Expression and Distribution of Adhesion Molecule CD44 in Healing Corneal Epithelia

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PURPOSE. To study isoform expression and cellular distribution of CD44, a cell surface glycoprotein thought to be an adhesion molecule in cell-cell and cell-substratum interactions, during corneal epithelial wound healing.

METHODS. Reverse transcription-polymerase chain reaction was performed to determine alternatively spliced rat CD44 isoforms. In situ hybridization was carried out on frozen sections of the rat corneas obtained at different time points after epithelial debridement. 35S-Labeled sense and antisense cRNA that recognizes rat CD44 standard form was used as a probe. Immunofluorescence was used to assess expression and localization of CD44 in the rat corneas during reepithelialization.

RESULTS. Corneal epithelia contained several alternatively spliced CD44 variants. Four large CD44 variants with inserts V1 through V10, V2 through V10, V3 through V10, and V4 through V10 were differentially expressed in migratory epithelia. The silver grains, indicating CD44 transcripts, started to increase in the epithelial cells surrounding the wound margin 3 hours after wounding and peaked at 18 hours in the basal epithelial cell layers, at which time the epithelia were actively migrating. As the cells began proliferation after wounding, the density of CD44 mRNA label declined but was still significantly higher than that in control specimens. The label returned to basal level as epithelial cells reverted to their normal phenotype. The location of CD44 on cell surfaces during corneal reepithelialization was consistent with the pattern of mRNA production. In the corneas at 18 hours after wounding, CD44 immunoreactivity was elevated in the entire epithelium, from the leading edge to the limbal-corneal border. As happened for the mRNA, the cell surface CD44 declined as cells differentiated to reestablish the multilayered epithelium.

CONCLUSIONS. The expression of CD44 correlates with corneal reepithelialization, suggesting that CD44 may be involved in cell-cell interactions that provide adhesive strength for the much-stressed epithelial sheet and in the cell-substratum interactions that mediate cell migration during reepithelialization. (Invest Ophthalmol Vis Sci. 1998;39:710–717)
was elevated in healing corneal epithelia and that wounding resulted in increased expression of CD44 in the basal and suprabasal layers. Furthermore, correlation was shown between the increased expression of CD44 and reepithelialization. The results of this study provided evidence for possible involvement of CD44 in reepithelialization and corneal wound healing.

MATERIALS AND METHODS

Corneal Wounding by Epithelial Debridement

Procedures involving experimental animals were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. A 3-mm diameter central area of the corneas of Sprague-Dawley rats weighing 150 to 175 g was demarcated with a dulled trephine, and the epithelium within this region was removed by gentle scraping with a scalpel. This procedure has been shown, at the electron microscopic level, to leave the basement membrane underlying the wounded area morphologically intact. The wounds were allowed to heal in vivo for various periods. At the end of each interval, the animals were euthanatized by CO2 inhalation, and the corneas were removed.

RNA Isolation and Amplification of CD44 Variants

The epithelial layers were scraped from all corneas and were transferred immediately to 1.5-ml microtubes cooled in an ethanol–dry ice bath. Total RNA was extracted from isolated corneal epithelia by the guanidine thiocyanate-phenol method. In general, eight wounded and eight normal corneas were used for RNA preparation, from which 30 to 60 µg of total RNA may be obtained. First-strand cDNA was generated in a 20-µl reaction volume in the presence of 0.5 µg oligo(dT) from 4 µg total RNA with reverse transcriptase (SuperScript II; Life Technologies, Gaithersburg, MD). The paired primers adjacent to the splicing site were GACCTTCCGACITTCAAGGCC and CTTCGGAAATCGATGATGCC for amplifying CD44 variants. A commercial system (Expand High Fidelity PCR; Boehringer Mannheim, Indianapolis, IN) was used with 1 µl cDNA as a template, and reverse transcription-polymerase chain reaction (RT-PCR) was performed according to the manufacturer's instructions. The PCR products were examined on agarose gels by ethidium bromide staining. To confirm the identity of the isoforms, the PCR products, separated on agarose gels, were recovered in two groups, one representing the predominant band (marked s, Fig. 1A) and one representing all others. Each group was cloned into pCR-Blunt Vector (Invitrogen, Carlsbad, CA). At least two clones with inserted cDNA fragments corresponding to each band in size (Fig. 1A) were sequenced (Sequenase II; United States Biochem, Cleveland, OH). To generate probes for in situ hybridization, the paired primers, GACCTTCCGACITTCAAGGCC and CTTCGGAAATCGATGATGCC were used to amplify rat CD44 cDNA. The PCR-generated DNA fragment corresponding to the cDNA encoding CD44 was cloned into TA cloning vector (Invitrogen) and was sequenced for molecular identity.

In Situ Hybridization

In situ hybridization was performed, according to a previously described method.21 Rat cDNA corresponding to the entire coding sequence of CD44 was used as a template for the synthesis of 35S-uridine triphosphate-labeled cRNA probes. This probe hybridized with all spliced mRNA isoforms expressed in cells. The size of the probes was adjusted to approximately 150 bp by treating cDNA with 0.2 M sodium carbonate (pH 10.2) for 50 minutes at 60°C. Cryostat sections (approximately 6 µm, three to four sections on each slide and three slides for each hybridization condition) of adult mouse eyes were fixed with 4% paraformaldehyde, treated with 1 µg/ml proteinase K, and acetylated. RNA probe (0.2 µg/ml per kilobase) was added to 60 µl hybridization solution containing 0.3 M NaCl, 10 mM Tris–HCl (pH 7.6), 5 mM ethylenediaminetetraacetic acid, 0.02% (wt/vol) Ficoll 400 (Pharmacia, Uppsala, Sweden), 0.02% (wt/vol) polyvinylpyrrolidone, 0.02% (wt/vol) bovine serum albumin, 50% deionized formamide, 10% dextran sulfate, 10 mM dithiothreitol, and 0.1 mg/ml yeast tRNA. Hybridization was carried out in a humid chamber over-
Alternative splicing of the CD44 gene transcript gives rise to wounded corneal epithelia (Fig. 1A). Primers that spanned the phosphate-buffered saline (PBS) for 10 minutes. Monoclonal goat immunoglobulin G (IgG) and 1% bovine serum albumin in CD44 Isoforms Expressed in Healing Corneal ImmunoResearch, West Grove, PA) for 1 hour at room temperature in a moist chamber. The bound antibodies were visualized after incubation with a 1:250 dilution of fluorescein isothiocyanate goat antimouse IgG (Jackson Immunoresearch, West Grove, PA) for 1 hour at room temperature in a moist chamber. After washing, coverslips were mounted with medium (Vectashield Mounting; Vector, Burlingame, CA). As a negative control, secondary antibodies alone were incubated with tissue sections. The sections were examined with a fluorescence microscope (Axiphot; Carl Zeiss, Thornwood, NY) or a confocal laser scanning microscope (TCS 4D; Leica, Nussloch, Germany) equipped with the excitation and barrier filters (515-545 nm) for fluorescein. The sections from different stages of wound healing were viewed consecutively at the same instrument settings. Corneas were sampled by collecting serial images, spaced 1 μm apart, through the full thickness of the cryostat sections. One optical section was chosen from each time point, and the confocal images were printed (XLS8000 PS printer; Eastman Kodak, Rochester, NY).

RESULTS

CD44 Isoforms Expressed in Healing Corneal Epithelium

Alternative splicing of the CD44 gene transcript gives rise to various CD44 mRNA and protein products in normal and malignant cells.10,11 reverse transcription-polymerase chain reaction amplification of rat corneal epithelial cDNA was used to detect and compare alternative splicing in control and wounded corneal epithelia (Fig. 1A). Primers that spanned the single splice site produced nine distinct bands from healing and five from normal corneal epithelia. Among the PCR products detected, the most abundant isoform was the smallest one observed without a V1 to V10 insertion (CD44), along with four minor forms in normal and healing corneal epithelia. In contrast, four large bands appearing as abundant forms were detected in the healing cells but not in the stationary corneal epithelial cells, indicating differential expression of these isoforms in migratory epithelial cells. To reveal the molecular identity of CD44 isoforms detected in healing corneal epithelia, the PCR products were subcloned. At least two clones with inserted DNA fragments corresponding to each band in size (Fig. 1A) were sequenced. Only one species of CD44 transcripts was identified from each band shown in Figure 1A. It should be mentioned that this result would be expected 50% of the time if any two similar sized transcripts were presented in equal amount. Furthermore, this approach would not allow detection of a lower amount of splicing variants in the same band. All nine isoforms detected and sequenced from healing corneal epithelia contained an exon encoding for 23 amino acids that was downstream of V10 (Fig. 1B). This exon was reported only in mouse, rat, and hamster,1,2,22,23 suggesting that it is a rodent-specific exon. Because it was not seen in the pMeta-1 clone that derived from a rat pancreatic carcinoma cell line,24 we suggested that this may be another alternatively spliced exon, which we referred to as V11. The alternative splicing was found at V1, V2, V3, V4, V6, V8, and V9. In all but one specimen, the 5' exon was accompanied by all downstream exons, yielding transcripts of varying size. The isoforms using splicing sites V1, V2, V3, and V4 (bands 1–4, respectively) encoded four major forms found in healing corneal epithelia. Isoforms using splicing sites V6 (band 5), V1 and V9 (band 6), two splicing sites, V1 and V9, were used; V1 splicing ended at the 3' site of V2, V8 (band 7), and V9 (band 8) encoded minor forms present in normal and healing corneal epithelium (Fig. 1).

Expression and Localization of CD44 mRNA during Corneal Reepithelialization Determined by In Situ Hybridization

To correlate CD44 expression with the process of reepithelialization, the presence of CD44 mRNA in corneal epithelia was determined by in situ hybridization. Figure 2 shows the distribution of CD44 mRNA-specific labeling during epithelial wound closure. A comparison of Figure 2A with Figure 2B shows that there were more silver grains in normal epithelium probed with antisense cRNA than were seen with a probe of sense cRNA, suggesting the presence of CD44 transcripts in epithelial cells in normal cornea. CD44 mRNA-specific labeling in the epithelial layer of corneas 3 hours after wounding was not uniformly distributed and was particularly abundant around the wound margin (large arrowhead, Fig. 2C) when compared with that in normal cornea (Fig. 2B). In corneas examined 8 hours after wounding (Figs. 2D, 2d), epithelial cells had begun to move into the wound bed with a well-defined leading edge (large arrows); high density of silver granules could be seen in the basal epithelial cell layers, relative to more superficial layers. The migrating epithelial sheet covered a much larger area at 18 hours after wounding (Figs. 2E, 2e); the density of silver grains in the basal epithelial cell layers was markedly high at this time point (Fig. 2E). The increase in CD44 mRNA-specific labeling in certain epithelial cells in wounded corneas indicated increased expression of CD44 transcripts in these cells.

In Figure 3, the expression and distribution of CD44 transcripts is shown after epithelial wound closure. As is shown in Figure 3A, the epithelial cell layers and cell density were reduced in the just-closed wound when compared with the morphology of the normal cornea (Figs. 2a, 2b). CD44 mRNA-specific labeling in the basal cell layers at this time point was still heavy (Fig. 3A), suggesting a continued increase in CD44 transcripts. One day after wound closure (2 days after wounding), the epithelial cells were at a hyperproliferative stage, evidenced by the increase in cell layers (compare Fig. 3b with Figs. 2a, 2b) and in cell number. CD44-specific labeling at this time point (Fig. 3B) was still higher in basal epithelial cell layers than that in normal control specimens (Fig. 2B). Four days after wounding, the corneas were morphologically similar to that of normal control specimens (Fig. 3C). Taken together, we concluded that the increased expression of CD44 transcripts detected by in situ

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hybridization correlated with corneal reepithelialization and restratification.

Expression and Localization of CD44 during Reepithelialization Assessed by Immunohistochemical Analysis

To evaluate CD44 expression at the protein level, immunohistochemical analysis was performed in frozen sections of normal corneas and those 18 hours after wounding (Fig. 4). Typical cell surface staining of CD44 was observed in the basal epithelia of conjunctiva and limbus (Fig. 4D) but not in normal corneal epithelium (Figs. 4C, 4D). A similar result was obtained when a different CD44 antibody was used.25 Remarkably, the basal epithelial cell layers in the healing cornea from the leading edge to the limbus-cornea junction was intensively labeled with CD44 antibody (Fig. 4A), indicating that the levels of CD44 protein in those cells were increased.

Expression and cellular distribution of CD44 at protein levels during reepithelialization were also examined (Fig. 5) with confocal laser scanning microscope. CD44 immunoae-
FIGURE 3. In situ hybridization of CD44 mRNA in rat corneal epithelia in the late phase of reepithelialization. Antisense CD44 cRNA (35S-labeled) corresponding to the entire coding sequence of CD44 was hybridized with corneal cryostat sections obtained from corneas 1 day (A, a), 2 days (B, b), and 4 days (C, c) after wounding. CD44 transcripts were noted in the basal epithelial layers of hyperproliferating corneas (A and B small arrows) but at background levels in the cornea with normal morphology (C, small arrowheads). EP, epithelium; ST, stroma. Bar, 50 μm.

Activity in the control normal corneal epithelium (Fig. 4) was undetectable above background (compare Fig. 5A with 5B). It is notable that despite the increase in CD44 transcripts, staining for CD44 protein in corneas at 3 hours after wounding was negative. At this time, the wounded epithelium was in the latent phase with no cell migration (Fig. 5C). CD44 immunoreactivity started to appear 8 hours after wounding on the surface of several epithelial cells surrounding the leading edge (arrow, Fig. 5D) and reached a remarkable level 18 hours after wounding (Fig. 5E). Strong CD44 staining was continually observed in the basal side of the epithelium after wound closure (Fig. 5F) and when the cells were hyperproliferating (Fig. 5G). In addition, CD44 staining was also observed in the apparently repopulating stroma keratocytes, indicating a pos-
Possible role of the protein in keratocytes cell proliferation, migration, or both. In Figures 5E, 5F, and 5G, the immunoreactivity of CD44 was localized across the entire cell surface. It was seen as interstitial staining along the basement membrane and was differentially distributed among the epithelial cell layers. There was strong staining in basal and suprabasal cell layers, moderate staining in wing cell layers, and no staining in apical cell layers. At day 4, when CD44 transcripts were at the basal level, a low level of surface staining of CD44 was still seen at the cell surface in two basal cell layers but not along the basement membrane (Fig. 5H), suggesting that CD44 may not be required for cell-substratum interaction at that time point.
DISCUSSION

CD44 comprises a group of cell surface glycoproteins that bind to various extracellular matrix components and modulates cell adhesion, cell migration, lymphocyte homing, and hematopoiesis.\(^6\)\(^7\) We hypothesized that CD44, through its interactions with hyaluronan and extracellular matrix components, may participate in epithelial sheet migration overlying the denuded basement membrane in a corneal debridement wound. In doing so, CD44 may have increased levels of CD44 mRNA and protein and altered isoform expression or cellular distribution in healing, compared with that in normal corneal epithelial cells. We are reporting the identification of nine alternatively spliced isoforms of CD44 expressed in healing and five in normal corneal epithelia. Our findings imply that these molecules have multiple functions in the corneal epithelium and in wound repair. We showed a marked increase in the level of total CD44 mRNA in healing corneal epithelium compared with that in stationary epithelia. Further, we documented a delay of several hours between the detection of CD44 mRNA and its protein’s appearance as cell surface molecules in healing corneal epithelia, which suggests possible regulation at translational and posttranslational levels. The spatial and temporal expression of CD44 in healing corneal epithelia is consistent with the hypothesis that CD44 plays multiple roles in mediating cell-cell and cell-matrix interaction during corneal reepithelialization.

The most striking features of the CD44 glycoprotein is the extent of its molecular heterogeneity, which is now recognized as arising mainly from alternative splicing.\(^6\)\(^7\) In the rat, as many as 11 alternative exons (V1-V11) may be inserted in tandem within a region of the mRNA that codes for the membrane-proximal portion of the extracellular domain.\(^10\)\(^-\)\(^12\) We detected nine isoforms detected in healing corneal epithelia. The two large bands detected (bands 1 and 2; Fig. 1) are the longest CD44 isoforms ever detected. Additional long sequences provide additional glycosylation sites and an attachment sequence for sulfated glycosaminoglycans (located at V3 for heparin or chondroitin sulfate), thereby further increasing its molecular heterogeneity.\(^7\)\(^,\)\(^26\)\(^-\)\(^27\) Thus, the unusually high complexity in the alternatively spliced isoforms provides CD44 with a high degree of structural and functional heterogeneity in the corneal epithelium.

What biologic role does CD44 play in corneal reepithelialization? It is likely that some CD44 isoforms are involved in cell-cell interaction and that they provide tensile strength for healing epithelial cells, whereas the others may serve as an adhesion molecule that is required in cell-matrix interactions for effective cell migration, including the lateral movement observed during wound closure and the upward movement seen during epithelial cell hyperproliferation. To date, it is unclear which splicing variants participate in the cell-cell or cell-substratum interaction.\(^7\) In corneal epithelium, in addition to desmosomes,\(^28\)\(^-\)\(^30\) tight junctions and adherent junctions—containing the tight junction-associated protein ZO1, a focal adhesion associated phosphoprotein Paxillin, or both—were also seen.\(^31\)\(^,\)\(^32\) Compared with adhesion in these structures, CD44-mediated cell-cell adhesion may be more transient. Furthermore, CD44 molecules are known to be connected to the actin cytoskeleton through the ezrin-radixin-moesin family of proteins,\(^33\) whereas desmosomes may be dissociated from intermediate filaments in migratory epithelial cells.\(^3\)\(^-\)\(^34\) CD44-mediated adhesion should facilitate epithelial sheet migration and hyperproliferation. Therefore, our data showing that the wound-induced CD44 are localized and accumulate at the interstitial space of the epithelial sheet of healing cornea support a role of CD44 in epithelial cell-cell adhesion during wound healing as has been shown for apican, a heparan-chondroitin sulfate proteoglycan form of CD44-mediating keratinocyte cell–cell adhesion.\(^35\)

In normal corneas, the epithelial sheet is linked to the basement membrane through hemidesmosomes that include \(\alpha 6\beta 4\) integrin.\(^3\)\(^6\)\(^-\)\(^38\) A manifestation of altered epithelial phenotype during reepithelialization is the loss of tenacious binding between the epithelium and its underlying extracellular matrix that results from the dissolution of hemidesmosome.\(^36\)\(^-\)\(^38\) The presence of CD44 immunoreactivity beneath the epithelial sheet correlates with the disappearance of syndecan 1\(^29\) and the redistribution of integrin \(\alpha 6\beta 4\).\(^36\)\(^-\)\(^38\) CD44 has been implicated in cell-matrix adhesion of normal and transformed cells.\(^40\)\(^-\)\(^42\) In our study, the observation of CD44 localized in the basal side of the epithelial sheet in the wound and the appearance of immunostaining of CD44 in epithelial cells, coincident with the disappearance of hemidesmosomes,\(^36\)\(^-\)\(^38\) are consistent with a role of CD44 in cell-substrate adhesion. Thus, CD44 may serve as an adhesion molecule that is required in cell-matrix interactions for effective epithelial cell migration, as has been demonstrated in fibroblasts and keratinocytes.\(^5\)\(^-\)\(^44\)

Recently, CD44 was selectively suppressed in keratinocytes of the skin and in epithelium of the cornea of mice bearing an antisense CD44 transgene driven by a bovine keratin-5 promoter.\(^45\)\(^,\)\(^46\) In these transgenic mice, CD44 expression is not detected in skin keratinocytes and corneal epithelium, and abnormal hyaluronate accumulation is seen in the superficial dermis and beneath the corneal epithelium.\(^46\) Distinct morphologic alterations in basal keratinocytes and in corneal epithelium of the transgenic mice occur, indicating a major biologic role for CD44 in these tissues.\(^46\) In addition to the developmental role of CD44 cells demonstrated by the use of the transgenic mice, our results provide evidence of a role of CD44 in reepithelialization. Reepithelialization, an essential step in many forms of wound repair, is a complex process relying on cooperative action of many adhesion systems. CD44 may be an important player in this process: A full description of the behavior of this intriguing molecule awaits further study.

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