However, pelvic radiation is frequently associated with morbidity in the short as well as long term. A panorama of complications including the following: delayed wound healing, perineal wound infections, postoperative abscesses, fistulae, intestinal obstruction, perforations and bleeding are described. Negative effects on functional outcome like diarrhoea, urgency and faecal incontinence are also observed (4).

Irradiation of intestinal mucosa may cause an altered and/or decreased gut microbiota leading to an unfavourable growth of opportunistic microorganisms. This process is thought to enhance mucosal damage and intestinal inflammation related to gut radiotherapy (5-8).

Matrix metalloproteinases (MMPs) are endopeptidases belonging to a family of zinc-dependent enzymes with proteolytic properties capable of degrading all components of both the basement membrane and the extracellular matrix. This quality has been suggested to be of importance in the process of enhancing tumour cells ability to invade and spread (9-10).

MMPs are induced by interactions between cells or extracellular matrix (ECM) but also by cytokines and growth factors. Radiation-induced morbidity might be associated with an imbalance in remodeling ECM. ECM has been shown to be remodeled by ionizing radiation which causes an extensive oxidative damage at the cellular level and on numerous enzymes, for example transforming growth factor receptor beta (TGF-β) and matrix metalloproteinases (9).

Malignant epithelial cells are unique to express MMP-7, matrilysin, a protease that increase in metastatic colorectal cancer (*9*).

In a murine model both MMP-7 and TGF-β were upregulated by irradiation (*11*). RT is also found to induce an elevation of MMP-7 in humans (*9, 12*). During wound healing activation of MMPs is required in several of the steps leading to restored tissue integrity.

The aim of this prospective study was therefore to analyse the effects of irradiation on MMP-7 and TGF-β in patients with rectal cancer undergoing neoadjuvant RT.

Material and methods

Patients

The study was planned and designed as a case-controlled study comprising 77 patients diagnosed with rectal cancer and treated at the University Hospital of Malmö between 2003 and 2007. All patients were managed according to clinical protocol of the department and assessment of the local multidisciplinary treatment board which adheres to national guidelines. The study cohort consisted of 53 patients. 24 patients were excluded due to the following reasons: revised pathological diagnosis showing high grade dysplasia (8 patients), impaired general condition (8 patients), declining to participate (4 patients), synchronous colonic tumours (3 patients) and one patient for logistical reasons. Seventeen patients were female (32,1%) and 36 were male (67,9%). The study was approved by the Ethics Committee of Lund University, Sweden (ref 144/2004, amendment 597/2006) and written consent was obtained from each patient after oral and written information. Staging was performed according to the TNM system (13). Exclusion criteria included previous RT to the pelvic region, inflammatory bowel disease, neoadjuvant chemotherapy as well as ongoing steroid, immunosuppressive and/or antibiotic therapy. Three treatment groups were defined: one group receiving short term preoperative RT of 25 Gray (5 X 5 Gy), 20 patients; another group treated with long term preoperative RT of 50 Gy (25 X 2 Gy), 21 patients; and a control group undergoing surgery alone without RT, 12 patients.

Surgical procedures performed were either anterior resection or abdominoperineal resection, by the total mesorectal excision technique. A rigid rectosigmoidoscopy was performed before and after irradiation/before start of surgery. Two millimeter punch biopsies were obtained from tumour tissue as well as from normal mucosa not less than 2 centimeters from the tumour edge within the irradiated field. Three biopsies were taken: at inclusion before RT prior to start of surgery to eliminate possible effects of surgical trauma and ischaemia on MMP expression; at surgery and from the excised specimen. All tissue samples were instantly formalin fixed and paraffin embedded.

Tissue microarrays

Tissue microarray (TMA) were performed in two series; one biopsy TMA with 1 x 1 mm cores were taken out of biopsies from normal tissue as well as from tumour tissue, and one TMA from the complete surgical specimens through which 2 x 1 mm cores were drawn from areas comprising viable, non-necrotic tumour, and nearby microscopically benign rectal mucosa, respectively. This was performed by using a manual arraying device (MTA-1; Beecher Inc., Sun Prairie, WI, USA) and mounted in a recipient block.

Immunohistochemistry and staining evaluation

The TMAs were sectioned into 4 micrometer (μm) samples pretreated in the DAKO PT-link module using a standard protocol and buffer supplied by the manufacturer. Thereafter slides were stained in a DAKO Autostainer-plus using the EnVision™ FLEX including Peroxidise-Blocking Reagent (DAKO, Glostrup, Denmark) with monoclonal antibodies MMP-7 Santa Cruz mouse (clone MM0022-4C21) sc-101566 dilution 1:50 and TGF-beta Abcam rabbit poly (ab6603) dilution 1:200. Immunohistochemistry was performed by an automated staining machine (Ventana Medical Systems, Inc., Tucson, AZ, USA).

Three research scientists jointly annotated cytoplasmic expression of inflammatory markers for each core, in tumour tissue and mucosa. Annotation of absolute percent of positive cells was multiplied with annotated intensity (0-3) of stained cells, and a mean expression score was subsequently calculated for each patient at each time point. Discrepant cores were discussed until consensus was reached.

Statistics

Spearman's Rho and χ^2 tests were used to investigate RT groups and patient characteristics (Table 1). Mann-Whitneys U-test and Wilcoxon Z-test (Z) were used to investigate MMP7 expression differences between tissue from baseline before RT, after RT prior to surgery, and after resection, in the RT subgroups. All statistics were performed using SPSS version 21.0 (SPSS Inc, Chicago, IL, USA). P-values over 0.05 was considered significant.

Results

The characteristics of the patients enrolled in the study according to sex, age, stage and procedure performed are presented in Table 1. The majority of patients in the different groups were male younger than 75 years old. Disease stages were equally distributed among groups with the exception of stage IV with a very low number of cases enrolled. Anterior rectal resection was the mostly performed procedure followed by abdominoperineal resection.

MMP-7 showed a significant progressive increase compared to baseline values after short term RT with 25 Gy and furthermore a more significant raise just after surgery. After exposition to 50 Gy in the long term course, the expression of MMP-7 presents before surgery insignificant variations compared to baseline. A significant raise in MMP-7 expression was instead observed after surgery compared to baseline in all groups. When analysing the control patients not receiving RT, they show a trend similar to that observed in the 50 Gy group with stable values at baseline and before surgery, but which rise significantly after surgery. Normal mucosa analysis demonstrated very low levels of MMP-7 with no effect of RT when comparing baseline to preoperative samples in all groups. Only surgery induced a significant increase in MMP-7 in normal mucosa surrounding the tumour. The results of MMP-7 expression are presented in Table 2 and in Figure 1.

Similarly a homogenous trend was observed in the expression of TGF-beta in tumour before and after RT compared to baseline. In the three groups no signifi-

cant increase of TGF-beta was observed just before surgery. TGF-beta only showed significant 2 to 3 fold increase after surgery, having its peak in the 25 Gy radiated group (Table 3).

Discussion

The expression of MMP-7 m-RNA in humans has a high specificity for malignant epithelial cells of colorectal cancer with only weak expression in normal colorectal mucosa with a progressive trend from normal mucosa to cancer. Varied expressions with increasing grade of dysplasia and inflammation have been observed (9, 14-16). In cancer immunology, a clear role of MMP-7 and of other MMPs has been shown for tumour growth and invasion (14, 17-22). Increased MMP-7 expression in tumour tissue, in serum, lymph nodes and in peritoneal liquid generally correlates with worse prognosis, tendency for metastatic disease and reduced overall survival (15, 23-29). This suggests a possible role for MMP7 in the determination of locally advanced cancer in resected specimens, in staging and in planning for eventual adjuvant therapy showing the potential role of MMP-7 as prognostic factor and tumour marker (30).

The effect of RT on matrilysin gene in human rectal cancer was in vivo first investigated by Kumar et al. who observed an over expression of MMP-7 in radiated rectal cancer tissue compared to normal irradiated rectal tissue (9). Due to the strict correlation between surgery and RT in the current multimodal management of rectal cancer, the effect and the consequent possible modulation of radiation induced MMP-7 over-expression, remains a current field of investigation which is still far from the complete due to its complex features and of the several molecules and pathways involved.

Although there is a wide literature available dealing mainly with short term RT, to our knowledge there are no previous publications comparing in rectal cancer patients the effects of short and long course RT on MMP-7 expression. For this reason the present study must be considered as a pilot investigation on the modulation of different RT courses in this setting. Nevertheless the main limit of the present research is the low number of patients enrolled in the different groups.

The expression of MMP-7 and of other metalloproteinases is usually a result of specific immunological stimuli due to physiological and pathological processes leading to proliferation and remodelling in tissue growth and differentiation. This is seen in tissue repair and wound healing after inflammation or after various types of injury and trauma (11, 31-33).

In our clinical setting there is a double factor potentially acting on ECM remodelling since RT alone, surgery alone or both in combination, clearly affected MMP-7 expression. Non irradiated control cases presented significantly higher values only after surgical rectal resection. In 50 Gy irradiated cases the effect of RT itself seemed not to influence MMP-7 expression, however there was a tendency of radiation to decrease its concentration, probably due to severe effects of elevated cumulative dosage. Only after surgery did these irradiated patients present with significantly higher values of MMP-7 comparable to the 25 Gy group which had already after RT alone a significant increase in matrilysin expression before surgery. These values showed a further significant increase after the operation. The analysis of the above mentioned results demonstrate that MMP-7 is over-expressed after different kind of stimulations. Surgery alone is effective in this up-regulation and in combination with the procedure effects of short term 25 Gy RT leads to a further over-expression of MMP-7 after the radiation treatment. Higher doses of RT (50 Gy) with delayed surgery after more than a month didn't show any increases in MMP-7 at the time of surgery compared to level of protein observed at baseline. The explanation of this picture might be ascribed to the waiting time after sample collection since patients, according to the neoadjuvant protocol, undergo surgery four to six weeks after the end of the RT which is a sufficient time for the effects of the over-expression induced to be phased out. Another possible explanation might be the severe effects of high cumulative dosage RT with persistent oedema diluting the concentration of MMP-7 or probably due to necrosis with loss of tumoral cells and consequently of their protein products including matrilysin (34).

The over-expression of MMP-7 after RT in neoadjuvant and/or adjuvant radiotherapy, given the role of matrilysin in abnormal tissue remodelling after radiotherapeutic injury (9, 22, 26) and its relation to the progression and metastatic spreading of colorectal cancer leads to the rationale of investigating potential preventive or therapeutically use of specific tissue inhibitors to MMP`s (TIMP) in rectal cancer treatment with the intent of containing the increased morbidity mediated by metalloproteinases (9).

It is not possible to eliminate the surgical trauma in the treatment of rectal cancer but at least the preventive vascular ligation and the prompt excision of the specimen might limit the influence of surgery on the effects of elevated MMP-7 as this effect is equally observed in all the examined groups, without favour for any of them. According to our results, RT instead affects differently MMP-7 expression in relation to the long or short course radiation treatment used. This preliminary observation might support a more favourable use of long course therapy, for its limited effect on MMP-7 expression, which might act in containing tumour progression during RT.

The limited effect on normal mucosa of both surgery and RT in our setting confirms that previously shown by other authors and in a previous publication from our group attesting a progressive expression of MMP-7 from normal mucosa to cancer through different grades of dysplasia with low matrilysin expression also in normal mucosal specimens but with levels approximately 10-fold lower compared with those seen in the paired tumour samples (9, 14-15).

A homogenous trend was observed in the expression of TGF-beta, as a marker of inflammation and fibrosis, in tumour before and after treatment compared to baseline. In the three groups no significant increase of TGF-beta was observed after 25 and 50 Gy RT just before surgery. TGF-beta showed however significant 2 to 3 fold increases only after surgery, having its maximum increase in the 25 Gy radiated group. TGF-beta participates in the remodelling of the ECM, but has many other functions such as suppression of the immune system and regulation of cell growth. It was shown also to act both as an inhibitor of tumour growth and as a promoter

of tumour progression (35).

The activation of TGF-beta can be elicited by endogenous agents, for example plasmin or MMP-9 but also exogenous factors such as irradiation and radiation-activated TGF-beta may be involved in the mechanisms behind fibrosis (36).

However some studies have found a decrease or no effect on the TGF-beta levels after radiotherapy.

A previous investigation from our group in fact showed that radiotherapy and surgery induce depression of TGF-beta in rats in the first postoperative week followed by an up-regulation in the late period (35, 37). Similarly other authors showed lower active TGF-beta levels in rectal cancer irradiated patients both in tumour tissue and rectal mucosa (35). Evidence points toward a radiation induced activation of latent TGF-beta but this activation may only be seen in a limited window time, most probably in the later effect of TGF-beta induced fibrosis which may explain why in our study as previously similarly observed (35) it was not seen at the time of the biopsy after termination of RT.

Conclusions

Our study tried to further define the scenario of the modulation of MMP-7 expression by RT according to different regimens of treatment available in the clinical practice. We found that 50 Gy preoperative radiotherapy induces significantly less MMP-7 over-expression compared to short term 25 Gy irradiation at surgery. Surgery has an overriding effect of up regulation of MMP-7.

Further studies are needed to validate these findings and to better investigate the pathway involved in cancer growth and spreading through ECM remodelling under the effect of RT. Clinical use of matrilysin inhibitors as a useful preventive or therapeutic adjunct to radiotherapy in rectal cancer has to be investigated.

	n	**Age**		**Gender (M:male;F:Female)**		**Disease Stage**					**Operative procedure**			
	n (%)	<75	≥75	M	F	I	II	III	IV	Missing	Rectum resection	Abdomino-perineal resection	Hartmann´s procedure	Missing
No RT	12	8 (66.7)	4(33.3)	9(75.0)	3(25.0)	3(25.0)	4(33.3)	4(33.3)	1(8.3)	0(0)	9(75.0)	2(16.7)	1(8.3)	0(0)
Short-term RT	20	15(75.0)	5(25.0)	14(70.0)	6(30.0)	7(35.0)	3(15.0)	8(40.0)	2(10.0)	0(0)	11(55.0)	8(40.0)	1(5.0)	0(0)
Long-term RT	21	16(76.2)	5(23.8)	13(61.9)	8(38.1)	4(19.0)	7(33.3)	7(33.3)	1(4.8)	2(9.5)	9(42.9)	8(38.1)	3(14.3)	1(4.8)
p-value										0.628				0.506

Table 1. Patients's characteristics

Group	***n***	**mean rank**	**p-value**	**Z-value**
25 Gy RT	*20*			
Baseline	*12*	9.04		
Before surgery	*10*	18.70	0.030	-2.594
After surgery	*18*	29.14	<0.000001	-4.561
50 Gy RT	*21*			
Baseline	*14*	21.14		
Before surgery	*5*	15.00	0.391	-1.298
After surgery	*14*	36.71	0.00360	-3.003
No RT	*12*			
Baseline	*9*	10.00		
Before surgery	*8*	10.25	0.963	-0.172
After surgery	*11*	21.27	0.00164	-3.208

Table 2. Difference in tumour expression of MMP7 before and after treatment compared to baseline

Group	*n*	TGF-b mean rank	p-value	Z-value
25 Gy RT	*31*			
Baseline	*11*	8.91		
Before surgery	*7*	11.86	0.375	-0.958
After surgery	*20*	28.00	<0.000001	-4.404
50 Gy RT	*24*			
Baseline	*10*	10.60		
Before surgery	*2*	11.50	1.000	0.000
After surgery	*14*	26.71	0.000012	-3.862
No RT	*19*			
Baseline	*8*	8.12		
Before surgery	*5*	5.80	0.435	-0.897
After surgery	*11*	18.73	0.000185	-3.399

Table 3. Difference in tumour expression of TGF-β before and after treatment compared to baseline

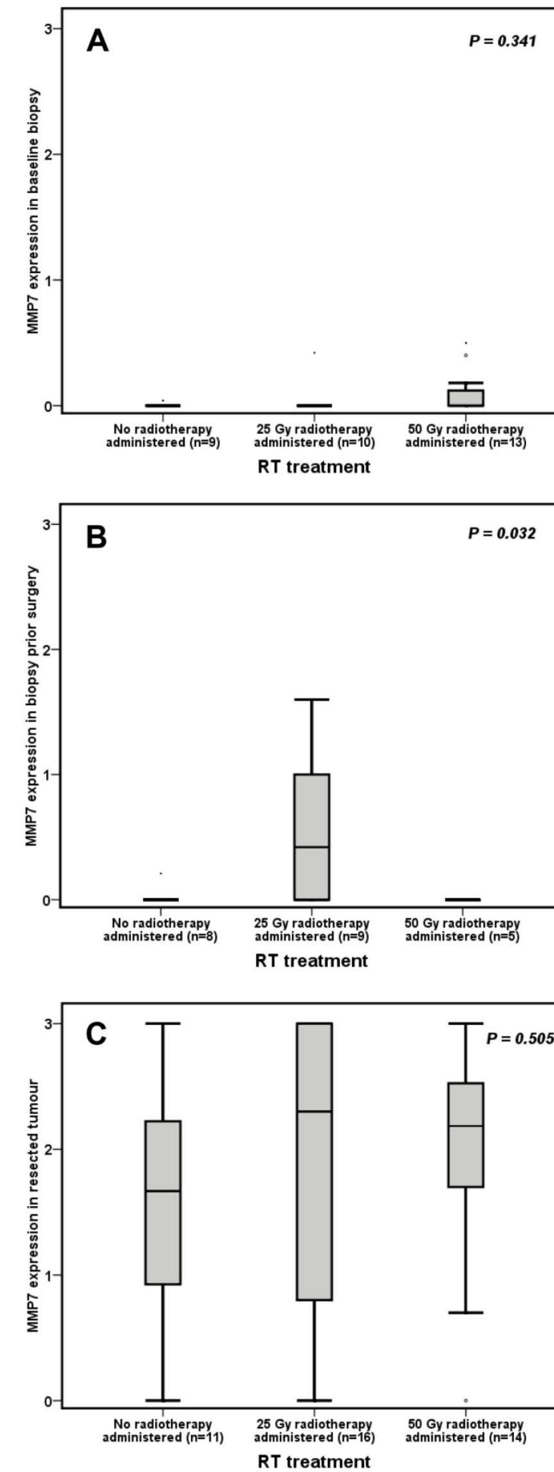

Figure 1. MMP7 tumour expression at baseline, in preoperative biopsy and in the specimen

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