A Novel Polypeptide Derived From Human Lactoferrin in Sodium Hyaluronate Prevents Postsurgical Adhesion Formation in the Rat

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Objective: The objective of the study was to evaluate whether a peptide derived from human lactoferrin, PXL01 could act safely to reduce the formation of peritoneal adhesions in the rat model and to map the molecular mechanisms of its action.

Summary Background Data: Adhesion formation is a significant problem within every surgical discipline causing suffering for the patients and major cost for the society. For many decades, attempts have been made to reduce postsurgical adhesions by reducing surgical trauma. It is now believed that major improvements in adhesion prevention will only be reached by developing dedicated antiscarring products, which are administrated in connection to the surgical intervention.

Methods: Anti-inflammatory as well as fibrinolytic activities of PXL01 were studied in relevant human cell lines. Using the sidewall defect-cecum abrasion model in the rat, the adhesion prevention properties of PXL01 formulated in sodium hyaluronate were evaluated. Large bowel anastomosis healing model in the rat was applied to study if PXL01 would have any negative effects on intestine healing.

Results: PXL01 exhibits an inhibitory effect on the most important hallmarks of scar formation by reducing infections, prohibiting inflammation, and promoting fibrinolysis. PXL01 formulated in sodium hyaluronate markedly reduced formation of peritoneal adhesions in rat without any adverse effects on wound healing.

Conclusions: A new class of synthetically derived water soluble low molecular weight peptide compound, PXL01 showed marked reduction of peritoneal adhesion formation in an animal model without any negative effects on healing. On the basis of these data, a comprehensive adhesion prevention regimen in clinical situation is expected.

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Peritoneal adhesions are fibrous tissue connections forming between abdominal structures after surgical trauma or other types of injury. General abdominal, vascular, gynecological, urological, and orthopedic surgery may lead to adhesion formation in up to 95% of the patients.[1–3] Postsurgical adhesions are considered the main cause of small bowel obstruction,[4] a well-known etiology of secondary infertility in females[5] as well as a possible cause of postoperative pain.[6] More than 30% of the individuals undergoing lower abdominal surgery are readmitted for disorders related to adhesion formation at some period during their life time.[1,2]

For many decades, attempts have been made to reduce postsurgical adhesions by reducing surgical trauma (avoiding desiccation, gentle tissue handling, and meticulous hemostasis) as well as by avoiding contamination of the abdominal cavity with foreign materials (using starch-free gloves, lint-free gauze, and absorbable sutures).[3,7,8] Importantly, the laparoscopic techniques have shown not to be sufficient to overcome the problem of postoperative adhesion formation.[9,10] Thus, intraperitoneal adhesions remain a major clinical issue, and it is now believed that future improvements may only be marginally influenced through superior surgical technique. Instead, the focus is to develop dedicated products for prevention of adhesion formation, which are administrated in connection to the surgical intervention.

Most of the therapeutic strategies tested in prevention of adhesions are medical device products. Different types of physical barriers have been evaluated, where the biodegradable films applied during the intervention are used to keep the injured abdominal surfaces separated during the critical period of peritoneal healing. The 2 most widely used adhesion-reducing barriers are Seprafilm (Genzyme, Cambridge, MA) and Interceed (Johnson & Johnson MedicalInc., Arlington, TX). Seprafilm, composed of sodium hyaluronic acid and carboxymethylcellulose (CMC), forms a viscous gel approximately 24 to 48 hours after application, and is slowly resorbed within 1 week.[11,12] Seprafilm has been shown to reduce postsurgical adhesion in clinical situation.[13–15] However, the device is difficult to apply, as it adheres to gloves and organs, and as it is brittle.[16] Additionally, Seprafilm increases the risk of sequelae associated with anastomosic leakage and is not compatible with laparoscopic procedures.[17] Interceed, composed of oxidized regenerated cellulose, is transformed into a gelatinous mass covering the injured peritoneum and has shown efficacy in adhesion-prevention in several clinical studies.[18–20] However, application of Interceed requires complete hemostasis as even small amounts of intraperitoneal bleeding negates any beneficial effect of this barrier.[16] A general limitation of using the physical barriers is the site-specificity of the product, requiring the surgeon to predict where adhesions will occur and where they would most likely cause clinical problems.

As an alternative to physical barriers, different fluids for intra-abdominal instillation such as icodextrin (Adept, Baxter Healthcare Corporation, IL) or lactated Ringers's solution, have been administrated after the surgery in volumes sufficient to allow floatation of the abdominal structures and thus preventing the injured surfaces from adhering to each other.[17,21–24] However, it has been shown that laparoscopically instilled fluids are absorbed more rapidly from the abdominal cavity than the time required for peritoneal healing.[25]

A limited number of pharmacologically active compounds have been tested for prevention of postsurgical adhesions. For

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example, the inflammatory components as well as fibroblast proliferation of the wound healing cascade have been a target of pharmacotherapy by using steroid drugs and cytotoxic drugs, respectively. However, these agents have shown ambiguous efficacy and potentially serious side effects.[26–29]

Because of the limited efficacy and difficult handling of the tested therapies, most of surgical interventions performed in the abdominal cavity today, do not include any products to prevent adhesion formation. The postsurgical adhesions thus continue to cause suffering for the patients and present a major cost for society.[30,31] Here we describe a novel approach to prevent formation of intra-abdominal adhesions using a synthetic peptide derived from human lactoferrin, PXL01. PXL01 exhibits an inhibitory effect on the most important hallmarks of scar formation by reducing risk for infections, prohibiting inflammation, and promoting fibrinolysis. The peptide is formulated in naturally occurring hydrophilic polymer sodium hyaluronic acid, which provides slow release properties of the drug and also contributes per se to the final results by physical barrier effects. In the present investigation the aim was to study PXL01 regarding adhesion prevention in animal model and to map the molecular mechanisms of its action.

MATERIALS AND METHODS

Antimicrobial Activity

The antimicrobial effect of PXL01 against *Staphylococcus aureus* (*S. aureus*; CCUG 1800, Culture Collection, University of Göteborg, Sweden), *Pseudomonas aeruginosa* (*P. aeruginosa*; ATCC 15442, American Type Culture Collection, Manassas, VA), and *Escherichia coli* (*E. coli*; CCUG31246) was determined by MMC (minimal microbicidal concentration) assay. Bacteria were cultured in brain-heart infusion medium (BHI, Difco, BD Diagnostics, Franklin Lakes, NJ) on a shaker over night at 37°C. The culture was thereafter diluted 1:10 in fresh BHI and incubated for additional 2 hours to reach log-phase growth. The bacteria were pelleted and suspended in 0.04% BHI to a concentration of 1×10^7 cells/mL as estimated by measuring optical density at 600 nm. The peptides were serially diluted by 2-fold steps from 200 to 1.56 μg/mL in 0.04% BHI, and 200 μL of the peptide dilutions were mixed in duplicate with 10 μL bacterial suspension in a 96-well culture plate (Nunc, Roskilde, Denmark). The microplate was incubated at 37°C for 2 hours. Five microliter of the suspension was aspirated from each well and added as a drop onto blood agar plates (Columbia agar; Oxoid, Basingstoke, UK) supplemented with 5% defibrinated horse blood (National Veterinary Institute (SVA), Uppsala, Sweden), and the plates were incubated over night. The minimal PXL01 concentration causing a 99% reduction of bacteria was defined as the MMC_{99}. The concentration of the bacterial suspension used in the assay was confirmed by a viable count estimation on blood agar plates.

In Vitro Assessment of Anti-Inflammatory Effects

The human acute monocytic leukemia cell line (THP-1) (ATCC TIB-202, ATCC, Manassas, VA) corresponding to human monocytes was maintained in Roswell Park Memorial Institute medium (RPMI) 1640 (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories GmbH, Pasching, Austria), 1 mM sodium pyruvate (Sigma-Aldrich, St. Louis, MO), and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (PAA Laboratories GmbH, Pasching, Austria). The cell density was adjusted to 1×10^6 cells/mL and 500 μL of the cell suspension was added per well to 24-well cell culture plates (Sarstedt, Nümbrecht, Germany). The cells were treated with 10 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, MO) for 48 hours to differentiate the monocytes into macrophage-like cells. After 48 hours the cells were stimulated by addition of 0.1 ng/mL lipopolysaccharide (LPS; *E. coli* serotype O55:B5; Sigma-Aldrich, St. Louis, MO) into the medium specified above except of containing 5% heat inactivated FBS. The LPS concentration used was selected based on in vitro titration experiments to give a close to maximum release of cytokines into the cell culture medium over a period of 6 hours. The indicated concentrations of PXL01 were added 30 minutes after addition of LPS. The cell supernatants were collected after 6 hours of incubation, centrifuged and frozen in −20°C, and kept frozen until analyzed for tumor necrosis factor (TNF-α), IL-1β, IL-6, and IL-8 production by enzyme-linked immunosorbent assay (ELISA, R&D Systems, Minneapolis, MN).

Regulation of Plasminogen Activator Inhibitors-1 Expression in Vitro

The mesothelial cell line MeT-5A (ATCC CRL-9444, ATCC, Manassas, VA) was cultured in Medium 199 containing Earle's salts, L-glutamine, and sodium bicarbonate (Gibco; Invitrogen, Carlsbad, CA) supplemented with 10% FBS (PAA Laboratories GmbH, Pasching, Austria), 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (PAA Laboratories GmbH, Pasching, Austria), 400 nM hydrocortisone (MP Biomedicals, Solon, OH), 870 nM insulin (Sigma-Aldrich, St. Louis, MO) and 3.3 nM epidermal growth factor (EGF; ImmunoKontact; AMS Biotechnology, Oxon, UK). When the cells reached 90% to 100% confluency, the spent media was removed, the cells were washed in D-PBS (without Ca^{2+} and Mg^{2+}; Gibco, Invitrogen, Carlsbad, CA) and detached from the surface by incubating in Trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco, Invitrogen, Carlsbad, CA) for 5 minutes. Cells were resuspended in complete M199 at a density of 6 to 8×10^4 cells/mL and 1 mL of the cell suspension was added to 24-well cell culture plates (Sarstedt, Nümbrecht, Germany). Cells were allowed to attach and proliferate for 72 hours, reaching a density of about 500 000 cells/well. Cells were washed in D-PBS and stimulated by addition of 0.1 ng/mL IL-1β (R&D Systems, Minneapolis, MN) into the medium specified above except of containing 5% heat inactivated FBS. The optimal IL-1β concentration used in the assay was selected based on titration experiments to give a close to maximum secretion of plasminogen activator inhibitor (PAI)-1 into the cell culture medium over a period of 6 hours. The indicated concentrations of PXL01 were added directly after addition of IL-1β. The cell supernatants were collected after 6 hours of incubation, frozen in aliquots at −20° until assayed for PAI-1 production by ELISA (TintElize, Trinity Biotech, Bray, Ireland).

Cytotoxicity

THP-1 and MeT-5A cells were cultured, stimulated, and treated with PXL01 as described above. After collection of cell supernatants after 6 hours of stimulation, the cells were detached from the surface by incubating in Trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco; Invitrogen, Carlsbad, CA) for 5 minutes. Cells were resuspended in culture medium containing 10% FBS (PAA Laboratories GmbH, Pasching, Austria). Cell viability was measured by Nucleocounter System (ChemoMetec A/S, Allerød, Denmark), according to manufacturer's instructions.

Preparation of PXL01 in Sodium Hyaluronate Hydrogels

PXL01 dissolved in sodium chloride solution was added to 2.5% sodium hyaluronate solution (molecular weight 3.8–8.1 MD; Bohus Biotech, Strömstad, Sweden) at a volume ratio of 2 of 5 PXL01 solution and 3 of 5 sodium hyaluronate solution, to obtain 1.5 or 6 mg/mL PXL01 in 1.5% sodium hyaluronate. The solutions

were homogenized by drawing the mixtures several times through 2.1 mm diameter needles.

PXL01 concentration and homogeneity in the sodium hyaluronate were determined by high performance liquid chromatography with UV detector (Agilent model 1100) at 220 nm. The analytical column used was a Vydac 218TP (C18, 5 μm, 250 $\times$ 4.6 mm). The mobile phases used (0.1% trifluoroacetic acid [TFA] in water containing 1% acetonitrile [solvent A] and 0.1% TFA in acetonitrile [solvent B]) were run at a gradient with a flow rate of 1.0 mL/min. Diluted PXL01 standards were applied to create calibration curves. Samples were prepared by adding hyaluronidase solution (hyaluronidase from *Streptomyces hyalurolyticus*; Sigma-Aldrich, St Louis, MO) with an enzyme activity of 500 U/mL to sample solutions. The mixtures were agitated for 2 hours at room temperature, and samples were diluted as needed with TFA in water, followed by additional mixing. The samples were centrifuged at 7000 rpm for 5 minutes before injection to the column.

In Vitro Release System Setup

A volume of 0.25 mL of the formulated product was placed into the well of the tissue culture plate (24-Flat Well Tissue Culture Plate, Techno Plastic Products AG, Trasadingen, Switzerland), resulting in a thin film of approximately 1.3 mm. The plates were placed into thermostat (37°C) for 1 hour to allow the product to reach the temperature of 37°C. 0.5 mL of the release medium (PBS, pH 7.4) re-equilibrated at 37°C was carefully layered over the surface of the gel and the tissue culture plates were transferred into a thermostatic shaker (60 rpm, 37°C). At predetermined time intervals, 10 μL aliquots of the aqueous solution were withdrawn from the release media. The concentration of PXL01 released was monitored at wavelength of 230 nm using a spectrophotometric measurement. Because the measurement of absorbance at 230 nm could detect the peptide as well as dissolved sodium hyaluronate in the release medium, a control release medium was used which has the same amount of sodium hyaluronate without any PXL01 as that of sodium hyaluronate with the drug.

Animal Model for Assessment of Postsurgical Adhesion Prevention

Female Sprague-Dawley rats (200–250 g, Charles River Laboratories, Sulzfeldt, Germany) were kept in a 12 hours light-dark cycle and were cared for in accordance with regulations for the protection of laboratory animals. The study was performed after previous approval from the local ethical committee.

Cecum abrasion as well as excision of the abdominal wall were performed to induce de novo adhesions as described previously.[32] Briefly, the rats were anesthetized with isoflurane (Isobavet, Shering-Plough Animal Health, Farum, Denmark) and buprenorfin (48 μg/kg, Temgesic, Shering-Plough, Brussels, Belgium) was given for postoperative pain relief. A 5 cm long midline incision of the abdomen was performed and a rectangle full thickness injury (5 $\times$ 25 mm) was made on the peritoneal wall through both the parietal peritoneum and the muscular fascia. Also, an area of the serous membrane on the both sides of the cecum, approximately 10 $\times$ 15 mm, was gently rubbed using cotton gauze until petechial hemorrhages seemed. The rats were randomized to untreated control group or treated groups. Excessive blood from the injury was removed and PXL01 in distilled water or in sodium hyaluronate was applied over the abraded areas using a syringe. In case of repeated administration, 2 additional doses of PXL01 in distilled water were given 24 and 48 hours postsurgery by intraperitoneal injection. The laparotomy wound was closed with a continuous suture (4-0 monocryl, Y3100H, Ethicon Inc. St-Stevens, Woluwe, Belgium) and the skin was closed with staples (Appose ULC35W, TycoHealthcare Group LP, Norwalk, CT). The animals were killed 6 days after surgery with an overdose of pentobarbital sodium (Pentobarbital vet, APL, Stockholm, Sweden). The abdomen was opened and the adhesions were scored by an evaluator blinded to the treatment. The incidence of adhesions between abdominal incision and the abraded cecum was quantified as a percentage of animals developing wall to wall adhesions connecting these injuries, in each group. Additionally, to comprehensively evaluate the total number of adhesions formed in the abdominal cavity, including the adhesions remote from the surgical trauma, 2 different grading schemes were used. The cumulative scoring scale described by Bothin et al[33] assigns the total number of adhesions present in the abdominal cavity: 1 point is given to each adhesion observed and the points are added to form the score. The adhesion scoring scale according to Nair et al[34] incorporates both the total number of adhesions, and the incidence of adhesions between target organs, whereas a higher grading is given to the latter one (0, no adhesions; 1, single band of adhesions from the viscera to the target organ; 2, 2 bands of adhesions from the viscera to the target organ; 3, more than 2 adhesive bands from the viscera to the target organ, 4, viscera directly adherent to abdominal wall, irrespective of number and extent of adhesive bands). Finally, the percentage of rats free from any abdominal adhesions was assessed in each group. Any possible signs of peritoneal inflammation (erythema and/or edema) or disrupted wound healing were recorded in connection to the necropsies. As a general marker for well being, the body weights of animals before and 6 days after the surgery were compared.

Large Bowel Anastomosis Model in the Rat

Female Sprague Dawley rats (200–250 g, Charles River Laboratories, Sulzfeldt, Germany) were kept at a 12 hours light-dark cycle and were cared for in accordance with regulations for the protection of laboratory animals. The study was conducted after previous approval from the local ethical committee.

Anesthesia was induced with isoflurane (Isobavet, Shering-Plough Animal Health, Farum, Denmark) and the rats received buprenorfin (48 μg/kg; Temgesic, Shering-Plough, Brussels) intramuscularly for postoperative pain relieve and Bimotrim (80 mg/kg; Bimeda, UK) subcutaneously before the surgery.

The abdominal wall was shaved and a midline laparatomy of approximately 3 cm was performed. The colon was exposed and transected 2 cm distal of cecum. A seromuscular end-to-end anastomosis was performed with 8 interrupted sutures using 6/0 monocryl (Y432H, Ethicon Inc, St-Stevens, Woluwe, Belgium) thread. A macaroon was placed in the colon at the anastomosis site as a stent during suturing. The rats were randomly divided into groups receiving PXL01 (6 mg/mL) in 1.5% sodium hyaluronate covering the anastomosis and surrounding peritoneum (n = 8) or no treatment (n = 8). The abdomen was closed with a continuous suture (4–0 monocryl, Y3100H, Ethicon Inc.) in the muscular layer and with staples (Appose ULC35W, TycoHealthcare Group LP, Norwalk, CT) in the skin. A total of 2 mL isotonic saline was administered subcutaneously to prevent dehydration.

The animals received additional doses of buprenorfin (24 μg/kg; Temgesic, Shering-Plough, Brussels) subcutaneously 2 times per day for 2 days after surgery. The animals were killed 7 days after surgery with an overdose of pentobarbital sodium (Pentobarbital vet, APL, Stockholm, Sweden). The abdomen was opened, and a 4 cm long intestinal segment was resected with the anastomosis site located in the middle. A tube connected to a pressure monitor was inserted into 1 side of the intestinal segment and the other side was ligated at the end. The intestinal segment was placed immediately under isotonic sodium chloride, saline stained with green food color was infused through the tube into the intestinal segment, and the intraluminar pressure was monitored using a Grass recorder (Grass Instruments Co, Quincy, OH). The maximum pres-

sure before anastomotic burst was recorded as the burst pressure. The appearance of stained saline around the anastomosis provided a visual indication of the time point for the burst. The evaluator was blinded to the treatment each animal had received.

RESULTS

Antimicrobial and Anti-Inflammatory Effects of PXL01

The extent of inflammatory response in peritoneum caused by surgical trauma or infection, is known to positively correlate with excessive formation of intra-abdominal adhesions.[35] PXL01 was shown to exhibit microbicidal activity against *S. aureus* (Gram-positive), *E. coli,* and *P. aeruginosa* (Gram-negative) bacteria with 12.5 to 25 μg/mL of the peptide being required for 99% killing of the strains (Table 1). Thus, the antimicrobial capacity of PXL01 was markedly higher compared with the parent molecule, lactoferrin, where concentrations >1000 μg/mL are required for 99% killing of these bacterial species.[36,37] Anti-inflammatory effect of PXL01 was studied in macrophages derived from the human monocyte cell line THP-1. PXL01 demonstrated a significant and similar reduction in LPS-induced release of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6, as well as the pro-inflammatory chemokine IL-8 (Fig. 1A). There was an obvious plateau in the action of PXL01 where increasing the concentration of the peptide above 200 μg/mL did not result in any further reduction in cytokine production. In these experiments, PXL01 was added to the cell culture medium 30 minutes after addition of LPS. Based on previous findings, this time interval should be sufficient for LPS to bind to the cell receptor and thus, to exclude that the peptide would neutralize the effect of LPS on cytokine production only by scavenging this agent.[38,39] To rule out the possibility of the reduction in cytokine production by PXL01 being related to drug induced decline in cell viability, the cell survival was measured after 6 hours of LPS-stimulation. The amount of viable cells after PXL01 treatment (highest concentration 400 μg/mL) was more than 95% of cells treated with LPS only, indicating that addition of PXL01 did not result in any significant cytotoxic effect (data not shown).

TABLE 1. Antibacterial Potency of PXL01 Compared With Lactoferrin

	Escherichia coli MMC (99%; μg/mL)	*Staphylococcus aureus* MMC (99%; μg/mL)	*Pseudomonas aeruginosa* MMC (99%; μg/mL)
PXL01	12.5	12.5	25
Lactoferrin*	>1000	>1000	>1000

*MMC values for lactoferrin against *Escherichia coli* (strain: O6:K5:H-), *Staphylococcus aureus (strain:* MRS3526), or *Pseudomonas aeruginosa* (strain: CCUG 551).[36]

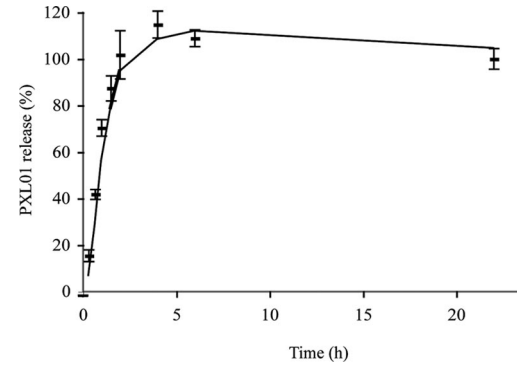

FIGURE 2. Release of PXL01 from sodium hyaluronate gels at 37°C. The concentration of PXL01 is 6 mg/mL in 1.5% sodium hylauronate solution. The cumulative drug released was expressed as the % drug released at time t. The data are shown as mean ± SD of 3 independent product preparations with the moving average trend line added.

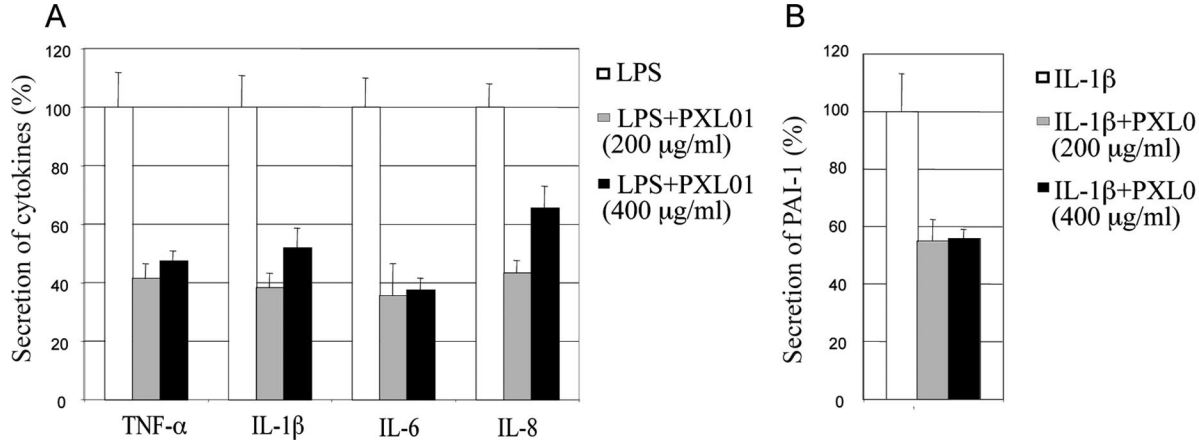

FIGURE 1. PXL01 regulation of inflammatory mediator production and fibrinolytic capacity in vitro. A, PXL01 inhibited secretion of pro-inflammatory markers from LPS-induced PMA treated THP-1 cells. PXL01 was added to cells in triplicate 30 minutes after the addition of LPS (0.1 ng/mL). Cytokine/chemokine levels were measured in the cell supernatants by ELISA after 6 hours of stimulation. Data are presented as mean ± SEM with LPS-stimulated cytokine/chemokine levels without peptide added set to 100%, whereas basal expression level of the corresponding marker is set to 0. B, PXL01 reduced IL-1β stimulated PAI-1 secretion in MeT-5A cells. PXL01 was added to cells in triplicate immediately after the addition of IL-1β (0.1 ng/mL). PAI-1 levels were measured by ELISA after 6 hours of stimulation. Data are presented as mean ± SEM with IL-1β stimulated PAI-1 levels without peptide added set to 100%, whereas basal secretion of PAI-1 is set to 0. Results are from one representative experiment of at least 2 independent experiments.

Regulation of Fibrinolysis by PXL01

The formation of postsurgical adhesions is to a large extent determined by the balance between fibrin deposition and degradation and the reduction in fibrinolytic activity, controlled by PAI-1, is known to positively correlate with adhesion formation.[40–42] IL-1β stimulated secretion of PAI-1 in human mesothelial cells was significantly reduced by PXL01 (Fig. 1B). No cytotoxicity of PXL01 was observed in mesothelial cells: incubation with 400 μg/mL of PXL01 resulted in more than 95% survival compared with cells treated with IL-1β only (data not shown).

PXL01 Release in Sodium Hyaluronate

PXL01 dissolved in sodium chloride solution was mixed with the sodium hyaluronate solution resulting in homogenous PXL01-containing hydrogel. The in vitro release experiments revealed a burst release of PXL01 from the sodium hyaluronate gel formulation with approximately 70% of PXL01 released within 1 hour (Fig. 2). Release behavior characterized by an initial burst is already demonstrated for other soluble compounds formulated in sodium hyaluronate.[43] This may have a functional use in providing an initial dose during drug delivery, minimizing any lag period. Importantly,

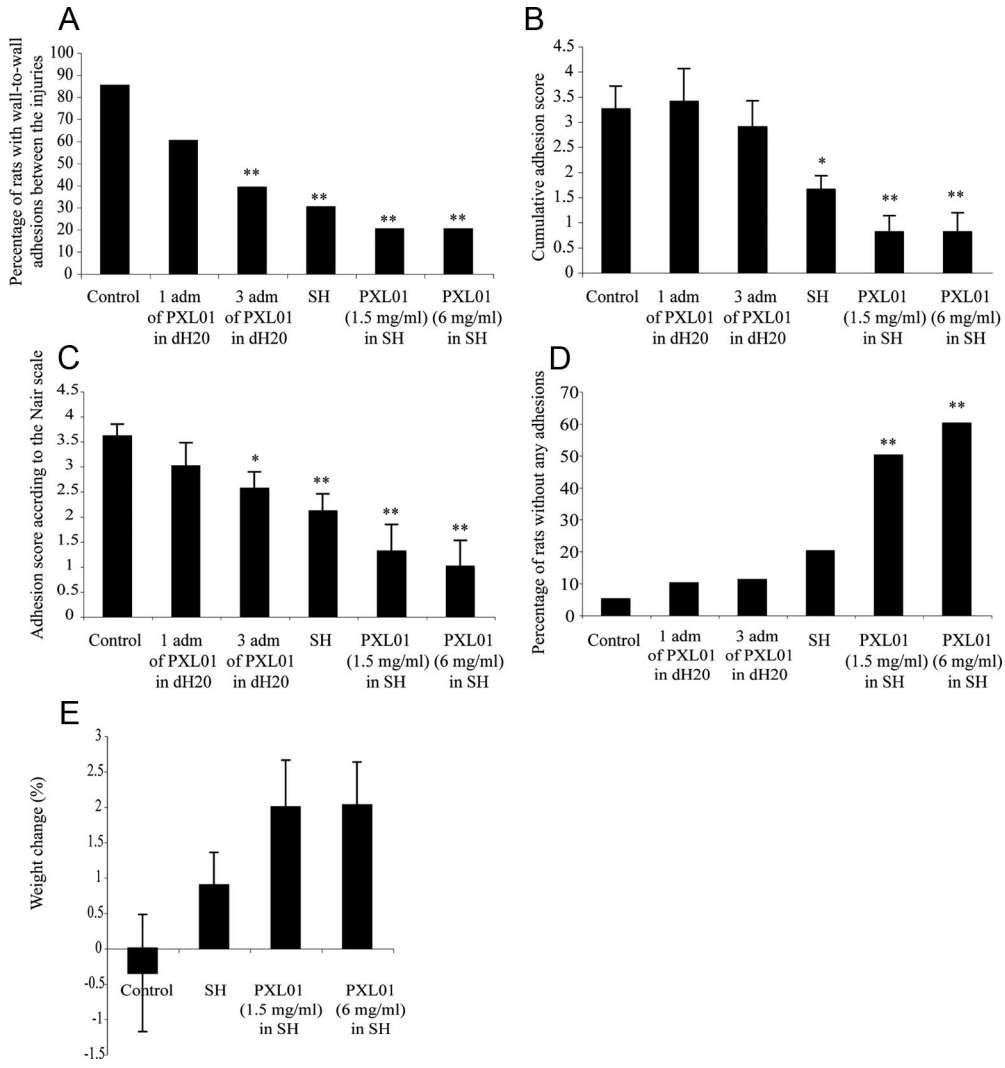

FIGURE 3. PXL01 in reduction of adhesion formation in rat model of abdominal surgery. A, The incidence of adhesion formation between the injury sites of abdominal wall and cecum is presented as a percentage of animals developing wall to wall adhesion connecting these injuries in each group. B, The cumulative scoring scale shows the total number of adhesions found in the abdominal cavity presented as mean ± SEM. C, The adhesion scores according to the Nair scale[34] are presented as mean ± SEM (scoring criteria listed in Materials and Methods). D, Percentage of animals without any adhesion formation in the abdominal cavity in each group. E, Weight change during the 6 survival days after surgery presented as percentage of initial weight. Data presented as mean ± SEM. n (control) = 20, n(1 administration of 0.5 mL PXL01 [6 mg/mL]) = 10, n(3 administrations of 0.5 mL PXL01 [2 mg/mL] in connection to the operation and 24 and 48 hours postsurgery by intraperitoneal injection) = 18, n(1 administration of 1.5% sodium hyaluronate) = 20, n(1 administration of 1 mL PXL01 [1.5 mg/mL] in 1.5% sodium hyaluronate) = 10, n(1 administration of 1.5 mL PXL01 [6 mg/mL] in 1.5% sodium hyaluronate) = 10). Statistical significance was estimated by Fisher's exact test (A, D) or by nonparametric Mann-Whitney *U* test (B, C). *$P < 0.05$; **$P < 0.01$ indicate statistical difference compared with the surgical control group of animals. Adm indicates administration; SH, sodium hyaluronate; dH_20, distilled water.

the release profiles of PXL01 from the formulated products prepared at 3 independent occasions were largely overlapping indicating that preparation of PXL01-loaded sodium hyaluronate gels is reproducible (Fig. 2).

Prevention of Peritoneal Adhesions by PXL01

The sidewall defect-cecum abrasion model in rat used in the present study produced reproducible adhesions between the 2 injured surfaces if no treatment was given, with 85% of the rats in the control group developing direct cecum-peritoneal wall adhesions (Fig. 3A). No significant reduction in adhesion formation was observed when 3 mg of PXL01 in water solution was administrated as a single dose in connection to the surgery (Figs. 3A–D). However, animals treated with 3 doses of 1 mg of PXL01 in water solution demonstrated marked reduction in adhesion formation compared with the control group of rats (Figs. 3A, C). These results indicate that slow release of PXL01 in the surgical area is beneficial, compared with the single treatment with the water solution of the peptide.

Sodium hyaluronate was chosen as carrier to achieve controlled release of PXL01. PXL01 is readily soluble and sufficiently stable in sodium hyaluronate, also the PXL01-containing sodium hyaluronate hydrogel is bioadhesive and easy to apply to the surgical area using a syringe. When PXL01 was applied in 1.5% high molecular weight sodium hyaluronate formulation, the formation of abdominal adhesions was significantly reduced, compared with the control group. Application of 6 mg/mL PXL01 in sodium hyaluronate resulted in a 4-fold reduction according to the cumulative adhesion scoring scale (Fig. 3B) and a 3.5-fold reduction of the adhesion score according to Nair (Fig. 3C). About 60% of animals treated with 6 mg/mL PXL01 in sodium hyaluronate were completely free from adhesions compared with 5% of the animals in control group and 20% of animals in the group treated with sodium hyaluronate (Fig. 3D). By several scoring scales, sodium hyaluronate per se was shown to reduce adhesion formation, presumably because of the physical barrier effect. Previous studies evaluating the effect of sodium hylaruonate in the rat models of intraperitoneal adhesion formation have reported variable results ranging from no positive effect[44,45] to significant reduction of adhesions.[46,47] Compared with the previously tested compositions, a higher concentration (1.5%) of high molecular weight fiber sodium hylaruonate was used in the present study. This is expected to result in a prolonged duration of sodium hyalruonate in the abdominal cavity and correspondingly, improved barrier effect.

No treatment-related adverse effects were recorded during the study regarding the wound healing or peritoneal inflammation assessed during necropsies. Also, the average body weight of the rats in the treatment groups was increased compared with their presurgical weights; although the difference compared with the control group did not reach statistical significance (Fig. 3E). Importantly, PXL01 in sodium hyaluronate administered around the intestinal anastomosis did not reduce the healing potential as estimated by the burst pressure of anastomosis measured 7 days after the surgery (burst pressure for the treatment group [n = 8] 206 ± 14 mm Hg vs. 197 ± 9 mm Hg in the sham group [n = 8]).

DISCUSSION

Lactoferrin, an iron binding glycoprotein present in milk, mucosal secretions, and secondary granules of neutrophiles, is an innate defense factor exhibiting antimicrobial and anti-inflammatory properties (reviewed in[48,49]). In the present study it was found that the synthetically produced peptide PXL01 derived from the N-terminal bactericidal domain of the lactoferrin molecule efficiently prevented postsurgical adhesion formation in the abdominal cavity.

The wound healing cascade begins with hemostasis and fibrin deposition, leading to an inflammatory response, followed by attraction and proliferation of fibroblasts, and finally, remodeling by collagen cross-linking.[50] Excessive accumulation of scar tissue in the form of postsurgical adhesions permanently connecting anatomic structures may result if any part of this healing sequence is altered. We demonstrated that PXL01 interferes with the intraperitoneal adhesion formation by simultaneously modulating several key processes in the wound healing cascade (Fig. 4).

Extravascular fibrin deposition is considered to be crucial in intra-abdominal adhesion formation. After peritoneal injury, a serosanguinous exudate forms at the wound site, which is quickly transformed into fibrinous adhesions, which may be lysed by plasmin. The injury of the peritoneum causes loss of its fibrinolytic activity, resulting in fibrin failing to be cleared and permanent collagen-containing adhesions between the structures are formed.[51,52] The fibrin clearance capacity is primarily regulated by

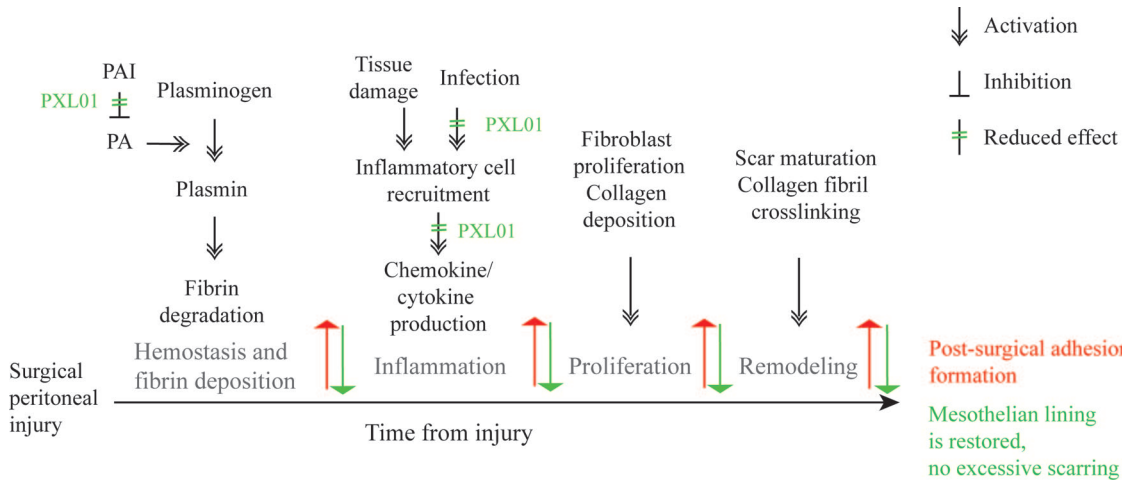

FIGURE 4. The sequence of events during the healing of surgical trauma. The formation of postsurgical adhesions versus wound healing without any excessive scarring is determined by the balance between fibrin deposition, inflammatory response, fibroblast proliferation and tissue remodelling stage, with the relative down-regulation of these processes leading to relatively less scar building. The sites of action of PXL01 are indicated. PA, plasminogen activators.

local activation of plasmin by plasminogen activators, which is counteracted by PAI produced by mesothelial lining cells of the abdominal cavity.[40–42,53] We demonstrated that PXL01 significantly reduces the expression of PAI-1, the main inhibitor of plasminogen activators, in a human mesothelial cell line, suggesting that PXL01 regulates the scarring process by locally increasing the fibrinolytic capacity in the abdominal cavity.

Peritoneal surgery may lead to inflammatory reactions, resulting in a release of chemokines and cytokines from local macrophages and recruitment of inflammatory cells. Prolonged inflammation in the peritoneal cavity wound healing cascade leads to the persistence of adhesions, whereas down-regulation of the inflammatory response is thought to be sufficient to restrict the following phases such as proliferation, maturation, and remodeling, leading to prevention of scar formation.[35,54] PXL01 reduces LPS-mediated pro-inflammatory mediator synthesis, including TNF-α, IL-1β, IL-6, and IL-8, as demonstrated in in vitro experiments using macrophages derived from a human monocytic cell line. The anti-inflammatory functions of PXL01 are expected to contribute to its ability to reduce excessive scarring (Fig. 4).

Microbial microenvironment has been shown to play an important role in adhesion formation, most probably by inducing adhesions through activation of the immune system.[33,55,56] Therefore, the ability of PXL01 to inhibit the growth of several bacterial strains may be of additional benefit in abdominal surgery.

We have used a previously validated sidewall defect-cecum abrasion model in rat[32,57] to establish whether PXL01 could act safely to prevent scar tissue formation after abdominal surgery in vivo. Tissue damage, particularly mesothelial abrasion caused by manipulative tissue contact with surgical instruments, is implicated as a primary factor in promoting postoperative adhesions in clinical situations.[58] In the rat model the injury on the peritoneal wall was combined with cecum abrasion, the injured surfaces were coated with the product, and the comprehensive adhesion grading was performed 6 days postsurgery. The time for evaluation was chosen based on previous publications demonstrating that adhesions do not continue to form after remesothelization of the abdominal wall has been completed by postsurgery day 3.[59,60] At the same time, adhesions present after 1 week are usually persistent even at 6 months after the surgery.[61,62]

No obvious adverse events such as listlessness, peritoneal inflammation, or inhibition of wound healing were observed in animals treated with PXL01 at any dose level. At the time of sacrifice all treatment groups had maintained or exceeded their presurgery weights (Fig. 3E). The ability of PXL01 to prevent adhesions was limited in water solution (Fig. 3A–D), possibly because of the fact that the peptide is rapidly eliminated from the peritoneum. However, the peptide was highly effective when formulated in sodium hyaluronate (Fig. 3A–D), causing significant reduction of adhesions according to different grading scales encompassing both the adhesions formed between the 2 injured surfaces as well as in the abdominal areas remote from the site of application. Sodium hyaluronate, a natural component of the extracellular matrix, is catabolized locally or transported to lymph nodes and later the systemic circulation, from where it is cleared by the endothelial cells of the liver.[63,64] Sodium hyaluronate is likely to enhance the effect of PXL01 by maintaining local concentrations of the drug through a controlled release. In vitro experiments indicate a relatively brief period of PXL01 release from sodium hyaluronate (Fig. 2) suggesting that the duration of the drug release required for adhesion prevention in vivo may be rather limited. This is in line with the previous evidence that the critical events in adhesion formation in the abdominal cavity occur within the first 36 hours.[32,65,66] Taken together, the sodium hyaluronate produces an area where the effects of local exposure to PXL01 and the physical barrier occur simultaneously, which results in significant and marked reduction of postsurgical adhesions. Importantly, PXL01 in sodium hyaluronate administered around the intestinal anastomosis did not interfere with the healing potential of the anastomosis.

In summary, we present a new class of synthetically derived water soluble low molecular weight peptide compound which simultaneously targets the pro-inflammatory mediators, bacterial infections, and excessive fibrinogenesis after the surgical trauma, resulting in significant reduction in scar formation. PXL01 formulated in sodium hyaluronate gel is easy to handle and administer and is compatible with either laparatomy or laparoscopy. On the basis of these properties of the product, it is expected to obtain a comprehensive adhesion prevention regimen within the abdominal cavity and preventing not only the adhesions which form at sites of operative procedures, but also adhesions that may form at sites not directly involved in surgery because of unintentional tissue injury during surgical manipulation.

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