An FDA Approved Neurokinin-1 Receptor Antagonist is Effective in Reducing Intraabdominal Adhesions when Administered Intraperitoneally, But Not Orally

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Abstract

Introduction Postoperative adhesions pose a continued healthcare problem. We previously demonstrated that intraperitoneal (IP) administration of a neurokinin-1 receptor antagonist (NK-1RA) at surgery reduces intraabdominal adhesions in rats. The NK-1RA aprepitant (Emend™, Merck) is clinically approved for preventing postoperative nausea and vomiting; however, its effects on adhesion formation are unknown. Thus, we determined the effects of IP and oral administration of aprepitant on adhesion formation in a rat model.
Methods Adhesions were surgically induced in rats that were randomized to receive either one or five oral preoperative doses or a single intraoperative IP dose of aprepitant (50 mg/kg). Adhesions were scored at 7 days. In similar experiments using IP dosing, animals were sacrificed at 24 h and peritoneal fluid, and tissue were collected to assess fibrinolytic activity and tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1) mRNA levels, respectively.
Results IP aprepitant reduced adhesion formation by 33% ($p<0.05$) compared with controls while oral aprepitant had no effect. Compared to controls IP aprepitant reduced tPA activity by 55% ($p<0.05$), increased PAI-1 mRNA levels by 140% ($p<0.05$), and had no affect on tPA mRNA levels.
Conclusion These data suggest that aprepitant maybe a useful pharmacologic agent for reducing adhesion formation clinically.

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Introduction

Postoperative adhesions comprise a source of morbidity for patients undergoing intraabdominal surgery. Some of the complications caused by intraabdominal adhesions include pain, infertility in women,[1] small bowel obstruction,[2,3] bowel ischemia, and possibly death. The end result is increased hospital visits and increased cost of care for the postsurgical patient.[4–6]

Postoperative adhesions result from the injury and inflammation to the peritoneal lining of the abdominal cavity. Examples of peritoneal injury caused by surgery include cutting, crush, cautery, infection, ischemia, and rough handling of the peritoneal surfaces.[7] Areas of subperitoneal extracellular matrix are denuded followed by platelet adhesion and degranulation.[8] Inflammatory mediators such as transforming growth factor-β-1, prostaglandins, leukocyte chemotaxins, and fibrinogen are released and aggregate in

the wound,[8] leading to the formation of fibrin bands. When these fibrin bands form between two opposing surfaces, an adhesion may form. The fibrin bands can act as scaffolding for inflammatory cell in growth.[7] The wound is first populated by neutrophils, followed by macrophages, angioblasts, and fibroblasts. Fibroblasts deposit collagen into the fibrin band leading to permanent adhesion formation by 7 days following surgery.[7,8]

Under normal circumstances, the fibrin bands are degraded by plasmin prior to recruitment of inflammatory cells. However, under postoperative conditions, plasminogen activator activity is decreased and the fibrin bands persist and progress to adhesion maturation.[7] Peritoneal fibrinolytic activity is closely associated with levels of tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1).[9] tPA functions by activating plasmin while PAI-1 acts by directly inhibiting tPA. Previous studies have demonstrated that modulation of tPA activity and PAI-1 levels can affect adhesion formation; downregulation of the fibrinolytic system results in increased adhesions; while upregulation results in decreased adhesions.[10–12]

Many methods for preventing postoperative intraabdominal adhesions have been investigated in the past, such as antibiotics, anti-inflammatory medications, and physical barriers.[13–16] To date, no pharmacologic compounds have been approved for use in preventing adhesions. Currently approved anti-adhesion methods are limited to physical barriers only. By physically separating two inflamed surfaces, adhesion formation can be reduced. However, the anti-adhesion activity of barrier-based methods is likely limited to where they are placed, potentially leaving adhesiogenic surfaces. Placement of a solid barrier is also limited to open abdominal procedures. A pharmacologic method may allow for treatment of all adhesiogenic surfaces as well as allow adhesion prevention during laparoscopic operations.

One class of pharmacologic compounds previously investigated by this laboratory is the neurokinin-1 receptor antagonists (NK-1RA). The neurokinin-1 receptor (NK-1R) is a high affinity receptor that is activated by binding tachykinins of which the proinflammatory neuropeptide substance-P is the best studied. Our laboratory has demonstrated that both substance-P and NK-1R are upregulated during peritoneal injury.[17] We also showed that intraperitoneal administration of an NK-1RA increases peritoneal tPA activity and decreases adhesion formation.[18] This drug family shows promise for decreasing intraabdominal adhesions.

Currently, the only FDA-approved NK-1RA is aprepitant (Emend™, Merck, Rahway, NJ, USA), which is in use for postoperative and chemotherapy-related nausea and vomiting. The perioperative usage of this drug and its pharmacologic activity create a unique opportunity for study of its potential anti-adhesion effects. Our goal was to evaluate the effect of orally or intraperitoneally administered aprepitant on the reduction of abdominal adhesion formation in a rat model.

Methods

Materials All chemicals were obtained from Sigma (St. Louis, MO, USA) unless otherwise noted. The highly specific, nonpeptide NK-1RA 5-[[(2R,3S)-2-[(1R)-1-[3,5-bis(trifluoromethyl)phenyl]ethoxy]-3-(4-fluorophenyl)-4-morpholinyl] methyl]-1,2-dihydro-3H-1,2,4-triazol-3-one (aprepitant) was used in this study (Merck, Rahway, NJ, USA). This antagonist is highly specific for the NK-1R and has no affinity for the NK-2 or NK-3 receptors.[19] Sterile 100% dimethyl sulfoxide (DMSO) was used to dissolve the pure aprepitant for intraperitoneal delivery while a solution of 2% carboxymethylcellulose (CMC) was used for delivery of the oral formulation (Emend™) as slurry.

Animals Male Wistar rats weighing 175–200 g (Charles River Labs, Wilmington, MA, USA) were used for all experiments. Rats were housed at a constant room temperature of 25°C, with 12-h light and dark cycles, and were provided standard rodent chow (Purina, 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.

Sialogogic assay A sialogogic assay was performed as described by Leeman et al. to determine whether oral and intraperitoneal administration of aprepitant effectively blocks NK-1R activity in rats.[20] Rats were gavaged or intraperitoneally injected with aprepitant (50 mg/kg) or vehicle alone ($N=3$ per group). Animals were anesthetized and intravenously injected with SP (0.1 μg/100g) 3 h after oral gavage or 30 min after intraperitoneal injection. Saliva from the sublingual salivary gland was collected immediately with a Pasteur pipette and weighed to determine volume.

Induction of intraabdominal adhesions Intraabdominal adhesions were induced in rats using our previously described model.[18] Briefly, animals were anesthetized using continuous isoflurane 2–4% in 100% oxygen. A midline laparotomy was performed, and three ischemic buttons were created along the paracolic gutters bilaterally, spaced 1 cm apart. The abdomen and skin were closed using braided absorbable sutures and clips, respectively. Animals received a subcutaneous dose of buprenorphine (0.1 mg/kg

body weight) at the time of operation, and then every 12 h as needed for up to 72 h postoperation.

Experimental design The first group of rats received an intraoperative 1 ml peritoneal lavage with either aprepitant (50 mg/kg, $N=14$), or vehicle control (100% DMSO, $N=12$), followed by gentle abdominal massage to distribute the solutions throughout the peritoneal cavity. A second group received oral gavages of 1 ml (50 mg/kg) of aprepitant suspended in 2% CMC or the vehicle alone. Two dosing schedules were used: one dose given 3 h preoperatively (aprepitant $N=8$, vehicle $N=7$), or five doses given preoperatively every 12 h over 60 h (aprepitant $N=13$, vehicle $N=14$). Since a single oral dose of aprepitant effectively blocked the sialogogic response suggesting that this dose may be globally effective in the rat, we chose to determine if one oral dose of aprepitant was effective in reducing adhesion formation. However, given the fact that the oral formulation of aprepitant is less than 60% bioavailable, we decided to significantly increase the number of doses of aprepitant the rats received to five preoperative oral doses. To determine the effect of aprepitant on the intraperitoneal fibrinolytic system and gene expression, the experimental design described above for intraperitoneal administration was used with the addition of a nonoperative group receiving only an intraperitoneal injection of aprepitant or DMSO. For this second set of experiments, the rats were anesthetized with a ketamine–xylazine solution (75 and 5 mg/ml) at 24 h after surgery at which time a second laparotomy was performed to collect peritoneal fluid and ischemic button tissue for analysis of tPA activity and mRNA expression levels, respectively. Rats were then euthanized by a combination of pneumothorax and cardiac puncture. The peritoneal fluid was collected by rinsing the peritoneal cavity with 2 ml of 37°C phosphate buffer containing heparin (1 IU/ml). Approximately 1 ml of the fluid was recovered and added to an equal volume of 0.2 M sodium acetate, pH 3.9, and cellular debris was removed by microcentrifugation (2,000 rpm × 1 min). The resulting supernatants were immediately frozen in liquid nitrogen and stored at −80°C until assayed for tPA activity. Ischemic button tissue was removed within a 1-mm rim of surrounding tissue and frozen in liquid nitrogen and stored at −80°C until ready for reverse transcription polymerase chain reaction (RT-PCR) analysis.

Evaluation of fibrinolytic activity in peritoneal fluid Acetate-treated peritoneal fluid samples were acidified with 0.2 volumes 0.375 N HCl and then diluted tenfold with distilled water. The fibrinolytic activity due to tPA in each sample was assayed in duplicate by adding 50 μl of the diluted sample to wells of a 96-well microtiter plate containing 50 μl of tPA stimulator (0.6 mg/ml cyanogen bromide digested fibrinogen, American Diagnostica, Stamford, CT, USA). Next, 150 μl of assay buffer [16.7 μg/ml human plasminogen (Athens Research and Technologies, Athens, GA, USA), 667 μM S-2251 substrate (American Diagnostica, Inc.), and 20 mM Tris, pH 8.3] was added to each well and gently mixed. Cleavage of the S-2251 substrate by tPA-activated plasmin produces a yellow color that absorbs at 405 and 490 nm (calibration blank). The change in absorbance was measured at 37°C over a 6-h period with a Spectra Max 250 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The activity of tPA in each sample was determined by extrapolation from a tPA (human; Calbiochem, San Diego, CA, USA) standard curve. In a separate control experiment designed to determine if the aprepitant or DMSO interfered with tPA activity assays, the above protocol was followed with the addition of a known concentration of pure tPA (2.5 U/ml) to peritoneal fluid samples before the addition of the S-2251 substrate.

RNA isolation and quantitative RT-PCR Ischemic buttons were homogenized by pulverization at –70°C on a liquid nitrogen-cooled plate. Total RNA was isolated from peritoneal ischemic button tissue (50 mg) with the SV Total RNA Isolation System (Promega), and RT-PCR was conducted with the Gene-AMP RNA PCR system (Applied Biosystems), as described by Reed.[17] The following primer sets were used to amplify tPA and PAI-1 (28 cycles of 95°C, 60°C, and 70°C for 30 s each): tPA, 5′TCTGACTTCGTCTGCCAGTG-3′ (sense) and 5′-GAGGCCTTGGAT-GTGGTAAA-3′ (antisense); PAI-1, 5′-ATCAACGACTGGGTGGAGAG-3′ (sense) and 5′-AGCCTGGTCATGTTGCTCTT-3′ (antisense). Real time PCR was performed on an Applied Biosystems 7000 Sequence Detection System machine using SYBR green. Levels of mRNA were normalized to glyceraldehyde 3-phosphate dehydrogenase, a constitutively expressed gene that did not vary among treatment groups.

Statistical analysis Data were analyzed with the Sigma Stat program (SPSS, Inc., Chicago, IL, USA) with one-way analysis of variance (ANOVA). When significant effects were detected ($p < 0.05$), the difference between specific means was determined by the Student–Newman–Keuls test. If a test of normality failed, Dunn's test of ANOVA by ranks was used. Differences were considered to be statistically significant if $p < 0.05$. All data are expressed as mean ± SEM.

Results

Intraperitoneal administration of aprepitant during surgery reduced intraabdominal adhesion formation The sialogogic assay demonstrated that aprepitant effectively blocked the
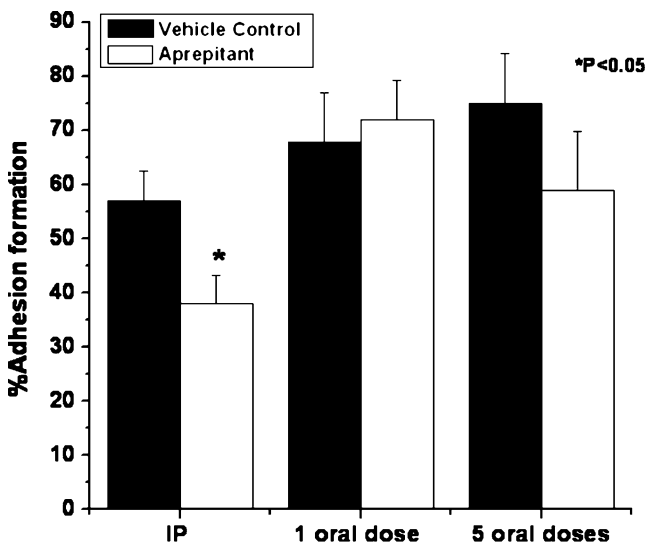
Figure 1 Intraperitoneal (*IP*) administration of aprepitant (50 mg/kg, $N=14$) at the time of operation shows decreased adhesion formation 7 days later compared with controls ($N=12$). Oral dosing with a single 3-h preoperative dose of aprepitant (50mg/kg; $N=8$) shows no significant difference in adhesion formation at 7 days postoperation when compared to vehicle control (2% CMC; $N=7$). Five oral doses of aprepitant (50 mg/kg; $N=13$), starting 2 days preoperation given every 12 h, shows no significant difference at postoperative day 7 when compared to vehicle control (2% CMC; $N=14$). Data are shown as mean ± SEM.

NK-1R by both enteral and intraperitoneal routes. Rats administered oral aprepitant showed a significant decrease in saliva production ($p<0.05$) compared to the vehicle (0.06±0.01 vs. 0.0003±0.0002 ml/200 gm body weight, respectively). Rats that received intraperitoneal aprepitant also showed a significant decrease in saliva production ($p<0.05$) compared to vehicle controls (0.11±0.03 vs. 0.02± 0.001 ml/200 gm body weight, respectively).

To test the anti-adhesion effects of aprepitant, the drug was first delivered locally (IP) at the time of surgery. Intraperitoneal administration of aprepitant decreased intra-abdominal adhesion formation significantly ($p<0.05$) at postoperative day 7. Rats given the NK-1RA had a mean intraabdominal adhesion formation score of 38.1±5.1% compared to 56.9±5.6% in the vehicle control group (Fig. 1). Neither schedule of oral aprepitant showed a significant effect on adhesion formation at postoperative day 7. Rats receiving a single dose 3 h prior to surgery had mean adhesion scores of 72±7% (aprepitant) and 68±9% (vehicle control), while rats receiving five doses preoperatively, once every 12 h over 60 h, had mean adhesion scores of 59%±11 (aprepitant) and 75±9% (vehicle control; Fig. 1).

Intraperitoneal administration of aprepitant during surgery decreased tPA activity Total tPA activity at 24 h post-operation was significantly decreased ($p<0.05$) in peritoneal

fluid from rats treated with a single intraoperative dose of aprepitant compared to vehicle controls. There was no effect on tPA activity when aprepitant was injected into non-operated animals 24 h prior to sacrifice (Fig. 2). In order to evaluate for potential interference of the aprepitant or DMSO with the tPA activity assay, 2.5 U/ml of tPA was added to peritoneal fluid from non-operated, aprepitant, and DMSO-treated animals. The assays were performed as described above. The addition of 2.5 U/ml increased tPA activity as expected, suggesting that aprepitant and DMSO do not inhibit the tPA assay (Table 1).

Intraperitoneal administration of aprepitant increased PAI-1 mRNA expression but and had no effect on tPA PAI-1 mRNA expression levels were 133% greater ($p<0.05$) in peritoneal adhesion tissue from rats treated with a single intraoperative dose of aprepitant compared to vehicle controls at 24 h postoperation. There was no difference in tPA mRNA expression levels in these same tissue samples (Fig. 3).

Discussion

This study demonstrates that intraperitoneal administration of aprepitant is effective in reducing adhesion formation while oral administration is not. One possible explanation for this observation is that despite the fact that orally administered aprepitant antagonizes the NK-1R, as demon-
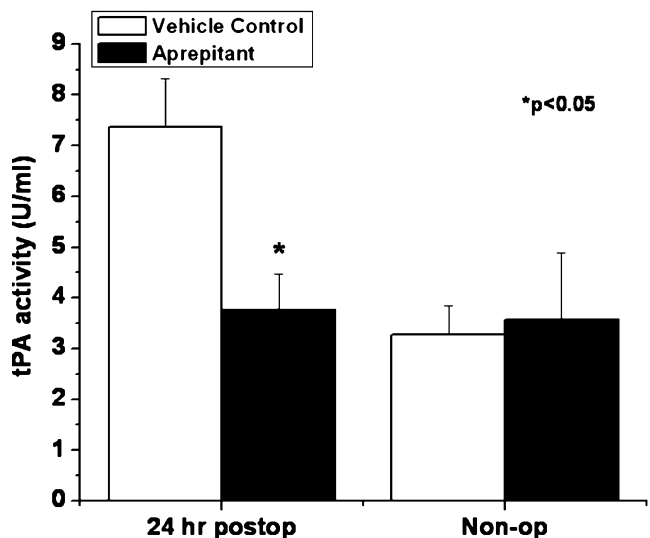

Figure 2 Twenty-four-hour postoperative (*postop*) tPA activity decreases in rats receiving IP aprepitant compared with vehicle controls (DMSO; $N=6$ per group). Non-operative (*nonop*) rats receiving IP injections of aprepitant or vehicle DMSO ($N=4$ per group) showed no difference between the two groups in tPA activity at 24 h post injection. Data are shown as mean ± SEM.

Table 1 tPA Activity Following the Addition of tPA to Peritoneal Fluid Samples

tPA Added (U/ml)	tPA Activity (U/ml)		
	Non-op	DMSO	Aprepitant
0	0±0.4	8.0±1.5	4.5±0.0
2.5	1.3±0.5	10.7±1.4	7.6±0.1

Data are shown as mean ± SEM

Non-op non-operated, *DMSO* dimethylsulfoxide vehicle

strated by the sialogogic assay, adequate peritoneal concentrations of aprepitant may not have been achieved at a critical time to prevent adhesion formation. Thus, the continuation of oral aprepitant administration after surgery would be of no further benefit to adhesion reduction. Our previous data suggest that there is a temporal window in which the NK-1RA is effective in reducing adhesion formation. We determined that the NK-1RA, CJ-12,255, must be available in the peritoneum no later than 5–6 h postoperation in order to reduce adhesion formation.[21] Other studies have also failed to show anti-adhesion effects of orally administered drugs that were effective by intraperitoneal administration. Oral doses of atorvastatin did not reduce adhesion formation in rats while intraoperative doses did.[22] Data presented here further support that the intraperitoneal administration of a pharmacologic agent during surgery may be the best method for adhesion prevention due to direct delivery to target tissues. This may be beneficial since a single intraperitoneal dose of a drug may limit the potential side effects associated with longer oral dosing regimens.

The tPA activity assays yielded unexpected results. Our previous work with the NK-1RA, CJ-12,255, showed increased postoperative tPA activity and increased *tPA* gene transcription compared to saline controls at 24 h.[18] However, intraperitoneally administered aprepitant decreased tPA activity compared to the DMSO vehicle. This correlated with the PCR results that showed tPA mRNA expression remained unchanged while PAI-1 mRNA expression increased, resulting in an overall downregulation of the fibrinolytic system. The tPA assays performed with an added known concentration of tPA demonstrate that neither aprepitant nor DMSO interfere with the tPA activity assay, eliminating this as a possible reason for the decreased measurement. Thus, the observed decrease in tPA activity is likely due to increased expression of PAI-1, not to changes in tPA expression.

In contrast to our current observations, several studies suggest that agents which elevate postoperative peritoneal fibrinolytic activity have the ability to reduce adhesion formation. In animal models, pharmacologic upregulation of peritoneal fibrinolysis, across a broad range of drug

types, is associated with decreased adhesion formation. Such drugs include the NK-1RA CJ-12,255, methylene blue, atorvastatin, pentoxifylline, and octreotide.[18,21–30] Studies in both humans and animals demonstrate the ability of the peritoneal fibrinolytic system to regulate adhesion formation. Patients with the most severe adhesions have decreased postoperative tPA activity as well as increased levels of PAI-1.[31] In animals, increasing postoperative peritoneal fibrinolytic activity by either inhibition of PAI-1 or IP administration of tPA reduces adhesion formation.[32,33] Furthermore, Sulaiman et al. demonstrated that tPA-null mice have increased postoperative adhesion formation compared to their wild-type counterparts.[12]

While the fibrinolytic system has been demonstrated to have a strong relationship with adhesion formation, the current data suggest that aprepitant may reduce adhesion formation via other mechanisms. Possible alternative mechanisms include modulation of proinflammatory mediators such as cyclooxygenase-2 (COX-2) and transforming growth factor (TGF)-β. The COX-2 enzyme has been demonstrated to have proinflammatory, proangiogenic, and pro-fibroblastic effects, all of which may promote adhesion formation, and inhibition of the COX-2 enzyme, which is expressed by adhesion fibroblasts,[34] has been shown to reduce adhesion formation in rodents.[14,35,36] Overproduction of TGF-β, a multifunctional growth factor, correlates with adhesion formation in both humans and animals,[37–39] and in animal models, blocking TGF-β activity has been shown to reduce adhesion formation.[40,41] In support of a possible link between aprepitant and COX-2 and/or TGF-β, the NK-1R ligand substance P has been shown to upregulate COX-2 expression in cultured human umbilical vein endothelial cells and colonic epithelial cells,[42,43] and
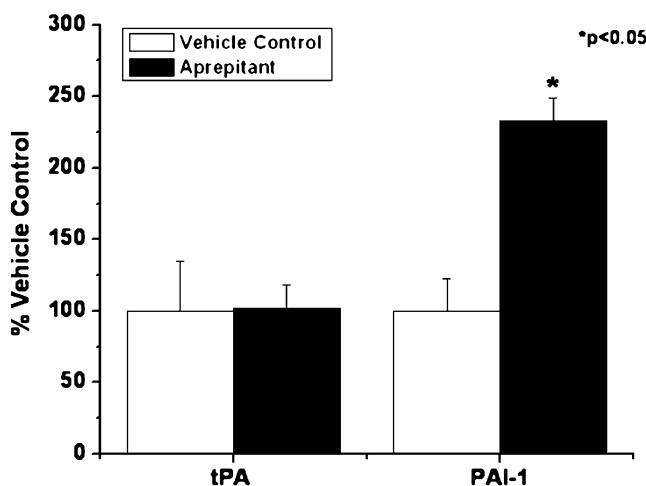
Figure 3 Twenty-four-hour postoperative rats showed a 233% greater PAI-1 mRNA level than vehicle controls (DMSO; *N*=3 per group). tPA mRNA levels were not statistically changed by aprepitant administration. Data are shown as mean ± SEM.

substance P has been shown to increase TGF-β expression in rat fibroblasts.[44] IP administration of aprepitant at surgery may also directly affect wound healing processes. Substance P released at sites of tissue injury, in addition to promoting inflammation, is thought to stimulate proliferation of epithelial, vascular, and connective tissue cells as part of the wound healing process.[45,46] Substance P may induce tissue fibrosis via augmentation of cytokine-induced fibroblast proliferation, effects on collagen organization, and regulation of matrix metalloproteinase expression.[47–50] We have previously reported that administration of the NK-1RA CJ-12,255, in addition to reducing adhesion formation, increases matrix metalloproteinase expression and activity in the postoperative peritoneum.[51] In the current study, aprepitant may reduce adhesion formation by modulating the inflammatory and wound-healing processes that begin with the onset of surgery well before the changes in peritoneal fibrinolytic activity occur. A possible scenario is that administration of aprepitant reduces postoperative fibrin band formation, thereby diminishing the need for fibrinolysis. Further investigation is needed to delineate the mechanisms by which aprepitant, as well as other NK-1RAs, reduce adhesion formation.

Conclusion

Intraperitoneal administration of the FDA approved NK-1RA, aprepitant, is effective in reducing intraabdominal adhesions while oral administration is not. The peritoneum may act as a selective barrier preventing orally administered or systemic drugs from significantly affecting processes in the peritoneum including adhesion formation. Thus, intraoperative peritoneal administration of a pharmacologic agent appears to be the most effective method for drug delivery to target adhesiogenesis. The results also suggest that the anti-adhesion effects of aprepitant may be via an alternative pathway other than the peritoneal fibrinolytic system. Although further investigation into the mechanism(s) of N K-1RA effects on adhesion formation is warranted, these studies support the use of an NK-1RA in adhesion prevention.

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Discussion

Margo Shoup, M.D. (Maywood, IL): I would like to congratulate the authors for trying to tackle a difficult problem that we all face with our patients with adhesion formation in a small bowel obstruction, and we really haven't made much headway in this in the last couple of decades. The authors in this paper attempt to study the effects of HA/CMC, or seprafilm, and neurokinin-1 receptor antagonist. The adhesions were measured at seven days postoperatively and placement of the buttons, and the Tpa was measured 24 hours after laparotomy. And we know that the synergistic effects of NK1RA and seprafilm is evident, but we are not really sure what is going on with the Tpa during all that. So I have a few questions for you.

Have you first looked at dose escalation studies with increasing NK1 receptor antagonists to evaluate the effects on Tpa, because this would clarify whether this is truly the mechanisms through which this is working. Also, you checked the Tpa levels 24 hours after surgery and, like I said, the adhesions at seven days. Have you looked at different time frames for both of these to see if there is more of a correlation? And at this point do you have any information on the status of the soluble seprafilm that is available in Europe, and if so, where do you think this may impact your study?

Thank you.

Rizal Lim, M.D. (Boston, MA): In terms of dose escalation of our antagonist, going back to the original parent compound, it is actually based off of a drug called ezlopitant. When we received this compound as a gift, the doses were actually based on the then maximum recommended dose of 25 mg/kg, which we used. But in earlier studies, as we started off with 5 mg/kg and then went to 10 mg/kg, we saw a progressive increase in adhesion prevention from those two doses.

In terms of the different time frames, looking at adhesions with this model specifically, our personal experience and data we have collected in the past have shown that when we look at adhesion formation beyond 7 days, we haven't really seen much of a difference in terms of severity. The same is true for tPA. In fact, what we have seen in previous studies is that tPA immediately post-op, at least within the rat, drops significantly and hits its nadir at approximately 24 hours and following that period of time begins to slowly rise back towards normal levels. So we chose that simply because it gives us a general idea of what the fibrinolytic activity within the abdomen is doing at its worst case scenario. We have also shown that giving the drug at 24 hours, we can alter that fibrinolytic activity.

And the final question, in terms of the soluble and gel forms of various barrier compounds, I am not firmly sure as to how far the various companies have progressed in terms of getting that approved within the U.S. But some of the implications which it may convey are that currently some of the biggest limitations of using HA/CMC barriers involve its actual application. It is a brittle, stiff material. It is difficult to use in certain cases such as laparoscopy, and I think that progressing to more of a gel type of device would improve its utility.