

Two Subpopulations of Differentiated Chondrocytes Identified with a Monoclonal Antibody to Keratan Sulfate

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ABSTRACT We have prepared a monoclonal antibody, named MZ15, that specifically binds keratan sulfate. Immunofluorescence studies showed that the distribution of keratan sulfate in articular cartilage was not uniform: the amount of keratan sulfate increased with distance from the articular surface. Two subpopulations of chondrocytes could be distinguished after isolation from cartilage by the presence or absence of cell surface keratan sulfate. Keratan sulfate-negative chondrocytes were shown to come from the upper cartilage layers. There was therefore a direct correlation between biochemical heterogeneity of cartilage matrix and heterogeneity within the chondrocyte population. During growth in monolayer culture, superficial chondrocytes began to synthesize keratan sulfate, but the cells could still be distinguished from cultures of deep or unfractionated chondrocytes by their reduced substrate adhesiveness and tendency to remain rounded.

Mature articular cartilage is avascular and lacks innervation. It contains a single differentiated cell type, the chondrocyte, which secretes a matrix predominantly of type II collagen and specific proteoglycans. Despite this apparent simplicity, however, the tissue is far from homogeneous. Both the ultrastructure of the chondrocytes and the composition of their extracellular matrix vary with distance from the articular surface.

Several distinct zones of articular cartilage can be distinguished on the basis of chondrocyte morphology and distribution. The superficial zone contains a thin layer of elongated chondrocytes. Beneath this, the middle zone contains oval or rounded cells distributed at random. The third, deep, zone contains groups of large, rounded cells, sometimes arranged in columns perpendicular to the tissue surface. Finally, there is a thin layer of calcifying cells adjacent to bone.

The packing and orientation of collagen fibers vary with depth in articular cartilage, and fiber diameter is greatest in the middle and deep zones (9, 26, 27). The amount of proteoglycan, its monomer size, glycosaminoglycan content, and degree of aggregation with hyaluronic acid all change with distance from the articular surface (1, 8, 20, 27). Such variation in biochemical composition in turn accounts for changes in the physical properties of the tissue with depth (17, 18).

One explanation for the observed matrix heterogeneity is that it reflects intrinsic differences between the chondrocytes

of different zones. However, until now, direct evidence for this has been lacking. In the present study we used a monoclonal antibody to keratan sulfate, MZ15, to identify two distinct subpopulations of differentiated chondrocytes, both in cartilage and after isolation from the tissue. We separated keratan sulfate-positive and -negative chondrocytes and grew them in culture, to discover whether the keratan sulfate phenotype is stable.

MATERIALS AND METHODS

Chondrocyte Isolation and Culture: Cartilage was dissected from the larynx and trotters of 9-mo-old bacon pigs, rinsed in PBS, and finely chopped. Laryngeal cartilage was stripped of perichondrium before chopping. Chondrocytes were released from surrounding matrix by sequential digestion at 37°C with the following enzymes (Sigma Chemical Co., St. Louis, Mo): 0.01% hyaluronidase (20 min); 0.1% pronase (1 h); 0.25% collagenase (1 h); and 0.25% collagenase (1 h). Any cells released by hyaluronidase or pronase were discarded. Cells obtained from the two incubations with collagenase were pooled and passed through a screen of nylon filter cloth (60- μ m aperture; Cadisch and Sons, London) to remove undigested matrix. Cells were considered viable if they excluded a 0.25% solution of trypan blue.

The chondrocyte culture medium was Dulbecco's modified Eagle's medium (DME)¹ containing 10% fetal calf serum. For monolayer culture, 10⁶ cells were plated out per 35-mm-diam petri dish (Falcon Labware, Oxnard, CA), and glass coverslips were added if required for immunofluorescence. Chondrocytes

¹Abbreviation used in this paper: DME, Dulbecco's modified Eagle's medium.

for immunization were incubated overnight in spinner culture. Chondrocytes were also grown in suspension in 1.3% methyl cellulose (a generous gift of Colorcon Ltd., Orpington, England; reference 30) in DME containing one-third of the normal concentration of calcium ions, in order to reduce cell clumping. Suspended cells were harvested by centrifugation after dilution of methyl cellulose with phosphate-buffered saline (PBS).

Preparation and Screening of Monoclonal Antibodies: After overnight incubation in spinner culture, laryngeal chondrocytes were collected by centrifugation and washed once in PBS. We resuspended the cells in PBS and complete Freund's adjuvant (Difco Laboratories Inc., Detroit, MI) and injected 2.5×10^7 cells at multiple subcutaneous sites into each BALB/c mouse. Mice received two booster injections, each of $1.5\text{--}2.0 \times 10^7$ cells, in PBS alone, and were killed 3 d after the final injection.

Spleen cells from an immunized mouse were fused with NS1 myeloma cells, using conventional procedures (19). We tested supernatants from the hybrid cultures for the presence of antibodies reacting with glutaraldehyde fixed pig chondrocytes, but not pig dermal fibroblasts or lymphocytes, in an enzyme-linked immunosorbent assay. Selected clones were subcloned by limiting dilution and retested for chondrocyte-specific reactivity. Clones were further propagated in ascites in Pristane (Aldrich Chemical Co., Ltd., Gillingham, England) primed mice. MZ15, the antibody described in this report, was shown to be a class 1 IgG by immunodiffusion against class and subclass-specific antisera.

Preparation of Proteoglycans and Proteoglycan Fragments: Proteoglycans were extracted from pig laryngeal cartilage with 4 M guanidinium chloride in 0.05 M sodium acetate, pH 5.8, with added proteinase inhibitors, as described previously (12). We performed associative equilibrium density gradient centrifugation on the extract and recentrifuged the bottom fraction (A1) containing proteoglycan aggregate to give a more purified proteoglycan aggregate preparation (AIAI). Proteoglycan binding region, keratan sulfate-rich region, and the stubs of chondroitin sulfate chains attached to peptide were obtained by digestion of the AIAI preparation with chondroitinase ABC (Miles Laboratories Inc., Elkhart, IN) and trypsin (Sigma Chemical Co.). The fragments were separated by chromatography on Sepharose CL6B (Pharmacia Fine Chemicals, Uppsala, Sweden) (16) and binding region was further purified as described by Ratcliffe and Hardingham (28).

Proteoglycan monomer was separated from other components of proteoglycan aggregate by equilibrium density gradient centrifugation under dissociative conditions (12), the bottom fraction containing purified proteoglycan monomer (AIAIDI). Reduction and alkylation of the monomer was performed as described by Hardingham et al. (14). Proteoglycan core protein was prepared by digestion of the monomer with chondroitinase ABC and purified by chromatography on Sepharose CL2B (Pharmacia Fine Chemicals; reference 16). Proteoglycan monomer fragments and bovine corneal keratan sulfate (Miles Laboratories Inc.) were digested with keratanase (Miles Laboratories Inc.), essentially as described by Oike et al. (24). Proteoglycan monomer from rat chondrosarcoma was isolated by the method of Oegema et al. (23).

Radioimmunoassay: Inhibition radioimmunoassay was performed as described previously (28), with the following modifications: the iodinated antigen was ^{125}I -AIAIDI; antibody-antigen complexes were incubated with *Staphylococcus aureus* for 2 h; *S. aureus* was suspended in distilled water and complexes bound to *S. aureus* were washed with distilled water. MZ15 ascitic fluid could precipitate >80% of ^{125}I -AIAIDI. A 1:25,000 dilution, which bound

40% of the ^{125}I -AIAIDI, was used in the radioimmunoassay, and the minimum amount of material that could be detected was 5 fmol.

We compared the inhibitory activity of proteoglycan fragments on a molar basis. We calculated concentrations of inhibitors by assuming the following protein molecular weights: proteoglycan monomer, 2×10^5 ; binding region, 5.5×10^4 ; keratan sulfate region, 4×10^4 ; and chondroitin sulfate peptides, 5×10^3 .

Immunofluorescence: For frozen sections, unfixed tissue was chilled rapidly in hexane to $<-70^\circ\text{C}$; 8–10- μm sections were then cut with a cryostat and air-dried at room temperature before staining. All tissues were from mature animals except for those isolated from 17-d chick embryos. For cell surface staining, cultured chondrocytes in suspension were fixed in 3.7% formaldehyde in PBS at room temperature for 8 min. For intracellular staining of chondrocytes grown on glass coverslips, formaldehyde fixation was followed by incubation in absolute methanol on ice for 5 min.

Cells or tissue sections were incubated in MZ15 (ascitic fluid diluted 1:1,000 in PBS) or rabbit anti-pig type II collagen (diluted 1:20; antiserum generously provided by V. Duance, Meat Research Institute, Bristol) at room temperature for 30 min, washed in PBS, and incubated in fluorescein-conjugated rabbit anti-mouse IgG or fluorescein-conjugated goat anti-rabbit IgG (1:16 dilution; Miles Scientific) as before. After further washing in PBS, we examined specimens under a Zeiss photomicroscope III, using incident illumination. When specimens were incubated with second antibody alone we observed no staining. Digestion of cartilage sections with 0.5 U/ml chondroitinase ABC in PBS at 37°C for 1 h, before incubation with MZ15, did not enhance antibody binding.

We determined the proportion of chondrocytes that stained positively with MZ15 or type II collagen antiserum from phase and fluorescence photographs of several microscopical fields selected at random.

RESULTS

MZ15 Binds to Keratan Sulfate

Injection of whole chondrocytes into BALB/c mice and fusion of the spleen cells with NS1 cells yielded many hybrid clones that secreted antibodies that bound to whole chondrocytes but not to dermal fibroblasts or lymphocytes. One hybridoma secreted IgG1 antibodies, designated MZ15, that recognized cartilage proteoglycan monomer (AIAIDI) in an enzyme-linked immunosorbent assay.

The core protein of cartilage proteoglycan has three separate domains: a highly folded globular region that forms the hyaluronic acid-binding site; a short region that contains most of the attachment sites for keratan sulfate; and a larger chondroitin sulfate-rich region (13). To locate the antibody binding sites of MZ15 more precisely, we used an inhibition radioimmunoassay to determine the ability of various unlabeled proteoglycan fragments to compete with iodinated AIAIDI for antibody binding (Fig. 1).

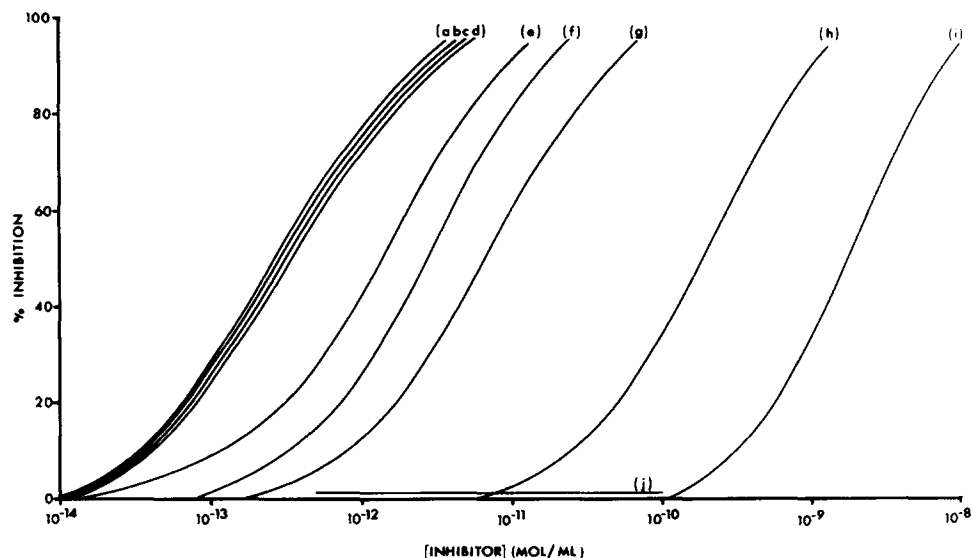


FIGURE 1 Competitive inhibition radioimmunoassay, showing ability of unlabeled proteoglycan fragments to compete with ^{125}I -AIAIDI for binding to MZ15. (a) Keratan sulfate-rich region; (b) reduced and alkylated AIAIDI; (c) AIAIDI after digestion with chondroitinase ABC; (d) AIAIDI; (e) AIAIDI after keratanase digestion; (f) keratan sulfate-rich region after keratanase digestion; (g) hyaluronic acid-binding region; (h) hyaluronic acid-binding region after keratanase digestion; (i) chondroitin sulfate peptides; (j) rat chondrosarcoma AIAIDI.

Reduction and alkylation of proteoglycan monomer, which destroys disulfide bonds that maintain the tertiary structure of the hyaluronic acid-binding region, did not affect the ability of AIAIDI to compete effectively for MZ15 binding (Fig. 1, curves *b* and *d*), and isolated binding region had only 5.5% of the inhibitory activity of intact monomer (Fig. 1, curve *g*). The inhibitory activity of chondroitin sulfate peptides was 0.02% of AIAIDI (Fig. 1, curve *i*), and since removal of chondroitin sulfate with chondroitinase ABC did not reduce the antigenicity of the monomer (Fig. 1, curve *c*), chondroitin sulfate could not be the antigen recognized by MZ15. In contrast, the keratan sulfate-rich region of proteoglycan monomer did retain inhibitory activity (Fig. 1, curve *a*), and keratanase digestion of either AIAIDI (Fig. 1, curve *e*) or the keratan sulfate-rich region (Fig. 1, curve *f*) caused a significant reduction in their ability to compete for MZ15 binding. The inhibitory activity of the hyaluronic acid-binding region, which contains a small amount of keratan sulfate, was reduced to 0.2% that of monomer by treatment with keratanase (Fig. 1, curve *h*). Furthermore, proteoglycan from rat chondrosarcoma, which contains no keratan sulfate, did not bind to MZ15 at all (Fig. 1, curve *j*).

These results suggested that the specificity of MZ15 was towards keratan sulfate. To confirm this, we tested free keratan sulfate chains in the inhibition radioimmunoassay. As shown in Fig. 2, keratan sulfate was a strong inhibitor of I^{125} -AIAIDI binding to MZ15. However, digestion of keratan sulfate chains with keratanase reduced its inhibitory activity to <1%. Thus, the antigenic determinant recognized by MZ15 is part of the keratan sulfate chain that is sensitive to keratanase digestion.

Tissue and Species Cross-reactivity

Keratan sulfate contains a repeating disaccharide of *N*-acetyl-glucosamine and galactose, and is classified into two types on the basis of its linkage to protein: keratan sulfate I occurs in cornea and keratan sulfate II is found in cartilage and intervertebral disc (22). We tested frozen sections of various tissues from different species for MZ15 binding. As Table I and Fig. 3 show, the antibody had wide species cross-reactivity and recognized both skeletal and corneal keratan sulfate.

Although MZ15 bound to mouse cornea, it did not stain mouse intervertebral disc or costal cartilage (Table I), and proteoglycans from these tissues did not have inhibitory activity in the radioimmunoassay (results not shown). This supports the conclusion, from biochemical analysis, that mouse cartilage, unlike cartilage of the other species tested, does not contain keratan sulfate (31).

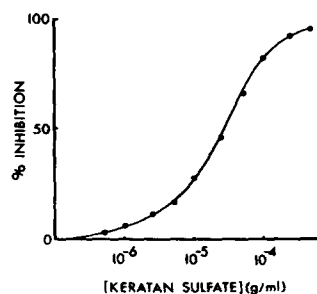


FIGURE 2 Competitive inhibition radioimmunoassay shows the ability of intact corneal keratan sulfate to compete with I^{125} -AIAIDI for binding to MZ15.

Distribution of Keratan Sulfate in Cartilage

In articular and laryngeal cartilage of the species tested (Table I) the distribution of keratan sulfate revealed by immunofluorescence with MZ15 was not uniform. In each case, there were areas of matrix surrounding chondrocytes near the tissue surface that did not bind the antibody (Fig. 3*a*). These

TABLE I. Tissues and Cells Stained with MZ15

Animal	Tissue/cells	MZ15 staining
Pig	Articular and laryngeal cartilage and isolated chondrocytes	+
	Dermal fibroblasts	-
	Lymphocytes	-
Human	Articular cartilage and isolated chondrocytes	+
Rabbit	Skin (epidermis and dermis)	-
	Isolated articular chondrocytes	+
Dog	Cornea	+
	Articular cartilage	+
Chick	Sternal cartilage	+
Mouse	Cornea	+
	Costal cartilage	-
	Lumbar intervertebral disc	-
	Cornea	+

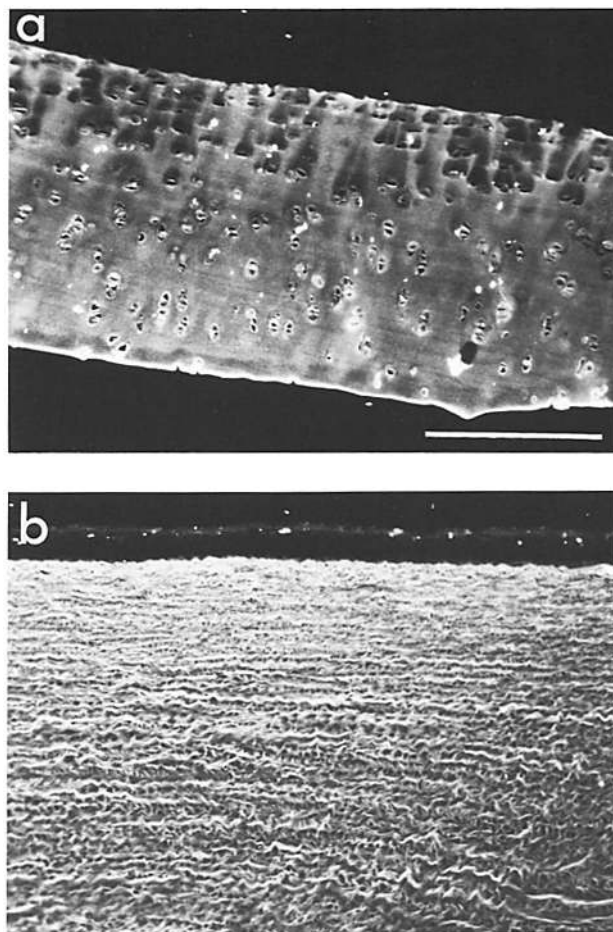


FIGURE 3 Frozen sections of unfixed tissue, stained with MZ15. (a) Pig articular cartilage; (b) rabbit corneal epithelium and part of underlying stroma. Bar, 200 μ m. \times 115.

regions extended beyond the pericellular matrix and resembled teardrops in shape. This prompted us to examine whether chondrocytes isolated from different layers of cartilage showed a corresponding variation in the amount of cell surface keratan sulfate.

Keratan Sulfate on Freshly Isolated Chondrocytes

Chondrocytes from full thickness pig articular cartilage were stained with MZ15 immediately after isolation. 50–60% of cells had surface keratan sulfate, some cells staining more intensely than others (Table II, Fig. 4*a*). All staining was abolished by treating chondrocytes with trypsin or pronase before fixation; this indicates that the keratan sulfate was attached to protein.

There was a distinct correlation between cell size and positive staining in freshly isolated chondrocytes: most of the cells that did not bind the antibody were small. Since the upper layers of cartilage contain smaller cells and less keratan sulfate than do the deeper zones (Fig. 3*a*), we investigated whether small MZ15-negative cells came from near the cartilage surface. The upper 20–30% of articular cartilage was removed by dissection and the chondrocytes were isolated. As Fig. 4*b* illustrates, these superficial chondrocytes were, indeed, smaller than chondrocytes from the deeper layers of cartilage, and most of them did not bind MZ15 (Table II). Chondrocytes were also isolated from cartilage that remained after removal of the upper layers ("deep" chondrocytes). However, because of the undulating surface of the cartilage, the upper layers had not been removed completely, and the proportion of keratan sulfate-positive cells was not significantly increased (Table II).

When superficial and deep chondrocytes were incubated in suspension overnight to allow resynthesis of matrix components (6) the intensity of staining with MZ15 increased (Fig. 4*c*). The proportion of cells with surface keratan sulfate also increased, but there were still fewer superficial cells that stained positively than there were deep cells (Table II). In contrast, the proportion of cells that stained positively with antiserum to type II collagen was the same in both populations (Table II, Fig. 4*d*).

The low proportion of superficial cells with surface keratan sulfate could not be explained by poor viability or loss of biosynthetic capacity, because superficial and deep chondrocytes excluded trypan blue to the same extent (>95% immediately after isolation), and there was no difference in the proportion of cells synthesizing type II collagen after overnight

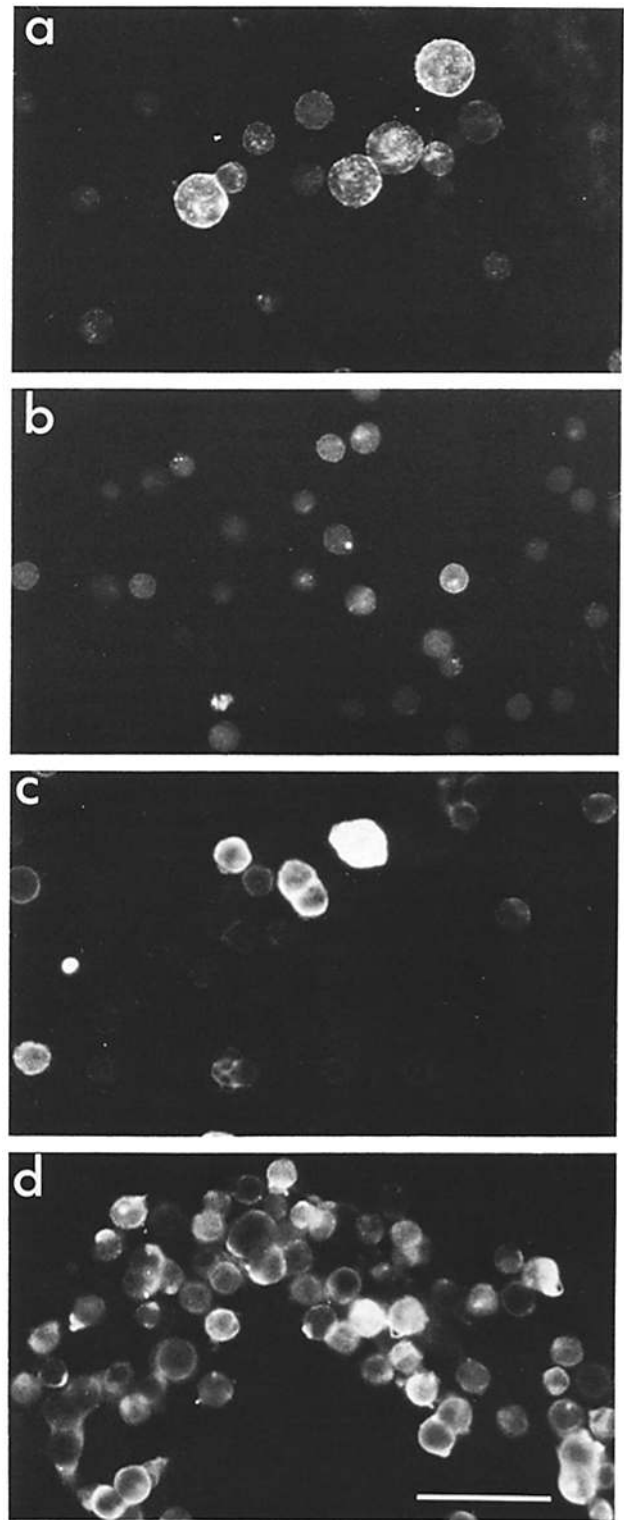


FIGURE 4 Pig articular chondrocytes in suspension. (a) Unfractionated, freshly isolated; (b) superficial, freshly isolated; (c and d) superficial, overnight incubation. Stained with: (a–c) MZ15, and (d) anti-type II collagen. Bar, 50 μ m. \times 363.

incubation in suspension (Table II). Furthermore, superficial chondrocytes were active in proteoglycan synthesis: proteoglycans from the upper layer of cartilage had the same monomer size and degree of aggregation, determined by chromatography on Sepharose 2B (13), as proteoglycans from deep or unfractionated cartilage (results not shown). The presence

TABLE II. Chondrocytes in Suspension Stained with MZ15 or Antiserum to Type II Collagen

Cells*	Antibody	% Positive	No. cells counted
Unfract. fresh	MZ15	54.5	1,057
Superf. fresh	MZ15	15.5	1,324
Deep fresh	MZ15	54.9	1,123
Superf. o/n	MZ15	36.4	618
Deep o/n	MZ15	62.6	952
Superf. o/n	Anti-type II collagen	83.7	602
Deep o/n	Anti-type II collagen	82.8	458

*Cells were isolated from full-thickness (unfract.) or superficial (superf.) cartilage, or from cartilage remaining after removal of superficial cells (deep), and prepared for immunofluorescence immediately after isolation (fresh) or after overnight incubation in suspension (o/n).

or absence of surface keratan sulfate on chondrocytes therefore reflects true heterogeneity within the cell population, and is related to the cells' position within the different layers of cartilage.

Keratan Sulfate in Monolayer Cultures of Chondrocytes

When freshly isolated articular chondrocytes were plated out on glass coverslips or tissue-culture plastic, they attached, and spreading was complete within 2 d. Cells that had begun to spread sometimes had a surface "cap" of keratan sulfate (Fig. 5a) which appeared to be discarded when spreading was complete, because patches of keratan sulfate were visible on the substrate, some distance from fully spread cells (Fig. 5b). However, new matrix synthesis was initiated in monolayer culture (7) and keratan sulfate was found at cell margins in the center of young colonies (Fig. 5, c and d). MZ15 also decorated a region of the cytoplasm close to the nucleus, corresponding to the Golgi region (Fryer, P. R., and A. Ratcliffe, unpublished observations). A few cells with intracellular staining had no surface keratan sulfate (Fig. 5c).

To discover whether the keratan sulfate phenotype of superficial and deep chondrocytes was stable during monolayer culture, the proportion of cells from each population with intracellular or surface keratan sulfate was assessed at intervals after plating. Under the culture conditions used, chondrocytes continued to synthesize aggregatable proteoglycan of the same monomer size as in cartilage for at least 21 d (results not shown). Cell number per dish doubled within 7 d and remained constant thereafter.

Within two days of plating, most of the chondrocytes derived from the upper cartilage layers stained positive with MZ15. By confluence, the proportion of keratan sulfate-positive cells appeared to be the same in superficial and deep cultures and remained unchanged at about 60–70% for up to 21 d in culture (Fig. 6, a and c). Although the two cell populations could no longer be distinguished by MZ15 staining when grown in monolayer culture, they did show consistent differences in culture morphology. Superficial chondrocytes adhered less strongly to the culture substrate during the first 2 d after plating, and tended to remain more rounded than did deep or unfractionated chondrocytes throughout the 21-d culture period (Fig. 6, b and d).

DISCUSSION

Until recently, localization of specific glycosaminoglycans by microscopy relied on indirect methods, such as histochemical staining (21, 29) or a combination of autoradiography and digestion with specific enzymes (15). Now, however, monoclonal antibodies to individual glycosaminoglycans are available (4, 5) and these allow precise and rapid localization by immunofluorescence. This paper describes MZ15, a monoclonal antibody that recognizes both skeletal and corneal keratan sulfate and has wide species cross-reactivity. The antigenic site is sensitive to keratanase digestion, which cleaves keratan sulfate chains predominantly into disaccharides (24). Thus, MZ15 binding depends on glycosaminoglycan chain length. This antibody appears to have similar properties to one recently described by Caterson et al. (4).

We have used MZ15 to demonstrate directly the distribution of keratan sulfate in articular cartilage. The results confirm previous histochemical (21, 29) and biochemical (1, 20)

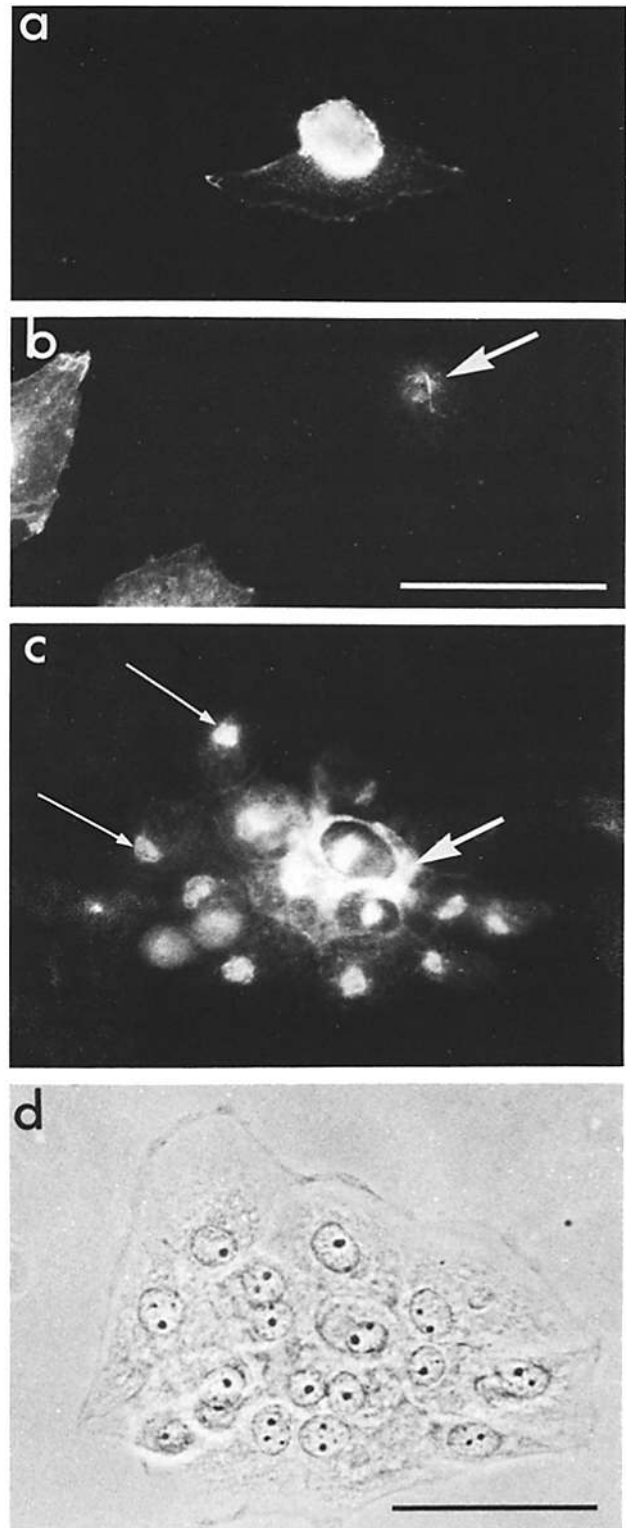


FIGURE 5 (a) Chondrocyte spreading. Note "cap" of keratan sulfate. (b) Keratan sulfate on culture substrate (arrow). (a and b) Bar, 50 μ m. \times 540. (c and d) Small colony of chondrocytes. Note pericellular matrix (thick arrow) and Golgi regions (thin arrows). Bar, 50 μ m. \times 560.

evidence that the amount of keratan sulfate in cartilage increases with depth. This, in turn, has been correlated with an increase in tissue stiffness with distance from the articular surface (18).

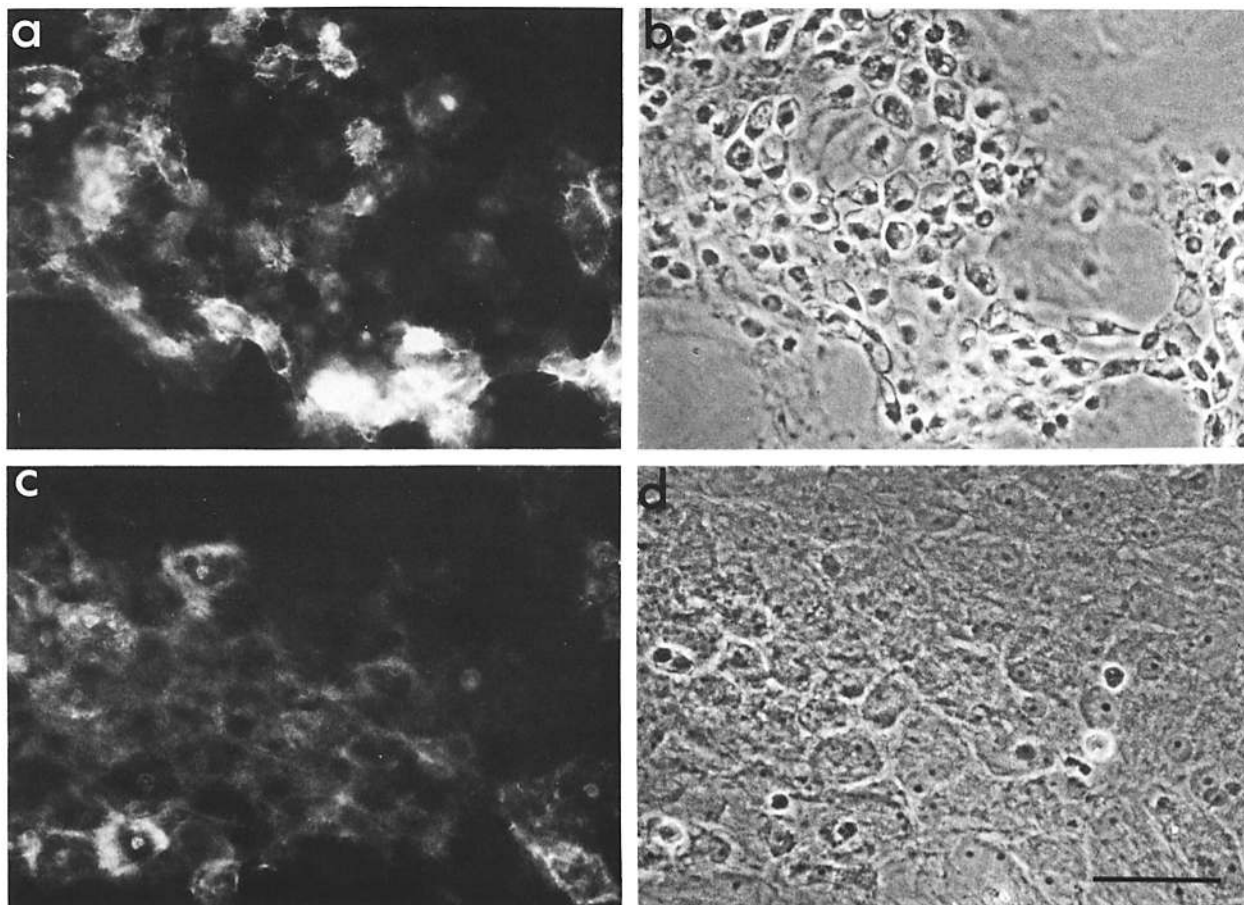


FIGURE 6 Chondrocytes grown in monolayer culture for 14 d were stained with MZ15. (a and b) Superficial and (c and d) deep chondrocytes. Bar, 50 μ m. \times 335.

The pattern of MZ15 staining in articular cartilage suggested that individual chondrocytes differ in their capacity for keratan sulfate synthesis. This was confirmed by the finding that only 50–60% of chondrocytes, either freshly isolated or allowed to recover overnight in suspension, had surface keratan sulfate, as compared with 80–90% that stained positively for surface type II collagen. Furthermore, the cells that lacked surface keratan sulfate came from the upper layers of cartilage.

The difference between superficial and deep chondrocytes cannot be explained by differences in viability or overall capacity for proteoglycan and type II collagen synthesis. Rather, it represents intrinsic heterogeneity within the cell population, which is reflected in the amount of keratan sulfate in different zones of articular cartilage. A similar correlation between heterogeneity of matrix composition and chondrocytes has been found in the different types of collagen synthesized by cells isolated from calcifying and noncalcifying regions of chick cartilage (3, 10).

To determine whether the keratan sulfate phenotype is stable when chondrocytes are grown in monolayer culture, superficial and deep chondrocytes were seeded at high density and stained with MZ15 at intervals after plating. At confluence, the proportion of keratan sulfate–positive cells was the same in both cell populations. This suggests that superficial chondrocytes are capable of synthesizing keratan sulfate, but that synthesis is normally inhibited in the cartilage environment.

When chondrocytes are placed in monolayer culture, they

can divide and spread, make extensive contacts with one another, and initiate new synthesis of matrix components (6, 7). It is perhaps not surprising, therefore, that the cells no longer synthesize the same matrix components as they do in situ in cartilage. It would clearly be of interest to determine culture conditions under which superficial chondrocytes did not initiate keratan sulfate synthesis. In this context, it is interesting that Vertel and Barkman (32) have noted individual chick chondrocytes in culture that do not contain keratan sulfate, even though they are synthesizing chondroitin sulfate proteoglycan.

Although superficial and deep chondrocytes ceased to differ with respect to keratan sulfate synthesis during monolayer culture, some difference in culture morphology was evident. Superficial chondrocytes appeared to attach less strongly to the culture substrate and showed less tendency to spread. Maintenance of rounded morphology has been linked to stability of the differentiated phenotype of chondrocytes (2, 11, 25, 33) and it remains to be determined whether the two subpopulations differ in this respect.

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