EXPERIMENTAL MODELS OF THE HUMAN PERITONEAL ENVIRONMENT:

EFFECTS OF TGF-β AND HYALURONAN

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Omslagsbild:

Mikroskopbild av odlade mesotelceller
(Foto P. Falk 2003)

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"Alla modeller har sina fel och brister och det är inget fel,
felet uppkommer om man inte beaktar dessa när man drar sina slutsatser"

Känd Professor

Till
Carina, Johan and Erik

ABSTRACT

BACKGROUND

Post surgical adhesion formation is still an unsolved problem and occurs when there is an imbalance between fibrin deposition and fibrin clearing capacity in the abdominal cavity. Transforming growth factor beta (TGF-β) is associated with fibrosis and hyaluronan has in several studies been showed to reduce adhesions. There are limitations to study mechanisms in humans, thus experimental models are needed. This work used *in vivo* and *in vitro* models to study effects of TGF-β and hyaluronan, and may further elucidate their involvement in peritoneal repair.

MATERIAL & METHODS

TGF-β_1 and fibrinolytic components were measured in peritoneal tissue in patients (I). In models response to increased levels of TGF-β_1 on fibrinolytical components in cultured mesothelial cells (MC) were investigated (II). Measurements of fibrinolytic components and proliferation by hyaluronan were investigated in MC (III & IV). TGF-β isoforms and fibrinolytic components were assessed in peritoneal fluid and plasma during surgery, together with mesothelial proliferation *in vitro* (V).

RESULTS

Increased TGF-β_1 levels in adhesion tissue were associated with adhesion formation and TGF-β_1 correlated to plasminogen activator inhibitor-1 (PAI-1). Increasing levels of TGF-β_1 decreased production of tissue plasminogen activator (t-PA) and increased PAI-1 release into the culture media dose dependently in cultured MC. The *in vitro* studies of hyaluronan on MC indicated an increase in fibrinolytic capacity and an increase in proliferation when added. In peritoneal fluid during surgery elevated fractions of TGF-$\beta_{1\text{-}2}$ were found compared to plasma. The levels of TGF-β_1 in peritoneal fluid correspond to the levels found to increase MC proliferation in vitro.

CONCLUSION

Increased levels of TGF-β_1 in peritoneal tissue seem to be associated with adhesions, which in part might be explained by local decrease in fibrinolytic response from mesothelial cells. The clinical anti-adhesion effect of hyaluronan is unclear, but might partially be explained by increased fibrinolytical capacity and increased mesothelial proliferation. Low levels of active TGF-β_1 might increase mesothelial regeneration *in vivo* in combination with remained local fibrin degradation capacity found in the abdominal cavity during surgery. These findings might be of importance in the understanding of peritoneal repair.

Key words: Adhesion formation, experimental model, cell culture, mesothelial cells, peritoneum, fibrinolytic system, transforming growth factor beta, hyaluronan, proliferation

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CONTENTS

LIST OF PUBLICATIONS

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

I. Overproduction of transforming growth factor-β1 (TGF-β1) is associated with adhesion formation and peritoneal fibrinolytic impairment.
Holmdahl L, Kotseos K, Bergström M, Falk P, Ivarsson M-L, Chegini N
Surgery 2001;129:626-32

II. Differential regulation of mesothelial cell fibrinolysis by transforming growth factor beta 1.
Falk P, Ma C, Chegini N and Holmdahl L
Scand J Clin Lab Invest, 2000;60:439-448

III. Sodium hyaluronate increases the fibrinolytical response of human peritoneal mesothelial cells exposed to tumor necrosis factor alpha.
Reijnen M, van Goor H, Falk P, Hedgren M and Holmdahl L
Arch Surg, 2001;136:291-296

IV. The antiadhesive agent sodium hyaluronate increase the proliferation rate of human peritoneal mesothelial cells.
Reijnen M, Falk P, van Goor H and Holmdahl L
Fertil Steril, 2000;74:146-51

V. Studies of TGF-β_{1-3} in peritoneal serosal fluid during abdominal surgery and their effect on human mesothelial cell proliferation *in vitro*.
Falk P, Bergström M, Palmgren I, Holmdahl L, Breimer M and Ivarsson M-L
in manuscript

ABBREVIATIONS

bp	Base pair
BSA	Bovine serum albumin
CAPD	Continuous ambulatory peritoneal dialysis
cDNA	Complementary deoxyribonucleic acid
DC	Direct current
E199	Cell culture medium 199, with Earl's salts
ECGF	Endothelial cell growth factor
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
FDP	Fibrin degradation products
FITC	Fluorescein isothiocyanate
HA	Hyaluronic acid (Hyaluronan)
HPMC	Human peritoneal mesothelial cells
LPS	Lipopolysaccharide
MC	Mesothelial cells
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
nm	nanometer
PAI-1	Plasminogen activator inhibitor type 1
PAI-2	Plasminogen activator inhibitor type 2
PBS	Phosphated buffered saline
PEST	Penicillin-Streptomycin
rRNA	Ribosomal ribonucleic acid
Rpm	Revolutions per minute
TF	Tissue factor
TGF-β	Transforming growth factor beta
TIFF	Tagged image file format
TIMP	Tissue inhibitor of metalloproteinase
TNF-α	Tumour necrosis factor alpha
t-PA	Tissue plasminogen activator
tRNA	Transfer ribonucleic acid
uPA	Urokinase plasminogen activator
UV	Ultraviolet

INTRODUCTION

A problem

The formation of post-surgical adhesions continues to be a clinical problem. One reason for adhesions in the peritoneal cavity is prior surgery (1). Early observations indicate that up to 90% of the patients undergoing abdominal surgery develop adhesion (2). Not all patients with adhesions will have clinical symptoms. However, some patients are later admitted to hospital with bowel obstruction (2-5). Adhesions can be filmy and easily divided or dense, fibrotic, vascularised tissue that requires sharp dissection. Different scoring systems have been used to classify adhesions, depending of quality and quantity (6-8). The presence of adhesions may cause pain for the patient (9) and contribute to increased costs, surgery time, prolonged hospital stay and greater consumption of health care resources (10, 11).

Peritoneal adhesions are an abnormal attachment that forms between tissues and organs within the abdominal cavity. However, these abnormal attachments may have had a "positive" influence during the evolution of humans. The primary function has likely been to seal any leakage, repair and prevent further damage to the individual. Post surgical adhesion formation has been described in the literature for more than a hundred years, but is still not completely understood (12-14).

This thesis will focus on different experimental models that can be used to explore some of the mechanisms that may be involved in post-surgical adhesion formation and to explore the influence of surgery and other factors in the development of adhesions. It is noteworthy that many of these factors are key components of the normal healing process as well.

The peritoneal cavity

Peritoneum

The body cavities are covered with a serous membrane. Besides the pleura and the pericardium, the peritoneum is the largest serous membrane with an area of approximately $2m^2$ in an adult (15-17). The parietal peritoneum covers the abdominal wall and the visceral peritoneum covers the organs within the abdominal cavity. The peritoneum consists of one loose connective part containing elastic fibers and a superficial part known as the

mesothelium, which consists of a thin layer of mesothelial cells resting on a basal membrane. The primary function of the mesothelium has historically been thought to minimize friction and to facilitate movement between organs. However, during recent years, the mesothelium has emerged as a cellular membrane responsible for many important functions including transport through the peritoneum (18) and secretion of extracellular matrix (ECM) proteins, growth factors and cytokines (19, 20). In addition, the mesothelium has a function in host defence against bacteria, the trafficking of cells and antigen presentation (16, 21) and it's trans-membrane exchange is used in continuous ambulatory peritoneal dialysis (CAPD) (22, 23).

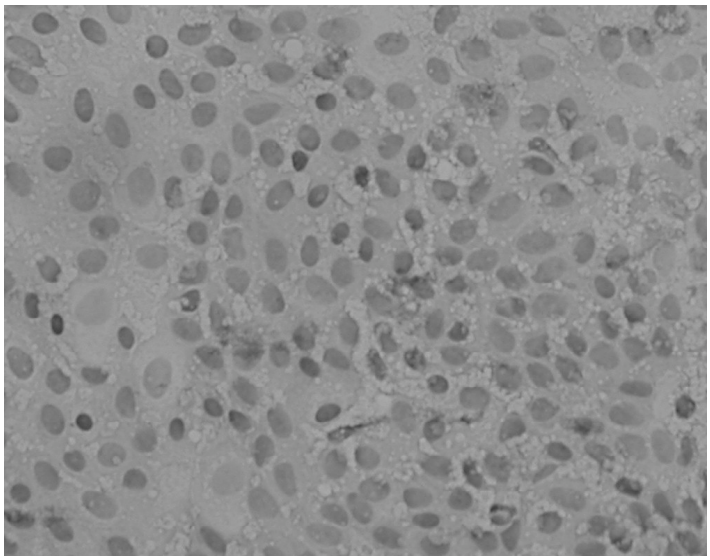

Figure 1: *En face* Häutchen preparation of the human peritoneal surface. The mesothelial surface of the peritoneum was captured on a pre-frozen glass slide. After removing the slide from the surface, glass slide was dried and the mesothelial surface were fixed and stained in Haematoxyllin/Eosin, Photo with Nikon E800 Eclipse, P. Falk (2004)

Mesothelial cells

The basal membrane of the peritoneum is covered by a thin monolayer of mesothelial cells, mostly elongated and flat cells that face the abdominal cavity (Figure 1). These cells, approximately 25µm in diameter, have the cytoplasm raised over a nucleus that is central round or oval (24). They can be divided into squamous-like or cuboidal cells according to their ultra structure. The predominantly mesothelial cell is a squamous-like cell. Cuboidal cells can be found in the folds of the liver and spleen, at the "milky spots" of the omentum and in the area of the diaphragm associated with the lymphatic lacunae (17, 25-27).

The surface of the mesothelial cell is covered by microvilli that vary in length, shape and density. They increase the functional peritoneal surface up to 40 m^2 (17, 28). Variable numbers of microvilli may be seen between different organs, different groups of cells and

even between single cells. The surface of a microvilli is covered by glycosaminoglycans with the most common being hyaluronan (also known as hyaluronic acid). Hyaluronan has been demonstrated both on the cell surface and in pinocytic vesicles. It has been discussed if hyaluronan is derived from the systemic circulation. However, hyaluronan seems to be produced and secreted locally. Mesothelial cells have a sophisticated system of vesicles and vacuoles and like microvilli the concentration of these vesicles and vacuoles varies between cells and sites. Vesicles are involved in the transport of fluid and particulate matter from the peritoneal cavity through the peritoneal surface. Experimental studies indicate that particles up to 100 nm can be transported through the mesothelial cell via micropinocytosis. The interstitial area between the mesothelial cells is complex with adjacent cells often overlapping and widely spread junctions and desmosomes between cells. In response to an activated immune system is likely that an inflammatory response on the mesothelial surface results in the release of cytokines. This action polarizes the mesothelial surface and promotes trans-mesothelial migration of white blood cells, including neutrophils and monocytes (17, 24, 26, 27, 29-33).

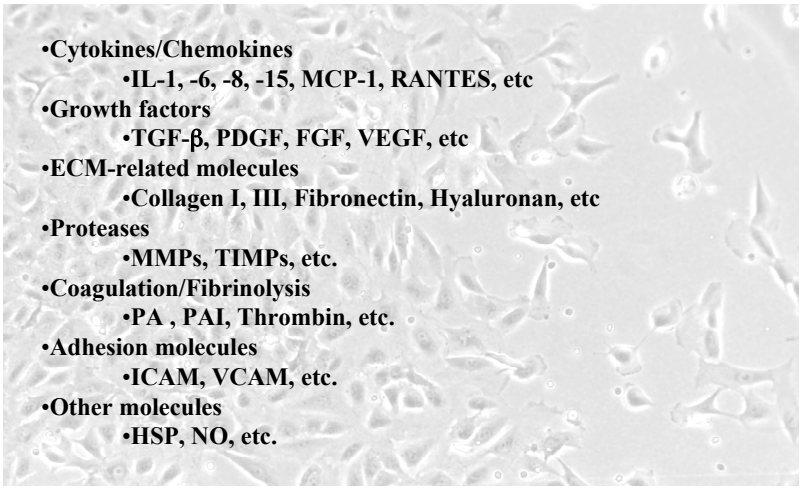

Figure 2: Mesothelial cells are under normal or stimulating conditions able to produce one or more of the following factors as reviewed by S. Mutsaers 2002. Cultured mesothelial cells, Photo by P. Falk (2003)

Furthermore, under normal or stimulated conditions, mesothelial cells have the capacity to produce several factors such as inflammatory proteins and ECM molecules involved in inflammation and tissue repair. In certain conditions, this process is regulated by growth factors, such as transforming growth factor-beta (TGF-β) (34). In experimental settings mesothelial cells are capable of producing interleukin-15 which is important in the process of antigen presentation to mononuclear cells (21). Mesothelial cells are also important in maintaining and balancing the fibrinolytic clearing capacity, which affects formation of

adhesions, by the production and secretion of plasminogen activators and their inhibitors (24, 35, 36). Mesothelial cells have the capacity to produce a variety of factors and some of them are summarized in Figure 2 (24).

Peritoneal repair and regeneration

The exact mechanism behind peritoneal and mesothelial repair is not fully understood. In 1919 Hertzler found that small and large peritoneal defects healed at the same rate (37). It was concluded that there must be other healing processes in place in addition to proliferation and migration from the wound edges (as in dermal wound healing). Under normal conditions regenerative properties of the peritoneum are remarkable, with rapid simultaneous epithelialization of the entire surface in a short period of time.

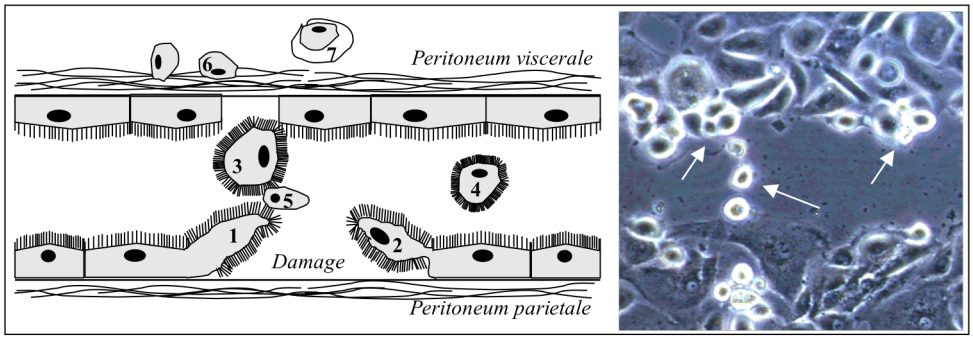

Figure 3: (Left) Schematic presentation of suggested origins of the regenerating mesothelium. 1) Migration of mesothelial cells close to the edge of damage. 2) Exfoliation of cells adjacent to the damage or 3) or from opposing surfaces. 4) Free-floating cells. 5) Transformation of macrophages. 6) Submesothelial mesenchymal derivated precursor cells. 7) Bone marrow derived circulating precursors (Modified from Mutsaers, 2002). (Right) Cultured human mesothelial cells, 24 hours after a predefined damage on the mesothelial surface. In a single layer of cells migrating and proliferating cells close to the damage surface could be seen (white arrows, Photo by P. Falk, 2000).

Trauma stimulates structural cell changes to facilitating repair. After trauma to the peritoneum in the form of a lesion, the damaged area is invaded and infiltrated by inflammatory cells within the next 36 hours. Due to increased vascular permeability, caused by histamine, an inflammatory response results in the release of active components. Polymorphonuclear (PMN) cells, macrophages and platelets together with fibrinogen in the peritoneal exudation forms a primary fibrin clot. Under normal conditions fibrinolytic activity will degrade the fibrin resulting in the formation of fibrin degradation products (FDP) (16). A number processes have been proposed which result in recruitment of new mesothelial cells, including migration of nearby cells, exfoliation of cells from adjacent or opposing surfaces, free floating cells, transformation of serosal macrophages or underlying fibroblasts, submesothelial mesenchymal cells or bone marrow derived precursors (24) (Figure 3). During ideal conditions both small and large defects on the peritoneum will regenerate within a week after

the damage (16, 38) (Figure 3). Although regeneration can lead to restoration of the peritoneal lining this is not always the case after surgery. Damaged surfaces can adhere to each other and form a postoperative adhesion, which in turn can cause complications.

Adhesion formation

The formation of postoperative peritoneal adhesions is specific to the serosal response to injury. Post surgical adhesions typically occur when there is an imbalance between deposition and degradation of fibrin resulting in persistent remnants of the fibrin matrix. Histological studies have shown that the healing process is similar to normal healing at the first, but a few days post surgery the fibrin matrix is then gradually invaded by reparative cells and the fibrin is replaced by connective tissue containing macrophages and fibroblasts. The adhesions are often covered with mesothelial cells and are also containing blood vessels. After one week fibroblasts produce collagen bundles and elastin (39). Nerve fibers have also been detected in abdominal adhesion (38, 40).

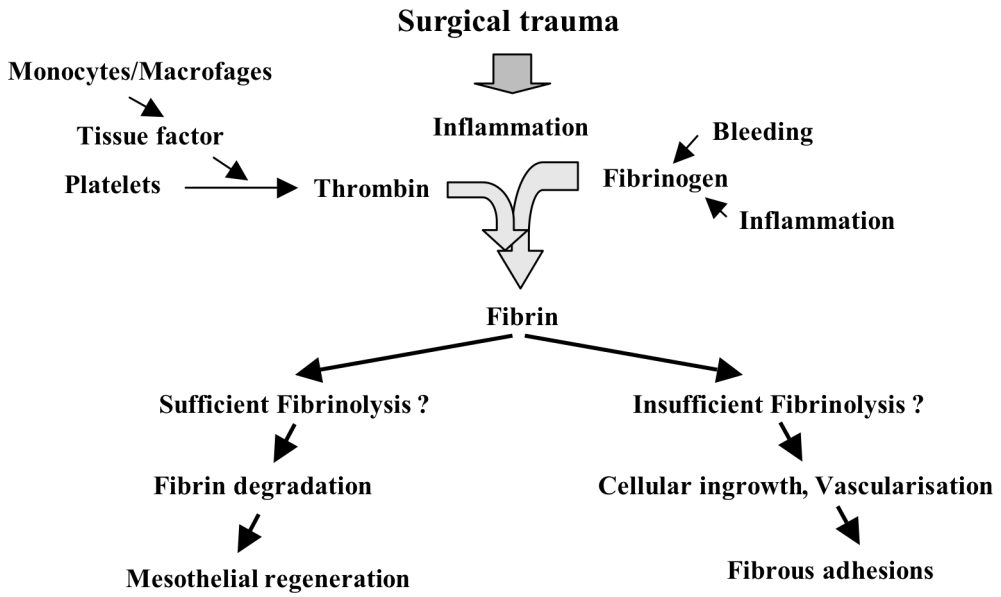

Figure 4: Schematic illustration of the process in adhesion formation.

The fibrinolytic system has a major impact in the early phase of postoperative adhesion formation. Under certain conditions, an imbalance of one or more components in this system, normally involved in the healing of the peritoneal surface, may leave an excess of fibrin matrix in the wound area thus contributing to adhesion formation (Figure 4).

The fibrinolytic system

In many biological systems, it is critical to maintain equilibrium between stimulators and inhibitors. The degradation of fibrin is highly regulated by the fibrinolytic system. Briefly, at the end of the coagulation cascade fibrinogen is converted into fibrin in the presence of thrombin. Fibrin together with aggregated platelets form the primary clot that stops the initial bleeding. When this function is complete the clot needs to be lysed to restore vascular patency. The fibrinolytic system is capable of lysing the primary clot by converting plasminogen into plasmin. This conversion is balanced by plasminogen activators and their inhibitors. Complete resolution of the fibrin clot results in fibrin degradation products (FDP) (41). Plasmin has the ability to resolve fibrin completely. However, extended fibrin generation with limited or no degradation results in a permanent structure by conversion of fibrin mesh into connective tissue.

The fibrin clearing capacity in the peritoneal cavity is similar to that of the systemic circulation. Similar to the endothelium, cells covering the peritoneal surface are capable of producing and secreting tissue-type plasminogen activator (t-PA) and urokinase plasminogen activator (uPA) and the plasminogen activator inhibitor -1 and -2 (PAI-1, PAI-2) (35). A balance between plasminogen activators and their inhibitors is crucial for the peritoneal fibrin clearing capacity (42, 43) (Figure 5).

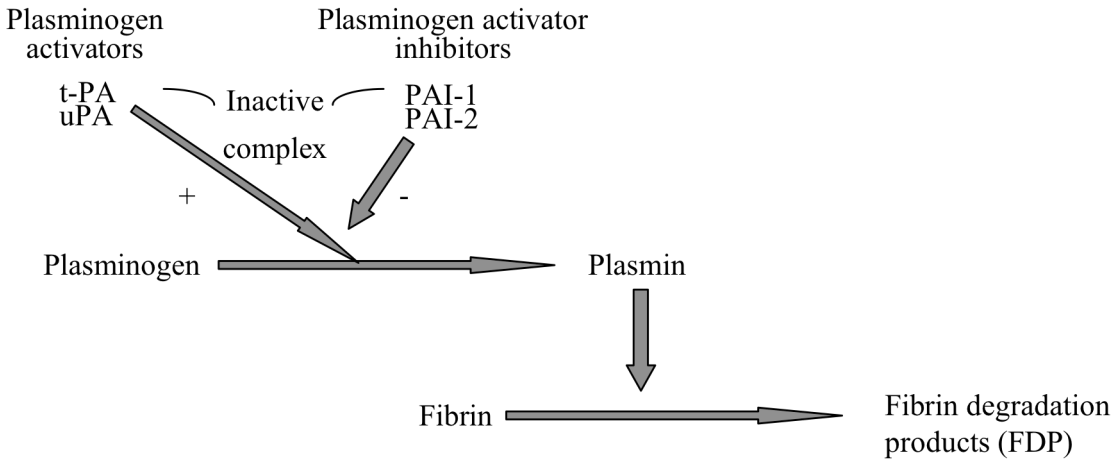

Figure 5: A schematic illustration on the components in the fibrinolytic system

Plasminogen activators

Plasmin and plasminogen activators are members of a glycoprotein family (serine proteases) and are present as active forms or as complex bound to one of their inhibitors.

t-PA is the main plasminogen activator in plasma and has been identified in many types of human tissue (35, 44, 45). It is highly fibrin specific and t-PA activity may be increased up to 1000-fold in the presence of fibrin (46). Active t-PA will be rapidly inactivated by 1:1 complex binding to the inhibitor PAI-1 (47, 48). The main source for t-PA production is believed to be the vascular endothelium. However, there is evidence that other cells like macrophages (49) and mesothelial cells (35, 36) also have the capacity to produce and secrete t-PA. Hence, in the peritoneal cavity, the mesothelium is likely to be a major source of t-PA.

uPA is another plasminogen activator, present in plasma and tissue and is the main activator in urine (50, 51). It is likely that uPA and t-PA are equally efficient in terms of fibrin degradation capacity (52). There is evidence that uPA can be present in the abdominal cavity (53) released from mesothelial cells (36) and it may be of importance in peritoneal wound healing. It is also reported that uPA plays a role in inflammation and spreading of metastatic cancer (54, 55) by facilitating the migration of cancer cells into other tissues. The role of uPA and its receptor in ovarian cancer (56), as well as surface bound plasminogen activation in tumour growth (57) has also been documented. However, since t-PA is highly fibrin specific (46, 58) and more than 95% of the fibrinolytical capacity in the peritoneal cavity is exerted by t-PA (59, 60), the role of uPA in the peritoneal tissue repair is poorly understood.

Plasminogen activator inhibitors

PAI-1 is the primary inhibitor of both t-PA and uPA and is produced and secreted by endothelial cells in the vascular wall (61-63). Moreover, PAI-1 has also been shown to be produced or secreted by platelets (64), macrophages (65), fibroblasts (66), and by mesothelial cells (35, 36, 67). Several stimuli including, inflammatory mediators or endotoxin (36), may also promote secretion of PAI-1 in cell culture systems. PAI-1 can inactivate t-PA in plasma within a few minutes (63). During inactivation PAI-1 forms inactive complexes with both t-PA and uPA.

PAI-2 was first found in placenta (68) and was known as "the placenta inhibitor", for a long period of time. In pregnant women plasma concentrations of PAI-2 increased during pregnancy, with a peak and an immediate decrease associated with the delivery (69). Later it was demonstrated that PAI-2 was present in amniotic fluid (70), in plasma during sepsis (71), and in the peritoneal cavity and secreted by macrophages (72) and mesothelial cells (36). A role of PAI-2 in ovarian cancer has also been described (73). PAI-1 is more fast acting than the PAI-2 and it has been suggested that PAI-1 and PAI-2 have different biological functions (74). Although PAI-2 has been detected in the peritoneal environment (74) its role in peritoneal tissue repair is unclear. Like PAI-1, PAI-2 also forms inactive complexes with the plasminogen activators t-PA and uPA.

Other active plasmin inhibitors in blood are α2-macroglobulin, α2-antiplasmin and α1-antitrypsin. Of these, α-2 antiplasmin is the most specific to plasmin. Since these inhibitors act more slowly than plasminogen activator inhibitors, it is conceivable that they could have different biological functions, and the role of plasmin inhibitors is poorly understood in the context of peritoneal fibrinolysis (63, 75).

Factors in the peritoneal environment

There are additional components in the peritoneal environment that can affect peritoneal tissue repair directly, or indirectly, via the fibrinolytic system. This would include factors that are likely to be produced or secreted into the abdominal cavity.

Transforming growth factor beta

TGF-βs are multifunctional cytokines and have unique abilities to initiate activities resulting in net synthesis of new connective tissue. TGF-βs can interact with interleukins and other cytokines in the activation of the immune system. They also have the capacity to stop the proliferation of cells, and initiate differentiation in a variety of cell systems such as endothelial cells, keratinocytes and some malignant cells (76).

TGF-β belongs to a superfamily of polypeptide molecules, and are designated TGF-$\beta_{1\text{-}5}$. There are three mammalian forms of TGF-βs (TGF-$\beta_{1\text{-}3}$). TGF-β_4 is found in chickens, and TGF-β_5 is found in frogs (77, 78). TGF-$\beta_{1\text{-}3}$ are secreted in a latent form from cells including

platelets (79) as a precursor. This latent form can be converted and activated in several ways, including proteolytic cleavage by denaturing agents such as urea or guanidine hydrochloride (79), extreme pH and temperature changes. These mechanisms have been demonstrated *in vitro* (80, 81) and similar mechanisms are likely to be of importance *in vivo* (82). In cell proliferation, the effect of TGF-β is determined by its influence on the cell cycle and the effects on growth arrest, proliferation and apoptosis. In most epithelial, endothelial and hematopoetic cells, TGF-β inhibits cell proliferation by blocking the cell in G1 phase (83). However, the effect of TGF-β on the peritoneal mesothelial lining is not fully elucidated.

TGF-β also interacts with the ECM, which is important in the wound healing processes. TGF-β normally stimulates ECM production by mesenchymal and epithelial cells, at the transcriptional level (84). The stimulatory role of TGF-β in scar formation, is well documented (85). TGF-β can promote ECM production by increasing synthesis of matrix proteins, including collagen type I, II, III, IV, V, VII and fibronectin (86, 87) together with an inhibiting of ECM proteolysis and degradation. These mechanisms combined leads to a net accumulation of ECM. This is accomplished by the inhibitory effect of TGF-β on the secretion of matrix degrading enzymes such as matrix metalloproteinases (MMPs), specifically MMP-1 and -3 and the effect on plasminogen activators (88). Additionally, the expression of inhibitors of proteolytic enzymes is increased by TGF-β such as PAI-1 and tissue inhibitor of matrix metalloproteinases-1 and 2 (TIMP-1, -2) (89). Not surprisingly, TGF-β_1 plays a central role in wound healing and in scar formation by stimulating fibrosis (87, 90, 91). Antibodies against TGF-β_1 have been shown to reduce the scarring process (92) in subcutaneous wounds in animals.

The effect of TGF-β on key mechanisms in peritoneal tissue repair has only been partially evaluated. TGF-β_1 has been reported to downregulate mRNA expression of t-PA (93) and increase the mRNA expression of PAI-1 in human bronchial epithelial cells (94) and in transformed mesothelial cells (95). In human pleural cells, TGF-β_1 has been observed to affect fibrinolytic components (20). However, it is unclear if these effects are similar in human peritoneal mesothelial cells. In experimental models of adhesion formation it has been demonstrated that the addition of TGF-β_1 can increase the numbers and severity of intra abdominal adhesions after surgery (96). Moreover, by using a neutralizing antibody to TGF-β_1, adhesions could be reduced (97) in animals supporting a role of TGF-β in peritoneal repair.

There might be multiple sources for TGF-β in the abdominal cavity; degranulation of platelets at sites of tissue injury, from surrounding tissue and from the mesothelium itself. It is reasonable to assume that if TGF-β is present in the abdominal cavity this would affect the fibrin degradation capacity locally, in favor of the formation of adhesions.

Hyaluronan

To be consistent, this work refers to hyaluronic acid, hyaluronan or sodium hyaluronate as hyaluronan.

The microvilli on the mesothelial cells are covered by glycosaminoglycans. The most common is hyaluronan (24, 30), a high molecular weight polysaccharide first described and found in the vitreous fluid (hyalos) in the eye. Hyaluronic acid, later introduced as hyaluronan (98), has important biological functions (99) in tissues. The concentration of hyaluronan is high in tissues undergoing healing processes (100, 101). Hyaluronan has also been reported to affect inflammatory response (102, 103). There is a relationship between hyaluronan and mitosis, as well as the detachment and movements of cells (104-106). It has also been described that an environment rich in hyaluronan provides a hydrated matrix that facilitates cell migration (107).

Hyaluronan, or hyaluronan based agents to reduce the formation of post-surgical adhesions have been used both in experimental animal models (108-111) and in clinical settings (112-117). Carboxymethylcellulose and hyaluronan (HA-CMC) is one of the most studied materials in this context. In patients undergoing colectomy the use of a of HA-CMC membrane (114) was effective in reducing adhesion formation. Moreover, in gynaecological patients undergoing laparotomy use of a hyaluronan solution as a precoating solution, was reported to be effective in reducing the formation of adhesions (115). The clinical outcome as reviewed by Reijnen *et al* (118) was that the incidence of severe adhesions was reduced by approximately 40 per cent.

There are several mechanisms that might explain why hyaluronan-based agents can reduce adhesion formations. The mechanical separation of the peritoneal surfaces during the first post-operative days may be the most important mechanism. General improvement of the

healing process due to the present hydrated matrix might also be of importance. However, there might be local effects on the mesothelial lining, but this is poorly understood.

Other factors in the peritoneal environment

Besides TGF-β and hyaluronan other factors are present in the peritoneal cavity. They could also influence the repair processess. Both matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) have been demonstrated in the peritoneal environment and are likely involved in peritoneal tissue repair. Both of these are affected by TGF-β resulting in a net accumulation of connective tissue. Since the function of MMPs is to completely or partially degrade the extra cellular matrix, an imbalance of MMPs may influence in the healing process as well (14, 81, 87, 89).

Cytokines are found in the peritoneal environment and several mediators may be of importance in the inflammatory response. Both tumour necrosis factor-alpha (TNF-α) and lipopolysaccharide (LPS) are likely to be involved in normal peritoneal healing and during peritonitis. Interestingly, these factors have been reported to affect the fibrin clearing capacity in the peritoneal cavity (35, 36). Other factors such as interleukins and adhesion molecules are also most likely involved in the peritoneal healing process as reviewed (14, 119).

In the present study, the focus has been on two factors; TGF-β and hyaluronan.

The use of experimental models

There are several important reasons for using experimental models in the study of peritoneal tissue repair. Firstly, the identification and isolation of mechanisms influencing various biological functions could be performed and monitored in a controlled environment. Secondly, the use of controlled experimental settings allows the study of these effects over time, which would be difficult to perform in a clinical setting. Thirdly, in the clinical setting the peritoneal cavity is available for only a short period of time during surgery, which could influence interpretation of results. Finally, experimental models are important in the understanding of biological effects likely to occur during abdominal surgery since experimentation on clinical subjects poses limitations.

AIM OF THE THESIS

The aim of this thesis was to investigate the effects of transforming growth factor beta and hyaluronan using different experimental models in order to further understand mechanisms of peritoneal repair.

The specific aims were:

- To investigate the presence of transforming growth factor-beta 1 (TGF-β_1) in peritoneal tissue in patients with adhesion formation and its possible relationship with fibrinolytic components (Paper I)

- To determine whether TGF-β_1 influences the expression of fibrinolytic components in cultured human peritoneal mesothelial cells (Paper II)

- To determine whether hyaluronan affects the expression of fibrinolytic components in cultured human peritoneal mesothelial cells (Paper III)

- To determine whether hyaluronan affects proliferation rate in cultured human peritoneal mesothelial cells (Paper IV)

- To a) investigate the presence and activation profile of TGF-β_1, TGF-β_2 and TGF-β_3 in the human peritoneal cavity and to compare with that of plasma during surgery in the clinic; b) investigate the effect of different TGF-β isoforms and concentrations on the mesothelial proliferation rate in experimental models (Paper V)

METHODOLOGICAL CONSIDERATIONS

General considerations

Sampling of human material and isolation of human peritoneal mesothelial cells was approved by the Regional Ethics committee at Göteborg University, Göteborg, Sweden. All subjects consented prior to participation.

The initial human study (Paper I)

Question

The question in the first study was whether transforming growth factor-beta 1 (TGF-β_1) is present in the peritoneal tissue in patients, and if so investigate the possible relationship of TGF-β_1 with fibrinolytical components.

Human subjects

In the first study, the role of TGF-β_1 in human peritoneal tissue and its possible relationship with decreased fibrinolytic capacity was investigated. For this reason, peritoneal biopsies were taken from twenty-two patients undergoing abdominal surgery for a colorectal disease. All patients (12 men and 10 women) had previously undergone surgery and all had pre-existing adhesions to varying degrees at the operation. Patients were excluded if any intra-abdominal infections or disseminated cancer was present. The mean age of subjects was 68 (range 37-91) years at the time of surgery.

Tissue sampling

A 5x5 mm biopsy was excised from the parietal peritoneum by dissecting the peritoneum from the underlying tissue. Furthermore, from some patients it was possible to sample adhesion tissue during adhesiolysis. During sampling, care was taken not to include extraneous tissue to minimize confounding factors. For this reason it was only possible to sample adhesion tissue from 10 out of 22 patients. Since blood include the factors of interest all tissue samples were quickly rinsed in saline solution to remove blood. Thereafter samples were put in pre-labelled air tight tubes and frozen at -70°C. Biopsies were stored frozen until processed further with homogenisation and biochemical assays.

Tissue homogenisation and protein extraction

Both peritoneal and adhesion samples were homogenised in the same manner. The method was developed to assay protein concentration in tissue and has been previously described in detail (43). All tissue samples were homogenised in batches to minimise effects of systematic multiple processing errors in tissue handling. Briefly, both peritoneal and adhesion tissue samples were rinsed in ice cold (0°C) homogenisation buffer (PBS pH 7.4, sodium chloride 0.5 mol/L, Triton X-100, 0.01%), weighed, cut into smaller pieces and transferred to an ice-cold tube to a final concentration of 40 mg tissue/mL buffer. Homogenisation was performed using an Ultra Turrax homogenizer which was gradually increased in speed to 24 000 rpm. The homogenate was aspirated on 0°C ice into an Eppendorf tube for centrifugation at 10 000g for 3 min at 4°C. The supernatant was aliquoted in several small 250µL tubes and stored in -70°C until biochemical assay.

Several types of extraction buffers have been used for the extraction of proteins from tissues. Depending on the factor assay, a range of buffers can be used for a particular assay. The buffer used in the present work was selected because of its lack of interference with subsequent assays. This buffer has been used to process tissue from peritoneum and adhesion tissue (120-122), colon (123), appendix (124), and certain tumours (125-127) with reproducible results. The homogenisation technique have been reported to be used to extract proteins including snap-freezing of tissue followed by crushing, detergent lysis, or controlled mechanical homogenisation by using an electric homogenizer (128, 129). The current method using an electric homogenizer is convenient since it is useful for both protein extraction and RNA extraction. Moreover, the homogeniser can easily be cleaned, and even autoclaved if needed, minimizing systemic errors.

Efficiency of extraction

A potential confounding factor could be the effectiveness of protein extraction. In the present experimental studies on repeated extractions on the same tissue sample, it was observed that almost all of the protein fraction (>97%) was extracted from the tissue during the first process. Thus, the technique was efficient and results obtained likely to accurately reflect tissue concentrations.

However, a disadvantage of the method is that the homogenizer has a limited homogenate volume of approximately 1-3 mL. We have previously observed that an optimal weight/volume ratio is about 40 mg tissue per mL buffer (130). Because of this, the maximum amount of tissue that can be in each sample is approximately 120 mg, which provides sufficient homogenate volume for several assays. On the other hand, we have also discovered that the minimal tissue weight is 10 mg by doing serial dilutions of the same sample. These experiments showed that samples less than 10 mg increased the error substantially (unpublished observation). Samples less than 10 mg have therefore been avoided, and none of the biopsies used in the study were below 40 mg each.

Biochemical assays

In the initial study, the detection of proteins was performed using commercial available enzyme-linked immunosorbent assay (ELISA). By using an antibody-antigen reaction, as well as an enzyme reaction, this technique usually converts a peroxidase sensitive substrate into a colour. Light absorption at a certain wavelength is then quantified using a spectrophotometer to assess the concentration. Internal standards of known concentrations are used to quantify the optical densities of the test samples. The antibody-antigen reaction in the initial step makes these assays very specific to the target. However, depending on what epitope the antibody is directed to, the assays cannot always discriminate different conformations of a protein such as a latent or active form.

In the first study, levels of t-PA antigen, uPA antigen and PAI-1 antigen were assayed with commercial assay kits from Biopool (Umeå, Sweden). The inactive complex between t-PA and PAI-1 (t-PA/PAI-complex) was measured using an assay from Novo Nordisk (Bagsvaerd, Denmark).

A method from Promega was used to measure both the active and total amount of TGF-β_1. First, the active form was assayed. Then the latent TGF-β_1 form was assayed following activation by acidification of an additional sample with HCl (1 mol/L) to pH 2.7-3.0 in a separate tube followed by neutralising and further dilution. The total amount can then be analysed on a separate immuno plate in an additional assay as described by the manufacturer. In all assays the detected protein was normalised to the wet weight of the homogenised tissue. This method was well established in the laboratory and has been observed to be comparable to normalizing to total protein content or to DNA (120).

Variability of the assays and quality control

Intra-assay variations reflect the variations between several samples within the same ELISA plate and inter-assay variations, the variations between several plates. These variations are known as the coefficient of variation (CV) and are expressed in % as the product of the standard deviation divided with the mean. For the assays in the present study the inter-assay variations for t-PA and uPA antigen was 10% and for PAI-1 9% according to the manufacturer. The inter-assay variation for the t-PA/PAI-1 complex was 10%, and the intra-assay CV was below 9% for all fibrinolytical factors. For TGF-β_1 the inter-assay variation between several immuno plates was 1.6% and the within assay variation was 11.6% according to the manufacturers specification when 20 determinations were performed with 4 different operators. The intra-assay variation for the TGF-β_1 assay is comparatively high. However, this assay was evaluated in the lab before, during and after this study and a value between 5 and 10 % was more likely to be applicable when 3 operators were involved.

The number of operators was kept to a minimum to standardize these procedures and to minimize intra-operator variability. All tissue homogenisations and protein extractions were done by a single operator, and the ELISA assays were performed by two persons. Quality control of these assays included samples with known concentrations (usually seven different concentrations) and a reagent blank on each plate. Additionally, each plate had several control samples (both at high and low concentrations) that were used in between several ELISA plates to assess the inter assay variability. Unexpected values or values that differed more than 10% between the replicates were reanalysed. A comprehensive quality tracking system was established from start of assay to the final dataset. Finally, a standard operating procedure for each assay was established. These procedures were used for all assays performed at the laboratory, including this thesis (Paper I to V).

Scoring of adhesions

It is well known that adhesion formation is variable and that the quality of adhesions can be different. In order to categorize pre-existing adhesions, a scoring system which has been used during pelvic surgery (8) was adapted for scoring abdominal adhesions. Because of the inherent challenges in categorizing adhesion formation, the system was simplified to describe objective findings rather than to utilize quality assessments. For example, extent of adhesions

was categorized into either the site of previous surgery (to the site) or to well beyond the surgical site (extensive). The quality of adhesion was simplified into two groups reflecting the potential clinical impact as filmy (separated by blunt dissection) or vascular (separated by sharp dissection and causing bleeding). Lastly, an assessment was made whether an adhesion distorted tissue planes to the extent there was a risk of organ damage during adhesiolysis (120). All assessments were made by experienced surgeons.

The first mesothelial study (Paper II)

Question

In the second paper, the question was whether different concentrations of TGF-β_1 influenced the expression of fibrinolytic components in cultured human peritoneal mesothelial cells by measuring the effect at the protein and mRNA level.

Cell culture

A cell culture model using mesothelial cells had been established. Cell culture and other experimental models to study local mechanisms *in vivo* have been developed and utilized for a number of years in the lab with both endothelial cells derived from human umbilical cords (HUVEC) and from adult veins (HAVEC) (131-134) and from cultured fibroblasts derived from human abdominal skin tissue (135). Culture conditions and techniques for identification are similar between endothelial cells and mesothelial cells. The methodological principles for isolation and general culture of mesothelial cells will be described further.

Mesothelial cell culture

The first mesothelial cell culture model established in the laboratory was based on the work done by van Hinsbergh *et al* (35). However, the Dutch group used omentally derived mesothelial cells and the present work uses cells derived from another source. Different techniques for isolating mesothelial cells and the identification of cultured mesothelial cells have been described in detail (36). The mesothelial cells used in the present study were isolated from a total of four patients undergoing elective surgery for colorectal reasons. None of the patients had any ongoing infections or peritonitis.

The cells were isolated as previously described (36). In brief, aspirated peritoneal fluid was put in a 50 mL tube and centrifugated at 650g in 20°C for 10 min. The supernatant was discarded and the pellet suspended in 5 mL complete E199 culture medium. This medium contained; Medium E199, Foetal Calf Serum (FCS), L-glutamine, antibiotics (PEST), ECGF prepared according to Maciag *et al* (136) and Heparin. The cells were then put into a 25cm^2 culture bottle (Cell+, Sarstedt, Germany) and incubated (Forma, Thermo Fisher) at 37°C with a 5% concentration of CO_2 to maintain pH of the culture medium at pH 7.3-7.4.
The suspension of cells was left to attach to the bottle and rinsed with fresh culture medium the next day. All cultures were monitored using an inverted microscope (Axiovert 25, Carl Zeiss AG). Complete culture medium was changed every second or third day. The cultures were further sub-cultivated with trypsin/EDTA solution when a 75-85% confluence was reached. A split ratio of 1:2-1:4 was used depending on the size of the seeding area and the experimental setup.

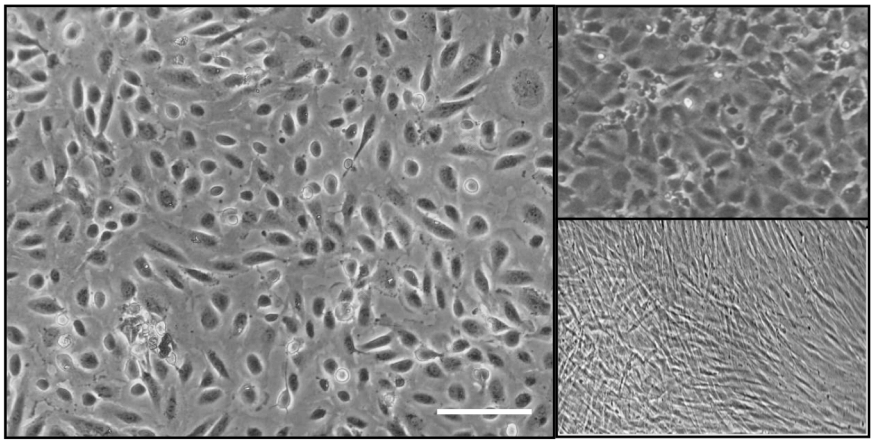

Figure 6: (Left) Phase contrast microscopy of human primary mesothelial cells in culture. Mesothelial cells in culture are elongated and form monocell layers (Size marker 100µm). In comparison to human adult vein endothelial cells (HAVEC) that forms a more ”cobblestone” like appearance (Upper right) and human skin fibroblasts that is not contact inhibited in culture, growing in several layers (Lower right). Photo by P. Falk, (2003, 1996 and 1999)

Identification of mesothelial cells

Identification of mesothelial cells was performed using an immunofluoroscence technique based on previously described methods (35, 36, 137, 138). Briefly, established parallel cultures were assessed visually by inverted microscopy for their morphologic appearance. Mesothelial specificity was assessed using primary antibodies against intra cellular cytokeratin -8, -18, -19, vimentin, von Willebrand factor and fibroblast specific antibodies, together with FITC-labelled secondary antibodies and an UV-light source on the microscope. Morphological elongated appearance (Figure 6) and positive immunostaining for cytokeratin and vimentin in cells, which were negative for endothelial and fibroblast antigen indicated

mesothelial cell cultures. Using this methodology it was confirmed by electron microscopy that these cells had microvilli and thus matched the characteristics of mesothelium (36).
As previously mentioned, primary cultured mesothelial cells lose their specific mesothelial phenotype after the fourth passage in culture (36) and a regression towards a more fibroblast like cell was seen when cells were cultured beyond the fourth passage. For that reason, no experiments using cells were performed after the third passage.

Sources of mesothelial cells

It has been reported that by homogenizing biopsies from human omental tissue, cells could be isolated during extraction and subsequent centrifugations (35). Since the omentum is rich in capillaries, potential contamination with endothelial cells, is a possibility. Thus, it is imperative to rule out contamination with endothelial cells. In the present thesis cells originated from the peritoneal fluid. When different mesothelial isolation techniques were compared the use of peritoneal fluid seemed preferable since it was a repeatable and less invasive method than extracting a piece of tissue from the omentum and at the same time minimized risk of contamination from endothelial cells.
Another potential source could be a transformed cell line. During initial experimental set-up the transformed cell line, Met-5A, was used to establish growth time, correct split ratio, etc. before the main experiments were performed. However, it was soon discovered that this particular cell line did not express fibrinolytic components to the same extent as human primary isolated cells (unpublished observation) when assessed after stimulation with proinflammatory agents (TNF-alpha) or endotoxin (LPS) (35, 36). Thus, the use of a commercial available transformed mesothelial cell line for these investigations did therefore not seem to be an attractive option.
To enable a steady supply of cells for multiple experiments cryopreservation was used. Parallel cultures from some of the isolated cell lines were stored in cryo tubes submerged in liquid nitrogen for use in confirmatory or complementary tests. The liquid nitrogen storage did not affect culture conditions or immunological specificities when compared to the results from the original cultures (unpublished observation).

Biochemical assays

As with the initial study, all of the assays used were commercial assays; t-PA antigen was assayed using Imulyse t-PA, PAI-1 and PAI-2 antigen was assyed using TintElize PAI-1 and TintElize PAI-2 (Biopool, Sweden) and urokinase antigen levels were detected by EUMIX-5 (Monozyme, Denmark). The active fraction of t-PA and the complex between t-PA and PAI-1 were detected using Funktionell tPA and Funktionell tPA/PAI-1 complex (NovoNordisk, Denmark), respectively.

In contrast to the ELISAs used the active fraction of t-PA is measured by using an antibody to bind the t-PA molecule to the bottom of the assay plate, without blocking the active site of the protein. As previously described, the *in vivo* function of t-PA is to convert the present inactive plasminogen into active plasmin, a reaction that is enhanced by the presence of fibrin. In the "Funktionell t-PA" assay, plasminogen and fibrin dimer is added to initiate the reaction. In conjunction with a plasmin sensitive substrate the reaction results in a measurable color.
In the present paper, biochemical assays were normalized to cell count and expressed as concentration/10^6 cells to reduce errors due to difference in cell density.

TGF-β_1 treatment and experimental settings

The concentrations selected for TGF-β_1 stimulation were from the same as previously reported under similar conditions (93, 95). TGF-β_1 used for this study was purchased commercially (R&D Systems) and diluted to 0.1, 1 and 10 ng/mL according to instructions from the manufacturer.
Human mesothelial cells from the second passage were seeded into 3.83 cm^2 12 well dishes. Freshly trypsinated cells were suspended into fresh culture medium at a cell density of $0.9x10^5$ cells/well. Cells were further cultured until confluent. In order to synchronize the cell cycles of the cultured cells, an additional 48 hours exposure to reduced serum concentration of FCS (10%) was used. Following this, the conditioned media were removed and replaced with medium containing the different TGF-β_1 concentrations (0.1-10 ng/mL) and with a further reduction in FCS (0.2%) to minimize impact of potential TGF-β_1 contamination from FCS. Cells with no addition of TGF-β_1 but medium only served as untreated controls. After 24 hours, conditioned medium were collected and frozen in aliquots for protein detection.

RNA preparation

In order to protect samples from RNA degradation, all chemicals and materials used for cell culture and molecular biology were purchased RN:ase free and handled aseptically. Total cellular RNA was isolated using Trizol (Life Technologies) followed by centrifugation with chloroform and isopropyl alcohol. The aqueous phase, containing the total RNA, was then washed with alcohol and dried. The RNA pellet was dissolved in water and a small sample was diluted and measured as optical density at wavelength 260 and 280nm ($A_{260/280nm}$ ratio) in order to examine RNA/DNA purity. RNA degrades easily and the $A_{260/280nm}$ ratio of degraded RNA has a ratio that is often less than 1.6. Ideally, this ratio should be between 1.6-2.0, as described by the manufacturer. In the present study, the ratio of the first experimental set-up measured between 1.4-1.6. However, in the later experiments the preparations generated ratios above 1.7. Total RNA with a ratio above 1.7 was used for additional experiments.

Detecting the mRNA levels

The technique for detecting mRNA for the fibrinolytical targets used in the present work was established at the Institute for Wound Research, University of Florida (Gainesville, FL, USA). Individual training on laboratory techniques was done on three separate occasions and methods were subsequently implemented in the laboratory in Göteborg. This paper was the first one from our group describing the development of the quantitative competitive reverse transcription polymerase chain reaction (Q-RT-PCR) technique (Paper II).
For detection of fibrinolytic mRNA by this technique, an external synthetic multiprimer cDNA standard was used generously provided by Prof. Chegini at the Institute for Wound Research. The technique using an external cRNA template has been described previously (139, 140). A more detailed description of the cDNA standard is given in Paper II. The procedure is based on the insertion of sequences of t-PA, uPA, PAI-1, PAI-2, uPA receptor and G3PDH into a plasmid.

Table 1: Base pair sizes and primer sequences for the different factors used in the Q-RT-PCR. Differences in base pair sizes varied from 81 to 242 base pairs between sample and template sizes in the different factors. Primer sequnces have been previously described (Dou *et al* 1996, Dou *et al* 1999).

Factors	Sample size (bp)	Template size (bp)	Diff (bp)	Primer sequences
PAI-1	359	259	100	Sense: 5´- CAGCAGGTGG CGCAGGCCTC - 3´ Antisense: 5´ - ACCCAGTCAT TGATGATGAA - 3´
PAI-2	300	219	81	Sense: 5´- TCCACCATGG CCATGGTCTA - 3´ Antisense: 5´ - GCTCGCAGAC TTCTCACCAA - 3´
uPA	348	233	115	Sense: 5´- CATCGAGAAC CAGCCCTGGT - 3´ Antisense: 5´ - GGCAGGCAGA TGGTCTGTAT - 3´
t-PA	833 or 351	591	242 or 240	Sense: 5´- GCCCAGAACG CCTACAGCGG - 3´ Antisense: 5´ - CACACTCCGT CCAGTCCGGC - 3´
uPA receptor	531	417	114	Sense: 5´- CGTCCAAAGG ATGACCGC - 3´ Antisense: 5´ - GGTCCAGAGG AGAGTGCC - 3´
G3PDH	598	424	174	Sense: 5´- CCACCCATGG CAAATTCCAT GGCA - 3´ Antisense: 5´ - TCTAGACGGC AGGTCAGGTC CACC - 3´

In order to obtain a sufficient amount of total RNA, several cell culture wells were pooled together in two groups of three each. The total RNA was isolated from the wells (Figure 7) and 2 μg of total cellular RNA subjected to a standard reverse transcriptase reaction to develop a cDNA library. In separate small PCR-tubes, equal amounts of unknown cDNA sample were added together with one of several dilutions of external cRNA standard (10^3-10^8 template copy numbers). All tubes were then subjected to a standard PCR reaction with 1.5 minutes at 94°C, 2 minutes at 58°C and 3 minutes at 72°C for a total of 40 cycles as previously described (140). The resulting PCR products were loaded together with a 100 bp (base pair) molecular marker in a submarine gel electrophoresis on a 1.8% agarose gel containing ethidium bromide 0.1% and then connected to a voltage source at 120 volts DC for 30-40 minutes. Target and sample products from the PCR reaction were separated according to their base pair sizes (Table 1). All gels were then illuminated with UV-light, photographed, scanned in a flat-bed scanner and finally saved as tiff-files on a personal computer.
Using an image software program (NIH Image v1.54) different band intensities were determined and normalised to the different base pair sizes. For each reaction, the intensities of band ratios, between the unknown samples and the known template, within each lane (for each PCR tube), were plotted (Y-axis) on a log-log standard curve against the template copy number (X-axis). When the ratio between the sample and the internal standard is equal (ratio=1) on the Y-axis, the concentration could be read on the X-axis expressed as concentration of mRNA copies, as previously described (Figure 7) (140). The presence of the house-keeping-gene G3PDH was used as a positive control in all cDNA samples.

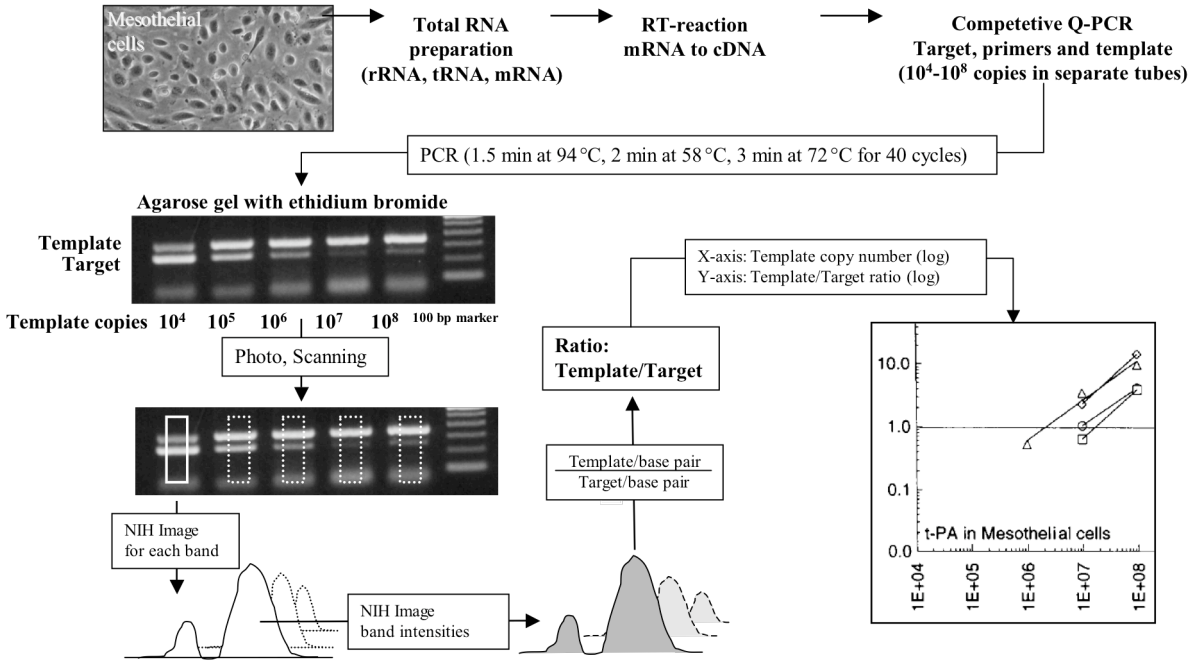

Figure 7: Schematic illustration of the methodological principles on the Q-RT-PCR technique from cultured cells to the graph. With Trizol cells are lysated and total RNA is extracted and a cDNA library is constructed in the RT-reaction. Multiple PCR reactions are performed with known template concentrations together with equal amount of the unknown sample. After PCR-reaction the different products are separated with agarose gel and photographed and band intensities were determined and related to base pair sizes for each template and sample. The template/sample ratio is plotted against the template concentration and unknown sample concentration is determined were the ratio is equal to 1. *Some figures originally published in Scandinavian Journal of Clinical and Laboratory Investigation, Differential regulation of mesothelial cell fibrinolysis by transformong growth factor beta 1; 60; 439-448, Copyright Informa Healthcare, (2000) <http://www.informaworld.com/journals>*

This technique was one of the first methods for quantification of a PCR result against a known amount from an external standard template. There are several ways for semi-quantitative detection, where the target gene is compared with a normal gene or a house-keeping gene normally expressed in all living cells. However, in the present study, the method is a quantitative detection method where the target primers competitively bind to the target gene in the unknown sample cDNA or the cDNA template with known amount of template copy numbers. Since the template with high concentration external standard will bind more primer than the unknown sample, the specific bands for the sample will increase in intensity in the same way that the band intensities for the template decrease (Figure 7).

This method was implemented in Göteborg in 1997 and used for several years, but has during recent years been replaced by a less labor intensive, more sensitive and reproducible real-time PCR (SmartCycler, Cepheid, USA) as previously described (141).

The second mesothelial study (Paper III)

Question

The third paper addressed the question whether hyaluronan affects expression of fibrinolytic components in cultured human peritoneal mesothelial cells at the protein and mRNA level.

Hyaluronan based agents have been used in clinical settings to reduce the formation of post-surgical adhesion and the mechanism of action is believed to be a physical separation of tissues. However, it is reasonable to assume that there might be a local effect on the mesothelial lining, and on fibrinolytic capacity since has biological effects. In this third work, the mesothelial cell culture model was further used, as well as assay systems for detecting proteins in culture media and intra cellular mRNA.

Mesothelial cell culture

Human peritoneal mesothelial cells were isolated, cultured and characterized in the same manner described earlier (Paper II). For this experimental model, mesothelial cells were isolated from five patients undergoing colorectal surgery for non-infectious reasons. Cells from the third passage were used for all experiments.

The experimental model

Hyaluronan has been reported to reduce intra-abdominal adhesions and abscesses in an animal adhesion model of peritonitis (142). From this study it was hypothesized that hyaluronan could achieve these effects by modulating local fibrinolytic capacity at the peritoneal surface. For this reason, an experimental model was designed to study possible local mechanisms. The use of TNF-α to simulate an ongoing inflammation and LPS to simulate an established infection, has been used in different experimental models (35, 36). Frequently used concentrations are 10μg/mL (for LPS) and 500 U/mL (for TNF-α) and these concentrations were used for this experiment.

Mesothelial cells in the third passage were cultured until confluent layers were established. Following TNF-α (Genzyme, Cambridge, MA, USA) addition to a final concentration of 500 U/mL in culture media, hyaluronan was immediately added. The concentration of hyaluronan (Genzyme, Cambridge, MA, USA) when used in a clinical setting is 0.4%. Several concentrations (0.1, 0.2 and 0.4%) of hyaluronan were used to investigate a possible dose-response. Control cells received medium only.

After 24 hours of incubation, the conditioned media were collected from each well and stored in several aliquots at -80°C. When all culture media were aspirated, Trizol reagent was added in order to prepare the total RNA in the same manner as previously described (Paper II). Since t-PA can be stored intracellularly it was of interest to investigate whether cultured cells contained t-PA or other fibrinolytic components that could be measured in the cell lysate. In preparation to assay for intracellular proteins, the cells were frozen to -80°C and then thawed in the incubator at 37°C. This cycle was repeated 3 times to disrupt the cell membranes. After the last cycle the cell suspensions were stored in several aliquots at -80°C until assayed. Since not both total RNA and intracellular proteins could be assayed on the same cultured cells, cells were divided in two equal groups where half of the cells were for total RNA preparation and the other half for the intracellular proteins measurements.

Biochemical assays

The assays used in the present study were similar to the ones presented in the first and second study. Plasminogen activators and inhibitors were analysed using commercially available ELISA kits. The levels of t-PA and PAI-1 antigen were analysed with kits from Biopool and uPA antigen levels were analysed using a kit from Monozyme (Hoersolm, Denmark). Since the cell count per cm^2 did not differ between wells, the measured values (ng/mL) were recorded and not normalised to cell count.

Detecting the mRNA levels

In order to investigate levels of mRNA for t-PA, uPA and PAI-1, the same methodological techniques were used as described in Paper II. In summary, total cellular RNA from treated or non-treated human mesothelial cells were used for RT-reaction and standard PCR with increasing internal cRNA (10^4 to 10^8) copies per reaction. Each PCR product was separated on an agarose gel, photographed and scanned. Using NIH-Image software, the band intensities were measured and the base-pair corrected ratio between the template and sample was plotted against the template copy number in a log-log graph. The concentration in the unknown samples is defined where the ratio is equal to 1. Found concentrations are expressed as mRNA copies per total µg RNA.

The third mesothelial study (Paper IV)

Question

The fourth paper addresses the question whether increasing concentrations of hyaluronan affect the rate of proliferation of cultured human peritoneal mesothelial cells.

The anti-adhesion effects of hyaluronan have been discussed. The beneficial influences of hyaluronan on wound healing and its important regulatory effects in the inflammatory response, together with its effect in cell migration and attachment by interactions with cell surfaces have been described (99, 102, 103). Hyaluronan may have similar effects in peritoneal repair and therefore the action of hyaluronan based adhesion barriers is not solely restricted to mechanical properties. To further investigate the role of hyaluronan in peritoneal repair, the effect on mesothelial proliferation rate was investigated.

Mesothelial cell culture

Human peritoneal mesothelial cells were isolated, identified and cultured as described in Paper II and III. All experiments were done before the third passage of cells.

Mesothelial cell proliferation

In order to have an understanding of impact of mesothelial repair, a first step was to investigate the proliferation rate of cultured cells. Options to measure proliferation rate included counting viable cells after staining with a vital dye, to measure DNA synthesis, or counting cells by automated counters which rely on dyes and cellular activity at different time points during the experimental process. In this experimental model, a specific colour substrate based on the sodium salt of 2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt or XTT (Sigma) was used. This XTT-method is based on the cleavage of the tetrazolium ring of XTT, by mitochondrial dehydrogenase in viable cells resulting in water soluble orange formazan crystals. The orange solution is measured spectrophotometrically at a wavelengt of 450 nm (143, 144) indicating an increase or decrease in viable cell numbers between different wells.

This method was established and utilized in a 96-well cell culture system to enhance optical density readings at 450 nm in a multi well plate reader utilizing computer software (V-max

and SoftMax Pro, Molecular Devices, USA). Cell culture medium without phenol red was used to reduce the color background in the proliferation assays. When a proliferation assay was performed, the remaining medium was removed and replaced with complete culture medium E199, without phenol red, containing 200 µg/mL XTT for an additional 4 hours in a culture incubator. The optical density at 450 nm was measured with a reference wavelength of 690 nm subtracted according to the manufacturer, and expressed as a per cent of the untreated control.

The experimental model (1) – Absence of inflammatory mediators

Rapid mesothelization is favourable in peritoneal healing since it restore tissue integrity earlier. A peritoneal defect is considered healed when mesothelization is completed. The origin of mesothelial cells that contribute to the mesothelial repair is not fully understood. It has been shown that mesothelial cells migrate from the edges of the denuded area, differentiate from underlying cells or originating from a population of "free-floating" cells that can form islands of mesothelium in the defect (Figure 3) (16, 38). In the experimental models described below, remesothelization was simulated both with non-attached cells and attached cells to reflect different mechanisms of remesothelialization.

For this reason, the first experiment (Figure 8, 1a) investigated the effect of hyaluronan on non-attached cells. Mesothelial cells were detached from the culture flasks using Trypsin-EDTA solution, centrifuged, resuspended in fresh culture medium and then counted in a Bürker chamber. Cells were transferred at a density of 30 000 cells/well into a 96-well multi-well plate. Hyaluronan was added to the medium in concentration of 0.05, 0.1, 0.2 and 0.4% and incubated. Control cells received medium only. The colorimetric proliferation assay with XTT was performed after 4 and 24 hours of incubation.

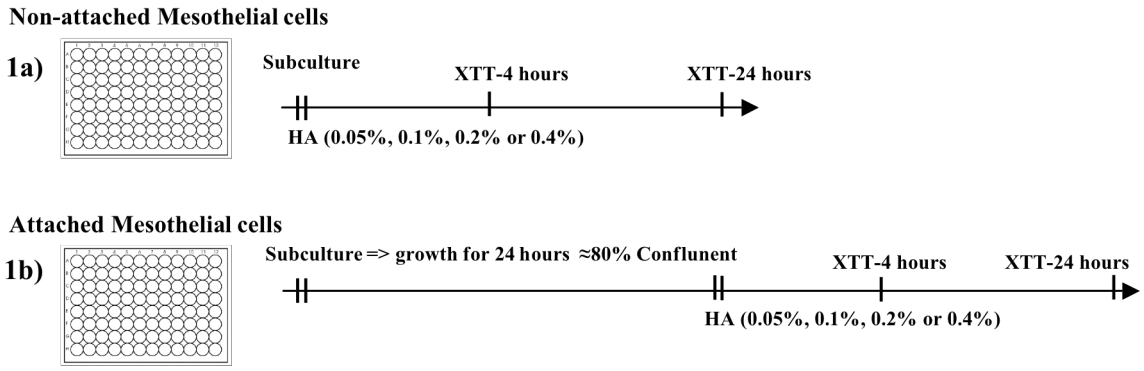

Figure 8: Schematic presentation of the two experimental models in absence of inflammatory mediators. In (1a) the hyaluronan was added immediately after seeding of mesothelial cells (non-attached cells) and in (1b) the cells were left to grew for 1 day (attached cells) prior to addition of hyaluronan (HA=hyaluronan).

The second experiment (Figure 8, 1b), investigated the effect of hyaluronan on attached cells. Mesothelial cells were detached in the same manner as described, but the cells were allowed to attach to the surface and to each other. During this time, the cells reached an approximate confluence of 80%. The cells were then exposed to the same concentrations of hyaluronan as described above and colorimetric proliferation assay with XTT was performed after 4 and 24 hours of incubation.

The experimental model (2) – Presence of inflammatory mediators

In conjunction with abdominal surgery or an intraabdominal infection, mesothelial cells are likely to be exposed to inflammatory mediators. In order to simulate this clinical situation, two additional experimental models were prepared. In the first experimental setting (2a), intraoperative contamination of gastrointestinal contents was simulated. The second experiment (2b), simulated an established peritonitis, where the mesothelium has been exposed to inflammatory mediators for a longer period of time.

In the second experiment (Figure 9, 2a), attached mesothelial cells with a confluence of approximately 80 % were treated with either of LPS 10 μg/mL, TNF-α 500 U/mL or a combination of both. Immediately after this treatment, hyaluronan was added to half of the wells at a final concentration of 0.2 % without removing the LPS or TNF-α. Medium without hyaluronan and LPS/TNF-α served as untreated controls. Separate cell cultures were incubated 4 and 24 hours, followed by determination of the proliferation rate.

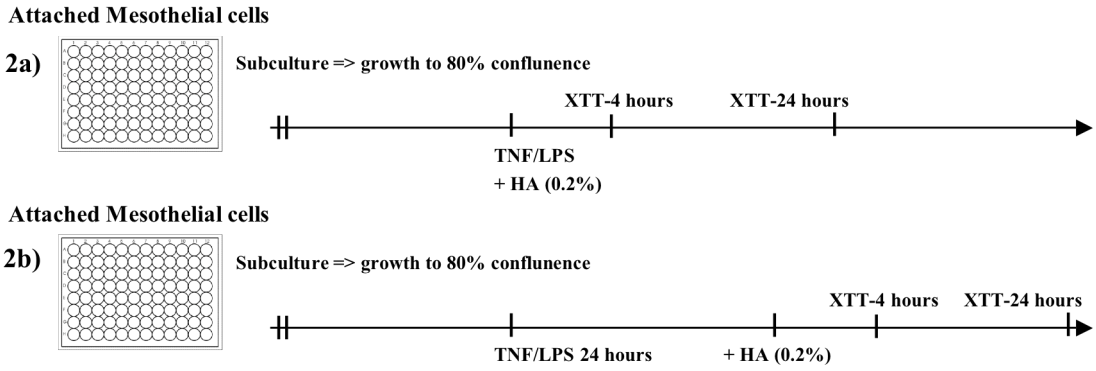

Figure 9: Schematic presentation of the two experimental models in the presence of inflammatory mediators. In 2a mesothelial cells were grown to 80% and then treated with either 10 µg/mL LPS, 500 U/mL TNF-a or the combination of both. Hyaluronan were immediately added and proliferation rate was measured using XTT-assay after 4 and 24 hours. In 2b) mesothelial cells were grown to 80% and then a preincubation for 24 hours with 10 µg/mL LPS, 500 U/mL TNF-a or the combination of both, was performed following addition of hyaluronan and XTT-assays at 4 and 24 hours (HA=hyaluronan).

In the last part of experiment (Figure 9, 2b), attached mesothelial cells with a confluence of 80 % were pre-incubated with the LPS and TNF-α combination for 24 hours, followed by addition of hyaluronan 0.2 % to half of the wells, without removing the media containing the inflammatory mediators. Similar to 2a, the control cells received medium only and separate cell cultures were incubated for 4 or 24 hours. Colorimetric proliferation assay with XTT was performed after 4 and 24 hours of incubation.

TGF-β isoforms in vivo and effects on cell proliferation in vitro (Paper V)

Question

The fifth and final paper, investigates the presence and activation profile of all TGF-β isoforms, compared with that of plasma during surgery. The effect of different TGF-β isoforms and their concentrations on mesothelial proliferation rate was also studied.

In a wound healing rat model, the most prominent isoform was TGF-β_1 followed by -β_2 and -β_3 (145). The presence of TGF-β_1 and -β_3 has also been demonstrated to be present in peritoneal- and adhesion-tissue in humans (146). However, little is known about the presence of TGF-βs or the activation profile in peritoneal fluid during surgery. Therefore, the first step was to investigate different TGF-β isoforms and activation profiles *in vivo*.

It would also be of interest to determine the effect on proliferation by TGF-β isoforms. A decrease in proliferation rate in both epithelial cells and transformed mesothelial cells of TGF-β_1 have been reported, but the effect of the isoforms TGF-β_2 and -β_3 of human mesothelial cells has not been described. The second step was therefore, to investigate the effect of different TGF-β isoforms on mesothelial proliferation rate *in vitro*.

Human subjects

The presence and activation profile of TGF-β_1, -β_2 and -β_3 in the peritoneal fluid was investigated in this study. Twenty-three patients undergoing elective open surgery for colorectal cancer were included. None of the patients had any infection, or signs of disseminated disease at the time of surgery. The patients were equally distributed with regard to gender (Male/Female: 11/12) and age at surgery (median age Male/Female; 70/71 years). Most of the surgical procedures were performed by the same surgeons.

Samples from peritoneal serosal fluid and plasma

Peritoneal serosal fluid was obtained by enclosing the small bowel in a sterile plastic bag within the peritoneal cavity during surgery (53). This technique protects the small bowel from damage during surgery and prevents desiccation. Accumulated serosal fluid from the bag was collected at the end of surgery (53). None of the collected samples demonstrated blood macroscopically. The mean volume of fluid from the bag was 20 mL, with a range of 5-67 mL. Time from skin incision to collection of fluid ranged from 35 to 264 minutes, with a mean of 146 minutes.

The expression in peritoneal fluid was compared to corresponding venous blood sampled during the surgery. Samples were collected in three different tubes; Sodium citrate, Diatube (CTAD) (BD, USA) and Stabilyte™ (Biopool) to optimise the different biochemical assays that was later to be performed. Both blood and serosal fluid samples were kept on ice (0°C) until centrifugation for 10 minutes at 10 000g at +4°C. All supernatants were stored in pre-labelled aliquots at -80°C until further analysed.

Biochemical assays

In order to compare levels of fibrinolytic components and TGF-β isoforms from peritoneal fluid and plasma, some mechanism had to be devised to correct for potential dilution. The final choice was total protein content in the fluid. This was performed using the DC Protein Assay (BioRad, Hercules, CA, USA). No difference was observed in mean protein content between the peritoneal serosal fluid 42.3±10.7 mg/mL and plasma 43.4±6.9 mg/mL (Mean±SD), respectively. As this method of normalization allows for comparison across different bodily compartments all subsequent enzyme concentrations or activities were normalised to the total protein content of each sample.

Assays used in the present study were similar those previously described for earlier studies. The levels of t-PA, uPA and PAI-1 antigen were analysed using kits from Biopool. The active fraction of t-PA was measured using Chromolize tPA (Biopool). This method was also modified for general plasmin detection (Figure 10).

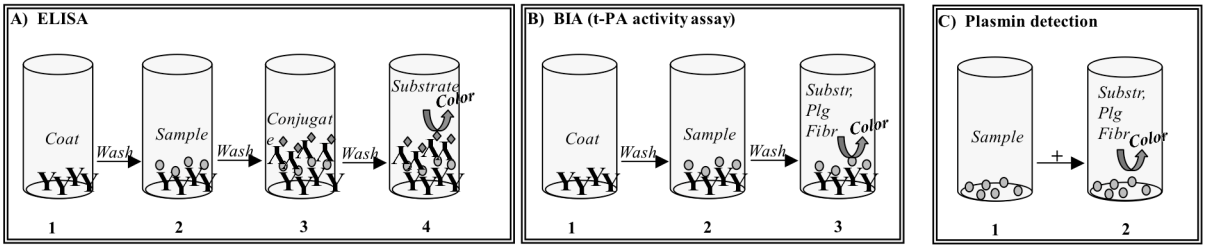

Figure 10: Schematic principles of different assays for protein detection: **A)** Enzyme-linked immunosorbent assay (ELISA). Capture antibodies is coated in the bottom of an 96-well plate **(1)**. Following wash, the samples, standards and internal controls are added **(2)**. After incubation, wells are washed and secondary antibody conjugated with an enzyme **(3)** that converts the present substrate that results in a coulor change in the last step **(4)**, where the colour change is proportional to the amount of antigen bound. **B)** Bio-functional immunosorbent assay (BIA) is similar to ELISA in the first steps **(1,2)**. An antigen is bound initially without blocking the active sites of the protein of interest which could convert a plasmin sensivity substrate **(3)**. The change in colour is proportional to the amount of t-PA converted plasminogen (example: Chromolize t-PA). **C)** Plasmin detection by adding the sample **(1)** together with a plasmin sensitive substrate **(2)** that detects converted plasminogen generated from both t-PA and uPA (Plg=Plasminogen, Fib=Fibrinmonomer, Substr=Plasmin sensitive substrate).

Likewise, commercially available methods were used for detecting TGF-β isoforms. The method from Promega was used (Paper I) for detecting TGF-β_1. TGF-β_2 was also analysed using a method from Promega. According to the manufacturer, the lower detection limit for TGF-β_2 was 32 pg/mL. The intra-assay variations were 7.4% at 767 pg/mL and 9.2% at 229 pg/mL. The inter-assay variations were 6.1% at 756 pg/mL and 27% at 94 pg/mL, indicating the method was less sensitive at low concentrations, close to the lower detection limit. For

TGF-β_3 a commercially available ELISA from R&D Systems (Abingdon, UK) was used. No lower detection limit or intra/inter assay variation was specified by the manufacturer. Therefore, the lower detection limit was defined as the lowest level that could be distinguished from the blank sample, typically 3 standard deviations above the mean optical density of the blank. The intra- and inter-assay variations were determined by analysing one sample with low and one sample with high concentrations, in replicates (n=4-6) within the same plate (intra-assay CV%), and with repeated measures between several plates (inter assay CV%). The lower detection limit thus calculated for the TGF-β_3 was 25 pg/mL and the intra- and inter-assay variations (CV) were 2-4% and 5-12%, respectively, depending on high or low concentrations. Higher concentrations gave a lower variability in CV%, in contrast to the lower concentrations that frequently varied between 10-12%. Measurements of active and total amounts of TGF-β isoforms were similar among the different assays. The active fractions could be measured directly in the ELISA plate using the kits provided. However to measure the total amount of TGF-β isoforms each sample needed to be acidified to pH 3.0 using 1 mol/L HCl, followed by a 15-min incubation at 22°C, resulting in activation of all TGF-β. To neutralise samples, 1 mol/L NaOH was supplemented before additional dilution and application to a second ELISA plate, according to the manufacturer's instructions. All results were normalized to total protein content using the DC protein assay.
When analysing venous blood for t-PA, Stabilyte™ plasma was used for t-PA activity, and for PAI-1 antigen the Diatube (CTAD) plasma was used. For the t-PA antigen, uPA antigen and TGF-β assays citrated plasma was used.

Mesothelial cell proliferation

With an experimental model to study human mesothelial cell proliferation in place (Paper IV), the effect of several concentrations of different TGF-β isoforms on mesothelial cell proliferation was studied. Concentrations of TGF-β isoforms found during surgery were used for this experiment. In the previously described experimental model, both attached and non-attached cells were used, simulating different stages in mesothelial repair. In this model, only attached mesothelial cells were used to simulate the reaction from intact mesothelial layer in presence of different TGF-β isoforms. The XTT based technique to measure mesothelial cell proliferation was previously described in detail (Paper IV). Cell culture medium without phenol red was used to reduce the colour background. Using a plate reader and computer based software optical density was determined and calculated as percent of untreated control.

The experimental model; Effects of TGF-β isoforms on mesothelial proliferation

All three TGF-β isoforms used in the mesothelial proliferation model and all isoforms were purchased from R&D Systems, and were suspended in sterile HCl (4 mmol/L) and BSA (0.1%) (Sigma) to activate the TGF-β isoforms. Additional dilutions (from $1x10^{-4}$ to $1x10^{4}$ pg/mL) of activated (acidified) TGF-β_{1-3} were done in culture medium without phenol red and with 1% FCS only. The highest concentration was chosen according to what has previously been used (Paper II) and lowest concentrations were similar to that found during surgery.

As previously described in Paper II-IV, primary isolated human peritoneal mesothelial cells were cultured until the second or third passage. From the second passage, mesothelial cells were subcultured to 96-well cell culture plates with a cell density of $2x10^{4}$ cells/well and were maintained for 3-4 days until cultures were 70-80% confluent. Mesothelial cells were preincubated for 24 hours with medium containing 1% FCS only and then stimulated with increasing concentrations (from $1x10^{-4}$ to $1x10^{4}$ pg/mL) of acidified and activated TGF-β_1, -β_2 and -β_3 for 16 hours in culture medium with 1% FCS only and without phenol red. Cells cultured with medium only, served as untreated controls. Mesothelial cell proliferation (XTT-assay) was measured by adding substrate to a concentration of 200 μg/mL in medium without phenol red. The formed Formazan salt product was measured by light absorbance at 450 nm in a 96-well plate reader. A reference wavelength of 690 nm was used to subtract background. Proliferation was calculated for all TGF-β isoforms at each concentration as percent of untreated cells on the same cell culture plate. For each TGF-β isoform experimental models were run in duplicates, and expressed as Mean±SD of six replicates at each concentration, in each experiment. Data is presented in bar graphs, where proliferation is expressed as percent of untreated cells. A positive bar represents an increase compared to control and a negative bar for a decrease in cell proliferation.

Correlation between TGF-β and fibrinolytic components

It has been proposed that plasminogen activators, t-PA and uPA alone or in combination, can activate TGF-β through the plasmin system (147, 148). However, it is not known if this is relevant in the abdominal cavity during surgery. Levels of t-PA activity in peritoneal serosal

fluid were therefore correlated with corresponding active TGF-β_{1-3} isoforms to determine possible associations between these components.

Statistics

Statistical methods and calculations used in the different papers are presented in this section. The outcome and interpretation of the statistical evaluations will be discussed in the *Results and Discussion* section. Statistical significance was defined as $p \leq 0.05$ and all tests were 2-tailed. Statistical calculations were performed using the StatView package (Abacus Concepts, Berkeley, CA, USA).

Paper I

In the first study, the majority of figures are presented as Box-Whisker plots, where the median value and inter quartile range are given, together with the 10^{th} and 90^{th} percentile. Hence, 80% of all data points are presented in the graphs. Some of the variables measured were normally distributed which was confirmed by Kolmogorov-Smirnov test for normality. However, since not all variables were normally distributed and the total number of patients limited, non-parametric statistics (Mann-Whitney U test) was used to analyse differences between groups. Linear regression was used to investigate correlations. Linear or 4-parameter regression was used to calculate assay concentrations for the biochemical parameters.

Paper II

In the second study, results are reported as means±standard error of the mean (Mean±SEM) in 6 replicates, presented per 10^6 cells. For the protein detection assay, a statistical analysis was made in two steps. A non-parametric Kruskal-Wallis test was first use to determine whether there was an overall difference between the separate groups. If results achieved statistical significance, the non-parametric Mann-Whitney U test was used to compare different concentrations to untreated control. The least square linear regression method was used to calculate the fit of the serial dilutions in the Q-RT-PCR reactions. In order to investigate whether t-PA or PAI-1 influenced the concentration of t-PA/PAI-1 complex a multiple linear regression was performed. Linear or 4-parameter regression was used to calculate assay concentrations for all biochemical parameters.

Paper III

Non-parametric statistics were used (Kruskal Wallis and Mann-Whitney U test) similar to the previously described. Results are reported as Box-Whisker plots. Linear or 4-parameter regression was used to calculate assay concentrations for all biochemical parameters.

Paper IV

The proliferation rate was measured as optical and expressed as per cent of untreated cells (100%). Results are presented as mean and standard error of the mean (Mean±SEM) of 8 replicates. The non-parametric Kruskal-Wallis and Mann-Whitney U test was used for determination of differences.

Paper V

Comparison of age and gender and of the total protein content between the two compartments (peritoneal fluid/plasma) was done with non-parametric (Mann-Whitney U) statistics. The analysis between the serosal fluid and plasma within the same patient were done with the paired non-parametric Wilcoxon signed rank test. Linear or 4-parameter regression was used to calculate assay concentrations for all biochemical parameters. Correlations were done using the non-parametric Spearman Rank Correlation test.

RESULTS AND DISCUSSION

The initial human study (Paper I)

The study

In the first study, the role of TGF-β_1 in peritoneal tissue of patients with adhesion formation and its relationship in tissue with decreased fibrinolytic capacity was investigated. Levels of active and total TGF-β_1 were measured in the extracted tissue, and correlated with assayed fibrinolytical factors. Finally, severity of adhesions was scored.

Results

All 22 patients undergoing surgery had pre-existing adhesions. Thirty-one of 32 biopsies had detectable TGF-β_1 levels. On average, the adhesion contained higher levels of both active and total fraction of TGF-β_1 compared to the levels in the peritoneum itself (Figure 11). There were also higher levels of the active fraction of TGF-β_1 in tissue sampled from patients with severe adhesions compared to those with less severe adhesions. The levels of total TGF-β_1 was similar in the groups, indicating that a greater fraction of TGF-β_1 was in its active form in patients with more dense and extensive adhesions (Figure 11).

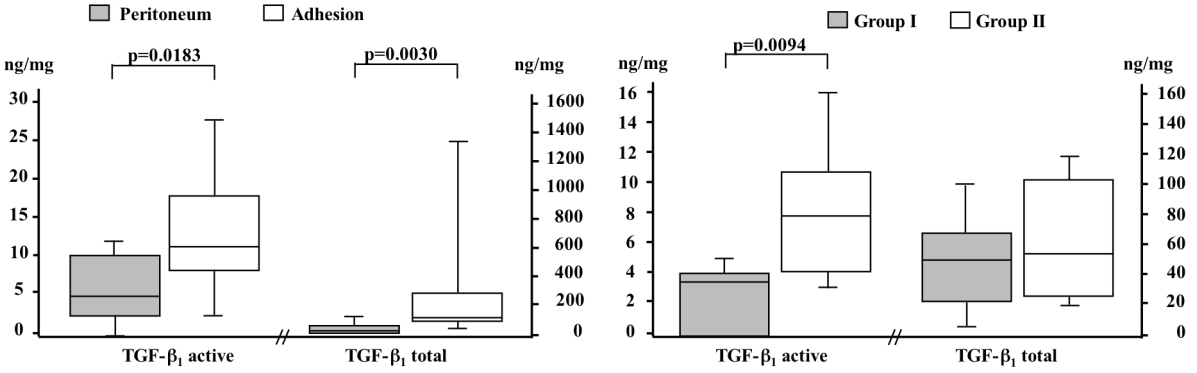

Figure 11: (Left) Peritoneal concentrations of TGF-β_1 in peritoneal and adhesion tissue. The active fraction (left axis) and total fraction (right axis) of TGF-β_1 (ng/mg of tissue). Both active and total TGF-β_1 were higher in adhesion tissue. **(Right)**: Peritoneal concentrations of TGF-β_1 in patients with limited or extensive adhesion formation after previous surgery. Active fraction (left axis) and total fraction (right axis) of TGF-β_1 in peritoneal tissue (ng/mg of tissue). Data is presented as box-whisker-plots representing median (horizontal line), inter quartile range (boxes) and 10th and 90th percentiles (error bars). *Data from this figure was originally published in Surgery, 129, Overproduction of transforming growth factor beta (TGF-β) is associated with adhesion formation and peritoneal fibrinolytic impairment, 626-32, Copyright Elsevier (2001).*

In order to investigate a possible association with the plasmin system, measured values of TGF-β_1 were correlated with plasminogen activators and the inhibitor PAI-1 (Figure 12). There was no correlation between uPA and t-PA with the active or total fraction of TGF-β_1 in adhesion tissue. Similar results were found in peritoneal tissue. However, there was a correlation in peritoneal tissue between PAI-1 and the active fraction of TGF-β_1 and to some extent between PAI-1 and the total fraction of TGF-β_1 in adhesion tissue. All further results from the fibrinolytic measurements have been previously described (120).

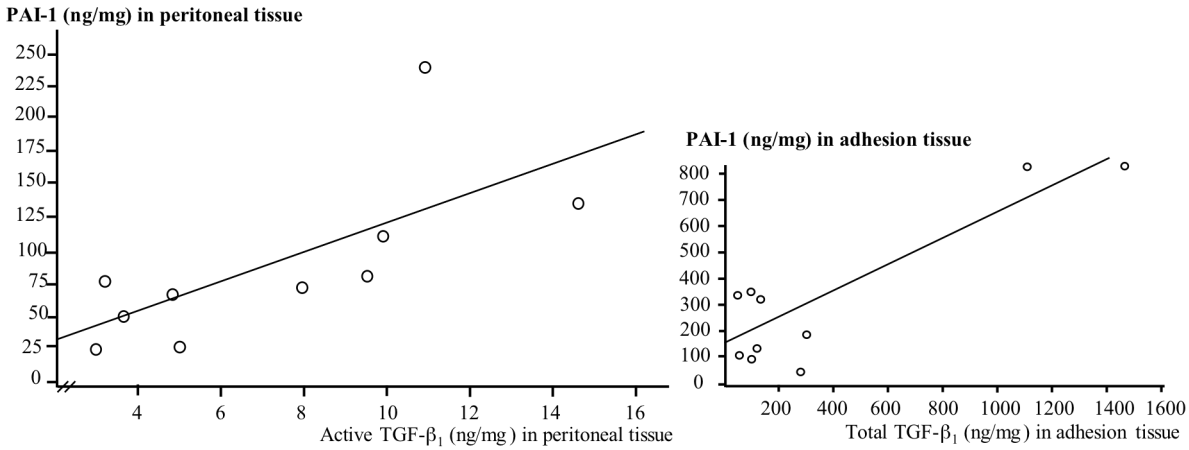

Figure 12: (Left) Correlation between PAI-1 and active TGF-β_1 in peritoneal tissue. The concentration of PAI-1 correlated significantly with the active fraction of TGF-β_1 (r^2=0.481, p=0.262, n=10) suggesting that TGF-β_1 affected the PAI-1 concentration. **(Right)** Correlation between PAI-1 and total TGF-β_1 in adhesion tissue. The concentration of PAI-1 correlated significantly with the total fraction of TGF-β_1 (r^2=0.761, p=0.0010, n=10) suggesting that TGF-β_1 was involved in the PAI-1 expression. *Data from this figures was published in Surgery, 129, 626-32, Copyright Elsevier (2001).*

When patients were scored for severity of adhesions eight of the included patients were categorised as having less severe adhesions and 14 patients were assigned to the severe adhesion formation category. Several of these had experienced adhesive small bowel obstruction.

To our knowledge, this was the first study that describes the findings of transforming growth factor in human adhesion tissue. Although the sample size was small (n=22), it showed that TGF-β_1 was present and that increased levels of TGF-β_1 seemed to be associated with increased PAI-1 in the tissue. These findings supports a role of TGF-β in adhesion formation and extend findings in experimental models (149).

Additional methodological considerations

Early on the protein content was normalised to the wet weight of peritoneal tissue. However, this method does not take into account differences in cellularity. Later, a method for measuring the total protein content was developed for both tissue extraction (125-127) and *in vitro* systems (150). Both methods correlated well with each other (120). Normalizing to the total protein content was later preferred since it was useful in different types of tissue, and enable comparisons between compartments (Paper V).

It is important to be aware that homogenised samples represents an average tissue concentration with contributions from multiple sources including, mesothelial cells, fibroblasts, healthy and diseased cells, normal and reactive cells, extracellular matrix, inflammatory cells, blood vessels and blood. In some cases it is acceptable to have the combined expression of a tissue such as in this case when a tissue surface could present a more or less adhesiogenic surface. In other cases, it might be important to generate information from a subgroup of cells. The laser capture microdissection (151) could be used, or experimental models of isolated cells (35, 131, 141) as reviewed by Ericsson *et al* (129).

A linear regression was used to determine the correlation between active and total TGF-β_1 and the PAI-1 level. However, a parametric linear method is always very sensitive to outliers, which is the case for this regression analysis. Two high concentrations of the TGF-β_1/PAI-1 could to some extent, affect the outcome of the correlation and significance testing. It is debatable whether a non-parametric analysis would have affected the outcome of the correlation, since conclusions drawn from 10 individuals are limited. However, when the same regression analysis was done with non-parametric statistics (Spearman Rank Correlation) some time after the publication of the paper, the correlation remain significant between the active fraction of TGF-β_1 and PAI-1 in peritoneal tissue (ρ_s=0.755, $p<0.05$), while the correlation in the adhesion tissue no longer was statistically significant (ρ_s=0.409, p=0.219). This could be either due to the outliers, or to the small sample size (beta error). However, robust correlation between the active fraction of TGF-β_1 and PAI-1 in the peritoneal tissue supports the hypothesis that expression of TGF-β_1 is associated with PAI-1 expression which is likely to have an impact on ECM deposition, and on factors in the plasmin system (38, 152).

The first mesothelial study (Paper II)

The study

In the second study the role of increasing concentration of TGF-β_1 on fibrinolytic expression in human cultured peritoneal mesothelial cells was investigated. Levels of plasminogen activators and plasminogen activator inhibitor were measured in conditioned culture media, thus reflecting the secretion of proteins from mesothelial cells. This was combined with an investigation of mRNA expression in order to study transcriptional regulation for these proteins.

Results

Cultured mesothelial cells secreted t-PA, uPA, PAI-1 and PAI-2 into the conditioned culture media. The conditioned medium also contained t-PA/PAI-1 complex indicating that a complex formation between t-PA and PAI-1 had occurred.

Both Rougier *et al* (95) and Tietze *et al* (93) have used TGF-β_1 in different cell culture models. Rougier (95) used from 0.1 to 10 ng/mL of TGF-β_1 in an immortalized mesothelial cell line. Tietze (93) used, TGF-β_1 in concentrations ranging from 0.0001 to 10 ng/mL, when investigating it's effect on omentally derived mesothelial cells. Therefore, this study used a concentration between 0.1 and 10 ng/mL TGF-β_1. Cultured mesothelial cells decreased the t-PA release with increasing concentrations of TGF-β_1 in the culture media ($p<0.01$), in a dose dependent manner (Figure 13). At a concentration of 0.1 ng/mL of TGF-β_1 t-PA secretion was significantly decreased to 52% of untreated control cells ($p<0.01$), and continued down to 63% of control at 10 ng/mL.

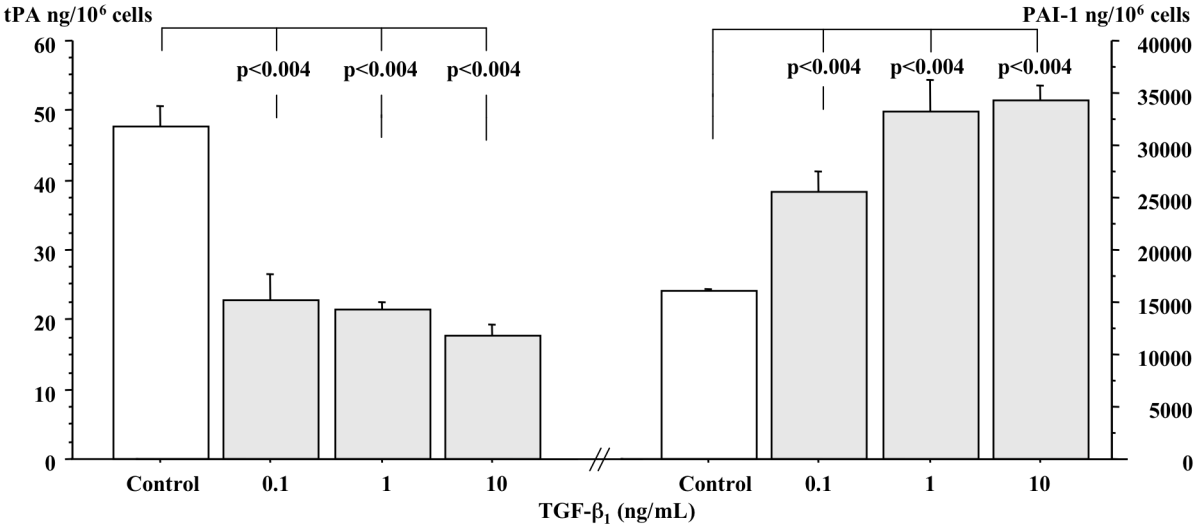

Figure 13: Protein levels in conditioned culture media 24 hours after stimulation with increasing levels of TGF-β_1. **(Left)** A dose dependent action of TGF-β_1 was seen with decreasing levels of t-PA and **(Right)** increasing levels of PAI-1 secretion from cultured mesothelial cells. Results presented as Mean±SEM (n=6). *Data from this figures was originally published in Scand J Clin Lab Invest, 60; 439-448, Copyright Informa Healthcare, (2000) <http://www.informaworld.com/journals>*

The opposite was observed for PAI-1, with an overall dose dependent significant increase in secretion of PAI-1 ($p<0.001$) with increasing concentrations of TGF-β_1 (Figure 13). At the lowest TGF-β_1 concentration (0.1 ng/mL) PAI-1 release increased 59% compared to untreated control ($p<0.01$), and at 1 ng/mL, the increase was more than 100% compared to the control ($p<0.01$). No further increase was detected at the 10 ng/mL concentration of TGF-β_1.

Interestingly, the concentrations of t-PA/PAI-1 complex followed the secretion of t-PA antigen levels with a significant overall decrease in formed t-PA/PAI-1 complex ($p<0.001$) (Figure 14). A significant 50% reduction compared to untreated control was seen at the lowest concentration of 0.1 ng/mL TGF-β_1 ($p<0.01$). A multiple linear regression was performed to find out whether the decrease in t-PA/PAI-1 complex was influenced by either t-PA or PAI-1, and it was found that the decrease was influenced by the levels of t-PA antigen ($r^2=0.84$, $p<0.001$), while PAI-1 antigen levels did not correlate at all. This is likely a reflection of rapid inhibition of t-PA secreted into the culture media by the higher concentrations of PAI-1.

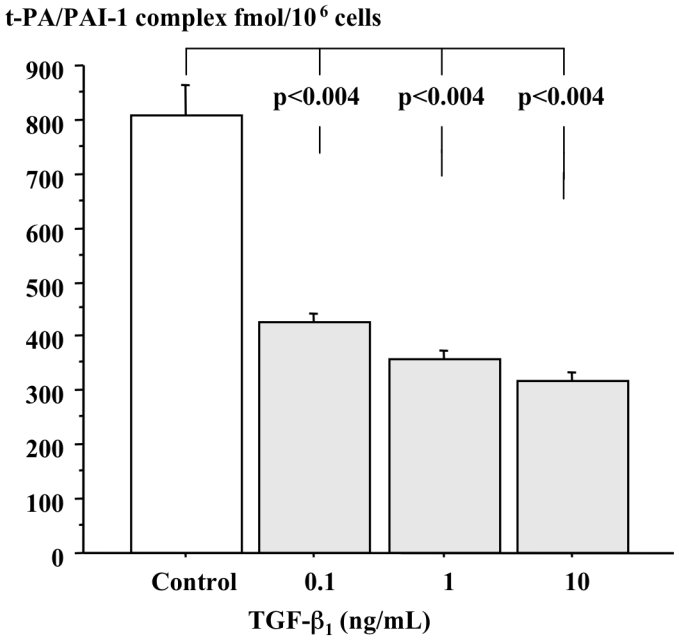

Figure 14: Protein levels in conditioned culture media 24 hours after stimulation with increasing levels of TGF-β_1. A dose dependent action of TGF-β_1 was seen with decreasing levels of t-PA/PAI-1 complex in the culture media from cultured mesothelial cells. Results presented as Mean±SEM (n=6). *Data from this figure was originally published in Scand J Clin Lab Invest, 60; 439-448, Copyright Informa Healthcare, (2000) <http://www.informaworld.com/journals>*

These findings contrast with other findings where the t-PA/PAI complex increased in conditioned media with increasing concentrations of TGF-β_1 (95). The reason for this is not known. One explanation could be differences in cell phenotypes since other work was done on omentally derived mesothelial cells and the present study used mesothelial cells from peritoneal fluid. Omentally derived cells did not secrete any uPA or PAI-2 (95), which were secreted by mesothelial cells in the present study indicating a phenotype difference. Both uPA and PAI-2 were secreted into the culture media during unstimulated conditions and were unaffected by increasing concentrations of TGF-β_1.

It was not possible to measure any active t-PA in culture media using the t-PA activity method. The reason for not detecting active t-PA could have several explanations. It was possible to measure the inactive t-PA/PAI-1 complex in a dose dependent manner in the culture medium. The presence of the complex indicates a rapid complex formation between t-PA and PAI-1, and quenching of t-PA activity is likely to have occurred. The PAI-1 antigen level in the culture media was much higher than the level of t-PA antigen, indicating an overabundance of PAI-1, which would support this notion. Interestingly, if these findings accurately reflect the human condition, the presence of high amounts of intraperitoneal TGF-β_1 has the potential to quench fibrinolytic activity.

In the present study, human peritoneal mesothelial cells also expressed t-PA, uPA, PAI-1, PAI-2 and uPA receptor mRNA during unstimulating conditions. Increasing concentrations of

TGF-β_1 decreased mRNA for t-PA in a dose-dependent manner, while it upregulated mRNA expression for uPA and uPA receptor. There was low or no effect of TGF-β_1 on PAI-1 (Figure 15) and PAI-2 mRNA expression at the time point measured. However, since there was an accumulation of PAI-1 over time, transcription is likely to have occured at some other time point.

The downregulating effect of TGF-β_1 on t-PA production is seen in less expression of mRNA copies (Figure 15), as well as a decreased secretion of t-PA in culture media. This observation confirms earlier findings (93, 95) and extends the observation that the local effect of TGF-β_1 on mesothelial cells is not only dose dependent, but also has profound effects even at low doses. Thus, TGF-β_1 has the potential to play an important role by significantly depressing local fibrin clearing capacity intraperitoneally.

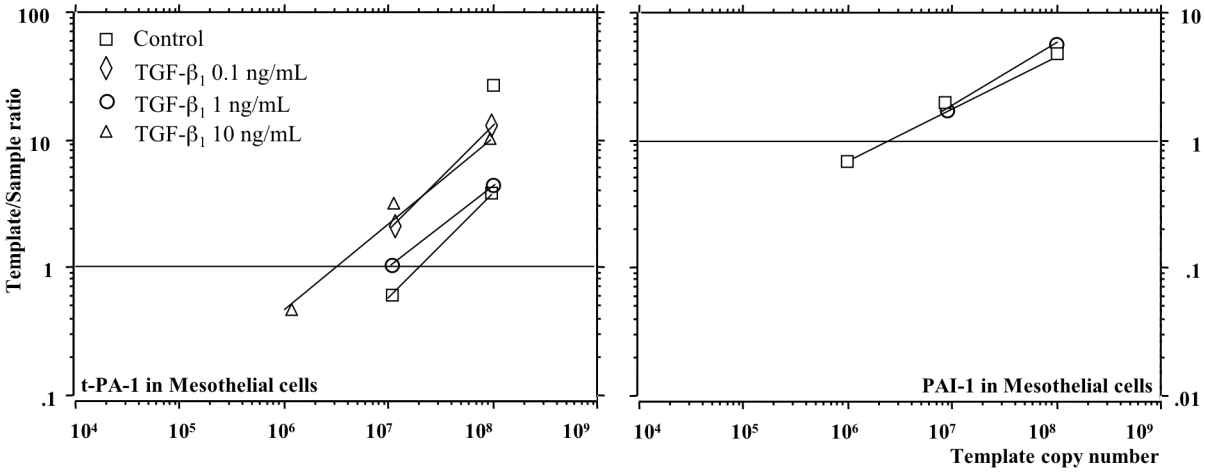

Figure 15: Messenger RNA expression/µg total RNA after treatment with TGF-β_1 0.1, 1 and 10ng/mL compared to untreated control. Results are presented as regression lines with the ratios at different template concentrations. When the ratios between the sample and the internal standard are equal (ratio=1) on the Y-axis, the concentration could be read on the X-axis as mRNA concentration. TGF-β_1 treatment of mesothelial cells reduced the levels of t-PA mRNA expression, while low or no effect was seen on the PAI-1 expression. *Data from this figures were originally published in Scand J Clin Lab Invest, 60; 439-448, Copyright Informa Healthcare, (2000) <http://www.informaworld.com/journals>*

The effect of TGF-β_1 on PAI-1 is complex. Although the present study demonstrated a dose-dependent increase in protein secretion into the culture media, a similar pattern was not seen in the expression of mRNA for PAI-1. This is in part, in conflict with other findings where an increase in mRNA expression was observed in human bronchial epithelial cells (94) and immortalized mesothelial cells (93, 95). The reason for this is unclear. However, the cell phenotype used in these studies is not the same as the cells used in the present study. It may be that different cell phenotypes react differently. Furthermore, the time point for total RNA preparation varied among the studies. In the present study (Paper II), RNA preparation took

place after sampling from the culture media, which was 24 hours after stimulation. In other studies time points between 4 and 12 hours (93, 95) were used. Thus, a rapid increase in PAI-1 mRNA after TGF-β_1 stimulation, might have been unnoticed with the present study design.

This experimental model suggests that the effect of TGF-β_1 is not limited to promotion of tissue fibrosis, but also affects peritoneal fibrin clearing capacity by differentially regulate local fibrinolysis with decreasing t-PA and increasing the inhibitor PAI-1 secretion. These observations further support a role for TGF-β_1 in adhesion formation.

The second mesothelial study (Paper III)

The study

In the third study, using an inflammatory experimental model, the role of increasing concentrations of hyaluronan on human cultured mesothelial cells was investigated. Secreted levels of plasminogen activators and plasminogen activator inhibitor were measured in conditioned culture media. Intracellular mRNA expression was investigated for the same reasons previously described in Paper II. Since t-PA can be stored intracellularly, proteins were also measured in mesothelial cells lysates.

Results

Cultured mesothelial cells secreted t-PA, uPA and PAI-1 in both the conditioned media and cell lysate. TNF-α and different concentrations of hyaluronan did not significantly change t-PA concentration in the culture media. However, when levels of t-PA antigen were measured in cell lysates, the concentrations were significantly increased in a dose dependent manner with increasing concentrations of hyaluronan. The amount of mRNA copies for t-PA, measured at 24 hrs after stimulation, did not change in the presence of hyaluronan.

The intracellular amount of t-PA increased with increasing concentrations of hyaluronan (Figure 16), suggesting mesothelial intracellular pooling, which has previously been described as occurring in endothelial cells (153). One mechanism could be that hyaluronan affects t-PA release at a post-translational level. However, since no effect was seen on the mRNA

expression for t-PA, there was no indication that t-PA was transcriptionally regulated by hyaluronan.

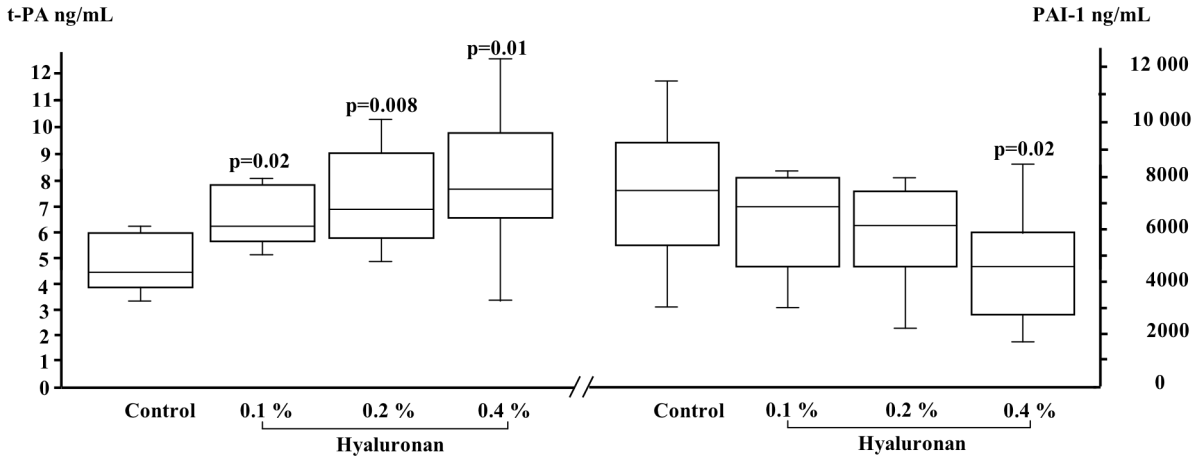

Figure 16: (Left) Levels of t-PA antigen in cell lysates. Increased concentrations of hyaluronan increase the levels of t-PA antigen in lysated cells and decrease the levels of PAI-1 antigen in culture media **(Right)**. Results are illustrated as median (horizontal line) interquartile range (boxes) and 10th and 90th percentiles (error bars). *Data from this figures was originally published in Archives of Surgery, March, 136; 291-296, Copyright© (2001) American Medical Association. All rights reserved.*

When uPA antigen levels were measured in culture media, their concentrations decreased significantly in a dose dependent way, with increasing concentrations of hyaluronan. However, uPA levels did not change when measured in the cell lysate. When the mRNA for uPA was measured with the Q-RT-PCR method, an increase in messenger RNA copies from $2x10^2$ to $4x10^5$ was observed after treatment with hyaluronan, which is a 1000-fold increase in the hyaluronan treated cells compared with the untreated control.

Hyaluronan decreased PAI-1 antigen levels in conditioned culture media compare to untreated controls, with the most profound effect at a hyaluronan concentration of 0.4% (Figure 16). There was no difference in PAI-1 levels in cell lysate compared to expression in medium. At the mRNA level, hyaluronan caused a 500-fold decrease in PAI-1 mRNA expression, from $6x10^4$ to $1x10^2$ copies per microgram of total RNA.
This is in part, in contrast with the findings of Tarhan *et al* (111) where the effects of a HA-CMC gel (Seprafilm) on the fibrinolytic system was investigated in a rat model. In that study, uPA was significantly decreased in peritoneal tissue in the HA-CMC group compared to control, and no difference were seen between the two groups for t-PA, PAI-1 or PAI-2 (111). The reason for this is unclear, however the experiments were performed using rat peritoneal

tissue, whereas human mesothelial cells were used in the present paper (Paper III) and the experimental conditions were different.

Despite the seemingly contradictory effect on the plasminogen activators it is likely that the net effect might be an increase of fibrinolytic capacity, since intracellularly pooled t-PA is ready to be released when cells are appropriately stimulated. Moreover, the decrease in PAI-1 concentrations in the culture medium would also favour an increased fibrin degradation capacity. This could potentially translate into a beneficial effect of hyaluronan in the peritoneal environment, and lends support to a biological effect.

The third mesothelial study (Paper IV)

The study

In the fourth study, the role of increasing concentrations of hyaluronan on the proliferation rate of attached and non-attached human peritoneal mesothelial cells, with or without inflammatory mediators, was investigated.

Results - Proliferation in the absence of inflammatory mediators

When proliferation was measured in non-attached cells, increasing concentrations of hyaluronan increased the proliferation rate in a dose dependent manner at both 4 and 24 hours (Figure 17). The most profound effect was found at the 0.4% concentration of hyaluronan (4 hours $p<0.01$, 24 hours $p<0.005$), (experimental model 1a in Paper IV).

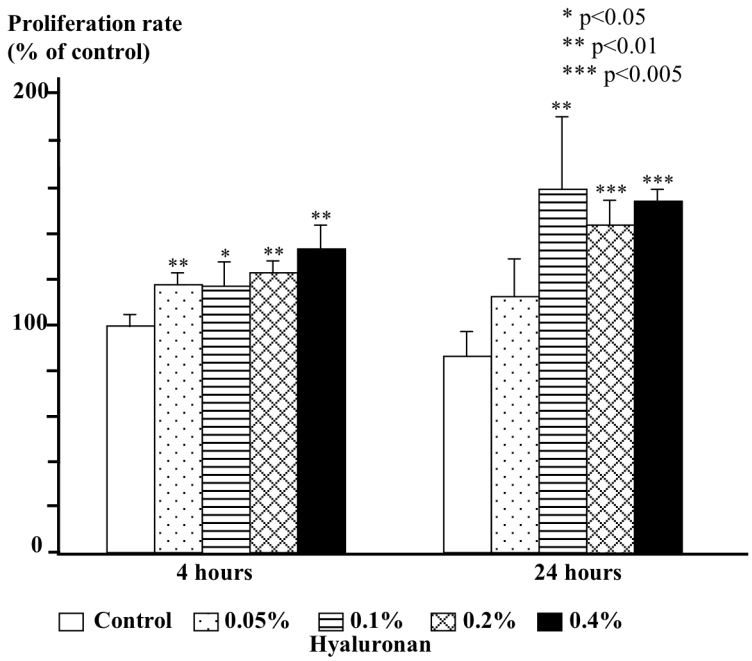

Figure 17: Proliferation rate, measured as optical density (at 450 nm), of non-attached human peritoneal mesothelial cells incubated with either 0.05, 0.1, 0.2 or 0.4% hyaluronan, at 4 or 24 hours after the addition of hyaluronan (n=8 each). Controls are considered as 100%. Results are presented as mean and SEM. *Data in the figure reprinted from Fertility and Sterility, 74, Reijnen M, Falk P, van Goor H, Holmdahl L, The antiadhesive agent sodium hyaluronate increases the proliferation rate of human peritoneal mesothelial cells, 146-151, Copyright 2000, with permission from Elsevier*

Similar to non-attached cells, the presence of hyaluronan increased the proliferation rate when measured at 4 and 24 hours. At 4 hours the difference was statistically significant using 0.2% hyaluronan ($p<0.01$) and at higher concentrations. At 24 hours, the difference was significant already at 0.05% ($p<0.01$) and gradually increased in a dose dependent manner (experimental model 1b in Paper IV).

When the proliferation rate was compared between the non-attached and attached cells, the rate was significantly increased in the attached cells. This suggests that cells needs to be close to each other or to a surface for this effect to be observed. Furthermore, when the proliferation rate was compared between the two time points, the proliferation rate was reduced to 50% in control cells at 24 hours compared to the rate at 4 hours. The reason for this is not known, but it is likely the cells reached a confluent state within the experimental time period, since the cells were already approximately 80% confluent when the experiment started and further proliferation was inhibited. Similar contact inhibition was not seen when the transformed mesothelial cell line Met-5A was used. This suggests that primary isolated human peritoneal cells are capable of regulating their own proliferation rate and reduce or end it when mesothelization is complete.

Proliferation in the presence of inflammatory mediators

In order to investigate different inflammatory conditions in the peritoneal cavity, shorter (inflammation model) and longer (peritonitis model) exposure time of inflammatory mediators was examined. Based on previous observations all experimental models were performed on attached mesothelial cells.

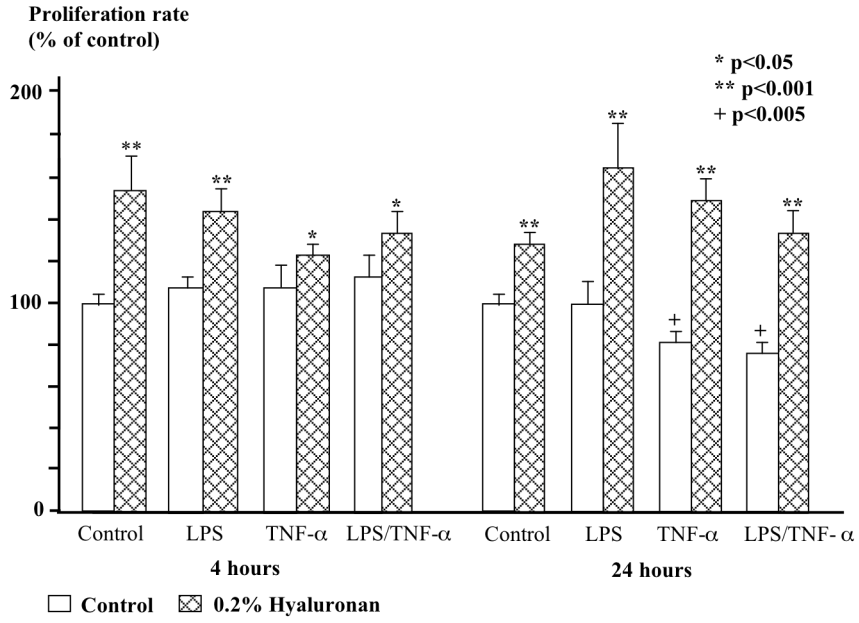

Figure 18: Proliferation rate of attached human peritoneal mesothelial cells treated with either LPS, TNF-a or a combination of both, and incubated with either no (open bars) or 0.2% (hatched bars) hyaluronan, 4 and 24 hours after incubation (n=8 each). Controls are considered as 100%. Results are presented as Mean and SEM. *Data in the figure reprinted from Fertil Steril, 74, 146-151, Copyright 2000, with permission from Elsevier*

In the inflammation model, exposure of LPS, TNF-α or their combination for four hours did not affect mesothelial proliferation (Figure 18). However, exposure of TNF-α alone or in combination with LPS for 24 hours reduced the proliferation rate to 75-80% compared to the untreated control ($p<0.005$).

When hyaluronan was added immediately after exposure to LPS, TNF-α or the combination, the proliferation rate was increased at both the 4 ($p<0.05$) and 24 ($p<0.001$) hour time points.

In the peritonitis model, 24 hours preincubation with inflammatory mediators resulted in a significant decrease in mesothelial cell proliferation (Figure 19) with the most profound effect by TNF-α and TNF-α in combination with LPS. However, exposure of cells to hyaluronan for 4 hours in addition to 24 hours preincubation had a protective effect and increased the proliferation rate of mesothelial cells preincubated with LPS, TNF-α or a combination of both ($p<0.001$) compared to untreated controls.

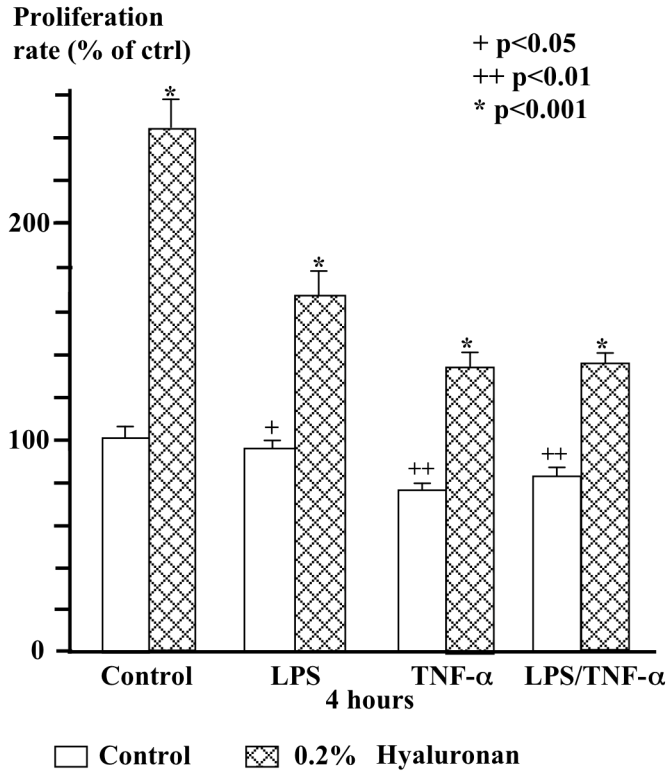

Figure 19: Proliferation rate of attached human peritoneal mesothelial cells preincubated with either LPS, TNF-a or a combination of both, and incubated with either no (open bars) or 0.2% (hatched bars) hyaluronan for additional 4 hours after incubation (n=8). Controls are considered as 100%. Results are presented as Mean and SEM. *Data in the figure reprinted from Fertil Steril, 74, 146-151, Copyright 2000, with permission from Elsevier*

The mesothelial cell cultures did not survive an exposure to the inflammatory mediators for 48 hours (24 hours preincubation with inflammatory mediators and additional 24 hours with or without hyaluronan).

With the above-mentioned experiments it could be demonstrated that hyaluronan increased the mesothelial cell proliferation *in vitro* even in the presence of inflammatory mediators. It could also be demonstrated that prolonged exposure to inflammatory mediators had a detrimental effect on mesothelial cell proliferation rate.

There are several ways by which hyaluronan can affect mesothelial cell proliferation. It might have effects on extra cellular matrix synthesis or structure (104) as well as modulation of growth factors, stimulation of surface receptor expression, or it could affect cell proliferation directly through a receptor (154). The CD44 receptor is a surface receptor that is involved in hyaluronan signalling pathway (155, 156) and a has possible role in cell-cell signalling and signal transduction (155). The cultured mesothelial cells in the present study expressed the CD44 surface antigen when incubated with a FITC labelled mouse anti-human CD44 antibody (Sigma, St.Louis, MO, USA) and exposed to UV-light in the inverted cell microscope. It is possible that the effect in mesothelial cell proliferation is mediated through the CD44 receptor, but this remains to be shown.

The main principles of action for hyaluronan based adhesion barriers is considered to be a mechanical separator of damaged peritoneal surfaces. As several of the hyaluronan-based agents on the market are modified in some way it is possible that their effect may differ. However, these findings suggest that a hyaluronan based adhesion barrier also could have biological effects in the peritoneal cavity.

TGF-β isoforms in vivo and effects on cell proliferation in vitro (Paper V)

The study

In the fifth and last study the presence and activation profile of TGF-β was further investigated and extended to include all three isoforms of TGF-β_{1-3}. Since TGF-β_1 was found in peritoneal tissue during surgery (Paper I) and had effects on mesothelial fibrin clearing capacity (Paper II) it was of interest to further characterise all present isoforms. This was done together with measurements of fibrinolytical factors in both peritoneal serosal fluid and plasma during surgery. Additionally, in an experimental model the effect of TGF-β_{1-3} on human mesothelial cell proliferation was studied.

Results – Presence and activation profiles of TGF-β_1, TGF-β_2 and TGF-β_3

In the first part of the study peritoneal serosal fluid and plasma levels of TGF-β isoforms were investigated. In both serosal fluid and plasma we found detectable levels of total and active fractions of the TGF-β_{1-3} isoforms (Table 2).

The active fraction of TGF-β_1 was higher in peritoneal serosal fluid compared with the corresponding plasma samples ($p<0.01$) (Table 2). In contrast, the levels of total TGF-β_1 in serosal fluid did not differ from the levels in plasma. This indicates that an increased activation of TGF-β_1 had occurred in the peritoneal environment.

Similar to TGF-β_1, the active fraction of TGF-β_2 was higher in serosal fluid compared with that of plasma ($p<0.01$), but the concentration of total TGF-β_2 was similar in the two

compartments (Table 2). Again, this indicates that an activation had occurred in the peritoneal compartment.

In contrast, active fraction of TGF-β_3 did not differ between the two compartments (Table 2). Moreover, the levels of total TGF-β_3 were higher in the serosal fluid ($p<0.01$) compared with the concentrations found in plasma. However, the amount of total TGF-β_3 was lower compared to the other isoforms in both set of samples. It therefore appears as TGF-β_3 is differentially affected in comparison to the other isoforms.

Table 2: Concentration of active and total TGF-β_1, -β_2 and -β_3 in peritoneal serosal fluid and plasma during open abdominal surgery. Values expressed as (Mean±SD) pg/mg total protein content. Statistical significant difference between the peritoneal and plasma concentrations has been calculated using Wilcoxon Signed rank test and a p-value ≤0.05 is considered statistically significant.

TGF-β isoforms (pg/mg protein)	Serosal fluid	Plasma	p-value
Active TGF-β_1	3.9 ± 7.3	0.5 ± 1.8	p<0.01
Total TGF-β_1	93.9 ± 40.0	94.0 ± 43.0	p=0.693
Active TGF-β_2	9.6 ± 7.8	5.8 ± 4.4	p<0.01
Total TGF-β_2	104.1 ± 41.9	96.6 ± 45.2	p=0.743
Active TGF-β_3	4.0 ± 7.3	2.9 ± 1.2	p=0.322
Total TGF-β_3	8.9 ± 2.8	6.4 ± 1.9	p<0.01

The pattern with increased active fraction of both TGF-β_1 and TGF-β_2 is comparable to what has been described in tear fluid in humans (157). The authors reported rather low, but detectable levels of the active fractions of TGF-β_1 and TGF-β_2, with substantially higher concentration of total TGF-β_1 and TGF-β_2.

Presence of fibrinolytic factors

Both serosal fluid and plasma showed detectable levels of t-PA, uPA and PAI-1 (Table 3). t-PA was measured both as an active fraction (t-PA activity) and as a total antigen (t-PA antigen). The levels of t-PA and uPA were higher in the peritoneal serosal fluid samples compared with the levels in plasma. This is similar with the findings described earlier (53) but with the difference that also uPA was increased in the peritoneal serosal fluid. In contrast to the activators, PAI-1 levels were lower in serosal fluid compared with plasma.

Table 3: Concentration of plasminogen activators (t-PA, uPA) and the inhibitor (PAI-1) in peritoneal serosal fluid and plasma during open abdominal surgery. Values are expressed as (Mean±SD) per mg total protein content. Statistical significant difference between the peritoneal and plasma concentrations has been calculated using Wilcoxon Signed rank test and a p-value ≤0.05 is considered statistically significant.

Assays per mg total protein	Serosal fluid	Plasma	p-value
t-PA antigen (pg/mg)	960 ± 560	310 ± 90	p<0.001
t-PA activity (IU/mg)	0.09 ± 0.12	0.01 ± 0.01	p<0.01
uPA antigen (pg/mg)	110 ± 130	10 ± 10	p<0.01
PAI-1 antigen (pg/mg)	620 ± 800	920 ± 530	p<0.01

A possible cause for the increased levels of plasminogen activators in the peritoneal cavity during surgery might be the release of activators from the mesothelial layer. Previously it could be demonstrated that endothelial cells could store t-PA intracellularly (153). In the present work (Paper III) mesothelial cells seemed to have a similar storage function. It might be that a similar mechanism could be involved *in vivo* enabling a rapid release into the cavity.

Correlations of TGF-β and fibrinolytic components

It has been proposed, based on experimental models (147, 148, 158) that plasminogen activators can activate TGF-β through the plasmin system. To investigate this, a correlation (Spearman Rank Correlation) was made between t-PA activity in serosal fluid (Table 3) with corresponding active TGF-$\beta_{1\text{-}3}$ isoforms (Table 2). A positive trend was detected with all three isoforms, however none of these reached statistical significance. In addition, no other correlation was seen between t-PA antigen or uPA antigen with any of the active TGF-β isoforms. Hence, there does not seem to be a direct relationship between TGF-βs and plasminogen activators in peritoneal serosal fluid.

However, the positive trend suggested some kind of relationship. Therefore additional experiments were done. This preliminary data indicate that plasmin itself may be related to the intraperitoneal activation of TGF-β. Plasmin activity can be measured using a plasmin sensitive substrate, identical to the one described for the t-PA activity method, but without using the t-PA specific capture antibody. The differences in assay techniques between ELISA, and these assays are schematic shown in Figure 10.

When plasmin concentrations were compared to the active TGF-β isoforms, there was a significant correlation between the levels of plasmin and the active fractions of the TGF-β isoforms. The active fractions of TGF-β_2 were positive correlated ρ_s=0.74 ($p<0.001$) as were the active fraction of TGF-β_3 with ρ_s=0.60 ($p<0.05$). A positive correlation was also found between plasmin and the active fraction of TGF-β_1, however it did not reach statistical significance ρ_s=0.42 (p=0.061).

These preliminary observations should be interpreted with caution. The concentrations in this experimental model were calculated against a t-PA standard, and it can be argued that a plasmin standard could be more appropriate to use. Furthermore, due to limited remaining sample volume only samples from 15 of 23 patients could be used. Nevertheless, these findings indicate that the resulting plasmin seems to be of greater importance than t-PA or uPA alone. Locally generated plasmin might thus be a mechanism by which activation of TGF-β in the abdominal cavity occurs, but further research is needed.

Effect of TGF-β_{1-3} on proliferation rate of mesothelial cells

The effect of the TGF-β_{1-3} isoforms on the proliferation rate of cultured human peritoneal mesothelial cells were tested by the XTT-assay as a marker of viable cells (Paper IV) (144). In the figure the effect of different concentrations of each TGF-β isoform on the proliferation rate of the mesothelial cells is expressed as percent of untreated controls.

An increase in proliferation is indicated by a bar graph above 0%, and a decrease is indicated with at bar below 0% (Figure 20). Similar to what has been described earlier (78, 159, 160) for TGF-β_1 a 50% reduction in cell proliferation was found in the present study when cell cultures were exposed to high concentrations ($5x10^3$-$1x10^4$ pg/mL) of TGF-β_{1-3} isoforms.

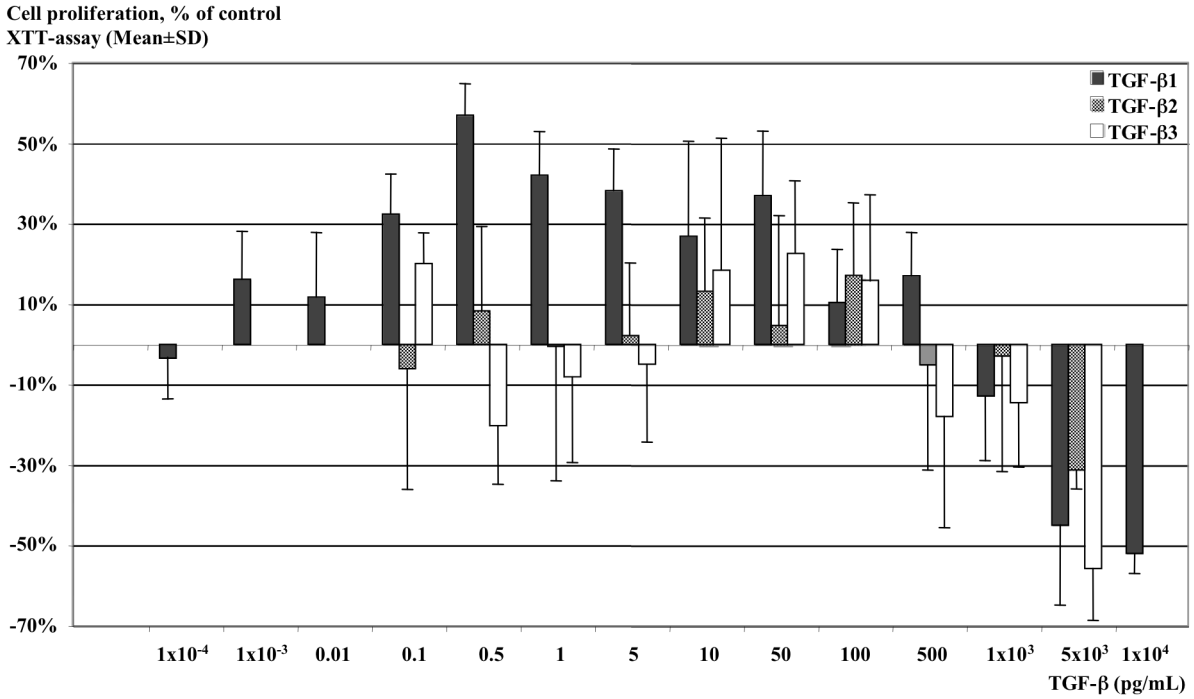

Figure 20: Proliferation studies on cultured human peritoneal mesothelial cells. Optical density measured at 450 nm at each concentration compared to cells with medium only. The XTT-assay indicates increased proliferation in the presence of TGF-β_1 at low concentrations compared to untreated control. All TGF-β isoforms decrease proliferation at concentrations from $1x10^3$ to $1x10^4$ pg/mL. The TGF-β_2 and -β_3 isoforms had no clear influence on the cell proliferation below $1x10^3$ pg/mL. Figure show representative data in 2 separate experiments on each TGF-β isoform, Mean±SD (n=6) in each experiment.

Interestingly, there was an increase in mesothelial cell proliferation in the presence of TGF-β_1 at 0.1 to 50 pg/mL compared to untreated control (Figure 20). Moreover, the TGF-β_1 effect was present at concentrations found in the peritoneal serosal fluid from patients during abdominal surgery. TGF-β_2 and TGF-β_3 isoforms had no consistent influence on the cell proliferation rate.

The increase in mesothelial proliferation at low concentrations of TGF-β_1 has to our knowledge not been shown previously. Other studies have reported different results and have used concentrations in different ranges (78, 159, 160), but not as low as in the present study. TGF-β has the ability to affect the cell cycle, proliferation and apoptosis, and in most epithelial cells TGF-β is an inhibitor of cell proliferation (83). The mechanism leading to increased proliferation therefore remains to be understood.

The concentrations used in this study were found in the surgical setting, and the observations are therefore likely to be clinically relevant. This suggests a new role for TGF-β_1 in a surgical context by enabling a rapid remesothelization during peritoneal repair.

FURTHER DISCUSSIONS AND FUTURE PERSPECTIVES

Experimental models of the human peritoneal environment

Experimental models and mesothelial cells

It could be discussed how representative the mesothelial cell cultures are for a surgical situation, in particular since different sources of mesothelial cells seem to give different results. Several research groups have been using omentally derived mesothelial cells, others are using transformed cell lines and in the present work we have used mesothelial cells derived from peritoneal fluid. It is not possible to evaluate what types of cells that best represent conditions *in vivo.* However, it is reasonable to assume that the free-floating mesothelial cells used in the present work, in early passages, is likely to be phenotypically similar to intact peritoneum. However, cell culturing is an artificial situation and results should therefore be interpreted with caution.

Even if the importance of the mesothelial cell has been increasingly appreciated during recent years, some mechanisms regarding regulating functions remain unclear. Experimental models of the mesothelial surface continue to be attractive tools to study isolated mechanisms of different biological functions in a controlled environment. Controlled experimental settings enhance the possibilities to study effects over a longer period of time that might be difficult to perform in the clinical setting. During surgical procedures, the peritoneal cavity is available only during a short period of time and mechanisms before and after are therefore difficult to follow. Thus, experimental models are important in the understanding of biological effects in a surgical context.

Models have been used to reflect different mechanisms in human situations as the vascular wall (62, 131, 132, 134, 153), in bone or cartilage (86, 88), in the eye (85), the lung (66) and wound healing (157, 161).

Moreover, experimental models have been used studying the mesothelial layer in the abdominal cavity. Isolation, culturing and characterizing of mesothelial cells have been described (35, 36, 137, 138). Models to study different mechanistic effects have been used for fibrinolytic components (20, 35, 36, 93, 95, 141, 150), response to inflammatory mediators

(35, 36, 93) (Paper III), growth factors (95) (Paper II and V), mechanisms in cell adhesion (33) and cell proliferation (159) (Paper IV and V). Experimental models are critical to the understanding of human biology, and could be very useful for the future development of surgery. Examples of this are models developed to mimic different surgical techniques including laparoscopy (141, 150).

During the development of mesothelial cell models it was noted that cells could not be cultured in too many passages without regressing into a fibroblastic phenotype. Due to this observation, we believed it was important to conduct the experiments as close to the primary isolated culture as possible. Since it was discovered that the morphological and immuno specific characteristics for a mesothelial cell type gradually disappeared starting in passage four, it was decided to perform all experiments before the fourth passage. However, it is noteworthy that, for some of the mesothelial cells the characteristics did not disappear, even after passage 10, 15 or even more. These findings were remarkable since the cells could be subcultivated repeatedly and still retain their mesothelial phenotype. None of these extraordinary cells were included in any of the experimental models, since they did not behave as expected. This kind of mesothelial cells have been suggested to be stem cells, that might be important candidates in transplantation of mesothelial cells in peritoneal damage (162, 163) or even general tissue engineering (164).

TGF-β in experimental models

In the beginning of this work, we sought to investigate if TGF-β_1 was present in peritoneal tissue during surgery and further investigate local fibrinolytic effects on the mesothelial cell layer. Given the fibrotic effect of TGF-β_1, together with reduced fibrinolytic capacity, a reasonable approach to reduce adhesion formation seemed to be to reduce the expression of TGF-β in the peritoneal cavity. However, the final paper suggests that TGF-β has a multitude of effects and that this might not be an entirely correct conclusion.

The biological effects of TGF-β include an ability to initiate synthesis in new tissue, interaction with interleukins and cytokines in the immune system, effects on cell proliferation, and initiation of cell differentiation in a variety of cell systems (76).
The different biological functions of TGF-β_1 have been investigated in a variety of cell culture models. Idell *et al* (66) described a model of the injured lung where cultured

fibroblasts decreased their fibrinolytic capacity by increasing the levels of PAI-1 when TGF-β_1 was added and concluded that TGF-β_1 favoured extravascular fibrin deposition. Moreover, Overall *et al* (88) described a model with calvarial bone cells together with fibroblast and found that not only the secretion of PAI-1 was increased, but also the release of TIMP, together resulting in net accumulation of connective tissue.
When the release of MMPs and TIMPs from the mesothelial lining in the abdominal cavity was investigated in cultured mesothelial cells and monocytes Ma *et al* (160) found that both MMPs and TIMPs were differentially regulated by TGF-β_1. Furthermore, Saed *et al* (165) investigated fibrosis and adhesion formation in a hypoxia model of the peritoneal cavity by culturing mesothelial cells with a minimum of oxygen present. The authors found an increase in Collagen III release from the mesothelial cells after TGF-β_1 treatment. Interestingly, the authors also found an increase in TGF-β_1 release in the hypoxia model indicating a pronounced effect of TGF-β_1 during hypoxic conditions, which is most likely to occur during tissue ischemia. Ischemia was early on identified as a strong stimulus for adhesion formation (166).

TGF-β_1 has a central role in wound healing and scar formation by stimulating fibrosis. This has also been investigated further using experimental models in animals. Roberts *et al* (87) found that granulomas were formed together with collagen production from fibroblasts when TGF-β_1 was injected in newborn mice. During formation of adhesions this is further supported in an experimental rat model described by Williams *et al* (96) where the number and severity of adhesions after addition of TGF-β_1 were increased during abdominal surgery. The role of TGF-β_1 in adhesion formation has been further described by Lucas *et al* (97). The authors found that a standardized surgical procedure induced adhesion formation, and by adding an antibody directed specifically to TGF-β_1 adhesions could be reduced. However, when using an antibody specifically directed to TGF-β_2, the adhesions remained. This indicates that TGF-β_1 is more important in this context than TGF-β_2. This is in part in contrast with the work done by Gorvy *et al* (149) where an experimental mouse model was developed. The authors manipulated all different TGF-β isoforms to see which isoform of TGF-β was the most important in adhesion formation. Their results demonstrated that by blocking both TGF-β_1 and TGF-β_2, adhesion formation could be reduced. The reason for this discrepancy is not known, however, Gorvy *et al* (149) suggested that the release of the different isoforms is taking place at different time points that is not reflected in the previous study (97).

When different TGF-β isoforms were investigated in humans, Chegini *et al* (146) found that both adhesion tissue and serosal tissue of various peritoneal organs express TGF-β_1 and TGF-β_3 differently during surgery. The authors concluded that TGF-β_1 was more critical in altering the outcome of adhesion formation and that the involvement of TGF-β_3 remained to be further investigated. In the present study (Paper V), all TGF-β isoforms were measured and it was notable that active fractions of TGF-β_{1-2} were increased in the peritoneal serosal fluid compared to plasma samples.

In cell proliferation, TGF-β_1 has ability to affect the cell cycle, growth arrest, proliferation and apoptosis. Additionally, TGF-β_1 has previously been reported to inhibit cell proliferation (83). In an experimental model with omentally derived mesothelial cells, Pierro *et al* (159) demonstrated that addition of TGF-β_1 decreased the proliferation rate more than 60% compared to untreated control. This could also be confirmed by Matsuo *et al* (167) when the long term effects of CAPD was investigated and the authors found a decrease in mesothelial proliferation with increasing concentrations of TGF-β_1. Similar patterns have also been described by Zhong *et al* (168). However, Mutsaers *et al* (169) reported that TGF-β_1 did not have any effect on the proliferation rate.

Hence there are conflicting reports regarding effects of TGF-β isoforms on mesothelial cell proliferation. A possible explanation might be that the different TGF-β isoforms have different biological functions depending on the concentration. The results obtained during this work would support such a notion. Various concentrations of TGF-β_1 have been used, together with different sources of mesothelial cells potentially explaining the divergent results. To summarize these thoughts graphically, a schematic overview is presented representing the published literature and results from the current thesis (Figure 21). The x-axis in the centre demonstrates the concentrations of TGF-$\beta_1$together with different biological functions in relation to concentration.

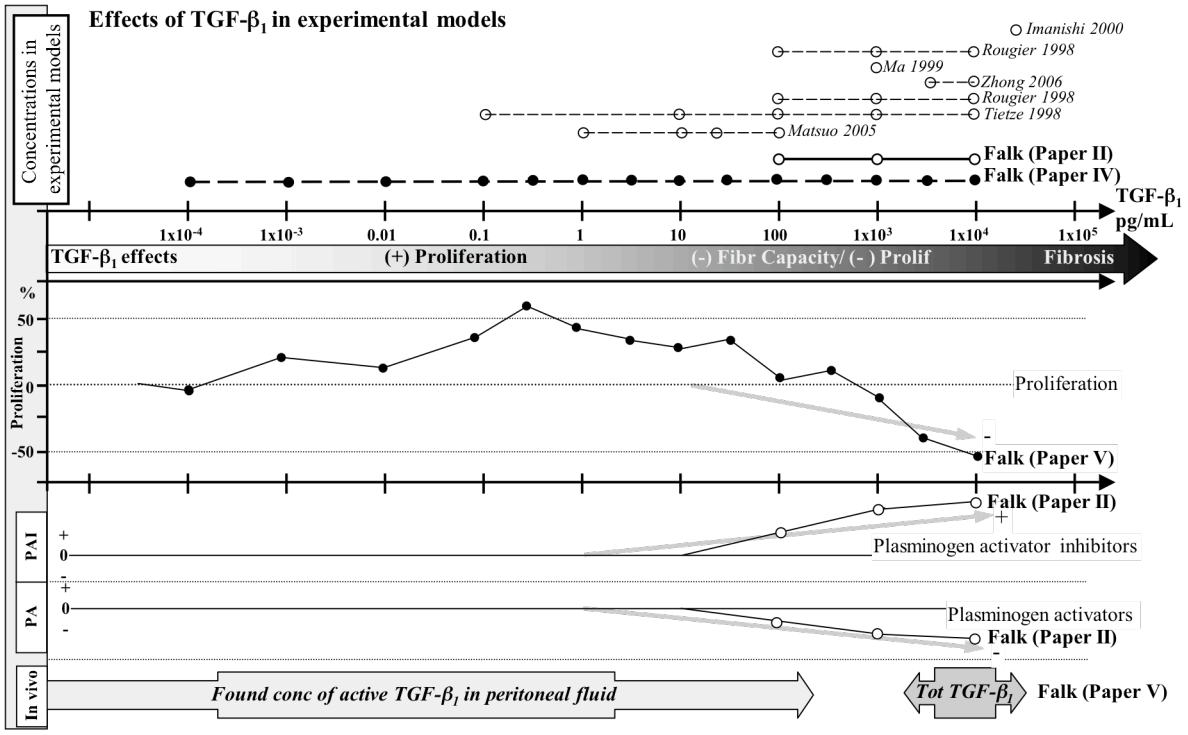

Figure 21: Effects of different TGF-β_1 concentrations used in experimental models. **(Top):** Used concentrations of TGF-β_1 in different experimental models. Increasing concentration from left to right (pg/mL). **(Middle):** Effects of different TGF-β_1 concentrations in mesothelial proliferation rate (gray decreasing arrow indicate found effects in literature). **(Down):** Effects of different TGF-β_1 concentrations on fibrinolytic components (gray arrow indicate increase in PAI and decrease in PA found in literaure). **(Bottom):** Found concentrations of TGF-β_1 in peritoneal fluid during surgery. (PAI=Plasminogen activator inhibitors, PA=Plasminogen activators)

A decrease in mesothelial proliferation rate at certain concentrations was confirmed in the present study and the results also indicate that this effect is concentration dependent. For the higher concentrations, a 50% reduction in cell proliferation was observed when cell cultures were exposed to TGF-$\beta_{1\text{-}3}$ isoforms. However there was an increase in mesothelial cell proliferation at lower concentrations. Importantly, this concentration is similar to the TGF-β_1 concentration found in the peritoneal serosal fluid from patients during abdominal surgery. It is therefore likely that, in a surgical context, TGF-β_1 would have a stimulatory effect on cell proliferation.

The role of uPA in the peritoneal cavity is poorly understood. Since the main plasminogen activator in the peritoneal cavity is t-PA, the role of uPA might not be so important in clearing fibrin deposits from the cavity. However, a possible role for uPA in the peritoneal cavity might be, alone or in combination with t-PA, local activation of small amounts of TGF-β through the plasmin system.

Hyaluronan in experimental models

Hyaluronan is present in most tissues and body fluid and has many biological functions (99). One of these is enhancement of the healing process with typically a high concentration of hyaluronan in the healing wound (100, 101). Moreover, hyaluronan has been described to induce mitosis and detachment and movements of cells (104-106) as it provides a hydrated matrix that facilitates cell migration (107).
The use of hyaluronan based agents in the prevention of adhesions has been used in both experimental models in animals (108-111) and in clinical use with a documented reduction of adhesions (112-117).

Glycosaminoglycans including hyaluronan have been reported to enhance movement and locomotion of transformed cells (106). It is also known that hyaluronan is important in angiogenesis (154). Different molecular weights have been reported to have different effects. Suzuki *et al* (102) investigated in an experimental inflammation model, the effects on macrophage function and reported dose dependent inhibition of phagocytosis with increasing concentrations and molecular weight of hyaluronan. The results indicate that hyaluronan with the highest molecular weight had the most profound effect. Reijnen *et al* (142) described an experimental model of peritonitis in the rat where adhesion formation was evaluated. The authors concluded that both adhesion formation and abscess formation was reduced when using a 0.4 % hyaluronan solution. Hence, it seems as hyaluronan can affect tissue or host response to an inflammatory stimuli.

In this thesis, a similar observation was made in Paper III where it was observed that hyaluronan could modulate the cellular response to inflammatory mediators. The most profound effect was seen with increasing levels of t-PA in cell lysates, and decreasing levels of PAI-1 in the conditioned culture media. In the present study, high molecular weight hyaluronan was used and it might be that hyaluronan with different molecular weight have different effects, in analogy with TGF-β isoforms.

In the experimental models (Paper IV), the mesothelial proliferation rate was increased with increasing concentrations of hyaluronan. Influence of inflammatory mediators for a longer period of time decreased cell proliferation. However, the presence of hyaluronan reduced this

effect, indicating that presence of a hyaluronan based adhesion barrier can have beneficial biological effects, even in the presence of inflammation.

SUMMARY AND CONCLUSIONS

In this thesis, the focus has been on the use of different experimental models to study effects of TGF-β isoforms and hyaluronan during conditions that would reflect common conditions in a surgical setting. From these experimental models it can be concluded:

- TGF-β_1 is present in peritoneal tissue and higher levels are associated with adhesions.

- TGF-β_1 can reduce mesothelial cell fibrinolytic capacity *in vitro* by decreasing the production of t-PA and by increasing the secretion of the inhibitor PAI-1.

- Hyaluronan can affect mesothelial cell fibrinolysis *in vitro* by increasing t-PA and decreasing the secretion of the inhibitor PAI-1.

- Hyaluronan can affect mesothelial cell proliferation in both attached and non-attached cultured human mesothelial cells.

- All three TGF-β isoforms are present in the human peritoneal cavity during surgery, Detected concentrations of TGF-β_1 had a stimulatory effect on cultured mesothelial cell proliferation rate, whereas the effect of the two other isoforms were more ambiguous. In general, a greater proportion of TGF-β isoforms were in an active configuration, indicating that an activation occurred in the peritoneal environment.

- Finally, although TGF-β isoforms, in particular TGF-β_1, generally promotes fibrosis, and in this work has been demonstrated to reduce mesothelial fibrinolytic capacity and thereby facilitate the formation of post-surgical adhesions, its effects may not be entirely negative. This thesis also indicates that TGF-β_1 can increase mesothelial cell proliferation rate, which might be beneficial in the post-surgical repair phase.

Sammanfattning på svenska

Experimentella modeller av den humana peritoneala miljön: Effekter av TGF-β och hyaluronan

Sammanväxningar (adherenser) efter bukkirurgi är ett stort och ännu olöst problem. Balans mellan enzymsystem bestående av bland annat plasminogen aktivatorer (t-PA, uPA) och plasminogen inhibitorer (PAI) är av betydelse för postoperativ peritoneal läkning/adherensbildning. Dessa enzymer frisätts bland annat lokalt av mesotelceller i bukhålan, vilka har en viktig roll vid reglering av den fibrinolytiska kapaciteten. Transforming growth factor beta (TGF-β) är en cytokin med många funktioner och effekter däribland fibrosbildning och har i djurmodeller visat sig påverka bildningen av adherenser. Hyaluronsyra (hyaluronan) förekommer naturligt i kroppen samt har även i kliniska studier visat sig reducera förekomsten av adherenser. Hur TGF-β påverkar fibrinolysen i bukhålan, samt vilka mekanismer som förklarar den adherensreducerande effekten av hyaluronan är oklara. Att studera dessa processer på människa har begränsningar och det är därför nödvändigt med experimentella modeller.

Syftet med dessa studier var att analysera lokal peritoneal fibrinolys samt i olika experimentella modeller studera effekter av TGF-β och hyaluronan på odlade humana mesotelceller.

Studie I: I human peritoneum studerades nivåerna av TGF-β_1 och fibrinolyskomponenter (t-PA, uPA och PAI-1) samt deras relation till adherenser efter tidigare kirurgi. Resultatet visade att det fanns ett samband mellan ökade nivåer av TGF-β_1 och ökad svårighetsgrad av adherenser. Dessutom fanns ett samband mellan ökade nivåer av TGF-β_1 och minskad fibrinolytisk kapacitet, genom ökad PAI-1.
Studie II: I experimentella modeller odlades humana mesotelceller vilka utsattes för stigande koncentrationer av TGF-β_1. Frisättningen av fibrinolyskomponenter i odlingsmediet, samt mRNA uttryck från cellerna visade dosberoende minskning av t-PA produktion, samtidigt som frisättning av PAI-1 ökade med stigande koncentration av TGF-β_1.
Studie III: I en liknande modell (Studie II) studerades effekten av stigande koncentrationer hyaluronan på odlade mesotelceller. Resultaten visade en ökad fibrinolytisk kapacitet genom ökande aktivatorer (t-PA) och minskande hämmare (PAI-1) hos odlade mesotelceller.

Studie IV: För att ytterligare undersöka effekten av hyaluronan studerades cellproliferation hos mesotelceller. Resultaten visade att cellproliferationen ökade med stigande koncentration hyaluronan samt att denna tycktes motverka en negativ proliferativ effekt av inflammatoriska mediatorer.
Studie V: Med kunskap från de tidigare studierna samt tidigare beskrivna modeller studerades TGF-β_1, -β_2, och -β_3 i human peritonealvätska. Vidare studerades cellproliferation med dessa isoformer hos odlade mesotelceller. I jämförelse med respektive plasmanivåer visade det sig att de aktiva fraktionerna av TGF-β_1 och -β_2 var förhöjda i peritonealvätska. Celler som stimulerades med koncentrationer av TGF-β_1 som motsvarar de normalt förekommande i bukhålan ökade cellproliferationen jämfört med kontroll, medan högre koncentrationer hade en hämmande effekt.

Konklusion: Vi fann att TGF-β_1 förekom i peritoneum samt i adherensvävnad. En påvisad korrelation med PAI-1 kan utgöra en bidragande faktor vid post-operativ adherensbildning, detta kunde senare stödjas med experimentella modeller på odlade celler. Fynden talar för att i bukhålan befintliga nivåer av TGF-β_1 kan stimulera mesotelcellers proliferation samt bidra till att bibehålla den fibrinolytiska kapaciteten i bukhålan, en viktig kunskap för ökad förståelse av postoperativ adherensbildning.
De experimentella studierna av hyaluronan kan till viss del vara en förklaringsmodell som stöder kliniska observationer avseende dess adherensförhindrande effekt. Tänkbara bakomliggande mekanismer kan vara ökad lokal fibrinolytisk kapacitet tillsammans med ökad cellproliferation.

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