Sodium Hyaluronate Increases the Fibrinolytic Response of Human Peritoneal Mesothelial Cells Exposed to Tumor Necrosis Factor α

Michel M. P. J. Reijnen, MD; Harry van Goor, MD, PhD; Peter Falk, BSc; Maria Hedgren, BSc; Lena Holmdahl, MD, PhD

Hypothesis: Sodium hyaluronate interferes with the fibrin degrading capacity of human peritoneal mesothelial cells exposed to tumor necrosis factor (TNF) α.

Design: Controlled laboratory experiment.

Intervention: Human peritoneal mesothelial cells were harvested from 5 patients undergoing laparotomy and cultured in vitro. Cells were treated with TNF-α, a cytokine typically involved in peritoneal inflammation, and sodium hyaluronate was added in a final concentration of 0.1%, 0.2%, or 0.4%. Controls received medium only. After 24 hours’ incubation, tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA), and plasminogen activator inhibitor type 1 (PAI-1) were measured in the medium and cell lysates using enzyme-linked immunosorbent assay techniques. Specific gene transcripts in cells treated with 0.4% sodium hyaluronate and controls were determined using a quantitative reverse transcription polymerase chain reaction.

Main Outcome Measures: Concentrations of tPA, uPA, and PAI-1, and their specific gene transcripts.

Results: Sodium hyaluronate significantly increased tPA concentration in cell lysates without affecting its gene expression as determined after 24 hours (P = .02). The uPA concentration was significantly decreased by sodium hyaluronate in the medium but not in cell lysates (P < .0001). The uPA messenger RNA expression was 1000-fold increased compared with control. Sodium hyaluronate significantly decreased PAI-1 concentration in the medium and reduced its gene expression 500-fold (P = .04), while PAI-1 concentration in cell lysates did not change.

Conclusion: Sodium hyaluronate affected the fibrinolytic response of TNF-α–stimulated human peritoneal mesothelial cells, most notably by decreasing PAI-1 transcription and release. This observation indicates that sodium hyaluronate counteracts the fibrinolytic decline induced by TNF-α and suggests a biological mechanism of action for sodium hyaluronate intra-abdominally.


Peritoneal injury from a variety of causes leads to peritoneal inflammation, including exudation of fibrinogen and fibrin formation into the abdominal cavity. These fibrin deposits may in turn become fibrous adhesions due to collagen deposition by invading fibroblasts. In an infectious environment, adhesions may form as a part of an abscess. Both intra-abdominal adhesions and abscesses cause significant clinical morbidity and mortality.

Intra-abdominal fibrin is degraded by the fibrinolytic system through the action of plasmin, which is enzymatically converted from plasminogen. By producing both plasminogen activators and inhibitors, mesothelial cells are crucial in the regulation of fibrinolytic capacity. Tissue-type plasminogen activator (tPA) is the main plasminogen activator, responsible for approximately 95% of plasminogen activator activity. A second plasminogen activator is urokinase-type plasminogen activator (uPA), which seems to play an important role in tissue remodeling, but its role in intra-abdominal adhesion formation is not well defined. Their activity is restricted by plasminogen activator inhibitor type 1 (PAI-1) and type 2 (PAI-2). A reduced peritoneal fibrinolytic capacity is induced by abdominal surgery and infection, predominantly resulting from high PAI-1 concentrations and to a lesser extent from low tPA levels. A high peritoneal PAI-1 concentration has been associated with severe adhesion formation.

It has been demonstrated that postsurgical adhesion formation was reduced by a sodium hyaluronate solution when administered in the peritoneal cavity during surgery. The effect of hyaluronate was attributed to protection of peritoneal surfaces from surgical trauma. We, however, showed a reduction of intra-abdominal adhesions and even abscesses in a rat peritonitis model, wherein the hya-
MATERIALS AND METHODS

MESOTHELIAL CELLS

Human peritoneal mesothelial cells were isolated from peritoneal lavage performed on 3 patients undergoing colorectal surgery for noninfectious reasons by use of the method described by Ivarsson et al.13 Meticulous hemostasis during opening of the abdomen prevented blood spill into the lavage fluid. We transferred the lavage fluid into 50-mL tubes and centrifuged the fluid at 650g for 10 minutes. The supernatant was withdrawn, and the pellet was resuspended in 5 mL of culture medium.

Cells were cultured at 37°C in 5% CO2 in air with a humidity of 80% to 90%, in tissue culture flasks (25 cm2) (Sarstedt, Newton, NC) in a medium that consisted of E199 (Sigma, St Louis, Mo), with the addition of 1-L-glutamine, 1.1 mmol/L (Sigma); penicillin-streptomycin, 30 IE/mL (Sigma); fetal bovine serum, 20% (Sigma); endothelial cell growth factor, 30 µg/mL, prepared according to Maciag et al14; heparin, 14 IU/mL (Lovens Lakemedel, Malmö, Sweden); and 0.3 µg/mL hydrocortisone (Sigma). All cells used were from the third passage. The mesothelial origin from the cells was verified by their typical cobblestone appearance in phase-contrast microscopy and by immunohistochemical analysis, as previously described.13

STUDY DESIGN

Human peritoneal mesothelial cells were cultured until a confluent monolayer was formed. The TNF-α (TNF-H; Genzyme, Cambridge, Mass) was added to the medium in a final concentration of 500 U/mL. Immediately after, sodium hyaluronate (molecular weight, 1.8-2.4×106 d) (Genzyme), diluted in the medium, was added in a final concentration of 0.1%, 0.2%, or 0.4%, which is the clinically used concentration. Controls received the medium without sodium hyaluronate. After 24 hours’ incubation, the medium was collected and stored in aliquots at −80°C until assayed. Because tPA can be stored intracellularly,15 cell lysates were used to measure intracellular pooling. Half of the cultured HPMC were thus used for protein measurements, and the other half for determination of gene transcripts. For determinations of intracellular pooling, cells were lysed by 3 cycles of freezing and thawing. Then the cell suspension was stored in aliquots at −80°C until measurements were performed. For detection of mRNA content the sodium hyaluronate, treated (0.4%) and untreated HPMC and several dilutions of known complementary RNA (cRNA) standard (1010 to 108 molecules per reaction) was subjected to a standard PCR reaction. The cellular RNA from treated or untreated HPMC was quantified via competition by the known cRNA. To exclude amplification of genomic DNA, total RNA was amplified without the quantitative reverse transcription PCR step as a negative control. The different products were separated on agarose gels containing ethidium bromide, photographed (Figure 1), scanned, and stored as TIFF computer files. The band intensities were determined using a computer program (NIH-Image version 1.54), and their intensity values were normalized to their molecular weight. The intensities of the band ratios within each lane were plotted against the copy number of the template per reaction in a log-log diagram. The quantity of the message was determined where the ratio of template/target mRNA molecules was equal to 1. The final quantity of mRNA expression was calculated and reported as the number of mRNA molecules per microgram of total RNA. Glyceraldehyde-3-phosphate dehydrogenase expression served as a housekeeping gene and a positive control, as previously described.6-10

STATISTICAL ANALYSIS

Values are given as median and interquartile range. Statistical analysis was made with the nonparametric Kruskal-Wallis test and the Mann-Whitney U test. The Kruskal-Wallis test determined overall differences, and if statistically significant, the Mann-Whitney U test was used to compare a treated group with the control. Statistical significance was defined as P<.05. All tests were 2-tailed. To calculate the fit of the serial dilution in the quantitative reverse transcription PCR, the linear regression was used.

RESULTS

TISSUE-TYPE PLASMINOGEN ACTIVATOR

The tPA concentration in the medium was not significantly influenced by treatment with sodium hyaluronate (P=.7) (Figure 2). In contrast to the medium, tPA concentration in cell lysates was significantly increased by treatment with sodium hyaluronate (P=.02) (Figure 3), but there was no significant difference in

BIOCHEMICAL ASSAYS

Assays of plasminogen activators and inhibitors were performed using commercially available enzyme-linked immunosorbent assay (ELISA) kits. Levels of tPA and PAI-1 were assessed using kits from Biopool (Immylse; Biopool, Ulmea, Sweden) and uPA using a kit from Monezyme (Horsholm, Denmark). The assay characteristics have been described previously.13,18

GENE EXPRESSION

Total cellular RNA was isolated from cell cultures to determine the expression of tPA, PAI-1, and uPA mRNA. To obtain enough mRNA, the total RNA were pooled from 6 different cell cultures in the same treatment group. Expression for tPA, PAI, and uPA were analyzed using a quantitative reverse transcription polymerase chain reaction (PCR).6-10 Briefly, 5 µg of total cellular RNA from treated or untreated HPMC and several dilutions of known complementary RNA (cRNA) standard (1010 to 108 molecules per reaction) was subjected to a standard PCR reaction. The cellular RNA from treated or untreated HPMC was quantified via competition by the known cRNA. To exclude amplification of genomic DNA, total RNA was amplified without the quantitative reverse transcription PCR step as a negative control. The different products were separated on agarose gels containing ethidium bromide, photographed (Figure 1), scanned, and stored as TIFF computer files. The band intensities were determined using a computer program (NIH-Image version 1.54), and their intensity values were normalized to their molecular weight. The intensities of the band ratios within each lane were plotted against the copy number of the template per reaction in a log-log diagram. The quantity of the message was determined where the ratio of template/target mRNA molecules was equal to 1. The final quantity of mRNA expression was calculated and reported as the number of mRNA molecules per microgram of total RNA. Glyceraldehyde-3-phosphate dehydrogenase expression served as a housekeeping gene and a positive control, as previously described.6-10

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tPA concentration between groups treated with different concentrations of sodium hyaluronate. The tPA mRNA expression in controls was $10^3$ mRNA copies per microgram of total RNA per cell. Incubation with 0.4% sodium hyaluronate did not change tPA mRNA expression (Figure 4).

**UROKINASE-TYPE PLASMINOGEN ACTIVATOR**

Sodium hyaluronate significantly decreased uPA concentration in the medium compared with control ($P < .0001$) (Figure 5). Treatment with 0.4% sodium hyaluronate significantly lowered uPA concentrations in comparison with 0.1% and 0.2% sodium hyaluronate ($P < .001$ and $P = .001$, respectively). In cell lysates, sodium hyaluronate did not significantly affect uPA concentration compared with controls (Figure 6).

The uPA mRNA expression in control cells was $2 \times 10^2$ mRNA copies per microgram of total RNA. Treatment with 0.4% sodium hyaluronate caused a 1000-fold increase in uPA mRNA expression to $4 \times 10^5$ mRNA copies per microgram of total RNA (Figure 4).

**PLASMINOGEN ACTIVATOR INHIBITOR TYPE 1**

Incubation with sodium hyaluronate significantly decreased PAI-1 concentration in the medium ($P = .04$) (Figure 7). Treatment with 0.4%, but not 0.1% and 0.2% sodium hyaluronate, significantly decreased PAI-1 concentrations compared with controls ($P = .02$). There was no significant difference in PAI-1 concentration between groups treated with different concentrations of sodium hyaluronate. In cell lysates, sodium hyaluronate did not significantly affect PAI-1 levels compared with controls (Figure 8).

The PAI-1 mRNA expression in control cells was $6 \times 10^4$ mRNA copies per microgram of total RNA per cell. Treatment with 0.4% sodium hyaluronate caused a 500-fold decrease in PAI-1 mRNA expression to $1 \times 10^2$ mRNA copies per microgram of total RNA (Figure 4).

**COMMENT**

Hyaluronan-based agents prevent adhesions after abdominal surgery. Becker et al. described a significant reduction in postoperative adhesion formation in patients undergoing colectomy and ileal pouch anal anastomosis with temporary diverting loop enterostomy for ulcerative colitis or familial polyposis, using a biodegradable membrane consisting of hyaluronan and carboxymethylcellulose. Similar results were found using this membrane in a prospective multicenter randomized trial.
of gynecologic patients undergoing myomectomy. Moreover, Diamond reported the efficacy of sodium hyaluronate, used as a precoating solution, in prevention of de novo adhesions in patients undergoing laparotomy for various benign gynecologic disorders. The adhesion-reducing capacity of hyaluronan-based bioresorbable membrane has been attributed to mechanical properties, separating damaged peritoneal surfaces that tend to adhere during the first days after surgery. A solution of hyaluronan, applied at abdominal entry, is thought to prevent inadvertent peritoneal trauma during surgery, thereby reducing the formation of adhesions.

We demonstrated that intraperitoneal treatment with sodium hyaluronate solution reduced adhesion and abscess formation in rats with bacterial peritonitis. In the study the solution was administered 24 hours after induction of a bacterial peritonitis, thus the reduction in adhesions and abscesses cannot be attributed to properties related to protective coating. Modulation of the fibrinolytic response in the abdominal cavity was hypothesized as a possible explanation for those findings.

Fibrin depositions occur in the abdominal cavity in response to trauma such as surgery or infection and are considered as the basis of both adhesions and abscesses. The local balance between fibrin deposition and degradation is decisive in adhesion formation, as recently reviewed. Reduction in fibrin-degrading capacity has been observed during abdominal operation and in peritonitis. Elevated PAI-1 concentrations in peritoneal tissue and fluid, and to a lesser extent a decline in the main fibrinolytic stimulator tPA, are responsible for the fibrinolytic impairment. Consequently, down-regulation of PAI-1 in particular would increase the fibrinolytic capacity and might restore the balance between fibrin formation and degradation.

A common denominator in the postoperative period and in peritonitis is an increase in intra-abdominal concentration of proinflammatory cytokines, most notably TNF-α. Because fibrin deposition is a prerequisite for abscess and adhesion formation, we chose to investigate the influence of hyaluronate on the fibrin degradation capacity in the presence of TNF-α. Human peritoneal mesothelial cells, harvested by peritoneal lavage and cultured in vitro, react to TNF-α exposure by an increase in PAI-1 and a decrease in tPA production, consequently depressing plasminogen acti-
Urokinase-type plasminogen activator antigen concentrations remain unaffected by treatment with TNF-α. Sodium hyaluronate decreased PAI-1 mRNA with a concomitant significant decline in PAI-1 levels in the medium, suggesting a transcriptional regulation. However, an increased message turnover could also explain the finding. Therefore, the exact mechanism cannot be concluded from the present study. Because PAI-1 overexpression is conceived to be a major reason for the decrease in peritoneal fibrin degradation capacity and demonstrated to be a marker of increased propensity for the development of adhesions, a regulatory effect on mesothelial PAI-1 expression could therefore have a major influence in the series of events leading to adhesions or abscesses. The effect of sodium hyaluronate on tPA expression seemed to be limited but nevertheless resulted in increased intracellular concentration, probably due to intracellular pooling, as is previously described for endothelial cells. This suggests that sodium hyaluronate affected the tPA production pathway at the posttranslational level. Alternatively, intracellular tPA degradation may be inhibited, a phenomenon that has been reported earlier for chloroquine and receptor-associated protein. Although there is no indication that the increased intracellular storage of tPA was transcriptionally regulated, this cannot be ruled out with the current experimental design, and further studies investigating this are warranted. As for uPA, on the other hand, there was an increased transcription that did not result in increased intracellular or extracellular concentration of the protein, suggesting a posttranscriptional block. Thus, the effects of sodium hyaluronate on mesothelial cell production in a proinflammatory environment seem complex, but nevertheless result in an increased plasminogen activation by decreasing the extracellular expression of the main inhibitor PAI-1.

The mechanism by which sodium hyaluronate affects mesothelial fibrinolysis is unknown. Sodium hyaluronate may interfere with TNF-α stimulation on the mesothelial cells by direct binding to TNF-α or by competing at the TNF-α receptor level. Blocking transforming growth factor β (TGF-β) at either its signal transduction receptors or its TNF-α-dependent production by the mesothelial cells might be another explanation for the decrease in PAI-1 caused by sodium hyaluronate. The TGF-β influences PAI-1 production of the mesothelial cells at the transcriptional level and is associated with elevated PAI-1 levels in peritoneal tissue. A toxic effect of sodium hyaluronate on the mesothelial cells is not likely because cells were viable and proliferating 24 hours after administration of sodium hyaluronate. Moreover, it was previously demonstrated that sodium hyaluronate causes mesothelial cell proliferation, even in the presence of TNF-α and/or lipopolysaccharide. Finally, sodium hyaluronate may exert its effect by binding to hyaluronan receptors as CD44, receptor for hyaluronate-mediated motility, and intercellular adhesion molecule-1. Mesothelial cells have the CD44 receptor on their cell surface. This receptor has a role in inflammatory conditions, allograft rejection, and tumor migration. An association, however, between this receptor and the production of components of the fibrinolytic system has to our knowledge not been demonstrated.

Abdominal surgery and infection cause extensive damage to the peritoneum, including the mesothelial monolayer. An episode of peritoneal damage is followed by repair mechanisms, including repopulation of peritoneal surfaces with mesothelial cells and the lysis of intra-abdominal fibrin by the abdominal fibrinolytic system. Results from the present study suggest that the biology of postsurgical peritoneal repair is modulated by hyaluronan. By increasing the fibrinolytic response of mesothelial cells, the instillation of hyaluronan in the abdominal cavity might be useful in clinical situations wherein excess fibrin is considered to be harmful. Hyaluronan used as a peritoneal instillate, or as a lavage solution, may prevent adhesions and abscesses in bacterial peritonitis, as was earlier demonstrated. Patients on continuous ambulatory peritoneal dialysis (CAPD) might benefit from lavage with hyaluronate when experiencing CAPD peritonitis. This type of peritonitis induces intra-abdominal adhesions, which in turn decrease the peritoneal surface needed for dialyzing and ultimately may cause CAPD failure. Further studies are needed to determine the benefit of hyaluronate in these clinical conditions.

Figure 7. Levels of plasminogen activator inhibitor type 1 (PAI-1) antigen in conditioned media. Results are illustrated as median (horizontal line) interquartile range (boxes) and 10th and 90th percentiles (error bars).

Figure 8. Levels of plasminogen activator inhibitor type 1 (PAI-1) antigen in cell lysates. Results are illustrated as median (horizontal line) interquartile range (boxes) and 10th and 90th percentiles (error bars).
Sodium hyaluronate increases the fibrinolytic response of human peritoneal mesothelial cells exposed to tumor necrosis factor α. Results described in the article indicate that hyaluronate, currently used in antiadhesive barrier materials, influences the fibrinolytic activity of the peritoneum under inflammatory conditions. The fibrinolytic activity of human peritoneal mesothelial cells is enhanced by treatment with hyaluronate, particularly by suppression of plasminogen activator inhibitor. An increased fibrinolytic activity in the abdominal cavity is considered beneficial for the reduction of intra-abdominal adhesions and abscesses. Postsurgical adhesions are the most common cause of small bowel obstruction and secondary female infertility and may be related to chronic abdominal pain. Adhesive small bowel obstruction is the reason for 1% of admissions to general surgery. Intra-abdominal abscesses are an important cause of morbidity and mortality in patients after (generalized) peritonitis.

Enhancement of the peritoneal fibrinolytic activity is particularly indicated in clinical situations wherein excess fibrin is formed. Examples hereof are generalized peritonitis, repeated laparotomies and continuous ambulatory peritoneal dialysis peritonitis. Use of hyaluronate, for example in peritoneal perioperative lavage fluid may, based on its property to enhance the fibrinolytic activity of peritoneal mesothelial cells, play a role to reduce adhesion and abscess related complications.

In summary, in an inflammatory environment, mesothelial cell fibrinolysis is affected by sodium hyaluronate most notably by a down-regulation of PAI-1 at the gene and protein level. This finding gives further support to the notion that barrier materials designed to reduce adhesion formation do not act solely through a mechanical action but might have a profound impact on peritoneal biology. Furthermore, the observation suggests that sodium hyaluronate may in part reduce adhesions and abscesses through increased fibrinolysis by reducing PAI-1 expression in the peritoneal cavity.

This work was supported by grants from the Surgical Infection Society of Europe (Dr Reijnen) and the Swedish Medical Research Council (project K98-17X-12650) (Dr Holmdahl).

This sodium hyaluronate was generously donated by the Genzyme Corporation, Cambridge, Mass.

Corresponding author and reprints: Michel M. P. J. Reijnen, MD, Department of Surgery, University Hospital Nijmegen, PO Box 9101, 6500 HB Nijmegen, the Netherlands (e-mail: MMPJ.Reijnen@worldonline.nl).

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