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Mechanical compression modulates matrix biosynthesis in chondrocyte/agarose culture

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SUMMARY

This study focuses on the effect of static and dynamic mechanical compression on the biosynthetic activity of chondrocytes cultured within agarose gel. Chondrocyte/agarose disks (3 mm diameter) were placed between impermeable platens and subjected to uniaxial unconfined compression at various times in culture (2-43 days). [^{35}S]sulfate and [^{3}H]proline radiolabel incorporation were used as measures of proteoglycan and protein synthesis, respectively. Graded levels of static compression (up to 50%) produced little or no change in biosynthesis at very early times, but resulted in significant decreases in synthesis with increasing compression amplitude at later times in culture; the latter observation was qualitatively similar to that seen in intact cartilage explants. Dynamic compression of ~3% dynamic strain amplitude (≃30 μm displacement amplitude) at 0.01-1.0 Hz, superimposed on a static offset compression, stimulated radiolabel incorporation by an amount that increased with time in culture prior to loading as more matrix was deposited around and near the cells. This stimulation was also similar to that observed in cartilage explants. The presence of greater matrix content at later times in culture also created differences in biosynthetic response at the center versus near the periphery of the 3 mm chondrocyte/agarose disks. The fact that chondrocyte response to static compression was significantly affected by the presence or absence of matrix, as were the physical properties of the disks, suggested that cell-matrix interactions (e.g. mechanical and/or receptor mediated) and extracellular physicochemical effects (increased [Na^+], reduced pH) may be more important than matrix-independent cell deformation and transport limitations in determining the biosynthetic response to static compression. For dynamic compression, fluid flow, streaming potentials, and cell-matrix interactions appeared to be more significant as stimuli than the small increase in fluid pressure, altered molecular transport, and matrix-independent cell deformation. The qualitative similarity in the biosynthetic response to mechanical compression of chondrocytes cultured in agarose gel and chondrocytes in intact cartilage further indicates that gel culture preserves certain physiological features of chondrocyte behavior and can be used to investigate chondrocyte response to physical and chemical stimuli in a controlled manner.

Key words: cartilage, chondrocyte, proteoglycan, matrix biomechanics, agarose, stress-mechanical

INTRODUCTION

Articular cartilage is the dense connective tissue that functions as a bearing material in synovial joints. Adult articular cartilage is avascular, aneural and alymphatic; cell nutrition is derived primarily from the synovial fluid (Mankin and Brandt, 1984). Nutrition in immature cartilage is aided by the presence of vascular canals. The chondrocytes are responsible for the synthesis, maintenance, and gradual turnover of an extracellular matrix (ECM) composed principally of a hydrated collagen fibril network enmeshed in a gel of highly charged proteoglycan (PG) molecules. The composition and architecture of the matrix (Maroudas, 1979) and the tissue's high water content (70-80% of wet weight) enable cartilage to withstand complex compressive, tensile and shear forces in joints (Hodge et al., 1986; Mow et al., 1984).

Cartilage extracellular matrix can remodel to meet the functional demands of loading. For example, repulsive electrostatic (osmotic) interactions between the proteoglycan constituents of the ECM enable cartilage to resist compressive loads (Buschmann and Grodzinsky, 1995; Buschmann, 1992; Grodzinsky, 1983; Maroudas, 1979). Animal studies have shown that PG content is higher in cartilage that is habitually loaded (Caterson and Lowther, 1978; Kiviranta et al., 1988; Salter et al., 1980), while immobilization of joints leads to a decrease in PG synthesis and total PG content (Akeson et al., 1973; Kiviranta et al., 1987; Olah and Kostenszky, 1972; Palmoski et al., 1979). There do appear to be limits in the ability of chondrocytes to respond to altered functional demands. Increasing intensity of normal usage (without experimental modification of normal joint anatomy) can eventually result in a reduction in PG content (Arokoski et al., 1993; Jurvelin et al.,

1990; Kiviranta et al., 1992). Animal models of osteoarthritis in which an abrupt change in joint motion is surgically introduced lead to profound attempts at adaptation but eventually result in cartilage deterioration and malfunction (Inerot et al., 1991). The mechanisms that mediate the biological response to mechanical loads in cartilage are not well understood.

Biosynthesis, metabolism, and turnover of cartilage matrix components have been studied extensively in vivo and using cell and tissue culture models (Kuettner et al., 1986). Recent in vitro studies have shown that mechanical loads can also induce metabolic responses in cartilage organ cultures. Static compression has been found to decrease proteoglycan and protein synthesis (Gray et al., 1988; Sah et al., 1989; Schneiderman et al., 1986), while dynamic compression at certain frequencies and amplitudes stimulated synthesis of these matrix constituents (Korver et al., 1992; Parkkinen et al., 1992; Sah et al., 1989). These data imply that the presence of physiologic mechanical loading forces may be necessary for the proper assembly and maintenance of a matrix that can function in the joint.

Chondrocytes cultured in an agarose gel maintain the expression of aggrecan (cartilage-specific large aggregating PG) and collagen types characteristic of articular cartilage (predominantly collagen II as well as types IX and XI) (Aydelotte et al., 1988; Benya and Shaffer, 1982; Sun et al., 1986). The maintenance of chondrocyte phenotype during long-term culture in agarose gels allowed the development of a mechanically functional cartilage-like matrix (Buschmann et al., 1992). The equilibrium modulus, dynamic stiffness, and oscillatory streaming potential rose to many times (>5×) their initial values at the start of the culture; the hydraulic permeability decreased to a fraction (~1/10) that of the cell-laden porous agarose at the beginning of the culture. In the present study we addressed the question of whether mechanical loading of chondrocyte/agarose cultures could affect the rate of synthesis of matrix macromolecules. We report that chondrocytes in agarose gel respond biosynthetically to static and dynamic mechanical loads in a manner similar to that of intact organ culture. However, the response to compression was more pronounced at later times in culture after the level of matrix development was more advanced. Application of mechanical loads in a culture environment may therefore significantly alter the long-term development of this tissue, and may shed light on the physiological events and cellular mechanisms by which chondrocytes respond to mechanical signals in vivo.

MATERIALS AND METHODS

Isolation of chondrocytes and agarose gel culture

Saddle sections (~8 kg) from 1-2 week old calves were obtained from a local abattoir (A. Arena, Hopkinton, MA) within 3-4 hours after slaughter. The intact femoropatellar groove was isolated as described previously (Sah et al., 1989). The region between the lateral and medial ridges of the groove was removed as one piece from the underlying bone. After being cleaned of any bone and fibrous material, the tissue (19-26 g) was diced into approximately 1 mm^3 pieces and cultured in feed medium (DMEM, high glucose, supplemented with 0.1 mM nonessential amino acids, 0.4 mM proline, 2 mM glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, 10 μg/ml ascorbate (Gibco), and 10% FBS (Hyclone, Logan, UT)) at 37°C in 5%CO_2:95% air. Medium (8 ml/g tissue) was changed daily and 4-6 days later cells were extracted by sequential pronase/collagenase digestion (Kuettner et al., 1982). Cartilage pieces were immersed in DMEM with 1% (w/v) pronase (Sigma P5147) and shaken gently for 1 hour at 37°C. They were then washed in PBS (with 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$) and shaken gently in DMEM with 5% serum and 0.4% (w/v) bacterial collagenase (Worthington, type 2) for 4 hours at 37°C. Cells were isolated from the digest by centrifugation for 10 minutes at 100 ***g***, resuspended, and passed through a 100 μm nylon filter (Spectrum, Los Angeles). The cells were centrifuged and resuspended again, and passed through a 20 μm filter. Cells were centrifuged again and resuspended in feed medium (but with no ascorbate) in a 300 ml non-vented T-flask so that no air was present in the flask. The flask was stored overnight at 4°C.

Total cell number was determined using a Coulter counter, and cell viability was determined with Trypan Blue exclusion on a hemocytometer. Chondrocytes were then centrifuged, resuspended in DMEM and mixed with an equal volume of PBS containing low melting temperature agarose (SeaPlaque agarose, FMC Bioproducts, Rockland, ME) (Aydelotte et al., 1986; Sun et al., 1986) at 37°C to yield ~2×10^7 cells/ml in 2% or 3% (w/v) agarose and cast between slab gel electrophoresis plates separated by 1 mm thick Teflon spacers (Buschmann et al., 1992). After gelling at 0°C for 5 minutes followed by 4°C for 2-6 hours, approximately 100 disks, 16 mm in diameter by 1 mm thick, were cored from the slab gels using a stainless steel punch. The chondrocyte/agarose disks were subsequently cultured on top of 1 mm pore size nylon mesh (Spectrum, Los Angeles) to promote nutrient diffusion from below and above. Each disk was fed 2 ml per day of medium (as above but with 50 μg/ml ascorbate) up to day 16, then 2.5 ml per day up to day 22, 3 ml up to day 32, and then 4 ml up to day 47. Agarose disks without chondrocytes were prepared and maintained in a similar manner. The data presented below represent a compilation of three cultures, of length 47 days, 35 days, and 28 days. Chondrocytes were cast in 2% and 3% agarose for the 47 and 35 day cultures while only 3% gels were made for the 28 day culture. The cell population for each culture was obtained from the two femoropatellar grooves of one animal.

Chambers for compression-radiolabel experiments

The chambers for static and dynamic compression, shown schematically in Fig. 1, have been described previously (Sah et al., 1989). The compressed thickness of the chondrocyte/agarose (CA) disks in the static chamber was set by placing teflon spacers between the chamber lid and base and applying a weight to the chamber lid, forcing the lid, spacer, and base together. These chambers could accommodate 12 specimens compressed to the desired thickness. A range of compression levels was achieved by using multiple chambers with spacers of different thicknesses. The static chambers were used in a tissue culture incubator and had small lateral channels in their bases allowing incubator gas to equilibrate with the medium. The total time to assemble and load each chamber was ~5-10 minutes.

For the dynamic chamber, compression was applied with a mechanical spectrometer (Dynastat, IMASS, Hingham, MA). A sterile tissue culture environment was maintained inside the chamber by recirculating the medium through a controlled heat exchanger (37°C) and by passing a humidified mixture of 5% CO_2:95% air through a 0.22 μm filter into the chamber above the fluid level. The dynamic chamber accommodated 24 specimens, 12 of which were statically compressed to 0.73 mm thickness (from 1.00 mm) using a set of pins fixed to the chamber lid. The other 12 specimens were compressed using the mechanical spectrometer via sliding rods in the chamber lid. They were initially compressed to the 0.73 mm static offset over which a sinusoidal displacement of 30 μm amplitude was superimposed. The dynamic chamber was slightly modified for experiments with chondrocyte/agarose disks: (1) the dynamic compression rods were hollowed out to ensure that the weight of the rods alone would not significantly compress the specimens; and (2) the medium was stirred inside the chamber using a motor-driven stir bar. The stirring helped

to eliminate temperature and pH gradients within the chamber and stagnant layers at the perimeter of the CA disks.

The mechanical Spectrometer was interfaced to a computer and a frequency synthesizer (Model 5100, Rockland Systems, West Nyack, NY) to control the applied compressive displacement (accuracy <1 μm). During culture experiments, the displacement and resulting load were recorded on a chart recorder and/or digitized and stored on a computer. Fourier decomposition of the load signal into fundamental and higher harmonic components enabled quantitative assessment of non-linearities. The total time to load CA disks into the dynamic chamber under sterile conditions, seal the chamber, mount it in the spectrometer, and begin compression was ~30 minutes.

Compression and radiolabeling protocols

Twelve 3 mm diameter CA disks were cored out of each 16 mm disk used in compression-radiolabel experiments with a stainless steel dermal punch (Miltex Instruments, Lake Success, NY). These 3 mm disks were then placed in culture dishes with prelabel medium (DMEM, low glucose, 10% FBS, 10 mM HEPES, 0.1 mM nonessential amino acids, 0.4 mM proline, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 20 μg/ml ascorbate). The dishes were placed in a standard incubator for 16-30 hours prior to compression.

Static mechanical compression

Forty-eight 3 mm disks cored from four larger 16 mm disks were removed from the prelabel dishes and loaded into 4 different static compression chambers with medium as above (0.78 ml per well; 1 disk per well) but containing 10 μCi/ml [^{35}S]sulfate and 20 μCi/ml [5-^{3}H]proline to assess the rates of GAG synthesis and amino acid uptake and protein synthesis, respectively. The static chambers were assembled with spacers to achieve final compressed thicknesses between 0.4 mm and 1.0 mm. After culturing the disks for 16 hours under compression, the specimens were removed from the chambers and analyzed for rates of radiolabel incorporation, GAG content and DNA content (see below). Free-swelling disks (1.00-1.09 mm thickness depending on the day of culture) were also incubated in 48-well dishes, 1 disk per well, alongside the compressed specimens.

Dynamic mechanical compression

Twenty-four 3 mm disks cored from two 16 mm disks were removed from the prelabel dishes and loaded into the dynamic chamber containing labeled medium (as above but with 20 ml in the single well containing all 24 3 mm disks) to provide 12 experimental dynamically compressed specimens and 12 controls. Experimental disks were subjected to oscillatory compression of 30 μm amplitude (60 μm peak-peak) superimposed on a static offset compression of 0.73 mm at one of the following frequencies: 0.001 Hz, 0.01 Hz, 0.1 Hz, 1.0 Hz. Control disks were held statically at 0.73 mm. Dynamic compression in the presence of radiolabel continued for 10 hours at which time the disks were removed from the chamber and analyzed for rates of incorporation, GAG content and DNA content (see below). Zero-displacement control experiments were also performed in which no sinusoidal displacement was applied to the dynamic compression rods.

Biochemical analysis of rates of incorporation, GAG content, DNA content

After each compression experiment in the presence of radiolabel, the disks were washed 6 times over 2 hours in cold PBS (with ~1 mM unlabeled sulfate and proline but without $CaCl_2$ or $MgCl_2$). In one set of experiments designed to quantify the spatial variation in radiolabel incorporation, 2 mm diameter centers were cored after washing the 3 mm CA disks and analyzed separately from the 3 mm rings. Disks were then lyophilized and digested in 1 ml/disk papain (Sigma P3125, 125 μg/ml in 0.1 M sodium phosphate, 5 mM Na_2-EDTA, and 5 mM cysteine-HCl, pH 6.5) for 16 hours at 60°C. The agarose was melted at 70°C for 10 minutes, immediately followed by vortexing. Portions (100 μl) of the digest were analyzed for radioactivity by mixing with 100 μl of 10% SDS and 2 ml Scinti-Verse Bio-HP (Fisher) and measuring [^{3}H]cpm and [^{35}S]cpm in a liquid scintillation counter (Rack-Beta 1211, LKB, Turku, Finland), with corrections for spillover and dilution quenching. For some of the disks, the macromolecular fraction of incorporated sulfate was determined by applying 0.5 ml of the digest to a PD10 column of Sephadex G-25 (M_r cutoff of 1,000-5,000; Pharmacia, Piscataway, NJ) and eluting with 2 M guanidine-HCl. Fractions (0.5 ml) were collected and counted, or alternatively, the macromolecular (V_0) and low molecular mass component peaks were pooled and counted. The content of sulfated GAG remaining in the disks was assessed by reaction of 20 μl portions with 200 μl Dimethylmethylene Blue dye (DMB) solution (Farndale et al., 1986) in 96-well microplates (Nunc) and spectrophotometry (model Vmax plate reader, Molecular Devices, Menlo Park, CA); chondroitin sulfate (0-2 μg of shark cartilage, Sigma) was used as a standard. The DNA content within the disks was measured by reaction of 100 μl portions of the digest with 2 ml Hoechst 33258 dye solution in an acrylic cuvet and fluorometry, using calf thymus DNA as a standard (Kim et al., 1988). GAG lost to the medium during culture was assessed by reacting 20 μl portions of feed medium (every second day up to day 37) in 200 μl DMB solution as above; no interference from serum or medium was observed. Aliquots of the labeled medium from static compression experiments were also separated on PD10 columns to obtain macromolecular counts in the medium which diffused out during the 16 hour labeling period.

Histological analysis

Whole 16 mm disks were removed from culture and fixed in 2% (v/v) glutaraldehyde solution buffered with 0.05 M sodium cacodylate, and containing 0.7% (w/v) of ruthenium hexaammine trichloride (RHT) (Hunziker et al., 1982). Fixation was effected initially at ambient temperature (6 hours) and subsequently at 4°C (12 hours). Disks were then washed in 0.065 M NaCl and 0.1 M sodium cacodylate (pH 7.4) and stored in 70% ethanol at 4°C. Following dehydration in a graded series of increasing ethanol concentrations, specimens were embedded in Epon 812 which was polymerized at +60°C. Semi-thin (1 μm) sections, prepared for analysis in the light microscope, were stained with Toluidine Blue 0. Thin sections stained with uranyl acetate and lead citrate were utilized for electron microscopic analysis in a Hitachi H7100-B electron microscope.

Physical characterization

The mechanical and electromechanical properties of 13 mm chondrocyte/agarose disks and control disks with no cells were measured as described in (Buschmann et al., 1992). Briefly, 13 mm diameter disks were tested in confined compression geometry using the mechanical spectrometer. The disk was placed in a tight-fitting confining cylindrical well inside a nonconducting chamber filled with PBS and equipped with silver/silver-chloride disk electrodes to measure the compression-induced uniaxial streaming potential. A porous compressing platen was placed on top of the specimen and brought into contact with the upper post of the spectrometer. Both equilibrium and dynamic mechanical tests were performed. A sequence of step compressions (0.5%) was applied from 0-20% compression. Stress-relaxation between steps was allowed to equilibrate before collecting the equilibrium load and proceeding to the next step. Once a 20% static offset was obtained, a small amplitude (5-7 μm) sinusoidal displacement was applied at frequencies between 0.01 and 1.0 Hz. The amplitude and phase of the displacement, load, and streaming potential were determined from the digitized data.

RESULTS

Time evolution of GAG and DNA content and biosynthetic rates

The rates of GAG and DNA accumulation within the disks

during the 3% agarose 47 day culture are shown in Fig. 2. These data are from one of the three culture experiments described here, but are representative of the 3% agarose specimens from all three cultures. GAG concentration (mg/ml-disk-volume) increased many-fold and DNA concentration increased by ~35% over the 47 day culture period. By 30-45 days in culture, both GAG and DNA concentration had reached ~1/4 that of the 'parent' calf cartilage tissue.

The measured [^{35}S]sulfate and [^{3}H]proline incorporation during 16-hour radiolabeling of these same free-swelling disks are shown versus time in culture in Fig. 2. The corresponding rates of sulfate and proline incorporation are also shown (nmol/10^6 cells per day), calculated assuming identical intracellular and extracellular specific activities and normalized to cell number using 7.7 pg DNA/cell (Kim et al., 1988). In general, the sulfate and proline incorporation levels in chondrocyte/agarose culture were initially higher (before day 30) and subsequently similar to that in the intact articular cartilage (Fig. 2). For the 47 day culture where both 2% and 3% agarose specimens were prepared, the incorporation rates in 2% agarose (not shown) were higher (~50%) than those in 3% agarose (Fig. 2) on day 3 but by day 12 the rates were not significantly different in cultures from the same animal. The rate of release of GAG to the medium (not shown) from the large 16 mm disks increased approximately linearly from day 0 to day 10, after which time the rate remained constant at (36.2±4.9) μg/disk per day for 2% agarose and (31.3±4.3) μg/disk/day for 3% agarose (mean ± s.d.; n=12 for each). The reduced rate of release in 3% agarose compared to 2% agarose seemed to compensate for the lower initial synthetic rate, and later in culture (day 20-47) was consistent with a slightly higher GAG concentration in the 3% agarose disks (Fig. 2) by 14±5% compared to 2% agarose specimens (not shown). The disks swelled in the axial direction by 5-10% over 47 days with no apparent difference between 2% and 3% agarose specimens (not shown). This swelling is most likely the result of the deposition of proteoglycan and its associated electrostatic swelling forces (Buschmann and Grodzinsky, 1995).

Morphological development

Chondrocytes fixed within a few hours after seeding (day 0) in agarose were homogeneously dispersed, essentially denuded of matrix, and structurally disorganized (Fig. 3A and B, and Fig. 4A and B). After 1 day of culture, the cells regained a normal morphological appearance, had synthesized a thin halo of pericellular matrix, and had expanded in volume (Fig. 3C and D, and Fig. 4C and D). By 6 days of culture, the cell-associated matrix had grown substantially and continued to expand thereafter (Fig. 5). Even though Toluidine Blue stained light microscopic sections suggested an apparent absence of matrix in the lightly stained interterritorial areas at later times (e.g. Fig. 5), preliminary electron microscopic evaluation (not shown) revealed the presence of matrix granules in this region. In addition the agarose seemed to be excluded from the darkly stained pericellular region and pushed away from the expanding matrix coat into a compact form immediately outside the pericellular coat. The lightly stained matrix outside this region appeared to be integrated with the agarose.

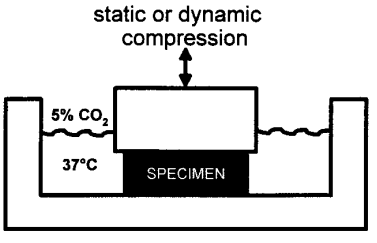

Fig. 1. Schematic of chambers used in the compression-radiolabel experiments.

Physical properties

The confined compression equilibrium modulus, H_A (a measure of the static equilibrium stiffness of the tissue) and the hydraulic permeability k (a measure of the ease of fluid flow through the material) were obtained from measurements of the dynamic stiffness of 13 mm diameter disks in the frequency range 0.01-1.0 Hz, as described by Buschmann et al. (1992). Fig. 6 shows that the modulus increased and the permeability decreased as the culture progressed and matrix was deposited in 3% agarose specimens. The streaming potential is a measure of the intratissue electric field produced by compression-induced fluid flow convecting counterions past fixed charge groups on the proteoglycans. The magnitude of these electric fields increased several fold during the 47 day culture (Fig. 6). The time evolution of these physical properties is qualitatively similar to that seen in (Buschmann et al., 1992) for 2% agarose. By day 28, the modulus and streaming potential were ~1/5 those of the 'parent' cartilage and the permeability was about twice that of the 'parent' tissue. The average modulus of the 3% control gel disks with no cells was 17.7±2.7 kPa (Fig. 6)

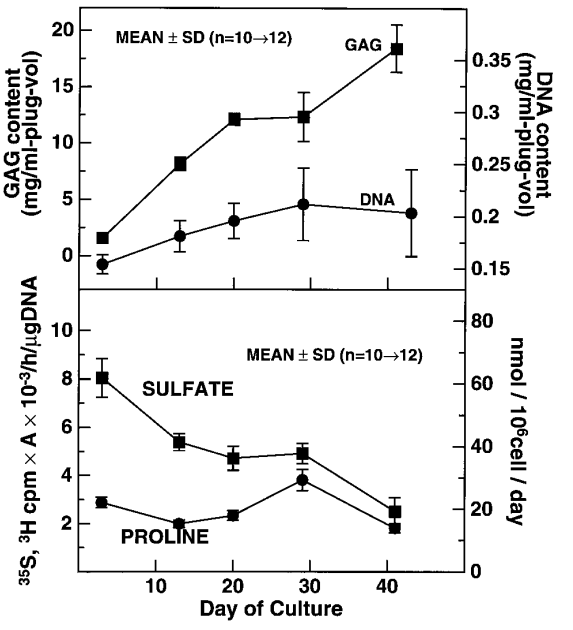

Fig. 2. Top: GAG and DNA concentration in 3 mm diameter chondrocyte/agarose disks versus time during a 47 day culture, in mg per ml of disk volume using 3% agarose. Disk volume increased by 5% to 10% during culture. Bottom: Sulfate and proline incorporation versus time in culture using 3% agarose. A = 1.36±0.04 for sulfate, 0.285± 0.008 for proline.

compared to 13.1±2.8 kPa for the 2% controls shown by Buschmann (1992) (mean ± s.d.; n=4,5). The hydraulic permeability of the 3% agarose control gels was $36.9\pm4.6\times10^{-15}$ $m^4/(N.s)$ compared to $51.4\pm8.5\times10^{-15}$ $m^4/(N.s)$ for 2% agarose control gels.

Biosynthetic response to static and dynamic compression in whole 3 mm diameter disks

The following results pertain to chondrocytes in 3% agarose unless otherwise indicated. At early times in culture (day 2) when little matrix has yet accumulated (Fig. 2), static compression of CA disks from a free swelling thickness of 1.00 mm down to 0.52 mm did not significantly alter the rate of [^{35}S]sulfate incorporation (Fig. 7). After 41 days of culture and the accumulation of ~1/4 the matrix content of 'parent' disks, there was a dose-dependent decrease in [^{35}S]sulfate incorporation rate with increasing compression (Fig. 7). The incorporation rate at 0.52 mm disk thickness was 22±13% lower than disks held at 0.95 mm and significantly lower than that of disks taken from day 2 of culture and compressed to the same 0.52 mm thickness. [^{3}H]proline incorporation followed similar trends. At later times, the accumulation of matrix in CA disks

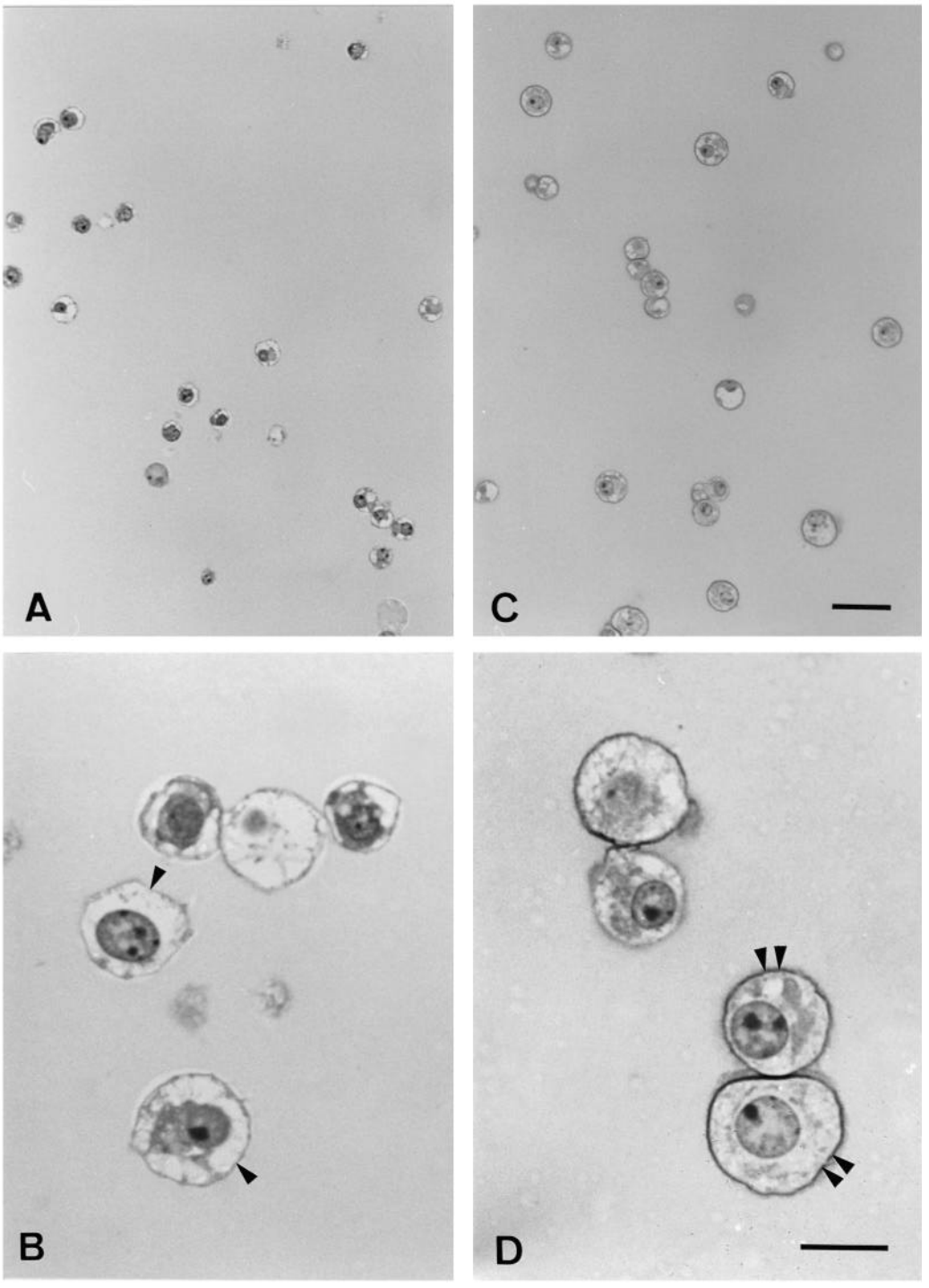

Fig. 3. (A and B) Agarose gels containing freshly seeded chondrocytes were fixed within 4 hours after gels were cast (day 0). (C and D) Agarose gels containing chondrocytes which were fixed on day 1 of culture, approximately 24 hours after seeding. Cells are homogeneously dispersed as individual cells or in groups of 2 or 3 cells after gel casting ($\simeq15\times10^6$ cells/ml). Immediately after casting chondrocytes appear shrunken and contain a disorganized cytoplasm (A and B). After 1 day of culture (C and D) a thin matrix coat has been generated and the cells are expanded with a more structured cytoplasm. Single arrowheads - cell membrane with no identifiable matrix coat. Double arrowheads - cell membrane with associated matrix coat. Thick sections (1 μm) stained with Toluidine Blue-O. (A and C) Same magnification: bar, 25 μm. (B and D) Same magnification: bar, 10 μm.

allowed for greater compression since the presence of matrix helped to retain disk integrity. At early times, compression further than 0.5 mm resulted in cracking of the agarose. Static compression of chondrocytes in 2% agarose showed very similar results (not shown).

PD10 analysis of papain digested 3 mm diameter disks

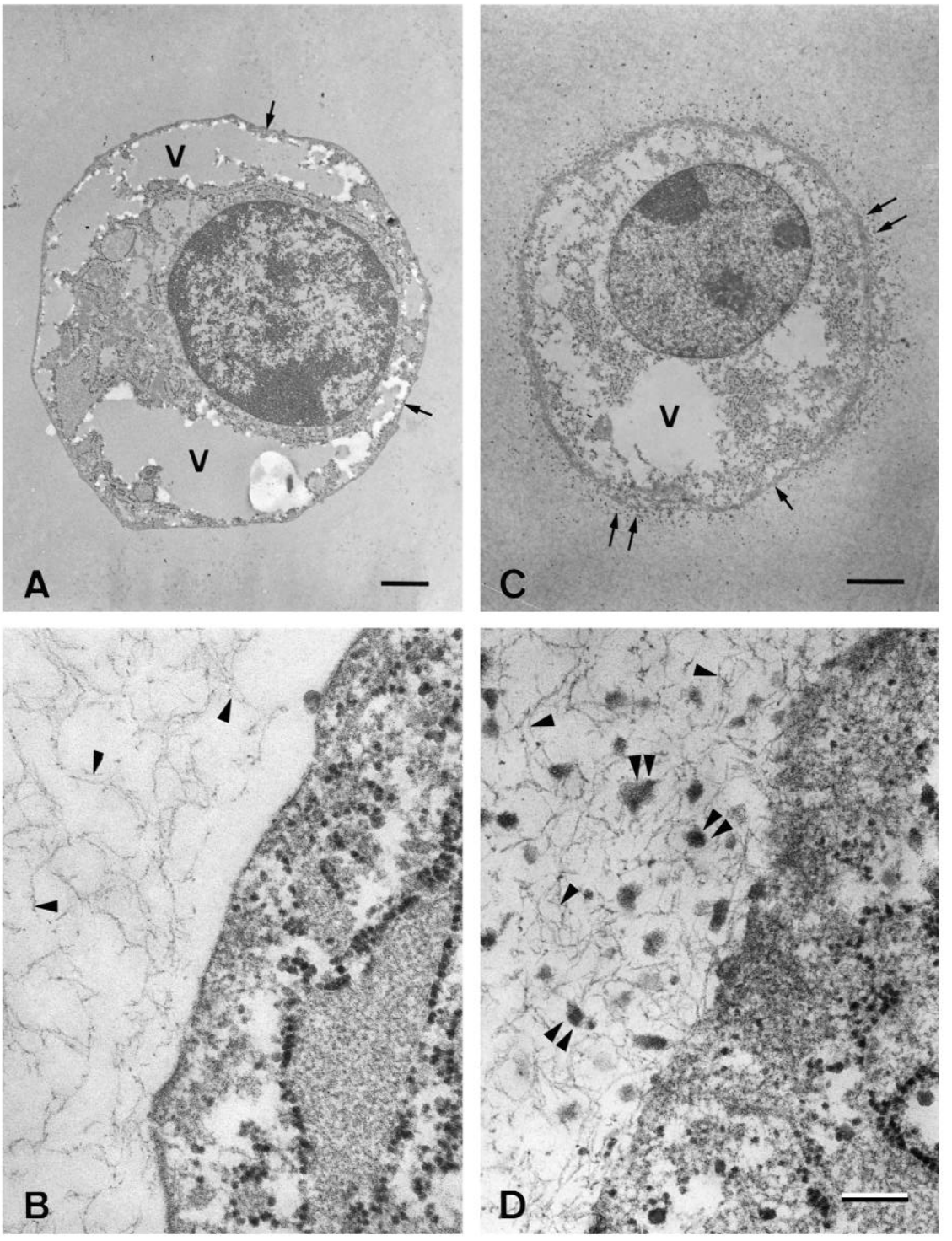

Fig. 4. Electron micrographs of individual cells on day 0 (A) and day 1 (C) of culture and the cell/matrix interface on day 0 (B) and day 1 (D) of culture. The cell fixed immediately after seeding (A) contains more prominent vacuolar space (V) than after 1 day of culture (C). The enzymatic digestion to isolate chondrocytes from cartilage has entirely removed any cell-associated matrix (B). Granules of newly synthesized matrix (paired arrowheads) have been incorporated into the agarose gel (single arrowheads) surrounding the chondrocytes after 1 day of culture (D). Single arrow - plasmalemma. Double arrows - pericellular matrix. Glutaraldehyde fixation in the presence of RHT. Section staining with uranyl acetate and lead citrate. (A) Bar, 1 μm. (C) Bar, 2 μm. (B and D) same magnification: bar, 0.2 μm.

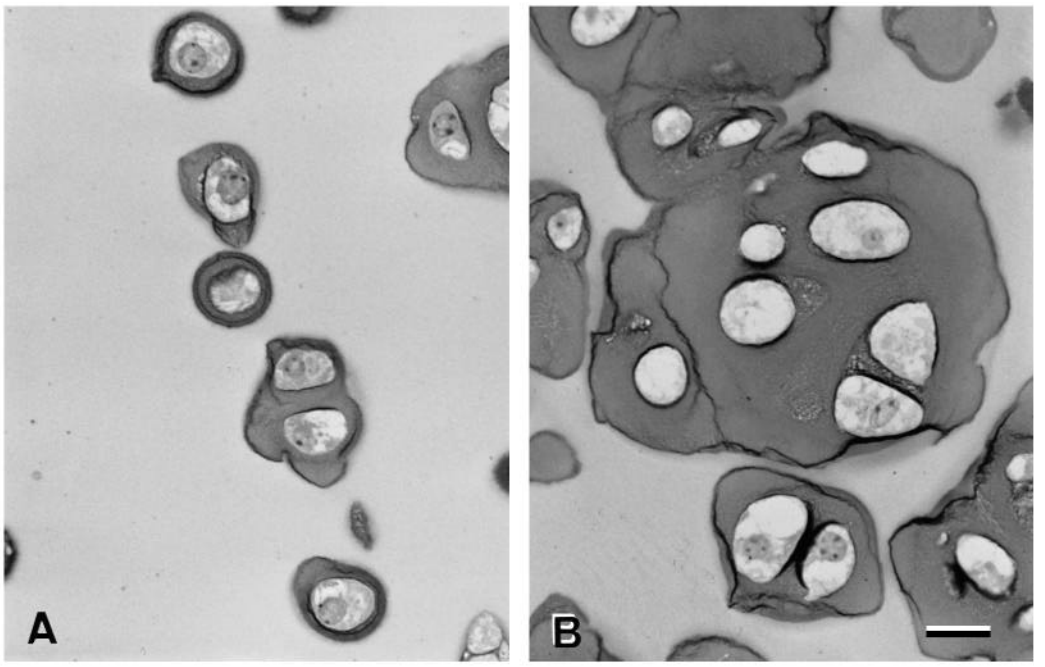

Fig. 5. Chondrocyte/agarose cultures after 6 (A) and 27 (B) days of culture. Representative light micrographs of Toluidine Blue stained 1 μm sections from early and late times in culture when mechanical compression experiments were performed. The more highly developed matrix on day 27 is required for the transduction of mechanical compression of these gels to a cellular biosynthetic response. (A and B) Same magnification: bar, 15 μm.

indicated that 99.2±0.5% of the total [^{35}S]sulfate in the disks was macromolecular (n=4). PD10 analysis of the media from disks that were statically held at 1.00 mm indicated that 1.4±0.6% of the total incorporated macromolecular [^{35}S]sulfate had diffused out into the medium during the 16 hour label.

At early times in culture (days 2-5) there was a small increase (6-13%) in the rate of [^{35}S]sulfate incorporation in the dynamically compressed CA disks when compared to controls held at the same static offset thickness (Fig. 8). At later times the rate of [^{35}S]sulfate incorporation in the dynamically compressed CA disks was 15%-25% greater than that of the static controls. Proline incorporation followed similar trends with levels of stimulation being somewhat higher than sulfate, 10-20% at early times and 10-35% at late times. The apparent increase in stimulation with frequency seen in Fig. 8 could be partially due to the 1.0 Hz experiment being done at 41 days (in the 47 day culture) compared to 23 days for the 0.1 Hz and 0.01 Hz experiments (in the 35 day culture). Only two experiments were performed at 0.001 Hz. A day 13 experiment resulted in a stimulation of sulfate incorporation of 1.128±0.118 (P<0.05) in dynamically compressed specimens relative to static controls, while a day 24 experiment revealed an inhibition of 0.937±0.70 (P>0.05). During the 10 hour compression-labeling period, ~36,000 cycles were completed at 1 Hz, ~3,600 at 0.1 Hz, ~360 at 0.01 Hz, and ~36 at 0.001 Hz. For 3% agarose specimens, the amplitude of the measured sinusoidal load decreased during the 10 hour constant compression-radiolabel experiments due to fatigue of the specimens. At early days of culture the load amplitude decreased by 15-50% during the 10 hours of applied compression, and at later times it decreased by 10-30%. The nonlinearity in the measured load increased from ~5% THD (total harmonic distortion) at the beginning of compression to ~50% THD after 10 hours at early times in culture. At later times in cultures, the final value of the THD was ~30%. The response of chondrocytes in 2% agarose to dynamic compression was variable, perhaps due to its lower stiffness and greater fatigue during repeated compression. Analysis of GAG and DNA

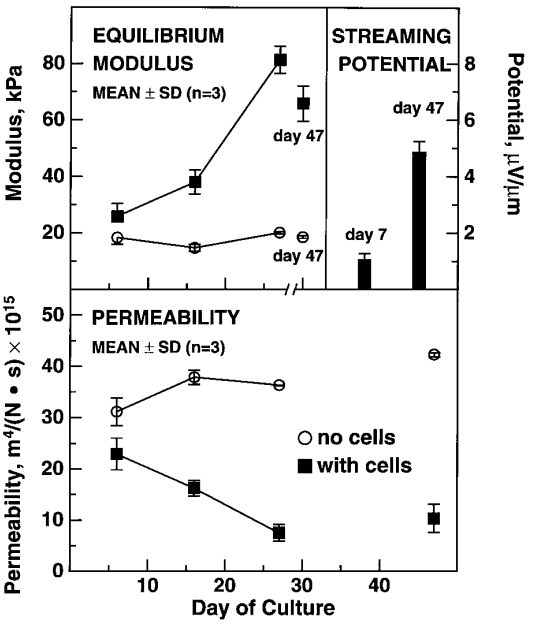

Fig. 6. Equilibrium confined compression modulus, H_A, and hydraulic permeability, k, of chondrocytes in 3% agarose disks versus time in culture, compared to control 3% agarose disks with no cells. The points connected by lines are from a 28 day culture while the day 47 points are from a different 47 day culture. These were calculated from the dynamic stiffness measured in the frequency range (0.01 Hz-1.0 Hz) using a model (Frank and Grodzinsky, 1987) based on the linear KLM biphasic theory (Mow et al., 1980). The streaming potential of chondrocyte/agarose disks at two different days from the 47 day culture are also shown.

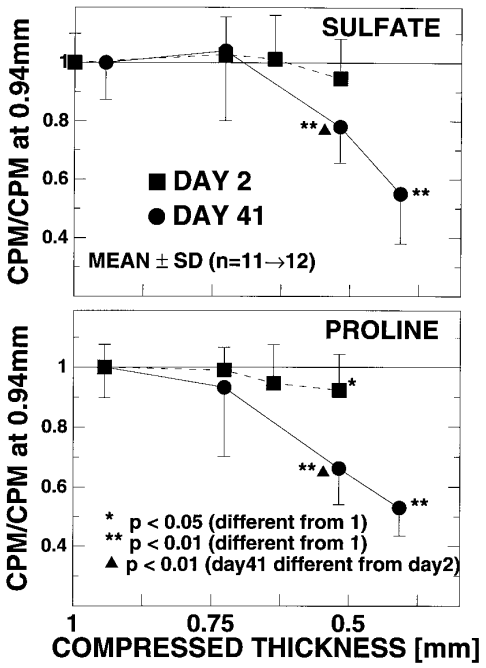

Fig. 7. Rate of incorporation of [^{35}S]sulfate and [^{3}H]proline in 3 mm diameter chondrocyte/3% agarose disks subjected to increasing levels of static compression. Chondrocyte/agarose disks were from day 2, before the accumulation of matrix, and day 41 after the accumulation of matrix (Fig. 2). The compression was applied using the chamber in Fig. 1, in unconfined geometry with impermeable platens.

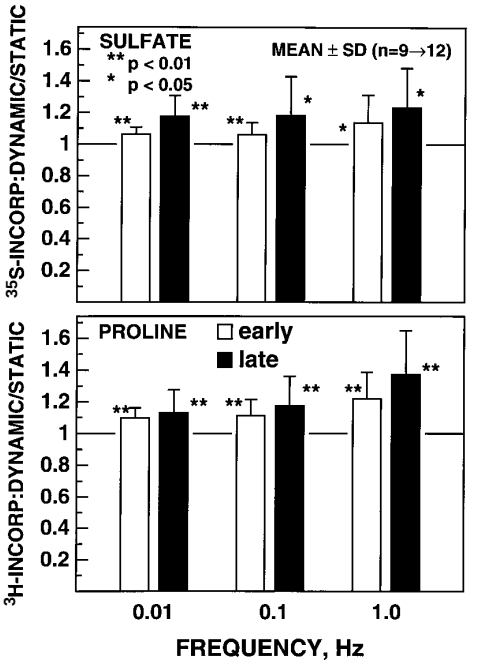

Fig. 8. Rate of [^{35}S]sulfate and [^{3}H]proline incorporation in 3 mm chondrocyte/3% agarose disks subjected to a 30 μm dynamic oscillatory displacement, normalized to disks held statically at the offset thickness of 0.73 mm. Disks were from early time (day 5 for 0.01 Hz and 0.1 Hz, day 2 for 1.0 Hz) in culture - open bars - and late time (day 23 for 0.01 Hz and 0.1 Hz, day 42 for 1.0 Hz) - solid bars. *p* values were computed using the two tailed *t*-test for dynamically compressed specimens compared to controls (static). The stimulation in [^{35}S]sulfate incorporation at later times of culture for dynamically compressed disks at 0.01 Hz is significantly higher than the stimulation at early time (p<0.05).

content showed no significant differences between the dynamically compressed disks and control disks, for all experiments, with 2% and 3% agarose.

Zero-displacement controls using the dynamic chamber were performed with the dynamic chamber in an identical fashion, but with no applied displacement. In two such experiments, the relative rates of [^{35}S]sulfate incorporation in the disks under the dynamic pins and those under the static pins were 1.006±0.112 and 1.048±0.047. For [^{3}H]proline the relative rates were 1.066±0.042 and 1.057±0.069 (mean ± s.d.; n=10-12).

Spatial variation of biosynthetic response to static and dynamic compression: 2 mm center vs 3 mm ring

In order to elucidate possible mechanisms involved in the inhibition of biosynthesis under static compression and the stimulation of biosynthesis with dynamic compression, experiments were done in which the 2 mm center and 3 mm outer ring of each 3 mm diameter disk were analyzed separately for incorporation rates, DNA content and GAG content. Fig. 9 shows that the inhibition in [^{35}S]sulfate incorporation rate produced by static compression was greater in the center of each disk than in the outer ring. The incorporation rates plotted in Fig. 9 are those of the center of each disk relative to the center held at 0.95 mm and those of the rings of each disk relative to the ring held at 0.95 mm. The total incorporation rates for the whole 3 mm disk were calculated from the center and ring of each disk. The inhibition in the center and the difference between the center and the ring increased with time in culture for experiments done on day 3, day 13, and day 24, in a dose dependent manner. At the levels of compression shown in Fig. 9 the incorporation rates in the ring were not affected while on day 24 the center was depressed by 20±18% when compressed to 0.637 mm. Incorporation rates in free-swelling specimens were not different from those held between impermeable platens at 0.95 mm thickness, near the free-swelling thickness of 1.00-1.09 mm. [^{3}H]proline incorporation followed very similar trends (data not shown). 2 mm centers were not cored on day 3 from disks compressed to 0.64 mm since small cracks in the disks prevented accurate coring. At later times disk integrity was maintained due to the accumulation of matrix.

The magnitude of stimulation of synthesis in dynamically compressed specimens was greater at later times than earlier times as seen in Fig. 8 and it was also greater in the outer ring than in the center. The difference between the ring and center increased with culture time and deposition of matrix. In Fig. 10 the rate of incorporation of the ring relative to the center of each disk is plotted for dynamically compressed specimens and static controls on day 4, 14, and 25. At early times (day 4) there is no difference in sulfate incorporation rate between the ring and the center in either the experimental or control disks. On day 25 the incorporation rate is higher in the ring for both the experimentals and controls. The 9% increase in the ring relative to the center on day 25 for the control held at a static

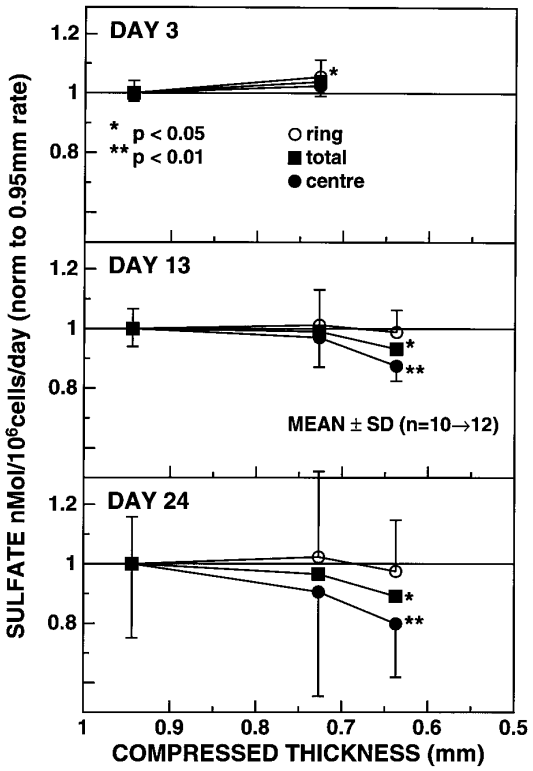

Fig. 9. Rate of incorporation of [^{35}S]sulfate in the 2 mm diameter centers and 3 mm diameter rings of chondrocyte/3%agarose disks subjected to increasing levels of static compression. Chondrocyte/agarose disks were from day 3, day 13, and day 24 of culture. The incorporation rates of the centers on each day are normalized to that of the center from the disk compressed to 0.95 mm on that day. The rings on each day are normalized to the incorporation rate of the ring from the disk compressed to 0.95 mm on that day. Proline incorporation rates showed similar trends.

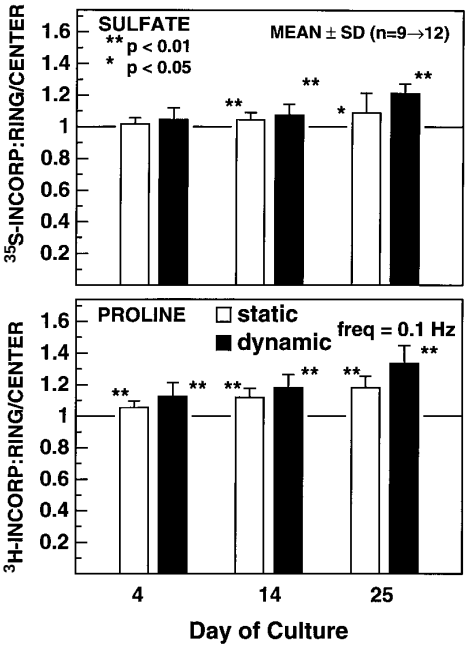

Fig. 10. Rate of incorporation of [^{35}S]sulfate and [^{3}H]proline in the 3 mm-diameter ring normalized to the 2 mm diameter center of chondrocyte/3%agarose disks subjected to a 30 μm dynamic oscillatory displacement at 0.1 Hz and disks held statically at the offset compression of 0.73 mm. Chondrocyte/agarose disks were from day 3, day 13, and day 24 of culture.

offset thickness of 0.73 mm is consistent with static compression experiments shown in Fig. 9. For the experimental disks the incorporation rate in the ring was 21% higher than the center, a difference which was only partially (~1/2) accounted for by the greater inhibition in the center with a static compression of 0.73 mm. The rings were then preferentially stimulated by dynamic compression compared to centers.

DISCUSSION

Articular chondrocytes grown in three-dimensional agarose gel cultures exhibit a biosynthetic response to deformation of the gel-cell-matrix system. The intensity of the response increases with increasing time of culture and therefore with the degree of matrix development, suggesting a fundamental role of the ECM in signal transduction. Static (equilibrium) compression results in a depression in synthesis of proteoglycans and proteins while small amplitude oscillatory dynamic compression stimulates synthetic rates. Intact cartilage explants respond to these loading regimes in a qualitatively similar manner (Sah et al., 1989). Chondrocytes cultured in agarose gel therefore not only express molecular and morphological characteristics of the chondrocyte phenotype but also respond to mechanical loading in a way similar to that of chondrocytes in organ culture and in vivo. In view of the fact that matrix development tends to be localized to within a few cell diameters around cells or cell-groups in agarose, it is suggested that the minimal system exhibiting typical responses to mechanical loading for chondrocytes includes the cell and its immediate pericellular matrix. The flexibility of the agarose system is particularly suited to the localization of transduction events within this tissue region.

Biosynthetic response to static compression

The inhibition of sulfate and proline incorporation rates caused by static compression was dependent on the day of culture and, therefore, the presence of matrix in chondrocyte/agarose disks. Without matrix, at early times in culture, little or no change in response to compression was observed (Figs 7 and 9). As matrix developed, the inhibition in biosynthesis was enhanced (Figs 7 and 9). Comparison with a previous study (Sah et al., 1989) using the calf 'parent' cartilage explants shows that by 41 days in culture when the GAG concentration in chondrocyte/agarose disks was ~1/4 that of the 'parent' cartilage disks, the relative depression in biosynthesis with compression was also ~1/4 of that seen in the calf cartilage disks. However, the slopes of the two curves of synthetic rate vs compressed thickness are similar in the regions where inhibition of synthesis is occurring. The chondrocyte/agarose system may require some initial compression to attain a 'physiological static offset' after which the response is more quantitatively similar to intact cartilage. The spatial variation in biosynthetic

response also exhibited a dependence on the level of matrix present in the disks. The 2 mm diameter central region of 3 mm disks was more strongly affected than the 3 mm diameter ring, and this difference became accentuated as the culture progressed and more matrix was deposited (Fig. 9). This spatial variation has also been observed with cartilage (Kim et al., 1994b).

Possible mechanisms involved in the transduction of static compression to a signal sensed by chondrocytes may be categorized as: (1) cell deformation, (2) transport-related, (3) physicochemical, and (4) cell-matrix interactions. Previous studies (Freeman et al., 1994; Lee and Bader, 1994) and ongoing work by us indicates that deformation of the agarose gel does result in cell-deformation. However, changes in cell shape and volume tend to be larger when less matrix is present (i.e. at early times in culture), opposite to the change in magnitude of the biosynthetic response with time in culture observed here. Numerous previous studies have also examined the possible role of transport limitations resulting from a reduction in the average pore size of the matrix under compression (Kim et al., 1994; Schneiderman et al., 1986; Tomlinson and Maroudas, 1980). The majority of these results to date have suggested that limitations in the availability of nutrients (e.g. oxygen, sulfate, growth factors etc.) is not solely responsible for the observed inhibition of biosynthesis in compressed cartilage. Transport related mechanisms may also appear less likely in light of Fig. 9. There were no center/ring differences in disks held at 0.95 mm thickness at any time in culture, early or late. Furthermore, there was no difference in biosynthetic rates between free-swelling disks and those with the top and bottom surfaces covered at 0.95 mm thickness (data not shown). If transport limitations were significant, some center/ring differences could be expected to appear in the specimens held at 0.95 mm as the culture progressed. Physicochemical mechanisms are related to the decrease in hydration with compression and the resulting increase in fixed charge density (FCD) associated with the presence of ionized charge groups on the proteoglycans. This increase in negative FCD increases intra-disk concentrations of cations such as Na^+ and H^+, thereby increasing the osmotic pressure and reducing the pH. Reduced extracellular pH has been seen to reduce cellular biosynthetic rates (Gray et al., 1988); however, longer term effects of pH changes and tissue compression were seen to be different (Boustany et al., 1994). The importance of physicochemical events in the chondrocyte/agarose system cannot be definitively ascertained on the basis of these studies and our present work.

Fig. 4 shows the beginnings of a substantial development in pericellular matrix. The granules in these electron micrographs are most likely condensed proteoglycans precipitated by the actions of the cationic dye ruthenium hexaammine trichloride and the chemical fixative. Proof of a collagen fibrillar network is difficult to find at the EM level in these micrographs due to the disruptive effects of the fixative. However, previous biochemical characterization of similar cultures has indicated that collagen, predominantly type II, is synthesized and deposited in the gel (Buschmann et al., unpublished results). We also have preliminary evidence using high-pressure freezing fixation that a fine fibrillar network is present. It is possible that specific connections between the chondrocyte and this pericellular matrix are required for the cellular response to mechanical stimuli. The increased responsiveness of the chondrocyte/agarose cultures with increasing time in culture could be partially a result of the development of the necessary transduction machinery at the cell-matrix interface, and potentially throughout the matrix (Fig. 5). It may be reasonable to assume that the chondrocyte itself is in a state of mechanical 'readiness' after day 1 given its apparently normal morphology after this time (Fig. 3). Although the molecular constituents of the chondrocyte-matrix interface are only beginning to be characterized, some components have been identified: β_1 integrins including α_2, α_3, α_5, α_6 and α_v (Durr et al., 1993; Enomoto et al., 1993) and the hyaluronan receptors, CD44, RHAMM, and TSG-6 (Flannery and Sandy, 1994; Hardingham and Fosang, 1992; Knudson and Knudson, 1993). Cell-matrix interactions could have important roles in the transduction of mechanical signals, analogous to those seen in development and differentiation (Adams and Watt, 1993). The role of integrins in mechanotransduction of other cell types is well-established (Ingber, 1991). It remains to be elucidated which if any of these receptors or intracellular structural alterations are important in the mechanical feedback system operant in cartilage.

Biosynthetic response to dynamic compression

A small stimulation in biosynthesis (6-13%) was observed in CA disks at early times in culture. At later times in culture, the level of stimulation in sulfate incorporation increased to 15-25%. The level of stimulation seen in calf cartilage disks under similar conditions was 20-40% (Sah et al., 1989). Therefore the stimulation in biosynthesis under dynamic compression appears to be enhanced when more matrix is present. The data of Fig. 10 also show an increase in stimulation in the outer ring compared to the center when more matrix is present.

Some possible mechanisms involved in the transduction of small amplitude dynamic compression to a cellular response are: (1) altered fluid pressure; (2) enhanced fluid flow (transport); (3) induced streaming potentials; (4) cell-matrix interactions; and (5) growth factor release from matrix binding sites or by cells. Physicochemical changes are probably not significant, since the disk volume is nearly constant with compression amplitudes of ~3%. Oscillatory compression will generally increase fluid pressure more in the center than in the ring while fluid velocities will be higher in the ring than in the center due to the unconfined geometry (Kim et al., 1994, 1995). The outer ring showed greater stimulation in radiolabel incorporation than the center. Thus, it is unlikely that altered pressure is responsible for the stimulation of biosynthesis in dynamically compressed CA disks, whereas mechanisms related to fluid flow are consistent with a greater stimulation in the ring. The maximum fluid pressures induced in dynamic compression are approximately equal to the measured stress (Kim et al., 1995). For CA disks at late times, the measured stress was $\simeq$30 kPa for a 30 μm displacement imposed on a 1 mm thick specimen. This is relatively small compared to the levels of pressure which have been observed to affect chondrocyte synthetic rates (>1 MPa) (Hall et al., 1991) and is approximately 10-20% that in calf cartilage disks. The level of stimulation of biosynthesis seen here is of the same order as that seen in cartilage disks, but with compression-induced fluid pressures about an order of magnitude lower.

The magnitude of the relative fluid velocity corresponding to the known values of frequency, displacement, and disk

thickness is proportional to the product of the modulus and the hydraulic permeability, $H_A \times k$ (Kim et al., 1995). From the data of Fig. 7, this product was calculated to be relatively constant at 0.5×10^{-9} m^2/s during the entire culture (up to 47 days). The product $H_A \times k$ for parent cartilage explants is also of the same magnitude. Therefore fluid velocities within explant and CA disks are of the same order of magnitude. Since the stimulation of synthesis by dynamic compression for both CA and explant disks is in the 10-40% range, mechanisms dependent on bulk fluid flow within the disk could play a role. For example, convective transport of mobile solutes in the extracellular fluid could be enhanced by dynamic compression. Given fluid velocities of ~1 μm/s over dimensions of ~30 μm, the Peclet number (i.e. the ratio of convective-transport/diffusive-transport for a given solute) is 3×10^{-7} cm^2 s^{-1}/D, where D is the solute diffusion coefficient. Therefore, transport of small molecules (M_r<500) with D $\geq10^{-7}$ cm^2/s will not be significantly enhanced by compression induced convection. However, transport of larger molecules may be affected. In addition to modulating transport, fluid flow can also generate shear stress at the cell surface, an important factor in the response of endothelial cells to luminal flow (Dull and Davies, 1991). Fluid flow can also redistribute and deform ECM components through viscous forces. Hence the discussion of cell-matrix interactions for static compression applies here as well. Electric fields (streaming potentials) are generated by fluid flow convecting mobile counterions past ionized charge groups on immobilized macromolecules (Grodzinsky, 1983). Streaming potential fields are proportional to both fluid velocity and matrix fixed charge density. In this context, an increase in stimulated biosynthetic response at later times in culture would be consistent with a greater fixed charge density even though bulk fluid velocities may not increase significantly with time in culture (at constant applied dynamic compression amplitude).

In summary, chondrocytes cultured in agarose synthesize, assemble and maintain an ECM containing macromolecules characteristic of the chondrocyte phenotype. The mechanical properties of these CA disks develop during culture to approach those of intact articular cartilage. We have further shown here that chondrocytes in agarose which have synthesized a cell-associated matrix exhibit a biosynthetic response to compression which is similar to that of explanted cartilage. Current evidence points towards the predominance of cell-matrix interactions, and fluid flow with its associated electric fields as important players informing the chondrocyte of its mechanical environment. Based on these findings, we believe that the flexibility of the agarose system should enable more detailed characterization of transduction pathways in the cellular response to mechanical signals. Such information will be of use in understanding normal physiology, pathologies (i.e. osteoarthritis), and development of cartilage.

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