A neurokinin 1 receptor antagonist decreases postoperative peritoneal adhesion formation and increases peritoneal fibrinolytic activity

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Contributed by Susan E. Leeman, May 7, 2004

Fibrous adhesions remain a major sequela of abdominal surgery. The proinflammatory peptide substance P (SP), known to participate in inflammatory events, may play a key role in adhesion formation. This hypothesis was tested by using an antagonist, CJ-12,255 (Pfizer), that blocks the binding of SP to the neurokinin 1 receptor (NK-1R). Adhesion formation was surgically induced in the peritoneum of rats receiving daily doses of the NK-1R antagonist (NK-1RA; 5.0 or 10.0 mg/kg per day) or saline. On postoperative day 7, both low and high doses of NK-1RA significantly (P < 0.05) reduced adhesion formation by 45% and 53%, respectively, compared with controls. Subsequently, the effect of NK-1RA administration on peritoneal fibrinolytic activity was investigated to determine a potential mechanism for SP action in the peritoneum. Samples were collected from nonoperated controls and from animals 24 h post-surgery that were administered either NK-1RA or saline. Fibrinolytic activity in peritoneal fluid was assayed by zymography, and expression of tissue plasminogen activator (tPA) and plasminogen activator inhibitor 1, both regulators of fibrinolytic activity, was assessed in peritoneal tissue and fluid by RT-PCR and bioassay, respectively. NK-1RA administration led to a marked (P < 0.05) increase in tPA mRNA levels in peritoneal tissue compared with nonoperated and saline-administered animals. Likewise, NK-1RA administration significantly (P < 0.05) increased tPA in the peritoneal fluid. These data suggest that activation of the NK-1R promotes peritoneal adhesion formation by limiting fibrinolytic activity in the postoperative peritoneum, thus enabling fibrous adhesions to persist.

Intraperitoneal adhesions are a costly, long-term sequela associated with abdominal surgery (1) and lead to increased postoperative morbidity, including small-bowel obstruction, difficult reoperative surgeries, infertility, and chronic pelvic pain (2–4). Several approaches have been used to prevent adhesions, including antiinflammatory agents (5), antibiotics (6), and both chemical (7, 8) and physical (9, 10) barriers. Unfortunately, none of these measures has proven uniformly effective under all surgical conditions. An understanding of the molecular and cellular mechanisms underlying the pathophysiology of adhesion formation should expedite the development of safe and efficient methods of adhesion prevention.

Studies in humans and in animal models have shown that surgical trauma initiates an inflammatory reaction that leads to deposition of a fibrin-rich matrix on peritoneal surfaces that is capable of forming attachments to adjacent viscera (11, 12). If the matrix organizes into fibroblast-containing fibrin bands, permanent adhesions can form as early as 7 days after abdominal operations (13). However, normal restitution of peritoneal surfaces will occur if the fibrinous exudate is degraded within 2–3 days of surgery (14). Fibrin is degraded primarily by plasmin, a protease converted from inactive plasminogen by two plasminogen activators (PA), tissue-type PA (tPA) and urokinase-type PA (uPA) (15). The primary PA in the peritoneum is tPA, which is inhibited when a 1:1 complex is formed with PA inhibitor (PAI)-1 (16). Surgical trauma has been shown to impair peritoneal fibrinolytic activity through reduction in PA activity and/or increases in PAI-1 activity (15, 17). The importance of tPA and PAI-1 in adhesion formation is underscored by studies showing that patients with the most severe adhesions overexpress PAI-1 and have decreased tPA activity (18). Furthermore, tPA gene knockout mice are more susceptible to adhesion formation after surgery than are uPA-deficient or wild-type mice (19). The molecular and biochemical mechanisms underlying the rapid changes in fibrinolytic activity after surgery have not been elucidated.

Peritoneal fibrinolytic activity and postoperative adhesion formation may be regulated, in part, by the proinflammatory peptide substance P (SP) (20, 21). SP is known to play an important role in inflammatory (22, 23), proliferative (24), and wound healing (25) processes. Known SP effects range from increasing inflammatory cytokine mRNA expression and secretion (26) to stimulating angiogenesis (27) and proliferation of fibroblasts (28). Neurons are the primary source of SP, but lymphocytes (29), monocytes (30), macrophages (31), and eosinophils (31) have been identified as additional sources. SP is present in peritoneal fluid (32), and SP-containing sensory neurons have been found in peritoneal adhesions (33).

SP belongs to the tachykinin family of peptides that also includes neurokinins A and B, hemokinin 1, and endokinins A and B (21, 34, 35). The broad range of biological effects associated with SP and the other tachykinins is mediated by means of binding to one of three neurokinin (NK) receptors [NK-1 receptor (NK-1R), NK-2 receptor, or NK-3 receptor] (36). SP has the highest selectivity for the NK-1R (21); however, the other tachykinins are also capable of functionally binding to the NK-1R (21, 34, 35).

Previous results from this laboratory show that mRNA levels for SP and NK-1R significantly increase in peritoneal adhesion tissue by 3 days after surgery to induce adhesion formation (37). To test the hypothesis that activation of the NK-1R promotes peritoneal adhesion formation, we determined the effects of a highly specific NK-1R antagonist (NK-1RA), CJ-12,255 (Pfizer), on adhesion development in a rat model. Potential mechanisms underlying the NK-1RA effects on adhesion formation were subsequently investigated. Specifically, the effects of NK-1RA administration on peritoneal fibrinolytic activity and tPA mRNA expression levels were measured in peritoneal fluid and tissue, respectively. The results indicate that agonists of the NK-1R may promote postoperative adhesion formation by decreasing peritoneal fibrinolytic activity, a finding that may lead to therapeutic methods of adhesion prevention.

Abbreviations: PA, plasminogen activator; tPA, tissue PA; uPA, urokinase-type PA; PAI, PA inhibitor; SP, substance P; NK, neurokinin; NK-1R, NK-1 receptor; NK-1RA, NK-1R antagonist.

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Materials and Methods

Materials. All chemicals were obtained from Sigma unless otherwise noted. The highly specific, non-peptide, NK-1RA (3R,4S,5S,6S)-6-diphenylmethyl-5-(5-isopropyl-2-methoxybenzylamino)-1-azabicyclo[2.2.2]octane-3-carboxylic acid (CJ-12,255) was used in this study. This antagonist is a structurally related analog of the parent compound CJ-11,974 (Ezlopitant, Pfizer), which has been shown to be highly specific for the NK-1R with no affinity for the NK-2 or NK-3 receptors (38). Furthermore, CJ-12,255 has been shown to completely block an SP-induced salivary response in rats (39).

Animals. Male Wistar rats (200–250 g; Charles River Breeding Laboratories) were used for all experiments. The animals were housed at a constant room temperature, with 12-h light and dark cycles, and were provided standard rodent chow (Purina, catalog no. 5001) and water ad libitum. The Institutional Animal Care and Use Committee at Boston University School of Medicine approved these studies, and all procedures and animal care were performed in accordance with recommendations outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Assessment of NK-1RA on Peritoneal Adhesion Formation. Peritoneal adhesions were created in adult Wistar rats as described in ref. 37. Briefly, a laparotomy was performed through a midline incision, and four ischemic buttons, spaced 1 cm apart, were created on both sides of the parietal peritoneum by grasping 5 mm of peritoneum with a hemostat and ligating the base of the segment with 4-0 silk suture. To assess the effects of NK-1RA on adhesion formation, peritoneal adhesions were induced in 42 animals that were randomized to experimental groups receiving the specific non-peptide NK-1RA, CJ-12,255, or vehicle (sterile saline). In the initial study, animals in the experimental group (n = 21) received 0.2-ml i.p. injections of 2.5 mg/kg twice a day for 2 days before the surgical procedure. At the time of surgery, one intraoperative bolus dose of antagonist (1 ml, 0.75 mg/ml) was given as a peritoneal lavage, and the animals then received i.p. injections for the 7 days of the study. Control animals (n = 21) were similarly injected/lavaged with sterile saline. This experiment was repeated with 10 mg/kg NK-1RA per day administered by an Alzet osmotic pump (Durect, Cupertino, CA) that continuously delivered 1 ml/h NK-1RA (∼125 mg/ml) or saline (n = 6 animals per group). All animals were killed at 7 days, and the adhesions were quantified in a blinded fashion. Each animal received a percent adhesion score based on the number of ischemic buttons with attached adhesions.

Assessment of NK-1RA on Peritoneal tPA and PAI-1 Expression and Activity. The temporal expression pattern of tPA and PAI-1 mRNA in peritoneal tissue collected from within a 0.5-cm radius of the ischemic buttons was determined by RT-PCR analysis at 0 (nonoperated controls), 1, 3, and 7 days after surgery (n = 6 per time point). Based on the results of this experiment, the effects of NK-1RA administration on tPA and PAI-1 mRNA and protein levels were determined at postoperative day 1 in peritoneal tissue and fluid by RT-PCR analysis and bioassay, respectively. Animals received 5.0 mg/kg NK-1RA or saline (n = 6 per group) per day as described previously. Control samples were also collected from six nonoperated animals. All samples were immediately frozen in liquid nitrogen and stored at −80°C until use.

RNA Isolation and Relative RT-PCR. Total RNA was isolated from peritoneal tissue (50 mg) with the SV Total RNA Isolation System (Promega), and RT-PCR was conducted with the GeneAmp RNA PCR System (Applied Biosystems) as described in ref. 37. The following primer sets were used to amplify tPA and PAI-1 (28 cycles of 95°C, 60°C, and 72°C for 30 s each): tPA, 5′-TCTGACCTTCGTCTGCACTG-3′ (sense) and 5′-AGAG-GCCCTTGAGTTGTTGAAA-3′ (antisense); PAI-1, 5′-AT-CAACGACTGGGGTGAGAGAC′ (sense) and 5′-AGCGGC- GTACTTGTCCTCTT-3′ (antisense). PCR products (15 μl) were subjected to electrophoresis on 2% agarose gels containing 0.03 μg/ml ethidium bromide, and quantitation of transcript level was carried out by analysis of scanned photographs of gels with imaging software (Scion, Frederick, MD) (40). Levels of mRNA expression were normalized to GAPDH, a constitutively expressed gene that did not vary among treatment groups.

Total tPA and PAI-1 Levels in Peritoneal Fluid. The Coaset t-PA kit (DiaPharma, Columbus, OH) and the Zymutest Rat-PAI-1 Activity kit (DiaPharma) were used according to the manufacturer’s instructions to measure total levels of tPA and PAI-1 in peritoneal fluid samples.

Fibrinolytic Activity in Peritoneal Fluid. In a subsequent experiment, tPA fibrinolytic fluid was collected in 5 mM citrate and 0.1 M acetate (final concentration) from 12 saline-administered, 12 NK-1RA-administered (10.0 mg/kg per day), and 12 nonoperated control animals for assessment of fibrinolytic activity caused by tPA activation of plasminogen (Athens Research and Technology, Athens, GA). Peritoneal fluid samples (1.0 ml) were run on 10% SDS polyacrylamide gels containing 0.1% gelatin and 0.002% plasminogen. After electrophoresis, gels were washed twice in 2% Triton X-100 and incubated overnight at 37°C in 0.1 M glycine, pH 8.3. Gels were then stained with a 0.25% Coomassie blue solution, and PA activity was visualized as clear bands produced by plasmin lysis of gelatin. For some experiments, 10 mM serine protease inhibitor PMSF was added to the developing buffer to determine the contribution of serine proteases. To identify the zones of lysis corresponding to tPA activity, tPA and/or uPA were immunoprecipitated from peritoneal fluid samples. Peritoneal fluid (10 μl) was diluted with an equal volume of buffer (40 mM phosphate, pH 7.5/1 M NaCl/0.2% SDS/2% Igepal CA-630/1% deoxycholate), and then 1 μg of tPA and/or uPA antibody (American Diagnostica, Greenwich, CT) was added and incubated at 4°C overnight. The next day, 10 μl of a 50% UltraLink protein A/G slurry (Pierce) was added to each sample and incubated overnight at 4°C. Samples were centrifuged at 16,000 × g for 1 min at 4°C, and the supernatant (50%) was analyzed by zymography for comparison to human recombinant tPA (0.01 IU; DiaPharma) and uPA (0.0002 units; Calbiochem) standards. The zone of lysis corresponding to tPA was quantified by using Scion imaging software.

Statistical Analysis. Data were analyzed by one-way ANOVA with SIGMASTAT software (SPSS, Chicago). When significant effects (P < 0.05) were detected, the differences between specific means were determined with the Student–Newman–Keuls test. Groups were deemed to be significantly different from one another when P < 0.05.

Results

NK-1RA Administration Reduces Adhesion Formation. On postoperative day 7, 72.2 ± 4.7% of the ischemic buttons created in the saline controls had formed adhesions that attached to various structures in the peritoneum including the small intestine and liver (Fig. 1A). Adhesion formation was significantly (P < 0.05) reduced by 45% and 53% when SP action was blocked by administration of the NK-1RA (Fig. 1) at 5.0 and 10.0 mg/kg per day, respectively (5.0 mg/kg per day, 39.9 ± 5.6%; 10.0 mg/kg per day, 31.0 ± 9.55%). Although the few quantifiable adhesions that did form in the NK-1RA-administered animals were less dense and filmer than those in the saline control animals.
NK-1RA Administration Increases tPA mRNA Expression Levels in Peritoneal Tissue. Because peritoneal fibrinolytic activity is an important determinant of adhesion outcome, the effects of NK-1RA administration on the relative mRNA expression levels of tPA and PAI-1 in peritoneal tissue at postoperative days 0, 1, 3, and 7 were assessed by semiquantitative RT-PCR (Fig. 2A). The tPA mRNA expression levels at day 1 were not different from day 0 control levels; however, on days 3 and 7 tPA mRNA levels were significantly increased \((P < 0.05)\) compared with control. PAI-1 mRNA levels increased significantly \((P < 0.05)\) at 1 day compared with controls and remained elevated at days 3 and 7. Based on these results, the effects of NK-1RA administration on tPA and PAI-1 mRNA levels were assessed 24 h post surgery. Administration of the NK-1RA caused a >2-fold \((P < 0.05)\) increase in tPA mRNA levels compared with both nonoperated controls and saline-administered animals (Fig. 2B) but had no effect on the postoperative increase in PAI-1 mRNA levels (Fig. 2C).

**NK-1RA Administration Increases tPA Activity in Peritoneal Fluid.** The levels of tPA and PAI-1 protein in peritoneal fluid on postoperative day 1 were measured with commercially available bioassay kits (Fig. 3). Total tPA levels significantly \((P < 0.05)\) increased in peritoneal fluid from the saline-administered (2.2-fold) and NK-1RA-administered (4.3-fold) animals compared

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Fig. 1. NK-1RA administration reduces postoperative adhesion formation. (A) By postoperative day 7, saline-administered animals had developed substantial adhesions (Upper). In comparison, adhesion formation was reduced in animals administered 5.0 mg/kg NK-1RA per day (Lower). Arrows indicate ischemic button sites. (B) Postoperative adhesions were significantly \((P < 0.05)\) reduced in rats administered NK-1RA CJ-12,255 at 5.0 mg/kg per day \((n = 21)\) and at 10.0 mg/kg per day \((n = 6)\) when compared with saline-administered control animals \((n = 27)\). The data shown are the means \(\pm\) SEM of percent adhesion formation. * \(P < 0.05\) when compared with controls.

Fig. 2. NK-1RA administration increases tPA mRNA expression levels in peritoneal tissue on postoperative day 1. (A) The relative mRNA expression levels of PAI-1 (open circles) and tPA (filled squares) in peritoneal tissue collected on postoperative days 1, 3, and 7 \((n = 6\) per time point) were determined by semiquantitative RT-PCR. Because PAI-1 mRNA levels increased on day 1 but tPA mRNA levels did not, the effects of NK-1RA administration \((5.0\) mg/kg per day) on tPA \((B)\) and PAI-1 \((C)\) mRNA expression were determined in postoperative day 1 peritoneal tissue \((n = 6\) per group). All data were normalized to GAPDH values and are expressed as percent of nonoperated controls. Data shown are the means \(\pm\) SEM. * \(P < 0.05\) when compared with nonoperated controls.
with nonoperated controls (Fig. 3A). The increase in tPA levels in the NK-1RA-administered animals was significantly \( P < 0.05 \) higher than in the saline-administered animals. PAI-1 levels were significantly \( P < 0.05 \) increased (up to 17-fold) in operated animals compared with nonoperated controls, but no significant effect of NK-1RA administration, compared with saline, was observed (Fig. 3B).

To determine whether the NK-1RA-induced increases in tPA mRNA and protein levels corresponded to biologically relevant changes in tPA activity, fibrinolytic activity in the peritoneal fluid was assessed by gelatin–plasminogen zymography. Several zones of lysis were evident on the zymographic gels (Fig. 4A) and were determined to be serine proteases by the fact that all lysis was blocked by addition of the serine protease inhibitor PMSF (data not shown). Fibrinolytic activity caused by tPA was significantly \( P < 0.05 \) increased in NK-1RA-administered animals when compared with nonoperated controls and saline-administered animals (Fig. 4B). The activity of tPA increased by 1.6-fold in saline-administered animals and by 2.1-fold in NK-1RA-administered animals compared with controls. Similar to results obtained with the bioassay kit, the increase in tPA levels in the NK-1RA-administered animals was significantly \( P < 0.05 \) higher than in the saline-administered animals. Verification that the 55-kDa lytic zone was caused by tPA activity was accomplished by size comparison to recombinant human tPA and uPA standards (C). Molecular mass markers (kDa) are indicated beside A and C. PF, peritoneal fluid; IP, immunoprecipitation.
human tPA and uPA standards and by immunoprecipitation of tPA and uPA from peritoneal fluid samples (Fig. 4C). The 55-kDa band of lysis was determined to be caused by tPA activity based on its comigration with the recombinant human tPA standard and by the fact that this band was abolished by tPA, but not uPA, immunoprecipitation.

Discussion

The data presented in this study demonstrate that interfering with the NK-1R significantly decreases postoperative adhesion formation in a rat model, and that this effect may be mediated, in part, by increasing peritoneal fibrinolytic activity. These data provide the first evidence that activation of the NK-1R by tachykinins such as SP is involved in adhesion formation. The participation of the NK-1R in promoting adhesions is not surprising, because binding of an agonist such as SP to the NK-1R can initiate a wide range of potentially adhesogenic effects, including production of inflammatory cytokines (41), stimulation of fibrosis (24), increased chemotaxis of neutrophils and macrophages (42, 43), and mitogenesis of fibroblasts (28).

Adhesion formation begins with trauma to the peritoneum, which elicits an inflammatory response, and results in the deposition of a fibrin-rich exudate. Newly formed fibrinous material in the peritoneum that overwhelms the local fibrinolytic potential, allowing adhesions to develop to an irreversible state. Interestingly, we did not see an increase in tPA mRNA levels in the saline-administered animals on postoperative day 1, a result also reported by Rout and Diamond (50). Therefore, the early postoperative increase in peritoneal tPA that we and others have observed may be caused by tPA release from intracellular stores.

In this study, administration of the NK-1RA CJ-12,255 increased peritoneal tissue tPA mRNA levels and peritoneal fluid tPA levels and activity on postoperative day 1, with no effect on the inhibitor of tPA, PAI-1. The NK-1RA effects on fibrinolytic activity are evident only after the surgical procedure to induce adhesions because administration of NK-1RA to nonoperated animals did not alter peritoneal tPA or PAI-1 expression or activity (data not shown). These data using the NK-1RA suggest that activation of the NK-1R promotes adhesion formation and that the mechanism of action may be by inhibiting synthesis and/or activity of tPA.

It is unclear from these studies to what extent SP, as opposed to other tachykinins, contributes to activation of the NK-1R in the postoperative peritoneum with subsequent effects on tPA expression and activity. SP has been shown, in other systems, to modulate expression of tPA and PAI-1. SP administration increased total PA and PAI protein and mRNA levels in synovial tissues of rabbits (51), and, in humans, i.v. injection of SP caused a dose-dependent increase in plasma tPA activity with no effect on plasma PAI levels (52). The fact that our data suggest that activation of the NK-1R reduces peritoneal tPA activity may be explained by tissue-specific differences. Regulation of tPA expression has been shown to differ between cell types. For example, tumor necrosis factor α stimulation increases tPA expression in fibroblast cells (53), whereas it decreases expression in mesothelial (54, 55) and endothelial (56) cells.

Other potential NK-1R agonists include hemokinin 1 and endokinins A and B. All three functionally bind the NK-1R with affinity that is similar to but less than that of SP (35). Hemokinin 1 is expressed outside of neuronal tissue predominantly in immune cells (34), and Weinstock (57) has recently reported that hemokinin 1 is produced at sites of inflammation in the gut lumen. Endokinins A and B are also expressed outside of neuronal tissue with high levels in the adrenal gland. Page et al. (35) determined that the hemodynamic response of rats to SP is closely mimicked by endokinins A and B. Together, these results led the authors to propose that hemokinin 1 and endokinins A and B are SP-like agonists that act in the periphery where SP is not present (35). It remains to be determined which NK-1R agonist is most relevant to adhesion formation.

In conclusion, these data demonstrate that administration of a NK-1RA can decrease postoperative adhesion formation and that a possible mechanism of action may be by increasing peritoneal fibrinolytic activity through increased expression and activity of tPA. The data presented in this study lead to a better understanding of the complex biochemical pathways of adhesionogenesis and suggest new therapeutic targets for adhesion prevention.
