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ARTICLES

Collagen in Tissue-Engineered Cartilage: Types, Structure, and Crosslinks

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Abstract The function of articular cartilage as a weight-bearing tissue depends on the specific arrangement of collagen types II and IX into a three-dimensional organized collagen network that can balance the swelling pressure of the proteoglycan/water gel. To determine whether cartilage engineered in vitro contains a functional collagen network, chondrocyte-polymer constructs were cultured for up to 6 weeks and analyzed with respect to the composition and ultrastructure of collagen by using biochemical and immunochemical methods and scanning electron microscopy. Total collagen content and the concentration of pyridinium crosslinks were significantly (57% and 70%, respectively) lower in tissue-engineered cartilage that in bovine calf articular cartilage. However, the fractions of collagen types II, IX, and X and the collagen network organization, density, and fibril diameter in engineered cartilage were not significantly different from those in natural articular cartilage. The implications of these findings for the field of tissue engineering are that differentiated chondrocytes are capable of forming a complex structure of collagen matrix in vitro, producing a tissue similar to natural articular cartilage on an ultrastructural scale. J. Cell. Biochem. 71:313–327, 1998.
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Key words: articular cartilage repair; tissue engineering; collagen type II; collagen type IX; collagen network; pyridinium crosslinks

Collagen accounts for the major fraction of the dry weight of articular cartilage (50–80%) [Mow et al., 1992]. The three-dimensional (3D) arrangement of collagen fibrils, which mainly consist of collagen type II (90%) [Kuettner, 1992], noncovalently immobilizes highly hydrated negatively charged proteoglycans [Buckwalter and Mankin, 1997] (Fig. 1A). It is the unique structure of this extracellular matrix that determines the load-bearing function of articular cartilage, which can withstand loads of several times body weight [Mow et al., 1992]. Fibrocartilage, which consists of dedifferentiated chondrocytes and mainly collagen type I (98%) [Eyre and Wu, 1983], is inefficient as a weight-bearing tissue [Minas and Nehrer, 1997]. Therefore, collagen type II is not only a marker of differentiated chondrocytes but also a prerequisite for a mechanically functional collagen network.

Because the collagen network is under constant tension even in the unloaded state due to the swelling pressure of the proteoglycan/water gel [Maroudas, 1976], collagen fibrils must not only possess tensile strength but must also be interconnected [Eyre and Wu, 1995]. Interfibrillar connections are thought to be mediated by collagen type IX [Mueller-Glauser et al., 1986; Diab, 1993], which is covalently linked to the surface of the collagen fibril [Eyre et al., 1987; Wu and Eyre, 1984] (Fig. 1B). Collagen type IX is, in addition to collagen type II, a marker for differentiated chondrocytes [Morrison et al., 1996]. The tensile strength of the collagen fibril depends on intermolecular crosslinks between collagen molecules within the collagen triple helix (Fig. 1C) [Eyre et al., 1992] and on fibril diameter. In mature articular cartilage, the pre-

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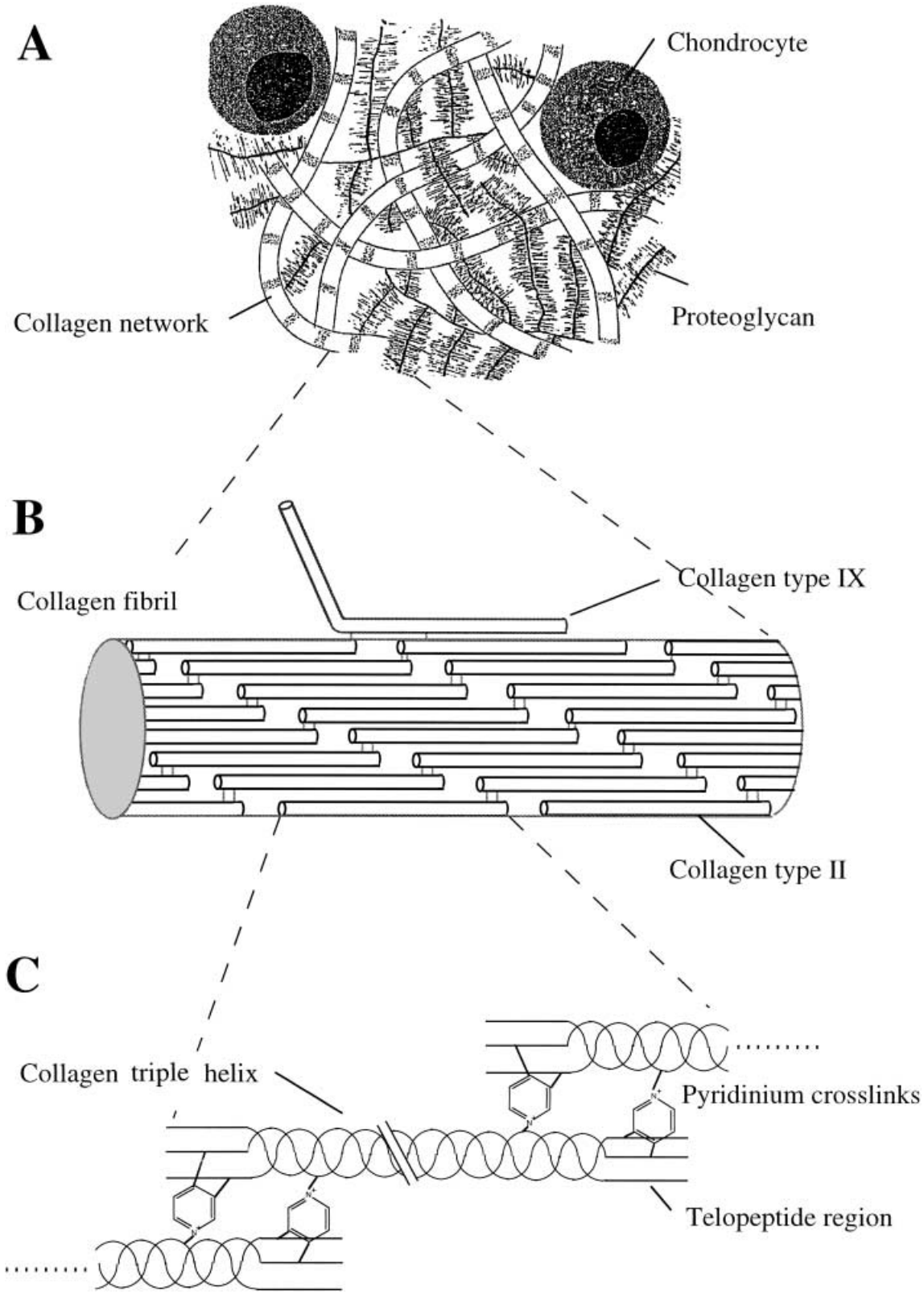

Fig. 1. **A:** Schematic representation of the arrangement of collagen fibrils, proteoglycan, and chondrocytes in articular cartilage. **B:** Collagen fibrils are mainly composed of a staggered collagen type II triple helix, with minor amounts of collagen type XI (not shown), and are interconnected with each other by collagen type IX. **C:** Pyridinium crosslinks interconnect single molecules of collagen type II, typically arranged as a triple helix [Fig. 1A adapted from Berkenblit et al., 1995].

dominant type of crosslink (>90%) is based on pyridinoline (Pyr), a pyridinium crosslink [Eyre et al., 1988]. As compared with other tissues such as tendon, articular cartilage has a two-fold higher number of crosslinks per unit collagen [Eyre, 1987].

Cartilaginous tissue has been engineered in vitro by using articular chondrocytes cultured on 3D biodegradable scaffolds in tissue culture bioreactors [Freed and Vunjak-Novakovic, 1995a]. The long-term goal of these studies is to use engineered cartilage for clinical applications, i.e., repair of articular cartilage defects that could potentially benefit an estimated one million patients per year [Langer and Vacanti, 1993]. The same model system is currently being used to study chondrogenesis under controlled conditions and cartilage-related diseases with respect to tissue response to potential therapeutics [Freed and Vunjak-Novakovic, 1998].

Cartilaginous matrix developed over the time of in vitro cultivation [Freed et al., 1998] and its structure was found to depend on the conditions of cell seeding and tissue cultivation [Vunjak-Novakovic et al., 1996]. Biochemical and histochemical characterizations of engineered constructs showed that the highest fractions and most homogeneous distributions of proteoglycan and collagen were obtained when scaffolds were first seeded with cells in well-mixed flasks and then cultured in rotating bioreactors (Fig. 2) [Freed and Vunjak-Novakovic, 1998]. In such engineered constructs, collagen type II represented the dominant fraction of total collagen [Freed et al., 1998]. However, it was unknown whether the collagen molecules within the fibrils were crosslinked and whether collagen types II and IX were arranged into a functional collagen network.

In the present study, chondrocyte–polymer constructs cultivated in rotating bioreactors were compared with natural articular cartilage with respect to collagen types II and IX, collagen-specific pyridinium crosslinks, and collagen network ultrastructure.

MATERIALS AND METHODS

Unless otherwise noted, all reagents and materials were purchased from Sigma (St. Louis, MO).

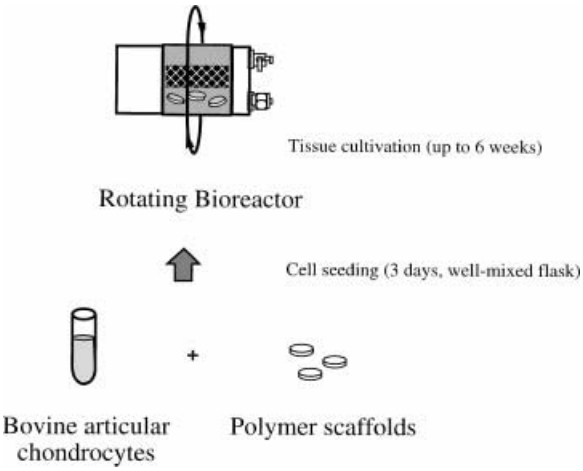

Fig. 2. Model system for tissue-engineering of cartilage. Chondrocytes were isolated from full-thickness bovine calf articular cartilage, seeded on biodegradable polymer scaffolds (fibrous polyglycolic acid, 97% void volume, 5 mm diameter × 2-mm-thick disks) in mixed flasks for 3 days, and cultured in rotating bioreactors for up to 6 weeks.

Cell Culture

Full-thickness articular cartilage was harvested aseptically from the femoropatellar grooves of knee joints of 2–3-week-old bovine calves obtained from a local abattoir within 8 h of slaughter. Chondrocytes were isolated by digestion with type II collagenase [Freed et al., 1993] and resuspended in Dulbecco's modified Eagle medium containing 4.5 g/l glucose, 584 mg/l glutamine, 10% fetal bovine serum (FBS), 50 U/ml penicillin, 50 µg/ml streptomycin, 10 mM N-2-hydroxyethylpiperazine N′-2-ethanesulfonic acid (HEPES), 0.1 mM nonessential amino acids, 0.4 mM proline, and 50 µg/ml ascorbic acid.

Scaffolds were produced as previously described [Freed et al., 1994] at Albany International (Mansfield, MA) by extruding polyglycolic acid into 13-µm fibers and processing these into a porous mesh (97% void volume, 5 mm diameter × 2 mm thick discs). Scaffolds were seeded with freshly isolated chondrocytes (5×10^6 cells/scaffold) in well-mixed spinner flasks (Bellco, Vineland, NJ) as previously described [Vunjak-Novakovic et al., 1996, 1998]. Briefly, 12 scaffolds per flask were threaded onto needles (four needles per flask, three scaffolds per needle) and seeded with 120 ml of cell suspension containing 5×10^5 cells/ml at 50 rpm. After 3 days, cell–polymer constructs were transferred into rotating bioreactors as previously described [Freed and Vunjak-Novakovic, 1995b]

and cultured for up to 6 weeks at 37°C, 10% CO_2. Each rotating vessel (RCCV-110, Synthecon, Houston, TX) consisted of two concentric cylinders that were rotated as a solid body and contained 11 constructs freely suspended in 110 ml of culture medium. Culture medium was replaced at a rate of 50% every 2–3 days, i.e., at a rate of 3 ml per construct per day. Gas exchange was provided by continuously pumping incubator air through the inner cylinder that was covered with a silicone membrane. Constructs used for analysis of collagen types [sodium dedecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE), Western blot, and dot blot] and pyridinium crosslinks were made in a separate experiment carried out under the exactly same conditions. Natural cartilage used as a control was obtained from middle regions of full-thickness plugs of bovine calf articular cartilage.

Biochemical Analyses

Samples of natural or engineered cartilage for biochemical analyses were frozen, lyophilized, and digested with proteinase K in the presence of protease inhibitors [Hollander et al., 1994]. Briefly, 4–10 mg dry weight of sample were digested with 1 ml proteinase K solution (1 mg/ml) containing 50 mM Tris, 1 mM EDTA, 1 mM iodoacetamide, and 10 μg/ml pepstatin A at 56°C for 15 h. The number of cells was assessed from DNA content by using Hoechst 33258 dye (Polysciences, Warrington, PA) and a spectrofluorometer (PTI QM-1, South Brunswick, NJ) according to the method of Kim et al. [1988]. Cell mass was calculated by using a conversion factor of 7.7 pg DNA/cell [Kim et al., 1988] and 10^{-10} g dry weight/chondrocyte [Freed et al., 1994]. Sulfated glycosaminoglycan (GAG; the polysaccharide component of proteoglycan) content was determined spectrophotometrically after reaction with dimethylmethylene blue dye (Aldrich, Milwaukee, WI) by using bovine chondroitin sulfate as a standard [Farndale et al., 1986]. Total collagen content was determined from the hydroxyproline content after acid hydrolysis (6 N HCl at 115°C for 18 h) and reaction with p-dimethylaminobenzaldehyde (Fischer, Paris, KY) and chloramine T (Mallinckrodt, Fair Lawn, NJ) [Woessner, 1961] by using a ratio of hydroxyproline to collagen of 0.1 [Hollander et al., 1994].

SDS-PAGE, Western Blot, Dot Blot, and Enzyme-Linked Immunosorbent Assay (ELISA) Analyses

Analysis of collagen type II using SDS-PAGE of cyanogen bromide (CNBr)-solubilized samples. The amount of collagen type II was measured by SDS-PAGE of CNBr-solubilized samples by using the ratio of the densities of the α1(II)CB10 to the α1(I)CB7, 8 and α1(II)CB11 bands, and standardization with pure collagens type I and II [O'Driscoll et al., 1985, 1995]. Briefly, 6–15 mg of the lyophilized sample of natural or engineered cartilage (or 2 mg of purified collagen type I or II) were incubated in 1 ml 70% (v/v) formic acid for 1 h at 60°C. Cyanogen bromide in 70% (v/v) formic acid was added to a final concentration of 50 mg/ml, and the solution was de-oxygenated by bubbling with nitrogen and incubated for 20 h at room temperature with constant stirring. After lyophylization, the residue was dissolved in SDS reducing sample buffer (Bio-Rad, Richmond, CA) to a concentration of 2.5 mg/ml and incubated at 80°C for 10 min. The solution was loaded on a 15% polyacrylamide gel (20 μl/well) and the SDS-PAGE was run for 1 h at 200 V [Laemmli, 1970].

After staining with Coomassie blue for 45 min, the gel was destained for 16 h by using a dye-absorbing sponge, imaged with a linear array CCD camera (Raiser, Germany), and subjected to densitometric analysis using NIH Image 1.56 software (gel blotting macro). Density calibration (using the uncalibrated OD option) was verified by using laser densitometer analysis (UltroScan XL, Pharmacia LKB, Uppsala, Sweden) and the GelScan XL 2.1 software (SCAN option, peak width set at 1). For each lane, the inner 80% of the width was analyzed, and the areas under the peaks corresponding to α1(II) CB10, α1(I) CB7, 8, and α1(II) CB11 bands were integrated. The fraction of collagen type II was determined as the ratio of the peak areas CB10/(CB7, 8 + CB11).

Analysis of collagen types II, IX, and X using SDS-PAGE, Western blot, and dot blot of pepsin-extracted samples. Samples of natural or engineered cartilage were extracted with 4 M guanidinium hydrochloride (GuHCl) in 50 mM Tris, pH 7.5, at 4°C for 20 h with shaking by using 10–120 mg dry weight/15 ml GuHCl. Samples were then partly digested with pepsin (0.6 mg/ml) in 0.5 M acetic acid at 4°C for 48 h with shaking at a ratio of pepsin to

dry weight of 1:10 [Wu and Eyre, 1984]. After lyophilization, the solubilized collagen was dissolved in 0.01 M HCl to a concentration of 3 mg/ml. The collagen chains were separated by SDS-PAGE (Ready-gel, BioRad) on a 7.5% acrylamide gel (nonreducing conditions) according to Laemmli [1970] and compared with collagen type II and type IX standards. Purified pepsin-treated collagen type II standard was obtained from Sigma. Collagen type IX standard was isolated from parent articular cartilage after GuHCl and pepsin extraction followed by fractionated salt precipitation, as described by Wu and Eyre [1984].

For Western blot analyses, pepsin-extracted samples separated by SDS-PAGE were blotted [25 mM Tris, 192 mM glycine, 0.1% SDS, 20% (v/v) methanol, for 80 min at 80 V at $\leqslant$20°C] onto a nitrocellulose membrane (Bio-Rad) by using a electrophoretic transfer cell (Mini Trans-Blot Electrophoretic Transfer Cell; Bio-Rad). Nonspecific binding sites were blocked with 2% bovine serum albumin (BSA) in 20 mM Tris, 500 mM NaCl, pH 7.5, for 45 min, and the membrane was incubated with a primary monoclonal antibody to collagen type II, IX, or X in 0.1% BSA, 20 mM Tris, 500 mM NaCl, 0.05% polyoxyethylene sorbitan monolaurate (Tween-20), pH 7.5, for 14 h. The incubation was followed by the secondary alkaline-phosphatase-conjugated antibody (Bio-Rad) for 1 h and 5-bromo-4-chloro-indolyl phosphate and nitro-blue tetrazolium to develop color (Immun-Blot Assay Kit; Bio-Rad). Monoclonal antibodies against collagen types II [Irwin et al., 1985], IX [Ye et al., 1991], and X [Girkontaite et al., 1996] were obtained from NeoMarkers (Fremont, CA), Developmental Studies Hybridoma Bank (Iowa City, IA), and K. von der Mark (University Erlangen, Germany) and were used at dilutions of 1:100, 1:25, and 1:10, respectively.

For dot blot analyses, 1 µl of pepsin-digested sample was applied to a nitrocellulose membrane (Bio-Rad). Nonspecific binding sites were blocked with BSA as described above, and collagen types II, IX, and X were detected by incubation with the appropriate primary monoclonal antibody and the secondary alkaline-phosphatase-conjugated antibody as described above for Western blot analysis. For semiquantitative analysis, dot blots were scanned (gray mode; Scan Jet IIcx, Hewlett Packard, Camas, WA), and the area under the peak was integrated by using NIH Image 1.56 software (gel blotting macro). To estimate the amounts of collagen types II and IX from dot blots, standard curves were established by using a series of dilutions of pepsin-solubilized natural cartilage samples (r^2 = 0.99 and 0.89 for anti-collagen types II and IX, respectively).

Analyses of collagen types II and IX using ELISA of proteinase K-solubilized and pepsin-extracted samples. Collagen type II was quantified by using proteinase K-digested samples after inactivation of the proteinase K (by boiling the digest for 12 min) and a monoclonal antibody-based inhibition ELISA [Hollander et al., 1994; Freed et al., 1998]. Collagen type IX was quantified by using a direct ELISA as follows. Ninety-six-well plates were coated with 100 µl of the pepsin-extracted samples (diluted to 5 µg/ml total collagen in 0.15 M K-phosphate, pH 7.6) for 16 h at 4°C and were incubated with the primary monoclonal antibody (2 h at room temperature) and the secondary alkaline-phosphatase-conjugated antibody (1 h, 30 min at room temperature) as described for Western blot analysis. Detection was done by the addition of 100 µl of substrate solution (16 mM p-nitrophenyl phosphate, 4 mM Mg^{2+}, 274 mM manitol, pH 10.2; ALP 10 solution) and incubation for 50 min at 37°C. Absorbance was measured at 405 nm by using a microplate reader (MR5000, Dynatech, Chantilly, VA).

Analysis of Collagen-Specific Mature Crosslinks

Collagen pyridinium crosslinks [pyridinoline (Pyr) and deoxy-pyridinoline (Dpyr)] were analyzed by using the methods of Eyre et al. [1984] and Black et al. [1988], with modifications. Samples of natural or engineered cartilage were hydrolyzed in 6 M HCl (10 mg wet weight/ml) for 24 h at 110°C. After removing the insoluble residue with centrifugation (2 min at 10,000*g*), the hydrolysate was applied to 6-ml fibrous cellulose columns for Pyr separation. The sample (0.6 ml) was mixed with 3.6 ml of the mobile phase (acetic acid:water:1-n-butanol, 1:1:4, v/v); after three column washes, the crosslinked amino acids were eluted with water, frozen, and lyophilized. Samples were then solubilized in 80 µl of 1% n-heptafluorobutyric acid (HFBA), and 20 µl of the sample were applied to a C-18 reverse phase column (Nova-Pack, 150 $\times$ 3.9 mm, 60 Å, 4 µm; Waters, Milford, MA), equilibrated with 14% acetonitrile containing 0.01 M HFBA, and eluted at a flow rate of 1 ml/min. Purified Pyr and Dpyr (Metra Biosystems, CA)

were used as standards, and the absorbance at 297 nm was monitored (490 UV/Vis HPLC-detector; Waters). Under these conditions, the typical retention times for Pyr and Dpyr were 11.5 min and 13.4 min, respectively. Results are expressed in moles of Pyr or as Dpyr per mole collagen triple helix, the latter of which was determined biochemically from hydroxyproline content, as described above.

Scanning Electron Microscopy (SEM)

Samples were fixed in 2.5% glutaraldehyde (Polysciences)/0.1 M cacodylate, pH 7.4, for 4 h at room temperature, washed with 0.15 M cacodylate, pH 7.4, cut with a razor, and dehydrated using graded ethanols (50, 70, 90, and 100% ethanol for 10 min each). After critical-point drying (Balzers Union, Balzers, Lichtenstein) and evaporation coating with carbon and gold/palladium (for all microstructural studies) or sputter coating with gold (for measurement of collagen fibril diameter; Technics, Inc., Alexandria, VA), the samples were examined in a 6320 FV field emission SEM (JEOL, Peabody, MA) operated at 1 kV or in an environmental SEM (Electroscan, Wilmington, MA) operated at 10 kV. To verify that proteoglycans were completely removed during the sample preparation procedure, smooth appearance of the collagen fibrils [Clarke, 1971a,b] was checked for several samples after incubation with hyaluronidase (4,000 U/ml) in 0.15 M cacodylate, pH 6.0, for 24 h at 37°C followed by α-chymotrypsin (400 U/ml) in 0.15 M cacodylate, pH 7.4, for 18 h at 37°C. Enzymatic treatments were carried out before the dehydration step. The diameter of the collagen fibrils was determined by averaging measurements taken on 10 randomly selected fibrils on two different SEMs.

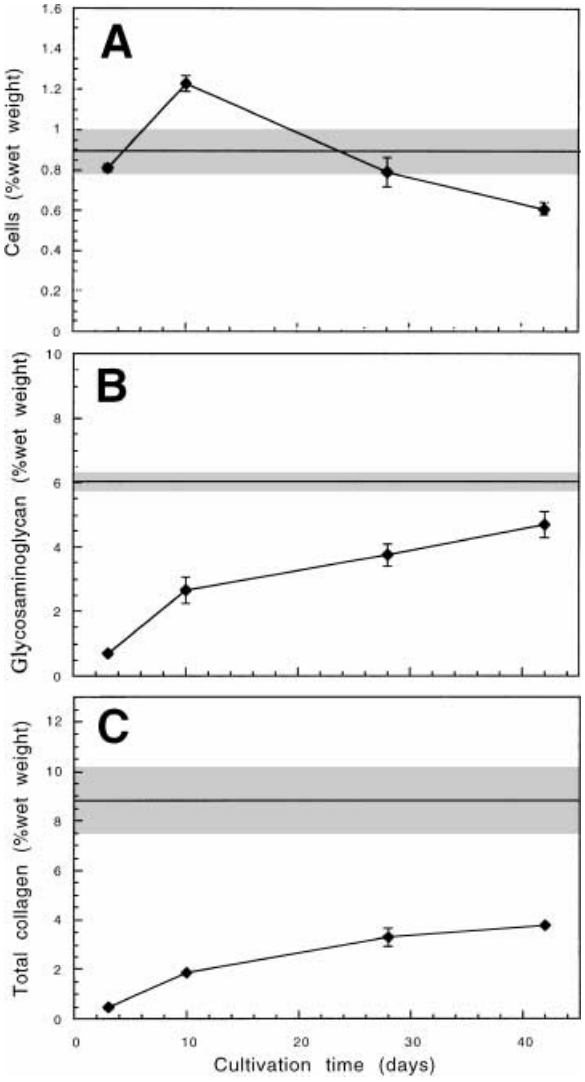

Fig. 3. Biochemical compositions of natural and engineered cartilage. Effect of cultivation time on the wet weight fractions of cells, glycosaminoglycan, and total collagen. Data are the mean ± SD of six independent measurements. Shaded areas represent the ranges measured for bovine calf articular cartilage (n = 6).

RESULTS

Biochemical Composition of Tissue-Engineered Cartilage

After 3 days of cell seeding, engineered constructs had an average wet weight of 78 mg and contained on average 0.66 mg cells, 0.56 mg GAG, and 0.38 mg total collagen. After 6 weeks of in vitro culture, these amounts increased to 237 mg, 1.44 mg, 11.16 mg, and 8.15 mg, respectively. Cell fraction per unit wet weight ranged from 69% to 138% of the value measured for natural cartilage (Fig. 3A). Wet weight fractions of GAG and total collagen increased from 3 days to 6 weeks of cultivation by seven- and eightfold and reached 78% and 43%, respectively, of the corresponding values measured for bovine calf articular cartilage (Fig. 3B,C). The water content of the constructs decreased from 92% after 3 days to 90% after 6 weeks in culture as compared with the value of 85% measured for natural cartilage. As compared with natural cartilage on a dry weight basis, 6-week constructs contained 107% cells, 128% GAG, and 63% collagen.

Collagen Analysis on a Molecular Level

Collagen types II and IX. Quantitative data from inhibition ELISA with a monoclonal antibody showed that constructs and natural articular cartilage contained comparable fractions of collagen type II (percentage of total collagen) at all time points studied. For constructs cultured for 3 days, 10 days, 4 weeks, and 6 weeks, the collagen type II fractions were 84 ± 5%, 89 ± 15%, 90 ± 16%, and 105 ± 5%, respectively, versus 90 ± 18% for natural cartilage. The fractions of collagen type II determined by inhibition ELISA were verified for 4-week constructs by a semiquantitative PAGE-based method using CNBr fragments of the collagen chains. The resulting fractions of type II collagen in 4-week constructs (86 ± 7%) and natural cartilage (93 ± 12%) were slightly but not significantly different from each other or from values determined by inhibition ELISA (Table 1).

SDS-PAGE and Western blot analyses of natural and engineered cartilage showed no apparent difference with respect to the patterns of pepsin-digested collagen chains. In particular, Figure 4A shows the presence of cartilage-specific collagen types II and IX in 4-week constructs and natural cartilage (type II: lanes 1, 4, 5–7; type IX: lanes 8–10). Collagen type IX contained low- and high-molecular-weight chains COL1 and COL2/3, respectively (Fig. 4A, lane 2). The presence of COL1 in natural and engineered cartilage was clearly shown by Western blot analysis (Fig. 4A, lanes 8–10) but not by SDS-PAGE due to the low fraction of this component (about 1% of the fraction of collagen type II; Fig. 4A, lanes 3–4). In addition, because the patterns of the collagen chains obtained for natural and engineered cartilage appeared indistinguishable, collagen type I did not appear to represent a significant fraction of the total collagen in either natural cartilage or engineered constructs (Fig. 4A, lanes 3–4).

Constructs cultured for 4 weeks and natural cartilage contained comparable amounts of collagen types II and IX, as determined by dot blot analysis (Table 1; Fig. 4B). Dot blot results of the collagen type II and IX fractions were not significantly different from the results obtained by ELISA (Table 1). Dot blot staining for collagen type IX was comparable to that for collagen type II (Fig. 4B), although the fraction of collagen type IX in the tissue is approximately 100-fold lower and the same amount of total collagen was applied on the membrane. The equivalent level of staining is presumably due to the higher affinity of the antibody against collagen type IX.

Collagen type X. A small amount of collagen type X was detected in natural cartilage, and only traces were detected in engineered cartilage (Fig. 4B), indicating that hypertrophic chondrocytes were essentially absent in 4-week constructs.

TABLE I. Collagen Type II and IX Fractions of Engineered Cartilage[a]

Method	Type II	Type IX
ELISA[b]	100 ± 18	105 ± 8
Dot blot	98 ± 8	73 ± 18
SDS-PAGE of CNBr fragments	92 ± 8	

[a]Data represent mean ± SD. Collagen fraction in 4-week constructs are presented as the percentage of the average value measured for natural cartilage. ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, sodium dodecylsolfate–polyacrylamide gel electrophoresis; CNBr, cyanogen bromide.
[b]Type II data by inhibition ELISA; type IX data by direct ELISA.

Collagen-Specific Mature Crosslinks

The content of pyridinoline crosslinks (Pyr) for constructs cultured for 4 weeks (0.28 ± 0.03 mol Pyr/mol collagen triple helix) was 30% of that measured for natural calf cartilage (0.92 ± 0.24 mol Pyr/mol collagen triple helix). Deoxypyridinoline crosslinks were not observed in natural or engineered cartilage. The reduced crosslink content of collagen in constructs appeared to be associated with the increased susceptibility to degradation by proteases: 47 ± 4% of the collagen in 4-week constructs was recovered in pepsin extracts versus 32 ± 9% for natural cartilage.

Ultrastructural Analysis of Engineered Tissue

Appearance of chondrocytes and cartilaginous matrix. SEM analysis was used to visualize chondrocytes and the 3D structure of collagen in engineered constructs. Removal of proteoglycans from tissue samples, which was verified by using proteoglycan degrading enzymes hyaluronidase and α-chymotrypsin, allowed the collagen network to be analyzed separately from other extracellular matrix components.

After 3 days of seeding, chondrocytes attached to polymer fibers and started to form extracellular matrix (ECM; Fig. 5A–D). More

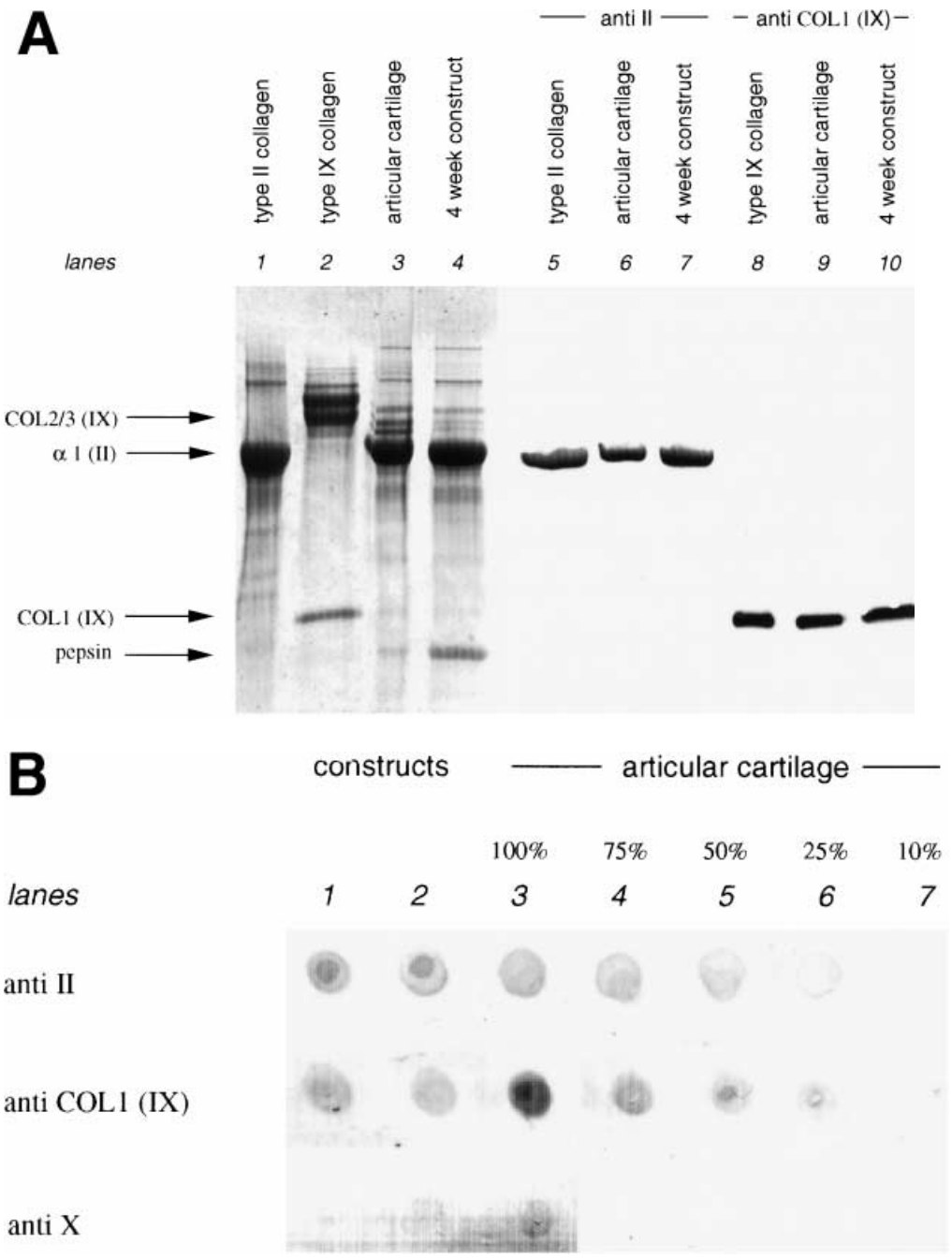

Fig. 4. Collagen typing. **A:** Sodium dedecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE; **lanes 1–4**) and Western blot (**lanes 5–10**) analysis of pepsin-solubilized samples of type II collagen (lanes 1, 5) and type IX collagen (lanes 2, 8) standards, bovine articular cartilage (lanes 3, 6, 9), and engineered cartilage constructs cultured for 4 weeks (lanes 4, 7, 10). The extracted collagens were separated on a 7.5% nonreducing SDS-polyacrylamide gel, transferred to a nitrocellulose membrane for Western blot analysis, and detected with monoclonal antibodies against types II and IX collagen. The positions of the migration of different chains and the band corresponding to the remaining pepsin from the extraction procedure are indicated. COL1 (IX) and COL2/3 (IX) refer to the low and high molecular weight forms, respectively, of collagen type IX. **B:** Dot blot analysis of two pepsin-solubilized samples of 4-week constructs and bovine articular cartilage using monoclonal antibodies against collagen types II (upper row), IX (middle row), and X (lower row). **Lanes 1** and **2:** Extracts of constructs containing 3 mg/ml total collagen. **Lane 3:** Extracts of natural articular cartilage containing 3 mg/ml total collagen. **Lanes 4–7:** Extracts of natural articular cartilage diluted to 75%, 50%, 25%, and 10% of the original concentration of collagen, respectively.

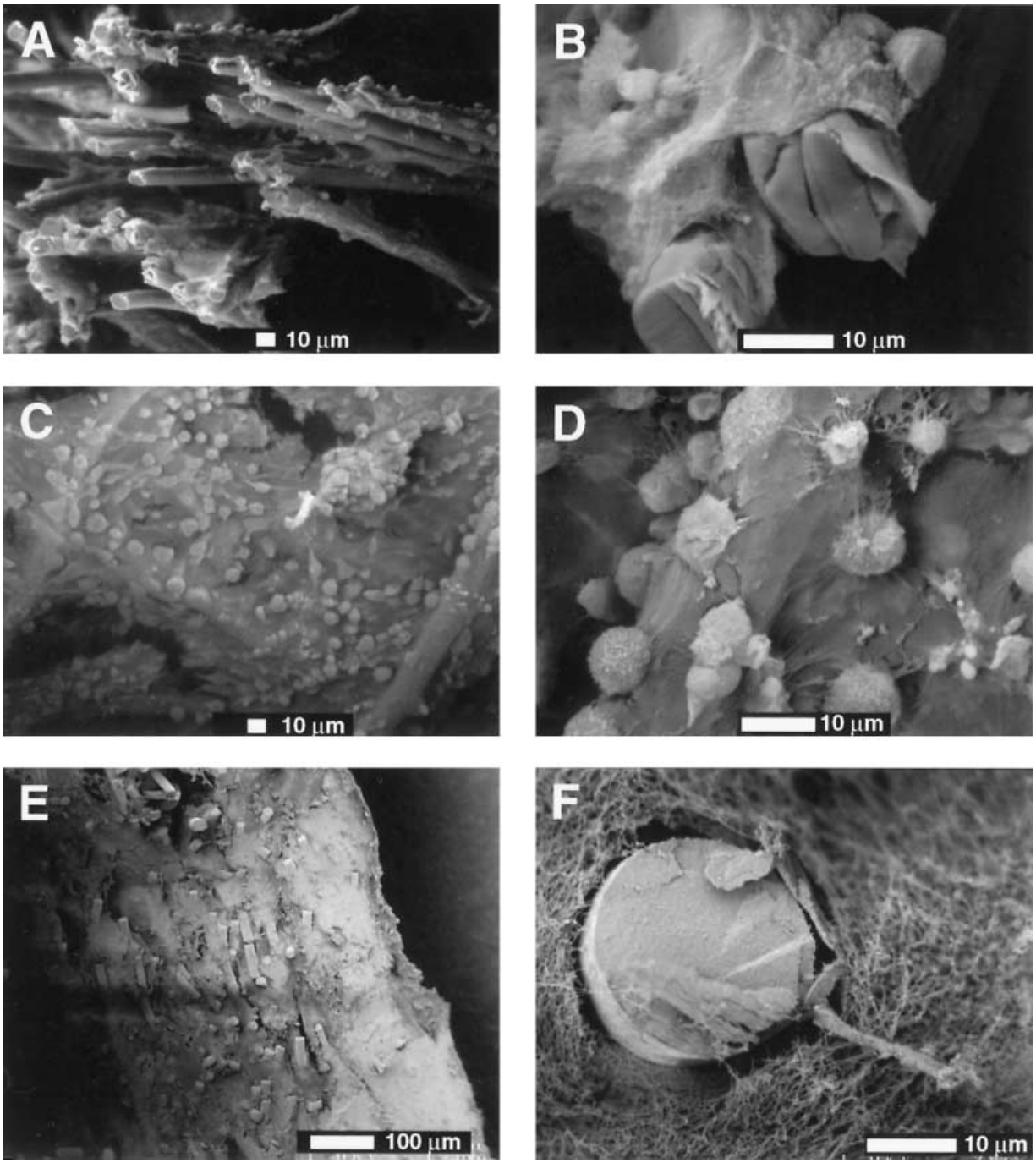

Fig. 5. Low magnification scanning electron micrographs: 3-day construct (**A, B:** interior, **C, D:** exterior), 10-day construct (**E:** interior and exterior, **F:** interior). In addition to small-diameter collagen fibrils, large-diameter polyglycolic acid (PGA) fibers (13 μm) can be seen.

ECMwas observed at the construct surface (Fig. 5C,D) than in the interior (Fig. 5A,B). Chondroctyes retained a spherical shape that is indicative of the expression of a well-differentiated phenotype (Fig. 5D) [von der Mark et al., 1977; Benya and Shaffer, 1982].

The interior of the construct, which was sparsely populated by cells after 3 days (Fig. 5A), began to accumulate ECM components after 10 days (Fig. 5E,F) and developed a continuously cartilaginous ECM after 6 weeks in culture (not shown).

Structure of the collagen network. Figure 5 compares the collagen networks in tissue-engineered cartilage after 3 days and 6 weeks of cultivation with those in natural cartilage. After 3 days, little organized collagen network structure could be seen. After 6 weeks, collagen fibrils appeared interconnected, and the structure of the collagen network resembled that in natural cartilage. In particular, a 3D network of thin collagen type II fibrils was present in both engineered and natural cartilage, in contrast to the linearly organized and thicker collagen type I fibers present in fibrocartilage (inset, Fig. 6C). No major differences in collagen network organization were observed between natural articular cartilage and constructs cultured for 10 days (Fig. 7), 4 weeks, or 6 weeks (data not shown).

Collagen Fibril Diameter

The diameter of the collagen fibrils in engineered constructs increased with culture time from 57 ± 13 nm at 10 days to 89 ± 36 nm at 6 weeks but was not significantly different from the fibril diameter of 53 ± 17 nm measured for natural cartilage. Actual fibril diameters are smaller than those determined by SEM because of the metal coating.

DISCUSSION

Tissue-engineered cartilage could potentially provide functional tissue equivalents for the replacement of damaged or lost cartilage. Isolated chondrocytes cultured on 3D polymer scaffolds in bioreactors have been shown to regenerate ECM that had cartilaginous appearance and biochemical composition [Freed and Vunjak-Novakovic, 1995a; Freed et al., 1998]. The present study has demonstrated that the composition and ultrastructure of the collagen network of cell–polymer constructs grown in rotating bioreactors resembled that of natural articular cartilage and not that of fibrocartilage and that chondrocytes retained their differentiated state.

Because fractions of collagen types II and IX are markers of the differentiation state of chondrocytes in tissue-engineered cartilage, the use of a range of different methods to analyze the matrix was very important. Accordingly, collagen type II was assessed with three independent methods: a quantitative inhibition ELISA, a semiquantitative dot blot, and a semiquantitative SDS-PAGE method based on CNBr fragments. Collagen type IX was determined by semiquantitative dot blot and direct ELISA analyses. All these methods showed that fractions of collagen types II and IX in engineered cartilage did not differ significantly from those in natural cartilage and that the predominant

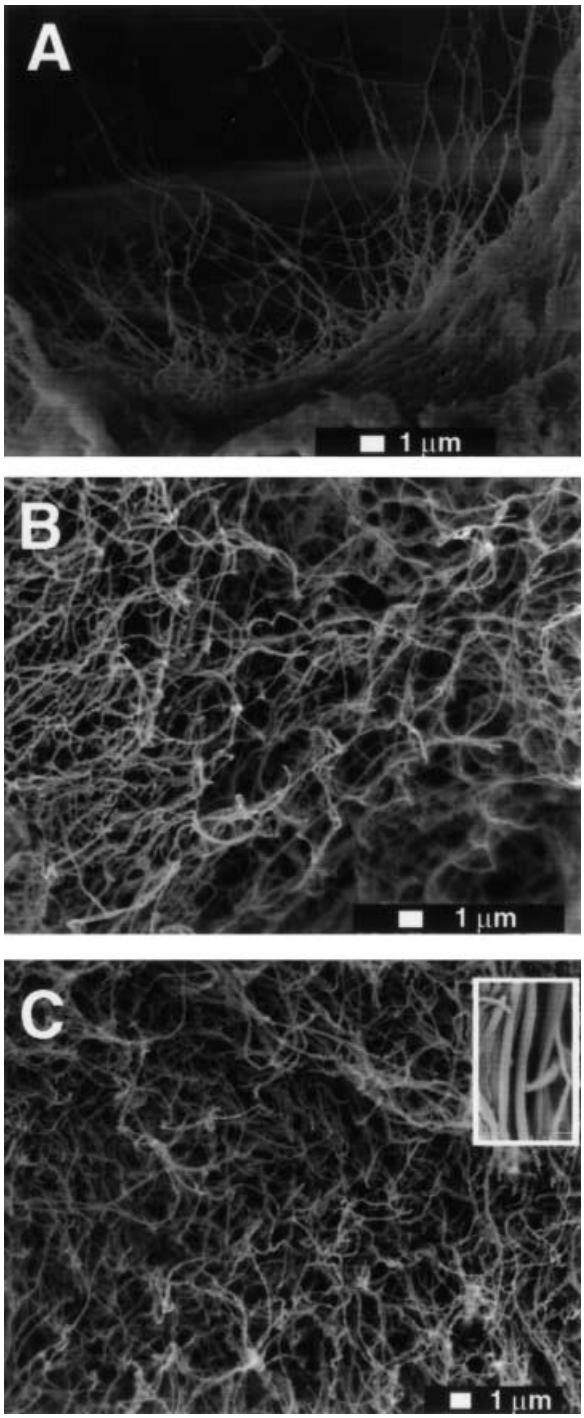

Fig. 6. Collagen network. Scanning electron micrographs of (**A**) 3-day construct, (**B**) 6-week construct, and (**C**) natural articular cartilage. Inset shows bovine meniscal cartilage at the same magnification.

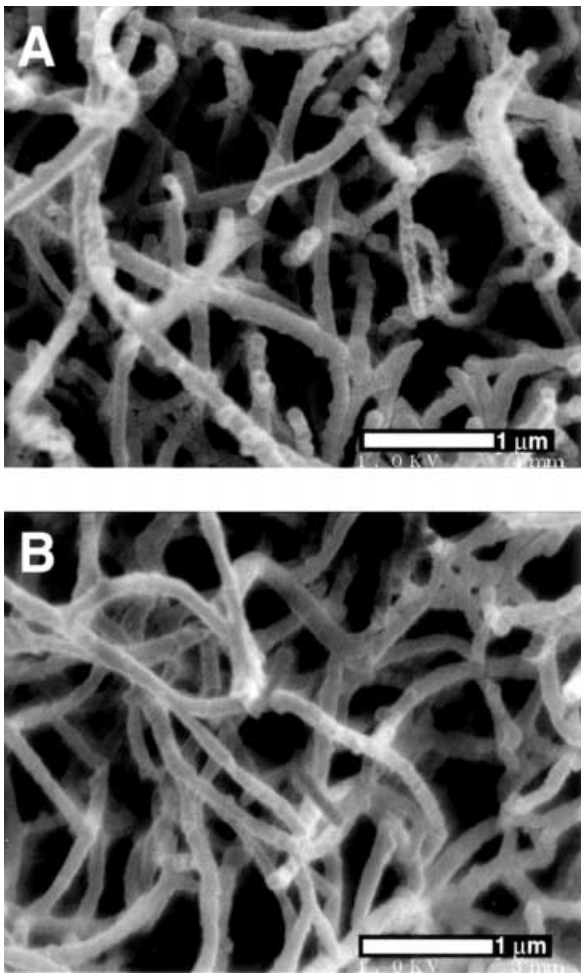

Fig. 7. Collagen fibrils. High magnification scanning electron micrographs of (**A**) 10-day construct and (**B**) natural bovine calf articular cartilage.

fraction (>84%) of total collagen in engineered and natural cartilage was collagen type II (Table 1; Fig. 4A,B). Some of these methods are classified as semiquantitative because of the incomplete solubilization of the cartilaginous tissue: pepsin solubilized 32% and 47% of the collagen in natural and engineered cartilage, respectively, whereas CNBr solubilized 93% and 95%, respectively. Collagen type II fractions determined by dot blot analyses of pepsin digests were virtually identical to those obtained from inhibition ELISA analyses of proteinase K digests (Table 1). Therefore, collagen fractions solubilized by pepsin are representative of the actual tissue composition.

Spherical cell shape, which is indicative of differentiated chondrocyte phenotype (Fig. 5D) [von der Mark et al., 1977] was consistent with high fractions of collagen type II (Table 1) and the absence of collagen type I (Fig. 4A) in tissue-engineered cartilage. Although the presence of collagen types II and IX in the matrix formed by bovine and human chondrocytes in alginate and high density cultures has been demonstrated [Ruggiero et al., 1993; Bonaventure et al., 1994; Petit et al., 1996], the present study is, to the best of our knowledge, the first comprehensive analysis and quantitation of collagen composition and network structure in engineered cartilage.

Collagen types II and IX are markers of differentiated chondrocytes and prerequisites for a functional collagen network in articular cartilage. That is, collagen type II mainly forms a 3D mesh of fibrils that are presumably interconnected by collagen type IX to balance the proteoglycan swelling pressure and provide the shape and tensile strength in articular cartilage (Fig. 1) [Eyre et al., 1992]. The crucial importance of collagen type II for articular cartilage function is illustrated by low fraction of collagen type II in osteoarthritic fibrocartilage and by the fact that mutations of the collagen type II gene cause severe cartilage diseases in humans [Eyre and Wu, 1995]. The relevance of collagen type IX in stabilizing the collagen network is demonstrated by the effect of the matrix metalloprotease (MMP) stromelysin in osteoarthritis, where cleavage of collagen type IX results in depolymerization of the collagen network [Kuettner, 1992]. The cartilage in mice lacking the gene for the α1(IX) collagen chain shows differences in structure and organization of the collagen network as compared with natural cartilage [Mallein-Gerin et al., 1995].

The finding that 4-week constructs contained essentially no collagen type X is consistent with the negligible amount of collagen type X in healthy natural cartilage (Fig. 4B) [Morrison et al., 1996; Rucklidge et al., 1996]. In contrast, increased amounts of collagen type X are an indicator of osteogenic differentiation or osteoarthritic tissue [Kuettner, 1992].

In contrast to the comparable fractions of collagen types II and IX, constructs cultured for 4 weeks had only 30% as many pyridinium crosslinks as compared with those in natural cartilage. The values measured for bovine calf articular cartilage were similar to those previously reported [Eyre and Oguchi, 1980]. Engineered constructs and the collagen matrix formed by chondrocytes in alginate beads contained comparable amounts of pyridinium crosslinks after 4 weeks in culture [Petit et al., 1996; Beekman et al., 1997]. The intermolecu-

lar pyridinium crosslinks significantly influence the tensile strength of collagen fibrils [Eyre et al., 1988, 1992; Schmidt et al., 1987]. Deoxypyridinoline crosslinks that are normally present in bone tissue were not observed in natural or engineered cartilage.

At least two possibilities need to be considered as a cause for the lower crosslink content of the constructs. Lysyl oxidase, which is involved in crosslink formation by catalyzing the first step in the cascade leading to mature crosslinks, may be inhibited by some factor in the in vitro culture environment. Alternatively, full crosslink formation may require more than 4 weeks. The crosslink content increases by a factor of approximately two during natural chondrogenesis (fetal and adult bovine cartilage contain 0.7 and 1.5 mol Pyr/mol collagen, respectively) [Eyre, 1987]. Accordingly, crosslink formation in engineered cartilage may continue to proceed with further in vitro cultivation or after implantation into articular cartilage defects. In vivo the maturation of reducible into nonreducible pyridinium crosslinks takes about 2 weeks [Eyre, 1981].

A decreased fraction of crosslinks may render collagen more susceptible to proteolytic degradation [Ronziere et al., 1990; Grynpas et al., 1994]. In the present study, engineered cartilage was shown to be 1.5-fold more susceptible to the protease pepsin. Therefore, increased collagen degradation caused by a reduced crosslink content may be responsible for the lower total amount of collagen in engineered cartilage as compared with that in natural cartilage (Fig. 3C; 43% of the wet weight fraction). Proteases potentially responsible for collagen degradation include MMPs, although these are significantly inhibited by 10% fetal calf serum and membrane-type MMPs (MT-MMP) [Ohuchi et al., 1997].

Another possible cause of the low collagen content in constructs is the inhibition of collagen fibril formation. Glucose present at a 25 mM concentration (4.5 g/l) used in the culture medium was shown to inhibit collagen fibril formation in vitro and in wound healing in diabetic patients in vivo [Lien et al., 1984]. However, several cell culture studies using rat endothelial and mesangial cells found no effect of glucose concentration in the range of 25 mM on the amount of collagen type I formed [Spiro et al., 1995; Takeuchi et al., 1995].

Further studies are needed to determine whether the reduced fraction of crosslinks, inhibited fibril formation, and/or reduced ratio of synthesis to degradation of collagen were responsible for the observed lower collagen content of engineered cartilage. Assessment of MMPs, MT-MMPs, and tissue inhibitor of MMPs may be crucial in that respect. Furthermore, we are aware that molecules not analyzed in the present study, such as proteoglycan, cartilage matrix protein, cartilage oligomeric matrix protein, and collagen types XI and VI, can contribute to the structural and mechanical properties of the cartilaginous tissue.

The relatively high water content of engineered cartilage (90–92%) versus natural cartilage (85%) is consistent with the lower pyridinium crosslink content in constructs, which is in turn likely to be associated with lower tensile strength of collagen fibrils. However, some of the differences in water content may be due to the decreased osmolality of culture medium as compared with the natural tissue, i.e., explants of cartilage cultured in the same medium showed an 1.1-fold increase in water content [Urban et al., 1993].

Our structural studies of engineered cartilage showed the architecture of collagen network and allowed the comparison of engineered constructs at various stages of cultivation with natural cartilage. The potential for 3D analysis is a unique feature of SEM as compared with analysis by histology or transmission electron microscopy (TEM). SEM analysis showed that the collagen network organization, density, and fibril diameters in constructs were comparable to those in natural articular cartilage (Figs. 6, 7). In particular, an organized 3D network of thin collagen type II fibrils was observed in the constructs, in contrast to the linearly organized and thicker collagen type I fibers in fibrocartilage (Fig. 6C). Construct ultrastructural appearance was consistent with the biochemically determined high fraction of collagen type II (Table 1) and the absence of collagen type I (Fig. 4A). The presence of collagen types II and IX suggest the heterotypic nature of the collagen fibrils in both engineered and natural cartilage [Mendler et al., 1989].

The densities of the collagen networks of natural and engineered cartilage appeared comparable by SEM (Fig. 6), although a twofold difference in the wet weight fractions of total

collagen was measured biochemically (Fig. 3C). These findings might be attributed to a lower density of collagen per fibril in engineered cartilage due to the reduced number of pyridinium crosslinks. The overall collagen network organization in constructs was indistinguishable from that in natural cartilage, which correlates well with the finding of similar fractions of collagen type IX (Table 1), which is pivotal for the development of a stable interconnected 3D collagen mesh [Eyre et al., 1987]. Collagen fibril diameters measured by SEM for engineered and natural cartilage were not significantly different from each other but were approximately two- to fourfold higher than those previously measured by TEM [Freed et al., 1997]. This result may be explained by the metal coating on the SEM samples. However, all values reported were within the range reported for immature articular cartilage [Stockwell, 1979]. The finding of relatively higher amounts of ECM at the surface as compared with the interior in 3-day constructs is consistent with the finding of a previous study [Freed et al., 1998].

In summary, the wet weight fraction of total collagen in engineered cartilage after 6 weeks of cultivation was significantly (57%) lower than that in bovine calf articular cartilage. However, the present study shows for the first time that collagen type II, IX, and X fractions, collagen network organization, and collagen fibril diameter in tissue-engineered cartilage were not significantly different from those in natural articular cartilage. The implications for the field of tissue engineering are that well-differentiated chondrocytes are capable of forming a complex collagen network in vitro, producing a tissue similar to natural cartilage on a ultrastructural scale. However, the concentration of pyridinium crosslinks in the constructs was only 30% of that in natural articular cartilage. These differences may account for the less favorable biomechanical properties of engineered constructs as compared with natural articular cartilage [Freed at al., 1997]. Future studies should include efforts to increase the crosslink content of constructs to improve their biomechanical properties, which is of relevance for potential clinical applications.

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