Degradation and Remodeling of Small Intestinal Submucosa in Canine Achilles Tendon Repair

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Background: Extracellular matrix derived from porcine small intestinal submucosa is used for the repair of musculotendinous tissues. Preclinical evaluation and clinical use have suggested that small intestinal submucosa extracellular matrix degrades rapidly after implantation and can be replaced by host tissue that is functionally and histologically similar to the normal tissue.

Methods: The present study analyzed the temporal degradation of a ten-layer multilaminate device of small intestinal submucosa extracellular matrix used for the repair of canine Achilles tendon and examined the corresponding histological appearance of the remodeled tissue during the course of scaffold degradation. Devices were fabricated from small intestinal submucosa extracellular matrix labeled with ^{14}C. The amount of ^{14}C remaining in the remodeled graft was measured by liquid scintillation counting at three, seven, fourteen, twenty-eight, sixty, and ninety days after surgery. Blood, urine, feces, and other parenchymal tissues were also harvested to determine the fate of scaffold degradation products. Tissue specimens were prepared for routine histological analysis to examine the morphology of the remodeled graft at each time-point.

Results: The small intestinal submucosa extracellular matrix graft degraded rapidly, with approximately 60% of the mass lost by one month after surgery, and the graft was completely resorbed by three months after surgery. The graft supported rapid cellular infiltration and host tissue ingrowth. By ninety days after surgery, the remodeled small intestinal submucosa extracellular matrix consisted of a dense collagenous tissue with organization, cellularity, and vascularity similar to that of normal tendon.

Conclusions: Small intestinal submucosa extracellular matrix is rapidly degraded after implantation for the repair of a musculotendinous tissue in this canine Achilles tendon repair model and is replaced by the deposition and organization of host tissue that is histologically similar to that of normal tissue.

Clinical Relevance: The present study provides insight into the degradation and remodeling of extracellular matrix derived from porcine small intestinal submucosa, a biologic scaffold that has been used clinically for musculotendinous applications.

Naturally derived extracellular matrix scaffolds are used for the repair of musculotendinous tissues, including the Achilles tendon and the rotator cuff[1,2]. Porcine small intestinal submucosa is one such scaffold material that has been evaluated extensively in preclinical studies for cardiovascular, urological, dermal, and musculoskeletal applications[3-11], and it has been used clinically in patients for reconstruction of damaged or missing tissues in many body systems[1,2,12-15]. Several preclinical animal studies have demonstrated that extracellular matrix derived from porcine small intestinal submucosa can successfully remodel into functional tendon or ligament tissues[8,11,16-18], but the rate of scaffold degradation in load-bearing musculotendinous applications has not been determined.

Although five naturally derived extracellular matrix scaffolds, including Restore (DePuy Orthopaedics, Warsaw, Indiana), CuffPatch (Arthrotek, Warsaw, Indiana), GraftJacket (Wright Medical Technology, Arlington, Tennessee), TissueMend (TEI Biosciences, Boston, Massachusetts), and Zimmer Collagen Repair Patch (Zimmer, Warsaw, Indiana) are currently on the market, the only quantitative assessment of the rate of degradation for any extracellular matrix scaffold was performed for native small intestinal submucosa extracellular matrix in the context of urinary bladder repair by integrally labeling the scaffold with ^{14}C[19]. The ^{14}C is an effective tracking

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biomolecule in an animal model because of its stability and the development of sensitive detection methods[20,21]. In the present study, ^{14}C-labeled small intestinal submucosa extracellular matrix scaffold was used to determine the rate of degradation and the fate of the degradation products when the scaffold was used as an interpositional graft in the canine Achilles tendon. In addition, histological analysis was performed to determine the temporal changes in the appearance of the small intestinal submucosa extracellular matrix scaffold during the remodeling process.

Materials and Methods

The production of ^{14}C-labeled extracellular matrix derived from small intestinal submucosa and the surgical procedure performed as part of this study were approved by the University of Pittsburgh Radiation Safety Committee and the Institutional Animal Care and Use Committee of the University of Pittsburgh. The animal care complied with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Preparation of ^{14}C-Labeled Small Intestinal Submucosa Extracellular Matrix

The methods for labeling the small intestinal submucosa extracellular matrix with ^{14}C have been reported previously[19]. Briefly, piglets were given weekly intravenous injections of 10 μCi of ^{14}C-labeled proline (256 mCi/mmol, 50 μCi/mL; Amersham Life Science, Piscataway, New Jersey) beginning at three weeks of age and continuing until the time the animal was killed. At approximately twenty-six weeks of age, the animals were killed and the small intestine was harvested. The small intestine was mechanically abraded to remove the tunica muscularis externa and the majority of the tunica mucosa. The remaining tunica submucosa and basilar portion of the tunica mucosa was then disinfected and decellularized in a 0.1% peracetic acid solution followed by two rinses each in phosphate-buffered saline solution and deionized water[22].

To produce each device for the Achilles tendon repair, a ten-layer construct of the hydrated sheets of ^{14}C-labeled small intestinal submucosa extracellular matrix was created such that the longitudinal axis of each sheet of the small intestinal submucosa was oriented in the same direction. The small intestinal submucosa has a preferred collagen fiber orientation along the long axis of the small intestine[23]; therefore, creating the construct in this uniaxial orientation provided increased strength in the direction aligned with the repaired tendon. The ten-layer construct was then laminated by a vacuum-pressing technique[22]. The construct was placed between two perforated stainless-steel sheets, and the stainless-steel plates were placed between sheets of sterile gauze. The entire construct was then sealed in vacuum bagging and was subjected to a vacuum of 710 to 735 mmHg (94.8 to 98.2 kPa) for approximately eight hours. The multilaminate device was cut into 2 × 3-cm sheets, with the preferred fiber direction corresponding to the 2-cm axis. The final construct was terminally sterilized by exposure to ethylene oxide.

Study Design

Twelve adult female mongrel dogs weighing approximately 20 kg were divided into six groups of two dogs each. All dogs were subjected to segmental resection of 1.5 cm of the Achilles tendon, and the defect was repaired with the bioscaffold composed of ^{14}C-labeled small intestinal submucosa extracellular matrix. One group of animals was killed at each of the following time-points: three, seven, fourteen, twenty-eight, sixty, and ninety days after surgery.

Surgical Procedure

A surgical plane of anesthesia was produced in each dog by induction with 12 to 25 mg/kg of sodium thiopental administered intravenously followed by intubation and maintenance with 2% to 3% isoflurane. The animals were prepared for surgery by shaving the surgical site and scrubbing with Betadine (povidone-iodine) solution. An antibiotic (cephalexin) was administered intramuscularly preoperatively and then postoperatively twice a day for seven days. For each dog, the Achilles tendon of a randomly selected hind limb was dissected free of surrounding tissue and a 1.5-cm segment was excised from the mid-tendinous region. The tendon defect was repaired with a 2 × 3-cm graft of the ^{14}C-labeled scaffold folded accordion style, such that 2 to 3 mm of the 2-cm length overlapped each end of the tendon defect, with use of 4-0 Prolene (polypropylene) suture to mark the ends of the defect (Fig. 1). The paratenon was sutured with 4-0 PDS (polydioxanone) suture, and the skin incision was closed with use of standard surgical technique. The animals were placed in a tube splint (a modified Robert Jones bandage) to allow immediate partial weight-bearing without allowing the tendon unit to stretch. The splint was removed after twenty-eight days, and the dogs were allowed to walk freely without external support.

Sample Collection

At regular time-intervals after surgery, urine, blood, and fecal samples were collected to determine the route of elimination of the degraded ^{14}C-labeled materials from the body. At the time the animals were killed, the remodeled graft and the contralateral, uninvolved control Achilles tendon were harvested for histological examination and quantification of the ^{14}C concentration within the tissue. The specimen prepared for histological analysis contained remodeled normal tendon and remodeled extracellular matrix so that the transition between the two tissues could be evaluated. The specimen for ^{14}C analysis was taken from the middle of the remodeled graft. Tissue samples were collected from the skin, skeletal muscle, mesenteric fat, spleen, liver, kidney, pancreas, lymph nodes, lung, heart, and brain to determine whether ^{14}C had collected in these organs.

^{14}C Analysis

The radioactivity in each sample was measured by liquid scintillation counting with use of a B-counter (model LS 1800; Beckman Coulter, Somerset, New Jersey). For urine, 0.2 mL of urine was added directly to 10 mL of scintillation fluid (Ul-

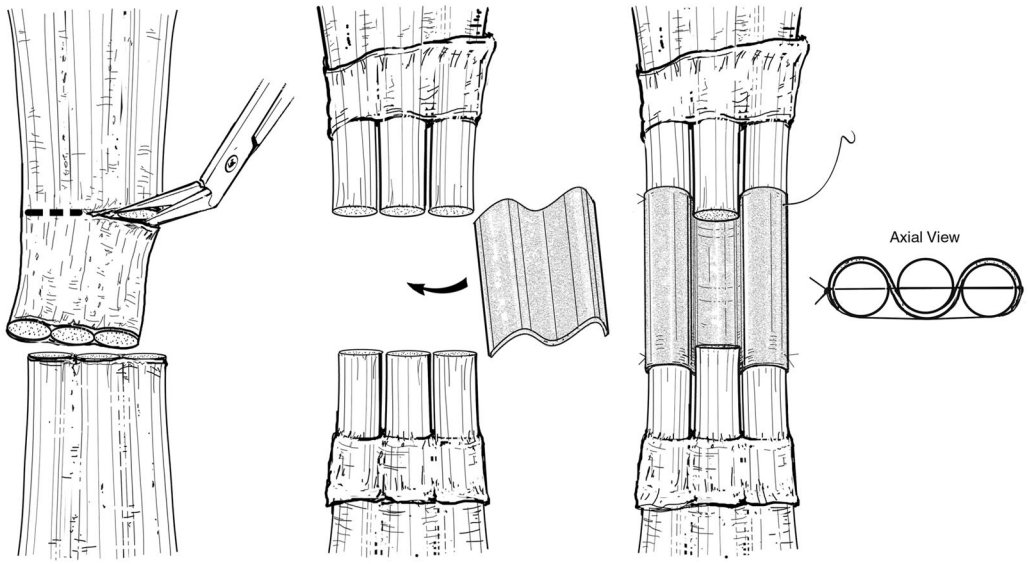

Fig. 1

Surgical placement of the small intestinal submucosa extracellular matrix graft in the Achilles tendon defect. The 2 x 3-cm graft was folded, accordion style, and woven through the bundles of the Achilles tendon and fixed with a mattress suture.

tima Gold; PerkinElmer, Boston, Massachusetts). The radioactivity in the urine was reported as counts per minute per milliliter (cpm/mL). For the extracellular matrix graft at time-zero, the remodeled tendon, and all other harvested tissues, approximately 80 mg of tissue was incubated with 1 mL of tissue solubilizer (Solvable; Packard Instruments, Meriden, Connecticut) at 50°C for two to four hours. Ten milliliters of scintillation fluid was then added to each sample, and the radioactivity was determined. The tissue radioactivity was reported as counts per minute per gram (cpm/g).

Blood samples were incubated with a 1:1 mixture of tissue solubilizer to isopropanol (Fisher Scientific, Pittsburgh, Pennsylvania) at 60°C for thirty minutes. The samples were then treated with 30% H_2O_2 (Spectrum, Gardena, California), to quench endogenous peroxidase activity, and were incubated at 60°C for an additional thirty minutes. Fifteen millili-

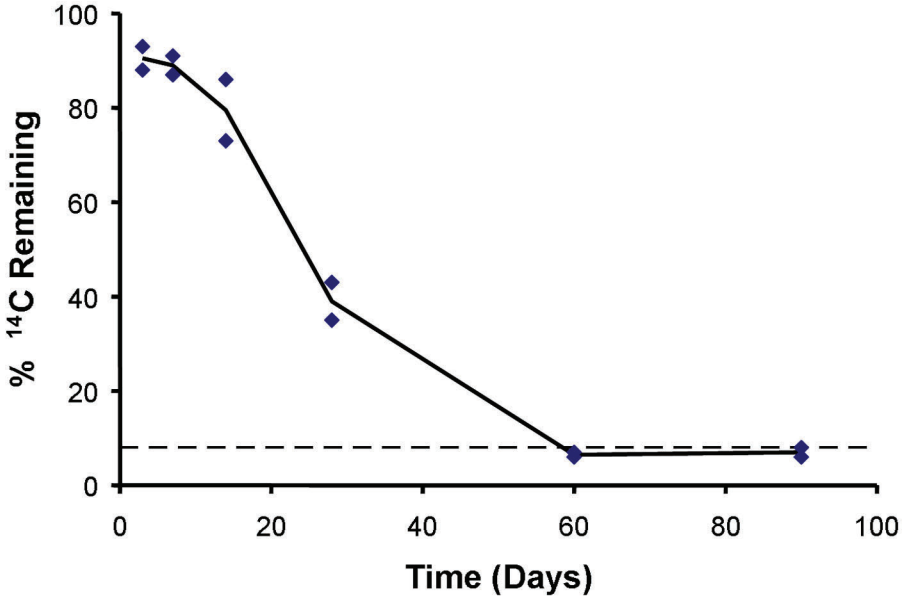

Fig. 2

Graphic representation of the average percentage of ^{14}C remaining at the graft site at each time-point. The dashed line indicates the background ^{14}C level observed at approximately 8%. The actual measurements for the percentage of ^{14}C are included next to each data point.

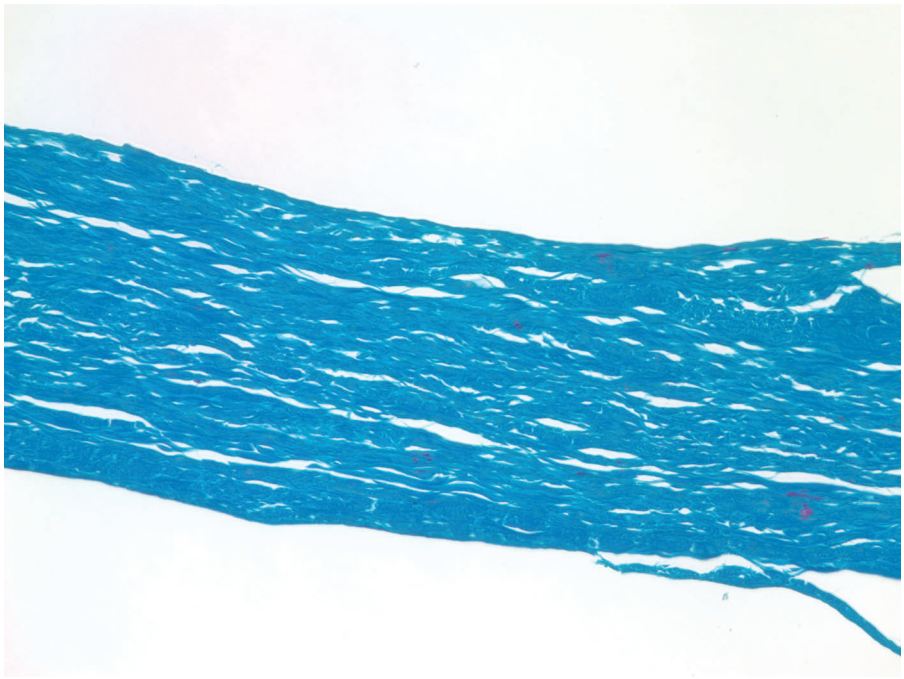

Fig. 3

Histological section of the small intestinal submucosa extracellular matrix device prior to implantation. The section shows a laminate structure of dense organized collagen and no cellularity (×20).

ters of scintillation fluid was added to each sample, and the radioactivity was measured. The blood radioactivity was reported as counts per minute per milliliter (cpm/mL). To measure the radioactivity in a fecal sample, approximately 20 mg of feces was rehydrated with 0.1 mL of water for thirty minutes at room temperature. One milliliter of tissue solubilizer was added to each sample, and the samples were incubated in a 50°C oven for one to two hours. Isopropanol (0.05 mL) was added before the final two-hour incubation at 50°C. Peroxidases were quenched with 0.2 mL of 30% H_2O_2, and 10 mL of scintillation fluid was added. The radioactivity of the feces was reported as counts per minute per gram (cpm/g).

Histological Analysis

A portion of each tendon was harvested and trimmed to include the remodeled extracellular matrix graft and the adjacent native tendon proximal and distal to the remodeled graft. The tissue was fixed in 10% neutral buffered formalin, sectioned longitudinally, and was stained both with hematoxylin and eosin and with Masson trichrome for histological examination. The cellularity of the remodeled graft was assessed by obtaining five photomicrographs from each section at 40× magnification for subsequent counting with use of MetaVue software (Universal Imaging, Downingtown, Pennsylvania). The cells were classified as neutrophils or mononuclear cells. The data are presented as the average number of cells per high-power (40×) field. For statistical comparisons, the Student t test was used to compare differences between groups and significance was set at a p value of <0.05.

Results

Surgical Outcome

All animals recovered well from surgery, and they tolerated the splinting for one month. After removal of the splint, all animals returned to a normal gait. Gross evaluation of the repaired tendons after the animals were killed showed no evidence of tendon rupture at any time during the postoperative period. By visual inspection, there did not appear to be any change in the total tendon length or the length of the repair site.

^{14}C Analysis

The values for ^{14}C concentration of the remodeling extracellular matrix tissue for each dog are listed, and the average values at each time-point are plotted in Figure 2. Approximately 10% of the scaffold material had been degraded and removed from the site of remodeling by as early as three days after surgery. By fourteen days, approximately 20% of the scaffold material had been degraded, and, at twenty-eight days, approximately 60% of the scaffold material had been degraded. By sixty days after surgery, the amount of ^{14}C in the remodeled tissue was equal to background levels (<8%), indicating complete scaffold degradation and removal from the site of remodeling.

The only other tissues in which ^{14}C activity could be detected were blood and urine. The blood showed positive ^{14}C activity at three and seven days after surgery. The urine showed ^{14}C activity at three, seven, and fourteen days after surgery. All other tissue samples were negative (i.e., below background values) for ^{14}C activity at all time-points.

TABLE I Cellularity of Normal Tendon and Remodeled Graft*

Tissue	Mononuclear Cells	Neutrophils	Total Cells
Native tendon	54 ± 17	0	54 ± 17
Small intestinal submucosa device	0	0	0
Day 3	53 ± 15	49 ± 10†	102 ± 15†
	58 ± 8	86 ± 34†	144 ± 29†
Day 7	154 ± 35†	4 ± 6	158 ± 31†
	166 ± 30†	0	166 ± 30†
Day 14	377 ± 15†	14 ± 8†	390 ± 23†
	152 ± 38†	0	152 ± 38†
Day 28	105 ± 31†	0	105 ± 31†
	184 ± 24†	0	184 ± 24†
Day 60	116 ± 24†	0	116 ± 24†
	146 ± 67†	0	146 ± 67†
Day 90	63 ± 5	0	63 ± 5
	82 ± 24	0	82 ± 24

*All data are given as the average number (and standard deviation) of cells per five high-power (40×) fields. †Compared with the cellularity for the native tendon, the difference was significant ($p < 0.05$).

Histological Analysis

Histological examination of the extracellular matrix graft prior to implantation showed a laminate structure of dense, organized, collagenous tissue with no cellularity (Fig. 3).

Tissues harvested from the remodeling graft and tendon showed that, at three days after surgery, the acellular scaffold material had been infiltrated with a large number of host inflammatory cells characterized by an approximately equal number of neutrophils and mononuclear cells (Table I). Early evidence for scaffold degradation included the separation of

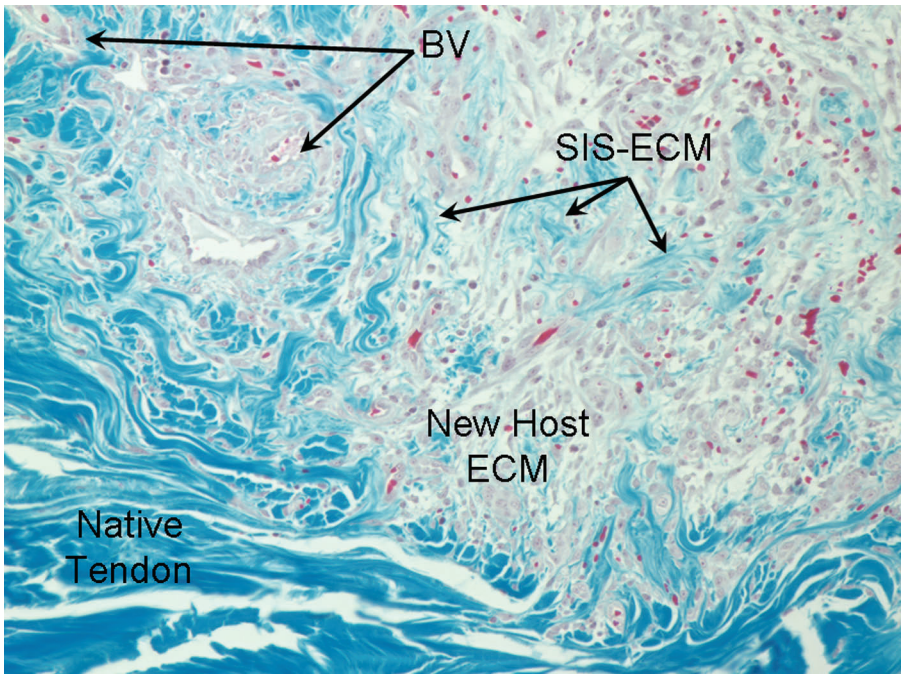

Fig. 4

Histological section of the interface between the remodeled graft (SIS-ECM) and the remodeled Achilles tendon at seven days after surgery (Masson trichrome, ×40). The section shows an abundant cellular infiltrate that consists almost exclusively of mononuclear cells surrounded by new host extracellular matrix. There was also evidence of angiogenesis, with blood vessels (BV). A moderate degree of scaffold degradation is indicated by the marked change in the architecture of the graft.

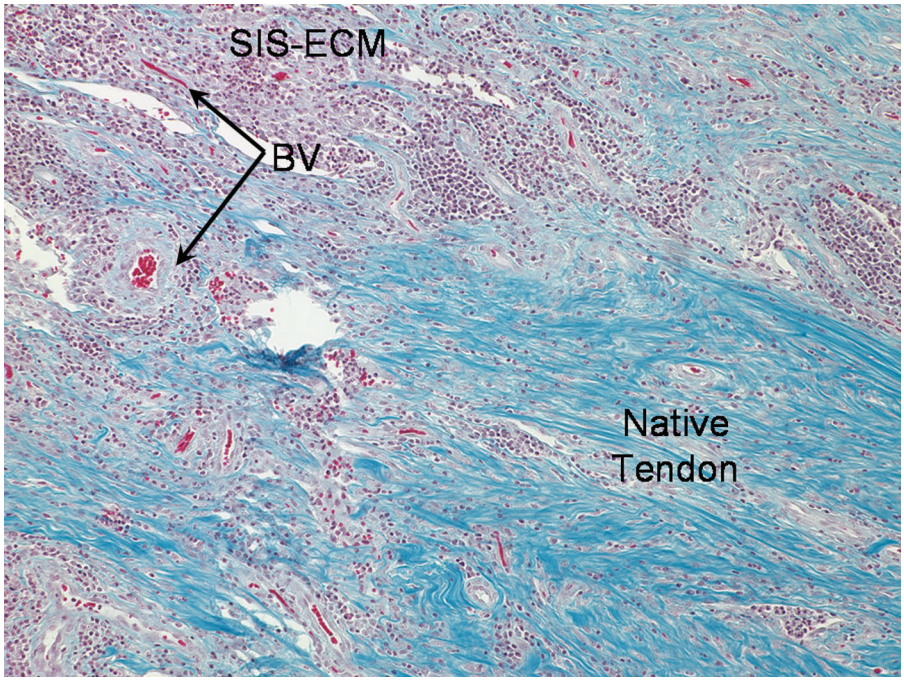

Fig. 5

Histological section of the interface between the remodeled graft (SIS-ECM) and the remodeled Achilles tendon at fourteen days after surgery (Masson trichrome, ×20). The section shows that the entire scaffold material is infiltrated with host cells that are almost exclusively mononuclear in morphology. There is also evidence of angiogenesis, with blood vessels (BV). The graft was well integrated with the newly deposited host tissue.

the individual sheets within the multilaminate structure by infiltrating host cells and a loss of distinct scaffold architecture at the periphery of the graft material. There was no histological evidence for deposition of new host extracellular matrix on the basis of the distinct margins of the graft and the cut end of the native tendon.

By seven days (Fig. 4), the cellular infiltrate had increased in amount and consisted almost exclusively of mononuclear cells (Table I). A moderate degree of scaffold degradation was present, and the host-cell infiltrate had progressed from the periphery of the graft material to the center of the graft material. New host extracellular matrix was present at the remodeling site and was represented by the presence of amorphous connective tissue between the native tissue and the graft material.

By fourteen days (Fig. 5), the entire scaffold material was infiltrated with host cells that were primarily mononuclear in morphology with only a few scattered neutrophils being present. This time-point showed the greatest cellularity over the course of remodeling with an average (and standard deviation) of 271 ± 133 cells per high-power (40×) field (Table I). Graft degradation was characterized by separation of the various layers of the multilaminate sheet and loss of a distinct boundary between newly deposited host extracellular matrix and the original graft material. Deposition of new host-derived extracellular matrix was present, especially at the periphery of the graft. The distinct line of demarcation between the scaffold material and the cut end of the native Achilles tendon to which it was attached was no longer evident by fourteen days.

By twenty-eight days (Fig. 6), there was a decrease in the cellularity similar to the numbers observed at seven days after implantation (Table I). The mononuclear cells were still abundant and were uniformly distributed throughout the remodeled extracellular matrix graft. No neutrophils were observed. There was loss of almost all morphologic evidence of the graft material, which was replaced by a relatively homogeneous deposition of new host-derived extracellular matrix material. The remodeled extracellular matrix was beginning to show regions of organization by this time-point.

At sixty days (Fig. 7), there was replacement of the graft material by organized, aligned host-derived extracellular matrix and spindle cells consistent with the morphologic appearance of fibroblasts. The total cellularity was not different from the cellularity at twenty-eight days (Table I). The remodeled graft was replaced by organized connective tissue, and the site could only be identified from the native tissue by the presence of the Prolene suture.

The ninety-day tissue samples (Fig. 8) showed a slight decrease in the number of spindle cells present within the graft material compared with the sixty-day sample, and the cellularity was no different from the normal Achilles tendon (Table I). The collagen fiber organization and vascularity were also qualitatively similar to those of normal tendon tissue.

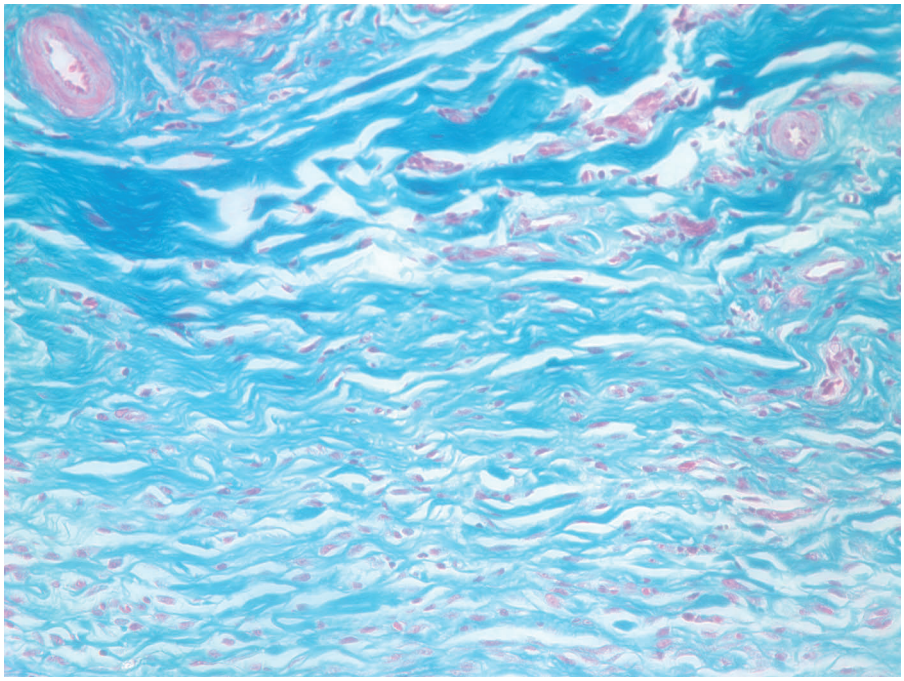

Fig. 6

Histological section of remodeled graft at twenty-eight days after surgery (Masson trichrome, ×20). Dense sheets of exclusively mononuclear cells have populated the remodeled extracellular matrix scaffold. There is a loss of morphologic evidence of the scaffold with relatively homogeneous deposition of new host-derived extracellular matrix that is disorganized in its orientation.

Fig. 7

Histological section of remodeled graft at sixty days after surgery (Masson trichrome, ×20). The section shows that the graft has been replaced with organized, aligned host-derived extracellular matrix and spindle cells consistent with the morphologic appearance of fibroblasts. The repair site between the graft and the native tissue cannot be identified and has been replaced by a uniform and organized connective tissue.

Fig. 8

Histological section of remodeled graft at ninety days after surgery (Masson trichrome, ×20). The section shows that the remodeled graft has been completely replaced with dense, organized, aligned host-derived extracellular matrix and spindle-shaped cells consistent with fibroblast morphology. The cellularity and vascularity are qualitatively similar to that of normal tendon tissue.

Discussion

The results of this study show that extracellular matrix derived from small intestinal submucosa, when used as a biologic scaffold for Achilles tendon reconstruction in this canine model, is rapidly degraded after implantation, with approximately 60% of the mass degraded and resorbed within four weeks. Degradation of the scaffold appears to be complete by three months. These findings are almost identical to the degradation rate of small intestinal submucosa extracellular matrix when this biologic scaffold was used for reconstruction of the urinary bladder[19]. The resorbed ^{14}C-labeled degradation products in the present study were eliminated from the body primarily by urinary excretion. No detectable ^{14}C was found in any of the parenchymal organs that were examined.

The nonlinear temporal degradation of the scaffold was associated with the extent and distribution of the host cellular infiltrate. At the time of implantation, no cells were present within the scaffold. As cells infiltrated the graft, the rate of scaffold degradation increased. By four weeks after surgery, the cell infiltrate had lessened and the rate of scaffold degradation had begun to diminish. The distinctive laminated architecture of the small intestinal submucosa extracellular matrix graft was no longer distinguishable by twenty-eight days, and the remodeled scaffold showed a relatively uniform accumulation of homogenous collagenous connective tissue.

The Achilles tendon is subjected to some of the highest stresses of any tendon in the body[24-27]. It would therefore seem logical that an extracellular matrix scaffold that is subjected to rapid degradation, such as small intestinal submucosa extracellular matrix, would be at risk of failure when used as a repair device for the Achilles tendon under physiologic loading, especially during the first four to eight weeks after surgery. In fact, this concern has been the rationale for chemically cross-linking many extracellular matrix scaffolds including one commercially available device derived from small intestinal submucosa extracellular matrix (CuffPatch; Arthrotek)[28-30]. In the present study, a tube splint that allowed for partial weight-bearing was utilized for the first month after implantation to prevent failure due to suture pull-out while still allowing the extracellular matrix scaffold to bear load. The application of load early in the remodeling process has been shown to be important for constructive tissue-remodeling[17]. After removal of the splint in the present study, the remodeled scaffold was sufficiently strong to withstand unrestricted cage activity in this animal model without evidence of rupture. The remodeled scaffold showed organized, aligned collagenous tissue after removal of the tube splint at twenty-eight days. Although mechanical testing was not performed in this study, a previous study with use of the same canine model of Achilles tendon repair showed that the strength of the remodeled Achilles tendon exceeded the strength of the insertion to the gastrocnemius muscle and the calcaneus by twelve weeks after repair with small intestinal submucosa extracellular matrix[16].

Recent studies have suggested that degradation of an extracellular matrix scaffold may be an essential component of a constructive remodeling response as opposed to scar-tissue deposition[31-34]. Low-molecular-weight peptides formed during the degradation of small intestinal submucosa extracellular matrix have been shown to have chemoattractant properties for several cell types in vitro and angiogenic potential in vivo[32]. Chimeric mouse models, including a model in which small intestinal submucosa extracellular matrix was used to repair the Achilles tendon, showed that bone marrow-derived cells are recruited to the site of healing and that they participate in the long-term remodeling of the scaffold[31,34]. In addition, in vitro studies have shown that degradation products of extracellular matrix scaffolds have antibacterial properties[33], but, in the absence of degradation, the extracellular matrix scaffolds support bacterial growth[35]. It is plausible that chemoattractance by degradation products contributed to the recruitment of host cells and, ultimately, to tendon remodeling. Stated differently, degradation of an extracellular matrix scaffold may be a requisite process with bioactive consequences that contribute to the overall remodeling events. Inhibition of scaffold degradation by chemically cross-linking the extracellular matrix may decrease or eliminate the beneficial effects of extracellular matrix degradation products.

A limitation of the present study is the small number of animals (two) that were evaluated at each time-point. The low numbers were a result of practical considerations, including the cost of the study and the complexity involved in producing the small intestinal submucosa extracellular matrix labeled with ^{14}C. Despite these limitations, this study showed rapid degradation of a small intestinal submucosa extracellular matrix scaffold used for the repair of a musculotendinous tissue, an application for which small intestinal submucosa extracellular matrix is currently in clinical use. This study also shows that, as degradation occurs, the graft remodels into dense collagen-rich connective tissue with an organization, cellularity, and vascularity similar to that of native tendon tissue. ■

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