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OSTEOARTHRITIS and CARTILAGE

Isolated chondrons: a viable alternative for studies of chondrocyte metabolism *in vitro*

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Summary

Objective: To develop and test a simple enzymatic procedure for isolating chondrons, which consist of the chondrocytes and their surrounding pericellular microenvironment.

Design: Chondrons were obtained by digesting adult human articular cartilage with a mixture of dispase and collagenase. Chondrons and chondrocytes were cultured in alginate beads, immunofluorescence labeled and examined by confocal microscopy.

Results: Comparison of freshly isolated chondrons with cryostat sections of cartilage revealed that type VI collagen, type II collagen and aggrecan were retained, but fibronectin and a unique chondroitin sulfate epitope recognized by the antibody, 7D4, were lost. Comparison of enzymatic and mechanical homogenization methods revealed subtle changes in chondron morphology and retention of fibronectin in mechanically isolated chondrons. Average yield of enzyme-isolated chondrons was slightly lower than that of chondrocytes isolated by pronase and collagenase digestion, but was much greater than that reported for mechanically isolated chondrons. Enzyme-isolated chondron viability was greater than 80% 1 day after isolation, and continued to be above 80% through 7 weeks of alginate bead culture. Viability of isolated chondrocytes was initially greater than 80% but fell to 60–80% with time in culture. Chondrons and isolated chondrocytes had a similar division rate except osteoarthritic chondrons were significantly slower after 2 weeks in culture. Cell division was more rapid for nonosteoarthritic chondrons than for osteoarthritic ones.

Conclusions: Enzymatic isolation of chondrons is relatively simple, gives better yield and viability than mechanical isolation, but comparable yield and viability of traditional chondrocyte isolation. Enzymatic chondron isolation allows the effect of the *in vivo*-formed pericellular matrix on chondrocyte metabolism to be studied *in vitro*.

Key words: Chondron, Chondrocyte, Articular cartilage, Type VI collagen.

Introduction

Cultured chondrocytes and explants from articular cartilage are widely used to study chondrocyte physiology and drug responses [1–3]. When chondrocytes are used in culture systems, they are typically stripped of all extracellular matrix interactions and of functional extracellular matrix receptors and then put into a foreign environment such as plastic, agarose or alginate. For *in vitro* studies, the composition and presence of extracellular matrix and the repetoire of cell-surface receptors has been shown to have a major effect on gene expression and response to growth factors [4, 5]. In cartilage, all molecules that pass to or from the chondrocyte must pass through the pericellular microenvironment. During their passage, some molecules, such as growth factors, are modified or retained [6]. Extracellular matrix molecules such as aggrecan may also be modified during their passage through the pericellular matrix which could participate in the time-dependent maturation of aggrecan complex formation with hyaluronan [7]. Because the extracellular matrix can have such a significant effect on the chondrocyte phenotype, a more physiologically representative model of chondrocyte metabolism *in vivo* is likely to be obtained by retaining the native pericellular matrix with the chondrocyte during isolation.

An alternative for studying chondrocyte metabolism is to isolate and culture chondrons. Chondrons are structural units in cartilage consisting of one or more chondrocytes, the surrounding pericellular matrix and an enclosing capsule [8–12]. While chondron structure and molecular anatomy have become increasingly well characterized, studies of chondron metabolism

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have received limited attention because the previously described method of mechanical isolation involved repeated low-speed homogenizations which compromised many chondrons and gave low yields of viable chondrons [12]. Studies with mechanically-isolated chondrons, however, have provided a wealth of information on chondron composition, showing them to be rich in proteoglycans and collagen types II, VI and IX [12–15], while chondrons isolated from osteoarthritic cartilage were shown to have a different morphology to those isolated from normal cartilage [15, 16]. When chondrons are isolated, the pericellular matrix is retained, but the extensive territorial and interterritorial matrices are removed. In addition, isolated chondrons retain the cellular relationships identified *in vivo* such as chondrocytes present in columnar chondrons typical of the deep zone of articular cartilage or in multiple chondron clusters typical of osteoarthritic cartilage.

In order to have easier access to chondrons for studies on the functional and metabolic properties of the native pericellular matrix, a simple enzymatic procedure for isolating chondrons has been developed. This procedure yields sterile, viable chondrons with a similar morphology and matrix composition to those found *in situ*. In previous work, we reported that chondrons differ from isolated chondrocytes in their pattern of proteoglycan synthesis when maintained in alginate bead culture [17]. In this study, we show that yield, viability, and longevity in bead culture are comparable for enzymatically isolated chondrons and isolated chondrocytes obtained from adult human articular cartilage, and that subtle morphological and compositional differences exist between mechanically and enzymatically isolated chondrons. Confocal microscopy was used to compare the structure and composition of the chondron *in situ* with that of the isolated chondron, while the enzymatic isolation of chondrons was compared with the previously published method of mechanical isolation [12]. The enzymatic isolation and culture of chondrons has previously been published as an abstract [18].

Materials and Methods

ISOLATION AND CULTURE

Chondrons and chondrocytes were isolated from osteoarthritic human knee and hip cartilage obtained at the time of joint replacement (21 individuals, age 41–78 years). Cartilage was harvested from both lesioned and non-lesioned areas and combined. Nonosteoarthritic human cartilage was obtained as surgical waste tissue at the time of surgery from knee, hip, shoulder, wrist and ankle joints (17 individuals, ages 18–93 years). These specimens had no grossly visible defects and are termed nonosteoarthritic rather than normal because some were obtained due to trauma, while for others, the joint may have been immobilized prior to surgery. For each specimen, the cartilage was minced before dividing into equal parts for chondron and chondrocyte isolation (procedures compared and summarized in Table I). Chondrocytes were isolated using pronase (Boehringer-Mannheim and Calbiochem) and collagenase (CLS-2, Worthington) as previously described [19]. Chondrons were obtained from minced articular cartilage using 0.3% dispase (a neutral protease classified as an amino-endo peptidase produced by *Bacillus polymyxa*) (GIBCO) and 0.2% collagenase (CLS-2, Worthington) in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline PBS at 37°C with shaking for 5 h. After straining through 70 μm nylon mesh (Falcon cell strainer) and centrifugation, enzymatically isolated chondrons were resuspended in Ham's F-12 tissue culture medium and counted with a hemacytometer. Isolated chondrocytes or chondrons were embedded in alginate beads, essentially as previously described,

Table I
Comparison of procedures for enzymatic isolation of chondrocytes and chondrons

Traditionally isolated chondrocytes (no pericellular matrix)	Chondrons (intact pericellular matrix)
Mince cartilage	Mince cartilage
Digest with 1320 PUK/ml pronase in DMEM-H plus 5% FBS for 1 h	Digest with 0.3% dispase plus 0.2% collagenase in PBS for 5 h
Digest with 0.4% collagenase in DMEM-H plus 5% FBS for 3 h	
Filter through 70 μm nylon mesh	Filter through 70 μm nylon mesh
Embed chondrocytes in alginate beads	Embed chondrons in alginate beads

DMEM-H, Dulbecco's modified essential medium with high glucose (4500 mg/ml); PUK, protein units; PBS, phosphate-buffered saline; FBS, fetal bovine serum.

[20] at densities ranging from $1–4\times10^6$ cells/ml of 1.2% alginate (Sigma) in 0.15 M NaCl. The alginate–cell suspension was extruded as drops from a 23 gauge needle into 102 mM $CaCl_2$ contained in 25 cm^2 flasks where the beads were subsequently cultured. The alginate bead cultures were fed every other day with Ham's F-12/DMEML [Delbecco's modified essential medium with low glucose (1000 mg/l)] containing 10% fetal bovine serum (FBS), penicillin/streptomycin and 25 μg/ml phospitan C (L ascorbic acid-2-monophosphate, generously provided by Showa Denko America, Inc., Tokyo).

MECHANICAL VERSUS ENZYMATIC ISOLATION PROCEDURE

Tibial cartilage was obtained at necropsy from mature healthy crossbred dogs aged 2–5 years. No evidence of osteoarthritic lesions were found in the five animals examined. Resected cartilage was pooled and finely diced, and the sample divided in two. One half was treated for enzymatic extraction as described above, and the enzymatically isolated chondrons were plated into agarose gel monolayers and processed as previously described [12]. The remaining half of the sample was processed by mechanical chondron extraction techniques as previously described [13, 21]. Specific and accurate regulation of homogenization speed is essential for mechanical extraction of chondrons. Briefly, diced cartilage in 20 ml PBS was serially homogenized at 4000 rpm for 60 s, the homogenate resuspended to 50 ml in PBS, and the large cartilage fragments allowed to settle for 15–30 s. The fine flocculent material was decanted and pooled, the preparation resuspended to 50 ml, and the settling decantation repeated. The homogenization and settling steps were serially repeated until the entire sample had been rendered into a flocculent suspension which was collected by centrifugation to yield a final preparation in 20 ml PBS. These preparations therefore contained all of the original cartilage sample, and presented by phase-contrast or DIC microscopy as intact viable chondrons, intact non-viable chondrons, capsular 'ghosts', small intact chips of articular cartilage containing five to 50 chondrons, and collagenous debris from the territorial and interterritorial matrices. This heterogeneous cartilage homogenate was finally mixed with agarose gel and cast as a monolayer before fixation in 2% paraformaldehyde in preparation for comparative immunohistochemical examination of mechanically versus enzymatically isolated chondron morphology and composition.

MEASURE OF VIABILITY AND CELL DIVISION

Viability of chondrons and chondrocytes were measured after overnight culture in alginate and at 2, 4 and 7 weeks of culture. While still in the alginate beads, cells were fixed in 3.7% formaldehyde in Hank's balanced salt solution with calcium and magnesium (HBSS+) for 20 min, rinsed 3×5 min and then labeled with Hoechst 33342 (10 μg/ml, Molecular Probes) and eosin-Y phloxine B solution (1:100, Sigma) in HBSS+ for 2–4 h. After rinsing, beads were placed individually on glass slides in a moist chamber, 15 μl of 55 mM Na citrate 50 mM NaCl was added to each bead. After the beads dissolved, a 22 mm^2 coverslip was placed over the cells and the edges were sealed with melted VALAP (1:1:2 by weight of Vaseline, lanolin and paraffin). When viewed with a fluorescence microscope using a Fura 2 filter set, the nuclei appeared a bright blue–green and the cytoplasm was a faint orange. The pericellular matrix of the chondrons did not stain with the eosin. Two beads were examined for each time point with a minimum of 100 chondrocytes/chondrocyte clusters or 50 chondron clusters being counted for each bead. Cells were judged to be viable on the basis of positive staining with Hoechst and an intact nucleus. Eosin staining and phase contrast were used to detect cells that were negative for Hoechst. Fluorescein diacetate (5 μg/ml) [12, 22] and propidium iodide (5 μg/ml) staining in conjunction with Hoechst 33342 staining of live cells were used to confirm the validity of using normal Hoechst staining as an indicator of viability. (Only 6% of cells that stained with Hoechst failed to retain fluorescein diacetate. Of these 6%, 60% were not stained with propidium iodide). This approach was necessary because the number of cells per chondron in multiple chondron clusters can be difficult to determine without nuclear labeling. The use of Hoechst-stained, fixed cells also allowed other parameters to be measured on the same specimens without losing the viability marker during observation (for instance, [22]).

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For immunofluorescence of cryostat sections, fresh human knee cartilage was cut from the bone. A piece approximately $2\times6\times6$ mm, was put into −20°C dimethyl sulfoxide:methanol (20:80 v:v) and stored at −20°C. Before sectioning, the cartilage piece was halved then immersed in PBS for 20–30 min. After freezing, 25 μm-thick sections were cut and immediately transferred to PBS on ice. Isolated chondrocytes and chondrons were

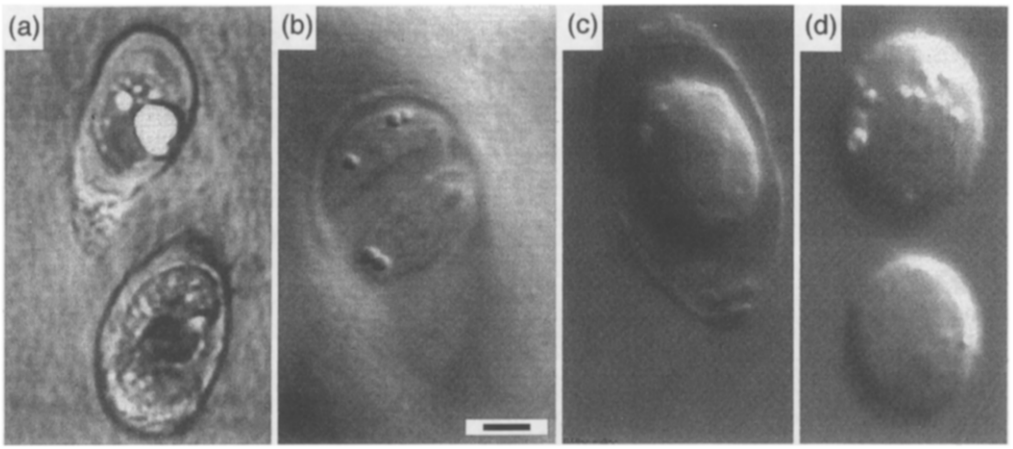

FIG. 1. Chondrons consist of chondrocytes surrounded by pericellular matrix which is enclosed in a rim or capsule. (a) Two chondrons in a toluidine blue stained glycol methacrylate section of human articular cartilage, (b) a chondron with two cells in a vibratome section of unfixed human articular cartilage, and (c) a chondron isolated enzymatically from human articular cartilage. (d) Chondrocytes isolated from human articular cartilage using pronase followed by collagenase. (b)–(d) are micrographs made with video-enhanced differential interference contrast (DIC or Nomarski) microscopy. All are at the same magnification, bar 6 μm.

fixed in alginate beads either as above in cold dimethyl sulfoxide:methanol (immediately after isolation) or with 3.7% formaldehyde in PBS for 20 min. After rinsing in PBS, the beads were dissolved as above. Melted 1% Seakem gold agarose (at 42°C) was added to the alginate–cell suspension. The agarose with cells was cast into a thin film and cut into 10 mm squares. The agarose-cell films and the cryostat sections were handled as free-floating sections for labeling. For comparison of mechanically versus enzymatically isolated chondrons, circular plugs were cored from the agarose monolayers and also treated as free-floating preparations, as previously described [12]. The following primary antibodies were used: a rabbit polyclonal to type II collagen (cat. #T59104R, Biodesign International, Kennebunk, ME, U.S.A.), Hfn7.1 to fibronectin (prepared by R. J. Klebe and obtained from the Developmental Studies Hybridoma Bank), H4C4 to CD44 (Prepared by J. E. K. Hildreth and J. T. August and obtained from the Developmental Studies Hybridoma Bank), 3C4 to type VI collagen (generously provided by Eva Engvall), 7D4 to chondroitin sulfate [23] and 5D4 to keratan sulfate [24]. The secondary antibodies were Cy3 labeled donkey anti-mouse and Cy3-donkey anti-rabbit (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, U.S.A.). The films and sections were mounted in PBS:glycerol (50:50 v:w) containing 0.01% sodium azide and sealed with clear nail polish. The specimens were imaged with a Zeiss confocal microscope using a ×100 1.3 NA oil immersion objective. The micrographs were made with contrast adjustments to reveal as much of the matrix material as possible rather than for quantitative comparisons.

Results

Chondrons enzymatically isolated from human articular cartilage retained a similar morphology to chondrons observed in cartilage sections (Fig. 1). The surrounding rim or capsule was evident with many of the isolated chondrons. Isolated chondrocytes did not retain any extracellular matrix material as determined by DIC microscopy [Fig. 1(d)] or by immunofluorescence (not shown). The chondron preparations contained occasional small pieces of acellular interterritorial matrix and a small proportion of isolated chondrocytes.

Enzymatic isolation of chondrons produced slightly lower average yields than that obtained with isolated chondrocytes (Table II). The percentage of chondron to chondrocyte yield averaged $83 \pm 10\%$ (mean $\pm$ S.E.) for the osteoarthritic specimens and $128 \pm 19.6\%$ for the non-osteoarthritic. The difference was not significant ($P > 0.4$) for the nonosteoarthritic but was significant ($P < 0.01$) for the osteoarthritic. At the end of the enzyme digestion for both techniques, a few partially digested cartilage pieces usually remained that were probably pieces left a little large during the mincing process. The yield of chondrons could be increased by incubating the cartilage for an additional hour with fresh enzyme solution, but this did not result in complete solubilization of the tissue. When overnight incubation of the human articular cartilage in the dispase–collagenase

Table II
Yield of chondrons and traditionally isolated chondrocytes

Tissue source	Cell yield (average ± S.E. per gram tissue) Chondrons	Chondrocytes	
Nonosteoarthritic human (17)	$2.9 \pm 1.0 \times 10^6$	$3.3 \pm 1.5 \times 10^6$	$P > 0.4$
Osteoarthritic human (29)	$1.9 \pm 0.2 \times 10^6$	$3.0 \pm 0.4 \times 10^6$	$P < 0.01$

Number of specimens is in parentheses.

solution was tried, full digestion of the cartilage was obtained, but the resulting chondrons did not have intact capsules (not shown). Comparable yields of enzymatically isolated chondrons were achieved with canine material (results not shown), while previous studies indicate low yields of viable chondrons (10–20%) were achieved by mechanical homogenization [21].

To determine what is lost from the chondron during the enzyme digestion of the cartilage, freshly isolated chondrons were compared with cryostat sections of cartilage from the same specimen of normal human knee articular cartilage (Figs 2 and 3). Both were fixed in cold dimethyl sulfoxide:methanol and labeled for immunofluorescence. Chondrons in the cartilage sections were defined by anti-type VI collagen and somewhat by 7D4, an antibody to chondroitin sulfate. Labeling for fibronectin, type II collagen and keratan sulfate was found throughout the cartilage matrix with no distinct boundaries for the chondrons. The freshly isolated chondrons retained type VI collagen, type II collagen and keratan sulfate. The greater labeling density for the type II collagen in the isolated chondrons [Fig. 3(c)] may be due to increased exposure of the epitope in enzyme isolated preparations. The 7D4 epitope and fibronectin were lost from the chondron matrix but many of the chondrocytes within chondrons showed intracellular labeling. Consistent with the intracellular presence upon isolation, fibronectin and the 7D4 epitope appeared in the chondron matrix within 24–72 h of culture (see below). Labeling for CD44, a hyaluronan receptor [25], was clearly evident on the cell surfaces in the cartilage sections [Fig. 2(f)], but was only partially retained on the cell surface in enzyme isolated chondrons [Fig. 3(f)], and was completely lost from the surface of isolated chondrocytes (not shown). CD44 was also found intracellularly upon isolation and was abundant on the cell surface after 24 h of culture (see below).

Structural comparisons of mechanically and enzymatically isolated canine chondrons are shown in Fig. 4. Type VI immunolabeling revealed intense compacted labeling around each chondrocyte in mechanically isolated chondrons, with reduced labeling in the interconnecting segments between adjacent chondrons [Fig. 4(a)]. Enzymatically isolated chondrons also showed intense pericellular labeling for type VI collagen, but appeared broader in dimension and more stippled than mechanically isolated preparations [Fig. 4(b)]. Fibronectin labeling of mechanically isolated chondrons appeared intense around both the chondrocytes and in the interconnecting segments [Fig. 4(c)]. However, pericellular fibronectin labeling was completely absent from enzymatically isolated chondrons at time zero; the weak reactivity present representing intracellular labeling [Fig. 4(d)]. The comparison of 5D4 labeling after each isolation is illustrated in Figs 4(e) and (f), and indicates pericellular retention of keratan sulfate, but subtle changes in its morphological distribution.

Matrix molecules and the hyaluronan receptor, CD44, which were lost during the enzymatic isolation, reappeared after 1–3 days of alginate bead culture (Fig. 5). After 3 days in culture fibronectin became abundant in the pericellular

Table III
Distribution of chondrons per cluster for freshly isolated chondrons (average percent ± S.E.)

Number of chondrons	Nonosteoarthritic	Osteoarthritic
1	70.5 ± 3.5	51 ± 5.2*
2	21.3 ± 2.4	27.4 ± 1.7**
3	4.4 ± 0.6	9.4 ± 1.3*
4–5	2.8 ± 0.9	7.7 ± 1.7*
>6	1.4 ± 0.9	4.6 ± 1.3*

*$P < 0.01$, **$P < 0.05$ (ANOVA).

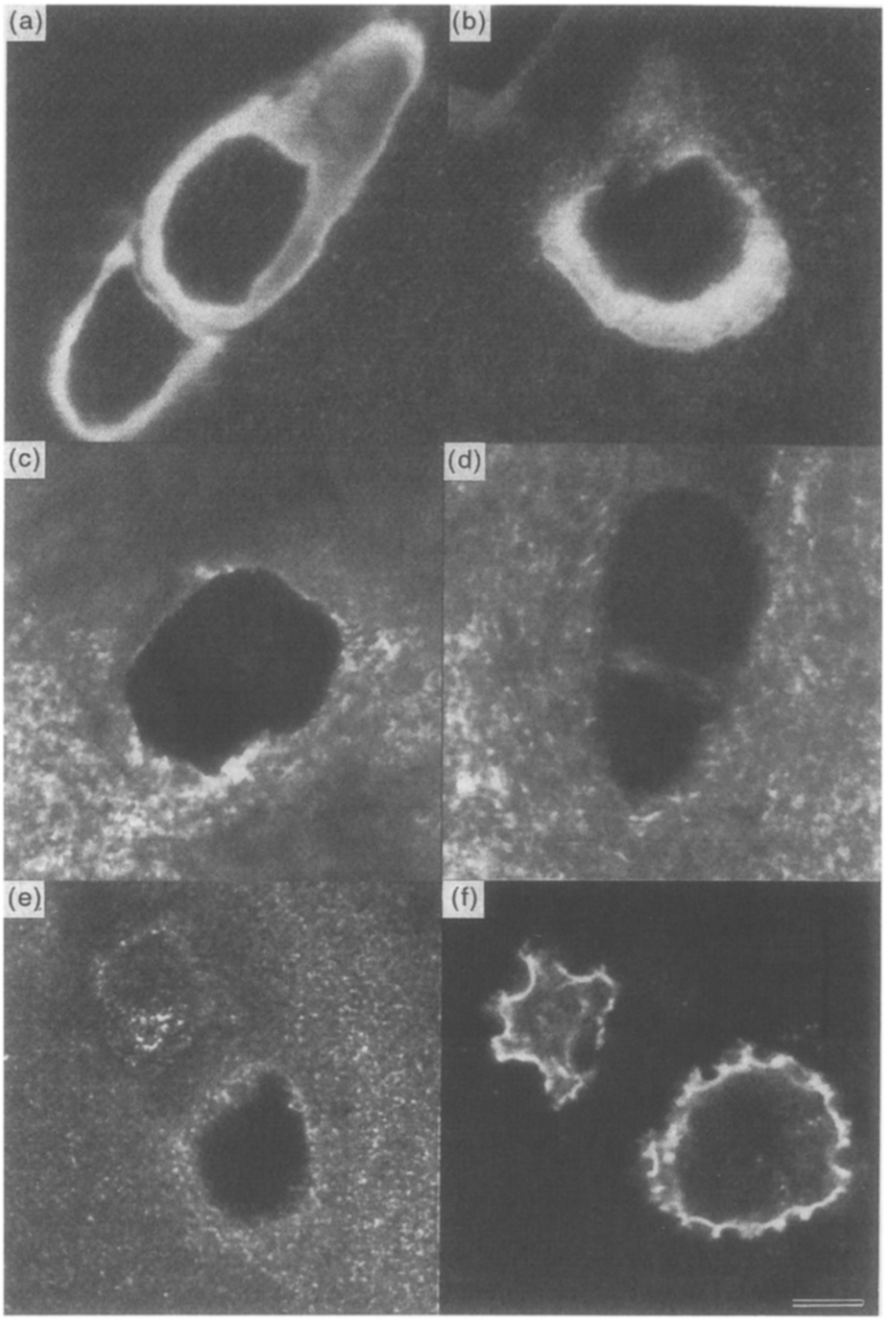

FIG. 2. Confocal micrographs of different matrix components in cryostat sections of normal knee articular cartilage obtained from a 60 year old at the time of amputation for non-healing of superficial burns. Sections were labeled by immunofluorescence for: (a) type VI collagen; (b) chondroitin sulfate (antibody 7D4); (c) type II collagen; (d) fibronectin; (e) keratan sulfate; and (f) CD44. The cells appear dark in the micrographs shown in (a)–(e). Only type VI collagen and 7D4 labeling appear to define the chondron. At the boundary of the chondron, there were subtle changes in intensity of label or texture with 5D4 and fibronectin but these are difficult to distinguish in the micrographs. The chondrocytes and/or the surrounding matrix underwent some shrinkage as is evident by the irregular appearance of the labeled plasma membrane in (f). The upper left cell in (f) is sectioned near the cell surface while the lower right cell is sectioned through its mid-region. Intracellular labeling was also present. Each micrograph is from a single optical section. Bar 5 μm.

matrix [Fig. 5(a)] but was not quite up to the density observed in cartilage [Fig. 2(d)] or in mechanically isolated chondrons [Fig. 4(c)]. CD44 was the fastest to reappear and many chondrocytes within chondrons had uniform plasma membrane labeling after only 1 day in culture [Fig. 5(b)]. The chondroitin sulfate epitope recognized by the antibody, 7D4, also reappeared after only one night

of culture in many chondrons. The expression of the 7D4 epitope was found to be highly variable between chondrons and between specimens.

Seventy per cent of chondron clusters isolated from nonosteoarthritic cartilage were unicellular with only 8% consisting of three or more chondrons (Table III). About half of the chondron clusters freshly isolated from osteoarthritic human cartilage contained a single chondron (Table III). There were also many doublets. Two to 4% of the chondron clusters contained eight to 22 cells. Chondron clusters with three to six cells were in two arrangements: loose clusters and single cell columns; clusters with greater than six cells

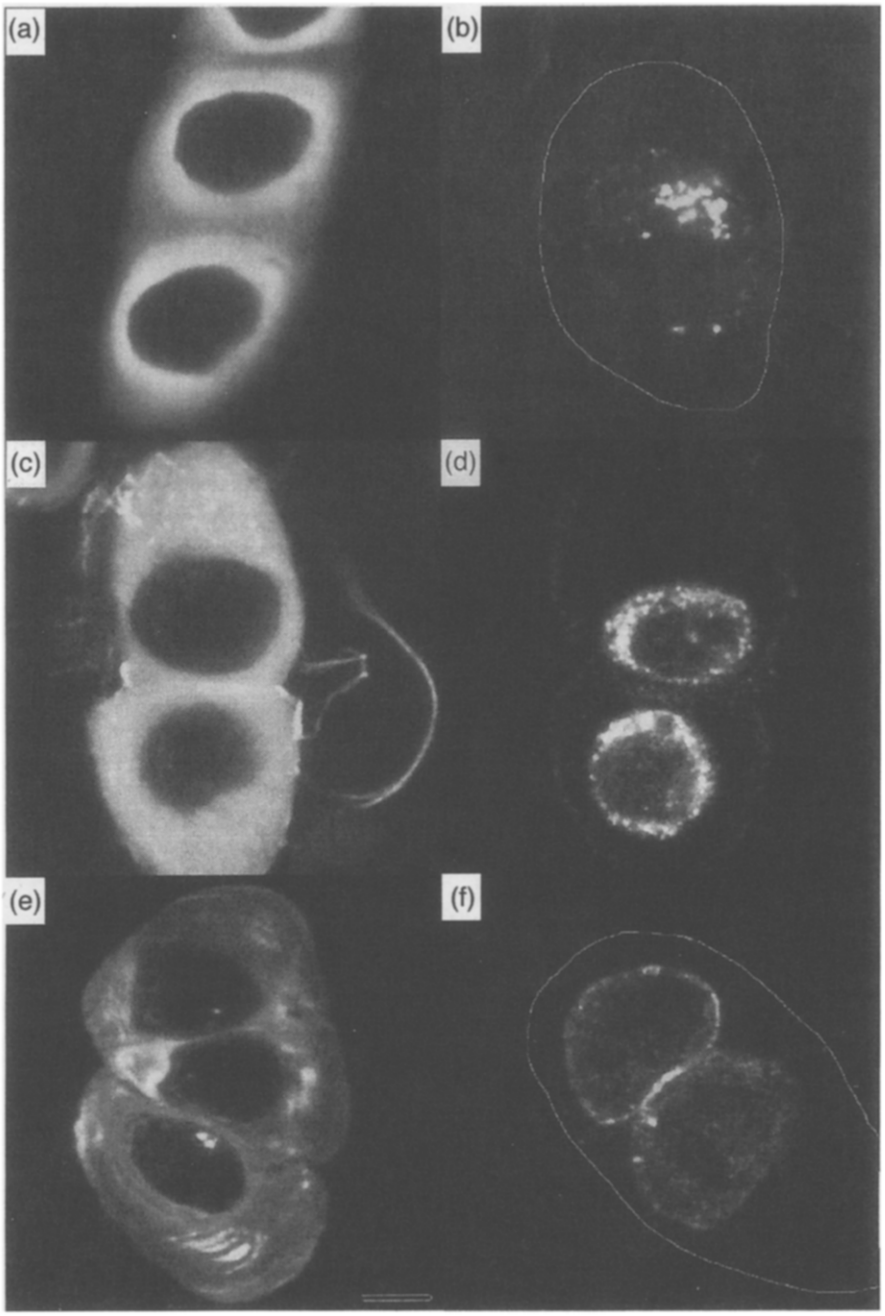

FIG. 3. Confocal micrographs of freshly isolated chondrons fixed immediately after embedding in alginate beads. The chondrons were isolated from the same specimen used for the cryostat sections shown in Fig. 3. (a) type VI collagen; (b) chondroitin sulfate (antibody 7D4); (c) type II collagen; (d) fibronectin; (e) keratan sulfate; and (f) CD44. The outlines in (b) and (f) show the outer edges of the chondrons; the labeling in these figures was primarily intracellular; the dark areas within the cells are the nuclei. The pericellular matrix in (d) was faintly labeled. Each micrograph was from a single optical section except for (c) where four optical sections taken 1 μm apart were combined. This was necessary to show the collagen fibrils which have a three-dimensional arrangement. Bar 5 μm.

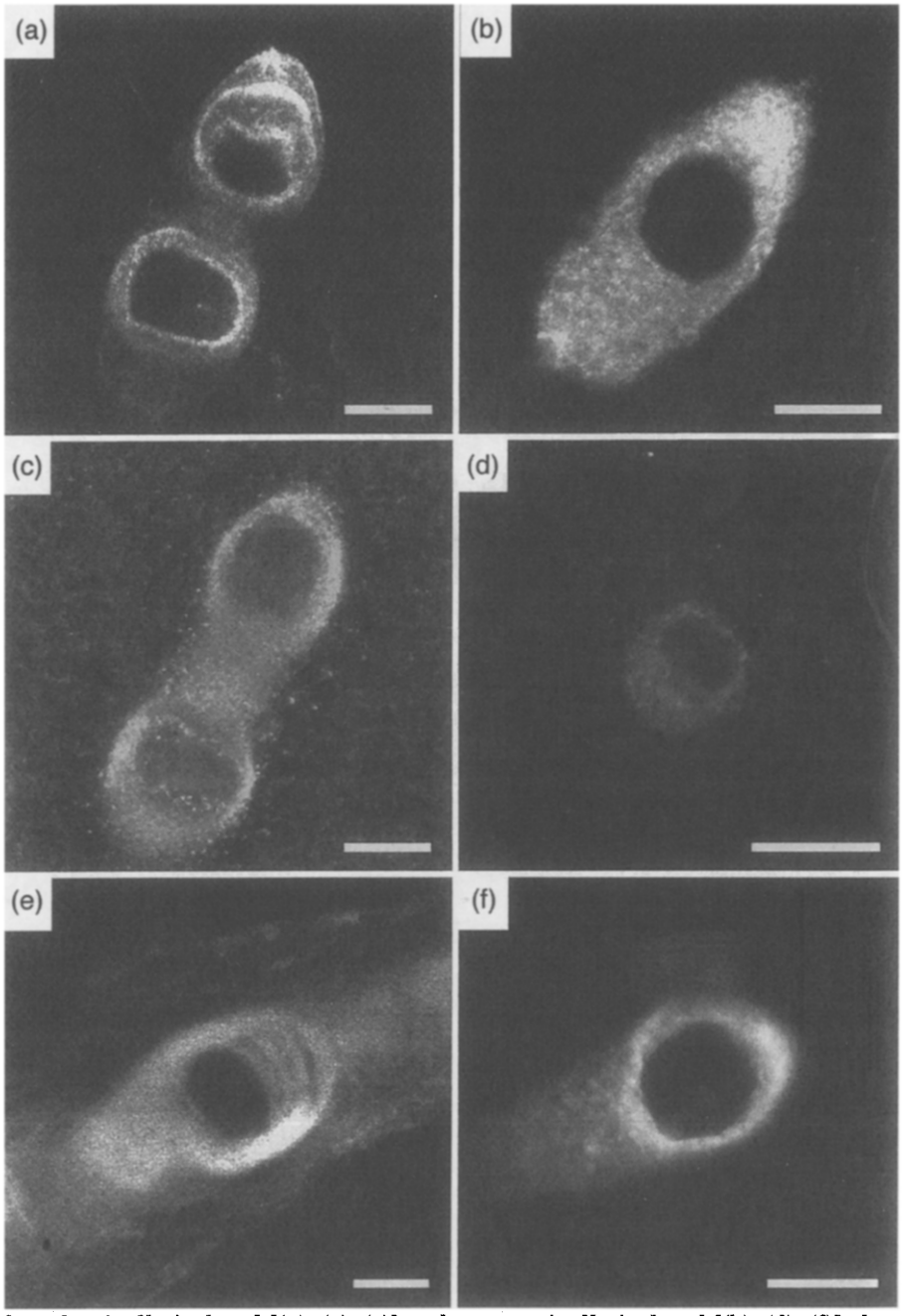

FIG. 4. Comparison of mechanically isolated [(a), (c), (e)] and enzymatically isolated [(b), (d), (f)] chondrons labeled for type VI collagen [(a), (b)] fibronectin [(c), (d)] and the keratan sulfate epitope (5D4) of aggrecan [(e), (f)]. The structure of mechanically isolated chondrons conformed to established patterns of immunolabeling with a strong reation in the pericellular matrix and capsule around each chondrocyte, and a generally weaker reaction in the tail and interconnecting segments [(a), (c), (e)]. In contrast, enzymatically isolated chondrons showed a broader more stippled labeling pattern for type VI (b), a complete absence of pericellular but weak intracellular fibronectin labeling (d) and apparently normal keratan sulfate distribution (f).

always had a loose arrangement. The distribution for the osteoarthritic and nonosteoarthritic samples were significantly different ($P < 0.01$, analysis of variance).

The viability of enzymatically isolated chondrons and chondrocytes 1 day after isolation from nonosteoarthritic and osteoarthritic cartilage was greater than 80%. There was no significant difference in viability for osteoarthritic and nonosteoarthritic chondrocytes at this time point. For the chondrons, non-viable cells were found in single, double and multiple chondron clusters. There appeared to be no relationship between number of chondrons per cluster and cell viability.

Clusters with chondrons ranging from four to 22 contained none, one or two non-viable cells. Only rarely were multiple chondron clusters found with all cells dead. Viability for chondrons continued to be high (≈80%) through 7 weeks of culture. The chondrocytes, on the other hand had lower average viability (60–80%) with longer times in culture (Fig. 6). After 4 months in culture, chondrons from one specimen of osteoarthritic knee cartilage had a viability of 80%.

Cell division was monitored by an increase in the number of cells per 100 chondron clusters or chondrocyte clusters. This approach was possible because isolated chondrocytes and chondrocytes within chondron clusters stay together when they divide in alginate (Fig. 7). This method of quantifying cell division was used instead of [^{3}H]thymidine incorporation because cell division is very slow in alginate bead culture for human chondrocytes from adults. (Cell division occurs at a much higher rate in monolayer cultures; Lee, unpublished observations; [26]). By determining changes in the number of cells per 100 clusters, a much longer window in time could be monitored. Per cent change in number of cells is partially dependent on cell death, as well as cell division, as only viable cells were included in the computations. For chondrons isolated from nonosteoarthritic cartilage specimens, the increase in cell number after 2 weeks was significantly ($P < 0.02$, analysis of variance) greater than with chondrons isolated from osteoarthritic cartilage (Fig. 8). The inhibition of cell division observed at the 2-week time point for the OA chondrons was not observed with the nonosteoarthritic specimens. The nonosteoarthritic specimens were from patients ranging in age from 18–86 years with a mean age of 50 years while the osteoarthritic had a range of 41–78 years with a mean age of 61 years. Although the nonosteoarthritic specimens were younger on the average, the four nonosteoarthritic specimens from individuals with ages over 70 years had an average increase in cell number of 15.8% (range of –2–67) at the 2 week time point, and there was no correlation of per cent change in cell number with age of patient for either the nonosteoarthritic ($r = -0.19$) or osteoarthritic ($r = -0.04$) specimens. There was no significant difference in cell division between the chondrocytes and chondrons from the nonosteoarthritic specimens.

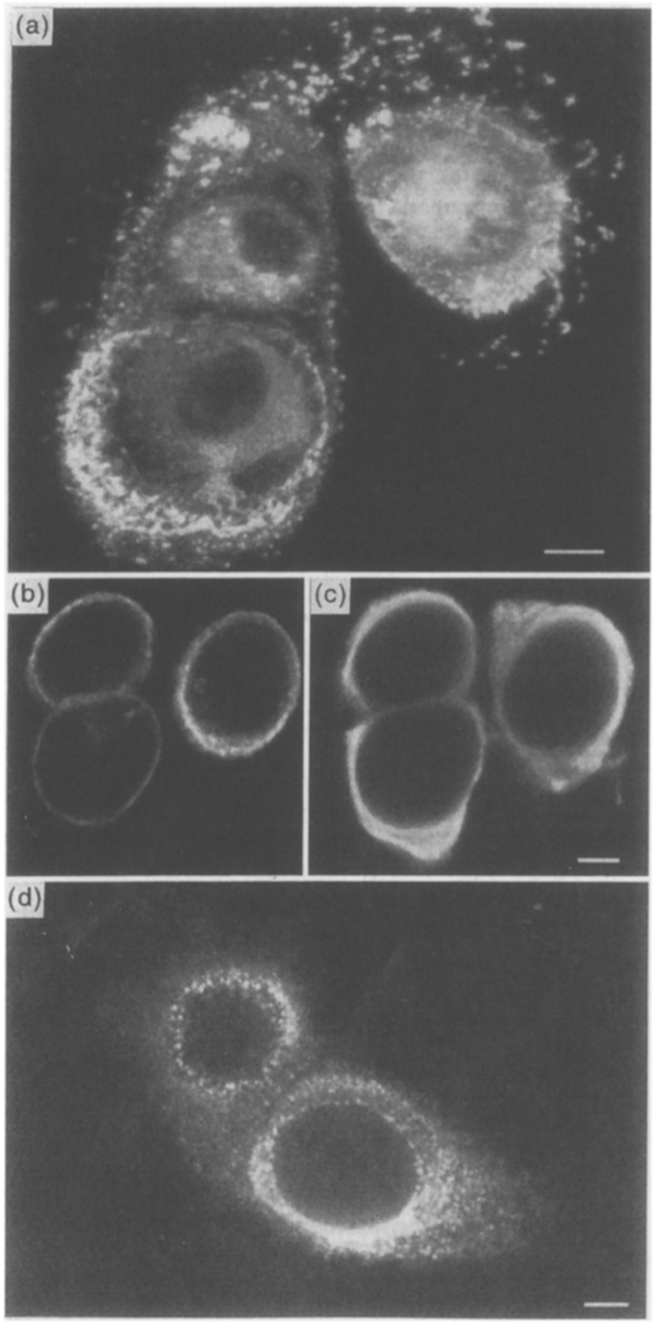

FIG. 5. Chondrons after 1–3 days in alginate bead culture regain matrix macromolecules lost during enzymatic isolation. The confocal micrographs are of chondrons labeled by immunofluorescence for: (a) fibronectin, a chondron doublet and single chondron isolated from normal hip cartilage and cultured for 3 days. Fibronectin labeling was found intracellular, within the pericellular matrix and extending out from the chondrons. (b) CD44, chondrons were isolated from normal hip cartilage and cultured overnight. (c) Type VI collagen, same chondrons shown in (b). (d) Chondroitin sulfate (7D4), a chondron doublet isolated from normal ankle cartilage and cultured overnight. The doublet was tilted relative to the optical axis so the focal level is not through the center of the upper chondrocyte. Each micrograph was from a single optical section except for (a) where seven optical sections taken 0.23 μm apart were combined to show the fibrils which have a three-dimensional arrangement. Bars 5 μm.

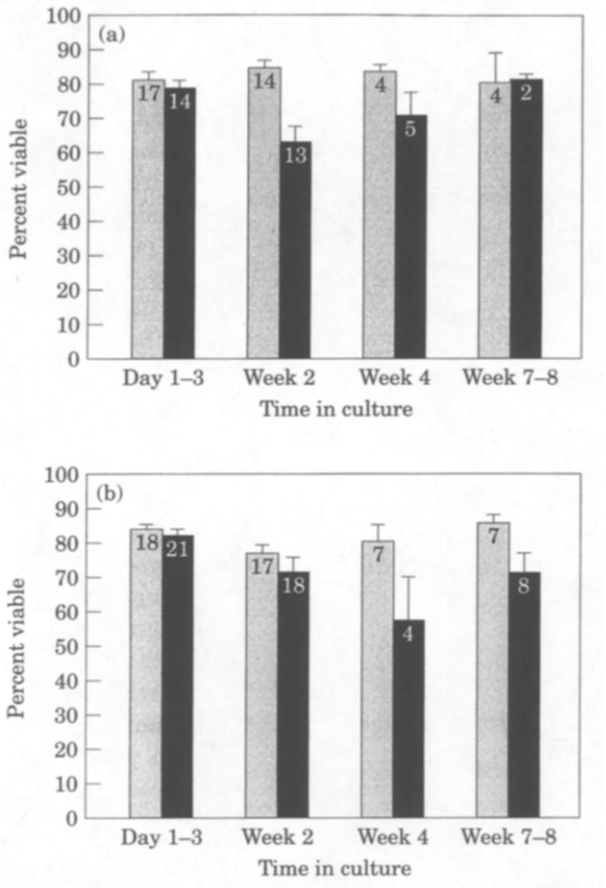

FIG. 6. Viability for chondrons and chondrocytes maintained in alginate bead culture for 7 weeks. (a) nonosteoarthritic specimens, (b) osteoarthritic specimens. Number of specimens is given on the bars. Viability was determined by Hoechst 33342 staining as described in Materials and Methods. (▤), Chondrons; (■), chondrocytes.

The original chondron morphology was lost with cell division. Freshly isolated, chondrons contained only rounded chondrocytes [Fig. 7(a)]. With cell division, the chondrocyte shape became more elliptical and the pericellular matrix no longer formed a uniform rim around individual cells [Fig. 7(c)]. After multiple rounds of cell division for both isolated chondrocytes and chondrons, elongated chondrocytes appeared around the edges of the large clusters [Fig. 7(d)].

Discussion

The use of enzymatically isolated chondrons as an *in vitro* model provides a valuable opportunity to study chondrocyte metabolism in the presence of the chondrocytes' native, three-dimensional pericellular matrix. The natural relationship between cells was maintained in that columns of chondrons and chondron clusters were preserved as structural units. In addition, plasma membrane proteins with extracellular domains are less affected by the more gentle enzymatic isolation procedure than by treatment with pronase or trypsin which are usually used to isolate chondrocytes. While structural studies of isolated chondrons have been greatly facilitated by mechanical extraction procedures, metabolic studies have proved more difficult, and have been limited by the low yield of viable chondrons. The new method of enzymatic chondron isolation presented in this study therefore offers significant advantages in that the same effort is required as that for chondrocyte isolation, and good yield of viable chondrons can be achieved. In addition, chondrocytes isolated as chondrons remained viable and maintained their chondron morphology for extended periods of alginate bead culture. Some differences, however, were found between nonosteoarthritic and osteoarthritic in that cell division was reduced for chondrons and isolated chondrocytes from osteoarthritic compared with nonosteoarthritic specimens.

The ability to isolate chondrons from cartilage matrix by enzyme digestion is not surprising when one considers that the chondron contains the majority of the small quantities of type VI collagen found in the cartilage matrix (Figs 2 and 3; [14]). Type VI collagen, however, is a somewhat unique collagen in that it is not degraded by matrix metalloproteinases or by bacterial collagenase but is digested by some serine proteases [27], and collagenase digestion has previously been used to obtain intact type VI collagen fibrils from skin [28]. Given its strong interaction with a range of cartilage matrix macromolecules including fibronectin [29, 30] and decorin [31], type VI collagen may therefore provide sufficient structure to retain other pericellular matrix components such as type II collagen and aggrecan within the chondron during mild enzymatic digestion. Indeed, the degradation of type II collagen was not complete since type II collagen was found within and associated with enzymatically isolated chondrons [Fig. 3(c)]. In addition, the capsule surrounding the chondron appears to also be retained during the mild protease digestion and may contribute to retaining the chondron structure and some of its components. The pericellular capsule has previously been identified in both canine and human articular cartilage with transmission electron microscopy [10, 32] and is partially composed of type VI collagen [14].

Two matrix macromolecules, however, were lost from the pericellular matrix during the isolation process. These included fibronectin, which is cleaved in several sites by proteases [33], and the

proteoglycans containing the chondroitin sulfate epitope recognized by antibody 7D4. In contrast, aggrecan epitopes reognized by 5D4 were retained after enzymatic extraction. The reason for the loss of the 7D4 epitope is unclear at this time as the proteoglycan with this epitope has not been identified. The pericellular matrix in articular cartilage has been shown to also contain perlecan [34], laminin [34, 35], biglycan [36] and type IX collagen [13, 15, 37]. The retention or loss of these molecules during enzymatic chondron isolation was not investigated in this study, although perlecan has been identified in the matrix of some freshly isolated chondrons (Lee, personal observation). The loss of type IX collagen from osteoarthritic chondron clusters has also recently been reported [15].

Compared with mechanical isolation using low-speed homogenization, enzymatic isolation offers several advantages for obtaining chondrons. The isolation process is much simpler, the yield of viable chondrons is five- to 10-fold higher [12], and sterility is fairly easy to maintain. There also appears to be less interterritorial matrix present in enzyme preparations of chondrons. The chondrons yielded by enzymatic isolation were similar to mechanically isolated chondrons regarding to keratan sulfate and type VI collagen organization [12, 14] but differed particularly regarding fibronectin distribution, which was initially absent from enzymatically isolated chondrons, but recovered during the early stages of culture. With regard to overall morphology, chondrons isolated by both procedures had a rim detectable with DIC microscopy, and included single and multiple chondron clusters. The main structural difference observed was that enzymatically isolated chondrons appeared slightly larger and less accurately defined, and frequently lacked the long tail region reported for mechanically isolated chondrons [12].

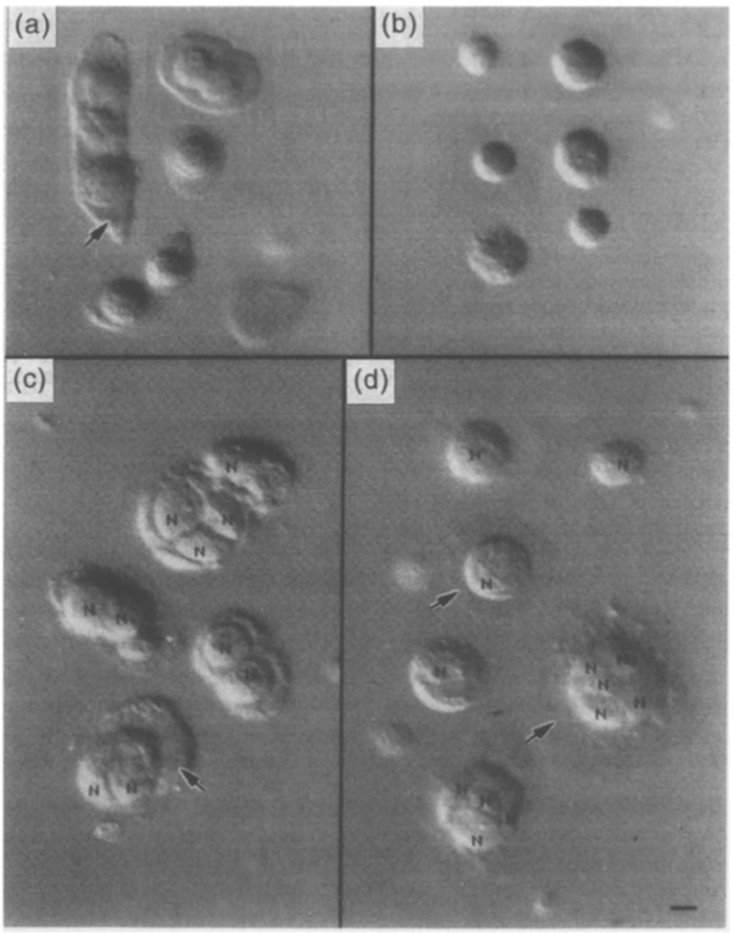

FIG. 7. Chondrocytes whether isolated or in chondrons stayed together in clusters after cell division. Chondrons [(a), (c)] and isolated chondrocytes [(b), (d)] from the same specimen of nonosteoarthritic cartilage. (a) and (b) were taken 1 day after isolation. (c) and (d) were taken after 1 month in alginate bead culture. Positions of nuclei were determined by staining with Hoechst [indiated in (c) and (d) by N]. The pericellular matrix is indicated by arrows. Chondrons and chondrocytes were fixed and embedded in agarose before viewing with DIC microscopy. Representative cells were montaged together. Bar, 6 μm.

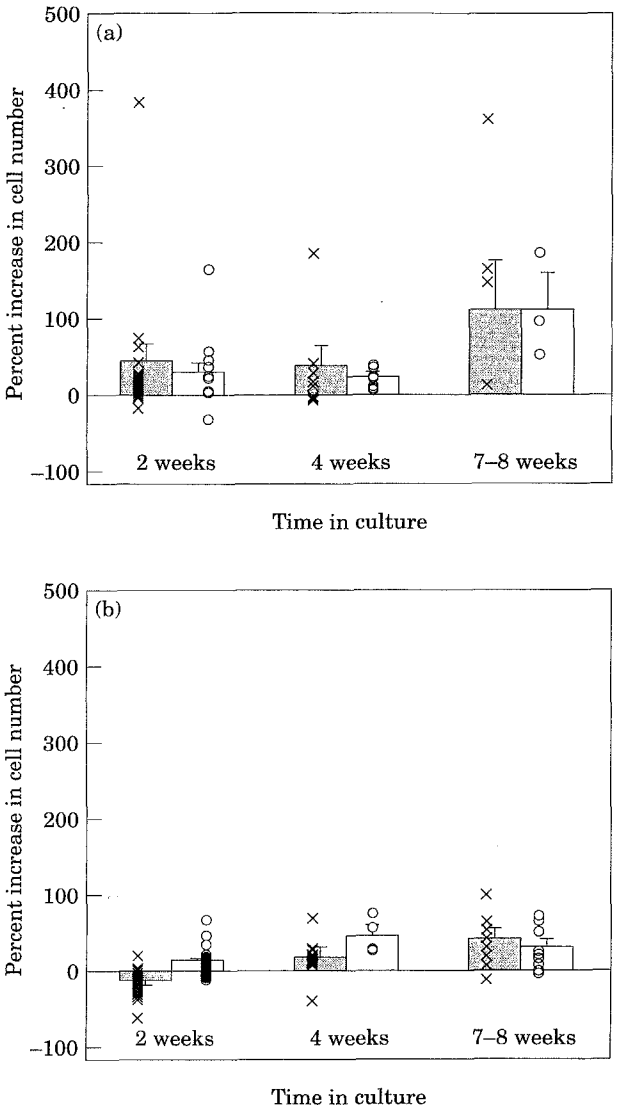

FIG. 8. To monitor cell division, the number of chondrocytes per 100 clusters was determined. The percent change in cell number is computed based on the day 1 counts. The bars show the mean + S.E. The data points are for individual specimens. (a) non-osteoarthritic, (b) osteoarthritic specimens. (▦) Chondrons; (□) chondrocytes; (×) chondrons; (○) chondrocytes.

Pulse chase autoradiographic studies in progress also indicate that enzymatically isolated chondrons retained significantly less [^{35}S]-sulfate labelled aggrecan within the pericellular microenvironment than mechanically isolated chondrons, and that the preferential pericellular sequestration of newly synthesized aggrecan was not maintained past 6 days in enzymatically isolated chondrons (Poole, unpublished observations).

The retention of the native pericellular matrix may be especially important when using *in vitro* models to determine changes in chondrocyte physiology that occur due to osteoarthritis, since the surrounding extracellular matrix is known to affect cell physiology [4], and the pericellular region has been shown to be altered in osteoarthritis [38–43]. Indeed, studies have also shown that some chondrons isolated from osteoarthritic cartilage have a greatly enlarged pericellular matrix relative to those isolated from non-osteoarthritic cartilage (Lee, unpublished observations; [15, 16]). This larger size was probably not due to swelling during the isolation procedure because chondrons with an enlarged pericellular matrix have also been reported in sections of osteoarthritic cartilage and in chondrons mechanically isolated from osteoarthritic canine material (unpublished obvervations; [15, 16]). In this study, we saw greater differences in the rate of cell division between the chondrons and isolated chondrocytes obtained from osteoarthritic cartilage than from nonosteoarthritic cartilage.

This study was primarily concerned with chondrons isolated from human articular cartilage, while comparison of mechanically versus enzymatically isolated chondrons were performed using adult canine material. However, cartilage from other species is frequently used to study chondrocyte metabolism *in vitro*, and thus it is of interest that chondrons can be isolated from other species. Poole and co-workers [16] reported that chondrons isolated from canine articular cartilage were morphologically identical to those isolated from human cartilage, and similar chondron structures have been identified in adult pig articular cartilage [13] and the intervertebral discs of bovine and human samples [44]. We have also found that chondrons can be isolated from mature bovine articular cartilage although preliminary data indicate that the chondrons are slightly smaller than those isolated from nonosteoarthritic human cartilage. When cultured in alginate beads, bovine chondrons produced more collagen fibrils than isolated chondrocytes [18], but similar studies have not yet been performed on canine material.

The culture of enzymatically isolated chondrons therefore offers several possibilities for studying the effect of the *in vivo*-formed pericellular matrix on chondrocyte metabolism and for studying the composition and structure of the pericellular microenvironment. New matrix components are secreted and assembled into the existing pericellular matrix within a 24-h period. The data presented here on viability and cell division, and preliminary data on proteoglycan synthesis [17] indicate that the retention of the *in vivo* pericellular matrix does affect chondrocyte metabolism *in vitro*. Isolated chondrocytes form a pericellular matrix in algi-

nate bead culture [26] but this pericellular matrix has a different structure than that of isolated chondrons [18] and appears to have a different effect on chondrocyte metabolism. Because contacts with extracellular matrix have a direct effect on cell metabolism and the pericellular matrix is altered in osteoarthritis, the osteoarthritic pericellular matrix would be expected to have a different effect on the chondrocyte than the pericellular matrix from normal cartilage. Therefore chondrons isolated from osteoarthritic cartilage should be considered as an alternative *in vitro* model for studying the metabolism of osteoarthritic chondrocytes. The enzymatic isolation of chondrons detailed in this study therefore provides a model system with which to study chondrocyte metabolism within the confines of its natural three-dimensional pericellular microenvironment without the encumbrance of the bulk cartilage matrix.

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