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Applications of hyaluronan in abdominal surgery

An experimental study

C.J.J.M. Sikkink

Stellingen behorende bij het proefschrift

APPLICATIONS OF HYALURONAN IN ABDOMINAL SURGERY

AN EXPERIMENTAL STUDY

- Humane mesotheelcellen, verkregen uit omentum, alsook uit peritoneaalvocht zijn geschikt voor het opkweken van cellijnen, zonder verlies van specifieke eigenschappen, zelfs na bevroren opslag. *(Dit proefschrift)*

- Cellen van het monocyten-macrophagensysteem moduleren de fibrinolytische capaciteit van humane peritoneale mesotheelcellen en interfereren met de hualuronzuur-geassocieerde veranderingen in de mesotheliale fibrinolytische capaciteit. *(Dit proefschrift)*

- Hyaluronzuur gel vermindert intra-abdominale adhesies en abcesvorming niet in een experimenteel peritonitismodel. *(Dit proefschrift)*

- Met gelabelde liposomen is het mogelijk om zowel adhesies als abcessen te detecteren. *(Dit proefschrift)*

- Een gecombineerde mesh van polypropyleen met hyaluronzuurmembraan benadert de ideale mesh voor herniaherstel het meest in een dierexperimenteel model. *(Dit proefschrift)*

- Hyaluronzuur heeft geen uitgesproken remmend effect op intraperitoneale tumor implantatie en groei in een diermodel. *(Dit proefschrift)*

- De immense populariteit van sociale netwerken zoals facebook en hyves staat in sterk contrast met de angst voor schending van de privacy door introductie van het landelijk EPD.

- Een goed arts neemt zijn patiënten en werk zeer serieus, zichzelf niet al te.

- Het goedkoopst is zelden synoniem met kwalitatief het beste. Toch wordt de indruk gewekt dat dit fenomeen in de zorg geen opgeld doet.

- Men moet patiënten niet opereren op hun sterfdag (uit De Heelmeesters, Abraham Verghese)

- Mensen die niets te bieden hebben, bieden hoop. (Arnon Grunberg)

- Sommige vragen zijn zo goed dat het jammer zou zijn ze met een antwoord te verknoeien. (Harry Mulisch)

APPLICATIONS OF HYALURONAN IN ABDOMINAL SURGERY

AN EXPERIMENTAL STUDY

C.J.J.M. Sikkink

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AN EXPERIMENTAL STUDY

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Promotor:
prof.dr. R.P. Bleichrodt

Copromotoren:
dr. H. van Goor
dr. M.M.P.J. Reijnen (Rijnstate Ziekenhuis)

Manuscriptcommissie:
prof. dr. W.F.J. Feitz (voorzitter)
prof. dr. J.F. Lange (Erasmus Medisch Centrum)
dr. X.F. Walboomers

Paranimfen:
Drs. H.M.E. Coveliers
Drs. J.P.J. Paas

Contents

CHAPTER 1
GENERAL INTRODUCTION

GENERAL INTRODUCTION

Intra-abdominal adhesion and abscess formation; clinical impact and pathophysiology

Abdominal surgery inevitably leads to peritoneal trauma. Peritoneal trauma, how insignificant it may seem, can lead to the formation of adhesions. Adhesions are responsible for considerable morbidity and even mortality and have an enormous impact on the health care burden. Adhesions are the main cause of intestinal obstruction in the Western world, accounting for 40% of cases and are responsible for over two thirds of the readmissions for small bowel obstruction[1]. After conventional colorectal surgery, approximately 20% of patients is readmitted within 4 years after the operation, for reasons directly or indirectly related to adhesions[2]. Other, well-known and frequent complications of adhesions are chronic abdominal and pelvic pain, secondary infertility in women and inadvertent enterotomy during relaparotomy[3-5].

Adhesions arise from peritoneal trauma, which can be induced by surgery, trauma or inflammation. Mesothelial cells are loosely interconnected and easily detach from their basal membrane, even after delicate trauma. Subsequently an inflammatory response activates the coagulation cascade, resulting in a cascade of events in order to achieve reepithelialization. On the damaged areas a fibrin matrix is formed. Under normal circumstances the fibrinolytic system will eventually lyse the fibrin deposits and new mesothelial cells will reepithelialize the denuded areas within a week[6, 7].

Beside activation of the coagulation cascade, surgery and infection will reduce fibrinolysis, mainly due to increased plasminogen activator inhibitor type-1 (PAI-1) levels and decreased tissue-type plasminogen activator (tPA) levels[7-11]. When fibrin deposits are not lysed, the clots are invaded by fibrocollagenous tissue, thus forming fibrous adhesions.

In case of intra-abdominal infection, bacteria can be caught in fibrin clots, protecting microorganisms against the immunologic defenses in the abdomen. These contaminated fibrin clots can act as a nidus for abscess formation. Intra-abdominal abscesses are a frequent complication of peritonitis and carry a high morbidity. Dense adhesions occur around abscesses, which makes early surgical interventions hazardous. In severe cases of peritonitis, multiple relaparotomies and the use of temporary closure devices may be necessary. These reinterventions aggravate the formation of adhesions and carry a high risk of complications such as serosal bleeding, inadvertent enterotomy and fistula formation[5]. So, adhesion formation after intra-abdominal sepsis is a major problem complicating future surgery.

Adhesion related complications underline the urge for optimal adhesion prevention during abdominal surgery. Nevertheless the interest in this topic is limited since the magnitude of the problem is often underestimated, the knowledge on the subject is limited and the efficacy of preventive measures is negligible. The awareness about the consequences of adhesions is also hampered because surgeons are seldom confronted with these complications in their own patients because most complications are acute and present some time after the initial procedure.

Hyaluronan

Although the majority of adhesions will have no clinical implications, the incidence and the severity of complications caused by adhesions are much too high to ignore. Many studies have focused on prevention of adhesions and intra-abdominal abscesses. Apart from good surgical technique and avoiding adhesiogenic foreign materials, much attention is paid to the use of antiadhesive agents. Until now, hyaluronan-based antiadhesive agents are among the most intensive studied products, and probably are the most successful as well.

Hyaluronan is a polysaccharide of repeating disaccharide units of sodium glucuronate and N-acetyl-glucosamine linked by glycosidic bonds. Initially it was described as hyaluronic acid, a polysaccharide isolated from the vitreous fluid of the eye (hyalos) that contained uronic acid[12]. Later the name hyaluronan was more generally used[13]. Hyaluronate is another frequently used synonym.

Hyaluronan is found in almost all tissues and fluids of vertebrates, especially in connective tissue[14]. Hyaluronan is generally known to be associated with tissue repair and wound healing. In healing tissues the concentration of hyaluronan is high[15, 16]. Its biological functions appear to be the result of both its physicochemical properties and its biological interactions[17]. Hyaluronan is a hygroscopic macromolecule, which makes hyaluronan solutions highly osmotic and important for water homeostasis. Hyaluronan can modulate the inflammatory respons by influencing cytokine production, and facilitation of adhesion of cytokine-activated lymphocytes. In a next phase cell migration and proliferation are facilitated[17-19]. Hyaluronan also plays a role in the downregulation of the inflammatory response. Its free-radical scavenging and antioxidant properties and its supposed inhibiting effect on proteinases, seem to be responsible for this downregulation, stabilizing the granulation tissue during the healing process. These processes and the influence of hyaluronan on these processes are complex and much is still unclear. This is equally true for the role of hyaluronan in adhesion and abscess prevention.

Despite the widespread use of hyaluronan-based antiadhesive agents, there is much debate on the exact mechanism of action of hyaluronan in adhesion prevention. Mechanical separation of traumatized peritoneal surfaces is probably one of the most important mechanisms. Hyaluronan solutions are thought to create a pool in which the intestines float, keeping them separated from each other as well as from the peritoneal lining of the abdominal cavity. Due to this "hydroflotation", uninterrupted healing of the mesothelial lining is possible. The short half-life of hyaluronan solutions, however seems to interfere with optimal adhesion prevention. Hyaluronan-based gels and membranes have been developed to solve this problem. Membranes create a mechanical barrier, whereas the mechanism of gels is probably between creation of hydroflotation and creating a barrier.
Apart from the mechanical explanation, hyaluronan has a biological mechanism of action in adhesion prevention. Hyaluronan increases the proliferation of mesothelial cells in vitro[20, 21]. Part of the adhesion reducing capacity of hyaluronan might thus be explained by its ability to accelerate peritoneal healing due to stimulation of mesothelial cell proliferation. The impact of hyaluronan on fibrinolysis has been subject of many studies, but yet its influence is still unclear. In vitro, hyaluronan has shown to increase the fibrinolytic response of mesothelial cells, due to a decrease of PAI-1 release and an increase of the intracellular tPA concentration[22]. However, other in vitro and in vivo studies showed contradicting results[23-25]. Detailed information on the topic of hyaluronan and its effects on intra-abdominal adhesion and abscess formation was already described by Reijnen[26]. Although its working mechanism is not completely understood, hyaluronan is well-known for its antiadhesive properties, as stated above[27-33]. In clinical studies hyaluronan reduces the incidence, severity and extent of adhesions after abdominal surgery. However, the incidence of intestinal obstruction is not reduced. Two recent reviews support these conclusions[34, 35].

Hyaluronan and infection

Reijnen et al. were among the first to describe the use of hyaluronan in a peritonitis model in rats[36, 37]. Hyaluronan membrane failed to show a favourable effect, whereas hyaluronan solution reduced the severity of adhesions and the incidence of abscesses. High volumes of 0.2% and 0.4% hyaluronan solution proved to be most effective, suggesting that the "hydroflotation effect" was an important factor as well. In a similar experiment, Tüzüner et al. found similar results showing that hyaluronan solution was superior to hyaluronan membrane[38]. Hyaluronan solution reduced both incidence and severity of adhesions, whereas

hyaluronan membrane reduced only the severity. No differences were found with regard to morbidity or abscess formation. Unfortunately, withdrawal of the used hyaluronan solution from the market prohibits further research.
Tzianabos et al. proved that hyaluronan membrane is safe when used in rats with generalized peritonitis[39]. In a second experiment they used a modified hyaluronan membrane with glycerol in rats with generalized peritonitis and found no negative effects on morbidity and mortality. In contrast with these outcomes, ferric hyaluronan gel was found to have serious adverse events. In rats with generalized peritonitis, application of ferric hyaluronan gel resulted in almost 100% mortality compared to 49% in controls. Possible explanations pointed at presumed negative properties of iron under these circumstances. Unfortunately the effect on adhesion formation was not evaluated. Ghellai et al. described a trend towards increased abscess formation, using hyaluronan membrane in generalized peritonitis in rats[40]. Hyaluronan did not affect the amount or strength of adhesions after cecal ligation or cecal ligation and puncture in this study. Kayaoglu et al. also studied the earlier mentioned modified hyaluronan membrane, with glycerol added[41]. Its use resulted in increased adhesion formation during bacterial peritonitis. Uchida et al. used hyaluronan membrane in an experimental study in rats using a cecal ligation and puncture model[42]. Use of hyaluronan, did not show significant differences in serum inflammatory cytokine levels.
Several clinical studies using hyaluronan in the presence of contamination or overt infection have been performed. Tang et al. used ferric hyaluronan gel in patients undergoing elective colorectal surgery[43]. The study was terminated prematurely due to an increase of anastomotic leaks in the intervention group. Cohen et al. applied the hyaluronan/ glycerol membrane in patients undergoing restorative proctocolectomy and ileal pouch-anal anastomosis[31]. The hyaluronan/ glycerol membrane reduced the incidence, extent and severity of adhesions, but increased incidence of infectious complications. Vrijland et al. used hyaluronan membrane in a randomized controlled trial in patients who underwent a Hartmann procedure for sigmoid diverticulitis or obstruction[28]. 81% of patients had a peritonitis. Hyaluronan membrane did reduce the severity of adhesions, although the incidence was not significantly altered. Uchida et al. studied patients who underwent surgery for colorectal cancer[42]. The patients were retrospectively divided into two groups: in one group hyaluronan membrane was applied just before abdominal wall closure. In the other group no hyaluronan membrane was used. No adverse events were found in the hyaluronan group.
In conclusion, there are only limited data on hyaluronan and infection. The adverse results of the few studies preclude definite statements on the

effectiveness of hyaluronan, regarding adhesion and abscess prevention, under infectious conditions.

Hyaluronan and tumor

In abdominal surgery, a considerable amount of procedures is performed for malignant disease. Little is known about the effect of the application of hyaluronan during oncologic surgical procedures[32, 42, 44]. Although no negative side effects have been mentioned up to now, both positive and negative effects of hyaluronan on tumor anchorage and growth are imaginable. On one hand, hyaluronan may promote anchorage, independent growth, and invasiveness of cancer cells as described by Toole[45, 46]. On the other hand, the antiadhesive properties of hyaluronan and its presumed influence on peritoneal healing can support a theory of prevention of tumor adherence and growth. In conclusion, the data on the application of hyaluronan during oncologic surgical procedures are too scarce to draw any conclusions.

Aim of the studies

As described above, hyaluronan has acknowledged antiadhesive properties. Nevertheless the studies focusing on its use under the most challenging conditions with regard to adhesion prevention are scarce. Use of hyaluronan under inflammatory and infectious conditions warrants further research, focusing both on descriptive basal studies, as well on interventional studies. In chapter two we reviewed the use of hyaluronan-based antiadhesive agents in abdominal surgery.
Mesothelial cells play a central role in the the (patho)biology of the peritoneal cavity, in the maintenance of the peritoneal membrane, and in tissue repair. To study the effects of hyaluronan on mesothelial cells, in vitro studies are inevitable. Cultures of human cells are usually used for this purpose. However, little is known about the differences between mesothelial cells isolated from different sources. This and the effects of cell propagation and storage at low temperatures were the objectives of the study in chapter three. Effects of different stimuli and hyaluronan were studied.
The balance between fibrinolysis and coagulation after peritoneal trauma and infection is decisive in the development of adhesions and eventually abscesses. In chapter four, an in vitro study is described, focusing on the fibrinolytic response of human peritoneal mesothelial cells, after stimulation with lipopolysaccharide. Especially the influence of monocytes, macrophages and hyaluronan were evaluated.

Some of the earlier interventional studies showed a beneficial effect of hyaluronan solutions under contaminated conditions. In chapter five, a hyaluronan-based gel was compared to saline, in order to determine if a gel, that remains longer in the abdominal cavity, gives better results with respect to adhesion and abscess formation in a peritonitis model in rats.
Evaluation of the adhesion and abscess reducing capacities of antiadhesive agents is of utmost importance. Most data come from experimental studies. In human clinical studies, therapy evaluation is a problem. The study in chapter six investigated whether it was possible to use a new diagnostic technique, using ^{99m}Tc-PEG-liposomes, to detect adhesions and abscesses. Again a peritonitis model in rats was used and the earlier positive experiences with hyaluronan could now be evaluated once more, potentially even using a non-invasive technique.
Incisional hernias occur in 10-15% after laparotomy. Mesh repair is the preferred treatment of these hernias. These prosthetic materials generate a local inflammatory response that ultimately results in adhesion formation. The inflammatory respons depends on the chemical and physical properties of the meshes. The study in chapter seven focuses on adhesion formation after mesh repair and the potential prevention of adhesions by hyaluronan. Both combinations of antiadhesives with mesh and composite meshes were studied, again with a central role for hyaluronan.
The pathways of adhesion and abscess formation are, as stated earlier, common, resulting from an inflammatory cascade after injury. The mechanism of tumor implantation on the peritoneum after oncologic bowel surgery is presumed to be dominated by factors involved in wound healing and adhesion formation as well. Although use of hyaluronan during oncologic resections is controversial, the presumed similarities of these pathways, resulted in the hypothesis that the antiadhesive agent hyaluronan could reduce peritoneal tumor implantation. In the study in chapter eight we investigated the influence of hyaluronan membrane on tumor cell implantation in the abdomen of mice and rats. The study was not primarily designed to answer the question whether hyaluronan can be used safely during oncologic abdominal surgery, but focused on a possible “tumor implantation diminishing” effect.

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CHAPTER 2

HYALURONAN-BASED ANTIADHESIVE AGENTS IN ABDOMINAL SURGERY: APPLICATIONS, RESULTS, AND MECHANISMS OF ACTION

C.J.J.M. Sikkink
C.J. Zeebregts
M.M.P.J. Reijnen

Surg Technol Int. 2007; 16: 19-29.

Abstract

Postsurgical intra-abdominal adhesions cause significant morbidity and mortality, with small bowel obstruction being the most common complication. The urge to prevent adhesion formation has resulted in multiple experimental and clinical trials and the development of numerous antiadhesive agents. Through the years, hyaluronan-based antiadhesives have proved to be successful in the reduction of adhesion formation. Despite the obvious effectiveness of hyaluronan, there is still much debate on its clinical use and mechanisms of action. Various hyaluronan-containing products have been introduced and withdrawn from the market. The application of hyaluronan in combination with meshes for hernia repair appears to be a promising concept. Not all different applications of hyaluronan are well-known and its use in patients with a malignancy or abdominal infection remains controversial. Here an overview is given on the effects of hyaluronan-based antiadhesive agents in abdominal surgery, its use in infectious conditions, and its oncologic repercussions. The most important mechanism of action appears to be the mechanical separation of damaged peritoneal surfaces. However, the biological effects of hyaluronan, such as modulation of cell proliferation and peritoneal biology, might also be of influence.

Introduction

Peritoneal trauma during abdominal surgery and abdominal infection can lead to intra-abdominal adhesion formation (Fig. 1). Adhesions, especially when excessive, can cause severe complications and are responsible for considerable morbidity and mortality. Adhesions are the main cause of intestinal obstruction in the developed world and account for approximately 70% of readmissions for small bowel obstruction[1]. After conventional colorectal surgery, one out of five patients is readmitted for reasons directly or indirectly related to adhesions within 4 years after the operation[2]. The relative risk of adhesion-related complications in this group is 29.7 per 100 initial procedures over 4 years time. Furthermore, adhesions account for 15% to 20% of cases of secondary infertility in women and are associated with chronic abdominal and pelvic pain [3, 4]. Relaparotomies are complicated by the presence of adhesions as well; procedures are longer and the risk of inadvertent enterotomy is approximately 20%, which in turn is associated with a higher incidence of postoperative complications, an increased risk of admission to intensive care units, and prolonged hospital stays[5]. Complication rates may be even higher when adhesions are accompanied by abdominal infection and abscess formation. The high incidence of adhesion-related complications, their severity, and the obvious impact on the health care burden urge attention for the prevention of postsurgical adhesion formation.

Mechanical separation of adhesiogenic wound surfaces during the first phase of peritoneal healing – which takes 5 days to 7 days – is the most common concept of adhesion prevention. During the last decades, several mechanical barriers have been developed. Membranes of oxidized regenerated cellulose or expanded polytetrafluoroethylene have been demonstrated to decrease the incidence of adhesion formation[6-9]. However, oxidized regenerated cellulose was less effective in the presence of blood. Expanded polytetrafluoroethylene may not be the ideal antiadhesive, as it is a permanent device; it remains in situ on the injured site where it is placed, prone for device-related complications.

Hyaluronan (HA) is a polysaccharide made up of repeating disaccharide units of sodium glucuronate and N-acetyl-glucosamine linked by glycosidic bonds (Fig. 2). In 1934, Meyer and Palmer were the first to describe hyaluronic acid, a polysaccharide isolated from the vitreous fluid (hyalos) that contained uronic acid[10]. Later, the name hyaluronan was introduced[11]. The intraperitoneal application of HA derivates was considered a promising concept for the reduction of adhesions. Today HA is well-known for its antiadhesive properties and has been studied extensively. Currently, HA-based agents are the most

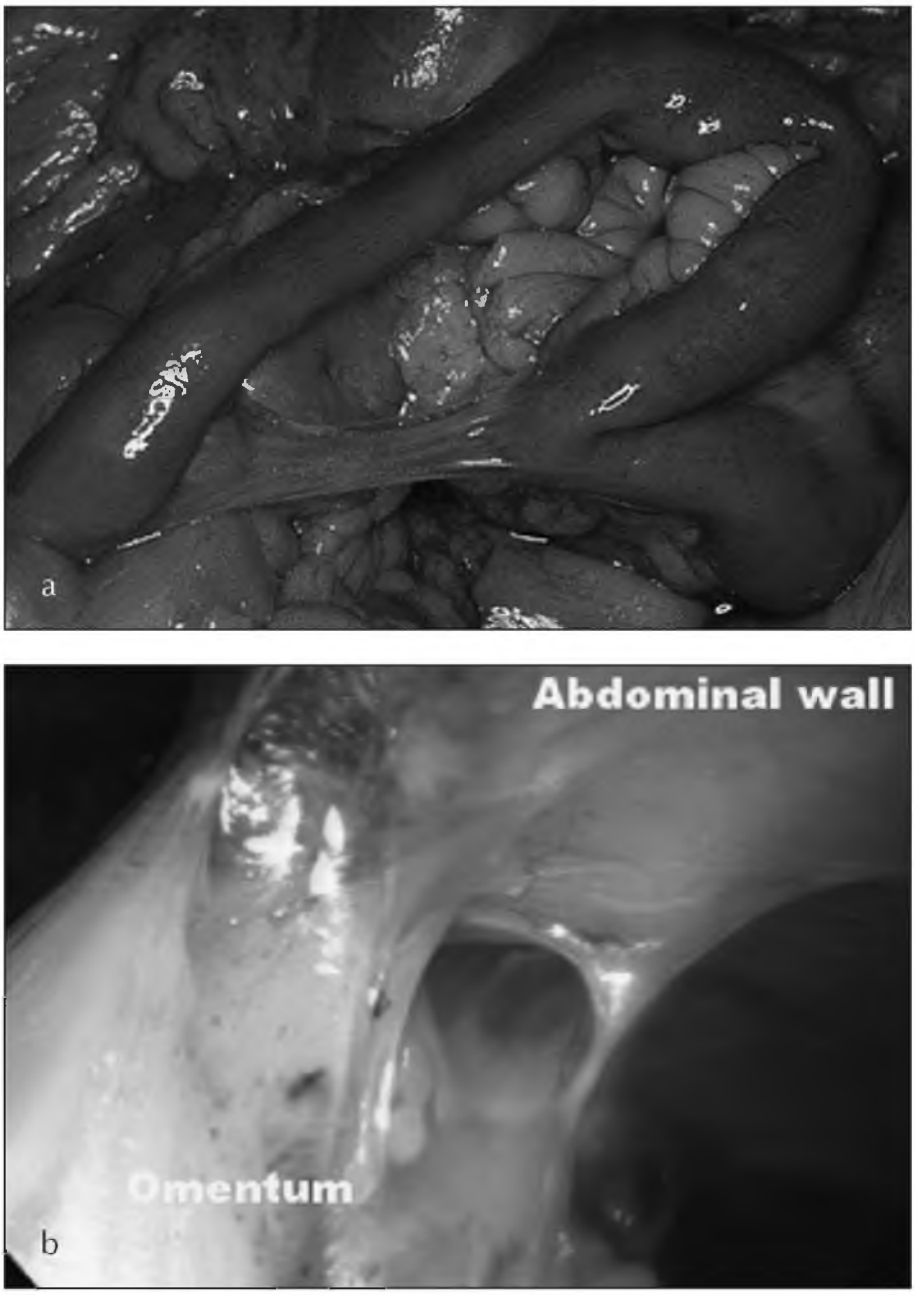

Figure 1. (a) Intra-abdominal adhesion between small bowel loops. (b) Intra-abdominal adhesion between omentum and the abdominal wall.

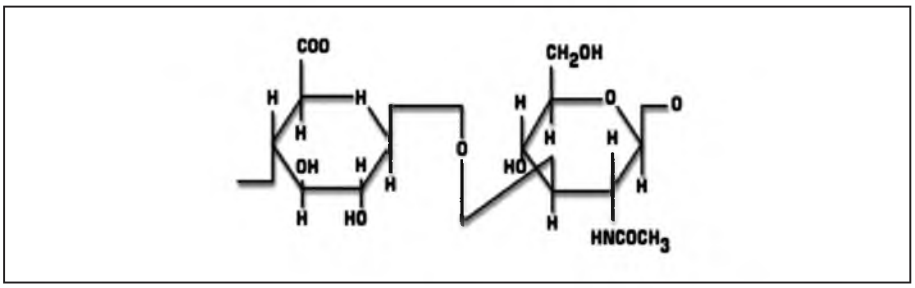

Figure 2. Hyaluronan is a polysaccharide made up of repeating disaccharide units of sodium glucuronate and N-acetyl-glucosamine linked by glycosidic bonds.

frequently used antiadhesive agents worldwide with an undisputed adhesion reducing effect. Use of HA under contaminated or infectious conditions and during oncologic procedures is described as well. However, its use under these conditions is still controversial. In this chapter, an overview is given on the role of HA-based antiadhesive agents in abdominal surgery, with attention for different application forms, mechanisms of action, results, and oncologic repercussions.

Hyaluronan-based antiadhesive agents

1. Sodium Hyaluronan Carboxymethylcellulose Membrane

The hyaluronan-carboxymethylcellulose (CMC) antiadhesive barrier, known as Seprafilm® (Genzyme, Cambridge, MA, USA), is a sterile, bioresorbable membrane (Fig. 3). The US Food and Drug Administration (FDA) approval of the agent was obtained in 1996. The membrane is applied to injured peritoneal spots, thereby acting as a physical barrier to separate traumatized peritoneal layers during the first phase of peritoneal wound healing. It is composed of two polysaccharides – sodium HA and CMC – and turns into a gel within 24 hours to 48 hours, remaining at the site of placement in this gel form for up to 7 days. Experimental studies concerning the use of this HA-CMC membrane are numerous. The report from Burns et al. in 1997 first describes its adhesion-reducing capacity in a rat cecal abrasion and sidewall injury model[12]. Hellebrekers et al. studied various antiadhesive barriers in a rat model, with HA-CMC showing superior results[9]. In another study by Kutlay et al., the HA-CMC membrane was more effective than heparin and aprotinin in a cecal abrasion model in rats[13]. Any presumed fibrinolytic activity of the HA-CMC membrane could not be supported by experimental studies from Reijnen et al. and Tarhan et al.[14, 15].

Figure 3. Application of the HA-CMC membrane in open surgery for digestive disease. A dry white Tyvek® sleeve, in which the transparant HA-CMC membrane is supplied, is used to position the membrane in the abdomen. The Tyvek® sleeve is removed once the hydrophilic HA-CMC membrane has been properly positioned. (Image kindly provided by Genzyme)

In a randomized clinical trial, Becker et al. studied the effect of the HA-CMC membrane in patients with ulcerative colitis or familial polyposis who were scheduled for ileal pouch-anal anastomosis with diverting-loop ileostomy[16]. The included patients were randomized into two groups: in one group, the HA-CMC membrane was placed under the midline incision prior to closure; and in the other group, no antiadhesive agent was applied. Adhesions were evaluated laparoscopically at the time of ileostomy closure 8 weeks to 12 weeks later. The data of 175 patients were analyzed. Only 5 (6%) of 90 control patients were free of adhesions versus 43 (51%) of 85 patients treated with the HA-CMC membrane ($p < 0.001$). The mean percent of incision length involved with adhesions was also significantly greater in the control group (63% versus 23%, $p < 0.001$). Furthermore, the percentage of patients with dense adhesions was significantly higher in the control group (58% versus 15%, $p < 0.001$). The use of the HA-CMC membrane was not related to an increased incidence of adverse events.

In another randomized trial, Diamond studied the effect of the HA-CMC membrane in women undergoing uterine myomectomy[17]. The 127 patients included in the trial were randomized to treatment with the HA-CMC membrane or to no adjunctive antiadhesive treatment at the end of the procedure. Adhesions were assessed during second-look laparoscopy. The mean number of sites adherent to the uterine surface was significantly lower in the HA-CMC membrane treated group (4.98 sites ± 0.52 sites) compared with the no-treatment group (7.88 sites ± 0.48 sites), as were the mean uterine adhesion severity scores (1.94 ± 0.14 versus 2.43 ± 0.10; all values treatment versus no treatment, respectively), mean extent scores (1.23 ± 0.12 versus 1.68 ± 0.10), and the mean area of adhesions (13.2 cm^2 ± 1.67 cm^2 versus 18.7 cm^2 ± 1.66 cm^2). Again, the use of the HA-CMC membrane was not associated with an increase in postoperative complications.

Vrijland et al. assessed the applicability of the HA-CMC membrane in patients requiring a Hartmann's procedure for sigmoid diverticulitis or obstructed rectosigmoid[18]. Patients were randomized to either HA-CMC membrane placement in the pelvis and under the midline incision or no additional antiadhesive treatment. Adhesions were evaluated laparoscopically at second-stage surgery for restoration of bowel continuity. In that study of 42 patients, although the incidence of adhesions did not differ significantly between groups, the severity of the adhesions was significantly reduced in the group treated with the HA-CMC membrane. There was no correlation between the use of the HA-CMC membrane and the incidence of postoperative complications.

In a worldwide trial focusing on the incidence of bowel obstruction, Fazio et al. recently reported on the efficacy of the HA-CMC membrane in patients who underwent intestinal resection[19]. Patients were randomized to be treated with the HA-CMC membrane, which was applied on adhesiogenic areas, or no treatment. The mean follow-up time was 3.5 years. Although the overall rate of bowel obstruction was similar in both groups, the incidence of adhesive small-bowel obstruction requiring reoperation was significantly lower in the group treated with the HA-CMC membrane: 1.8% versus 3.4% ($p < 0.05$). Two other randomized studies have shown the value of the HA-CMC membrane in reducing peristomal adhesions, facilitating early closure, and in reducing postoperative adhesions in pediatric patients[20, 21].

In a safety study by Beck et al., the use of this membrane was not associated with an increased incidence of abdominal or pelvic abscess, pulmonary embolism, or foreign-body reaction[22]. However, wrapping the suture or staple line of a bowel anastomosis with the HA-CMC membrane should be avoided, as this might increase the risk of detrimental sequelae associated with anastomotic leaks.

2. Glycerol Sodium Hyaluronan Carboxymethylcellulose Membrane

The HA-CMC membrane has been modified by adding glycerol (G). G-HA-CMC membrane, also known as Seprafilm II® (Genzyme) was developed to improve the handling characteristics of the HA-CMC membrane. The HA-CMC membrane is known to be brittle and sometimes it can be technically difficult to apply; contact of the membrane with an area other than the desired one can lead to a sticky mass, which is hard to replace. Moreover, the HA-CMC membrane is difficult to use in laparoscopic procedures due to its characteristics. The addition of glycerol made the membrane easier to apply. However, only a few studies evaluated its efficacy and safety.

Kayaoglu et al. studied the use of the G-HA-CMC membrane in a rat adhesion model under clean conditions and during peritonitis[23]. Surprisingly, the use of the membrane did not reduce adhesion formation under clean circumstances and even caused increased adhesion formation in bacterial peritonitis.

Recently, a clinical trial by Cohen et al. reported on the use of the G-HA-CMC membrane in patients undergoing restorative proctocolectomy and ileal pouch-anal anastomosis with diverting loop ileostomy[24]. Indications for surgery were ulcerative colitis and familial polyposis. The evaluation of adhesions was performed during ileostomy closure by means of laparoscopy. A significant reduction in incidence, extent, and severity of adhesions was observed in patients treated with the G-HA-CMC membrane. However, an increased incidence of infectious complications was noted, possibly due to the glycerol application. Further production and marketing was then stopped by the manufacturers.

3. Hyaluronan Solution

The use of solutions of HA to prevent adhesions was already described in the early 1990s. In this group of agents, Sepracoat® (Genzyme) is probably one of the best known products. It is a sterile-filtered, nonpyrogenic 0.4% solution of sodium HA in phosphate buffered saline.

Solutions containing HA have been studied extensively for their adhesion-reducing capacity. Burns et al. performed one of the most extensive experimental studies on this topic[25]. They described the adhesion-reducing capacity of different concentrations of HA solution in a rat cecal abrasion model. The use of the HA solution resulted in a reduced severity of adhesions, with 0.4% HA solution being most successful.

Only one clinical prospective randomized trial has been published to our knowledge describing the use of 0.4% HA solution for the reduction of postsurgical adhesion formation. Diamond assessed the use of 0.4% HA solution

in women who underwent various gynecologic procedures via laparotomy[26]. During the procedure, the peritoneum was repeatedly coated with the 0.4% HA solution or with phosphate-buffered saline, acting as a placebo. After 40 days, adhesions were assessed during second-look laparoscopy. The data of 277 women were available for safety evaluations and of 245 women for efficacy studies. The group treated with the 0.4% HA solution had a significantly lower incidence of adhesions when compared with the placebo group. The proportion of sites involved was lower (0.23 ± 0.02 versus 0.30 ± 0.02, respectively) and the percentage of patients without de novo adhesions was higher (13.1% versus 4.6%, respectively). Furthermore, the use of the 0.4% HA solution resulted in a significantly reduced adhesion extent and severity. In that study, no obvious adverse effects of 0.4% HA solution were described.
Nevertheless, in 1997 the FDA panel voted that Sepracoat® was not yet approvable due to the lack of proven clinical effectiveness. Further studies and evaluation were suggested. However, based upon market dynamics at the time the manufacturers chose not to pursue US market approval. This led to the complete withdrawal of the product from all markets due to business economics (personal communication).

4. 0.5% Ferric Hyaluronan Gel

In the abdominal cavity, an HA solution has a very short residence time and may disappear within hours after placement. Therefore, modifications of the HA solution have been brought to the market to increase its viscosity and prolong its residence time. Crosslinking of HA with ferric ions has resulted in the production of 0.5% ferric HA gel (Gynecare Intergel®, Ethicon, Somerville, NJ, USA), a sterile, nonpyrogenic, viscous solution. Initially, this gel was brought to the market under the name of Lubricoat®.
Few experimental studies on the use of 0.5% ferric HA gel have been published. In 1997, Johns et al. reported on an experimental study on various test formulations applied as peritoneal instillates in a sidewall and uterine horn model in rabbits[27]. Adhesion formation was assessed at 7 days and at 14 days. HA that was not ionically crosslinked was ineffective in reducing adhesions in these models even when applied at high viscosity, whereas the ionically crosslinked formulations of HA with trivalent iron were highly effective. Efficacy improved with increased levels of ionic crosslinking. In contrast, in more recent experimental studies the use of 0.5% ferric HA gel has shown no convincing adhesion-reducing effects in a laparoscopic adhesion model in rabbits and in rats[28, 29].

Thornton et al. assessed the safety and efficacy of 0.5% ferric HA gel in reducing adhesions in patients undergoing abdominal surgery by laparotomy with a planned second-look laparoscopy[30]. Women desirous of fertility, aged from 24 to 41, were randomized to receive either 300 ml of 0.5% ferric HA gel or lactated Ringer's solution at the completion of the procedure. Treated patients had significantly fewer intra-abdominal adhesions and the adhesions that did form in this group were significantly less extensive and less severe. Lundorff et al., who randomized patients undergoing a laparotomy for various gynecological procedures including myomectomy, ovariectomy, salpingostomy and adhesiolysis, performed a similar study[31]. Women were aged 18 to 46 and adhesions were evaluated at 24 sites at second-look laparoscopy 6 weeks to 12 weeks later. The ferric HA group had significantly fewer adhesions and again the adhesions that formed despite the use of ferric HA were significantly less extensive and less severe compared with controls. The American Fertility Society score for adnexal adhesions was reduced by 69% in the treatment group compared to controls. In a larger clinical trial by Johns et al., 281 women were randomized with 143 women in the treatment arm and 138 in the control arm[32]. Results were similar to the results in the other two studies. The American Fertility Society score for adnexal adhesions was now reduced by 59%. In all three studies, no obvious adverse effects of the use of the ferric HA solution were noted. However, a recently published trial conducted by Tang et al. examining the efficacy and safety of 0.5% ferric HA gel in colorectal resections had to be suspended because of high morbidity in the treatment group, mainly due to anastomotic dehiscence and prolonged postoperative ileus[33].

In 2003, the manufacturers withdrew Intergel® from the market. The product was intended to be used in conventional gynecological surgery to reduce postsurgical adhesions as an adjunct to good surgical technique. The voluntary withdrawal was conducted to complete an assessment of information obtained during post-marketing experience with the device, including adverse events associated with off-label use in laparoscopy and laparoscopic procedures such as hysterectomies. The post-market reports included late-onset postoperative pain and repeat surgeries following the onset of pain, noninfectious foreign body reactions, and tissue adherence (information provided by the FDA and manufacturers).

5. Hyaluronan Carboxymethylcellulose Gel

As described above, the HA-CMC membrane proved to be effective in adhesion reduction. However, as mentioned its handling characteristics may not be optimal, especially in laparoscopic surgery, where application of the membrane

can be difficult. Laparoscopic use of a solution or gel is much easier. HA-CMC gel (Sepragel®, Genzyme) was developed as an alternative to the HA-CMC membrane.

In 1996, Burns et al. described the use of HA-CMC gel for the prevention of adhesions in adhesion models in rats and rabbits[34]. Treatment with the gel resulted in an increased number of animals without adhesions by 70% in a rat cecal abrasion model and over 90% in a rabbit sidewall defect-bowel abrasion model. Leach et al. showed a reduction of adhesions using the HA-CMC gel in a rabbit uterine horn model[35]. The uterine horn model was shown to be adhesiogenic, with 29 (70%) of 42 untreated uterine horns found to have adhesions. After treatment with HA-CMC gel, 22 (55%) of 40 uterine horns were free of adhesions compared with 12 (30%) of 42 uterine horns in controls. Postsurgical adhesion formation was significantly reduced in animals treated with HA-CMC gel when compared with controls ($p < 0.05$). Separate analysis for extent, severity, and density showed significant differences for adhesion severity and density ($p < 0.05$). Clinical trials studying the safety and efficacy of HA-CMC gel have not yet been performed to our knowledge. HA-CMC gel has not been submitted for regulatory review in the United States or the European Union and is therefore not available for clinical use. According to the manufacturers, this is a clinical development project that is still under investigation (personal communication).

6. Auto-Crosslinked Hyaluronan Gel

Another novelty in the field of HA-based instillates was the development of auto-crosslinked HA gel (Hyalobarrier® gel, Fidia Advanced Biopolymers, Abano Terme, Italy). This highly viscous gel was obtained by means of an internal crosslinking reaction of pure HA in the absence of any chemical substance foreign to the native HA structure. The commercially offered auto-crosslinked HA gel had a concentration of 4% and a prolonged residence time in the abdominal cavity was claimed by the manufacturers.

De Iaco et al. described the use of auto-crosslinked HA gel in a laparoscopic adhesion model in rabbits[36]. Use of auto-crosslinked HA gel resulted in a significant reduction of adhesion formation: 35% of the treated animals had severe adhesions versus 66% in the control group. Furthermore the mean adhesion score was significant lower in the treated group. In a subsequent study, auto-crosslinked HA gel appeared to be effective in reducing adhesions in the presence of inadequate hemostasis as well[37]. In a rat model of laparotomy and uterine horn injury, Koçak et al. managed to reduce adhesions using auto-crosslinked HA gel[38]. Belluco et al. used an adhesion model in rabbits with

different concentrations of the gel[39]. Again, the adhesion-reducing effect was obvious, with the 4% concentration being the most efficient.

The only available clinical data to our knowledge come from studies in women undergoing abdominal surgery for gynecological indications; Acunzo et al. used the auto-crosslinked HA gel in women following hysteroscopic intrauterine adhesiolysis[40]. After 3 months, a significantly lower rate of intrauterine adhesions was observed in the auto-crosslinked HA treated group ($n = 43$) compared with controls ($n = 41$; 14% versus 32%, $p < 0.05$). Patients in the treated group showed significantly lower adhesion scores at follow-up in comparison with those in the control group (2.0 ± 0.0 versus 5.3 ± 0.2, $p < 0.001$). When an intrauterine adhesion staging was performed according to the American Fertility Society, all auto-crosslinked HA gel treated patients had mild (Stage I) adhesions. Among control patients, only 25% had mild adhesions and 75% had moderate (Stage II) adhesions. The same research group has performed a comparable study in women with a single surgically remediable intrauterine lesion[41]. Again, a significantly lower rate of intrauterine adhesions was observed in the auto-crosslinked HA gel treated group ($n = 67$) compared with controls ($n = 65$; 10% versus 26%, $p < 0.05$). The mean adhesion score was significantly lower as well (2.42 ± 0.78 versus 3.83 ± 0.98, $p < 0.05$). The adhesion severity according to the American Fertility Society demonstrated a significantly decreased adhesion severity in the treated group (86% mild adhesions, 14% moderate adhesions) compared with controls (24% mild, 76% moderate). No adverse gel-related effects were detected in the auto-crosslinked HA gel treated group.

In a recent study by Pellicano et al., the gel proved useful for reducing the incidence of postsurgical adhesions after laparoscopic myomectomy[42]. Auto-crosslinked HA gel-treated patients ($n = 18$) had a significantly lower rate of postsurgical adhesions in comparison with controls ($n = 18$; 28% versus 78%, $p < 0.01$). The rate of adhesions was significantly higher ($p < 0.05$) in patients treated with interrupted "figure-eight" sutures than in subjects treated with subserous sutures. Four out of nine patients (44%) in the auto-crosslinked HA gel-treated group and eight out of nine patients (89%) in the control group who were treated with so-called interrupted "figure-eight" sutures developed postoperative adhesions, whereas one out of nine (11%) auto-crosslinked HA gel-treated patients and six out of nine (67%) control patients who were treated with subserous sutures developed postoperative adhesions. No side effects of the applied gel were reported.

Currently, the auto-crosslinked HA gel is also no longer available for clinical use. It was withdrawn from the market after it failed to show its effectiveness in adhesion reduction on a large scale after clinical research (personal communication).

7. Other Hyaluronan-Based Products

A few other crosslinked HA products have been described in recent years. Haney et al. studied a barrier composed of chemically crosslinked HA (Incert®, Anika Therapeutics, Woburn, MA, USA)[43]. The product was used in a murine uterine horn model. Fewer adhesions were present when excision injuries were separated by the barrier (43% versus 88%), whereas the number of adhesions was unchanged after electrocautery injuries (54% versus 65%, N.S.). Despite human pilot trials to test the safety and effectiveness of this product for prevention of adhesions after spinal surgery, no trials studying the abdominal use are available to our knowledge. Until now, the barrier has not been approved by the FDA for use in the United States.

Jackson et al. focused on the use of a paclitaxel-loaded crosslinked HA film – HA crosslinked with water-soluble carbodiimide and containing 10% glycerol and 1% or 5% paclitaxel[44]. In a rat cecal abrasion model, both 1% and 5% paclitaxel film effectively reduced the formation of adhesions, with the 5% paclitaxel film being the most effective. However, this product led to excess fluid in the abdominal cavity at necropsy. No further studies have been reported at this time.

Li et al. described the development of a crosslinked HA hydrogel that contained a covalently bound derivative of the antiproliferative drug mitomycin C (MMC)[45]. The hydrogel was tested with 0.5% MMC and 2% MMC in vitro and in vivo. In vitro HA film loaded with 0.5% MMC inhibited proliferation when incubated with human T31 tracheal scar fibroblasts, whereas the 2% MMC films were cytotoxic. In vivo, the MMC films were implanted intra-abdominally in rats; the HA-MMC films reduced the thickness of fibrous tissue formed surrounding it. In a second study by the same group, HA-MMC films and gels were evaluated in a uterine horn model in rats[46]. Both films and gels were tested in several concentrations and were highly efficient in reducing adhesions. The results of the HA-MMC films were dose-dependent. The efficacy of the gels was highly correlated to the concentration, with the HA 0.625% MMC gel being the most effective. The use of this novelty is not yet described in clinical studies to our knowledge.

Hernia repair and hyaluronan containing meshes

Stimulated by the adhesion-reducing properties of HA in abdominal surgery, other applications have recently been explored, including its use in hernia repair. Adhesion reduction after abdominal wall reconstruction using prosthetic meshes is challenging. Incisional hernia after abdominal surgery is a common

complication and tension-free repair is considered a prerequisite for successful treatment. This requires the use of a prosthetic mesh. To allow optimal ingrowth and to prevent reherniation, the mesh should be macroporous and nonsoluble. Polypropylene is the most commonly used biomaterial for this purpose. However, a major drawback is the propensity of adhesion formation at the peritoneal side of this mesh, introducing the risk of bowel obstruction, fistula formation, and inadvertent enterotomy at relaparotomy. Experimental studies have shown a successful reduction of mesh-related adhesions using a combination of polypropylene mesh with a separate HA-CMC membrane[47-49]. To improve the handling characteristics, a polypropylene mesh was covered on one side with sodium HA-CMC (Sepramesh®, Genzyme). Greenawalt et al. were the first to describe results of this antiadhesive mesh[50].

The use of the composite mesh resulted in a reduction of mesh-related adhesions compared with a polypropylene mesh or polypropylene/ePTFE mesh in a rabbit model of incisional hernia. Van 't Riet et al. performed experiments in a rat incisional hernia model, comparing several antiadhesive meshes or combinations of mesh with antiadhesive agents[51]. The composite HA-CMC mesh was the most effective in adhesion reduction. This superior behavior with regard to adhesion reduction compared with other meshes was confirmed by a recent experimental study by Sikkink et al.[52]. Adhesion reduction was obtained without compromising reherniation and infection rates. Felemovicius et al. studied the use of the polypropylene HA-CMC mesh and polypropylene HA-CMC mesh combined with an additional HA-CMC membrane in a rat hernia model[53]. Polypropylene HA-CMC mesh reduced adhesions by roughly three-quarters compared to polypropylene mesh. The combination of the polypropylene HA-CMC mesh with the membrane nearly eliminated adhesion formation. Building on these successes, human studies should now further enlighten the clinical value of the polypropylene HA-CMC mesh.

Recently, the polypropylene HA-CMC mesh has been further developed into a polypropylene mesh co-knitted with polyglycolic acid (PGA) fibers (Sepramesh® IP, Genzyme) (Fig. 4). The PGA surface is coated with a bioresorbable, chemically modified sodium HA, CMC, and polyethylene glycol-based hydrogel. The co-knitting of polyglycolic acid in this mesh provides for a stronger bond between the permanent polypropylene mesh and the temporary adhesion barrier component as compared to the previous generation. A literature search does not reveal any clinical or preclinical data on this second generation product at this time, although the manufacturers claim the same level of benefit as the first generation product with substantially better handling characteristics and easier use in laparoscopic procedures (Fig. 5).

Figure 4. Polypropylene mesh co-knitted with polyglycolic acid fibers, coated with a HA-CMC polyethylene glycol based hydrogel. (Image kindly provided by Genzyme)

Figure 5. Handling characteristics of the polypropylene polyglycolic acid HA-CMC polyethylene glycol mesh: flexible and well-suited for laparoscopic procedures. (Image kindly provided by Genzyme)

The use of hyaluronan in infectious conditions

The use of HA-based antiadhesives under infectious conditions remains controversial. The majority of the available data at this time results from experimental studies and presents contradicting outcomes. Reijnen et al. described the use of the HA-CMC membrane in a peritonitis model in rats[54]. Although the contrary was hypothesized, the HA-CMC membrane did not reduce adhesions under these conditions. Tzianabos et al. stated that adhesion reduction devices might even potentiate intra-abdominal infection[55]. In a rat model, they used HA-CMC and G-HA-CMC membranes after the insertion of a bacterial inoculum in the peritoneal cavity. Both membranes did not increase abscess rates. Mortality data were only available for HA-CMC membrane, which did not increase mortality. In this study, the effect of the membranes on adhesion formation was not evaluated. Ghellai et al. described use of the HA-CMC membrane after cecal ligation and puncture or cecal ligation alone in rats[56]. Again, the HA-CMC membrane did not reduce the number or tenacity of adhesions. A trend toward increased abscess formation was associated with the use of the HA-CMC membrane in the cecal ligation group. Again using a cecal ligation and puncture model in rats, Tüzüner et al. found no differences between the control and HA-CMC membrane-treated groups regarding mortality, abdominal abscess formation, and median adhesion scores[57]. On the contrary, the use of the HA-CMC membrane led to significantly less dense adhesions. The increased adhesion formation after use of the G-HA-CMC membrane in bacterial peritonitis in the study of Kayaoglu et al. was already mentioned earlier in this chapter[23]. The only clinical prospective study that we are aware of describing the use of HA-CMC membrane in patients suffering from peritonitis is the above mentioned study of Vrijland et al.[18]. In this study, 81% of the patients treated with the HA-CMC membrane were diagnosed to have sigmoid diverticulitis with signs of peritonitis. Although the number of patients in this study was small, it is interesting to note that although the incidence of adhesions did not differ between groups, the severity of adhesions was reduced by the HA-CMC membrane.

In the above mentioned experimental study of Reijnen et al. showing no beneficial effect of the HA-CMC membrane, the 0.4% HA solution proved to reduce the incidence of adhesions and abscesses in a peritonitis model[54]. The median severity of adhesions was significant lower using the HA solution at Day 7 and at Day 21 postoperatively. At Day 21, none of the rats treated with the HA solution had an intra-abdominal abscess, in contrast to 4 out of 12 (33%) rats in the control group. These results were confirmed by a second, larger study on

this topic showing high volumes of 0.2% and 0.4% HA to be most effective, which may suggest a "hydroflotation" effect of the HA solution[58]. These superior results of HA solution compared to the HA-CMC membrane under infectious conditions were confirmed by the quoted study of Tüzüner et al.[57]. Unfortunately, withdrawal of Sepracoat® from the market has prohibited further clinical studies. There are no clinical data available on the use of ferric HA gel under contaminated and infectious conditions. The study of Tzianabos et al. describes the use of a preparation of ferric HA gel in an intra-abdominal model of infection in rats[55]. This study focused on a potential propagation of the device on intra-abdominal infection. The use of ferric HA gels potentiated bacterial peritonitis and led to significantly increased mortality rates of 90% to 100%, compared to 49% in controls. This could be explained by the known increase in virulence of bacterial species by iron. Furthermore, some virulent strains of human pathogens are able to use iron for the survival and replication of the organism. Sikkink et al. studied the use of auto-crosslinked HA gel in a rat peritonitis model[59]. Bacterial peritonitis was induced using a cecal ligation and puncture model, and the animals were randomized to receive 4% auto-crosslinked HA gel or phosphate buffered saline. Different amounts of the gel were used and the effects on adhesion and abscess formation were evaluated at different time points. In this study, a trend toward increased mortality due to fecal peritonitis with subsequent sepsis in the auto-crosslinked HA gel treated groups was observed. There were no significant differences in median total adhesion scores and abscess rates between groups.

Oncologic repercussions of hyaluronan

Little is known at this time about the use of HA during oncologic surgical procedures. Nevertheless, experimental studies have documented a critical role for HA in tumor growth and metastasis, as it interacts with cell behavior in various ways. The physical properties of HA contribute to tissue biomechanics. Furthermore, it acts as a template for the assembly of other pericellular macromolecules and interacts directly with cell surface receptors that transduce intracellular signals. Consequently, HA may promote anchorage, independent growth, and invasiveness of cancer cells as described by Toole[60, 61].
Haverlag et al. studied the influence of HA solution on tumor cell adhesion[62]. In vitro, mesothelial cells were cultured in monolayers and the effect of HA solution on adhesion of tumor cells evaluated. The use of HA solution showed an inhibitory effect on tumor adhesion. In a uterine abrasion model in rats, HA solution tended to increase tumor load. In a laparotomy model, the

mean total tumor scores did not differ significantly. Underwood et al. examined the effect of the HA-CMC membrane on tumor cell implantation at surgical wound and laparoscopic trocar sites in a hamster model[63]. It was concluded that the HA-CMC membrane neither had a protective nor an adverse effect on tumor implantation or growth. On the contrary, Tan et al. demonstrated that the HA solution significantly increased tumor cell proliferation and motility in vitro[64]. In vivo, a significantly higher total tumor nodule count was noted when using the HA solution in a rat model. Hubbard et al. examined the effect of the HA-CMC membrane on cancer cell growth and metastasis in a mouse model[65]. The HA-CMC membrane did not affect tumor metastasis. However, the placement of the membrane on nontraumatized peritoneum led to increased local tumor growth. It was concluded that not the HA but the traumatic placement of the membrane was responsible for local increased tumor growth. Pucciarelli et al. studied the effect of auto-crosslinked HA gel, native HA, and HA-CMC membrane on experimental intraperitoneal tumor implantation in mice[66]. Human HT29 colorectal cells were used, and the antiadhesives showed no negative impact on survival or tumor implantation. In an experimental laparoscopic study in mice by Sasaki et al., a protective effect of the HA-CMC membrane on port site metastasis was suggested[67]. Sikkink et al. recently studied the influence of the G-HA-CMC membrane on intraperitoneal tumor implantation and growth in a mouse and rat model of peritoneal trauma[68]. No major effects were found. However, a uniform conclusion cannot be drawn from these contradictory experimental results.

Human studies are even more scarce. Kusunoki et al. already described the use of the HA-CMC membrane in 1999 for the reconstruction of the pelvic floor after abdominoperineal rectal excision for oncologic reasons in three patients[69]. No remarks were made about the safety of HA in these cases. In a retrospective study in patients with colorectal excisions and short follow-up, Oikonomakis et al. found no adverse effects of the HA-CMC membrane[70]. More recently, Kusunoki et al. showed in a prospective randomized study that the use of the HA-CMC membrane had no adverse effects in a group of patients with rectal carcinoma who were treated with radiation therapy, two-stage surgery, and chemotherapy[71]. The median follow-up period was 43.6 months. The treatment with the HA-CMC membrane reduced adhesions in this chemoradiated group. Meanwhile, no other prospective randomized human studies have been performed to our knowledge.

Thus far, manufacturers have not promoted the use of HA-based agents in patients with malignancies. The product information for 0.5% ferric HA (Intergel®) clearly states that the product was not studied and is not recommended for

use in patients with cancer. The same accounts for other products, such as HA solution (Sepracoat®), HA-CMC membrane (Seprafilm®), G-HA-CMC membrane (Seprafilm II®), polypropylene HA-CMC mesh (Sepramesh®), and polypropylene polyglycolic acid HA-CMC polyethylene glycol mesh (Sepramesh® IP).

Mechanisms of action of hyaluronan

HA-based antiadhesive agents have proved to be effective in reducing intra-abdominal adhesions in both experimental and clinical studies. Although the use of these products is widespread, there is still much debate on the exact mechanisms of action. Mechanical separation of injured peritoneal surfaces is probably the most important. Once these surfaces have healed after a period of approximately five days, no de novo adhesions will form. HA-CMC membranes create a mechanical barrier between the adhesiogenic wound surfaces. This allows the peritoneal lining to heal without adherence to the adjacent structures. HA solutions create a medium in which the bowel floats (the hydroflotation hypothesis), thus separating the intestines and thereby allowing adhesion-free repair of the mesothelial lining. The mechanical mechanism of the action of gels is probably somewhere between acting as a barrier and creating hydroflotation.

Apart from these mechanical mechanisms, biological mechanisms of action may be involved as well. Present in virtually all tissues and body fluids of vertebrates and with a profound role in cell biology, HA might improve peritoneal healing and enhance fibrinolysis[72].

The relation between HA synthesis and cell proliferation has been studied thoroughly. The concentration of HA in healing tissues is high[73, 74]. Furthermore, HA synthesis facilitates cell detachment, mitosis, and locomotion[75-79]. An HA-rich environment provides an open, hydrated matrix that facilitates cell migration[80,81]. Exogenous HA may be beneficial in wound healing[82, 83]. Rapid, undisturbed restoration of the mesothelial lining of the traumatized peritoneum after surgery or infection should prevent adhesions, as stated previously. This concept is supported by the adhesion-reducing effect of intraperitoneal seeding of mesothelial cells or mesenchymal stem cells[84, 85]. Several studies have shown that HA increases the proliferation rate of mesothelial cells in vitro[86, 87]. This process implies that part of the adhesion-reducing capacity of HA may partly be explained by its ability to improve peritoneal healing due to the stimulation of mesothelial cell proliferation.

Abdominal surgery and peritonitis are known to disturb the equilibrium between the coagulation cascade and the fibrinolytic system, resulting in decreased

peritoneal fibrinolytic capacity and subsequently enhanced adhesion formation. The influence of HA on peritoneal fibrinolysis despite several studies is not completely clear. HA increases the fibrinolytic response of human peritoneal mesothelial cells, mainly by decreasing PAI-1 transcription and release but also by increasing the intracellular tPA concentration in vitro[88]. However, in vivo HA-based membrane and solution did not affect tissue tPA antigen or its activity in rat peritoneal biopsies after colonic surgery, with and without peritonitis[14]. Reijnen et al. discussed several explanations for these contradicting results in an earlier review[89]. However, a more recent in vitro study by Sikkink et al. did not show a significant effect of HA on the production of tPA by mesothelial cells either[90]. This study showed that cells of the monocyte-macrophage system modulate the fibrinolytic capacity of lipopolysaccharide-treated human peritoneal mesothelial cells by increasing PAI-1 and tPA. HA solution decreased PAI-1 production by mesothelial cells, an effect that was ameliorated by the presence of monocyte-like cells. Two other studies did not add clarity to this subject, as the use of HA showed no effect on tPA and PAI levels in one and even resulted in a decrease of tPA in the other[15, 91]. Perhaps sequential biopsies from the peritoneum in human studies could further enlighten the role of HA in fibrinolysis, although the harvesting of successive biopsies would not be feasible in humans. Other sampling techniques, including the use of a peritoneal chamber or microdialysis, might overcome this in the near future[92].

Conclusions

HA-based antiadhesive agents have proven to be effective reducers of postsurgical adhesions in both experimental and clinical studies. Unfortunately, several products have been withdrawn form the market before larger clinical trials could be initiated. The application of HA in composite meshes for the repair of abdominal wall hernia appears to be worthwhile as well. Data on the use of HA under infectious conditions are scarce and do not allow conclusions about its preventive potential and clinical safety under these conditions. However, the use of HA solution appears to be beneficial in experimental models. Data on oncologic repercussions using HA are also scarce and contradictory, troubling conclusions on the use of HA-based agents in patients with a malignancy. Widespread use of HA should therefore be restricted to noninfectious and benign conditions. Further elucidation of the involved mechanisms and the clinical application of HA in patients with peritonitis and patients with abdominal malignancies are challenges for future trials.

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CHAPTER 3

INTERCELLULAR ADHESION MOLECULE-1 AND GELATINASE EXPRESSION IN HUMAN PERITONEAL MESOTHELIAL CELLS DURING PROPAGATION IN CULTURE

C.J.J.M. Sikkink
M.M.P.J. Reijnen
B.A.R. Duffhues
B.M. de Man
R.M.L.M. Lomme
H. van Goor

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Abstract

Background: Mesothelial cells are involved in a variety of biological processes among which the formation of peritoneal adhesions. Cultures of human peritoneal mesothelial cells comprise an important tool to investigate behaviour and functions of mesothelial cells. Very little is known about differences between mesothelial cells isolated from different sources and of changes in specific functions as caused by cell propagation *in vitro* or resulting from storage of cells at low temperatures. The current study aims to characterize two particular cellular activities relevant for tissue repair, the expression of ICAM-1 and the gelatinase activity, and to assess the effect of hyaluronan, an antiadhesive agent, on these cellular activities.

Methods: Viable cell lines were established from both omentum and peritoneal lavage fluid from seven patients. ICAM-1 expression, by ELISA, and matrix metalloproteinase (MMP) bioactivity, by zymography, were measured in the second and fourth passage, the latter also after freezing and storage of cells in liquid nitrogen. The effects of IL-1β, TNF-α, phorbol myristate acetate (PMA) and hyaluronan were analysed.

Results: ICAM-1 was constitutively expressed and stimulated by IL-1β, TNF-α PMA. All cell lines produced both MMP-2 and MMP-9. Only the latter activity was affected by TNF-α and, especially so, PMA. Differences were found between the second and fourth passage, and between cells of different lineage, mostly so if the relative stimulation by the various agents was compared. Addition of sodium hyaluronate either to control cultures or to cultures together with any of the three stimuli examined did not significantly change either ICAM-1 expression or gelatinase activity. Freezing and storage of cells did not affect their functions.

Conclusions: Both the human omentum and peritoneal lavage fluid are good sources to establish mesothelial cell lines which can be propagated, also after freezing, without qualitative changes in their ability to express ICAM-1 and produce the gelatinases. For omental cells there is a differential effect of stimulation depending on whether the cells have been passaged 2 or 4 times. The presence of hyaluronan did not affect the expression of ICAM-1 or the gelatinases.

Introduction

Mesothelium lines the peritoneal, pleural and pericardial cavities, with visceral and parietal surfaces covering the internal organs and body wall, respectively. The peritoneal mesothelium consists of a monolayer of specialised epithelial-like mesothelial cells that rests on a basement membrane supported by connective tissue. In recent years it has been shown that mesothelial cells are involved in a variety of biological processes, including antigen presentation, tumor cell adhesion and growth, and inflammation and healing[1]. Thus, the peritoneal mesothelial cells play a central role in the (patho)biology of the peritoneal cavity, in the maintenance of the peritoneal membrane, and in peritoneal tissue repair including coagulation and fibrinolysis[2]. Importantly, mesothelial cells are involved in the formation of intra-abdominal adhesions, which are a common complication following laparotomy[3]. Also, it has been suggested that regulation of mesothelial cell functions may be pivotal in the optimal management of patients with peritonitis[4].

The mesothelial cells are loosely attached to the submesothelial layer and are easily removed or damaged due to peritoneal infection, pelvic inflammation, or tissue trauma induced during surgical procedures or peritoneal dialysis. They participate in initiating and resolving intraperitoneal inflammation and repair. In this respect, mesothelial cell intercellular adhesion molecule-1 (ICAM-1) expression and matrix metalloproteinase (MMP) activity are of interest. ICAM-1 expression facilitates the influx of leukocytes from the vascular compartment into the serosal space[5] while the MMPs, including the gelatinases MMP-2 and MMP-9, are involved in matrix homeostasis[6, 7]. Since peritoneal injury will lead to high local concentrations of inflammatory cytokines[2, 8, 9], any of their effects on these mesothelial cell functions will constitute a potential regulatory mechanism.

Research on specific functions of human peritoneal mesothelial cells is usually performed either with primary cultures obtained from omental tissue or with immortalized cell lines, the latter by definition phenotypically altered[10, 11]. It has also been suggested that peritoneal lavage fluid would be a superior source of mesothelial cells[10]. However, little is known about differences between mesothelial cells isolated from different sources and of changes in specific functions as caused by cell propagation in vitro or resulting from storage of cells at low temperatures. Such knowledge is necessary for performing studies on isolated cells in such a way that culture conditions do least affect the cellular phenotype.

Hyaluronan is a high molecular weight polyanionic polysaccharide and part of the extracellular matrix. There is considerable evidence that hyaluronan has

a beneficial influence on wound healing of several tissues. Besides a direct involvement in wound repair, hyaluronan is an important regulatory molecule in the inflammatory response[12-14], and modifies cell migration and attachment by interaction with cell surfaces[15]. Hyaluronan reportedly reduces intra-abdominal adhesion formation[16-19].
The current study aims to characterize two particular cellular activities relevant for repair, the expression of ICAM-1 and the gelatinase activity, as they depend on a variety of conditions mentioned above. The effect of hyaluronan on the expression of ICAM-1 and the gelatinase activity was assessed in addition.

Methods

Materials

Culture medium M199 (containing 25 mM HEPES, Earl's salts and L-glutamate), foetal bovine serum, penicillin-streptomycin, trypsin, ethylenediaminetetraacetic acid (EDTA) were obtained from Life Technologies, Breda, The Netherlands. Hydrocortisone, phorbol myristate acetate (PMA), gelatin (Type A: From Porcine Skin) and p-amino-phenylmercuric acetate (APMA) were purchased from Sigma Chemical (St Louis, MO, USA). Recombinant human interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) were from Peprotech (Rocky Hill, NY, USA). Sodium hyaluronate was from Genzyme (Cambridge, MA, USA). Human recombinant MMP-2 and MMP-9 were from Oncogene Research Products, Cambridge, MA, USA. A crude fraction of endothelial cell growth factors (ECGF) was kindly provided by the Department of Paediatrics, University Medical Center, Nijmegen, The Netherlands; heparin was obtained from Leo pharmaceutical products (Weesp, The Netherlands). Mouse anti-human cytokeratin 18 (M7010), rabbit anti-human von Willebrand factor (A082), FITC-labelled goat anti-mouse (F479), swine anti-rabbit (F205), horseradish peroxidase-conjugated goat-anti-mouse and orthophenyl diamine were from DAKO A/S, Glostrup, Denmark; mouse anti-human cytokeratin 19 (C7159) was from Sigma. Mouse anti-human ICAM-1 (clone HM-1) was from Hycult biotechnology (Uden, The Netherlands).

Isolation of cells

Source material for peritoneal mesothelials cells was obtained, after informed consent, from seven patients who underwent elective open abdominal surgery for non-infectious and non-tumorous conditions.
Peritoneal lavage fluid was obtained, immediately after opening of the peritoneal cavity, by distributing approximately 500 ml (37°C) saline solution and aspirating 100 ml fluid after 2 min. The fluid was centrifuged for 10 min at 350xg (4°C). The

sediment was washed twice in cold culture medium (see below) and finally resuspended and seeded onto 75 cm^2 tissue culture flasks.
From each patient approximately 5 g omental tissue was excised and collected in phosphate buffered saline (PBS). The tissue was washed twice with PBS and cells were detached by incubated under rotation in 16 ml of a solution containing 0.5 mg/ml trypsin and 0.2 mg/ml EDTA for 30 min at 37°C. Thereafter 16 ml culture medium was added, the resulting tissue was removed and the remaining suspension was centrifuged for 10 min at 350 x g (4°C), washed twice again with culture medium and the resulting cells were seeded onto 75 cm^2 tissue culture flasks. Institutional ethical board approval was obtained for this study.

Cell culture and identification

The cells were propagated in a humified incubator at 37°C under 5% CO_2/95% air atmosphere. The medium consisted of M199 completed with 20% (v/v) foetal bovine serum albumin, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.5 μg/ml hydrocortisone, 150 μg/ml crude ECGF and 14U/ml heparin. One day after seeding or passage, and then every three days the cultures were replenished with fresh medium. Confluent cultures were passaged by brief (2-3 min) trypsinization (0.5 mg/ml trypsin and 0.2 mg/ml EDTA) until approximately 80% cell detachment was observed by light microscopy. For both lavage and omentum isolates cells were denominated as passage 1 (P1) after their first trypsinization from the 75 cm^2 tissue culture flasks. All cell lines were cultured up to passage 4 (P4). For propagation of cells a split ratio of 1:2.5 was applied. Typically, cells were seeded at a density of 1-1.5 x $10^4/cm^2$. Cell functions were examined in the second and fourth passage. A batch of cells from passage 2 (P2) was frozen in liquid nitrogen and after storage propagated until P4 for comparison with cells that had not been through a cyclus of freezing/thawing.
The purity of isolated cells and their mesothelial phenotype were evaluated by phase-contrast microscopy of the monolayer morphology and by immunohistochemistry. For the purpose of the latter, cultures were grown to confluency in 24-well plates, fixed in 95% ethanol and processed for immunofluerescence as described[10]. Cells were treated with antibodies to the specific endothelial markers cytokeratin 18 and 19 and the endothelial marker von Willebrand factor. Characteristic staining was visualized after incubation with a second fluoresecent (FITC labelled) antibody.

Cell functions: ICAM-1 expression and gelatinase activity

Cells were seeded (5 x 10^3 cells/well) in gelatin-coated 96 well plates in complete medium and grown to confluence in 5 days. The medium was changed at the first and third day after seeding. On day 5 cells were washed and incubated for 24 h with culture medium containing only 0.1% (v/v) albumin in the presence of various agents (n=6 for each condition) such as IL-1β (0.1 and 10 ng/ml), TNF-α (0.1 and 10 ng/ml), PMA (25 ng/ml) and sodium hyaluronate (0.08%). Thereafter, cultures were analysed immediately for ICAM-1 expression and the medium from 6 wells was pooled and stored at –80°C for subsequent measurement of gelatinase activity.

The expression of ICAM-1 was determined by a modified ELISA[20]. Briefly, the cultures were washed with serum-free M199 to remove serum components, and fixed with a 0.025% glutaraldehyde solution in PBS. The tissue culture plates were then pre-incubated for 1 h at 37°C with murine monoclonal anti-ICAM-1 (clone HM2, 1:1000) in ELISA buffer (0.5% bovine serum albumin in PBS). After 4 washing steps, specific binding was detected by incubation with horseradish peroxidase-conjugated goat-anti-mouse (1:1000) for 1 h at 37°C, and enzyme activity was analysed by using orthophenyl diamine substrate and measuring the optical density at 490 nm on a Bio-Rad 550 plate reader.

The activity of the gelatinases MMP-2 and MMP-9 was measured by quantitative gelatin zymography, as decribed previously[21]. Briefly, pooled medium was mixed with an equal volume of sample buffer, containing 0.125 M Tris-HCl, pH 6.8, 17.4% (w/v) glycerol, 4% sodium dodecylsulphate (SDS) and 0.01% bromophenol blue. Five µl aliquots were loaded on a 7.5% (w/v) standard Laemmli SDS-polyacrylamide gel containing 2 mg/ml gelatin as a substrate. Quantification of the proteinase activities, which were expressed as arbitrary units on the basis of lysed area, was performed using a Sharp JX-330 scanner and Imagemaster ID software (Amersham Pharmacia Biotech, Uppsala, Sweden). In-between comparison of values obtained on different gels was performed using an internal standard. The presence of true MMP activity was confirmed by adding 10 mM EDTA or 1 mM 1,10'phenanthroline, both MMP inhibitors, to the buffers used after electrophoresis. Human recombinant MMP-2 (1 ng/lane) and MMP-9 (0.5 ng/lane) were added to compare their localisation with endogenous activities. Activation of recombinant MMPs was achieved by incubation with 1mM p-amino-phenylmercuric acetate (APMA) (Sigma, St. Louis, MO, USA).

Statistical analysis

The tests used, mostly a two-sided paired t-test, are mentioned where appropriate. Differences between groups are considered significant if $p<0.05$.

Results

Establishment of cultures

Omental biopsies and lavage fluid were obtained from all patients and viable cell lines were established from all, with the exception of one sample of lavage fluid. The growth rate of cell lines from both sources, from P1 onwards, was similar. During cell propagation cells were routinely screened by light microscopy and all retained typical polygonal (cobblestone) morphology. Contrast microscopy failed to disclose any evidence of contamination by fibroblasts, endothelial cells or smooth muscle cells. Cells stained positive for cytokeratin 18 and 19 with sharply delineated cell boundaries and stained negative for von Willebrand factor. The opposite was true for cultures of human umbilical vein endothelial cells, used as negative controls. Propagation until the 10^{th} passage was possible without any apparent changes in morphology as observed by light microscopy. Mesothelial cell lines that had been stored in liquid nitrogen showed features being equal to those of their counterparts that had not gone through this procedure.

ICAM-1 expression

Both omental and lavage cells from P2 constitutively expressed ICAM-1, but expression in the control medium was significantly higher in the cells cultured from lavage fluid (figure 1). Expression was increased by incubation with IL-1β, TNF-α and PMA for both cell sources, but more clearly so in the omental cells. In the lavage cells, average values after incubation with 0.1 ng/ml IL-1β or TNF-α, or PMA, were higher than in controls but not significantly so. As a consequence, the degree of stimulation by the various agents was significantly higher in omental than in lavage cells.

Figure 2 shows that the basal ICAM-1 expression in P4 omental cells was significantly higher than in P2 cells. Although expression in P4 was still enhanced ($p<0.05$) by both IL-1β and TNF-α, the relative increase was significantly less than in P2 for all stimuli examined. Such a difference was not observed in lavage cells (data not shown): cells in P4 reacted in a similar fashion as those in P2 (figure 1B).

Cells that had been propagated after storage in liquid nitrogen (and thus could only be evaluated in P4) did behave similarly to the corresponding cells that not had been frozen. This is illustrated in figure 3 for the omental cells. Here, the stimulation by 10 ng/ml IL-1β seemed even more explicit in the cells cultured from frozen samples. Addition of sodium hyaluronate either to control cultures or to cultures together with any of the three stimuli examined did not significantly change ICAM-1 expression.

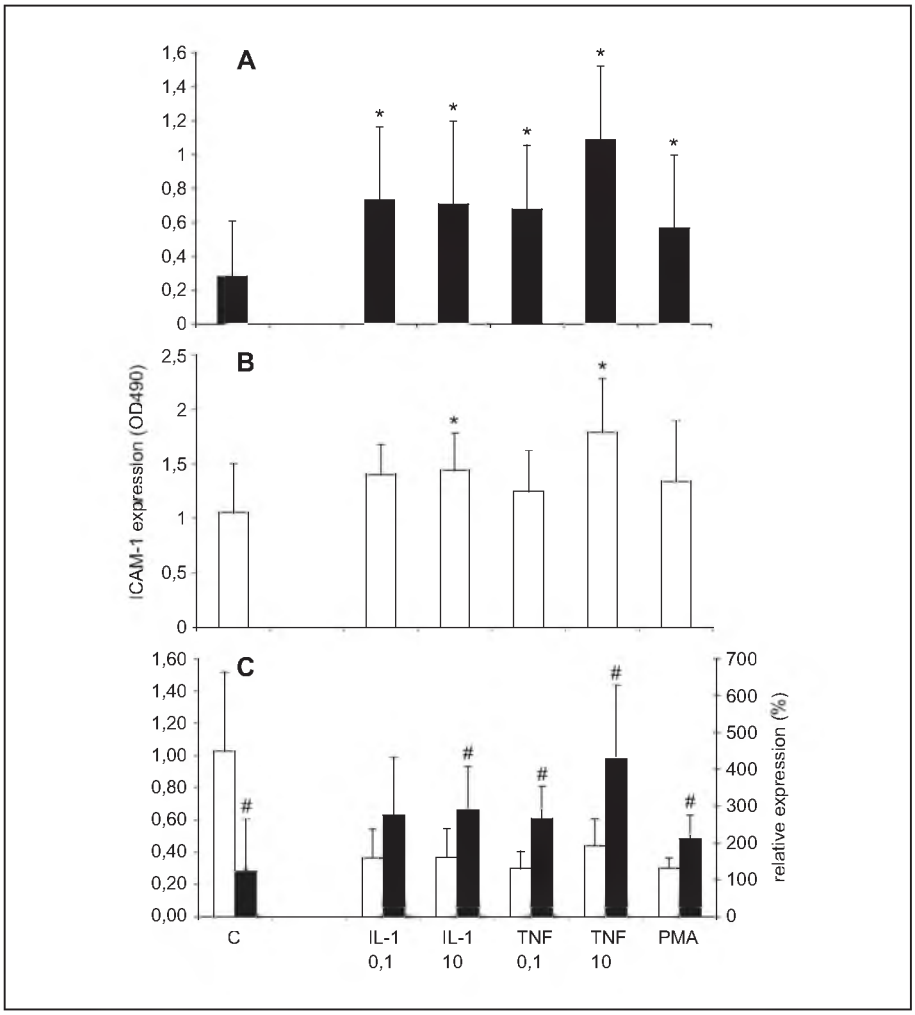

Figure 1. ICAM-1 expression on mesothelial cells from the second passage. Average (+SD) expression in omental (A) and lavage (B) cells in control medium and in media with IL-1β (0.1 and 10 ng/ml), TNF-α (0.1 and 10 ng/ml) or PMA (25 ng/ml). Panel C represents the relative expression with respect to control medium (100%) for both omental (black bars) and lavage (open bars) cells. * $p<0.05$ vs control (two-tailed paired t-test); # $p<0.05$ omentum vs lavage (unpaired t-test with welch correction).

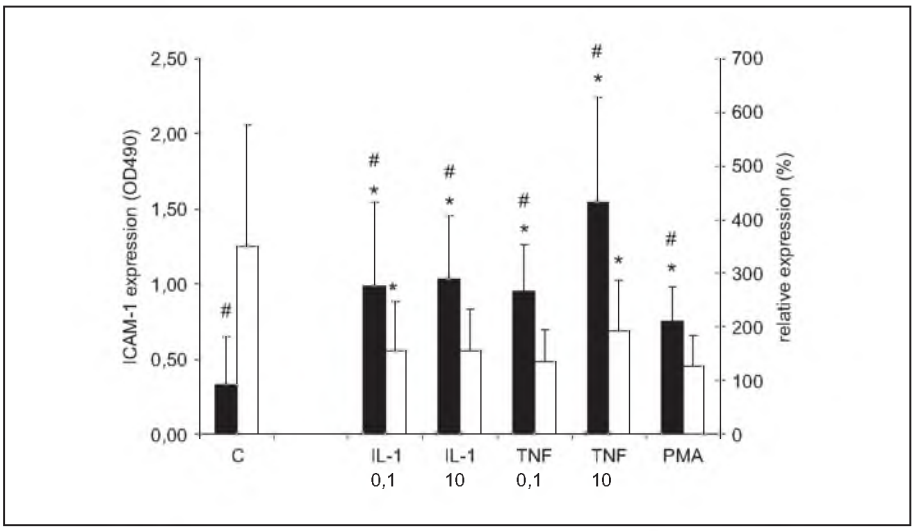

Figure 2. ICAM-1 expression on mesothelial cells from omentum: effect of passage. Average (+SD) absolute expression in control medium C (left axis) for P2 (black bars) and P4 (open bars) cells. Relative expression (right axis) with respect to control medium (100%) for media with IL-1β (0.1 and 10 ng/ml), TNF-α (0.1 and 10 ng/ml) or PMA (25 ng/ml). * $p<0.05$ vs control; # $p<0.05$ P2 vs P4 (both: two-tailed paired t-test).

MMP-2 and MMP-9 activity

All cell lines produced both the gelatinases MMP-2 and MMP-9: Figure 4 shows typical examples demonstrating the presence only of the pro-enzymes and not the activated (lower molecular weight) forms. The secreted activity of MMP-2 was far higher than that of MMP-9 and mean activities in all cell lines appeared the same, independent of either passage or origin (table 1). In cells from the lavage lineage, MMP-9 activity was significantly lower in P4 than in P2.

Table 1. GELATINASE ACTIVITY IN MESOTHELIAL CELLS

		MMP-2	MMP-9
Omentum	P2	97 ± 42 (7)	2.36 ± 1.34 (7)
	P4	82 ± 39 (7)	2.46 ± 1.27 (7)
Lavage	P2	93 ± 52 (6)	2.39 ± 0.84 (6)*
	P4	72 ± 38 (6)	1.38 ± 0.68 (6)

MMP activity in the culture medium from unstimulated cells is expressed in arbitrary units and shown as mean ± SD.
* P2 vs P4, $p<0.05$, unpaired t-test.

In none of the cell lines did incubation with IL-1β, TNF-α or PMA lead to significant changes in MMP-2 activity. This is illustrated in figure 4 and further quantified for P2 in figure 5. Addition of hyaluronic acid to any of the incubation media did not affect activity (data not shown).

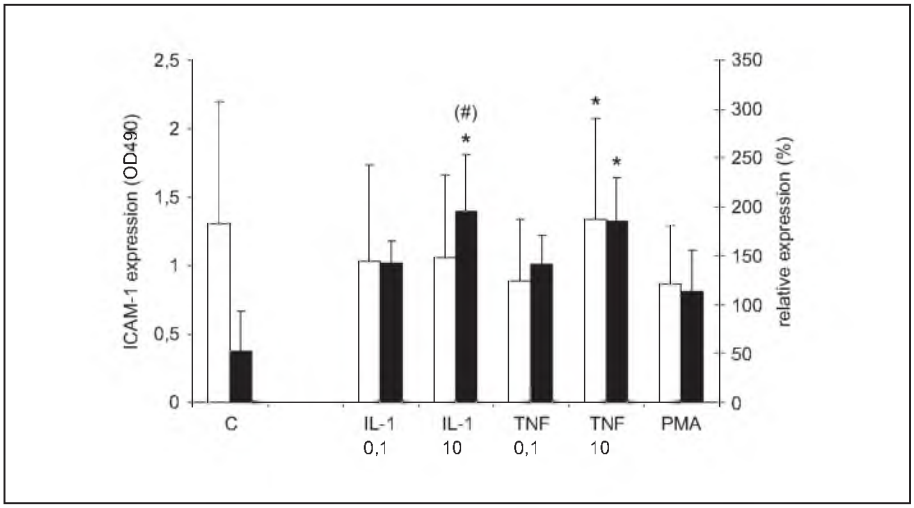

Figure 3. ICAM-1 expression on mesothelial cells from omentum: effect of freezing. Average (+SD) absolute expression in P4 in control medium C (left axis) for cells which had been frozen (black bars) and those which had remained unfrozen during propagation (open bars). Relative expression (right axis) with respect to control medium (100%) for media with IL-1β (0.1 and 10 ng/ml), TNF-α (0.1 and 10 ng/ml) or PMA (25 ng/ml). * $p<0.05$ vs control; (#) $p= 0.07$ P2 vs P4 (both: two-tailed paired t-test).

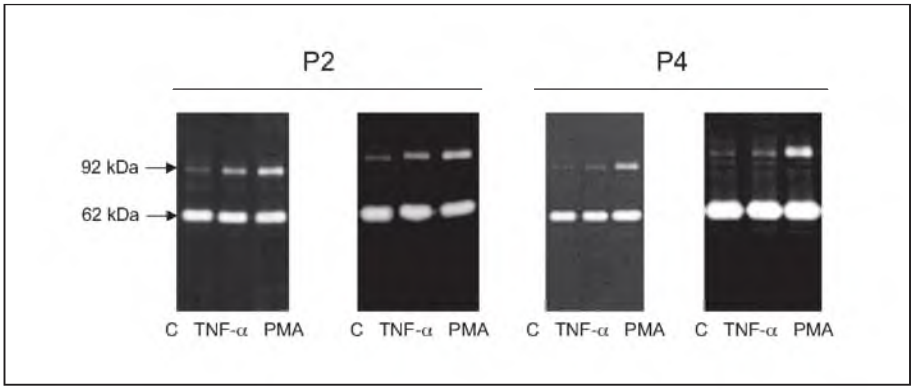

Figure 4. Zymogram. Typical examples of gelatinases produced by two different omental cell lines in P2 and P4, both in control medium and after stimulation with TNF-α and PMA.

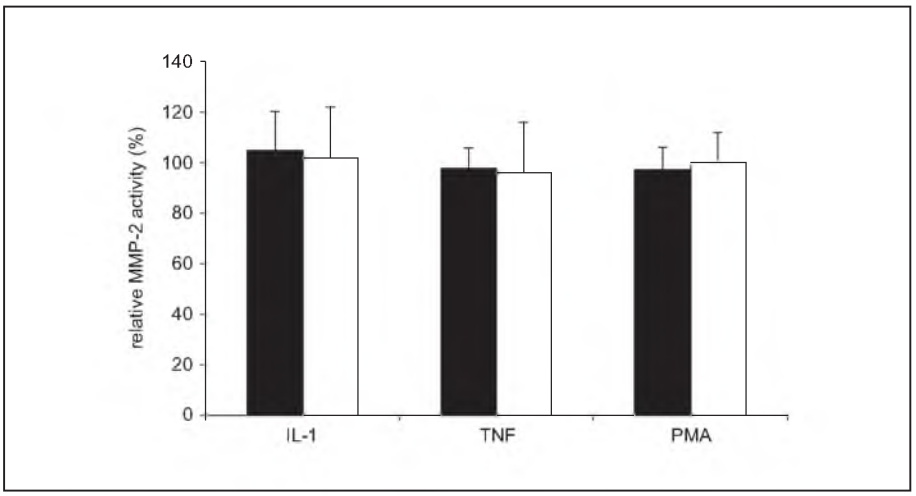

Figure 5. MMP-2 activity in mesothelial cells from the second passage. Average (+SD) activity in P2 cells from omentum (black bars) and lavage cells (open bars). Results are given as relative activity vs control medium (100%) in media incubated with IL-1β (10 ng/ml), TNF-α (10 ng/ml) or PMA (25 ng/ml).

The MMP-9 activity was affected by TNF-α and, especially so, by PMA (figure 6). In cells from the second passage the relative stimulation was significantly higher in omental cells than in lavage cells (figure 6). In P4 this difference only existed for stimulation by TNF-α, although the average stimulation in omental cells of 44% failed to reach significance. When comparing the relative activities in P4 to those in P2, no significant differences were found (two-tailed paired t-test). As with MMP-2, addition of sodium hyaluronate to any of the incubation media did not affect MMP-9 activity at all.

Discussion

Viable human mesothelial cell cultures can easily be established with cells obtained both from omentum and lavage fluid. The two cell functions investigated, ICAM-1 expression and gelatinase activity, are largely retained after propagation into the fourth passage. The differences observed appear to be quantitative only: stimulation by mediators remains intact, though in the case of omental cells to a lesser degree. Although the primary function of the mesothelium is to act as a protective non-adhesive surface, mesothelial cells are also actively involved in a variety of processes such as antigen presentation, inflammation and repair, coagulation and fibrinolysis and tumor cell adhesion. Peritoneal mesothelial cells play a key role in peritoneal healing processes and adhesion formation[22]. Thus, their

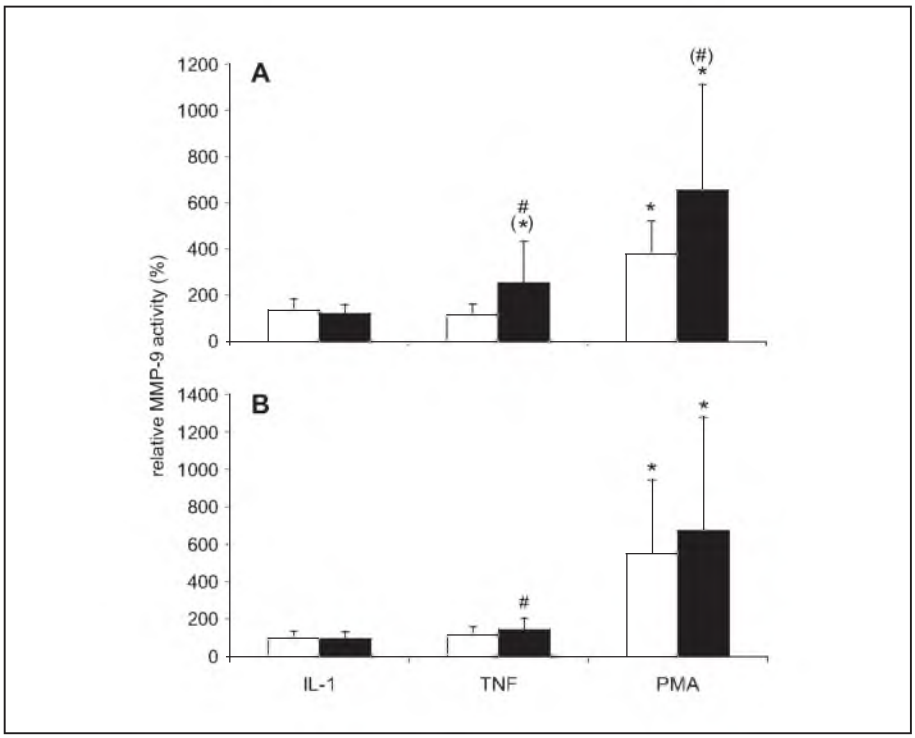

Figure 6. MMP-9 activity in mesothelial cells. Average (+SD) activity in P2 (A) and P4 (B) cells from omentum (black bars) and lavage cells (open bars). Results are given as relative activity vs control medium (100%) in media incubated with IL-1β (10 ng/ml), TNF-α (10 ng/ml) or PMA (25 ng/ml). * $p<0.05$ and (*) $p=0.06$ vs control; # $p<0.05$ and (#) $p=0.07$ omentum vs lavage (all: two-tailed paired t-test).

functions are increasingly studied in culture under conditions thought to mimic e.g. repair after surgery, pelvic inflammatory disease, peritonitis or peritoneal dialysis. The expression of ICAM-1 and the production of gelatinases are two cellular functions that are thought to be generally involved in repair after a variety of insults.

While the most explicit stimulation of cell functions in the present study is seen with omental cell lines in P2, significant stimulation of ICAM-1 expression by both IL1-β and TNF-α is still observed in P4, also if cell lines had been deep-frozen. The latter result would suggest that mesothelial cells obtained from one patient can be harvested, propagated and stored, to be used at any suitable time for study of cellular functions. This would greatly facilitate studies on the properties of human mesothelial cells. However, one should realize that results

may depend on the passage number. In our study for example ICAM-1 expression in P2 omental cells was significantly higher than in P4 cells, even though ICAM-1 expression remained high in all conditions tested.
If investigated in P2, the earliest moment after harvesting considered practical for the purpose of studies into multiple cellular functions, cells from the lavage lineage appear to be less responsive to stimuli than omental cells. Both ICAM-1 expression and MMP-9 activity are significantly stronger increased in the latter by the various mediators investigated. Still, lavage cells are responsive and remain so during further propagation. It has been reported that mesothelial cells from lavage fluid and omentum are also similar with respect to their production of components from the fibrinolytic system[10]. Thus, lavage fluid appears to be a feasible alternative as a source for mesothelial cells if insufficient omental tissue would be available. Still, it should be kept in mind that the mesothelial cells found in the lavage fluid may be either somehow damaged and thereby detached or recruited from the mesothelial lining for a purpose. It has been shown recently that free-floating mesothelial cells attach to injured serosal surfaces, proliferate and incorporate into the regenerating mesothelium[23]. They thus may have an essential role in peritoneal healing and therefore certain functional differences between these free-floating cells and cells present in the mesothelial lining may be expected.
It has been well established that mesothelial cells constitutively express ICAM-1. Expression of this adhesion molecule represents an important function of the mesothelial cell. For instance, ICAM-1 is essential in the peritoneal immune response against invading pathogens[24, 25] and is involved in the adhesion of tumor cells during the development of peritoneal metastases[26]. The cell lines described in the present study all express ICAM-1. Also, they consistently show upregulation of ICAM-1 expression by both IL-1β and TNF-α.
MMP expression appears to be another important function of the mesothelial cell. A certain amount of matrix degradation is intrinsic to tissue repair. Thus, MMPs are involved in healing processes in general and it has been demonstrated that mesothelial cells have the capacity to produce MMPs, although data are relatively scarce, certainly those obtained on non-transformed cells. Pleural mesothelial cells have been found to constitutively express MMP-1,2,3 and -9 activity[7, 27]. With regard to human peritoneal cells, an immortalized cell line has been found to produce both MMP-2 and MMP-9 activity[28], while cell lines grown from omentum and analysed in the second or third passage expressed MMP-2 and MMP-3 constitutively, but MMP-9 only after incubation with IL1-β[29]. In our hands, mesothelial cells always express MMP-2, but incubation with IL-1β, TNF-α or PMA does not affect the activity found in the culture medium. MMP-9 is also

invariably found, though at a far lower activity. There is very little stimulation by TNF-α and none with IL-1β, while PMA strongly stimulates the production of MMP-9. Each time, only the (inactive) proform of MMP-9 is found. Thus, while our data represent consistent findings in separate cell lines obtained from 7 different subjects, they do not completely agree with the few reports published so far. Possibly, this emphasizes the fact that some cellular functions may vary strongly depending on cellular culture and propagation conditions. This implicates that results from in vitro experiments may be affected by specific culture and propagation conditions. Testing with various cell lines and origins may reduce this possibility.

We have also investigated the effects of hyaluronan on ICAM-1 expression and gelatinase activity. Hyaluronan synthesis and degradation are thought to be involved in mesothelial repair[30] and to influence the wound healing processes and inflammation in general. Hyaluronan-based agents reduce the formation of postsurgical adhesions, most likely due to both physical and biological properties[22]. Hyaluronan has been shown to modulate the fibrinolytic response of peritoneal mesothelial cells to TNF-α[31, 32] and to affect both MMP-2 and MMP-9 release into the medium of cultured cells such as macrophages[33] and tumor cells[34]. Hence, in neither of the cell lines examined here, did sodium hyaluronate affect ICAM-1 expression under any of the conditions investigated. This result is in accordance with a very recent report investigating its potential for therapeutic intervention in mesothelial-tumor adhesion[35]. In addition, in none of the cell lines, sodium hyaluronate changed MMP activity, either in the presence or absence of the various other agents added as potential stimuli. Thus it appears that both cellular repair functions investigated will remain unchanged by fluctuations in hyaluronan levels.

To our knowledge, there are hardly any studies reporting in a comprehensive manner on mesothelial cell functions as affected by culture and propagation conditions. Experiments on isolated cells are mostly done on primary cultures or immortalized cell lines. In the latter case, it is often unclear to what extent these cells retain their original characteristics. The current study emphasizes the fact that omental tissue is probably the best source for the isolation and culturing of human mesothelial cells since, in some respects, they remain more responsive than cells obtained from peritoneal lavage fluid from the same subject. For omental cells there is a differential effect of stimulation depending on whether the cells have been passaged 2 or 4 times. It is important to realize that isolated cells may be stored in liquid nitrogen and propagated again without any apparent loss of the functions studied here. The use of such cultures might limit the need to collect tissue from human subjects.

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CHAPTER 4

INFLUENCE OF MONOCYTE-LIKE CELLS ON THE FIBRINOLYTIC ACTIVITY OF PERITONEAL MESOTHELIAL CELLS AND THE EFFECT OF SODIUM HYALURONATE

C.J.J.M. Sikkink
M.M.P.J. Reijnen
P. Falk
H. van Goor
L. Holmdahl

Fertil Steril 2005; 84 Suppl 2: 1072-7.

Abstract

Objective: To determine whether the presence of cells of the monocyte-macrophage system affects the fibrinolytic response of peritoneal mesothelial cells to lipopolysaccharide (LPS) in presence and absence of sodium hyaluronate.
Design: Controlled laboratory experiment.
Setting: Cell cultures in an academic laboratory research environment.
Cells: Human peritoneal mesothelial cells were harvested from patients undergoing a laparotomy for non-infectious reasons and cultured in vitro. Co-cultures were formed by adding U-937 human monocyte-like cells to a monolayer of mesothelial cells.
Interventions: After 24 hours, cultures were treated with 10 ng/ml LPS and sodium hyaluronate was added in a final concentration of 0.2%. Controls received medium without sodium hyaluronate.
Main outcome measures: After 24 hours incubation, tissue Plasminogen Activator (tPA), urokinase Plasminogen Activator (uPA) and Plasminogen Activator Inhibitor-1 (PAI-1) levels were determined in medium and cell lysates using ELISA techniques.
Results: In medium of co-cultures, tPA and PAI-1 concentrations were significantly increased compared to monocultures ($p<0.05$ and $p<0.005$, respectively), while uPA concentration was significantly decreased ($p<0.01$). In cell lysates of co-cultures, PAI-1 concentration was significantly increased compared to monocultures ($p<0.01$), while tPA and uPA were unaffected. Treatment with sodium hyaluronate significantly decreased PAI-1 ($p<0.05$) and uPA ($p<0.01$) concentrations in medium of monocultures, but decreased uPA concentration only in medium of co-cultures ($p<0.01$), compared to controls.
Conclusion: Cells of the monocyte-macrophage system modulate the fibrinolytic capacity of LPS treated human peritoneal mesothelial cells and interfere in the hyaluronan associated changes in mesothelial fibrinolytic capacity.

Introduction

Intra-abdominal infection, caused by perforation of viscera or leakage of a bowel anastomosis, is a critical and potential lethal condition. Peritonitis elicits an inflammatory response, accompanied by the production and release of a broad spectrum of biologically active proteins and exudation of protein rich fluid. The peritoneal exudate contains large amounts of fibrinogen. The activated coagulation cascade will result in the formation of thrombin that triggers conversion of fibrinogen into fibrin.

Under normal circumstances, fibrin is lysed by the fibrinolytic system. After abdominal surgery and infection, however, the equilibrium between coagulation and fibrinolysis is disturbed, in favor of the coagulation system[1-3]. Thus, fibrin will form deposits that are a matrix for ingrowth of fibrocollagenous tissue. Moreover, fibrin clots protect bacteria against the immunological defense of the abdominal cavity. Following this pathway, intra-abdominal fibrin may eventually lead to adhesion and abscess formation[1, 2, 4].

By producing both plasminogen activators and inhibitors mesothelial cells are decisive in the genesis of adhesions and abscesses. Tissue-type plasminogen activator (tPA) is the main plasminogen activator. A second, but less potent plasminogen activator is urokinase-type plasminogen activator (uPA), which also may play a role in tissue remodeling processes[5]. Their activity is restricted by plasminogen activating inhibitors, predominantly type 1 (PAI-1).

The monocyte-macrophage system may influence peritoneal fibrinolysis by the production of a variety of cytokines. Cytokines have been reported to alter the production of both plasmin activators and its inhibitors by mesothelial cells. Cells of the monocyte-macrophage system are attracted to the abdominal cavity 48 to 72 hours cells after bacterial infection by inflammatory mediators, and thus might alter mesothelial cell functions. These inflammatory cells are triggered by bacterial compounds, including lipopolysaccharide (LPS), which are released during colorectal surgery and peritonitis.

Hyaluronan is a substance of the extracellular matrix, which influences wound healing processes and inflammation[6-9]. Hyaluronan reportedly reduces intra-abdominal adhesion formation[10-12]. Moreover it may reduce the incidence of intra-abdominal abscesses in experimental peritonitis[13, 14]. We have earlier demonstrated that sodium hyaluronate modulates the fibrinolytic response of peritoneal mesothelial cells to TNF-α[15].

We hypothesized that cells of the monocyte-macrophage system affect the fibrinolytic response of human peritoneal mesothelial cells, and that effects of hyaluronan might differ in the presence and absence of these cells.

Materials and Methods

Cell culturing

Human peritoneal mesothelial cells were isolated from peritoneal lavage performed in 5 patients undergoing colorectal surgery for non-infectious reasons, according to Ivarsson et al.[16]. Meticulous hemostasis during opening of the abdomen prevented blood spill in the lavage fluid. Lavage fluids were transferred into 50 ml tubes and centrifuged at 650 g at 20ºC for ten minutes. The supernatant was withdrawn and the pellet resuspended in 5 ml culture medium. Cells were cultured at 37°C in 5% CO_2 in air with a humidity of 80-90%, in tissue culture flasks (25cm^2) (Sarstedt, Newton, NC, USA) in medium which consisted of E199 (Sigma, St.Louis, MO, USA), with the addition of L-glutamine 1.1 mmol/l (Sigma), penicillin-streptomycin 30 I.E./ml (Sigma), fetal bovine serum 20% (Sigma), endothelial cell growth factor 150 µg/ml prepared according to Maciag[17], heparin 14 IU/ml (Lövens Läkemedel, Malmö, Sweden) and 0.5 µg/ml hydrocortisone (Sigma). Cells were passaged two times at the most. The mesothelial origin from the cells was controlled by their typical cobblestone appearance in phase contrast microscopy and by immunohistochemistry, as previously described[16]. In addition, the presence of cytokeratin 18 and 19, typically expressed by mesothelial cells, was verified using a mouse anti-human cytokeratin 18 antibody (DAKO A/S, Glostrup Denmark, M7010, diluted 1:20), and a mouse anti-human cytokeratin 19 antibody (DAKO A/S, M0888, diluted 1:20) that were FITC-labeled.
U-937 human monocyte-like cells (ATCC, Rockville, MD, USA) were cultured in medium that consisted of RPMI 1640 (Sigma) with the addition of L-glutamine 1.1 mmol/l (Sigma), penicillin-streptomycin 30 I.E./ml (Sigma) and 10% fetal bovine serum (Sigma), as specified by the manufacturer.
The experiments were approved by the Institutional Review Board of the Sahlgrenska University Hospital/Östra, Göteborg University, Göteborg, Sweden, and informed consent was obtained prior to the cell harvesting.

Study design

Mesothelial cells were cultured until a complete monolayer was formed. In order to form co-cultures U-937 cells were added to half of the cultures one day prior to the experiments in a final concentration of 10^6 U-937 cells/well. Medium of both mono- and co-cultures consisted of 50% E199 medium and 50% RPMI 1640 medium. Pilot studies have demonstrated that this composition of medium is optimal for the growth of both cell types (data not published).
To mimic the clinical situation of colorectal surgery and bacterial peritonitis,

all cultures were treated with 10 ng/ml LPS (Sigma, St.Louis, MA, USA). For each study group eight cultures were used (n=8). Four experimental groups were created;

1. Mesothelial cells with regular medium.
2. Co-cultures of mesothelial cells and U-937 cells with regular medium.
3. Mesothelial cells with medium containing 0.2% (w/v) sodium hyaluronate (Genzyme Corporation, Cambridge, MA, USA).
4. Co-cultures of mesothelial cells and U-937 cells with medium containing 0.2% (w/v) sodium hyaluronate.

After 24 hours incubation, medium was collected and stored in aliquots at –80°C until measurements. Fresh medium was added to the cells and they were lysated by freezing and thawing three times. Cell lysates were stored in aliquots at –80°C until assayed.

Biochemical assays

Determinations of plasminogen activators and inhibitors were done using commercially available ELISA-kits. Levels of tPA-antigen, and PAI-1-antigen were assessed using imulyse kits from Biopool (Umeå, Sweden) and uPA-antigen using a kit from Monozyme (Horsholm, Denmark).

Statistics

Values are given in box plots (median, interquartile range and 10^{th} and 90^{th} percentiles) in several figures. Analysis of differences was performed using the Mann-Whitney U test. Statistical significance was set at $P<0.05$. All tests were 2-tailed.

Results

tPA-antigen

The presence of U-937 cells significantly increased tPA concentration in the media of cultures not treated with hyaluronate (group 1 vs. group 2, $p<0.05$) (Fig. 1). The addition of sodium hyaluronate to the medium ameliorated the effect of the U-937 cells (group 3 vs. group 4).

Treatment with sodium hyaluronate did not affect tPA concentrations in the media of both mono- and co-cultures (group 1 vs. group 3 and group 2 vs. group 4, respectively).

There was no significant difference between tPA concentrations measured in cell lysates of mono- and co-cultures (group 1 vs. group 2 and group 3 vs. group 4, respectively) (Fig. 2).

Treatment with sodium hyaluronate did not significantly affect tPA concentration measured in cell lysates of both mono- and co-cultures (group 1 vs. group 3 and group 2 vs. group 4, respectively).

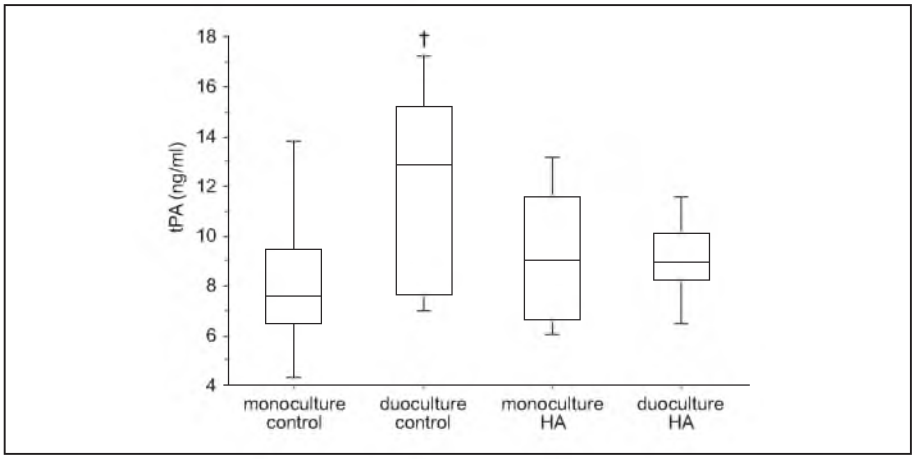

Figure 1. Levels of tPA-ag in medium of monocultures and co-cultures. Results are illustrated as median (horizontal line) interquartile range (boxes) and 10th and 90th percentiles (error bars). † = $p<0.05$ monocultures versus co-cultures.

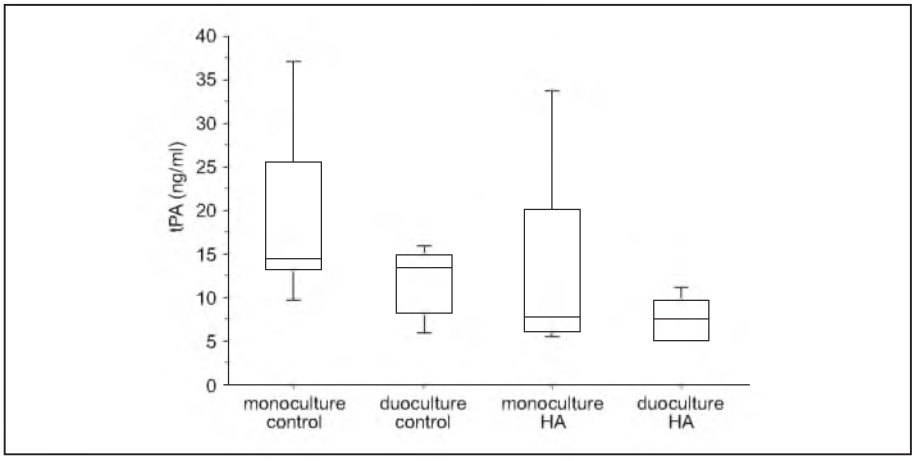

Figure 2. Levels of tPA-ag in cell lysates of monocultures and co-cultures. Results are illustrated as median (horizontal line) interquartile range (boxes) and 10th and 90th percentiles (error bars).

uPA-antigen

The presence of U-937 cells significantly decreased uPA concentrations in the media of cultures not exposed to sodium hyaluronate (group 1 vs. group 2, $p<0.01$) and the media of cells treated with sodium hyaluronate (group 3 vs. group 4, $p<0.01$) (Fig. 3).

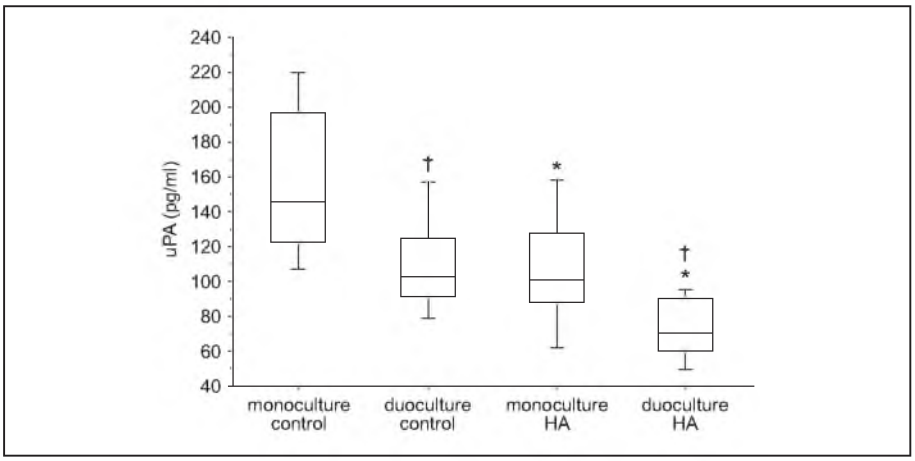

Figure 3. Levels of uPA-ag in medium of monocultures and co-cultures. Results are illustrated as median (horizontal line) interquartile range (boxes) and 10th and 90th percentiles (error bars). † = $p<0.01$, monocultures versus co-cultures, * = $p<0.01$ sodium hyaluronate (HA) versus control.

Sodium hyaluronate significantly decreased uPA concentration measured in medium of both monocultures (group 1 vs. group 3, $p<0.01$) and co-cultures (group 2 vs. group 4, $p<0.01$).

There was no significant difference in uPA concentrations measured in cell lysates of monocultures and co-cultures, regardless the presence or absence of sodium hyaluronate (group 1 vs. group 2 and group 3 vs. group 4) (Fig. 4).

Treatment with sodium hyaluronate did not have a significant effect on uPA concentration in cell lysates of both monocultures and co-cultures (group 1 vs. group 3 and group 2 vs. group 4, respectively).

PAI-1-antigen

PAI-1 concentration in medium was significantly increased in co-cultures compared to monocultures in both media from cultures not treated with sodium hyaluronate (group 1 vs. group 2, $p<0.005$) and media from cells exposed to sodium hyaluronate (group 3 vs. group 4, $p<0.005$) (Fig. 5).

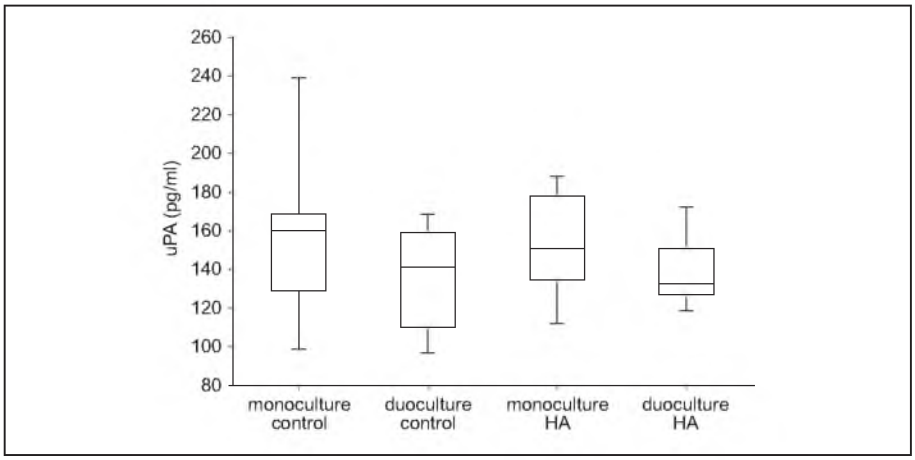

Figure 4. Levels of uPA-ag in cell lysates of monocultures and co-cultures. Results are illustrated as median (horizontal line) interquartile range (boxes) and 10th and 90th percentiles (error bars).

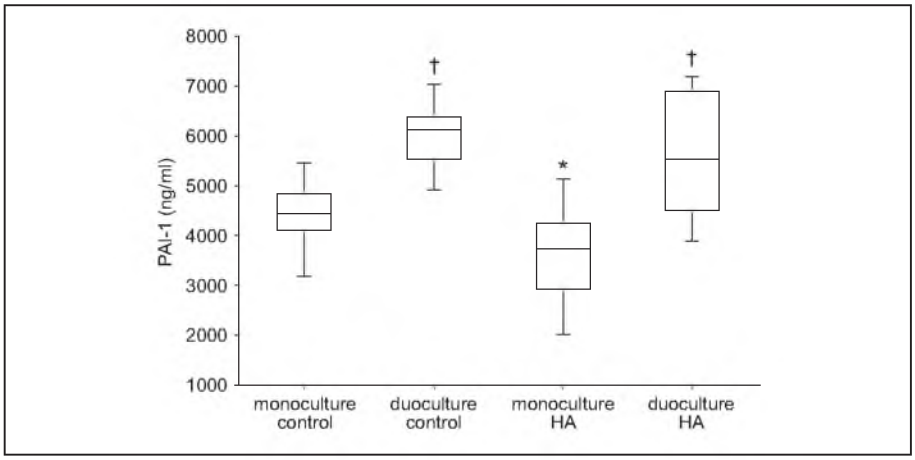

Figure 5. Levels of PAI-1-ag in medium of monocultures and co-cultures. Results are illustrated as median (horizontal line) interquartile range (boxes) and 10th and 90th percentiles (error bars). † = $p<0.005$, monoculture versus co-culture. * = $p<0.05$, sodium hyaluronate (HA) versus control.

Treatment with sodium hyaluronate significantly decreased PAI-1 concentration in medium of monocultures (group 1 vs. group 3, $p<0.05$). In contrast, exposure to sodium hyaluronate did not affect PAI-1 levels in media of co-cultures (group 2 vs. group 4).

In cell lysates, the PAI-1 concentration was significantly increased in co-cultures compared to monocultures, both in the absence and presence of sodium hyaluronate (group 1 vs. group 2, $p<0.01$ and group 3 vs. group 4, $p<0.05$, respectively) (Fig. 6).
Treatment with sodium hyaluronate did not affect PAI-1 concentration in cell lysates of both mono- and co-cultures (group 1 vs. group 3 and group 2 vs. group 4, respectively).

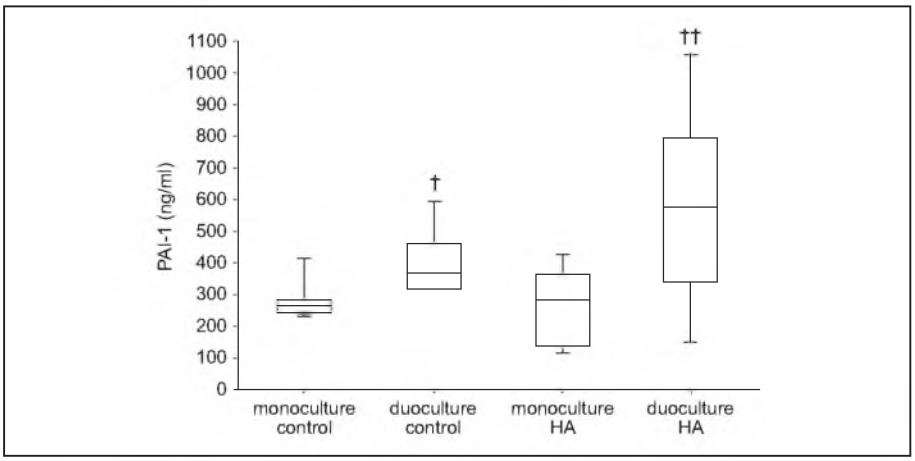

Figure 6. Levels of PAI-1-ag in cell lysates of monocultures and co-cultures. Results are illustrated as median (horizontal line) interquartile range (boxes) and 10th and 90th percentiles (error bars). † = $p<0.01$, †† = $p<0.05$ monoculture versus co-culture.

Discussion

In the present study we have demonstrated that cells of the monocyte-macrophage system have a significant influence on the fibrinolytic response of human peritoneal mesothelial cells exposed to LPS and that the effect of sodium hyaluronate on this response differs in presence or absence of monocyte-like cells. The occurrence of U-937 cells in the cultures increased both tPA and PAI-1 in medium, while the uPA levels decreased. Monocytes/macrophages can produce a broad scale of inflammatory cytokines, including TNF-α and TGF-β[18, 19]. Both cytokines are known regulators of peritoneal fibrinolysis, mainly by increasing the production of PAI-1 by mesothelial cells[16, 20]. It seems unlikely that the U937 cells themselves have contributed to the increased PAI-1 levels since these cells do not seem to produce PAI-1[21].

If the U937 cells have contributed to the increased tPA levels cannot be concluded from the present study. However, previous experiments of our group have shown that these cells do not produce detectable amounts of tPA under these conditions (data not published). The presence of U-937 cells in the cultures did not seem to affect the intracellular storage of tPA in mesothelial cells. This may indicate that the monocytes mainly affect tPA release from mesothelial cells.

The U-937 cells decreased the secretion of uPA into the medium, but did not affect its intracellular storage. This suggests that the reduced expression of uPA in the media may not be an effect of internalization of uPA, but rather a decreased synthesis.

In peritonitis the peritoneal fibrinolytic capacity is depressed[2, 3]. Experimental data have demonstrated that the period of hypofibrinolysis is followed by a phase of increased activity after several days[22, 23]. This is in accordance with the time period wherein cells of the monocyte-macrophage system invade the peritoneal cavity. The increased tPA concentrations found in co-cultures suggest that monocyte-like cells might be involved in this rebound phenomenon, either by stimulating mesothelial tPA production or by producing it themselves. However, in the presence of monocyte-like cells, the PAI-1 expression was equally increased, which will rapidly inactivate the surplus of tPA by forming one-on-one complexes. The role of monocyte-like cells in peritoneal repair mechanisms therefore requires further investigation.

Several studies have demonstrated that intra-peritoneal treatment with hyaluronan-based agents reduces postsurgical adhesion formation[10-12]. Moreover, it is reported to reduce abscesses in experimental peritonitis[13, 14]. The principle mode of action of hyaluronan agents is considered to be separation of injured serosal layers and protection of the peritoneal surface from surgical damage. Hyaluronan, however, has also documented biological functions[2]. We previously demonstrated that hyaluronan modulates the fibrinolytic capacity of human peritoneal mesothelial cells treated with TNF-alpha, mainly by decreasing the production of PAI-1[15].

In the present study sodium hyaluronate also decreased PAI-1 levels produced by mesothelial cells exposed to LPS, most likely due to a decreased synthesis. This observation gives further support to the notion that sodium hyaluronan may, in part, reduce adhesions and abscesses through increased peritoneal fibrinolysis. The presence of monocyte-like cells, however, abolished the reduction in PAI-1, indicating that hyaluronan may become less efficient when cells of the monocyte-macrophage system have already invaded the abdominal cavity.

The presence of LPS has profound influence on mesothelial cell fibrinolysis. It decreases tPA and increases PAI-1 expression of these cells[16]. Moreover, it stimulates the production of various cytokines by monocyte-like cells that, in turn, also have an effect on mesothelial fibrinolysis. Both monocytes and mesothelial cells carry hyaluronan binding surface receptors, including CD44 and ICAM-1[7, 24, 25]. Binding of hyaluronan to the CD44 receptor modulates the cytokine expression of the monocyte, which might have changed the production of fibrinolytic factors by mesothelial cells in our study. The mechanisms involved in this system are complex though and require additional studies.

The presence of monocyte-like cells in the cultures increased both the release of PAI-1 and its intracellular pool, which suggests either an enhanced synthesis or a diminished degradation of PAI-1. High levels of PAI-1 have been correlated with various clinical situations, including adhesion formation and cancer cell invasion and angiogenesis[26, 27]. Peritonitis, caused by a perforated colorectal carcinoma, is correlated with a poor prognosis. In this clinical situation large amounts of inflammatory cells are present in the peritoneal cavity, which is associated with high PAI-1 levels. Increased PAI-1 concentrations, subsequently, might affect the intraperitoneal behavior of colorectal tumor cells. This, however, warrants further research.

It is concluded that monocyte-like cells modulate the fibrinolytic activity of human peritoneal mesothelial cells by increasing both tPA and PAI-1. Sodium hyaluronate decreased PAI-1 production by mesothelial cells, which effect was ameliorated by the presence of monocyte-like cells.

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CHAPTER 5

AUTO-CROSS-LINKED HYALURONIC ACID GEL DOES NOT REDUCE INTRA-ABDOMINAL ADHESIONS OR ABSCESS FORMATION IN A RAT MODEL OF PERITONITIS

C.J.J.M. Sikkink
B. de Man
R.P. Bleichrodt
H. van Goor

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Abstract

Background: Prevention of adhesion and abscess formation would decrease mortality and morbidity after peritonitis. In this study the effect of a new antiadhesive, auto-cross-linked hyaluronic acid polysaccharide (ACP) gel, on adhesion and abscess formation was studied in a rat peritonitis model.
Study Design: In experiment 1, bacterial peritonitis was induced in 24 Wistar rats, using a cecal ligation and puncture model. Animals were randomized to receive 4ml ACP gel (4%) or 4 ml phosphate buffered saline (PBS). After two weeks animals were killed and adhesions and abscesses were scored. In experiment 2, 72 rats underwent the same procedure but were randomized to receive 2 ml ACP gel, 4 ml ACP gel or 4 ml PBS. After one and three weeks, respectively, half of the animals in each group were killed and adhesions and abscesses were scored.
Results: The median total adhesion score was 12 (range 3-20) in the ACP group and was 9 (range 6-12) in the PBS group (not significant) in experiment 1. 91% of rats in the ACP group developed abscesses, versus 90% in the control group. There were no significant differences in abscess size or number of abscesses. In experiment 2, total adhesion scores in the 2 ml ACP group, 4 ml ACP group and PBS group were 4 (range 2-20), 6 (range 1-11) and 6 (range 1-18) respectively (not significant) after one week and 3.5 (range 1-8), 5 (range 2-15) and 4 (range 0-9), respectively (not significant) after three weeks. All rats in the 2 ml ACP group and the PBS group and 83% of the 4 ml ACP group had developed abscesses after one week. After three weeks these percentages were 80, 75 and 73, respectively. There were no significant differences in size or number of abscesses between groups both after one and three weeks.
Conclusion: Auto-cross-linked hyaluronic acid polysaccharide does not reduce adhesion and abscess formation in a rat peritonitis model.

Introduction

Secondary peritonitis is a common and serious problem in surgical practice associated with high morbidity and mortality. Its most severe causes are bowel perforation and intestinal anastomotic dehiscence. Emergency surgery is required and potentially life saving. Surgery is frequently complicated by abscess formation requiring percutaneous drainage or relaparotomy. In severe cases of peritonitis, more than one laparotomy and the use of temporary closure devices are inevitable. These relaparotomies may easily be complicated by severe adhesion formation and dissection of the adhesions may cause serosal bleeding, inadvertent enterotomy and fistula formation[1, 2]. Diffuse adhesion formation is reason to postpone restoration of intestinal continuity or abdominal wall reconstruction in these patients for six to twelve months. Prevention of adhesions and abscesses, which share a pathophysiological pathway of formation, would be of great help to reduce mortality and morbidity after peritonitis.

In the last years, numerous antiadhesives have been studied, most, however, in elective surgery and non-infectious conditions[3-6]. It is notable that adhesion prevention after peritonitis is hardly studied, whereas peritonitis and subsequent surgery are the ultimate challenge for testing the effectiveness of antiadhesives. Some antiadhesives are not registered for infectious conditions or even aggravate infection, which in part explains the paucity of data[7].

Hyaluronic acid (HA) membrane reduces adhesions in experimental and clinical studies[3-6]. The adhesion reducing capacity of HA seems to be the result of being a physical barrier limiting tissue approximation and biological effects on peritoneal repair mechanisms[8-11]. We previously reported beneficial effects of HA solution in experimental peritonitis[8, 10]. Liquid HA was used to assure spreading of the antiadhesive throughout the abdomen. Drawback of the HA solution was the short residence time. An antiadhesive agent that spreads through the whole abdomen and remains for a period long enough to allow peritoneal healing would be most ideal and a new hyaluronic acid derivate (auto-cross-linked polysaccharide (ACP) gel (Hyalobarrier® gel) seemed to meet these demands.

ACP is a highly viscous suspension in sterile distilled water of pure hyaluronic acid, which has been internally cross-linked without use of foreign molecules. ACP gel reduces intra-abdominal adhesion formation in experimental adhesion models[12-15].

Following the previous experiences with HA solution it was the aim of this study to evaluate the effect of a viscous pure hyaluronic acid derivate on adhesion and abscess formation in a rat model of secondary peritonitis.

Materials and Methods

Study design

The effect of ACP gel on adhesion and abscess formation was studied in an experimental model of peritonitis in rats, closely mimicking the clinical situation. In the first experiment ACP was compared to control (PBS). In the second experiment two volumes of ACP were used and results were scored on two time points. The study protocol was approved by the Animal Ethics Review Committee of the Faculty of Medicine, Radboud University Nijmegen, the Netherlands

Animals

Male Wistar rats (Harlan Nederland, Zeist, The Netherlands), weighing between 260 and 320 gram were housed at 21° Celsius with a day-night cycle of 12 hours. They had free access to water and standard rodent chow (Hope Farms BV, Woerden, The Netherlands).

Antiadhesive agent

Auto-cross-linked polysaccharide (ACP) gel (Hyalobarrier® gel, Fidia Advanced Biopolymers, Abano Terme, Italy) is a highly viscous gel, based on pure hyaluronic acid. ACP is obtained by means of an intern cross-linking reaction of pure hyaluronic acid, in the absence of any chemical substance foreign to the native hyaluronic acid structure. The commercially available ACP gel has a concentration of 4%.

Experiment 1: Effect of ACP gel on adhesion and abscess formation in a rat peritonitis model

A cecal ligation and puncture (CLP) procedure was done to induce bacterial peritonitis in 24 rats. This is a well-elaborated model in our laboratory with mortality rates between 5 and 30 percent mimicking the clinical situation of secondary perforation peritonitis and subsequent surgical source control, and postoperative intra-abdominal adhesion and abscess formation[8, 10]. Food was withheld from the animals 12 hours before the first operation. On day zero, rats were weighed and anaesthetized with a fluothane, nitrous-oxide-oxygen mixture. Before operation the abdomen was shaved and disinfected with 70% alcohol. Via a 3 cm midline laparotomy, the cecum was dissected without damaging the vascularization and was filled backwards with feces. The rat cecum has a saccular shape extending below the level of the ileocecal valve. The cecum was ligated just distal to the ileocecal valve, with a 3.0 polyglactin

suture (Vicryl®, Ethicon, Norderstedt, Germany), without interfering with the passage of stools from the ileum to the colon. The antimesenterial site of the cecum was punctured once with a 19-Gauge needle. The abdominal wall was closed in 2 layers with a 3.0 polyglactin suture. Immediately after operation, rats received a single dose of gentamicine sulfate (Centrafarm Services BV, Etten-Leur, The Netherlands), 6mg/kg, intramuscularly and buprenofine hydrochloride (Temgesic®, Reckitt & Colman Products Ltd., Amstelveen, The Netherlands), 0.1 mg/kg, subcutaneously. All animals were resuscitated with 10 ml of isotonic sodium chloride solution administered subcutaneously.
On day one, the abdomen was reopened under anaesthesia, and in half of the animals peritoneal fluid samples were taken and collected for culture. The abdominal cavity was rinsed with 10 ml of isotonic sodium chloride solution and the cecum was resected.
Before closure of the abdomen, animals were randomly assigned to receive 4 ml ACP gel 4% instilled in the abdominal cavity (ACP group; n=12) or 4 ml phosphate-buffered saline (PBS group; n=12). After two weeks, the animals were weighed and killed using carbon dioxide asphyxiation. Adhesions were scored in a blinded manner according to the method of Zühlke et al., whereby grade zero means no adhesions and grade IV means very strong adhesions, only dissectible with sharp instruments with organ damage almost unavoidable[16]. Sites of adhesions scored included the midline, the upper abdomen (liver), the parietal peritoneum, the omentum, and between bowel loops. The total score of these five locations was noted as the total adhesion score (0-20). If present, the size of abscesses was noted. An abscess was defined as a walled off collection containing purulent material. In half of the animals samples were taken from the abscesses for microbiological examination.

Experiment 2: Effect of two volumes of ACP gel on adhesion and abscess formation in a rat peritonitis model, measured at one and three weeks

Bacterial peritonitis was induced in 72 rats according to the same protocol as applied in experiment 1. Animals were randomised into three groups of 24 rats and treated with 2 ml ACP gel 4%, 4 ml ACP gel 4% or 4 ml PBS (control). After one and three weeks, respectively, half of the animals in each group (n=12) were killed, and adhesions and abscesses were scored as described above. One and three weeks were chosen in conjunction with two weeks in experiment 1 to control for spontaneous resolution of adhesion and abscess formation in time.

Bacterial cultures

Samples of peritoneal fluid were cultured semiquantitatively in aerobic and anaerobic conditions. Colombia III agar with 5% sheep blood (Beckton Dickinson, Etten-Leur, The Netherlands), Levine eosin-methylene blue agar (Oxoid, Haarlem, The Netherlands) and fastidious anaerobic agar (Tapley, Bury, UK) with or without kanamycin, were used for cultures. After 24 and 48 hours of incubation at 37°, bacteria were identified using standard procedures.

Statistical analysis

Statistical analysis of the data in experiment 1 was performed using the non-parametric Mann-Whitney U test and Fisher's exact test. Results of experiment 2 were analysed using the Kruskal-Wallis test for multiple comparisons, with Dunn's post-tests and the Chi-squared test for independence. A p-value < 0.05 was considered statistically significant.

Results

Sepsis and mortality

Following the cecal ligation and puncture procedure rats demonstrated symptoms of intra-abdominal sepsis, including apathic behaviour, ocular exudates, pilo-erection and diarrhoea. These symptoms resolved after 2-3 days after the resection of the ligated and punctured cecum and abdominal lavage in most rats. Three rats (12%) prematurely died in experiment 1; one in the ACP group, two in the PBS group (p=1.00). Death was probably due to inadvertent enterotomy during the surgical procedure in one case. Cause of death was unclear in two cases. 22 Rats (31%) prematurely died in experiment 2; 9 in the 2 ml ACP group, 10 in the 4 ml ACP group and 3 in the PBS group (p=0.06). Fecal peritonitis with subsequent sepsis seemed to be the cause of death in all but one cases.

Cultures

Both in experiment 1 and 2, bacterial cultures taken at the day of cecal resection, revealed a mixed aerobic and anaerobic flora of *Escherichia coli*, coagulase negative *Staphylococcus*, *Proteus* species, *Streptococcus viridans*, *Enterococcus* species, (gram-negative and -positive rods), anaerobic gram-negative and -positive rods and anaerobic gram-positive cocci. A similar flora was found in the abscesses at two weeks in experiment 1.

Adhesions and abscesses

The median adhesion scores at the five different locations and the total adhesion score in experiment 1 are shown in table 1. The quality of adhesions did not differ, except for the omentum at which site adhesions were thicker in the ACP group (p=0.01). 91% of rats in the ACP group developed abscesses, versus 90% in the control group (p=1.00). There was no significant difference in abscess size (p=0.22) or number of abscesses (p=0.76) between the ACP and PBS group.

Table 1. DEATH, ABSCESS RATE AND ADHESION SCORES OF RATS TREATED WITH 4 ML ACP 4% GEL COMPARED TO CONTROLS (PBS). ADHESION SCORES AFTER 2 WEEKS ARE GIVEN AS MEDIAN AND RANGE. * P=0.01 ACP 4ML VS PBS 4ML

	ACP, 4ml	PBS, 4ml
N	12	12
Premature death (N)	1	2
% of rats with abscess	91	90
Adhesion score midline	3 (0-4)	3 (0-4)
Adhesion score liver	0 (0-4)	0 (0-2)
Adhesion score omentum	3 (0-4)*	1.5 (0-3)
Adhesion score peritoneum	3 (0-4)	2 (0-3)
Adhesion score bowel loops	4 (0-4)	3 (2-4)
Total adhesion score	12 (3-20)	9 (6-12)

Similarly to experiment 1, differences between groups with regard to adhesion scores at one and three weeks in experiment 2 were not significant except for the omentum, at which site adhesions were thicker in the 4 ml ACP group at three weeks (difference between groups; p=0.02, post tests: p<0.05 ACP 4 ml vs PBS) (Table 2). 100% of rats in both the 2 ml ACP group and PBS group and 83% of the 4 ml ACP group had developed abscesses after one week (p=0.27). There were no significant differences in abscess size (p=0.66) or number of abscesses (p=0.57). After three weeks, 80% of the 2 ml ACP group, 75% of the 4 ml ACP group and 73% of the PBS group had developed abscesses (p=0.93). Again, both abscess sizes (p=0.34) and number of abscesses were not different (p=0.63).

Table 2. DEATH, ABSCESS RATE AND ADHESION SCORES OF RATS TREATED WITH 2 ML OR 4 ML ACP 4% GEL, COMPARED TO CONTROLS (PBS). ADHESION SCORES AFTER 1 AND 3 WEEKS ARE GIVEN AS MEDIAN AND RANGE. * P<0.05 ACP 4ML VS PBS 4ML

	Scores after 1 week			Scores after 3 weeks		
	ACP, 2ml	ACP, 4ml	PBS, 4ml	ACP, 2ml	ACP, 4ml	PBS, 4ml
N	12	12	12	12	12	12
Premature death (N)	7	6	2	2	4	1
% of rats with abscess	100	83	100	80	75	73
Adhesion score midline	0 (0-4)	0 (0-1)	1 (0-4)	0 (0-2)	0 (0-4)	0 (0-2)
Adhesion score liver	0 (0-4)	2 (0-4)	0 (0-4)	0 (0-4)	1 (0-4)	0 (0-3)
Adhesion score omentum	1 (0-4)	1 (0-2)	1 (0-3)	1 (0-3)	1.5 (1-3)*	0 (0-2)
Adhesion score peritoneum	2 (0-4)	0 (0-2)	1.5 (0-4)	0 (0-1)	1 (0-4)	0 (0-2)
Adhesion score bowel loops	2 (1-4)	3 (1-4)	2.5 (1-4)	2 (1-4)	1.5 (1-4)	2 (0-4)
Total adhesion score	4 (2-20)	6 (1-11)	6 (1-18)	3.5 (1-8)	5 (2-15)	4 (0-9)

Discussion

Hyaluronic acid based agents have shown to be successful reducing postsurgical adhesions after elective surgery[3, 5, 6, 17]. Installing hyaluronic acid into an infected abdomen or pelvis is far less studied and the effectiveness is unclear. The lack of interest in prevention of adhesions in peritonitis or pelvic infection is surprising, considering the fact that adhesions after surgery in these conditions cause serious problems. First, there is a great risk of serosal bleeding, inadvertent enterotomy and fistula formation at dissecting adhesions during relaparotomies[1, 2]. Second, intra-abdominal abscess formation, the result of bacterial entrapment in fibrinous adhesions, is a major complication frequently encountered after treatment for severe peritonitis. Third, diffuse adhesions complicate surgical procedures such as restoration of intestinal continuity and secondary closure of the abdominal wall, which often have to be postponed for several months in order to deal with 'more friendly' adhesions. Finally, there is an association between an increased incidence of adhesive small bowel obstruction and emergency abdomino-pelvic surgery for infection. This anti-adhesive agent with good characteristics for usage in peritonitis was expected to prevent diffuse adhesion formation and abscesses in an experimental rat model of peritonitis closely mimicking secondary peritonitis caused by bowel perforation. However, pure hyaluronic acid cross-linked gel showed no beneficial results.

There is debate on the optimal application form of an agent in postsurgical adhesion prevention. Membranes, sprays, viscous gels and liquid solutions all have been propagated. The advantage of a membrane or a spray is the possibility to selectively cover adhesiogenic spots and not the uninjured peritoneal tissue or "risk areas" of adhesion prevention such as fresh bowel anastomoses[18]. A further advantage is the prolonged residence time of membranes, allowing for separation of traumatized surfaces during the period of mesothelial healing, estimated to be at least 5 days[4, 19]. Disadvantage is the technical difficulty using membranes in laparoscopic surgery. A spray would overcome this disadvantage but is not broadly available. A liquid solution seems more appropriate when broader areas of the peritoneum are damaged such as in generalized peritonitis and in laparoscopic surgery. Disadvantages, however, are the short residence time[6], the inability to control for interference with fresh anastomoses, which are increasingly made in peritonitis and a potential drawback on wound healing. Viscous solution was expected to combine the advantages of both membrane and solution; slow spread throughout the whole abdomen and a longer residence time in the abdominal cavity, allowing adhesion free peritoneal healing. Despite its potential, based on its properties and earlier studies[12-15], viscous ACP gel did not reduce adhesions or abscesses. In contrast adhesions and abscesses were more severe around the omentum and there was a trend towards higher mortality due to abdominal sepsis.

We have previously showed that hyaluronic acid solution reduces adhesions and abscesses in the same experimental model[8, 10]. These results were attributed to a 'floating' effect on the abdominal organs and biologic activity of hyaluronic acid. The results were remarkable, given the fact that HA solution disappears from the abdominal cavity long before peritoneal healing is completed and originally was designed to protect peritoneal tissue during surgical trauma[6]. Hyaluronic acid membrane, another format of HA, did not have a beneficial effect in the same peritonitis model and a trend towards increased abscess formation has also been observed[20]. The similarity between the results of the membrane and the ACP gel in peritonitis leads to the assumption that the format and not the substance itself determines the action in an infected abdominal cavity. This is further supported by similar findings with use of other HA based antiadhesive gels and gels, viscous solutions and membranes not containing hyaluronic acid[7, 21].

In adhesion prevention studies under sterile conditions, concentrations of 1, 2, 4 and 6% ACP gel were tested[14, 15]. The concentration and cross-linking level of the ACP gel determine its viscosity, with higher viscosity at higher levels of these two determinants[22]. Although all concentrations reduced the number and

quality of adhesions significantly, 4% seemed to be the optimal concentration and was chosen in this study.
The negative results may be explained in several ways. First, the gel stays at the site of instillation at the right lower abdomen and does not spread throughout the abdominal cavity due to its high viscosity in combination with a disturbed peritoneal fluid circulation and bowel paralysis during peritonitis. Second, compartimentalization of the abdominal cavity by rapid fibrin formation disturbs proper distribution of the gel. Third, bacteria are entrapped in the viscous gel and growth is relatively unaffected by immune cells and antibiotic therapy, resulting in abscess formation. Indeed, at sacrifice of the animals the majority of adhesions and abscesses were in the proximity of the cecal resection spot surrounded by omentum. In vitro studies would enlighten the question whether the substance itself influences bacterial growth or the gel physically contains the bacteria in a similar way it is hypothesized for a membrane. The containment theory also explains the similar findings with different volumes of ACP gel. Finally, the gel obstructs the diaphragmatic pores, impeding clearance of pathogens via diaphragmatic lymphatic absorption, which is considered an important host defence mechanism in peritonitis[23].
The present study raises concern on the safety of ACP gel under contaminated conditions in terms of mortality due to abdominal sepsis. Results on safety and effectiveness in elective surgery may not automatically be extrapolated to contaminated or infectious procedures. The present unexpected safety results, increased bacterial infection in particular, should be kept in mind when bringing new antiadhesive products to the clinic.
It could be argued that the number of animals in the experiments has influenced the negative results as well. We were interested in a clinically relevant reduction compatible with at least 50% reduction of adhesions and abscess formation in animals. In the control group almost all rats developed adhesions and abscesses, so even with these group sizes a strong reduction would have been noticed. Furthermore, adhesions and abscesses were scored on several time points with similar results. It is known that abscesses induced by peritonitis resolve spontaneously in most rats after a certain time period. This implies that a possible effect of ACP gel is more easily detected after a short time span and a negative effect is more obvious after a longer period. Again, similar abscess rates were seen for all time intervals.
It is concluded that ACP gel does not reduce adhesions and abscesses in a relevant experimental rat model of peritonitis. Adequate and safe prevention of adhesions in contaminated conditions remains a challenge. Seeking for optimal viscosity, volume and residence time is crucial in the development of new antiadhesive products.

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CHAPTER 6

TC-99M-PEG-LIPOSOMES TARGET BOTH ADHESIONS AND ABSCESSES AND THEIR REDUCTION BY HYALURONATE IN RATS WITH FECAL PERITONITIS

C.J.J.M. Sikkink
M.M.P.J. Reijnen
P. Laverman
W.J.G. Oyen
H. van Goor

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Abstract

Background: Abdominal adhesions and abscesses are a major source of morbidity and mortality after abdominal surgery and peritonitis. Adhesions are hard to detect with standard imaging techniques. Liposomes, coated with polyethylene glycol (PEG), represent an agent developed for infection imaging. This study investigated the capacity of ^{99m}Tc-PEG-liposomes to localize early adhesion formation after peritonitis. Additionally, the value of ^{99m}Tc-PEG-liposomes for therapy evaluation of hyaluronan solution, which reduces adhesion and abscess formation in experimental peritonitis, was assessed.

Methods: In 24 rats a bacterial peritonitis was induced by performing a cecal ligation and puncture procedure. The animals were treated with sodium chloride solution or 0.4% hyaluronan solution intra-abdominally. One week later, scintigraphy was performed, using ^{99m}Tc-PEG-liposomes and abnormal focal uptake in the abdomen was scored. Hereafter autopsy was performed and adhesions and abscesses were scored.

Results: A significant correlation was found between the total adhesion score and the scintigraphic score ($p<0.01$, $r=0.65$). Treatment with hyaluronan significantly reduced the total adhesion score ($p=0.01$). The size of abscesses significantly correlated with the scintigraphic score ($p<0.01$, $r=0.62$). Treatment with hyaluronan reduced the size of abscesses ($p<0.05$).

Conclusion: ^{99m}Tc-PEG-liposomes are able to detect early adhesions and abscesses and may be used for therapy evaluation of agents that reduce adhesions and abscesses.

Introduction

Intra-abdominal adhesions and abscesses are a major source of morbidity and mortality. They are mainly caused by abdominal surgery and peritonitis. Adhesions are the foremost cause of intestinal obstruction in the Western world and account for approximately 70% of readmissions for small bowel obstruction[1]. They are responsible for 15% to 20% of cases of secondary infertility in women and are associated with chronic abdominal and pelvic pain[2, 3]. Moreover, adhesions are associated with surgical complications during relaparotomy and a higher incidence of postoperative complications, relaparotomies, admissions to the intensive care unit, and a prolonged hospital stay[4].

Early dense peritoneal adhesion formation is frequent after peritonitis. Adhesion networks may entrap bacteria resulting in intra-abdominal abscesses. Prevention of these networks might reduce residual abscesses after peritonitis and facilitate relaparotomy, which is regularly indicated during treatment of severe peritonitis. Computed tomography (CT) scanning and magnetic resonance imaging (MRI) are not sufficiently accurate to demonstrate early dense adhesion formation.

Liposomes, small lipid vesicles coated with polyethylene glycol (PEG) have been developed for infection imaging[5]. PEG-liposomes can be labelled with ^{99m}Tc in a rapid and efficient procedure, providing images of high quality at low cost and with low radiation dose[6]. When injected intravenously the liposomes accumulate in areas with an increased vascular permeability such as infiltrates and abscesses. Experimental and clinical studies have shown excellent targeting in various models of infection and inflammation, including the cecal ligation and puncture (CLP) model[5-13]. PEG-liposomes localizing early adhesions following peritonitis have never been investigated.

The first aim of the present study was to evaluate the value of ^{99m}Tc-PEG-liposomes visualizing early adhesion formation after experimental peritonitis. Secondly, the feasibility of ^{99m}Tc-PEG-liposomes for evaluation of intra-peritoneal treatment with hyaluronate solution in peritonitis was studied. Hyaluronan-based agents are known to successfully reduce post-surgical intra-abdominal adhesions in both experimental and clinical trials[14-17] and adhesion and abscess formation in experimental peritonitis[18, 19].

Materials and Methods

Animal model

All experiments were carried out in accordance with the guidelines of the local Animal Welfare Committee. Twenty-four male, randomly bred Wistar rats (Harlan Nederland, Zeist, The Netherlands), weighing 270-310 gram, were accustomed to laboratory conditions for one week before experimental use. Animals were housed at 21°C with a day-night cycle of 12 hours. They had free access to water and standard rodent chow (Hope Farms B.V., Woerden, The Netherlands). A bacterial peritonitis was induced by CLP, as described by Wichterman et al.[20]. Briefly, the animals were fasted for 12 hours prior to the first operation. On day zero, rats were weighed and anaesthetized with a mixture of fluothane (Zeneca, Cheshire, United Kingdom), nitrous oxide and oxygen. Prior to the operation the abdomen was shaved and disinfected with 70% ethanol. Via a three cm midline laparotomy, the cecum was dissected and filled backwards with feces. Thereafter, the cecum was ligated just distal of the ileocecal valve, with a 3.0 polyglactin Vicryl suture (Ethicon, Norderstedt, Germany) and its antimesenterial site was punctured once with a 19-Gauge needle. The abdominal wall was closed in two layers with 3.0 Vicryl suture. Immediately after operation, rats received one single dose of 6 mg/kg body weight gentamicin (Centrafarm Services B.V., Etten-Leur, The Netherlands) intramuscularly, and 0.1 mg/kg body weight buprenorfine (Temgesic®, Reckitt & Colman Products Ltd., Amstelveen, The Netherlands) subcutaneously for analgesia. For resuscitation, all animals received 10 ml isotonic sodium chloride solution subcutaneously. At day one, the abdomen was reopened under anaesthesia and peritoneal fluid was taken and collected in a BBL™ Port-A-Cul™ envelope (Becton Dickinson, Cockeysville, Maryland, USA) for microbiological examination. The abdominal cavity was rinsed with 10 ml of isotonic sodium chloride solution and the ligated and perforated cecum was resected. Before closure of the abdomen, animals were randomly assigned to receive 8 mL of isotonic sodium chloride solution (n=12), or 8 mL of 0.4% hyaluronate solution (Sepracoat™, Genzyme Corporation, Cambridge, MA, USA) (n=12) instilled throughout the whole abdominal cavity. The abdominal wall was closed in two layers as described above. Rats received 10 ml isotonic sodium chloride solution and 0.1 mg/kg body weight buprenorfine subcutaneously.

Study design

On day 8, seven days after resection of the cecum, rats were injected via the tail vein with 15 MBq ^{99m}Tc-PEG-liposomes. At 2, 4 and 24 hours, the animals were anaesthetised with a mixture of fluothane, nitrous oxide and oxygen, and

were placed prone on a single headed gamma camera equipped with a parallel-hole, low-energy collimator. Images (30,000 counts/animal) were obtained and stored in a 256 x 256 matrix (Fig. 1). The scintigraphic images were evaluated for increased uptake, and the images at 24 hours appeared to show abnormal abdominal uptake most obvious. The abnormal uptake in the images at 24 hours was scored by comparing the activity with other regions, as described by Schölmerich et al.[21]: 0 = no uptake; 1 = less than bone marrow; 2 = more than bone marrow, but less than liver; 3 = more or equal to liver.

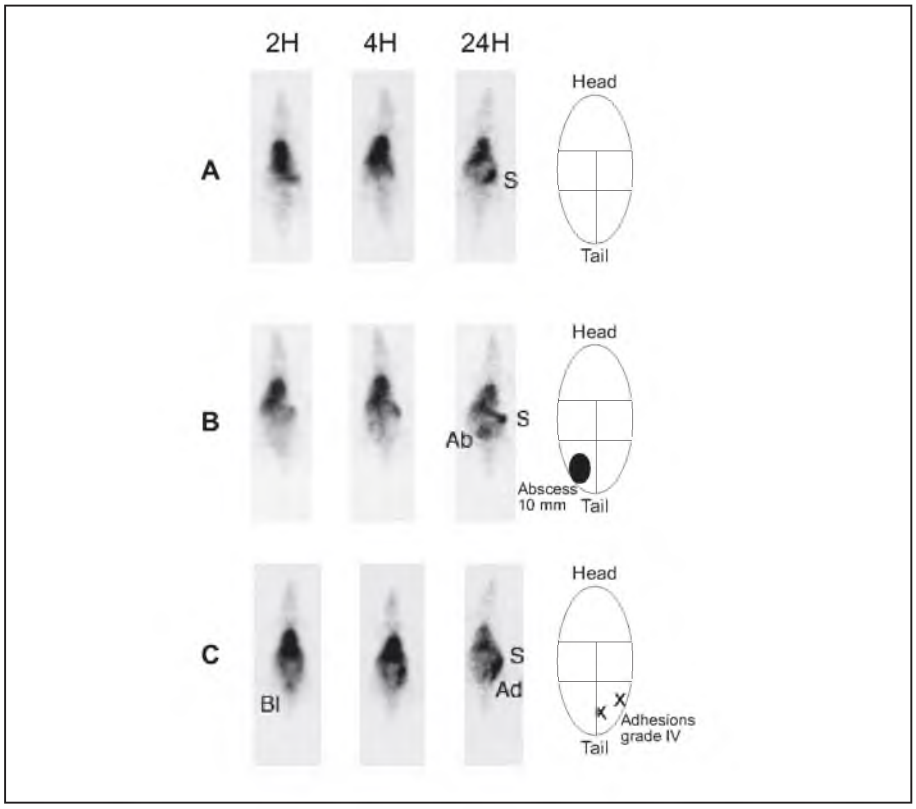

Figure 1. Scintigraphic images of rats 2, 4 and 24 hours after injection of ^{99m}Tc-PEG-liposomes, and a schematic drawing of the observations at postmortem investigation. A: rat without adhesions or abscesses, B: rat with abscess, C: rat with adhesions.
S = spleen, Bl = bladder, Ab = abscess, Ad = adhesions. Note the physiologic high uptake at the spleen and bladder region. The development of adhesions or abscesses is best seen in the images 24 hours after injection.

After recording of the final image, rats were killed by carbon dioxide asphyxiation. At autopsy, macroscopic abnormalities were assessed without knowledge of the imaging results. Adhesions were scored in a blinded manner by one author (H.v.G.) according to the method of Zühlke et al.[22], whereby grade zero means no adhesions and grade IV means firm extensive adhesions that are only dissectable with sharp instruments, with organ damage almost unavoidable. Sites of adhesions scored included the midline, the upper abdomen (liver), the parietal peritoneum, and between bowel loops. Total score of these four locations was noted as the total adhesion score (0-16). If present, the size of abscesses was noted. An abscess was defined as a walled off collection containing purulent material. Samples were taken from the abscesses for microbiological examination. The locations of the adhesions and abscesses were noted in a standardized drawing and compared to the scintigraphic images.

Radiopharmaceutical

^{99m}Tc-PEG-HYNIC-liposomes were prepared as described previously[6]. The liposomes were composed of the polyethyleneglycol-2000 derivative of distearoylphosphatidyl-ethanolamine (PEG-DSPE), partially hydrogenated egg-phosphatidylcholine (PHEPC), cholesterol and the hydrazino-nicotinamide derivative of distearoylphosphatidyl-ethalonamine (HYNIC-DSPE) in a molar ratio of 0.15:1.85:1:0.07. The particle size distribution was determined by dynamic light scattering with a Malvern 2000 system equipped with a 25 mW Neon laser (Malvern, UK). The mean size of the liposome preparations was 80-85 nm with a polydispersity index of < 0.1. Preformed HYNIC-PEG liposomes were labelled with ^{99m}Tc by adding 0.25 ml liposomes (75 μmol phospholipid/ml) to a mixture of 10 mg N-[Tris(hydroxymethyl)-methyl]glycine (Tricine, Fluka), 10 μg stannous chloride in 0.5 ml saline and $^{99m}TcO_4^-$ in saline (15 MBq/μmol phospholipid). The mixture was incubated for 15 min at room temperature. Without any further purification, labeling efficiency was >95%. Fifteen MBq ^{99m}Tc-liposomes were injected intravenously per rat.

Bacterial cultures

Samples of peritoneal fluid and abscesses were cultured semiquantitatively in aerobic and anaerobic conditions. Colombia III agar with 5% sheep blood (Becton & Dickinson, Etten-Leur, The Netherlands), Levine Eosin Methylene Blue agar (Oxoid, Haarlem, The Netherlands) and fastidious anaerobic agar (Tapley, Bury, United Kingdom) with or without kanamycin, were used for cultures. After 24 and 48 hours of incubation at 37°C, bacteria were identified using standard procedures.

Statistical analysis

Two-tailed statistical analysis of differences between groups was performed using the non-parametric Mann-Whitney U test and the Fisher's Exact test. Correlation between adhesions or abscesses and focal uptake on the images was assessed using the Spearman rank correlation test. A p-value <0.05 was considered statistically significant.

Results

All rats demonstrated symptoms of intra-abdominal sepsis, including apathic behaviour, ocular exudates, pilo-erection and diarrhoea following CLP. These symptoms resolved within two days after resection of the ligated and perforated cecum and peritoneal lavage. There were no differences in body weights between groups. One rat, treated with hyaluronan solution, prematurely died due to an unknown cause.

Bacterial cultures of the peritoneal fluid, taken at the day of cecal resection, revealed a mixed aerobic and anaerobic flora of Escherichia coli, Enterococcus species, Proteus species, coliforme gram-negative bacilli, anaerobic gram negative and positive rods and Staphylococcus species in concentrations of 10^5-10^9 colony forming units/mL. Cultures from the abscesses revealed a mixed aerobic and anaerobic flora, similar to those found on the day of cecal resection, in concentrations of 10^7-10^9 colony forming units/mL

Scintigraphy with ^{99m}Tc-PEG-liposomes, adhesions and abscesses

At autopsy 18/23 (78%) rats had adhesions and 12/23(52%) rats had abscesses. Nine of twenty-three (39%) had adhesions without abscesses. Two rats had no intra-abdominal abscesses or adhesions. Five of eleven (45%) hyaluronan treated rats were free of adhesions compared to none of twelve (0%) control rats ($p=0.01$; Fig 2). Four of eleven (36%) hyaluronan treated rats had adhesions requiring sharp dissection (grade 3 and 4) compared to eleven of twelve (92%) control rats ($p<0.01$).

The median focal uptake score of ^{99m}Tc-PEG-liposomes at 24 hours after injection was 1.0 (range 0-3) in rats treated with hyaluronan and 2.5 (range 0-3) in control rats ($p=0.09$)(Fig. 3). There was a significant correlation between the focal uptake score and the total adhesion score ($p<0.01$, $r=0.65$) (Fig.4). Analysis of the subgroup of eleven rats, that had no abscesses demonstrated a significant correlation between the total adhesion score and the focal uptake score ($p<0.05$, $r=0.70$) (Fig 5.).

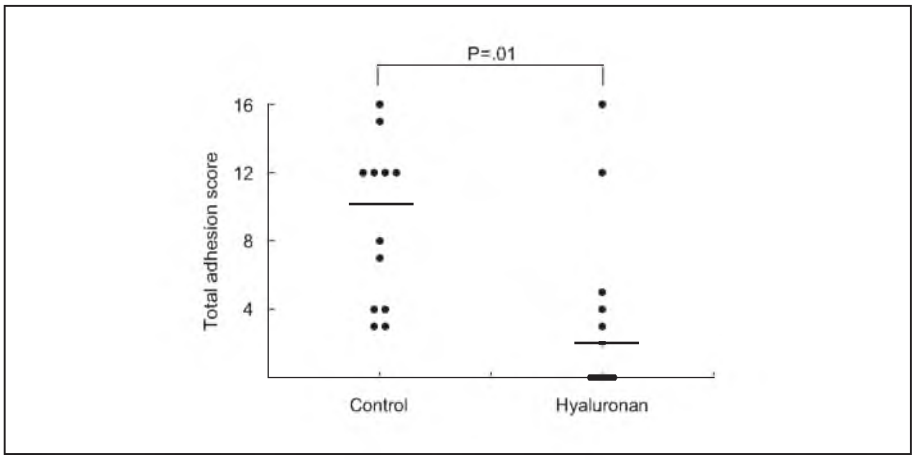

Figure 2. Total adhesion score one week after cecal ligation and puncture in rats treated with 0.4% hyaluronan solution and controls. Points represent adhesion score in individual animals, with bars indicating median levels.

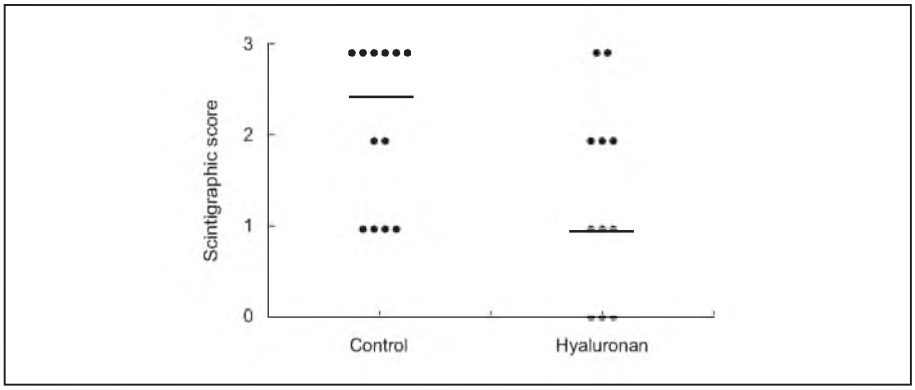

Figure 3. The scintigraphic score one week after cecal ligation and puncture in rats treated with 0.4% hyaluronan solution and controls. Points represent scintigraphic score in individual animals, with bars indicating median levels.

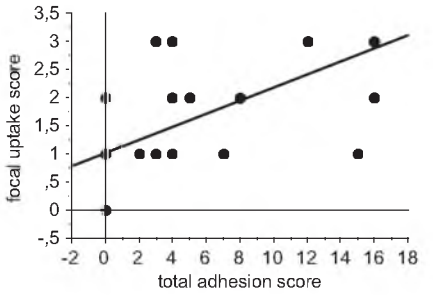

Figure 4. The relationship between the total adhesion score and focal uptake score ($p<0.01$, $r=0.65$).

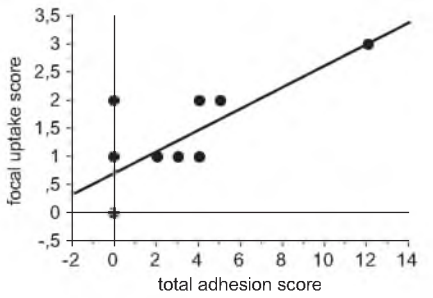

Figure 5. The relationship between the incidence of adhesions without abscess formation and focal uptake score ($p<0.05$, $r=0.70$).

Three of eleven (27%) hyaluronan treated rats had an intra-abdominal abscess compared to nine of twelve (75%) control rats ($p<0.05$; Fig 6). The size of abscesses significantly correlated with the total adhesion score ($p<0.001$, $r=0.74$) (Fig. 7). At scintigraphy, there was a significant correlation between the focal uptake score and the size of abscesses ($p<0.01$, $r=0.62$) (Fig. 8).

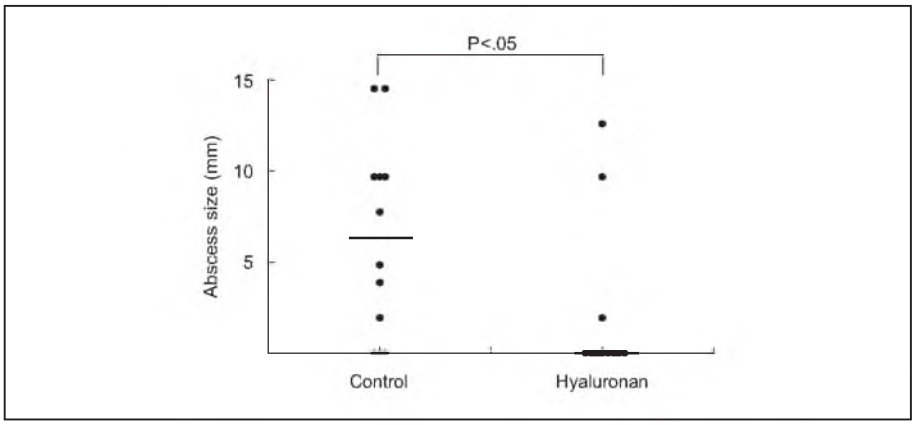

Figure 6. Mean abscess size one week after cecal ligation and puncture in rats treated with 0.4% hyaluronan solution and controls. Points represent abscess size in individual animals, with bars indicating median levels.

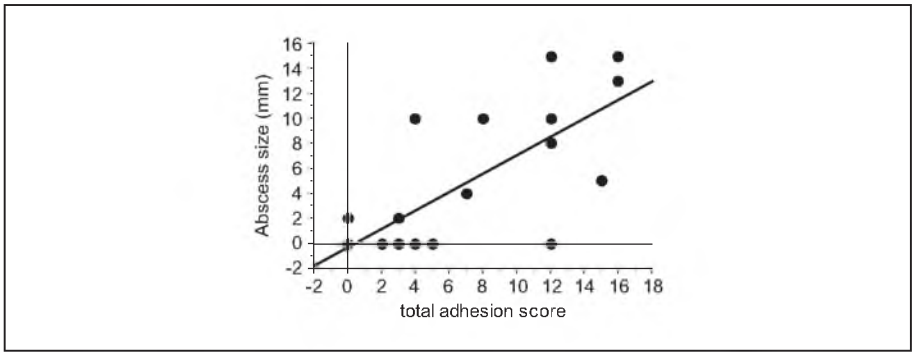

Figure 7. The relationship between the total adhesion score and the abscess size ($p<0.001$, $r=0.74$).

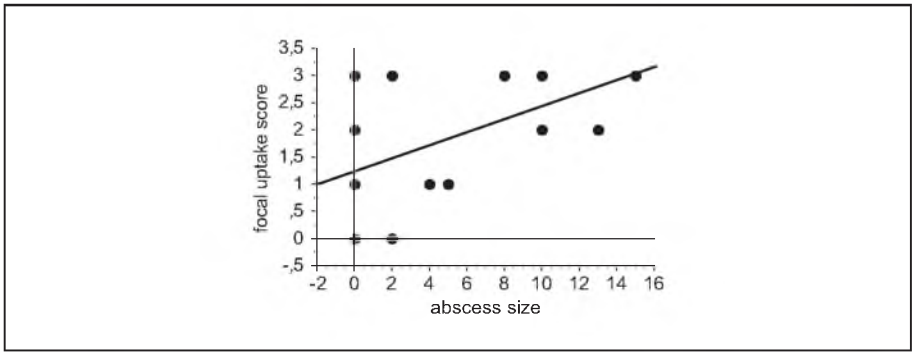

Figure 8. The relationship between the abscess size and focal uptake score ($p<0.01$, $r=0.62$).

Discussion

It was previously shown that ^{99m}Tc-PEG-HYNIC-liposomes are able to detect intra-abdominal abscesses in experimental peritonitis[10]. The present study demonstrates the capability to image adhesions without the presence of infection as well. Reduction in adhesion and abscess formation by hyaluronate solution was apparent by lower focal uptake of the radiopharmaceutical.

Results of the present study are of importance for several reasons. First, this is the first study suggesting a role for ^{99m}Tc-PEG-HYNIC-liposomes in the detection of early adhesions after generalized peritonitis. This observation may be of clinical interest since these adhesions frequently complicate relaparotomies[4], which are commonly performed in patients suffering from bacterial peritonitis. The detection and localization of these early inflammatory adhesions may contribute to the safety of these procedures, reducing for example the rate of inadvertent enterotomies, a frequent complication of postsurgical adhesions. Thusfar, anatomical imaging techniques such as ultrasonography, CT and MRI have yielded disappointing results for the detection of intra-abdominal adhesions. Ultrasonography may only detect adhesions with the anterior parietal peritoneum[23, 24] while CT scanning does not seem to be of value in the detection of intra-abdominal adhesions. Cine MRI holds promise but information especially regarding deeply located adhesions is scarce[25]. An explanation for the accumulation of liposomes in early adhesive tissue is the locally increased vascular permeability of the peritoneal tissue after peritonitis. Since hyperpermeability and angiogenesis are also present after elective surgery it is expected that ^{99m}Tc-PEG-HYNIC-liposomes may detect postsurgical adhesions as well. Using these liposomes it does not seem to be possible to differentiate between early inflammatory adhesions and other infectious processes such as abscesses.

Second, ^{99m}Tc-PEG-liposomes may be of additional value in abscess imaging. Currently, ultrasonography and computed assisted tomography are considered the modalities of choice for the detection of intra-abdominal abscesses. These techniques, however, fully depend on morphological abnormalities that may be absent in early stages of adhesion and abscess formation, or equivocal in postoperative patients. Using ^{99m}Tc-PEG-liposomes would indicate areas of inflammation but the technique is limited by the fact that it is not possible yet to differentiate between vascularized early adhesions and early abscesses.

Third, the use of ^{99m}Tc-PEG-liposomes offers an opportunity to evaluate adhesion and abscess reducing therapies, such as treatment with hyaluronan-based antiadhesives. At present, second look procedures or long follow-up

periods are needed to demonstrate effect of these agents. Scintigraphy after ^{99m}Tc-PEG-liposomes injection may provide a non-invasive method to obtain relevant information within a shorter time period. It is noteworthy that ^{99m}Tc has optimal characteristics for scintigraphic imaging with a half-life of 6 hrs and a low radiation burden, which offers the opportunity of several follow-up scans. Whether the technique is also useful for the detection of permanent adhesions after the period of acute inflammation remains to be elucidated, although vascularization and the presence of inflammatory cells in long existing adhesions have been described[26]. Moreover, additional studies on the assessment of early adhesions generated using a non-infectious model are warranted.

As demonstrated earlier, hyaluronate solution effectively reduced adhesion and abscess formation in experimental fecal peritonitis[18, 19]. In these previous studies, the incidence of adhesions that required sharp dissection was reduced by approximately 40%. Moreover, the hyaluronan solution reduced the incidence of intra-abdominal abscesses by 50%. In contrast, the use of hyaluronan-carboxymethylcellulose membrane demonstrated a trend towards increased abscess formation in experimental peritonitis[18, 27]. The mechanism of action is still unclear, but is most likely based on combined physical and biological properties of hyaluronan, as previously reviewed[28]. The potent effects of hyaluronan suggest a new implication for clinical practice in patients with secondary peritonitis, using it as a lavage solution during surgery. Additionally, patients on continuously ambulatory peritoneal dialysis (CAPD) might benefit from lavage with hyaluronan when suffering from CAPD peritonitis. This type of peritonitis induces intra-abdominal adhesions that, in turn, decrease peritoneal surface needed for dialyzing and ultimately may cause CAPD failure. Further studies seem warranted to determine the benefit of hyaluronan in these clinical conditions.

The high focal uptake of liposomes in infectious foci holds promise for its use in abscess and adhesion preventive therapy by means of targeted drug delivery. Liposomes may be labelled by technetium-99m for scintigraphic imaging, but also agents with an abscess and adhesion reducing capacity may be encapsulated such as tissue plasminogen activator (tPA). Intra-abdominal application of tPA reduces the formation of adhesions and abscesses in various experimental models[29, 30]. It was never tested in a clinical trial since intra-peritoneal application of tPA during surgery might cause bleeding complications. This potential complication may be prevented by intravenous administration as tPA-PEG-liposomes. Moreover, liposomes facilitate sequential administrations of antiadhesive agents.

It is concluded that Tc-99m-labeled HYNIC-PEG-liposomes may detect early

adhesion formation and abscesses. It has potential for the evaluation of new agents reducing adhesions and abscesses. Further research is warranted to evaluate the value of this technique in adhesion formation after non-infectious surgery.

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CHAPTER 7

ADHESION FORMATION AND REHERNIATION DIFFER BETWEEN MESHES USED FOR ABDOMINAL WALL RECONSTRUCTION

C.J.J.M. Sikkink
T.S. de Vries Reilingh
A.W. Malyar
J.A. Jansen
R.P. Bleichrodt
H. van Goor

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Abstract

Background: Incisional hernia is a common surgical problem, frequently requiring prosthetic mesh repair. The demands of the ideal mesh seem conflicting; ingrowth at the mesh-fascia interface, without development of adhesions at the visceral mesh surface. Various antiadhesives combined with macroporous mesh and composite meshes were studied for prevention of adhesions to mesh and ingrowth into the fascia.

Methods: In 60 rats an abdominal wall defect was created and repaired with underlay mesh. Rats were divided in six groups and treated with polypropylene mesh (PPM, control), PPM with auto-cross-linked polymers gel (ACP), PPM with fibrinogen glue (FG), polypropylene/ expanded polytetrafluoroethylene (ePTFE) mesh, polypropylene/ sodium hyaluronate/ carboxymethylcellulose (HA/CMC) mesh, and polypropylene-collagen/polyethylene-glycol/glycerol (CPGG) mesh. Mesh infection was assessed in the postoperative period, adhesions and reherniations were scored at sacrifice two months after operation and tensile strength of the mesh-tissue interface was measured.

Results: Six rats developed mesh infection, half of them were treated with PPM/ePTFE. The PPM/HA/CMC group showed a significant reduction in the amount and severity of adhesions. In animals treated with PPM/ACP and PPM/FG, severity of adhesions was reduced as well. Reherniation rate in the PPM/ACP group was 50% and significant higher than that in other groups. Rats in the PPM/HA/CMC had the highest tensile strength.

Conclusion: PPM/HA/CMC approaches the demands of the ideal mesh best, having superior antiadhesive properties, no reherniation and no infection in this rat model of incisional hernia.

Introduction

Incisional hernias occur in approximately 10-20% of patients after abdominal surgery[1, 2], causing signs varying from mild discomfort and pain to incarceration and strangulation of bowel loops. Prerequisite for successful treatment of incisional hernias is tension-free repair, which often demands the use of prosthetic meshes to bridge the abdominal wall defect. Polypropylene is the most commonly used biomaterial in incisional hernia repair. This macroporous mesh shows good anchorage to the fascia by ingrowth of fibroblasts[3]. Great drawback is the propensity of adhesion formation at the peritoneal side of the mesh[3-5], introducing risk of bowel obstruction and fistula formation. Microporous meshes, like expanded polytetrafluoroethylene, were developed to withstand formation of adhesions and associated complications. However, ingrowth of fibrocollagenous tissue appeared to be reduced in such order that reherniation occurred[6, 7].
At present two concepts are elaborated in an attempt to meet the demands of an ideal mesh; good fibroblast ingrowth and anchorage at the fascial side and no adhesion formation at the peritoneal side. These concepts include a double-layer mesh, having a macroporous and a microporous or nonporous side, and a traditional mesh combined with an antiadhesive agent either incorporated in the mesh or added separately in the peritoneal cavity. Double-layer meshes are increasingly applied in the clinical situation, but despite very encouraging experimental results[8], high mesh infection rate[9] and decreased tensile strength at the mesh-tissue interface[3] have been reported as well. Mesh combined with antiadhesives has good anchorage and shows significant reduction of adhesions in the experimental setting[4, 5, 10-12].
We studied various types of antiadhesive agents combined with a macroporous mesh and compared adhesion formation, reherniation and infection with standard and double-layer meshes in a rat model of large incisional hernia.

Materials and Methods

Animals

Male Wistar rats (Harlan, Zeist, the Netherlands), weighing between 165 g and 225 g were housed at 21°C with a day-night cycle of 12 hours. They had free access to water and standard rodent chow (Hope Farms BV, Woerden, the Netherlands). Study protocols were approved by the Animal Ethics Review Committee of the Faculty of Medicine, University of Nijmegen, the Netherlands.

Meshes

Meshes used were polypropylene mesh (PPM) (Prolene®, Ethicon, Norderstedt, Germany), which served as control mesh, polypropylene/ expanded polytetrafluoroethylene mesh (PPM/ePTFE) (Bard®Composix®, Bard, New Jersey, USA), polypropylene mesh/ sodium hyaluronate/carboxymethylcellulose (PPM/HA/CMC) (Sepramesh®, Genzyme Corporation, Cambridge, USA), and polypropylene-collagen/polyethylene-glycol/glycerol mesh (PPM-CPGG) (Parietene® Composite, Sofradim International, Trévoux, France).

Antiadhesive agents

Antiadhesive agents used were auto-cross-linked polymers (ACP) Gel (Hyalobarrier® gel, Fidia Advanced Biopolymers srl, Abano Terme, Italy) and Fibrinogen glue (FG) (Tissucol®, Baxter Healthcare Corporation, Deerfield, USA).

Study design

60 rats were anaesthetized with an isoflurane (Abbott Labaratories Ltd., Queenborough, UK) nitrous-oxide oxygen mixture. After shaving and disinfection of the abdomen, a midline laparotomy was performed. Subsequently a 2,0 x 3,0 cm full thickness abdominal wall defect was created, which represents approximately 25% of the abdominal wall, without excision of the skin[7]. Rats were randomised in 6 treatment groups (Table 1). In group 1 (controls), the abdominal hernia was treated by using a polypropylene mesh. In groups 2 and 3 polypropylene mesh was combined with use of the antiadhesive agents ACP gel (group 2) and FG (group 3). In group 4-6, composite meshes were used; a mesh having a macroporous and a microporous side, consisting of polypropylene combined with ePTFE (group 4), a mesh having a polypropylene layer combined with a hyaluronan-based antiadhesive layer (group 5) and a mesh having a collagen-based antiadhesive layer added to a polypropylene one (group 6). Meshes of 2,5 x 3,5 cm were placed using underlay technique and were sutured to the fascia with 8 interrupted, non-resorbable 4/0 polypropylene (Prolene®, Ethicon, Norderstedt, Germany) sutures. 4 ml ACP (4%) gel and 2 ml of FG was applied over the abdominal organs under the mesh, before the last two sutures were tied in group 2 and 3, respectively. The underlay technique was chosen because of claimed superiority preventing reherniation above inlay or onlay techniques[13]. Skin was closed using staples.

Mesh infection was thoroughly monitored in the postoperative period and at premature death. Mesh infection was defined as any pus discharge from the wound resulting in wound dehiscence and uncovered mesh. After two months, animals were killed using carbon dioxide asphyxiation. The abdomen was opened

Table 1. DIFFERENT TREATMENT GROUPS IN AN ABDOMINAL WALL HERNIA MODEL IN RATS.

Group	Rats (no.)	Treatment	Separate anti-adhesive agent	Description
1	10	PPM	No	Control
2	10	PPM + ACP-gel	ACP-gel	Mesh + antiadhesive
3	10	PPM + FG	FG	Mesh + antiadhesive
4	10	PPM/ePTFE	No	Double layer mesh
5	10	PPM/HA/CMC	No	Mesh including antiadhesive
6	10	PPM/CPGG	No	Mesh including antiadhesive

PPM = polypropylene mesh
ACP = auto-cross-linked polymers
FG = fibrinogen glue
ePTFE = expanded polytetrafluoroethylene
HA/CMC = sodium hyaluronate/ carboxymethylcellulose
CPGG = collagen/ polyethylene-glycol/glycerol

using a lateral incision, aside from the mesh. The presence of reherniation was noted and the percentage of mesh covered by adhesions was scored. The severity of adhesion formation to the mesh was classified using the Zühlke criteria, whereby grade zero means no adhesions and grade 4 means very dense adhesions, only dissectable with sharp instruments with organ damage almost unavoidable[14]. Subsequently the mesh including 1,0 cm of surrounding fascia was harvested. Sutures were removed and tensile strength of the mesh-fascia specimen was determined.

Tensile testing

After collection of the mesh with surrounding tissue, the mesh was divided in two parts, leaving surrounding tissue on three sides. Subsequently the tissue on two sides was removed, and the width of the remaining mesh-tissue interface was measured (mm). Both ends of the mesh-tissue specimen were fixed in metal clips of a tensile tester (Instron, Canton, USA). Tension on the specimen was increased until rupture of the mesh-tissue interface or fascia itself occurred. Tests were performed with a rate of strain of 1 cm/min. Maximal tensile force was expressed in Newton per square mm of specimen.

Statistical analysis

Statistical analysis between groups was performed using the Chi-squared test for independence for comparison of numbers of events, and the Kruskal-Wallis test (two-tailed) for comparison of continuous variables. The Kruskal-Wallis test was extended with post-tests in case of significant differences. $P < 0.05$ was considered significant.

Results

Mesh infection

The total number of animals who developed mesh infection was 6; one rat in the PPM/ACP group, three rats in the PPM/ePTFE group and two rats in the PPM/CPGG group. Mesh infection led to premature death in one rat (PPM/CPGG group). Differences between groups were not significant ($p=0.114$) (Table 2).

Adhesion formation

The percentage of the mesh covered by adhesions differed significantly between groups ($p=0.03$), with PPM/HA/CMC having the lowest percentage of adhesions (10%) (Table 2). Post-tests revealed that the difference in mesh coverage was significant for PPM/HA/CMC compared to control (PPM) ($p<0.05$). One rat in the PPM/HA/CMC group had zero adhesions. Severity of adhesion formation differed significantly between groups ($p<0.002$). Scores in the PPM/HA/CMC group (2, range 0-4) and PPM/ACP group (2, range 1-2) were significant lower than those in the PPM group (3, range 2-4; $p<0.05$ and $p<0.01$, respectively) (Table 2). The number of animals having dense adhesions (grade 3 and 4) differed between groups ($p<0.002$). One rat in the PPM/HA/CMC and in the PPM/FG group developed dense adhesions and zero rats in the PPM/ACP group (Table 2). Three PPM/ePTFE rats with infection had 100% mesh coverage with dense adhesions (grade 3).

Reherniation and tensile strength

Reherniation rate was significantly higher in PPM/ACP rats (50%) compared to PPM/FG rats (10%) and rats in all other groups (0%) ($p<0.001$) (Table 2).
The interfaces between fascia and PPM/HA/CMC had the highest tensile strength ($p<0.005$) (Fig. 1). PPM/HA/CMC and PPM/ePTFE mesh-fascia interfaces demonstrated significantly higher maximal tensile strength ($p<0.01$ and $p<0.05$, respectively) than the PPM/ACP mesh interface.

Table 2. INFECTION, ADHESIONS AND REHERNIATION AFTER ABDOMINAL WALL RECONSTRUCTION WITH DIFFERENT MESHES IN A RAT MODEL.

Mesh	No. of rats at end of study	No. of rats with mesh infection	% of mesh covered by adhesions median+range	Zühlke score median+range	No. of rats with Zühlke score 0-2 †	No. of rats with Zühlke score 3-4 †	No. of rats with reherniation ‡
PPM	10	0	40 (10-80)	3 (2-4)	3	7	0
PPM/ACP	10	1	35 (15-90)	2 (1-2)**	10	0	5
PPM/FG	10	0	30 (5-90)	2 (2-3)	9	1	1
PPM/ePTFE	10	3	22.5 (10-100)	3 (2-3)	4	6	0
PPM/HA/CMC	10	0	10 (0-35)*	2 (0-4)*	9	1	0
PPM/CPGG	9	2	40 (5-90)	2 (1-3)	6	3	0

*p<0.05 PPM/HA/CMC vs PPM
**p<0.01 PPM/ACP vs PPM
†p<0.002 between groups
‡p<0.001 between groups

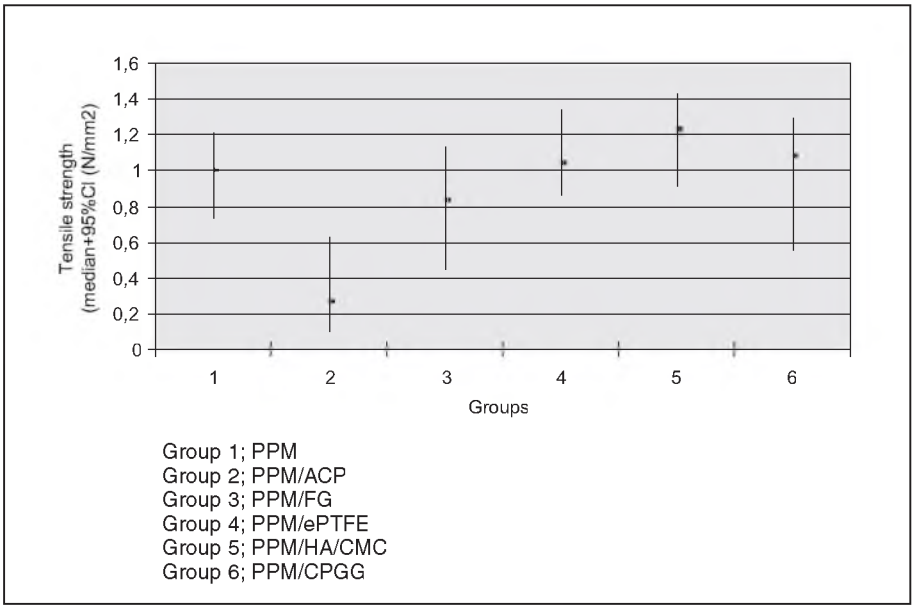

Figure 1. Tensile strenghts of the mesh-fascia interfaces.

Discussion

Reconstruction of large incisional hernias, compelling the use of prosthetic material, is a challenge in general surgery. Mesh infection, adhesive bowel obstruction, fistula formation and hernia recurrence are devastating early and late postoperative complications. Much research has focused on finding the ideal mesh preventing these complications. The ideal mesh should well incorporate fibrocollagenous tissue and anchor firmly to adjacent fascia, be unsusceptible for infection and cause no adhesion formation. In the present study PPM/HA/CMC approached the demands of an ideal mesh most, having superior antiadhesive properties, good anchorage to the fascia and no infection in this rat model of incisional hernia.

There are several animal models to investigate mesh repair of incisional hernia including rat, rabbit and porcine model. No model has proven superiority mimicking the human situation. We choose rats because of our large experience with these animals and the reproducible model of incisional hernia repair and evaluation of adhesions.

HA(/CMC) has demonstrated experimental and clinical efficacy reducing postoperative adhesion formation in various types of abdominal-pelvic operations[15, 16]. The adhesion reducing capacity is attributed to mechanical separation of injured serosal layers. A contributing biological effect of the hyaluronan component on peritoneal repair mechanisms has recently been suggested[17, 18]. Encouraged by the good clinical results, investigators used a HA/CMC sheet underlying a PP mesh in incisional hernia repair, leading to a reduction of adhesion formation[4, 10]. The composite mesh of PP, coated on the peritoneal side with a hyaluronan-based bioresorbable membrane demonstrated similar adhesion reduction but does not exhibit the difficult handling characteristics like the separate HA sheet[5, 11]. Adhesions that developed by using PPM/HA/CMC were not only reduced in number but were more filmy, which is of clinical importance reducing enteric fistula formation and inadvertent enterotomy on re-entry of the abdomen. More filmy adhesions were also seen to the other meshes that were combined with antiadhesives. Nevertheless, this study did not reproduce the results of recent studies, in which the use of presumed antiadhesive substances as fibrinogen, collagen and hyaluronate gel, led to a marked reduction in amount and density of adhesions[8, 12, 19-24].

The PPM/ePTFE mesh with very small pore size at the peritoneal side was designed to withhold ingrowth of cells involved in adhesion formation and yet failed to do so. Massive dense adhesions to the meshes were particularly seen in three animals with a mesh infection, a finding similarly done in the two animals with an infected PPM/CPGG mesh. Stimulation of the coagulation

cascade by bacteria invading the mesh is a first step in fibrin deposition and adhesion formation. The overwhelming inflammatory response accompanying infection stimulates exudation of fibrinogen-rich fluid in the peritoneal fluid and attracts peritoneal inflammatory cells, processes known to be key factors in adhesion formation[25, 26]. An inflammatory response elicited by the foreign body as such, has been demonstrated with the use of ePTFE[27] and is a possible additional factor inducing adhesions. With regard to prevention of adhesion formation to the mesh the concept of preventing inflammation-induced fibrinous attachments and rapid coverage with mesothelial cells might be more valid than that of small pore size or no pores at all preventing cell ingrowth. Hyaluronate, present in ACP gel and PPM/HA/CMC mesh has documented anti-inflammatory properties[17, 18]. Use of fibrinolytic agents or mesothelial cell layers are alternatives preventing deposition of products following peritoneal inflammatory response[28, 29]. Increased mesh infection in the present study is in concordance with the clinically known susceptibility of ePTFE to bacterial invasion and outgrowth giving infection that almost always require mesh excision. This might be explained by the size of host defence cells, which prevents their penetration of the microporous ePTFE structure, while bacteria, which are smaller in size, are able to invade the mesh.

Although auto-cross-linked hyaluronate showed great potential reducing the density of adhesions to the PP mesh, the high rate of reherniation makes it unsuitable for use in hernia repair. Protrusion of the visco-elastic substance when suturing the mesh to the fascial edges combined with the prolonged residence time may adversely affect the healing process at the mesh-fascia interface. Wound-healing problems reflected by fascial dehiscence or incisional hernia are not known from scarce data available on the gel.

Fibrin glue has shown beneficial effects reducing adhesions attributed to rapid sealing of lymph and blood vessels at traumatized peritoneal surfaces preventing ongoing fibrinogen exudation[23, 24]. In the present study, use of PPM/FG decreased adhesion density. The large clinical experience with fibrin glue in other surgeries and the easy handling of the spray form makes it an attractive alternative to HA/CMC in clinical hernia repair.

Although PPM/HA/CMC mesh was superior to other composite meshes and PP meshes combined with antiadhesives in this model, complete adhesion prevention was not achieved. Laparoscopic placement of the mesh could further decrease adhesion formation as laparoscopic surgery has been associated with diminished inflammatory reaction[30]. For laparoscopic use a more versatile mesh (Sepramesh IP®, Genzyme Corporation, Cambridge, USA) has recently be designed because the one used in the experiments cracks when applied through laparoscopic trocars.

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CHAPTER 8

HYALURONAN-BASED ANTIADHESIVE MEMBRANE HAS NO MAJOR EFFECT ON INTRAPERITONEAL GROWTH OF COLONIC TUMOUR CELLS

C.J.J.M. Sikkink
V.A. Postma
M.M.P.J. Reijnen
B. de Man
R.P. Bleichrodt
H. van Goor

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Abstract

Background: A relationship between postsurgical adhesion formation and peritoneal tumour implantation has been proposed. Hyaluronan (HA)-based agents reduce adhesion formation, but the effect on peritoneal tumour is not established. This study investigated the influence of a HA containing agent on intraperitoneal tumour in an experimental model.
Methods: 66 Balb/c mice underwent laparotomy and damage was inflicted to the parietal peritoneum. The animals were randomized into 5 groups. Groups 1 and 2 received HA-carboxymethylcellulose bioresorbable membrane and no treatment, respectively. Mice in groups 3-5 were injected intraperitoneally with 10^5 colon 26-B cells after the laparotomy. Treatment consisted of HA membrane, no HA agent and placement of HA membrane on the non-traumatized peritoneal wall, respectively. Animals were killed after 14 days; adhesions were scored in groups 1 and 2, and tumour mass in groups 3-5.
45 Wag/Rij rats underwent the same procedures and treatment as mice in groups 3-5. In rats 10^6 CC-531 cells were injected. Rats were killed after 3 weeks and tumour mass was scored.
Results: HA membrane resulted in a significant reduction of adhesions, but had no major effect on intraperitoneal tumour mass in mice and rats.
Conclusion: HA-carboxymethylcellulose bioresorbable membrane has no major effect on intraperitoneal tumour implantation and growth in an experimental model.

Introduction

The most common sites for colorectal cancer recurrence are the resection site and the peritoneal surfaces[1, 2]. Peritoneal carcinomatosis is seen in approximately 25-40% of all recurrences[1, 2]. In about 10 percent of patients, peritoneal dissemination is present at diagnosis of the primary tumour[3]. The prognosis of patients with peritoneal carcinomatosis is unfavourable, having a median survival of six months[4].

Tumour penetration through the bowel wall and tumour spill during the primary operation are considered to be the causes of peritoneal metastasis[5]. The mechanism of tumour implantation in the peritoneum after surgery is presumed to be dominated by factors involved in wound healing and adhesion formation[6-9].

In recent years, the experimental and clinical application of antiadhesive products has received much attention. Hyaluronan (HA) derivates reduce postsurgical adhesion formation[10-12]. The adhesion reducing capacity of this polyanionic polysaccharide seems to be the result of mechanical separation, as well as biological effects on peritoneal repair mechanisms[12-14].

Based on the properties, it was hypothesized that HA reduces peritoneal tumour implantation and growth. The hypothesis was tested using both a mouse and rat model of peritoneal injury, tumour implantation and growth. In addition, adverse effects of HA on tumour growth were evaluated.

Materials and Methods

Study design

The effect of HA-carboxymethylcellulose on adhesion formation (mouse model) and implantation and growth of tumour cells (mouse and rat models) was studied in two rodent models of surgical peritoneal injury. Study protocols were approved by the Animal Ethics Review Committee of the Faculty of Medicine, University of Nijmegen, the Netherlands.

Animals

Balb/c mice (Charles River, Sulzfeld, Germany), weighing between 20 and 26 g and WAG/Rij rats (Charles River, Sulzfeld, Germany), weighing between 224 and 282 gram were housed at 21°C with a day-night cycle of 12 hours. They had free access to water and standard rodent chow (Hope Farms BV, Woerden, the Netherlands).

Tumour

Balb/c mouse model

A murine adenocarcinoma cell line colon 26-B, which is derived from a chemically induced colon carcinoma in a Balb/c mouse was used. Cells were grown in the thigh of a Balb/c mouse. The tumour nodulus was collected at growth of approximately 1 cm in diameter and sliced in pieces (<1 mm). RPMI medium (5 ml), Collagenase (20 mg) and DNase (5 µl of a 20 mg/ml solution) were added. The solution was rotated for 2 hours at 37°C, centrifuged (4 minutes at 1500 r.p.m.), washed twice with phosphate buffered saline (PBS), centrifuged again and resuspended in PBS. Viability was measured by trypan-blue exclusion. Viability of cells always exceeded 95%.

WAG/Rij rat model

CC-531 tumour is a moderately differentiated, weakly immunogenic colonic adenocarcinoma induced in WAG rats by 1,2dimethylhydrazine. The tumour was maintained in cell culture in RPMI-1640 medium, supplemented with 5% fetal calf serum, penicillin (100U/ml), streptomycin (100mcg/ml) and glutamin (200 mmol). All supplements were obtained from Life Technologies. Before their use in vivo, cells were trypsinized and washed with PBS. Viability of cells always exceeded 95%.

10^5 Colon 26-B cells in 0.5cc NaCl were used for the selected animals in experiment 1 (mice) and 10^6 CC-531 cells in 1cc NaCl were used in experiment 2 (rats). These doses were based on dose-finding studies, aiming at about 80% of animals having macroscopic tumour load. In these studies, the aimed tumour loads were obtained after a period of two weeks in mice and three weeks in rats.

Hyaluronan derivate

The second generation HA-carboxymethylcellulose-USP glycerol bioresorbable membrane (Seprafilm II™, Genzyme Corporation, Cambridge, USA) was used as antiadhesive agent. This membrane was kindly donated by Genzyme Corporation, Naarden, the Netherlands.

Control groups received no treatment. HA membrane on the non-traumatized peritoneum served as control for possible tumour stimulating activity of HA.

Experiment 1: Effect HA on intra-abdominal adhesion formation and growth of intraperitoneal tumour cells in mice

Sixty-six mice were anaesthetized with a fluothane (Zeneca, Cheshire, United Kingdom) nitrous-oxide oxygen mixture. After shaving and disinfection of the abdomen, a 2 cm midline laparotomy was performed. Peritoneal trauma was

inflicted by a 1 cm incision on the right parietal peritoneum, 1.5 cm lateral from the midline incision. The peritoneal incision was closed using three Prolene 5/0 (Ethicon, Norderstedt, Germany) sutures, dividing the trauma area in eight areas of 12.5%. Mice were randomized in 5 groups and treated as described in Table 1. The midline was closed using Prolene 4.0 (Ethicon, Norderstedt, Germany) for the fascia and staples for the skin. After the procedure, mice in groups 3-5 were injected with tumour cells intraperitoneally.

Fourteen days after the operative procedure mice were killed using CO2 asphyxia. The extent and quality of the adhesions were scored in group 1 and 2 mice. The extent was quantified by estimation of the involvement of the eight areas of 12.5% on the traumatized peritoneum, as previously described[15]. The quality of adhesions was scored using the Zühlke classification[16]; type 1 adhesion: filmy adhesion, easy to separate by blunt dissection; type 2: stronger adhesion; blunt dissection possible, partly sharp dissection necessary, beginning of vascularisation; type 3: strong adhesion, lysis possible by sharp dissection only, clear vascularisation; type 4: very strong adhesion, lysis possible by sharp dissection only, organs strongly attached with severe adhesions, damage of organs hardly preventable.

Table 1. DIFFERENT TREATMENT GROUPS IN EXPERIMENT 1 AND 2.

	Group	No. of animals	Tumour cells	HA membrane traumatized peritoneum	HA membrane laparotomy wound	HA membrane non-traumatized peritoneum
Exp. 1 (mice)			colon 26b	0.7x1.5 cm	1x2.5 cm	0.7x1.5 cm
	1	15	no	yes	yes	no
	2	15	no	no	no	no
	3	12	10^5	yes	yes	no
	4	12	10^5	no	no	no
	5	12	10^5	no	yes	yes
Exp. 2 (rats)			CC-531	1x2.5 cm	1x6 cm	1x2.5 cm
	1	15	10^6	yes	yes	no
	2	15	10^6	no	no	no
	3	15	10^6	no	yes	yes

In group 3-5, the intraperitoneal tumour growth was scored by determining the percentage of the traumatized area occupied by tumour, using the peritoneal cancer index (PCI) according to Steller[17] and by weighing the total tumour mass after excision. The PCI ranges from 0-5; a score of 0 means no tumour growth, a score of 1 indicates a tumour diameter less than 0.5 cm, a score of 2 a tumour diameter between 0.5 and 1 cm, a score of 3 a tumour diameter between 1 and 2 cm, a score of 4 a tumour diameter between 2 and 3 cm and a score of 5 a tumour diameter exceeding 3 cm.

Experiment 2: Effect HA on adhesion and growth of intraperitoneal tumour cells in rats

Forty-five rats received a similar peritoneal injury as described in experiment 1. However, the midline incision was 5 cm and the peritoneal incision was 2cm, 2 cm lateral from the midline incision. Rats were randomized in three groups and treated as described in Table 1. The abdomen was closed in two layers, using Prolene 4/0 and staples. Afterwards tumour cells were injected intraperitoneally. In rats, no groups without tumour were included to control for the adhesion reducing capacity of the HA membrane.
Three weeks after the operative procedure the rats were killed by CO2 asphyxia. Intraperitoneal tumour growth was scored as described in experiment 1.

Statistical analysis

Statistical analysis between groups was performed using the Mann-Whitney U test (nonparametric, two-tailed). Statistical significance was defined as $p < 0.05$.

Results

Experiment 1: Effect HA on intra-abdominal adhesion formation and growth of intraperitoneal tumour cells in mice

No mice died in group 1 and 2 before the end of the experiment. The use of HA membrane resulted in a significant reduction of the percentage of adhesions at the injured peritoneal wall (12.5% (range 0-100), vs 75% (0-100), $p < 0.01$). Zühlke score was also significant lower when a HA membrane was used compared to controls (2(0-4) vs 4(0-4), $p < 0.05$).
In group 3-5, no mice died before the end of the experiment. All mice but two (both in group 4; no HA membrane) developed tumour growth. As expected, tumour growth was almost exclusively located at the site of peritoneal trauma and the laparotomy wound. HA membrane at the traumatized area, the laparotomy wound, or the non-traumatized peritoneal wall did not significantly

affect the percentage of (traumatized) area covered by tumour, the PCI or the tumour weight (Table 2).

Table 2. RESULTS OF HA MEMBRANE TREATMENT IN A PERITONEAL DISSEMINATION MODEL IN MICE.

Group	n	% tumour trauma area	PCI R	PCI L	PCI ML	tumour weight R	tumour weight L	tumour weight ML	total tumour weight
3	12	62.5 (37.5-100)	2 (1-3)	0 (0-0)	3 (1-4)	273 (93-637)	0 (0-0)	537 (98-794)	769 (191-1431)
4	12	75 (0-100)	2 (0-4)	0 (0-0)	3 (0-4)	313 (0-793)	0 (0-0)	397 (0-1250)	697 (0-2043)
5	12	43.8 (0-100)	2 (0-3)	0 (0-3)	2 (1-4)	160 (0-627)	0 (0-943)	352 (118-751)	550 (118-1585)

Values are median (range). Group 3; HA membrane, group 4; no additives, group 5; HA membrane contralateral. PCI R; peritoneal cancer index right peritoneum, L=left peritoneum, ML= peritoneum at the site of the midline incision. Tumour weights are in milligrams. Differences between groups are not significant.

Experiment 2: Effect HA on adhesion and growth of intraperitoneal tumour cells in rats

Two of 45 rats died before the end of the experiment. One rat died on the second postoperative day due to peritonitis after accidental puncture of the colon. One rat was killed on the third postoperative day because of eventration. Eight of 43 rats did not develop tumour growth; three in group 1 (HA membrane), five in group 2 (no HA) and one in group 3 (HA contralateral). As in mice, tumour growth was predominantly located at the traumatized peritoneum and the laparotomy wound. The results are shown in Table 3. There was no significant difference in tumour growth between rats treated with or without HA membrane, at any side of injury.

Table 3. RESULTS OF HA MEMBRANE TREATMENT IN A PERITONEAL DISSEMINATION MODEL IN RATS

Group	n	% tumour trauma area	PCI R	PCI L	PCI ML	tumour weight R+ML	tumour weight L	total tumour weight
1	14	100 (0-100)	5 (0-5)	0 (0-0)	5 (0-5)	9.9 (0-16.5)	0 (0-0)	9.9 (0-16.5)
2	14	93.8 (0-100)	3.5 (0-5)	0 (0-0)	4 (0-5)	4.6 (0-20.1)	0 (0-0)	4.6 (0-20.1)
3	15	100 (0-100)	5 (0-5)	0 (0-1)	5 (0-5)	7.7 (0-20)	0 (0-4.7)	7.7 (0-20)

Values are median (range). Group 1; HA membrane, group 2; no additives, group 3; HA membrane contralateral. PCI R; peritoneal cancer index right peritoneum, L=left peritoneum, ML= peritoneum at the site of the midline incision. Tumour weights are in grams. Differences between groups are not significant.

Discussion

Modified HA derivates are presently used to reduce the number and severity of postsurgical adhesions[10-12]. Question has been raised as whether these derivates would prevent tumour implantation and growth at the traumatized peritoneum, because of the presumed similarity between pathogenesis of adhesions and tumour implantation[5, 8]. The present study has shown that HA-carboxymethylcellulose bioresorbable membrane does not prevent tumour implantation and growth in a model of surgical trauma and peritoneal dissemination of colonic cancer cells, whereas adhesions were significantly reduced. Although the use of HA membrane did not result in a major reduction of tumour implantation and growth at the site of trauma, it neither seemed to promote tumour growth.

There are several possible explanations for the fact that adhesions were reduced by HA, while no evident reduction of tumour implantation and growth was found. First, HA reduced adhesions, but total prevention was not accomplished, leaving areas with adhesions and tumour cell implants. Second, tumour cells remain present in the peritoneal cavity in high concentrations and implantation takes place several days after tumour cell injection at a moment that the HA film has loosened from the injured peritoneal wall but healing of the peritoneal mesothelium is not complete[18]. The HA bioresorbable membrane is reported to remain in the abdominal cavity in gel form for about 7 days[19]. It is, however, uncertain whether active substance remains present for a sufficient long period, at the injured site where the HA membrane is initially placed.

In both mice and rats experiments some animals did not develop tumour growth. The most likely explanation is "metastatic inefficiency"; not all tumour cells lead to metastasis[5]. Both immunological defence mechanisms and other factors involved in wound healing and adhesion formation influence peritoneal tumour implantation and growth after surgery[6-9]. In dose-finding studies (data not shown), the number of animals without tumour growth increased when lower amounts of tumour cells were injected. There might be a critical amount of tumour cells needed to overcome the host defence mechanisms.

We used both a rat and mouse model. Main reason was to control for inter-species variability. Another reason was the concern that the results in mice were influenced by the limited intraperitoneal space. More tissue-handling and tissue-contact seems unavoidable in comparison with rats, which creates unintended large areas of serosal damage. Furthermore, the relatively greater mass of HA derivates in the smaller paracolic gutter of mice might influence the fluid movements in the abdominal cavity. Tumour cells follow these fluid

movements from the pelvis via the paracolic gutters towards the subhepatic and subdiaphragmatic spaces[18].

In contradiction to the hypothesis of this study, it has been suggested that HA derivates increase tumour implantation and growth. The design of the experiments, with placement of HA membrane on non-traumatized peritoneum, allowed to study this in addition. The effect of HA itself seems dualistic based on its interactions with the surface receptor CD44, which is present on a variety of cancer cells, including colon 26-B and CC-531 cells[20, 21]. Zeng et al. demonstrated that tumour growth can be inhibited in vivo by HA oligomers, probably due to an effect on the interaction of endogenous HA and CD44[22]. Through this interaction, exogenous HA may bind spilled tumour cells via their CD44 receptors, thereby prohibiting direct attachment to traumatized peritoneum, after which the immunologic system can clear these tumour cells. It is also possible that disruption of the endogenous HA-CD44 interactions leads to inhibition of signal transduction necessary for tumour cell proliferation. On the contrary, HA derivates may attract tumour cells to traumatized areas, bind the CD44 receptors and promote local implantation and growth. Our results show that HA membrane itself has no major tumour promoting effect, strongly suggesting that peritoneal trauma is a key factor in enhanced tumour growth.

Only a few other experimental studies have been performed, with regard to the use of HA in an intra-abdominal tumour model[23-26] (Table 4). Haverlag et al. studied whether the use of a 0.4% HA solution affected the adhesion of CC-531 colonic carcinoma cells[23]. Their in vitro studies showed an inhibitory effect of HA solution on adhesion of tumour cells. However, experiments in rats showed a tendency towards higher tumour load when HA solution was used in an uterine abrasion model. In a laparotomy model, mean total tumour scores did not differ significantly. We feel that HA solution is unsuitable for studying tumour growth reduction, because of the short lifetime (<48-72 hours) in the abdominal cavity[27].

Underwood et al. examined the effects of a HA membrane on GW-39 human colon cancer implantation at surgical wound and laparoscopic trocar sites in a hamster model[24]. They concluded that HA membrane neither had a protective nor an adverse effect on tumour implantation and/or growth.

On the contrary, Tan and associates performed in vitro studies, using human colorectal tumour cell lines SW480, SW620 and SW707, and demonstrated that HA solution significantly increased tumour cell proliferation and motility[25]. In vivo, the use of HA solution resulted in a significantly higher total tumour nodule count in a laparotomy model in rats, using DHD/K12 tumour cells intraperitoneally. The use of a nodule count as method of tumour load assessment in this study is questionable.

Table 4. STUDIES CONCERNING THE EFFECT OF HA ON PERITONEAL DISSEMINATION (IN VIVO RESULTS).

Reference	Year	Animal	Model	Tumour	HA derivate	Outcome
Haverlag et al.	1999	WAG rat	Laparotomy and uterine horn abrasion or laparotomy only, i.p.seeding of tumour	CC-531	HA 0.4% (Sepracoat™)	No effect of HA
Underwood et al.	1999	Syrian hamster	Laparotomy and trocar placement, i.p. seeding of tumour	GW-39	HA membrane (Seprafilm™)	No effect of HA
Tan et al.	2001	BD IX rat	Laparotomy, i.p. seeding of tumour	DHD/K12	HA 0.4%	HA enhances tumour growth
Hubbard et al.	2002	BALB/c mouse	Laparotomy, i.p. seeding of tumour	KM12-L4	HA membrane (Seprafilm™)	No effect of HA?
Sikkink et al.	2003	BALB/c mouse WAG rat	Laparotomy, peritoneal wall incision, i.p. seeding of tumour	colon 26-B CC-531	HA membrane (SeprafilmII™)	No major effect of HA

I.p.= intraperitoneal

Hubbard and Burns examined the effect of the first generation HA membrane on human colon cancer cell growth and metastasis in a nude mouse model[26]. They concluded that HA-carboxymethylcellulose did not affect tumour metastasis. However, placement of the membrane on non-traumatized peritoneum led to increased local tumour growth. They attributed this outcome to trauma, as result of placement of the biomaterial, and not to its composition. In our experience placement of the membrane is not a traumatic procedure and their explanation given is questionable.

A uniform conclusion can not be drawn from the results of these studies, due to the contradicting results, variability in models and tumour cell lines used. It is concluded from the present study that HA-carboxymethylcellulose membrane has no major effect on intraperitoneal tumour implantation and growth.

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CHAPTER 9

SUMMARY AND FUTURE DIRECTIONS

Summary

In chapter 1 the clinical impact and pathophysiology of intra-abdominal adhesion and abscess formation, which have a common pathophysiological base via the fibrinogenesis and fibrinolysis pathway, are summarized. Postoperative adhesions cause considerable morbidity and even mortality. Readmissions for small bowel obstruction after abdominal surgery are frequent and lead to survival intervention in a considerable amount of cases. Adhesions complicate relaparotomies and increase the incidence of inadvertent enterotomy.
Adhesions develop after peritoneal trauma, which may be induced by surgery or inflammation. A fibrin matrix is formed on the damaged peritoneal areas. Normally the fibrinolytic system will lyse these clots, however, peritoneal fibrinolytic activity is impaired after surgery and infection. Many studies have focused on methods to diminish fibrin formation or increase fibrinolysis in order to reduce adhesions and intra-abdominal abscess formation.
Hyaluronan, a polysaccharide, is a frequently studied antiadhesive substance. The working mechanism of hyaluronan is twofold: mechanical separation of traumatized peritoneal surfaces and biological activity. Hyaluronan plays a role in tissue repair and wound healing, modifies the inflammatory response and facilitates cell migration and proliferation. Fibrin deposits may trap bacteria and thus form a nidus for abscess formation. Prevention of fibrin formation or early lysis of these deposits in patients with intra-abdominal infections may be an attractive method to prevent abscess formation. Since hyaluronan is effective in adhesion prevention and has distinct activity on the fibrinolytic cascade, its use in the treatment of intra-abdominal sepsis is most challenging. There is hardly any study on this topic.
In chapter 2 the literature of hyaluronan-based antiadhesive agents in abdominal surgery is reviewed. The urge to prevent adhesion formation has resulted in numerous experimental and clinical trials. Despite the effectiveness of hyaluronan, there is much debate on its clinical use and mechanisms of action. Various hyaluronan-containing products have been introduced but are withdrawn from the market. The application of hyaluronan in combination with meshes for hernia repair appears to be a promising concept. Not all different applications of hyaluronan are well studied and the use in patients with a malignancy or abdominal infection remains controversial. An overview is given on the effects of hyaluronan-based antiadhesive agents in abdominal surgery, the use in infectious conditions and in malignant disease. The different possible mechanisms of action of hyaluronan are discussed as well.

Peritoneal mesothelial cells are involved in a variety of biological processes among which the formation of peritoneal adhesions. Cultures comprise an important tool to investigate human mesothelial cell behaviour and functions. The objective of the study in chapter 3 was to evaluate the effects of cell propagation and freezing and storing, when using mesothelial cells from different sources, and the effect of hyaluronan on cell function. Viable cell lines were obtained from both omentum and peritoneal lavage fluid of seven patients. Intercellular adhesion molecule-1 (ICAM-1) expression, by ELISA, and matrix metalloproteinase (MMP) bioactivity, by zymography, were measured in the second and fourth passage. MMP bioactivity was additionally measured after freezing and storage of cells in liquid nitrogen. The effects of different stimuli, including interleukin 1beta (IL-1β), tumor necrosis factor-alpha (TNF-α), phorbol myristate acetate (PMA) with and without hyaluronan were determined. ICAM-1 was constitutively expressed and stimulated by IL-1β, TNF-α and PMA. All cell lines produced both MMP-2 and MMP-9. Only the latter activity was positively affected by TNF-α and, especially so, PMA. Differences were found between the second and fourth passage, and between cells of different lineage (i.e. lavage cells vs omental cells), mostly so if the relative stimulation by the various agents was compared. Addition of sodium hyaluronate either to control cultures or to cultures together with any of the three stimuli examined did not significantly change ICAM-1 expression and gelatinase activity. Freezing and storage of cells did not affect cell functions. From this study it was concluded that both the human omentum and peritoneal lavage fluid are good sources of mesothelial cells for in vitro culturing. Cells can be propagated, also after freezing, without qualitative changes in their ability to express ICAM-1 and to produce gelatinases. For omental cells there are differential effects of stimulation depending on whether the cells have been passaged 2 or 4 times. The presence of hyaluronan did not affect the expression of ICAM-1 or gelatinases.

The objective of the study in chapter 4 was to determine whether the presence of cells of the monocyte-macrophage system affects the fibrinolytic response of peritoneal mesothelial cells to lipopolysaccharide (LPS) in the presence and absence of sodium hyaluronate. Human peritoneal mesothelial cells were harvested from patients undergoing a laparotomy for non-infectious reasons and cultured in vitro. Co-cultures were formed by adding U-937 human monocyte-like cells to a monolayer of mesothelial cells. After 24 hours, cultures were treated with 10 ng/ml LPS and sodium hyaluronate was added in a final concentration of 0.2%. Controls received medium without sodium hyaluronate. After 24 hours incubation, tissue Plasminogen Activator (tPA), urokinase Plasminogen Activator (uPA) and Plasminogen Activator Inhibitor-1 (PAI-1) levels

were determined in medium and cell lysates using ELISA techniques. In medium of co-cultures, tPA and PAI-1 concentrations were significantly increased compared to monocultures ($p<0.05$ and $p<0.005$, respectively), while uPA concentration was significantly decreased ($p<0.01$). In cell lysates of co-cultures, PAI-1 concentration was significantly increased compared to monocultures ($p<0.01$), while tPA and uPA were unaffected. Sodium hyaluronate significantly decreased PAI-1 ($p<0.05$) and uPA ($p<0.01$) concentrations in medium of monocultures and uPA concentration only in medium of co-cultures ($p<0.01$). It was concluded that cells of the monocyte-macrophage system modulate the fibrinolytic capacity of LPS treated human peritoneal mesothelial cells and interfere dualistic in the hyaluronan associated changes in mesothelial fibrinolytic capacity.

From chapter 3 and 4 no unequivocal conclusions about the presumed biological properties of hyaluronan could be drawn, although results presented in chapter 4 confirmed earlier observations that hyaluronan may affect peritoneal fibrinolysis.

The aim of chapter 5 was to determine whether auto-cross-linked hyaluronic acid polysaccharide (ACP) gel could reduce adhesion and abscess formation in a rat peritonitis model. In the first experiment, bacterial peritonitis was induced in 24 Wistar rats, using a cecal ligation and puncture model. Animals were randomized to receive 4 ml ACP gel (4%) or 4 ml phosphate buffered saline (PBS). After two weeks animals were killed and adhesions and abscesses were scored. In the second experiment, 72 rats underwent the same procedure but were randomized to receive 2 ml ACP gel, 4 ml ACP gel or 4 ml PBS. After one and three weeks, respectively, half of the animals in each group were killed and adhesions and abscesses were scored. In the first experiment, the median total adhesion score was 12 (range 3-20) in the ACP group and was 9 (range 6-12) in the PBS group (not significant). 91% of rats in the ACP group and 90% in the control group developed abscesses. There were no significant differences in abscess size or number of abscesses between groups. In the second experiment, total adhesion scores after one week in the 2 ml ACP group, 4 ml ACP group and PBS group were 4 (range 2-20), 6 (range 1-11) and 6 (range 1-18) respectively (not significant), and 3.5 (range 1-8), 5 (range 2-15) and 4 (range 0-9), respectively (not significant) after three weeks. All rats in the 2 ml ACP group and the PBS group and 83% of the 4 ml ACP group had abscesses after one week. After three weeks these percentages were 80, 75 and 73, respectively. There were no significant differences in size or number of abscesses between groups both after one and three weeks. It was concluded that ACP gel does not reduce adhesion and abscess formation in a rat peritonitis model.

In chapter 6, the use of ^{99m}Tc-polyethylene glycol (PEG)-liposomes for detection of early adhesion formation after peritonitis was studied. Additionally, the value of ^{99m}Tc-PEG-liposomes for therapy evaluation of hyaluronan solution was assessed. It was previously shown that this solution reduces both adhesions and abscesses in a model for secondary peritonitis. A bacterial peritonitis was induced in 24 rats by performing a cecal ligation and puncture procedure. The animals were treated with sodium chloride solution or 0.4% hyaluronan solution intra-abdominally. One week later, a scintigraphy was performed, using ^{99m}Tc-PEG-liposomes and abnormal focal uptake in the abdomen was scored. Hereafter autopsy was performed and adhesions and abscesses were scored. A significant correlation was found between the total adhesion score and the scintigraphic score ($p<0.01$, $r=0.65$). Treatment with hyaluronan significantly reduced the total adhesion score ($p=0.01$). The size of abscesses significantly correlated with the scintigraphic score ($p<0.01$, $r=0.62$). Treatment with hyaluronan reduced the size of abscesses ($p<0.05$). It was concluded that ^{99m}Tc-PEG-liposomes are able to detect early adhesions and abscesses and may be used for therapy evaluation of agents that reduce adhesions and abscesses. It was, however, not possible to distinguish between adhesions or abscesses. The antiadhesive and abscess reducing capacity of hyaluronan was reconfirmed. After abdominal surgery, and especially after secondary peritonitis, incisional hernia is a frequent complication. Hernia repair usually requires the use of prosthetic mesh. The demands of an ideal mesh are conflicting; ingrowth at the mesh-fascia interface, without ingrowth at the visceral mesh surface by adhesions. In chapter 7, various antiadhesives combined with macroporous mesh and composite meshes were studied for prevention of adhesions to mesh and ingrowth into the fascia. An abdominal wall defect was created in 60 rats and repaired with underlay mesh. Rats were divided in six groups for repair with polypropylene mesh (PPM, control), PPM with auto-cross-linked polymers gel (ACP), PPM with fibrinogen glue (FG), polypropylene/ expanded polytetrafluoroethylene (ePTFE) mesh, polypropylene/sodium hyaluronate/ carboxymethylcellulose (HA/CMC) mesh, and polypropylene-collagen/polyethylene-glycol/glycerol (CPGG) mesh. Endpoints were mesh infection, adhesions, reherniations and tensile strength of the mesh-tissue interface at sacrifice after two months. Six rats developed mesh infection, half of them after repair with PPM/ePTFE. The PPM/HA/CMC group showed a significant reduction in the amount and severity of adhesions. In the groups with PPM/ACP and PPM/FG, severity of adhesions was reduced as well. Reherniation rate was significantly higher in the PPM/ACP group (50%) compared to other. Rats in the PPM/HA/CMC

had the highest tensile strength. It was concluded that PPM/HA/CMC approaches the demands of the ideal mesh best, in a rat model of incisional hernia.
A relationship between postsurgical adhesion formation and peritoneal tumor implantation has been suggested in previous studies[1-3]. The aim of the study described in chapter 8 was to investigate the influence of a hyaluronan containing agent on intraperitoneal tumor in an experimental tumor model. 66 Balb/c mice underwent a laparotomy and damage was inflicted to the parietal peritoneum. Animals were randomized into 5 groups. Groups 1 and 2 received hyaluronan-carboxymethylcellulose bioresorbable membrane and no treatment, respectively. Mice in groups 3-5 were injected intraperitoneally with 10^5 colon 26-B cells after the laparotomy. Treatment consisted of hyaluronan membrane, no hyaluronan agent and placement of hyaluronan membrane at the non-traumatized parietal peritoneum, respectively. Animals were killed after 14 days; adhesions were scored in groups 1 and 2, and tumor mass in groups 3-5. This tumor experiment was also performed in 45 Wag/Rij rats. In rats 10^6 CC-531 cells were injected. Rats were killed after 3 weeks and tumor mass was scored. Hyaluronan membrane resulted in a significant reduction of adhesions, but had no major effect on intraperitoneal tumor mass in both experimental models.

Future directions

Although progress has been made in the attempts to reduce postoperative adhesion formation, the problem is far from being resolved. The optimal antiadhesive agent should safely and completely prevent adhesions. Hyaluronan-based agents promote fibrinolysis and mesothelial healing processes, thereby possibly preventing adhesion formation. The (clinical) importance of these biological properties in adhesion prevention, however, remains unclear, and the barrier function of hyaluronan might be far more relevant. Various experimental studies have shown the capacity of hyaluronan-based agents to reduce adhesions, which was confirmed in this thesis. Clinical use of hyaluronan-based agents has shown a reduction of the incidence, severity and extent of adhesions as well[4-10]. There is, however, less evidence that hyaluronan-based agents reduce adhesion related complications[11, 12]. Studies on clinical endpoints of adhesive problems are difficult to design and carry out, and need large financial resources. Further experimental and clinical research should focus on prevention of postsurgical adhesions, without interference with the peritoneal healing processes and without side effects.

Until now, it has been shown that hyaluronan-based film is able to prevent adhesions to the midline incision in at least 50% of patients undergoing midline laparotomy[4]. However, the increased risk of anastomotic leakage when these films are wrapped around anastomoses, is a major drawback, illustrating that hyaluronan may disturb the normal healing of bowel anastomosis[6].

In experimental studies, the composition and the amount of the used hyaluronan-based agent seem important[13-15]. The use of membranes, gels and solutions, despite their base of hyaluronan, leads to different results. Addition of other substances, such as iron or glycerol, negatively affects safety of these antiadhesive agents[8, 16-18]. These observations give valuable information for further research regarding the effect of viscosity on adhesion prevention and on the safety profile. Antiadhesives should not potentiate infection, nor stimulate tumor adhesion and growth, nor impair healing.

Results of the in vitro study in chapter 4 shed light on a possible influence of timing of hyaluronan administration. It was noticed that hyaluronan decreased PAI-1 levels produced by mesothelial cells exposed to LPS. The presence of monocyte-like cells, however, abolished the reduction in PAI-1. Hypothesis is that hyaluronan is less efficient with regard to its fibrinolytic effects, when cells of the monocyte-macrophage system have already invaded the abdominal cavity. Consequence is to interfere early with hyaluronan-based products in the response to surgical trauma or peritonitis. This is feasible in elective surgery, but more difficult in peritonitis because the inflammatory response is well underway before treatment has commenced. Data from experimental studies only show moderate effect on intra-abdominal abcess formation[5, 8, 13-20]. These data combined with the data in chapter 5 make it questionable whether it is worthwhile to put much effort in further research on this subject.

As found in our study reported in chapter 6, intravenously administered liposomes accumulate in areas with an increased vascular permeability such as infiltrates, abscesses and adhesions. This finding gives the opportunity to use liposomes loaded with antiadhesive agents such as hyaluronan, tissue plasminogen activator (tPA), or anti-PAI antibodies for targeted therapy.

Prosthetic mesh repair is the gold standard for abdominal hernia repair. The demand to avoid adhesion formation to the mesh has led to the development of multiple meshes. In our model of incisional hernia, the polypropylene mesh combined with hyaluronan proved to be the best with regard to adhesion prevention. The combined mesh of polypropylene with hyaluronan not only showed the best antiadhesive properties, but optimal ingrowth at the mesh-fascia interface as well. The long-term effects of this mesh, however deserve attention. The presence of the hyaluronan part is only temporary and the

presence of intra-abdominal polypropylene may be potentially harmful at the long run. It is obvious that randomized clinical trials comparing different meshes with antiadhesive properties are necessary in order to find out which one is best.

Few studies have focused on the use of hyaluronan during oncologic abdominal surgery[9, 20, 21]. Unfavorable oncologic effects of hyaluronan have not been reported until now. In our experimental study hyaluronan had no major effect on intraperitoneal tumor growth, either. The used number of animals, however, was chosen anticipating on a significant decrease of tumor growth. It is possible that negative side effects of hyaluronan in this experimental oncologic model were not found due to small numbers of animals. Widespread use of hyaluronan in oncologic abdominal procedures seems premature, as studies on safety are not available. Further studies are warranted to answer the question whether hyaluronan can be used without reserve in combination with various abdominal malignancies. The follow-up of patients in whom hyaluronan has been used under these circumstances seems of utmost importance.

In conclusion hyaluronan has undeniable antiadhesive properties. Not all hyaluronan-based agents are equally successful. Future challenges lie in a further unraveling of the different mechanisms involved in adhesion formation and finding more effective ways to reduce adhesions. Further progress in this field might lay in optimalization of the composition of the hyaluronan-based agents, combinations with other agents and more sophisticated ways of administration.

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Chapter 10

SAMENVATTING EN PLANNEN VOOR DE TOEKOMST

SAMENVATTING

In hoofdstuk 1 zijn de klinische impact en de pathophysiologie van intra-abdominale verklevingen en abcessen, welke een gemeenschappelijke basis hebben via de fibrinogenese en fibrinolyse route, samengevat. Postoperatieve verklevingen resulteren in een aanzienlijke morbiditeit en zelfs mortaliteit. Heropnames in verband met darm-obstructie na abdominale chirurgie zijn veel voorkomend en leiden niet zelden tot chirurgische reïnterventie. Verklevingen compliceren relaparotomieën en verhogen de incidentie van iatrogeen darmletsel. Verklevingen ontstaan door peritoneaal letsel, hetgeen kan ontstaan door trauma of ontsteking. Op de beschadigde oppervlakken wordt een fibrine-neerslag gevormd. Ofschoon normaliter het fibrinolytische systeem deze neerslagen oplost, kunnen chirurgie en infectie leiden tot een verminderde fibrinolyse. Veel onderzoek heeft zich gericht op het vinden van manieren om fibrinevorming te verminderen of fibrinolyse te versterken, om zo verklevingen en intra-abdominale abcessen te verminderen.
Hyaluronzuur, een polysaccharide, is een veelvuldig onderzochte anti-adhesieve substantie. Het werkingsmechanisme van hyaluronzuur lijkt terug te voeren op mechanische scheiding van beschadigde peritoneale oppervlakken, en is verder terug te voeren op biologische eigenschappen van hyaluronzuur. Hyaluronzuur speelt een rol bij weefselherstel en wondgenezing, beïnvloedt de inflammatoire respons en faciliteert cel migratie en proliferatie. Fibrine deposities kunnen bacterieën afkapselen en zo een bron voor abcedering vormen. Preventie van fibrinevorming of vroeg oplossen van deze deposities bij patiënten met intra-abdominale infecties zou een aantrekkelijk methode kunnen zijn om abcesvorming te voorkomen. Aangezien hyaluronzuur effectief is in het voorkomen van verklevingen en duidelijke invloed heeft op de fibrinolytische cascade, is het gebruik ervan bij de behandeling van intra-abdominale sepsis het meest uitdagend. Er is vrijwel geen enkele studie op dit gebied.
Hoofdstuk 2 bestaat uit een review van de literatuur over op hyaluronzuur-gebaseerde anti-adhesieve middelen, toegepast in abdominale chirurgie. De noodzaak om verklevingen tegen te gaan heeft geresulteerd in vele experimentele en klinische trials. Ondanks de effectiviteit van hyaluronzuur bestaat er nog veel discussie over de klinische toepassing en het werkingsmechanisme. Verschillende hyaluronzuur-bevattende producten zijn geïntroduceerd en ook weer teruggetrokken van de markt. De toepassing van hyaluronzuur in combinatie met matjes bij herstel van buikwandbreuken lijkt een veelbelovend concept te zijn. Niet alle toepassingen van hyaluronzuur zijn uitgebreid onderzocht en het gebruik ervan onder infectieuze condities of bij patiënten

met een maligniteit blijft controversieel. Een overzicht wordt geschetst van de effecten van hyaluronzuur-bevattende anti-adhesieve middelen toegepast in abdominale chirurgie, het gebruik ervan onder infectieuze omstandigheden en bij maligne ziekte. De verschillende mogelijke werkingsmechanismen van hyaluronzuur worden eveneens besproken.
Peritoneale mesotheelcellen zijn betrokken bij verschillende biologische processen, waaronder de vorming van peritoneale verklevingen. Kweken van humane peritoneale mesotheelcellen vormen een belangrijk middel om het gedrag en de functies van mesotheelcellen te onderzoeken. Het doel van de studie in hoofdstuk 3 was om de effecten te onderzoeken van het opkweken van cellen en het invriezen en opslaan ervan, gebruikmakend van mesotheelcellen van verschillende bronnen. De invloed van hyaluronzuur op celfuncties werd eveneens bekeken. Vitale cellijnen werden verkregen van zowel omentum als peritoneale lavagevloeistof van zeven patiënten. Intercellulair adhesie molecuul-1 (ICAM-1) expressie, middels ELISA, en matrix metalloproteïnase (MMP) bio-activiteit, middels zymographie, werden gemeten in de tweede en vierde passage. MMP bio-activiteit werd ook gemeten na invriezen en opslag van cellen in vloeibare stikstof. De effecten van verschillende stimuli, te weten interleukine 1beta (IL-1β), tumor necrosis factor-alpha (TNF-α) en phorbol myristaat acetaat (PMA), met en zonder hyaluronzuur werden geanalyseerd. ICAM-1 werd tot expressie gebracht en gestimuleerd door IL-1β, TNF-α en PMA. Alle cellijnen produceerden zowel MMP-2 als MMP-9. Alleen de laatste activiteit werd positief beïnvloed door TNF-α en met name PMA. Verschillen werden gevonden tussen de tweede en de vierde passage, en tussen cellen van verschillende afkomst (lavagevloeistof cellen versus omentale cellen), met name als de relatieve stimulatie door de verschillende middelen vergeleken werd. Toevoeging van hyaluronzuur aan controle kweken of aan kweken samen met een van de drie onderzochte stimulantia resulteerde niet in een significante verandering van ICAM-1 expressie of gelatinase activiteit. Invriezen en opslag van cellen beïnvloedde hun functies niet. Op grond van deze studie werd geconcludeerd dat zowel humaan omentum als peritoneale lavagevloeistof goede bronnen vormen om mesotheliale cellijnen te verkrijgen, die kunnen worden opgekweekt, ook na invriezen, zonder kwalitatieve veranderingen in hun vermogen om ICAM-1 tot expressie te brengen en gelatinasen te produceren. Voor omentumcellen bestaat er een verschillend effect van stimulatie, afhankelijk van het feit of de cellen uit passage 2 of 4 kwamen. De aanwezigheid van hyaluronzuur beïnvloedde de expressie van ICAM-1 of de gelatinasen niet.
Het doel van de studie in hoofdstuk 4 was om uit te zoeken of de aanwezigheid van cellen van het monocyt-macrophaag systeem de fibrinolytische respons

van peritoneale mesotheelcellen op lipopolysaccharide (LPS) in de aan- en afwezigheid van hyaluronzuur beïnvloedt. Humane peritoneale mesotheelcellen werden verkregen bij patiënten die een laparotomie ondergingen voor niet-infectieuze redenen en werden in vitro gekweekt. Co-culturen werden gevormd door U-937 humane monocyt-achtige cellen toe te voegen aan een monolaag van mesotheelcellen. Na 24 uur werden kweken behandeld met 10 ng/ml LPS en hyaluronzuur werd toegevoegd in een uiteindelijke concentratie van 0.2%. Controles kregen medium zonder hyaluronzuur. Na 24 uur incubatie werden de waarden bepaald van tissue Plasminogeen Activator (tPA), urokinase Plasminogeen Activator (uPA) en Plasminogeen Activator Inhibitor-1 (PAI-1) in medium en cellysaten, gebruikmakend van ELISA technieken. In medium van co-culturen waren de tPA en PAI-1 concentraties significant verhoogd vergeleken met monoculturen ($p<0.05$ en $p<0.005$, respectievelijk), terwijl de uPA concentratie significant verlaagd was ($p<0.01$). In cellysaten van co-culturen, was de PAI-1 concentratie significant verhoogd vergeleken met monoculturen ($p<0.01$), terwijl tPA en uPA onveranderd waren. Behandeling met hyaluronzuur gaf een significante verlaging van PAI-1 ($p<0.05$) en uPA ($p<0.01$) concentraties in medium van monoculturen, maar verlaagde alleen de uPA concentratie in medium van co-culturen ($p<0.01$). Geconcludeerd werd dat cellen van het monocyt-macrophaag systeem de fibrinolytische capaciteit van met LPS behandelde humane peritoneale mesotheelcellen moduleren en dualistisch interfereren in de hyaluronzuur-gerelateerde veranderingen in mesotheliale fibrinolytische capaciteit.

Uit hoofdstuk 3 en 4 kunnen geen eenduidige conclusies over de veronderstelde biologische eigenschappen van hyaluronzuur getrokken worden, hoewel de resultaten uit hoofdstuk 4 eerdere bevindingen ondersteunen over de mogelijke invloed van hyaluronzuur op peritoneale fibrinolyse.

In hoofdstuk 5 was het doel om te bepalen of auto-cross-linked hyaluronzuur polysaccharide (ACP) gel verklevingen en abcesvorming zou kunnen verminderen in een peritonitismodel bij ratten. In het eerste experiment werd een bacteriële peritonitis geïnduceerd bij 24 Wistar ratten, gebruikmakend van een model waarbij het coecum wordt geligeerd en gepuncteerd. Dieren werden gerandomiseerd en ontvingen 4 ml ACP gel (4%) of 4 ml phosphate buffered saline (PBS). Na 2 weken werden de dieren gedood en werden verklevingen en abcessen gescoord. In een tweede experiment ondergingen 72 ratten dezelfde procedure, maar ontvingen nu na randomisatie 2 ml ACP gel, 4 ml ACP gel of 4 ml PBS. Na 1 en 3 weken respectievelijk werd de helft van de dieren in iedere groep gedood en werden verklevingen en abcessen gescoord. In het eerste experiment was de mediane totale adhesie-score 12

(range 3-20) in de ACP groep en 9 (range 6-12) in de PBS groep (niet significant). 91% van de ratten in de ACP groep en 90% van de controle groep ontwikkelde abcessen. Er waren geen significante verschillen in abcesgrootte of aantal abcessen. In het tweede experiment waren de totale adhesie-scores in de 2 ml ACP groep, 4 ml ACP groep en PBS groep 4 (range 2-20), 6 (range 1-11) en 6 (range 1-18) respectievelijk (niet significant) na 1 week en 3.5 (range 1-8), 5 (range 2-15) en 4 (range 0-9), respectievelijk (niet significant) na 3 weken. Alle ratten in de 2 ml ACP groep en de PBS groep en 83% van de 4 ml ACP groep hadden abcessen na 1 week. Na 3 weken waren deze percentages 80, 75 en 73, respectievelijk. Er bestonden geen significante verschillen wat betreft de grootte of het aantal abcessen tussen de groepen na 1 en 3 weken. Geconcludeerd werd dat ACP gel verklevingen en abcesvorming in een peritonitis model in ratten niet vermindert.

In hoofdstuk 6 werd het gebruik van ^{99m}Tc-polyethylene glycol (PEG)-liposomen voor de detectie van vroege verklevingen na peritonitis bestudeerd. Verder werd de waarde van ^{99m}Tc-PEG-liposomen voor therapie-evaluatie van hyaluronzuur-oplossing geëvalueerd. Bij 24 ratten werd een bacteriële peritonitis geïnduceerd middels het ligeren en puncteren van het coecum. De dieren werden behandeld met natrium/chloride oplossing of 0.4% hyaluronzuur-oplossing, intra-abdominaal toegediend. Een week later werd scintigraphie verricht, gebruikmakend van ^{99m}Tc-PEG-liposomen en werd abnormale focale uptake in het abdomen gescoord. Hierna werd autopsie uitgevoerd en werden verklevingen en abcessen beoordeeld. Een significante correlatie werd vastgesteld tussen de totale adhesie-score en de scintigraphie-score ($p<0.01$, $r=0.65$). Behandeling met hyaluronzuur leidde tot een significante vermindering van de totale adhesie-score ($p=0.01$). Ook was er sprake van een significante correlatie tussen de grootte van abcessen en de scintigraphie-score ($p<0.01$, $r=0.62$). Behandeling met hyaluronzuur verminderde de grootte van abcessen ($p<0.05$). Geconcludeerd werd dat ^{99m}Tc-PEG-liposomen in staat zijn om vroege verklevingen en abcessen te detecteren en gebruikt zouden kunnen worden voor therapie evaluatie van middelen met een adhesie- en abcesverminderend effect. Het was evenwel niet mogelijk om verklevingen en abcessen van elkaar te onderscheiden. Verder werd de capaciteit van hyaluronzuur om verklevingen en abcessen te verminderen wederom bevestigd.

Na abdominale chirurgie, en met name na secundaire peritonitis, is het optreden van een littekenbreuk een veel voorkomende complicatie. Het herstel van breuken vereist doorgaans het gebruik van een kunststof mat. De vereisten van de ideale mat lijken tegenstrijdig; ingroei op de overgang mat - fascie, zonder het ontwikkelen van verklevingen aan het viscerale oppervlak van de

mat. In hoofdstuk 7 worden verschillende anti-adhesieve middelen gecombineerd met een macroporeuze mat en samengestelde matten onderzocht, waarbij gekeken werd naar het vermogen om verklevingen aan de mat te voorkomen en tevens gelet werd op de ingroei ter plaatse van de fascie. Bij 60 ratten werd een buikwanddefect gemaakt en hersteld met een underlay mat. Ratten werden verdeeld in 6 groepen en behandeld met polypropylene mesh (PPM, controle), PPM met auto-cross-linked polymers gel (ACP), PPM met fibrinogen glue (FG), polypropylene/ expanded polytetrafluoroethylene (ePTFE) mesh, polypropylene/ sodium hyaluronate/ carboxymethylcellulose (HA/CMC) mesh, en polypropylene-collagen/polyethylene-glycol/glycerol (CPGG) mesh. Mat-infecties werden gedurende het postoperatieve beloop bijgehouden, verklevingen en reherniaties werden gescoord na het termineren na 2 maanden, waarbij ook de trekkracht van de mat-weefsel overgang werd gemeten. Zes ratten ontwikkelden een mat-infectie, waarbij de helft hiervan behandeld werd met PPM/ePTFE. In de PPM/HA/CMC groep werd een significante reductie gezien van de hoeveelheid verklevingen, alsook van de ernst ervan. In de groepen met PPM/ACP en PPM/FG, was de ernst van de verklevingen eveneens verminderd. Het aantal reherniaties was significant hoger in de PPM/ACP groep (50%). De mat-weefsel overgangen van ratten in de PPM/HA/CMC groep hadden de hoogste trekkracht. Geconcludeerd werd dat PPM/HA/CMC de vereisten van de ideale mat het dichtst benadert, in een littekenbreukmodel in ratten.
Een relatie tussen postchirurgische vorming van verklevingen en peritoneale tumorimplantatie is geopperd[1-3]. Het doel van de studie in hoofdstuk 8 was om de invloed van een hyaluronzuurbevattend middel op intraperitoneale tumor te onderzoeken in een experimenteel model. 66 Balb/c muizen ondergingen een laparotomie en schade werd gemaakt op het pariëtale peritoneum. Dieren werden gerandomiseerd in 5 groepen. Groepen 1 en 2 kregen behandeling met een hyaluronzuurbevattende, oplosbare membraan en geen behandeling, respectievelijk. Muizen in de groepen 3-5 werden intraperitoneaal geïnjecteerd met 10^5 colon 26-B cellen na de laparotomie. Behandeling bestond uit hyaluronzuur membraan, geen hyaluronzuurbevattend middel en plaatsing van de hyaluronzuur membraan op de niet beschadigde peritoneale wand, respectievelijk. Dieren werden gedood na 14 dagen; verklevingen werden gescoord in de groepen 1 en 2 en tumormassa in groepen 3-5. Het tumorexperiment werd ook uitgevoerd, gebruikmakend van 45 Wag/Rij ratten. Bij de ratten werden 10^6 CC-531 cellen geïnjecteerd. Ratten werden gedood na 3 weken en de tumormassa werd gescoord. Gebruik van hyaluronzuur membraan resulteerde in een significante reductie van verklevingen, maar had geen evident effect op de intraperitoneale tumormassa bij muizen en ratten in deze experimentele modellen.

Plannen voor de toekomst

Ondanks de vooruitgang die is geboekt bij het reduceren van postoperatieve verklevingen, is het probleem verre van opgelost. Het optimale anti-adhesieve middel zou veilig en volledig verklevingen moeten voorkomen. Middelen op basis van hyaluronzuur stimuleren fibrinolyse en mesotheliale genezingsprocessen, waardoor ze mogelijk verklevingen voorkomen. Het (klinische) belang van deze biologische eigenschappen bij adhesie-preventie blijft evenwel onduidelijk, en de barrière functie van hyaluronzuur zou veel relevanter kunnen zijn. Verschillende experimentele studies hebben het vermogen van middelen op hyaluronzuur-basis om verklevingen te verminderen aangetoond, hetgeen bevestigd werd in dit proefschrift. De klinische toepassing van middelen op hyaluronzuur-basis heeft eveneens geleid tot een reductie van incidentie, ernst en uitgebreidheid van verklevingen[4-10]. Er is echter minder bewijs dat middelen op hyaluronzuur-basis adhesie-gerelateerde complicaties verminderen[11, 12]. Studies die zich richten op klinische eindpunten van problemen gerelateerd aan verklevingen zijn moeilijk op te zetten en uit te voeren, en hebben royale financiële ondersteuning nodig. Toekomstige experimentele en klinische onderzoeken zouden zich moeten richten op preventie van postchirurgische verklevingen, zonder beïnvloeding van de peritoneale genezingsprocessen en zonder bijwerkingen. Tot nu toe is aangetoond dat een hyaluronzuur membraan in staat is om verklevingen met de midline incisie te voorkomen bij ten minste 50% van de patiënten die een midline laparotomie ondergingen[4]. Echter, het verhoogde risico op naadlekkage wanneer deze membranen rondom anastomosen worden gelegd, is een groot nadeel, dat laat zien dat hyaluronzuur de normale genezing van een darmnaad kan verstoren[6].
In de experimentele studies lijkt de samenstelling en hoeveelheid van het gebruikte middel op hyaluronzuur-basis van belang[13-15]. Het gebruik van membranen, gels en oplossingen kan, ondanks de gemeenschappelijke basis van hyaluronzuur, leiden tot verschillende resultaten. Het toevoegen van andere substanties, zoals ijzer of glycerol, heeft een negatieve invloed op de veiligheid van deze anti-adhesieve middelen[8, 16-18]. Deze bevindingen geven waardevolle informatie voor verder onderzoek gericht op de invloed van viscositeit op adhesiepreventie en op het veiligheidsprofiel. Anti-adhesieve middelen zouden infectie niet moeten verergeren, zouden tumor-adhesie en -groei niet moeten stimuleren en zouden genezing niet moeten belemmeren.
De in vitro studie in hoofdstuk 4 suggereert dat de timing van hyaluronzuur toediening ook cruciaal zou kunnen zijn. Er werd vastgesteld dat hyaluronzuur zorgt voor een verlaging van PAI-1 levels, geproduceerd door mesothelcellen die werden blootgesteld aan LPS. Echter, de aanwezigheid van monocyt-

achtige cellen, deed de reductie van PAI-1 teniet. Dit zou erop kunnen duiden dat hyaluronzuur minder effectief is wat betreft de fibrinolytische effecten, wanneer cellen van het monocyt-macrophaag systeem al aangekomen zijn in de buikholte. Dit betekent dat men vroeg zou moeten interveniëren met middelen op hyaluronzuur-basis in de respons op chirurgisch trauma of peritonitis. Dit is haalbaar bij electieve chirurgie, maar moeilijker bij peritonitis, daar de inflammatoire respons al op gang is, voordat de behandeling begint. De data van experimentele studies laten slechts bescheiden effecten zien op intra-abdominale abcesvorming[5, 8, 13-20]. Deze data gecombineerd met de data uit hoofdstuk 5 maken het discutabel of het de moeite waard is om veel energie te steken in verdere onderzoeken naar dit onderwerp.
Zoals werd aangetoond in onze studie in hoofdstuk 6, accumuleren liposomen na intraveneuze injectie in gebieden met een toegenomen vasculaire permeabiliteit zoals infiltraten, abcessen en verklevingen. Deze bevinding biedt de kans om liposomen met daaraan gekoppeld anti-adhesieve middelen zoals hyaluronzuur, tissue plasminogeen activator (tPA), of anti-PAI antilichamen, te gebruiken voor doelgerichte therapie.
Herstel middels een kunststof mat is de gouden standaard bij de behandeling van littekenbreuken. Met het doel om verklevingen aan de mat te voorkomen zijn vele matten ontwikkeld. In ons model van littekenbreuken bleek de polypropyleen mat gecombineerd met hyaluronzuur de beste te zijn. De gecombineerde mat van polypropylene met hyaluronzuur liet niet alleen de beste anti-adhesieve eigenschappen zien, maar had ook de beste ingroei op de mat-fascie overgang. De lange termijn resultaten van deze mat verdienen desalniettemin aandacht. De aanwezigheid van het hyaluronzuur deel is slechts tijdelijk en de aanwezigheid van polypropyleen in de buikholte is potentieel schadelijk. Het is duidelijk dat gerandomiseerde klinische trials nodig zijn, waarin verschillende matten met anti-adhesieve eigenschappen worden vergeleken, om erachter te komen welke het beste is.
Slechts enkele studies zijn gericht op het gebruik van hyaluronzuur tijdens oncologische abdominale chirurgie[9, 20, 21]. Ongunstige oncologische effecten van hyaluronzuur zijn tot op heden niet gemeld. In onze experimentele studie had hyaluronzuur evenmin een evident effect op intraperitoneale tumorgroei. Het gebruikte aantal dieren was echter gekozen uitgaande van een significante vermindering van tumorgroei. Het is mogelijk dat negatieve bijwerkingen van hyaluronzuur in dit experimentele oncologische model niet werden vastgesteld door te kleine aantallen dieren. Wijdverbreid gebruik van hyaluronzuur tijdens oncologische abdominale procedures lijkt prematuur, gezien het feit dat er nog geen veiligheidsstudies beschikbaar zijn. Verdere studies zijn noodzakelijk om

de vraag te beantwoorden of hyaluronzuur zonder terughoudendheid gebruikt kan worden in combinatie met abdominale maligniteiten. De follow-up van patiënten, bij wie hyaluronzuur onder deze omstandigheden gebruikt is, lijkt van groot belang.
Geconcludeerd kan worden dat hyaluronzuur ontegenzeggelijk anti-adhesieve eigenschappen heeft. Toekomstige uitdagingen liggen in een verdere ontrafeling van de verschillende mechanismen die betrokken zijn bij adhesievorming en in het vinden van effectievere manieren om verklevingen tegen te gaan. Verdere vooruitgang op dit gebied zou geboekt kunnen worden door optimalisatie van de samenstelling van de middelen op hyaluronzuur-basis, combinaties met andere middelen en meer geavanceerde manieren van toediening.

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CURRICULUM VITAE AUCTORIS

Curriculum vitae auctoris

Kees-Jan Sikkink was born on February 17th, 1971 in Hoensbroek, The Netherlands. He completed the secondary school at the Bisschopelijk College in Sittard in 1989. In that year he started his medical education at the University of Nijmegen. He passed his final exams in 1996 and his first job as surgical resident was at the Martini Hospital in Groningen. In 1999 he returned to Nijmegen and in 2000 his surgical training started at the University Medical Centre Nijmegen, under supervision of prof. dr. R.J.A. Goris, and later prof. dr. R.P. Bleichrodt. In 2003 the second part of his training started at the Rijnstate Hospital in Arnhem under supervision of dr. J.H.G. Klinkenbijl. After completion of his surgical training, he went to Amsterdam for further vascular specialisation at the VU Medical Centre, under supervision of prof. dr. W. Wisselink. Since July 2007, he is member of the surgical staff of Orbis Medical Centre in Sittard. Recent fusion of the surgical staff of Orbis Medical Centre and the surgical staff of Atrium Medical Centre Heerlen has led to the institution of Maatschap Heelkunde Zuid-Limburg. During his surgical training, the research on hyaluronan and its various applications was started, which finally resulted in this thesis. The research was performed under supervision of dr. H. van Goor, with an important contribution of dr. M.M.P.J. Reijnen as well.
Kees-Jan Sikkink is married to Véronique Sep, with whom he has three children: Tuur, Fleur and Rosa.