Expression of adhesion molecule CD44 on human corneas

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Abstract

Aims—This study was undertaken to confirm the distribution and expression of the molecule CD44 on human corneas under normal and pathological conditions.

Methods—Fifty eight corneal buttons from adult patients suffering from various corneal diseases and four normal corneas were included in this study. Frozen sections were stained immunohistochemically with monoclonal antibodies against human CD44 using an APAAP method and observed under a light microscope.

Results—In normal corneas CD44 was predominantly expressed on the membranes of basal epithelial cells and on the keratocytes, as well as on the vascular endothelial cells of the corneal limbi, but was not expressed on corneal endothelial cells. Enhanced expression of CD44 was observed on the epithelium of corneas with inflammation and allograft rejection. In a number of abnormal conditions including allograft rejection, corneal trauma, primary and secondary corneal endothelial decompensation the remaining endothelial cells stained positively for CD44. However, in some corneas of keratitis, keratoconus, and dystrophy the endothelium which appeared relatively integral in morphology and amount remained CD44 negative.

Conclusions—These results suggest that CD44, the hyaluronate receptor, may play an important role in corneal cell–cell and cell–matrix interactions. Its regulation is closely related to corneal inflammatory reactions. The induction of CD44 on corneal endothelium might play a potential role in compensatory processes when corneal endothelial cells are injured.

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CD44, the hyaluronate receptor,[1] is a transmembrane glycoprotein which was recognised as a cell adhesion receptor that takes part in cell–cell and cell–matrix interaction such as cell migration, lymphocyte homing and activation, and tumour growth and metastasis.[2–4] The main haematopoietic form of CD44 has a molecular weight of 85–95 kDa.[5 6] There are some variant CD44 isoforms (CD44v) induced by alternate splicing of multiple exons into the extracellular domain and by different post-translational modifications in different cell types.[3 7] CD44 may bind its ligand hyaluronan in response to antigenic stimuli and may participate in the effector stage of immunological responses,[8] and be capable of binding fibronectin, laminin, and collagen I.[6 9]

In corneal disorders such as keratitis and corneal allograft rejection some cell adhesion molecules have been shown to be involved in the pathological processes.[10–13] However, whether CD44 participates in the processes of corneal diseases is less well known. There have been very few studies of CD44 in human corneas and the existing reports on the distribution of CD44 on corneal tissues differ. Alho and Underhill reported that CD44 was absent from human corneal epithelium,[14] and Foets and colleagues showed that corneal endothelial cells were negative for CD44, but also reported a 'garland-like' positive pattern of expression in corneal endothelial flat mounts of organ cultured corneas.[15] However, on rabbit corneas Asari *et al* observed that corneal keratocytes were positive for CD44, in addition to epithelial cells and the apical borders of endothelial cells.[16] To confirm further the distribution and expression of CD44 on the corneas we performed an immunohistochemical study on human corneas with an APAAP staining technique.

Material and methods

SPECIMENS

Fifty eight corneal buttons from adult patients suffering from various corneal diseases were collected during penetrating keratoplasty. They were classified into inflammation related or non-inflammation related groups according to the aetiology (Table 1). Four normal corneas from donor eyes (age 56–74, mean 66 years) which did not meet the rigid standards of the Kiel cornea bank were used as controls. One quarter of each of the corneas was snap frozen in Tissue-Tek OCT by immersion in liquid nitrogen. Frozen sections 6 μm thick were prepared with a cryostat, air dried overnight at room temperature, then fixed in acetone for 10 minutes and stored at −20 °C.

IMMUNOHISTOCHEMISTRY

The monoclonal antibodies used for detecting CD44 were F10-44-2 (Biozol, Germany) and J173 (Dianova, Germany). Both of them were diluted 1:50 with phosphate buffered saline containing 1% bovine serum. The alkaline phosphatase anti-alkaline phosphatase (APAAP) method was used with modifications.[17] Briefly, the sections were postfixed with chloroform for 10 minutes and then incubated

Table 1 Clinical data of the patients involved in the study

Clinical diagnosis	No	Mean age/range (years)	Sex (M/F)	Interval (range)* (months)
Inflammation related:				
Keratitis	9	67.2/58–76	4/5	127.4 (10–360)
Graft rejection	8	53.5/20–84	3/5	46.6 (3–204)
Alkaline burn	3	52.3/42–65	3/0	146.7 (77.5–420)
Corneal trauma	2	28.5/23–36	1/1	10.5 (0.5–10)
Non-inflammation related:				
Pseudophakic bullous keratopathy	15	68.7/30–83	8/7	48.5 (3–118)
Graft endothelial failure	2	51/50–52	2/0	40.5 (33–48)
Fuchs' endothelial dystrophy	6	71.8/65–79	2/4	n
Keratoconus	9	31.1/22–45	6/3	13.2 (9–18 years)†
Scrofulous scar	2	56/37–75	2/0	n
Lattice dystrophy	1	67	F	n
Meesmann dystrophy	1	64	F	n

*Time periods (months) from the first clinical event (for keratitis, alkaline burn, and corneal trauma) or previous surgery (for graft rejection, graft failure, and pseudophakic bullous keratopathy) to the corneal allografting.
†Time period (years) from the initiation of spectacle wearing to the keratoplasty.
n = not confirmed.

successively with the primary antibody for 30 minutes, a rabbit anti-mouse immunoglobulin (Dako, Germany, diluted 1:50) for 30 minutes, and the APAAP complex (Dako, Germany) for 30 minutes. Finally, the alkaline phosphatase reaction was developed with the substrate naphthol AS-BI phosphate/new fuchsin (Sigma, Germany). The slides were counterstained with haematoxylin and then mounted with glycergel. All incubation steps were performed at room temperature in a humid chamber and followed by thorough washing of the slides with three changes of TRIS buffered saline (pH 7.6). Positive controls were obtained using the antibodies on cryosections of human tonsil. Negative controls were prepared with normal mouse serum (Dako, Germany) instead of the primary antibody. The intensity of staining was evaluated qualitatively by two independent observers under a Zeiss light microscope as: − no cells stain; + part of the cells stain; ++ all cells or nearly all cells stain.

Results
Staining with the two monoclonal antibodies F10-44-2 and J173 showed identical results.

Table 2 Expression of CD44 on human corneal tissue

Clinical diagnosis	No	Epithelium	Stroma cells	Endothelium
Normal	4	+	$++$[A]	–
Inflammation related:				
Keratitis	9	$+^{2}++^{6}$ (1)	$++$[BC]	$-^{7}$[D] $++^{2}$
Graft rejection	8	$++^{7}$ (1)	$+^{2}++^{6}$[BC]	$+^{5}++^{1}$ (2)
Alkaline burn	3	++	$++$[C]	$++^{1}-^{2}$[D]
Corneal trauma	2	++	$++$[BC]	++
Non-inflammation related:				
Pseudophakic bullous keratopathy	15	$+^{9}++^{4}$ (2)	$+^{2}++^{13}$	$+^{3}++^{5}$ (7)
Graft endothelial failure	2	+	++	+
Fuchs' endothelial dystrophy	6	+	$+^{1}++^{5}$	$+^{2}++^{2}$ (2)
Keratoconus	9	+	$+^{2}++^{7}$	$-^{9}$[D]
Scrofulous scarring	2	+	$+^{1}++^{1}$	$-$[D]
Lattice dystrophy	1	+	++	$-$[D]
Meesmann dystrophy	1	+	++	$-$[D]

Numbers in parentheses indicate the number of cases in which no cell layer existed on the slide. Superscript numbers show the number of patients with positive staining at different levels of staining intensity as indicated.
A = CD44 positive vascular endothelium in the corneal limbal stroma.
B = CD44 positive neovascular endothelium.
C = CD44 positive infiltrate.
D = endothelial cells relatively normal in morphology and amount.

No cell staining was found on the negative controls. The results are summarised in Table 2.

EXPRESSION OF CD44 ON CORNEAL EPITHELIUM
In normal corneas, CD44 was preferentially expressed on the basal layer of epithelial cells with a integral membrane staining pattern (Fig 1A). The staining of wing cells was related to the corneal location. In general, there were fewer CD44 positive wing cells in central epithelium than in peripheral epithelium. In corneal limbus, almost all epithelial layers stained strongly for CD44 (Fig 1B). A higher intensity of CD44 staining, by which all epithelial layers stained, was observed on the epithelium of all corneas with allograft rejection, corneal trauma, alkaline burn, corneas with keratitis except two cases, and four of 15 with pseudophakic bullous keratopathy. This was especially obvious in regions where graft and receptor corneas adjoin, where Bowman's membrane was destroyed, and where epithelium showed abnormal thickening (Fig 1C and D). There were no evident differences in CD44 expression between control corneas and those with keratoconus, Fuchs' endothelial dystrophy, scrofulous scarring, nine of 15 specimens of pseudophakic bullous keratopathy, except that some pathological cases showed varying extents of granular staining pattern on the epithelial membranes (Fig 1E).

EXPRESSION OF CD44 ON CORNEAL STROMA
CD44 was constitutively expressed on most keratocytes in normal corneas. Vascular endothelium in corneal limbal stroma expressed CD44. Neovascular endothelium in the stroma of corneas with keratitis, allograft rejection and corneal trauma displayed strong intensity of CD44 staining (Fig 1F). In these conditions, CD44 was expressed intensely on a varying number of infiltrating cells within the stroma. No apparent alterations of CD44 staining on the keratocytes in the diseased corneas were observed.

EXPRESSION OF CD44 ON CORNEAL ENDOTHELIUM
In normal corneas, the endothelial cells were definitely negative for CD44 (Fig 1G). In diseased corneas CD44 expression on endothelial cells showed a close association with the endothelial alterations in morphology and in amount. Under the light microscope, the endothelial cell layer was apparently attenuated and partially denuded in the cases of allograft rejection, graft endothelial failure, Fuchs' endothelial dystrophy, corneal trauma, pseudophakic bullous keratopathy, and two specimens of keratitis. In these cases CD44 was expressed on the remaining endothelial cells (Fig 1H and I). No CD44 staining was detected on the corneal endothelial cells of the remaining patients including seven cases of keratitis, two of alkaline burn, nine of keratoconus, two of scrofulous scarring, two of Meesmann and lattice dystrophy, in which the endothelial cells
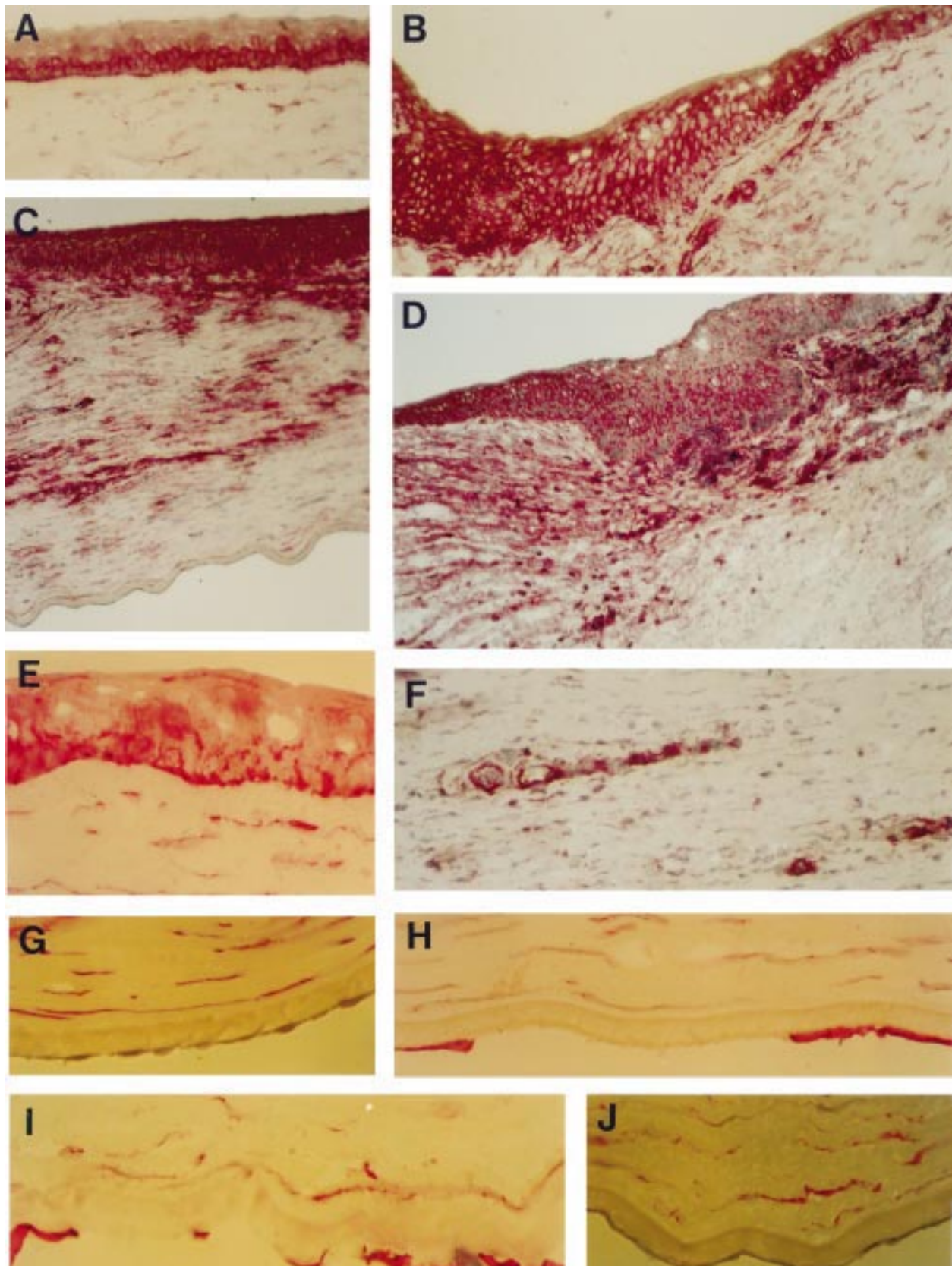
Figure 1   Alkaline phosphatase anti-alkaline phosphatase immunostaining for CD44 in human corneal sections. Original magnification × 200 for (A), (B), (C), (D) and (F); and × 400 for the others. (A) Epithelial cell staining in normal central region; (B) epithelial cell staining in normal limbus; (C) section from a cornea with keratitis showing intense staining in all epithelial layers, but no staining on endothelial cells; (D) section from a cornea with allograft rejection showing the staining of epithelium in the region where graft and receptor corneas adjoin; (E) epithelial cell staining in Fuchs' endothelial dystrophy; (F) CD44 staining on neovascular endothelium and infiltrating cells in cornea with keratitis; (G) normal corneal endothelial cells showing no staining for CD44; (H) section from a cornea with pseudophakic bullous keratopathy showing positive staining on the remaining endothelial nells; (I) section from a cornea with Fuchs' endothelial dystrophy showing the CD44 staining on the remaining endothelial cells; and (J) section from a cornea with keratoconus showing negative staining of endothelial cells for CD44.

appeared relatively normal in morphology and in amount (Fig 1J and C).

Discussion

CD44 is an transmembrane adhesion molecule that has a complex structure and function. Its 'standard' form (CD44s), which is a hyaluronan receptor, exists on haematopoietic cells and many other cell types.[5 6] In this study, CD44s was detected on the membranes of the corneal epithelial cells. This finding is in keeping with the reports that CD44 exists on epithelial cells of various human tissues including skin, intestine, lung, and kidney.[4 18] The different extents of CD44 expression may be a result of a different differentiation pattern related to the epithelial location, and may indicate that CD44 is related not only to the maintenance of normal tissue structure, but also to the proliferative potency of the epithelium, as suggested by some studies.[4 14 19] Our study showed that normal corneal keratocytes expressed CD44. This is probably also associated with the maintenance of stroma tissue structure. The finding that normal corneal endothelium did not express CD44 is in agreement with the report of Foets and colleagues,[15] and may suggest a cell type specific expression on corneal tissue. However, whether there is CD44 isotype specific expression on corneal endothelial cells should be confirmed in further studies.

In pathological cases the corneal tissues displayed various expressions of CD44. In keratitis, allograft rejection, corneal trauma, and alkaline burn the expression of CD44 was upregulated on the epithelial layers. This may indicate an association of CD44 with inflammation related conditions, although the significance of the regulation is unclear. The pathological meaning for the alteration of a granular pattern of CD44 staining on epithelial cells by some non-inflammation related specimens needs further study. However, we did not find evidence of upregulation of CD44 on keratocytes in the same cases. This might be explained by: (i) all keratocytes stain already in the normal cornea. Compared with the epithelium, there are no more layers and no other differentiation stadia in the stroma; (ii) a cell type specific regulation; (iii) a disease specific regulation. This is not very likely since CD44 is expressed on most epithelial layers, while the expression of keratocytes stays the same in the same diseased cornea. The expression of CD44 on infiltrating leucocytes and neovascular cells might play different roles during inflammation and graft reaction.

Our most striking finding is that CD44 was produced on corneal endothelial cells. The basis for the CD44 production on the corneal endothelium is unknown. However, our results showed that CD44 production is apparently associated with the integrity of the endothelial layer. It was expressed on the remaining endothelium in Fuchs' endothelial dystrophy, pseudophakic bullous keratopathy, and late stages of corneal allograft rejection, in which the endothelial cells are consistently related to or causative of the pathological process. In the conditions, the endothelial cells (probably no longer normal) have to spread their cell bodies to compensate for the loss of cells and to maintain the endothelial barrier by keeping the Descemet's membrane covered with cells. CD44, however, was not detected on the endothelial cells in the corneas of keratoconus, Meesmann, and lattice dystrophy where it is known that the endothelial cells are not involved in the disease specific pathological processes. It was interesting that the endothelial cells of seven corneas with keratitis remained negative for CD44, although the specimens may represent an end stage of the disease process. This might be explained by the non-involvement of the endothelial layer in these cases, since they appeared still relatively integral. These results suggest an association between CD44 and injury and the consequent compensatory processes of the endothelium. Besides serving as the hyaluronate receptor, CD44 was confirmed to be capable of binding some components of the extracellular matrix, including fibronectin, laminin, and collagen I,[6 9] which are important mediators of endothelial functions such as adhesion, spreading, migration, and proliferation.[20] The induction of CD44 on the remaining endothelial cells might be essential for mediating corneal endothelial cell–cell and cell–Decemet's membrane adherence in cases of endothelial cell damage.

The upregulation of CD44 in inflammatory processes of the skin, rheumatoid arthritis, and sympathetic ophthalmia has been studied previously.[21–24] The association of CD44 with inflammation was further supported by some in vitro studies in which CD44 was upregulated by some inflammatory cytokines on various cell types such as NK cells,[25] epidermal Langerhans cells,[26] endothelial cells, myelomonocytic cell lines, and some epithelial cell lines.[4 27] It seems likely that the CD44 regulation is disease and isoform specific, as suggested by Rosenberg and colleagues.[28] Their cytochemical study on biopsy samples from various types of human colitis showed significantly increased epithelial expression of CD44 isoforms containing the v6 and v3 epitopes. The implication was that varying CD44 isoform expression could potentially help to differentiate ulcerative colitis from Crohn's disease and other forms of colonic inflammation. In addition, a recent study revealed that CD44 expression increased significantly on cytomegalovirus infected human embryonic fibroblasts, but decreased in herpes simplex virus 1 and varicella zoster virus infected human embryonic fibroblasts,[29] also suggesting a disease specific regulation of CD44.

CD44 has been known to be associated with the activation and migration of lymphocyte to secondary lymphoid tissue via adhesion to high endothelial venules.[30–32] However, the actual mechanism by which CD44 plays roles in the immune activity related to tissue transplantation remains uncertain. Evidence is accumulating to demonstrate the participation of stimulated cells expressing high levels of CD44 in

immunological responses in vivo. Mobley and Dailey observed the appearance of a minor Mel-14 negative, high CD44+ population of CD8+ cells in lymph nodes draining an allograft.[33] Mobley *et al* found that late after rejection of minor histocompatibility Ag disparate skin grafts, the majority of memory CD8 T cells express CD44.[34] A recent study using sheep renal allografts revealed that alternatively spliced CD44 mRNA appeared as early as day 1 after transplantation and peaked between days 3 to 5.[35] Similarly, in a rat small bowel graft model significantly increased CD44 expression was detected in the lamina propria on days 5 to 7, and it was suggested that CD44 expression on enterocytes may provide an early indicator of a developing rejection response.[36] Recently, some studies noticed a relation between CD44 modulation and cytotoxic activity of NK cells,[37 38] indicating that CD44 may be involved in the effector stage of immune responses.

In this report we have demonstrated the distribution and expression of CD44s on human corneal tissues. The various expressions of CD44 we observed suggests that the hyaluronate receptor might be closely associated with the pathological processes of some corneal diseases, although the most specimens investigated in this study were samples of the end stage of the diseases. Further investigations on the expression and function of various CD44 isoforms on human corneas need to be done. Future studies should also focus on the time course and functional relation with other biological regulating systems.

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