Modulation of native chondroitin sulphate structure in tissue development and in disease

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Summary

Chondroitin sulphate proteoglycans are synthesised by different tissues and cell types, and the chondroitin sulphate chains are variably sulphated. Three monoclonal antibodies 3B3, 7D4 and 6C3 that recognise different native chondroitin sulphate epitopes have been used to investigate changes in structure during embryonic tissue development in the chick and in the response of mature canine articular cartilage during experimental osteoarthritis. Strong focal expression of the epitopes was seen during development of chick bursa, which was different for the three epitopes and which changed during 5 days of development. In embryonic chick limb, although chondroitin sulphate is present throughout the cartilage, the 3B3 epitope, which is at the non-reducing terminus of chains, was only expressed on chondroitin sulphate within one region of the sub-articular cartilage. In mature canine articular cartilage the expression of this epitope on proteoglycans was very low, but when determined 3 or 6 months after induction of experimental osteoarthritis the level was greatly increased in all joints tested (23/23). The abundance of the other two native chondroitin sulphate epitopes was also increased in this experimental disease. The results show that expression of the chondroitin sulphate epitopes detected by the monoclonal antibodies changes during cellular differentiation and development and suggests that it is closely controlled by the cells synthesising chondroitin sulphate chains.

Key words: chondroitin sulphate, monoclonal antibodies, osteoarthritis.

Introduction

Chondroitin sulphate, a common component of vertebrate tissues, is a glycosaminoglycan that is covalently attached via a neutral trisaccharide linkage region to specific proteins to form proteoglycans that may occur intracellularly, on cell surfaces, or, more abundantly, within extracellular matrices (Heinegård and Paulsson, 1984; Höök et al. 1984; Hardingham, 1986; Ruoslahti, 1988). The primary component of chondroitin sulphate is a repeating disaccharide sequence, of glucuronic acid and N-acetyl galactosamine residues, that can be differentially sulphated on the 4- or 6- positions of the galactosamine, or can be non-sulphated. Previously, monoclonal antibodies were prepared against chondroitin sulphate proteoglycans that had been digested using chondroitinase ABC (Caterson et al. 1985), an eliminase that digests the chondroitin sulphate chains and that leaves a product containing a terminal unsaturated glucuronate residue (Caterson et al. 1985). Use of these antibodies, following chondroitinase ABC digestion, shows interesting variations in the tissue distribution of predominantly 4-, 6- or unsulphated disaccharide repeats. Use of these monoclonal antibodies has lead to the discovery that these structures occur in chondroitin sulphates obtained from a variety of sources, but that relative expression differs widely and depends upon the species and tissue sources and, moreover, within the same tissue, such as cartilage, varies with development and in experimental joint disease.
Materials and methods

Monoclonal antibodies to chondroitin sulphate

Monoclonal antibody 3B3 was produced and characterised as previously described (Couchman et al. 1984; Caterson et al. 1987) using chondroitinas AB-digested rat chondrosarcoma proteoglycan (R-CS-PG) as antigen (Christner et al. 1980). Monoclonal antibodies 7D4 and 6C3 were produced using 17-day embryonic chicken bone marrow proteoglycan (C-BM-PG) as antigen (without chondroitinaside digestion) as described by Sorrell et al. (1982a, 1990). Monoclonal antibody 2B6, which recognises only the neoepitope created by chondroitinase ABC digestion of chondroitin sulphate containing an unsaturated hexuronate and 4-sulphated N-acetyl galactosamine was used as previously described (Christner et al. 1980; Caterson et al. 1985).

Specificity of antibodies 7D4 and 6C3

Reaction of antibodies 7D4 and 6C3 with chondroitin sulphate proteoglycans (CS-PG) were tested by antibody dilution in a direct ELISA assay (Caterson et al. 1983). In brief, the wells of microtitre plates were coated by incubation with either (1) embryonic chick epiphyseal cartilage proteoglycan, (2) bovine nasal cartilage proteoglycan or (3) shark cranial cartilage proteoglycan (all at 25 µg·ml⁻¹). Assays were then performed using serial 1:3 dilutions of the ascites fluid of 7D4 or 6C3 that was already diluted 1:100 in phosphate-buffered saline (0.1 M NaCl, 0.05 M NaH₂PO₄, pH 7.4). PBS) containing 0.03% (w/v) sodium azide and 1% (w/v) bovine serum albumin. Colour development was with a peroxidase-conjugated goat anti-mouse immunoglobulin and O-phenylenediamine substrate. Titres reported are the reciprocal of the dilution giving half-maximum absorbance (492 nm).

Sensitivity of 6C3 and 7D4 epitopes to digestion with chondroitinase ABC

Comparison of the sensitivity of 7D4 and 6C3 epitopes on different CS-PG antigens to digestion with chondroitinase ABC was tested on the ELISA plates described above. Serial dilutions (1 to 5) of chondroitinase ABC digested at 0.1 unit·ml⁻¹ were added to the wells for 5 min at room temperature. The wells were washed (×5 in cold PBS) and ELISA assays were carried out with antibody 2B6 (diluted 1 in 1000), antibody 7D4 (diluted 1 in 10000) and antibody 6C3 (diluted 1 in 8000).

Periodate sensitivity of 3B3, 7D4 and 6C3 epitopes

The sensitivity of epitopes to periodate oxidation was tested by incubation of antigens absorbed on nitrocellulose sheets with peroxidase-conjugated rabbit anti-mouse immunoglobulin and DAB reagent, (3,3'-diaminobenzidine/CoCl₃/NH₄NiSO₄) (De Blas and Cherwinski, 1983). The intensity of stained bands in parallel tracks of experimental OA and control samples were compared by densitometric scans in a Shimadzu CS-930.

Results

Chondroitin sulphate structure

Three different anti-chondroitin sulphate monoclonal antibodies used in these studies, each of which recognise a distinct epitope in native chondroitin sulphate. Two of these antibodies, 6C3 and 7D4, were raised against native chondroitin sulphate proteoglycans obtained from embryonic chicken bone marrow (Sorrell et al. 1990). They reacted with chick bone marrow proteoglycan and all reaction was abolished after digestion with chondroitinase ABC. A third antibody, originally raised against a chondroitinase-digested neo antigen (Caterson et al. 1985), has been previously shown also to interact with a saturated terminal glucuronate residue adjacent to 6-sulphated galactosamine and therefore could recognise this structure when it was present in a native chain (Caterson et al. 1985). As summarised in Table 1, analysis indicated that these three antibodies reacted differently with chondroitin sulphates obtained from different sources (Caterson et al. 1989; Hardingham et al. 1989). Furthermore, the antigenicity of the native epitope detected by antibody 3B3, but not the epitopes detected by antibodies 6C3 or 7D4, was destroyed by periodate oxidation. Under the conditions used in this study, only structures located at the non-reducing terminus are affected by periodate oxidation (Woodward et al. 1985). Thus, it was confirmed that the native 3B3 epitope is located at the non-reducing terminus, in contrast to the other two epitopes, which are

Table 1. Specificity of monoclonal antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Antigen</th>
<th>Chick</th>
<th>Bovine</th>
<th>Shark</th>
<th>Epitope characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>7D4</td>
<td>IgM</td>
<td>C-BM-PG</td>
<td>&gt;660000</td>
<td>110000</td>
<td>510000</td>
<td>Epitope content reduced by digestion with chondroitinase ABC; insensitive to periodate oxidation (Caterson et al. 1987) and characterised as previously described using chondroitinas AB-digested rat chondrosarcoma proteoglycan (R-CS-PG) as an antigen (Christner et al. 1980). Antibodies 7D4 and 6C3 were produced using 17-day embryonic chick bone marrow proteoglycan (Sorrell et al. 1988) (C-BM-PG) as antigen. Antibodies 7D4 and 6C3 were shown to react with chondroitin sulphate (CS-PG) from different sources in a direct ELISA assay. Results show the reciprocal dilution giving 50% of maximum colour development with each antigen. The epitope recognised by mAb 3B3 was sensitive to periodate (5 min) at room temperature for 1 h. The epitopes recognised by mAbs 7D4 and 6C3 were unaffected by periodate at up to at least 20 min.</td>
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Fig. 1. The age-related expression of native chondroitin sulphate epitopes recognised by antibody 6C3 in the embryonic chick bursa of Fabricius. (A) At day 13 of incubation, the native 6C3 epitope is present throughout connective tissues and is also present in newly emerging epithelial follicles (arrows), which serve as sites of B-lymphocyte differentiation and maturation. (B) By day 18 of incubation, native 6C3 epitope expression is now largely confined to lymphoid follicles (arrows), and is generally absent from connective tissue regions, except perivascular sites (not shown). Indirect immunoperoxidase staining on section of paraffin-embedded tissues that were subsequently counterstained with hematoxylin (Sorrell et al. 1988a). ×140.
suggested to be periodate-resistant structures, or to be non-terminal chondroitin sulphate chain sequences.

A second study was performed to gain further evidence for the location of 6C3 and 7D4 epitopes in chondroitin sulphate chains. Chondroitin sulphate proteoglycans from three different sources, chick cartilage, bovine nasal cartilage and shark cartilage, were tested for their content of epitope after brief digestion with increasing amounts of chondroitinase ABC, an enzyme that progressively shortens chondroitin sulphate chains from their non-reducing terminal end (Saito et al. 1968; Fosang and Hardingham, unpublished results). As a measure of the extent of digestion the monoclonal antibody 2B6 was used as a standard (Christner et al. 1980; Caterson et al. 1984, 1985). This antibody recognises only the unsaturated structure generated by chondroitinase ABC digestion, i.e. it would only be created as non-reducing chain terminal structures were removed and the chains were shortened. The results with different degrees of chondroitinase digestion (Table 2) showed that all preparations were very sensitive to chondroitinase ABC digestion, as very little enzyme was required to generate 25% of maximum 2B6 epitope. In contrast it required 5–100 times more enzyme (with different proteoglycans) to reduce the content of 6C3 epitope by more than 50%, or 5–3000 times more enzyme (with different proteoglycans) to reduce the content of the 7D4 epitope by more than 50%. These results suggest that the 7D4 epitope is a more chondroitinase-resistant structure than the 6C3 epitope, or it is distributed further from the non-reducing termini of the chondroitin sulphate chains than the 6C3 epitope in the chicken and bovine proteoglycan preparations.

These results appear most likely to reflect the separate and independent distribution of 6C3 and 7D4 epitopes within chondroitin sulphate chains of different origin. Although it was also possible that the 7D4 and 6C3 epitopes were expressed on a minority of chains that are relatively poor enzyme substrates, this was suggested to be unlikely, as, in similar digests, it was shown that all chains were progressively shortened (Fosang and Hardingham, unpublished results). The 6C3 and 7D4 epitopes therefore appear to be distributed within chondroitin sulphate chains rather than at the non-reducing ends of the chains as identified for the 3B3 epitope. These data (Tables 1, 2) indicate that the three native chondroitin sulphate epitopes studied are structurally distinct and occur in different amounts in chondroitin sulphate from different origins.

### Developmental expression of chondroitin sulphate epitopes

Application of antibody 6C3 to localising epitopes within native chondroitin sulphate chains in tissue sections of the developing chicken bursa of Fabricius showed remarkable regional and developmental changes in the expression of the chondroitin sulphate epitopes (Fig. 1). At day 13 of incubation, the 6C3 epitope was present throughout connective tissue and was also present in newly emerging epithelial follicles, which serve as sites of B-lymphocyte differentiation and maturation. By day 18 of incubation, the 6C3 epitope was now largely confined to lymphoid follicles, disappearing from the surrounding connective tissue (Fig. 1B). In contrast, the 7D4 epitope was detected within both follicles and connective tissue throughout this period of development. Thus, the expression of these two chondroitin sulphate epitopes is differentially modulated by cells in both an age-dependent and a tissue-specific manner.

In another study antibody 3B3 was effective in identifying a native chondroitin sulphate epitope associated with cartilage development in embryonic chick leg bones (Fig. 2A). Furthermore, the distribution of proteoglycans bearing the native epitope was confined to specific locations within developing chick leg bones. These were the regions occupied by flattened chondrocytes of the growth plate, where it was first detected in an intracellular, juxtanuclear compartment. In the zone of hypertrophied chondrocytes of more mature cartilage the epitope was primarily located in extracellular spaces. In contrast, chondrocytes in the epiphyseal, articular and joint spaces produced chondroitin sulphate proteoglycans, but without the native 3B3 epitope (Fig. 2B). The native 3B3 epitope was not exclusively found in the leg rudiment, but has also been identified in pelvic cartilage and vertebral cartilage during chick development (results not shown). Therefore, it is possible that this epitope may be a regional marker for some stages of embryonic cartilage development in the chick. Bone marrow is the only non-cartilaginous tissue in which this native chondroitin sulphate epitope has been detected (Sorrell et al. 1988b).

### Table 2. Comparison of the sensitivity of 6C3 and 7D4 epitopes on different CS-PG antigens to chondroitinase ABC digestion

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Chondroitinase ABC (units ml⁻¹) required for the generation of 25% of maximum neo epitope in different CS-PG antigens</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Chick</td>
</tr>
<tr>
<td>2B6</td>
<td>0.00003</td>
</tr>
<tr>
<td>6C3</td>
<td>0.004</td>
</tr>
<tr>
<td>7D4</td>
<td>0.10</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Native epitope loss (% of total) after incubation with chondroitinase ABC at 0.10 units ml⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chick</td>
</tr>
<tr>
<td>6C3</td>
<td>100</td>
</tr>
<tr>
<td>7D4</td>
<td>100</td>
</tr>
</tbody>
</table>

ELISA assay of different proteoglycans from chick, bovine and shark cartilage as described in Materials and methods. (A) Monoclonal antibody 2B6 only recognises a chondroitinase ABC-generated neoantigen and the results demonstrate the low exposure to enzyme that is sufficient to begin digesting the chondroitin sulphate chains. (B) The epitope recognised by monoclonal antibody 6C3 is more rapidly lost on chondroitinase ABC digestion than that of 7D4 but the sensitivity also varies with the antigen source. This difference is also shown in C, where the proportion of epitope lost after digestion with a high concentration of enzyme is compared for the different antigens.

### Modulation of chondroitin sulphate epitope expression in tissue pathology

Other studies indicate that specific native chondroitin sulphate epitopes may be uniquely expressed in certain pathological conditions. In experimental canine OA, in which the cruciate ligament is sectioned (Pond and Nuki, 1973), there is a series of responses in the articular cartilage that occur in the succeeding months and resemble the early changes characteristic of natural OA.
Monoclonal antibodies that recognise carbohydrate structures in native (non-enzyme digested) chondroitin sulphate have revealed an additional level of structural organisation for these glycosaminoglycans. In the present study, three different monoclonal antibodies identified three structurally distinct epitopes within chondroitin sulphates. More significantly, the presence and, possibly, the location of these structures within the chondroitin sulphate chains varies with the source, developmental status and pathological status of cells that produce these molecules.

Structural and spatial specificity were demonstrated using chemical and enzymatic methods that selectively modify or remove different components of chondroitin sulphate chains. Periodate oxidation, under the conditions used in the present study, modifies structures located at the non-reducing terminus (Woodward et al. 1985), and results in the loss of antigenicity for antibody 3B3. This indicates that the native 3B3 epitope is exclusively located at the non-reducing termini of those chondroitin sulphate chains that express this epitope. However, this epitope is present only on select populations of chondroitin sulphates, which means that cells can regulate its presence within the chain. Periodate oxidation does not destroy antigenicity of either the 6C3 or 7D4 epitopes, indicating that the carbohydrate structures comprising these epitopes are periodate-resistant or are located in non-terminal sequences of chondroitin sulphate chains.

The structures responsible for the 6C3 and 7D4 epitopes, depending upon the source of the proteoglycan, can be either totally or partially removed from chondroitin sulphate chains by chondroitinase ABC digestion. In all proteoglycans tested, the 6C3 epitope is completely removed by chondroitinase; however, the 7D4 epitope, on some proteoglycans, is only partially removed, which suggests that these two epitopes are both structurally and spatially distinct.

Non-random distribution of specific types of disaccharides has been shown in shark cartilage chondroitin sulphate chains by Uchiyama et al. (1987), using chemical modification and limited chondroitinase ACI digestion. A high proportion of the 4-sulphated and unsulphated disaccharides were found near the linkage region, whereas 6-sulphated and oversulphated disaccharides were concentrated nearer the non-reducing terminus. A comparable situation has been reported from the analysis of heparin chains. Radoff and Danishefsky (1984) selectively removed segments from the non-reducing terminus of heparin chains, separated these chains according to length and measured the antithrombin III binding activity. They found that longer chains contained greater binding activity and concluded that the sequences responsible are concentrated near the non-reducing terminus. Non-random distribution of disaccharides has also been reported in keratan sulphate (Oeben et al. 1987) and in heparan sulphate (Lyon et al. 1987). These data, and those presented here, argue that cells can regulate the enzymes that modify sugar residues within glycosaminoglycan chains and this results in distinct sequences of chain structure.

During development cells also regulate the expression of
Fig. 3. Immunolocalisation with antibodies 3B3 and 7D4 of proteoglycans from experimental canine OA and control articular cartilage after separation by electrophoresis on composite agarose/polyacrylamide gels. Equal amounts of proteoglycan (5 μg) were electrophoresed and transferred to nylon membranes and immunolocalised as described in Materials and methods. The results showed stronger expression of native 3B3 epitope in proteoglycans from experimental OA cartilage 3 months after induction compared with contralateral control cartilage. The expression of 7D4 was stronger in control cartilage than 3B3, but it again was increased in experimental OA proteoglycans.

selected epitopes independently. This is seen in the development of the embryonic chick bursa of Fabricius (Sorrell et al. 1988a) and thymus (J. M. Sorrell, unpublished results). Expression of the 6C3 epitope becomes confined to the bursal follicles, and sites of B-lymphocyte development, but the same epitope is not expressed in regions of T-lymphocyte development in the chick thymus. The pattern of 7D4 epitope expression is different, being present throughout the bursa (Sorrell et al. 1988a), but being concentrated in the thymic cortex where T-lymphocytes are developing (J. M. Sorrell, unpublished results). The native 3B3 epitope is also expressed in embryonic chick, but here the expression is confined to bone marrow and cartilage (Sorrell et al. 1988b). Although all chondrocytes in developing chick leg bones produce chondroitin sulphate, only chondrocytes in the growth and hypertrophic zones express the native 3B3 epitope. This epitope is first detected intracellularly in a juxtanuclear site that is probably the Golgi apparatus. Another recent study (Mark et al. 1989) has also demonstrated a transient expression of a native chondroitin sulphate epitope during fetal rat development. Therefore, modulation of the structure of chondroitin sulphate during development may be a general phenomenon. The alteration in epitope expression that occurs in experimental OA may also be related to these developmental changes, as in the cartilage
response there is a re-initiation of a high level of chondroitin sulphate synthesis, which, in some respects, resembles what occurs in early development.

Although the structures of the three epitopes recognised by the monoclonal antibodies used here have yet to be fully defined, some evidence for unusual structures in chondroitin sulphate chains have previously been reported. A monoclonal antibody was described with specificity for a disaccharide containing glucuronate 2-sulphate and N-acetylgalactosamine 6-sulphate, a structure that was shown to vary greatly in content in chondroitin sulphate from different animal sources (Yamagata et al. 1987). Another monoclonal antibody has been produced that is reported to recognise an epitope containing the 4,6-sulphated disaccharide found in chondroitin sulphate E (Kongtawelert and Ghosh, 1990). Investigation of newly synthesised chondroitin sulphate in embryonic rat and chicken cartilage showed that N-acetyl galactosamine 4-sulphate or 4,6-disulphate was the preferred non-reducing terminal sugar (Otsu et al. 1987). These studies indicate that carbohydrate domains that contain unusual sulphated patterns might be antigenic in mice. In this regard, the atypical carbohydrate structures that are antigenic may be somewhat analogous to the pentasaccharide domain of the antithrombin III binding region of heparin, which is also known to contain an unusual trisulphated disaccharide (Thunberg et al. 1982).

The present results can be usefully compared with the developmental changes that are expressed in glycosylation patterns of glycoproteins and glycolipids, which have thereby been termed onco-developmental antigens (Feizi, 1985). Another example is the disease-related change in patterns of glycosylation that has been reported for immunoglobulins (Parekh et al. 1985). The principle that carbohydrate structures expressed by cells can be modulated during biological processes may thus also be extended to glycosaminoglycan chain structure.

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References


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