Localization of Glycosaminoglycans and CD44 in the Human Lacrimal Gland

Kenji YOSHIDA, Tohru NITATORI and Yasuo UCHIYAMA

Departments of Ophthalmology1 and Anatomy2, Iwate Medical University School of Medicine, Morioka; and Department of Cell Biology and Anatomy3, Osaka University Medical School, Suita, Osaka, Japan

Received October 8, 1996

Summary. Recent analyses of tears indicate the presence of glycosaminoglycans as their components, but their origin remains unknown. To further understand the origin of these tear components, we investigated by immunohistochemical techniques the localization of glycosaminoglycans and CD44 in human lacrimal glands obtained from 20 cadavers at autopsy. Monoclonal antibodies to CD44, a receptor for hyaluronic acid, dermatan sulfate, chondroitin sulfate, and keratan sulfate were applied to the tissue. Hyaluronic acid binding region was also used for the staining of hyaluronic acid. By light microscopy, immunoreactivity for CD44 was mostly detected on the baso-lateral membrane of acinar and ductal cells, and the vascular endothelium in the interstitium. Positive staining of hyaluronic acid was associated intensely with the basal membrane of acinar and ductal cells and weakly, faintly or not at all with their lateral membrane. Positive staining of hyaluronic acid and immunoreactivity for dermatan sulfate were detected in interstitial fibrous structures; particularly, the former was intense in the perivascular fibrous structures, and the latter along the periparenchimal fibrous structures. Immunoreactivity for chondroitin sulfate and keratan sulfate was seen in some acinar cells and the acinar and ductal lumen. By electron microscopy, immunogold particles indicating chondroitin sulfate or keratan sulfate labeled secretory granules of the acinar cells.

Considering the fact that CD44 is a receptor molecule for hyaluronic acid, the association of hyaluronic acid with the basal membrane and weakly or faintly with the lateral membrane of acinar and ductal cells may be attributed to the expression of CD44 on the baso-lateral membrane of the cells. Moreover, the presence of immunoreactivity for chondroitin sulfate and keratan sulfate in secretory granules of acinar cells and their lumens suggests that tears from the lacrimal gland contain these glycosaminoglycans.

Glycosaminoglycans are broadly distributed polysaccharides that are known to play an important role in the maintenance of various tissues. Hyaluronic acid is a linear high-molecular weight polysaccharide in connective tissue and a non-sulfated glycosaminoglycan, while chondroitin sulfate, dermatan sulfate, and keratan sulfate are highly anionic proteoglycan constituents because of the presence of sulfate radicals. In the ocular tissue, glycosaminoglycans are known to be involved in the development and maintenance of the cornea and probably with the corneal transparency (ASARI et al., 1992b).

Tears exist in the ocular surface—which consists of three layers; lipid, aqueous and mucoid layers—while they play important roles in the protection, metabolism and immune system for this surface (MISHIMA, 1966; IWATA and KABASAWA, 1971; IWATA, 1973). Until recently, carbohydrates in tears were believed to be secreted from the goblet cells existing in the conjunctiva. Histochemical studies using various types of lectins or specific staining for glycoconjugates have shown the presence of sugar chains in the mammalian lacrimal gland, including humans (JENSEN et al., 1969; JENSEN, 1970; AHMED and GRIERSON, 1989). Moreover, hyaluronic acid and chondroitin sulfate have been demonstrated in human tears collected from the corneal surface (MIYAUCHI et al., 1996), although the source of these products remains unknown. The possible source of glycosaminoglycans in tears is the lacrimal gland, but direct evidence for the presence of glycosaminoglycans has not been demonstrated here.

The hyaluronic acid binding region of proteoglycans specifically binds to hyaluronic acid molecules (HARDINGHAM and MUIR, 1972; HASCALL and HEINEGÅRD, 1974; ASARI et al., 1992a, b). It has been shown that a biotinylated hyaluronic acid binding
region is available for the histochemical demonstration of hyaluronic acid in various tissues, including the ocular tissue (ASARI et al., 1992a, b). On the other hand, CD44 is a type I transmembrane glycoprotein involved in homo- and heterotypic cell adhesion and cell-matrix interaction. Due to alternative splicing of the CD44 gene, its molecules are structurally complicated and have CD44 variants with altered adhesion properties (GUNTHERT et al., 1993). Because of this structural complexity, the CD44 molecules act as receptors for various molecules such as sulfated proteoglycans, fibronectin and type I and IV collagen (CARTER and WAYNER, 1988; JALKANEN and JALKANEN, 1992; TOYAMA-SORIMACHI and MIYASAKA, 1994), although the most selective ligand for CD44 is hyaluronic acid (STAMENCOVIC et al., 1989). Because of this structural complexity, the CD44 molecules act as receptors for various molecules such as sulfated proteoglycans, fibronectin and type I and IV collagen (CARTER and WAYNER, 1988; JALKANEN and JALKANEN, 1992; TOYAMA-SORIMACHI and MIYASAKA, 1994), although the most selective ligand for CD44 is hyaluronic acid (STAMENCOVIC et al., 1989). In addition to specific antibodies against sulfated proteoglycans, histochemical and immunohistochemical techniques for hyaluronic acid and CD44 are suitable for the analysis of whether glycosaminoglycans are present in secretory granules of acinar cells in the human lacrimal gland and/or localized in the tissue.

The present study examined the specific localization in the human lacrimal gland of glycosaminoglycans, including hyaluronic acid, dermatan sulfate, chondroitin sulfate and keratan sulfate as well as CD44 using histochemical and immunohistochemical techniques.

**MATERIALS AND METHODS**

Human lacrimal glands were obtained from cadavers at autopsy in Iwate Medical University Hospital: the subjects were 12 males and 8 females, and their ages ranged between 16 and 90 years.

**Light microscopy**

Lacrimal glands were immersed in 4% paraformaldehyde-0.2% glutaraldehyde buffered with 0.1 M cacodylate-HCl buffer (pH 7.2) at 4°C for 1 h, and then further in 4% paraformaldehyde with the same buffer at 4°C for 1 h. After washing thoroughly with the same buffer containing 7.5% sucrose, the samples were dehydrated through a graded series of ethanol concentrations and embedded in LR-White in a gelatin capsule. Thin sections were cut with an ultramicrotome (LKB2088, Sweden) and mounted on nickel grids. Thin sections were incubated with 5% normal rabbit serum at room temperature for 20 min. They were then incubated with the following first antibodies at 4°C for 36 h: monoclonal anti-chondroitin sulfate (1:100) and anti-keratan sulfate (1:100). Gold-labeled rabbit anti-mouse IgG (12 nm in diameter) was applied at room temperature for 1 h. Between each step, the grids were rinsed with 0.02 M Tris-HCL buffered 0.5 M NaCl (pH 7.4) containing 0.1% bovine serum albumin. After staining with a saturated aqueous solution of uranyl acetate and lead citrate, they were observed with an electron microscope (Hitachi H-7100, Tokyo, Japan).

**RESULTS**

**Immunostaining for CD44 and histochemical staining for hyaluronic acid**

Immunoreactivity for CD44 was detected in parenchymal and interstitial cells (Fig. 1a, c, e). Its positive staining was clearly demonstrated on the baso-lateral membrane of acinar and ductal cells (Fig. 1a, c), while 100; 3B3, 1 : 100) (Seikagaku Co., Tokyo Japan), and monoclonal anti-keratan sulfate (1 : 100) (Seikagaku Co., Tokyo Japan). A biotinylated hyaluronic acid binding region extracted from bovine nasal cartilage (2 μg/ml) was also applied to the staining of hyaluronic acid. Further incubation was carried out with biotinylated rabbit anti-mouse or rat IgG for 1 h at room temperature and with streptavidin-peroxidase (HISTOFINE; Nichirei, Tokyo, Japan) for 30 min at room temperature. Between each step, the cryosections were rinsed thoroughly with 0.1 M phosphate buffered 0.5 M NaCl (pH 7.2). Staining for peroxidase was carried out with 0.0125% diaminobenzidine (DAB) and 0.002% H2O2 in 0.05 M Tris-HCl buffer (pH 7.6) at room temperature for 10 min. Immunostained cryosections were observed and photographed with a Nomarski differential interference contrast microscope (Olympus; Tokyo, Japan).
Fig. 1. Photomicrographs of the human lacrimal gland stained for CD44 (a, c, e) and hyaluronic acid (b, d, f). Immunoreactivity for CD44 is apparently localized on the baso-lateral membrane of acinar and ductal cells (a, c), and on the vascular endothelium (arrows) and some interstitial cells (e). On the other hand, positive staining of hyaluronate appears intensely on the basal membrane of acinar and ductal cells and weakly, faintly of lacking on their lateral membrane (b, d), while it is also detected in the interstitial fibrous structures, especially in those located in the perivascular region (b, f). a, b: ×90; c: ×450; d: ×550; e, f: ×330
Fig. 2 a. and b. Photomicrographs of the human lacrimal gland immunostained for dermatan sulfate. Immunostaining is demonstrated on interstitial fibrous structures, especially on those located in the peri-acinar and ductal regions. a: ×90; b: ×450

Fig. 3 a and b. Photomicrographs of the human lacrimal gland immunostained for chondroitin sulfate. Immunoreactivity appears intensely in the lumen of the acini and ducts, while it is weakly seen in the acinar cells. In the interstitium, positive staining appears on fibrous structures (arrow). a: ×90; b: ×450

Fig. 4 a and b. Photomicrographs of the human lacrimal gland immunostained for keratan sulfate. The staining pattern of keratan sulfate is similar to that for chondroitin sulfate (see Fig. 3). In addition to intense staining in the lumen of the acini and ducts, strongly immunopositive acinar cells can be seen (arrows), while fine granular immunodeposits are also seen in the apical region of the cells (arrowheads). a: ×90; b: ×450
Fig. 5. Immunocytochemical staining of chondroitin sulfate in acinar cells of the human lacrimal gland. Immunogold particles indicating chondroitin sulfate label electron-dense (a) and -lucent (b) secretory granules of the cells. N nucleus. a: ×9,000; b: ×14,000

Fig. 6. Immunocytochemical staining of keratan sulfate in acinar cells of the human lacrimal glands. Immunogold particles indicating human keratan sulfate are associated with several electron-lucent secretory granules in some acinar cells (a), while they are more densely localized in secretory granules in another cell (b). N nucleus. a: ×9,000; b: ×14,000
it also appeared on the vascular endothelium and some fibroblasts in the interstitium (Fig. 1e). Positive staining of hyaluronic acid by the biotinylated hyaluronic acid binding region was seen in parenchymal and interstitial cells, and interstitial fibrous structures (Fig. 1b, d, f). Reaction products were distinctly detected on the basal membrane of acinar and ductal cells, but were weak, faint, or lacking on their lateral membrane (Fig. 1b, d). In the interstitium, positively stained fibrous structures were prominent at the perivascular region (Fig. 1f), but reaction products were not detected on the vascular endothelium.

**Immunostaining for dermatan sulfate**

When immunostaining for dermatan sulfate was applied to the lacrimal gland, the staining pattern resembled that of hyaluronic acid (Fig. 2a, b). Basically, immunolabelings of dermatan sulfate were associated with fibrous structures and interstitial fibroblast-like cells (Fig. 2). In the peri-acinar and -ductal regions, fibrous structures close to the parenchymal cells were positively stained with anti-dermatan sulfate (Fig. 2b). In contrast with the staining pattern of hyaluronic acid, no staining appeared on the plasma membrane of the acini and ducts at all. Intertissial fibrous structures and fibroblasts were densely associated with dermatan sulfate immunoreactivity (Fig. 2a, b).

**Immunostaining for chondroitin sulfate and keratan sulfate**

As opposed to the staining patterns of hyaluronic acid and dermatan sulfate in the lacrimal gland, immunoreactivity for chondroitin sulfate and keratan sulfate was detected mostly in some acinar cells and the acinar and ductal lumen (Figs. 3, 4). Intense staining was seen in the acinar and ductal lumen, while the immunoreactivity was relatively weak in acinar cells (Figs. 3, 4). In the positively stained acinar cells, immunodeposits appeared granular in some cases, especially those for keratan sulfate (Figs. 3b, 4b). In addition, the positive staining of chondroitin sulfate was seen in fibrous structures located in some parts of the interstitium (Fig. 3a).

To confirm immunoreactivity for chondroitin sulfate and keratan sulfate in some acinar cells, we further analyzed the localization of those glycosaminoglycans using immuno electron microscopy. Immunogold particles indicating chondroitin sulfate or keratan sulfate clearly labeled secretory granules of acinar cells (Figs. 5, 6). In such positively labeled acinar cells, the gold particles were not detected in all secretory granules, but some granules were negative; particularly, those indicating keratan sulfate often appeared restricted to several secretory granules of the acinar cells (Fig. 6a). Chondroitin sulfate-immunopositive secretory granules were electron-dense (mucous) or -lucent (serous) (A or B type granules) (Fig. 5), while keratan sulfate-immunopositive ones were mostly electron-lucent (mucous) (A type granules) and irregular in shape (Fig. 6).

In the present study we examined the localization of CD44 and various types of glycosaminoglycans in the human lacrimal gland obtained from 20 cadavers including 12 males and 8 females aged 16 to 90 years. The localization patterns of CD44 and glycosaminoglycans examined did not show any sexual or age-dependent differences.

**DISCUSSION**

The present study demonstrated that hyaluronic acid, dermatan sulfate, chondroitin sulfate, and keratan sulfate, all major constituents of glycosaminoglycans, were present in the human lacrimal gland, though showing differing localization patterns. Moreover, we also revealed that CD44, a receptor for hyaluronic acid, was localized on the basal-lateral membrane of acinar and ductal cells and the vascular endothelium in the interstitium.

In the human lacrimal gland, there are two types of acinar cells: the type A (K or mucous) and type B (G or serous) cells (KUHNEL, 1968; ALLEN et al., 1972). Type A cells are the source of lysozyme, contributing more to gland volume than the B (ALLEN et al., 1972). Because of the difficulty in collecting tears in adequate amounts for biochemical analysis, histochemical and organ culture methods have been applied to examine the chemical properties of secretory products of acinar cells in the lacrimal glands. The orbital lacrimal glands of rodents and humans examined by the PAS reaction are known to show granules of acinar cells comparable to the granules in the parotid gland, suggesting the presence of glycoproteins (LEBLOND, 1950; TAKAGI and TAKEMURA, 1954). Histochemical staining has further exhibited that there are high amounts of sialic acid and small amounts of acid sulfated glycoconjugates in the acini of the human lacrimal gland (JENSEN et al., 1969; JENSEN, 1970).

By histochemical and organ culture studies, ALLEN et al. (1972) have demonstrated that the type B granules are sources of neutral and acid glycoproteins in tears, the latter being a sulfomucin, while the type A granules contain sialic acid and/or acid sulfated glycoproteins.
It is interesting that the localization patterns of glycosaminoglycans are different. As Jansen et al. (1969) and Allen et al. (1972) have indicated the presence of acid sulfated glycoproteins in the human lacrimal gland, we found in the present study that immunoreactivity for chondroitin sulfate and keratan sulfate appeared weakly in some acinar cells, but intensely in the acinar and ductal lumen. By electron microscopy, immunogold labeling was clearly associated with secretory granules of some acinar cells, although it was not deposited in all secretory granules of the cells, especially in the case of keratan sulfate immunostaining. Moreover, immunogold particles indicating chondroitin sulfate appeared on both A (mucous) and B (serous) type granules, while those showing keratan sulfate appeared mostly on A type granules. These results suggest that acid sulfated glycoproteins in secretory granules of acinar cells in the human lacrimal gland can be attributed to the presence of chondroitin sulfate and keratan sulfate.

At present it remains unknown why the immunoreactivity for chondroitin sulfate and keratan sulfate became intense in the lumen of the acini. Accumulation of these glycosaminoglycans may occur in the lumen of the acini or their epitopes may be exposed by mixing with other secretory components in the lumen. However, judging from the fact that chondroitin sulfate and keratan sulfate are present in the secretory granules of acinar cells and in the acinar and ductal lumen, it seems likely that human tears contain these glycosaminoglycans.

In contrast with the localization pattern of chondroitin sulfate and keratan sulfate, immunolabelings of hyaluronic acid and dermatan sulfate appeared on interstitial fibrous structures, especially those of dermatan sulfate, although chondroitin sulfate immunoreactivity was weakly detected on interstitial fibrous structures. Using a biotinylated hyaluronic acid binding region, Asari et al. (1992) have demonstrated that proteoglycan aggregates including chondroitin sulfate, dermatan sulfate, and keratan sulfate are associated with collagen molecules via hyaluronic acid molecules in articular cartilage. In the interstitial matrix of the human lacrimal gland, therefore, dermatan sulfate proteoglycan may be closely associated with collagen fibrils via hyaluronic acid. According to Gregory et al. (1982), dermatan sulfate proteoglycan contains chondroitin sulfate in rabbit corneal tissue. In comparison with the intense immunoreactivity for dermatan sulfate in the interstitium, that for chondroitin sulfate was only very weakly present in the interstitial fibrous structures, suggesting that dermatan sulfate proteoglycan in the interstitial matrix tissue of the human lacrimal gland may contain chondroitin sulfate.

CD44 is a cell sulfate surface glycoprotein found on epithelial and lymphoid cells, having various molecular forms because of the presence of splice variants (Aruffo et al., 1990; Kenzel et al., 1993). Quantitative analysis of the distribution of CD44 in mice has shown that the spleen, thymus, liver, intestine, uterus and choroid of the eye are the major sites of expression (Kenzel et al., 1993). In rabbit eye, CD44 is expressed in the corneal epithelium and endothelium (Asari et al., 1992b). In the present study, CD44 immunoreactivity was distinctly found on the basolateral membrane of acinar and ductal cells. As stated above, histochemical analysis using a biotinylated hyaluronic acid binding region revealed that hyaluronic acid was associated with the basal membrane of the acinar and ductal cells and weakly, faintly or not at all with the lateral membrane. Considering the fact that CD44 is a receptor protein of hyaluronic acid, the strong association of hyaluronic acid with the basal membrane and weak or faint one with lateral membrane of epithelial cells in the human lacrimal gland may be attributed to the expression of CD44 on the baso-lateral membrane of the epithelium.

Hyaluronic acid and chondroitin sulfate have recently been demonstrated in rabbit tears using high performance liquid chromatography (Miyauchi et al., 1996). By the biotinylated hyaluronic acid binding region, we could not find any positive staining of hyaluronic acid in the acini and their lumen of the human lacrimal gland, although chondroitin sulfate immunoreactivity was distinctly detected in the secretory granules of acinar cells. Hyaluronic acid chain elongation at the reducing end has been suggested to occur in plasma membranes by the alternate addition of the chains to the substrates (Prehm, 1983), while its synthetase activity in oligodendroglialoma cells has been shown to be co-fractionated with a plasma membrane marker (Philipson and Schwartz, 1984). These data may explain the difficulty in the histochemical demonstration of the intracellular localization of hyaluronic acid. According to Asari et al. (1992b), positive staining of hyaluronic acid is present in the rabbit corneal epithelium, being associated with the expression of CD44. Although the origin of hyaluronic acid in the corneal epithelium remains unknown, Asari et al. (1992b) have suggested that hyaluronic acid may be produced in the epithelial cells, since it is also localized in the limbal epithelial and skin epidermal layers (Tammi et al., 1988; Sorrelli et al., 1990; Asari et al., 1992b). Therefore, the hyaluronic acid in rabbit tears demonstrated by Miyauchi et al. (1996) may not originate from the lacrimal
gland but from the corneal epithelium. Of course, we cannot deny the possibility that hyaluronic acid is released from acinar or ductal cells in the lacrimal glands of humans as well rabbits.

It is well known that the lacrimal gland is one of target sites for lymphocyte infiltration closely associated with SJÖGREN’s syndrome (CLAIR et al., 1992). In the present study, the lacrimal gland examined did not show lymphoid cell infiltration even in the tissues obtained from older subjects, while by histochemical and immunohistochemical analyses of the localization of CD44 and glycosaminoglycans, no differences were detected in staining patterns among samples. Although CD44 is usually present on epithelial and lymphoid cells (KENNEL et al., 1993), we also found that CD44 immunoreactivity was localized on the vascular endothelium and some fibroblasts in the interstitium of the lacrimal gland, in addition to the epithelial expression of CD44. At present we do not know the precise roles of CD44 in the human lacrimal gland. However, considering the specific association of CD44 with hyaluronic acid in the lacrimal gland, further studies on the relationship between the two molecules under pathological conditions such as SJÖGREN’s syndrome may be important to reveal the roles of these molecules in the gland.

Acknowledgements. We with to thank Prof. Yutaka TAZAWA, Department of Ophthalmology, Iwate Medical University School of Medicine, for his valuable information and advice, and Dr. Akira ASARI, Tokyo Institute, Seikagaku Corporation, for his technical assistance.

REFERENCES


Prof. Yasuo UCHIYAMA
Department of Cell Biology and Anatomy I
Osaka University Medical School
2-2 Yamadaoka, Suita
Osaka, 565 Japan

内山 安男
565 吹田市山田丘 2-2
大阪大学医学部
解剖学第一教室