Addition of Nimesulide to Small Intestinal Submucosa Biomaterial Inhibits Postsurgical Adhesiogenesis in Rats

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Abstract: Adhesion formation is a common complication in abdominal surgery with incidence as high as 93% and small bowel obstruction a common complication. Because the extracellular matrix material, small intestinal submucosa (SIS), is commonly used in various surgical procedures, methods to inhibit adhesiogenesis are of great interest. This study was undertaken to determine if incorporation of nimesulide (NM), a selective cyclooxygenase (COX)-2 inhibitor, could reduce the extent and tenacity of intraabdominal adhesion formation associated with SIS implantation. Female Sprague–Dawley rats underwent a cecal abrasion surgical procedure to induce adhesiogenesis. Rats were either left untreated or treated by direct application over the injured cecum with polypropylene mesh (PPM); SIS; SIS containing a low dose of NM; or SIS containing a high dose of NM. Rats were euthanized 21 days later, and adhesion extent and tenacity were evaluated using standard scales (0 = minimal adhesiogenesis; 4 = severe adhesiogenesis). Addition of NM to SIS resulted in a significant ($p < 0.05$) reduction in adhesion extent and in a similar reduction in adhesion tenacity for SIS containing a low dose of NM. Adhesions typically extended from the abraded cecal surface to the body wall and were characterized histologically by fibrous tissue adherent to the cecal wall. In conclusion, addition of the nonsteroidal anti-inflammatory, COX-2 selective drug, NM, to SIS attenuates adhesion extent and tenacity when compared with surgical placement of SIS or PPM alone. © 2010 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 93B: 18–23, 2010

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INTRODUCTION

Porcine small intestinal submucosa (SIS), a naturally occurring extracellular matrix (ECM), has found wide application in a number of clinical situations.. For example, SIS is used in multiple abdominal sites such as urethral and vaginal prolapse repair, colon and rectal prolapse repair, reconstruction of the pelvic floor, bladder support, tissue repair, and hernia repair, indicating a compatibility with the abdominal environment.

SIS is isolated and disinfected using proprietary methods that preserve bioactive molecules and much of the native structure of the ECM,[1,2] and it has been shown to contain collagens I, III, IV, and VI, the growth factors FGF-2 and TGF-β, fibronectin, laminin, sulfated glycosaminoglycans, heparan sulfate proteoglycan, and hyaluronic acid.[1–7] The three-dimensional structure of SIS combined with its cytokine content facilitates cellular ingrowth and proliferation, resulting in a rapid replacement of SIS by endogenously produced tissue.[5–8]

Animal and clinical studies have demonstrated that SIS induces host cell infiltration, site-specific remodeling, and repair following implantation.[9–11] However, this naturally derived medical device still has the propensity to occasionally form adhesions following implantation,[12,13] which is the main reason that the SIS biomaterial is contraindicated for use in close proximity to the bowel or to reinforce soft tissues in close proximity to the uterus. In this regard, methods to overcome adhesion formation would improve the clinical utility of SIS.

A major factor in the formation of postsurgical adhesions is decreased peritoneal fibrinolytic activity. Fibrinolytic activity is dependent on the balance between

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fibrinolysis activators, including tissue plasminogen activator and urokinase plasminogen activator; and plasminogen activator inhibitors (PAI-1 and PAI-2).[14,15] The equilibrium of these activators is disrupted after injury,[16] during inflammation,[16–18] and in response to ischemia,[19] allowing the formation of fibrinous adhesions between injured surfaces.[20] This shift in the fibrinolytic equilibrium is exacerbated by the localized release of inflammatory cytokines[21] and prostaglandins, and the arrival of PAI-1 secreting macrophages and polymorphonuclear leukocytes. Additionally, the release of inflammatory cytokines stimulates fibroblasts in the area to release additional PAI-1.[22] In such an inflamed state, the fibrinous adhesion matrix persists and provides a scaffold for infiltration by collagen-secreting fibroblasts and angiogenic cells. Permanent fibrous adhesions are the result.[23]

Although the inflammatory pathways involved in adhesiogenesis are not entirely defined, cyclooxygenase (COX)-2 may plan an important role. Fibroblasts isolated from human adhesion tissue had a distinct phenotype, which included increased COX-2 expression compared to normal peritoneal fibroblasts.[24] Furthermore, there was no difference observed in COX-1 levels between normal and adhesion fibroblasts.[25] Taken together, these data suggest that inhibitors of COX-2 activity might inhibit adhesiogenesis.

Pharmacological intervention has largely focused on compounds, which are either nonselective inhibitors of COX activity or specifically inhibit COX-2 versus COX-1. In this regard, nonsteroidal anti-inflammatory drugs (NSAIDs) have been extensively evaluated as nonselective COX inhibitors; however, inconsistency in methods and results makes difficult an overall interpretation of efficacy.[26–33] In contrast to nonselective COX inhibitors, administration of selective COX-2 inhibitors specifically targets a pathway with import to adhesion formation. For example, rofecoxib administered orally inhibited adhesion formation in a rat model.[34] A comparative study involving orogastric gavage of compounds in a murine adhesion model showed that the selective COX-2 inhibitors, celecoxib and rofecoxib, significantly reduced adhesion formation compared to nonselective COX inhibitors.[35]

Nimesulide (NM), *N*-(4-nitro-2-phenoxyphenyl) methanesulfonamide, is an extensively characterized NSAID that selectively inhibits COX-2 mediated inflammation.[36,37] NM is well tolerated by the liver, lungs, kidneys, and blood, with no prolongation of bleeding times or alteration of other hemostatic variables.[38] NM administered topically to the traumatized uterine horn inhibited adhesion formation in a rat model.[39] Conceivably, because the COX pathway is important in adhesiogenesis, addition of NM to implanted biomaterials could decrease adhesion formation.

In the study described here, we used a rat cecum abrasion model[40] for adhesion formation to investigate the ability of NM to inhibit postsurgical adhesiogenesis when combined with SIS.

MATERIALS AND METHODS

Addition of NM to SIS

Strips of lyophilized 2-layer SIS (Cook Biotech, West Lafayette, IN) measuring 6 cm × 2 cm were prepared as either: (1) SIS (mean mass 79.6 ± 7 mg), (2) high-dose NM-augmented SIS (74.9 ± 10 mg), and (3) low-dose NM-augmented SIS (75.2 ± 11 mg). The high- and low-dose NM-augmented SIS constructs were prepared by soaking the SIS strips for 1 h in 800 or 200 μM solutions of NM (Sigma Chemical Company, St. Louis, MO) in DMSO (Sigma), respectively, at room temperature with moderate agitation. The samples were removed from solution, frozen, and relyophilized. All samples were sterilized with ethylene oxide gas before implantation.

Elution studies using gas chromatography/mass spectrometry to quantify the loading of NM onto SIS using DMSO were performed. NM-loaded SIS samples were eluted in methanol overnight. The capillary gas chromatography/mass spectrometry analyses were carried out using a GCQ mass spectrometer system (FinniganMAT/ThermoElectron Corporation, San Jose, CA). Typical electron energy was 70 eV with the ion source temperature maintained at 210°C. The individual components were separated using a 15 m × 0.25-mm SPB-1701 capillary column. The film thickness was 0.25 μm. The initial column temperature was set at 200°C, held for 0.1 min, and heated to 280°C at 10.0°C per minute. The injector temperature was set at 230°C. The mass spectra were obtained by scanning from m/z 41 to m/z 400. NM concentration was determined by comparing area under peak readings between m/z 344 and m/z 358 against a standard curve of known NM concentrations in methanol.

Cecal Abrasion Model for Postsurgical Adhesions

The studies described here were approved by the University of Notre Dame Institutional Animal Care and Use Committee and conducted in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care International. Specific pathogen-free 250–300-g female Sprague–Dawley rats (Harlan, Indianapolis, IN) were used for these studies. Animals were maintained on Purina Rodent Lab Chow (Purina, Richmond, IN) and tap water provided *ad libitum*. Rats were anesthetized with an intramuscular dose of ketamine hydrochloride (90 mg/kg) and xylazine (10 mg/kg) and prepared for aseptic abdominal surgery.

Using established techniques,[40] the ceca of the rats were exteriorized, abraded with a nylon brush to the point of producing mild petechial hemorrhage, and replaced into the abdominal cavity. In addition to the cecal abrasion, the peritoneal cavity was mildly abraded with a standard nylon toothbrush. Following replacement of the cecum, the abdominal wall was closed with 4–0 silk sutures, and the skin was closed with surgical staples. An additional three

TABLE I. Adhesion Grading Scale

	Score	Characteristic
Extent	0	No adhesions
	1	Adhesions on up to 25% of the abraded cecum
	2	Adhesions on up to 50% of the abraded cecum
	3	Adhesions on up to 70% of the abraded cecum
	4	Adhesions on up to 100% of the abraded cecum
Tenacity	0	No resistance to separation
	1	Mild resistance
	2	Moderate resistance
	3	Marked resistance
	4	Sharp dissection required for separation

Adhesion extent was characterized numerically based upon the percentage of the abraded portion of the cecum, which was covered with adhesion tissue, and adhesion tenacity was characterized numerically based upon the degree of resistance to separation.

rats underwent a sham operation without abrasion of the cecum or peritoneal wall. Rats received 1.0 mg/kg butopharnol tartarate intramuscularly and 5.0 mL of sterile saline subcutaneously following surgery.

Experimental Design

Following induction of anesthesia and preparation for aseptic surgery, rats underwent the cecal abrasion procedure. In seven rats (control), the cecum was placed back into the abdominal cavity without any additional treatment. In treatment groups (six rats per group), cecal abrasion was followed by implantation of either polypropylene mesh (PPM; Prolene®, Ethicon, Somerville, NJ) or SIS. For implantation, a 6 cm × 2-cm strip of sterile PPM or SIS (no added NM; low-dose NM; or high-dose NM) was placed over the abraded cecal surface and secured to itself and to mesenteric fat overlying the cecum with nonabsorbable nylon suture. Care was taken to avoid perforation of the bowel. Twenty-one days after surgery, the rats were euthanized by inhalation of carbon dioxide. The extent and tenacity of adhesions were evaluated using a scale adapted from similar studies of adhesiogenesis (Table I).[40,41] Adhesion tenacity was regarded as the resistance of the tissue to separation, and adhesion extent was regarded as the degree to which the adhesion tissue covered the abraded cecal surface. Tissue samples from adhesions were placed in 10% neutral buffered formalin for fixation prior to sectioning and staining the hematoxylin and eosin for microscopic evaluation.

Statistical Analysis

Data were analyzed by nonparametric one-way ANOVA (Kruskal–Wallis) to test for overall group differences with respect to the extent and tenacity of adhesions. In the presence of an overall significant effect, Wilcoxon matched pair tests were used to establish significance between individual treatments. Differences were considered significant when $p < 0.05$.

RESULTS

Addition of NM to SIS

Elution studies using gas chromatography/mass spectroscopy to quantify the loading of NM onto SIS using a DMSO vehicle were performed. These experiments demonstrated that samples of SIS incubated in 800 μM NM had a mean concentration of 6.91 ± 1.6 ng NM/mg SIS, whereas SIS incubated in 200 μM NM had a mean concentration of 2.61 ± 1.8 ng NM/mg SIS. Extrapolation of these drug concentrations yielded a total dose range of 410–654 ng for the high-dose samples and 152–236 ng for the low-dose samples. These concentrations were both well below the rat intraperitoneal LD50 for NM of 163 mg/kg.[42]

Effect of NM-augmented SIS on Adhesiogenesis

All the rats survived the initial surgery, though acute deaths (within 48 h) occurred in all groups except controls, with one death in the PPM group, two in the SIS group, two in the low-dose NM SIS group, and one in the high-dose NM SIS group. Gross necropsy revealed lesions consistent with bowel infarction in most cases. No additional deaths occurred during the remainder of the 21-day study.

NM is a selective COX-2 inhibitor and we therefore predicted that addition of NM to SIS would inhibit adhesiogenesis. All rats in all groups developed adhesions. As shown in Figure 1, a significant ($p < 0.05$) decrease in the extent of adhesion was found between groups receiving NM incorporated into SIS, versus groups without NM.

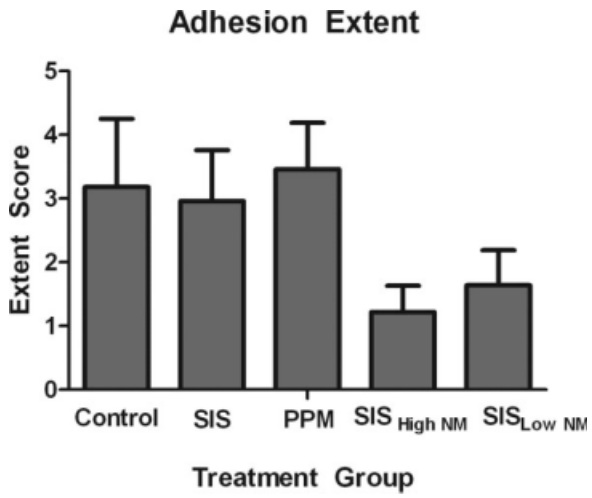

Figure 1. Mean extent of postsurgical adhesions. There was a significant ($p < 0.05$) decrease in adhesion extent scores between groups with nimesulide versus groups without nimesulide. There was no significant difference in adhesion extent scores between the group treated with a high dose of NM incorporated into SIS ($SIS_{High\ NM}$) and the group treated with a low dose of NM incorporated into SIS ($SIS_{Low\ NM}$). Other groups included untreated controls; animals treated with SIS having no added NM; and animals treated with polypropylene mesh (PPM). Error bars are standard error of the mean.

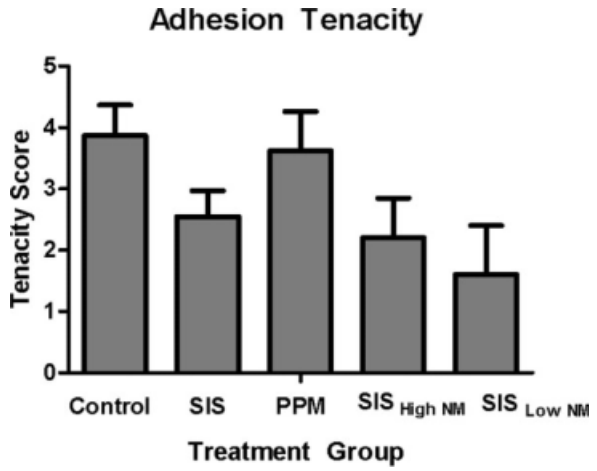

Figure 2. Mean tenacity of postsurgical adhesions. There was a significant ($p < 0.05$) decrease in tenacity scores between the animals in the untreated control group and those treated with polypropylene mesh (PPM) when compared with animals treated with SIS having no added NM, animals treated with a high dose of NM incorporated into SIS ($SIS_{High\ NM}$) and animals treated with a low dose of NM incorporated into SIS ($SIS_{Low\ NM}$). Error bars represent standard error of the mean.

Similarly, Figure 2 highlights a significant ($p \leq 0.05$) reduction in adhesion tenacity in groups receiving NM incorporated into SIS compared to the untreated control and PPM groups. Furthermore, there was a significant ($p < 0.05$) reduction in adhesion tenacity between the group treated with SIS and that treated with SIS containing a low dose, but not a high dose, of NM. Adhesions typically extended from the abraded surface of the cecum to the abdominal wall. Microscopically, adhesions were similar between all groups and consisted of fibrous connective tissue in which the mucosal and muscular layers of the cecum were firmly embedded (Figures 3 and 4).

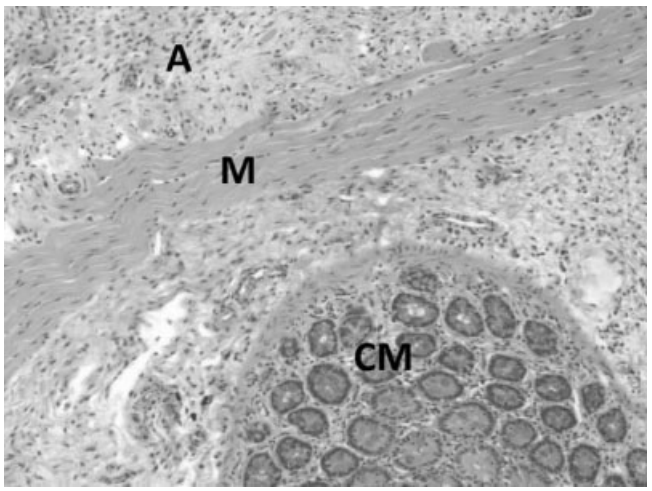

Figure 3. Photomicrograph of typical cecal adhesion in rat treated with SIS containing no added NM. Both the cecal mucosa (CM) and cecal muscle layer (M) are firmly embedded within the adhesion tissue (A), which consists of fibroblasts and fibrous connective tissue. Magnification 400×.

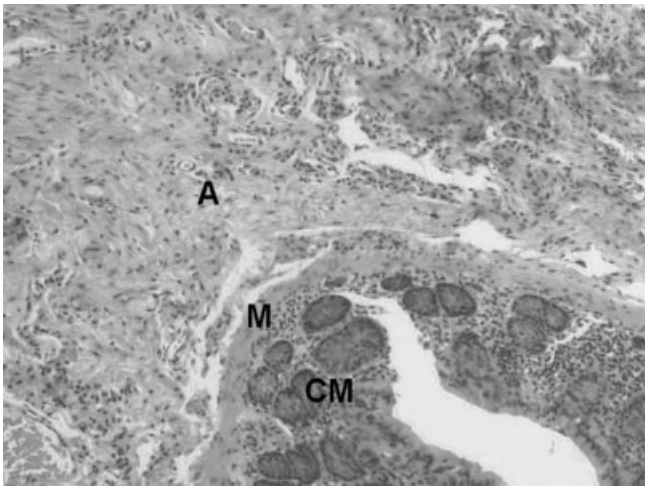

Figure 4. Photomicrograph of cecal adhesion in rat treated with SIS containing a high dose of NM. The cecal mucosa (CM) and cecal muscle layer (M) are closely adhered to the adhesion tissue (A), which consists of fibroblasts and fibrous connective tissue. No difference in the histological character of adhesions was noted between any groups of rats. Magnification 400×.

DISCUSSION

Postsurgical adhesiogenesis appears to be due to disruption in the local fibrinolytic activity of the injured peritoneum.[16] The common pathway from injury to adhesion is a local inflammatory response of the peritoneum that decreases fibrinolysis and enables the persistence of a fibrin scaffold at the site of injury, providing a nidus for cellular infiltration and remodeling.[15,43] Surgical injury is a combination of cutting, abrasion, dessication, ischemia, and coagulation trauma,[44,45] each of which is sufficient to elicit a local inflammatory response that can induce adhesion formation by muting the fibrinolytic system.

The critical period in peritoneal wound healing, in which fibrinous deposits become more organized, occurs at 2–5 days postoperatively.[16] If the fibrinolytic equilibrium of the injured peritoneum can be shifted in favor of fibrinolysis by limiting local peritoneal inflammation through this critical period, postsurgical adhesions can be reduced or prevented.

Prostaglandin release by peritoneal macrophages and fibroblasts stimulates adhesion formation,[46] and inhibition of prostaglandin by aspirin has been demonstrated to reduce adhesions in a rat model.[47] COX enzymes are the first in a cascade of enzymes that convert arachidonic acid from phospholipid membranes into prostaglandins. COX-2 is primarily produced by macrophages and fibroblasts during inflammation and is responsible for further upregulation of prostaglandin synthesis. Attenuation of the inflammatory response would likely reduce the persistence of the fibrin scaffold and reduce adhesion formation. A recent paper by Saed et al.[24] suggests another potential mechanism of action for COX-2 inhibitors in the reduction of postsurgical adhesions. Those authors found that COX-2 was expressed

by fibroblasts from adhesions but not from adjacent normal tissues, suggesting that COX-2 is involved in the induction of an "adhesion phenotype" for proliferative fibroblasts. Furthermore, because COX-2, and not COX-1, is expressed on new angiogenic epithelial cells, it may be that COX-2 inhibitors may selectively inhibit angiogenesis associated with newly forming adhesions.[35,48]

The experiments described here were performed to test the hypothesis that addition of a selective inhibitor of COX-2 to a degradable ECM-based biomaterial already widely used for soft tissue repair (SIS) would reduce postsurgical adhesion formation that is sometimes associated with implantation of SIS.

Adhesion formation following surgical placement of SIS has been noted in several animal studies. In rats, adhesions were present in most animals following repair of full-thickness abdominal wall defects with SIS,[12] though they were not as extensive or as strong as the adhesions seen in defects repaired with PPM. Additional studies in the rat have rated adhesions formed after SIS implantation as less severe compared to those seen with PPM.[13] In a model designed to examine the feasibility of using SIS as a tubular ureter graft in the rabbit, all subjects demonstrated mild intra-abdominal adhesions to the SIS graft material.[49] In the rabbit uterine horn, SIS was used to cover the area of surgical injury following aggressive ablation of the uterine horn tissue.[50] Compared to the control side, SIS barrier placement resulted in a reduction but not complete elimination in the number of adhesions present, from a median of eight for control animals to four for animals treated with SIS.

This study demonstrates that incorporation of NM into SIS, a commonly used biomaterial for tissue repair, effectively inhibits postsurgical adhesiogenesis. Significant reductions in both adhesion extent and tenacity were found in rats, which were treated with SIS that had either a high or low level of NM incorporated into it, with the exception of a lack of statistically significant difference in tenacity between adhesions in rats treated with SIS and those treated with SIS containing a high dose of NM. It is unclear why the SIS containing a low dose of NM demonstrated a greater reduction in adhesion tenacity than that containing a high dose of NM, although the difference was not significant, and it is possible that studies using greater numbers of animals would mitigate this difference, because no adjustment was made for multiple comparisons. Alternatively, there may have been a dose-dependence in the response, which was not detectable in this study. The data do not suggest if inhibition of adhesiogenesis by SIS containing NM is confined only to the period during which NM is eluted from the SIS. Indeed, it may be the case that the adhesiogenic process could resume once eluted NM has been physiologically cleared. Further studies will need to investigate this possibility.

It should be noted that all acute deaths occurred within 48 h of surgery. On the basis of gross necropsy, we believe that the likely cause of death was bowel ischemia, rather than postsurgical adhesions. Because there were no deaths in the control group, it is possible that blood vessels were entrapped by the sutures used to secure the implanted material, resulting in ischemic damage to the bowel.

In summary, addition of the selective COX-2 inhibitor, NM, to SIS significantly attenuated adhesion extent and tenacity. The encouraging results of this study merit further investigation of NM augmentation of SIS to reduce the formation of postsurgical adhesions when SIS is used in the treatment of soft tissue injury.

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