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ACHILLES TENDON REPLACEMENT BY A COLLAGEN FIBER PROSTHESIS: MORPHOLOGICAL EVALUATION OF NEOTENDON FORMATION.

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Abstract

Reconstituted type I collagen was processed into fibers which were subsequently severely dehydrated and cyanamide cross-linked. Fibers prepared by this method were stronger and more resistant to degradation than uncrosslinked fibers. When used as a tendon replacement prosthesis, morphological events occurred which were observed by light, scanning, transmission electron microscopy and electron histochemistry.

Resorption was the initial host response to the prosthesis and involved gradual biodegradation. Formation of a host-replacement tendon was the second response. Increased collagen fibril diameters and a transition in the proteoglycan/collagen fibril interactions occurred in the newly developing connective tissue between 3 and 10 weeks post-implantation. These extracellular matrix transitions were major events occurring during wound healing and led to the assembly of a mature connective tissue.

When used as a tendon prosthesis, these collagen fibers rapidly resorb while allowing simultaneous formation of aligned connective tissue. The fibers may have other applications in the fields of Orthopaedic Surgery, Neurosurgery and Biomaterials Research.

KEY WORDS: Tendon prosthesis, neotendon, connective tissue, proteoglycan, regeneration, collagen, biomaterials, Achilles tendon.

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Introduction

The use of natural and synthetic resorbable materials for the repair of hard and soft tissues in the human body is the focus of continued research in Orthopaedic Surgery, Neurosurgery and Biomaterials Science. Collagen, a naturally occurring biopolymer, is widely used in surgical applications (Chvapil, 1980; Parsons, 1985; Pachence et al., 1987). *In vitro*, collagen is used to culture epithelial cells (Doillon et al., 1984, 1988), fibroblasts (Gey et al., 1974; Bell et al., 1983; Reid and Jefferson, 1984; Ricci et al., 1984; Doillon et al., 1988; Law et al., 1989), cardiac cells (LeFurgy et al., 1989) and neurons (Iversen et al., 1981; Manthrope et al., 1983; Shine et al., 1985; Wasserman, et al., 1988). An excellent review of collagenous matrices used as a substratum for cells grown in culture is provided by Hodges (Hodges et al., 1985). *In vivo,* collagen is used as a dressing for wound repair (Oluwasanmi and Chvapil, 1976; Bell et al., 1981; 1983; Leipziger et al., 1985; Yannas, 1988; Doillon et al., 1986; Wasserman et al., 1988), a nerve conduit (Colin and Donoff, 1984; De La Torre et al., 1984; Satou et al., 1986; Yannas et al., 1987; Yannas, 1988; Madison et al., 1985, 1988; Wasserman et al., 1989b) and a tendon prosthesis (Kato et al., 1988; Wasserman et al., 1988, 1989a; Law et al., 1989; Goldstein et al., 1989).

In this paper the problem of tendon replacement is addressed. Damaged tendons can be replaced with devices prepared from synthetic non-resorbable materials including; silk (Lang, 1902), metal wire (Arkin and Siffert, 1953; Hausner, 1970), nylon (Sarkin, 1956; Hall, et al., 1975), Teflon (Williams, 1960), Dacron (Bowen and Dyer, 1962; Hall et al., 1975), silicone/polyester (Bader and Curtin, 1968; King et al., 1974) and carbon

(Goodship et al., 1980; Jenkins and McKibbin, 1980; Alexander et al., 1981; Aragona et al., 1981; Mendes et al., 1985; Goodship and Cooke., 1986). A review of materials used as artificial tendons is provided by Murray and Semple (1979) and Goodship and Cooke (1986). Synthetic materials are generally regarded as unacceptable since with time they fail due to fatigue or premature breakdown of the material. Consequently, the use of natural biological materials for tendon replacement was investigated.

Parsons (1985) reviews the use of collagenous tissues as allografts, xenografts and autografts for tendon/ligament replacement. Numerous problems are associated with the use of these materials. For example, both allografts and xenografts (obtained from bovine tendon or cadaver) create antigenicity problems (McMaster et al., 1976; Parsons, 1985; Goodship and Cooke, 1986). The antigenicity of graft material can be reduced by crosslinking with glutaraldehyde or isocyanates (McMaster et al., 1976; Chvapil 1980; Parsons, 1985; Goodship and Cooke, 1986). However, elongation of such material can occur resulting in joint instability (Friedman et al., 1985; Alexander et al., 1986). Furthermore, glutaraldehyde fixed tissues remain acellular for long periods (Tauro et al., 1989; Gendler et al., 1984) resulting in extended graft resorption. This may affect neotendon development and tissue remodeling. Autografts are most desirable because of their biocompatibility but necessitate a second surgery with attendant complications and are therefore of limited availability. Additionally, this procedure can result in dysfunction of the donor joint from which the graft was removed.

A final viable option for tissue augmentation and replacement are natural resorbable biological materials. Reconstituted type I collagen is ideally suited for this purpose because it has low antigenicity (Parsons, 1985; Tauro et al., 1989), is biodegradable (Weadock, et al., 1984; Law et al., 1989) and can be engineered into various forms to suit specific biomedical problems (Chvapil, 1980; Pachence et al., 1987). For example, purified reconstituted type I collagen can be formed into fibers for use as a prosthesis suitable for tendon and ligament replacement (Kato et al., 1988; Wasserman et al., 1988, 1989a; Law et al., 1989).

In this paper, we report on the production of collagen fibers, their physical characteristics and their use as a prosthesis for replacement of rabbit Achilles tendons. The tissue responses to the prosthesis will be defined morphologically by light, scanning, transmission electron microscopy and electron histochemistry using quinolinic blue. Quantitative changes in collagen fibril diameters are known to occur in developing connective tissues (Scott, 1984; Scott et al., 1981; Parry et al., 1978; Parry and Craig, 1984; 1988). For this reason, changes in collagen fibril diameters as well as organizational changes between proteoglycans and collagen fibrils of the accumulating matrix will be reported as they relate to wound healing.

Materials and Methods

Preparation and implantation of collagen fibers treated by severe dehydration and exposure to cyanamide.

Insoluble type I collagen obtained from bovine hide corium was used for fiber production. The corium was limed, fragmented, swollen in acid, precipitated, washed in distilled water and isopropanol, lyophilized and frozen at -30°C for future use. Lyophilized collagen (1.2 g) was added to 120 ml of HCL solution (pH 2.0) and blended in an Osterizer at a speed of 10,000 revolutions per minute for 4 minutes. The resulting 1% collagen dispersion was then pumped to eliminate air bubble entrapment. The dispersion (room temperature) was then transferred to a 30 ml syringe and stored at 3°C.

To produce the collagen fibers, the dispersion was extruded through polyethylene tubing with an inner diameter of 0.28 mm into a 37°C, pH 7.5, fiber formation buffer. The buffer was composed of 135 mM $NaCl_2$, 30 mM TES(N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid) and 30 mM sodium phosphate dibasic. After 45 minutes in the buffer, the fibers were rinsed in isopropyl alcohol for 4 hrs followed by distilled water for 20 minutes. The fibers were then air-dried under tension and dehydrothermally crosslinked by treating them in a vacuum oven at 100 mtorr and 110°C for 72 hrs.

Following this procedure, the dehydrothermal crosslinked collagen fibers were

exposed to cyanamide vapors (20 g of cyanamide dissolved in 5 ml of distilled water) for 1 day at room temperature. Two hundred and fifty fibers were coated with a 1% (w/v) uncrosslinked collagen dispersion to form a 1 cm x 20 cm ribbon prosthesis.

The prostheses were implanted into 12 mature New Zealand white rabbits weighing 6-7 lbs. Light microscopic studies were performed at 5 weeks on 5 of the tendon prostheses and electron microscopic studies were done on 1 of the prostheses. At 10 weeks, light microscopy was done on an additional 5 prostheses and electron microscopy on the 1 remaining prosthesis. To implant the devices, the animals were anesthetized and maintained under gas anesthesia. The implantation of the tendon prosthesis was done under sterile conditions. The Achilles tendon was exposed and the gastrocnemius portion of this tendon totally removed. A collagen fiber prosthesis was first woven into the musculotendinous junction and then into the tendocalcaneal junction. Both ends were secured with horizontal mattress sutures of 4-0 Dexon (Davis and Geck, Pearl River, N. Y.). The final length of the prosthesis was about 3 cm.

Devascularized control tendons were studied by light microscopy in 10 additional animals. Light microscopic studies were performed at 5 weeks on 5 of the tendons and at 10 weeks on the remaining 5 tendons. The procedure involved excising a 3 cm segment of Achilles tendon then reanastomosing the excised tissue by a modified Kessler repair. For electron microscopic studies, 1 Achilles tendon was removed from a normal animal and prepared for conventional scanning and transmission electron microscopy, and electron histochemistry using quinolinic blue.

Mechanical and physical properties of reconstituted collagen fibers.

Mechanical properties were determined in tension at a strain rate of 50% per minute using an Instron Tester Model 1122 (Canton, MA). Fibers were mounted on a paper frame using 5 minute epoxy adhesive (Devron Corp., Danvers, MA) placed at the fiber ends with a 2 cm gauge length window. Prior to testing, the fibers were immersed in phosphate buffered saline (PBS) pH 7.5 for at least 15 minutes. Fiber diameters were measured both in dry and wet states at three arbitrary locations along the gauge length using a calibrated eyepiece of a Leitz Laborlux 12 Pol microscope, and averages were calculated to obtain original areas assuming that the cross-sections were circular. Load-extension curves obtained from the chart recorder were used to calculate ultimate stress (peak force/cross-sectional area) and ultimate strain (change in length/initial length). Since the stress-strain curve of these collagen fibers is non-linear, the modulus varied according to the stress or strain being applied. The chord modulus was calculated by drawing a best fit secant line at the upper region of the stress-strain curve to obtain an average stiffness of these fibers.

The ability of collagen fibers to resist *in vivo* resorption and *in vitro* degradation by collagenase were tested. Dehydrothermal and cyanamide crosslinked fibers, were tested *in vivo* by subcutaneous implantation into 2 separate rats and replacement of Achilles tendon in 4 separate rabbits. *In vitro* testing of dehydrothermal and cyanamide crosslinked collagen fibers and uncrosslinked collagen fibers was performed as follows. Twenty-five mm lengths of 3 individual fibers from each of the 2 groups were placed into polystyrene dishes. Three ml of collagenase (1000 units/ml) were added to each dish and covered. The dishes were incubated at 37°C and checked at 10 minute intervals until the collagen fibers were digested (Librizzi, 1989).

Light and electron microscopy and measurements of collagen fibril diameters.

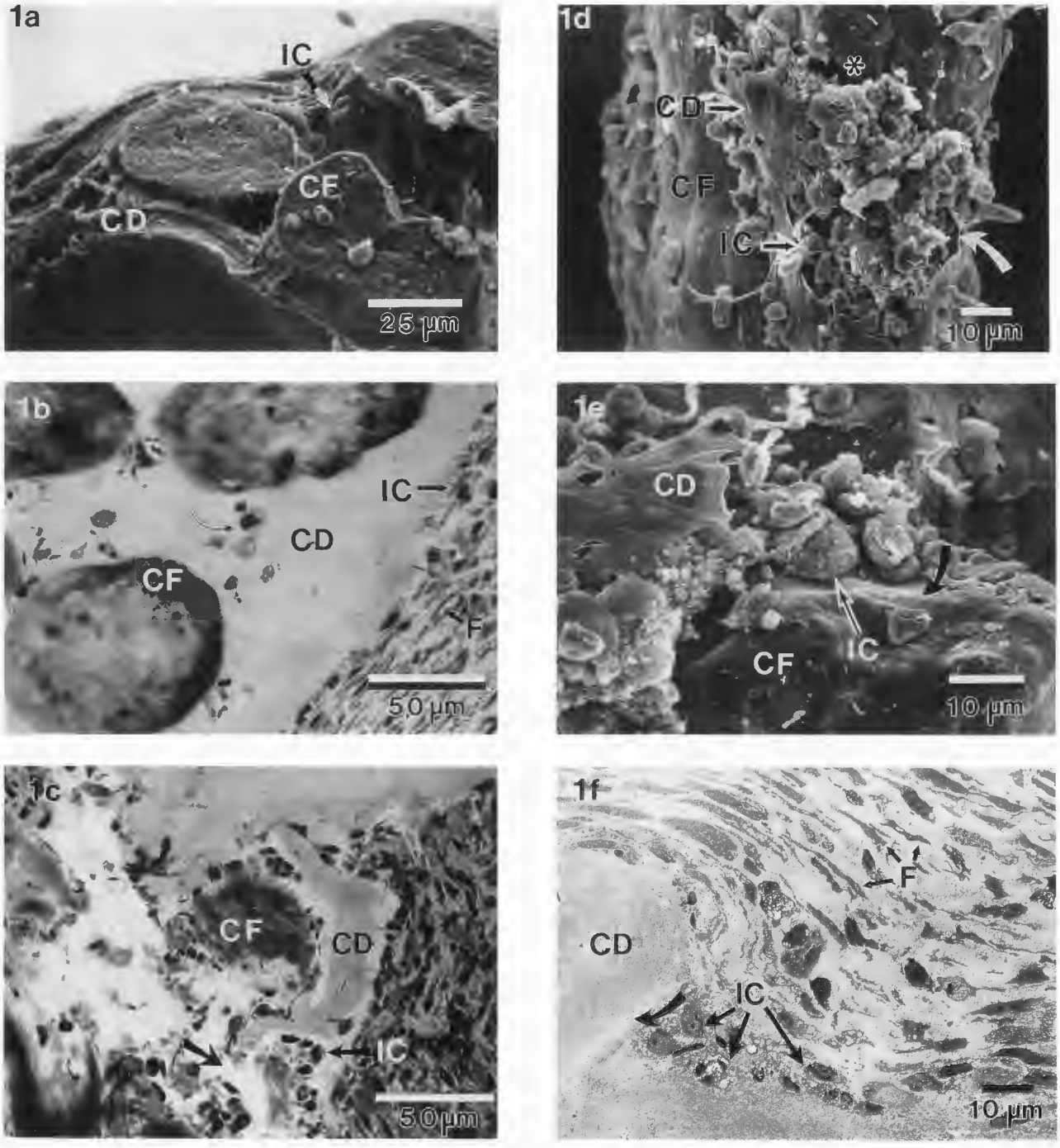
For light microscopic observations, a total of 20 prostheses were examined: 5 prostheses at 3 and 10 weeks each and 5 devascularized controls at 3 and 10 weeks each (as described in the "Preparation and implantation..." section). Tissues were fixed in Carson's fixative, dehydrated under a series of alcohols, paraffin embedded, sectioned, and stained using Sirius Red and Masson's Trichrome. Light microscopic observations of thick sections obtained from Polybed 812 resin (Polysciences, Warrington, PA) embedded specimens prepared for transmission electron microscopy were also made. These sections were stained with 1% Toluidine Blue in 1% sodium carbonate. Light photomicrographs were made of both paraffin and resin embedded sections.

For scanning and transmission electron microscopy the 3 and 10 week prostheses and control tendon (as described in the "Preparation and implantation..." section) all received the same fixation and histological

treatments as follows. The freshly dissected tissues were immersed in 4°C fixative, diced into cubes and transferred to fresh fixative for an additional 90-120 mins. The fixative was composed of 1.5% glutaraldehyde (v/v)-4% paraformaldehyde (v/v; Polysciences, Warrington, PA) containing 4 mM $CaCl_2$, buffered by 0.1 M cacodylate (pH 7.4). The tissues were post-fixed at 4°C in 1% OsO_4 (pH 7.4), followed by block staining in 1% uranyl acetate (w/v) in 50 mM sodium acetate. Tissues were dehydrated, infiltrated, and embedded in Polybed 812 resin (Polysciences, Warrington, PA). Samples prepared for proteoglycan staining were fixed at room temperature and a pH of 5.7, in 2.5% glutaraldehyde (v/v) in a critical electrolyte solution of 25 mM sodium acetate-0.3 M $MgCl_2$ with 0.05% quinolinic blue (QB; w/v) (Scott, 1980; Scott and Orford, 1981; Scott 1985). QB samples were only primary fixed and were block stained in 0.5% sodium tungstate (v/v; aqueous). Tissues were dehydrated through a series of alcohols. Sodium tungstate (0.5%, w/v) was included in the 50% ethanol (v/v) stage. Tissues were infiltrated and embedded in Polybed 812 resin (Polysciences, Warrington, PA). Thin sections from conventional samples were double stained in uranyl acetate (saturated or 2% aqueous or saturated ethanolic) and lead citrate (Reynolds, 1963). Sections from QB samples were cut at 80-150 nm, mounted on formvar-carbon coated slot grids or mesh grids, double stained in 1% (w/v; aqueous) phosphotungstic acid and 10% uranyl acetate (w/v; aqueous). Alternatively, sections were stained in 2% uranyl acetate (aqueous) and lead citrate (Reynolds, 1963). Sections on mesh grids were carbon coated before viewing. For scanning electron microscopy, the tissues were critical point dried after dehydration, mounted on aluminum stubs with silver paint, sputter-coated with 40 nm of gold-palladium and examined at 5 or 10 kV on an AMRAY 1400 scanning electron microscope.

Determination of collagen fibril diameters were made from 3 and 10 week developing connective tissue and neotendon, respectively. Statistical analyses for bimodality were determined by the methods of Haldane (1952), Cox (1966) and Everitt and Hand (1981). Measurements were taken directly from transmission electron micrographs using an optical loop.

Figure 1. Morphology of collagen fiber prosthesis and newly formed connective tissue at 3 weeks post-implantation. **(a)** Scanning electron micrograph showing the uncrosslinked collagen dispersion (CD) and dehydrothermal and cyanamide crosslinked collagen fibers (CF). In this area the prosthesis is largely intact. Inflammatory cells are attached to the collagen fibers (IC). These inflammatory cells are also seen in figures 1b, c, d and e in various stages of prosthesis resorption. **(b)** Light photomicrograph showing intact collagen dispersion (CD) and intact collagen fibers (CF) surrounded by a layer of inflammatory cells (IC) and fibroblasts (F) which are arranged circumferentially around the prosthesis. Notice that some of the inflammatory cells (curved arrow) are within the matrix of the dispersion. **(c)** Light photomicrograph showing area of prosthesis undergoing early stages of resorption. Inflammatory cells (IC) have degraded a portion of the collagen dispersion (CD) (arrow) and reached a collagen fiber. Both the dispersion and fibers are partially degraded. **(d)** Scanning electron micrograph showing advanced biodegradation of collagen dispersion (CD) by inflammatory cells (IC). In this profile no newly formed tissue is shown. Inflammatory cells have formed a large hole in the dispersion (curved arrow). A collagen fiber (CF) can be seen in the background. Area with asterisk is shown at higher magnification in (1e). **(e)** Scanning electron micrograph showing higher magnification and horizontal profile of area with asterisk in (1d). Note the numerous inflammatory cells (IC) which have resorbed a substantial portion of the collagen dispersion. Compare thickness of the dispersion pictured here to (a). The inflammatory cells have begun to resorb an underlying collagen fiber (CF) of the prosthesis. Note that under 2 of the cells are resorbed areas, represented by depressions, on the surface of the collagen fibers (curved arrow). **(f)** Transmission electron micrograph showing a portion of intact collagen dispersion (CD) surrounded by a layer of inflammatory cells (IC) and fibroblasts (F) which are arranged circumferentially around the prosthesis. (Compare to figure 1b). The inflammatory cells have started to erode a pocket in the dispersion similar to that shown at the arrow in (1c).



Results

Mechanical and physical properties of reconstituted collagen fibers.

Reconstituted collagen fibers of the implant had an average diameter of 50 to 55 µm prior to implantation. As shown in Table 1, crosslinking fibers by means of dehydrothermal treatment for 3 days and exposure to cyanamide vapor for 1 day (DHT3-C1) reduced fiber swelling by 86% and increased the strength of the fibers significantly. Although the ultimate strain was similar for both types of collagen fibers, the ultimate stress of crosslinked fibers was approximately 7 times that of uncrosslinked fibers. Crosslinked fibers were roughly 10 times stiffer than uncrosslinked fibers.

Table 1. Ultimate mechanical properties of reconstituted collagen fibers. Fibers were tested under uniaxial tension at a strain rate of 50% per minute after hydrating in phosphate buffered saline. The modulus was calculated by drawing a best fit secant line at the upper region of the stress-strain curve. UNX denotes uncrosslinked fibers. DHT3-C1 denotes fibers crosslinked by dehydrothermal treatment for 3 days and subsequent exposure to cyanamide vapor for 1 day. Uncrosslinked fibers swelled 68% more than crosslinked dehyrothermal and cyanamide treated fibers. The latter were also 10 times more stiff.

Fiber Type	Diameters			Stress	Strain	Modulus
	Dry	Wet	% Swell	MPa	%	MPa
DHT3 C1 n=12	53.00 ±3.15 μm	80.80 ±6.98 μm	52	17.40 ±3.08	22.20 ±2.79	131.00 ±32.03
UNX n=8	52.60 ±9.96 μm	125.00 ±22.40 μm	138	2.40± 0.46	24.00 ±3.03	13.20 ±2.89

Table 2. *In vivo* and *in vitro* stability of reconstituted collagen fibers. UNX denotes uncrosslinked fibers. DHT3-C1 denotes fibers crosslinked by dehydrothermal treatment for 3 days and subsequent exposure to cyanamide vapor for 1 day. The DHT3-C1 collagen fibers lasted longer than the uncrosslinked collagen fibers whether used as implants (*in vivo*) or etched by collagenase (*in vitro*).

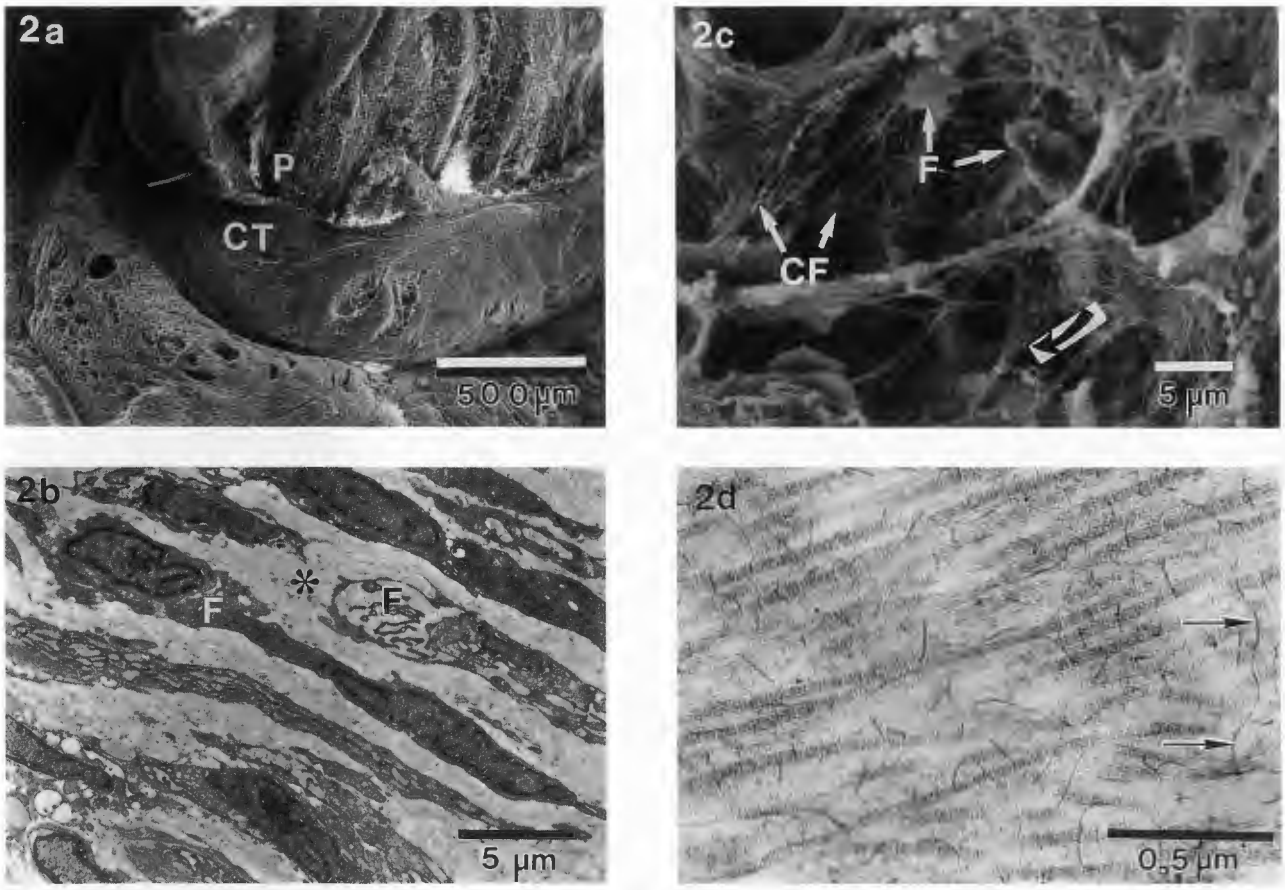
Fiber Type	# of Animals or fibers	Implant Model Used	Stability of Implant
DHT3 C1	n=2	Rat Sub-cutaneous (*In Vivo*)	Resorbed by 2 wks
	n=4	Rabbit Achilles Tendon (*In Vivo*)	Resorbed by 10 wks
	n=3	Collagenase Etching (*In Vitro*)	Resorbed after 195.3 ± 9.5 min
UNX	n=3	Collagenase Etching (*In Vitro*)	Resorbed after 29.7 ±1.5 min

Figure 2. Morphology of newly formed connective tissue at 3 weeks post-implantation. **(a)** Scanning electron micrograph showing newly formed connective tissue (CT) around the collagen fiber prosthesis (P). Some retraction of the tissue has occurred during routine biological preparation. **(b)** Transmission electron microscopy of newly formed connective tissue. Numerous fibroblasts (F) are oriented circumferentially around the prosthesis (not shown). The cells are actively synthesizing new connective tissue matrix which appears here as an amorphous matrix (asterisk) between the cells. A higher magnification view of this matrix is shown in (2d). **(c)** Scanning electron microscopy of newly formed connective tissue surrounding the prosthesis. Note the frail collagen fibrils (CF) which have not yet formed bundles. In some areas the fibrils appear to be clustered (curved arrow). Interspersed among the fibrils are numerous fibroblasts (F). **(d)** Transmission electron microscopy showing quinolinic blue staining (specific for proteoglycans) of newly formed connective tissue. Shown are small diameter collagen fibrils among disorganized proteoglycans. Note the very long proteoglycan (arrows) which extends over 7 collagen fibrils.

As shown in Table 2 the dehydrothermal and cyanamide crosslinked collagen fibers implanted subcutaneously into rats were completely resorbed by 2 weeks. In the rabbit Achilles tendon model this group of collagen fibers were completely resorbed by 10 weeks, although in at least 1 specimen, vestiges of the collagen fibers were identified. *In vitro* testing of the dehydrothermal and cyanamide crosslinked collagen fibers, by etching the fibers with collagenase, showed they were completely digested after 195.3±9.5 mins. The uncrosslinked fibers were completely resorbed in 29.7±1.5 mins, nearly 6.5 times faster than the dehydrothermal and cyanamide treated fibers.

Morphology of 3 and 10 week prostheses and newly formed connective tissue.

At 3 wks, the uncrosslinked collagen dispersion and dehydrothermal and cyanamide crosslinked collagen fibers were in various states of degradation. Figures 1a and



1b show an area of the prosthesis where the reconstituted fibers and dispersion were still intact. In other areas, the dispersion and collagen fibers of the prosthesis were in the process of degrading (Figures 1c, d and e). Careful inspection of the dispersion showed that some inflammatory cells had "worked" their way through the dispersion (by phagocytosis) and were approaching the collagen fibers (Figure 1b). In other areas, the dispersion and fibers had undergone considerable resorption by inflammatory cells (Figures 1c, d and e). It appeared that the inflammatory cells initially formed a layer around the prosthesis, actively phagocytosing the highly degradable layer of dispersion. Figure 1f shows inflammatory cells penetrating the edge of the collagen dispersion. Once the cells reached the collagen fibers, they started to degrade these structures too (compare to figure 1a for position of dispersion and collagen fibers).

At the 3 week time point a layer of fibroblasts was also observed around the prosthesis (Figures 1f and 2b). This layer was actually juxtaposed to the layer of inflammatory cells which were phagocytosing the dispersion (Figure 1f). The fibroblasts were separated by an amorphous matrix (Figures 1f and 2b). This matrix consisted of newly formed connective tissue and had a variable thickness which appeared to range from 30-130 µm (Figure 2a). This tissue contained many capillaries. Examination at high magnification showed the matrix was composed of frail and loosely knit collagen fibrils connected by a meshwork of large disorganized proteoglycans (Figures 2c and 2d). The proteoglycans frequently appeared as long filamentous ribbons, some of which measured up to 0.5 µm in length (Figure 2d). In many profiles these ribbons crossed over as many as 7 collagen fibrils (Figure 2d). Proteoglycans were oriented parallel, oblique and perpendicular to collagen fibrils. The approximate spacing of the filaments where they attached to the collagen was 62.5 nm. Proteoglycans were frequently unassociated with collagen fibrils. The mean diameter of collagen fibrils in the developing connective

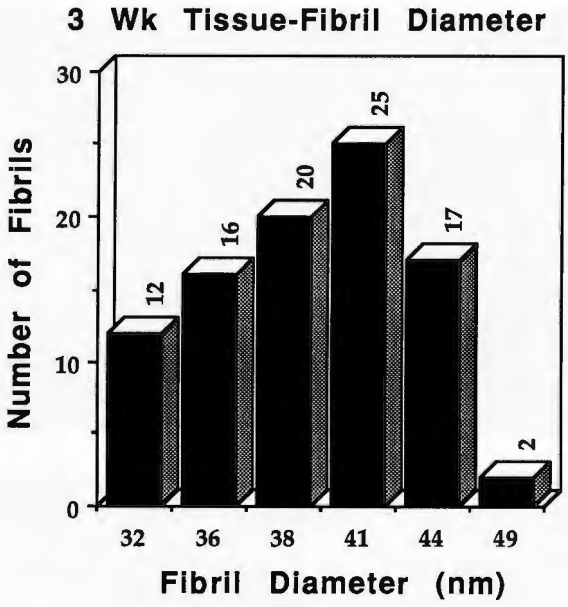


Figure 3. Three week connective tissue fibril diameters. At 3 weeks there is a unimodal distribution with a mean fibril diameter of 39 nm.

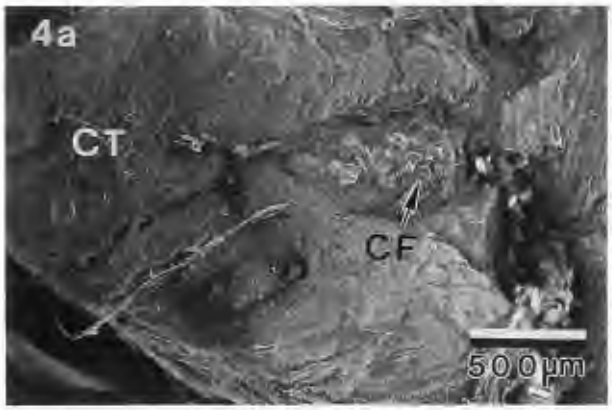
Figure 4. Morphology of 10 week neotendon. (**a**) Scanning electron microscopy of large portion of neotendon. Shown is newly formed connective tissue (CT). Note an area containing unresorbed remnants of collagen fibers from the prosthesis (CF). (**b**) Scanning electron micrograph showing highly organized and tightly packed collagen fibrils (CF). (**c**) Transmission electron micrograph showing organization of collagen fibrils into bundles (CFB) at 10 weeks. Note the fibroblasts (F) among the bundles of collagen. Collagen oriented tangentially (arrows) represent crimp formation in the neotendon. (**d**) Transmission electron micrograph showing quinolinic blue staining (for proteoglycans) of neotendon. Shown are large diameter collagen fibrils and smaller highly organized proteoglycans (arrow).

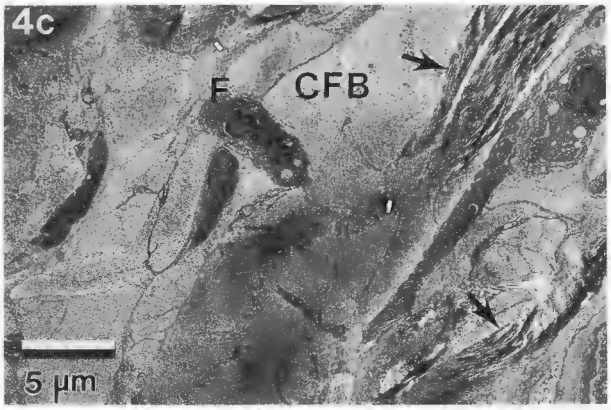
tissue was 39 nm. The distribution of fibril diameters is shown in Figure 3.

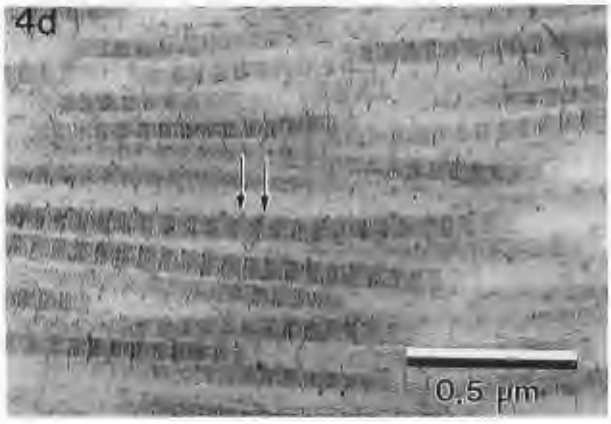
By 10 weeks, an appreciable neotendon that was well vascularized had formed (Figure 4a). In the 5 samples examined by light microscopy, no signs of the collagen fiber prosthesis could be found. In the 10 week sample examined by scanning electron microscopy, remnants of collagen fibers from the prosthesis could be seen (Figure 4a). The neotendon was composed of collagen fibrils which were now more highly organized and tightly packed than at 3 weeks (Figure 4b). Tightly packed bundles of collagen fibrils were arranged in longitudinal orientation relative to the implanted prosthesis. Some collagen fibril bundles were also observed in oblique orientations within a single plane of section (Figure 4c). Interspersed between the bundles were fibroblasts identified by their long thin processes. There was an appreciable attenuation of these cells compared to the 3 week group. The proteoglycans appeared highly organized (Figure 4d). The approximate spacing of the filaments where they attached to the collagen was 62.5 nm. Proteoglycans were always associated with collagen fibrils. At 10 weeks, the mean diameter for collagen fibrils in the neotendon was 72 nm (Figure 5). A bimodal distribution of the collagen fibrils was apparent based on their diameters. The lower end of this distribution ranged from 59 to 68 nm and had a mean diameter of 65 nm. The upper portion of this distribution ranged from 74 to 88 nm and had a mean of 78 nm.

Autograft or devascularized tendons underwent a period of fibroblast necrosis which was observed by light microscopy at 3 weeks. The collagen of these tendons appeared normal. Fibroblasts appeared to repopulate the tendon by 10 weeks and the collagen still looked like normal tendon. No inflammatory response involving resorption of the collagen was observed at 3 weeks (morphology of these samples not shown).

Ultrastructural observations of normal rabbit tendon showed distinct bundles of tightly packed collagen fibrils (Figure 6a). The collagen fibrils within the bundles were of variable diameters, highly organized and tightly packed (Figure 6b). Fibroblasts were interspersed between the bundles (not shown). Proteoglycans in control tendon appeared as thin filaments or dots in longitudinal sections (Figure 6b). A thorough description of collagen fibril diameters and proteoglycans in normal connective tissues is provided by Scott (Scott, 1980), Scott and co-workers (Scott et al., 1981; Scott and Orford, 1981) and Parry and Craig (1977, 1988).







Discussion

Autogenous tendon is the most desirable biomaterial for grafting. Observations on devascularized controls demonstrated that, except for a transient diminution of the fibroblast population, there is virtually no resorption of the collagen graft. The eventual repopulation of the tendon by host fibroblasts means the tissue will be sustained for extended periods. Because of limitations on autogenous tendon availability and the complications associated with obtaining these tissues, a more reliable tendon replacement is needed. Goodship and Cooke (1986) suggest that the ideal biomaterial should be resorbable while still allowing fibroblast infiltration and the gradual establishment of a neotendon that is comparable to the host's original tendon in biomechanical and morphological properties.

Our studies performed on a reconstituted collagen fiber prosthesis indicated this material may be an acceptable tendon replacement. The combined virtues of strength, resorbability and induction of

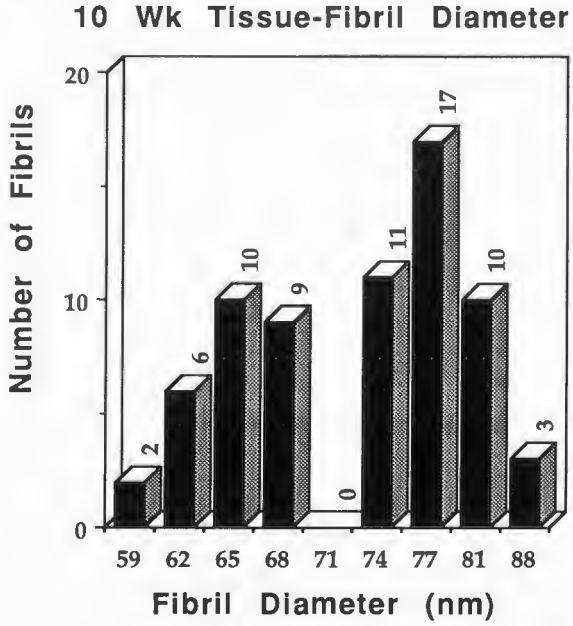
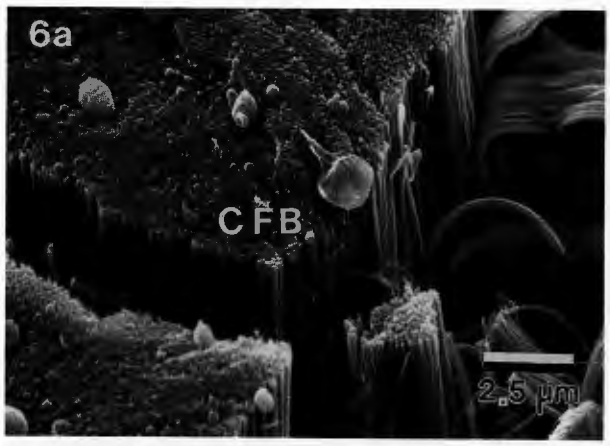


Figure 5. Ten week neotendon collagen fibril diameters. At 10 weeks there was a bimodal distribution with a mean fibril diameter of 72 nm.

aligned host connective tissue were major reasons which lead to the use of this fiber for Achilles tendon replacement. Reconstituted type I collagen fibers crosslinked by dehydrothermal and cyanamide treatment were both stronger and more stable than uncrosslinked collagen fibers. When used as an Achilles tendon replacement, they lasted up to 10 weeks. Subcutaneous implantation of this fiber in rats results in complete resorption by 6 weeks (Law et al., 1989). In the present study, the collagen fiber prosthesis induced the formation of new collagen which was aligned parallel and oblique to the orientation of the prosthesis. Law and co-workers (1989) placed reconstituted collagen fibers parallel to the medial collateral ligament and found newly developing collagen aligned parallel to the reconstituted collagen fibers. The development of longitudinally aligned and oblique collagen fibril bundles by 10 weeks are confirmed by other studies which actually implanted collagen fibers for tendon replacement (Goldstein at al., 1989).

Achilles tendon replacement with a collagen fiber prosthesis induced 2 categories of host-elicited responses. The first was resorption and involved a classic inflammatory response. This response included an initial phase in which the collagen dispersion biodegraded. Phagocytosing macrophages predominate by 5 days post-trauma in tendon injuries (Gillman, 1968). The collagen dispersion was more vulnerable to degradation than the fibers because it was not crosslinked. This conclusion is supported by the stability tests which showed uncrosslinked collagen was degraded by collagenase nearly 6.5 times more rapidly than the fibers. Inflammatory cells, in addition to being actively phagocytic, release collagenase (Lazarus, 1968a, 1968b; Harris et al., 1969; Oronsky et al., 1973; Harris and Krane, 1974; Wahl, 1975) which would enzymatically attack the dispersion. A second phase of resorption was degradation of the collagen fibers. Degradation of the dispersion and the fibers must be considered separate phases because the rate of fiber resorption was dependent upon the method of crosslinking. The dispersion was never crosslinked and consistently degraded the fastest. Dehydrothermal and cyanamide crosslinked fibers degraded more slowly. At the other extreme are fibers crosslinked by glutaraldehyde. These collagen fibers implanted subcutaneously into rats are reported to persist past 6 months (Law et al., 1989). When used as a rabbit Achilles tendon replacement, glutaraldehyde crosslinked fibers are still present at 20 weeks, however, dehydrothermal and cyanamide crosslinked fibers are mostly resorbed by 10 weeks (Goldstein et al., 1989). The latter



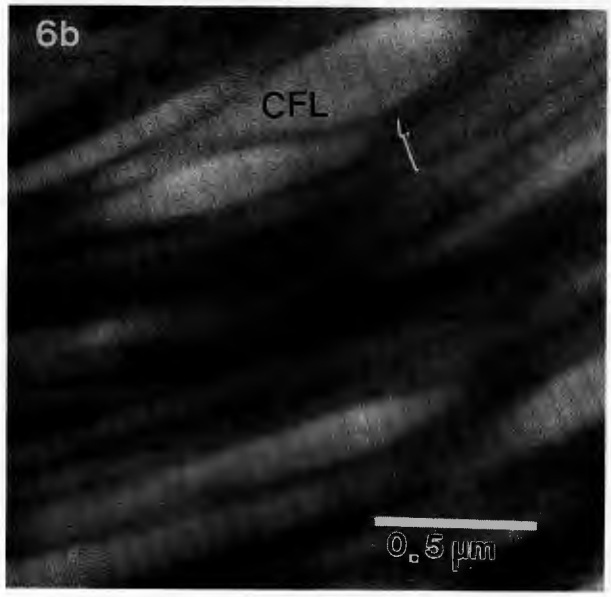


Figure 6. Morphology of control tendon. (**a**) Scanning electron micrograph showing tightly packed collagen fibril bundles (CFB). (**b**) Transmission electron microscopy showing quinolinic blue staining (for proteoglycans). Shown are tightly packed collagen fibrils of variable diameters. Note extremely large collagen fibrils (CFL). Proteoglycans appear as highly organized dots at sides of fibrils (arrow).

is corroborated in the present study and demonstrates differences in the rate of resorption depending upon the method of crosslinking.

The second category of host-elicited responses involved a sequence of chronological matrix events including successive macromolecular changes leading to the assembly of a mature connective tissue. About 5 days following a tendon injury the number of fibroblasts begins to increase (Goodship and Cooke, 1986) and reaches a maximum 3 weeks post-injury (Birdsell et al., 1966). In the present study, fibroblasts invaded the prosthesis and deposited a circumferential coating of new connective tissue around the device. Initially this matrix consisted of frail small diameter collagen fibrils connected by large proteoglycans. Between 3 and 10 weeks this matrix transformed into one containing highly aligned collagen fibrils with larger diameters. These fibrils were connected by more organized proteoglycans.

The fact that collagen fibril diameters increased over time may be the result of a gradual fusion of fibrils and accretion of new collagen. Parry and Craig (1988) propose that collagen fibrils grow in increments of 7 to 8 nm units. In the present study, the mean collagen fibril diameters at 3 and 10 weeks were 39 and 72 nm respectively, a difference of 33 nm. This is approximately a 4 fold increase based on 8 nm increments. An alternate way to analyze fibril growth is to consider the 3 week mean of 39 nm and 10 week (Figure 5) bimodal means of 65 (lower end) and 78 (upper end) nm. In this case, the differences were 26 and 13 nearly a 3 and 2 fold increase, respectively, based on 8 nm units. These observations on the growth of collagen fibrils during wound healing are important since they corroborate that which other workers (Scott, 1984; Scott et al., 1981; Parry and Craig, 1988) report for collagen fibril growth in normal developing connective tissues.

The organization of collagen fibers is reported to be a function of molecular constraints imposed by proteoglycans (Borcherding et al., 1975). The size of collagen fibers may be controlled by chondroitin sulfate (Flint et al., 1984). In developing rat tail tendons, increasing collagen fibril diameters are correlated with a decline in the proteoglycan/collagen ratio due to a loss of chondroitin sulfate (Scott, 1984; Scott et al., 1981). Perhaps, the matrix organizational changes seen in the present study were also related to specific biochemical (PG) changes.

These results emphasize similarities between matrix morphogenesis during wound healing and that which occurs during normal developmental biology. In the present study a reorganization of the matrix, including an increase in collagen fibril diameters and proteoglycan changes takes place. This leads to the restoration of a nearly normal tissue. However, it must be pointed out that not all connective tissue matrices heal the same way. For example, a tear in the cornea will result in the deposition of large diameter and highly disorganized collagen fibrils resulting in an optically opaque scar. This is dramatically different from the small and highly organized fibrils laid down during normal development which are thought to be responsible for optical translucence (Maurice, 1957; Anseth and Fransson, 1969; Cintron and Kublin, 1977). Some biological systems may retain the ability to heal as they develop while others lose it. This could be a reflection of an inability to retain the original matrix synthesizing machinery. Perhaps the application of an appropriate biomaterial scaffold to injured tissues which have lost their original morphogenetic patterning capabilities, will allow the synthesis of connective tissues which mimic the morphological and biomechanical properties of the original tissue.

Summary

Dehydrothermal and cyanamide crosslinked collagen fibers when used as a tendon replacement prosthesis provide a scaffold for the formation of new connective tissue. This prosthesis induces a sequence of host-elicited responses. These responses have been defined morphologically and categorized as prosthesis resorption and connective tissue induction. Prosthesis resorption involves an inflammatory response leading to the complete removal of the device. Connective tissue induction involves a progressive maturation of the newly synthesized extracellular matrix including, an increase in collagen fibril diameters and a transition in collagen/proteoglycan interactions, resulting in a well developed neotendon. These

dehydrothermal and cyanamide crosslinked fibers are ideally suited for use in tissue augmentation and/or replacement and may have applications to biological systems where wound healing is compromised or otherwise altered from its original patterning.

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Discussion with Reviewers

M. Chvapil: (a) What is the final pH of the strong acid extruded collagen fiber?

(b) How did the authors verify the degree of cross-linking of collagen fibers?

(c) The finding of bimodal distribution of the fibers diameter at 10 weeks needs mathematical proof, based on probability, that there is factual "biguassian distribution". How many repeats were done?

(d) This reviewer has difficulty to see "old" and new collagen fibers in Figure 4b compared to Figure 6a. Why are collagen fiber bundles in Figure 4b newly formed, not stemming from the implant? It has been the experience of this reviewer that the newly formed collagen fibers within a tendon are oriented parallel to the axis of mechanical stresses....even at early periods (8-12 weeks). You show the presence of "transverse and oblique orientations", which may reflect the original fibers of the implant.

Authors: (a) The final pH of the fiber has not been determined. The tissue ingrowth in and around the fibers reflects their biocompatibility. One must assume they are physiological in pH or else toxicity and severe inflammatory reactions would be observed.

(b) Strength and stability characteristics of dehydrothermal and cyanamide crosslinked vs. uncrosslinked collagen fibers show the former fibers are stronger and longer lasting than the later (Table 1). This indicates that dehydrothermal and cyanamide crosslinked collagen fibers are more highly crosslinked than control uncrosslinked fibers. For a more extensive examination of collagen fiber crosslinking techniques the reviewer is referred to a paper on the subject by Weadock and co-workers (1984).

(c) Three different methods are used to evaluate the modality of the frequency distribution for the 10 week fibril diameters. The first two techniques presented by Haldane (1952) and modified by Cox (1966) involve the tabulation of the second central difference and their standard errors. The resultant data is then inspected for two points of inflection which are indicative of bimodality and bitangentiality. The technique is based on geometric properties of the sample frequency curve. These are considered informal methods and the degree of significance is hard to assess (Cox, 1966). However, using both the methods presented by Cox (1966) and Haldane (1952) there is evidence of two points of inflection in the frequency distribution thus indicating that the distribution is bimodal.

A more analytical method is reported by Everitt and Hand (1981). They establish that a sufficient condition, such that there exists significant values of p, for which a mixture of distributions is bimodal, is given by:

$$\left(\mu_2 - \mu_1\right)^2 > \frac{8\,\sigma_1^2\,\sigma_2^2}{\left(\sigma_1^2 + \sigma_2^2\right)}$$

where, m is the mean of the peak value with the subscript 1 signifying the lower peak and the subscript 2 indicating the upper peak. The symbols indicate the standard deviation of the respective peaks as indicated by the subscripts. The mean of the lower peak was measured as 65.04 ± 3.0 and the mean of the upper peak as 77.17 ± 4.4 micrometers. The result of this evaluation indicates that the

square of the difference of the means equals 147.2 and the right side of the above equation equals 51.6, consequently 147.2 > 51.6 satisfying the sufficient condition for bimodality as presented by Everitt and Hand (1981).

(d) Based on extensive light microscopic examination of these neotendons it is apparent that by 10 weeks there is virtually no remaining prosthesis or "old collagen". A small vestige of collagen fibrils from the prosthesis is seen in the center of the sample examined by scanning electron microscopy (Figure 4a) but this is the exception. It is apparent that the structures shown in Figure 4b represent new collagen which has become organized into collagen fibril bundles. This is also shown in Figure 4c by transmission electron microscopy. To be certain that this "newly synthesized" collagen doesn't contain vestiges of the "old (prosthesis)" collagen we will perform gold labeled anti-body studies directed at bovine type I collagen. This should determine if there is any bovine collagen from the prosthesis left among the new collagen fibrils of the rabbit neotendon. Additional support that these fibril bundles are synthesized by the host (rabbit) and are not part of the prosthesis comes from the fact that the newly formed connective tissue fibril bundles contain fibroblasts which are never present within or among the collagen fibers and fibrils of the prosthesis (Figure 4c). Transverse or oblique bundles of collagen fibrils as seen at 10 weeks (Figure 4c) may represent collagen forming a crimp which is characteristic of tendon. Crimp development in neotendon is well documented by this laboratory using polarized light microscopy (Goldstein et al., 1989)

C. W. Kischer: Would you care to speculate as to why collagen fibrils are arranged in fascicles or bundles? What would be the limiting size? What factors or structures determine the division into bundles?

Authors: Functionally, collagen bundles and fascicles provide maximum tensile strength to a tendon. Strength requirements or stress may account for the evolution of collagen into fascicles rather then randomly oriented single collagen fibrils. The hierarchical organization of the extracellular matrix is regulated by the cells of the matrix, from synthesis of the collagen molecule to collagen fibril formation and ultimately the formation of macroaggregates (Trelstad and Hayashi, 1979). Matrix cells contain specific collagen fibril bundle synthesizing compartments which directly regulate the configuration of the final collagen macroaggregate (Trelstad and Hayashi, 1979; Birk and Trelstad, 1986). The geometry of the matrix suprastructure can therefore be traced back to the morphology and physiology of the matrix specific cell. Cells of the matrix are genetically pre-programmed for the synthesis and subsequent organization of the connective tissue matrix.

The constraints placed on collagen fibril, bundle and fascicle size may be related to more than 1 parameter. The cells of the matrix regulate the formation of the collagen fibrils (Trelstad and Hayashi, 1979; Trelstad and Silver, 1981; Birk and Trelstad, 1984, 1985). The propeptides on the procollagen molecules have also been implicated in the regulation of collagen fibril formation (Weistner et al., 1979; Fleischmajer, 1981; Miyahara et al., 1982), however, cleavage of propeptides may be a function of cellular processes. In addition, mechanical properties of the tissue or physical forces applied to tissues are directly related to collagen fibril diameters (Parry et al., 1978; Merilees and Flint, 1980; Parry and Craig, 1988). Finally, the interaction between proteoglycans, glycosaminoglycans and collagen fibrils regulates collagen fibril diameter (Borcherding et al., 1975; Parry et al., 1982; Scott and Orford, 1981; Scott, 1984; Scott et al., 1981). With maturity, it is known that collagen fibril diameters increase (Parry and Craig, 1977; Parry et al., 1978; Scott et al., 1981; Flint et al., 1984). It is likely one or more of the factors described above plays a role in limiting the overall size of the collagen fibril, fiber and fascicle which occurs during development and wound healing. For additional information on the assembly and support of connective tissues an excellent summary is provided by Brodsky and Eikenberry (Brodsky and Eikenberry, 1985).

E. Bell: It is not clear how the authors distinguish between the implant and what is presumed to be laid down by the host cells. They may not be aware that the process of acid extracting collagen can result in the swelling and twisting of collagen fibrils but that these distortions are reversible. Basing a discrimination on what the initial appearance of the implant looks like is insufficient since the implant itself can change with time.

Collagen marking by some more rigorous technique is essential for an accurate accounting of the sequence of morphological changes that the authors attempt to interpret. The statement for example "These cells have deposited a circumferential coating of collagen....." is extremely difficult to defend since there is no evidence whatever that what they point to is the product of the fibroblasts they see in the neighborhood. In fact, they don't even show that the material is collagen.

Authors: At 3 weeks the collagen fiber implant is still intact but shows signs of resorption (inflammatory cell degradation). It is unlikely that the connective tissue ring which surrounds the prosthesis is a portion of the prosthesis since its morphological characteristics are completely different. For example, the connective tissue ring contains fibroblasts (Figures 1f, 2b and 4c). These were never identified in the prosthesis, either before implantation or after explantation. In addition, the new connective tissue contains enormous proteoglycans as demonstrated by quinolinic blue staining. These are also not found in the prosthesis (Garg et al., 1988). Finally, the new connective tissue is composed of frail, disorganized, widely spaced and small diameter collagen fibrils that show no sign of collagen fibril bundles as are seen in the prosthesis (Wasserman et al., 1988). It is clearly connective tissue in the formative stages (Figures 2b, 2c and 2d) and should not be confused with the prosthesis.

Our statement that "These cells deposit a circumferential coating of collagen around the prosthesis" is based on 3 significant observations. First, this "coating of collagen" is composed of fibroblasts which are known to synthesize collagen. Second, the fibroblasts are embedded in an amorphous material which at high magnification is composed of fibrils having a striation with a periodicity of approximately 62 nm and an approximate diameter of 39 nm. These dimensions are characteristic of collagen Third, when stained for proteoglycans an incredible network of filamentous material is found intertwined among the fibrils. Proteoglycans are reported to associate with collagen fibrils (Borcherding et al., 1975; Parry et al., 1982; Scott, 1984; Scott and Orford, 1981; Scott et al., 1981). All of these results suggest this is a developing connective tissue matrix containing collagen fibrils and proteoglycans.

At 10 weeks, extensive light microscopy observations show that rarely a few fragments of collagen fibers from the prosthesis remain. The fibers are almost degraded by inflammatory cells. Dr. Bell's contention is that it is difficult to "distinguish between the implant and what is presumed to be laid down by the host cells" As we explained above, at 3 weeks the morphological characteristics of the new connective tissue are so different from the prosthesis that it is easy to distinguish one from the other. However, at 10 weeks, the prosthesis is resorbed and it would be difficult to "distinguish" between new collagen and vestiges of the collagen fiber prosthesis. With this challenging problem, Dr. Bell stimulates an interesting question. Could the prosthesis become incorporated into the collagen of the developing neotendon? Dr. Bell states that "collagen marking by some more rigorous technique is essential for an accurate accounting of the morphological changes" that take place during prosthesis degradation and neotendon formation. We agree with this and it is likely that through the use of collagen specific gold probes this provocative question can be answered. These are ongoing studies which will be reported in a future communication.

Additional References

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