in vivo

COLLAGENOUS BIOCOMPOSITES FOR THE REPAIR OF SOFT TISSUE INJURY

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Abstract:

Results of implantation studies in a variety of animal tissue models demonstrate that the rate of biogradation of a collagen scaffold should parallel the rate of wound healing observed in particular anatomic sites. This rapid degradation maximizes tissue regeneration and minimizes encapsulation of the implant. The following paper reviews the effects of crosslinking on the rate of tissue ingrowth and regeneration. In addition, preliminary mechanical data on newly developed soluble type I collagen fibers is presented as a possible advance in the production of high strength collagen based tissue scaffolds.

Introduction:

Collagen is one of the major scaffolding elements produced by nature. It is found in various biochemical forms and in different tissue architectures. Tendon/ligament contains quarter-staggered collagen molecules that are laterally bundled into fibrils, bundles of fibrils and fibers (1). In comparison, skin contains a wavy "biaxial" array of collagen fibers that are found within a plane almost parallel to the surface. Peripheral nerve contains collagenous sheaths or tubes that surround groups of axons. Dura mater is a bilayer containing collagen fibers parallel to the midline of the brain that is sandwiched on top of a layer of collagen fibers perpendicular to the midline.

Collagenous biocomposites that mimic the structural components found in the tissues listed above have been investigated including: (a) a collagen tendon/ligament prosthesis consisting of parallel aligned type I collagen fibers in an amorphous type I collagen matrix; (b) a dermal replacement consisting of collagen fibers freeze dried into a planar open pore sponge; (c) a dural replacement composed of a collagen film containing planar collagen fibers; and (d) a collagen fiber substrate for axon elongation in peripheral nerve. In all of these applications the degradation rate of the implant should match the rate of tissue repair to optimize the rate of wound healing and at the same time it should act as a scaffold that mimics normal tissue architecture.

It has been known since the 1950s that collagen molecules can be self assembled in neutral salt solutions to form fibrils that are identical to those found in tissues. Our laboratory has developed collagenous biocomposites, using the self assembly technique, that mimic connective tissue found in tendons/ligaments, skin, nerve and dura mater.

In all cases the reconstituted collagen biocomposite has been stabilized through crosslinking processes. These crosslinking processes consist of one of either of two methods. The first method involves exposing the collagen to a water soluble carbodiimide, resulting in the formation of peptide bonds. This method by itself or combined with severe dehydration (dehydrothermal crosslinking) results in a lightly crosslinked biocomposite. Alternately, reconstituted collagenous materials are stabilized by exposure to glutaraldehyde vapor producing a highly crosslinked matrix (11).

The following review summarizes the results of a variety of different studies involving reconstituted collagen fabricated to provide a scaffold for tissue ingrowth and regeneration. Table I summarizes the results of these studies.

Tendon/ligament tissue analog:

The first phase of the development of a tendon/ligament replacement involved the production and characterization of a reconstituted collagen fiber. Kato et al. tested the

Table I: Summary of Collagen Based Scaffolds for Tissue Repair.

Tissue	Tissue Structure	Tissue Analog	Animal Model	Implant Duration	Tissue Compatiblity	Reference
---------	---------	Reconstituted collagen fiber: 1% w/v dispersion extruded through thin P.E. tubing. Crosslinked C. or Glut.	Subcutaneous implant in Rats	C. (1&2 weeks) Glut. (2&4 weeks)	C. 2 week subacute inflammation, majority degraded. Glut. 4 week subacute inflammation, minor degradation.	(12)
Tendon	Hierarchically structured system of aligned collagen fibrils arranged in bundles.	Reconstituted collagen fiber composite: 200-250 fibers/ prosthesis. Crosslinked C. or Glut.	Rabbit Achilles tendon	3,10, 20 & 52 weeks	C. 20 week neotendon formation, cellularity comparable to normal tendon. Minor inflammation at early time points. C. 52 week neotendon formation, implant resorbed, crimp comparable to autogenous graft. Glut. 52 week implant intact, chronic inflammation, formation of fibrous capsule.	(10,13)
Ligament	Aligned collagen fibrils arranged in bundles with a slight axial twist.	Reconstituted collagen fiber Composite: 250 fibers/prosthesis Crosslinked C. or Glut.	Rabbit ACL	4 & 20 weeks	C. 20 week implant resorbed with neoligament formation. Glut. 20 week implant intact, and chronic inflammatory response.	(9)
Skin	Random array of collagen fibers within a plane.	Freeze dried collagen sponge impregnated with fibronectin and coated with thin layer of silicone. Crosslinked C.	Guinea pig full thickness dermal wound	6, 9 & 12 days	Collagen fibers deposited within sponge oriented with collagenous material of sponge. Fibronectin enhances adhesion and alignment of fibroblasts.	(2)
Dura mater	Random array of collagen fibers within a plane.	Type I collagen film Crosslinked C.	Dural replacement in rabbit	16, 28, 42, & 56 days	Films replaced by day 42 with new tissue comparable to dura mater, minimal inflammation at early time points.	(1)
Peripheral nerve	Axially oriented nerve tissue surrounded by connective tissue sheath.	Silicone tube containing longitudinally aligned reconstituted collagen fibers with variable crosslink density.	Sciatic nerve replacement in rats	4 & 6 weeks	Rapidly degrading collagen fibers increased myelinated and unmyelinated axons at 6 weeks.	(16)

Note: C. = dehydrothermal and carbodiimide crosslinking, Glut. = Glutaraldehyde crosslinking.

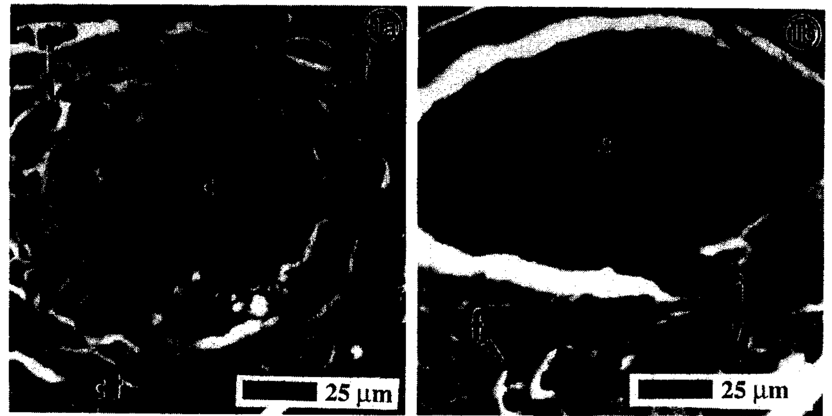

Figure 1. Fig. 1a shows a light micrograph of a carbodiimide crosslinked collagen fiber (c) implanted subcutaneously for 2 weeks. Note the disrupted edge of the fiber indicating degradation. The implant is surrounded by new connective tissue (ct), fibroblasts (f), and inflammatory cells (i). Fig. 1b is a light micrograph of a glutaraldehyde vapor crosslinked collagen fiber (g) implanted subcutaneously in a rat for 2 weeks. The implant shows no evidence of degradation, and is surrounded by new connective tissue, as well as fibroblasts and inflammatory cells.

mechanical properties of reconstituted collagen fibers produced by extruding a type I collagen dispersion through thin polyethylene tubing into a fiber formation bath (11,12). The results of these studies indicated that it was feasible to produce a crosslinked collagen fiber with strength characteristics comparable to isolated fibers from rat tail tendon.

Subsequent subcutaneous implantation of bundles of these fibers in a rat model (12), indicated that the carbodiimide treated collagen induced a subacute inflammatory response at one week post-implantation with minor degradation of the implant. However, at two weeks post surgical implantation, virtually all the implanted collagen fibers were degraded and replaced by new connective tissue and inflammatory cells. The glutaraldehyde treated fibers were explanted at two and four weeks. In comparison to the carbodiimide crosslinked fibers, the glutaraldehyde crosslinked materials remained intact at two and four weeks with an associated subacute inflammatory response. At four weeks the glutaraldehyde crosslinked fibers showed a minimal degree of degradation. Figure 1 shows a light micrograph comparing the two treatment methods.

Preliminary success with subcutaneous implants prompted the evaluation of crosslinked collagen fibers in a rabbit Achilles tendon model (10). 200 - 250 collagen fibers crosslinked with either carbodiimide or glutaraldehyde were implanted for 3, 10, and 20 weeks. Following explantation the prostheses were mechanically tested in tension as well as evaluated for biocompatiblity. The autogenous grafts were infiltrated with fibroblasts and capillaries and partially replaced by 20 weeks. In comparison, at three weeks the collagen fiber implants were infiltrated with new connective tissue. At ten weeks, reorganization of the connective tissue was evident around the implanted collagen fibers, in particular the carbodiimide crosslinked collagen had been replaced with neotendon. The heavily crosslinked glutaraldehyde fiber prostheses were resorbed more slowly and were surrounded by more inflammatory cells than the carbodiimide treated fibers. Although the initial strength of the glutaraldehyde treated prosthesis was comparable to the strength of the autogenous controls, the strength of the graft decreased markedly by the twentieth week of implantation. The strength of the grafted carbodiimide crosslinked prosthesis increased as the implant resorbed and the neotendon reorganized over the course of twenty weeks of implantation.

A one year study was conducted to evaluate the long term biocompatibility of the tendon ligament prosthesis crosslinked with either carbodiimide or glutaraldehyde (13). Histological evaluation of the explanted prostheses indicated that the carbodiimide crosslinked fibers were

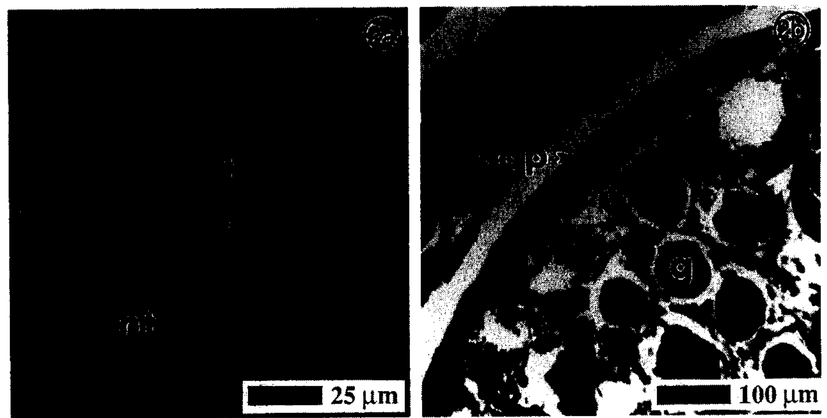

Figure 2. Fig. 2a and 2b are light micrographs of tendon protheses explanted from an rabbit Achilles tendon model after 1 year. Fig. 2a shows the histological results of a prosthesis crosslinked with carbodiimide. The implant is completely degraded and replaced with neotendon (**nt**), and infiltrated with fibroblasts (**f**). In comparison, the glutaraldehyde crosslinked prosthesis (**g**) was still present after one year and is surrounded by a fibrous tissue capsule (**cap**).

completely resorbed and replaced with neotendon having cellularity and morphological characteristics comparable to the autogenous control and normal tendon, although the cell density was greater in the graft materials than the control. The glutaraldehyde crosslinked prosthesis was encapsulated with evidence of the original prosthesis still present in the tissue. Figure 2 shows a histological comparison between the two implant types at 52 weeks. This study concluded that the lightly crosslinked carbodiimide treated prosthesis promoted resorption and replacement of the prosthesis with neotendon formation.

In addition to the Achilles tendon model, an intra articular site of implantation was investigated in rabbits (9). Bundles containing 225 crosslinked collagen fibers were implanted as replacements for the anterior cruciate ligament of a rabbit and harvested after 4 and 20 weeks of implantation. Mechanical tests were performed on the harvested anterior cruciate ligament, femoral and tibial condyle system. In addition, the explants were evaluated histologically. At four weeks, the materials crosslinked with either carbodiimide or glutaraldehyde were infiltrated with fibroblasts and inflammatory cells. At this time the carbodiimide crosslinked implant showed signs of resorption. By 20 weeks the carbodiimide crosslinked prosthesis was completely resorbed and replaced by an organized neoligament. The glutaraldehyde crosslinked prosthesis was only partially degraded and in addition was surrounded by a fibrous capsule with evidence of chronic inflammation.

The results of these studies suggest that neotendon and neoligament formation is facilitated in the presence of a tissue like scaffold that biodegrades at the same rate as an autograft. Prolongation of resorption of the implant results in encapsulation and a chronic foreign body response similar to that observed with synthetic polymers.

Dermal tissue analog:

Large dermal defects present an interesting challenge for tissue ingrowth scaffolds. The effects of a reconstituted collagen sponge on fibroblasts were investigated by Doillon et al. (3). In this study type I collagen sponges were prepared by dispersing insoluble type I collagen isolated from bovine corium in acid distilled water followed by freeze drying in a plastic pan. The sponges were stabilized by dehydothermal treatment and carbodiimide crosslinking.

Fibroblast and collagen sponge interactions were studied in-vitro, in cell culture, and in-vivo by implantation onto full thickness dermal wounds produced on the backs of guinea pigs

(3). After 10 days in culture and after 6, 9, and 12 days of implantation, the sponges were harvested and processed for light and scanning electron microscopy. Results of the in-vitro study indicated that fibroblasts grown on collagen sponges near the edges were elongated and they synthesized a relatively disorganized connective tissue matrix. Fibroblasts that had invaded the interior of the sponge were more elongated and appeared to orient their long axis parallel to neighboring fibroblasts. As a result of this orientation, the newly synthesized collagen fibers were more organized. In some instances the new collagen fibrils were laid down parallel to each other. In the presence of fibronectin the fibroblasts spread and attached themselves to the matrix to a greater extent.

Results of the in-vivo dermal studies indicated that the fibroblast interactions with the collagen sponge could be generalized to occur via two processes. The first involved invasion of fibroblasts into the channels and pores of the sponge from the periphery of the wound, while the second involved the formulation of granulation tissue below the sponge.

At 6 days post implantation, fibroblast ingrowth was observed along with a few inflammatory cells, with the inflammation reducing by the 9th day. The reduction in inflammatory cells was accompanied by an increase in the density of fibroblasts. By the 12th day of implantation, the sponge was completely infiltrated with fibroblasts, and in some instances the sponge was replaced by granulation tissue. Vascularization of the implanted sponge was also seen by the 12th day.

The fibril structure of the newly synthesized collagen after 6 days of implantation was relatively thin (1μm in diameter), and appeared to be randomly arranged. By day 9 the fibril diameter had increased to 6 μm and fibrils were more organized particularly in the sponges containing fibronectin. The granulation tissue beneath the implant was characterized by collagen fibers oriented in the plane of the skin.

In summary, based on both cell culture and full thickness dermal implantation, it was evident that fibroblasts invade and grow in the pores of the sponge from the edges of the wound and subsequently elongate and spread, orienting their long axis parallel to neighboring fibroblasts. In the absence of a biological scaffold in a wound, our results suggest that an artificial collagen fibrillar network acts as a template which organizes the spatial deposition of newly synthesized collagen fibers. Fibronectin promotes and enhances the migration of fibroblasts within the sponge as well as enhances the orientation of these fibroblasts and increases the diameter of the newly synthesized collagen fibers.

A number of studies have investigated the ability of hyaluronic acid (HA) and fibronectin (FN) to enhance the process of wound healing (3,4,6,7). Sponges prepared as noted above were impregnated with FN and HA prior to freeze drying. It was found that a combination of 1% FN and 1% HA maximized the rate of fibroblast and tissue ingrowth. Above this concentration the ingrowth potential of the implant was decreased.

In addition to FN and HA, the effects of glycosaminoglycans and glycoproteins on fibroblasts and epidermal cells cultured on analogs of the extracellular matrix were evaluated (8). The results of this study indicated that a crosslinked type I collagen sponge and non-collagenous connective tissue macromolecules can improve cell growth properties on extracellular matrix analogs.

Marks et al. (14) devised a study to investigate the effects of cell seeding and coating with bFGF on the ingrowth characteristics of fibroblasts in full thickness dermal wounds. The degree of collagen sponge degradation was determined based on staining intensity differences between collagen fibers in the sponge and the newly synthesized collagen fibers deposited within the implant. Results of these studies indicate wounds treated with fibroblast seeded or bFGF treated collagen matrices, showed more rapid wound healing and an associated increase in the synthesis of new tissue. The rapid degradation and replacement of the implanted sponge may be associated with the increased cellularity or fibroblast cell density. During the early stages of implantation (15 days) the ultimate tensile strength of the wound tissue was increased over the untreated sponge, and can be correlated with the development of collagen networks within the tissues.

Dura mater tissue analog:

Cranial neurosurgical procedures often result in damage to the dura mater, the connective tissue sheath encasing the brain and spinal column. This often requires the replacement of the dura with an artificial substitute or processed cadaver dura. These options present additional complications, including inflammation, adhesions, and in the case of cadaver dura, the

possibility of disease transmission (2). The necessity for a convenient dural substitute led us to hypothesize that a reconstituted collagen film containing crosslinked type I collagen fibers would be useful as a dural substitute (2). These films were implanted as replacements for dura extracted from a large cranial defect, and compared to autogenous dura. Animals were sacrificed at 16, 28, 42, and 56 days post implantation and evaluated histologically.

Experimental observations indicated that the reconstituted collagen dural substitute exhibited minimal inflammation during the early stages of implantation with progressive resorption and replacement of the implant with new connective tissue. By the 56th day post-implantation the dural scaffold was completely resorbed and the neodura exhibited connective tissue and fibroblast cellularity comparable to the natural dura and the autogenous dural graft.

Peripheral nerve analog:

Collagen has been investigated as a substrate for peripheral nerve regeneration as described in a recent review Yannas et al. (19). However, in most of these systems the collagenous substrate is not an aligned system of collagen fibers that may promote linear elongation of severed axons. Preliminary work on the efficacy of reconstituted collagen fibers as a substrate for neural regeneration was conducted by Wong et al. in 1990 (18). In this in-vitro model, cortical neurons isolated from fetal rats were grown in cell culture on etched collagen fibers. The results indicated that cortical neurons elongate with their axis parallel to the axis of the collagen fibers. In addition, neurite outgrowth from these cells also paralleled the fiber axis. It was suggested that this model system could be used to study the effects neurotrophic factors, as well as the optimization of an extracellular matrix substrate for nerve regeneration.

The results of the cell culture studies prompted the investigation of aligned reconstituted collagen fibers in-vivo as a scaffold for sciatic nerve repair within a rat model (15). In this investigation reconstituted type I collagen fibers were placed within a silicone tube. Upon transection of the sciatic nerve, a 1 centimeter gap was produced and the proximal and distal ends of the nerve inserted within the silicone tube. As a result, the reconstituted collagen fibers were aligned with the axis of the regenerating nerve. Implants were harvested at 4 and 6 weeks post-implantation and processed for transmission electron microscopy. The rapidly degrading fibers induced greater myelinated and unmyelinated axon densities at 6 weeks compared to the slowly degrading fibers. The study concluded that the implanted collagen fiber can enhance or inhibit the regeneration of sciatic nerve tissue depending on the rate of biodegradation. It also suggests that the aligned collagen fiber facilitates axonal elongation toward the distal segment, through the alignment of migrating fibroblasts and Schwann cells during the early stages of regeneration.

Advances in Collagen Self Assembly:

During the last several years it has been possible to self assemble collagen fibers in vitro from collagen molecules. Although these fibers cannot be detected visually during the extrusion process for about 30 minutes at 37°C, it is now possible to form these fibers with diameters as small as 20μm. An unexpected result of these studies was that the alignment of molecules that resulted from self assembly in vitro equaled or exceeded that observed in rat tail tendon fibers. In vitro, self assembled fibers have strength to weight ratios that exceed those found for metal alloys (see Table II). As our knowledge of the self assembly process improves so will the ability to develop a new generation of high strength fibers. Tensile strengths of reconstituted fibers have been achieved as high as 442 MPa (dry) and 80 MPa (wet); these values that exceed those reported for collagen fibers derived from tendon (see Table II).

Conclusions:

Rapid implant degradation and ingrowth with new connective tissue seen in the lightly crosslinked matrices is associated with neotissue formation. In the case of the highly crosslinked matrices, encapsulation and in some cases a chronic foreign body response is observed. This implies that rapid tissue ingrowth and degradation of the implant material

Table II: Mechanical Properties of Axially Aligned Triply-Periodic Structures

Tissue	Strain at Failure (%)	Ultimate Tensile Strength (MPa)	Strength/Weight (MPa/g/cc)
Rat tail tendon	31.4 (7.1)	375.2 (41.9)	293 (36.1)
Reconstituted fibers from insoluble collagen	23.0 (12.3)	218.6 (14.2)	170.8 (12.2)
Reconstituted fibers from soluble collagen	19.2 (10.4)	442.0 (80.7)	345.0 (69.6)
Stainless Steel, Co, and Ti Alloys	10.0 - 50.0	500 - 800	~100

Note: Values for rehydrated fibers dehydrothermally crosslinked in parentheses.

promotes tissue repair and regeneration. Rapid degradation is associated with an initial inflammatory response that is resolved during wound healing. The development of high strength collagen fibers produced by the extrusion of soluble type I collagen molecules, will prove beneficial in the construction of the next generation of high strength, biocompatible collagenous biocomposites as well as serve as a basis for the design of new biodegradable synthetic polymers.

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