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Glycosaminoglycan Content of Small Intestinal Submucosa: A Bioscaffold for Tissue Replacement

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ABSTRACT

Small intestinal submucosa (SIS) is a resorbable biomaterial that induces tissue remodeling when used as a xenogeneic tissue graft in animal models of vascular, urologic, dermatologic, neurologic, and orthopedic injury. Determination of the composition and structure of naturally occurring biomaterials such as SIS that promote tissue remodeling is necessary for the greater understanding of their role in wound healing. Since glycosaminoglycans (GAGs) are important components of extracellular matrix (ECM) and SIS is primarily an ECM-based material, studies were performed to identify the species of glycosaminoglycans present in SIS. Porcine SIS was chemically extracted and the extracts were analyzed for uronic acid. The extractable uronic acid content was determined to be 47.7 μmol/g (approximately 21 μg GAG/mg) of the dry weight of the SIS tissue. Using electrophoretic separation of GAGs on cellulose acetate membranes, hyaluronic acid, heparin, heparan sulfate, chondroitin sulfate A, and dermatan sulfate were identified. Digestion of specific GAGs with selective enzymes confirmed the presence of these GAG species. Two GAGs common to other tissues with large basement membrane ECM components, keratan sulfate and chondroitin sulfate C, were not detected in the SIS extracts. Identification of specific GAGs in the composition of the ECM-rich SIS provides a starting point toward a more comprehensive understanding of the structure and function of this naturally occurring biomaterial with favorable *in vivo* tissue remodeling properties.

INTRODUCTION

SMALL INTESTINAL SUBMUCOSA (SIS) is a resorbable xenogeneic bioscaffold that has induced constructive remodeling in animal models of arterial[1–3] and venous[1,4] vascular grafts, urinary bladder repair,[5] tendon[6] and ligament[7] repair, dura meter replacement,[8] and the treatment of skin wounds[9] and body wall defects.[10] Following implantation, host cells proliferate and differentiate into site-specific connective tissue structures, which appear to replace the SIS material within 90 days.[6] The remodeling process has been strongly associated with angiogenesis, cell migration, and differentiation, and deposition of extensive extracellular matrix (ECM).[1] The response is unique because the remodeled tissue is structurally and functionally similar to the injured tissue it was meant to replace.

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The mechanisms by which such cellular and tissue remodeling occurs in response to the SIS scaffold are unknown. Since SIS consists primarily of extracellular matrix, it is logical that naturally occurring structural proteins, glycosaminoglycans, proteoglycans, glycoproteins, and associated cell signaling factors are present in the material. These components, in conjunction with growth factors, the local healing environment, oxygen tension and other external factors, and the internal milieu of the host, compose a complex web of interactions that results in a healing response unique to SIS-treated wounds.

Glycosaminoglycans (GAGs) are essential components of the extracellular matrix that serve both structural and functional roles. In addition to providing structural integrity to the ECM, GAGs modulate the healing of soft tissues in several different ways. Such modulation includes organizing the deposition of collagen fibers,[11,12] stimulating angiogenesis,[13] inhibiting coagulation,[13,14] and initiating cell and tissue proliferation[15] and differentiation.[12]

During wound healing, growth factor–GAG interactions abound. Heparin chains may directly stimulate angiogenesis or may act as a part of a proteoglycan to stimulate the angiogenic effects of FGF-2.[16] Dermatan sulfate, as a component of several different proteoglycans, interacts with TGF-β1[17] and may help to control matrix formation and remodeling during the later phases of healing. In addition to regulating the function of TGF-β1, dermatan sulfate-containing proteoglycans regulate the structure of the ECM by controlling collagen fibril size, orientation, and deposition.

The purposes of the present study were to quantify the amount of GAGs in the porcine SIS biomaterial and to identify the types of GAGs that contribute to the total GAG content. Since much of SIS is ECM, it is logical that GAGs represent an important component of this biomaterial and serve several different important biological functions in the SIS-induced healing response.

MATERIALS AND METHODS

Reagents

Standard preparations of chondroitin sulfate A (CSA), chondroitin sulfate B (CSB), chondroitin sulfate C (CSC), hyaluronic acid (HA), and heparin (HEP) were purchased from Sigma, St. Louis, MO. Heparin sulfate (HS) standard was purchased from ICN Pharmaceuticals, Costa Mesa, CA. Type XIV bacterial protease, hyaluronidase (EC 4.2.2.1), chondroitinase AC (EC 4.2.2.5), chondroitinase B (no EC number), heparinase I (EC 4.2.2.7), and heparinase III (EC 4.2.2.8) were purchased from Sigma, St. Louis, MO. Titan III cellulose acetate membranes were purchased from Helena Labs, Beaumont, TX.

Extraction of GAGs from SIS

Glycosaminoglycans were extracted from porcine SIS following the method of Breen et al.[18] with minor modifications. Briefly, samples of SIS were frozen in liquid nitrogen, pulverized using a mortar and pestle, and then lyophilized. The SIS powder was weighed and was placed in a solution of chloroform–methanol at 4°C for 24 h with constant stirring. After 24 h, the liquid was poured off, the chloroform–methanol solution was changed, and the procedure was repeated. After 48 h, the suspension was centrifuged at 1400*g* (Beckman model GPR) for 20 min and the supernatant was discarded. The resulting precipitate was dried under vacuum pressure and was stored at −20°C until further use.

Each 50 mg sample of dried, defatted tissue was resuspended in 2 ml of 0.5 *M* sodium acetate buffer (pH 7.5), placed in a boiling water bath for 20 min, and incubated with protease (5 μg/mg tissue) for 12 h at 37°C. Additional enzyme was added to the digest to yield a concentration of 10 μg/mg tissue, and digestion was allowed to proceed for 48 more h. Calcium chloride (10 m*M*) was added to the digest to yield a calcium concentration of 1.0 m*M* and the samples were placed in a shaking incubator at 50°C for 24 h. The tissue digest was cooled to 4°C and trichloroacetic acid was added to a final concentration of 5%. The solution was allowed to sit for 10 min before being centrifuged at 17,300*g* (Beckman model J2-21) and 4°C for 20 min. The supernatant was saved and the precipitate was treated with 2 ml of 5% trichloroacetic acid and recentrifuged. The supernatants were pooled and were treated for 24 h at 4°C with three volumes of 5% potassium acetate in 100% ethanol. The suspension was centrifuged for 20 min at 17,300*g* and 4°C and the supernatant was discarded. The precipitate was treated sequentially with 2 ml of 100% ethanol, 2 ml of

a 1:1 v/v mixture of ethanol–ether, and 2 ml of 100% ether (with centrifugation between treatments). The ether was removed under continuous positive pressure air flow. The dried precipitate was resuspended at a concentration of 2 mg/ml in 0.075 *M* NaCl for immediate use, or was stored dry at −20°C.

Quantitation of GAG Amount

The total amount of GAGs in SIS isolate was evaluated according to the uronic acid analysis developed by Blumenkrantz and Asboe-Hansen[19] with minor modifications. Briefly, 200 μl of a sample of SIS isolate (2 mg/ml in 0.075 *M* NaCl) was added to 1.2 ml of 0.0125 *M* potassium tetraborate in concentrated sulfuric acid. The mixture was heated to 100°C for 5 min and was cooled in ice water. The cooled samples were treated with 20 μl of a solution of 3 mg *m*-hydroxydiphenyl in 10 ml of 0.5 *N* NaOH. Absorbance was read at 520 nm (Perkin Elmer Lambda 3B spectrophotometer) after 10 min.

Enzyme Degradation of Isolated GAG Chains

Enzymatic degradation of GAG chains was performed using the general procedures reported by Breen et al.[18] and Linhardt.[20,21] A sample of GAG isolate was resuspended at 2 mg/ml in 0.075 *M* NaCl and 50-μl aliquots of the solution were treated with enzyme as described below.

Digestion with hyaluronidase. The GAG isolate was suspended in 50 μl of sodium acetate–sodium chloride buffer, pH 5.4, containing 0.15 *M* each of sodium acetate and sodium chloride and 0.07 units of hyaluronidase. The solution was incubated for 1 h at 37°C in a shaking water bath. The solution was boiled for 1 min to denature the enzyme and was then cooled to room temperature for electrophoresis.

Digestion with chondroitinase AC and chondroitinase B. The GAG isolate was suspended in 50 μl of Tris–chloride–acetate buffer containing 0.05 *M* each of Tris, sodium acetate, and sodium chloride, adjusted to pH 8.0. To the buffer were added 1.5 μmol of albumin and 0.07 units of enzyme. The solution was incubated for 1 h at 37°C in a shaking water bath. The solution was boiled for 1 min to denature the enzyme and was then cooled to room temperature for electrophoresis.

Digestion with heparinase I and III. The GAG isolate was suspended in 50 μl of 5 m*M* sodium phosphate buffer, pH 7.6, containing 200 m*M* sodium chloride, 0.01% (w/v) albumin, and 3.75 units of enzyme. The solution was incubated for 8 h at 30°C in a shaking water bath. The solution was boiled for 1 min to denature the enzyme and was then cooled to room temperature for electrophoresis.

Electrophoretic Separation of Isolated GAGs

Electrophoresis was performed on Titan III cellulose acetate membranes. Each membrane was immersed in water to a height of 1.5 cm, and the opposite end was immersed in the buffer to be used during the run. A thin 2–4 mm band was left between the buffer soak and the water soak. Samples (2 mg/ml) containing a trace of phenol red were applied to the membrane in 1.0-μl aliquots. The membrane was placed in the electrophoresis chamber and was subjected to a constant voltage of 200 V for 2–3 min, until a thin, yellow line was visible at the boundary between the buffer and the water. The plate was then submerged in the electrophoresis buffer and allowed to soak for 2 min.

Electrophoresis was performed in one of three different buffer systems to optimally separate the different GAG species. Electrophoresis using a 0.05 *M* LiCl–0.01 *N* HCl buffer (pH 2.0, 20 min, 12 mA) was used to separate the chondroitin sulfate group of GAGs from heparan sulfate, heparin, and hyaluronic acid. It was possible to separate chondroitin sulfate A from the other GAGs in the tissue using a 0.05 *M* phosphate buffer system (pH 7.2, 15 min, 10 mA). The presence of hyaluronic acid and chondroitin sulfate B was confirmed using a buffer system containing 0.2 *M* $ZnSO_4$ (pH 5.1, 75 min, 6 mA).

Following electrophoresis, the separated GAGs were stained using a 10% (w/v) solution of alcian blue in 3% acetic acid (pH 2.5) for 10 min. After blotting excess stain, the membrane was destained for 5 min in an aqueous solution containing 5% acetic acid and 10% ethanol. If the background was not clear, the destaining solution was changed and the procedure repeated. The membrane was dried in room air under a ventilated hood at 25°C.

RESULTS

The GAG isolation protocol yielded 3.5 ± 1.3 mg of extract from each 50 mg sample of dried, defatted SIS. Analysis of the uronic acid present in the isolated sample showed that the uronic acid content of the extract was 47.7 μmol/g dry tissue weight. These values correspond to a total GAG content of 21 μg/mg of the dry weight of the porcine SIS tissue. The identical procedures performed using canine aorta as the source tissue yielded values for uronic acid of 28.2 μmol/g (12.4 μg GAG/mg dry tissue). These values correlate well with reports of GAG amounts in other tissues (Table 1).

Following extraction, GAGs were separated and identified using cellulose acetate electrophoresis. Because the structural differences in GAG types cause different GAG species to migrate at different rates in different buffer systems, three different buffer systems were used to optimally separate and identify all of the GAG types in the extraction mixture. An LiCl buffer system was used to separate HS, HEP, and HA from the chondroitin sulfates (Fig. 1A), and a phosphate buffer system was used to separate the chondroitin sulfate groups from each other (Fig. 1B). It should be noted that the presence of chondroitin sulfate B was confirmed using a buffer system containing zinc sulfate, because CSB and HEP migrate similarly in the phosphate buffer (data not shown).

Following initial separation and identification, GAG types were confirmed using selective enzyme digestion and comparative electrophoresis. Samples of extracts were subjected to treatment with heparinase or heparitinase. Using an LiCl buffer system for electrophoresis, it was possible to confirm the presence of heparin (Fig. 2A) and heparan sulfate (Fig. 2B) in the SIS-derived sample.

Although heparinase and heparitinase selectively cleave heparin and heparan sulfate GAG chains, they are not totally selective for one GAG species or the other. The primary substrate for heparinase is heparin, while the primary substrate for heparitinase is heparan sulfate. Because of the similarity in structure of the GAG chains, cross-digestion occurs. Evidence of cross-digestion can be seen in Figure 2A and B but it does not interfere with the interpretation of the results.

A $ZnSO_4$ buffer system was used to confirm the presence of CSB (Fig. 3A) and HA (Fig. 3B) in the SIS isolate. To prove the presence of CSB in the matrix, a sample of GAG extract was treated chondroitinase B, an enzyme selective for the dermatan sulfate GAG chain; it does not digest CSA or CSC. Hyaluronidase treatment of an SIS extract similarly confirmed the presence of HA in the material. It should be noted that is not possible to separate CSA and HEP in this buffer system because of the similarity in their mobilities.

A phosphate buffer system was used to separate CSA from the other components in the SIS–GAG iso-

TABLE 1. REVIEW OF GAG CONTENT OF VARIOUS TISSUES

Tissue	*Method of analysis*	*μmol UA/HXA/g dry tissue*	*μg GAG/mg dry tissue*	*Reference*
Fetal skin	Hexosamine	18.9	8.0	Breen[18]
Fetal skin	Uronic acid	19.2	8.4	Breen[18]
Adult skin, abdominal	Hexosamine	5.5	2.3	Breen[18]
Adult skin, neck	Hexosamine	6.36	2.7	Breen[22]
Adult skin	Uronic acid	2.14 μg UA/mg	5.4	Tajima[23]
Adult skin, diseased	Uronic acid	3.08 μg UA/mg	7.7	Tajima[23]
Lateral meniscus	Hexosamine	8.94	3.8	Adams[24]
Medial meniscus	Hexosamine	12.18	5.2	Adams[24]
Articular cartilage	Uronic acid	Not reported	8.5	McNicol[25]
Fetal sclera	Hexosamine	14.2	6.1	Breen[18]
Adult sclera	Hexosamine	59.2	25.3	Breen[18]
Adult cornea	Hexosamine	122	52.1	Breen[18]
Canine lat meniscus	Uronic acid	20.0 μg UA/mg	50	Adams[26]
Canine med meniscus	Uronic acid	10.2 μg UA/mg	25.5	Adams[26]
Small intestinal submucosa	Uronic acid	47.7	21	
Canine aorta	Uronic acid	28.2	12.4	

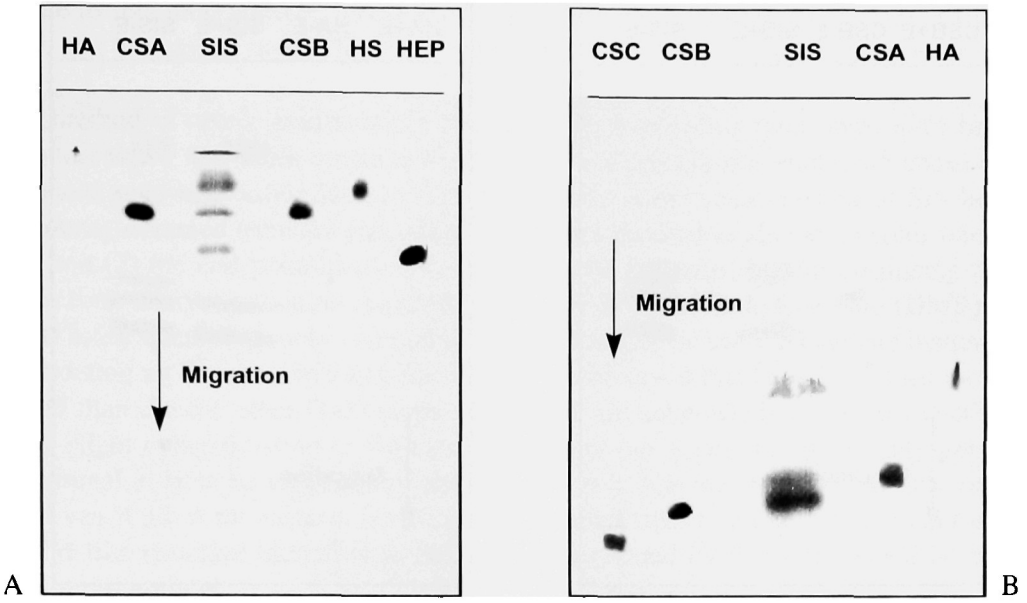

FIG. 1. Cellulose acetate electrophoresis of the SIS–GAG isolate with preparations of GAG standards. (A) LiCl buffer system. (B) Phosphate buffer system. CSA, chondroitin sulfate A; CSB, chondroitin sulfate B (dermatan sulfate); CSC, chondroitin sulfate C; HA, hyaluronic acid; HS, heparan sulfate; HEP, heparin; SIS, small intestinal submucosa GAG extract.

late. To confirm the presence of this GAG in the material, the sample was treated with chondroitinase AC. This enzyme selectively digests the CSA and CSC GAG chains, but leaves CSB intact. In addition to confirming the presence of CSA in the material, it was also possible to determine that CSC was absent from the SIS–GAG isolate (Fig. 4).

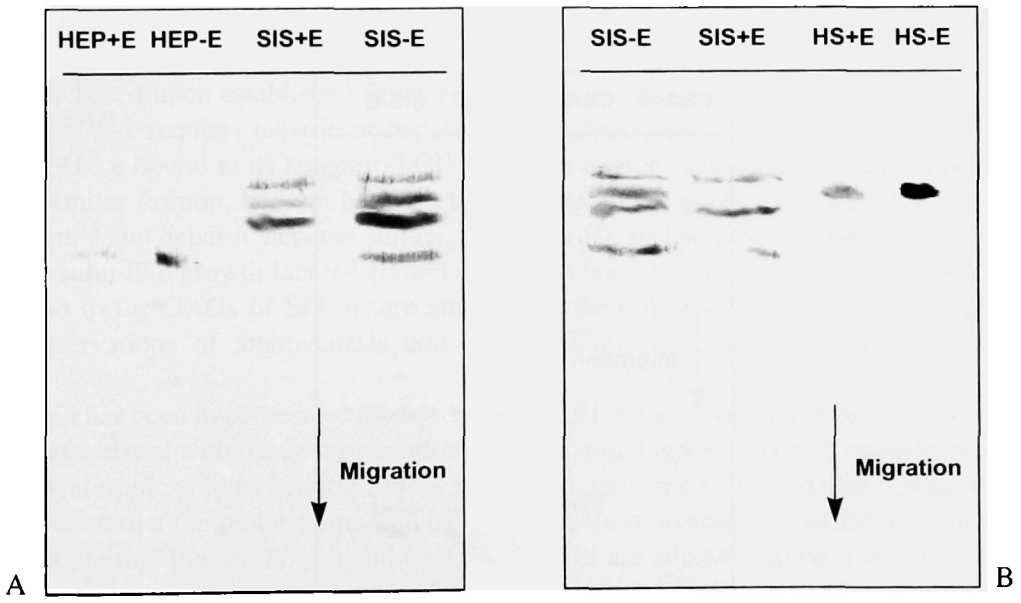

FIG. 2. Cellulose acetate electrophoresis of the SIS–GAG isolate using an LiCl buffer system to confirm the presence of heparin and heparan sulfate in the material. (A) Lane 1, HEP standard digested with heparinase (EC 4.2.2.7); lane 2, HEP standard; lane 3, SIS–GAG isolate digested with heparinase; lane 4, SIS-GAG isolate. (B) Lane 1, SIS–GAG isolate; lane 2, SIS-GAG isolate digested with heparitinase (EC 4.2.2.8); lane 3, HS standard digested with heparitinase; lane 4, HS standard.

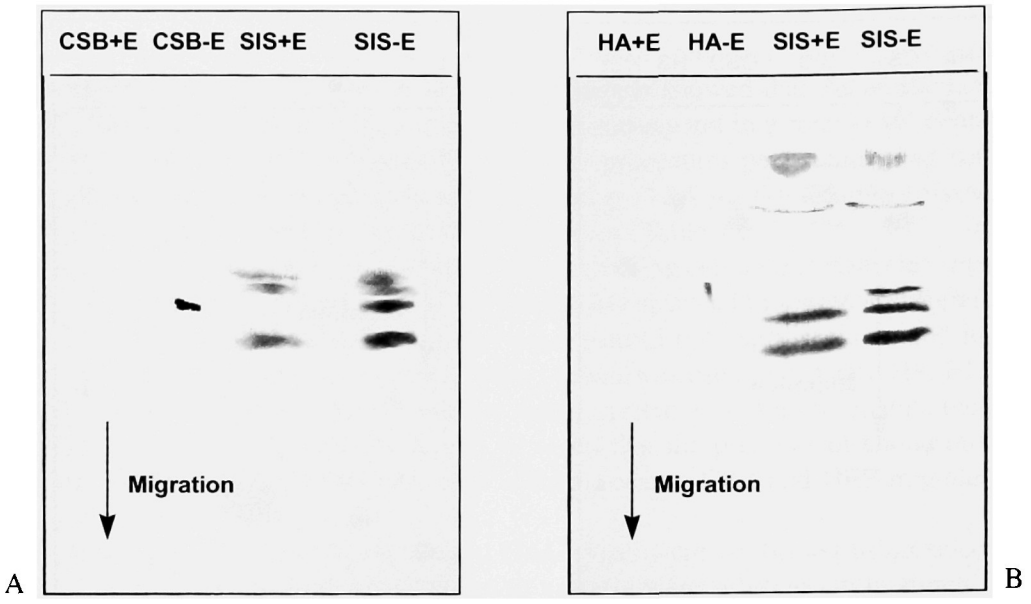

FIG. 3. Cellulose acetate electrophoresis of the SIS–GAG isolate using a $ZnSO_4$ buffer system to confirm the presence of hyaluronic acid and chondroitin sulfate B in SIS. (A) Lane 1, CSB standard digested with chondroitinase B (no EC number); lane 2, CSB standard; lane 3, SIS-GAG isolate digested with chondroitinase B; lane 4, SIS–GAG isolate. (B) Lane 1, HA standard digested with hyaluronidase (EC 4.2.2.1); lane 2, HA standard; lane 3, SIS–GAG isolate digested with hyaluronidase; lane 4, SIS–GAG isolate.

DISCUSSION

The analysis of uronic acid or hexosamine is recommended as the method of choice for obtaining the total GAG concentration of an unknown sample.[18] We have quantitated the uronic acid content of porcine SIS and have determined it to be 47.7 μmol/g dry tissue weight. This value corresponds to a total GAG

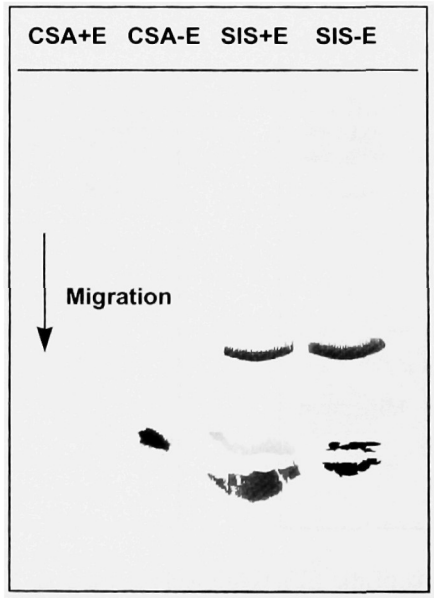

FIG. 4. Cellulose acetate electrophoresis of the SIS–GAG isolate using a phosphate buffer system to confirm the presence of chondroitin sulfate A in the SIS. Lane 1, CSA standard digested with chondroitinase AC (EC 4.2.2.5); lane 2, CSA standard; lane 3, SIS-GAG isolate digested with chondroitinase AC; lane 4, SIS-GAG isolate.

content in the tissue of 21 μg/mg dry tissue weight. We have also determined that five different species of GAGs contribute to this total amount. These GAGs are chrondroitin sulfate A, dermatan sulfate, heparin, heparan sulfate, and hyaluronic acid. The relative amounts of the different species were not determined in the present study.

The specific method of uronic acid analysis of tissues has evolved as procedures have been developed that are more quantitative and more sensitive than previous assays. In this study, we chose to employ the method of Blumenkrantz and Asboe-Hansen[19] to evaluate the uronic acid content of SIS because (1) the high degree of purity obtained from the extraction procedures resulted in almost no color interference from neutral sugars, and (2) the end point assay was more reliable (reproducible) in estimating the total GAG content than the time-course evaluation required by the 1,9-dimethylmethylene blue (DMB) assay. In addition, the DMB assay quantitates only sulfated GAGs, and cannot be used to evaluate hyaluronic acid levels.[27] While validating the uronic acid assay for our use, we observed that heparin yielded more chromogen per unit of GAG than did the other GAG types. Therefore, we acknowledge that the heparin in our samples results in a slight overestimation of the GAG content of our tissue, but we readily point out that interference is minimal at best, as estimates of heparin in whole porcine intestine are only on the order of 0.22 μg/mg.[28] Even if all of the heparin commercially isolated from hog intestine is found in the specific, trilaminate, ECM-like structure identified as SIS, the error created by this total would be minor in comparison to the overall amount.

Since SIS represents a specific trilaminate structure of the small intestine, the composition of SIS must be established independently from any other reports specifying the overall GAG content of hog intestine. This study indicates that several types of GAGs are present in SIS, a structure that consists of the tunica submucosa, the tunica muscularis mucosa, and the less well developed stratum compactum.[1]

It is not surprising that a wide variety of GAG types are found in this tissue since the structure, especially the superficial layers, essentially serves as the basement membrane for the rapidly dividing cell population of the tunica mucosa. The amount of GAG in SIS corresponds well with the amounts reported in other basement membrane containing tissues, such as canine meniscal tissue[26] and adult sclera.[18] Adult cornea, which consists almost entirely of basement membrane, contains significantly more GAGs,[18] while skin contains significantly less.[18,23]

The remodeling phenomenon that occurs in mammals following implantation of xenogeneic SIS includes rapid neovascularization and early mononuclear cell accumulation. Mesenchymal and epithelial cell proliferation and differentiation are observed by 1 week after *in vivo* implantation; extensive deposition of new ECM occurs almost immediately. The role of the SIS GAGs in these processes is unknown, but speculation can be made based upon established findings of others.

For example, FGF-2 requires heparin or heparan sulfate containing molecules for high affinity binding to its receptor.[16] Once bound to its receptor, FGF-2 induces angiogenesis, cell differentiation, and cell proliferation. In a similar fashion, heparin has also been shown to potentiate EGF and PDGF-induced fibroblast proliferation,[15] and heparin, heparan sulfate, and dermatan sulfate have recently been shown to inhibit the binding of insulin-like growth factor-I (IGF-1) to its binding proteins.[29] It is plausible that such growth factors are bound to the GAGs of SIS, or are attracted to the GAGs of SIS after implantation, given the morphologic observations of angiogenesis and cell proliferation reported in numerous *in vivo* studies.[2–7,9,10,30]

Hyaluronic acid has been hypothesized to sequester TGF-β1 in the extracellular matrix.[31] In fetal wounds, it has also been associated with tissue regeneration and the rapid, highly organized deposition of collagen.[12] High levels of hyaluronic acid in healing tissues have been associated with scarless wound repair,[12] leading to the postulation that the sequestering of TGF-β1 by hyaluronic acid may inhibit the formation of scar tissue. It is plausible that the levels of hyaluronic acid in SIS are adequate to bind the amount of TGF-β1 locally released in response to injury, thus explaining the paucity of scar tissue deposition and the subsequent tissue remodeling seen in response to SIS implantation *in vivo*.

Dermatan sulfate can interact with growth factors as part of a proteoglycan,[17] but can also act independently as an antithrombotic agent by inhibiting the thrombin-induced aggregation of platelets and may activate the fibrinolytic pathway by causing the release of tissue phasminogen activator (tPA).[13] Dermatan sulfate can act as an anticoagulant by inhibiting thrombin formation, either directly through heparin cofac-

tor II or antithrombin II or indirectly through protein C activation.[14] In previous vascular studies in which SIS was used as large- and small-diameter arterial and venous grafts, thrombosis was not a significant problem when the stratum compactum was used as the blood contact surface.[2–4] It is plausible that dermatan sulfate or a dermatan sulfate-containing proteoglycan present in the material contributes to the thromboresistant properties that have been observed.

Since GAGs represent the posttranslational glycosylation of proteoglycan core proteins, it is logical that a variety of proteoglycans will be found in SIS. The relative role in tissue remodeling for the ECM GAGs reported in this study and the other ECM constituents that are certain to be found in SIS remains to be determined.

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