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Detection of age-related changes in the distributions of keratan sulfates and chondroitin sulfates in developing chick limbs: an immunocytochemical study

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Summary

A panel of four separate monoclonal antibodies, all known to specifically recognize epitopes on keratan sulfate glycosaminoglycans, were employed in an immunocytochemical study of developing chick hind limbs. In addition, two monoclonal antibodies specific for epitopes on chondroitin/dermatan sulfate glycosaminoglycans were employed on equivalent sections to determine the degree of colocalization of keratan and chondroitin/dermatan sulfates. The spatial distributions of keratan sulfate and chondroitin/dermatan sulfate differed to some extent. In younger embryos, high extracellular concentrations of keratan sulfate occurred in joints and articular cartilages, with diminishing amounts being present in epiphyseal and diaphyseal regions. The high concentration of keratan sulfate in joints and articular cartilage corresponded to equally high concentration of chondroitin-6 sulfate. With advancing age, the above mentioned distribution was modified, most notably by increased amounts of keratan sulfate within diaphyseal regions. Finally, the use of four different anti-keratan sulfate monoclonal antibodies made it possible to compare keratan sulfate epitope expression. Differences in keratan sulfate epitopes were noted in some regions of bones, mostly in diaphyseal regions of younger bones and epiphyseal regions of older bones. This pattern of keratan sulfate expression suggests that different types of keratan sulfate may be present and their expression may be developmentally regulated.

Key words: keratan sulfate, chondroitin sulfate, immunocytochemistry, limb development.

Introduction

Keratan sulfate is a glycosaminoglycan that has been identified as a matrix component of two types of connective tissues, corneal stroma and cartilage matrix (Hascall & Hascall, 1981; Hassell *et al.* 1986). These keratan sulfates are substituted to core proteins that may, particularly in cartilage, also be substituted with chondroitin sulfates (Hassell *et al.* 1986). More recently, the application of immunochemical and immunocytochemical methods has resulted in the identification of keratan sulfate in a much wider variety of tissues (Hyldahl *et al.* 1986; Funderburgh *et al.* 1987; Sorrell *et al.* 1988*b*,*c*). Furthermore, use of anti-keratan sulfate monoclonal antibodies has revealed differences in epitope expression among keratan sulfates (Caterson *et al.* 1986; SundarRaj *et al.* 1986; Funderburgh *et al.* 1987, 1988). Developmental studies, in which more than one anti-keratan sulfate monoclonal antibody was used, have demonstrated both age- and tissue-specific differences in keratan sulfates. SundarRaj *et al.* (1986) have shown that keratan sulfate epitopes appear in a developmentally defined manner in the rabbit eye. Funderburgh *et al.* (1988) found that keratan sulfates in scar tissue from corneal wounds contained a structurally different form of keratan sulfate than that found in normal cornea. Differences in epitopes may be related to variations in sulfation patterns within glycosaminoglycans (Mehmet *et al.* 1986; Tang *et al.* 1986).

In the present study, four separate anti-keratan sulfate monoclonal antibodies were used to immunocytochemically identify keratan sulfate in developing chick hind limbs. These antibodies were used, not only to identify keratan sulfate, but to determine whether there were spatial-, age-, or epitope-specific differences. Keratan sulfate was identified as early as day 5·5 of incubation. At that age, it was largely confined to joints and articular cartilages, with lesser amounts in epiphyseal cartilages. These results correspond to the identification of keratan sulfates in tendon, ligament, and other noncartilaginous components of joints (Craig *et al.* 1987*b*; Vogel & Evanko, 1987; Daniel & Mills,

1988). With advancing age, this distribution of keratan sulfate gradually changed. Extracellular keratan sulfate now appeared in central regions of bones.

The four anti-keratan sulfate monoclonal antibodies differed in their ability to detect keratan sulfate in some regions of developing bones. This was particularly evident in the intracytoplasmically identified keratan sulfate of younger bones. However, differential keratan sulfate epitope expression continued to be present in cartilage of older bones. Thus, this study indicates that keratan sulfate is an early component of developing chick bones and that its expression, like that of chondroitin sulfates (Kimata *et al.* 1974; Stocum *et al.* 1979; Shinomura *et al.* 1984), appears to be developmentally regulated.

Table 1. *Antigen specificity for anti-keratan sulfate monoclonal antibodies*

Antibody	Source of antigen	Relative competitive inhibition (nanograms giving 50 % inhibition)	
		KSI	KSII
5D4	Human articular cartilage	430	33
1B4	Bovine nasal cartilage	500	500
2D3	Bovine nasal cartilage	115	13
4A1	Bovine nasal cartilage	45	52

All four antibodies were raised against purified cartilage proteoglycan obtained from the sources indicated. Their epitope specificities were compared in a competitive radioimmunoassay using corneal keratan sulfate type I (KSI) and skeletal keratan sulfate type II (KSII) as competing (non-labelled) antigen.

Materials and methods

Antibodies

All four anti-KS monoclonal antibodies, 5D4, 1B4, 2D3, and 4A1, used in this study were raised against purified mammalian cartilage proteoglycans. The four anti-keratan sulfate antibodies were characterized using a competitive radioimmunoassay in which ^{125}I-bovine nasal cartilage-ABC-core was the labelled antigen and keratan sulfate type I (Sigma) and keratan sulfate type II (Martin & Mathews NIH standard) were the unlabelled antigens (Caterson *et al.* 1983). The data were expressed as nanograms of unlabelled antigen required to give a 50 % inhibition.

The two monoclonal, anti-chondroitin/dermatan sulfate antibodies, 2B6 and 3B3, were raised against chondroitinase ABC-digested cartilage proteoglycans. These antibodies recognized chondroitin-4- and dermatan sulfates and chondroitin-6-sulfate, respectively (Caterson *et al.* 1985, 1986).

Tissue preparation and immunocytochemistry

Tissues were taken from outbred lines of White Leghorn chick embryos at the ages indicated in the results section. At earlier stages, three embryos were used at each time point; two each were used for later stages. Tissue processing and indirect immunoperoxidase immunocytochemistry were performed as previously published (Sorrell *et al.* 1988*a*). None of the immunostained samples that are present in the text were counterstained. However, other samples were counterstained with hematoxylin to facilitate localization of intracellular epitopes.

Controls

Separate controls were performed for each antibody and each tissue. These included sections in which the primary antibody incubation step was omitted and sections in which the primary antibody incubation was performed, but in the presence of $1\,\mathrm{mg\,ml^{-1}}$ of purified corneal keratan sulfate (type I). No immunostain was detected in either situation.

Results

The distribution of keratan sulfate glycosaminoglycans was studied, using an indirect immunoperoxidase method, in embryonic chick hind limbs. Four separated monoclonal antibodies, each antibody recognizing epitopes present in both keratan sulfate type I and type II, were used. Each antibody varied in its ability to recognize epitopes on these two forms of keratan sulfate (Table 1). This suggests that these antibodies may recognize subtle epitope differences within glycosaminoglycan chains.

All four antibodies identified what appeared, at low magnification, to be discrete foci of immunostain in day 5·5 and day 6·5 limbs (not shown). At higher magnification, these foci were found to correspond to joints and articular surfaces of the knee (see below), tibiotarsal, and metatarsophalangeal joints (not shown). Lesser, diminishing amounts of keratan sulfate were detected in epiphyseal and diaphyseal regions of both femurs and tibias.

Others have already described the distribution of keratan sulfate in the metatarsophalangeal joint (Craig *et al.* 1987*a*); therefore, this study has concentrated on results obtained from the knee joint. The structure of the anterior region of a knee joint obtained from a day 6·5 embryo is shown in Fig. 1. The joint capsule, articular cartilages, femoral and tibial heads are identified. The joint cavity has not yet formed; consequently, the femur and tibia are still connected by cells that will subsequently degenerate and die to form the joint space. This latter is termed the interzone region (Holder, 1977).

All four anti-keratan sulfate monoclonal antibodies identified high levels of keratan sulfate in the joint region (Fig. 2A). Intense zones of immunostain were confined to articular cartilages and to a band, which apparently connected the femoral and tibial heads, that extended along the apical margin of the joint capsule. This band may represent either ligament or developing patellar tissue (Fell & Canti, 1934). Both the interzone region and the region immediately subjacent to the immunostained apical band lacked detectable keratan sulfate. Also, no keratan sulfate was detectable in the posterior aspect of the joint capsule (not shown). Lesser, but detectable, levels of keratan sulfate were present in epiphyseal cartilages. This nonuniform distribution of keratan sulfate did not reflect the distribution of all glycosaminoglycans, since immunostaining using anti-chondroitin sulfate antibodies 3B3 and 2B6 pro-

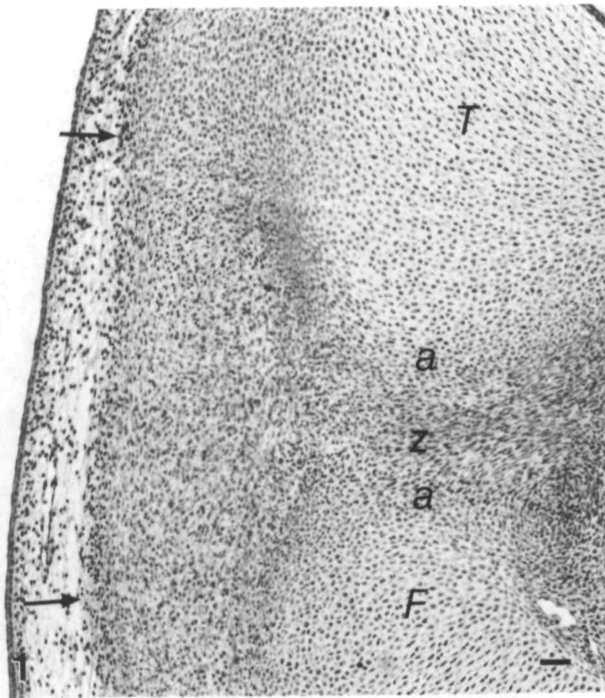

Fig. 1. The anterior portion of a knee joint from a day 6·5 embryo is shown. The femoral (F) and tibial (T) heads are covered by articular cartilage (a). These two bones are still connected by cells forming the interzone region (z). Arrows indicate the anterior margin of the joint capsule. Hematoxylin and eosin, ×115, bar = 35 μm.

duced very different patterns (Fig. 2B,C). Chondroitin-6 sulfate (3B3) was intensely detected in joint and articular regions, including regions where there was no detectable keratan sulfate (compare Fig. 2A and B). In contrast, chondroitin-4/dermatan sulfates appeared to be generally absent from these sites (Fig. 2C). These results suggest that some chondroitin sulfate proteoglycans in the joint may be substituted with both chondroitin and keratan sulfate glycosaminoglycans, while others may be substituted with only chondroitin sulfates. It also indicates that a differential distribution of chondroitin and keratan sulfates occurs at this early stage of development.

The abrupt transition between articular and epiphyseal cartilages was due to the amount of extracellular keratan sulfate. In contrast to the articular zone, only a thin halo of pericellular keratan sulfate was seen surrounding many epiphyseal chondrocytes (Fig. 3). In the diaphyseal region, even this pericellular immunostain was not detected by any of the anti-keratan sulfate antibodies (bottom of Fig. 3 and Fig. 4). Based upon studies of counterstained samples (not shown), keratan sulfate epitopes were restricted to a single juxtanuclear site. This was not evident in all cells, possibly due to the fact that this is sectioned tissue and that some juxtanuclear sites may have been out of the plane of section (Fig. 4). Three out of four anti-keratan sulfate antibodies identified this intracellular keratan sulfate (see below). This pattern of keratan sulfate distribution translated, at lower magnifications, to a gradient in keratan sulfate content. As in the joint region, the apparent lack of extracellular keratan sulfate in diaphyseal regions was not due to the lack of proteoglycans, since anti-chondroitin sulfate antibodies readily detected extracellular glycosaminoglycans in this zone (Fig. 5).

The absence of extracellular keratan sulfate in the diaphyseal region appeared to be age-related, since extracellular keratan sulfate could be detected within this region of bones obtained from older embryos. By day 9·5, both pericellular and extracellular keratan sulfate were detected by all four anti-keratan sulfate antibodies (Fig. 6). In fact, by day 11·5, keratan sulfate was intensely detected in diaphyseal regions (Fig. 7). In other words, the high content of diaphyseal keratan

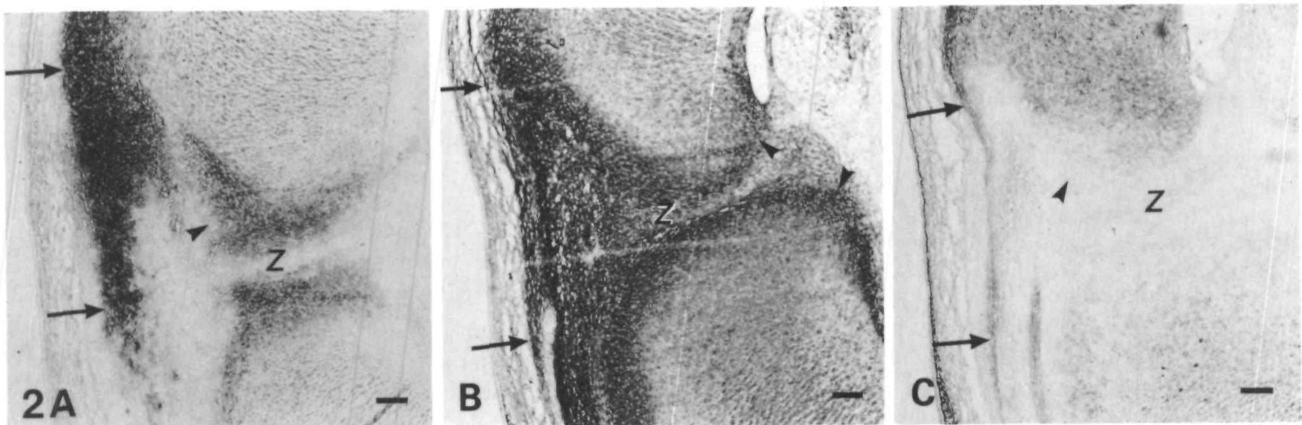

Fig. 2. (A) Keratan sulfate (antibody 4A1) is intensely immunostained in articular cartilage (arrowheads) and in a band extending along the apical margin of the joint capsule (arrows). Epiphyseal cartilages of femoral and tibial heads is less intensely immunostained. No keratan sulfate is detected in the interzone (z) region. (B) Unlike keratan sulfate, chondroitin-6 sulfate (antibody 3B3) is present throughout the joint capsule, including the interzone region. It is also readily detected in articular and epiphyseal cartilages. (C) Chondroitin-4/dermatan sulfate (antibody 2B6) distribution is very different from both keratan sulfate and chondroitin-6 sulfate. It is almost completely absent from the joint region and from articular cartilages, but is detected in epiphyseal cartilages. ×90, bar = 40 μm.

Fig. 3. Extracellular keratan sulfate (antibody 4A1) is readily detected in articular cartilage (a) from a day 6·5 femur. However, an abrupt transition occurs at the articular-epiphyseal boundary. In the epiphyseal region (e), keratan sulfate is restricted to a pericellular region around most chondrocytes (arrowheads). No pericellular keratan sulfate is detected around any chondrocyte (arrows) in the proximal portion of the diaphysis (d). ×380, bar = 13 μm.

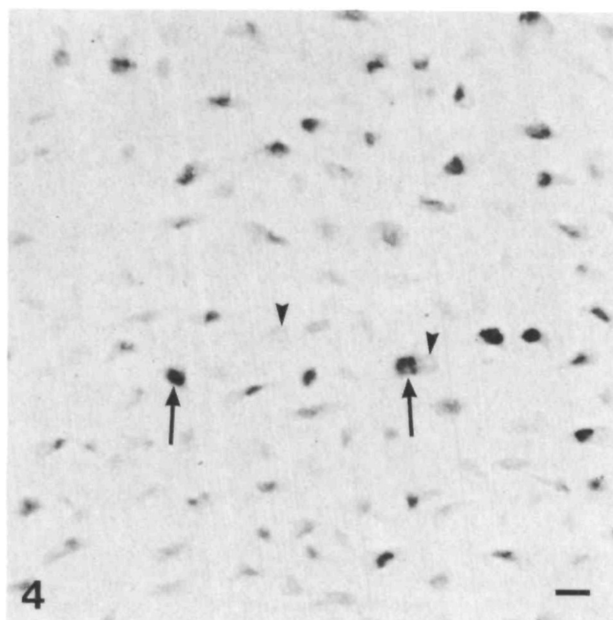

Fig. 4. At higher magnifications no extracellular or pericellular keratan sulfate can be detected in mid-diaphyseal regions (same bone as shown in Fig. 3). Cell surfaces of chondrocytes lack detectable pericellular immunostain (arrowheads). However, intracellular keratan sulfate (antibody 4A1) is present in many chondrocytes (arrows). ×600, bar = 8 μm.

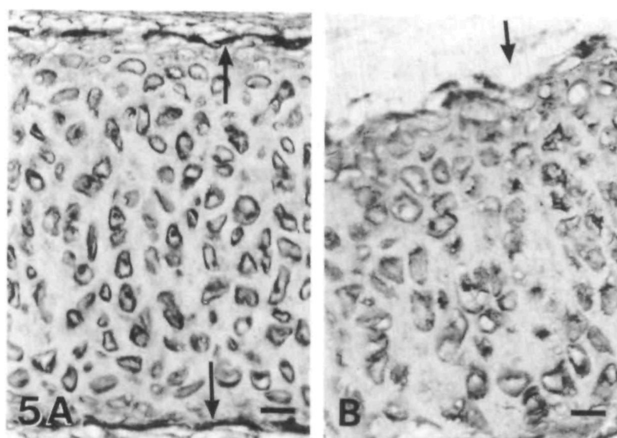

Fig. 5. Extracellular and pericellular chondroitin sulfates are present in the diaphyseal regions of bones from day 6·5 embryos. The distributions of different chondroitin sulfates are similar except that (A) chondroitin-4/dermatan sulfate (antibody 2B6) is detected in osteoid (arrows), but (B) chondroitin-6 sulfate (antibody 3B3) is not detected (arrow). ×320, bar = 16 μm.

sulfate persisted in bones of older embryos, as did the high content of keratan sulfate in articular cartilages and joint tissues, such as the meniscus (Fig. 8). Thus, the distribution of keratan sulfate appears to be both spatially and age-related.

Differences in epitope expression were detected by comparing the results of the four anti-keratan sulfate monoclonal antibodies. In younger embryos, all four monoclonals detected epitopes in joints, articular and epiphyseal regions. However, there were distinct differences in keratan sulfate epitopes detected in middle regions of bones. Antibody 4A1 provided the strongest immunocytochemical detection of intracytoplasmic epitope; while antibodies 2D3 and 5D4 both provided intermediate detection; and, finally, antibody 1B4 barely detected epitope (Fig. 9). These results strongly suggest that a differential expression of keratan sulfate occurs at this site. Similarly, differences in keratan sulfate epitope expression were noted in bones obtained from older embryos. As shown in Fig. 10, antibodies 2D3 and 5D4 differentially identified keratan sulfate in femoral condyle from a day 18 embryo. Both monoclonals detected cell-associated epitope, but only antibody 2D3 detected extracellular epitope, particularly in articular cartilage. Thus, different forms of keratan sulfate likely exist and can be discriminated immunologically. Furthermore, their expression appears to be related to both the location of the chondrocyte producing the epitope and the developmental age of that chondrocyte.

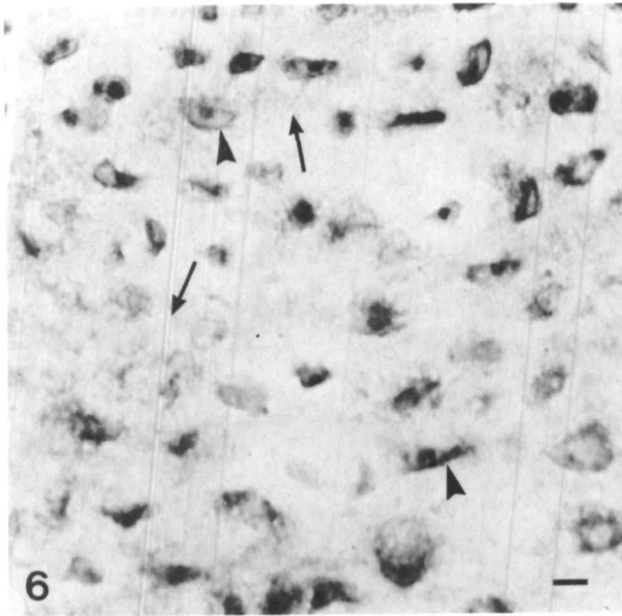

Fig. 6. Extracellular (arrows) and pericellular (arrowheads) keratan sulfate (antibody 2D3) is detectable in the diaphyseal regions of bones from day 9·5 embryos (note the differences in the immunostaining pattern shown in this bone compared with that shown in Fig. 4). ×585, bar = 9 μm.

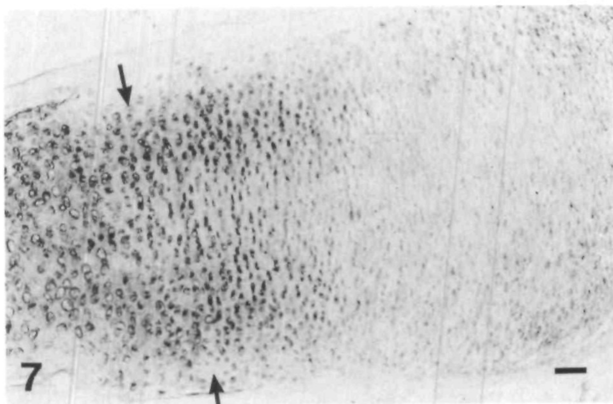

Fig. 7. Extracellular keratan sulfate is readily detected in the mid-diaphyseal region (arrows) of bones older than that shown in Fig. 6 (antibody 5D4, day 11 embryo). ×75, bar = 53 μm.

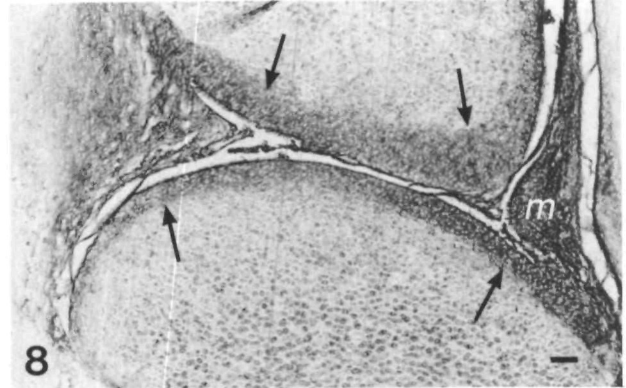

Fig. 8. Articular cartilages (arrows) and joint tissues of the knee, such as the meniscus (m), continue to be intensely immunostained by anti-keratan sulfate antibodies (antibody 2D3) in older (day 9·5) embryos. ×95, bar = 42 μm.

Discussion

The most striking feature observed in developing hind limbs of chick embryos was the high content of keratan sulfate, as determined by immunocytochemical analyses. Even more interestingly, keratan sulfate was not uniformly distributed throughout cartilage, but was concentrated within joints and developing articular cartilage surfaces. In contrast, chondroitin/dermatan sulfates were more widely distributed throughout all regions of cartilage. The highest extracellular concentrations of keratan sulfate corresponded to regions that also contained a high content of chondroitin-6 sulfate, but low chondroitin-4/dermatan sulfate content. Taken together, these observations indicate that metabolic heterogeneity, in terms of glycosaminoglycan synthesis, occurs, and that these different populations of cells are highly organized into identifiable regions.

Similar conclusions, based upon data obtained from biochemical studies of cartilages, have been reached by others (De Luca *et al.* 1977; Garg *et al.* 1981; Pacifici *et al.* 1981; Webber *et al.* 1987). The relative contents of chondroitin and keratan sulfate were found to vary according to location and to depth within cartilage (Franzen *et al.* 1981; Zanetti *et al.* 1985; Williams *et al.* 1988). Vasan & Lash (1978) found that articular cartilage of embryonic chick tibias contained a higher keratan sulfate content than did epiphyseal cartilage. This was confirmed in this study; furthermore, the present data indicate that equally high contents of keratan sulfate were located in some of the noncartilaginous tissues of the developing joint.

In bones from younger embryos, a gradient in keratan sulfate content was observed that, upon closer inspection, proved to be related to the amount of extracellular keratan sulfate. In bones from younger embryos, extracellular keratan sulfate was detected in articular and epiphyseal regions. Even between these latter two zones remarkable differences in the amounts of keratan sulfate were noted; considerably more extracellular keratan sulfate was present in the articular zone. In the zones containing flattened cells and hypertrophic cells, keratan sulfate was restricted to intracytoplasmic, juxtanuclear regions, presumably corresponding to the Golgi apparatus, as has been previously suggested (Vertel & Barkman, 1984). Vertel & Barkman (1984), in a study of cultured embryonic chick chondrocytes, found that, while all of these cells produced extracellular chondroitin sulfate, only some produced detectable extracellular keratan sulfate. However, keratan sulfate could be detected in some of those cells that did not display this glycosaminoglycan extracellularly. From this they concluded that chondroitin and keratan sulfate synthesis and secretion were inde-

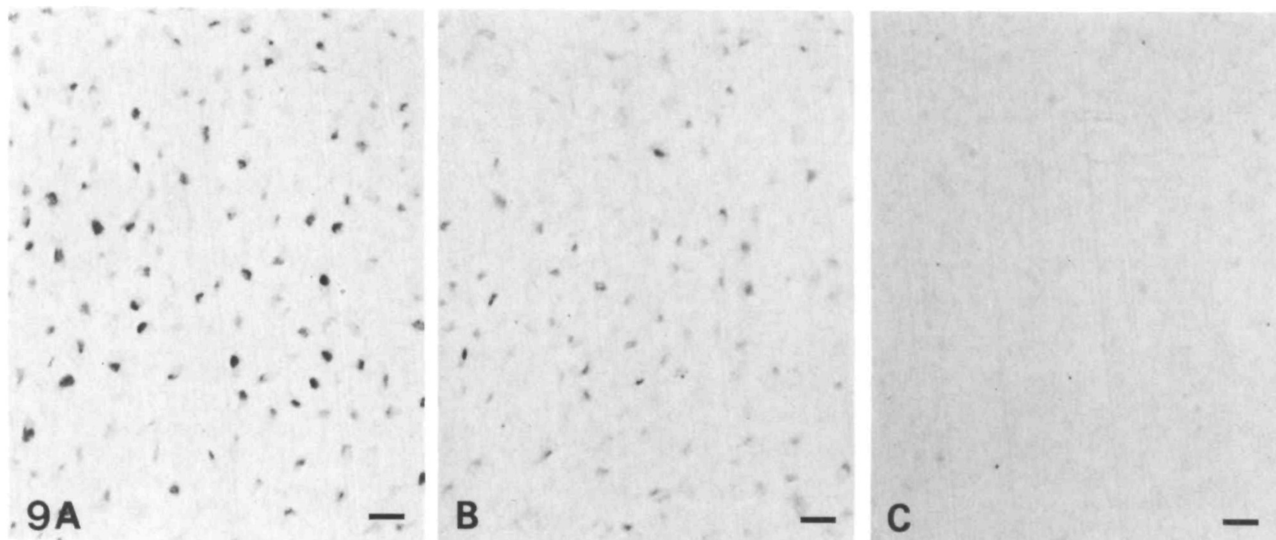

Fig. 9. Sections obtained from diaphyseal region of the same day 6·5 tibia have been immunostained using antibodies 4A1 (A), 2D3 (B), and 1B4 (C) and have been presented for comparison. Note that there are significant differences in epitope detection. Antibodies 4A1 and 2D3 (also antibody 5D4, not shown) detect epitope which is located intracellularly (see Fig. 4). In contrast, antibody 1B4 fails to detect epitope in this region. ×340, bars = 15 μm.

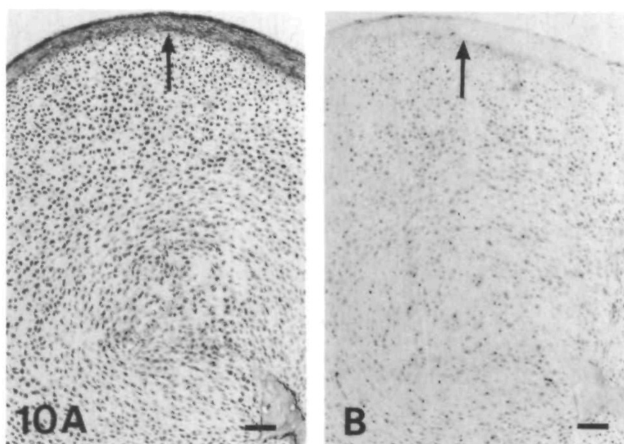

Fig. 10. Sections of a day 18 femoral condyle have been immunostained using antibodies 2D3 (A) and 5D4 (B). Note that, in both figures, epiphyseal chondrocytes are darkly immunostained, indicating the presence of keratan sulfate closely associated with these cells. In contrast, keratan sulfate within extracellular matrix, especially that within articular cartilages (arrows), is identified by antibody 2D3 (A), but not by antibody 5D4 (B). ×65, bars = 60 μm.

pendently regulated. Zanetti *et al.* (1985), in a separate study, reached similar conclusions. In intact bones, a similar independent regulation also appears to exist, but here, regulation seems to depend upon the location of chondrocytes within the developing bone. These results indicate that the differential ability of chondrocytes to store or to secrete keratan sulfate relates to developmental age.

Substantial, age-related increases in keratan sulfate content have been documented in both mammalian and avian cartilages. This increase is accomplished by the substitution of more, and longer, keratan sulfate chains to chondroitin sulfate proteoglycans (De Luca *et al.* 1977; Garg *et al.* 1981; Pacifici *et al.* 1981; Webber *et al.* 1988). In the chick embryo, increased keratan sulfate was immunocytochemically detected by day 9·5. At that time, extracellular keratan sulfate was noted in the diaphyseal regions of both femurs and tibias. Extracellular keratan sulfate persisted in all regions of cartilage in older embryos. Thus, the present data indicate that most chondrocytes are capable of synthesizing keratan sulfate, as evidenced by its intracytoplasmic detection. However, the secretion of keratan sulfate, and/or its extracellular metabolism, may be independently regulated.

With the development of anti-keratan sulfate monoclonal antibodies has come the realization that keratan sulfate is much more widely distributed than had previously been expected (Funderburgh *et al.* 1987). Recent immunocytochemical studies have identified keratan sulfate in such noncartilaginous tissues as tendons, ligaments, and other joint tissues (Craig *et al.* 1987*a*,*b*). In addition, the development of significant numbers of different anti-keratan sulfate monoclonal antibodies has revealed that considerable heterogeneity exists within keratan sulfate chains, indicating that keratan sulfate glycosaminoglycan structure is not simply related to keratan sulfate type I or type II (SundarRaj *et al.* 1986; Funderburgh *et al.* 1987, 1988). At least some of this heterogeneity is likely related to variations in sulfation patterns that occur within these chains (Mehmet *et al.* 1986; Tang *et al.* 1986; Funderburgh *et al.* 1987). Direct evidence for differences in keratan sulfate epitopes has been documented for antibodies 5D4 and 1B4. Antibody 5D4 appears to recognize a more highly sulfated epitope than does antibody 1B4 (Mehmet *et al.* 1986). Others have also

suggested that sulfation of keratan sulfate is an important factor in tissue and developmental distribution of keratan sulfate (SundarRaj *et al.* 1986; Funderburgh *et al.* 1988). The differential detection of keratan sulfate by monoclonal antibodies in chick cartilage suggests that the types of keratan sulfate are developmentally related. Too little is yet known to make predictions about the role that different forms of keratan sulfate might have in influencing the structure of extracellular matrices.

In conclusion, keratan sulfate glycosaminoglycans are present in embryonic chick limbs at very early stages. Their synthesis and distribution, like that of chondroitin/dermatan sulfate glycosaminoglycans (Shinomura *et al.* 1984), appears to be both spatially- and age-related. Presumably, this differential keratan sulfate content has important influences on the structure and function of extracellular matrices present in different locations.

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