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Light microscopic histochemical and immunohistochemical localisation of sulphated glycosaminoglycans in the rooster comb and wattle tissues

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ABSTRACT

Comb and wattle tissues, which consist of layers of epidermis, dermis and central connective tissue, are known to contain sulphated glycosaminoglycans (GAGs) including dermatan sulphate and chondroitin sulphate–dermatan sulphate copolymers. Little is known about distribution of these CAGs in each tissue. The objective of this study was to localise sulphated GAGS in the comb and wattle tissues from mature roosters. Monoclonal antibodies 6D6, CS-56 and AH12 specific to dermatan sulphate proteoglycan (decorin), chondroitin sulphate and keratan sulphate, respectively, were used. In both tissues, 6D6 epitope was found to be more concentrated in the superficial layer of dermis and the central connective tissue than in the intermediate layer of dermis containing fibromucoid tissue. The staining pattern for 6D6 epitope was similar to that for collagen fibres. In contrast, CS-56 epitope was uniformly distributed in most parts of the dermis and the central connective tissue. The stratum germinativum in the epidermis was the major tissue showing positive staining with AH12, haematoxylin and safranin-O.

Key words: *Gallus domesticus*; proteoglycan; decorin; chondroitin sulphate; keratan sulphate.

INTRODUCTION

Comb and wattle, the male dominated excrescences in chickens are testosterone-sensitive tissues. They are composed of 2 tissues each comprising 3 layers (Lucas & Stettenheim, 1972) including epidermis, dermis and central connective tissue. The epidermis contains 2 layers, stratum corneum and stratum germinativum. The dermis can be divided into superficial and intermediate layers. The latter contains fibromucoid tissue occupying a large part of the comb and wattle structures of roosters. Studies of the chemical composition of comb and wattle tissues have shown that collagen and hyaluronic acid, a nonsulphated glycosaminoglycan (GAG) are the major constituents in these tissues (Schiller & Dorfman, 1956; Swann, 1968; Ng Kwai Hang & Anastassiadis, 1980; Nakano & Sim, 1989). They also contain relatively small amounts of sulphated GAGs including dermatan sulphate (DS) and chondroitin sulphate-dermatan sulphate (CS-DS) copolymers (Nakano & Sim, 1992*a*). More recently, a disaccharide epitope of keratan sulphate (KS) has been detected immunochemically in comb and wattle tissues (Nakano & Sim, 1994). It is known that hyaluronic acid is concentrated in the fibromucoid tissue (Balazs et al. 1959). However, limited information is available concerning distribution of other constituents including collagen and sulphated GAGs. Sulphated GAGs are covalently attached to core proteins to form proteoglycans, but the type of proteoglycan in the comb and wattle is unknown.

Immunohistochemical staining with specific monoclonal antibodies (MAb) if any, is a useful technique to localise a constituent molecule in tissues. A histochemical method is less specific compared with an immunohistochemical method, but useful in combination with the latter. The present report describes light microscopic histochemical and immunohisto-

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chemical studies of comb and wattle tissues from mature roosters. Three MAbs capable of detecting epitopes on proteoglycans were used to localise sulphated GAGs in different layers.

MATERIALS AND METHODS

Fresh comb and wattle tissues were obtained post-mortem from 4 52-wk-old Single Comb White Leghorn roosters. Samples for histological staining were collected from the anterior, central and posterior portions of each comb, and central portion of each wattle. They were approximately 1 cm wide strips cut vertically to the base of comb or wattle. Samples were fixed in 4% buffered formalin pH 7.3 containing 0.5% cetylpyridinium chloride (Williams & Jackson, 1956), dehydrated with ascending concentrations of ethanol, cleared in benzene, and embedded in paraffin (Drury & Wallington, 1967). Sections were cut at 5 µm, deparaffinised and stained with haematoxylin and eosin (H&E), Gomori trichrome, Alcian blue (pH 2.5), and safranin-O and fast green (Lillie, 1965; Drury & Wallington, 1967). Sections were then digested with chondroitinase-ABC (Seikagaku America, Inc. Rockville, MD, USA) (0.1 unit/ml) in 0.1 M sodium acetate −0.1 M Tris-HCl, pH 8.0 at 37 °C for 1 h) and stained with safranin-O and fast green.

The MAbs used for immunohistochemical staining were 6D6 (Pringle et al. 1985; Scott et al. 1993), CS-56 (Sigma Chemical Co., St Louis, MO, USA) (Avnur & Geiger, 1984) and AH12 (Nakano et al. 1993). The MAb, 6D6 was raised against bovine skin decorin (proteoglycan having a single DS chain), and stained chicken skin (Pringle et al. 1985), suggesting that chicken decorin has the 6D6 epitope. The MAbs, CS-56 and AH12 recognise disaccharide epitopes of CS and KS, respectively, both of which are detectable immunochemically in GAG fractions isolated from comb and wattle (Nakano & Sim, 1992*b*, 1994).

For immunostaining, deparaffinised sections were treated with 2% (vol/vol) hydrogen peroxide in methanol for 30 min to eliminate endogenous peroxidase activity. Sections to be incubated with 6D6 and AH12 were treated either with chondroitinase-ABC (see above) or testicular hyaluronidase (type V from sheep testis) (10 mg/ml in TBS: 0.05 M Tris-HCl−0.15 M NaCl, pH 7.6 at 37 °C for 1 h) to digest CS and hyaluronic acid which might otherwise mask tissue antigens. Sections to be incubated with CS-56 were not treated with either enzyme. Sections were then soaked in normal rabbit serum (diluted 1 in 20 in TBS) for 30 min and incubated at 4 °C overnight with diluted ascites fluid (CS-56, 1:200 and AH12, 1:1000, respectively) or hybridoma culture supernatant (6D6, 1:10). Control sections were incubated with and without normal ascites fluid derived from mouse myeloma cells or myeloma cell culture supernatant. Sections were washed with TBS, and incubated for 45 min with rabbit antimouse Ig (G, A and M) (Zymed Laboratories, San Francisco, CA, USA) diluted 1:50. Final incubation was carried out using mouse-peroxidase-antiperoxidase complex (Nordic Immunology, Tilberg, The Netherlands) diluted 1:300. Colour was developed with diaminobenezidine tetrahydrochloride-hydrogen peroxide (Pringle et al. 1985).

RESULTS

The morphology of the epidermis was in general similar to that of skin (Cormack, 1987) both in comb and wattle. No blood vessels were found in the 2 layers, stratum corneum and stratum germinativum. Thus nutrient supply to and waste removal from these layers appear to be by diffusion. Staining patterns for

Table 1. *Histochemical staining of comb tissues from mature roosters*[1]

Layers	Haematoxylin	Trichrome	Alcian blue	Safranin-O
Epidermis				
Stratum corneum	−[2]	−[3]	−	−[4]
Stratum germinativum	++	+	−	+++
Dermis				
Superficial layer	+	+++	++	+
Intermediate layer containing fibromucoid tissue	−	++	+++	+
Central connective tissue	−	+++	+	+

[1] Staining reactions were graded from negative (−) to highly positive (+++) according to intensity. [2] Highly eosinophilic in the stratum corneum. [3] Green colouration for collagen was graded. [4] Intense staining with fast green. In general, similar staining patterns for collagen and GAG were observed in wattle with the exception of weaker safranin-O staining in wattle than in comb.

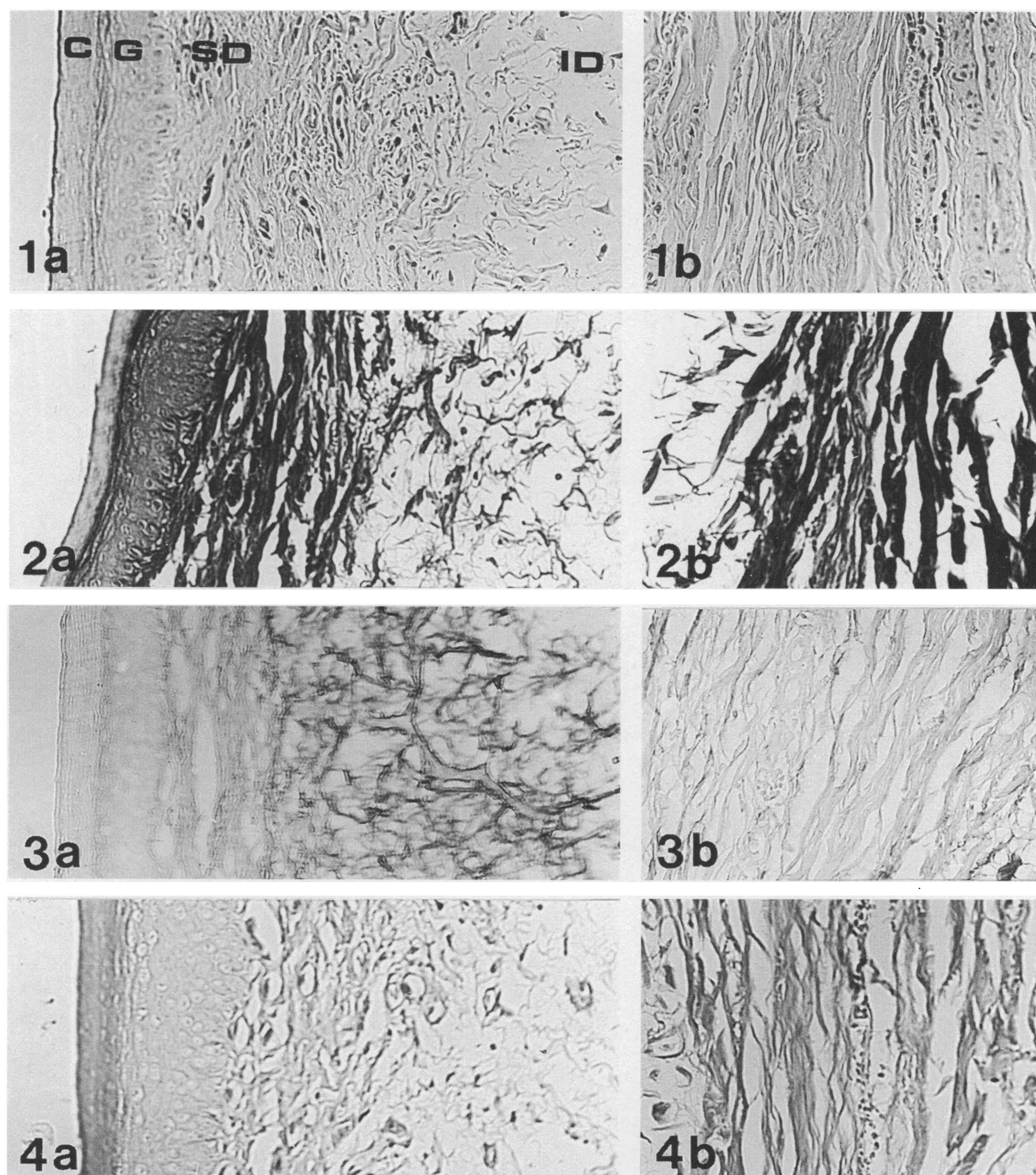

Fig. 1. H & E staining of comb. (*a*) Section showing stratum corneum (C), stratum germinativum (G), surface layer of dermis (SD), and intermediate layer of dermis (ID) containing fibromucoid tissue. (*b*) Section containing central connective tissue. Both ×250.
Fig. 2. Comb sections stained with Gomori trichrome. ×250.
Fig. 3. Comb sections stained with Alcian blue. ×250.
Fig. 4. Comb sections stained with safranin-O and fast green. ×250.

collagen and GAG were in general similar among sampling sites of comb and between comb and wattle tissues with the exception of the lower intensities of safranin-O and AH12 staining in the latter (see below).

The results of histochemical staining of comb and wattle are shown in Table 1 and Figures 1–8. The stratum corneum was highly eosinophilic with few nuclei following H & E staining, and stained purplish red with Gomori trichrome. Since Gomori trichrome stains collagen fibres green, the results suggested that collagen is not the major protein in the stratum corneum, which is probably a keratin-rich layer as is the stratum corneum in the skin (Cormack, 1987). The stratum germinativum was basophilic with H & E and had a pinkish grey or greyish colouration with trichrome, suggesting presence of noncollagenous acidic material in this layer. On the other hand, there

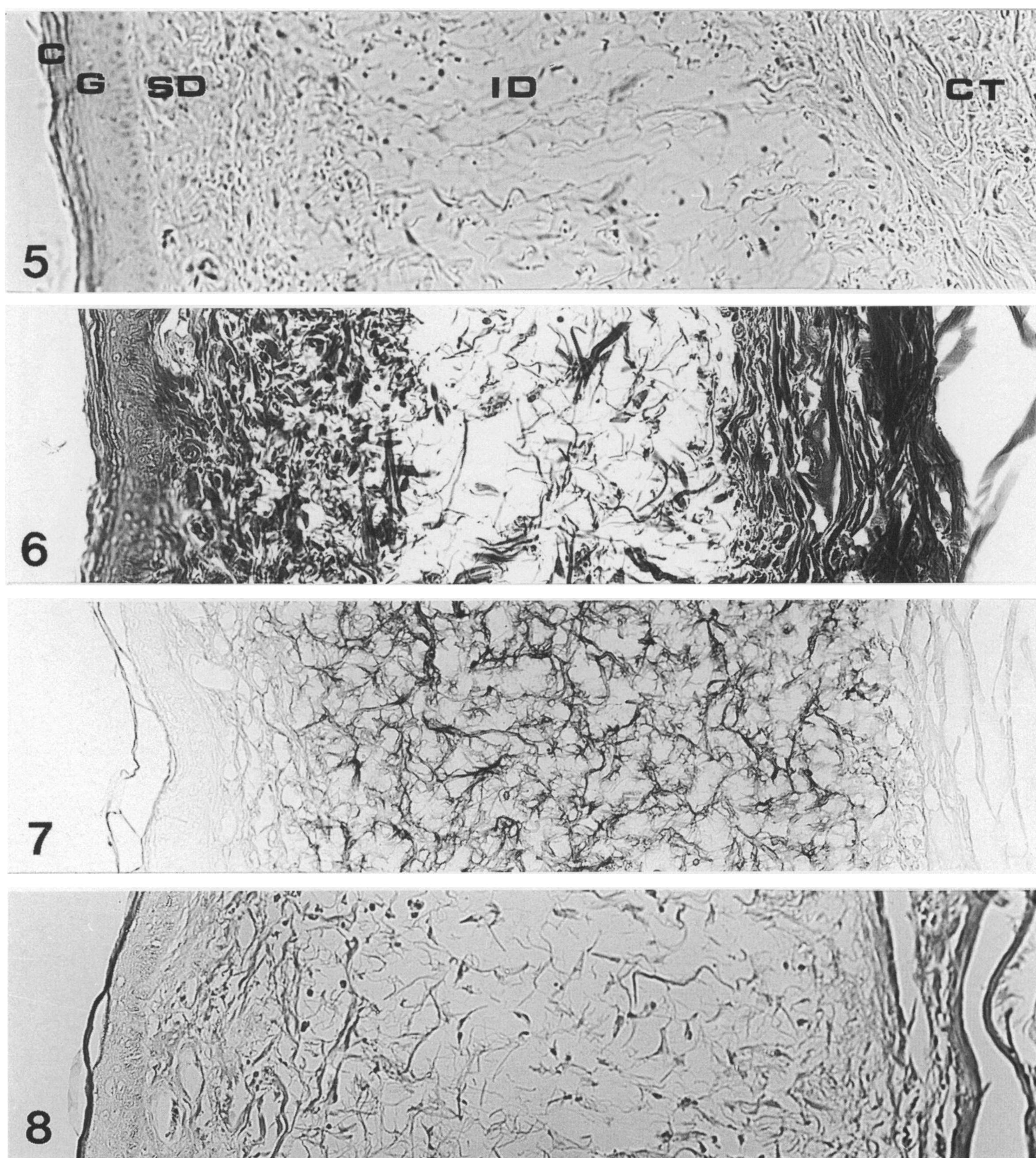

Fig. 5. H & E staining of wattle. The section contained stratum corneum (C), stratum germinativum (G), surface layer of dermis (SD), intermediate layer of dermis (ID), and central connective tissue (CT). ×250.
Fig. 6. Wattle section stained with Gomori trichrome. ×250.
Fig. 7. Wattle section stained with Alcian blue. ×250.
Fig. 8. Wattle section stained with safranin-O and fast green. ×250.

were abundant collagen fibres with green colouration in the dermis and the central connective tissue, where the intensity of haematoxylin staining was reduced. In the dermis, however, the proportion of collagen was less in the intermediate layer containing hyaluronic acid-rich fibromucoid tissue than in the superficial layer. The staining pattern for GAG differed between Alcian blue and safranin-O. The intensity of Alcian blue staining was highest in the intermediate layer of dermis and higher in the superficial layer of dermis than in the central connective tissue. Little staining was seen with this dye in the epidermis. In contrast, the intensity of safranin-O staining was highest in the stratum germinativum. This was consistent with increased intensity of haematoxylin staining in this layer (see above). Intense safranin-O staining was still seen in the stratum germinativum after treatment of sections with chondroitinase-ABC (results not

Table 2. *Immunohistochemical staining of comb tissues from mature roosters*[1]

Layers	Monoclonal antibodies[2]		
	6D6	CS-56	AH12
Epidermis			
Stratum corneum	−	−	+
Stratum germinativum	+	−	+++
Dermis			
Superficial layer	++	++	+
Intermediate layer containing fibromucoid tissue	+	+++	−
Central connective tissue	++	+++	+

[1] Staining reactions were graded from negative (−) to highly positive (+++) according to intensity. [2] Raised against bovine skin decorin (6D6), chondroitin sulphate (CS-56) and keratain sulphate (AH12). In general, similar staining patterns were observed in wattle with the exception of weaker immunostaining with AH12 in wattle than in comb.

shown). Little staining was seen with safranin-O in the stratum corneum which was stained intensely with fast green. Safranin-O staining was weaker in the other layers including intermediate layer of dermis and the central connective tissue, where positive staining was seen in small numbers of fibres and blood vessels with and without chondroitinase-ABC treatment. Staining intensity of safranin-O was greater in comb than in wattle.

Control staining either with or without normal ascites or myeloma cell culture supernatant showed negative results both in comb and wattle (results not shown). The capability of each MAb to immunostain sections of comb is summarised in Table 2 and sections stained with 3 MAbs are shown in Figures 3 (for comb) and 4 (wattle).

No MAbs with the exception of AH12 (see below) showed positive immunoreactivity in the stratum corneum either from comb or wattle. Relatively weak but positive immunostaining with 6D6 was seen in the stratum germinativum, the dermis and the central connective tissue. The intensity was greater in the superficial layer of dermis and the central connective tissue than in the stratum germinativum and the intermediate layer of dermis containing fibromucoid tissue. The staining pattern for 6D6 epitope was similar to that of Gomori trichrome for collagen (Table 1, Figs 2, 6), suggesting close association between collagen and decorin recognised by 6D6 in the tissue examined.

In contrast to 6D6 staining, intense immunostain with CS-56 was seen in most part of dermis and central connective tissue. However, the intensity of CS-56 staining tended to be slightly reduced in narrow regions of the superficial layer of the dermis adjacent to the stratum germinativum. The CS-56 staining ranged from negative to weak in the stratum germinativum from either tissue. Compared with 6D6 or CS-56 epitopes, AH12 epitope was localised in more restricted areas of both comb and wattle. The stratum germinativum was the major tissue showing highly positive immunostaining with AH12. The stratum corneum, superficial layer of dermis, and central connective tissues were also found to be weakly but positively stained with AH12 both in comb and wattle. AH12 staining tended to be weaker in wattle than in comb.

DISCUSSION

We have previously reported that the sulphated GAG of comb and wattle consists of hybrid GAGs including DS with high iduronic acid contents ($>60\%$ of total uronic acid) and CS-DS copolymers with high glucuronic acid contents ($>60\%$) (Nakano & Sim, 1992*a*). The DS accounted for approximately 47 and 52% of total sulphated GAG from comb and wattle, respectively. Corresponding values for copolymeric GAG were 42 and 38%. We also found that the MAb, CS-56 was highly reactive to the CS-DS copolymers but not to the DS (Nakano & Sim, 1992*b*). From these findings, it is suggested that the DS is mainly derived from tissues containing 6D6 epitope (e.g. superficial layer of dermis and central connective tissue), and the CS-DS copolymers from those containing CS-56 epitope (whole thickness of dermis and central connective tissue).

Decorin is a low-molecular weight proteoglycan (MW $\sim$ 100 kDa) which can bind to collagen fibrils and transforming growth factor-β (TGF-β) through its protein core (Kresse et al. 1994). Thus the similarity in distribution of 6D6 epitope and collagen stained by Gomori trichrome (see above) is expected. Roles of decorin in regulating collagen fibrillogenesis and neutralising the effect of TGF-β have been suggested (Kresse et al. 1994). No attempt was made in this study to detect biglycan, a proteoglycan containing DS and CS, in the comb and wattle. Biglycan has been found in various tissues such as skin and cartilage (Choi et al. 1989; Kresse et al. 1994).

From the present results, it appears that hyaluronic acid, a major GAG in the intermediate layer of dermis containing fibromucoid tissue (Balazs et al. 1959) has higher affinity to Alcian blue than to safranin-O. The latter likely has higher affinity to sulphated GAGs including KS. It has been reported that safranin-O staining reflects KS staining in bovine fibrocartilage

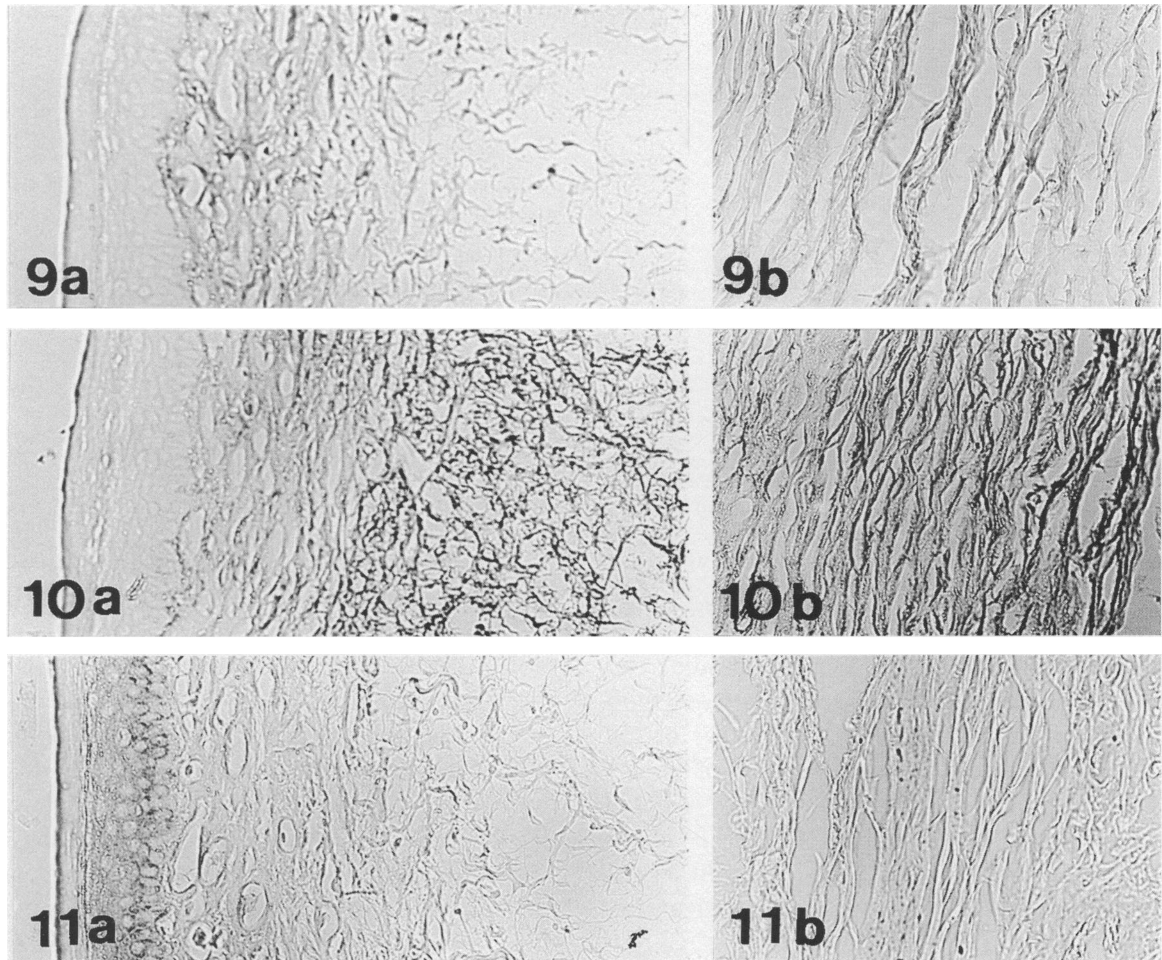

Fig. 9. Comb sections stained with antidecorin MAb, 6D6. ×250.
Fig. 10. Comb sections stained with anti-CS MAb, CS-56. ×250.
Fig. 11. Comb sections stained with anti-KS MAb, AH12. ×250.

tissue (Nakano et al. 1993). This is consistent with the present results of positive immunostains with anti-KS Mab, AH12 (Table 2, Figs 11*a*, 14) observed in the stratum germinativum and positive safranin-O staining of sections treated with and without (Table 1) chondroitinase-ABC in the same tissue. Chondroitinase-ABC can digest galactosaminoglycans and hyaluronic acid but cannot digest KS. The results also confirm our previous findings (Nakano & Sim, 1994) with ELISA showing the presence in comb and wattle of AH12 epitope, which was highly susceptible to KS degrading enzymes, endo-β-galactosidase and keratanase (Nakano & Sim, 1994). Keratan sulphate is a uronic acid free GAG more commonly found in corneal and cartilaginous tissues (Stuhlsatz et al. 1989) and the occurrence of this GAG in comb and wattle was not anticipated. Keratan sulphate has, however, been localised immunohistochemically in other tissues including chick tendon (Craig et al. 1987) and human skin (Willen et al. 1991). In the latter, positive reactions with anti-KS MAbs were found in the epidermis including both the stratum corneum and the stratum germinativum, which were consistent with the present results with AH12.

The prominent AH12 staining observed in the stratum germinativum of comb or wattle, where there was no blood supply (see above), appear to support the previous proposal that galactose containing GAG, KS, is a functional substitute for glucuronic acid containing GAG, CS, under conditions of oxygen lack (Scott & Haigh, 1988). The biosynthesis of CS requires oxygen in the stage from UDP glucose to UDP glucuronic acid, while the biosynthesis of KS does not require oxygen in making UDP galactose from UDP glucose (Scott & Haigh, 1988).

The differences observed in tissue distribution among the 3 epitopes in this study suggest the presence of structurally different proteoglycans recognised by 6D6, CS-56 and AH12, respectively. However, since 6D6 recognises the protein core of decorin, but CS-56 and AH12 recognise GAG chains, it is not clear whether or not proteoglycans having CS-56 or AH12

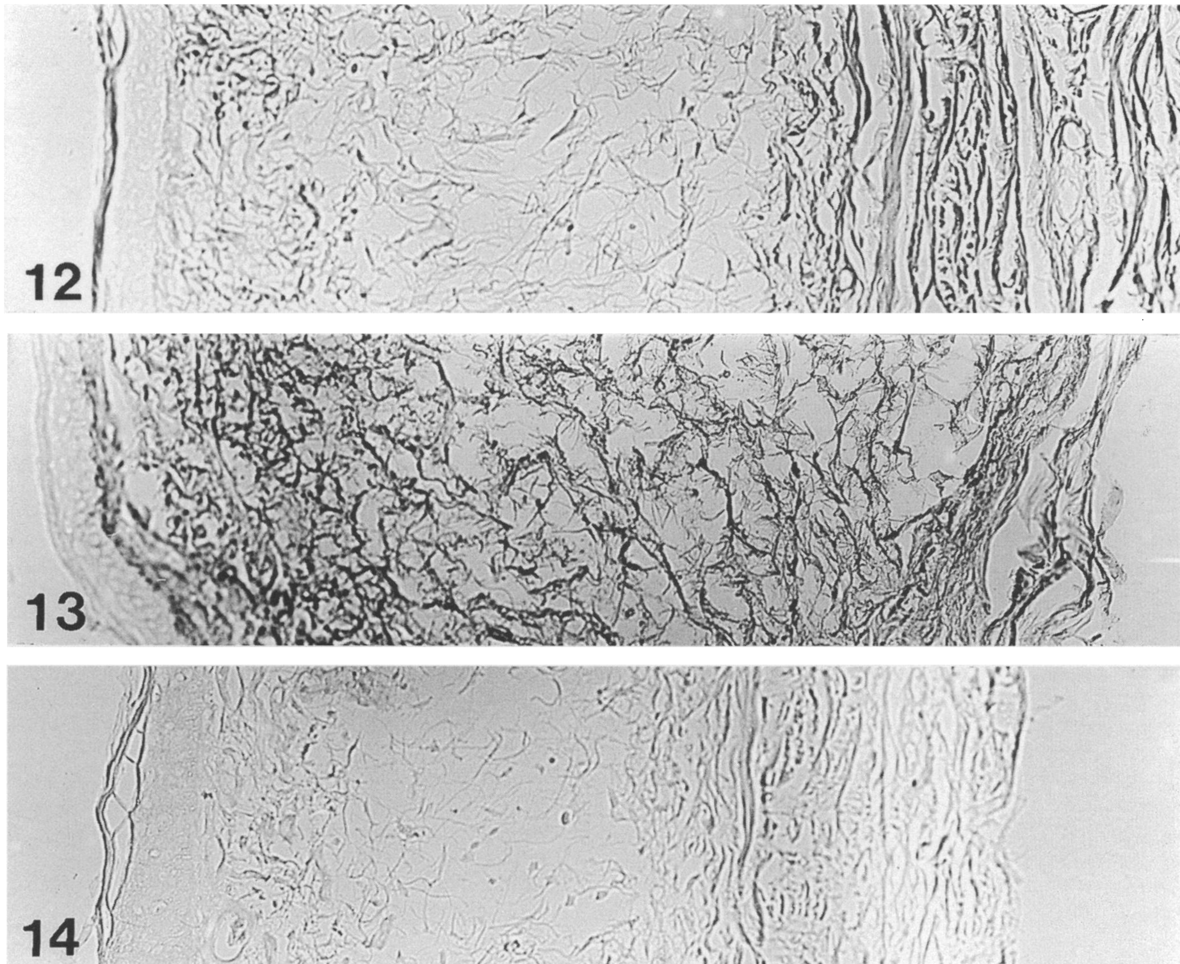

Fig. 12. Wattle section stained with anti-decorin MAb, 6D6. ×250.
Fig. 13. Wattle section stained with anti-CS MAb, CS-56. ×250.
Fig. 14. Wattle section stained with anti-KS MAb, AH12. ×250.

epitope contain a distinct protein core not reactive to 6D6. This problem will be clarified by purifying 2 proteoglycans from the epidermis (KS containing proteoglycan) and the dermis (proteoglycan containing CS-DS copolymers) of comb and wattle, and by analysing that these proteoglycans are not reactive to 6D6. We have recently found positive immunostaining of comb and wattle with the antibody raised against the protein core of chondroitin sulphate proteoglycan from bovine sclera, in that the tissue distribution of chondroitin sulphate proteoglycan was similar to that of chondroitin sulphate recognised by CS-56 (Nakano et al., unpublished results). This suggested presence of chondroitin sulphate proteoglycan in the comb and wattle tissue.

The present results provided the immunohistochemical evidence for the presence of previously reported sulphated GAGs including DS, CS-DS copolymers and KS in the comb and wattle tissues, and further showed that tissue distribution differed between the GAGs. Purification of chondroitin sulphate proteoglycan and keratan sulphate proteoglycan from comb and wattle is under progress in our laboratory to characterise their protein cores.

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