Differently charged polypeptides and their impact on peritoneal and pleural postoperative adhesion formation

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Prevention of Adhesions by PL/PG after Adhesiolysis

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Abstract

Background: Previous studies of differently charged polypeptides, Poly-L-lysine (PL) and Poly-L-Glutamate (PG) have shown promising results, reducing postsurgical adhesions. This study aimed to investigate the possible anti adhesion effect of those combined polypeptides, after adhesiolysis. The concentration of tPA, PAI-1 and active TGFβ1 in biopsies from adhesions, unharmed peritoneum before and after adhesiolysis, was also investigated.

Materials and methods: A total of 24 male rats were divided in three groups A (N=8), B (N=8) and C (N=8). All rats underwent primary adhesiolysis at day 0, and adhesiolysis at day 7. Adhesions were evaluated at day 7 and 14, where group B received PL/PG after surgery at day 0 and after adhesiolysis at day 7, and group C received PL/PG after adhesiolysis at day 7. Tissue plasminogen activator (tPA), Plasminogen activator inhibitor 1(PAI-1) and active transforming growth factor beta 1(TGF-β1) were collected from biopsies of adhesions and normal peritoneum at day 0, 7 and 14.

Results: Significant reduction of adhesions p<0.05 was seen in group B at day 7 after primary surgery, and at day 14 after adhesiolysis. Significantly p<0.05 reduction of adhesions was seen at day 14 after adhesiolysis in group C. Some variations were seen in tPA, PAI-1 and active TGFβ1.

Conclusions: PL/PG may be used to prevent adhesion formation after adhesiolysis. The process of fibrinolysis and fibrosis was not affected, after PL/PG prophylaxis and adhesiolysis.

Keywords: Abdominal adhesions; Prevention; Polypeptides; Adhesiolysis

Introduction

Abdominal adhesions constitute major health related problems, both for the individual patient, and globally due to large expenditures for the healthcare [1,2]. Most of the abdominal adhesions form due to previous peritoneal damage, mainly during abdominal surgery, and may end up causing intestinal obstructions, pain and female infertility, along with other complications [3,4].

The overall picture of how abdominal adhesions develop is quite clear, although some parts remain to be elucidated.

Abdominal adhesions form as a result of peritoneal injury. Peritoneum is a serous delicate organ, lining the abdominal cavity, with both protective and restorative functions [5]. Peritoneum consists of a single layer of loosely attached mesothelial cells, resting on a basal lamina and a submesothelial area. The submesothelial area contains resident cells, capillaries and lymphatic vessels [6].

Mesothelial cell detachment is an immediate consequence of peritoneal damage of any kind, leading to serosanguinous leak in the area, forming a fibrin mesh between injured peritoneal sites. The fibrin is usually resolved due to local fibrinolysis, however during extensive trauma and local tissue hypoxia, the fibrin strands may persist, leading to stable adhesions through deposition of collagen and other extracellular matrix proteins [7-9].

One important factor initiating fibrinolysis via the serine protease plasmin in previous damaged peritoneum is the Tissue plasminogen activator (tPA), stored in great quantities in endothelial and mesothelial cells on the peritoneum [10]. Plasminogen activator inhibitor (PAI-1), on the other hand, is an important factor impairing fibrinolysis through inhibition of tPA [11,12].

The production of collagen and other extracellular matrix proteins in damaged peritoneum is mostly governed by fibroblasts and active transforming growth factor beta 1(TGF-β1) [13].

Many attempts have been made during the past years to prevent postsurgical adhesions, although none have been feasible in every aspect. The group has focused on combining positively charged polymers, Poly-L-lysine (PL) with negatively charged polymers, Poly-L-Glutamate (PG), forming a bioactive, nontoxic, degradable polymer that seals of the injured peritoneal site, prohibiting fibrin deposition and eventually, the development of postsurgical abdominal adhesions. Previous studies have focused on comparing the anti adhesion effects in the abdomen after the primary surgery, when administering the PL/PG complex [14-16].

However, a huge amount of patients undergoes repeated surgical procedures in the abdomen, causing extensive adhesions, which often forces the surgeons to perform intra abdominal adhesiolysis, thereby increasing the risk of complications such as bleedings, fistulas, abscesses and more [17-20].

In this study, the examination of the potential anti adhesion effect of PL/PG after adhesiolysis was focused. Key parameters i.e. tPA, PAI-1 and active TGFβ1 in biopsies from normal peritoneum and biopsies

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of adhesions were measured, before and after adhesiolysis, in order to disclose possible differences in the aspects of fibrinolysis or fibrosis.

Materials and Methods

Animals

A total of 24 male Sprague Dawley rats (Charles River, Sulzfeld, Germany), weighing approximately 250 g each, were used for induction of peritoneal adhesions. The animals were kept under standardized conditions and had free access to water and pellets. The local ethical committee at Lund University approved the adhesion study (Lund Sweden), and the animals received the best animal care, in compliance with the guidelines of the Swedish Government and Lund University, Sweden.

Chemicals

The chemicals Poly-L-lysine MW>30 kDa (PL) and Poly-L-Glutamate MW 15-50 kD (PG) (Sigma Aldrich™, St. Louis, Missouri, USA) were freshly prepared at the day of the experiment, with 2.54% glycerol and water, in order to create an osmotic balanced solution to a final concentration of 0.5% (5 mg/ml). They were put in separate bottle atomizers that administrated 0.5 ml volume with one dose.

Model

The animals were anesthetized with 50 mg/kg Ketalar (Parker Davis™ Detroit, Michigan, USA) and Xylazine 6 mg/kg (Rompun Vet, Bayer AB, Gothenburg, Sweden), by an intramuscular injection. The abdomen was opened through a midline incision, during sterile conditions. A standardized animal adhesion model, lateral incision of the peritoneum, was used, described and validated previously [21]. In brief, a 10 mm long incision was made in the lateral abdominal wall, including the whole parietal peritoneum through to the muscle underneath. The incision was stitched with interrupted sutures (60 Prolene™ Ethicon, Somerville, NJ, USA). Treatment was then applied by first spraying the PL and thereafter, PG followed by abdominal closure, using a running suture (Prolene™ 4-0, Ethicon, Somerville, NJ, USA) in two layers. Controls received saline NaCl. The animals received subcutaneous saline (0.9%, 5 ml) for resuscitation, and buprenorphine (0.01 mg/kg, Butorphanol, Davis™ Detroit, Michigan, USA) and Xylazine 6 mg/kg (Rompun Vet, Bayer AB, Gothenburg, Sweden), by an intramuscular injection.

Statistical analysis

Results were shown as mean ± Standard Error (SE). Mann Whitney ´ U test were used to determine differences between the groups, both regarding adhesions, as well as tPA, PAI-1 and active TGFβ1. P<0.05 were considered statistically. The statistical analyses were performed with SPSS used for analysis (SPSS v17.0, SPSS Inc., Chicago, Ill., US).

Results

Adhesions

Adhesions were significantly lower at day 7, p=0.001; and day 14, p=0.007; in group B compared to group A (Figure 1). Significantly less adhesions were observed at day 14 in group C compared to group A, p=0.003 (Figure 1).

tPA

Normal peritoneal biopsies: Differences of tPA levels in biopsies from normal peritoneum were seen at day 0, 7 and 14 between group A, B and C, although none of these were significant (Figure 2).

Biopsies from adhesions: Significantly higher levels of tPA from adhesions were seen at day 0 and day 7 in group B compared to group A, p<0.05; and in group C compared to group A and B at day 14, p<0.05 (Figure 3).

PAI-1

5.3.1 Normal peritoneal biopsies: Significantly higher levels of PAI-1 in biopsies from normal peritoneum were seen in group A and

<table>
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Table 1: Peritoneal biopsy collecting IPA, PAI-1 and TGFβ1*, adhesion biopsy collecting IPA, PAI-1, TGFβ1** Adhesions evaluation***
C compared to group B at day 7, $p<0.05$ (Figure 4). Significantly lower levels of PAI-1 were seen in group B at day 14 compared to group A and C, $p<0.05$ (Figure 4).

**Biopsies from adhesions:** Significantly higher levels of PAI-1 from adhesion biopsies were seen at day 7 in group A and C compared to group B, $p<0.05$ (Figure 5). Significantly higher levels of PAI-1 were seen in group C compared to group B and A, $p<0.05$ at day 14 (Figure 5).

**Active TGF-β1**

**Normal peritoneal biopsies:** Levels of active TGF-β1 from normal peritoneum were significantly higher at day 7 in group A and group C compared to group B, $p<0.05$. At day 14, group A and C had significantly higher levels of active TGF-β1 compared to group B, $p<0.05$ (Figure 6).

**Biopsies from adhesions:** Active TGF-β1 from biopsies of adhesions in group B were significantly higher than in group A and C at day 14, $p<0.05$ (Figure 7).

**Adhesion score**

Significant lower adhesion score were seen at day 7 in group B compared to group A and C, $p<0.05$ (Figure 8). Significant lower adhesion score were seen in group C compared to group A at day 14 (Figure 8).
Histology

Histology shows less inflammatory cells surrounding the PL/PG complex (Figure 9). Macroscopical appearance is also disclosed (Figure 10).

Discussion

In this article, it was demonstrated that PL/PG administered locally on peritoneum after adhesion creating surgery at day 0 and after adhesiolysis at day 7 in group B, significantly reduced adhesion...
Histology of adhesion area, with and without PL/PG complex. A
Animal treated with saline (A) and PLPG treated animal (B) at
similar patterns in all three groups (Figure 2).

Peritoneum (opposite site to primary surgery and adhesiolysis) showed
adhesions. (contralateral to the operated peritoneal site) and in biopsies of formed
fibrosis (TGF-β1) were measured,
groups, the substances involved in fibrinolysis (tPA and PAI-1) and
formed within 7 to 8 days postoperatively [25,26].

It was hypothesized based on this and the data that the gradually
increasing tPA levels seen in the peritoneal biopsies of unharmed
peritoneum, might represent a source of tPA to the contralateral side
subjected to surgery. It was further speculated that PL/PG did not affect
the levels of tPA in the peripheral peritoneum.

Adhesions differed between the groups, not only in amount, but
also in quality (Figure 8). In group A, they were solid and hard, but in
group B, they were filmy and smooth, both at day 7 and 14, and loose
and filmy in group C at day 14.

Levels of tPA in biopsies from adhesions before and after
adhesiolysis were increased at day 7 in group B compared to A, and at
day 14 in group C compared to group A (Figure 3). It was concluded
that there might have been a more pending fibrinolysis in group B at
day 7, compared to group A and C at the same time. The higher levels of
tPA at day 14 in group C to group A could represent a rebound effect in
fibrinolysis, supported in literature [31]. The reason for lower levels of
tPA at day 14 in group B compared to C could be due to a partial sealant
effect of the PL/PG in group (Figure 3).

PAI-1 is located in the submesothelial area in a normal resting state
of peritoneum. Whenever peritoneal injury occurs, PAI-1 is increased,
produced by several cells involved in the injury and restorative
peritoneal process. Some of those are mesothelial cells and PMN cells
activated by cytokines, such as IL1 and TNFα [32,33].

In biopsies from normal peritoneum, increasing levels of PAI-
1 in all three groups was seen. It was concluded that the unharmed
peritoneum might have been affected indirectly by the trauma from
the contralateral side, thereby the elevating levels. Interestingly, lower
levels were seen in group B at both day 7 and 14, which could reflect a
lower impact of the surgical trauma in that group (Figure 4).

In biopsies from peritoneal adhesions, similar levels at day 7
between group A and 7 were seen, which could represent the similar
amount and quality of adhesions (Figure 1 and 8). This is in conjunction
with lower levels of PAI-1 in group B at this time. The higher level of
PAI-1 at day 14 is hard to explain, although it could represent a result
of higher tPA levels, since PAI-1 is known to form complex with tPa
[7,11]. In fact, higher levels of tPA were seen at day 14 in group C that
paralleled the higher PAI-1 levels at this time (Figure 3). It was concluded
that there might have been a more pending fibrinolysis in group B at
day 14 in group C compared to group A (Figure 3).

In order to detect possible variations, before and after adhesiolysis,
regarding the restorative process of peritoneum between the three
groups, the substances involved in fibrinolysis (tPA and PAI-1) and
fibrosis (TGF-β1) were measured, both from normal peritoneum
(contralateral to the operated peritoneal site) and in biopsies of formed
adhesions.

tPA in biopsies from normal peritoneum taken at contralateral
peritoneum (opposite site to primary surgery and adhesiolysis) showed
similar patterns in all three groups (Figure 2).

Previous immunohistochemistry studies of peritoneum have shown evidence that tPA is stored in large quantities in endothelial
cells, located in submesothelial capillaries, and might be released
whenever peritoneum is harmed, even though the damage may be
located elsewhere in the peritoneum [27-30].

Interestingly, PL/PG also significantly reduced adhesions in group
C at day 14, when administered after adhesiolysis at day 7 (Figure 1).

The results indicate that PL/PG may be used both as adjuvant
therapy during primary surgery, to avoid future adhesions, but also in
relaparotomy after adhesiolysis, to diminish further adhesions.

In group B, slightly fewer adhesions were seen at day 14, compared
to day 7. Although the difference between these time points was not
significant, it was speculated that larger groups would show a significant
difference, which would imply an existence of some synergistic effect in
readministering the PL/PG, if the relaparotomy is performed within a
week (Figure 1). This is an interesting theory, since most adhesions are
formed within 7 to 8 days postoperatively [25,26].

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An important factor to consider when measuring peritoneal
adhesions is the remodelling of Extracellular Matrix (ECM), since this
is a central part in wound healing. TGF-β1 has been shown to be a key
regulator in remodelling of ECM [34]. TGF-β1 exists in three latent
isoforms (TGF-β1-3) that are activated through proteolysis, during
peritoneal injury. Here, active TGF-β1 was measured, that is known to
play a key role in the formation of adhesions [35].

Peritoneal biopsies from unharmed peritoneum in our three groups
also showed some variations, within and between the groups. When the
biopsies were taken from unharmed peritoneum at different time
points, they had to be taken a bit separated from each other, in order to
avoid fibrosis and possible false to high levels of active TGF-β1. Thus,
it was interpreted that the different levels of active TGF-β1 showed in
figure 6 may have partly, been a result of separated biopsies [36]. It
was further hypothesized that the smaller amounts of active TGF-β1

Figure 9: Histology of adhesion area, with and without PL/PG complex. A
shows less fibrosis (treatment with PL/PG), than B.

Figure 10: Animal treated with saline (A) and PLPG treated animal (B) at
day 14. In the control, the cecum is totally adhered to the lateral abdominal
wall, whereas in the treated animal a smooth and healed peritoneum is noted
(white/transparent area in picture).
in group B at day 7 and 14 compared to group A and C, might have represented a lower fibrotic state in that group, seen as smaller amounts of adhesions (Figure 1).

Biopsies from adhesions showed different levels of active TGF-β1, however, none were significant. Group B and C had filmy and loose adhesions at day 7 and 14. Group A had crude and hard adhesions at day 7 and 14 (data not shown). It was speculated (based on this) that active TGF-β1 failed to show the difference in quality of adhesions between the groups.

Despite lower adhesions in group B and C at day 14 levels of active TGF-β1, in biopsies from adhesions showed higher levels than the control group A at the same time. It could only be speculated that one of the reasons for this could be due to activation through the tPA substance, within the adhesions, since the serine proteases are known to be important activators of latent TGF-β1 [37], and tPA is known to be the largest activator of the serine protease plasmin. Higher levels of tPA were in fact seen in at day 14 in both group B and C compared to group A (Figure 3). Biopsies from unharmed peritoneal tissue were not performed in this study, since previous experiments did not reveal any affection of levels of the key parameters studied [38,39].

Conclusions

In summary, this article shows that PL/PG may be used as an adjuvant after adhesiolysis, performed in the abdomen, in order to avoid future adhesions. The PL/PG complex does not seem to interfere with the normal process of fibrinolysis and fibrosis, when investigated before and after adhesiolysis.

References


