Osteoarthritis and Cartilage (2002) **10,** 297–307
© 2002 OsteoArthritis Research Society International
doi:10.1053/joca.2002.0517, available online at http://www.idealibrary.com on IDEAL®
1063–4584/02/040297+11 $22.00/0

Osteoarthritis and Cartilage
Journal of the OsteoArthritis Research Society International

The influence of the pericellular microenvironment on the chondrocyte response to osmotic challenge

W. A. Hing, A. F. Sherwin, J. M. Ross and C. A. Poole
Division of Anatomy with Radiology, Faculty of Medical and Health Sciences, The University of Auckland, Auckland, New Zealand

Summary

Objective: To examine whether differences in the pericellular microenvironment of different chondron preparations influence the chondrocyte volume regulatory response to experimental osmotic challenge.

Design: Mechanically extracted chondrons (MC), enzymatically extracted chondrons (EC) and isolated chondrocytes (IC) were seeded into agarose and sampled at 1, 3 and 7 days. Samples mounted in a perfusion chamber were subjected to osmotic challenge. The cross-sectional areas of the chondrocyte and pericellular microenvironment were measured under isotonic, hypertonic and hypotonic conditions, and percentage change calculated. Separate samples were immunolabeled for type VI collagen and keratan sulfate.

Results: Initially, the microenvironment of MC represented 60% of the chondron area and was occupied by type VI collagen and keratan sulfate. In EC, the microenvironment comprised 18% of the chondron area with narrow bands of type VI collagen and keratan sulfate. IC had no visible microenvironment, with small amounts of type VI collagen and keratan sulfate present. All preparations sequestered additional pericellular macromolecules during culture. Under isotonic conditions, the EC and IC chondrocytes were larger than those of MC. All chondrocytes shrank under hypertonic conditions and swelled under hypotonic conditions. MC were the least responsive, displaying the most efficient volume regulation. IC showed the largest response initially but this decreased with time. EC exhibited intermediate responses that decreased as the microenvironment increased in size.

Conclusions: The composition and structural integrity of the pericellular microenvironment do influence the cellular response to experimental osmotic challenge. This suggests that the microenvironment functions *in situ* to mediate the chondrocyte response to physicochemical changes associated with joint loading. © 2002 OsteoArthritis Research Society International

Key words: Chondron, Chondrocyte, Pericellular microenvironment, Volume regulation.

Introduction

The chondrocyte and its pericellular microenvironment form the chondron[1], the primary structural, functional and metabolic unit of articular cartilage. The chondron is thought to be osmotically robust with the pericellular microenvironment acting to protect the chondrocyte from deformation under load[2]. However, the role of the pericellular microenvironment in protecting the cell and regulating the chondrocyte response to osmotic challenge has not been extensively investigated.

Articular cartilage is uniquely structured to resist a range of non-uniform compressive, tensile and shear forces by redistributing fluid, regulating volume and minimizing osmotic fluctuations[3]. The interaction between water and ions in the matrix influences the load bearing performance of cartilage. Mechanical loading results in changes in the ionic and osmotic environment of chondrocytes, since it causes the expression of interstitial fluid from the matrix thus increasing the local proteoglycan concentration and the local osmotic pressure[4,5]. The properties of the tissue are largely dependent on the composition and organization of the extracellular matrix and tissue fluid that form 90–95% of the tissue volume. The remainder comprises the chondrocytes, the cells responsible for the synthesis, assembly, maintenance and repair of the extracellular matrix[6].

The ability of articular cartilage to resist load is not only dependent on the composition and organization of the extracellular matrix[7] but also on the relationship between the chondrocyte and its pericellular microenvironment[2]. The microenvironment of the chondron comprises two integrated parts: the pericellular glycocalyx, which is rich in hyaluronan[8,9], aggrecan[10] and fibronectin,[11] and the pericellular capsule, which is composed of fibrillar collagen types II, IX and XI[12,13], microfibrillar collagen type VI[14] and laminin[15]. The chondrocyte is intimately linked to the pericellular microenvironment via a range of cell-specific receptors that include several integrins and CD44[16]. These receptors are closely linked to the chondrocyte cytoskeleton and play a major role in signaling matrix changes to the cell[17–19]. It has been argued that the high concentration of aggrecan in the pericellular microenvironment increases the fixed charge density close to the cell, creating a large osmotic potential and swelling tendency[20–22] within the chondron. This is counteracted by the tightly woven

Received 29 March 2001; revision requested 31 July 2001; revision received 29 October 2001; accepted 2 January 2002.
Source of support: Arthritis Foundation of New Zealand, the Health Research Council of New Zealand, and the Auckland University of Technology.
Address for correspondence: W. A. Hing, Division of Anatomy with Radiology, Faculty of Medical and Health Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand. Tel: (09) 373 7599 x6066; Fax: (09) 373 7484; E-mail: w.hing@auckland.ac.nz

network of pericellular collagens which forms an isotropic capsule around the chondrocyte, entrapping proteoglycans within the microenvironment and limiting their expansion to create an osmotically robust chondron[10,23]. The aim of this study was to establish whether the pericellular microenvironment regulates the cellular response to the biomechanical and physicochemical matrix changes which occur under load[24,25].

The mechanisms by which chondrocytes respond to changes in their environment have been investigated using isolated chondrocytes subjected to a range of biomechanical or physicochemical forces *in vitro*. For example, mechanical loading[26–30], changes in extra- and intra-cellular pH[31,32] and hydrostatic pressure[33–35] have been shown to promote changes in matrix synthesis. Moreover, alterations to the osmotic and ionic properties of the culture media not only influence matrix synthesis[32,36–40] but also the chondrocyte volume regulatory response[19,41–44]. The volume regulatory mechanisms of the chondrocyte are mediated through a number of transmembrane fluxes that include voltage-activated ion channels, passive exchangers, and ATP-dependent ion pumps[45–49]. It is therefore possible to study the physiological response of cultured chondrocytes to osmotic challenge by manipulating the properties of the nutrient medium.

This study utilized three chondrocyte preparations that differed markedly in the integrity and composition of their pericellular microenvironments: mechanically isolated chondrons (MC)[50], enzymatically isolated chondrons (EC)[51] and enzymatically isolated chondrocytes (IC)[52]. All three preparations were subjected to osmotic challenges to determine whether differences in the integrity of the pericellular microenvironment mediate the chondrocyte response to physicochemical changes designed to mimic joint loading.

Materials and methods

MATERIALS

Dulbecco's phosphate-buffered saline (DPBS), Dulbecco's Modified Eagle's Medium (DMEM), foetal calf serum (FCS), Earle's Balanced Salt Solution, ascorbic acid and dispase were obtained from Life Technologies Inc., New Zealand. Agarose (Type IX-A Ultra-low gelling temperature), penicillin and streptomycin sulfate were obtained from Sigma Chemical Co., Australia. Collagenase CLS-2 was obtained from Worthington Biochemical Corp., U.S.A. Pronase was obtained from Boehringer Mannheim NZ Limited. 5-chloromethylfluorescein diacetate (CMFDA) and ethidium homodimer-1 were obtained from Molecular Probes Inc., U.S.A.

ISOLATION AND PREPARATION OF CHONDRONS AND CHONDROCYTES

Tissue preparation

Articular cartilage was resected from the medial and lateral tibial plateaux of four mature, healthy, cross-bred dogs (2–5 years) euthanased by the Auckland City Pound under veterinary supervision. Canine tibial cartilage was used in this study since more than 95% of the chondrons and chondrocytes originate from the middle and deep layers[53], where the chondron microenvironment is most highly differentiated[22,54]. The cartilage was diced, washed and stored in DMEM plus 20% FCS at 37°C until processing.

Mechanically isolated chondrons (MC)

Chondrons were extracted by low speed serial homogenization, as previously described[2,55]. Diced cartilage was homogenized at 4000 rpm for 1 min and large chips collected by gravity sedimentation. The supernatant was decanted and the remaining cartilage chips re-homogenized. The process was repeated until the cartilage sample was completely disrupted. Supernatants were pooled and filtered to remove fragments >500 μm^2, producing a heterogeneous mixture of single chondrons, chondron columns and cartilage microchips which was resuspended in DMEM plus 20% FCS in preparation for agarose plating.

Enzymatically isolated chondrons (EC)

Chondrons were enzymatically isolated using a mixture of dispase and collagenase[51]. EC were collected and washed by centrifugation, and resuspended in DMEM plus 20% FCS in preparation for agarose plating.

Isolated chondrocytes (IC)

Chondrocytes were isolated using sequential digestion with pronase and collagenase[52]. Cells were collected and washed by centrifugation, and resuspended in DMEM plus 20% FCS in preparation for agarose plating.

Preparation of agarose gel constructs

MC, EC and IC preparations were mixed 1:1 with an 8% solution of low melting point agarose prepared in Earle's Balanced Salt Solution[52]. These solutions were cast to a depth of 5 mm and gelled at 4°C for 20 min. Plugs, 5 mm diameter, were cored from the agarose constructs and cultured for up to 7 days in DMEM supplemented with FCS (20%), penicillin (100 U/ml), streptomycin sulfate (100 μg/ml) and ascorbate (50 μg/ml). Plugs were monitored daily and media replaced on alternate days.

Immunohistochemistry

Samples were collected for immunohistochemistry on days 1 and 7 and labeled with CMFDA to identify viable cells[56] prior to fixation in 4% paraformaldehyde plus 2% sucrose in DPBS (15 min, 37°C). Immunolabeling was performed using 5D4, a monoclonal antibody raised against keratan sulfate[9] and a polyclonal antibody raised against type VI collagen[55]. Biotinylated secondary antibodies and streptavidin-linked Texas Red were used for detection. Slides were examined on a Leica TCS 4D confocal microscope (Leica, Germany) and a series of optical sections collected through the entire depth of the selected chondrons and chondrocytes. Images were processed using Adobe Photoshop 4.0 and assembled into plates using Adobe Pagemaker 6.5.

ENVIRONMENTAL PERFUSION CHAMBER AND IMAGING SYSTEM

Functional studies were performed using a new environmental perfusion chamber which provides a narrow viewing

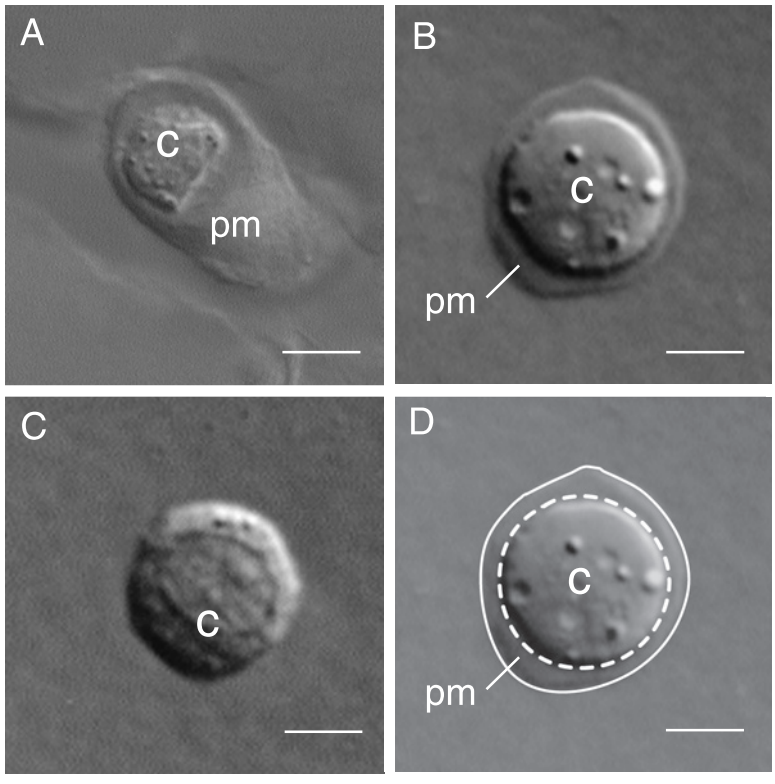

Fig. 1. Digital DIC micrographs of a mechanical chondron (A), enzymatic chondron (B) and isolated chondrocyte (C) at day 1 in agarose culture. The chondrocyte (D) and pericellular microenvironment (pm) regions are indicated. Panel D shows an example of how MetaMorph software was used to define the chondrocyte and pericellular microenvironment of an EC. Scale bars=5 μm.

cavity for imaging dynamic cellular behavior[57]. Agarose plugs containing MC, EC or IC sampled at 1, 3 and 7 days were placed in the perfusion chamber and this was mounted on the stage of a Zeiss Axioplan-2 microscope (Zeiss, Germany) equipped with Differential Interference Contrast (DIC) objectives and a Newvicom VE1000 video camera (Dage-MTI Inc., U.S.A.). Media perfusion through the viewing cavity was maintained at 5000 μl/h using Melsungen AG syringe pumps (B. Braun, Germany) and temperature maintained at 37°C[57]. All preparations were examined at high magnification (100×/1.30NA) using time-lapse, computer-enhanced video microscopy (CEVM). Images were captured, processed and analysed using MetaMorph software (Universal Imaging Corp., U.S.A.).

OSMOTIC PERFUSION PROTOCOLS

Preparation of media

Supplemented DMEM (316.2±14.9 mOsm) was used as the isotonic medium for all experiments. This was rendered hypertonic by the addition of NaCl and hypotonic by dilution with distilled water[37]. The osmolality of each solution was measured using a Wescor 550 Vapour Pressure Osmometer (Wescor Inc., U.S.A.) and averaged 170.8±17.4 mOsm for hypotonic media and 633.2±55.4 mOsm for hypertonic media.

Perfusion protocol

Initially, samples of each preparation were equilibrated in isotonic medium for 30 min. Single chondrons or chondrocytes were selected, mapped relative to one another and the first DIC images captured at their largest cross-sectional profile. One selected cell was monitored throughout the procedure using time-lapse CEVM. Hypertonic perfusion was performed for 45 min and then all mapped cells were imaged. The selected cell was monitored again as the sample was perfused with isotonic medium for 30 min until equilibrium and the mapped cells imaged for the third time. Hypotonic medium was then perfused through the chamber for 45 min and the mapped cells imaged. The selected cell was monitored for a further 30 min while isotonic conditions were restored to equilibrium before the final images were recorded for all mapped cells. Multiple plugs were analysed at each time point.

ANALYSES

Cell viability

To ensure that imaged chondrocytes remained viable throughout the perfusion protocol, ethidium homodimer-1 was added to the final isotonic perfusate. Viable cells exclude this dye, and data from cells showing positive nuclear fluorescence were not included in the analyses.

Area analysis

Perimeter lines were drawn around both the chondrocyte and the chondron at their largest cross-sectional profile using Metamorph [Fig. 1(D)]. The pixel area was calibrated using precision graticules, and the cross-sectional areas

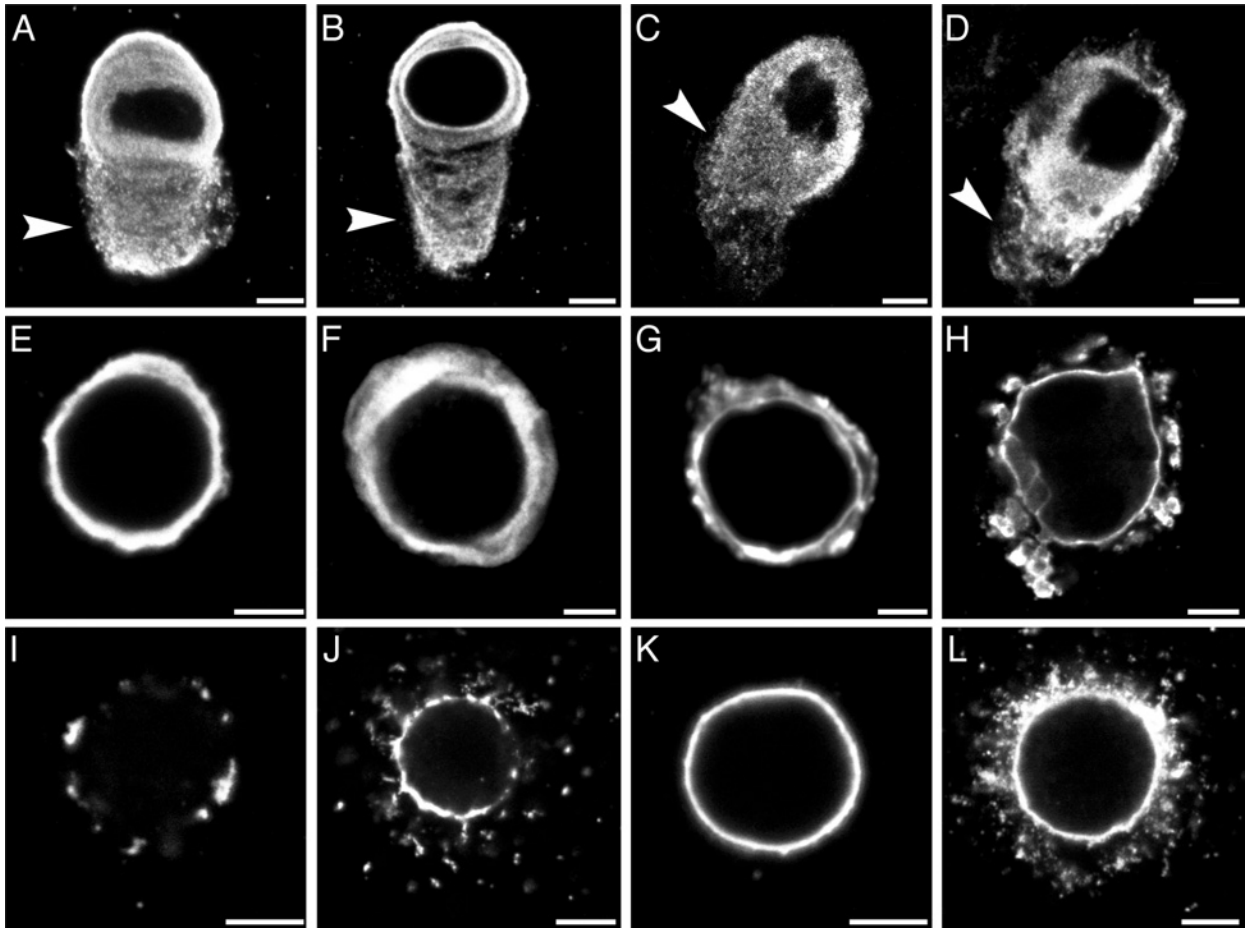

Fig. 2. Single optical sections showing the distribution of type VI collagen (A), (B), (E), (F), (I), (J) and keratan sulfate (C), (D), (G), (H), (K), (L) in MC (A–D), EC (E–H) and IC (I–L) at day 1 (A), (C), (E), (G), (I), (K) and day 7 in agarose culture (B), (D), (F), (H), (J), (L). Arrowheads indicate chondron tail regions. Scale bars=5 μm.

calculated for the chondron and the chondrocyte, and by subtraction, the pericellular microenvironment. The cross-sectional areas of MC, EC and IC were measured under isotonic, hypertonic and hypotonic conditions after 1, 3 and 7 days of culture and the percentage change in cross-sectional area calculated.

Statistical analysis

The data were subjected to three-way analyses of variance (ANOVA) using the general linear model procedure within the SAS software package (SAS Institute, Cary, NC, U.S.A.). Type I error rate was set at α=0.05. The significance of differences among means were examined post-hoc, at the 95% Confidence Level (P<0.05), using an appropriate set of contrast coefficients.

Results

STRUCTURE AND COMPOSITION

DIC images from MC, EC and IC preparations under isotonic conditions are shown in Fig. 1. At day 1, MC showed chondrocytes surrounded by an extensive and clearly defined pericellular microenvironment [Fig. 1(A)], while in EC, the chondrocytes appeared larger with a narrower microenvironment [Fig. 1(B)]. In IC, no pericellular microenvironment was observed around the chondrocytes [Fig. 1(C)]. Cell viability was evident by the intracellular movement of cytoplasmic organelles throughout the experiment, and the majority of cells were viable at the end of the perfusion protocol.

The distribution of type VI collagen and keratan sulfate in the pericellular microenvironment of each preparation is shown in Fig. 2. In MC at day 1, both type VI collagen [Fig. 2(A)] and keratan sulfate [Fig. 2(C)] were circumferentially organized around the chondrocyte with staining also evident in the tail region of the microenvironment. By day 7, staining for type VI collagen had increased at the cell surface [Fig. 2(B)], while the intensity of keratan sulfate increased throughout the microenvironment [Fig. 2(D)].

In EC at day 1, both type VI collagen [Fig. 2(E)] and keratan sulfate [Fig. 2(G)] formed a narrow band around the chondrocyte but few tails were evident. Increased pericellular sequestration of both molecules had occurred by day 7 [Fig. 2(F),(H)], with filaments of keratan sulfate extending outwards from the pericellular region [Fig. 2(H)].

In IC preparations at day 1, small punctate deposits of type VI collagen [Fig. 2(I)] were evident over the cell surface while keratan sulfate [Fig. 2(K)] formed a thin calyx around the chondrocyte. By day 7, pericellular staining for both molecules had increased, with some granular

Table I
The mean values for the cross-sectional area of the chondrocyte, the pericellular microenvironment and chondron for all three preparations over the 7 day culture period

		Cross-sectional area under isotonic conditions (μm^2)					
	Preparation	Time in Culture					
		Day 1		Day 3		Day 7	
MC	Chondrocyte	95.4±8.3	(17)	62.3±3.8	(17)	51.6±3.6	(18)
	Pericellular microenvironment	142.9±12.6	(17)	140.9±9.0	(17)	167.2±8.4	(18)
	Chondron	250.3±12.3	(17)	204.0±9.6	(17)	220.1±10.0	(18)
EC	Chondrocyte	141.8±4.9	(33)	145.8±7.8	(14)	206.4±11.1	(24)
	Pericellular microenvironment	31.5±3.6	(33)	34.5±8.2	(14)	68.5±8.7	(24)
	Chondron	171.7±4.9	(33)	181.4±11.0	(14)	270.0±16.3	(24)
IC	Chondrocyte	141.5±7.1	(22)	139.1±6.6	(17)	207.2.±13.8	(33)
	Pericellular microenvironment	—		—		73.9±14.2	(33)
	Chondron	—		—		291.7±29.0	(33)

The values are expressed as mean±S.E.M. Sample number in brackets.
The following comparisons were statistically significant ($P<0.05$). Non-significant comparisons are omitted.

Chondrocyte
Day:day
MC: 1 vs 3; 1 vs 7
EC: 1 vs 7; 3 vs 7
IC: 1 vs 7; 3 vs 7

Preparation:preparation
Day 1: MC vs EC; MC vs IC
Day 3: MC vs EC; MC vs IC
Day 7: MC vs EC; MC vs IC

Pericellular Microenvironment
Day:day
MC: 1 vs 7; 3 vs 7
EC: 1 vs 7; 3 vs 7

Chondron
Day:day
MC: 1 vs 3
EC: 1 vs 7; 3 vs 7

Preparation:preparation
Day 1: MC vs EC
Day 7: MC vs EC; MC vs IC

extensions evident from the pericellular region [Fig. 2(J),(L)].

CROSS-SECTIONAL AREA ANALYSIS UNDER ISOTONIC CONDITIONS

The mean values for the cross-sectional area of the chondrocyte, the pericellular microenvironment and the chondron for all three preparations are summarized in Table I. The area of chondrocytes in MC decreased from days 1 to 3 with no further change by day 7. At all three time points, the chondrocytes of MC were significantly smaller than the chondrocytes of EC and IC. No significant differences were found between the size of EC and IC chondrocytes at any time point, despite these cells increasing in area by 41% and 49% respectively, between days 3 and 7.

At day 1, the pericellular microenvironment in MC was significantly larger than EC or IC, and represented 60.5± 2.9% of the chondron cross-sectional area. In contrast, the microenvironment in EC represented only 17.8±1.9% of the chondron area. During the first 3 days in culture, the microenvironment of MC and EC showed no change. However, between day 3 and 7 the microenvironment of MC increased by 19% while that of EC increased by 98%. In IC, no microenvironment could be detected by DIC microscopy at days 1 and 3 of culture, but by day 7, the chondrocyte had elaborated a new microenvironment that was similar in area to that of EC.

The chondron area of MC decreased slightly from days 1 to 3, but showed no statistical difference when analysed over the whole culture period. EC showed no difference in chondron cross-sectional area between days 1 and 3, but had increased by 49% at day 7. The cross-sectional area of IC at day 7 was similar to that of EC. Comparisons of chondron area showed that MC were significantly larger than EC at day 1, but not statistically different at day 3. By day 7, increases in both the microenvironment and the cell size in EC and IC resulted in a significantly larger chondron cross-sectional area compared to MC.

CHONDROCYTE RESPONSE TO OSMOTIC CHALLENGE

The shrinking and swelling responses of the chondrocytes of MC, EC and IC under isotonic, hypertonic and hypotonic conditions are presented in Fig. 3, where direct comparisons of the different preparations and their responses to osmotic media are illustrated. The mean percentage changes in cross-sectional areas are presented in Fig. 4. Dynamic intracellular movement of lipid droplets, Golgi vesicles and the nucleus were observed in all chondrocytes under isotonic and hypotonic conditions. This dynamic behavior was reduced or stopped as the chondrocytes shrank under hypertonic conditions, but returned to normal as the cells re-equilibrated to isotonic conditions.

CROSS-SECTIONAL AREA ANALYSES UNDER HYPERTONIC CONDITIONS

All chondrocytes shrank in hypertonic medium [Fig. 4(A)]. The chondrocytes of MC showed the least degree of shrinkage and IC the greatest, with the response of EC chondrocytes intermediate between the two. MC and EC showed no change in response between days 1 and 7, but IC displayed significantly less shrinkage at day 7 in comparison to day 1.

The percentage change in chondron cross-sectional area of MC was minimal in hypertonic media at all time points [Fig. 4(B)]. Although EC shrank slightly in size, the responses were not significantly different over the culture

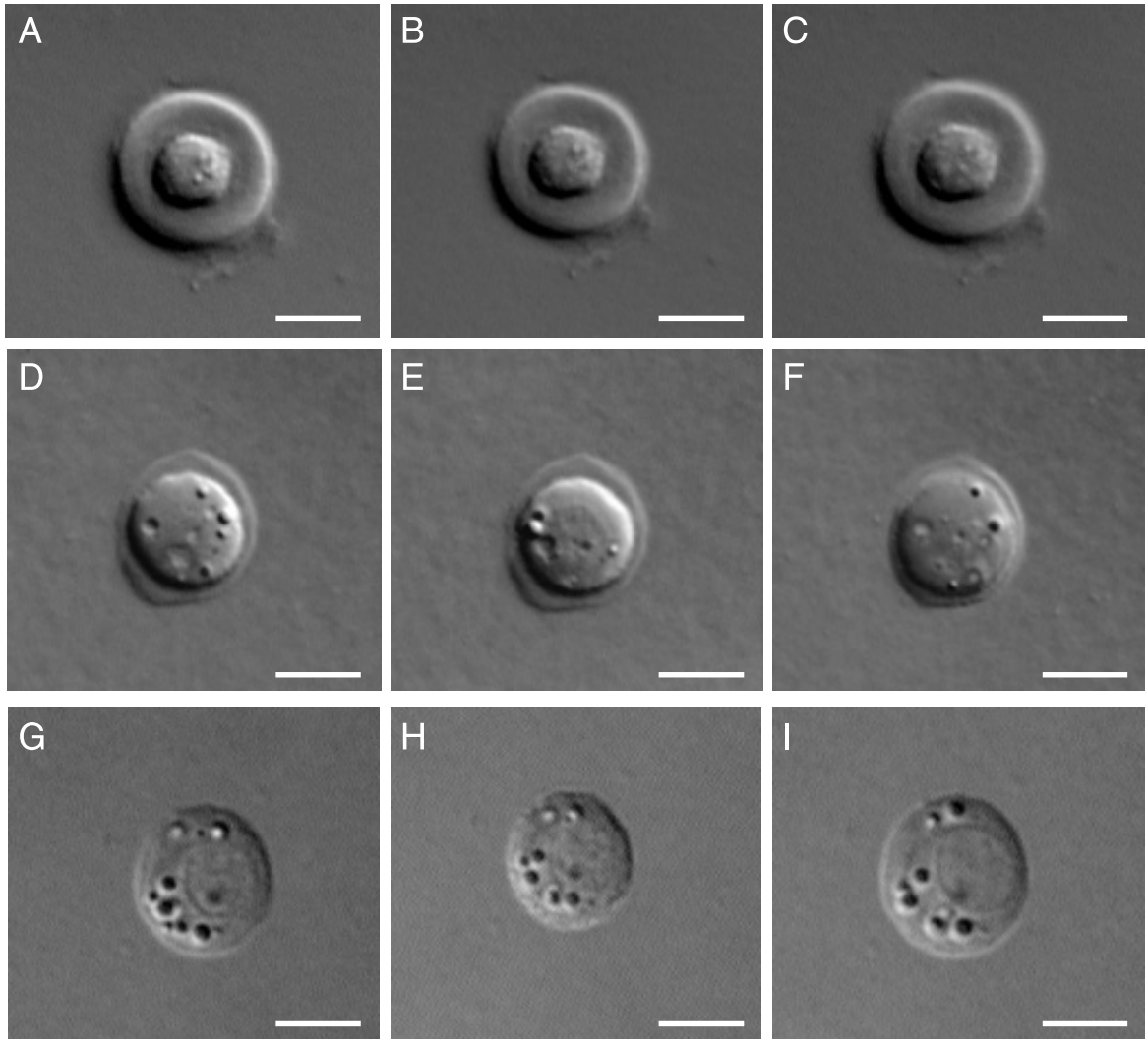

Fig. 3. Digital DIC micrographs showing the effect of osmotic challenges at day 1 on MC (A)–(C), EC (D)–(F) and IC (G)–(I) under isotonic (A), (D), (G) hypertonic (B), (E), (H) and hypotonic (C), (F), (I) conditions. Direct comparisons of the different preparations and their responses to osmotic media are illustrated. Scale bars = 5 μm.

period. In IC however, the newly formed chondron evident at 7 days shrank markedly under hypertonic conditions.

CROSS-SECTIONAL AREA ANALYSES UNDER HYPOTONIC CONDITIONS

All chondrocytes swelled significantly in hypotonic medium. The response was greatest in IC, less in EC, and least in MC preparations [Fig. 4(C)]. The response of chondrocytes in MC did not change over time in culture, while the chondrocytes in both EC and IC showed a reduced swelling response at day 7 when compared to day 1.

The percentage change in the chondron cross-sectional area of MC was minimal in response to hypotonic media at all time points [Fig. 4(D)]. EC swelled more than MC at day 1, but this response reduced over 7 days in culture. The newly formed chondron in IC preparations at day 7 swelled significantly under hypotonic conditions.

Discussion

Structural, immunohistochemical and functional studies have been used to characterize the influence of the pericellular microenvironment in mediating the chondrocyte response to osmotic challenge. The dynamic behavior of chondrocytes within chondrons isolated by mechanical homogenization or enzymatic extraction was compared with isolated chondrocytes under isotonic, hypertonic and hypotonic conditions. The results show that differences in the size, structure, composition and integrity of the pericellular microenvironment can markedly influence the chondrocyte response to osmotic challenges designed to mimic cartilage loading.

MC preparations differ fundamentally from EC and IC since no enzymatic digestion is used during the extraction procedure. The distribution patterns for type VI collagen and keratan sulfate are consistent with previous chondron studies[55,58,59], and correlate closely with chondron structure *in situ*[1]. Moreover, the pericellular microenvironment of

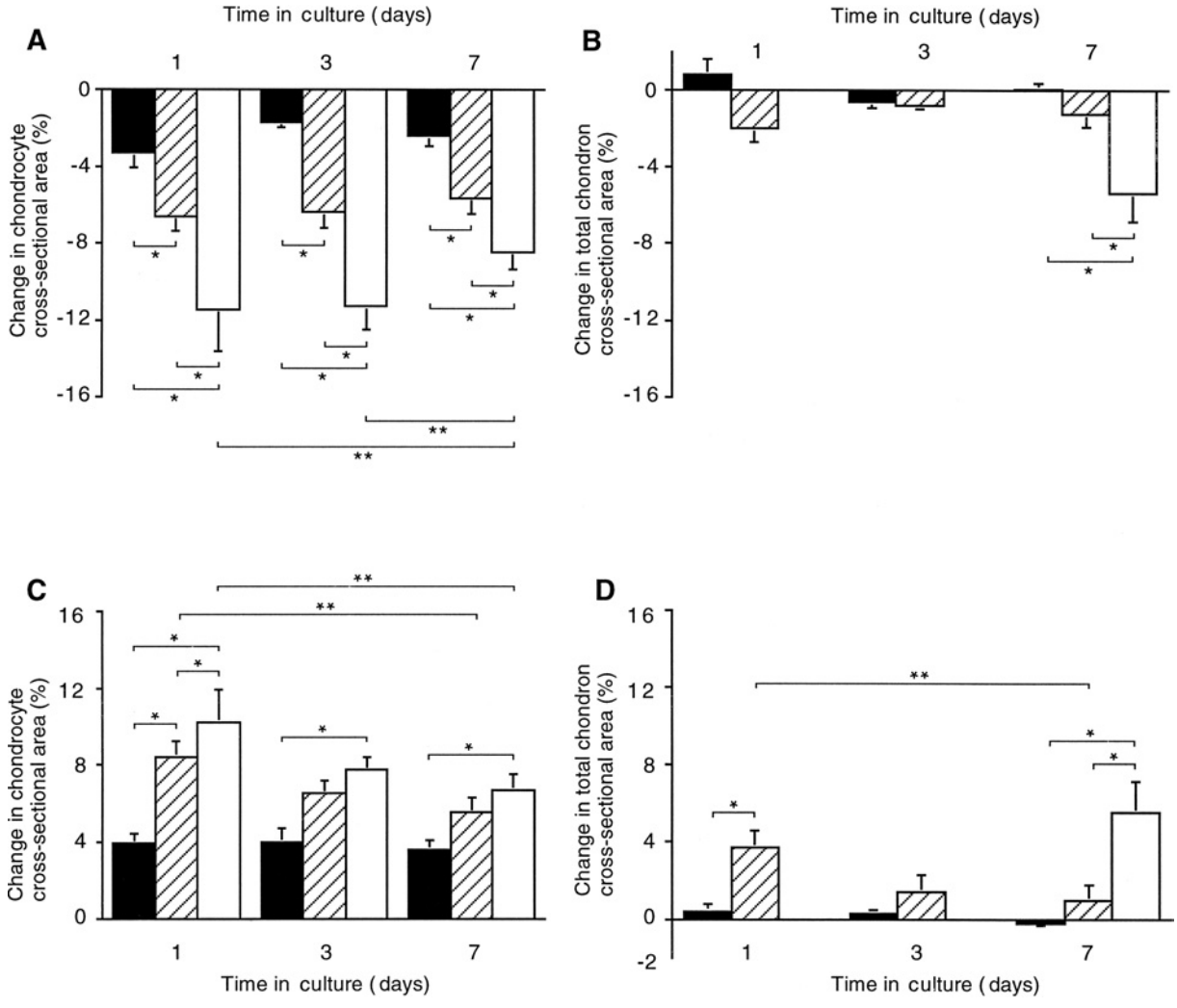

Fig. 4. Relative percentage changes in cross-sectional areas of the chondrocyte (A), (C) and the chondron (B), (D), at days 1, 3 and 7, under hypertonic (A), (B) and hypotonic (C), (D) challenge. MC (black), EC (striped), IC (white). Values represent sample means ± S.E.M. Statistically significant differences ($P<0.05$) have been indicated for post-hoc comparisons *between days* (*) and *between preparations* (**). The *N* values for MC, EC and IC respectively are: 14, 31, 14 (day 1); 17, 14, 17 (day 3); 18, 24, 32 (day 7).

MC represents the major proportion (60%) of the chondron cross-sectional area, and most accurately represents the chondrons observed in adult canine articular cartilage.

In contrast, enzymatic chondron extraction uses a mixture of dispase and collagenase to release the chondrocyte and its microenvironment from the matrix[51]. Previous studies on mature human cartilage showed strong retention of collagenase-resistant type VI collagen, but reduction or loss of other critical matrix macromolecules including aggrecan and fibronectin[51]. Adaptation of these methods for use on adult canine cartilage produced EC preparations which retained a narrow microenvironment containing type VI collagen and keratan sulfate, but lacked type II collagen and fibronectin (unpublished data). These structural and compositional differences in the EC microenvironment reduced its size to 18% of the chondron area, and provided a markedly different chondron preparation for comparative studies.

Chondrocytes isolated by sequential pronase and collagenase digestion showed no evidence of a pericellular microenvironment at the time of isolation. However, pericellular type VI collagen and keratan sulfate were evident by confocal immunohistochemistry within 24 h of culture, and the new microenvironment which formed around IC was consistent in structure and organization with that reported in other studies[52,60,61]. In terms of size, structure and composition, the pericellular microenvironment of IC and EC preparations shared similarities by the end of the culture period, but still varied markedly from the pericellular microenvironment of MC preparations.

These marked differences in the structure and extent of the microenvironment influenced chondrocyte behavior under isotonic conditions. The chondrocytes of IC and EC remained similar in size to each other throughout the culture period, and were consistently larger than those of MC.

The large increase in size of both EC and IC chondrocytes at day 1 represents a direct volume regulatory response to isotonic media, since isolated chondrocytes have previously been reported to increase in size after isolation due to changes in ambient osmolality[5,7]. The larger size of both IC and EC chondrocytes could perhaps be explained by their comparative lack of constraint since MC chondrocytes have a more structurally complex

pericellular microenvironment surrounding them[1,2]. The fact that EC chondrocytes behaved in a similar manner to IC suggests that the presence of some pericellular macromolecules is not sufficient to protect the chondrocyte. Furthermore, the increase in size of both IC and EC chondrocytes by day 7 correlated with the increased size and complexity of the newly elaborated microenvironment. Previous research has shown that isolated chondrocytes increase in size over 7 days of culture in agarose and that this increase is thought to be related to a higher level of metabolic activity[5,7,52]. In contrast, the chondrocytes of MC preparations decreased slightly in size as new macromolecules were added into the existing microenvironment. One explanation may be that the low maintenance requirements of an intact pericellular microenvironment cause the MC chondrocytes to have a lower metabolic activity than EC or IC, and hence a slower rate of synthesis in culture. The presence of a pericellular microenvironment has previously been suggested to down-regulate chondrocyte metabolism *in vitro*[62].

Taken together, all these observations indicate that it is not merely the presence or absence of specific matrix macromolecules, but the nature of their structural integration which provides the physical restraint of the pericellular microenvironment and contributes to the chondrocyte volume regulatory response under isotonic conditions.

All cells have adapted mechanisms to avoid excessive volume fluctuation by adjusting osmotic imbalances between their internal and external environments[63]. The unique ability of articular cartilage to minimize large changes in cell volume, despite being frequently subjected to the osmotic fluctuations associated with joint loading, make it one of the most efficient volume regulatory tissues[5]. Previous studies have investigated the osmotic response of IC under a variety of *in vitro* conditions[43,44,64,65], but this is the first study to show that the chondrocytes of MC and EC also respond according to classical osmotic principles by swelling in hypotonic media and shrinking in hypertonic media. More recently, investigation of the osmotic response of chondrocytes *in situ* under hypo-and hypertonic conditions[66] has shown that chondrocytes respond to varying degrees depending on which layer they are in (superficial, middle or deep), and that this can be attributed to the influence of their extracellular matrix.

The most efficient response to osmotic challenge was observed in MC where minimal change in chondrocyte cross-sectional area occurred under all experimental conditions. This suggests that the structural and compositional integrity of the MC microenvironment forms an osmotic buffer between the chondrocyte and the extracellular medium. High concentrations of hyaluronan and aggrecan are retained within the MC microenvironment[1] and these, together with intact fibrillar and microfibrillar collagens, are likely to maintain the mechanical stiffness of the microenvironment and its osmotic buffering capacity. Similarly, the relationship between the chondrocyte and its pericellular microenvironment, and the signaling mechanisms involved in their dynamic interaction, should also be minimally affected in these preparations. Our observations are also consistent with biomechanical compression studies in agarose in which MC showed negligible deformation when compressed to 20% strain indicating an estimated pericellular stiffness in excess of 25 kPa[24]. The osmotic responses of the MC preparations were therefore consistent with the hypothesis that cell-matrix and matrix-matrix interactions in the microenvironment are minimally disrupted during mechanical extraction, and that this chondron model can maintain a physicochemical responsiveness *in vitro* which is likely to mimic its behavior in intact tissue.

The morphological differences between MC and EC preparations were also reflected in the response of EC to osmotic challenge. The chondrocyte response of EC gradually diminished as new matrix macromolecules were sequestered into the existing microenvironment. This indicates that a return towards structural and compositional integrity correlates directly with a more efficient response to osmotic challenge. Similar behavior was seen in IC preparations which showed the greatest change in chondrocyte response at day 1, but were able to regulate volume more efficiently as a new microenvironment was assembled. This data is consistent with biomechanical studies in which the behavior of EC and IC were shown to differ under load. Both preparations increased their biomechanical stiffness as further pericellular macromolecules were sequestered into the microenvironment[24]. Our results are also consistent with studies using micropipette aspiration to assess the deformation properties of the EC and IC microenvironments[67]. The Young's modulus calculated for the pericellular microenvironment of human EC (1.29±1.06 kPa), was significantly greater than that estimated for IC (0.79±0.72 kPa), but still several orders lower than the cartilage extracellular matrix (~1 MPa)[25]. Further studies have shown a relationship between cytoskeletal organization and the elaboration of a collagen-rich microenvironment *in vitro*[68] suggesting a functional relationship between the intracellular and extracellular tensegrity networks. Together, these results indicate that the chondrocytes of EC and IC resynthesize matrix macromolecules lost during the extraction procedures, sequester these macromolecules within the pericellular microenvironment, and finally organize their three-dimensional assembly to form a more functionally effective barrier against mechanical and osmotic forces resulting from joint loading.

Cell volume regulation in response to osmotic challenge is a complex process involving an array of membrane pumps, voltage-activated and mechanosensitive ion channels, passive exchangers and co-transporters that control the flux of osmotically active molecules, and are closely related to the cytoskeletal network[47,69–72]. Furthermore, the volume regulatory response of chondrocytes has been linked to alterations in tension of the actin cytoskeleton which is capable of signaling changes in chondrocyte volume to the cell nucleus in response to altered osmolarity[19,73].

In addition to defining the physicochemical environment of the chondrocyte, the pericellular microenvironment interacts directly with the chondrocyte through cell-surface receptors such as integrins and CD44. These receptors bind to specific matrix macromolecules such as fibronectin, hyaluronan, and pericellular collagens[74–76] and all play key roles in the integration of the matrix with intracellular components, including the cytoskeleton[17–19,73,77]. Cell–matrix interactions are involved with 'outside-in signaling' and the translation of forces acting on the cartilage matrix into signals which regulate chondrocyte function, cell shape[78] and the volume regulatory response of the chondrocyte. It is likely that these cell–matrix linkages are compromised or lost in both EC and IC preparations during the extraction procedures. This disruption and loss of specific macromolecules must severely compromise normal signaling mechanisms and contribute to the changes in volume regulatory response observed in this study. The continued recovery of volume regulatory response during the culture period implies recovery of some of the signaling

mechanisms as cells resynthesize receptors and matrix ligands responsible for pericellular matrix assembly.

Recent studies have suggested that the viscoelastic properties of the pericellular matrix play an important role in defining the mechanical environment of the chondrocyte and that theoretical models which lack pericellular microenvironment data are seriously flawed[79]. Future models investigating chondrocyte deformation and physicochemical function should therefore include details of the structure and properties of the pericellular microenvironment. The responses to osmotic challenge reported in this study provide the first evidence that the pericellular microenvironment can influence the volume regulatory response of the chondrocyte. The data suggest that the composition and integrity of the microenvironment influence the ability of the chondrocyte to respond to osmotic challenge and indicate that the intact pericellular microenvironment functions efficiently *in vivo* to minimize the exposure of the chondrocyte to dynamic osmotic challenges that could compromise function.

Acknowledgments

The authors would like to thank Dr Denis Loiselle for his kind assistance with the statistical analyses. The research was funded by grants from the Arthritis Foundation of New Zealand (WH, CAP), the Health Research Council of New Zealand (CAP, JMR, AFS), and the Auckland University of Technology (WH).

References

1. Poole CA. Articular cartilage chondrons: form, function and failure. J Anat 1997;191:1–13.
2. Poole CA, Flint MH, Beaumont BW. Chondrons extracted from canine tibial cartilage: preliminary report on their isolation and structure. J Orthop Res 1988;6:408–19.
3. Ratcliffe A, Mow VC. Extracellular matrix. In: Comper WD, Ed. Articular Cartilage. Australia: Harwood Academic Publishers 1996:224–302.
4. Maroudas A. Physico-chemical properties of articular cartilage. In: Freeman MAR, Ed. Adult Articular Cartilage. London: Pitman Medical 1979:215–90.
5. Urban JPG. The chondrocyte: a cell under pressure. Br J Rheum 1994;33:901–8.
6. Buckwalter JA, Rosenberg LC, Hunziker EB. Articular cartilage: Composition, structure, response to injury, and methods of facilitating repair. In: Ewing JW, Ed. Articular Cartilage and Knee Joint Function: Basic science and arthroscopy. New York: Raven Press Ltd 1990:19–56.
7. Muir H. The chondrocyte, architect of cartilage: Biomechanics, structure, function and molecular biology of cartilage matrix macromolecules. Bioessays 1995; 17(12):1039–48.
8. Mason RM. Recent advances in the biochemistry of hyaluronic acid in cartilage. In: Deyl Z, Adam M, Eds. Connective Tissue Research: Chemistry, Biology and Physiology. New York: Alan R Liss 1981:87–112.
9. Poole CA, Glant TT, Schofield JR. Chondrons from articular cartilage: IV. Immunolocalization of proteoglycan epitopes in isolated canine tibial chondrons. J Histochem Cytochem 1991;39(9):1175–87.
10. Winter GM, Poole CA, Ilic MZ, Ross JM, Handley CJ. Identification of district metabolic pools of aggrecan and their relationship to type VI collagen in the chondrons of mature bovine articular cartilage explants. Conn Tiss Res 1998;37(3-4):277–93.
11. Glant TT, Hadhazy CS, Mikecz K, Sipos A. Appearance and persistence of fibronectin in cartilage. Specific interaction of fibronectin with collagen type II. Histochemistry 1985;82:149–58.
12. Poole CA, Gilbert RT, Herbage D, Hartmann DJ. Immunolocalisation of type IX collagen in normal and osteoarthritic canine tibial cartilage and isolated chondrons. Osteoarthritis Cart 1997;5(3):191–204.
13. Wotton SF, Jeacocke RE, Maciewicz RA, Wardale RJ, Duance VC. The application of scanning confocal microscopy in cartilage research. Histochem J 1991; 23:328–35.
14. Poole CA. Chondrons: the chondrocyte and its pericellular microenvironment. In: Kuettner KE, Schleyerbach R, Peyron JC, Hascall VC, Eds. Articular Cartilage and Osteoarthritis. London: Raven Press 1992:201–20.
15. Durr J, Lammi P, Goodman SL, Aigner T, Von der Mark K. Identification and immunolocalization of laminin in cartilage. Expt Cell Res 1996;222:225–33.
16. Von der Mark K, Conrad G. Cartilage cell differentiation. Clin Orthop Rel Res 1979;139:185–205.
17. Banes AJ, Tsuzaki M, Yamamoto J, Fischer T, Brigman B, Brown T, *et al*. Mechanoreception at the cellular level: the detection, interpretation, and diversity of responses to mechanical signals. Biochem Cell Biol 1995;73:349–65.
18. Chen CS, Ingber DE. Tensegrity and mechanoregulation: from skeleton to cytoskeleton. Osteoarthritis Cart 1999;7:81–94.
19. McCauley PG, Winlove CP, Bailey AII, Gratzer GB, Pinder JC, Urban JPG. The effect of changes in chondrocyte volume on tension of the actin cytoskeleton (Abstract). Trans Orthop Res Soc 1998; 23:516.
20. Benninghoff A. Form und bau der Gelenkknorpel in ihren Beziehungen zur Funktion: Zeiter teil: Der Aufbau des Gelenkknorpels in seinen Beziehungen zur Funktion. Zschr Zellforch 1925;2:783–862.
21. Szirmai JA. The concept of the chondron as a biomechanical unit. In: Hartmann F, Ed. Biopolymer und Biomechanik von Bindegewebssytemen. New York: Academic Press 1974:87–91.
22. Poole CA, Flint MH, Beaumont BW. Chondrons in cartilage: Ultrastructural analysis of the pericellular microenvironment in adult human articular cartilage. J Orthop Res 1987;5:509–22.
23. Poole CA. The structure and function of articular cartilage matrices. In: Woessner JF, Howell D, Eds. Joint Cartilage Degradation. New York: Marcel Dekker Inc 1993:1–35.
24. Knight MM, Ross JM, Sherwin AF, Lee DA, Bader DL, Poole CA. Chondrocyte deformation within mechanically and enzymatically extracted chondrons compressed in agarose. Biochim Biophys Acta 2001; 25167:1–6.
25. Guilak F, Jones WR, Ping Ting-Beall H, Lee GM. The deformation behavior and mechanical properties of chondrocytes in articular cartilage. Osteoarthritis Cart 1999;7:59–70.
26. Buschmann MD, Gluzband YA, Grodzinsky AJ, Hunziker EB. Mechanical compression modulates

matrix biosynthesis in chondrocyte/agarose culture. J Cell Sci 1995;108:1497–508.
27. Freeman PM, Natarajan RN, Kimura JH, Andriacchi TP. Chondrocyte cells respond mechanically to compressive loads. J Orthop Res 1994;12:311–20.
28. Lee DA, Bader DL. Compressive strains at physiological frequencies influence the metabolism of chondrocytes seeded in agarose. J Orthop Res 1997;15: 181–8.
29. Toyada T, Saito S, Inokuchi S, Yabe Y. The effects of tensile load on the metabolism of cultured chondrocytes. Clin Orthop Rel Res 1999;359:221–8.
30. Knight MM, Lee DA, Bader DL. Distribution of chondrocyte deformation in compressed agarose gel using confocal microscopy. Cellular Eng 1996;1:97–102.
31. Wachtel E, Maroudas A. The effects of pH and ionic strength on intrafibrillar hydration in articular cartilage. Biochim Biophys Acta 1997;1381:37–48.
32. Wilkins RJ, Hall AC. Control of matrix synthesis in isolated bovine chondrocytes by extracellular and intracellular pH. J Cell Physiol 1995;164:474–81.
33. Hall AC. The differential effects of hydrostatic pressure on cation transport pathways of isolated articular chondrocytes. J Cell Physiol 1999;178:197–204.
34. Lammi MJ, Inkinen R, Parkkinen JJ, Hakkinen T, Jortikka M, Nelimarkka LO, *et al*. Expression of reduced amounts of structurally altered aggrecan in articular cartilage chondrocytes exposed to high hydrostatic pressure. Biochem J 1994;304:723–30.
35. Lane Smith R, Rusk SF, Ellison BE, Wessells P, Tsuchiya K, Carter DR, *et al*. In vitro stimulation of articular chondrocyte mRNA and extracellular matrix synthesis by hydrostatic pressure. J Orthop Res 1996;14:53–60.
36. Borghetti P, Salda LD, De Angelis E, Maltarello MC, Maraldi NM, Borghetti AF. Adaptive cellular response to osmotic stress in pig articular chondrocytes. Tissue & Cell 1995;27:173–83.
37. Hall AC. Volume-sensitive taurine transport in bovine articular chondrocytes. J Physiol 1995;484: 755–66.
38. Palmer G, Chao PG, Raia F, Mauck R, Valhmu W, Hung C. Osmotic loading regulates chondrocyte cell size and aggrecan gene expression (Abstract). Trans Orthop Res Soc 2000;25:0924.
39. Parker A, Boutell J, Beer D, Powell S, Maciewicz R. A microarray based analysis of chondrocyte gene expression in response to altered osmolarity (Abstract). Trans Orthop Res Soc 2001;26:0031.
40. Urban JPG, Hall AC, Gehl KA. Regulation of matrix synthesis rates by the ionic and osmotic environment of articular cartilage chondrocytes. J Cell Physiol 1993;154:262–70.
41. Deshayes CMP, Hall AC, Urban JPG. Effect of extracellular osmolarity on porcine articular chondrocyte volume. J Physiol 1993;467:214P.
42. Errington RJ, Hall AC. Volume regulatory properties of porcine articular chondrocytes measured in catilage explants using confocal microscopy. J Physiol 1995;482.P:12–13.
43. Hall AC, Armitage AJ, Bush PG. Adaptive changes of isolated articular chondrocytes to changes in osmolarity (Abstract). Trans Orthop Res Soc 1999;24: 0724.
44. Hung CT, Jamieson KV, Roy R, Wong DD, Chao PG, Sun DD, *et al*. Comparison of transient chondrocyte swelling and shrinking behaviour (Abstract). Trans Orthop Res Soc 2001;26:0559.
45. de Angelis E, Petronini PG, Borghetti P, Borghetti AF, Wheeler KP. Induction of betaine-γ-aminobutyric acid transport activity in porcine chondrocytes exposed to hypertonicity. J Physiol 1999;518:187–94.
46. Erickson G, Guilak F. Osmotic stress initiates intracellular calcium waves in chondrocytes through extracellular influx, the inositol phosphate and a G-protein mediated pathway (Abstract). Trans Orthop Res Soc 2000;25:0923.
47. Mobasheri A, Mobasheri R, Francis MJO, Trujillo E, Alvarez de la Rosa D, Martin-Vasallo P. Ion transport in chondrocytes: membrane transporters involved in intracellular ion homeostasis and the regulation of cell volume, free [Ca^{2+}] and pH. Histol Histopathol 1998;13:893–910.
48. Urban JPG, Bayliss MT. Regulation of proteoglycan synthesis rate in cartilage in vitro: influence of extracellular ionic composition. Biochim Biophys Acta 1989;992:59–65.
49. Yellowley CE, Donahue J. Hypotonic swelling increases internal calcium concentration and activates membrane ion channel currents in bovine articular chondrocytes (Abstract). Trans Orthop Res Soc 2000;25:0328.
50. Poole CA. Chondrons extracted from articular cartilage: methods and applications. In: Maroudas A, Kuettner KE, Eds. Methods in Cartilage Research. Academic Press, London, 1990:78–83.
51. Lee GM, Poole CA, Kelley SS, Chang J, Caterson B. Isolated chondrons: a viable alternative for studies of chondrocyte metabolism in vitro. Osteoarthritis Cart 1997;5:261–274.
52. Knight MM, Lee DA, Bader DL. The influence of elaborated pericellular matrix on the deformation of isolated articular chondrocytes cultured in agarose. Biochim Biophys Acta 1998;1405:67–77.
53. Arokoski JPA, Hyttinen MM, Helminen HJ, Jurvelin JS. Biomechanical and structural characteristics of canine femoral and tibial cartilage. J Biomed Mat Res 1999;48:99–107.
54. Poole CA, Flint MH, Beaumont BW. Morphological and functional interrelationships of articular cartilage matrices. J Anat 1984;138:113–30.
55. Poole CA, Ayad S, Schofield JR. Chondrons from articular cartilage: I. Immunolocalization of type VI collagen in the pericellular capsule of isolated canine tibial chondrons. J Cell Sci 1988b;90:635–43.
56. Poole CA, Brookes NH, Gilbert RT, Beaumont BW, Crowther A, Scott L, *et al*. Detection of viable and non-viable cells in connective tissue explants using the fixable fluoroprobes 5-chloromethylfluorescein diacetate and ethidium homodimer-1. Conn Tiss Res 1996;33:233–41.
57. Hing WA, Poole CA, Jensen CG, Watson M. An integrated environmental perfusion chamber and heating system for long term, high resolution imaging of living cells. J Microsc 2000;199:90–5.
58. Chang J, Poole CA. Sequestration of type VI collagen in the pericellular microenvironment of adult chondrocytes cultured in agarose. Osteoarthritis Cart 1996; 4:275–85.
59. Poole CA, Ayad S, Gilbert R. Chondrons form articular cartilage. (V) Immunohistochemical evaluation of

type VI collagen organisation in isolated chondrons by light, confocal and electron microscopy. J Cell Sci 1992;103:1101–10.
60. Chang J, Nakajima H, Poole CA. Structural colocalisation of type VI collagen and fibronectin in agarose canine cultured chondrocytes and isolated chondrons extracted from adult canine tibial cartilage. J Anat 1997;190:523–32.
61. Idowu BD, Knight MM, Bader DL, Lee DA. The organisation of cytoskeletal components in isolated chondrocytes seeded in agarose (Abstract). Trans Orthop Res Soc 1999;24:0717.
62. Handley CJ, Lowther DA. Extracellular matrix metabolism by chondrocytes; III Modulation of proteoglycan synthesis by extracellular levels of proteoglycan in cartilage cells in culture. Biochim Biophys Acta 1977;500:132–9.
63. Lang F, Waldegger S. Regulating cell volume. American Scientist 1997;85:456–463.
64. Erickson G, Minkhorst R, Guilak F. The calcium transients elicited in chondrocytes as a result of exposure to osmotic pressure change are not due to simple membrane deformation (Abstract). Trans Orthop Res Soc 2001;26:0555.
65. Gu WY, Lai WM, Mow VC. A mixture theory for charged-hydrated soft tissues containing multi-electrolytes: passive transport and swelling behaviours. J Biomech Eng 1998;120:169–80.
66. Bush PG, Hall AC. The osmotic sensitivity of isolated and in situ bovine articular chondrocytes. J Orthop Res 2001;19:768–78.
67. Jones WR, Ting-Beall HP, Lee GM, Kelley SS, Hochmuth RM, Guilak F. Mechanical properties of human chondrocytes and chondrons from normal and osteoarthritic cartilage (Abstract). Trans Orthop Res Soc 1997;22:199.
68. Idowu BD, Knight MM, Bader DL, Lee DA. Confocal analysis of cytoskeletal organisation within isolated chondrocyte sub-populations cultured in agarose. Histochem J 2000;32:165–74.
69. Erickson G, Guilak F. Dissociation and remodeling of the actin cytoskeleton of chondrocytes in response to hypo-osmotic stress (Abstract). Trans Orthop Res Soc 2001;26:0180.
70. Hall AC, Horwitz ER, Wilkins RJ. The cellular physiology of articular cartilage. Exp Physiol 1996; 81:535–45.
71. Janmey PA. The cytoskeleton and cell signalling: component localization and mechanical coupling. Physiol Rev 1998;78:763–81.
72. Pritchard S, Erickson G, Guilak F. Osmotically induced calcium transients in IVD cells are dependent on the actin cytoskeleton (Abstract). Trans Orthop Res Soc 2001;26:0032.
73. Benjamin M, Archer CW, Ralphs JR. Cytoskeleton of cartilage cells. Microsc Res Tech 1994;28:372–7.
74. Knudson CB. Hyaluronan receptor-directed assembly of chondrocyte pericellular matrix. J Cell Biol 1993;120:825–34.
75. Sommarin Y, Larsson T, Heinegard D. Chondrocyte-matrix interactions: attachment to proteins isolated from cartilage. Exp Cell Res 1989;184:181–92.
76. Von der Mark K, Mollenhuer J. Annexin V interactions with collagen. Cell Mol Life Sci 1997;53:539–45.
77. Loeser RF. Chondrocyte integrin expression and function. Biorheol 2000;37:109–16.
78. Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. Cell 1992;69:11–25.
79. Guilak F, Mow VC. The mechanical environment of the chondrocyte: a biphasic finite element model of cell-matrix interactions in articular cartilage. J Biomech 2000;33:1663–73.