Effects of Polylysine and Polyglutamate on Inflammation and the Normal Process of Peritoneal Healing After Surgery

Daniel Åkerberg, Karolin Isaksson, Monica Posaric-Bauden, Roland Andersson and Bobby Tingstedt

Abstract

Introduction: Intraperitoneal adhesions are common after abdominal surgery and may lead to serious clinical complications. Previous studies have investigated the possible effects of the polypeptides poly-L-lysine (αPL) and poly-L-glutamate (PG) forming a polymer complex that prohibits local peritoneal adhesions after surgery. The aim of this study was to examine whether the normal process of peritoneal healing was affected by PL/PG polymer matrix.

Material and methods: Male rats (Sprague Dawley) (n=84) underwent abdominal wall surgery and suturing. Rats were randomized in groups according to evaluation time (2, 4, 6, 8, 24 hours and 7 days) with corresponding control groups. Controls received saline (0.9%) and the experimental groups received PL/PG on the surgery site. tPA, PAI-1, IL-6 and active TGFb1 were analyzed at given time points postoperatively in peritoneal lavage. Adhesions were evaluated after seven days. Significant differences were considered to be p<0.05.

Results: At a few individual time points small differences were seen between the groups (control and experiment) comparing levels of tPA, PAI-1, IL-6 and active TGFb1. When comparing levels of substances from all time points no statistical differences were seen between the groups as a total. Adhesions were significantly decreased on day 7, p=0.002.

Conclusion: Despite significant reduction in adhesions PL/PG administered intraperitoneally as an anti-adhesion agent locally on surgically traumatized area does not seem to affect the normal process of peritoneal healing.

Keywords: Abdominal adhesions; Prevention; Polypeptides; Tissue plasminogen activator; Plasminogen activator inhibitor-1

Introduction

Abdominal adhesions develop mainly after surgery and cause significant health-related problems both for the individual patient and for society [1]. Abdominal adhesions form on the peritoneum. The peritoneum covers the intra-abdominal cavity and is a smooth protective functional unit consisting of a single mesothelial cell layer resting on a basement membrane with a submesothelial area beneath. The submesothelium is a loose connective tissue harvesting capillaries and lymphatic vessels. During abdominal surgery the trauma involving mesothelial cells and submesothelial area cause changes that might lead to stable adhesions [2,3]. The formation of adhesions is believed to begin with local hypoxia at the site of the peritoneal injury, leading to the release of inflammatory cells (macrophages and neutrophils) from local damaged capillaries followed by fibrin depositions, decreased fibrinolysis and the proliferation of local and remote fibroblasts and mesothelial cells, contributing to increased fibrosis and eventually to stable fibrin strands that are replaced by collagen polymers [4-8]. Components from coagulation tissue plasminogen activator (tPA), plasminogen activator inhibitor (PAI-1) and cytokines transforming growth factor beta (TGFβ1) and interleukin-6 (IL-6) are examples of important and central factors involved in the formation of adhesions. tPA is the major initiator of fibrinolysis through the serine protease plasmin. PAI-1, TGFβ1 and IL-6 are important substances involved in peritoneal damage and healing and have the capacity to reduce the local peritoneal fibrinolysis [9-11].

Many previous attempts have been made to find an anti-adhesive agent. Recent studies have focused on preventing local abdominal adhesions by administering differently charged polymers, the polycation α-poly-L-lysine (αPL) and the polyanion poly-L-glutamate (PG), which when combined together form a degradable, non-toxic protective biofilm accumulated on the injured peritoneal surface [12].

We have hypothesized that the differently charged polymers acts as a goal seeking internal sealing that covers the injured peritoneal areas by way of electrostatical forces [13,14]. The present study aimed to determine whether this internal sealing of damaged peritoneum by the combined polymers (αPL and PG) in any way interfered with the process of inflammation and fibrosis in a rat peritoneal adhesion model. In this study we focused on measuring the central parameters that are known to induce fibrosis and fibrinolysis i.e., tPA, PAI-1, IL-6 and activeTGF-b1 in peritoneal fluid pre- and post-operatively in rats.

Material and Methods

Animals

Eighty-four Sprague-Dawley rats (Taconic Farms, Inc., DK) weighing about 250 g each were used. The animals were kept under standardized conditions with free access to pellets and tap water and libitum. The animals also received animal care in compliance with the guidelines of the Swedish Government and University of Lund, Sweden. The study was approved by the local ethical committee at Lund University. The animals were allocated to groups according to

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were measured with a caliper up to one-tenth of a millimeter and data
lengths of the incisions as well as the adhesions covering the wound
experimental wound or to another intra-abdominal organ. The
Adhesions were considered as tissue (bowels or fat) adherent to the
model
UK) concentration.
Sweden), IL-6 and active TGFb1 (R&D Systems Europe, Abingdon,
with ELISA to measure tPA, PAI-1 (Labinova AB, Upplands Väsby,
peritoneal lavages were immediately aliquoted to smaller volumes (400
units. The abdomen was closed
containing 0.9% NaCl (control group) or 1 ml αPL immediately
sutures polypropylene 4-0. This area was then sprayed with an atomizer
lateral abdominal wall. The incision was sutured with 4 interrupted
frozen to -70°C. Thereafter peritoneal adhesions were created with
adhesion procedure), aliquoted to smaller volumes (400 μL) and snap
lavage with 2 ml pre-warmed PBS was collected at time 0 h (before
Anesthetic drugs were prepared prior to the experiment: Ketamine
60 mg/kg (Ketalar, Pfizer, New York, USA) was mixed together with
Xylazine 16 mg/kg (Rompun Vet, Bayer AB, Gothenburg, Sweden)
and later injected intramuscularly. Sodium Chloride (NaCl 0.9%) was
used as a control (Baxter Medical AB, Kista, Sweden) and Phosphate
Buffered Saline (PBS) (Sigma Aldrich, St. Louis, Mo, USA) was used
for peritoneal lavage.

Equipment
Surgical instruments (scissors, scalpels, forceps, drapes and
sponges), sutures polypropylene 3-0 and 4-0, with curved needles
(Ethicon, Somerville, NJ, USA) and syringes (Beckton Dickinson,
Helsingborg Sweden) were used.

Surgical model
The animals were anesthetized by intramuscular injection and the
abdomen was shaved and disinfected with an alcohol swab. A midline
incision was made and the abdominal cavity was entered, peritoneal
lavage with 2 ml pre-warmed PBS was collected at time 0 h (before
adhesion procedure), aliquoted to smaller volumes (400 μL) and snap
frozen to -70°C. Thereafter peritoneal adhesions were created with
an established method [6] via a sharp incision, 15 mm long, on the
lateral abdominal wall. The incision was sutured with 4 interrupted
sutures polypropylene 4-0. This area was then sprayed with an atomizer
containing 0.9% NaCl (control group) or 1 ml αPL immediately
followed by 1 ml PG (experimental group). The abdomen was closed
using a running suture, PDS 4-0 in two layers.

Using a small incision in the midline, peritoneal lavage (with
2 ml pre-warmed PBS) was made at times 2, 4, 6, 8, 24 h and 7 days
after the adhesion-creating procedure in both groups (Table 1). The
peritoneal lavages were immediately aliquoted to smaller volumes (400
μL) and snap frozen to -70°C. The peritoneal lavage was then analyzed
with ELISA to measure tPA, PAI-1 (Labinova AB, Upplands Väsby,
Sweden), IL-6 and active TGFb1 (R&D Systems Europe, Abingdon,
UK) concentration.

Adhesions were also evaluated on day 7 according to a validated
model in a blinded manner. Following the lavage, the abdomen
was opened through a U-shaped incision with its base to the right.
Adhesions were considered as tissue (bowels or fat) adherent to the
experimental wound or to another intra-abdominal organ. The
lengths of the incisions as well as the adhesions covering the wound
were measured with a caliper up to one-tenth of a millimeter and
data
were expressed as the percentage of the wound covered by adhesions.
The distances were measured at the peritoneal level. Other adhesions
between intra-abdominal organs were also noted. After collecting
peritoneal fluid and measuring adhesions, the animals were euthanized
in accordance to AVMA [15].

Table 1: Experimental design.

<table>
<thead>
<tr>
<th>Groups (time in hours for control of tPA, PAI-1, TGFb1 and IL-6 in peritoneal lavage)</th>
<th>Control (NaCl), number of animals</th>
<th>PL/PG, number of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (before adhesion procedure)</td>
<td>42 animals from all groups</td>
<td>42 animals from all groups</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>6</td>
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<td>6</td>
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<tr>
<td>8</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>24</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>7days</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

Statistical analysis
Analyses were made with a non-parametric Mann-Whitney U test
to determine statistical differences between 2 groups. A Kruskal-Wallis
non-parametric test was used to determine differences between several
groups. P values below 0.05 were considered significant. Concentrations
were presented as mean ± SE (Standard Error) on Figures 1-4. SPSS
was used for analysis (SPSS v17.0, SPSS Inc., Chicago, Ill., US).

Results

1. tPA
After the adhesion procedure the tPA levels increased at 2 and
4 h similarly for both the control and experiment groups (Figure 1).
A significantly lower tPA concentration in the experimental group
compared to the controls was seen at 6 h (p=0.002). Decreasing levels
for both the experimental and control groups were seen at 6 h,
followed by increasing levels at all further times up to 7d (Figure 1). The
tPA values were all significantly raised compared to time 0 h (p<0.05)
(Figure 1).

1. PAI-1
PAI-1 levels were significantly raised (p=0.019) at 4 h in controls
as compared to the experimental group (Figure 2). At 7d, PAI-1 levels
were significantly lower in controls than in the experimental group
(p=0.026). Levels of PAI-1 elevated above physiological range were
seen at 4 h for the control and at 6 h for the PL/PG. Thereafter, the
reduction in concentration displayed similar patterns in both groups
(Figure 2).

1. IL-6
IL-6 levels were steeply elevated after the adhesion procedure (in
both the control and experimental groups) at 4 h (Figure 3). Thereafter,
a gradual decline in concentrations could be seen in both groups to 7d
(Figure 3). A minor significant difference in IL-6 concentration was
seen between the groups at 7d (p=0.041) (Figure 3).

1. TGF-b
A gradual incline of the active TGFβ1 concentration (in both
experimental and control groups) could be seen after the adhesion
procedure (Figure 4). Elevated levels (in both groups) were seen at 7d
(Figure 4).

1. Adhesions
Adhesions were significantly reduced on day seven, measured as a
percentage of the inflicted wound, p=0.002, (Figure 5).
Applied together with a negatively charged polyanion (PG), they form a neutral charged PL/PG complex which may operate as a degradable biofilm, preventing adhesions from developing in the wounded area [17]. However, the question of PL/PG whether the matrix interferes with factors involved in the processes of inflammation and fibrosis has not been investigated. In this study we focused on measuring the tPA, PAI-1, IL-6 and TGF-b in a pre- and post-operative state in rats in the peritoneal fluid. The time points were measured continually in order to detect a potential dynamic pattern of tPA, PAI-1, IL-6 and TGF-b. The substances were chosen to be analyzed since they (among others) play a pivotal role in the healing process after peritoneal injury as pointed out by other authors [5,18].

Previous studies have stated that there is a diffusion of substances between the peritoneal fluid and the submesothelial space during normal physiological equilibrium. Substances such as PAI-1 and others are known to have a circadian rhythm and may thus vary in concentrations throughout the day in the blood. This might be one of the reasons for the concentrations slightly above zero for both PAI-1 and IL-6 prior to the experiment (see time 0 in Figure 2 and 3) [19,20].

Discussion

In this study we demonstrated that there were no significant differences in fibrotic or inflammatory factors as measured by tPA, PAI-1, TGF-b and IL-6 in peritoneal fluid, between the control group and the PL/PG group, before and after surgery.

PL/PG has, in previous experiments, decreased the formation of peritoneal adhesions [16]. It has been locally applied on the damaged surface of the peritoneum to form a non-toxic polymer that accumulates and partially seals the injured area from the surrounding tissues [13] and peritoneal fluid and thereby might diminish the incorporation of permanent adhesion strands formed on the wounded peritoneal surface after surgery. Previous research has stated that negative charges, such as the injured peritoneal surface, might interact electrostatically with a positively charged polycation (αPL) hence the accumulation.
The levels of tPA followed a similar pattern in both groups when analyzed postoperatively. However, there was a dip in tPA concentration at 6 h in the PL/PG group which was not seen for the control group. The tPA is known to be an important initiator of the fibrinolysis and we hypothesized that the general lower levels, however non-significant, of tPA in the peritoneal fluid of the PL/PG group was a combination of the sealing effect of the PL/PG complex and smaller amounts of adhesions. One could speculate that the reason for higher levels of tPA in the peritoneal fluid of the control group could be due to higher amounts of fibrin residues in the peritoneal wound, thereby causing more pending fibrinolysis in this group, which would render higher tPA levels. Endothelial cells are known to harvest both plasmin and its major initiator tPA, and the endothelial cells are believed to be a major contributor of tPA to the peritoneal fluid [21]. The endothelial cells of the peritoneum are located in the submesothelial space in the capillaries. The sealant effect of PL/PG might be one of the explanations for generally smaller amounts of tPA secretion from endothelial cells from the submesothelial space to the peritoneal fluid.

It was previously shown that tPA in peritoneal lavage was increased in the beginning of the peritoneal injury to induce fibrinolysis and that its levels fluctuate and usually decrease during the first hours. Thereafter, these levels increase again and are usually elevated beyond 7d days post-operative. This is thought to be due to a rebound effect in fibrinolysis after peritoneal trauma [22]. In our study we noted higher values of tPA at 7d in both the PL/PG group and the controls which might point to a normal fibrinolytic process at this point (7d) in both groups.

PAI-1 levels of peritoneal fluid were elevated and reached levels considered above the normal physiological range in the control group at 4 h, then in the PL/PG group, at 6 h (Figure 2). It is speculated that one of the reasons for the delay in the elevated levels of PAI-1 in the PL/PG as compared to the control group could be due to the partial sealant effect that the PL/PG complex exerts on the injured tissues. Previous studies have stated that the effect of PAI-1 is primarily located in the submesothelial space under both normal and inflammatory conditions [23]. In our surgical adhesion model we exposed the submesothelial space and thereby speculate that the reasons for smaller adhesions could be due to a sealed submesothelial space and thereby a partially sealed PAI-1 substance. Some of the known contributing causes for elevated PAI-1 concentration are the early inflammatory cells such as polymorphonuclear leukocytes (PMN cells) and macrophages that invade the injured peritoneum during the first days after the peritoneal injury [24]. Both PMN and macrophages are known to be involved in the secretion of cytokines such as TNF-α, IL-1 and IL-6 [25-27]. The cytokines are known to induce high levels of PAI-1 in the first hours after peritoneal injury [28]. In our study PAI-1 had, as previously mentioned above, a 2 h later raise in concentration in the PL/PG group as compared to the controls. We draw the conclusion that one of the reasons for later elevation in PAI-1 levels in the PL/PG group could be due to the sealant effect at the wounded peritoneal space, which might delay the invasion of inflammatory cells to the area from the surrounding tissues. Further time sequential histology studies will have to be made to investigate this issue.

IL-6 is a very adhesiogenic cytokine and has been shown to be important in the adhesion developing process [29]. We did not detect any significant changes between the groups although some local variations between the groups were seen and any possible decrease in inflammation could not be shown by measuring IL-6 in peritoneal fluid.

Three days after the peritoneal injury the PMN cells normally start to decrease in number and are gradually replaced by mesothelial cells [30,31]. We speculated that the fluctuating (with some delay in the PL/PG group) levels of IL-6 in both groups seen during the first week after the surgery followed a normal pattern of inflammation in both groups.

TGF-b, a cytokine consisting of 3 isoforms, is known to increase the risk of fibrosis in the long term and thereby increase the risk of peritoneal adhesions [32,33]. Different isoforms of TGFb have been shown to have different distributions in the peritoneum [34]. The biological activity of TGFb resides both in the total and active form. The most important form regarding formation of abdominal adhesions is the active TGFb1 [35]. Here we noted smaller amounts of active TGFb1 in the beginning of the experiment (2 to 24 h) and higher levels at 7d. This is consistent with previous data [20] and we concluded that active TGFb1 factor is excreted similarly in both the control and the PL/PG groups and that the normal fibrotic capacity was similar for both groups.

Conclusion

In summary, while reducing adhesions we could not demonstrate any significant difference in the concentrations of tPA, PAI-1, IL-6 and active TGFb1 in peritoneal fluid between the control and PL/PG groups after adhesion-inducing surgery. Even though some results could point to a delay in the release of some of the studied factors by the PL/PG complex sealing effect, this study cannot show this. We therefore conclude thus that the normal processes of inflammation and fibrosis during the healing of the peritoneum does not seem to be affected by the PL/PG complex which is in concordance clinical results in animals in previous studies on the PL/PG complex.

References


