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

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

Table 1: Experimental design.

table 1, with 6 animals for each time-point. In the last groups adhesions were evaluated before obtaining fluids for analysis.

Chemicals

Osmotic balanced (2.54 wt% glycerol) aqueous solutions (0.05%) of αPL and PG (Sigma Aldrich, St. Louis, Mo, USA) were freshly prepared on the day of the experiment and stored in the refrigerator until used. 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, scalpel, 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[8] 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].

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

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).

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).

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).

TGF-b

A gradual incline of the active TGFb1 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).

Adhesions

Adhesions were significantly reduced on day seven, measured as a percentage of the inflicted wound, $p=0.002$, (Figure 5).

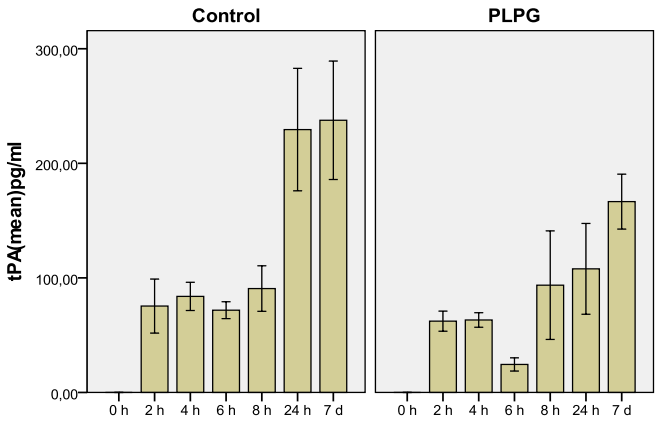

Figure 1: Peritoneal concentrations of tPA (pg/ml) in controls (NaCl) and experimental group (PL/PG) before (0h) and after surgery (2h-7d) No significant difference in concentrations were seen between the PL/PG group and controls as a total. A significant difference in tPA concentration between PL/PG and controls were seen at 6h (p=0.002). Graphs show tPA concentration ±SE.

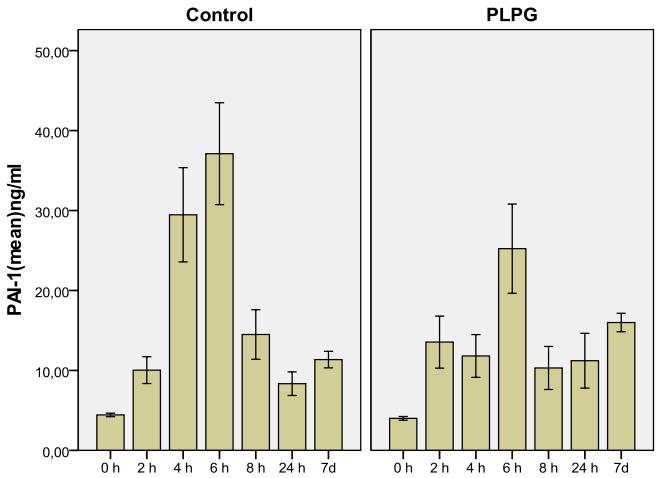

Figure 2: Peritoneal concentrations of PAI-1 (ng/ml) in controls (NaCl) and experimental group (PL/PG) before (0h) and after surgery (2h-7d) No significant difference in concentrations were seen between the PL/PG group and controls as a total. A significant difference in PAI-1 concentration between PL/PG and controls were seen at 4h (p=0.019) and 7d (0.026). Graphs show PAI-1 concentration ±SE.

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. 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].

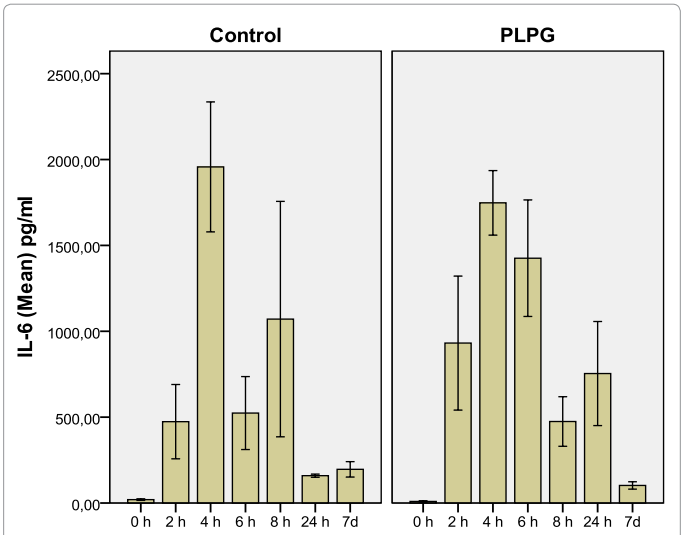

Figure 3: Peritoneal concentrations of IL-6 (Pg/ml) in controls (NaCl) and experimental group (PL/PG) before (0h) and after surgery (2h-7d) No significant difference in concentrations were seen between the PL/PG group and controls as a total. A small significant difference in IL-6 concentration between PL/PG and controls were seen at 7d (p=0.041). Graphs show IL-6 concentration ±SE.

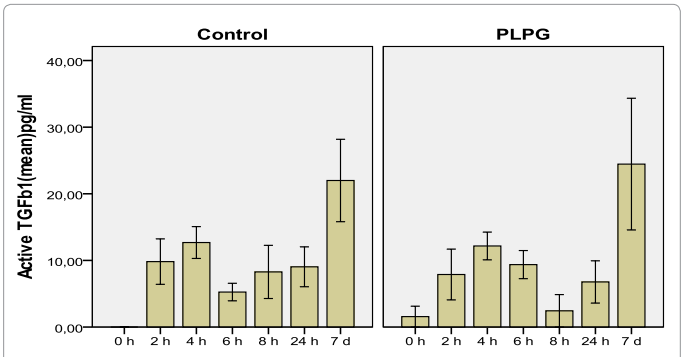

Figure 4: Peritoneal concentrations of Active TGFb1 in controls (NaCl) and experimental group (PL/PG) before (0h) and after surgery (2h-7d) No significant difference in concentrations were seen between the PL/PG group and controls as a total. Graphs show Active TGFb1 concentration ±SE.

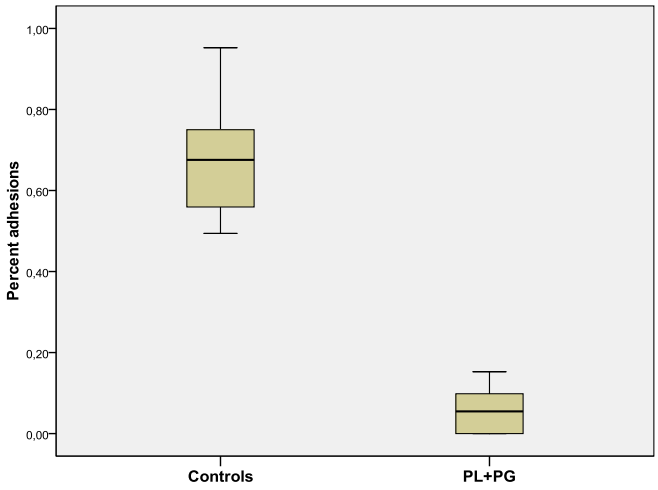

Figure 5: Adhesions were significantly reduced in treated animals, p=0.002.

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[39] 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.

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