# Evaluation of the porcine intestinal collagen layer as a biomaterial

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Received 8 October 1999; accepted 29 December 1999

**Abstract:** The submucosal layer of the small intestine has been investigated as a source of collagenous tissue with the potential to be used as a biomaterial because of its inherent strength and biocompatibility. In this study we utilized a novel method for processing the tissue to generate an acellular intestinal collagen layer (ICL). This nondetergent, nonenzymatic chemical cleaning protocol removes cells and cellular debris without damaging the native collagen structure. Multilayer laminates of ICL crosslinked with a water-soluble carbodiimide (EDC) were evaluated as a tissue repair material in a rabbit abdominal hernia model. The ICL laminates provided the requisite physical properties and did not lead to adhesion formation. No immune response to the porcine collagen was detectable, and this material did not show any calcification in either the rabbit model or in the juvenile rat model. © 2000 John Wiley & Sons, Inc. J Biomed Mater Res, 51, 442–452, 2000.

**Key words:** collagen biomaterial; porcine submucosa; soft-tissue repair

## INTRODUCTION

The clinical need for strong, biocompatible materials that encourage integration while minimizing adverse tissue reactions, such as adhesion formation, is apparent. Yet, the design of optimal surgical repair materials to reinforce or replace soft tissues remains problematic. In the repair of abdominal wall defects and hernias, for example, conventional prosthetic materials such as knitted polypropylene mesh suffer from a number of complications, including mesh extrusion, bowel adherence, fistula formation, wound infection, and seroma development.[1–3] Despite their limitations, these surgical repair materials are required in order to reduce the tension on the wound when the defect is large and/or the abdominal tissue lacks sufficient strength for primary closure.[3] These "tension-free" methods using prosthetic materials have reduced hernia recurrence rates substantially.[1,2,4,5] Problems with permanent repair materials have lead to the development of synthetic resorbable materials that are not associated with a continuing foreign-body reaction. Ideal degradable materials would persist long enough in the patient to act as a scaffold for appropriate host tissue deposition but eventually would be replaced.

Thus far, synthetic degradable materials have not provided sufficient strength during the degradation process.[6,7] In both degradable and nondegradable synthetic materials, the suture line remains a weak area due to the lack of tissue ingrowth at the periphery of the patch, which can lead to re-herniation.[1,8]

Natural collagenous materials are being investigated for surgical repair because of their inherent low antigenicity and their ability to integrate with surrounding tissue.[8–11] Degradable collagenous materials have shown potential but may lose strength *in vivo* if they are not adequately crosslinked.[11,12] The submucosal layer of the small intestine, a thin collagenous connective tissue, has been used with promising results for experimental abdominal wall repair.[13,14] This tissue also has been evaluated as a biomaterial for a variety of other tissue repair or replacement applications, including tendon[15] and bladder[16,17] repair. The intestinal submucosa appears to possess the requisite biocompatibility and strength for surgical repair and can be remodeled by the host tissue.[18]

In the above applications, the submucosal layer was isolated by manually scraping off the mucosal and muscular layers of porcine small intestines. However, this method generates a tissue containing variable amounts of cellular debris, glycosaminoglycans and lipids. While it is difficult to predict the impact of this

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material heterogeneity for all applications, the variability does influence the fabrication of constructs, and residual cellular components have been implicated as nodes for calcification.[31] Consequently we have developed a method for generating an acellular layer of intestinal collagen from the porcine submucosa without compromising the native collagen structure. In this report the preparation and characterization of this porcine intestinal collagen material are described, and the potential utility of this material is evaluated in a rabbit abdominal hernia repair model.

## MATERIALS AND METHODS

### ICL preparation

The intestinal collagen layer (ICL) was prepared from the intestines of large (at least 205 kg) swine from a closed herd (Parsons Farm, Hadley, Massachusetts). The small intestines were harvested (Adams' Farm, Athol, Massachusetts) and shipped on ice to our facility (Organogenesis Inc., Canton, Massachusetts). The cleaning processes were initiated within 4 h of harvest. The mesenteric and outer membrane layers manually were removed from the small intestine. The intestines then were processed mechanically through a series of rollers (Ernest Bitterling Model M34, Nottingham, England) under a constant flow of hot water (40°C) to remove the inner mucosal and outer muscular layers. After mechanical cleaning, the intestine was slit longitudinally between the lymphatic vessels and cut into 15-cm sections that were processed through a series of chemical cleaning steps. The v/v ratio of each chemical cleaning solution to tissue was 100:1, and the incubations all were carried out at ambient room temperature. The tissue was first incubated for about 16 h in a solution of 100 m*M* of ethylenediaminetetraacetic acid (EDTA) in 10 m*M* of sodium hydroxide (NaOH) with a pH of 11–12. The second incubation in 1*M* of hydrochloric acid (HCl) in 1*M* of sodium chloride (NaCl) at pH 0 to 1 was carried out for 6–8 h. This was followed by an incubation in 1*M* of sodium chloride and 10 m*M* of phosphate-buffered saline (PBS) at pH 7–7.4 for 16 h and then a 2-h incubation in 10 m*M* of PBS at pH 7–7.4. Finally, the tissue was rinsed in sterile water at pH 5.8–7.0 for at least 2 h. The resulting ICL material then was packaged for storage at −80°C and thawed as needed. All lots of material were tested for sterility and residual endotoxin levels prior to use.

### ICL characterization

Following mechanical and chemical cleaning, 5 μm sections of ICL were processed for histology and stained with hematoxylin and eosin to determine the extent of cellular remnants and to assess the structure of the collagen fibers. The collagen amount and type were determined by measuring hydroxyproline content and by SDS-PAGE, as described previously.[19,20] Residual glycosaminoglycans (GAGs) were assayed following digestion of the ICL with papain (Sigma Chemical Co., St. Louis, Missouri). GAGs were extracted using DOWEX cation exchange resin (Sigma Chemical Co.) and separated on cellulose acetate sheets by electrophoresis. The bands were stained with alcian blue (Sigma Chemical Co.), cut out, dissolved in DMSO, and their absorbance measured on a spectrophotometer at 678 nm. The DNA concentration was determined using a DNA/RNA isolation kit (US 73750, Amersham Life Sciences, Cleveland, Ohio), following the standard protocol. The lipid content of dry ICL was determined by methylene chloride extraction. Briefly, ICL was dried and the percent of the solids was determined on a CEM analyzer (model AVC-80, CEM Corp., Matthews, North Carolina). Methylene chloride was added to the round-bottom flask of a Soxhlet distiller/condenser (Kontes Brand, Fisher Scientific, Pittsburgh, Pennsylvania) and placed in a heated water bath. The ICL was placed in a porous thimble above which the organic solvent condensed and extracted the lipids from the ICL. After boiling off the condensed methylene chloride, the weight and percent of extracted material were determined. The thermal stability of the ICL was measured with a differential scanning calorimeter (model DSC12E, Mettler Toledo Co., Highstown, New Jersey) before and after chemical cleaning. Samples weighing 5–10 mg were cut from the ICL, sealed in 20-μL aluminum crucibles, and heated from 35° to 100°C at a rate of 10°C/min. Subsequently, $T_{onset}$, the temperature associated with the onset of the denaturation peak, was determined from the thermogram using analysis software (DSC-Mettler Toledo TA 89A).

### Laminate construction

ICL laminates were constructed by placing the desired number of ICL sheets on top of each other with the layers aligned parallel to the long axis of the intestines. The laminates were dried and crosslinked with ethyl-3(3-dimethylamino) propyl carbodiimide (EDC) at the indicated concentration. The ICL laminates were rinsed in 0.1% peracetic acid, vacuum-sealed into hermetic packaging, and terminally sterilized by gamma irradiation (25–35 kGy).

### Laminate characterization

The thermal stability, suture retention, strength, and stiffness of the patch material were measured before and after gamma sterilization to characterize the physical properties and determine the effects of irradiation. The thermal stability was measured as described above. The following tests were performed on moist samples using a servohydraulic

material testing machine (MiniBionix 858, MTS Systems, Minneapolis, Minnesota) using TestWare SX and TestStar2 software. The suture retention was measured using a rectangular test sample 12.5 mm wide by 50 mm long. Each sample was held in the lower grip and threaded with a 5-0 Surgilene (polypropylene) suture 2 mm from its edge. The two ends of the suture were attached to the upper grip and pulled at a constant rate of 125 mm/min. The suture retention was defined as the peak force obtained during this procedure. The stiffness and strength were obtained from constant strain rate (0.03 $s^{-1}$) uniaxial tensile failure tests on 12.5-mm wide strips of the ICL constructs. The gauge length (~28 mm) was defined at a tare load of 0.5 g; the direction of loading was perpendicular to the long axis of the intestines. The load was normalized to sample width (tension) but not thickness (stress) since the *in vivo* loading is in the plane of the tissue and must be supported by the graft regardless of the thickness of the material. The stiffness was defined as the maximum slope of the tension (force/width) versus strain (Δ length/gauge length) curve. The strength was defined as the maximum tension (N/cm).

## Rabbit hernia model

The ICL laminates used for hernia repair were crosslinked with either 100 m*M* of EDC in 50% acetone/50% deionized water or with 1 m*M* of EDC in 2-[N-Morpholino]ethanesulfonic acid (MES) buffer (Pierce Biochemical, Rockford, Illinois). New Zealand white rabbits (4.0 kg) were used for the *in vivo* studies. The animals were caged and kept on a 12-h light/dark schedule at ~22°C. All procedures were performed at the Tufts New England Medical Center (Boston, Massachusetts) in compliance with the Animal Care and Use Committee (ACUC) guidelines and in accordance with the NIH guidelines for the use and care of laboratory animals (NIH Publication #85-23 Rev. 1985). Prior to surgery, the animals were medicated with 3.0 cc of xylazine and 1.0 cc of acepromazine. (Vedco Inc., St. Joseph, Missouri). General anesthesia was induced by an intramuscular injection of 40 mg/kg of Ketamine (Phoenix Pharmaceuticals Inc., St. Joseph, Missouri). After depilation of the surgical site, the area was wiped down with 70% isopropanol and iodine. A full-thickness defect approximately 5 cm long was made in the anterior abdominal wall through the center of the *rectus abdominis* muscle and the underlying peritoneum. The ICL patch was trimmed to the size of the defect and attached to the *rectus* sheath with an uninterrupted, nonresorbable 3-0 Prolene suture. The skin was closed with 3-0 Dexon and 3-0 Ethibond sutures. All suture material was obtained from Roboz Surgical (Rockville, Maryland).

At the end of the indicated implant period, anesthesia was induced, as described above. After examination for any gross reaction or re-herniation of the defect, the abdomen was opened to expose the peritoneal surface of the patch. Adhesions were graded using the standard scale in which a score of 0 indicates no adhesions, 1 represents thin and filmy adhesions easily separable by blunt dissection, 2 indicates firmly attached adhesions requiring aggressive blunt or mild sharp dissection, and a score of 3 indicates adhesions integrated with viscera that require aggressive sharp dissection.[21,22] The grafts also were evaluated for tissue integration, surface vascularization, fibrous capsule formation, and inflammation. Immediately following gross examination, the animal was euthanized with potassium chloride. A full-thickness section of host tissue with the implanted patch was removed and fixed with 10% formalin and then processed for evaluation. A median strip, including the patch, horizontal to the *rectus abdominis* muscle of approximately 1 cm in width was stained with hematoxylin and eosin (H&E) for histologic analysis of cell infiltration, inflammatory response, and collagen morphology or with Alizarin red to identify any sites of calcification.

## Immunologic analysis

A 10-mL sample of whole blood was collected from each animal at the time of explant to assay for antibodies to the porcine collagen. Porcine Type I collagen (WAKO Co., China) was coated onto Nunc Maxisorb 96-well plates (100 μL per well at a concentration of 1 μg/mL) overnight at 4°C. The plates were then blocked using 250 μL per well of 3% fish gelatin (Sigma Chemical Co.) for 2 h at 37°C. The plates were rinsed with PBS and 50 μL of rabbit serum was added. Purified rabbit anti-porcine collagen (Biodesign, Kennebunk, Maine) and normal rabbit serum were used as positive and negative controls, respectively. After incubation at 37°C for 2 h, the plates were washed four times with PBS + 0.1% Tween-20. Fifty μL of biotinylated goat anti-rabbit Ig (Jackson Immunoresearch, West Grove, Pennsylvania) were added to each well at a final concentration of 1 μg/mL. Plates were incubated at 37°C for 1 h and then washed, as above. Fifty μL of streptavidin–alkaline phosphatase (Jackson Immunoresearch) diluted 1:2000 then were added to each well, incubated for 30 min at 37°C, and washed. Plates were developed using 100 μL of a 1-mg/mL solution of p-nitrophenylphosphate, and antibody binding was determined using a SpectroMax 250 plate reader (Molecular Dynamics, Sunnyvale, California). The sensitivity of antibody detection is 1-3 nanograms/mL, which is approximately twice background.

## Mechanical characterization of explants

Uniaxial failure tests were performed on three 180-day-old explants and three corresponding control rabbit abdominal specimens. From each explant, a 19 to 23-mm wide strip approximately 50 mm in length was cut parallel to the predominant muscle fiber direction. The sample consisted of patch, incorporation region, and abdominal tissue. A control sample was cut parallel to the patch sample from the host abdominal tissue. The patch portion of each specimen was

placed in the upper grip, and the abdominal muscle was placed in the lower grip. The grip-to-grip spacing was approximately 25 mm at a tare load of 0.5 g. For the control tissues both grips held abdominal muscle. All specimens were tested at room temperature and kept moist with isotonic saline spray. The samples were loaded in uniaxial tension at a strain rate of 0.03 $s^{-1}$ until failure. As with the pre-implanted material, strength (N/cm) rather than ultimate tensile stress ($N/cm^2$) was reported because the implant and attachment region must withstand the forces experienced by the abdominal wall regardless of the thickness of the material. Stiffness of the explants was not reported since the deformation of the host tissue and the attachment region cannot be separated from deformation of the patch material itself. Similarly, the explant integration strength is not a direct measure of the strength of the remaining graft material since it was not gripped from both sides.

### Rat calcification model

A one-layer ICL sheet was prepared using sterile technique, crosslinked with 1 m*M* of EDC in deionized water for 16 h, and terminally sterilized by gamma irradiation (25–35 kGy). Four-week-old Sprague–Dawley rats were used for the *in vivo* analysis; the studies were carried out at Pine Acre Rabbitry/Farm (Norton, Massachusetts) in compliance with ACUC and NIH guidelines for the use and care of laboratory animals (NIH Publication #85-23 Rev. 1985). Prior to surgery each animal was anesthetized with ketamine and xylazine. A full-thickness incision was created in the skin on the ventrum. A 1-$cm^2$ piece of ICL was folded and inserted subcutaneously between the skin and the *rectus abdominis* muscles. The ICL was then stapled to the fascia and the wound was closed with staples and dressed with a sterile bandage. Using the same protocol, bovine heart valves fixed with 0.625% glutaraldehyde for 3 days were implanted as the positive control. At 7 and 28 days postsurgery, the animals were anesthetized, as previously described. The ICL and valves were explanted, fixed in 10% formalin, and processed for histologic evaluation. Calcification was assessed by Alizarin red staining.

## RESULTS

### ICL preparation and characterization

In our early studies exploring the use of this collagenous biomaterial in soft tissue repair and vascular prosthetics, the submucosal layer was obtained from the porcine small intestine after mechanical cleaning, using a customized, commercial gut-cleaning machine. We demonstrated that a six-layer laminate crosslinked with EDC, a water-soluble carbodiimide, and sterilized with peracetic acid was effective as a hernia repair patch in the rabbit model. The resulting biomaterial, GraftPatch™, received 510K clearance from the FDA for use in surgical repair.[23] Although these studies were promising, it became clear that the material obtained after mechanical cleaning was heterogeneous. The amount of residual cellular material in the submucosal layer varied between preparations from different donor pigs as well as within different sections of the small intestine. In particular, mechanical cleaning was not adequate for removing the endothelial cells in the vasculature of the tissue. Consequently, a chemical cleaning process was needed that would remove cells and cellular debris while maintaining the native collagen structure. To minimize damage to the collagen, we developed a nonenzymatic, nondetergent procedure for removing noncollagenous components of the tissue while at the same time controlling the swelling of the collagen fibers.

The first step in this process uses an alkaline chelating solution to saponify lipids and disrupt the basement membrane components of the cellular material that remains attached after mechanical cleaning. Although EDTA or EGTA [ethylenebis(oxyethylenitrilo)-tetraacetic acid] can be used, EDTA in NaOH at a pH of 11–12 was the most effective. The second incubation used an acidic salt solution; hydrochloric acid (HCl), acetic acid, or sulfuric acid at concentrations of 0.5 to 2*M* with NaCl added at concentrations of 0.1 to 2.0*M* to control swelling of the tissue. HCl is preferred for this step because it breaks up DNA into fragments of fewer than six base pairs. The next step involves exposure to a salt solution at physiologic pH (7.0 to 7.4). This solution helps neutralize the material and minimize swelling. We have found that the minimal effective amount for the chemical cleaning of the tissue is a ratio of 100:1 v/v of solution to tissue and that agitation of the solutions improves penetration of the chemicals. The required incubation times vary with the thickness of the tissue and were established at room temperature although the temperature can range from 4.0° to 45.0°C. The final step uses injection-grade water to rinse the tissue free of the cleaning agents so that the endotoxin levels can be measured.

The efficacy of this chemical cleaning process is illustrated in Figure 1. The photomicrograph of tissue sections stained with H&E shows the presence of residual cellular material after mechanical processing [Fig. 1(A)], which was removed by chemical cleaning [Fig. 1(B)] without visible damage to the collagen fibers. Biochemical analysis of the ICL obtained after this chemical processing confirmed that the tissue was predominantly Type I collagen with less than 0.7% lipids and undetectable levels of GAGs (<0.6%) and DNA (<0.1 ng/μL). In addition to the morphologic and biochemical analysis of the ICL, thermal stability

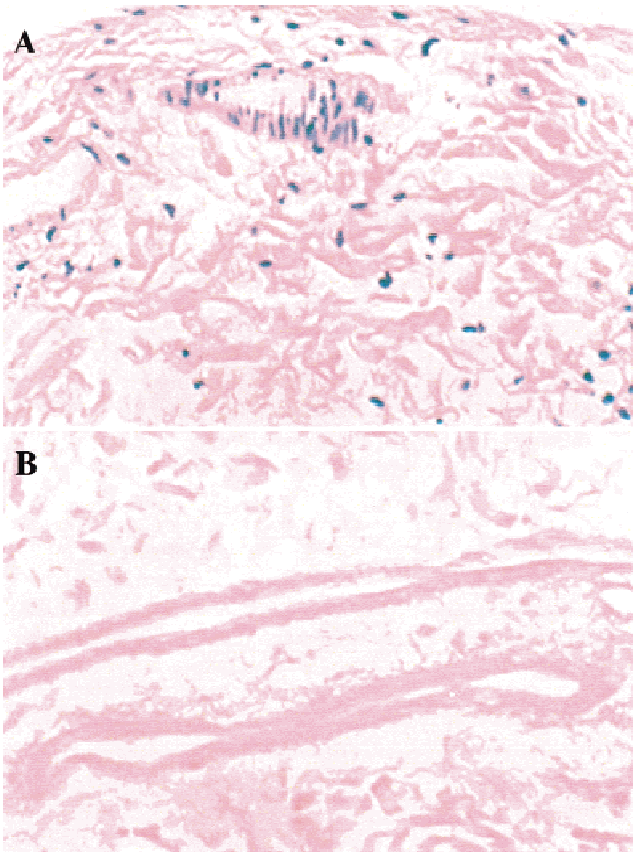


**Figure 1.** The effect of chemical cleaning on porcine submucosal tissue. The submucosal layer of the porcine small intestine was evaluated using hematoxylin and eosin (H&E) staining after (A) mechanical cleaning and after (B) both mechanical and chemical cleaning. In the absence of chemical cleaning, cells and cellular debris can be seen throughout the tissue. However, no residual cellular material is seen after the chemical cleaning procedure. Original magnifications, ×20.

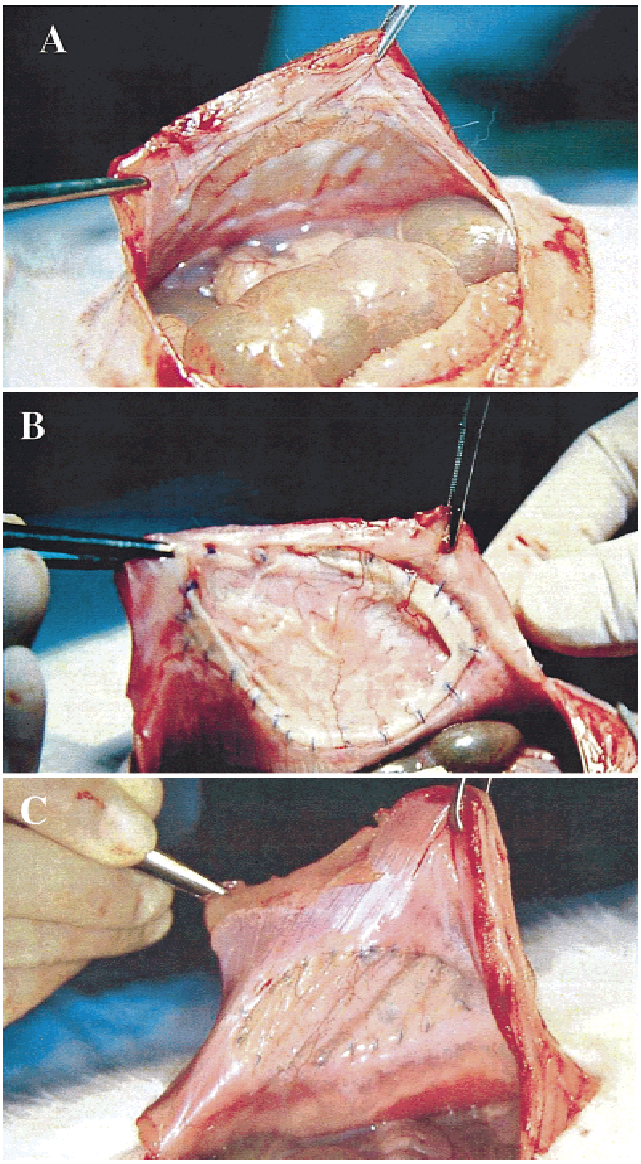


**Figure 2.** Gross morphology of the ICL hernia repair patches. Six-layer ICL laminates crosslinked with 100 m*M* of EDC were used to repair defects created in the rabbit abdominal wall. The rabbits were evaluated after 1, 2, 3, and 6 months ($n$ = 3 at each time point). No adverse reaction or seroma development was observed in any of the animals. (A) The patches were well integrated and had developed a mesothelial-like covering within 1 month. At 1 month, some neovascularization was observed. After (B) 3 months and (C) 6 months, the morphologies were similar, with more extensive vascularization on the visceral surface of the patch.

was assessed to evaluate the effect of the material processing. The denaturation onset temperatures were 68.4° ± 0.4°C before and 68.3° ± 1.3°C after chemical cleaning. This lack of alteration of the thermal stability was consistent with the native collagen triple helical structure not having been disrupted by the tissue processing.

### *In vivo* function of ICL laminates

Having developed a process that could generate an acellular collagen layer without damaging the collagen fibers, the ICL material was tested for *in vivo* function in the rabbit abdominal hernia model. This study was designed to determine if the chemical processing changed the *in vivo* performance of the GraftPatch™ laminate. In the first set of experiments, a six-layer laminate crosslinked with 100 m*M* of EDC in 50% acetone was tested for implant periods ranging from 1 to 6 months, with three rabbits evaluated at each time point (30, 60, 99, 180 days). Full-thickness abdominal wall defects were created and then repaired, as described in the Materials and Methods section.

All animals underwent an uneventful postoperative course, with no swelling, herniation, or inflammation at the repair site of the abdominal wall. As shown in

Figure 2, gross examination at the time of the explant indicated that the inner surface of the patch was covered with a glistening mesothelial-like tissue layer that appeared to be continuous with the parietal peritoneum. A time-dependent contraction of the grafts was observed but not quantified in this study. In ten of the animals, the implant areas were free of any adhesions. One of the rabbits at 30 days had a grade 1 adhesion to ~30% of the suture, and at 180 days one rabbit had a grade 1 adhesion attached to two points along the suture line.

By 30 days neovascularization was visually evident on the peritoneal surface extending from the peripheral attachment approximately halfway to the center of each patch. At 66, 99, and 180 days, the surface of the patch was completely vascularized and there was no macroscopic evidence of any gross tissue reaction. Histologic examination of the explanted patches indicated that after 30 days inflammatory cells typical of a foreign-body response were present throughout the explant. This moderate inflammatory response consisted mostly of focal lymphocytic aggregation with few neutrophils. A number of macrophages and multinucleated giant cells were detected. Newly deposited fibrous tissue was loosely organized on the visceral side and moderately organized on the skin side, and both sides were mildly populated with fibroblasts. Very little infiltration of fibroblasts was observed between the layers of the patch, however; the layers of the patch were compact and the collagen did not appear to be degraded [Fig. 3(A)]. After implantation for 3 months, the histology was similar but with fewer inflammatory cells, and the patches had begun to incorporate into the native abdominal wall tissue. Blood vessels were evident on both sides of the patch. The visceral collagen layer was populated with more fibroblasts and was more organized than at 30 days. Limited ICL digestion was observed in the surface layers of the implant and some fibroblast infiltration was observed, but the layers of the laminate still were relatively compact [Fig. 3(B)]. Patches explanted after 6 months again showed some infiltration of host fibroblasts, but the extent of remodeling was not discernibly different from what it was at 99 days [Fig. 3(C)]. At this 6-month time point, one patch was becoming encapsulated whereas the other two patches had fibroblasts infiltrating between at least two of the layers.

### Immunologic response to ICL

The low immunogenicity of this porcine Type I collagen was demonstrated in studies that analyzed the antibody response of rabbits that received the GraftPatch™. As depicted in Figure 4, ELISA analysis of serum samples taken from grafted rabbits showed little or no production of antibodies to Type I porcine collagen relative to normal rabbit serum. This lack of response was confirmed by Western blot analysis using purified porcine Type I collagen (data not shown).

### Physical properties of the GraftPatch™ laminate

The mechanical properties of the six-layer ICL laminates used for this first *in vivo* study are summarized in Table I. These data reflect the thermal stability, suture retention, strength, and stiffness of this construct after terminal sterilization using gamma irradiation. Not unexpectedly, gamma irradiation did have some effect on the mechanical properties of the ICL laminate. The onset temperature decreased from 75.1° ± 0.8°C to 72.5° ± 1.1°C following irradiation; the denaturation peaks from the thermograms were smooth and roughly symmetrical in both cases. The suture retention of the constructs decreased from 8.7 ± 2.2*N* to 6.6 ± 1.6*N*. This value still is well within the general surgical specification of greater than 2*N*. The uniaxial tension–strain curves were nonlinear, characteristic of collagenous connective tissues. Gamma sterilization decreased the stiffness of the constructs from 131.7 ± 27.5*N*/cm to 83.8 ± 4.9*N*/cm. The strength of the laminates also was decreased by irradiation at this dosage level; the peak tension values were 26.0 ± 4.0*N*/cm and 19.6 ± 1.9*N*/cm before and after irradiation, respectively.

Mechanical testing of the explanted patches was more complicated to perform and interpret than it was for the pre-implant constructs. It was difficult to determine which portion of the graft/tissue complex was the weakest since samples failed in the host tissue, at the suture line, and in the patch material. Regardless of failure mode, there was no significant difference in the strength of the patch/abdomen complex at 180 days (27.8 ± 5.6*N*/cm) and the host abdominal wall (28.1 ± 14.6*N*/cm). Furthermore, all samples were above the 16*N*/cm level reported to be important for human abdominal repair.[24] Thus it appears that the GraftPatch™ material retains adequate strength up to 180 days in the rabbit abdomen and is generally stronger than the integration region and the local abdominal wall. These data, however, are preliminary and cannot be directly compared to the pre-implantation strength since the explant tests measure integration strength, not strength of the graft material itself.

### Effect of crosslinking on *in vivo* function

While the results from the first study confirmed that the chemical cleaning did not adversely affect Graft-

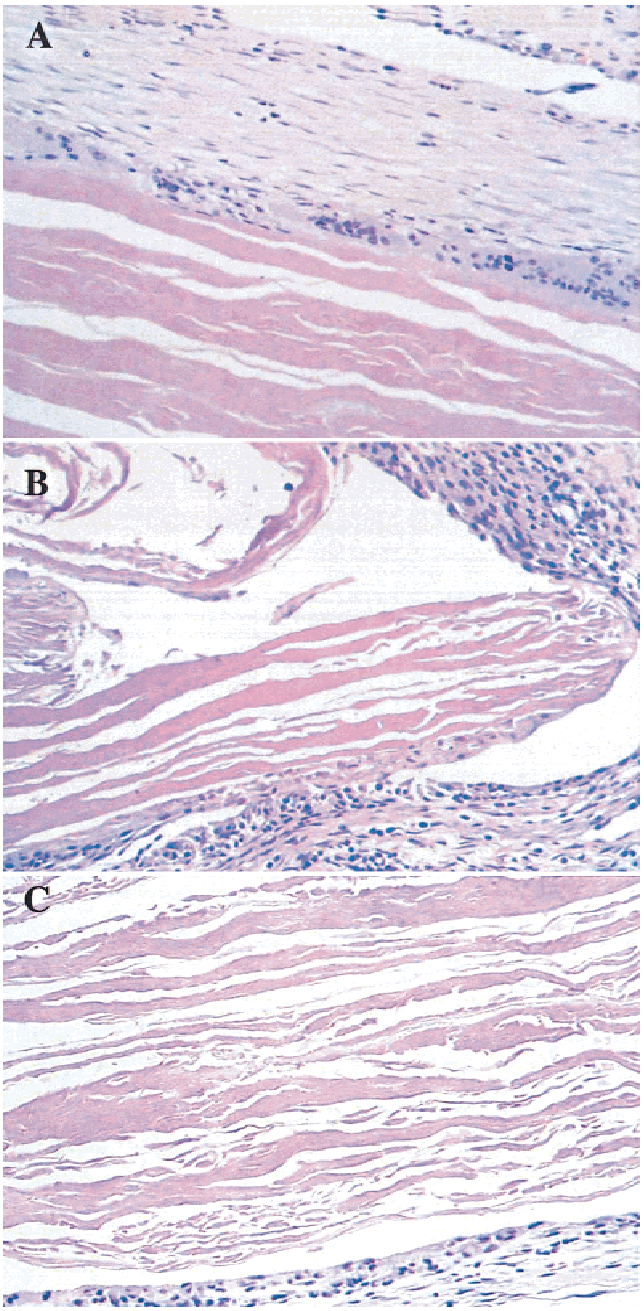


**Figure 3.** Histologic evaluation of explanted patches. The ICL patches were explanted at the indicated times and processed for histologic evaluation using H&E staining. (A) One month postimplantation the ICL collagen is clearly visible, and while some inflammatory cells have presented adjacent to the implant, little patch degradation is evident. (B) At 3 months blood vessels can be seen on both sides of the patch and fibroblasts have begun infiltrating into the ICL. A moderate inflammatory response is still present. (C) Remodeling, inflammation, and infiltration after 6 months postimplantation are very similar to the 3-month samples. Original magnifications, ×20.

Patch™ performance in the rabbit hernia model, the patches were not extensively remodeled by the end of this 6-month study period. Therefore the time course of this study was not sufficient to determine if the

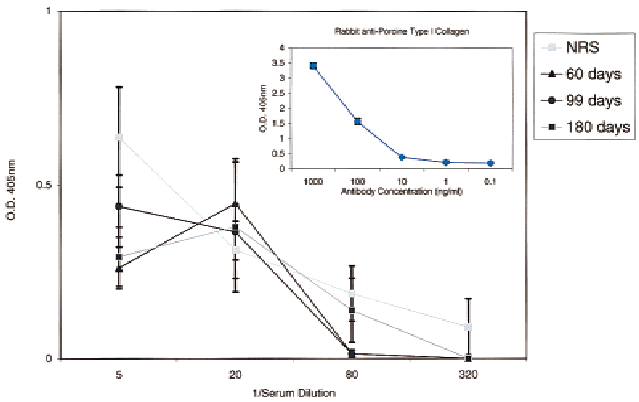


**Figure 4.** Immune response to the porcine ICL. Blood samples were taken from both control rabbits and those with ICL patch implants. These samples were assayed for the presence of antibodies to porcine collagen using standard ELISA methods. None of the implant animals demonstrated a measurable antibody response. The standard curve for this assay is shown in the inset.

patches would maintain the required strength throughout the remodeling process. Related research investigating the effects of crosslinking conditions used in the construction of ICL laminates demonstrated that the strength, stiffness, and *in vivo* remodeling of the laminates are related to the concentrations of EDC and acetone.[25] Crosslinking generally is used to decrease the antigenicity of xenogeneic tissues and to increase their stability to proteolytic degradation.[26] Unlike more common fixatives, such as glutaraldehyde, EDC increases collagen biostability and reduces antigenicity while preserving cell compatibility.[27,28]

**TABLE I**
**Mechanical Properties of the ICL Laminates**

| Analysis | 100 m*M* of EDC/50% Acetone (6-Layer) | 1 m*M* of MES (4-Layer) |
|---|---|---|
| Ultimate Tensile Strength (N/cm) | 19.6 ± 1.9 | 17.2 ± 3.3 |
| Stiffness (N/cm)* | 83.8 ± 4.9 | 62.4 ± 5.2 |
| Suture Retention (N) | 6.6 ± 1.6 | >2.0 |
| $T_{onset}$ ± (C)* | 72.5 ± 1.1 | 70.2 ± 0.3 |

Mechanical characterization was performed for both the six-layer ICL laminate crosslinked with 100 m*M* of EDC in 50% acetone/50% deionized water and the four-layer ICL laminate crosslinked with 1 m*M* of EDC in deionized water. Stiffness and strength were measured using 12.5-mm-wide strips with a gauge length of ~28 mm at a tare load of 0.5 g. Strength was calculated as the tension (force/width). Stiffness was defined as the maximum slope of the tension–strain curve. Suture retention was determined by pulling a 5-0 Surgilene suture with a 2-mm bite through the material at a constant rate and measuring the peak force. $T_{onset}$ was determined by differential scanning calorimetry.

*Denotes statistical difference, $p < 0.05$ after $t$ test was performed.

However, the acellular nature of the ICL decreases the need for crosslinking to minimize the antigenicity of residual cellular material.

Based on the results from these previous crosslinking studies, the second *in vivo* study using the rabbit hernia model evaluated the performance of four-layer laminates crosslinked with 1 m*M* of EDC in deionized water without acetone. The physical properties of these constructs are shown in Table I. As expected with less stringent crosslinking conditions, the $T_{onset}$ was lower. These patches were implanted in the rabbit hernia model for 3 months. The gross examination at explant showed results similar to the previous study. The patches were well healed with no evidence of either seroma or adhesion formation. However, the lower crosslinking did allow faster remodeling. As illustrated in Figure 5, there was a substantial amount of cellular infiltration and remodeling of the collagen after 90 days. Thus even under conditions in which this collagenous biomaterial is remodeled and replaced by host tissue, the repair function does not appear to be compromised.

### ICL calcification

Alizarin red staining for calcium showed no evidence of calcification in the patch material in any of the animals. Small focal areas of calcification in the host tissue were associated with the suture material but did not extend into the implant. This result was encouraging since one of the motivations for developing a reproducible, acellular material was to minimize the potential for calcification. To further investigate the likelihood of calcification of this material, the juvenile rat model was used to compare the response to ICL and glutaraldehyde-fixed bovine heart valve leaflets. This is a widely used, aggressive model for assessing calcification *in vivo*.[29] The tissues were implanted for 7 or 28 days, with three rats evaluated at each time point. All six of the rats that received glutaraldehyde-treated valve leaflets showed extensive calcification, as determined by Alizarin red staining. In contrast, even after 28 days, no calcification was detectable in the ICL (Fig. 6).

## DISCUSSION AND CONCLUSIONS

When using a biologic tissue as the base material for a surgical repair material, the purity and reproducibility of the material are of utmost importance. Remnant cell components in xenografts may contribute to calcification and/or immunogenic reactions.[30] In particular, lipids and DNA fragments are likely to play a role in calcification.[31] In addition, removal of cellular components from a potential biomaterial may reduce the antigenicity and potential for protracted inflammation. Based upon the lack of adverse tissue reactions to untreated SIS in numerous studies,[18] collagen scaffolds from the porcine intestinal tract appear to be less antigenic than other tissues, such as bovine pericardium. The desire to generate a well characterized and reproducible biomaterial from SIS led to the development of a chemical process that would produce an acellular collagenous tissue.

Characterization of the ICL material following our chemical cleaning process confirmed that there was no detectable ultrastructural damage or change in the thermal stability of the collagen fibers. In addition, the chemical cleaning method described herein does not involve the use of any proteolytic enzymes or detergents. Detergents such as sodium dodecyl sulfate have been suggested to inhibit cellular repopulation of collagenous substrates.[32] Having established a method for producing acellular ICL, this material was used to create GraftPatch™, a six layer laminate that has received FDA clearance for use in soft tissue repair.[23] The results of a 6-month study in the rabbit abdominal hernia model confirmed that the additional processing of the material did not affect its performance in this surgical repair application. Consistent with our previous rabbit studies as well as with reports using rodent and canine models,[13,14] minimal adhesion formation was observed. In contrast, postsurgical adhesion formations are a common complication associated with the use of synthetic materials.[21] The lack of adhesions may be due to the rapid reformation of a neomesothelial layer on the peritoneal surface of the patch.[33]

Unlike synthetic materials, GraftPatch™ became well integrated with the host tissue along the suture line, as shown by histology and the lack of suture line herniation. The mechanical data demonstrate that at 6 months the integration region was as strong as the adjacent abdominal tissue. In a previous study, Clark et al. showed the inherent strength of the untreated intestinal tissue by demonstrating that two-layer, nonfixed intestinal submucosal implants did not herniate in a large animal model.[13] In contrast, resorbable synthetic repair materials often degrade too quickly for the host tissue to form appropriate connective tissue and lead to graft failure.[6,7]

The multilayered laminates that we have evaluated were crosslinked with carbodiimide. Unlike the more traditional tissue stabilization methods that use fixatives such as glutaraldehyde, this process does not prevent cell ingrowth *in vivo*.[28] Although it is a widely used fixative, glutaraldehyde generally does not allow

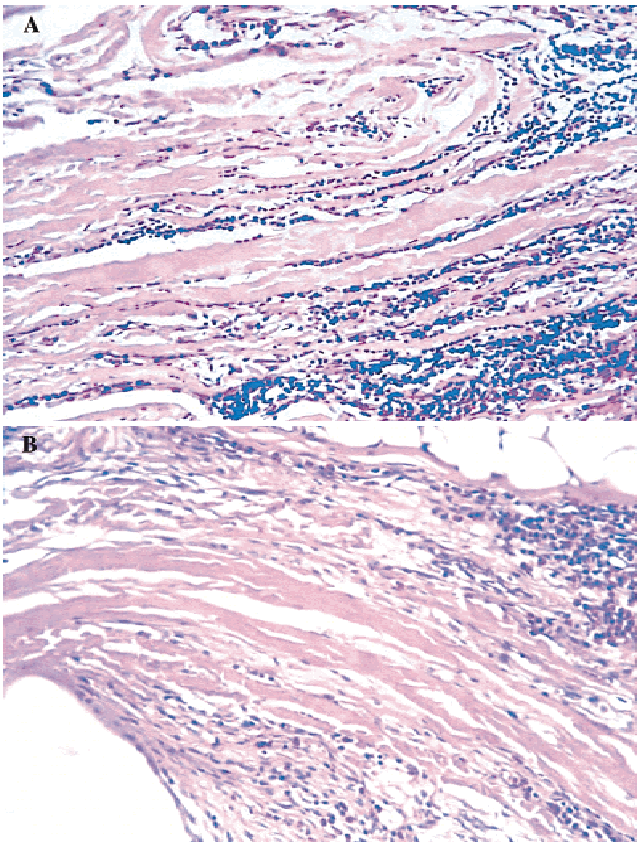


**Figure 5.** Effect of chemical crosslinking on cell infiltration. In this study, four-layer ICL patches were crosslinked with 1 m*M* of EDC and then implanted in the rabbit hernia model for 3 months. Explants were stained with H&E, as described. In contrast to the more highly crosslinked patches illustrated in Figure 3, cellular infiltration was much more extensive, as seen in these two representative samples. Original magnification, ×20.

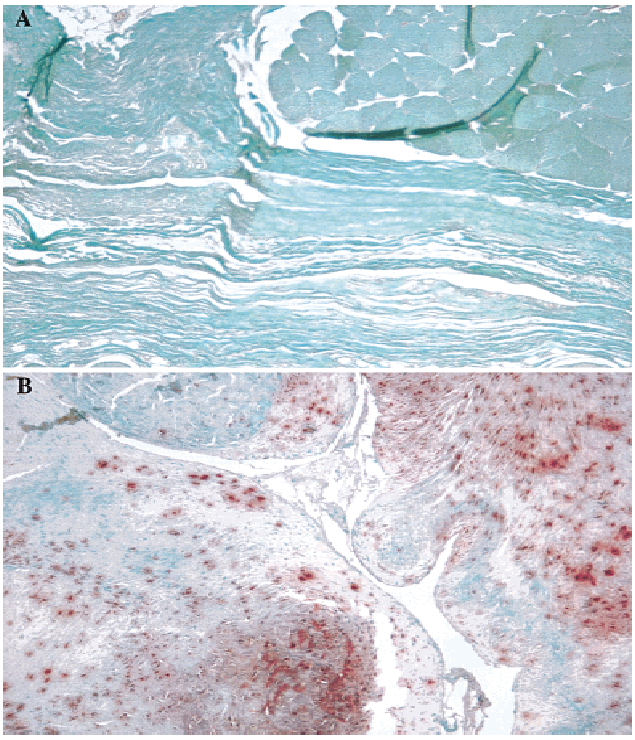


**Figure 6.** Assessment of ICL calcification. None of the explanted patches in the hernia study showed any calcification, as measured by Alizarin red staining. To evaluate the potential of this ICL material for calcification, a one-layer patch crosslinked with 1 m*M* of EDC was implanted subcutaneously in the juvenile (4 weeks) rat for 7 and 28 days ($n$ = 3 at each time point). As illustrated by the 28-day sample (A), no calcification was seen in any of the specimens. In contrast, glutaraldehyde-treated bovine heart valve leaflets (B) showed extensive calcification after 28 days using this model.

remodeling of the tissue, generates cytotoxic residuals, and is associated with calcification.[34,35] Initially, acetone was used to increase the efficiency of EDC crosslinking[36] and to increase the strength of adherence between layers of the patch. The high lamination strength may inhibit the layers from separating during implantation and thus eliminate subsequent seroma formation. However, the strong adherence between the collagen layers also may slow the remodeling by inhibiting interlayer cellular infiltration. In the hernia repair model, decreasing the crosslinking did increase remodeling of the collagen but did not compromise efficacy in that no reherniation or seroma formation was seen. Clearly, the desired physical properties and rate of remodeling of ICL constructs should be tailored to the requirements of the specific tissue repair/replacement application.

The ICL laminates provide an "off the shelf" biomaterial that can be sterilized using gamma irradiation and is easy to handle. Both the six-layer and the four-layer patches maintained the required strength in the rabbit hernia repair model and showed an extremely low incidence of adhesion formation. No calcification of the material was observed in the rabbit studies or in the more aggressive juvenile rat model. Furthermore, serologic analysis indicated that none of the rabbits showed any evidence of an immune response to the porcine ICL. The low antigenicity of this material is not surprising given the highly conserved nature of collagen and the lack of residual porcine cellular material.

In addition to hernia repair, ICL laminates may be suitable for other soft tissue repair and general applications, such as staple line reinforcement. Other applications could include cardiovascular procedures and materials, such as atrial and ventricular septal defect repair, vascular conduits (small diameter grafts, enlargement of right ventricular outflow tract, aortic root enlargement), vascular patches, or heart valve cusps. In fact, ICL tubes have been used successfully as small diameter vascular grafts in a rabbit carotid model.[37]

Although each application will have specific mechanical and biologic requirements, its biocompatibility, mechanical strength, and low adhesion formation make ICL a promising surgical repair material.

We would like to thank Dr. Joseph Laning for the immunologic analyses, Jeff Crews, Reuben Chevere, and Jonathan Thayer for assistance with the histologic evaluation and calcification studies, and Dr. Nathaniel Bachrach for insightful discussion during the preparation of this manuscript. We also appreciate the efforts of Dr. Raymond Connolly (Tufts New England Medical Center), who provided the hernia repair model.

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