

**REMODELLING OF EXTRACELLULAR MATRIX
IN RECTAL CANCER AND PREOPERATIVE
RADIOTHERAPY**

Eva Angenete



UNIVERSITY OF GOTHENBURG

Göteborg 2009

Department of Surgery
Institute of Clinical Sciences at Sahlgrenska Academy
University of Gothenburg

© Eva Angenete 2009
ISBN: 978-91-628-7733-0
Printed by Geson Hylte Tryck, Gothenburg, Sweden 2009



To Johan and Ester

ABSTRACT

BACKGROUND

Preoperative radiotherapy reduces local recurrence due to rectal cancer, but increases postoperative morbidity. The aim of this thesis was to study the impact of radiotherapy on extracellular matrix (ECM) remodelling enzymes and growth factors after radiotherapy and also their possible use as surrogate markers for complications. A secondary aim was to explore the use of these enzymes and growth factors as markers for tumour classification and risk predictors of metastases and death of rectal cancer.

MATERIALS AND METHODS

In paper I-III 91-110 patients undergoing surgery with or without preoperative radiotherapy were studied through biopsies taken from tumour tissue and adjacent mucosa as well as plasma samples during surgery. Clinical parameters were registered and the patients were followed yearly. Paper IV encompasses 32 patients with sequential biopsies before treatment and from the surgical specimen. Twenty of them received preoperative radiotherapy. Protein levels of matrix metalloproteinase (MMP)-1, -2, -9 (Papers I and IV), urokinase plasminogen activator (uPA) (Papers III-IV), plasminogen activator inhibitor-1 (PAI-1) (Papers III-IV), transforming growth factor- β 1 (TGF- β 1) (Papers II and IV), tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) (Paper IV) and calprotectin (Paper IV) were determined by ELISA. Biopsies from irradiated and non-irradiated peritoneal areas were also analysed (Paper IV) for tissue-type plasminogen activator (tPA). The localisation of calprotectin in mucosa was determined by immunohistochemistry (Paper IV).

RESULTS

MMP-2 and PAI-1 levels were higher after radiotherapy in both mucosa and tumour whereas uPA and calprotectin were higher in mucosa after radiotherapy. High levels of MMP-2 correlated to wound-infections and fistula formation. Peritoneal biopsies displayed lower levels of tPA in irradiated patients indicating a reduced fibrinolytic capacity. Levels of MMP-1, -2, -9, uPA, PAI-1, total TGF- β 1 and calprotectin were higher in tumour tissue compared to mucosa. High levels of total TGF- β 1 in tumour tissue correlated to the presence of metastases and high levels of PAI-1 in tumour tissue were associated with lateral spread irrespective of radiotherapy. PAI-1 in tumour tissue was also associated with an increased risk of death due to rectal cancer in multivariate analysis.

CONCLUSIONS

The ECM remodelling proteases and growth factors mirror to some extent the response to radiotherapy and may be involved in the pathogenesis of radiotherapy associated morbidity. MMP-2 can be related to clinically evident complications after surgery and radiotherapy and could be explored further for use as a clinical marker. To further improve selection of patients for radiotherapy the levels of TGF- β 1 and PAI-1 in tumour tissue could be of use in preoperative assessment.

Keywords: Rectal cancer, Radiotherapy, Extracellular matrix, MMP-2, PAI-1, uPA, Calprotectin, TGF- β 1

TABLE OF CONTENTS

INTRODUCTION	1
<i>Rectal cancer</i>	1
<i>Treatment strategies for rectal cancer</i>	4
<i>Extracellular matrix remodelling</i>	8
AIMS OF THIS THESIS	14
METHODOLOGICAL CONSIDERATIONS	15
<i>Patients</i>	15
<i>Clinical follow-up</i>	15
<i>Plasma and tissue sampling and processing</i>	16
<i>Laboratory work</i>	17
<i>Statistical methods and considerations</i>	18
<i>Ethical considerations</i>	19
RESULTS AND COMMENTS	20
<i>Matrix metalloproteinases</i>	20
<i>Transforming growth factor -β1</i>	21
<i>The plasminogen system</i>	22
<i>Calprotectin</i>	24
GENERAL DISCUSSION AND FUTURE PERSPECTIVES	25
<i>Reactions to radiotherapy</i>	25
<i>Extracellular matrix remodelling and rectal cancer</i>	26
<i>Future perspectives</i>	27
CONCLUSIONS OF THIS THESIS	28
ACKNOWLEDGEMENTS	29
REFERENCES	31
PAPERS I-IV	39

LIST OF PUBLICATIONS

This thesis is based on the following publications and manuscripts, which are referred to in the text by their Roman numerals (I-IV).

- I. Matrix metalloproteinases in rectal mucosa, tumour and plasma: response after preoperative irradiation.
Angenete E, Langenskiöld M, Falk P, Ivarsson ML,
Int J Colorectal Dis 2007;22(6):667-74
- II. Transforming growth factor beta-1 in rectal tumour, mucosa and plasma in relation to radiotherapy and clinical outcome in rectal cancer patients.
Angenete E, Langenskiöld M, Palmgren I, Falk P, Öresland T, Ivarsson ML,
Int J Colorectal Dis 2007;22(11):1331-8
- III. uPA and PAI-1 in Rectal Cancer-Relationship to Radiotherapy and Clinical Outcome.
Angenete E, Langenskiöld M, Palmgren I, Falk P, Öresland T, Ivarsson ML,
J Surg Res 2008 Apr 7 (Epub ahead of print)
- IV. Preoperative radiotherapy and extracellular matrix remodelling in rectal mucosa and tumour. Matrix metalloproteinases and plasminogen components.
Angenete E, Öresland T, Falk P, Breimer M, Hultborn R, Ivarsson ML,
Submitted for publication

ABBREVIATIONS

APR	abdominoperineal resection
AR	anterior resection
BRCA1	breast cancer gene 1
CRM	circumferential resection margin
CSS	cancer specific survival
CT	computer tomography
CT-PET	computer tomography-positron emission tomography
CV	coefficient of variation
DNA	deoxyribonucleic acid
ECM	extracellular matrix
ELISA	enzyme linked immunosorbent assay
EMVI	extramural vascular invasion
EUS	endorectal ultrasound
FAP	familiar adenomatous polyposis
Gy	gray, the absorption of one joule of energy by one kilogram of matter
LAP	latency associated peptide
LTBP	latent transforming growth factor- β 1 binding protein
MMPs	matrix metalloproteinases
MRI	magnetic resonance imaging
MV	megavolt
PAI	plasminogen activator inhibitor
PBS	phosphate buffered saline
SBU	Swedish Council of Technology Assessment in Health Care
TGF- β 1	transforming growth factor- β 1
TIMPs	tissue inhibitors of matrix metalloproteinases
TME	total mesorectal excision
TNM	classification of malignant tumours, developed by the International Union Against Cancer (UICC) (Union International Contre le Cancer)
tPA	tissue-type plasminogen activator
uPA	urokinase plasminogen activator
uPAR	urokinase plasminogen activator receptor
VEGF	vascular endothelial growth factor

INTRODUCTION

RECTAL CANCER

Epidemiology, aetiology and risk factors

Epidemiology

Rectal cancer is among the ten most common cancers in the Western World (1) with approximately 1800 new cases each year in Sweden (2). It is more frequent among the elderly population and in men. According to the Swedish Rectal Cancer Registry the 5-year cancer-specific survival for rectal cancer was about 62% between 1999 and 2003 (3). There has been a substantial improvement in rectal cancer survival over the last couple of decades (4, 5) and this is also true for local recurrence rates that have been reduced from historical figures of up to 30-40 % to about 5-10% through meticulous surgery and the addition of preoperative radiotherapy (6, 7). A multidisciplinary approach has also been important in the development of rectal cancer treatment (8).

Aetiology

It is generally believed that a rectal cancer develops from normal epithelium that transforms to dysplasia and eventually into an invasive adenocarcinoma. Some patients carry an inherited increased risk of rectal cancer, generally in an autosomal dominant manner, such as familial adenomatous polyposis (FAP) or hereditary non-polyposis colorectal cancer (Lynch syndrome) (9).

The time required to develop a rectal cancer is unknown, but studies regarding tumour kinetics indicate that adenocarcinomas often are slow growing tumours with a monoclonal origin starting several years prior clinical symptoms and subsequent diagnosis (10). It has been suggested that almost all types of cancers require the same type of capabilities during tumour development. Simply they can be described as: self-sufficiency in growth signals as well as avoidance of anti-proliferative signals, evasion of apoptosis, sustained angiogenesis, tissue invasion and metastasis and unlimited replication (11).

Risk factors

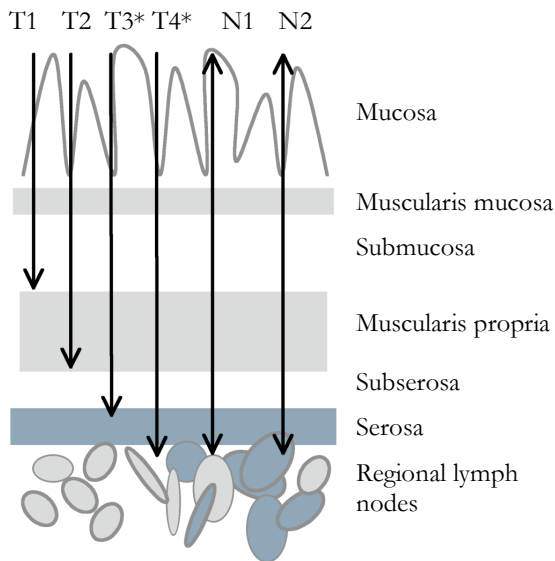
The involvement of genetic, environmental and dietary factors in rectal cancer pathogenesis are established, however many studies include both colon and rectal cancer, and it can not be totally certain that all these factors apply to rectal cancer alone. It has been suggested that red and processed meat and high intake of alcohol may increase the risk for rectal cancer and high intake of calcium may decrease this risk. Less physical exercise and a high BMI are associated with a higher risk of colon cancer, but this has not convincingly been shown for rectal cancer (8).

Diagnosis and imaging of rectal cancer

Diagnosis and tumour classification

Upon suspicion of a rectal cancer, a digital examination of the tumour is performed. Macroscopic form, relative position to the surrounding anatomy and tumour fixity are evaluated. This is followed by rigid rectoscopy, where the height of the tumour is estimated and multiple biopsies are taken for microscopic evaluation and confirmation of the diagnosis. Previously, a couple of decades ago, these examinations were the basis for

treatment decisions. However, to select patients for the optimal treatment a more thorough preoperative assessment is desired. Today several risk factors for a more advanced disease have been identified. Apart from the height of the tumour, the lateral spread (T classification), the nodal status, (N classification) and the circumferential resection margin (CRM) are of prognostic importance for the risk of local recurrence and metastases (12, 13) (TNM classification, Figure I). More recently preoperatively visualized extramural vascular invasion (EMVI) has been considered to be important as predictor of the risk of metastases, local recurrence and death of rectal cancer (14).



**sub classification*

T3a	minimal invasion	< 1 mm below m propria
T3b	slight invasion	1-5 mm below m propria
T3c	moderate invasion	>5-15 mm below m. propria
T3d	extensive invasion	>15 mm below m. propria
T4a	tumour grows onto other organs	
T4b	tumour perforates visceral peritoneum	

Furthermore patients with distant metastases (M1) or synchronous colonic tumours must be identified. Approximately fifteen percent of patients presenting with rectal cancer have distant metastases at diagnosis (15), and up to nine percent of patients with a colorectal tumour have synchronous carcinomas in the colon/rectum (9).

Imaging

Today there are several methods in use to provide information about the tumour and to assess risk factors before treatment. In table I are listings of some of the available preoperative assessments, their use today and the estimated accuracy of these different imaging modalities.

Figure I The TNM classification for rectal cancer.

The T classification depicts the lateral spread of the tumour as visualised in the figure.

The N classification is related to regional nodes, including the superior, middle, and inferior rectal (haemorrhoidal), inferior mesenteric, internal iliac, mesorectal, lateral sacral, and presacral nodes.

It is divided into:

N0 (no lymph nodes involved)

N1 (<4 lymph nodes involved)

N2 (>4 lymph nodes involved).

The M classification is divided into:

M0 = no metastases

M1 = metastases

Table I A display of imaging modalities in rectal cancer (8, 9, 14, 16-21).

A. LOCAL TUMOUR ASSESSMENT			
	Endorectal ultrasound (EUS)	Magnetic resonance imaging (MRI)	Computer tomography (CT) CT-Positron emission tomography (CT-PET)
Lateral spread	Accurate in early rectal cancer (T1+T2). User dependent.	Less specificity than EUS in T1-T2, more accurate in advanced tumours.	No advantages compared to EUS and MRI.
CRM	Too little data to evaluate.	Sensitivity of up to 94%, specificity 85%.	Requires more advanced techniques than the standards used today.
Lymph node involvement	Difficult to assess after preoperative treatment. User dependent.	Slightly better sensitivity and specificity than EUS. Assess nodes throughout the pelvis and the abdomen.	As the size is the main selection criteria small tumour nodes may be overlooked. Data similar to MRI in meta-analysis.
EMVI	Too little data to evaluate.	Only used in a few centres, data are promising but scarce.	Too little data to evaluate.
B. DISTANT METASTASES			
	Abdominal ultrasound	MRI	CT CT-PET
Liver	Operator dependent, but accurate, enhanced with contrast. Superior if used intra-operatively.	Superior to both CT and US using special contrast methods.	With special programs the sensitivity is about 93%. Good for surveillance, easy to compare examinations.
Thorax	Recommended for primary survey.	Too little data to evaluate.	Recommended to use if suspicious lesion is found on chest x-ray.
C. SYNCHRONOUS TUMOURS			
	Colonoscopy	Barium enema	CT-colonography
Colon	Enables intervention, user dependent. Recommended to exclude adenomas.	Sufficient in most cases to exclude larger tumours. Not recommended for diagnosis of adenomas.	Sensitivity at least equal to barium enema. Under evaluation and may be used more frequently in the future.

TREATMENT STRATEGIES FOR RECTAL CANCER

Surgery

There are two main abdominal procedures for rectal cancer today: the abdominoperineal excision (APR) and the anterior resection (AR). Local procedures for less advanced rectal cancers (T1), such as transanal endoscopic microsurgery are also available.

The AR is performed in tumours where the height of the tumour is enough to provide a sufficient tumour-free margin to the pelvic floor. It accounts for approximately 50% of rectal cancer surgery in Sweden. The technique was improved during the 1980s when Heald suggested following the embryological planes, a total mesorectal excision (TME) (22). This has shown reduced recurrence rates (6) and is widely accepted today. Care is taken to avoid damaging the hypogastric and pelvic nerves. The rectum is divided two to five centimetres below the tumour, and after rinsing the rectum with water the anastomosis is often achieved by a circular stapling instrument.

If the patient has a poor sphincter function prior surgery or the risk of anastomotic dehiscence is considered too high, the patient can be recommended an AR without an anastomosis, commonly referred to as the Hartmann's procedure. This procedure is performed in about 10-15% of rectal cancer patients in Sweden.

The APR was introduced by Ernest Miles in 1908. It has been refined over the years and remains a widely used technique today. It is performed when the tumour is so low that the oncologic result may be compromised with an anastomosis. In addition to an abdominal TME, a perineal dissection takes place, including the levators in the resection. A little more than 30 % of surgery for rectal cancer in Sweden is performed as an APR. The prognosis for patients with low rectal cancers undergoing APR is worse than for patients with higher tumours undergoing an AR (23, 24), and a more radical perineal approach has been introduced, where a cylindrical resection is performed (25). It involves a termination of the abdominal dissection at a higher level, reducing the risk of dissecting too close to the tumour in the pelvis. There are today no large randomised trials proving this technique superior to previous surgical approaches.

Postoperative morbidity and mortality

According to the Swedish Rectal Cancer Registry the 30-day post-operative mortality is approximately 2.2-2.5% after rectal cancer surgery (3). The frequency of complications is about 35% all together, where cardiovascular complications and non-surgical infections attribute for 14% of these complications.

A quarter of the patients have surgical complications such as wound sepsis, wound rupture, bleeding or anastomotic dehiscence (3). The frequency of anastomotic dehiscence is almost 10% and has not been affected by alterations of treatment between 1995 and 2003. It is a feared complication often resulting in increased morbidity and mortality. Identified risk factors are among others male gender, a low anastomosis, intra-operative adverse events and preoperative radiotherapy (26, 27). Studies have shown that a diverting loop-ileostomy decreases the number of symptomatic anastomotic dehiscence (28) and it is recommended for patients with an estimated increased risk.

Chemotherapy

In general chemotherapy can be given in three different settings, neoadjuvant i.e. prior surgery (often in adjunct to radiotherapy) or as an adjuvant treatment with the aim of targeting micro-metastases and thus reduce the risk of future metastases and subsequent death of rectal cancer. It can also be administered with a palliative intent, where patients receive chemotherapy after the discovery of an advanced disease.

The neoadjuvant approach will briefly be discussed in relation to radiotherapy in coming sections. Regarding adjuvant treatment there is no compelling evidence showing a clear benefit for rectal cancer patients. There are indications that adjuvant chemotherapy, using similar selection criteria as for colon cancer, may be beneficial for rectal cancer, but this remains to be proven in larger series (29). It has been suggested that patients responding well to neoadjuvant treatment may benefit more from adjuvant chemotherapy (30). There is an ongoing study in Europe, the SCRIPT-study, which may answer some of these questions, but presently adjuvant chemotherapy for rectal cancer patients treated with radical surgery remains controversial.

In patients with advanced rectal cancer most studies include both colon and rectal cancer. It is evident that palliative chemotherapy increases survival, and one study indicates that treatment should be initiated as soon as the advanced disease is known, although the patient may be asymptomatic (29). There is yet no consensus on this issue.

Radiotherapy

Indications

The choice of preoperative radiotherapy treatment is based upon the preoperative evaluation described above. However, there are different stratification schedules in different countries (31). The current recommendations in Sweden (2009) are presented in table II, where the main focus has been on reduction of local recurrence, more than on systemic disease.

Table II Indications for radiotherapy in Sweden 2009. Patients with suspected lymph node involvement (N1-N2) are recommended radiotherapy regardless of T classification. All criteria must be fulfilled to belong to the “good” group, one criteria sufficient for upgrading to the “bad” or “ugly” group. Source: The national treatment guidelines.

	Height	T	N	CRM	EMVI	Implications for treatment	Incidence in Sweden
“good”	mid/ upper rectum	T1- T3a	N0	clear	no	No pre-operative treatment	30-40% of all new cancer
“bad”	mid/ upper rectum	T3b- T3d, T4b	N1- N2	threatened	yes	Short term pre-operative radiotherapy	40-60% of all new cancers
	low rectum	T1- T3d, T4b	N1- N2	threatened	yes		
“ugly”	any	T4a	any	involved	any	Neoadjuvant radiotherapy & chemotherapy	10-20% of all new cancers
	any	T3- T4	any	involved	any		

Patients are recommended short course preoperative radiotherapy if the tumour is considered to belong to the “bad” group (This thesis studies patients with this short course regime.). Patients with “ugly” tumours are recommended longer course radiotherapy with the intent to cause shrinkage of the tumour and allow resection. In adjunct to this, chemotherapy is often given and studies indicate an improved local

tumour control and that this treatment may render non-resectable tumours resectable (15, 32).

Background

Radiotherapy has been studied as an adjunct to rectal cancer therapy for a couple of decades. It is possible to administer preoperatively as well as postoperatively. There are advantages of preoperative administration as tumour cells that are well oxygenated have a better radio sensitivity. However, it may also cause over-treatment, as the tumour may be overestimated and thus the patient receives preoperative radiotherapy without any benefit.

The Swedish Council of Technology Assessment in Health Care (SBU) presented a systematic review of radiation therapy trials in 2003 and found preoperative radiotherapy more efficient than postoperative radiotherapy in reducing local recurrence (15). Furthermore there was evidence for improved survival due to preoperative radiotherapy. However, radiotherapy does not compensate for a positive CRM margin (≤ 1 mm) (33). Many studies regarding radiotherapy were done before the introduction of more advanced preoperative imaging. With the better assessment of today some of these patients are excluded from radiotherapy treatment, as their risk of local recurrence is considered so low that the benefit may be less than the disadvantages related to treatment-associated morbidity.

Radiotherapy administered as short course (fraction of 5 Gy for 5 days) or long course (1,8-2 Gy fractions up to 46-50,4 Gy) with or without chemotherapy are the most common preoperative treatments given to rectal cancer patients. Whether chemotherapy should be added to short course radiotherapy or long course radiotherapy is not certain today (8). Studies are ongoing trying to find the optimal therapeutic approach, both regarding radiotherapy fractions, timing of surgery and addition of chemotherapy.

There is no general worldwide acceptance for preoperative radiotherapy mainly based on the difficulties of preoperative staging leading to risk of over-treatment.

Irradiation techniques

The clinical target volume for the standard preoperative treatment 5x5 Gy is concentrated to the fields where a locoregional recurrence is most probable. This includes the primary tumour as well as the primary lymph nodes in the mesorectum (8). It also includes the closest secondary lymph nodes outside the mesorectum i.e. the tissue at the perimeter of the planned resection margins. The anal canal is excluded if the planned operation is an AR. The planning of the clinical target volume requires a CT scan to be precise. The daily target dose is 5 Gy, and the optimal energy source is 8-15 MV. The treatment should try to avoid surrounding tissue if possible, and the optimal approach for this is a four-field technique.

Morbidity and toxicity of rectal radiotherapy

Clinical manifestations

There is strong evidence that preoperative radiotherapy for rectal cancer has adverse side effects, and some of the acute effects as well as late adverse effects emerging over the years are briefly shown in table III.

Table III *Acute and late adverse effects after preoperative radiotherapy for rectal cancer (26, 27, 34-43).*

Acute adverse effects	Comments
Increased risk or anastomotic dehiscence	Shown in retrospective studies, not in randomised trials. Remains disputed.
Impaired perineal wound healing	Supported by experimental data. Dependent on dose, fractionation and time between surgery and radiotherapy.
Late adverse effects	Comments
Anal and rectal dysfunction	Seen regardless of surgical technique.
Increased risk of small bowel obstruction	Possible that the current improved irradiation technique will ameliorate this problem.
Increased risk of fistulas	Seen in the Stockholm trials.
Impaired sexual function	Both male and females, surgery has a negative effect, and radiotherapy may aggravate sexual dysfunction especially in women.
Increased risk of secondary cancers	Shown in one study, the absolute risk is low and the gain in reduced local recurrence may still be of more importance.
Urinary tract dysfunction	Has been shown, but most studies do not have convincing data.
Increased risk for thromboembolic disease	One study has shown increased risk of cardiovascular disease, no other convincing data.
Increased risk of hip fractures	Rarely seen after rectal cancer radiotherapy, but has been shown in one report.
Decreased quality of life?	Few studies address this subject, and no conclusions can be drawn, although no major differences have been seen.

Molecular and pathological changes after rectal radiotherapy

Many of the adverse effects referred to in table III are related to unfavourable effects in the normal tissue. Radiotherapy has an ionizing effect and releases free radicals that cause DNA-damage or apoptosis. In relation to the tumour cells, this is the desired effect.

In the surrounding tissue however, this leads to an activation of the coagulation system, for example resulting in inactivation of thrombomodulin, probably both by genetic down-regulation and increased release into the circulation (44). Transcription factors are activated (45) and the effect on mast cells and endothelial cells leads to a vasodilatation and an increased permeability. This microvascular injury most certainly depicts much of the initial response and release of proinflammatory and profibrotic cytokines (46). Contrary to regular wound healing this is not later inhibited by anti-inflammatory cytokines, leading to a more persistent activation (36, 47). Interestingly, depending on doses and fractionation an anti-inflammatory response can be elicited by radiotherapy (36).

In patients subject to rectal radiotherapy there are also indications of a systemic response with effects on leucocyte count as well as hemoglobin count (48, 49), and a decreased leucocyte count has been associated with postoperative complications (49, 50).

The reaction to radiotherapy is differential; the individual reaction is related to genetic susceptibility, co-factors such as age and co-morbidity (51-53). Thus there may be a considerable patient-to-patient variation in the normal tissue response.

The pathological changes after radiotherapy are commonly divided into three entities: acute, consequential (related to persistent acute damage) and late effects (months to years after treatment) (47). In the rectum the acute effects are seen in the mucosa (54) and they often disappear within treatment or shortly thereafter (47).

The intestinal tract is susceptible to consequential effects, and the epithelial damage, i.e. barrier breakdown, is of importance (55). It is probable that amelioration of acute effects, perhaps by protecting the endothelial and mucosal barriers will reduce the consequential late effect.

The late effects that emerge over several years are mainly situated in the submucosa and over time an excessive deposition of interstitial collagens, i.e. fibrosis, occurs. The fibrosis may cause strictures and reduced compliance and is represented by large inhomogeneous fibrin deposits and atypical fibroblasts (56). It has been suggested that the effects of radiotherapy could be referred to as a “complex wound” (36) indicating that extracellular matrix remodelling and the following events are central in radiotherapy injury.

EXTRACELLULAR MATRIX REMODELLING

Extracellular matrix

The extracellular matrix (ECM) is a structural entity that supports the cells of the human body. The matrix differs somewhat between different tissues. It is produced by epithelial, endothelial and mesenchymal cells and consists of the basement membrane and an interstitial matrix. Both these parts consist of collagen, but also of adhesive glycoproteins such as laminin, receptors such as integrins and interacting molecules such as proteoglycans and hyaluronan (Figure II).

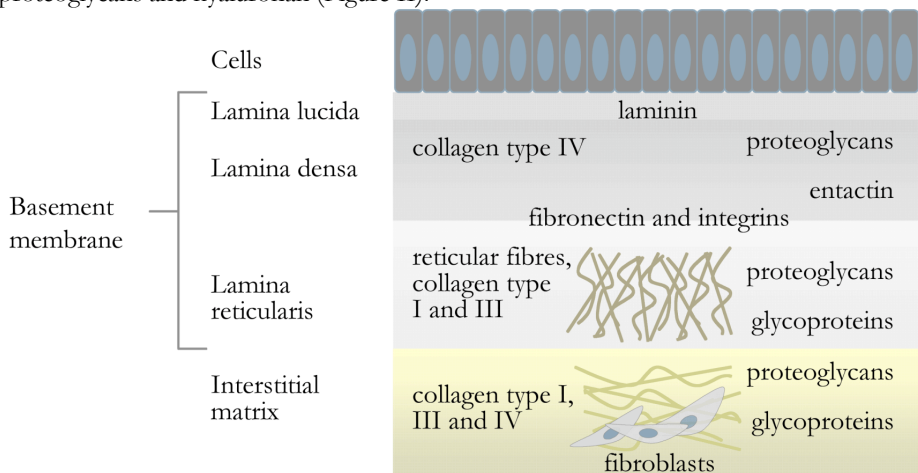


Figure II The extracellular matrix in a schematic picture, only some of the interacting molecules are mentioned.

The cells in the extracellular matrix, for example fibroblasts, myofibroblasts and chondrocytes, all synthesize collagen. There are more than 20 different types of collagen, where type I collagen is the most common collagen in the body, found everywhere

except in cartilage. Type IV collagen is the main structural component of the basement membrane and forms a network for laminin, entactin, growth factors and proteases (57). The ECM is under constant remodelling with the help of proteases, such as the matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), the plasminogen system, thrombin and plasmin (58, 59). Transforming growth factor- β 1 (TGF- β 1) also takes part in the remodelling of the ECM. All of these interact, and an outline of their interactions is shown in figure III. A more detailed review is presented in the upcoming sections.

The ECM and especially the basement membrane are of importance both in inflammation, normal development, wound healing and cancer. Not only does the ECM hold important receptors and growth factors, the components themselves, such as collagen and laminin, can after cleavage promote cell migration (59, 60).

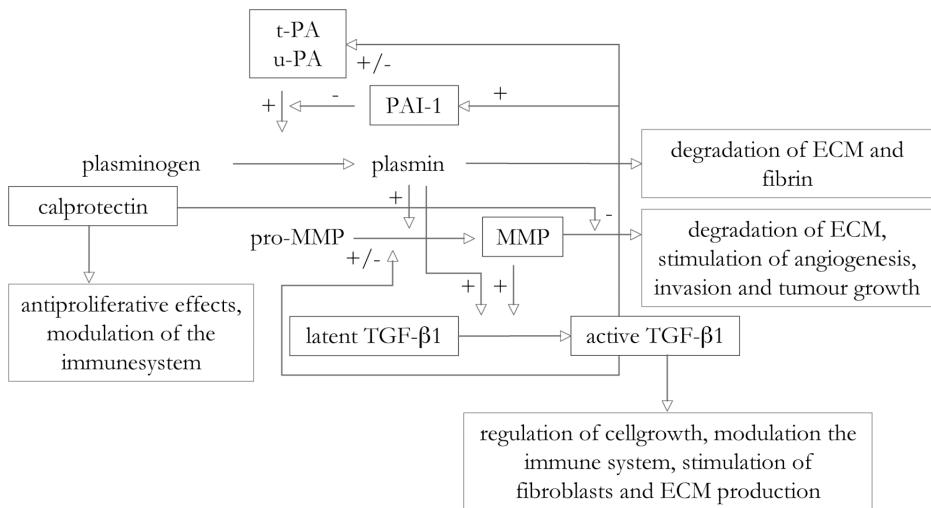


Figure III The extracellular matrix remodelling proteases and cytokines work together in an intricate system. This figure shows some of their interactions (60-65).

Matrix metalloproteinases

Introduction

Matrix metalloproteinases (MMPs) are a family of zink-dependent enzymes that first were discovered responsible for the dissolution of the tadpole tail 40 years ago by Jerome Gross (65). The family consists of more than 20 different MMPs that are divided into groups according to specificity for different ECM components but they are also numbered in order of discovery.

The MMP activity is controlled at the transcription level, by proteolytic activation of the zymogen and through inhibition by a variety of inhibitors such as tissue inhibitor of metalloproteinases 1 and 2 (TIMP-1 and -2) and α 2-macroglobulin. (As shown in figure IV TIMP-1 also interacts with TGF- β 1 in fibrosis development.) Most MMPs can be activated by each other, but also by serine proteases, or in a combination with inhibitors. The MMPs degrade parts of the ECM as well as activate other proteases and growth factors, and these abilities make them important in human development, cancer and wound-healing (60). Higher levels of MMPs are found in defective wound-healing and

fistula formation (66, 67) and it has been shown that MMPs are of importance in infectious disease (68, 69).

MMPs and cancer

The clinical use of matrix metalloproteinases (MMPs) has been studied extensively in relationship to tumour classification, metastasis and prognosis of many types of cancers including colorectal cancer (63, 70-75). Although it is clear that MMPs play an important part in cancer development the use of MMPs as clinical markers requires further study.

Many or most of the steps during carcinogenesis involve MMPs (57, 60, 76-79) rendering them vital for tumour development. However, although they later may be recruited to the tumour cell membrane, MMPs are mainly produced by fibroblasts, endothelial cells and leucocytes (70). There are exceptions to this, such as MMP-7, which is produced by tumour cells.

MMPs in rectal cancer radiotherapy

Both in vitro and in vivo studies have shown increased levels of MMPs, especially MMP-2 and MMP-9, after radiotherapy (80-85). However, the results are somewhat conflicting, where some studies only have shown an increase in MMP-2, and most studies are not performed on rectal cancer patients.

TIMP-1 mRNA is increased in human radiation enteritis (80), and TIMP-1 is also increased in rat colon and ileum after radiotherapy, but TIMP-2 is unaffected (84, 86). In human rectal tumour TIMP-1 does not increase after radiotherapy (87).

It has been proposed that radiotherapy increases the tumour's aggressiveness by this increase in MMPs, both by induction of angiogenesis (88, 89) and by increased invasive potential (90-93).

Although not shown in irradiated rectal cancer patients, a study of anastomotic dehiscence in humans suggests an association with increased levels of MMPs (94). Furthermore, experimental data indicate that inhibition of MMPs increase anastomotic strength (95-97). Thus increased levels of MMP-2 could be related to the possible increased risk of anastomotic dehiscence following radiotherapy (26, 27).

Transforming growth factor- β 1

Introduction

Transforming growth factor- β 1 (TGF- β 1) is one of at least three isoforms of TGF- β . It is a cytokine with many functions that mainly involve inhibition of cell proliferation, regulation of the immune system and increased ECM deposition (76).

TGF- β 1 was originally found in platelets and placenta tissue, but it is now known that many other cells such as fibroblasts can produce TGF- β 1.

It is secreted as a latent complex bound to latency associated peptide (LAP) or the latent TGF- β 1 binding protein (LTBP) and is stored in the ECM. The latent forms require a proteolytic cleavage or conformational change to be activated, and the activated TGF- β 1 has a short half-life. It is thought that plasmin, integrins, decorin and MMPs activate TGF- β (64, 76, 98). It can also be activated by an acidic environment or ionizing radiation (76). TGF- β 1 interacts with the ECM by binding to large proteoglycans at the cell surface.

The actions of TGF- β 1 are mediated through its receptors type I and II, and subsequent phosphorylation of target proteins.

TGF- β 1 and cancer

In non-malignant tissue TGF- β 1 exerts an inhibitory effect on the cell cycle. It has been suggested that the decreasing gradient of TGF- β 1 in colon toward rectum may explain why the incidence of colonic cancers is higher more distally in the colon (99). Evidence also points to this cytokine having a tumour suppressing function in early stages of malignancy, probably through effects on the surrounding stroma and the immune system.

In more advanced tumours the tumour cells themselves produce TGF- β 1 and in this setting it does not have a tumour suppressing effect (100), instead TGF- β 1 facilitates avoidance of anti-proliferative signals and provides the tumour with self-sufficiency in growth factors. The mechanisms behind this change in roles are probably by reduced receptor expression as well as effects on the post receptor signalling (100, 101). The tumour-associated TGF- β 1 seems to facilitate invasion in part through activation of plasminogen activators (100) and by induction of epithelial-mesenchymal-transition. TGF- β 1 also suppresses the immune system facilitating tumour spread and stimulates myofibroblasts producing among others MMPs (102).

TGF- β 1 in rectal cancer radiotherapy

Although TGF- β 1 is activated by radiotherapy (103), higher levels of TGF- β 1 have not been shown in human irradiated rectal tissue, and a decrease in immunoreactivity has been displayed in rectal tumour (104). An early decrease has also been shown in animal studies after a combination of radiotherapy and surgery (105). However, a few months after radiotherapy TGF- β 1 has been found in irradiated colon (106) and in the vessels of irradiated rectum (107). This is consistent with the belief that TGF- β 1 is one of the most important cytokines in radiation-induced fibrosis, which is part of the late effects of irradiation (103). Plasma TGF- β 1 is also one of the most evaluated potential markers for radiation induced morbidity, although it has not yet reached much clinical use (108).

It is suggested that TGF- β 1 enhances the radiation-induced fibrosis through at least three pathways; induction of collagen production and inhibition of collagenase activity, induction of fibroblast proliferation and terminal differentiation of fibroblasts and other structural cells of the ECM (109) (Figure IV).

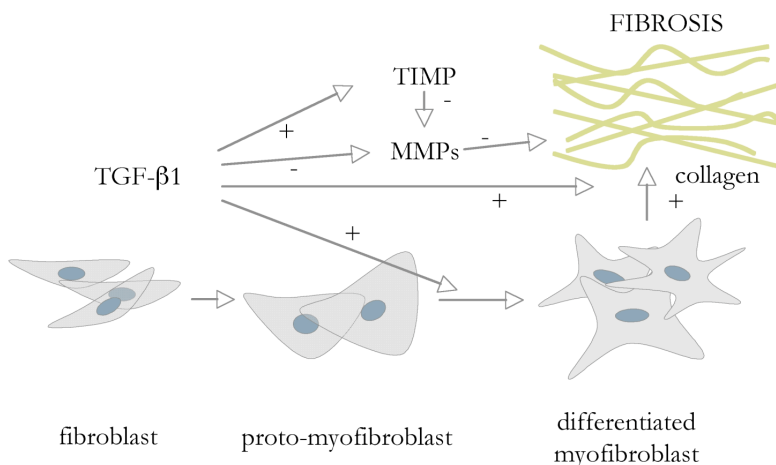


Figure IV TGF- β 1 affects fibrosis development through many different actions.

The plasminogen system

Introduction

The plasminogen system (PS) consists of two activators, tissue-type plasminogen activator (tPA) and urokinase plasminogen activator (uPA) and two inhibitors, plasminogen activator inhibitor-1 and -2 (PAI-1 and PAI-2) as well as the precursor plasminogen and the urokinase plasminogen activator receptor (uPAR).

The activators have somewhat disparate functions where uPA is mainly involved in physiological and pathological tissue remodelling. uPA is produced by stroma cells such as fibroblasts and inflammatory cells, secreted as pro-uPA and activated by among others plasmin and kallikrein. To elicit cellular activities such as cell differentiation and migration uPA binds to the receptor uPAR (110, 111).

tPA's primary function is thrombolysis and it is the main plasminogen activator in plasma (111). It is produced by endothelial cells, macrophages and mesothelial cells, secreted as a precursor and mainly activated by fibrin. It has been shown to be of importance to postoperative adhesion formation, as lower levels of tPA indicate reduced fibrinolytic capacity (112).

Among the inhibitors PAI-1 is the primary inhibitor of both uPA and tPA (62). It has a short half-life and is only present in small concentrations under normal physiological conditions. During stress or trauma endothelial cells, fibroblasts, platelets and mesothelial cells produce PAI-1. It is present both as an active and a latent form, the active PAI-1 is in complex with vitronectin (a glycoprotein present in ECM and plasma), which stabilizes and possibly alters the specificity of PAI-1 (62, 113).

uPA and PAI-1 in cancer

uPA and PAI-1 have both been shown to have a prognostic impact in breast cancer, as well as other cancers (79, 113), predicting the risk of future metastases.

uPA bound to uPAR on the cell surface is primarily a proteolytic activator facilitating ECM remodelling during tumour invasion and takes part in creating a desmoplastic stroma around the tumour cells (114). However, it also regulates angiogenesis through plasmin-induced release of VEGF and by production of the angiogenesis inhibitor angiostatin through the cleavage of plasmin (79, 115).

PAI-1 is found in myofibroblasts and endothelial cells in colon cancer (116). It is thought that endogenous PAI-1 is essential to angiogenesis (117), and also that a high PAI-1 concentration counteracts these events (118). PAI-1 promotes cell detachment from the ECM as well as cell migration (79). Many of the functions of PAI-1 seem to be independent of its inhibitory functions of uPA, which may explain the paradox that both activator and inhibitor are associated with poor prognosis in cancer.

The plasminogen system in rectal cancer radiotherapy

Fibrosis is a side effect of radiotherapy and is mainly due to an excess accumulation of matrix in the interstitial tissue. PAI-1 inhibits the conversion of plasminogen to plasmin, which is one of the main degrading enzymes of this matrix. This inhibition may also reduce MMP activation, augmenting the decreased degradation (62). Studies have shown that both radiotherapy and TGF- β 1 can stimulate PAI-1 transcription (119, 120). Furthermore studies have shown that PAI-1 is upregulated in endothelial cells after rectal radiotherapy (121).

uPA has been found to be both elevated and reduced after radiotherapy (86, 104). The role of uPA in reactions to radiotherapy remains speculative. It is possible that it mainly mirrors an inflammatory response, although it has been suggested that reduced levels of uPA may reflect a lower invasive potential of the tumour (104).

Calprotectin

Introduction

Calprotectin consists of a non-covalent association between two proteins, S100A8 and S100A9 and is present in neutrophils, granulocytes and monocytes as well as some epithelial cells. It is calcium-dependent and when connected to calcium resistant to proteolysis and heat (122).

Calprotectin is regulated at the transcription level by several pro-inflammatory cytokines and interacts with proteoglycans such as heparan sulfate (123).

It is believed to have a regulatory function in inflammation, as well as antibacterial and antiproliferative effects. It is involved in leukocyte recruitment (124) and is found in abundance at inflammatory sites (125). Calprotectin can inhibit most MMPs, probably by sequestration of zink (61), which is interesting as some of the effects of calprotectin seems to be inhibited by zink (125). It has been suggested that the zink levels in plasma may be enough to inhibit calprotectin in the absence of an inflammatory response, but when calprotectin is increased it overcomes this inhibition.

Calprotectin in cancer

Calprotectin has been found in higher levels in colorectal tumour compared to mucosa (126, 127) and it has also been associated with breast cancer where the known tumour suppressor gene BRCA1 seems to interact at transcription level with calprotectin (123). It has been suggested that calprotectin takes part in a relatively novel concept referred to as formation of a pre-metastatic place, i.e. preparing the site of metastases for the arrival of tumour cells (79). It has been believed that faecal levels of calprotectin would be higher in colorectal patients than healthy controls, but a recent meta-analysis could not confirm this theory (128). Calprotectin also has an inhibitory effect on tumour cell growth, and can induce apoptosis in tumour cells, as well as in fibroblasts (125).

Calprotectin in rectal cancer radiotherapy

As calprotectin is related to the degree of inflammation, faecal calprotectin has gained use as a marker for active inflammatory disease (125, 128). It has also been tested for use as a marker of radiation-induced proctitis (48, 129, 130) but the results are contradictory and require further study before being implemented in clinical practice.

AIMS OF THIS THESIS

The aims of this thesis were to:

- Study the effect on extracellular matrix remodelling proteases and growth factors by preoperative radiotherapy for rectal cancer
- Study correlations between these proteases and growth factors and clinically evident complications after radiotherapy and surgery for rectal cancer
- Evaluate whether any of these proteases and growth factors could be used as surrogate markers for tumour classification, risk of developing metastases or cancer specific survival.

METHODOLOGICAL CONSIDERATIONS

PATIENTS

Paper I-III

This thesis summarizes four studies, where the first three in part include the same patients. The patients in paper I-III were consecutively included between 1999-2003. Exclusion was mainly due to logistic reasons at the surgical ward resulting in that biopsies were not taken, or participation in conflicting trials. There was no other patient selection. Some patients included in the studies were excluded from analysis due to pathology confirming the diagnosis adenoma or a different radiotherapy protocol. In paper I and III 91 patients remained for analysis, and in paper II 110 patients were analysed and followed.

The irradiated and non-irradiated groups were fairly similar in regard to gender. There was an age difference between the two groups, where the radiotherapy group had a slightly lower median age, which probably is due to selection of patients for radiotherapy treatment. Forty-nine percent of the patients received radiotherapy.

All surgical tumour specimens were classified according to TNM. As metastatic disease was an exclusion criterion for radiotherapy there was only one patient with metastatic disease (M1) in the irradiated group, this patient was later planned for liver resection and thus treated with a curative intent.

In a concomitant group of subjects that underwent treatment for rectal cancer during this period the distribution of differences in regard to tumour classification is similar to our study group, suggesting that our material mirrors the true picture at the clinic during the study period. It would have been expected to be more advanced tumours in the irradiated group, the lack of this is most probably due to insufficient preoperative classification of the tumour as neither MRI nor endoscopic ultrasound was available for all patients during this period. Also the treatment strategies described in the introduction are the current recommendations, and do not fully apply to the treatment approach 1999-2003.

According to regional therapy standards patients with lymph nodes metastases were offered adjuvant chemotherapy, 5-fluorouracil and leucovorin, if they were under the age of 80 unless there was significant co-morbidity or post-operative complications. Some patients chose to decline chemotherapy.

Paper IV

The first three studies were performed as a comparison of patients with and without radiotherapy. To rule out as much of the patient-to-patient variation, in relation to radiotherapy, as possible and to exclude possible effects of surgical trauma the fourth study was carried out.

Forty-two patients were consecutively included in this study. Ten patients were excluded from analysis and thirty-two patients remained. Twenty patients received preoperative radiotherapy; the twelve patients not receiving radiotherapy were controls. There was no difference in age or gender, but more patients underwent APR in the irradiated group.

CLINICAL FOLLOW-UP

In paper I and II postoperative infections and healing difficulties, small bowel obstruction and anastomotic dehiscence were retrospectively reviewed. Some clinical

data may have been missed, as patient records only were obtained from the three hospitals included in the Sahlgrenska University Hospital, however as the patients were called for yearly visits an extra control of other events outside these hospitals was possible. Wound-infection was registered at thirty days. The yearly visits also included a rigid rectoscopy and a clinical evaluation. Suspicion of metastases or local recurrence was confirmed with biopsies, x-ray or MRI.

In paper III the Swedish Cause of Death Registry was reviewed for causes of death and this is dependent on accurate reporting to the Swedish Cause of Death Registry. However, all patient records were also reviewed in an attempt to ascertain the data in the registry.

PLASMA AND TISSUE SAMPLING AND PROCESSING

Plasma

Paper I-III

Venous blood samples were taken in a standardised manner during induction of anaesthesia. The samples were then centrifuged and the plasma collected and frozen until further processing.

Tissue sampling

Paper I-III

Mucosa and tumour biopsies

In the operating theatre, biopsies were taken from the tumour and the rectal mucosa. The rectal mucosal biopsies were taken 10 cm from the tumour, within the radiation field. The biopsies weighed approximately 20-40 mg (wet weight) and were frozen until further processed.

Paper IV

Mucosa and tumour biopsies

Biopsies prior treatment (baseline biopsies) were taken from both tumour and from adjacent macroscopically tumour-free mucosa using a rigid rectoscope and biopsy forceps. Some of the biopsies taken through the rectoscope were small, which may increase the risk of errors during further analysis (131). The biopsies were frozen until further processed.

During surgery, biopsies were taken in a similar manner as in paper I-III.

Peritoneal biopsies

At the start of the operation biopsies were taken from parietal peritoneum in the upper part of the abdomen, to ensure a non-irradiated area, together with biopsies from irradiated parietal peritoneum in the pelvis. The biopsies were taken in a standardised manner, however, peritoneal biopsies are sensitive to manipulation and it is difficult to ensure that the biopsies are fully comparable, both in size and degree of manipulation. This must be accounted for when interpreting the results. The biopsies were weighed and frozen until further processed.

LABORATORY WORK

Protein extraction

The biopsies were mixed with a phosphate buffered saline (PBS), as described earlier (112) to reach a concentration of 40 mg of tissue/mL of buffer. The samples were then homogenised (Ultra Turrax; Janke & Kunkel GmbH, Staufen, Germany) and to avoid proteolysis of the enzymes this process was performed during ice-cold (0° C) conditions. After centrifugation (10 000 g, 3 min) the supernatant was withdrawn and stored at -70°C until analysed in batches. The technique has been used extensively at our laboratory and our studies have estimated that most of the protein fraction (<97%) is extracted during this process.

Protein assay – Enzyme Linked ImmunoSorbent Assay (ELISA)

Figure V shows a schematic picture of the principles behind enzyme linked immunosorbent assay (ELISA). To standardise the results the concentrations of the proteins were normalized to total protein content based on the method by Lowry (132), with a protein assay from Bio-Rad (Hercules, CA, USA). The total protein content did not differ between irradiated and non-irradiated patients.

Quality control and variability

All assays were run in duplicate by two experienced laboratory technicians. Unexpected values or results where the coefficient of variation (CV) was high were reanalysed.

The CV is a statistical measure of the dispersion of data and represents the ratio of the standard deviation to the mean. For the assays used in these studies the inter-assay variation was approximately 5-10% according to the manufacturer, which may be of importance during interpretation of results, especially results where the statistical significance is close to $p=0.05$.

Matrix metalloproteinases

The homogenised samples were analysed for MMP-1, MMP-2 and MMP-9 using ELISA-kits from Amersham Pharmacia Biotech (Buckinghamshire, UK), as described previously (133).

Transforming growth factor- β 1

Analysis of TGF- β 1 was carried out with ELISA-kits from Promega (Madison WI, USA). Measurements of active and total amounts of TGF- β 1 were performed in separate

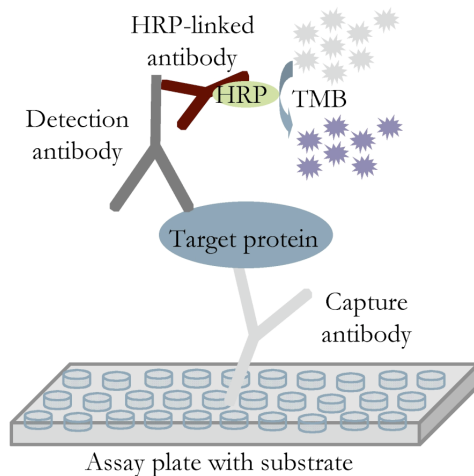


Figure V Enzyme linked immunosorbent assay (ELISA). An antibody-antigen reaction together with an enzyme reaction converts a peroxidase sensitive substrate into colour and the light absorption is then read in an automatic plate reader (*V-max* from Molecular Devices).

steps. The active fraction of TGF- β 1 was assayed directly in the ELISA plate using the kits provided. For measuring the total amount of TGF- β 1 additional samples were acidified to pH 3.0 using 1 mol/L HCl, followed by a 15 minute incubation at 22°C, resulting in activation of all TGF- β 1. To neutralise samples 1 mol/L NaOH was supplemented before application to the second ELISA plate.

The plasminogen system

The fibrinolytic parameters uPA and PAI-1 were analysed using ELISA-kits from Biopool (Umeå, Sweden).

Calprotectin

Calprotectin was analysed using ELISA-kits from Bühlmann Laboratories AG (Schönenbuch, Switzerland). As this analysis is designed to measure calprotectin in stool samples the tests were made more than twice and we also used other controls with known high inflammatory reaction, as calprotectin is present in inflammatory cells. The results were also studied by immunohistochemistry.

Histology

Biopsies taken for histological examination and immunohistochemistry were fixed in Bouin's solution (Sigma Diagnostica, St Louis, MO, USA) overnight. Following wash with PBS solution biopsies were dehydrated in increasing ethanol gradients and xylene prior to paraffin embedding. Four to six micrometer sections were deparaffinised and stained with Haematoxylin and Eosin for morphologic assessment.

Immunohistochemistry

The expression of calprotectin in the mucosa was evaluated with immunohistochemistry in selected biopsies. Primary mouse anti-human Macrophage L1 protein/Calprotectin antibodies diluted 1:10 (Ab 62227, Lot No: 430744, Abcam, Cambridge, UK) were used together with the DAKO Envision system (DAKO Cytomation, Glostrup, Denmark) and detected with diaminobenzidine. Incubations of tissue sections with mouse IgG directed towards an enzyme neither present nor inducible in mammalian tissue instead of primary antibodies (X-0931, DAKO Cytomation, Glostrup, Denmark) at the same concentration, served as negative controls. Slides were counterstained with Haematoxylin and evaluated using Nikon Eclipse 800 microscope together with Nikon Coolpix 995 digital photo equipment (Nikon Instruments Inc, Melville, N.Y., USA).

STATISTICAL METHODS AND CONSIDERATIONS

The statistical methods used in this thesis are non-parametric. The data has been tested with the Kolmogorov-Smirnov test against a normal distribution confirming that most data is non-parametrically distributed.

In paper I statistical calculations were performed using StatView (Abacus Concepts, Berkeley, Ca, USA) and in paper II-IV SPSS 11.0.4 and 13.0 (SPSS Inc., Chicago, Illinois, U.S.A.).

All graphs are presented as box-plots showing the median (horizontal line), interquartile range (boxes) and the 10th and 90th percentile (error bars). For paper I-III statisticians were consulted.

Univariate analysis

The Mann-Whitney U test was used for non-related samples and Wilcoxon signed rank test for comparing dependent samples. Chi-square or Fisher's exact test were used where

appropriate. The Kruskal-Wallis test was used to test several independent samples. Correlation analysis was calculated using Spearman's rank test.

Multiple analyses as performed in these papers are subject to the question of mass-significance. This can be dealt with using the Bonferroni correction. However, when using a Bonferroni correction there is also a possibility of not discovering results through a beta-error. It remains disputed whether a Bonferroni correction is required or not, using this type of correction may be considered statistically conservative. We have chosen not to use this method, but an awareness of the number of analyses made is important when interpreting the results presented.

Multivariate analysis

In paper II logistic regression was used for multivariate analysis; the odds ratio (OR) is displayed with 95% confidence interval (CI). In paper III Cox proportional hazard was used for multivariate analysis. Likelihood Ratios (LR) backward stepwise regression was used to identify significant variables. Hazard ratio (HR) is displayed with a 95% confidence interval (CI 95%).

It has been recommend to use the "rule of ten" when using the Cox proportional hazard model, which means that for each covariate there should be ten events. This is not the case in papers II and III. However, this is a recommendation, and has been questioned (134), but it is important to have in mind when interpreting multivariate statistics.

ETHICAL CONSIDERATIONS

The University of Gothenburg Local Ethics Committee approved these studies. All patients gave informed consent.

RESULTS AND COMMENTS

The main results of the studies included in this thesis are presented below. More results and graphs are found in the appended papers.

MATRIX METALLOPROTEINASES

To understand the pathologic process during and after radiotherapy which involves both a remodelling and degradation of ECM, with an emphasis on collagen turn-over as well as an effect on the basement membrane, MMP-1, -2 and -9 were investigated in biopsies taken during surgery from both irradiated and non-irradiated patients (paper I).

We found higher levels of MMP-1, -2 and -9 in tumour compared to mucosa, irrespective of preoperative radiotherapy or not (Figure VI) (unpublished observation). This was also confirmed in the baseline biopsies in paper IV.

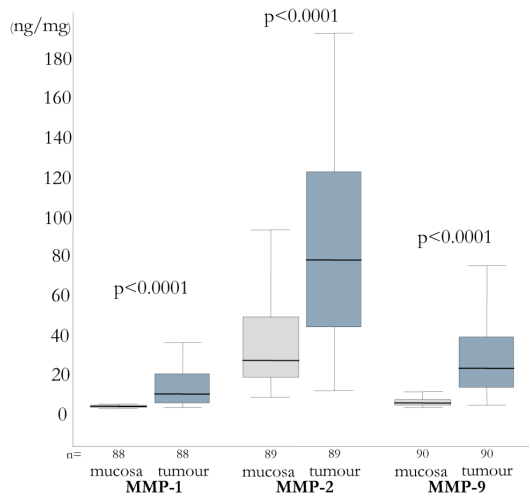
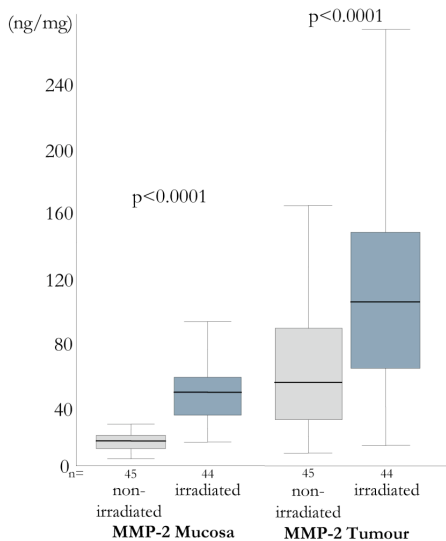


Figure VI MMP-1, MMP-2 and MMP-9 were all significantly higher in rectal tumour compared to mucosa.



When comparing irradiated patients with patients without radiotherapy we found significantly higher levels of MMP-2 in tumour tissue and this was also true for MMP-2 in mucosa (Figure VII), the same results were seen in paper IV. MMP-1 and -9 had the same pattern in mucosa in paper I but this could not be shown in paper IV.

Figure VII MMP-2 was significantly higher in irradiated patients, both in mucosa and tumour (Paper I). Data from this figure was originally published in *Int J Colorectal Dis* 2007;22(6):667-74. Printed with kind permission from Springer Science+Business Media.

Wound-infections did not differ between the irradiated and non-irradiated group, but when looking at the entire patient material there was a significantly higher level of MMP-2 in the eleven patients later developing a wound-infection (n=88, p=0.02). Fistulas in the perineal area were only present in irradiated patients and their levels of MMP-2 were higher in both tumour and mucosa compared to the rest of the patient material (Figure VIII).

Univariate analysis of MMP-1 in mucosa and tumour displayed higher levels in patients developing distant metastases (n=76; p=0.02 and n=74; p=0.04). MMP-2 in plasma in univariate analysis was found to be significantly higher in patients later developing metastases (n=77, p=0.007).

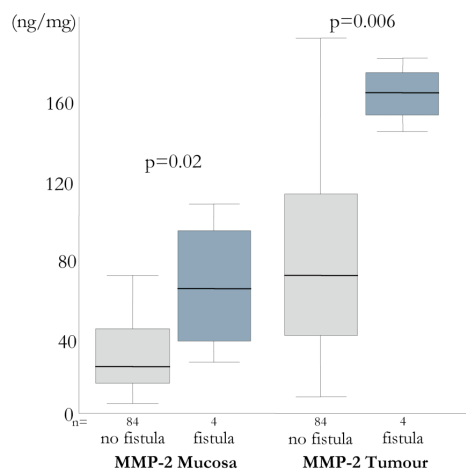


Figure VIII In patients developing fistulas MMP-2 levels were higher in both mucosa and tumour (Paper I). Data from this figure was originally published in *Int J Colorectal Dis* 2007;22(6):667-74. Printed with kind permission from Springer Science+Business Media.

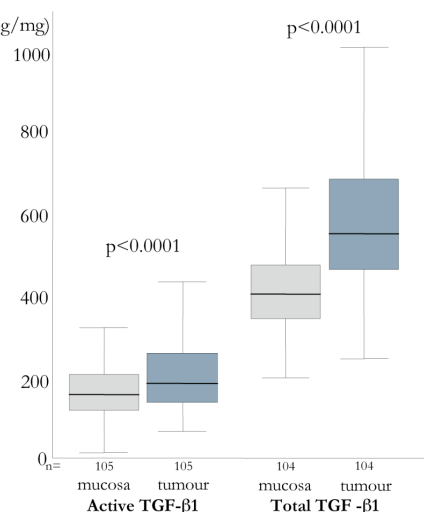
TRANSFORMING GROWTH FACTOR -β1

To further pursue the question of TGF-β1 in radiation fibrosis as well as the significance of TGF-β1 in rectal cancer, active and total levels of TGF-β1 were studied. Radiotherapy can induce fibrosis development, through remodelling of the ECM with an excessive deposition of collagen. TGF-β1 increases ECM deposition and is activated by radiotherapy (76).

Previous data have shown a decreased immunoreactivity in rectal tumour and no effect in rectal mucosa (104).

In paper II results displayed higher levels of both active and total TGF-β1 in tumour compared to mucosa (Figure IX). Interestingly active TGF-β1 was not higher in the baseline biopsies of tumours in the fourth study.

Figure IX Both active and total TGF-β1 levels were significantly higher in tumour compared to mucosa (Paper II). Data from this figure was originally published in *Int J Colorectal Dis* 2007;22 (11):1331-8. Printed with kind permission from Springer Science+BusinessMedia.



In paper II irradiated patients had lower levels of active TGF- β 1 in both tumour and mucosa, but no significant difference was seen in total TGF- β 1. These results are not sustained in paper IV.

The surgical specimen classification according to TNM classification did not correlate with neither active nor total TGF- β 1 in regard to T or N classification. Patients with metastases at diagnosis (M1) had higher levels of TGF- β 1 in tumour compared to M0 patients. (As metastasis was an exclusion criterion for radiotherapy, irradiated patients were excluded from this analysis.)

In univariate analysis active TGF- β 1 in plasma was associated with development of metastases. In a multivariate logistic regression with the co-variables T classification, N classification, radiotherapy and chemotherapy this was confirmed showing lower levels of active TGF- β 1 in plasma associated with development of metastases during the first three years ($p=0.02$, OR=0.832, 95% CI=0.708-0.976).

Univariate analysis also revealed higher levels of total TGF- β 1 in mucosa in patients with local recurrence ($p=0.04$). In multivariate analysis with the co-variables T, N and M classification, radiotherapy and chemotherapy, total TGF- β 1 in mucosa remained significant in patients with local recurrence ($p=0.01$, OR=0.986, 95% CI=0.974-0.997).

THE PLASMINOGEN SYSTEM

TGF- β 1 is together with PAI-1 associated with fibrosis and evidence point to a combined activation of PAI-1 in radiotherapy (62, 119). There is evidence that both the activator uPA and the inhibitor PAI-1 can be related to prognosis in different cancers (79, 113). To evaluate the role of parts of the plasminogen system in rectal radiotherapy pathogenesis and rectal cancer uPA and PAI-1 were analysed.

The levels uPA and PAI-1 were higher in tumour compared to mucosa in both irradiated and non-irradiated patients (Figure X) in paper III and this could also be seen in the baseline biopsies in the fourth study.

Irradiated patients displayed higher levels of PAI-1 in tumour tissue and mucosa compared to non-irradiated patients and uPA levels were higher in mucosa (Figure XI). These results were verified in paper IV.

In relation to the TNM classification, the surgical specimens with higher T classification and tumour-afflicted lymph nodes (N) had higher levels of PAI-1 in tumour tissue, but in subgroup analysis the latter could only be seen in irradiated patients. Patients with synchronous metastases (M1) had higher levels of PAI-1 in plasma ($n=45$; $p<0.0001$).

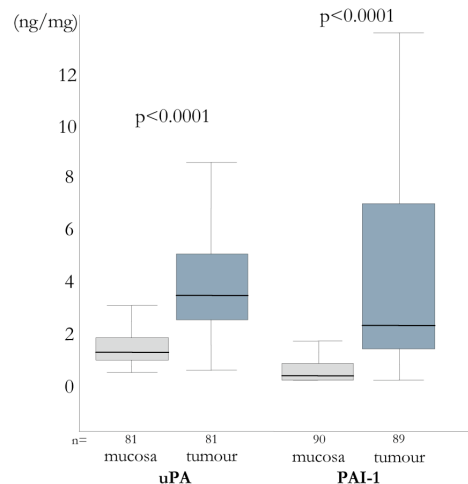


Figure X uPA and PAI-1 were significantly higher in tumour compared to mucosa (Paper III). Data from this figure was originally published in *J Surg Res* 2008 Apr 7. Reprinted with kind permission from Elsevier.

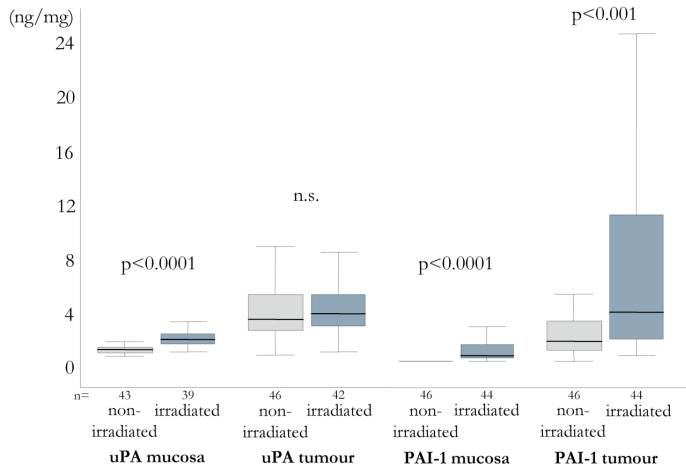


Figure XI PAI-1 was significantly higher in both tumour and mucosa in irradiated patients, a pattern that also was seen in mucosa regarding uPA (Paper III). Data from this figure was originally published in *J Surg Res* 2008 Apr 7. Reprinted with kind permission from Elsevier.

Univariate analysis revealed an increased risk of local recurrence in patients with high PAI-1 in tumour tissue, which was not confirmed in multivariate analysis.

In patients developing metastases (n=21) during the follow-up period a relationship was seen with the ratio of uPA/PAI-1 in tumour tissue (Table IV) as well as with chemotherapy.

Table IV Univariate and multivariate analysis regarding risk factors for development of metastases (Paper III). Data from this figure was originally published in *J Surg Res* 2008 Apr 7. Reprinted with kind permission from Elsevier.

Variable	Univariate	Multivariate	HR	95% CI
Age	p=0.877	p=0.957	0.998	0.934-1.067
Sex	p=0.765	p=0.354	0.631	0.238-1.671
Radiotherapy	p=0.401	p=0.142	0.432	0.141-1.325
Adjuvant chemotherapy	p<0.001	p<0.05	3.154	1.166-8.533
T1+T2/T3-T4	p<0.05	p=0.078	3.848	0.862-17.187
N0 vs N1+N2	p<0.01	p=0.783	0.825	0.209-3.260
PAI-1 in tumour	p<0.001	p=0.711	0.990	0.936-1.046
PAI-1 in plasma	p<0.01	p=0.376	1.593	0.569-4.459
uPA/PAI-1 tumour	p<0.005	p<0.01	0.520	0.296-0.914

Chemotherapy, M classification and PAI-1 in tumour were found to be significantly related to death of rectal cancer in both univariate and multivariate analysis (Table V).

Table V Univariate and multivariate analysis determining risk factors for death of rectal cancer (Paper III). Data from this figure was originally published in *J Surg Res* 2008 Apr 7. Reprinted with kind permission from Elsevier.

Variable	Univariate	Multivariate	HR	95% CI
Age	p=0.503	p=0.446	1.016	0.975-1.060
Sex	p=0.352	p=0.449	0.736	0.333-1.629
Radiotherapy	p=0.055	p=0.053	0.398	0.157-1.012
Adjuvant chemotherapy	p<0.0001	p<0.05	2.434	1.083-5.471
T1+T2/T3-T4	p<0.005	p=0.125	2.413	0.783-7.439
N0 vs N1+N2	p<0.001	p=0.936	1.044	0.363-3.001
M-stage	p<0.0001	p<0.001	5.992	2.159-16.631
PAI-1 in tumour	p<0.01	p<0.01	1.036	1.007-1.066
PAI-1 in plasma	p<0.001	p=0.682	1.150	0.588-2.250
uPA/PAI-1 tumour	p=0.169	p=0.455	0.900	0.683-1.187

PAI-1 as well as tPA are closely associated with adhesion formation. The increased risk of small bowel obstruction after radiotherapy (38) was the incentive to study peritoneal biopsies that were irradiated, and compare these with non-irradiated peritoneum. tPA levels were found to be lower in the pelvic biopsies of irradiated patients compared to controls (median 7.8 ng/mg compared to 17.7 ng/mg, p<0.05). It is possible that this reflects a reduced fibrinolytic activity in irradiated patients. However, it is somewhat puzzling that there is no difference between the pelvic and abdominal biopsy in the irradiated patients and it is possible that flaws in biopsy technique may influence these results.

CALPROTECTIN

Calprotectin in faeces has been suggested as a possible marker of mucosal radiation injury (130), and it is possible that it could be used as a surrogate marker for an aggravated inflammatory response to radiotherapy. We found higher levels of calprotectin in tumour tissue compared to rectal mucosa in the baseline biopsies, which confirms previous results and implicates that calprotectin may be important in rectal cancer (127).

The levels of calprotectin were significantly higher in irradiated mucosa compared to baseline biopsies, and were localized to inflammatory cells such as granulocytes and macrophages. As predicted calprotectin reflects the inflammatory response in the rectal mucosa, and this supports the idea of using calprotectin as an inflammatory marker in rectal radiotherapy.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

It is evident that radiotherapy as part of rectal cancer therapy is here to stay. The data on reduced frequency of local recurrence (7) and possibly even increased survival (15) are convincing. However, as survival has improved over the years preventing or ameliorating long-term side effects of radiotherapy have become increasingly important. It is probable that not all patients have a benefit from radiotherapy compared to their potential risk of increased morbidity after surgery. Thus a further understanding of the pathophysiology related to rectal cancer radiotherapy is essential and selection of patients prior to radiotherapy is necessary to provide individually tailored treatment.

This thesis aimed to evaluate effects of radiotherapy on mechanisms involved in ECM remodelling and subsequently improve the understanding of ECM remodelling effects on positive and negative outcomes after short-term radiotherapy. A further aim was to devise possible markers for better patient selection.

REACTIONS TO RADIOTHERAPY

The first three papers in this thesis compared patients undergoing surgery with or without prior radiotherapy. This has benefits in that the inclusion of patients is uncomplicated, patients are asked for participation, but no extra examinations than biopsies and blood samples taken during surgery are performed. However, a possible drawback is that it is difficult to totally rule out an effect of surgery on the components studied. Also it requires a fairly similar patient material, which in theory, would not be the case as there is an intended selection of patients for radiotherapy. There are also patient-to-patient variations in the response to radiotherapy (51-53), which may influence the results. However, the fourth paper in this thesis confirms the most significant events of the previous three papers, which is reassuring.

All of the events in the tissue after irradiation occur in different time frames, making it difficult to envision the entire picture in one single biopsy. It can only deliver a snapshot of the process. Thus one disadvantage of our human studies is the limitation of biopsies; it is not ethically possible to take numerous biopsies sequentially on newly operated patients. On the other hand it has the advantage of displaying reactions in human tissue, which cannot be done with in vitro or animal studies. Sequential biopsies have been studied in patients with radiotherapy due to prostate cancer (81), but these patients did not undergo rectal surgery. Also, even so it is still impossible to acquire biopsies that go deep, perhaps beyond the submucosa, which would be of interest in order to study the development of fibrosis.

This thesis supports that MMP-2 is higher in irradiated tissue. The findings with higher levels of MMP-2 in irradiated mucosa and tumour in paper I were confirmed in the concluding fourth paper. The clinical implications are many, but our studies have only shown a relationship between MMP-2 and clinical manifestations related to wound healing such as infections and fistulas. Evidence points to an increased risk of anastomotic dehiscence in patients treated with preoperative radiotherapy (26, 27). Although the risk of symptomatic anastomotic dehiscence can be reduced by a defunctioning stoma (28) no current strategy to reduce the problem itself has evolved. It is possible that addition of inhibitors of MMPs could be of use in patients undergoing preoperative radiotherapy.

Previous studies in rats have indicated an increase of the MMPs, in particular MMP-2 in the mucosa, as an early reaction to radiotherapy. This could represent epithelial denudation, and the somewhat later increase of TIMP-1 would then represent the restitution phase of this mucosal ECM remodelling (86). Our biopsies are taken up to 6 days after radiotherapy, which may explain why we did not see effects of radiotherapy on TIMP-1 levels.

That TGF- β 1 is involved in the pathogenesis of radiotherapy induced tissue injury seems well established (103). The activation of TGF- β 1 by radiotherapy (76) could not be demonstrated in our studies. In fact we found lower levels of active TGF- β 1 in irradiated mucosa in paper II, but no reaction at all in paper IV. The half-life of active TGF- β 1 is very short, and it may be that this affected the results. On the other hand it is also possible that the effect of TGF- β 1 is seen mainly in tissue subject to fibrosis development, and thus our biopsies would not be able to demonstrate any significant changes, as they do not include the submucosa.

As PAI-1 is involved in fibrotic processes throughout the body we surmised that PAI-1 would be increased in tissue subject to radiotherapy. Evidence by other authors also supported this theory (119, 121). The third paper showed a marked increase of PAI-1 in both mucosa and tumour, which also was supported in the fourth study. As the endothelial injury is initiated early by radiotherapy, this could explain why this fibrogenic component is shown in our studies in contrast to TGF- β 1.

EXTRACELLULAR MATRIX REMODELLING AND RECTAL CANCER

As discussed in the introduction, tumours, such as rectal cancer, evolve through a multitude of events. We have indirectly studied a part of one of many important events in carcinogenesis, the remodelling of the extracellular matrix.

Although the exact mechanisms cannot be elucidated by our studies it seems evident that MMP-1, -2 and -9, that together degrade the basement membrane, provide growth factors, stimulate angiogenesis and stimulate migration, are of relevance in rectal cancer biology. They were all elevated in tumour tissue compared to adjacent mucosa, indicating an increased production. Our results do not allow any further analysis regarding the mechanisms of MMP-1, -2 and -9 in rectal cancer. The results in paper I indicated that MMP-1 may be used as a prognostic factor, which of course could be of use in assessment of the individual patient's tumour. However, multivariate analysis was not performed, and the significance level also indicates that the results must be considered exploratory.

Transforming growth factor- β 1 is a very interesting cytokine in colorectal tumour biology. The decreasing gradient of TGF- β 1 in colon (99) supports theories regarding its importance, as tumours of the colon are more common in the distal part. We found higher levels of TGF- β 1 in tumour compared to mucosa, which also was confirmed in the baseline biopsies of the fourth study. The correlation with TGF- β 1 in tumour tissue and the presence of metastases is interesting as it may be possible to indicate the presence of metastatic disease by a rectal biopsy.

The results of active TGF- β 1 are a little more tentative as the number of events may challenge the multivariate statistics. Also active TGF- β 1 half-life offers an opportunity to question the possible clinical use as the timing of sampling would be crucial.

The plasminogen system with uPA and PAI-1 is already considered a prognostic factor in breast cancer and its use is being evaluated (135). The increased levels of uPA and PAI-1 in tumour compared to mucosa in our studies also indicate their importance although a deeper understanding of the pathophysiology of their actions cannot be obtained from

these results. PAI-1 is higher in tumours with a more advanced lateral spread indicating relevance in a more advanced disease. This was also somewhat confirmed by the multivariate analysis determining PAI-1 in tumour associated to death of rectal cancer and uPA/PAI-1 related to development of metastases, but again there must be caution in interpretation of the results due to a low number of events.

The plasma results with PAI-1 higher in metastatic disease are also a little dubious as the presence of PAI-1 in plasma is rather short lived. If this was to be used in clinical practice several studies with sequential plasma collections would have to be performed before its use could be evaluated.

FUTURE PERSPECTIVES

The aim of this thesis was to study some of the proteases and growth factors that are involved in the remodelling after radiotherapy. The results have revealed several areas of interest. To continue the pursuit for molecular markers of more aggravated inflammatory reactions to radiotherapy would be interesting. As the vascular injury is prominent in early onset, immunohistochemistry for PAI-1 as well as thrombomodulin could prove rewarding. TGF- β 1 is vital to fibrosis, and although our biopsies could not find any differences in total TGF- β 1 levels in relation to radiotherapy, further studies, perhaps with cell cultures of fibroblasts from rectal tissue, would be of interest.

Furthermore, a continued examination of calprotectin in rectal tissue with a larger patient material and correlation to rectal dysfunction, investigated both by clinical examination and questionnaires could prove to be rewarding. It is also possible that further studies could be carried out on archived material from larger randomised trials determining the effect of radiotherapy. This would remove the possible selection bias.

The decreased levels of tPA in irradiated peritoneal tissue may be a statistical result without any clinical value, but it may also be a clue to the peritoneal response to radiotherapy. A larger patient material to increase statistical power together with in vivo research on cell cultures of mesothelial cells could shed a light on this matter. Further knowledge of the peritoneal response could enable additional support for anti-adhesive products in patients treated with radiotherapy.

In relation to rectal cancer TGF- β 1 in tumour tissue and PAI-1 in tumour tissue are the most interesting findings that may help in the preoperative assessment process. Additional information could be obtained by a larger patient material, and would preferably be performed on preoperative biopsies to mimic the real clinical situation.

CONCLUSIONS OF THIS THESIS

- MMP-2, uPA, PAI-1 and calprotectin protein levels are increased by short-term radiotherapy for rectal cancer. These levels indicate an augmented remodelling of extracellular matrix after radiotherapy.
- MMP-2 levels in both mucosa and tumour are higher in patients with clinically evident complications after radiotherapy and surgery for rectal cancer. It is possible that high levels of MMP-2 could predict risk of complications.
- MMP-1, -2 -9, uPA, PAI-1, total TGF- β 1 and calprotectin all have higher protein levels in tumour compared to mucosa implicating their importance in rectal cancer.
- High PAI-1 protein levels in tumour tissue are related to the extent of lateral spread (T classification) as well as to death of rectal cancer. In the future it is possible that PAI-1 levels in tumour tissue could be used during preoperative assessment to identify patients in need of more appraisal or intensified treatment.
- High total TGF- β 1 levels in tumour tissue are associated with the presence of metastases and could thus also be of use in preoperative assessment in the future, although larger confirming studies are required.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to all friends, colleagues and co-workers who have contributed to this thesis. In particular I would like to thank:

Marie-Louise Ivarsson, my supervisor and friend, for invaluable support and enthusiasm. I admire you for your endless patience and thank you for your guidance and encouragement.

Tom Öresland, my co-supervisor, mentor and friend, for support and for having confidence in me. Your clinical and surgical skills make you a real role model.

Peter Falk, co-author and co-worker, for friendship as well as for generously sharing your knowledge and for helping me with everything from laboratory analysis to proof-reading.

Marcus Langenskiöld, close friend, colleague and co-author, for teaming up with me. I sincerely hope we will continue to work together in the future.

Ulf Angerås, head of the Surgical Department, Sahlgrenska University Hospital/Östra, for being an excellent leader and for making the writing of this thesis possible.

Michael Breimer, professor of Surgery and co-author, for your support and candid help, as well as for sharing your knowledge in scientific writing.

Ragnar Hultborn, professor of Oncology and co-author for inspiring discussions and ideas.

Ingrid Palmgren, lab technician and co-author, for outstanding laboratory work and support.

Maria Bergström, colleague and friend, for our friendship and your encouragement.

Lena Holmdahl, colleague and friend, who introduced me to the world of science and came up with the idea in the first place.

Eva Haglind, professor of Surgery, for encouragement, inspiration and guidance.

Svante Nordgren, professor of Surgery, for introducing me to colorectal surgery and surgical research.

Kristina Ticehurst, Per-Ola Park, Anders Rosemar and Anna Solberg, colleagues and fellow researchers at the lab, for support and inspiration.

Hillevi Björkqvist and Ann-Louise Helminen, research nurses, for excellent work and collection of patient material.

Pushpa Saksena, colleague at the Pathology Department, for help with interpretation of immunohistochemistry in paper IV.

Per-Göte Lindgren, head of ward 351B, and all my other **colleagues** and **co-workers** at the Surgical Department for help with these studies and for sharing their enthusiasm and knowledge of surgery with me. You create a great ambience at our workplace.

My parents **Anders** and **Marianne**, my sisters **Ingrid** and **Erika** with family, for never-ending support and help during this process and for believing in me.

Last, but certainly not least, **Johan**, my wonderful husband and **Ester**, my Princess! You bring endless light into my world and this could not have been possible without you.

Finally, this work was supported by grants from several sources. I would like to thank the following for generous support: The Swedish Medical Society (No: 18272 and 20842), Gothenburg Medical Society, Halland's Research Council, Assar Gabrielsson Research Foundation, King Gustav V Jubilee Clinic Cancer Research Foundation and the Swedish LUA/ALF Foundation (ALFGBG No:3033 and ALFGBG No:11365).

REFERENCES

1. Ferlay J, Autier P, Boniol M, Heanue M, Colombet M, Boyle P. Estimates of the cancer incidence and mortality in Europe in 2006. *Ann Oncol* 2007;18(3):581-92.
2. S.O.S. Cancer Incidence in Sweden 2007: The National Board of Health and Welfare; 2008.
3. Pahlman L, Bohe M, Cedermark B, Dahlberg M, Lindmark G, Sjudahl R, et al. The Swedish rectal cancer registry. *Br J Surg* 2007;94(10):1285-92.
4. Talback M, Stenbeck M, Rosen M, Barlow L, Glimelius B. Cancer survival in Sweden 1960-1998--developments across four decades. *Acta Oncol* 2003;42(7):637-59.
5. den Dulk M, Krijnen P, Marijnen CA, Rutten HJ, van de Poll-Franse LV, Putter H, et al. Improved overall survival for patients with rectal cancer since 1990: the effects of TME surgery and pre-operative radiotherapy. *Eur J Cancer* 2008;44(12):1710-6.
6. MacFarlane JK, Ryall RD, Heald RJ. Mesorectal excision for rectal cancer. *Lancet* 1993;341(8843):457-60.
7. Peeters KC, Marijnen CA, Nagtegaal ID, Kranenbarg EK, Putter H, Wiggers T, et al. The TME trial after a median follow-up of 6 years: increased local control but no survival benefit in irradiated patients with resectable rectal carcinoma. *Ann Surg* 2007;246(5):693-701.
8. Valentini V, Beets-Tan R, Borras JM, Krivokapic Z, Leer JW, Pahlman L, et al. Evidence and research in rectal cancer. *Radiother Oncol* 2008;87(3):449-74.
9. Keighley MRB, N.S W. *Surgery of The Anus, Rectum & Colon*. Third edition ed: Saunders Elsevier Ltd; 2008.
10. Friberg S, Mattson S. On the growth rates of human malignant tumors: implications for medical decision making. *J Surg Oncol* 1997;65(4):284-97.
11. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100(1):57-70.
12. Nagtegaal ID, Quirke P. What is the role for the circumferential margin in the modern treatment of rectal cancer? *J Clin Oncol* 2008;26(2):303-12.
13. Herrera L, Brown MT. Prognostic profile in rectal cancer. *Dis Colon Rectum* 1994;37(2 Suppl):S1-5.
14. Smith N, Brown G. Preoperative staging of rectal cancer. *Acta Oncol* 2008;47(1):20-31.
15. Glimelius B, Gronberg H, Jarhult J, Wallgren A, Cavallin-Stahl E. A systematic overview of radiation therapy effects in rectal cancer. *Acta Oncol* 2003;42(5-6):476-92.
16. Bipat S, Glas AS, Slors FJ, Zwinderman AH, Bossuyt PM, Stoker J. Rectal cancer: local staging and assessment of lymph node involvement with endoluminal US, CT, and MR imaging--a meta-analysis. *Radiology* 2004;232(3):773-83.
17. Wolberink SV, Beets-Tan RG, de Haas-Kock DF, Span MM, van de Jagt EJ, van de Velde CJ, et al. Conventional CT for the prediction of an involved circumferential resection margin in primary rectal cancer. *Dig Dis* 2007;25(1):80-5.
18. Sahani DV, Kalva SP, Hahn PF. Imaging of rectal cancer. *Semin Radiat Oncol* 2003;13(4):389-402.
19. Lahaye MJ, Engelen SM, Nelemans PJ, Beets GL, van de Velde CJ, van Engelshoven JM, et al. Imaging for predicting the risk factors--the circumferential resection margin and nodal disease--of local recurrence in rectal cancer: a meta-analysis. *Semin Ultrasound CT MR* 2005;26(4):259-68.
20. Karantanas AH, Yarmenitis S, Papanikolaou N, Gourtsoyiannis N. Preoperative imaging staging of rectal cancer. *Dig Dis* 2007;25(1):20-32.
21. Muthusamy VR, Chang KJ. Optimal methods for staging rectal cancer. *Clin Cancer Res* 2007;13(22 Pt 2):6877s-84s.

22. Heald RJ, Ryall RD. Recurrence and survival after total mesorectal excision for rectal cancer. *Lancet* 1986;1(8496):1479-82.
23. den Dulk M, Putter H, Collette L, Marijnen CA, Folkesson J, Bosset JF, et al. The abdominoperineal resection itself is associated with an adverse outcome: The European experience based on a pooled analysis of five European randomised clinical trials on rectal cancer. *Eur J Cancer* 2009.
24. Marr R, Birbeck K, Garvican J, Macklin CP, Tiffin NJ, Parsons WJ, et al. The modern abdominoperineal excision: the next challenge after total mesorectal excision. *Ann Surg* 2005;242(1):74-82.
25. Holm T, Ljung A, Haggmark T, Jurell G, Lagergren J. Extended abdominoperineal resection with gluteus maximus flap reconstruction of the pelvic floor for rectal cancer. *Br J Surg* 2007;94(2):232-8.
26. Jestin P, Pahlman L, Gunnarsson U. Risk factors for anastomotic leakage after rectal cancer surgery: a case-control study. *Colorectal Dis* 2008;10(7):715-21.
27. Matthiessen P, Hallbook O, Andersson M, Rutegard J, Sjodahl R. Risk factors for anastomotic leakage after anterior resection of the rectum. *Colorectal Dis* 2004;6(6):462-9.
28. Matthiessen P, Hallbook O, Rutegard J, Simert G, Sjodahl R. Defunctioning stoma reduces symptomatic anastomotic leakage after low anterior resection of the rectum for cancer: a randomized multicenter trial. *Ann Surg* 2007;246(2):207-14.
29. Ragnhammar P, Hafstrom L, Nygren P, Glimelius B. A systematic overview of chemotherapy effects in colorectal cancer. *Acta Oncol* 2001;40(2-3):282-308.
30. Collette L, Bosset JF, den Dulk M, Nguyen F, Mineur L, Maingon P, et al. Patients with curative resection of cT3-4 rectal cancer after preoperative radiotherapy or radiochemotherapy: does anybody benefit from adjuvant fluorouracil-based chemotherapy? A trial of the European Organisation for Research and Treatment of Cancer Radiation Oncology Group. *J Clin Oncol* 2007;25(28):4379-86.
31. Blomqvist L, Glimelius B. The 'good', the 'bad', and the 'ugly' rectal cancers. *Acta Oncol* 2008;47(1):5-8.
32. Vestermark LW, Jacobsen A, Qvortrup C, Hansen F, Bisgaard C, Baatrup G, et al. Long-term results of a phase II trial of high-dose radiotherapy (60 Gy) and UFT/l-leucovorin in patients with non-resectable locally advanced rectal cancer (LARC). *Acta Oncol* 2008;47(3):428-33.
33. Marijnen CA, Nagtegaal ID, Kapiteijn E, Kranenbarg EK, Noordijk EM, van Krieken JH, et al. Radiotherapy does not compensate for positive resection margins in rectal cancer patients: report of a multicenter randomized trial. *Int J Radiat Oncol Biol Phys* 2003;55(5):1311-20.
34. Ooi BS, Tjandra JJ, Green MD. Morbidities of adjuvant chemotherapy and radiotherapy for resectable rectal cancer: an overview. *Dis Colon Rectum* 1999;42(3):403-18.
35. Chadwick MA, Vieten D, Pettitt E, Dixon AR, Roe AM. Short course preoperative radiotherapy is the single most important risk factor for perineal wound complications after abdominoperineal excision of the rectum. *Colorectal Dis* 2006;8(9):756-61.
36. Denham JW, Hauer-Jensen M. The radiotherapeutic injury--a complex 'wound'. *Radiother Oncol* 2002;63(2):129-45.
37. Birgisson H, Pahlman L, Gunnarsson U, Glimelius B. Late adverse effects of radiation therapy for rectal cancer - a systematic overview. *Acta Oncol* 2007;46(4):504-16.

38. Birgisson H, Pahlman L, Gunnarsson U, Glimelius B. Late gastrointestinal disorders after rectal cancer surgery with and without preoperative radiation therapy. *Br J Surg* 2008;95(2):206-13.
39. Holm T, Singnomklao T, Rutqvist LE, Cedermark B. Adjuvant preoperative radiotherapy in patients with rectal carcinoma. Adverse effects during long term follow-up of two randomized trials. *Cancer* 1996;78(5):968-76.
40. Birgisson H, Pahlman L, Gunnarsson U, Glimelius B. Occurrence of second cancers in patients treated with radiotherapy for rectal cancer. *J Clin Oncol* 2005;23(25):6126-31.
41. Pollack J, Holm T, Cedermark B, Altman D, Holmstrom B, Glimelius B, et al. Late adverse effects of short-course preoperative radiotherapy in rectal cancer. *Br J Surg* 2006;93(12):1519-25.
42. Pollack J, Holm T, Cedermark B, Holmstrom B, Mellgren A. Long-term effect of preoperative radiation therapy on anorectal function. *Dis Colon Rectum* 2006;49(3):345-52.
43. Lange MM, Marijnen CA, Maas CP, Putter H, Rutten HJ, Stiggelbout AM, et al. Risk factors for sexual dysfunction after rectal cancer treatment. *Eur J Cancer* 2009.
44. Wang J, Zheng H, Ou X, Fink LM, Hauer-Jensen M. Deficiency of microvascular thrombomodulin and up-regulation of protease-activated receptor-1 in irradiated rat intestine: possible link between endothelial dysfunction and chronic radiation fibrosis. *Am J Pathol* 2002;160(6):2063-72.
45. Criswell T, Leskov K, Miyamoto S, Luo G, Boothman DA. Transcription factors activated in mammalian cells after clinically relevant doses of ionizing radiation. *Oncogene* 2003;22(37):5813-27.
46. Rodemann HP, Blaese MA. Responses of normal cells to ionizing radiation. *Semin Radiat Oncol* 2007;17(2):81-8.
47. Stone HB, Coleman CN, Anscher MS, McBride WH. Effects of radiation on normal tissue: consequences and mechanisms. *Lancet Oncol* 2003;4(9):529-36.
48. Larsen A, Bjorge B, Klementsens B, Helgeland L, Wentzel-Larsen T, Fagerhol MK, et al. Time patterns of changes in biomarkers, symptoms and histopathology during pelvic radiotherapy. *Acta Oncol* 2007;46(5):639-50.
49. Johnson LB, Adawi D, Sandberg S, Ottochian B, Albertsen C, Manjer J, et al. Peripheral leucocyte count variations in rectal cancer treatment. *Eur J Surg Oncol* 2009.
50. Johnson LB, Jorgensen LN, Adawi D, Blomqvist P, Asklof GB, Gottrup F, et al. The effect of preoperative radiotherapy on systemic collagen deposition and postoperative infective complications in rectal cancer patients. *Dis Colon Rectum* 2005;48(8):1573-80.
51. Bentzen SM. Preventing or reducing late side effects of radiation therapy: radiobiology meets molecular pathology. *Nat Rev Cancer* 2006;6(9):702-13.
52. Travis EL. Genetic susceptibility to late normal tissue injury. *Semin Radiat Oncol* 2007;17(2):149-55.
53. Bentzen SM, Overgaard J. Patient-to-Patient Variability in the Expression of Radiation-Induced Normal Tissue Injury. *Semin Radiat Oncol* 1994;4(2):68-80.
54. Leupin N, Curschmann J, Kranzbuhler H, Maurer CA, Laissue JA, Mazzucchelli L. Acute radiation colitis in patients treated with short-term preoperative radiotherapy for rectal cancer. *Am J Surg Pathol* 2002;26(4):498-504.
55. Dorr W, Hendry JH. Consequential late effects in normal tissues. *Radiation Oncol* 2001;61(3):223-31.
56. Fajardo LF. The pathology of ionizing radiation as defined by morphologic patterns. *Acta Oncol* 2005;44(1):13-22.

57. Engbring JA, Kleinman HK. The basement membrane matrix in malignancy. *J Pathol* 2003;200(4):465-70.
58. Bosman FT, Stamenkovic I. Functional structure and composition of the extracellular matrix. *J Pathol* 2003;200(4):423-8.
59. Liotta LA, Kohn EC. The microenvironment of the tumour-host interface. *Nature* 2001;411(6835):375-9.
60. Stamenkovic I. Extracellular matrix remodelling: the role of matrix metalloproteinases. *J Pathol* 2003;200(4):448-64.
61. Isaksen B, Fagerhol MK. Calprotectin inhibits matrix metalloproteinases by sequestration of zinc. *Mol Pathol* 2001;54(5):289-92.
62. Dellas C, Loskutoff DJ. Historical analysis of PAI-1 from its discovery to its potential role in cell motility and disease. *Thromb Haemost* 2005;93(4):631-40.
63. Wagenaar-Miller RA, Gorden L, Matrisian LM. Matrix metalloproteinases in colorectal cancer: is it worth talking about? *Cancer Metastasis Rev* 2004;23(1-2):119-35.
64. Fowlkes JL, Winkler MK. Exploring the interface between metallo-proteinase activity and growth factor and cytokine bioavailability. *Cytokine Growth Factor Rev* 2002;13(3):277-87.
65. Brinckerhoff CE, Matrisian LM. Matrix metalloproteinases: a tail of a frog that became a prince. *Nat Rev Mol Cell Biol* 2002;3(3):207-14.
66. Loo WT, Sasano H, Chow LW. Pro-inflammatory cytokine, matrix metalloproteinases and TIMP-1 are involved in wound healing after mastectomy in invasive breast cancer patients. *Biomed Pharmacother* 2007;61(9):548-52.
67. Kirkegaard T, Hansen A, Bruun E, Brynskov J. Expression and localisation of matrix metalloproteinases and their natural inhibitors in fistulae of patients with Crohn's disease. *Gut* 2004;53(5):701-9.
68. Okamoto T, Akuta T, Tamura F, van Der Vliet A, Akaïke T. Molecular mechanism for activation and regulation of matrix metalloproteinases during bacterial infections and respiratory inflammation. *Biol Chem* 2004;385(11):997-1006.
69. Elkington PT, O'Kane CM, Friedland JS. The paradox of matrix metalloproteinases in infectious disease. *Clin Exp Immunol* 2005;142(1):12-20.
70. Zucker S, Vacirca J. Role of matrix metalloproteinases (MMPs) in colorectal cancer. *Cancer Metastasis Rev* 2004;23(1-2):101-17.
71. Schwandner O, Schlamp A, Broll R, Bruch HP. Clinicopathologic and prognostic significance of matrix metalloproteinases in rectal cancer. *Int J Colorectal Dis* 2007;22(2):127-36.
72. Hilska M, Roberts PJ, Collan YU, Laine VJ, Kossi J, Hirsimäki P, et al. Prognostic significance of matrix metalloproteinases-1, -2, -7 and -13 and tissue inhibitors of metalloproteinases-1, -2, -3 and -4 in colorectal cancer. *Int J Cancer* 2007;121(4):714-23.
73. Mook OR, Frederiks WM, Van Noorden CJ. The role of gelatinases in colorectal cancer progression and metastasis. *Biochim Biophys Acta* 2004;1705(2):69-89.
74. Langenskiöld M, Holmdahl L., Falk P., Ivarsson M-L. Increased plasma MMP-2 protein expression in lymph node positive patients with colorectal cancer. *Int J of Colorectal Diseases* 2004;20(3):245-52.
75. Baker EA, Leaper DJ. The plasminogen activator and matrix metalloproteinase systems in colorectal cancer. relationship to tumour pathology. *Eur J Cancer* 2003;39(7):981-8.
76. Lawrence DA. Latent-TGF-beta: an overview. *Mol Cell Biochem* 2001;219(1-2):163-70.
77. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer* 2002;2(3):161-74.

78. Shapiro SD. Matrix metalloproteinase degradation of extracellular matrix: biological consequences. *Curr Opin Cell Biol* 1998;10(5):602-8.
79. Duffy MJ, McGowan PM, Gallagher WM. Cancer invasion and metastasis: changing views. *J Pathol* 2008;214(3):283-93.
80. Strup-Perrot C, Mathe D, Linard C, Violot D, Milliat F, Francois A, et al. Global gene expression profiles reveal an increase in mRNA levels of collagens, MMPs, and TIMPs in late radiation enteritis. *Am J Physiol Gastrointest Liver Physiol* 2004;287(4):G875-85.
81. Hovdenak N, Wang J, Sung CC, Kelly T, Fajardo LF, Hauer-Jensen M. Clinical significance of increased gelatinolytic activity in the rectal mucosa during external beam radiation therapy of prostate cancer. *Int J Radiat Oncol Biol Phys* 2002;53(4):919-27.
82. Kumar A, Collins HM, Scholefield JH, Watson SA. Increased type-IV collagenase (MMP-2 and MMP-9) activity following preoperative radiotherapy in rectal cancer. *Br J Cancer* 2000;82(4):960-5.
83. Zhao W, O'Malley Y, Wei S, Robbins ME. Irradiation of rat tubule epithelial cells alters the expression of gene products associated with the synthesis and degradation of extracellular matrix. *Int J Radiat Biol* 2000;76(3):391-402.
84. Strup-Perrot C, Vozenin-Brotans MC, Vandamme M, Linard C, Mathe D. Expression of matrix metalloproteinases and tissue inhibitor metalloproteinases increases in X-irradiated rat ileum despite the disappearance of CD8a T cells. *World J Gastroenterol* 2005;11(40):6312-21.
85. Riekkki R, Jukkola A, Sassi ML, Hoyhtya M, Kallioinen M, Risteli J, et al. Modulation of skin collagen metabolism by irradiation: collagen synthesis is increased in irradiated human skin. *Br J Dermatol* 2000;142(5):874-80.
86. Strup-Perrot C, Vozenin-Brotans MC, Vandamme M, Benderitter M, Mathe D. Expression and activation of MMP -2, -3, -9, -14 are induced in rat colon after abdominal X-irradiation. *Scand J Gastroenterol* 2006;41(1):60-70.
87. Unsal Kilic D, Uner A, Akyurek N, Erpolat P, Dursun A, Pak Y. Matrix metalloproteinase-9 expression correlated with tumor response in patients with locally advanced rectal cancer undergoing preoperative chemoradiotherapy. *Int J Radiat Oncol Biol Phys* 2007;67(1):196-203.
88. Giannopoulou E, Katsoris P, Hatzia Apostolou M, Kardamakis D, Kotsaki E, Polytarchou C, et al. X-rays modulate extracellular matrix in vivo. *Int J Cancer* 2001;94(5):690-8.
89. Jadhav U, Mohanam S. Response of neuroblastoma cells to ionizing radiation: modulation of in vitro invasiveness and angiogenesis of human microvascular endothelial cells. *Int J Oncol* 2006;29(6):1525-31.
90. Cheng JC, Chou CH, Kuo ML, Hsieh CY. Radiation-enhanced hepatocellular carcinoma cell invasion with MMP-9 expression through PI3K/Akt/NF-kappaB signal transduction pathway. *Oncogene* 2006;25(53):7009-18.
91. Speake WJ, Dean RA, Kumar A, Morris TM, Scholefield JH, Watson SA. Radiation induced MMP expression from rectal cancer is short lived but contributes to in vitro invasion. *Eur J Surg Oncol* 2005;31(8):869-74.
92. Qian LW, Mizumoto K, Urashima T, Nagai E, Maehara N, Sato N, et al. Radiation-induced increase in invasive potential of human pancreatic cancer cells and its blockade by a matrix metalloproteinase inhibitor, CGS27023. *Clin Cancer Res* 2002;8(4):1223-7.
93. Paquette B, Baptiste C, Therriault H, Arguin G, Plouffe B, Lemay R. In vitro irradiation of basement membrane enhances the invasiveness of breast cancer cells. *Br J Cancer* 2007;97(11):1505-12.

94. Stumpf M, Klinge U, Wilms A, Zabrocki R, Rosch R, Junge K, et al. Changes of the extracellular matrix as a risk factor for anastomotic leakage after large bowel surgery. *Surgery* 2005;137(2):229-34.
95. de Hingh IH, Siemonsma MA, de Man BM, Lomme RM, Hendriks T. The matrix metalloproteinase inhibitor BB-94 improves the strength of intestinal anastomoses in the rat. *Int J Colorectal Dis* 2002;17(5):348-54.
96. Siemonsma MA, de Hingh IH, de Man BM, Lomme RM, Verhofstad AA, Hendriks T. Doxycycline improves wound strength after intestinal anastomosis in the rat. *Surgery* 2003;133(3):268-76.
97. Syk I, Agren MS, Adawi D, Jeppsson B. Inhibition of matrix metalloproteinases enhances breaking strength of colonic anastomoses in an experimental model. *Br J Surg* 2001;88(2):228-34.
98. Stander M, Naumann U, Wick W, Weller M. Transforming growth factor-beta and p-21: multiple molecular targets of decorin-mediated suppression of neoplastic growth. *Cell Tissue Res* 1999;296(2):221-7.
99. Kushiyaama Y, Fukuda R, Suetsugu H, Kazumori H, Ishihara S, Adachi K, et al. Site-dependent production of transforming growth factor beta1 in colonic mucosa: its possible role in tumorigenesis of the colon. *J Lab Clin Med* 2000;136(3):201-8.
100. Reiss M. TGF-beta and cancer. *Microbes Infect* 1999;1(15):1327-47.
101. Wakefield LM, Roberts AB. TGF-beta signaling: positive and negative effects on tumorigenesis. *Curr Opin Genet Dev* 2002;12(1):22-9.
102. Massague J. TGFbeta in Cancer. *Cell* 2008;134(2):215-30.
103. Martin M, Lefaix J, Delanian S. TGF-beta1 and radiation fibrosis: a master switch and a specific therapeutic target? *Int J Radiat Oncol Biol Phys* 2000;47(2):277-90.
104. Richter KK, Fink LM, Hughes BM, Shmaysani HM, Sung CC, Hauer-Jensen M. Differential effect of radiation on endothelial cell function in rectal cancer and normal rectum. *Am J Surg* 1998;176(6):642-7.
105. Johnson LB, Adawi D, Agren MS, Jorgensen LN, Wittgren L, Mattsson S, et al. Combination of pre-operative radiotherapy and surgery suppresses local accumulation of collagen and TGF-beta1 in rats. *J Surg Res* 2006;133(2):136-42.
106. Canney PA, Dean S. Transforming growth factor beta: a promotor of late connective tissue injury following radiotherapy? *Br J Radiol* 1990;63(752):620-3.
107. Milliat F, Francois A, Isoir M, Deutsch E, Tamarat R, Tarlet G, et al. Influence of endothelial cells on vascular smooth muscle cells phenotype after irradiation: implication in radiation-induced vascular damages. *Am J Pathol* 2006;169(4):1484-95.
108. Okunieff P, Chen Y, Maguire DJ, Huser AK. Molecular markers of radiation-related normal tissue toxicity. *Cancer Metastasis Rev* 2008;27(3):363-74.
109. Hill RP, Rodemann HP, Hendry JH, Roberts SA, Anscher MS. Normal tissue radiobiology: from the laboratory to the clinic. *Int J Radiat Oncol Biol Phys* 2001;49(2):353-65.
110. Berger DH. Plasmin/plasminogen system in colorectal cancer. *World J Surg* 2002;26(7):767-71.
111. Dano K, Behrendt N, Hoyer-Hansen G, Johnsen M, Lund LR, Ploug M, et al. Plasminogen activation and cancer. *Thromb Haemost* 2005;93(4):676-81.
112. Ivarsson ML, Bergstrom M, Eriksson E, Risberg B, Holmdahl L. Tissue markers as predictors of postoperative adhesions. *Br J Surg* 1998;85(11):1549-54.
113. Andreasen PA, Egelund R, Petersen HH. The plasminogen activation system in tumor growth, invasion, and metastasis. *Cell Mol Life Sci* 2000;57(1):25-40.
114. Durand MK, Bodker JS, Christensen A, Dupont DM, Hansen M, Jensen JK, et al. Plasminogen activator inhibitor-I and tumour growth, invasion, and metastasis. *Thromb Haemost* 2004;91(3):438-49.

115. Westphal JR, Van't Hullenaar R, Geurts-Moespot A, Sweep FC, Verheijen JH, Bussemakers MM, et al. Angiostatin generation by human tumor cell lines: involvement of plasminogen activators. *Int J Cancer* 2000;86(6):760-7.
116. Illemann M, Hansen U, Nielsen HJ, Andreasen PA, Hoyer-Hansen G, Lund LR, et al. Leading-edge myofibroblasts in human colon cancer express plasminogen activator inhibitor-1. *Am J Clin Pathol* 2004;122(2):256-65.
117. Bajou K, Noel A, Gerard RD, Masson V, Brunner N, Holst-Hansen C, et al. Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. *Nat Med* 1998;4(8):923-8.
118. Bajou K, Maillard C, Jost M, Lijnen RH, Gils A, Declerck P, et al. Host-derived plasminogen activator inhibitor-1 (PAI-1) concentration is critical for in vivo tumoral angiogenesis and growth. *Oncogene* 2004;23(41):6986-90.
119. Hageman J, Eggen BJ, Rozema T, Damman K, Kampinga HH, Coppes RP. Radiation and transforming growth factor-beta cooperate in transcriptional activation of the profibrotic plasminogen activator inhibitor-1 gene. *Clin Cancer Res* 2005;11(16):5956-64.
120. Zhao W, Spitz DR, Oberley LW, Robbins ME. Redox modulation of the pro-fibrogenic mediator plasminogen activator inhibitor-1 following ionizing radiation. *Cancer Res* 2001;61(14):5537-43.
121. Milliat F, Sabourin JC, Tarlet G, Holler V, Deutsch E, Buard V, et al. Essential role of plasminogen activator inhibitor type-1 in radiation enteropathy. *Am J Pathol* 2008;172(3):691-701.
122. John B, Fagerhol MK, Lyberg T, Prydz H, Brandtzaeg P, Naess-Andresen CF, et al. Functional and clinical aspects of the myelomonocyte protein calprotectin. *Mol Pathol* 1997;50(3):113-23.
123. Gebhardt C, Nemeth J, Angel P, Hess J. S100A8 and S100A9 in inflammation and cancer. *Biochem Pharmacol* 2006;72(11):1622-31.
124. Passey RJ, Xu K, Hume DA, Geczy CL. S100A8: emerging functions and regulation. *J Leukoc Biol* 1999;66(4):549-56.
125. Yui S, Nakatani Y, Mikami M. Calprotectin (S100A8/S100A9), an inflammatory protein complex from neutrophils with a broad apoptosis-inducing activity. *Biol Pharm Bull* 2003;26(6):753-60.
126. Stulik J, Hernychova L, Porkertova S, Knizek J, Macela A, Bures J, et al. Proteome study of colorectal carcinogenesis. *Electrophoresis* 2001;22(14):3019-25.
127. Stulik J, Osterreicher J, Koupilova K, Knizek, Macela A, Bures J, et al. The analysis of S100A9 and S100A8 expression in matched sets of macroscopically normal colon mucosa and colorectal carcinoma: the S100A9 and S100A8 positive cells underlie and invade tumor mass. *Electrophoresis* 1999;20(4-5):1047-54.
128. von Roon AC, Karamountzos L, Purkayastha S, Reese GE, Darzi AW, Teare JP, et al. Diagnostic precision of fecal calprotectin for inflammatory bowel disease and colorectal malignancy. *Am J Gastroenterol* 2007;102(4):803-13.
129. Wedlake L, McGough C, Hackett C, Thomas K, Blake P, Harrington K, et al. Can biological markers act as non-invasive, sensitive indicators of radiation-induced effects in the gastrointestinal mucosa? *Aliment Pharmacol Ther* 2008;27(10):980-7.
130. Hille A, Schmidt-Giese E, Hermann RM, Herrmann MK, Rave-Frank M, Schirmer M, et al. A prospective study of faecal calprotectin and lactoferrin in the monitoring of acute radiation proctitis in prostate cancer treatment. *Scand J Gastroenterol* 2007;1-7.
131. Falk P. Experimental models of the Human Peritoneal Environment: Effects of TGF-beta and Hyaluronan. Göteborg: University of Gothenburg; 2008.

132. Lowry O, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951;193:265-275.
133. Falk P, Ivarsson ML. Examination gloves affect secretion of matrix metalloproteinases and their inhibitors from human abdominal skin fibroblasts. *Wound Repair Regen* 2003;11(3):230-4.
134. Vittinghoff E, McCulloch CE. Relaxing the rule of ten events per variable in logistic and Cox regression. *Am J Epidemiol* 2007;165(6):710-8.
135. Harbeck N, Schmitt M, Paepke S, Allgayer H, Kates RE. Tumor-associated proteolytic factors uPA and PAI-1: critical appraisal of their clinical relevance in breast cancer and their integration into decision-support algorithms. *Crit Rev Clin Lab Sci* 2007;44(2):179-201.