Characterization of proteoglycan accumulation during formation of cartilagenous tissue in vitro

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Summary

In order to study proteoglycan retention and accumulation, we optimized a chondrocyte cell culture system in which isolated bovine articular chondrocytes accumulate extracellular matrix to form a continuous layer of cartilagenous tissue. The tissue can attain a thickness of up to 110 μm by 35 days. The cells synthesize large keratan sulfate containing proteoglycans and type II collagen indicating that the chondrocytes maintain their phenotype in these culture conditions. Matrix accumulation is enhanced by increased cell density and the presence of serum and ascorbic acid. The amount of proteoglycans synthesized by the chondrocytes increases up to day 21 and then decreases to the same levels as are synthesized during the first week of culture. The percentage of newly synthesized proteoglycans retained in the matrix increases from 20% on day 6 to a maximum of 85% by day 35. The proteoglycan and collagen content in the tissue increases with time in culture. The changes in the percentage of proteoglycans retained parallels the increase in proteoglycan content. After day 35, there is no further increase in the amount of proteoglycans and collagen nor in the percentage of newly synthesized proteoglycans retained in the extracellular matrix. These studies demonstrate that the cultures are going through two phases: one of matrix accumulation and then one of maintaining the existing matrix. The period of matrix accumulation occurs between days 10–21 whereas matrix maintenance is observed after day 35. Using this culture system to study proteoglycan accumulation and maintenance during these culture periods may prove useful in identifying the mechanisms regulating these processes.

Key words: Cartilage, Chondrocytes, Proteoglycans, Collagen.

Introduction

Articular cartilage differs from other tissues in that it is avascular and the cells are surrounded by abundant extracellular matrix [1, 2]. Although each cell is a functional unit with little if any interactions between cells, there is a specific organization of matrix molecules, such as proteoglycans and collagen in the cartilage going from the superficial to the deeper zones [3–6]. The organization of cartilage matrix is a continuous process as there is turnover of matrix macromolecules in cartilage. The half life of proteoglycans in vitro is variable depending on the source of cartilage, and can range up to 30 days [7, 8]. Collagen is more stable and has a much longer half life, ranging from 300 days to years [9]. How the chondrocyte 'senses' that synthesis of matrix macromolecules is necessary and how they are retained and organized into this well-ordered structure is complex and difficult to examine. Although there is abundant information about chondrocyte biosynthesis of proteoglycans in the presence of growth factors or cytokines, and chondrogenesis in developing limbs, the process of matrix accumulation by articular chondrocytes has not been studied as extensively [8, 10–12]. Understanding these processes may identify molecules or the sequence of events regulating cartilage formation and maintenance. Ultimately, the conditions that favor regeneration of cartilage that has been lost due to disease or injury may be identified.

In this paper we describe a culture system in which bovine articular chondrocytes, plated on Millipore® filter inserts accumulate extracellular matrix to form a continuous layer of cartilagenous tissue. Proteoglycan synthesis and retention in the matrix were examined during the periods of tissue formation and maintenance.

Methods

CHONDROCYTE CULTURES

Filter inserts (Millicell CM®, Millipore Corp., Bedford, MA, U.S.A.) were coated with collagen
Chondrocytes in vitro (Type I or II, 0.5 mg/ml 0.012 N HCl) [Sigma Chemical Co., St. Louis, MO, U.S.A.] and dried overnight. They were UV sterilized for 30 min prior to use and then placed into the wells of the culture dish (24-well plate). Chondrocytes were obtained from calf articular cartilage from the metacarpophalangeal joints as described previously [13]. The cells were plated on the filter inserts at low density (1 × 10^6 cells/cm^2) or high density (3 × 10^6 cells/cm^2) and maintained in Ham's F-12 with 5% fetal bovine serum (FBS). In selected experiments, the serum concentration was increased to 20% on day 5. On day 7, ascorbic acid (final concentration 50 μg/ml for cultures maintained in 5% FBS and 100 μg/ml for cultures in 20% FBS) (Gibco/BRL, Burlington, ON, Canada) was added to the medium of all cultures. The cells were grown at 37°C in a humidified atmosphere supplemented with 5% CO2. Medium was changed every two days and fresh ascorbic acid was added with each change of medium. The cultures were harvested at 3, 6, 10, 21, 35 or 49 days. All experiments were repeated at least three times.

**HISTOLOGICAL EXAMINATION**

The chondrocyte cultures on the filter inserts were harvested at selected intervals and fixed in 10% formalin. They were paraffin-embedded and 5 μm sections were cut and stained with either hematoxylin & eosin, toluidine blue or picrosirius red. Toluidine blue stains sulfated proteoglycans and picrosirius red stains collagen in the extracellular matrix. Morphometric quantitation of these cultures were performed using light microscopy, a digitized tablet interfaced with an IBM computer and the Bioquant Image Analysis program. The culture thickness was determined by measuring 10 separate points in each section and three sections per culture were examined. The mean value for each time point was calculated by determining the mean value of triplicate cultures.

**DNA QUANTITATION**

The cultures at different time points were digested with papain (10 μg/ml) in 20 mM ammonium acetate, 1 mM ethylenediaminetetraacetic acid (EDTA) and 2 mM dithiotreitol (DTT) for at least 18 h at 65°C. The DNA content was measured using Hoechst 33258 dye (Polysciences Inc., Warrington, PA, U.S.A.) and fluorometry (emission wavelength: 365 nm and excitation wavelength: 458 nm) as described by Kim et al. [14]. Calf thymus DNA (Pharmacia, Montreal, Quebec, Canada) was used to generate the standard curve.

**COLLAGEN QUANTITATION**

An aliquot of the papain digest was hydrolysed using 12 N HCl overnight at 110°C. The amount of collagen in the extracellular matrix was determined by measuring hydroxyproline using high pressure liquid chromatography (C18 reverse column) and a Waters PicoTag amino acid analysis system.

**PROTEOGLYCAN QUANTITATION**

Proteoglycan content was determined by measuring the amount of glycosaminoglycan in the papain digests of each culture using the dimethyl-methylene blue dye binding assay (Polysciences Inc., Warrington, PA, U.S.A.) and spectrophotometry as described by Farndale et al. [15] and modified by Goldberg et al. [16]. The samples are diluted with 1% bovine serum albumin in phosphate buffered saline and the dye is diluted with 10 mM Tris-HCl, pH 7.4 immediately prior to the assay. The assay is performed in 96-well plates. Chondroitin sulfate (Sigma Chemicals, St. Louis, MO, U.S.A.) was used to generate the standard curve.

**PROTEOGLYCAN BIOSYNTHESIS**

To analyze proteoglycan biosynthesis, the cultures were incubated with [35S]sulfate (1-4 μCi per well) for 24 h prior to harvesting. Matrix proteoglycans were extracted with 4 M guanidine HCl in 50 mM sodium acetate, pH 5.8 containing 0.1 M 6-aminohexanoic acid, 50 mM benzamidine HCl, 10 mM EDTA and 5 mM N-ethylmaleimide for 24 h at 4°C. The proteoglycans from the matrix and supernatant were precipitated by addition of three volumes of ice-cold ethanol. After 24 h at 4°C the pellets were collected by centrifugation at 14 000 rpm for 30 min, washed three times with 70% ethanol [17]. The pellets were either dissolved in 8 M urea for agarose gel electrophoresis or 4 M guanidinium with protease inhibitors for column chromatography or for quantitating [35S]SO4 incorporation by counting in a β-scintillation counter. Bovine cartilage explant cultures which had been incubated with [35S]SO4 for 24 h served as controls. The proteoglycans from the explant cultures were extracted identically to the filter cultures.

**PROTEOGLYCAN ANALYSIS**

Prior to loading on the gel, an aliquot of the [35S] labeled matrix extracts re-suspended in 8 M urea was heated to 60°C for 1 h and an equal volume
of 2 × loading buffer added (60% glycerol, 0.04 M Tris-acetate, pH 6.3, 1 mM sodium sulphate and 0.05% bromphenol blue). The proteoglycans were separated on 0.8% submerged horizontal agarose gels. The gels were subsequently prepared for autoradiography or immunoblotting.

Selected gels were transferred to nitrocellulose for Western blot analysis. The blots were blocked with 5% bovine serum albumin and then incubated with a monoclonal antibody reactive with keratan sulfate (mabD1B2, generous gift from Dr M. Adams, University of Calgary, Canada). Reactivity was detected using affinity purified rabbit anti-mouse IgG antibody conjugated to alkaline phosphatase. Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) were added for substrate and colour reaction (GIBCO/BRL, Burlington, ON, Canada).

GEL FILTRATION CHROMATOGRAPHY

The proteoglycans extracted from the matrix of 21- and 49-day-old cultures were analyzed by Sepharose CL-2B column chromatography (1 × 100 cm) under dissociative conditions. Matrix extracts resuspended in 4 M guanidine HCl were eluted with 4 M guanidine HCl buffer containing protease inhibitors, as described above, at 4°C. Fractions (1.8 ml) were collected using a flow rate of 6 ml/h. The elution profile was analyzed for its partition coefficient, $K_{av}$ [$K_{av} = (V_e - V_0)/(V_t - V_0)$], where $V_0$ = void volume, $V_t$ = total volume, $V_e$ = elution volume of the fraction. $V_t$ was determined using $[^3]$SO$_4$ and $V_0$ was determined using dextran sulfate.

ANALYSIS OF COLLAGEN

The cultures were labeled with [¹⁴C]proline (4 μCi per well) for 24 h and then extracted with pepsin (100 μg/ml in 0.5 M acetic acid) (Worthington Biochemical Corp., Freehold, NY, U.S.A.) for 48 h at 4°C. The digestion was stopped by the addition of 4 × Laemmli's buffer. The pepsin extract was separated on a 7% sodium dodecyl sulfate-polyacrylamide gel and either prepared for autoradiography or transferred to nitrocellulose for Western blot analysis. The presence of type II or type I collagen was determined by immunoblot analysis using antibody reactive to type II collagen (dilation 1/100) or type I collagen (dilation 1/100) (Southern Biotechnology Associates Inc., Birmingham, AL, U.S.A.) The blots were washed three times and reactivity detected using anti-goat IgG antibody conjugated to alkaline phosphatase as described above.

Results

MORPHOLOGICAL APPEARANCE OF CULTURES

The chondrocyte cultures were examined histologically at 3, 6, 10, 21, 35 and 49 days after initiation of the cultures to assess matrix accumulation and organization. Precoating the filters with collagen was necessary for the attachment of the chondrocytes. Type I and type II collagen were equally effective as adhesive agents (data not shown). On day 6 the cells were multi-layered and surrounded by small amounts of pericellular proteoglycans as demonstrated by toluidine blue staining [Fig. 1(a)]. By day 10, which was 3 days after the addition of ascorbic acid, there was a significant increase in the amount of matrix accumulated such that a continuous layer of cartilagenous tissue had formed (data not shown). At 10 days, no cellular organization was evident. By 21 days [Fig. 1(b)] the superficial layer of chondrocytes was usually elongated and oriented parallel to the surface of the tissue. The deeper cells were spherical. The 21-, 35- and 49-day-old cultures were examined by polarized light microscopy following picrosirius staining. These cultures showed collagen organization similar to articular cartilage [18] in that the collagen in the superficial zone of the cultures was oriented parallel to the surface and the collagen in the deeper zone surrounded the chondrocytes [Fig. 1(c)].

QUANTITATION OF MATRIX THICKNESS AND DNA CONTENT

Cultures, plated at a cell density of $1 \times 10^6$ or $3 \times 10^6$ cells/cm$^2$, were compared for formation of cartilagenous tissue and cellularity up to 35 days in culture (Table I). The thickness of the tissue generated increased over time and was dependent

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<th>Culture duration (days)</th>
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The effect of cell density on chondrocyte culture thickness was examined. Chondrocytes were plated at $1 \times 10^6$ cells/cm$^2$ (low density) or $3 \times 10^6$ cells/cm$^2$ (high density) on filter inserts and harvested on days 6, 10, 21 and 35. The cultures were fixed in formalin and the thickness of the cultures measured morphometrically as described under the Methods. The results are from one representative experiment performed in triplicate, and are expressed as mean ± s.e.
on the initial plating density. Cells plated at the lower cell density ($1 \times 10^6$ cells/cm$^2$) formed tissue that by day 35 measured $34 \pm 2 \mu m$. When cells were plated at a higher density ($3 \times 10^6$ cells/cm$^2$), a thickness of $110 \pm 7 \mu m$ was obtained during the same time period.

There was no change in the DNA content of the cultures during the 35 days at either cell density, indicating that the increase in culture thickness was due to extracellular matrix accumulation and not to an increased number of cells. The average DNA content of the cultures was $11 \pm 1 \mu g$ and $33 \pm 5 \mu g$ (mean $\pm$ s.e.) for the low and high density cultures respectively.

**MAINTENANCE OF CHONDROCYTE PHENOTYPE: ANALYSIS OF COLLAGEN AND PROTEOGLYCAN IN THE MATRIX**

To ensure that the chondrocytes maintained their phenotype under the culture conditions, we examined whether chondrocytes were synthesizing cartilage type macromolecules such as type II collagen and large proteoglycans. Analysis of the [$^{14}$C]proline-labeled pepsin extracts of the matrix for 21-day-old cultures by gel electrophoresis and autoradiography showed the presence of a single major band with a mobility similar to type II collagen [Fig. 2(a)]. By Western blot analysis, this band was identified as type II collagen [Fig. 2(b)]. No type I collagen was detected in the pepsin extracts by either autoradiography or Western blot analysis using an antibody reactive with type I collagen [Fig. 2(b)].

By agarose gel electrophoresis, the proteoglycan monomers migrated slightly faster than proteoglycans extracted from the cartilage explants, suggesting that they were similar but not identical in size (Fig. 3). The proteoglycan, aggrecan, contained keratan sulfate, as demonstrated by Western blot analysis using an antibody reactive with keratan sulfate antibody (data not shown). Proteoglycan
monomer heterogeneity, when examined by agarose gel electrophoresis, did not vary over the 49 days (data not shown). When examined by molecular sieve chromatography under dissociative conditions, the newly synthesized proteoglycans retained in the matrix on days 21 and 49 were of similar size. The proteoglycans were large and heterogenous with a $K_v$ of 0.44 ± 0.02 (Fig. 4).

PROTEOGLYCAN AND COLLAGEN ACCUMULATION IN THE MATRIX

The proteoglycan and collagen content in the extracellular matrix of the cultures was quantitated over 49 days (Fig. 5). Only small amounts of proteoglycans and collagen were present in 6-day-old cultures. The proteoglycan and collagen content in the matrix increased linearly up to 35 days and then plateaued. The amount of collagen was extrapolated from the hydroxyproline content, as hydroxyproline comprises approximately 10% of the weight of collagen [19]. At 35 days the tissue contained approximately 280 μg collagen and 95 μg of glycosaminoglycans, representing a collagen to proteoglycan ratio of approximately 3:1.

PROTEOGLYCAN BIOSYNTHESIS AND RETENTION IN FILTER CULTURE

Proteoglycan synthesis was determined by quantitating the total amount of $[^{35}S]$SO$_4$ incorporated into proteoglycans in the matrix and supernatant (Fig. 6). Proteoglycan synthesis increased linearly between 6 and 21 days and remained high up to day 35. By 49 days, proteoglycan synthesis declined to levels similar to that observed in 6-day-old cultures.
Figure 4. Gel chromatography of proteoglycans extracted from the matrix of 21 (-----) and 49 (---) day old cultures. Proteoglycan hydrodynamic size was determined by Sepharose CL-2B chromatography under dissociative conditions (4 M GuHCl). This is a representative elution profile from one experiment which has been repeated four times.

Figure 7(a) demonstrates that the amount of newly synthesized proteoglycans retained in the matrix or secreted into the medium varied during the culture period. The amount of newly synthesized proteoglycans incorporated into the matrix increased up to 35 days, whereas the amount secreted into the supernatant declined approximately four-fold between days 10 and 49. An increase in the percentage retention of newly synthesized proteoglycans was observed between 6 and 35 days. At day 6 approximately 20% of the [35S]-labeled proteoglycans were retained in the matrix and this increased to 85% when measured on day 35 [Fig. 7(b)]. No further increase in retention occurred in cultures after 35 days. The increase in proteoglycan content in the extracellular matrix paralleled the increase in the percentage of proteoglycans retained in the matrix.

Discussion

During the period of growth and in the disease acromegaly [20], articular cartilage becomes thicker, suggesting that chondrocytes can synthesize and accumulate sufficient amounts of matrix macromolecules to increase the amount of cartilage. Yet chondrocytes cannot adequately accumulate and replace matrix which has been lost due to trauma or disease [21–23]. In the present study, we have optimized an articular chondrocyte culture system in which isolated cells placed on filter inserts accumulate extracellular matrix and form cartilaginous tissue. This system provides an in vitro model system to investigate the mechanisms regulating proteoglycan and collagen retention that result in matrix accumulation. While many methods for culturing chondrocytes...
FIG. 7. The amount of newly synthesized proteoglycans retained in the matrix was determined. (a) The amount of radioactivity in the extracellular matrix (–○–) and the supernatant (–■–) at varying times was quantitated as described under the Methods. The results are expressed as the mean ± s.e. The experiment was repeated four times. (b) The percentage of newly synthesized proteoglycans retained in the matrix at different times was calculated.

have been developed, maintaining chondrocytes on filter inserts has advantages over these other culture methods in that a continuous layer of tissue which is amenable to histological and biochemical assessment can be obtained in a reasonable amount of time. Other chondrocyte culture systems such as monolayer and suspension cultures do not accumulate sufficient extracellular matrix to form a continuous layer of tissue [13, 24–26]. Chondrocytes have been grown in alginate [10] or on hydroxyapatite [11] beads and under these conditions remain spherical and accumulate type II collagen and cartilage type proteoglycans but still do not form tissue. In 3-dimensional cultures when chondrocytes are embedded in collagen or polymers, cartilagenous tissue is produced [12, 27]. However, the exogenous macromolecules which provide the scaffolding for tissue formation might affect matrix organization.

Chondrocytes in filter culture produce cartilagenous tissue and maintain their phenotypic expression of proteoglycans and collagen. The proteoglycans are large and contain keratan sulfate. The average size of the proteoglycan monomers synthesized and incorporated in the matrix did not change over the time examined. The cells produced type II collagen throughout the culture period. The absence of type I collagen in the matrix is in keeping with chondrocytes maintaining their phenotype. Under optimal conditions, the cartilagenous tissue attains a thickness of 110 µm and shows organization of cells and collagen into superficial and deeper layers similar to that described previously for articular cartilage [1–6, 18]. The culture thickness, proteoglycan and collagen content in the extracellular matrix increases in parallel. The extracellular matrix contains approximately three-fold more collagen than proteoglycan, which is similar to in vivo cartilage. The cultures reach a point where there is no further change in proteoglycan and collagen content in the extracellular matrix. These data indicate that the cultures go through two phases, one of matrix accumulation which occurs between days 10 and 21 and one where the matrix is being maintained which occurs after 35 days. As the cartilagenous tissue produced by these cultures is sufficiently similar to in vivo cartilage, it can be used to study the mechanisms regulating cartilage matrix accumulation or maintenance.

The amount of [35S]SO₄ incorporated into proteoglycans increased during tissue formation and decreased during the period of tissue maintenance. When these studies were repeated with [3H]glucosamine instead of [35S]SO₄ (data not shown), similar changes occurred, suggesting that [35S]SO₄ incorporation reflects the changes in proteoglycan synthesis and not differences in proteoglycan sulfation. These data demonstrate that chondrocyte synthesis of proteoglycans is greater when matrix is accumulating. The changes in environmental conditions or matrix molecules responsible for regulating proteoglycan synthesis by chondrocytes have yet to be elucidated. It has been shown that chondrocyte synthesis of proteoglycans can be either stimulated or inhibited by extracellular proteoglycans or hyaluronate [28–36]. So it may be that the amount of proteoglycans synthesized in filter culture is being regulated by the concentration of macromolecules in
the pericellular matrix during tissue formation and maintenance.

In this culture system, the percentage of newly synthesized proteoglycans incorporated in the extracellular matrix increased during the period of matrix accumulation and maximal retention occurred during tissue maintenance. It is not evident why proteoglycan retention changes over time but it may be due to the developing tissue organization and/or the increasing amount of collagen in the matrix. This seems likely, as the collagen and proteoglycan content in the matrix of these cultures increase in parallel. This hypothesis is supported by other experimental work, which showed that when chondrocytes in culture are grown in the presence of ascorbate, which induces accumulation of collagen in the pericellular region, there is increased proteoglycan retention [37, 38]. Fibroblast cultures supplemented with L-ascorbic acid 2-phosphate, a long acting ascorbic acid derivative, can be stimulated to form a 3-dimensional structure [39]. Alternatively, as proteoglycan retention in cartilage has been attributed to proteoglycan aggregation with hyaluronate and its stabilization by link protein [40], it may be that link protein synthesis by chondrocytes in filter culture changes during the culture period. Plaas et al., using long-term rabbit chondrocyte cultures, have demonstrated a decrease in the amount of link protein synthesized with time in culture [41], which would alter proteoglycan aggregation and its retention. Preliminary data indicate that the proteoglycans synthesized in these culture conditions have the ability to aggregate with hyaluronic acid. The mechanism(s) regulating proteoglycan retention in the matrix requires further investigation.

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