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On Microvascular Inflammation and Intestinal Leakage in Radiation Enteropathy

Andrada Mihăescu

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Som med vederbörligt tillstånd av Medicinska Fakulteten vid Lunds Universitet för avläggande av doktorsexamen i medicinsk vetenskap kommer att offentligen försvaras i aulan, Clinical Research Centre i Malmö, Ingång 72, Skåne Universitetssjukhuset, Malmö, torsdagen den 9 december 2010 kl. 09.00

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Title and subtitle ON MICROVASCULAR INFLAMMATION AND INTESTINAL LEAKAGE IN RADIATION ENTEROPATHY		
<p>Abstract</p> <p>Gastrointestinal tract damage is an insidious feature in patients undergoing radiotherapy. Microvascular inflammation, including leukocyte and platelet recruitment as well as epithelial barrier dysfunction, are considered to constitute key components in the pathophysiology of radiation-induced enteropathy. The aim of this thesis was to investigate the basic mechanisms behind leukocyte-platelet-endothelial cell interactions in the colonic microcirculation and intestinal leakage in response to radiotherapy. It was found that immunoneutralization of P-selectin (CD62P) and P-selectin glycoprotein ligand-1 (CD162) abolished radiation-provoked leukocyte and platelet rolling in the colon. Moreover, inhibition of P-selectin and P-selectin glycoprotein ligand-1 also markedly decreased firm adhesion of leukocyte and platelets, suggesting that a rolling adhesive interaction is a prerequisite for subsequent leukocyte and platelet accumulation in response to radiation. However, inhibition of leukocyte and platelet recruitment or systemic depletion of leukocytes and platelets had no impact on radiation-induced intestinal leakage, suggesting that microvascular inflammation does not cause intestinal barrier dysfunction in radiation enteropathy. The role of p38 mitogen-activated protein kinase and Rho-kinase signalling was next examined by use of specific antagonists. Blocking p38 mitogen-activated protein kinase and Rho-kinase activity markedly decreased radiation-induced leukocyte and platelet recruitment in the colonic microvasculature as well as maintained intestinal integrity in response to radiotherapy. Taken together, this thesis elucidates detailed mechanisms regulating microvascular inflammation and intestinal dysfunction in radiation enteropathy. Thus, these findings not only increase our understanding of pathological tissue changes in response to radiotherapy but may also help to develop more effective and specific protective strategies for patients undergoing radiotherapy.</p>		
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On Microvascular Inflammation and Intestinal Leakage in Radiation Enteropathy

by

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LUND UNIVERSITY
Faculty of Medicine

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In memory of my Mother

*There is a crack in everything.
That's how the light gets in.*
Leonard Cohen

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Abbreviations

AJCC	American Joint Committee on Cancer
DSS	Dextran sulphate sodium
GTP	Guanosine triphosphate
Gy	Gray
ICAM	Intercellular adhesion molecule
IL	Interleukin
i.p.	intraperitoneal
i.v.	intravenous
KC	Cytokine-induced neutrophil chemoattractant
LAD	Leukocyte adhesion deficiency
LFA-1	Lymphocyte function antigen-1
LTB4	Leukotriene B4
MAPK	Mitogen-activated protein kinase
MIP-2	Macrophage inflammatory protein-2
MNL	Mononuclear leukocyte
MPO	Myeloperoxidase
OFR	Oxygen free radicals
PBS	Phosphate buffered saline
PAF	Platelet activating factor
PDGF	Platelet derived growth factor
PECAM	Platelet endothelial cell adhesion molecule
PMNL	Polymorphonuclear leukocytes
PSGL-1	P-selectin glycoprotein ligand-1
RT-PCR	Reverse transcriptase-polymerase chain reaction
TBI	Total body irradiation
TNF- α	Tumor necrosis factor- α
VCAM	Vascular cell adhesion molecule

Original Papers

This thesis is based on the following papers referred to in the text by their Roman numerals:

- I **Critical role of P-selectin and lymphocyte function antigen-1 in radiation-induced leukocyte-endothelial cell interactions in the colon.** Mihaescu A, Thornberg C, Mattsson S, Wang Y, Jeppsson B, Thorlaciuss H. *Diseases of the Colon and Rectum*. 2007;50(12):2194-202.
- II **Radiation-induced platelet-endothelial cell interactions are mediated by P-selectin and PSGL-1 in the colonic microcirculation.** Mihaescu A, Thornberg C, Santén S, Mattsson S, Jeppsson B, Thorlaciuss H. *Submitted to Radiation Research*.
- III **p38 mitogen-activated protein kinase signalling regulates vascular inflammation and epithelial barrier dysfunction in an experimental model of radiation-induced colitis.** Mihaescu A, Santén S, Jeppsson B, Thorlaciuss H. *British Journal of Surgery*. 2010;97(2):226-34.
- IV **Rho-kinase signalling mediates radiation-induced inflammation and intestinal barrier dysfunction.** Mihaescu A, Santén S, Jeppsson B, Thorlaciuss H. *British Journal of Surgery*. 2010; *In press*.

Introduction

Radiotherapy is often used as a curative or adjuvant method in cancer treatment, alone or combined with surgery, chemotherapy or different combinations of these three methods (Colorectal Cancer Collaborative Group, 2001). Most common cancer types can be treated with radiotherapy in some way. The precise treatment intent (curative, adjuvant or palliative) will depend on the type of the tumour, location and stage, as well as the general health of the patient. The target of radiation beam is localised to the region being treated. Unfortunately, radiotherapy harms nearby healthy tissue by inducing inflammatory and other cellular reactions, which sometimes makes it impossible for the affected cells to continue to grow and divide in a normal way (Molla, 2007).

Inflammation represents the tissue response to harmful stimuli. Since Celsus time (ca 30 BC–38 AD) a lot of research has been done about *inflammatio*. He was the first to describe four of the five cardinal signs of local inflammation: *rubor* (redness), *calor* (increased heat), *tumor* (swelling) and *dolor* (pain) while *functio laesa* (loss of function) was added later by Galen. The inflammatory response to various injuries is a complex cascade of events characterized by changes in the microcirculation. It is speculated that inflammation associated with irradiation – including leukocyte and platelet recruitment (Panes et al., 1995) and/or intestinal leakage (Nejdfors et al., 2000), are two of the most important components in the acute phase of radiation enteropathy. Fibrosis represents the clinical consequence of these two phenomena but exactly how these puzzle bits fall together is still not known.

Leukocyte recruitment is a multi-step process, well characterized by a coordinated sequence of ligand-receptor interactions between leukocytes and endothelial cells (Butcher, 1991). A prominent role in inflammation has been attributed to polymorphonuclear leukocytes (PMNLs) that dominate between all other types of leukocytes. PMNLs act as one of the first-responders inflammatory cells to migrate toward the site of inflammation and release cytotoxic substances when activated. Moreover, in a similar way, platelets seem to play an important role in microvascular inflammation (Braun et al., 2008).

Colorectal cancer is the second most common form of cancer and the third leading cause of cancer-related death in the Western world. Radiotherapy is not used routinely in colon cancer treatment because it is difficult to target specific portions of the colon. It is more common for radiation therapy to be used in rectal cancer, since the rectum does not move as much as the colon and is thus easier to target. Almost half of the patients diagnosed with rectal cancer undergo radiotherapy at some point during the course of the disease; for approximately 25% of the patients radiation therapy plays a curable role. The backside of radiotherapy is that it easily leads to enteropathy, which unfortunately may have grave and long time secondary effects for patients even if they are cured from the cancer (Berthrong and Fajardo, 1981).

However, the detailed mechanisms behind microvascular inflammation and intestinal leakage associated with radiation-induced enteropathy remain elusive. A more detailed understanding and ability to control these mechanisms may help the patients, protecting them against the consequences of radiotherapy. Therefore, the purpose of this thesis was to investigate the mechanisms regulating microvascular inflammation and intestinal leakage in radiation enteropathy.

Background

B.1 Radiation

Radiation therapy (also referred to as radiotherapy or irradiation) is the use of ionizing radiation as part of cancer treatment to control malignant cells. Radiation therapy can be administered externally via external beam radiotherapy or internally via brachytherapy. Radiotherapy is mainly used for curative or adjuvant cancer treatment. It can also be used as palliative treatment for local disease control or symptomatic relief (Colorectal Cancer Collaborative Group, 2001).

The effects of radiation therapy are mainly localised to the region being treated. Radiation therapy destroys cells in the area being treated, the "target tissue", by damaging their genetic material, making it impossible for these cells to continue to grow and divide. Although radiation damages both cancer cells and normal cells, most normal cells should recover from the effects of radiation and function properly. The goal of radiotherapy is to damage as many cancer cells as possible, while limiting harm to nearby healthy tissue. Hence, it is given in many fractions, allowing healthy tissue to recover between fractions. Radiation damage is a term associated with ionizing radiation.

Irradiation dosage to each site depends on a number of factors, including the radiosensitivity of each cancer type and whether there are tissues and organs nearby that may be damaged by the treatment. The radiation field may also include the draining lymph nodes if they are clinically or radiologically involved with tumour, or if there is thought to be a risk of malignant spread. It is necessary to include a margin of normal tissue around the tumour to allow for uncertainties in daily set-up and internal tumour motion. These uncertainties can be caused by internal movements (e.g. respiration and bladder filling) and movement of external skin marks relative to the tumour position. To spare normal tissues (such as skin or organs which radiation must pass through in order to treat the tumour), shaped radiation beams are aimed from several angles of exposure to intersect at the tumour, providing a much larger absorbed dose there than in the surrounding healthy tissue.

B.2 Cellular pathophysiology in radiation-induced tissue injury

The pathological processes induced by radiation injury starts immediately after exposure, although the clinical and histological features may not become apparent for weeks, months, or even years after treatment. In the lung, for example, changes detected 6 weeks after irradiation are mild even after a high dose but by 6 months there is widespread fibrosis (Stone et al., 2003). Radiation injury is commonly classified as acute, consequential, or late effects, according to the time before appearance of symptoms. Acute (early) effects are defined as those that are observed during the course of treatment or within a few weeks after treatment. Consequential effects (sometimes called consequential late effects) appear later and are caused by persistent acute damage (Dorr and Hendry, 2001). Late effects emerge months to years after radiation exposure. The terms acute and late have been used for convenience in radiation therapy but because the underlying molecular and cellular processes are complex and lead to a range of events, the definitions may be more operational than mechanistic (Denham et al., 2001). Early symptoms may not be apparent in some organs that develop late injury, such as the kidney, where trauma or surgery months or years after irradiation can precipitate acute breakdown of tissue that has been functioning normally (Stone et al., 2003). Acute radiation damage is most prominent in tissues with rapidly proliferating cells, such as in the alimentary tract and symptoms develop when functional cells are lost. The functional cells are not replaced because of the damage to the stem-cell compartment. In tissues such as the gut, there is compensatory proliferation within the stem cells, which are more tolerant to radiation than other types of cells, followed by replacement of functional cells and recovery. Symptoms therefore generally subside, often during the course of radiotherapy. The ionisation events and free radicals produced by radiation cause damage to vital cellular components. DNA damage from radiation commonly leads to death of cells in the first cell division after irradiation or within the first few divisions (Thompson and Suit, 1969).

Cell death during mitosis (mitotic death) is generally caused by unrepaired or improperly repaired chromosomal damage (Dewey et al., 1970). Cell death may also occur by apoptosis. Certain cell types, especially lymphocytes, spermatogonia, and serous cells in the salivary gland undergo apoptosis during interphase after irradiation (Hendry and West, 1997; Stephens et al., 1991). This type of death is rapid and is often associated with cells in specific locations within tissues, for example, in proliferative cells of intestinal crypts (Potten, 1992). Cells may also leave the reproductive pool by differentiation rather than proliferation (Steel, 2001). This senescence may be a particularly important response of fibroblasts, resulting in excess collagen deposition and fibrosis.

Radiation activates various cellular signalling pathways (Dent et al., 2003) that lead to expression and activation of proinflammatory and profibrotic cytokines (Chen et al., 2002; Rubin et al., 1995; Fu et al., 2001), vascular injury (Paris et al., 2001), and activation of the coagulation cascade (Hauer-Jensen et al., 1999). These changes may

be involved in the development of oedema, inflammatory responses and the initiation of wound-healing processes (Stone et al., 2003).

B.3 Colorectal cancer

The worldwide incidence of colorectal cancer has been increasing significantly during the last three decades. In Sweden, for example, each year more than 5500 new cases are diagnosed. One of the reasons is the increased age of the population. More than 70% of the rectal cancer patients are older than 65 year.

The mechanisms involved in colorectal cancer development are not well known. Genetic factors are believed to have an increased impact in developing the disease. The incidence varies geographically which makes the environment important as well. Another factor is adenoma, a neoplastic lesion that is precancerous in most colorectal cancers (Morson, 1974; Muto et al., 1975). The grades of dysplasia and histological types of adenomas are classified according to the criteria of the World Health Organization (Winawer et al., 1992). Dysplasia is usually graded as low or high grade on the basis of the degree of cytological atypia and the presence of any architectural abnormality in the gland. Severe dysplasia and carcinoma in situ are categorized as high-grade dysplasia, which in time may develop into cancer.

The treatment of colorectal cancer is complex including surgery associated with radio- or chemotherapy. In 1914 Symonds reported the first successful treatment of rectal cancer employing radiotherapy. So far, only trials using a total dose more than 20 Gy for preoperative radiotherapy have demonstrated lower recurrence rates compared with surgery alone (Wheeler et al., 1999). Radiotherapy indications for rectal cancer include neoadjuvant, adjuvant or palliative regimens (Glimelius, 2006). Neoadjuvant indication applies in order to decrease the risk of recurrence following surgery or to allow less invasive surgical approaches (such as a low anterior resection instead of an abdominoperineal resection). Adjuvant regimens are indicated when the tumour perforates the rectum or involves regional lymph nodes (AJCC T3 or T4 tumours or Dukes' B or C tumours) and palliative regimens are indicated to decrease the tumour burden in order to relieve or prevent symptoms.

The radiotherapeutic regimens used in Sweden for treatment of rectal cancer are: the short preoperative cure consisting of 25 Gy/week (5 Gy x 5 days) (Dahlberg et al., 1998), the long preoperative cure 50 Gy/5 weeks (2 Gy x 25 days) ± chemotherapy and long radiotherapy postoperative cure ± chemotherapy.

B.4 Microvascular inflammation

B.4.1 Leukocyte recruitment

The prototype of leukocyte recruitment

There are different types of leukocytes, including neutrophils (polymorphonuclear leukocytes, PMNLs), band cells (slightly immature neutrophils), T-type lymphocytes (T cells), B-type lymphocytes (B cells), monocytes, eosinophils, and basophils. All the types of white blood cells are reflected in the white blood cell count. During the acute phase of inflammation, PMNLs (aprox. 60% of leukocytes) are one of the first-responders of inflammatory cells to migrate to the site of inflammation, being recruited within minutes. Other leukocytes types, as T cells, are essential for innate and adaptive immune systems and potential important for immune surveillance against tumour cells (de Saint Basile et al., 2010). Thus, our focus in this thesis is on neutrophils and not on the T-cells.

Leukocyte recruitment from microvessels into tissues is a wellregulated and rate-limiting process governed by a remarkable complexity of molecular interactions. The cascade contains at least four steps: tethering, rolling, adhesion and transmigration. Each of these steps appears to be necessary for effective leukocyte recruitment, because blocking any of these steps may severely reduce leukocyte accumulation in the tissue.

Most of our current knowledge of the molecular mediators of leukocyte recruitment has been derived from adhesion assays and studies of the microcirculation. Three classes of adhesion molecules which are involved in the recruitment cascade are described: selectins, integrins and immunoglobulin supergene family (Ley, 1996).

Leukocyte rolling

As a part of the multistep process, the selectins promote the initial attachment (tethering) and subsequent movement (rolling) of leukocytes in microvessels where they become activated as a consequence of exposure to locally produced chemokines. Leukocyte rolling is the process when the white blood cells that normally move through microvessels, slow down 40-50 times and tend to move into a position close to the endothelial surface (Schmid-Schonbein et al., 1980) due to hemodynamic factors in the microcirculation. This allows the leukocytes to come into closer proximity of the endothelium and increases the possibility of interactions between the leukocyte and endothelium, which consequently increases the likelihood of leukocyte activation.

Leukocytes can be initiated to roll by many pro-inflammatory agents such as histamine, thrombin or oxygen free radicals (Thorlacius et al., 1994; Tedder et al., 1995). Rolling leukocytes are not always committed to either firmly adhere to the vessel wall or roll along the entire vessel length; they frequently detach and return to the mainstream of flowing blood.

The rolling process is predominantly mediated by the selectin family of adhesion molecules, which is represented by a class of calcium-dependent glycoproteins and

comprise three members, i.e. L-, E- and P-selectin. P-selectin seems to play a crucial role in leukocyte rolling in the intestine (Thorlacius et al., 1994; Mansson et al., 2000; Klintman et al., 2002; Wan et al., 2002).

P-selectin

P-selectin (CD62P, GMP-140 or PADGEM) has a molecular weight of 86-140 kDa depending of the amount of $-NH_2$ terminal glycosylation. It can be found on the surfaces of activated endothelial cells, which line the inner surface of blood vessels, and on activated platelets. In non-activated endothelial cells it is stored in Weibel-Palade bodies and in α -granules of non-activated platelets (Panes and Granger, 1998).

Upon stimulation, the initial response occurs within minutes from the pre-formed stores by fusion of the Weibel-Palade granules with the plasma membrane. Another peak of activation occurs several hours later and is a transcriptionally regulated event, if the inflammatory stimulus persists (Panes and Granger, 1998), with a higher impact compared to the initial one.

The importance of P-selectin-mediated leukocyte recruitment has been demonstrated in different experimental models such as DSS-colitis (Wan et al., 2002), LPS-induced liver damage (Laschke et al., 2007) and ischaemia-reperfusion of the intestine (Santen et al., 2007) where functional blockade with specific antibodies reduced leukocyte rolling and leukocyte adhesion significantly. Interestingly, Molla *et al.* (Molla et al., 2001) reported that radiation-induced leukocyte adhesion in the intestine was intact in P-selectin-deficient mice and concluded that single blockade of P-selectin may not be sufficient to inhibit radiation-induced leukocyte adhesion (Molla et al., 2001). More recent publications have shown that P-selectin-deficient mice are compensated by increased expression of other adhesion molecules, including vascular cell adhesion molecule-1 (VCAM-1) (Molla et al., 2003), which may explain the intact radiation-induced leukocyte adhesion responses observed in these animals. Thus, further studies on the role of P-selectin in mediating radiation-induced leukocyte-endothelium interactions are warranted.

PSGL-1

The ligands for the selectins are highly glycosylated proteins, bearing the tetrasaccharides sialyl Lewis X (sLe^x) and sialyl Lewis A (sLe^a), which present ligand activity for all selectins (Panes and Granger, 1998). The critical role of the selectin ligands is illustrated by the leukocyte adhesion deficiency syndrome II (LAD II) where no sLe^x is found on leukocytes. The clinical manifestations of LAD II are recurrent infections since PMNLs are unable to interact with vascular endothelial selectins, which in turn compromise the extravascular leukocyte recruitment (Etzioni et al., 1992; von Andrian et al., 1993).

PSGL-1 is a dimeric sialomucin glycoprotein and the best characterized high-affinity selectin ligand. It is expressed on almost all haematopoietic cells but in a functional state on a limited group of circulating cells, including neutrophils, basophils, eosinophils, dendritic cells and memory/effector T-cells. PSGL-1 is the only known high-affinity

ligand for P-selectin but has also affinity for L- and E-selectin (Sako et al., 1993). The importance of PSGL-1 has been investigated in ischaemia-reperfusion models of the heart (Hayward et al., 1999), liver (Dulkanchainun et al., 1998) and colon (Santen et al., 2007), where its blockade was shown to reduce tissue damage.

Leukocyte adhesion

Leukocyte rolling is a prerequisite to firm adhesion, as it was demonstrated *in vitro* by Lawrence and Springer using surrogate blood vessels and activated neutrophils (Lawrence and Springer, 1991). Firm adhesion of leukocytes, mediated by integrins, precedes their extravasations into the underlying tissue. The mechanism behind the activation of integrins in leukocytes is not clear, but it is thought to be regulated by intracellular calcium (Vandenberghe and Ceuppens, 1990). Chemokines, PAF, LTB₄, OLRs or C₅ are some of the factors incriminated to activate leukocyte integrins (Tonnesen, 1989).

LFA-1

Leukocyte adhesion to endothelial cells is mainly mediated by a group of heterodimers referred to as β_2 -integrins, which consist of a common β -subunit (CD18) but different α -subunits (CD11a–c) such as LFA-1 (CD11a), Mac-1 (CD11b) and p150 95 (CD11c). β_2 -integrins are located on the leukocyte surface and once activated bind to members of the immunoglobulin gene superfamily, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells thereby promoting strong adhesive interactions and complete arrest of rolling cells.

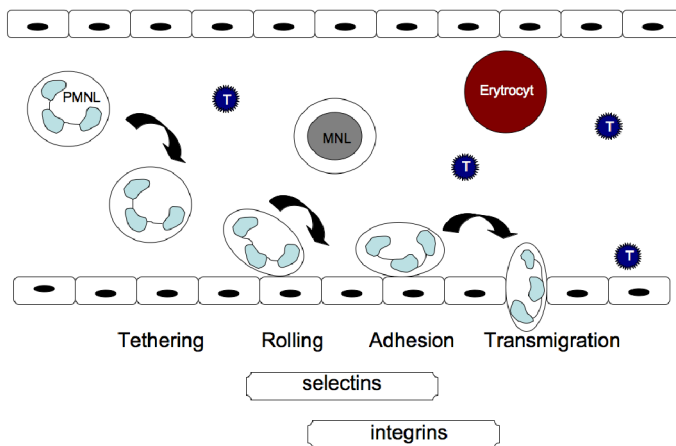
LFA-1 (CD11a/CD18) is constitutively expressed on leukocytes and upon activation there is no increased expression but a change from a “low avidity” latent state to a “high avidity” state by a molecular clustering. Additionally LFA-1 undergoes a conformational change, which also contributes to this “high avidity” state.

The significance and functionality of CD18 is illustrated in the clinic by the leukocyte adhesion deficiency I syndrome (LAD I), a rare autosomal recessive disorder characterized by a mutation in the gene encoding the common CD 18 subunit and results in the absence of β_2 -integrins (Anderson and Springer, 1987). The result of this mutation is the inability to recruit neutrophils. This appears because the initial rolling leukocytes are unable to switch to the “high-avidity” state for further ligand binding, which in turn makes leukocyte arrestment impossible (Kuijpers et al., 1997). Clinical signs of this disorder are leukocytosis and an increased susceptibility to bacterial infections (Harlan, 1993). Genetic manipulation of LFA-1 has greatly contributed to the understanding of the function of this adhesion molecule in leukocyte recruitment in liver endotoxemia (Li et al., 2004) and in ischaemia-reperfusion (Riaz et al., 2002). Although, the specific role of LFA-1 in radiation-induced enteropathy has not been clarified so far.

Leukocyte transmigration

The last step in the leukocyte recruitment process is transmigration into the inflamed tissue. The movement of adherent leukocytes towards the endothelial intercellular junctions is committed with the help of pseudopod-like extensions. Leukocytes are able to modulate their form in order to squeeze in between the endothelial cells and to pass into the extravascular space. This movement requires several endothelial cell adhesion molecules where PECAM-1 seems to play a critical role (Muller et al., 1993; Newman, 1997).

The paracellular pathway, described above, is considered to be the most common way for leukocytes to transmigrate into the inflamed tissue, although other routes are described, *i.e.* by crossing the endothelial cells either by transcytotic migration (Feng et al., 1998) or via pre-existing holes (Walker et al., 1995) in endothelial cells.



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B.4.2 CXC chemokines

The activation and migration of leukocytes into inflamed tissues are regulated by *chemo*-attractant *cytokines*, *i.e.* *chemokines*, a group of low molecular-weight proteins that are subdivided into four subfamilies: C, CC, CXC and CX3C chemokines, based on the number and spacing of the amino-terminal conserved cysteine residues. The biological effects of chemokines result from their binding to the cognate receptors, seven transmembrane G protein-coupled receptors. Over 40 chemokines and 19 receptors have been identified so far, for which complex ligand-receptor relationships are proposed with high redundancy (Yoshie et al., 2001).

The CXC chemokines such as MIP-2 and KC are thought to play a crucial role in neutrophil recruitment (Mantovani, 1999; Zlotnik et al., 1999) during different patho-

logical inflammatory conditions, including radiation-induced enteropathy (Matsumura and Demaria, 2010).

MIP-2 and KC

Mouse MIP-2 cDNA encodes a 100 amino acid residue precursor protein from which the amino-terminal 27 amino acid residues are cleaved to generate the mature mouse MIP-2. The protein sequence of mouse MIP-2 shows approximately 63% identity to that of mouse KC, another mouse alpha chemokine. Mouse KC cDNA encodes a 96 amino acid residue precursor protein from which the amino-terminal 19 amino acid residues are cleaved to generate the 77 amino acid residue mature KC. Based on these protein sequence similarities and since chemokines with protein sequence homology to human IL-8 have not been identified in mice, it has been suggested that the mouse KC and MIP-2 are functional homologues of human IL-8 in mice (Wuyts et al., 1996). A putative mouse homologue of the human IL-8 receptor beta (IL-8 Rb) has also been cloned. This receptor shows 71% identity to human IL-8 Rb and 68% identity to human IL-8 Ra. Both mouse KC and MIP-2 bind mouse IL-8 Rb with high affinity (Heinrich and Bravo, 1995).

Like human IL-8, mouse MIP-2 and KC exhibits potent neutrophil chemotactic activity and has been shown to be mediators of neutrophil recruitment in response to tissue injury and infection (Driscoll, 1994). Moreover, increased MIP-2 and KC expression has been found to be associated with neutrophil influx in various inflammatory conditions (Standiford et al., 1996; Su et al., 1996; Greenberger et al., 1996; Seebach et al., 1995) and in radiotherapy (Matsumura and Demaria, 2010; Schmal et al., 1996).

B.4.3 Platelet recruitment

Platelets are regularly-shaped anuclear cell fragments, 2-3 μm in diameter, derived from fragmentation of megakaryocytes in the bone marrow (Italiano and Shivdasani, 2003). The average lifespan of a platelet is normally just 5 to 9 days. Platelets play a fundamental role in haemostasis and thrombosis and lately emerging evidence show their crucial role in inflammation and tissue injury (von Hundelshausen and Weber, 2007) as well. Earlier studies suggest that platelets may undergo similar adhesive steps as leukocytes in the inflammatory process (Klinger, 1997b; Mordon 2002).

On activation, platelets exhibit the ability to release considerable quantities of secretory products and express a multitude of immune receptors on their membrane (von Hundelshausen and Weber, 2007). Some studies summarize the most important findings on the role of platelets according to their molecular key constituents with immune and/or inflammatory relevance, laying out a “toolbox” for the participation of platelets as immune cells and give an update of the intensively investigated involvement of platelets in inflammation and the immune system (von Hundelshausen and Weber, 2007).

Platelets are of particular interest in radiation-induced enteropathy as they have the capacity to exert both proinflammatory and profibrogenic effects (Klinger, 1997b; Mordon 2002). Previous reports have shown that anti-platelet agents, such as ticlopidine, clopidogrel and acetylsalicylic acid can ameliorate radiation-induced intestinal inflammation and injury (Akyurek et al., 2006; Wang et al., 2002; Mennie and Dalley, 1973), indirectly suggesting a role of platelets in the pathophysiology of radiation-induced enteropathy.

B.5 Intracellular signalling pathways

Major biological responses to inflammatory stimuli are integrated and conveyed by specific intracellular signals in radiation (Dent, 2003; Dent et al., 2003).

B.5.1 p38 MAPK

The mitogen-activated protein kinase (MAPK) cascade is a highly conserved module that is involved in various cellular functions, including cell proliferation, differentiation and migration and represent one of the major systems used by eukaryotic cells to transduce extracellular stimuli into intracellular and intranuclear responses (Kyriakis and Avruch, 2001). Mammals express at least four distinctly regulated groups of MAPKs, extracellular signal-related kinases (ERK)-1/2, Jun amino-terminal kinases (JNK1/2/3), p38 proteins (p38 α /beta/gamma/delta) and ERK5, that are activated by specific MAPKKs. Each MAPKK can be activated by more than one MAPKKK, increasing the complexity and diversity of MAPK signalling. Presumably each MAPKKK confers responsiveness to distinct stimuli including ionizing radiation, microbial infection and ischaemic injury (Kyriakis and Avruch, 2001).

The p38 MAPK was originally described as p38 α , a 38 kDa polypeptide that underwent Tyr phosphorylation in response to endotoxin treatment and osmotic shock (Han et al., 1994). Later on, three other forms, p38 β , p38 γ and p38 δ were identified (Freshney et al., 1994). p38 MAPKs are members of the MAPK family that are activated by a variety of environmental stress and inflammatory cytokines. Activation of the p38 MAPK pathway has been implicated in the formation of pro-inflammatory cytokines, such as tumour necrosis factor- α (TNF- α), interleukin-6 (IL-6) and the CXC chemokine IL-8 (Underwood et al., 2000; Klintman, 2005; Lee et al., 1994).

Pyridinyl-imidazole anti-inflammatory drugs, such as SB203580 or SB239063, has been extensively investigated in different inflammatory conditions (Lee et al., 1994; Underwood et al., 2000). Emerging data shows that inhibition of p38 MAPK successfully reduced radiation-induced fibroblast differentiation in a lung model, suggesting that p38MAPK blockade could be a potential strategy to disrupt fibrotic processes associated with radiotherapy (Park et al., 2010). Moreover, cell culture experiments showed that cytokine-provoked epithelial cell permeability is dependent on p38MAPK *in vitro*

(Werz et al., 2001), which in turn raised speculations that p38-MAPK may be involved in epithelial barrier dysfunction after radiotherapy.

B.5.2 Rho kinase

Intracellular signalling pathways mediated by small GTP-binding proteins has captured the attention in the molecular biology field during the last ten years (Fukata et al., 2001; Shimokawa and Takeshita, 2005) as important regulators of fundamental cellular processes including proliferation, differentiation, adhesion, migration and apoptosis (Narumiya et al., 1997; Etienne-Manneville and Hall, 2002). Important members of this group are the Rho GTPases family of proteins such as Rac, Rho, Cdc42 (Hall, 2005). Like all regulatory GTPases, these proteins exist as inactive GDP-bound and an active GTP-bound conformation (Hall, 2005).

One of the most studied and best characterized effectors of the Rho family are the Rho-kinases (ROCKs). Two ROCK isoforms have been identified, ROCK I and ROCK II (Leung et al., 1995; Ishizaki et al., 1996). The two isoforms are present in the human, rat and mouse tissues although ROCK I is mostly pronounced in the liver, testes and kidney where ROCK II is prominent in the brain and striated muscle (Ishizaki et al., 1996; Leung et al., 1996). ROCK inhibitors, such as Y-27632, which was used in our study, bind the kinase domain and inhibit both ROCKs with similar potency (Ishizaki et al., 1996; Breitenlechner et al., 2003). The molecular formula of ROCKs is characterized by a N-terminal catalytic kinase domain, a coiled-coil and C-terminal domain (Ishizaki et al., 1996; Shi and Wei, 2007) where the C-terminal-domain acting as a Rho-binding site upon activation (Ishizaki et al., 1996).

Initially, the Rho/ROCK pathway was investigated in cardiovascular diseases (Shimokawa and Takeshita, 2005; Shimokawa and Rashid, 2007) mostly because this particular intracellular pathway is correlated with angiotensin II, thrombin and platelet-derived growth-factor (PDGF). For example, pre-treatment with Y27632 in a coronary occlusion model, reduced the infarction area dose-dependently and attenuated the levels of KC and IL-6 (Bao et al., 2004). More recent studies show that the Rho-kinase signalling pathway seems to be involved in inflammatory processes with a particular role in chemokine expression, leukocyte-endothelial cell interaction associated to colonic ischemia-reperfusion (Santen et al. 2010) and LPS-induced platelet capture to the endothelium (Slotta et al., 2006 and 2008; Thorlacius et al., 2006). Clinical trials where patients have been treated with fasudil or Y27632 have shown promising results in pulmonary hypertension and to prevent cardiovascular injuries (Ishikura et al., 2006; Mohri et al., 2003).

Interestingly, it has been shown that ROCKs are involved in the maintenance of fibrosis in the bowel wall of patients with late radiation enteritis (Bourgier et al., 2005). Moreover, several studies suggest that Rho-kinase function plays an important role in regulating the integrity of epithelial barriers (Popoff and Geny, 2009). In this context, it is also interesting to note that inhibition of Rho-kinase activity ameliorates radiation-provoked fibrosis (Bourgier et al., 2005). Thus, we hypothesized in this thesis that Rho-

kinase signalling may play a central role in mediating microvascular intestinal inflammation and maintenance of epithelial barrier function in response to radiotherapy.

B.5.3 Oncological aspects on p38 MAPK and Rho kinase activity

During the last few years it has been speculated that different signalling pathways, such as p38 MAPK and Rho kinase, are involved in cancer disease by their direct implications in cell alterations (Cuenda and Rousseau, 2007; Sahai and Marshall, 2002). For example, it has been reported that p38 MAPK is involved in the growth-inhibitory signalling cascade of contact inhibition in fibroblasts. This novel physiological function of p38 MAPK in cell cycle control provides further support for the idea that p38 may act as suppressor of tumorigenesis (Faust et al., 2005). A clear example in this sense is the role of p38 MAPK in inducing terminal differentiation and inhibiting proliferation of rhabdomyosarcoma-derived cells, one of the more common solid tumours of childhood, as a consequence of defects in differentiation of muscle precursor cells. This defect is attributed to deficiency in p38 MAPK activity (Puri et al., 2000). Moreover, it has been proposed that p38 MAPK is involved in restraining uncontrolled cell proliferation. This was clearly established in a recent report demonstrating that a key function for the p38 MAPK in preventing tumorigenesis is to promote growth arrest and apoptosis specifically in response to reactive oxygen species (Dolado et al., 2007). In contrast, several other groups discussed the role of p38 MAPK pathway as a tumour suppressor, but only a few publications have provided evidence for an oncogenic potential of this pathway (Rennefahrt et al., 2005). This could involve supporting tumour growth and metastasis via the regulation of angiogenesis and cell invasion. Growth of tumours beyond a certain size results in hypoxia and requires the formation of new blood vessels for further growth, which is controlled through the production and secretion of angiogenic factors. p38 MAPK is activated by hypoxic conditions and is involved in the production of VEGF (Pages et al., 2000). Interestingly, it was shown that p38 MAPK also plays a role in the downstream signalling of VEGF leading to angiogenesis (Rousseau et al., 2000; Ringshausen et al., 2004).

It has been reported that Rho kinase signalling plays an important role in oncogenesis. For example, increased ROCK II levels has been shown in hepatocellular (Wong et al., 2009), colon (Vishnubhotla et al., 2007), and bladder (Kamai et al., 2003) cancer. A study of 41 pairs of hepatocellular carcinomas revealed that ROCK II is frequently overexpressed as compared to non-tumorous livers, while ROCK II expression is unaltered. Silencing of ROCK II by short-hairpin RNA reduces stress fiber formation, phosphorylation of MYPT1, migration and invasion *in vitro*, and lung metastasis *in vivo* (Wong et al., 2009). In contrast, ROCK I expression levels, but not ROCK II, are significantly higher in human mammary tumours and are associated with poor clinical outcome and overall survival of patients (Lane et al., 2008). Elevated ROCK I levels were recently reported to be involved in the transformation of hormone refractory prostate cancer (Aouadi et al., 2009).

Moreover, it has been shown that inhibition of the kinase activity of the Rho effector ROCK reduces the metastatic potential of tumour cells in animal models (Kiddera et al. 2010). In addition, it was shown that administration of a pharmacological inhibitor of ROCK activity prevents the intraperitoneal dissemination of hepatocellular carcinoma cells and causes them to revert to an expansive, less aggressive mode of growth in the liver (Itoh et al., 1999; Murata et al., 2001; Somlyo et al., 2000). The requirement for RHO/ROCK signalling in endothelial cells for cells to cross endothelial-cell barriers raises the possibility that RHO proteins might also be useful anti-tumour targets in the stromal cells; which has potential benefits because these cells are genetically normal and not prone to drug resistance.

It is known that Rho-protein function contributes to both the loss of growth control and the invasive phenotype of some tumour cells. So, Rho proteins might be particularly attractive targets for therapy, as they affect many aspects of tumourigenesis. However, some of the studies indicate that reduced Rho-protein function contributes to the morphological changes observed in tumour cells, which raises the dangerous possibility that inhibition of Rho proteins might promote a more aggressive tumour phenotype. Even if this is the case for some tumour types, antagonizing Rho function might block tumour-cell proliferation and therefore have clinical benefit. Such observations indicate that it might be necessary to target particular types or stages of tumours, or specific Rho-activating or Rho-effector pathways. The requirement for survival signals to prevent apoptosis in normal cells is reduced in tumour cells. Rho proteins have been implicated in both pro- and anti-apoptotic signalling, and in the apoptotic process itself (Coleman and Olson, 2002).

B.6 Intestinal permeability and fibrosis

In general, the concept of permeability relates to that property of a membrane that enables passage of a solute by unmediated diffusion. The diffusion of the solute across a simple membrane is determined both by the structure (composition, charge, thickness) of the membrane, the physicochemical properties of the solute and its interaction with the media (Bjarnason et al., 1995). Mucosal barrier injury is one of the most debilitating side effects of radiotherapy and chemotherapy treatment (Bellm et al., 2000). The intestinal crypt epithelium is sensitive to radiation, and within hours after a single exposure to even a low dose of radiation, there is an increase in the number of cells that progress to death (Potten and Booth, 1997). Morphologic alterations such as villi shortening, crypt depletion, disruption of tight-junctions between the epithelial cells, submucosal oedema, and increased infiltration of the lamina propria with inflammatory cells have been observed in the intestine, one to three days after exposure to gamma radiation (Porvaznik, 1979; Buell and Harding, 1989; Herrera et al., 1995). Functional effects, such as alterations in fluid and electrolyte transport, have been demonstrated in rat colon (Dublineau et al., 1998) and in canine ileum, (Herrera et al., 1995) three to four days after irradiation. The effects on the barrier properties of the intestinal mucosa

after ionizing radiation has also been studied on the human colorectal mucosa and it was found that ionizing radiation may inflict damage to the intestinal epithelial barrier function and that this is dose, rate and time dependent (Nejdfors et al., 2000).

The clinical consequence of the dysfunctional barrier is intestinal fibrosis, a chaotic wound healing process, which in time easily might give complications in form of strictures and/or perforation. Wound healing is a sequence of several basic processes including inflammation, cell proliferation, matrix formation and remodelling, angiogenesis, wound contraction and epithelialization. Irradiation may cause failure of this coordinated serial of events and this may cause complications including strictures, wound dehiscence, bowel perforation, fistula, abscess and hemorrhage. Increased intestinal permeability after exposure to radiation is the consequence of the acute inflammatory processes and may lead in time to a chaotic healing process. This is believed to be the direct consequence of radiation on mast cells (Park et al. 2010) and endothelial cells which, in turn, increase generation of thrombin, release of histamine and prostaglandins I₂ and E₂ and facilitated by neutrophil adhesion to the endothelial surface in the hours following radiation exposure (Dunn et al., 1986; Molla et al., 1999; Panes et al., 1995). Moreover, an increased barrier dysfunction may result in the passage of potentially harmful substances, which may explain some of the complications associated to irradiation such as bacterial passage into the abdominal cavity. The interrelationship between microvascular inflammation on one hand and intestinal leakage on the other hand is not known, and is one of the aims to elucidate in this thesis.

Aims

1. To study the mechanisms of leukocyte recruitment in colonic microvascular inflammation induced by radiation.
2. To investigate the mechanisms behind radiation-provoked platelet-endothelial interactions in the large intestine.
3. To define the role of p38 MAPK in colonic vascular inflammation and intestinal barrier dysfunction induced by radiation.
4. To examine the molecular mechanisms of Rho-kinase regulated leukocyte and platelet responses and intestinal leakage in the radiated colon.
5. To study the relationship between leukocyte and platelet recruitment on one hand and intestinal leakage on the other hand in radiation enteropathy.

Materials and Methods

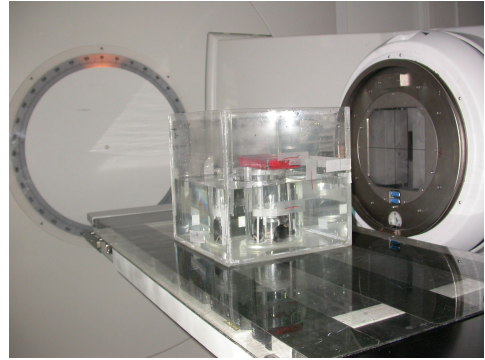
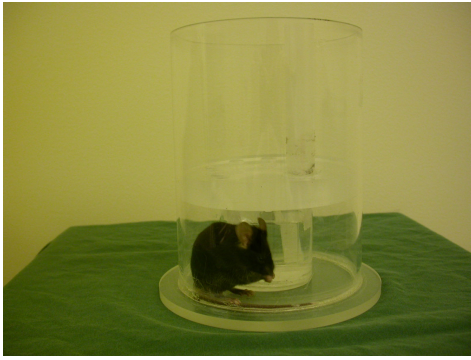
M.1 Animals

All studies have been performed on mice. Male C57BL/6J (I, II, III, IV) and LFA-1-deficient mice (I) weighing 22 to 26 g were kept under standard laboratory conditions during the experiments. Animals were maintained on a 12-hour light/dark cycle and allowed free access to animal chow and tap water. The accommodation period under these conditions was 1 week before use in the experiments. All experimental procedures were performed in accordance with legislation on the protection of animals and were approved by the Ethical Committee on Animal Experiments in Lund.

M.2 Experimental models

Total Body Irradiation

For the pilot study (I) we developed a protocol in collaboration with the Department of Radiation Physics, Malmö University Hospital. Animals were placed in a special designed cylinder, which was lowered into a cubic device containing water for immersion. Then TBI was performed by administration of a single bolus with a linear accelerator producing 6 MV photons (Elekta SL Series linear accelerator, Elekta, Crawley, UK) and indicated doses were given at 3.7 Gy per minute. This experiment was performed at the Department of Radiation Physics at Malmö University Hospital.



In the pilot experiment, we analysed leukocyte recruitment in intravital microscopy by evaluating different doses of radiation (0–20 Gy) at 16 h after exposure. Once 20 Gy had been established as a reproducible and effective dose, it was evaluated at different time points (0–16 h). We found that administration of 20 Gy caused maximal radiation-induced inflammation in the colon at 16 h after exposure and this protocol was used in all later experiments.

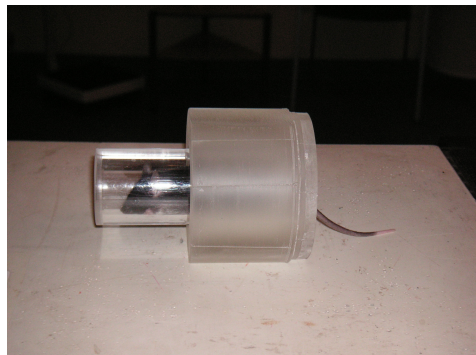
In order to further develop our experimental TBI model a refined protocol was used in study III and IV. TBI was performed in a Gammacell 40 Exactor (MDS Nordion, Ottawa, Ontario, Canada) in anaesthetized mice. This experiment was performed in the Animal Department at the Clinical Research Centre, Malmö. The administered dose of 20 Gy was given at 1.09 Gy per minute.



Abdominal Radiation

This protocol was also developed in collaboration with our colleagues at the Department of Radiation Physics, Malmö University Hospital, as an extension to our first protocol. Anaesthetized animals received abdominal irradiation as a single 20 Gy dose administered with the same linear accelerator producing 6 MV photons (Elekta SL Series linear accelerator, Elekta, Crawley, UK) and was given at 3.7 Gy/min. For this purpose, mice were placed in a special designed cylinder, which covered the area of the body

between processus xiphoideus and the tail with respect for the head, neck and thoracic cavity. The cylinder was designed to spread the radiation equally to the exposed abdominal surface (II).



Antibodies and Drugs

A mixture of 7.5 mg ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100 g body weight was administered intraperitoneally (i.p.) in order to induce anaesthesia and analgesia (I, II, III, IV). The animals were kept on a heating pad (37°C) during the experiments. All antibodies were administered intravenously (i.v.) in the tail vein (I, II).

Immunoneutralization of selectins or their ligands was achieved by injection of a monoclonal antibody directed against mouse P-selectin (RB 40.34, rat IgG, 40 µg /mouse, R&D Systems, Europe) or against mouse PSGL-1 (α-PSGL-1 ab, anti-mouse CD162, 40 µg/mouse, R&D Systems, Europe) immediately before exposure to 20 Gy radiation (I, II). We also used a control group, which was pretreated with an isotype-matched control antibody (R3-34, rat IgG, 40 µg/mouse, R&D Systems, Europe) (I, II).

As described in the second paper, in order to delineate the role of platelet depletion in radiation-induced intestinal leakage, mice were i.v. pretreated 2 h prior to irradiation with the anti-GP1bα antibody (rat IgG, 1 mg/kg, Emfret Analytics GmbH & Co.KG, Würzburg, Germany) and to define the contribution of leukocytes, mice received an i.v. anti-Ly-6G antibody (Gr-1, Rat IgG2b, 6 mg/kg, eBioscience, Frankfurt, Germany), divided in 3mg/kg at 24 h before radiation exposure and another 3mg/kg immediately prior to irradiation (II).

To investigate the role of p38MAPK activity in radiation-induced leukocyte rolling and adhesion, we used a highly selective p38 MAPK inhibitor, SB239063 (*trans*-1-[4-hydroxycyclohexyl]-4-[4-fluorophenyl]-5-[2-methoxypyridimin-4-yl]-imidazole) (Sigma Chemical CO., St Louis, USA). For this purpose, SB239063 (0.4 and 4.0 mg/kg) or vehicle (150 µl acidified 0.5 % tragacanth; Sigma Chemical Co.) was admin-

istered intravenously in the tail vein immediately before the animals were irradiated (III).

To characterize the role of Rho-kinase activity in radiation-induced leukocyte rolling and adhesion, 1 mg/kg and 10 mg/kg of the selective Rho-kinase inhibitor Y27632 (Sigma Chemical Co.) or vehicle (PBS) was administered i.p. immediately prior to irradiation (IV).

Leukocytes and platelets were stained by intravenous administration of 0.5 mg/ml rhodamine-6G (125 μ l/mouse; Sigma Chemical Co) into the retro-orbital sinus (I, II, III, IV).

M.3 Inverted intravital fluorescence microscopy

The colonic microcirculation was examined using an inverted Olympus microscope (IX70; Olympus Optical, Hamburg, Germany) equipped with different lenses ($\times 10$ /numerical aperture (NA) 0.25 and $\times 40$ /NA 0.60). The microscopic image was televised using a charge-coupled device video camera (FK 6990 Cohu; Pieper, Schwerte, Germany) and recorded on videotape (I) or converted via ADVC-55 (Canopus, Thomson, Paris, France) for recording on a computer for subsequent offline analysis (II-IV).

Anaesthetized animals were placed in the supine position on a heating pad (37°C) to maintain body temperature. Leukocytes and platelets were stained by intravenous administration of 0.5 mg/ml rhodamine-6G (125 μ l/mouse; Sigma Chemical). A midline laparotomy was performed and the colon gently exteriorized. After positioning under the microscope, an equilibration period of 5 min was allowed before starting microscopic observation of the colonic microcirculation 5 cm proximal to the anus. Analysis of leukocyte- and platelet-endothelium interactions (rolling and adhesion) was conducted in colonic venules (inner diameter 15–30 μ m) with stable resting blood flow. Quantification of microcirculatory parameters was performed offline by frame-to-frame analysis of the images. Leukocyte and platelet rolling was determined by counting the number of such cells passing a reference point in the venule per 20 s, and expressed as cells per minute. Firm adhesion was measured by counting the number of cells adhering to 200- μ m long segments of venular endothelium and remaining stationary for 20 s, and expressed as cells per millimetre venule. A total of five postcapillary venules were evaluated in each animal.

After intravital observation, the large bowel was collected and rinsed with phosphate-buffered saline (PBS); tissue was cut open along the antimesenteric border, weighed, snap-frozen in liquid nitrogen and stored at -80°C for later enzyme-linked immunosorbent and myeloperoxidase (MPO) assays as described below (III, IV).

At the end of the experiment, 30 μ l of blood was collected for analysis of systemic leukocyte differential (I,II,III,IV) and platelet count (II).

M.4 Laboratory analyses

RT-PCR

Gene expression of CD11a was used to confirm deficiency of LFA-1 (CD11a/CD18) in mutant mice. Thus, neutrophils were freshly isolated from bone marrow of wild-type and CD11a gene-targeted mice. The bone marrow was flushed aseptically out of the femurs and humeri bones with ice-cold PBS and neutrophils were then isolated by using Ficoll-Paque Research Grade (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The purity of bone marrow neutrophils was more than 70% as assessed by differential counting after staining with Turk's solution using a hemacytometer. Total RNA was extracted from bone marrow neutrophils using RNeasy Mini-kit (Qiagen GmbH, Hilden, Germany) and treated with RNease-free DNase (Amersham Pharmacia Biotech AB, Sollentuna, Sweden) to remove potential genomic DNA contaminants according to the manufacturer's handbook. RNA concentrations were determined by measuring the absorbance at 260 nm spectrophotometrically. Reverse-transcription polymerase chain reaction (RT-PCR) was performed with SuperScript One-Step RT-PCR system (GIBCO BRL Life Technologies, Grand Islands, NY, USA). The RT-PCR profile was 1 cycle of cDNA synthesis at 50°C for 30 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 10 minutes.

After RT-PCR, aliquots of the RT-PCR products were separated on a 2% agarose gel containing ethidium bromide and photographed. The primers sequences were as follows: CD11a (f) 5'-AGA TCG AGT CCG GAC CCA CAG-3'; CD11a (r) 5'-GGC AGT GAT AGA GGC CTC CCG-3'. Beta-actin (f) 5'-ATG TTT GAG ACC TTC AAC ACC-3'; Beta-actin (r) 5'-TCT CCA GGG AGG AAG AGG AT-3'. Beta-actin served as a house-keeping gene to control for the loading amount of cDNA (I).

ELISA

In order to quantify the CXC chemokine levels, MIP-2 and KC were analyzed in the supernatant of colonic tissue homogenized in PBS by using double antibody Quantikine enzyme-linked immunosorbent assay kits (R&D Systems Europe, Abingdon, UK) with recombinant MIP-2 and KC as standards. The minimal detectable protein concentration level was less than 1.5 pg/ml (III, IV).

MPO

Colon samples were thawed and homogenized in 1 ml 0.02 mmol/l buffered phosphate (pH 7.4). After 10 min centrifugation at room temperature at 14 000 rpm, the supernatant was discarded and the pellet homogenized in 1 ml 0.5% hexadecyltrimethylammonium bromide and cooled to -20°C. The sample was then freeze-thawed, sonicated for 90 s and kept in a 60°C water bath for 2 hours. After centrifugation at 13 148g for 10 min at room temperature, MPO activity was determined spectrophotometrically as

the MPO-catalysed change in absorbance in redox reactions with hydrogen peroxide (450 nm, with reference filter 540 nm, at 25°C). Values were expressed as MPO units per gram tissue (III, IV).

Intestinal permeability

A separate experiment was designed to estimate the degree of radiation-induced intestinal permeability (II-IV). At 13 hours after irradiation mice were gavaged with a solution containing 22 mg/ml FITC-conjugated dextran (FITC-dextran of average molecular weight 4.4 kDa; Sigma Chemical) in a total volume of 20 ml per kg bodyweight PBS (pH7.4). At 3 hours after gavage blood samples (150 µl) were obtained from the inferior vena cava, in order to analyse plasma FITC-dextran levels. Blood samples were centrifuged (3 000 rpm and 4°C) for 20 min and plasma (50 µl) was mixed with an equal volume of PBS and added to a 96-well microplate. The concentration of FITC-dextran was determined by spectrophoto-fluorometry with an excitation wavelength of 485 nm and an emission wavelength of 530 nm, using serially diluted samples of the marker as standards.

Systemic Leukocyte Count

At the end of the experiments, 20 µl blood was mixed with Turk's solution (0.2 mg gentian violet in 1 ml glacial acid acetic, 6.25% v/v) in a 1:10 dilution (I, II, III, IV). Leukocytes were differentiated as MNL and PMNL cells in a Bürker chamber.

Systemic Platelet Count

Another 10 µl of blood were mixed with Stromatrol (stromatolic agent for blood platelet count, Mascia Brunelli, Italy) solution in a 1:100 dilution and counted in a Bürker chamber. The results were expressed as cells per ml (II).

M.5 Statistical analysis

Statistical evaluations were performed by use of the Kruskal-Wallis one-way analysis of variance on ranks for unpaired samples (Dunn's post hoc test was used) for multiple groups (I, II, IV). The Mann-Whitney U test was used for the pilot study (I) and to determine differences between sham and positive control in paper II and IV.

For the third article, statistical evaluation was performed using one-way ANOVA followed by multiple comparisons *versus* control group (Holm-Sidak method) (III).

SigmaStat for Windows version 3.5" software was employed (Systat Software Inc., Chicago, Illinois, USA).

The results are presented as mean values (standard error of the means) (I, II, III) and as median with inter-quartile range (25th and 75th percentiles) (IV). *n* represents the number of animals per group. $P < 0.050$ was considered statistically significant.

Results and Discussion

R.1 Radiation-induced leukocyte and platelet responses

The low tolerance of the normal tissues continues to be an impediment to the optimal use of radiation therapy in the treatment of cancer (Stone et al., 2003). It has been demonstrated that leukocyte recruitment is a rate-limiting step in inflammatory bowel diseases (Wan et al., 2002; Santen et al., 2007) including radiation-induced enteropathy (Johnson et al., 2004). An early microvascular inflammatory response, beginning a few hours after radiation exposure is characterized by leukocyte infiltration into the irradiated organs. This pathophysiological process is regarded as one of the main determinants of radiation-induced organ damage (Buell and Harding, 1989; Panes et al., 1995). At the beginning of this thesis we developed a reproducible experimental model in order to study these early intestinal inflammatory responses associated with irradiation. For this purpose we used inverted intravital microscopy and a pilot study was designed as described in the Material and Methods. We found that radiation exposure increased leukocyte responses in a dose and time dependent manner in the colonic venules. The highest leukocyte response was observed 16 hours after administration of 20 Gy, which was used for all further studies. By means of intravital microscopic observations in colonic postcapillary venules we found that radiation increased the number of rolling and adhering leukocytes significantly (I, II, III, IV).

During the last years, several studies with main focus in inflammation, reconsider the role of platelets mainly because their content of potent proinflammatory substances, and thereby attribute them an important role as mediators and effector cells in this process (Klinger, 1997a). Although direct evidence for the role of platelets in radiation-induced enteropathy is still lacking, several studies have reported that anti-platelet agents, such as ticlopidine (Akyurek et al., 2006), clopidogel (Wang et al., 2002) and acetylsalicylic acid (Mennie and Dalley, 1973), exert a protective effect against intestinal damage associated with radiotherapy. Moreover, it has been shown that radiation causes a clear-cut shift towards a prothrombotic situation due to reduced expression of endothelial cell prostacyclin (Rubin et al., 1985) and thrombomodulin (Richter et al., 1997) as well as increased release of von Willebrand factor (Rubin et al., 1985).

Our intravital microscopy observations revealed that both abdominal and TBI significantly increased platelet rolling and adhesion in the colonic venules as compared with non-radiated animals (II, III, IV).

Considering that early microvascular injury, characterized by increased interactions between leukocytes/platelets and the endothelial cells lining the microvasculature, may be a rate-limiting process, we speculated this to be one of several central features in the pathogenesis of acute radiation-induced enteropathy, which in turn has been suggested to be responsible for the chronic and progressive nature of delayed radiation injury (Panes et al., 1995; Beck et al., 2004). Based on this, it was extrapolated that interference with leukocyte and platelet recruitment may be of therapeutic value to ameliorate intestinal side effects of radiation.

R.2 P-selectin mediates radiation-induced leukocyte and platelet recruitment

Leukocyte and platelet rolling along the vascular endothelium, an early step in the recruitment process of these cells, is critically dependent on endothelial cell-expression of P-selectin although other candidates have been suggested (Riaz et al., 2002). One object of the first two studies of this thesis was to clarify the mechanisms behind early leukocyte- and platelet-endothelial interactions in the colonic microvasculature after exposure to radiation. In our experiments we observed that inhibition of P-selectin decreased leukocyte recruitment significantly (I) and these data were later confirmed and presented in the second article (II).

By means of intravital microscopy, we found that inhibition of P-selectin function decreased leukocyte rolling by more than 83% (I), suggesting that P-selectin plays a predominant and nonredundant role in supporting leukocyte rolling in colonic venules in response to radiation. Moreover, we found that immunoneutralization of P-selectin attenuated radiation-induced leukocyte firm adhesion by more than 87% (I). Thus, our data suggest that P-selectin not only mediates leukocyte rolling but that P-selectin-dependent rolling is a prerequisite for subsequent firm leukocyte adhesion in radiation-induced leukocyte-endothelium interactions (I). P-selectin is not the only molecule involved in leukocyte adhesion process. For example, Son *et al.* (2001) (Son et al., 2001) reported that inhibition of firm leukocyte adhesion by interfering with either ICAM-1 or VCAM-1 can protect against pathologic intestinal inflammation provoked by radiotherapy, suggesting that there are several other molecules supporting radiation-provoked leukocyte adhesion.

In contrast with our data, Molla *et al.* (2001) (Molla et al., 2001) reported that radiation-induced leukocyte adhesion is intact in P-selectin-deficient mice and concluded that inhibition of P-selectin may not be sufficient to interfere with radiation-provoked leukocyte adhesion. One explanation to this observation could be that different radiation doses and protocols were used by our group as compared with Molla *et al.*

(2001). Moreover, knowing that VCAM-1 plays a significant role in supporting radiation-induced leukocyte adhesion (Molla et al., 2003), and that VCAM-1 is expressed at significantly higher levels in P-selectin-deficient mice compared with wild-type mice (Carrithers et al., 2002; Gironella et al., 2002), it is possible that the intact leukocyte adhesion observed in P-selectin-deficient mice reported by Molla *et al.* (2001) may be the result of compensatory mechanisms.

In addition, in our second paper, we demonstrated that P-selectin is a critical adhesion molecule supporting radiation-provoked platelet rolling in the colon (II). Immunoneutralization of P-selectin reduced radiation-induced platelet rolling by 87% (II). Moreover, we also found that inhibition of P-selectin blocked radiation-induced platelet adhesion by 63% (II), suggesting that platelet recruitment in radiation exposed colon is a multistep process similar to the already observed leukocyte responses, in which initial rolling is a prerequisite for subsequent firm adhesion in response to radiotherapy.

The crucial role of P-selectin in platelet recruitment was also reported by Laschke *et al.* (2008) who showed that immunoneutralization of P-selectin inhibited hepatic microvascular accumulation of platelets and leukocytes, and protected against bile duct ligation-induced cholestasis and provoked hepatocellular damage (Laschke et al., 2008; Braun et al., 2008). In addition, Braun *et al.* (2008) reported that P-selectin and PSGL-1 mediate early interactions between platelets and other cells, including endothelial cells and leukocytes, in inflamed femoral arteries (Braun et al., 2008).

R.3 PSGL-1 role in radiation-induced leukocyte and platelet recruitment

Radiation-induced inflammation causes the expression of cell adhesion molecules, such as P-selectin, on the surface of the blood vessel wall. P-selectin glycoprotein ligand-1 (PSGL-1) has been proposed as the main receptor for P-selectin (Sako et al., 1993). Showing that P-selectin plays a key role for cell recruitment in the colon exposed to radiation, one objective in the second study was to clarify the importance of PSGL-1 in leukocyte and platelet recruitment after exposure to radiation.

Pretreatment with a monoclonal antibody against PSGL-1 reduced radiation-provoked leukocyte and platelet rolling and adhesion by more than 77% and 83%, respectively, in the colon (II). This suggests that PSGL-1 *per se* plays a crucial role in both leukocyte- and platelet-recruitment associated with radiation injury. Our results are in line with previous data showing that in ischaemia-reperfusion of the heart, liver and colon (Hayward et al., 1999; Dulkanchainun et al., 1998; Santen et al., 2007) PSGL-1 plays a dominant role in mediating leukocyte rolling and adhesion. Moreover, it has been shown that immunoneutralization of CD162 (PSGL-1) decreased platelet primary and secondary capture as well as leukocyte rolling and adhesion after LPS/gal injection in a femoral vein model, significantly (Slotta et al., 2009). This further underlines that

PSGL-1 plays a central role in mediating platelet- and leukocyte-endothelium interactions in inflamed tissues.

R.4 Role of LFA-1 in radiation-induced leukocyte recruitment

LFA-1 is considered an important member of the integrin family of adhesion molecules which mediates leukocyte adhesion (Carlos and Harlan, 1994) but the importance of LFA-1 seems to vary depending on the stimulus, experimental model and organ studied (Yoshida et al., 2001; Issekutz and Issekutz, 1992; Argenbright et al., 1991). Based on this knowledge, we found an interest in studying the role of β_2 -integrin (CD11a) in radiation-provoked leukocyte recruitment in the colon, which was performed by using gene-targeted animals (I). Interestingly, we found that firm leukocyte adhesion triggered by radiation, was almost abolished in LFA-1-deficient-mice (I). Notably, the number of rolling leukocytes in LFA-1-deficient mice was not different from the wild-type mice, suggesting that LFA-1 is not an important molecule in the initial phase of leukocyte recruitment in this model (I). These observations are in line with similar results reported in a colonic model of ischaemia-reperfusion (Riaz et al., 2002). Importantly, it has been shown that LFA-1 may not be the only molecule supporting radiation-provoked leukocyte adhesion and that other adhesion molecules such as VLA-4 (very late antigen-4) (Son et al., 2001) or Mac-1 (Handschel et al., 1999) may also play an important role in this process. Moreover, it has been shown that Mac-1 (CD11b/CD18) and LFA-1 cooperate in a sequential manner to secure efficient adhesion of leukocytes on endothelial cells (Handschel et al., 1999), suggesting that targeting Mac-1 also may ameliorate radiation-induced leukocyte accumulation (Handschel et al., 1999). Interestingly, no compensatory adhesion mechanisms like the one founds in P-selectin deficient mice has been observed in LFA-1-deficient mice.

However, our results in the first study show a 94% reduction in leukocyte adhesion in LFA-1-deficient mice exposed to radiation compared to the control group. This indicates that LFA-1 is a dominant molecule in mediating stable adhesion of leukocyte-endothelial interactions in the colonic microvasculature in response to radiation. Our finding is supported by a previous study showing that ICAM-1, which is the main receptor of LFA-1 on endothelial cells, supports radiation-induced leukocyte adhesion in the colon (Molla et al., 2003; Son et al., 2001).

Taken together, the results from the first and the second study indicate that P-selectin and PSGL-1 are critical adhesion molecules supporting radiation-provoked leukocyte- and platelet-endothelium interactions in the large intestine. Our results show that inhibition of P-selectin and PSGL-1 not only blocked rolling but also radiation-induced leukocyte and platelet adhesion, suggesting that both leukocyte and platelet recruitment are multistep processes in which initial rolling is a prerequisite for subsequent firm adhesion in response to irradiation (I, II). Moreover, in the first study we

found that LFA-1-deficiency abrogates leukocyte adhesion after radiation indicating that LFA-1 is a key molecule in supporting radiation-induced leukocyte recruitment in the colon.

R.5 p38 MAPK activity and radiation-induced enteropathy

Once we established the role of cell adhesion molecules in leukocyte and platelet recruitment, we focused on the intracellular signalling mechanisms controlling leukocyte- and platelet-endothelial interactions in the large intestine.

MAPKs integrate and process various extracellular signals, controlling expression of cytokines and organizing cellular responses to non-specific stress stimuli (Kumar et al., 2003). Numerous studies have reported that radiotherapy provokes activation of MAPK pathways in different tissues (Dent, 2003; Wang et al., 2000; Lee et al., 2002).

In the third study, we showed that administration of SB239063, a second-generation p38 MAPK inhibitor (Barone et al., 2001), decreased leukocyte rolling and adhesion by more than 70% and 90%, respectively. Moreover, inhibition of p38 MAPK activity reduced tissue accumulation of leukocytes, illustrated by a 88% reduction in the colonic levels of MPO. These results suggest that radiation-provoked leukocyte recruitment is dependent on p38 MAPK signalling.

It has been reported that ionizing radiation upregulates the expression of chemokines in the intestine (Linard et al., 2004; Van der Meeren et al., 2005). Knowing that tissue resident cells, such as mast cells (Santen et al., 2008 and 2009) are able to release substances like TNF- α and CXC chemokines, which in turn activate leukocyte recruitment, we wanted to measure the levels of MIP-2 and KC in colonic tissue exposed to radiation. We found that 16 hours after exposure the levels of MIP-2 and KC increased by more than 400% and 300%, respectively. After pretreatment with SB239063 we noticed a significant decrease in the levels of CXC chemokines by more than 70%. This result could partly explain the inhibitory effect of SB239063 on leukocyte accumulation. Our data do not exclude that p38 MAPK regulate other important aspects of leukocyte-endothelial adhesion interactions, i.e. it has been reported that inhibition of p38 MAPK reduce endothelial cell expression of P-selectin, ICAM-1 and VCAM-1 (Tamura et al., 1998; Pietersma et al., 1997; Yan et al., 2002; Gao et al., 2002).

In addition, we showed that administration of SB239063 decreased radiation-provoked platelet-endothelium interactions by more than 70%. This indicates that radiation-induced platelet responses are dependent on p38 MAPK activity. So far to our knowledge, there are no similar studies in the literature investigating the relationship between platelets-endothelial interactions in relation to p38 MAPK pathway after irradiation.

Taken together, our findings suggest that p38 MAPK is a potent regulator of early vascular inflammatory changes in radiation-induced enteropathy.

R.6 Rho-kinase signalling pathway in radiation-induced enteropathy

During the last twenty years, several reports have underlined the importance of small GTP-binding proteins, where the Rho GTP-ase proteins and one of their effector, Rho kinase, seems to be important pawns in controlling intracellular signalling pathways (Fukata et al., 2001; Shimokawa and Takeshita, 2005). Rho kinase activity plays an important role in early inflammatory processes, influencing leukocyte-platelet-endothelium interactions as well as formation of cytokines and reactive oxygen species (Bokoch, 2005; Diebold and Bokoch, 2005; Laschke et al., 2009; Riento and Ridley, 2003). Thus, the main object of our fourth study was to investigate the role of Rho-kinase activity in acute radiation-induced enteropathy. Our results showed that Y-27632 protects against radiation-induced enteropathy in a dose dependent manner.

Intravital microscopy observations of the colonic microcirculation revealed that inhibition of ROCK reduced radiation-induced leukocyte- and platelet-endothelium interactions in the colon, i.e. leukocyte rolling by more than 30% and leukocyte adhesion by almost 100%. Moreover, Y-27632 significantly decreased radiation-induced platelet rolling and adhesion by 55% and 74%. Our observations are in line with previous data showing that microvascular inflammation plays a critical role in early intestinal injury associated with radiotherapy (Molla, 2007) and moreover, with other results reporting that inhibition of Rho-kinase activity by treatment with Y-27632 regulates leukocyte trafficking into inflamed liver tissue (Laschke et al., 2009) and colon (Santen et al., 2010). Interestingly, inhibition of Rho-kinase signalling not only reduced leukocyte and platelet interactions within the intravascular compartment but also decreased the MPO levels in the intestinal tissue, suggesting that Rho-kinase is an important regulator of pathological inflammation in radiation-provoked enteropathy.

Tissue accumulation and navigation of inflammatory cells are orchestrated by secreted tissue chemokines (Riaz et al., 2003). CXC chemokines, such as MIP-2 and KC, are particularly potent inducers of neutrophil activation and migration (Riaz et al., 2003). Under certain conditions, the Rho-kinase pathway has been forwarded to control pro-inflammatory cytokine formation after exposure to irradiation (Gervaz et al., 2009). In the fourth study we investigated if Rho-kinase signalling might regulate MIP-2 and KC expression in radiation-induced intestinal injury. We found that radiation exposure markedly increased intestinal formation of CXC chemokines by more than 300%. Administration of Y-27632 decreased colonic content of MIP-2 and KC down to 86% and 26%, respectively. These results suggest that Rho-kinase plays an important role in controlling CXC chemokine production in the irradiated colon. Knowing that secretion of CXC chemokines regulates leukocyte trafficking in the colon exposed to irradiation, our findings possibly explain the inhibitory effect of blocking Rho-kinase activity in radiation-induced leukocyte recruitment. These observations are also supported by recent findings showing that statins, which not only reduce cholesterol synthesis but also decrease Rho-kinase activity and exert pleiotrophic anti-inflammatory effects, inhibit radiation-induced leukocyte accumulation and tissue injury (Wang et al., 2007; Holler et al., 2009).

In conclusion, it appears that Rho-kinase signalling plays an important role in the early phase of radiation-induced intestinal inflammation and that targeting Rho-kinase activity may be a useful strategy to intervene in the early phase of radiation enteropathy.

R.7 Intestinal leakage in radiation enteropathy

Intestinal integrity is a fundamental component in maintaining the gut homeostasis. It has been reported from clinical investigations that radiotherapy destroys intestinal integrity and increases gut permeability (Nejdfors et al., 2000). Intact intestinal barrier function is pivotal for protection against translocation of bacteria and toxins (Nejdfors et al., 2000; Park et al., 2010).

The relationship between microvascular inflammation and intestinal leakage in radiation enteropathy is not known. It may be that microvascular injury causes increased epithelial permeability or that these two processes are inter-independent components in the pathophysiology of radiation-provoked enteropathy.

Our experiments show that radiation significantly increased intestinal permeability by more than three-fold compared with the control group (II). Taking in consideration that inhibition of P-selectin or PSGL-1 abolished radiation-provoked platelet and leukocyte interactions with the endothelium in the intestine exposed to radiation, we asked whether targeting P-selectin and PSGL-1 may reduce radiation-induced intestinal leakage. Interestingly, we found that immunoneutralization of P-selectin or PSGL-1 had no effect on radiation-provoked intestinal permeability, suggesting that neither platelet nor leukocyte recruitment in the microcirculation cause intestinal barrier dysfunction. To further investigate this observation, we depleted mice of platelets and leukocytes by systemic administration of specific antibodies. Notably, we found that depletion of platelets and neutrophils had no impact on intestinal leakage, suggesting that radiation-induced intestinal permeability is dependent on other mechanisms than leukocyte and platelet recruitment in the colon (II).

Knowing the importance of intestinal barrier function for protection against translocation of coliform bacteria and toxins (Hauer-Jensen et al., 2007; Luyer et al., 2004) it was interesting to observe that administration of SB239063 decreased radiation-induced epithelial barrier dysfunction, suggesting that p38 MAPK is a potent regulator in maintaining intestinal integrity (III). This observation is supported by cell culture experiments showing that cytokine provoked epithelial cell permeability is dependent on p38 MAPK signals *in vitro* (Werz et al., 2001). Based on our results we speculate that p38 MAPK may be involved in causing epithelial barrier dysfunction and in addition, we suggest that radiation enteropathy may be added to the list of conditions, including reperfusion injury (Gao et al., 2002; Santen et al., 2009), joint inflammation (Badger et al., 2001), acute liver failure (Klintman, 2005), asthma (Choudhury et al., 2002) and glomerulonephritis (Stambe et al., 2004), that can be protected against by targeting p38 MAPK signalling (III).

Interestingly, it has been suggested that Rho-kinase activity plays an important role in regulating the integrity of epithelial barriers (Popoff and Geny, 2009) and moreover, inhibition of Rho kinase activity seems to ameliorate fibrotic processes associated with radiotherapy (Bourgier et al., 2005). In the fourth study we investigated whether Rho-kinase signalling may be involved in radiation-induced epithelial cell dysfunction. We found that radiation increased colonic permeability by nearly six-fold (IV) where inhibition of Rho-kinase activity markedly reduced intestinal leakage by 81%, after exposure to irradiation. These results suggest that inhibition of Rho-kinase activity protects against pathological tissue damage in response to radiation. This effect of Rho-kinase has also been shown in cardiovascular diseases (Shimokawa and Rashid, 2007), endotoxaemic liver injury (Thorlacius et al., 2006) and septic lung damage (Tasaka et al., 2005). In this context, it is also interesting to note that a recent study showed that Rho-kinase signalling plays a key role in intestinal fibrogenesis, including formation of connective tissue growth factor and type I collagen in smooth muscle cells derived from the intestine (Bourgier et al., 2005). Thus, it appears that Rho-kinase signalling plays an important role in the early phase of irradiation characterised by vascular inflammation and intestinal barrier dysfunction but could also play a crucial role in the late phase of radiation-induced intestinal toxicity dominated by fibrogenic changes, suggesting that targeting Rho-kinase activity may be a useful strategy in both early and late phases of radiation-induced enteropathy (IV).

Our findings increased the understanding regarding the molecular mechanisms regulating microvascular inflammation and the complex interplay between different components of the tissue response to radiotherapy. We showed that leukocyte and platelet recruitment has no impact on radiation-induced intestinal leakage whereas inhibition of p38 MAPK or Rho-kinase signalling significantly attenuates epithelial barrier dysfunction in radiation enteropathy. Knowing that these signalling pathways can regulate epithelial cell apoptosis (Stenson, 2007) and that radiotherapy is a powerful inducer of epithelial cell apoptosis (Garin-Laflam et al., 2009; Gervaz et al., 2009) we may speculate that intestinal leakage in the radiated gut is more likely related to direct apoptotic mechanisms in the epithelium rather than to intravascular changes in terms of leukocyte and platelet recruitment. In this context, it is also important to note that the relationship between microvascular inflammation or intestinal leakage on one hand and intestinal fibrosis on the other hand is not known. Thus, although microvascular inflammation has no impact on intestinal integrity, leukocyte and platelet responses may still play an important role for later fibrogenesis in the intestinal tract. Moreover, future experiments could possibly delineate the relationship between intestinal leakage and fibrosis in radiation enteropathy.

The results of this thesis raised speculations on preventive treatment in patients undergoing radiotherapy where inhibition of leukocyte and platelet recruitment and/or interference with specific signalling pathways could prevent radiation-induced late negative effects, such as fibrosis. Future investigations need to be done on the balance between prevention of injuries on healthy surrounding tissues and treatment compliance on tumour cells.

Conclusions

1. Radiation-induced leukocyte rolling is mediated by P-selectin and PSGL-1. Firm leukocyte adhesion is supported by LFA-1 in radiation enteropathy.
2. P-selectin-dependent leukocyte rolling is a precondition for subsequent leukocyte adhesion in response to radiation.
3. P-selectin and PSGL-1 are key molecules in regulating radiation-induced platelet interactions in the colonic microcirculation.
4. p38 MAPK activity regulates microvascular inflammation and intestinal barrier dysfunction in radiation enteropathy.
5. Rho-kinase activity controls leukocyte- platelet-endothelial cell interactions and intestinal permeability in response to irradiation.
6. Radiation-induced intestinal leakage is independent of neutrophil and platelet recruitment in the colonic microvasculature.

Populärvetenskaplig sammanfattning

Joniserande strålning används ofta som behandling vid olika tumörsjukdomar i buk-
håla och bäckenregion men är ibland förenad med biverkningar. Dessa kan uppkomma
tidigt/akut, dvs i direkt anslutning till genomförd behandling eller sent, efter månader-
år. Besvären kan vara av mycket varierande och ofta lindrig övergående karaktär såsom
buksmärtor, diarré och blödningar från tarmen. På längre sikt finns det dock risk för
allvarliga biverkningar från tarmen såsom fistelbildning, perforationer samt tarmvred.
Dessa reaktioner medför ibland ett stort och livslångt lidande för de patienter som
drabbas och beror på att delar av tarmen exponerats oavsiktligt i strålfältet. Både de ti-
diga och sena biverkningarna anses ha ett nära samband med de akuta inflammatoriska
förändringar eller det vävnadsläckage som äger rum i direkt anslutning till strålbehand-
lingen. För att bättre kunna förhindra och behandla dessa biverkningar är det viktigt att
undersöka bakgrunden till och vilka mekanismer som reglerar dessa processer.

Inflammation beskrevs första gången av Celsus för 2000 år sedan som rodnad, svull-
nad, värme och smärta vilket är kroppens, och ffa kärlträdets, reaktion på utifrån kom-
mande stimuli såsom virus, bakterier och trauma i syfte att avgränsa, bekämpa och läka
den uppkomna skadan. Inflammatoriska reaktioner kan också uppstå av andra orsaker
t ex genom att vävnaden exponeras för joniserande strålning. Oavsett initierande agens
har alla inflammatoriska reaktioner vissa basala mekanismer som är gemensamma, t ex
rekrytering av immunceller samt läckage av vätska från blodkärl till drabbad vävnad,
vilka båda anses kunna bidra till ovan beskrivna biverkningar. Rekrytering av vita blod-
kroppar (leukocyter) och blodplättar (trombocyter) till det skadade området är en fler-
stegsprocess i vilken leukocyter/trombocyter rullar längs kärlväggens endotelceller med
hjälp av en specifik grupp molekyler kallad selektiner. Vidare aktiveras immuncellerna
av kemokiner och fastnar längs kärlväggen varvid leukocyterna sedan kan klämma sig
emellan endotelcellerna och vandra ut i vävnaden för att delta i den uppkomna inflam-
matoriska processen till gagn för individen. Det är oklart hur denna process ser ut i
detalj men tidigare publicerade data talar för att både leukocyt/trombocytrekrytering

alternativt endotelcellspåverkan, med efterföljande vävnadsläckage av vätska, kan vara av betydelse för utvecklingen av sena biverkningar i tarmen. Delarbetena i denna avhandling belyser några av de mekanismer som reglerar dessa tidiga processer vid strål-skada på tjocktarmen. Vidare klargörs sambandet emellan å ena sidan leukocyt/trombocytrekrytering, intakt tarmslemhinna och å andra sidan vävnadsläckage, inducerad av joniserande strålning.

Målet med de första och andra delarbetena var att klargöra vilka molekyler som förmedlar interaktionen mellan såväl leukocyter/trombocyter som mellan endotelceller på kärlväggen, i strålad tarm. Därtill undersöktes strålningens effekt på vävnadsläckage då försöksdjuren behandlats med antikroppar mot neutrofila granulocyter (ett slags leukocyter, vilka anses betydelsefulla i inflammatoriska processer) respektive trombocyter vilket utlöser neutropeni (brist på vita blodkroppar) respektive trombocytopeni (brist på trombocyter) i blodet. Djuren bestrålades och efter 16 timmar undersöktes mikrocirkulationen i tjocktarmen med hjälp av intravitalmikroskopi. Det noterades då att betydligt flera leuko/trombocyter rullade längs och satt fast (adhererade) på kärlväggen, vilket tyder på en inflammatorisk reaktion. Djuren förbehandlades med specifika antikroppar riktade emot P-selektin och PSGL-1 varvid både leuko/trombocytrullning och leuko/trombocytadhesion minskade betydligt. Vidare noterades att LFA-1, en viktig adhesionsmolekyl, reglerar leukocytadhesionen i tarmen. Det strålningsinducerade vävnadsläckaget av vätska befanns vara oberoende av P-selektin, PSGL-1 blockering och neutro/trombocytopeni.

Yttre stimuli, såsom osmotisk chock, bakteriegifter samt joniserande strålning aktiverar intracellulära signalsystem i olika vävnader. Ett av dessa system, p38 MAPK, har visat sig vara viktigt för reglering av genöverföring, utmognad och protein tillverkning för vävnadens integritet och överlevnad. SB 239063 är en drog som har visat sig vara en potent hämmare av p38 MAPK-aktivitet vilket studerades i det tredje arbetet. Då SB 239063 gavs till djuren, före bestrålning, noterades att både leuko/trombocytrullning respektive adhesion markant minskade. Därtill noterades även en minskning av MPO-aktiviteten, ett mått på förekomst av neutrofila granulocyter, i vävnaden. De vid leukocytrekrytering viktiga signalsubstanserna MIP-2 och KC, samt vätskeutträdet till vävnaden, minskade. Detta tyder på att bibehållen tarmintegritet är en viktig komponent för att förhindra vävnadsläckage.

Rho-kinaser är en familj av proteiner vilka anses vara viktiga för eukaryota cellers rörlighet och adhesionsmöjligheter. I det fjärde arbetet förbehandlades försöksdjuren med Y27632, en hämmare av Rho-aktivitet, vilket resulterade i minskad leuko/trombocyt rekrytering och lägre MPO/MIP-2/KC nivåer i vävnaden. Slutligen noterades en betydande reduktion av vävnadsläckaget.

Sammanfattningsvis beskrivs i denna avhandling viktiga mekanismer vilka reglerar leuko- och trombocytrekrytering samt vävnadsläckage vid exponering för joniserande strålning. Den har visat att strålnings framkallad leuko/trombocytrullning är P-selektin och PSGL-1-beroende samt LFA-1 reglerar leukocytadhesion i tjocktarmen. Interferens med p38-MAPK och Rho-kinas aktivitet skyddade emot leuko- trombocytrekrytering, minskade MPO-aktivitet samt tjocktarmens innehåll av de viktiga signalmolekylerna

MIP-2 och KC. I detta arbete visas också att vävnadsläckaget inte är beroende av det inflammatoriska vävnadssvaret, dvs leuko/trombocytrekrytering, utan är snarare beroende av bibehållen integritet i vävnaden. Resultaten i denna avhandling kan bidra till förståelse för de mekanismer som orsakar strålskada i tarmen, syftandes till att i framtiden kunna skydda patienter mot allvarliga biverkningar orsakad av strålbehandling.

Summary in Romanian – Rezumatul tezei

Iradierea ionizantă – radioterapia – este folosită adesea ca și modalitate de tratament în cazul diferitelor tumori maligne cu localizare abdominală sau pelvină. Din nefericire, radioterapia este adesea asociată cu reacții adverse; acestea pot apărea imediat – direct după expunerea la radiații, sau cu întârziere – la distanță de luni și chiar ani după terminarea tratamentului. Reacțiile adverse imediate pot avea manifestări precum diaree, dureri abdominale sau sângerări intestinale. Cu timpul însă, există un risc crescut de reacții adverse tardive cum ar fi apariția de fistule, perforații sau volvulus. Reacțiile adverse sunt rezultatul expunerii intestinale în câmpul de iradiere și reprezintă un dis-comfort pentru pacienți până la sfârșitul vieții. Se presupune că atât reacțiile adverse imediate cât și cele tardive sunt direct corelate cu inflamația acută și/sau cu leakage-ul intestinal, ambele fiind consecințe directe ale expunerii la iradiere.

Inflammatio – descrisă pentru prima dată de Celsus în urmă cu 2000 de ani este identificată prin prezența celor cinci semne cardinale: *rubor* (eritem), *dolor* (durere), *tumor* (edem), *calor* (hipertermie) și *functio laesa* (impotență funcțională). Aceasta apare inițial la nivelul vaselor sangvine ca reacție la factorii externi precum virusi sau bacterii dar și după expunerea la radioterapie. Scopul reacției inflamatorii este de a delimita și vindeca zona afectată. Indiferent de natura factorilor provocatori, mecanismele ce stau la baza inflamației acute sunt aceleași: apariția celulelor de apărare și a exudatului.

Apariția leucocitelor (globulele albe) în țesuturi este un proces complex în care, inițial leucocitele migrează de-a lungul endoteliului vascular fiind ajutate de un grup specific de molecule numite selectine. În pasul următor, se activează chemokinele, ceea ce are ca rezultat aderarea fixă a leucocitelor de-a lungul peretelui vascular. În ultimul pas al acestui proces leucocitele se strecoară printre celulele endoteliale în spațiul extravascular pentru a-și îndeplini misiunea în țesuturile inflamate. Trombocitele trec printr-un proces similar leucocitelor însă nu se știe exact dacă părăsesc sau nu spațiul intravascular. Aceste mecanisme nu sunt deocamdată înțelese în detaliu. Literatura de specialitate

speculează că apariția leucocitelor și/sau a trombocitelor împreună cu influențele directe asupra endoteliului vascular sunt importante în dezvoltarea efectelor adverse intestinale. Lucrările incluse în această teză de doctorat subliniază câteva din mecanismele ce reglează procesele imediate induse de radioterapie asupra colonului. Teza analizează legătura dintre radioterapie, pe de o parte și apariția în țesuturi a leucocitelor/trombocitelor, precum și rolul leakage-ului intestinal de cealaltă parte.

Scopul primelor două lucrări este de a investiga moleculele responsabile de intermedierea interacțiunii între leucocite/trombocite și celulele endoteliului vascular după expunerea la iradiere. În completare, s-a analizat efectul iradierii asupra leakage-ului intestinal cu ajutorul animalelor de laborator tratate cu un anticorp împotriva neutrofilelor respectiv a trombocitelor – ceea ce are ca rezultat leucopenia și trombocitopenia. Animalele au fost expuse iradierii cu 20 Gy iar după 16 ore, cu ajutorul microscopiei intravitale a fost analizată microcirculația intestinală. Această metodă a relatat o creștere clară în număr a leucocitelor și a trombocitelor ce migrează precum și a celor aderente la suprafața endoteliului vascular reprezentând un semn clar al reacției inflamatorii acute. Animalele au fost de asemenea tratate cu anticorpi specifici împotriva selectinei P sau a ligantului său specific PSGL-1. Acest tratament a scăzut semnificativ atât numărul de leucocite/trombocite ce migrează dar și a celor aderente la endoteliu. În continuare a fost observată importanța moleculei de adeziune numită LFA-1 în procesul de aderare al leucocitelor la peretele vascular. În mod deosebit s-a remarcat faptul că leakage-ul intestinal indus de radioterapie este independent de selectina P, PSGL-1 și leuco/trombocitopenie.

Stimuli externi precum șocul osmotic, toxinele bacteriene sau radiațiile ionizante activează căi de semnalizare intracelulară în diferite țesuturi. Unul din aceste sisteme, p38 MAPK, s-a dovedit a fi important în reglarea transcripțiilor genetice, diferențierea și sinteza de proteine în scopul păstrării integrității și a supraviețuirii țesuturilor. SB 239063 este o substanță care s-a dovedit potentă în blocarea activității sistemului p38 MAPK după iradiere, aceasta reprezentând scopul studiului în cea de-a treia lucrare a acestei teze. Administrarea de SB 239063 înaintea iradierii animalelor de laborator a avut ca efect diminuarea leucocitelor și trombocitelor ce migrează sau aderă la endoteliu. În plus, s-a remarcat o scădere marcată în activitatea mieloperoxidazei (MPO) – un instrument de măsură al acumulării neutrofilelor în țesuturi, precum și a nivelului chemokinelor MIP-2 și KC și a leakage-ului intestinal indus de expunerea la radiații ionizante.

În cea de-a patra lucrare inclusă în teză, animalele de laborator au fost tratate înaintea expunerii la iradiere cu Y 27632, o substanță inhibitoare a activității Rho-kinazelor. Rho-kinazele sunt o familie de proteine cu importanță în motilitatea și adeziunea celulelor eucariote. Efectul acestei inhibiții este scăderea numărului de leucocite și trombocite apărute în țesuturi precum și a activității MPO, MIP-2 și KC în colon. De asemenea a fost notată o diminuare semnificativă a nivelului leakage-ului intestinal.

În concluzie, această teză descrie mecanisme importante ce reglează leucocitele și trombocitele apărute în țesuturi precum și leakage-ul intestinal, ambele fiind consecința directă a expunerii la radiații ionizante. S-a demonstrat că procesul de migrare al leuco-

citelor și trombocitelor după iradiere este dependent de selectina P și PSGL-1 precum și că LFA-1 este responsabilă de aderența leucocitelor la peretele vascular, la nivelul colonului.

Interferența cu activitatea sistemului p38 MAPK și Rho-kinaze s-a dovedit că protejează împotriva apariției leucocitelor și trombocitelor în țesuturi, scade nivelul activității MPO împreună cu MIP-2 și KC.

În această teză s-a demonstrat de asemenea că leakage-ul intestinal este independent de răspunsul țesuturilor la inflamație, cu alte cuvinte de apariția leucocitelor și trombocitelor în țesuturi. În schimb, alte mecanisme cum ar fi apoptoza celulară, se pare că au importanță în prezervarea integrității interstițiale.

Rezultatele acestei teze de doctorat au importanță în înțelegerea mecanismelor ce stau la baza afectării intestinale după expunerea la radiații ionizante în scopul de a proteja pacienții împotriva reacțiilor adverse generate de radioterapie.

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