Experimental Colonic Obstruction and Anastomotic Healing

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2012

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Malmö 2012

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Lund University, Faculty of Medicine Doctoral Dissertation Series 2012:12
ISBN 978-91-86871-74-1
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2012

To my family

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List of Original Papers

This thesis is based upon the following papers, which will be referred to in the text by their Roman numerals:

I. PM Krarup, M Rehn, J Sand-Dejmek, R Ehrnström, MS Ågren and I Syk.
Rapid morphological changes and loss of collagen following experimental acute colonic obstruction.
Submitted to International Journal of Colorectal Disease

II. M Rehn, M S Ågren, I Syk.
Collagen levels are normalized after decompression of experimentally obstructed colon.
Colorectal Disease 2011; 13:e165-169 *

III. M Rehn, PM Krarup, LH Christensen, MS Ågren, I Syk.
Indiscriminate inhibition of matrix metalloproteinases and tumour necrosis-α converting enzyme impairs anastomotic healing in experimentally obstructed colon.
Manuscript

IV. B Pasternak, M Rehn, L Andresen, MS Ågren, AM Heegaard, P Tengvall, P Aspenberg.
Doxycycline-coated sutures improve mechanical strength of intestinal anastomoses.
International Journal of Colorectal Disease 2008; 23:271-276 **

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Abbreviations

ADAMTS	A Disintegrin and Metalloproteinase with Thrombospondin Motifs
APMA	Aminophenylmercuric acetate
ASA	American Society of Anesthesiologists
BMI	Body Mass Index
CRC	Colorectal cancer
ECM	Extracellular matrix
EGF	Epidermal Growth Factor
MMP	Matrix Metalloproteinase
MMPI	Matrix Metalloproteinase Inhibitor
NSAID	Non-Steroidal Anti-Inflammatory Drug
PDGF	Platelet-derived growth factor
PGE	Prostaglandin E
PGI	Prostaglandin I
TGF	Transforming growth factor
TIMP	Tissue Inhibitor of Metalloproteinases
TNF	Tumour necrosis factor
VEGF	Vascular endothelial growth factor

Introduction

> "Surgeons have now for some years been pleading for permission to have the sole control of cases of acute obstruction of the bowels; for they argue, and rightly, that to surgery alone can we look for cure. Surgery, they urge, ought to be the first resource and not the last. As a last resource, however, it still too often unfortunately remains, and nothing that surgeons can say seems likely to remove it from this position. Our subject for operation is probably in a pitiable plight. His vital powers, sufficiently tried by his disease, are further depressed by opium; his intestines already in turmoil and laden with secretions, are further worried by purgatives. His weary stomach, that has been for days patiently pouring up fluids and gases from the bowels, has in addition to return stuffs that we pour into it through the gullet. The pour body, equally harassed by disease and by treatment, is then handed over to the surgeon. For what? – I had almost said for slaughter. For such an operation the surgeon puts together his instruments with a heavy heart, for he knows that the resources at his command are not often successful on the dying."(1)
>
> **James Greig Smith**
> 1890

Modern medicine and surgery of the gastrointestinal tract is less troublesome but still remains unpredictable to some degree. Now we have the tools to identify obstruction early and make a safe operation but the outcome is still dependent on inherent factors related to the ability of uneventful healing.

Leakage of bowel content from an anastomosis is a feared complication in gastrointestinal surgery. The importance of the collagen-rich submucosa on the integrity of the newly formed anastomosis has been known since Halsted reported his experiments in 1887 (2). After proof of existence of an enzyme capable of degrading collagen in animals was established (3), reports started to appear demonstrating collagenolytic activity in the bowel (4, 5). The point of view was that collagenases were potentially dangerous enzymes being able to dissolve an important structural protein. Naturally these circumstances raised the interest in trying to manipulate the activity of the collagenases in order to enhance anastomotic healing (6).

Gastrointestinal Surgery

Several factors including anaesthesia, analgesia, aseptic technique and nutritional support are providing the basis for the ability to perform safe surgery on the gastrointestinal tract. One cornerstone is however the healing properties of wounded tissues. By using the intrinsic capacity of restoring tissue integrity it is possible to inflict injury by means of laparotomy and enteric resection and get a predictable outcome provided the cut edges are aligned by sutures or staples. Since Travers experimental suturing of divided dog small intestine in the early 19th century the understanding of the subject has increased tremendously (7). We are presently being able to perform safe surgery with excellent long-term outcome in a wide range of surgical disorders.

The most frequent reason for gastrointestinal resections is cancer. Other causes include inflammatory bowel disease, volvolus, colonic diverticular disease, obstruction, penetrating wounds, strangulated hernia and ischemia. Additionally the increasing prevalence of obesity in the population has made procedures comprising anastomotic surgery more common.

Colorectal cancer

Colorectal cancer is the 2nd (female) 3rd (male) most common malignancy in the world but incidence varies between different geographic regions (8). That environmental factors determine the rate of incidence is indirectly supported by the fact that incidence rise when people move from low to high incidence areas such as the industrial world. There is a linkage with dietary factors. Fat has a positive association with incidence but the role of dietary fibres is incompletely defined.

In Sweden the incidence of colorectal cancer is increasing among patients younger than 80 years of age but mortality is slightly decreasing. About as many men as women are diagnosed with colon cancer but for rectal cancers more men are affected. Median age at diagnosis for colorectal cancer is in the early 70ies (9-11).

Colorectal cancer is treated by resection and with radiotherapy and chemotherapy as adjunct treatments.

Surgery

In elective surgery the length of intestine removed harbouring the cancer is not as much determined by the preferred oncological safety margin as to the extent of the lymphadenectomy and the blood supply to the intestine. For a cancer tumour in the cecal or ascending part of the colon an ileocecal resection or a right hemicolectomy is performed. If the tumour is located in the hepatic flexure an extended right sided hemicolectomy can be carried out. Situated in the transverse colon a transverse colectomy is done. A segmental resection of the splenic flexure can achieve adequate surgery but if the tumour is located in the descending colon a left hemicolectomy is necessitated. Sigmoid cancer is treated by a segmental resection but a rectosigmoidal location requires an anterior resection. A more distal position of a tumour is either handled by a low anterior resection or an abdominoperineal resection.

In Sweden 91 % of the new cases of colon cancer (3951 patients) underwent some kind of surgery in 2009. In the elective setting half of the procedures were a right hemicolectomy reflecting the most common location for cancer tumours (9). The proportion of patients with rectal cancer (1804 new cases) having surgery was 82 %. The most frequent operation was anterior resection (10).

Anastomotic leakage

Normal recovery after gastrointestinal surgery implicate return to oral intake of food, recurrence of bowel function, waning pain experience and retrieval of autonomy leading to discharge from hospital. A clinical recovery diverging from the expected course is suggestive of complication and in the event of underlying anastomotic surgery leakage should be suspected.

In elective colonic surgery leakage is relatively uncommon with a reported frequency of 2-3 % (12-14). A comprehensive study has elucidated risk factors associated with leakage after resection and anastomosis for colon cancer. Emergency surgery due to obstruction, long duration of operation, male gender, ASA III-IV, BMI>30 and comorbidity (cardiac, hepatic, renal) were independent risk factors for developing leakage (14).

Further analysis of the same cohort of patients treated with resection and anastomosis for colon cancer have disclosed increased rate of secondary complications associated with the occurrence of anastomotic leakage. Consequently a prolonged hospital stay and increased in-hospital mortality followed compared to patients not having leakage. Additionally there was a poorer long term tumour-free survival and overall survival (15).

Patients operated on for rectal cancer in Sweden and undergoing anterior resection (653 patients) had a dehiscence rate of 10 % in 2009 (10). A recently published metaanalysis showed that anastomotic leakage after rectal cancer resection was correlated to a higher rate of local recurrence and a poorer oncological long term survival (16).

Colonic obstruction

The growth of the adenocarcinoma in the colon and rectum is distinguished by an intramural expansion followed by lateral progression in the transverse direction leading to circumferential involvement of the intestine. Obviously this circumstance is the cause behind the obstructive potential of colorectal cancer. An obstructing cancer of the colon requiring urgent surgical intervention is most likely situated in the left colon (17).

Every fifth case operated on for colon cancer in Sweden 2009 was accounted as emergency surgery of which 74 % was due to obstruction. Of all new cases 14 % (554 patients) had emergency surgery because of colonic obstruction (9). The reported anastomotic leakage rate after resection of colon cancer presenting as obstruction is around 6 % (14, 18, 19).

There are a few surgical options at hand dealing with colonic obstruction attributable to cancer (20). Normal treatment is primary resection, either as a subtotal or segmental colectomy, with anastomosis implying a convenient one-stage procedure. Hemicolectomy is usually standard procedure for right-sided colonic obstruction. For left-sided obstruction there are a couple of other strategies to consider, taken into account the option of performing anastomosis on an unprepared bowel with uncertain healing potential. For high risk patients a simple colostomy or resection with end colostomy (Hartmann's operation) probably is the safest alternatives. Factors associated with a higher risk for in-hospital mortality, in patients with large bowel obstruction, are age, ASA grade, operative urgency and Dukes stage (21).

Simple colostomy as part of a staged procedure for treatment of colon cancer is however no longer used. Hartmann's operation carries no risk of anastomotic dehiscence since no anastomosis is performed. On the other hand a second major operation is needed for reversal of the colostomy. The reversal rate varies between 19-71 % not differentiating between the underlying diagnosis (22). Actually, a reversal rate as low as 9 % is reported for colon cancer (23).

Colonic stenting

Yet another option is the concept of relieving the obstructed bowel by a minimally invasive technique, as a bridge to final surgery, represented by colonoscopic stenting.

Of all patients undergoing resectional surgery in Sweden 2009 for colonic cancer 3 % had a relieving procedure before the actual cancer operation. In one third of these procedures (26 patients) a stent was used as a bridge to surgery. In the rectal cancer group 9 % (118 patients) of all operated patients had a diverting stoma before the resectional surgery. For only 2 % of these 118 patients a stent was used for preoperative relief and accordingly much less frequently used in rectal than in colon cancer (9, 10).

If placement of a stent in colonic obstruction is successful, outcome of bridged resection and primary anastomosis can yield low figures on dehiscence (24, 25).

Anastomotic Healing

From the point of injury a complex course of events take place comprising specific cellular and molecular actions aiming at restored tissue integrity. The different events occur in a temporal and spatial orderly manner. Most of the understanding is derived from wound models in skin but can be translated to anastomotic healing as well.
The first response to injury is bleeding. Exposed extracellular matrix or cells expressing tissue factor stimulate clot formation and haemostasis is achieved by further platelet recruitment with the clot reinforced by fibrin and fibronectin. The initial vasoconstriction is followed by vasodilatation, mediated by PGE_2, PGI_2 and VEGF, with increased vascular permeability. This facilitates extravasation of recruited cells of haematopoietic origin. Chemoattractants such as PDGF and TGFβ are released from platelets and within 24 hours a large number of polymorphonuclear leukocytes are engaged in clearing the wound. Macrophages, originating mainly from circulating monocytes, assist in removing devitalized tissue and excess material. Within 2 days they are the dominant cell type if bacteria are absent. The macrophage is also an important source of cytokines and growth factors vital for continued healing. Fibroblasts are stimulated to proliferate as well as endothelial cells initiating angiogenesis. The provisional fibrin-fibronectin matrix is degraded and replaced by the regular constituents of the extracellular matrix. Randomly positioned collagen fibrils are gradually replaced by more organized fibres giving increased strength to the wound (26).
Epithelialisation is under favourable conditions rapid and accomplished by proliferation and migration of epithelial cells at the wound edge. In the presence of excess necrotic tissue epithelialisation is delayed and a more profound inflammatory process ensues resulting in deferred wound healing.

The colonic wall

There are four layers building up the bowel wall, namely the mucosa, submucosa, muscularis externa and the serosa.

Innermost is the mucosal layer, containing the epithelium supported by loose connective tissue, called lamina propria and the muscularis mucosae, a thin layer of smooth muscle.

The submucosa is a collagen-rich connective tissue layer between the mucosa and the muscularis externa (se below under separate heading).

The thick muscularis externa is responsible for the propulsive activity of the bowel. The smooth muscle is arranged in an inner circular layer and an outer longitudinal layer which in humans is modified to three longitudinal bands, the taenia coli. The taenias coalesce in the upper part of the rectum to a continuous layer around the circumference of the last part.

Outermost is a thin layer of connective tissue enfolding the muscularis externa. If the bowel is located intraperitoneal a simple squamous epithelium covers the connective tissue and the layer in those parts is called serosa. The rectum lacks the epithelial part of the serosa and has only the connective tissue, the adventitia, adhering to the surroundings.

The neural stimulation of the bowel is mediated by the enteric nervous system through the myenteric plexus, primarily responsible for peristaltic movements, and the submucosal plexus, directing localized blood flow among other functions. The two plexuses have multiple interconnections and activity is modified by sympathetic and parasympathetic nerves.

Submucosa

In many different aspects the submucosa functions not only as a supportive framework physically but just as important is its biological role in tissue remodelling. The collagen fibres, which are resistant to stretching forces, is embedded in a hydrated ground substance composed of mostly proteoglycans. They are typically compound molecules consisting of a core protein holding multiple polysaccharide side-chains belonging to the glycosaminoglycans (GAGs). The collagen fibres and proteoglycan molecules in the extracellular matrix is extensively bound to each other and the cells occupying it. The glycoprotein fibronectin has an important role in cell-matrix interaction presenting several binding sites in its dimer structure.

The elements in the extracellular matrix have immense impact on the cells residing in the matrix. They direct cell behaviour such as proliferation, migration and adhesion. They control activity of signals such as chemo attractants and growth factors. In resting states the activity in the matrix is low. However, in situations like wound healing there is a need for rapid degradation and remodelling of the matrix (27).

Collagen

The collagen in the submucosa of the large intestine is mainly represented by type I and III (28). They are fibril-forming and assembled into fibres with a width of about 5 μm.

The collagen molecule is made up by three polypeptide α-chains organized in a typical superhelix. Every third amino acid is represented by glycine enabling the three helical α-chains to pack tightly. The molecule is also rich with proline, some of which are hydroxylated to form hydroxyproline which is unique to collagen and

elastin. The hydroxyl group of this amino acid stabilizes the triple helix by hydrogen bonds between the α-chains and other hydroxylated proline and lysine residues. The hydroxyproline content of collagen is about 13 % (29).
After secretion the molecules are made insoluble by the action of procollagen C-peptidase and ADAMTS-2, ADAMTS -3, ADAMTS-14 proteolytic enzymes (MEROPS M12A and B subfamily respectively) where after the formed tropocollagen molecules aggregate into fibrils (30). Intermolecular covalent bonds between lysine residues strengthen the fibril, also described as collagen crosslinking. The enzyme lysyl oxidase is responsible for this final step in collagen maturation. Lysyl oxidase is activated extracellularly by procollagen C-peptidase (30). Collagen fibrillogenesis is also thought to be dependent on a fibonectin scaffold requiring interactions with cell-surface intergrins (31).
Tissues show different patterns of fibril arrangement depending on what mechanical stress is exercised on it. The submucosa of the human large intestine viewed with electron microscope reveals a honeycomb pattern of collagen fibres explaining the extensibility of the intestine despite the inextensible properties of collagen fibres themselves (32). The arrangement of fibres is not by chance but actively organized by fibroblasts (33, 34). The myofibroblast is a modified fibroblast and is commonly found in wound healing tissue. They show similarities with smooth muscle cells but also differ from them in that they do not have a basal lamina.
Under quiescent conditions the turnover rate of collagen is low. Fibroblasts are not only responsible for the synthesis of collagen but also for its degradation in mature resting tissues. After cleavage of the collagen fibril by the action of a membrane-bound protease (MMP 14 or MT1-MMP) the fragments are internalized by endocytosis and further degraded in lysosomes (35).

Matrix metalloproteinases

Until the early 1950s collagen was considered a very stable protein and not exposed to degradation after being synthesized and deposited in the various tissues in the body. Then a report was published showing how collagen was rapidly lost in postpartum rat myometrium suggesting reabsorption of collagen (36). In 1970 it was possible to demonstrate collagenolytic activity in homogenates from involuting rat uterus (37). Since then the knowledge in the field has increased dramatically and the presence of different matrix metalloproteinases in rat uterus after parturition has been confirmed as well (38).

The MMPs belong to the M10A subfamily of metalloendopeptidases, also known as the metzincins (39). They have an important role in the development, turnover and repair of different tissues. Moreover, they are also active in different pathophysiological conditions such as cancer, rheumatoid arthritis and periodontitis.

The latency of the secreted proenzyme is due to a cysteine switch in which a cysteine residue in the pro-domain is covalently bound to the catalytic site containing a zinc ion. They are activated by proteolytic cleavage of the pro-domain. This group of proteinases also shares a common structure with a Met-turn, conserved methionine C-terminal, below the active catalytic site contributing to structural integrity. The C-terminal domain is responsible for interactions with the ECM and determines substrate specificity (40). Six of the known 23 MMPs are membrane-bound enzymes employing their activity close to the cell surface. The MMPs show substantially ECM substrate specificity overlap even if some were regarded as true collagenases (MMP-1, MMP-8 and MMP-13) which cleave the collagen molecule into a 3/4-length piece from the N-end and a 1/4-length piece from the C-end subject to further degradation (41). Especially MMP-13 show wide substrate specificity towards several ECM components including fibronectin (42).

MMPs are induced by interactions between cells or cell-ECM but also by cytokines and growth factors. Activation is through the action of other MMPs or tissue and plasma proteinases. There are four different tissue inhibitor of metalloproteinases (TIMPs) exerting control of proteolytic activity by the MMPs but this is also accomplished by non-specific proteinase inhibitors such as α_2-macroglobulin (43). TIMPs are synthesized by fibroblasts and endothelial cells but also by several other cell types.

During wound healing activation of MMPs is required in several of the steps leading to restored tissue integrity. Degradation of the provisional wound matrix, creating space for cell migration, angiogenesis and ECM remodelling all require MMP action (44, 45). Effects of their activity also include biological active cleavage products and changing activity of signalling molecules (46).

Clinical experience

Stumpf et al presented in 2005 the results of a study looking into the possibility of an underlying disorder in collagen metabolism contributing to risk of developing anastomotic leakage. Samples from intact colon were prospectively sampled from patients having a colorectal anastomosis. Patients developing anastomotic leakage had a lower collagen I/III ratio suggesting poorer tissue quality since collagen type III is less cross-linked and consequently have less tensile strength. Furthermore they found a higher expression of mainly MMP-2 but also MMP-1 and MMP-9 in the mucosa and submucosa suggesting defective collagen metabolism as a specific patient related risk predisposing to anastomotic leakage (47).

In a small study Pasternak et al investigated levels of MMPs in intraperitoneal fluid from patients operated on with anterior resection. Patients developing anastomotic leakage had increased levels of MMP-8 and MMP-9. Whether this was the primary cause of leakage or secondary to other mechanisms could not be answered (48).

Sheridan et al administered aprotinin intravenously with reference to its collagenases inhibitory properties in a randomized, placebo-controlled clinical trial in 216 patients undergoing resection and anastomosis. The investigators had conflicting results. Aprotinin was beneficial in anterior resection but harmful in colocolic anastomosis where a higher leakage rate was found. No probable explanation for the latter finding was presented besides that the technical performance in one participating center was questioned (49).

MMPs have been studied in diseases with the potential to aggravate by perforation. In more severe appendicitis levels of MMP-1 and MMP-9 were upregulated. Since immunostaining for the endogenous inhibitor TIMP-1 was patchy and uneven as for MMP-9 it was proposed that local imbalance in hot spots in favour for MMP-9 could lead to tissue degradation and subsequent perforation (50). MMP-9 was also found to be most elevated closest to a perforation (51).

In complicated diverticular disease MMP-9 was however not found to be the dominant MMP type. In affected parts of resected sigmoid colon increased expression of MMP-1, MMP-2 and MMP-3 was found. This finding could however reflect ongoing tissue remodelling. Only a small proportion of the patient cohort was operated on for acute perforation (52).

Ulcers in colonic specimens from humans operated on for ischemic colitis showed migratory epithelial cells expressing MMP-1, MMP-7 and MMP-10 (53).

Experimental experience

Collagen and anastomotic healing

Ever since the experimental works of Hawley and Cronin the common view has been that loss of submucosal collagen in healing anastomoses is attributed to the action of collagenases and suggested to be the underlying mechanisms responsible for anastomotic dehiscence (5, 54). Loss of collagen in the early course of anastomotic healing was also found in rats premarked with tritiated proline from young age (55). Collagen concentration is temporarily decreased close to an anastomosis. This change in collagen coincides with a dip in anastomotic breaking strength reflecting the anastomotic segments' capacity to retain sutures when drawn apart in a testing machine (56).

Using an accurate technique with interrupted sutures in a single layer in animals showing an uneventful recovery the decrease, four days after anastomosis, was around 23 %. A more pronounced decrease of collagen concentration was related to anastomotic complications (57). This was however not related to any differences in mechanical strength (58, 59).

The lowered concentration of collagen in the vicinity of a colonic anastomosis was found to occur only a few hours after surgery and the lowering

rate was highest after 24 hours (60). This coincided with the rapid appearance of polymorphonuclear leukocytes (neutrophil granulocytes) in the anastomotic wound. Since the existence of a granulocyte derived collagenase was known the conclusion was that collagen degradation was neutrophil dependent (61). This enzyme was given the name collagenase-2 but is presently referred to as MMP-8. MMP-8 is stored in intracellular granules and released upon stimulation (42, 62). The expected decrease in anastomotic breaking strength was avoided in animals made neutropaenic (ileo-ileal anastomosis) (63) but when neutrophils were prevented to accumulate in the anastomotic wound (colo-colic anastomosis) no effect on breaking strength was found, however (64).

The clinical experience with higher incidence of anastomotic dehiscence in surgery of colonic obstruction led to experimental models to further look into the effect of decreased hydroxyproline on anastomotic healing. Adding to this interest was the results from a study using continuous suturing of the anastomosis complicated by narrowing with faecal stagnation and colonic dilation (57).

Consequently, when using an experimental model of obstruction for four days with suturing of anastomosis in an area of colon already depleted on 30-45 % of collagen, measured as hydroxyproline concentration, a complication rate of 27 % was found. The control animals in the same study, without obstruction, only had complications in 2 % of cases (65). These findings could be supportive for the idea that hydroxyproline concentration in the bowel wall is important for outcome of anastomotic healing considering that intact collagen provides the sutures a firm anchorage essential for anastomotic integrity in the early phase. Interestingly, there was no difference in anastomotic breaking strength between the obstructive group, depleted of collagen, and the reference group with anastomosis in normal colon, immediately after construction or the controls, on neither postoperative day.

MMPs and anastomotic healing

It was found that collagenase activity increased significantly between the 1st and 3rd day after anastomosis, assayed in culture medium from rabbit anastomotic colon samples (66). In analogy, the decrease in hydroxyproline did not develop until the 3rd day, which was in contrast to the findings of Hendriks et al (1985). In another study collagenolytic activity could be detected as early as 24 hours after anastomosis using an assay based on tissue extracts measuring degradation of fibrillar collagen (67). Tissue extracts were also used in a study looking at the presence of MMPs in uninjured rat colon and anastomotic segments. When extracts were run on gelatin gels increased levels of MMPs was observed already 24 hours after anastomosis. When latent MMPs were activated by APMA incubation the activity in the gels corresponded particularly to the presence of MMP-9 as read by the investigators (68, 69).

When localization of collagenases was performed by immunofluorescence in normal anastomotic healing the enzymes were found to be situated only close to

the cut bowel edges and in proximity to the sutures, which precluded any extensive collagen degradation (70, 71). Moreover, these findings were consolidated in a competent study looking into changes occurring in the direct proximity to the anastomosis in normal rat colon and more specifically around the sutures. It was found that hydroxyprolin levels were more decreased in the absolute vicinity of the sutures compared to the tissue in between or more distant. This was confirmed by histology showing loss of collagen around the suture channels. MMP-8 and MMP-9 was identified by immunohistochemistry in macrophages surrounding the sutures whereas the areas in between were significantly less infiltrated by these cells. When samples from the tissue around the sutures were incubated in vitro with physiologic substrate significantly increased collagenolysis was detected (72).

In another study using immunofluorescence for localization of MMPs the enzymes were identified at the suture line or close to the cut edges in the control group (73). The intervention group however, having an ischemic segment resected and the bowel anastomosed, demonstrated MMPs not only restricted to the anastomosis but also proximal and distal to it. The localization corresponded to abnormalities found on histology, such as oedema and haemorrhage in the submucosa and fragmentation and loss of striation pattern in the muscle layer, secondary to the preceding ischemia and proceeding until the 3rd day after anastomosis.

In another model with compromised anastomosis, 24 hours of ring obstruction followed by resection and anastomosis resulted in more aggravated changes in the bowel wall than obstruction and ring release alone. Histologically detected changes consisted of loss of epithelial border, submucosal oedema and an inflammatory cell infiltrate and corresponded to localization of MMPs by immunofluorescence. The abnormalities in the bowel wall were almost entirely resolved 3 days after release of the obstructing ring but continued longer after anastomosis which where suggestive for poorer healing in compromised anastomoses. In contrast to healing after resection and anastomosis in normal colon, MMPs were not only restricted to the proximity of the anastomosis but were found further upstream from the anastomosis (74). Looking into MMP activity in obstructed rat colon determined ex vivo by degradation of collagen actually found a significant increased activity compared to normal colon. Most of the activity was latent since 90 % was attributed to APMA treatment (75). This finding could be correlated to the previously mentioned investigation by Savage et al where the MMPs mainly were located intracellularly.

MMP inhibition and anastomotic healing

Rats have been used extensively in studies on anastomotic healing. A standard colonic anastomosis in a healthy rat usually heals uneventfully (76). Since

recovery is extremely good, a marker of healing such as anastomotic dehiscence is ineffective, which has necessitated the application of surrogate markers.

In an early study of anastomotic healing, using the proteinase inhibitor aprotinin, the results were suggestive of collagenase inhibition when improved bursting strength in rat colonic anastomosis was found (77). Yet another study showed increased breaking strength and collagen content on the 4th day after anastomosis (78). The phenomenon with improved mechanical strength was also found in subsequent studies using other agents with anti-collagenase qualities (79-84).

Thus, the attempts to improve anastomotic healing by means of MMP inhibition has typically been limited to assessing mechanical properties such as breaking strength or bursting pressure (85). Normally such treatment prevents most of the otherwise expected weakening of the anastomosis on the 3rd day. However, the difference has evened out on the 7th day. In the studies referred to no animal was followed longer than seven days and practically no complications were found (79, 82, 84, 86). Interestingly, no difference in anastomotic collagen could be demonstrated between control animals or treated animals. Suggested explanations were sampling technique too insensitive to detect small differences in the suture line or that MMPI treatment might preserve existing mature collagen in the submucosa.

Aims

The overall aim with the thesis was to investigate certain physiological factors believed to be important in healing of a compromised colonic anastomosis and how outcome of anastomotic healing could be influenced by manipulating the activity of collagen-degrading enzymes.

Specific aims:

- To define the sequential changes in collagen and morphology in the bowel wall following obstruction. (I)
- To study collagen changes in the bowel wall after decompression of obstruction. (II)
- To rule out the effect of a non-selective inhibitor of matrix metalloproteinases on healing of a compromised anastomosis. (III)
- To examine the effect on anastomotic healing of a locally applied inhibitor of matrix metalloproteinases. (IV)

Material and Methods

Animals

For all experiments Sprague-Dawley® rats were used and they were supplied by Taconic, Denmark. The animals´ weights were 210-434 gram. They had an acclimatization period for at least seven days before experiments. Standard food pellet and water was provided ad libitum. After decompression (II) and anastomosis in GM6001-treated animals (III) housing was in individual cages.

All experiments were approved by either the Ethical Committee on Animal Experiments in Lund (I-III) or the Danish National Experimental Animal Inspectorate (IV).

Experimental models

Study protocols

Paper I

A total of 40 animals were used. They were assigned to five different groups. From one group normal tissue was sampled. The other four groups had colonic obstruction, as described below, for 1, 2, 3 or 4 days before sampling.

Paper II

A total of 59 animals were used. 28 were subjected to obstruction for four days. They were allocated into three groups. One group was terminated immediately after ring removal and the other two received decompression and recovery for 3 and 10 days respectively. 23 animals were sham-operated without obstruction but had corresponding recovery periods. Normal tissue was harvested from eight animals not subject to any surgery.

Paper III

A total of 24 animals were used. They were randomly assigned to treatment with a non-selective MMP-inhibitor or placebo (vehicle alone). Thus each group included 12 animals. After four days of colonic obstruction, resection and anastomosis were

performed. Treatment started at initiation of obstruction and was discontinued on day 5 after anastomosis. The integrity of the anastomosis was radiologically assessed on days 1, 3 and prior to termination on day 7. Clinical and histopathological evaluations of anastomosis were made 7 days after surgery.

Paper IV

A total of 85 animals were used. The effect of a locally delivered MMP-inhibitor on anastomotic breaking strength was studied. Two experiments were performed within the study. In experiment A doxycycline-coated sutures were compared to carrier coated sutures. In experiment B carrier coated sutures were compared to unmodified sutures.

Surgery

The animals were anaesthetized with a combination of analgesics (fentanyl citrate and fluanisone), sedatives (midazolam) and an antiemetic (droperidol; IV). If postoperative analgesia was given, either an opioid (buprenorphine) or a NSAID (carprofen; IV) was used. Access to the abdominal cavity was through a midline incision of about 3 centimeters length. The abdominal wall was closed in two separate layers.

Construction of colonic anastomosis

Anastomosis was constructed with assist of an operating microscope (III) or magnifying lamp (IV). Either eight (IV) or nine (III) interrupted 6-0 (IV) or 7-0 (III) sutures, placed about 2 mm from the resection margin, were used for end-to-end anastomosis.

Colonic obstruction

Obstruction was initiated by positioning of a silicone ring around the distal colon about 3 cm above the peritoneal reflection (87). The ring had an inner width of 6.5 mm and a length of 5 mm. It efficiently caused faecal stagnation but possibly allowed some gas to pass at least in the beginning of the obstruction period. Obstruction was maintained for 4 days until removal of the ring leading to decompression (II) or until resection and anastomosis (III). In paper I obstruction was maintained for 1-4 days depending on subgroup.

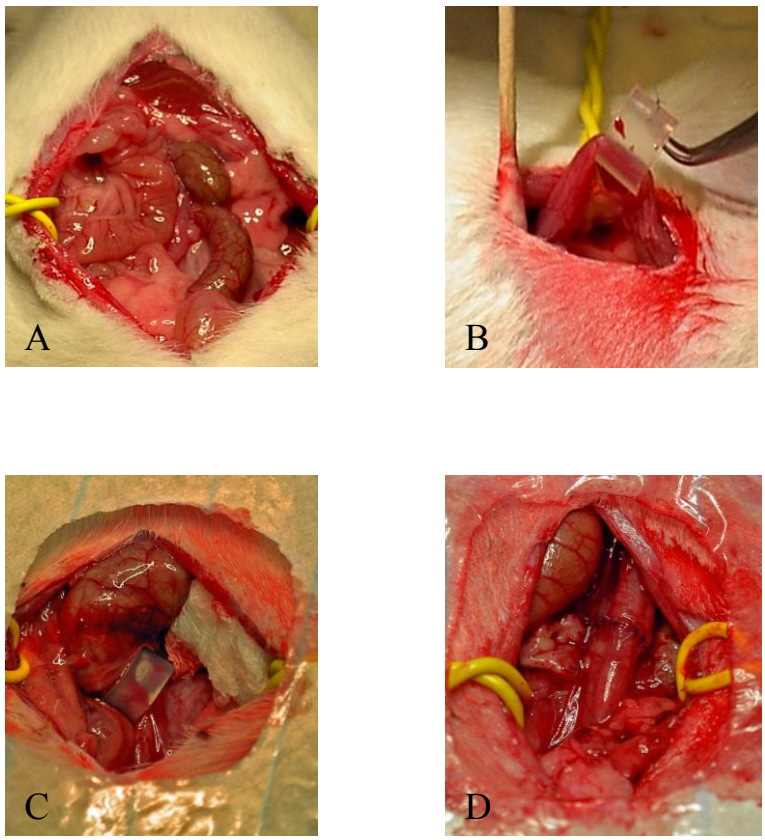


Figure 1
Pictures of the experimental model. Normal colon with faecal content (A). Ring around colon prior to obstruction (B). Dilated colon proximal to obstructing ring after 4 days (C). Anastomosis (D).

Non-selective MMP inhibition

GM 6001 (Galardin, Ilomastat) is a broad spectrum hydroxamate class of inhibitor of matrix metalloproteinases (88). Reported Ki values are in the low nM range.

Animals treated with GM 6001 got ten consecutively subcutaneous injections from the first day of obstruction until the fifth day after anastomosis (III). The daily dose was 30 mg/kg and the solution was prepared by suspending 1 mg of GM 6001 in 20 µl ethanol where after the mixture was diluted with phosphate buffered saline to a concentration of 5 mg/ml.

Other studies using GM6001 have used a dosage of 100 mg/kg in skin or peritoneal wounding models (89-91). The same dosage was found to abate the usual decrease in anastomotic breaking strength found in non-treated animals (86). In contrary to the latter study, reduced rates of weight gain were noticed in the former studies. They also continued the treatment for almost twice as long. Furthermore, musculoskeletal side effects are reported after eight days of treatment with a similar broad spectrum hydroxamate inhibitor, marismastat (BB-2516) (92).

In order to evade untoward side effects a lower dose than 100 mg/kg would be desirable. When choosing a dose of 30 mg/kg, we applied the results from an experimental study looking at the effect of different doses of GM6001 on breaking strength of colon anastomosis three days after surgery. Dosages of 25 mg/kg, 50 mg/kg and 100 mg/kg were as effective in regard to breaking strength, which in turn was significantly higher compared to animals treated with vehicle alone (86).

Coated sutures

Sterile 6–0 polybutester monofilament sutures (Novafil™) were activated during 20 s in a radio frequency plasma chamber (Plasmaprep 100, Nanotech, Sweden). The activated sutures were incubated for 30 min in 6% glutaraldehyde in phosphate-buffered saline (PBS), pH 9. The surfaces were extensively rinsed in PBS, pH 9. Ten layers of fibrinogen (Hyphen BioMed, Neuville-sur-Oise, France; molecular weight, 340 kDa; clottability, 98%) were prepared as follows (93): the glutaraldehyde-coated sutures were incubated for 30 min in 1 mg/ml fibrinogen dissolved in PBS at pH 7.4. The sutures were extensively rinsed in PBS followed by incubation during 30 min in PBS, pH 5.5, containing 0.2 M N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC; Sigma-Aldrich, St. Louis, MO, USA) and 0.05 M N-hydroxyl-succinimide (NHS; Sigma-Aldrich). Then a new 1 mg/ml fibrinogen solution was prepared in PBS buffer, pH 5.5, and the sutures were incubated for 30 min in this, rinsed in PBS and again incubated in the EDC–NHS solution. As the EDC solution is unstable at room conditions, new solutions were prepared every second hour. This procedure was repeated until ten fibrinogen layers were immobilized. The cross-linked fibrinogen surface was

subsequently incubated in EDC–NHS as described above and for 3 h in a 1-mg/ml solution doxycycline hyclate (Sigma-Aldrich) or for 3 h in PBS (control sutures) and finally rinsed in distilled water.

Thicknesses of the fibrinogen and doxycycline layers on the sutures were measured by null ellipsometry (Auto EL III, Rudolph Research, Flanders, NJ, USA) in air, calculated according to the McCrackin evaluation algorithm and converted into an approximate adsorbed amount per unit area by de Feijters formula (94, 95). The assumed refractive index of the protein and immobilized doxycycline film was nf=1.465 (96). During the measurements, 1 nm of adsorbed proteins equaled approximately 120 ng/cm2 (97).

The so aseptically prepared sutures for experiment A were stored at room temperature in the dark in a 0.5-mg/ml doxycycline PBS solution, pH 5.5, until use within 6 days. Fibrinogen-coated control sutures were stored in PBS, pH 5.5, under identical conditions. In experiment B, the fibrinogen-coated sutures were dried using nitrogen gas, stored at room temperature in sterile bags and used within 14 days after production. Uncoated sutures were packaged aseptically in identical sterile bags. The sutures were indistinguishable by visual inspection and physical handling such as elasticity and pliability.

Mechanical testing

After killing the rats, the abdomen was opened and the colon carefully freed from adhesions. A 4-cm segment with the anastomosis was resected and gently cleaned of fecal contents. The segment was mounted in a material testing machine (LF Plus, Lloyds Instruments, Fareham, UK) equipped with a 10-N load cell (XLC-0010-A1, Lloyds Instruments) with 10 mm between the clamps. The colon segments were stretched at a constant deformation rate of 10 mm/min until rupture. The maximal load (breaking strength) and the area under the curve to the breaking point (energy uptake at failure) were derived from the load–strain curve calculated by the software (Nexygen, Lloyds Instruments). The breaking point was defined by the maximum force value. The measurements were done with sutures in place.

Fluoroscopy

During the imaging procedures the animals were sedated with a mixture of Ketalar® (ketamine 75mg/kg) and Dormicum® (midazolam 5 mg/kg) injected subcutaneously. With the animal lying in prone position an 8 F balloon catheter was gently inserted through the anus and the tip of the catheter placed in the upper part of the rectum. The balloon was filled with 0.75 ml saline. The catheter was connected to a syringe pump (model 351, Sage Instruments, Cambridge, Mass.,

USA) and a pressure transducer. Pressure changes were recorded by a computer with Chart4 software connected to PowerLab® data acquisition system (ADInstruments, Castle Hill, Australia). Contrast enema (Omnipaque 240 mg I/ml, GE Healthcare) was instilled in a rate of 1 ml/min during continuous imaging with a fluoroscope (Exposcop CB7-D, Ziehm). Anastomotic integrity or degree of leakage was evaluated in anterior-posterior and lateral positions.

Clinical evaluation of anastomosis

Assessment of anastomosis was done in terminated animals on day 7 after anastomosis. The abdomen was opened and the anastomotic area carefully dissected and freed from adhesions. To standardize the evaluation a grading system was set up (table 1).

Table 1
Clinical assessment criteria of the anastomosis

Grade	Criteria
0	Normal anastomotic healing
1	Oedema and abundant adhesive bowel-loops but no abscess formation or leakage
2	Abscess formation in the bowel wall but no leakage
3	Intra-abdominal abscess secondary to anastomotic leakage
4	Intra-abdominal abscess secondary to anastomotic leakage leading to death

Histology and immunohistochemistry

Full-thickness samples were obtained from the colon, fixed in formalin, embedded in paraffin, sectioned at 5 μm and stained with haematoxylin and eosin. The bowel wall was evaluated on degree of oedema and inflammation after 0-4 days of obstruction (I) or degree of anastomotic healing (III).

For identification of myofibroblasts immunostaining for α-smooth muscle actin was done with monoclonal mouse anti-human smooth muscle actin (clone 1A4). For assessment of cell proliferation immunostaining was made with monoclonal mouse anti-rat Ki-67 antigen (clone MIB-5). Immunohistochemistry was performed with the Autostainer Link-system (Dako, Glostrup, Denmark) (III).

Collagen type I was stained using Alcian blue and van Gieson (III).

Laboratory analyses

Analysis of collagen

Measurement of hydroxyproline was done for analysis of collagen (98). The colonic samples were dried to constant weight at 100°C after which they were hydrolyzed in 6 M hydrochloric acid for 18 hours at 110°C. The hydrolysates were evaporated and residues were washed three times with deionized water with complete evaporation between each wash step to remove residual acid. Samples were then dissolved in 2.0 ml acetate-citrate buffer (1.2% acetic acid, 12% sodium acetate, 5% citric acid, 3.4% sodium hydroxide; pH 6.0) and ultra-sonicated. Chloramine-T was added (0.5 ml per 1.0 ml sample) followed by 20 minutes incubation in room temperature for oxidation of hydroxyproline where after 0.5 ml of 16.5% perchloric acid and 15% 4-dimetylaminbenzaldhyd in 1-propanolol were added followed by 15 minutes incubation in 60°C water bath during which the chromogen was formed. After chilling, colorimetric measuring of absorbance was carried out (99).

The optical density of the samples was measured in duplicates at 557 nm using the Versamax micro plate reader (Molecular Devices, Sunnyvale, CA, USA). The hydroxyproline concentrations of the samples were calculated, using the software (Softmax® Pro, Molecular Devices), by an L-hydroxyproline standard curve.

Determination of MMP activity

The total MMP activity in the obstructed colonic tissues was analyzed in tissue homogenates (75, 100). Tissues were immersed in 20 times volumes of 0.25% (vol/vol) Triton® X-100 (Sigma-Aldrich, St. Louis, MO, USA) containing 10 mM (final concentration) $CaCl_2$ and 1 mM Pefabloc® SC (Roche Applied Science, Mannheim, Germany). Tissues were disintegrated with Ultra-Turrax® T-25 (IKA Labortechnik, Staufen, Germany) homogenizer run at 20,500 rpm for 1 min at 0°C. The homogenates were centrifuged at 10,000 × g for 30 min at 4°C and supernatants removed. The pellets were resuspended in 20 volumes of 10 mM $CaCl_2$, 150 mM NaCl, 50 mM Tris-HCl (pH 7.6), 0.03 % Brij® 35, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.2 mg/ml NaN_3 and 1 mM phenylmethanesulfonyl fluoride. The resuspended pellet was aliquoted into two 400-µl portions to 2-ml polypropylene microcentrifuge tubes (Safe-T-Seal®, USA/Scientific Plastics (Europe) Limited). Aminophenylmercuric acid (APMA) at 1 mM was added to one aliquot to activate latent enzymes. The second aliquot served as blank by adding 2 mM (final concentration) 1,10-phenanthroline that completely blocks MMP activity by chelating zinc. Samples were incubated for 18 h at 37°C and then centrifuged at 10,000 × g for 30 min at 4°C. Supernatants and

pellets were analyzed for collagen content, measured as hydroxyproline as described above. Total MMP activity was defined as the percentage of degraded collagen in the APMA-treated sample minus the percentage of collagen present in the phenanthroline-treated sample (75).

Statistical methods

Paper I

The subgroups in paper I were small and to obtain normal distribution hydroxyproline levels were log-transformed. To investigate differences in mean, one-way ANOVA was used. To rule out what group significantly differed from day 0 the Newman-Keul post-hoc test was applied. Hydroxyproline data were given as geometric mean ± back transformed standard error. All other variables in paper I were shown as mean ± standard deviation.

To assess linear dependence Pearson's correlation coefficient was determined.

Paper II and III

In paper II and III the subgroups were small and assumption of normal distribution could not be made. Therefore the non-parametric Mann-Whitney U-test was used to assess differences between groups.

Spearman's rank correlation coefficient was used to measure dependence since data was not normally distributed (II).

Data are presented as median and interquartile range.

Paper IV

For analysis of difference in means between the subgroups on day 3 in experiment A and B respectively, the t-test was used since the variation in the compared groups was about the same. Data was assumed to be normally distributed.

Results are presented as mean ±standard deviation.

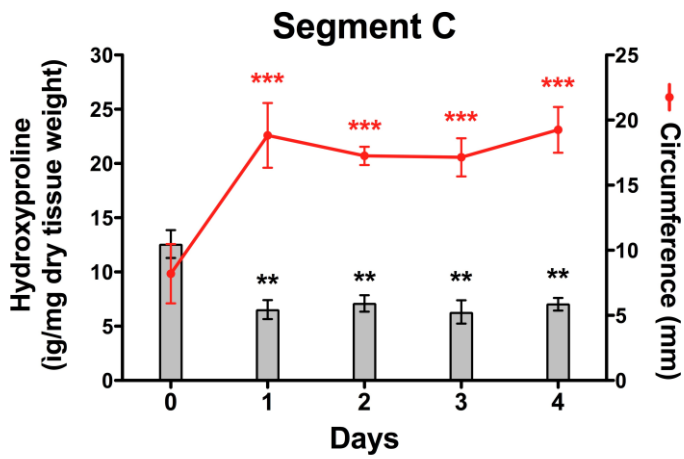
$P<0.05$ was considered statistically significant in all papers.

Results and General Discussion

Obstruction-related variations in collagen

The hydroxyproline concentration is known to be decreased after four days of obstruction (75, 87). However, when in time after obstruction this change occurs was not known, which lead to the experiments presented in paper I. We found that the drop in hydroxyproline concentration took place early after initiation of obstruction. Compared to normal colon the level of hydroxyproline proximal to the obstruction had fallen with about 40-45 % already after 24 hours. Further on no additional decrease was found. This latter finding could be attributable to increased collagen synthesis which has been shown to occur at least on day four after obstruction (87). Fibroblasts are known to respond with gene activation to mechanical forces resulting in connective tissue synthesis (101). In our experimental model of obstruction the colon was dilated to more than double its normal circumference in the same time frame as the decrease in hydroxyproline took place (figure 2).

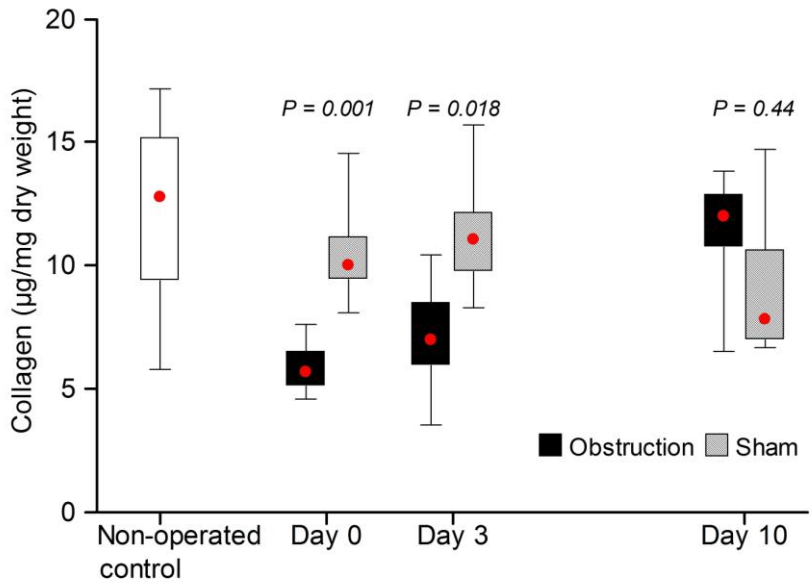
Figure 2
Colonic circumference (mean ± SD) and hydroxyproline concentration (geometric mean ± back transformed SE) in segment C, just proximal to the obstruction, after 0 to 4 days of obstruction. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to day 0.



Morphologically an inflammatory infiltrate predominantly consisting of neutrophils and macrophages was present proximal to the obstruction after 24 hours. A similarity to the characteristic cellular infiltrate seen in anastomotic healing is obvious (102). Moreover, a pronounced oedema was established in the submucosa from early on but was reduced after two days while the cellular infiltrate persisted.

Assuming that hydroxyproline concentration at time for construction of anastomosis is associated with healing potential, measures to normalize a reduced concentration would be interesting to investigate. The clinical parallel would be stenting and bridge-to-surgery in obstructing colon cancers. This interest led to the experiments presented in paper II. By using the same model for obstruction, we expected to obtain similar changes in the bowel wall as described above. The obstruction was relieved after four days when hydroxyproline levels were reliably depressed. It was found that the bowel could re-establish its healing capacity in terms of hydroxyproline which returned to preoperative levels after 10 days of recovery (figure 3).

Figure 3
Collagen (hydroxyproline) concentration in the colon of obstructed (filled boxes), sham-operated (cross-hatched) and nonoperated control groups (open box).



Unfortunately there is no reliable method to study breakdown of collagen in anastomotic wounds. Generally, decrease in hydroxyproline has been correlated to collagen degradation. But concerns have been raised on how to interpret changes of hydroxyproline concentration. A decrease could depend on true degradation but also be the result of dilution or a combination (103). Concentration is usually expressed as µg hydroxyproline/mg dry tissue. If the amount of collagen is actually unchanged an identified decreased concentration is due to increased dry weight. A correct evaluation of changes in concentration presume an adynamic milieu not responding to the cascade of signals triggered by the stress put on the tissue at wounding. A substantial inflammatory cellular infiltrate is the normal reaction, in anastomotic healing as well as in bowel obstruction, as described above. Besides the contribution to the dry weight by influx of cells, extracellular matrix components are synthesized and deposited. Hyaluronan is a large heavy-weight extracellular matrix molecule important in tissue healing (104). It is pro-inflammatory and facilitates cell migration probably due to its hygroscopic properties (105). Fibronectin is a high-molecular weight molecule also found early in anastomotic wounds and important for cell-matrix and matrix-matrix interactions in healing (106).

Supportive for the theory that decrease in hydroxyproline concentration reflects collagen lysis by the action of MMPs is the factual presence of these enzymes in stressed tissues such as anastomotic wounds and obstructed bowel (73, 75). But presence of MMPs does not preclude other effects of these enzymes than just being destructive (41).

Maybe the solution closest to the truth of what is going on with collagen is analyses of collagen content and synthesis. Analysis of synthesis requires isotope-based labelling techniques which is difficult and time-consuming. Measurements of the content of collagen in a tissue sample, expressed as µg hydroxyproline, require a standardized and meticulous sampling technique, or else comparisons will be inaccurate.

Gene expression and deposition of collagen gradually increases during the first week after wounding (106, 107). A decrease in collagen content in the early phase should then suggest collagen degradation. When collagen synthesis is well under way after 5-7 days the content should increase in analogy if enzymatic activity has subsided. Attempts have been made with competent experiments and the hypothesized pattern was actually found in anastomotic healing (108). Furthermore, colonic obstruction for 4 days resulted in increased collagen synthesis but unchanged collagen content proximal to the obstruction suggesting remodeling including collagen degradation as well (87).

As an integral part of anastomotic healing or obstruction an inevitable decrease in hydroxyproline concentration occurs in the early phase. Whether the restored collagen levels 10 days after decompression of colonic obstruction is a result of synthesis and replacement of old collagen or an indication of diminished inflammation, with reference to tissue dry weight, is not ruled out by the present

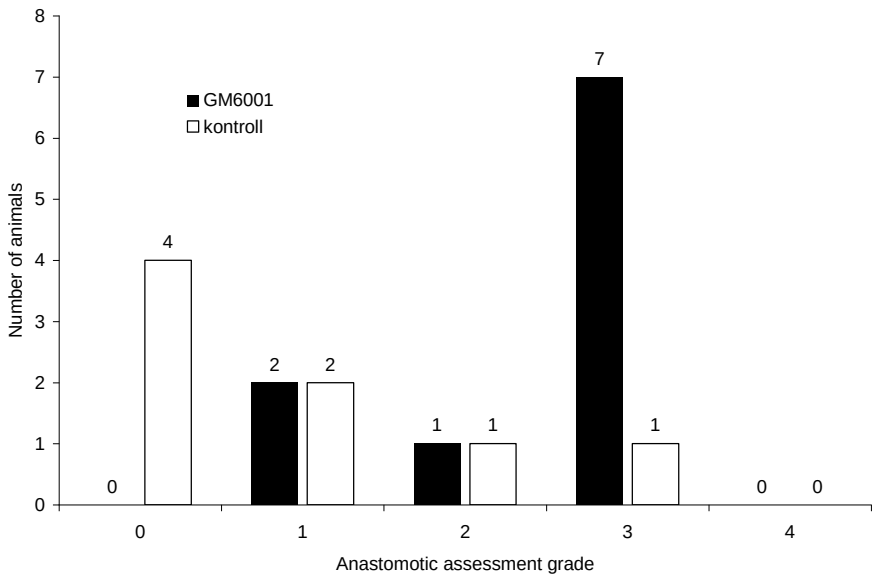
study (II). Presumably it could be read as an expression of re-established healing capacity.

MMP inhibition in compromised anastomosis

Previous experience with MMPI treatment is generally limited to studies with small numbers of animals making it impossible to detect any possible differences in frequency of anastomotic complications. The conceivable benefit of a MMP inhibitor would be more obvious in a model with a high complication rate. The experimental model used in paper III fulfils that requirement (figure 1, page 27). Rats exposed to colon resection and anastomosis after a 4 day long obstruction period developed anastomotic dehiscence or perianastomotic abscesses in 27 % of cases, probably in the early course of the 7 day long follow-up (65). Thus anastomotic leakage could be used as a primary endpoint, instead of an inferior surrogate marker, for the experiments in paper III.

The assessment criteria in table 1 proved applicable to discriminate differences in macroscopic appearance of the anastomosis. Unexpectedly the GM6001-treated animals demonstrated significantly poorer healing as presented in figure 4.

Figure 4
Anastomotic healing evaluated by clinical criteria on a scale from 0 to 4 as defined in Table 1 in rats treated with GM6001 (filled bars) or control (open). GM6001 (n = 10) versus control (n = 8), P = 0.006 (Mann-Whitney test).



The results indicate that MMP-mediated collagen degradation is of minor importance in the cause of anastomotic dehiscence. Supporting this remark is that Törnqvist et al found no difference in breaking strength or collagen content between anastomosis of obstructed or normal colon neither in the early course of healing or after seven days. Instead it was suggested that discrepancies in colon circumference could contribute to increased dehiscence rate. The results of imaging on day 1 do not exclude such an explanation (III). Three out of four anastomosis showing leakage on day 1 with fluoroscopic imaging were revealed to have leakage day 7 on clinical examination. As a comparison, in anastomosis of normal colon no leakage could be detected by contrast enema and fluoroscopy, not even as early as 1 hour after construction of anastomosis using eight interrupted inverting sutures (109).

The leakages detected by imaging on day 1 in paper III suggests different mechanisms leading to clinical leakage as evaluated day 7. First, the structural disparity with apparent different bowel circumferences could be causative, either MMP-inhibitory dependent or not. The circumference of the proximal end was almost twice as high compared to the distal bowel (19 vs. 10 mm; values from paper I). Second, leaking anastomoses, not demonstrating radiological leakage in the early course, possibly have another cause than structural and are probably related to disturbances in the normal events during healing inflicted by the GM6001 manipulation of MMP activity.

Further insight into the outcome of the experiments in paper III are provided by the histopathological evaluations. Firstly, collagen type I staining was of comparable amount in the two groups. GM6001 did not have an effect on the number of myofibroblasts in the granulation tissue. Myofibroblasts are, besides collagen synthesis, important for diminishing the size of wounds by the process of wound contraction. According to previous investigations collagen content is comparatively unchanged 7 days after anastomosis in obstructed colon. With sutures in place breaking strength was significantly increased (65). Breaking strength is also increased after one week in healing of normal colon but sutures does not contribute to the integrity of the anastomosis at this stage (110, 111).

Even if collagen seems to be unchanged as read from the histopathology one can not exclude influence on its fibrillation. The removal of the C-terminal propeptide requires the activity of procollagen C-peptidase which is zinc-dependent metallopeptidase closely related to MMPs. The procollagen N-terminal propeptide is removed by ADAMTS's, another metalloendopeptidase. Thus, GM6001 could possibly affect the activity of these enzymes' activity and impair collagen deposition in the wound. Unaffected activity of procollagen C-peptidase is also required for activation of lysyl oxidase which is responsible for collagen cross-linking (30). According to Törnqvist's work collagen synthesis is well under way already during obstruction but level out on a rate comparable to normal colon 4 days after anastomosis. It is difficult to rule out what impact GM6001 had on net collagen turnover in the present study since treatment was given throughout the

obstruction period and continued for 5 days after anastomosis. Reduced degradation as well as impaired collagen deposition is conceivable.

Removal of devitalized tissue is an important part of anastomotic healing and requires proteolytic activity. Besides the sloughing of the inverted bowel tips the bowel ends must reunite during a transition state when the wound gap is composed of a provisional matrix reinforced by a fibrin-fibronectin mesh. Consequently a significant wider wound gap and more necroses were found in the GM6001-treated animals suggesting impaired ability to discard devitalized tissue and gradually replace the granulation tissue with scar tissue. Contrasting this finding is the effect of GM6001 on anastomotic healing in normal colon where the wound gap was considerable narrower than in the controls on the 3rd day after construction of anastomosis (86). The findings in the present study nonetheless imply that degradation is an inevitable part of the healing process and if counteracted by means of MMP inhibition could lead to dehiscence.

The epithelialisation was defective in the GM6001-treated animals compared to controls in which partial or complete coverage was observed. In contrast, anastomotic wounds in normal colon showed no difference in epithelialisation when GM6001-treated animals were compared with controls. However, those animals were not followed for more than 3 days (86). Nevertheless, it was demonstrated in two separate studies that GM6001-treatment significantly impaired epithelialisation of cutaneous wounds (90, 112). In contrast and interestingly, GM6001 improved healing of skin incisions (89). The discrepancy could be explained by the fact that incisional wounds have a minimal epithelial defect compared with the much more extensive epithelial defect in excisional wounds.

A conceivable explanation for the disturbed re-epithelialisation is provided from the role of MMPs in cell migration. Epithelial cells must dissociate from their attachment to neighbouring cells and the ECM in order to move in over the provisional wound matrix. This MMP-dependent feature has been investigated in several wound models in skin, cornea, lung and mucosal epithelium (113).

One mechanism for the observed impaired epithelialisation could be correlated to disturbances in the action of ADAM17. This enzyme also belongs to the metalloendopeptidases and is inhibited by GM6001 (IC_{50} 320 nM) (114). ADAM17 release the soluble form of TNF from its cell membrane integrated form. In a skin wounding model re-epithelialisation was delayed in TNF-deficient mice and correlated to reduced MMP-9 levels (115). Explants in a human foetal gut wound healing model showed migrating epithelial cells positive for MMP-1 and MMP-10 assessed with in situ hybridization. Furthermore, when the explants were incubated with TNF-α the signal for MMP-10 and the number of migrating epithelial cells were increased (53).

The typical granulocytic wound infiltrate has normally disappeared by day 7 after anastomosis (103). In the GM6001-treated animals the infiltrate was still substantial after 7 days (III). The condition could be secondary to the deficient epithelialisation leaving the granulation tissue without protective barrier against

the hostile luminal environment rich in bacteria. Under these circumstances with an amplified inflammation delayed wound healing would be expected leaving the anastomosis vulnerable to dehiscence.

The negative impact of GM6001-treatment on anastomotic healing in compromised anastomosis seems to have no resemblance with anastomotic healing in normal colon. BB-94 and BB-1101 are broad-spectrum hydroxamate matrix metalloproteinase inhibitors with Ki values for several MMPs in the low nM range and as such demonstrates similarities with GM6001. Rats treated with BB-94 or BB-1101 developed no anastomotic dehiscence during 7 days (79, 82). One conceivable explanation is provided by the fact that the anastomotic wound is narrower in normal colon enabling adequate epithelialisation even under the impact of MMP inhibition.

Limited MMP inhibition in anastomotic healing

If collagen degradation is limited to the area in the direct proximity of the sutures as indicated by the results by Ågren et al it would be favourable to contain MMP inhibition to this part of the anastomosis (72). A route for local delivery of a inhibitory drug would possibly avoid unwanted effects on recognized MMP functions in wound healing.

Doxycycline is a tetracycline derivative and besides well known antibacterial qualities display collagenase inhibitory effect and have among several other MMP-inhibitors showed to improve anastomotic breaking strength (83). The mechanisms doxycyclin exercise its inhibitory effect through on MMPs is probably diverse (83, 116).

In paper IV the impact of sutures coated with doxycycline on anastomotic wound strength was investigated. Successful coating was determined by ellipsometry. It was estimated that 1 cm of doxycycline-coated suture held about 7 ng of doxycyclin.

When anastomotic healing is evaluated by means of mechanical testing one consistently find a temporarily decrease in breaking strength during the first few days being at its lowest on the 3rd day after anastomosis. Most of the reduction was prevented with the doxycyclin-coated sutures in parallel to the result when doxycyclin was given systemically.

The difference between the immediate controls and the carrier coated day 3 did not reach significance ($p=0.08$). Experiment B could however confirm that no effect was attributed to the carrier protein (cross-linked fibrinogen) itself (table 2).

Table 2 Results of the experiments in paper IV.
Locally administered doxyxycline increased anastomotic breaking strength on day 3 compared to control (carrier coated sutures), (mean ±SD).

experiment		n	breaking strength (N)		p-value
			day 0	day 3	
A	uninjured	5	2.93 ±1.07		0.026
	immediate	10	1.53 ±0.44		
	doxycyclin coated	15		1.47 ±0.2	
	carrier coated	15		1.25 ±0.26	
B	carrier coated	20		1.16 ±0.36	0.64
	uncoated	20		1.21 ±0.37	

The mechanism contributing to improved breaking strength is somewhat elusive. On histology the submucosa in the proximity of an anastomosis on day 3 show mainly oedema, mononuclear inflammatory cells and collagen fibres are dispersed within the submucosa (72, 103). No obvious differences are reported in the studies by Simonsma, Kiyama or Ågren (2011) comparing MMP-inhibited animals with controls. When examining more closely the suture zone, as in the study by Ågren et al (72), a picture resembling a foreign body reaction is perceived (117). Could the accumulation of macrophages and following degradation of collagen and possibly other ECM components around the sutures be responsible for the decreased suture holding capacity? In that case the condition is unavoidable if not completely inert biological materials are used. The tensile strength of anastomoses or any other wound is zero without sutures during the first few days after wounding (118). Gastrointestinal surgery is impossible if cut bowels ends are not realigned and hold together by sutures or staples. Experimental research has looked into the strength development of anastomosis after sutures being removed only 1 hour after construction. Breaking strength was measured during the first 24 hours and bursting pressure for one week. Strength development was linear as might have been expected (119).

The mechanism behind the increased breaking strength observed with doxycycline-coated sutures in paper IV could be the result of decreased local inflammation. MMP inhibition would expect to have an impact on several events occurring around the sutures such as decreased chemotactic gradient, less recruitment of inflammatory cells and activation of degradative enzymes. If mature collagen fibres are preserved around the sutures tensile strength would improve. Whether this effect is important is not known.

Since an anastomosis subject for testing of breaking strength is discontinued it will never be possible to relate a value of breaking strength to anastomotic

failure. Uncompromised anastomoses will almost always heal uneventfully without complications. When considering decreased breaking strength as unwanted with risk of dehiscence one does not take into account that one is perhaps just studying a fundamental imperative physiological process leading to complete healing. However, to strengthen the suture holding capacity in a compromised anastomosis, such as in obstructed colon, by local delivery of a MMP inhibitor through coated sutures, would possibly prevent broadening of the anastomotic wound following slack in the sutures and consequently lead to improved outcome.

Concluding Remarks

In the human context the relevance of results from experimental animal research could be questioned and caution is probably advisable if one is tempted to draw conclusions regarding human biology. Data obtained from animal research is nevertheless invaluable in pointing out the direction on how to understand biological processes in man. As for anastomotic healing, animal studies allow us to closely examine both normal physiology but also the pathophysiology during stressed conditions. Clinical studies allow us to identify patient specific risk factors associated with an adverse outcome such as anastomotic dehiscence. The anastomosis however, is inaccessible once the abdominal wall is closed.

To study the course of events taking place during experimental anastomotic healing different methods can be applied. Briefly they deal with mechanical properties, biochemical changes or histology. If experimental conditions are manipulated, changes in analysed parameters are usually understood as either improved or impaired healing capacity. How to correctly relate an increased breaking strength or a decreased concentration of collagen with reference to risk of anastomotic dehiscence is however uncertain. Intact collagen is nevertheless important for the bowels capacity to retain the sutures and consequently the anastomosis' integrity in the early phase of healing.

The results suggest that decreased collagen is of less significance with respect to leakage than other mechanisms dependent on unaffected MMP function. Targeted MMP inhibition however, does not preclude advantageous effects on anastomotic healing.

In summary the studies in this thesis have showed that:

- Substantial changes in bowel structure and collagen turnover occur rapidly after onset of colonic obstruction and that,
- these changes give a completely different foundation for healing than in uncompromised bowel, but also that
- reversal of obstruction leads to restored conditions after ten days.
- Non-selective systemic MMP inhibition have a wide impact on anastomotic healing not only limited to decreased collagen degradation, which
- leads to impaired healing and increased incidence of anastomotic complications in colonic obstruction, but
- MMP inhibition restricted to the suture zone is promising.

Acknowledgements

Ingvar Syk, principal supervisor, who invited me and introduced me to experimental research. Thank you for your encouragement and for giving me the opportunity to complete this project.

Magnus Ågren, supervisor, for your constant support and enthusiasm and great knowledge in the field.

Professor **Bengt Jeppsson**, who has been supportive throughout the project.

Peter-Martin Krarup, my co-author, who boosted the project.

Susanne Eiswohld, for practical help in the lab.

Dr **Ingrid Tengrup**, for guidance into my professional career.

Colleagues and **teammates**, for always having good laughs at hand.

Liselott, my beloved wife, who makes everything possible.

My beautiful children; **Julia**, the most outstanding girl; **Lukas**, with an awesome kick which soon will overthrow even me and; **Isak**, the master in air. You give me all the right bearing in life.

Populärvetenskaplig sammanfattning på svenska

De flesta patienter återhämtar sig väl efter ett kirurgiskt ingrepp, blir återställda och återgår till ett normalt liv. Men om komplikationer till det kirurgiska ingreppet inträffar, avvikelser från det förväntade läkningsförloppet, kan konsekvensen bli allt från lite extra antibiotika behandling eller kanske något längre sjukhusvård än planerat till bestående funktionsnedsättning eller för tidig död.

Den grundläggande förutsättningen för att kunna operera en patient är skadad vävnads förmåga att läka. Mycket av forskningen inom kirurgi har således intresserat sig för sårläkningens natur samt orsaker till och konsekvenser av fördröjd eller utebliven sårläkning.

Vid tarmoperationer skapar kirurgen i princip tre olika sår. För åtkomsten till bukhålan delas först huden vilket ger tillgång till bukväggen. Denna utgörs av muskler och stödjande bindvävsskikt. När dessa delats är bukhålans organ tillgängliga. Om patienten har en tumörsjukdom i tjocktarmen delas denna ovanför och nedanför tumören varefter den sjuka delen av tarmen tas bort. De två öppna tarmändarna, mellan vilka den borttagna delen låg, sys därefter ihop med varandra.

De kirurgiska såren i huden, bukväggen och tarmen behöver var för sig läka ihop för att funktionen i respektive vävnad och organ ska återställas. Vävnaden hålls ihop med stygn för att inte glida isär innan styrkan i såren är tillräckligt stor. Om läkningen i huden är påverkad kan det bli en glipa men det ger oftast inget större obehag för patienten förutom ömhet och behov av förband om såret vätskar sig. Det finns dock risk för sårinfektion som behöver behandlas med antibiotika.

Nedsatt sårläkning i bukväggen medför större konsekvenser för patienten. Om inte kontinuiteten i bindvävsskikten återställs uppkommer ett ärrbråck. Det är en utbuktning av bukhinnan genom en försvagning i bukväggen. Ärrbråck orsakar besvär för patienten och medför även risker varför ärrbråck oftast repareras.

Om läkningen av ihopkopplingen i tarmen (anastomosen) är försämrad och en glipa uppkommer blir konsekvenserna allvarliga. Det läcker då tarminnehåll rikt på bakterier ut i bukhålan med bukhinneinflammation som följd. Det är ett livshotande tillstånd som måste opereras akut. Som en följd av den påverkan kroppen utsätts för vid anastomosläckage uppkommer risker som har betydelse för hälsan även på lång sikt. Det är således av största vikt att skapa så goda förutsättningar som möjligt för normal anastomosläkning.

Under år 2009 fick 5755 personer i Sverige diagnosen cancer i tjocktarm eller ändtarm. De flesta av dem opererades med borttagande av tumören och

ihopkoppling av tarmen. Symtom talande för cancer i tjocktarm och ändtarm och som för patienten till sjukvården kan vara synligt blod i avföringen, blodbrist, förändrade avföringsvanor eller viktnedgång. Tumören hittas oftast när tarmen undersöks med böjlig slang med kamera (coloskopi) då även vävnadsprov tas som kan bekräfta diagnosen. Tumörsjukdomens utbredning fastställs med röntgen (datortomografi). Efter utredning genomgår patienten en planerad operation.

För ungefär var sjunde patient med cancer i tjocktarmen debuterar dock sjukdomen akut. Tumören har då inte gett sig till känna förrän den har dragit ihop tarmkanalen så att avföringen inte längre kan passera. Patienten är oförmögen att tömma tarmen på gaser och avföring och mår oftast mycket dåligt med buksmärta och kräkningar samt har symtom på vätskebrist. En akut operation är riskfylld men nödvändig.

Risken för läckage från en tarmanastomos är ungefär tre gånger högre om patienten har opererats akut på grund av stopp i tjocktarmen jämfört med en planerad operation. Detta återspeglar troligen en försämrad läkningsförmåga i tarm som har spänts ut ovanför en tumör som orsakat ett totalt hinder.

Sårläkningens natur och förlopp i en tarmanastomos går inte att studera på människa eftersom anastomosen är oåtkomlig inne i bukhålan. Forskning på människa är istället hänvisad till att hitta relevanta faktorer, före eller under operation, som kan ha betydelse för utgången. Faktorer hos patienten som kan påverka läkningen negativt är samtidig hjärt- och kärlsjukdom, undernäring, kortisonbehandling och akut operation på grund av tarmhinder. Faktorer av betydelse under operationen är till exempel om operationen är tekniskt svår och tar lång tid eller om tarmen kopplas ihop nära ändtarmsöppningen.

Forskning på djur gör det möjligt att inhämta kunskap hur sårläkning går till på cellnivå och man kan även utsätta läkningsprocessen för olika påfrestningar i syfte att hitta mekanismer som är av betydelse för läkningsförmågan.

Sårläkningsprocessen påbörjas omedelbart efter att ett sår uppstått, antingen som sårskada efter ett olycksfall eller till följd av kirurgens kniv. Processen följer en fastställd händelsekedja och börjar med att blodplättar ansamlas i såret och blödningen upphör. Olika signaler frisätts vilket lockar till sig vita blodkroppar specialiserade på att skydda kroppen mot skadliga bakterier. Sårkanterna består av skadad vävnad som behöver brytas ner och istället ersättas av speciell läkningsvävnad. De vita blodkropparna får hjälp av en annan sorts celler som förflyttat sig in i såret och kallas makrofager. Förutom att städa upp i såret lockar de till sig ytterligare celler, fibroblaster, endotelceller och glatta muskelceller, som kan bilda ny vävnad och blodkärl. Deras arbete leder till att sårkanterna överbryggas och kopplas ihop med varandra igen. Cellerna som är inblandade i sårläkningsprocessen bryter ner skadad och tillfällig läkningsvävnad och ersätter den med nybildad. Till det använder de sig av vävnadsnedbrytande enzymer, bland annat de så kallade matrix metalloproteaserna. Dessa enzymers aktivitet är noga balanserad eftersom okontrollerad aktivitet skulle få förödande konsekvenser. De har förmåga att bryta ner stödjevävnaden i tarmväggen.

Stödjevävnaden är rik på bindvävsproteinet kollagen som bildar ett nätverk av starka fibrer. Det har länge varit känt att kollagenfibrerna är den starka förankringen för stygnen, som förhindrar att tarmändarna glider isär under läkningsprocessen. Om kollagenfibrerna bryts ner förlorar tarmväggen sin förmåga att hålla fast stygnen, såret öppnar sig och tarminnehåll läcker ut i bukhålan.

Experimentell forskning har gett stöd för att kollagen bryts ner för mycket i tarm som varit utspänd på grund av en förträngning. Orsaken skulle kunna vara för hög enzymaktivitet. De undersökningar som redovisas i den här avhandlingen har fastställt att minskningen av kollagenhalten inträffar mycket tidigt i förloppet efter att tarmen blivit utspänd av ett hinder (I). Det är i och för sig troligt att flera faktorer samverkar till att anastomoser läker sämre om tarmen varit utspänd. Resultatet kan dock tolkas som att kapaciteten för tarmen att läka minskar redan tidigt i förloppet. Vi fann också att kollagenhalten ökar igen och blir normal under en återhämtningsperiod efter att hindret togs bort (II). Möjligen återställs även läkningsförmågan samtidigt. Man har i patientstudier visat att man kan få god läkning av tarmanastomoser även vid utspänd tarm om förträngningen först trycks tillbaka från insidan av tarmen med ett rör (stent). Tarmkanalen återställs därmed och patienten kan istället opereras planerat när tarmen har fått normal vidd igen och patienten i övrigt är i bättre skick.

En annan tilltalande metod, som alternativ till avlastning av utspänd tarm med hjälp av stent innan planerad operation, skulle vara att vid en akut operation behandla med läkemedel som hämmar aktiviteten av de vävnadsnedbrytande enzymerna. I ett av arbetena i avhandlingen studerades effekten av enzymhämmaren GM6001 vid läkning av experimentell tarmanastomos med hög risk för komplikationer. Hypotesen var att behandlingen skulle minska antalet anastomosläckage. Det visade sig att effekten blev motsatt (III). Således har troligen kollagennedbrytning mindre betydelse för risken att utveckla läckage än andra mekanismer beroende av opåverkad funktion av matrix metalloproteaser. Möjligen var en bidragande orsak till det försämrade resultatet att epitelcellerna i tarmens slemhinna hämmades att vandra ut och täcka sårvävnaden. Det ledde till en förstärkt sårinflammation som troligen var förödande.

För att prova om man kunde hämma enzymaktivitet och kollagennedbrytning utan att påverka viktiga funktioner för metalloproteaserna tillverkades stygn som täcktes med läkemedlet doxycyclin. På det sättet skulle man kunna få effekt direkt vid de viktiga kollagenfibrerna i tarmväggen utan att påverka epitelcellerna i slemhinnan. Stygnen användes till att sy anastomos i normal tarm som inte var utspänd. Det visade sig att styrkan i anastomosen, det vill säga förmågan för tarmväggen att hålla kvar stygnen, förbättrades (IV). Resultatet motiverar framtida studier med behandlade stygn i syfte att utröna om dessa är en användbar metod även när läkningsbetingelserna är försämrade som vid utspänd tarm.

References

1. Smith JG. A Discussion on the Surgical Treatment of Acute Intestinal Obstruction. The British Medical Journal. 1890 Oct 11:835.
2. Halsted W. Circular suture of the intestine - an experimental study. Am J Med Sci. 1887;188:436-60.
3. Gross J, Lapiere CM. Collagenolytic activity in amphibian tissues: a tissue culture assay. Proc Natl Acad Sci U S A. 1962 Jun 15;48:1014-22.
4. Riley WB, Jr., Peacock EE, Jr. Identification, distribution, and significance of a collagenolytic enzyme in human tissues. Proc Soc Exp Biol Med. 1967 Jan;124(1):207-10.
5. Hawley PR, Faulk WP, Hunt TK, Dunphy JE. Collagenase activity in the gastro-intestinal tract. Br J Surg. 1970 Dec;57(12):896-900.
6. Hogstrom H, Haglund U, Zederfeldt B. Beneficial effect on intestinal anastomoses of S-2441, a synthetic kallikrein-kinin antagonist. Experimental studies in the rat. Am J Surg. 1985 Sep;150(3):312-4.
7. Travers B. An inquiry into the process of nature in repairing injuries of the intestines: illustrating the treatment of penetrating wounds, and strangulated hernia. Longman, Hurst, Rees, Orme and Brown. 1812;London.
8. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011 Mar-Apr;61(2):69-90.
9. Swedish Colon Cancer Registry.
10. Swedish Rectal Cancer Registry.
11. Swedish Health and Welfare Statistical Databases. Cancer Register.
12. Schrock TR, Deveney CW, Dunphy JE. Factor contributing to leakage of colonic anastomoses. Ann Surg. 1973 May;177(5):513-8.
13. Jestin P, Nilsson J, Heurgren M, Pahlman L, Glimelius B, Gunnarsson U. Emergency surgery for colonic cancer in a defined population. Br J Surg. 2005 Jan;92(1):94-100.
14. Kube R, Mroczkowski P, Steinert R, Sahm M, Schmidt U, Gastinger I, et al. [Anastomotic leakage following bowel resections for colon cancer: multivariate analysis of risk factors]. Chirurg. 2009 Dec;80(12):1153-9.
15. Kube R, Mroczkowski P, Granowski D, Benedix F, Sahm M, Schmidt U, et al. Anastomotic leakage after colon cancer surgery: a predictor of significant morbidity and hospital mortality, and diminished tumour-free survival. Eur J Surg Oncol. 2010 Feb;36(2):120-4.
16. Mirnezami A, Mirnezami R, Chandrakumaran K, Sasapu K, Sagar P, Finan P. Increased local recurrence and reduced survival from colorectal cancer following anastomotic leak: systematic review and meta-analysis. Ann Surg. 2011 May;253(5):890-9.
17. Serpell JW, McDermott FT, Katrivessis H, Hughes ES. Obstructing carcinomas of the colon. Br J Surg. 1989 Sep;76(9):965-9.

18. Lee YM, Law WL, Chu KW, Poon RT. Emergency surgery for obstructing colorectal cancers: a comparison between right-sided and left-sided lesions. J Am Coll Surg. 2001 Jun;192(6):719-25.
19. Baccari P, Bisagni P, Crippa S, Sampietro R, Staudacher C. Operative and long-term results after one-stage surgery for obstructing colonic cancer. Hepatogastroenterology. 2006 Sep-Oct;53(71):698-701.
20. Trompetas V. Emergency management of malignant acute left-sided colonic obstruction. Ann R Coll Surg Engl. 2008 Apr;90(3):181-6.
21. Tekkis PP, Kinsman R, Thompson MR, Stamatakis JD. The Association of Coloproctology of Great Britain and Ireland study of large bowel obstruction caused by colorectal cancer. Ann Surg. 2004 Jul;240(1):76-81.
22. van de Wall BJ, Draaisma WA, Schouten ES, Broeders IA, Consten EC. Conventional and laparoscopic reversal of the Hartmann procedure: a review of literature. J Gastrointest Surg. 2010 Apr;14(4):743-52.
23. Zorcolo L, Covotta L, Carlomagno N, Bartolo DC. Safety of primary anastomosis in emergency colo-rectal surgery. Colorectal Dis. 2003 May;5(3):262-9.
24. Alcantara M, Serra X, Bombardo J, Falco J, Perandreu J, Ayguavives I, et al. Colorectal stenting as an effective therapy for preoperative and palliative treatment of large bowel obstruction: 9 years' experience. Tech Coloproctol. 2007 Dec;11(4):316-22.
25. Saida Y, Sumiyama Y, Nagao J, Uramatsu M. Long-term prognosis of preoperative "bridge to surgery" expandable metallic stent insertion for obstructive colorectal cancer: comparison with emergency operation. Dis Colon Rectum. 2003 Oct;46(10 Suppl):S44-9.
26. Madden JW, Peacock EE, Jr. Studies on the biology of collagen during wound healing. I. Rate of collagen synthesis and deposition in cutaneous wounds of the rat. Surgery. 1968 Jul;64(1):288-94.
27. Bosman FT, Stamenkovic I. Functional structure and composition of the extracellular matrix. J Pathol. 2003 Jul;200(4):423-8.
28. Epstein EH, Jr., Munderloh NH. Isolation and characterization of CNBr peptides of human (alpha 1 (III))3 collagen and tissue distribution of (alpha 1 (I))2 alpha 2 and (alpha 1 (III))3 collagens. J Biol Chem. 1975 Dec 25;250(24):9304-12.
29. Ignat'eva NY, Danilov NA, Averkiev SV, Obrezkova MV, Lunin VV, Sobol EN. Determination of hydroxyproline in tissues and the evaluation of the collagen content of the tissues. Journal of Analytical Chemistry. 2007;62(1):51-7.
30. Trackman PC. Diverse biological functions of extracellular collagen processing enzymes. J Cell Biochem. 2005 Dec 1;96(5):927-37.
31. Canty EG, Kadler KE. Procollagen trafficking, processing and fibrillogenesis. J Cell Sci. 2005 Apr 1;118(Pt 7):1341-53.
32. Lord MG, Valies P, Broughton AC. A morphologic study of the submucosa of the large intestine. Surg Gynecol Obstet. 1977 Jul;145(1):55-60.
33. Meshel AS, Wei Q, Adelstein RS, Sheetz MP. Basic mechanism of three-dimensional collagen fibre transport by fibroblasts. Nat Cell Biol. 2005 Feb;7(2):157-64.
34. Grinnell F. Fibroblast mechanics in three-dimensional collagen matrices. J Bodyw Mov Ther. 2008 Jul;12(3):191-3.
35. Lee H, Overall CM, McCulloch CA, Sodek J. A critical role for the membrane-type 1 matrix metalloproteinase in collagen phagocytosis. Mol Biol Cell. 2006 Nov;17(11):4812-26.

36. Harkness RD, Moralee BE. The time-course and route of loss of collagen from the rat's uterus during post-partum involution. J Physiol. 1956 Jun 28;132(3):502-8.
37. Ryan JN, Woessner JF, Jr. Mammalian collagenase: direct demonstration in homogenates of involuting rat uterus. Biochem Biophys Res Commun. 1971 Jul 2;44(1):144-9.
38. Manase K, Endo T, Chida M, Nagasawa K, Honnma H, Yamazaki K, et al. Coordinated elevation of membrane type 1-matrix metalloproteinase and matrix metalloproteinase-2 expression in rat uterus during postpartum involution. Reprod Biol Endocrinol. 2006;4:32.
39. Rawlings ND, Barrett AJ, Bateman A. MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Res. Jan;40(1):D343-50.
40. Tallant C, Marrero A, Gomis-Ruth FX. Matrix metalloproteinases: fold and function of their catalytic domains. Biochim Biophys Acta. 2010 Jan;1803(1):20-8.
41. Somerville RP OS, Apte SS. Matrix metalloproteinases: old dogs with new tricks. Genome Biol. 2003;4(6):216.
42. Ravanti L, Kahari VM. Matrix metalloproteinases in wound repair (review). Int J Mol Med. 2000 Oct;6(4):391-407.
43. Gill SE, Parks WC. Metalloproteinases and their inhibitors: regulators of wound healing. Int J Biochem Cell Biol. 2008;40(6-7):1334-47.
44. Page-McCaw A, Ewald AJ, Werb Z. Matrix metalloproteinases and the regulation of tissue remodelling. Nat Rev Mol Cell Biol. 2007 Mar;8(3):221-33.
45. Steffensen B, Hakkinen L, Larjava H. Proteolytic events of wound-healing--coordinated interactions among matrix metalloproteinases (MMPs), integrins, and extracellular matrix molecules. Crit Rev Oral Biol Med. 2001;12(5):373-98.
46. Mott JD, Werb Z. Regulation of matrix biology by matrix metalloproteinases. Curr Opin Cell Biol. 2004 Oct;16(5):558-64.
47. 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.
48. Pasternak B, Matthiessen P, Jansson K, Andersson M, Aspenberg P. Elevated intraperitoneal matrix metalloproteinases-8 and -9 in patients who develop anastomotic leakage after rectal cancer surgery: a pilot study. Colorectal Dis. 2010 Jul;12(7 Online):e93-8.
49. Sheridan WG, Shandall AA, Alexander-Williams J, Keighley MR, Boulos PB, Young HL. A multicenter trial of the use of the proteolytic enzyme inhibitor aprotinin in colorectal surgery. Dis Colon Rectum. 1989 Jun;32(6):505-8.
50. Solberg A, Holmdahl L, Falk P, Palmgren I, Ivarsson ML. A local imbalance between MMP and TIMP may have an implication on the severity and course of appendicitis. Int J Colorectal Dis. 2008 Jun;23(6):611-8.
51. Solberg A, Holmdahl L, Falk P, Willen R, Palmgren I, Ivarsson ML. Tissue proteolysis in appendicitis with perforation. J Surg Res. Aug;169(2):194-201.
52. Rosemar A, Ivarsson ML, Borjesson L, Holmdahl L. Increased concentration of tissue-degrading matrix metalloproteinases and their inhibitor in complicated diverticular disease. Scand J Gastroenterol. 2007 Feb;42(2):215-20.
53. Salmela MT, Pender SL, Karjalainen-Lindsberg ML, Puolakkainen P, Macdonald TT, Saarialho-Kere U. Collagenase-1 (MMP-1), matrilysin-1 (MMP-7), and

stromelysin-2 (MMP-10) are expressed by migrating enterocytes during intestinal wound healing. Scand J Gastroenterol. 2004 Nov;39(11):1095-104.
54. Cronin K, Jackson DS, Dunphy JE. Changing bursting strength and collagen content of the healing colon. Surg Gynecol Obstet. 1968 Apr;126(4):747-53.
55. Irvin TT, Hunt TK. Reappraisal of the healing process of anastomosis of the colon. Surg Gynecol Obstet. 1974 May;138(5):741-6.
56. Hendriks T, Mastboom W. Healing of experimental intestinal anastomoses: Parameters for repair. Diseases of the colon & rectum. 1990;33(10):891-901.
57. Jiborn H, Ahonen J, Zederfeldt B. Healing of experimental colonic anastomoses. The effect of suture technic on collagen concentration in the colonic wall. Am J Surg. 1978 Mar;135(3):333-40.
58. Jiborn H, Ahonen J, Zederfeldt B. Healing of experimental colonic anastomoses. II. Breaking strength of the colon after left colon resection and anastomosis. Am J Surg. 1978 Nov;136(5):595-9.
59. Jiborn H, Ahonen J, Zederfeldt B. Healing of experimental colonic anastomoses. I. Bursting strength of the colon after left colon resection and anastomosis. Am J Surg. 1978 Nov;136(5):587-94.
60. Hendriks T, Vereecken THLB, Hesp WLEM, Schillings PHM, de Boer HHM. Loss of collagen from experimental intestinal anastomoses: Early events. Experimental and Molecular Pathology. 1985;42(3):411-8.
61. Horwitz AL, Hance AJ, Crystal RG. Granulocyte collagenase: selective digestion of type I relative to type III collagen. Proc Natl Acad Sci U S A. 1977 Mar;74(3):897-901.
62. Hasty KA, Pourmotabbed TF, Goldberg GI, Thompson JP, Spinella DG, Stevens RM, et al. Human neutrophil collagenase. A distinct gene product with homology to other matrix metalloproteinases. J Biol Chem. 1990 Jul 15;265(20):11421-4.
63. Hogstrom H, Haglund U. Neutropenia prevents decrease in strength of rat intestinal anastomosis: partial effect of oxygen free radical scavengers and allopurinol. Surgery. 1986 Jun;99(6):716-20.
64. Törkvist L, Månsson P, Raud J, Larsson J, Thorlacius H. Role of CD18-dependent neutrophil recruitment in skin and intestinal wound healing. Eur Surg Res. 2001 Jul-Aug;33(4):249-54.
65. Törnqvist A, Blomquist P, Jiborn H, Zederfeldt B. Anastomotic healing after resection of left-colon stenosis: effect on collagen metabolism and anastomotic strength. An experimental study in the rat. Dis Colon Rectum. 1990 Mar;33(3):217-21.
66. Chowcat NL, Savage FJ, Lewin MR, Boulos PB. Direct measurement of collagenase in colonic anastomosis. Br J Surg. 1990 Nov;77(11):1284-7.
67. van der Stappen JW, Hendriks T, de Boer HH, de Man BM, de Pont JJ. Collagenolytic activity in experimental intestinal anastomoses. Differences between small and large bowel and evidence for the presence of collagenase. Int J Colorectal Dis. 1992 Jun;7(2):95-101.
68. Seifert WF, Wobbes T, Hendriks T. Divergent patterns of matrix metalloproteinase activity during wound healing in ileum and colon of rats. Gut. 1996 Jul;39(1):114-9.
69. de Hingh IH, Lomme RM, van Goor H, Bleichrodt RP, Hendriks T. Changes in gelatinase activity in the gastrointestinal tract after anastomotic construction in the ileum or colon. Dis Colon Rectum. 2005 Nov;48(11):2133-41.

70. Chowcat NL, Savage FJ, Hembry RM, Boulos PB. Role of collagenase in colonic anastomoses: a reappraisal. Br J Surg. 1988 Apr;75(4):330-4.
71. Shaper KR, Savage FJ, Hembry RM, Boulos PB. Regulation of matrix metalloproteinases in a model of colonic wound healing in a rabbit. Dis Colon Rectum. 2001 Dec;44(12):1857-66.
72. Ågren MS, Andersen TL, Mirastschijski U, Syk I, Schiodt CB, Surve V, et al. Action of matrix metalloproteinases at restricted sites in colon anastomosis repair: an immunohistochemical and biochemical study. Surgery. 2006 Jul;140(1):72-82.
73. Savage FJ, Lacombe DL, Boulos PB, Hembry RM. Role of matrix metalloproteinases in healing of colonic anastomosis. Dis Colon Rectum. 1997 Aug;40(8):962-70.
74. Savage, Lacombe, Hembry, Boulos. Effect of colonic obstruction on the distribution of matrix metalloproteinases during anastomotic healing. British journal of surgery. 1998;85(1):72-.
75. Syk I, Mirastschijski U, Jeppsson BW, Agren MS. Experimental Colonic Obstruction Increases Collagen Degradation by Matrix Metalloproteinases in the Bowel Wall. Diseases of the Colon and Rectum. 2003;46(9):1251-9.
76. Herrmann JB, Woodward SC, Pulaski EJ. Healing of Colonic Anastomoses in the Rat. Surg Gynecol Obstet. 1964 Aug;119:269-75.
77. Bary S, Kortmann H, Kopcke W. [Bursting pressure of colon in the rats and proteinase inhibition (author's transl)]. Res Exp Med (Berl). 1976 Aug 25;168(2):123-8.
78. Young HL, Wheeler MH. Collagenase inhibition in the healing colon. J R Soc Med. 1983 Jan;76(1):32-6.
79. 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 Feb;88(2):228-34.
80. Hogstrom H, Haglund U. Early decrease in suture line breaking strength. The effect of proposed collagenase inhibition. Res Exp Med (Berl). 1985;185(6):451-5.
81. Tani T, Tsutamoto Y, Eguchi Y, Araki H, Ebira Y, Ameno H, et al. Protease inhibitor reduces loss of tensile strength in rat anastomosis with peritonitis. J Surg Res. 2000 Feb;88(2):135-41.
82. 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 Sep;17(5):348-54.
83. 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 Mar;133(3):268-76.
84. Kiyama T, Onda M, Tokunaga A, Efron DT, Barbul A. Effect of matrix metalloproteinase inhibition on colonic anastomotic healing in rats. Journal of Gastrointestinal Surgery. 2001;5(3):303-11.
85. Ågren MS, Jorgensen LN, Delaisse JM. Matrix metalloproteinases and colon anastomosis repair: a new indication for pharmacological inhibition? Mini Rev Med Chem. 2004 Sep;4(7):769-78.
86. Ågren MS AT, Andersen L, Schiødt CB, Surve V, Andreassen TT, Risteli J, Franzén LE, Delaissé JM, Heegaard AM, Jorgensen LN. Nonselective matrix metalloproteinase but not tumor necrosis factor-α inhibition effectively preserves

the early critical colon anastomotic integrity. Int J Colorectal Dis. 2011;26(3):329-37.
87. Törnqvist A, Blomquist P, Ahonen J, Jiborn H, Zederfeldt B. The effect of stenosis on collagen metabolism in the colonic wall. Studies in the rat. Acta Chir Scand. 1988 May-Jun;154(5-6):389-93.
88. Grobelny D PL, Galardy RE. Inhibition of human skin fibroblast collagenase, thermolysin, and Pseudomonas aeruginosa elastase by peptide hydroxamic acids. Biochemistry. 1992;11(31):7152-4.
89. Witte MB, Thornton FJ, Kiyama T, Efron DT, Schulz GS, Moldawer LL, et al. Metalloproteinase inhibitors and wound healing: A novel enhancer of wound strength. Surgery. 1998;124(2):464-70.
90. Mirastschijski U, Haaksma CJ, Tomasek JJ, Agren MS. Matrix metalloproteinase inhibitor GM 6001 attenuates keratinocyte migration, contraction and myofibroblast formation in skin wounds. Experimental Cell Research. 2004;299(2):465-75.
91. Mirastschijski U, Johannesson K, Jeppsson B, Agren MS. Effect of a Matrix Metalloproteinase Activity and TNF-Alpha Converting Enzyme Inhibitor on Intra-Abdominal Adhesions. European Surgical Research. 2005;37(1):68-75.
92. Renkiewicz R, Qiu L, Lesch C, Sun X, Devalaraja R, Cody T, et al. Broad-spectrum matrix metalloproteinase inhibitor marimastat–induced musculoskeletal side effects in rats. Arthritis & Rheumatism. 2003;48(6):1742-9.
93. Tengvall P, Jansson E, Askendal A, Thomsen P, Gretzer C. Preparation of multilayer plasma protein films on silicon by EDC/NHS coupling chemistry. Colloids and Surfaces B: Biointerfaces. 2003;28(4):261-72.
94. McCrackin F. A Fortran Program for Analysis of Ellipsometer Measurements.: National Bureau of Standards; 1969.
95. De Feijter J, Benjamins J, Veer F. Ellipsometry as a tool to study the adsorption behavior of synthetic and biopolymers at the air-water interface. Biopolymers. 1978;17(7):1759-72.
96. Benesch J, Askendal A, Tengvall P. Quantification of adsorbed human serum albumin at solid interfaces: a comparison between radioimmunoassay (RIA) and simple null ellipsometry. Colloids and Surfaces B: Biointerfaces. 2000;18(2):71-81.
97. Stenberg M, Nygren H. The use of the isoscope ellipsometer in the study of adsorbed proteins and biospecific binding reactions. J de Physique Colloques. 1983;44(C10):83-6.
98. Woessner JF. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Archives of Biochemistry and Biophysics. 1961;93(2):440-7.
99. Stegemann H, Stalder K. Determination of hydroxyproline. Clin Chim Acta. 1967 Nov;18(2):267-73.
100. Woessner JF, Jr. Quantification of matrix metalloproteinases in tissue samples. Methods Enzymol. 1995;248:510-28.
101. Kessler D, Dethlefsen S, Haase I, Plomann M, Hirche F, Krieg T, et al. Fibroblasts in mechanically stressed collagen lattices assume a "synthetic" phenotype. J Biol Chem. 2001 Sep 28;276(39):36575-85.
102. Hesp WL, Hendriks T, Schillings PH, Lubbers EJ, de Boer HH. Histological features of wound repair: a comparison between experimental ileal and colonic anastomoses. Br J Exp Pathol. 1985 Oct;66(5):511-8.

103. Brasken P, Lehto M, Renvall S. Changes in the connective tissue composition of the submucosal layer of colonic anastomosis. An immunohistologic study in rats. Acta Chir Scand. 1989 Aug;155(8):413-9.
104. Weigel PH, Fuller GM, LeBoeuf RD. A model for the role of hyaluronic acid and fibrin in the early events during the inflammatory response and wound healing. J Theor Biol. 1986 Mar 21;119(2):219-34.
105. Chen WY, Abatangelo G. Functions of hyaluronan in wound repair. Wound Repair Regen. 1999 Mar-Apr;7(2):79-89.
106. Brasken P, Lehto M, Renvall S. Fibronectin, laminin, and collagen types I, III, IV and V in the healing rat colon anastomosis. Ann Chir Gynaecol. 1990;79(2):65-71.
107. Brasken P, Renvall S, Sandberg M. Fibronectin and collagen gene expression in healing experimental colonic anastomoses. Br J Surg. 1991 Sep;78(9):1048-52.
108. Oxlund H, Christensen H, Seyer-Hansen M, Andreassen TT. Collagen deposition and mechanical strength of colon anastomoses and skin incisional wounds of rats. J Surg Res. 1996 Nov;66(1):25-30.
109. Månsson P, Zhang X, Jeppsson B, Thorlacius H. Anastomotic healing in the rat colon: comparison between a radiological method, breaking strength and bursting pressure. International Journal of Colorectal Disease. 2002;17(6):420-5.
110. Blomquist P, Jiborn H, Zederfeldt B. Effect of diverting colostomy on breaking strength of anastomoses after resection of the left side of the colon. Studies in the rat. Am J Surg. 1985 Jun;149(6):712-5.
111. Mastboom WJ, Hendriks T, de Boer HH. Intestinal anastomotic healing in the absence of suture material: an experimental study in rats. Int J Colorectal Dis. 1991 Feb;6(1):33-7.
112. Lund LR, Romer J, Bugge TH, Nielsen BS, Frandsen TL, Degen JL, et al. Functional overlap between two classes of matrix-degrading proteases in wound healing. EMBO J. 1999 Sep 1;18(17):4645-56.
113. Chen P, Parks WC. Role of matrix metalloproteinases in epithelial migration. J Cell Biochem. 2009 Dec 15;108(6):1233-43.
114. Zhao Y, Yu J, Gu J, Huang W. The evaluation of inhibitive effectiveness of the tumour necrosis factor-alpha converting enzyme selective inhibitors by HPLC. J Enzyme Inhib Med Chem. 2011 Apr;26(2):181-7.
115. Scott KA, Arnott CH, Robinson SC, Moore RJ, Thompson RG, Marshall JF, et al. TNF-alpha regulates epithelial expression of MMP-9 and integrin alphavbeta6 during tumour promotion. A role for TNF-alpha in keratinocyte migration? Oncogene. 2004 Sep 9;23(41):6954-66.
116. Woessner JF, Jr. Matrix metalloproteinase inhibition. From the Jurassic to the third millennium. Ann N Y Acad Sci. 1999 Jun 30;878:388-403.
117. Anderson JM, Rodriguez A, Chang DT. Foreign body reaction to biomaterials. Semin Immunol. 2008 Apr;20(2):86-100.
118. Ballantyne GH. Intestinal suturing. Review of the experimental foundations for traditional doctrines. Dis Colon Rectum. 1983 Dec;26(12):836-43.
119. Wilker D, Sklarek J, Waldner H, Posel P. [Sutureless anastomoses in the rat, rabbit and pig]. Langenbecks Arch Chir. 1988;373(2):91-6.