Mature Full-thickness Articular Cartilage Explants Attached to Bone are Physiologically Stable over Long-term Culture in Serum-free Media

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Mature tissue explants containing the entire depth of articular cartilage, calcified and uncalcified, attached to a thin layer of subchondral bone were isolated from bovine humeral heads of 1–2-year-old steers. These explants were placed in defined serum-free culture medium for a period of 3 weeks to investigate their biological and mechanical stability and thus to determine their potential utility in studies of cartilage physiology. Tissue mass remained constant over the culture period and no evident tissue swelling or distortion was observed. Chondrocytes were viable in all zones at the time of tissue isolation and throughout the culture period, with the exception of a thin layer of cells at the articular surface and the cut radial edge of the disks. Proteoglycan metabolism attained a steady state after 5 days of culture when the rate of loss of proteoglycan to culture media was compensated by new synthesis to maintain a stable proteoglycan content. Collagen metabolism was also stable with a constant content of type II collagen and a constant content of denatured collagen II throughout culture; the content of the C-propeptide of type II procollagen as a measure of procollagen synthesis, dropped slightly during the first week to attain a steady state after that time. Dynamic and equilibrium mechanical properties of these explant disks were also stable confirming maintenance of these tissue properties during long-term culture. In addition, the disk geometry of the system, with the cut surface in the bone parallel to the intact articular surface, is well-suited to study tissue regulation by mechanical load. Taken together, the stability of these indicators of tissue physiology indicates the maintenance in serum-free conditions of normal metabolism for organ cultures containing full-depth mature articular cartilage attached to bone.

Keywords: Cartilage, chondrocyte, collagen, proteoglycan, biomechanics

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INTRODUCTION

Cartilage organ cultures are alternative model systems to cell cultures and animal models. Compared to animal models, organ cultures, or tissue explants, provide more defined systems to study these tissues, since several complicating systemic and environmental parameters are removed. The particular goals of an experiment must then be in line with the conditions of tissue culture. Compared to cell cultures, tissue explants maintain extracellular matrix components so that a more complete, and therefore more complex, physiology representative of the in vivo situation is allowed. In particular, chondrocyte phenotype according to morphological and biosynthetic characteristics is usually maintained in organ cultures, whereas it is seldom ensured in cell cultures. Thus, due to the maintenance of cell–extracellular matrix interactions in a controlled intact tissue system, tissue explants have been widely used to study biological regulatory mechanisms in cartilage.\textsuperscript{[1–14]}

Tissue explants of articular cartilage are prepared from joints shortly after slaughter, cutting tissue from the articular surface and placing it in small pieces in culture medium in an incubator. The resulting specimens can have a random or a regular geometry, depending on the way in which they are cut. For studies involving mechanical loads, a regular geometry in the form of a cylindrical disk is generally desired since defined loading conditions can then be applied. In addition to providing defined loading conditions, specialized cutting apparatus can prepare specimens which are either homogeneous due to selection of tissue from particular depths of young calf articular cartilage\textsuperscript{[9]} or represent the entire thickness of heterogeneous articular cartilage from a mature animal dissected from calcified cartilage and bone\textsuperscript{[11]} or can maintain the tidemark and part of the underlying subchondral bone in young small joints\textsuperscript{[15]} or neonatal mandibular condyles.\textsuperscript{[16,17]} These different cutting procedures can affect metabolism in culture, in part due to the selection of different types of cells and matrix, but also due to the location and extent of tissue disruption due to cutting, which can create uniform tissue swelling in young homogeneous explants\textsuperscript{[12]} or non-uniform swelling and distortion in more mature tissue.\textsuperscript{[18]} Thus, careful examination of the type of specimen desired for organ culture will have an impact on the type of cutting used in the isolation procedure.

After selection and isolation of tissue, in vitro metabolism is strongly influenced by culture conditions, in particular the choice of culture media where the main distinction to be made is that between serum-containing and defined serum-free medium.\textsuperscript{[19]} Studies using diced tissue from mature bovine joints concluded that Dulbecco’s modified Eagle’s medium needed 20% fetal calf serum supplement to maintain the content and the rate of synthesis of proteoglycan (PG) in long-term (2-week) explant culture.\textsuperscript{[20]} Some other studies, using larger tissue explant disks, have found stable PG synthetic rates over shorter periods in culture either with 0.1% of a synthetic serum substitute for young epiphyseal cartilage,\textsuperscript{[7]} or with no serum or serum substitute but with ascorbate and glutamine supplements for mature cartilage,\textsuperscript{[11]} thus indicating the potential for the use of defined media in tissue explant systems. Additionally, serum-supplemented medium has been found to have some undesirable effects on cartilage explants including the induction of cell outgrowth,\textsuperscript{[21]} selective stimulation of biosynthesis by chondrocytes in the tangential versus radial zone\textsuperscript{[15,22]} and reduced synthetic rates, reduced growth and induction of phenotypic instability in developing tissue models.\textsuperscript{[17]} Thus, the absence of serum in cartilage explant culture medium could furnish several advantages and improve the definition of experimental conditions, providing that a metabolically stable system can be achieved in its absence.

The goal of our studies was to develop a cartilage explant culture system having the following characteristics that would best approximate the in vivo situation: (1) full thickness mature articular cartilage attached to calcified cartilage and subchondral bone in a well-defined disk geometry, and (2) the explant system is physiologically stable, in defined
medium, in terms of cell viability, PG metabolism, collagen metabolism and functional mechanical properties.

MATERIALS AND METHODS

Explant Isolation and Culture

Shoulder joints with intact synovial membranes from 1- to 2-year-old steers were obtained from a local slaughterhouse 6–16 h post-mortem. The joint was surgically opened after which humidification was maintained with a constant irrigation of exposed articular surfaces with Hank’s buffered saline at 4°C (HBSS Gibco #14060 + 50 μg/ml gentamycin). An orthopaedic coring bit (#050.720, Straumann Canada, Toronto) with a 4 mm diameter core was used with a standard drill to create several cuts with the core axis perpendicular to the humeral articular surface (Fig. 1A). The core bit was cooled during drilling with HBSS at 4°C. The remaining attachment of the core to the bone was severed by cutting laterally with a bone saw to release 15–30

![Figure 1](image_url)

**FIGURE 1** Explant isolation. (A) A coring bit is used with a 4 mm diameter central core to cut cartilage/bone cylinders. Cylinders are removed from the bone by cutting horizontally with a bone saw. (B) The isolated cylinders are placed in a cutting apparatus designed to cut through the bone at a defined distance from and parallel to the articular cartilage surface. Irrigation with physiological saline is maintained throughout all steps of the procedure.
cartilage/bone cores per shoulder joint, each containing the entire articular cartilage thickness (1.0–1.3 mm) attached to a long (≥ 5 mm) bone stem. Cores were immersed in HBSS. A custom cutting jig (Fig. 1B) was used to remove most of the bone but to retain a thin layer of subchondral bone to preserve the natural cartilage/bone interface. The cores were mounted vertically in the jig and immobilized therein by compressing the bone end lightly via a screw to ensure flat contact of the articular surface with a guide surface. A rotating circular saw blade (#4400243, Fraisa, Bellach, Switzerland) attached to a dental saw (Volvere GX, Nakanishi Dental Co., Japan) was then passed through the bone laterally, in an orientation parallel to the guide surface and thus parallel to the articular surface, and at a vertical position adjusted to produce a total cartilage/bone thickness of 1.5 mm. Parallel orientation of the cutting surface to the guide surface and vertical positioning were achieved with units (models 423 and 443 translation stages, model RSP-1 rotation stage and MM-2 kinematic mirror mount) from Newport Corporation (Irvine, California). Constant irrigation of cartilage surfaces with HBSS was maintained. After cutting, the diameter of the disks was reduced from 4 to 3 mm using sterile biopsy punches (15-33-33 Miltex), except for cultures where collagen denaturation was analyzed which were left at 4 mm diameter to maximize the amount of material available. The resulting explants were 3 mm diameter and 1.500 ± 0.025 mm thick where 0.5–0.2 mm of subchondral bone was present, depending on the articular cartilage thickness of the particular core. Explanted disks were transferred into 48 well culture plates in a laminar flow hood where each disk was rinsed 5 times with agitation over 30 min in HBSS containing antibiotics to remove any non-sterile particles adhering to the tissue surfaces. Disks were then transferred to 96 well round-bottom microplates (#25850-96, Corning) and cultured in 250 μl/well of DMEM/F12 supplemented with 15 mM HEPES pH = 7.4 (D8900), 50 μg/ml gentamycin (G1272), 0.01% BSA (A8412) and 20 μg/ml ascorbate (A4034 all from Sigma) at 37°C in a 95% air, 5% CO₂. Media were changed every 24 h with ascorbate prepared fresh each day. Total culture periods were up to 21 days.

**Histology**

Histological analysis was performed on disks fixed immediately after their isolation using freshly prepared 4% w/v paraformaldehyde and 1% w/v glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3. Fixation proceeded for 4 h at room temperature followed by 16 h at 4°C. Disk segments were left undecalcified, washed in 0.1 M sodium cacodylate buffer alone, dehydrated in a graded ethanol series, and infiltrated and embedded in LR White acrylic resin (Marivac, Nova Scotia, Canada). The tissue-containing resin blocks were polymerized for 2 days at 58°C. Light microscope sections (1 μm thick) were prepared on a Reichert ultramicrotome, stained with toluidine blue, and photographed with a Zeiss Axiophot microscope. Some sections were additionally stained for mineral deposits in the tissue section with the von Kossa staining method, using 3% silver nitrate for 20 min under ultraviolet light.

**Cell Viability**

Viability of chondrocytes at the time of explant isolation, and over the 21-day culture period, was assessed using Calcein-AM for live cells and ethidium homodimer-1 for dead cells, found previously to be an optimal combination for live versus dead cell determination in vitro.[23] Calcein-AM is an uncharged non-fluorescent esterase substrate which diffuses freely into cells. Viable cells containing esterase activity convert Calcein-AM to Calcein which is charged and fluorescent and only retained in cells with an intact plasma membrane thereby producing a vivid green cytoplasmic stain in viable cells. Ethidium homodimer-1 has low membrane permeability, high affinity for DNA, and undergoes a 40-fold enhancement of fluorescence upon binding to DNA producing strong red nuclear fluorescence in non-viable cells.
Calcein-AM (C1430, Molecular Probes) stock solution was prepared at 1 mg/ml in DMSO and stored at −70°C. Ethidium homodimer-1 (E1169, Molecular Probes) stock solution was prepared at 1 mg/ml in 20% DMSO/80% H2O. The working solution for the viability assay was prepared immediately before use, by mixing 4 µl Calcein-AM stock and 2 µl ethidium homodimer stock in 4 ml PBS at pH 7.0 for final concentrations of 1 µM Calcein-AM and 0.6 µM ethidium homodimer-1. Either whole disks, or disk sections, were incubated in the working solution for a minimum of 30 min at room temperature. Disk sections were generated by immersing whole cartilage explant disks in liquid agarose (4% SeaPlaque from FMC Bioproducts solubilized in PBS) at 37°C with the bone explant surface contacting the stage of a Tissue Chopper (Mctwain Tissue Chopper, Brinkman). After gelling at room temperature, 50–75 µm thick vertical sections comprising a cross-section of full cartilage thickness and underlying bone were taken. Three disks isolated from different regions within the zone of compression were tested for viability on days 0, 1, 4, 7, 14, and 21 of culture. Fluorescent images were acquired using an inverted fluorescence Zeiss microscope equipped with a digital camera (SPOT, Diagnostic Instruments, Sterling Heights, Michigan, USA) and Northern Eclipse image-acquisition software (Empix Imaging, Mississauga, Ontario, Canada).

Proteoglycan Analysis

To estimate PG synthetic rate, groups of 5 explants were pulsed on days 1, 4, 7, 14 and 21 with 50 µCi/ml [35S]-sodium sulfate (#SJS 1A, Amersham, >1000 Ci/mmol) in culture media at 37°C (as above) for 3 h, followed by 4 washes over 30 min and a 30 min chase in unlabelled media. Explants were blotted, weighed, and digested in 1.00 ml/disk of 0.125 mg papain (#P3125 from Sigma, in 0.1M phosphate, 10 mM EDTA, pH 6.5) at 60°C for 16 h. The cartilage mass was estimated by subtraction of the weight of the residual bone after digestion. Aliquots of the digest were taken for scintillation counting and moles of incorporated sulfate was calculated by use of sulfate specific activity obtained by dividing the label activity (50 µCi/ml) by the concentration of sulfate in DME-F12 (409 nmol/ml) and assuming equal specific activity between media, extra- and intracellular sulfate pools. Previous work on similar systems has shown that >95% of incorporated label remaining is macromolecular and that the assumption of equal specific activities in the various pools is a reasonable one.[9]

PG content was assayed as total glycosaminoglycan (GAG) content using the dimethylmethylen blue dye (DMMB, #3610, Polysciences) as described previously.[24] Papain-digested samples were diluted 1:4 with PBE buffer before 10 µl aliquots were mixed with 250 µl of DMBB (46 µM in 40 mM glycine, 40 mM NaCl, pH 3.0) in 96 well assay plates and absorbances at 530 and 590 nm were read. The difference in absorbance at 530 and at 590 nm was compared to a standard curve derived from shark chondroitin sulfate (C4384, Sigma) made up in the PBE buffer. GAGs released to media were analyzed without further treatment in the same manner, except with standards made up in culture media.

Cell number was estimated using DNA content obtained with the fluorescent dye Hoechst 33258, assuming 7.7 µg DNA/106 cells.[25] Aliquots of 50 µl of papain digests were mixed with 250 µl of the Hoescht working solution (0.5 µg/ml in 0.01 M Tris, 0.001 M Na2EDTA. 0.1 M NaCl, pH 7.4, prepared fresh daily from a 1 mg/ml stock solution of Hoescht in water). Calf thymus DNA (#D1501, Sigma) was used from 50 to 600 ng (10 µg/ml stock) as a standard. Fluorescence was measured by excitation of samples at 360 nm monitoring emission at 460 nm.

PG content and cell number were normalized to cartilage mass. Loss of PG to media in each 24 h period was recorded as a fraction of total GAG content in the disk. PG synthetic rate was normalized to cell density and the number of hours during which sulfate label was present (3 h).
Collagen Analysis

Total and denatured type II collagen content were measured by an ELISA assay for the COL2-3/4m epitope.\[26] Briefly, explants were digested by overnight incubation at 37°C in 1 ml/50 mg of tissue of 1 mg/ml α-chymotrypsin (#C3142, Sigma) in 50 μM Tris containing 1 mM EDTA, 1 mM iodoacetamide and 10 μg/ml pepstatin A (all from Sigma). α-chymotrypsin can degrade non-helical (denatured) collagen,[27] without attacking the helical region or the COL2-3/4m epitope,[26] allowing measurement of denatured collagen in the explant. α-chymotrypsin was inhibited by adding 20 μl of 8 mg/ml tosyl-L-phenylalanine chloromethyl ketone (TPCK, #T4376, Sigma) per ml of digest. The residue was then digested overnight at 56°C with 1 mg/ml proteinase-K (#P0390, Sigma) as described for α-chymotrypsin. Proteinase-K was inactivated by boiling the samples for 10 min. Extracts were evaluated using an inhibition ELISA with antibody COL2-3/4m, shown to specifically recognize the CB11B epitope of the α1 chains of collagen II and the α3 chain of collagen XI with no cross-reactivity for collagen types I, III or X.[26] Total type II collagen was calculated as the sum of that measured in the two extracts and the percentage of denatured collagen II was the amount found in the α-chymotrypsin extract divided by the total amount.

Collagen synthetic rates were estimated by determining the content of the C-propeptide (CPII) of type II procollagen using a solution phase inhibition radioimmunoassay.[28] CPII content is representative of collagen synthesis since it is readily cleaved by C-proteinase after secretion, has relatively short half-life, and its content has been shown to correlate with conversion of [3H]-proline to [3H]-hydroxy-proline.[29] Explants were extracted in 4 M guanidine hydrochloride and assayed as described previously.[28,29]

Mechanical Properties

The equilibrium and dynamic stiffness of cartilage/bone explants were evaluated periodically over a 25-day culture period. Five disks were tested using the Mach-1 Mechanical Tester (BioSynTech Ltd., Laval, Quebec, Canada) placed in an incubator to maintain aseptic culture conditions during testing (37°C and 5% CO₂). On testing days (days 2, 5, 9, 12, 18, 25), individual disks were removed from the 96 well plate in culture and transferred to a sterile compression chamber containing culture media in a laminar flow hood. The closed chamber containing the disk was then transported to an incubator and placed on the mechanical tester. A sequence of 10 ramp displacements of 10 μm amplitude and 2 μm/s velocity were applied in uniaxial unconfined compression geometry to obtain a final total displacement of 100 μm corresponding approximately to a 10% total cartilage strain. After each ramp displacement, stress relaxation was allowed to occur until the time rate of change of the load was smaller than 1 g/min. Once equilibrium was reached after the last ramp compression, the 10% strain was maintained and a 8 μm sinusoidal displacement was applied at frequencies between 0.01 and 1.0 Hz. Each of the 5 disks was tested 6 times over 25 days. After testing on day 25, disks were sectioned with a tissue chopper and average uncalcified cartilage and average (calcified cartilage + bone) thickness in each disk were evaluated under a dissection microscope using a slide scale for calibration. Equilibrium modulus was calculated as the slope of a linear best fit of equilibrium force normalized to initial area versus displacement normalized to uncalcified cartilage thickness. Discrete Fourier transforms of load and position data were used to obtain the dynamic stiffness by dividing the fundamental component of force normalized to initial area by the fundamental component of position normalized to current uncalcified cartilage thickness.[30]

RESULTS

Histology

Cell and tissue architecture of the sampled disks showed morphological characteristics typical of bovine articular cartilage from animals in the age
range of 1–2 years. Chondrocytes near the articular surface were darkly stained and flattened, and occasionally displayed condensed nuclei (Fig. 2A), while cells in the deeper zones of the cartilage were more rounded and frequently occurred in pairs (Fig. 2B and C). Among the samples, either columns of chondrocytes, or chondrocyte pairs, were observed in the radial zone, reflecting the range of ages from which the tissue explants were derived. A layer of calcified cartilage, demarcated by a tidemark and confirmed by von Kossa staining (data not shown), was always present in the samples (Fig. 2C). Beneath the calcified cartilage, a variable amount of bone was present in the disks, with interdigitating prongs of bone inserting into the cartilage (Fig. 2D).

**Cell Viability**

Whole explants, as well as transverse disk sections obtained with a tissue chopper, were subjected to a viability assay on each day of culture, 3 disks per day on days 0, 1, 4, 7, 14 and 21. Articular chondrocyte viability at the time of explant isolation and during the entire 3-week culture period was near 100% throughout the thickness of articular cartilage, except for a thin layer of dead cells observed at the articular surface (Fig. 3A), and the cut disk edge (Fig. 3B). Occasionally, the cells at the articular surface stained green, but only in a few specimens. Cell viability after 21 days in culture was essentially unchanged from that observed at the time of

![Figure 2](image_url)

**FIGURE 2** Histological characteristics. Light micrographs of toluidine blue-stained sections of articular cartilage explant disks showing chondrocyte morphology and tissue characteristics at various depths from the articular surface. Most superficially (A), chondrocytes were flattened and condensed in appearance, while interiorly (B,C), cells were more rounded and appeared as pairs or columns. Calcification (*) of the cartilage was evident at the position of the tidemark (arrows in C) and by von Kossa staining (data not shown). Adjacent to the calcified cartilage, regions of subchondral bone could be clearly identified (D). AS = articular surface. Cart. = calcified cartilage.
FIGURE 3  Cell viability. Thick sections (50-75 μm) made with a tissue chopper were immersed in PBS containing Calcein-AM (green) as an indicator of live cells and ethidium homodimer-1 (red) as an indicator of dead cells. Cell viability displayed the same characteristics immediately following isolation as throughout the entire culture period. Chondrocytes are viable in all zones of the explant (A) with the exception of a thin layer of dead cells at the articular surface and the cut radial edge of the disk (B). The occasional dead cell in zones (B) other than the most superficial layer seen in some sections were found to be those cut by the tissue chopper in preparing the sections. (See Color Plate III.)

isolation (day 0), and interestingly, dead cell DNA was still observed in cells which were most likely non-viable since the start of the culture period (Fig. 3B). Viable cells seen below the tidemark were chondrocytes in islands of uncalcified cartilage while cells within the calcified cartilage and subchondral bone did not appear to take up either dye, perhaps due to diffusion barriers in these zones (Fig. 3A).
Proteoglycan Metabolism

Cartilage and bone masses within explants were stable at 9.8 ± 1.3 and 2.0 ± 1.2 mg (n = 20), respectively, over the 3-week culture period (Fig. 4A). Cell density estimated from DNA content was also stable over the culture period at 38.3 ± 8.3 million cells/g-cartilage (n = 28) (375 × 10^3 cells/disk) (Fig. 4B). During the first 4 days of culture immediately following explant isolation PG concentration in disks dropped from ~31 to ~21 μg/mg-cartilage (p = 0.03) and was thereafter relatively stable averaging 22.5 μg/mg (Fig. 5A). This estimated GAG loss in the disks during early culture was reflected in an increased amount of GAG found in the culture media during the first 4 days compared to later time points (Fig. 5B). The cumulative amount of GAG released to the media from days 1 to 4 accounted for the ~30% decrease found in the explant GAG concentration between days 1 and 4. During the course of culture, PG synthesis measured by [35S]-sulfate incorporation remained stable, averaging an intake of 0.89 nmol SO4^2- per million cells per hour (Fig. 5C). Assuming 1 mol of sulfate incorporated per 500 g GAG (MW of the chondroitin sulfate disaccharide) this value amounts to the synthesis of 4.0 μg GAG/disk per day, which would compensate for the steady-state loss to the media of 2% disk content per day (4.4 μg GAG/disk per day). Also, a total turnover time for PG can be estimated as 50 days.

Collagen Metabolism

Total type II collagen content displayed little variation over the 21 days of culture (Fig. 6A)
averaging 184 μg/mg-cartilage. Long-term culture did not significantly increase the amount of denatured collagen extracted with α-chymotrypsin, which remained in the region of 2.5% of total type II collagen content (Fig. 6B). The content of CPII of type II procollagen dropped slightly from 22 ng/mg-cartilage over the first week to a stable average value of 11.6 ng/mg from day 7 on (Fig. 6C). As an indicator of collagen synthesis, and using a half-life of 16 h measured in similar tissue,[29] this content of CPII can be shown to represent 24 ng/mg-cartilage of newly synthesized collagen every 24 h (using the ratio of collagen II to CPII molecular weight of 2.8 and a lifetime of ~ 32 h). Using a total content of type II collagen of 180 μg/mg-cartilage, the steady-state value of 11.6 ng-CPII/mg-cartilage represents 0.018% the molar concentration of type II collagen, and suggests a turnover time of 7,500 days or about 20 years, assuming a degradation rate equal to synthesis.

Mechanical Properties

Since the physiological function of articular cartilage is load-bearing, and this ability results from both the presence of, and proper interactions between, its biochemical constituents, it was important to evaluate the stability of mechanical behavior in tissue explant cultures. Equilibrium modulus was stable near the average value of 0.35 ± 0.1 MPa (mean ± s.d., n = 30) and dynamic stiffness evaluated at 1 Hz was relatively constant near its average value of 4.01 ± 1.3 MPa (mean ± s.d., n = 30).

DISCUSSION

Disks of uniform thickness containing full-depth articular cartilage attached to a thin layer of calcified cartilage and subchondral bone were isolated from 1- to 2-year-old bovine humeral heads and placed in serum-free culture conditions. Several physiological characteristics displayed reasonable stability over a 3-week culture period including cell viability, PG synthesis, content and loss to media, collagen II content, degree of denaturation and CPII content as an indicator of collagen II synthesis. In addition, dynamic and equilibrium mechanical behavior were stable in unconfined compression, as a measure of overall tissue functionality. Some observed behavior displayed a consistent dependence on culture time in repeated experiments including an early phase (days 1–4) of accelerated GAG loss to media before a steady state was obtained, and some diminution of CPII during the first week of culture as well. The stable content of collagen II, percentage of collagen II in the

![Graph of collagen metabolism](image_url)

**FIGURE 6** Collagen metabolism. Type II collagen content (A) and denaturation (B) are shown (assayed by ELISA on α-chymotrypsin and proteinase-K extracts). The concentration of the CPII of type II collagen (C) is an indicator of collagen II synthesis.
denatured state and collagen II CPII content are in agreement with previously reported values. Our average value of collagen II content (184 µg/mg) is similar to an average value obtained for human femoral cartilage of 140 µg/mg, where the percentage of collagen II in a denatured state was 1.5%, also similar to our average value of 2.5%. Combined with the observed stability in PG content, the lack of increased collagen denaturation in culture is consistent with observed stability in mechanical properties (Fig. 7). The latter also suggests that the presence of other cartilage matrix components and their interactions are not significantly disturbed in culture. Our value for CPII content of 11.6 ng/mg of tissue is lower than that of a freshly isolated highly synthetic fetal growth plate which is near 100 ng/mg but similar to the same type of fetal tissue placed in culture, et 1–15 ng/mg. Values for human cartilage ranged from 1 to 35 ng/mg in going from adult to fetal tissues. In terms of fetal bovine cartilage, the decrease seen in cultured tissue compared to fresh tissue is consistent with our finding of a small drop in tissue content over the first week in culture. We have also detected release of CPII to the media during culture in some preliminary experiments, with amounts released per day similar to the total content found in the disk, thus also supporting previous data showing CPII half-lives between 15 and 19 h. Collagen turnover rate estimated from CPII content and half-life appeared extremely slow in vitro requiring 20 years to replace the total collagen pool. Conversion of [3H]-proline to [3H]-hydroxyproline in young calf tissue indicated daily collagen synthesis near 0.8 mg/g of tissue (56 nmol of proline incorporated per g of tissue per hour with ~60% going into collagen and 100 proline per collagen molecule), suggesting a turnover time close to 1 year (200 mg-total collagen/0.8 mg/day = 250 days) rather than 20 years. The reason for the difference between these two turnover times, or rates of synthesis, is not clear.

To the best of our knowledge, no previous studies have characterized cell viability over long-term culture in vitro. Our observations of stable viability in all depth layers were encouraging despite some difficulty in maintaining the most superficial cells in viable state. The observation that these cells were occasionally viable after isolation may lead to isolation conditions which encourage their survival including reduced time from animal death to culture, or highly limited exposure to room air (oxygen and drying effects) or their viability could be dependent on factors found in synovial fluid. Cells present in the bone could not take up either dye, but were most likely not viable given their pycnotic histological appearance (Fig. 2) and likely dependence on a vascular oxygen supply. An interesting observation was that some columns of chondrocytes living in islands of uncalcified matrix but surrounded by calcified matrix were viable (green cells below the tidemark in Fig. 3A), an observation consistent with active aggrecan synthesis observed by autoradiography for these types of chondrocytes.

The rate of incorporation of [35S]-sulfate as an indicator of PG synthesis found in our study was
comparable to those determined previously in other serum-containing or serum-free systems. Our average rate of synthesis of 0.89 nmol-SO₄/million-cells/h represents 34 nmol/h/g of tissue, using the measured cell density (Fig. 4). This compares to 9 nmol/h/g for similar bovine tissue minced in DME medium containing 20% FBS,[20] and also approximately 10 nmol/h/g in bovine disk explants in serum-free MEM medium supplemented with glutamine and ascorbate.[11] A recent study[3] using bovine metacarpalphalangeal articular cartilage from 3 age groups (1–6 weeks, 2–6 months, 2–3 years) cultured in a basal medium of DME supplemented with 0.2% BSA alone found rates ranging from 1.5 to 27 nmol/h/g from old to young tissue. With 20% FBS these rates increased to 25–99 nmol/h/g while with a supplement of 20 ng/ml of recombinant insulin-like growth factor 1 rates were intermediate at 9–67 nmol/h/g. Our bovine tissue is from an age group similar to the oldest age in that study (but 1–2 years instead of 2–3 years). It is therefore interesting to note that our rates of [³⁵S]-sulfate incorporation in a serum-free medium are comparable to those of the latter study containing 20% FBS. Thus it is possible that certain parameters of our explant (less tissue disruption, less swelling, intact tidemark, presence of bone) and culture conditions (ascorbate supplement, DME/F12 versus DME) increase PG synthesis to levels comparable to those obtained only in the presence of 20% FBS using other systems. For example, trace elements (copper, zinc), fatty acids (linoleic acid), vitamins and amino acids contained in DME/F12 but not DME alone may be beneficial.[35,36] Also the multiple roles of ascorbate as a cofactor in collagen synthesis,[37] PG synthesis[38] and matrix assembly[39,40] despite its toxicity at high concentrations for some cell types,[41,42] and potential encouragement of phenotypic instability[43,44] needs to be considered.

We did perform some preliminary experiments exploring alternative culture conditions using our medium described here as the basal medium. The addition of 20 ng/ml of recombinant IGF-1 had no measurable effect on sulfate incorporation, while the addition of 10% FBS approximately doubled incorporation rates, most likely preferentially stimulating cells in the superficial and transitional zone.[15,22] We also mixed DME/F12 with a selenium[45,46] containing medium (MCDB104) but found no measurable effect on sulfate incorporation compared to DME/F12 alone, both supplemented with 20 μg/ml ascorbate and 0.01% BSA. Thus, in light of the various disadvantages of serum supplements (lack of definition and reproducibility, interference with experimental design and analysis) and physical distortion of heavily cut tissue explants, the metabolic stability of this geometrically well-defined full-depth mature cartilage disk explant in serum-free medium represents a useful model system in the study of cartilage physiology.

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