Effect of Crosslinking Method on Collagen Fiber–Fibroblast Interactions

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ABSTRACT: Collagen, the major structural protein of the extracellular matrix in animals, is a versatile biomaterial used in various tissue engineering applications. Crosslinking influences the mechanical properties, resorption kinetics, and biocompatibility of collagen-based biomaterials. In this study, we evaluated the effects of crosslinking on collagen fiber–fibroblast interactions *in vitro.* Collagen fibers were left untreated or crosslinked by ultraviolet (UV) irradiation, dehydrothermal (DHT) treatment (3 or 5 days), or hexamethylenediisocyanate (HMDIC) exposure. The initial attachment, proliferation (through 8 days), and morphology of human dermal fibroblasts were evaluated on control and crosslinked bundles of 200 collagen fibers *in vitro.* Initial attachment (number of fibroblasts at day 0) was increased on UV and DHT5-treated collagen fiber bundles. Fibroblast proliferation was similar for control, UV crosslinked, and DHT crosslinked fibers. In contrast, fibroblast attachment was significantly decreased and proliferation was delayed on HMDIC crosslinked fibers. These results, coupled with our previous studies, suggest that UV crosslinking of collagen fibers provides a combination of biocompatibility, mechanical properties, and strength retention suitable for various tissue engineering applications. © 1997 John Wiley & Sons, Inc. J Appl Polym Sci **63:** 1493–1498, 1997

Key words: Collagen fiber; crosslinking; fibroblast; biocompatibility; tissue engineering

INTRODUCTION

Tissue engineering is an emerging discipline that combines resorbable "scaffolds," cells, and cell signals to regenerate damaged tissue.[1] Collagen, the major structural protein of the extracellular matrix in animals, is a versatile biomaterial that has been used in various tissue engineering applications as a temporary scaffold to support new tissue formation. Our laboratory is developing collagen fiber-based scaffolds for anterior cruciate ligament (ACL) reconstruction.[2–5] Crosslinking of collagen fibers influences critical performance parameters including mechanical properties, resorption kinetics, and biocompatibility. Chemical crosslinkers[6–8] generally provide high strength, prolonged resorption, and poor biocompatibility. Our laboratory is investigating ultraviolet irradiation (UV) and dehydrothermal (DHT) treatment to covalently crosslink collagen without introducing potentially cytotoxic chemicals.[9,10]

We recently showed that both UV and DHT treatments strengthen collagen fibers[9]; however,

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DHT-treated fibers are largely denatured and resorb too rapidly in the presence of nonspecific proteases.[10] Our objective in this study was to evaluate the cellular compatibility of UV and DHT crosslinked collagen fibers using an *in vitro* fibroblast-seeding model.[5] Fibroblast attachment, morphology, and proliferation (through 8 days) were measured on UV and DHT crosslinked collagen fibers *in vitro*. Uncrosslinked and chemically crosslinked (hexamethylenediisocyanate; HMDIC) fibers were included as controls. Results of this biocompatibility study, combined with our previous strength[9] and resorption[10] studies, suggest that UV irradiation is a potentially useful collagen crosslinking method for various tissue engineering applications.

MATERIALS AND METHODS

Characterization of Collagen

Insoluble collagen derived from bovine corium was obtained from Devro, Inc. (Somerville, NJ). Amino acid analysis was conducted as previously described.[11] Collagen types were identified using $\alpha 1(I)$—CB2 and $\alpha 1(III)$—CB4 as marker cyanogen bromide peptides for type I and III collagens, respectively.[12] These analyses indicated that the starting material consisted essentially of pure collagen, predominantly type I and less than 3% type III.

Fabrication of Collagen Fibers

A 1% w/v collagen dispersion was prepared by blending milled collagen with HCl (pH = 2.4) as previously described.[9] Collagen dispersion was extruded at a constant rate through polyethylene tubing (inner diameter = 0.86 mm) into fiber formation buffer at 37°C, pH 7.4. After 45 min, the buffer was replaced with isopropyl alcohol. Alcohol was removed after 16 h and distilled water was added for 15 min. Collagen fibers (70 μm average dry diameter) were removed and allowed to air dry under tension.

Crosslinking of Collagen Fibers

Fibers were divided into five groups. The control group remained uncrosslinked (UNX), while other groups were crosslinked with either UV irradiation, DHT treatment (3 or 5 days), or hexamethylenediisocyanate (HMDIC).

Ultraviolet irradiation was applied by placing dry collagen fibers in an ultraviolet crosslinking chamber (Stratagene Stratalinker™, Model 2400). Fibers were exposed to a bank of five 15 watt ultraviolet bulbs for 30 min as previously described.[9] Dehydrothermal crosslinking was achieved by placing dry collagen fibers in a vacuum oven (Napco, Model 5851) and applying a vacuum of approximately 0.1 μm. One hour later the temperature was increased at a rate of 30°C per hour until the temperature reached 110°C. These conditions were maintained for 3 days (DHT3) or 5 days (DHT5). The oven was allowed to cool to room temperature prior to releasing the vacuum.

HMDIC crosslinking was performed by placing fibers in a 1% v/v solution of HMDIC and ethanol[13] for a total of four hours, fresh solution being added every hour. Fibers were rinsed in 100% ethanol three times (15 min each), 80% ethanol for 15 min, and 50% ethanol for 15 min. Fibers were rinsed extensively with distilled water and allowed to dry overnight.

Assembly of Collagen Fiber Bundles

Collagen fiber bundles were constructed by aligning 200 fibers in parallel and lightly coating with 1 % w/v collagen dispersion for handling purposes (dispersion was $<$ 10% of total bundle weight). Fiber bundles were submerged in fiber formation buffer at 37°C (15 min), then allowed to dry overnight under a weight of approximately 35 g to form a compact structure. All bundles were surface sterilized under a 30 watt ultraviolet lamp for 15 min/side and soaked for 24 h in sterile phosphate-buffered saline (PBS) with 2% fungizone. This brief surface sterilization procedure does not affect the mechanical properties of the fiber bundles (unpublished observations).

Mechanical Testing

Ten 200-fiber bundles from each experimental group were mechanically tested to determine structural and material properties. Fiber ends were epoxied to vellum paper for ease of handling and to prevent failure at the grips. Each fiber bundle (3 cm length) was immersed in PBS for at least 45 min prior to testing. Sample diameters were measured using a Z-MIKE Model 1202B laser micrometer and cross-sectional areas were calculated by assuming a circular cross-section. Samples were tensile tested on an Instron Model

4204 materials tester at a strain rate of 1000% strain per minute (gauge length = 10 mm; crosshead speed = 100 mm/min) until failure. Load vs. deformation curves were converted to stress vs. strain curves by considering sample dimensions. The ultimate tensile strength and modulus (slope of the stress–strain curve) were determined from the stress–strain curves of the collagen fiber bundles.

Fibroblast Seeding of Collagen Fiber Bundles

Human skin fibroblasts derived from the foreskin of a newborn male (HS68) were obtained from the American Type Culture Collection (Baltimore, MA). Cells were grown to confluence in 250 mL flasks in Dulbecco's Modified Eagle Medium (DMEM, Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum, 1% glutamine, 1% Hepes buffer, and 2% fungizone. Mycoplasma testing during culture was negative based on the Hoescht stain.[14] Sterile 200-fiber bundles were cut into 1 cm lengths and spread evenly on the bottom of 96-well tissue culture plates, and seeded with 5×10^4 fibroblasts. After approximately 30 min, cell-free media was added to bring the total well volume to 125 μL. Plates were incubated at 37°C (5% CO_2) and media was changed daily.

Determination of Fibroblast Attachment and Proliferation

Fibroblast attachment (4 h after seeding) and proliferation (2, 4, and 8 days) were measured using Cell Titer 96 (Promega Corp., Madison, WI), a colorimetric assay in which metabolically active cells react with a tetrazolium salt to produce a soluble formazan dye. This method has been used by others to evaluate fibroblast growth on collagenous substrates.[15]

Fibroblast-seeded collagen bundles ($n \geq 6$ for each crosslinking group at each time period) were removed from plates, rinsed twice in PBS, and transferred to 24-well plates. Cell Titer 96 (200 μL/well) was added to each well with 1 mL media supplemented with 5% fetal bovine serum. After 4 h incubation, the absorbance of the media was determined at 490 nm and compared to a standard curve to estimate viable cell number. Using this method, only viable cells are included in the count, and since fiber bundles were rinsed prior to testing, only cells attached to the fiber bundle were counted.

Table I UTS and Modulus of Collagen Fiber Bundles (Mean ± Standard Deviation)

Xlinker	UTS (MPa)	Modulus (MPa)
CONTROL	2.17 ± 1.3	9.13 ± 6.3
UV	7.14 ± 1.3	22.9 ± 5.4
DHT3	7.04 ± 1.0	32.3 ± 6.3
DHT5	8.23 ± 2.1	38.4 ± 20.4
HMDIC	13.8 ± 0.9	102 ± 21.3

Fibroblast Morphology

Fibroblast-seeded collagen bundles were examined by routine histology after 8 days *in vitro*. Bundles were fixed in Carson's buffered formalin, embedded in paraffin, and sections were stained with hematoxylin and eosin and viewed under a light microscope. Fluorescent microscopy was also conducted on several samples per group by labeling fibroblasts with PKH2-GL lipophylic dye (Zynaxis Cell Science, Malvern, PA) prior to seeding as previously described.[5]

Statistical Analyses

Analysis of variance (ANOVA) was performed using StatGraphics™ software to determine the effects of crosslinking method (uncrosslinked, UV, DHT3, DHT5, HMDIC) on fiber bundle mechanical properties, fibroblast attachment, and fibroblast proliferation. Differences between individual groups were considered significant for $p < 0.05$.

RESULTS

Mechanical Testing

All crosslinking methods significantly increased the ultimate tensile strength (UTS) and modulus of the collagen fiber bundles compared to uncrosslinked controls (Table I). Uncrosslinked fiber bundles were relatively weak and compliant; HMDIC crosslinked bundles were very strong and stiff. Intermediate strength and modulus values resulted from UV and DHT crosslinking. These results for 200-fiber bundles are consistent with our earlier mechanical testing results for individual collagen fibers.[9]

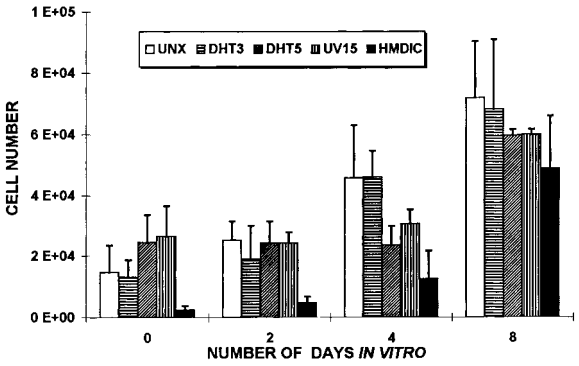

Figure 1 Fibroblast attachment (day 0; 4 h) and proliferation on collagen fiber bundles as a function of crosslinking method (mean with standard deviation; UNX = uncrosslinked control). Initial fibroblast attachment was highest on the UV crosslinked bundles. Cell numbers increased over time for all groups. Fibroblast attachment and proliferation were significantly decreased on HMDIC crosslinked fiber bundles.

Fibroblast Attachment, Proliferation, and Morphology

The number of fibroblasts attached to the collagen fiber bundles varied with the crosslinking method and time (Fig. 1). Initial fibroblast attachment (at 4 h; day 0 time point) was significantly increased on UV and DHT5 crosslinked fiber bundles. Significantly fewer fibroblasts were attached to HMDIC crosslinked bundles. In general, the number of fibroblasts found on the bundles increased with time. However, the number of fibroblasts observed on HMDIC crosslinked bundles at any time was significantly lower than all other groups.

Fibroblasts in monolayer culture (on tissue culture flasks) were well spread with no preferred orientation (Fig. 2). On collagen fiber bundles, fibroblasts were distributed along the length of individual collagen fibers within the bundle (Fig. 3). Fibroblasts were well spread and aligned along the long axis of the collagen fibers, typical of fibroblasts adhered to a cylindrical substrate having a diameter of less than 100 μm.[16] Histological sections showed fibroblasts attached to individual collagen fibers throughout the fiber bundle (Fig. 4). Fibroblast number, morphology, and distribution were similar for all except the HMDIC group, which had far fewer fibroblasts attached at any time period (consistent with results shown in Fig. 1). Nonviable cells were frequently found on and around the HMDIC crosslinked fiber bundles.

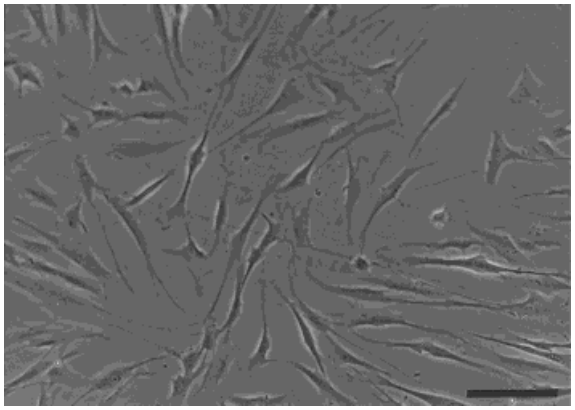

Figure 2 Human dermal fibroblasts in monolayer culture (on tissue culture flasks) were well spread with no preferred orientation. (Bar = 20 μm).

DISCUSSION

Collagenous tissues and processed tissue derivatives[17] in the form of coatings, sheets, sponges, gels, tubes, and fibers have been used as tissue culture substrates and implants. Although collagen is a "naturally" biocompatible protein, collagen–cell interactions are influenced by a number of variables including collagen type, secondary and tertiary structures, gross morphology, and the degree and type of crosslinking. Previously, we demonstrated that rabbit anterior cruciate ligament (ACL) and patellar tendon fibroblasts attach, proliferate, and secrete extracellular matrix on collagen fiber bundles *in vitro*.[5] In the present study, human dermal fibroblasts (potentially use-

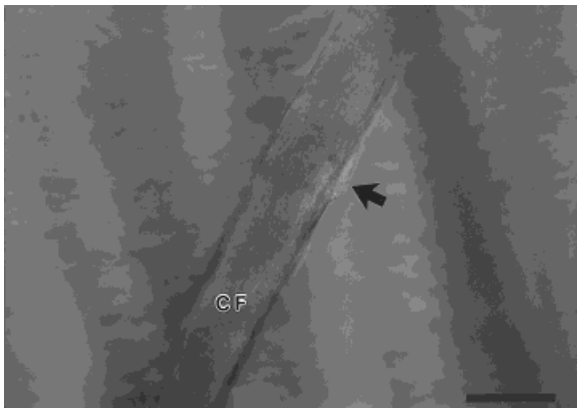

Figure 3 Human dermal fibroblasts (labeled with fluorescent dye; arrow) 4 h after seeding onto a UV crosslinked collagen fiber bundle *in vitro*. Fibroblasts were well distributed along the length of individual collagen fibers (CF) and tended to orient along the long axis of the fibers. (Bar = 100 μm).

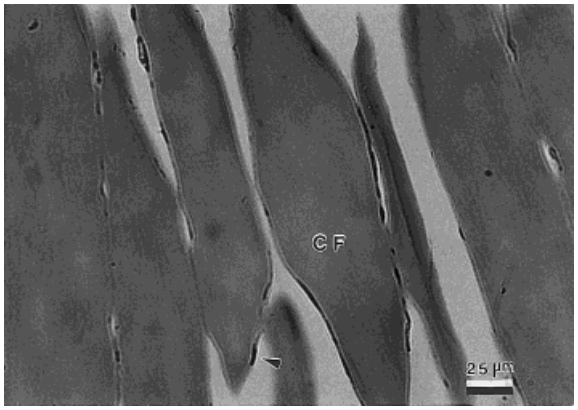

Figure 4 Longitudinal section of uncrosslinked collagen fiber (CF) bundle 8 days after seeding with fibroblasts (arrow) *in vitro.* Fibroblasts were distributed along the length of individual collagen fibers within the fiber bundle.

ful for "seeding" implants in a clinical setting) were used to evaluate collagen–cell interactions as a function of collagen crosslinking method.

We found no major differences in fibroblast growth on uncrosslinked, UV, and DHT crosslinked collagen fibers. UV irradiation and DHT treatment are crosslinking methods that do not introduce cytotoxic chemicals into the collagen fibers. UV irradiation is believed to crosslink collagen following free radical formation on aromatic residues. DHT treatment crosslinks by condensation reactions between carboxyl and amino groups following the exhaustive removal of bound water. Because an intact triple helical structure promotes integrin-mediated cell attachment to collagen,[18] we were concerned that the partial denaturation associated with DHT and UV treatments[10] might compromise fibroblast attachment. However, fibroblast attachment was increased on UV and DHT5 treated collagen fiber bundles. Several other investigators have suggested that UV irradiation enhances fibroblast attachment to collagen,[17,19] but the mechanism responsible for this effect is not clear.

In contrast, HMDIC crosslinked fiber bundles had consistently lower numbers of viable fibroblasts attached, consistent with previous reports.[20] HMDIC is a difunctional molecule that interacts with the R-NH_2 group on the collagen peptide, forming a covalent bond under elimination of water. The poor cell attachment and proliferation associated with this chemical crosslinker are probably due to leaching of cytotoxic hydrolysis products of HMDIC.[20] Cytotoxic products may decrease over time by rinsing, or be broken down by cellular enzymes. This may explain why after 8 days the number of cells on the HMDIC crosslinked fibers approached that of the other groups.

In summary, UV, DHT, and HMDIC treatments significantly improved the mechanical properties of the collagen fibers; however, the cytotoxicity associated with HMDIC in our hands renders it impractical for use in a tissue engineering device. UV irradiation and DHT treatment provided biocompatible collagenous substrates, supporting human fibroblast attachment and proliferation. UV irradiation would be the crosslinking method of choice over DHT treatment due to its ease of application (30 min as opposed to 3–5 days) and increased stability in the presence of proteolytic enzymes.[10] Implantation studies are underway to further characterize the effects of crosslinking methods on the biocompatibility, resorption rate, and strength retention of collagen fibers *in vivo.*

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