Localization of Corneal Epithelial Stem Cells in the Developing Rat

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A monoclonal antibody, 4G10.3, was developed that preferentially binds limbal basal cells in adult rat, rabbit, and human corneas. These cells were hypothesized to be the stem cells for the corneal epithelium. The antibody 4G10.3 was localized by immunofluorescence microscopy in rats 1 d and 1, 1.5, 2, 3, 4, and 6 wk of age. Until 1.5 wk, 4G10.3 bound intensely to all basal cells in the cornea and the limbus. At 2 wks, the basal cells at the central cornea abruptly changed their shape from flattened or ovoid to large and cuboidal and bound 4G10.3 with greatly reduced intensity. Increased stratification of epithelium also was seen. Cells binding 4G10.3 gradually became sequestered to the limbal area after 2 wk, concomitant with increased stratification. At 4 and 6 wk, 4G10.3 binding was identical to that in adult corneas with only limbal basal cells showing positive binding. Basal cells in the limbal epithelium did not decrease their intense binding of 4G10.3 or change their ovoid cellular shape from 1 d through adult life. These results suggest that, during development, stem or stem-like cells are localized throughout the basal layer of the corneal and limbal epithelium. As the cornea matures, these cells are sequestered in the limbus at the same time that stratification of the epithelium and shape changes occur in the basal cells. Invest Ophthalmol Vis Sci 33:2199-2206, 1992

Stem cells are thought to be present in all self-renewing tissues and are believed to be responsible for tissue homeostasis. During stress, such as accidental loss of cells, they amplify to replace the lost cells.1,2 In the cornea, the epithelium has been found to undergo a steady rate of cell mitosis and turnover.3 Epithelial cell migration as a primary response in epithelial wound healing has been studied.4-7 These observations, along with the studies of centripetal movement of corneal epithelial cells,8-10 have led to the hypothesis of stem cell existence in this tissue.

The exact location of corneal epithelial stem cells was unclear until it was hypothesized recently that the stem cells of the corneal epithelium are located in the basal layer of the limbal epithelium.11 This hypothesis was based on the finding that a 64-kD keratin, a marker for an advanced stage of corneal epithelial differentiation, is expressed in all corneal and limbal epithelial cells except limbal basal cells. This experiment used a monoclonal antibody, AE5, that was specific for the 64-kD keratin. Others subsequently supported this hypothesis by using 3H-thymidine labeling and showing the existence of slow-cycling limbal basal cells that can be stimulated preferentially to proliferate in response to wounding and a tumor promoter.12 However, positive identification of such stem cells has been difficult because of the lack of immunologic or biologic markers specific for limbal basal cells.

Recently, we developed a monoclonal antibody that binds to limbal basal cells in adult rat, rabbit, and human corneas.13 This monoclonal antibody, designated 4G10.3, specifically reacts against a 50-kD protein in western blots and binds to the limbal basal cells that are not bound by monoclonal antibody AE5.14 Reciprocal binding of AE5 and 4G10.3 was found in both human and rabbit limbus. Furthermore, in our wound model using rat corneas, the number of cells bound by 4G10.3 increased two- to threefold in response to a central epithelial defect or a corneal thermal burn.14 By contrast with simple epithelial defects, thermal burns damage underlying basement membrane and stroma and lead to epithelial hyperproliferation. This stress on the stem cells to renew the corneal epithelium resulted in a greater number of cells binding to 4G10.3. Therefore, 4G10.3 is a potential marker for either corneal epithelial stem cells alone or for both corneal epithelial stem cells and early-stage daughter cells that still carry 50-kD protein derived from their stem cells.

We analyzed changes in the stratification of epithelium, basal cell shape, localization, and binding inten-

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sity of 4G10.3 in the developing rat cornea. We investigated the stem or stem-like cell change during corneal epithelial maturation in the early developmental life of a rat. Our results support the concept that the limbal basal cells are stem cells of the corneal epithelium.

Materials and Methods

Antibody Preparation

We prepared the 50-kD protein-specific monoclonal antibody as previously described. This antibody is designated 4G10.3. As the source of antigens, a 0.1 mol/l ammonium acetate extract of limbal-to-limbal scrapes of rat corneal epithelium harvested 18 hr after scrape wounding was used. The antibody was prepared with standard hybridoma techniques. Hybridomas were screened by western blots, and one hybridoma that produced an antibody reactive with a 50-kD protein was detected. This hybridoma line was cloned twice by limiting dilution to ensure monospecificity.

Animal Model

Sprague-Dawley rats were examined at 1 d and 1, 1.5, 2, 3, 4, and 6 wk of age (four animals at each time) as were two adult rats, used as controls. Eyelid opening occurred at 2 wk. The rats were killed by an intraperitoneal injection of a lethal dose of sodium pentobarbital, and their eyeballs were enucleated. Rats younger than 2 wk of age required surgical excision of eyelid tissue. All experimental techniques adhered to the ARVO Resolution on the Use of Animals in Research.

Immunofluorescent Staining

The tissues were frozen in Tissue Tek II OCT Compound (LabTek, Naperville, IL); then 6-μm cryostat sections were placed on gelatin-coated slides and air dried overnight at 37°C. These slides were rehydrated in phosphate-buffered saline (PBS) and incubated in bovine serum albumin (BSA) 1% for 10 min. The primary antibody, 4G10.3, was applied at a 1:10 dilu-
tion. The slides were incubated for 1 hr in a moist chamber at room temperature, then blocked with BSA 1% for 10 min. Fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Jackson Immuno Research, Avondale, PA) was applied as a secondary antibody, and the slides were incubated again for 1 hr in a moist chamber. After washing in PBS for 10 min, the sections were mounted with a medium consisting of PBS, glycerol, and paraphenylenediamine. Negative control tissues were prepared by omitting the primary antibody from each antibody-binding study. The sections were viewed and photographed using an Axiophot III equipped for epiillumination (Carl Zeiss, Oberkochen, Germany). All photographs were taken with a 40× objective and an exposure time of 30 sec.

Observational Method

The ocular surface was divided into four locations: conjunctiva, limbus, peripheral cornea, and central cornea. Then, epithelial maturation, cellular shape of basal cells, binding intensity, and distributional change of cells binding 4G10.3 were observed. “Central cornea” was selected from the midarea of the cornea, and “peripheral cornea” was chosen from the area adjacent to the limbus. “Limbus” was distinguished by the presence of blood vessels and chamber angle; “conjunctiva,” an area far removed from the limbus, was distinguished by the presence of underlying ciliary muscle. The intensity of binding was graded from 0 (no binding) to 4+ (most intense binding).

Results

During epithelial maturation, until 1 week of age, one or two layers of cells were present in the central corneal (Figs. 1A–B), limbal (Figs. 2A–B), peripheral, and conjunctival epithelium (Figs. 3A–B). At 1.5 wk of age, epithelial thickness in all areas increased to two to three cell layers (Figs. 1C, 2C). At 2 wk of age, increased stratification to four to five cell layers was observed at the central cornea. Epithelial thickness of the central cornea was nearly doubled compared with that at 1.5 wk of age (Figs. 1C–D). At this time, no

Fig. 2. Limbus. At 1 day (A) and 1 week (B) of age, limbal epithelium consisted of one or two layers of cells. At 1.5 weeks (C), two to three layers of cells were present. At 2 weeks (D), three to four layers of cells were seen. Adult levels of stratification were reached at 3 (E) or 4 weeks; and 6 weeks (F). Regardless of epithelial maturation, basal cells in the limbal epithelium maintained slender and ovoid shape. Stain = Hematoxylin and eosin. Bar = 50 μm.
Fig. 3. Immunolocalization of 4G10.3 in conjunctiva. Basal cells showed intense binding (3-4+) at 1 day (A), 1 week (B), and 1.5 weeks (C). At 2 weeks (D), binding intensity dropped to 2-3+; (E) phase contrast of area shown in (D). At 3 weeks (F), occasional cells bound (2+) similarly to adult (G); (H) phase contrast of area shown in (G). Bar = 50 μm.

Major changes were observed at the other locations. At 3 wk of age, changes occurred in the peripheral cornea similar to those seen in the central cornea at 2 wk of age. The epithelial thickness of the peripheral cornea was twice that at 2 wk of age. Central corneal epithelium at this age became thicker, up to five to six cell layers (Fig. 1E). At 4 wk of age, cellular stratification nearly reached an adult level at all locations of conjunctiva, four to five cell layers (Figs. 3G-H); limbus, four to five layers (Fig. 2F); peripheral cornea, five to six layers; and central cornea, six to seven layers (Fig. 1F). After 6 wk of age, the superficial cell layer seemed to be more prominent than at 4 wk of age.

At 1 d and 1 week of age, it was observed that the 4G10.3 antibody bound intensely (4+) to all the basal cells in the cornea (Figs. 4A-B), limbus (Figs. 5A-B), and conjunctiva (Figs. 3A-B). At 1.5 wk of age, the binding intensity of 4G10.3 decreased in the central cornea (Figs. 4C-D), accompanied by a rounding of the basal cells. No changes were observed in other areas (Figs. 3C, 5C). At 2 wk of age, just before eyelid opening, the basal cells at the central cornea abruptly changed their shape from flattened or ovoid to large and cuboidal, and 4G10.3 binding was reduced greatly (Figs. 4E-F). Increased stratification to four to five layers of epithelial cells also was seen at the central cornea. At 3 wk of age, basal cells in the central cornea no longer bound 4G10.3, and their shape changed from cuboidal to columnar (Fig. 4G). In the peripheral cornea, shape changes similar to those seen in the central cornea at 2 wk of age were observed. The basal cells changed their shape from flattened or ovoid to large and cuboidal and lost their binding in-
Fig. 4. Immunolocalization of 4G10.3 in central cornea. 4G10.3 bound intensely (4+) to all the basal cells in the corneal epithelium at 1 day (A) and 1 week (B) of age. At 1.5 weeks (C), the binding intensity began to weaken in the central cornea (2-3+); (D) phase contrast of area shown in (C). At 2 weeks (E), basal cells bound to 4G10.3 with greatly reduced intensity (1+), and cellular shape changed to cuboidal; (F) phase contrast of area shown in (E). Basal cells in the central cornea no longer bound to 4G10.3 after 3 weeks (G); and 6 weeks (H). Bar = 50 μm.

Intensity from 2-3+ to 1+. The maturation process appeared to begin at the central cornea and then progress toward the peripheral cornea. The number of 4G10.3-positive basal cells decreased as the corneal epithelium became progressively thicker. After 4 wk of age, 4G10.3 binding was nearly identical to that in the adult cornea.

In the limbus, no change in the binding intensity of 4G10.3 was detected (Fig. 5). At all time (1 d of age to adult), basal cells in the limbus retained their flattened or ovoid shape, and the binding intensity of 4G10.3 remained constant (3-4+).

In conjunctival epithelium, the binding intensity of 4G10.3 decreased during development (Fig. 3). By contrast with the central corneal epithelium, occasional cells still bound 4G10.3 at a 2+ level in rats 3 wk of age (Fig. 3F) and older (Figs. 3G, 6).

Discussion
It has been hypothesized that corneal epithelial homeostasis involves centripetal migration from the periphery. These conjectures led to the current concept of stem cell localization in the basal cell layer of limbal epithelium. It was suggested that the movement of epithelial mass was a possible mode of corneal wound repair. This was explained by observations of the displacement of the limbal pigment line and of conjunctival goblet cells onto the cornea during epithelial healing of a corneal mustard-gas burn. Since this earlier
Fig. 5. Immunolocalization of 4G10.3 in limbal epithelium. Basal cells in the limbal epithelium bound 4G10.3 intensely (3-4+), and maintained their slender ovoid shape throughout all time points; 1 day (A), 1 week (B), 1.5 weeks (C); (D) phase contrast of area shown in (C), 2 weeks (E), 3 weeks (F), and 6 weeks (G); (H) phase contrast of area shown in (G). Bar = 50 μm.

Our current study was founded on the observation that the monoclonal antibody 4G10.3 reacts with limbal epithelium. All these studies were consistent with earlier results showing that limbal epithelium has a higher mitotic rate in culture than peripheral epithelium, with the peripheral epithelium rate higher than that of the central cornea.

Clinical evidence of stem cell location includes the finding that corneal and conjunctival intraepithelial neoplasia predominantly involves the limbal area. Also, the efficacy of limbal autografts used to treat chemically or thermally burned corneas, with apparent widespread ocular surface damage, provide additional clinical support for the limbal location of the stem cell. These results were corroborated in rabbit models.

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Fig. 6. Summary of changes in stratification, cell shape, and binding of 4G10.3. 4G10.3 bound intensely to all the basal cells in the ocular surface epithelium until 1.5 weeks. At 2 weeks, change of cellular shape, epithelial stratification, and loss of binding occurred at the central cornea first. Basal cell shape changed from ovoid to cuboidal, and binding intensity decreased from 2-3+ to 1+. Increased stratification of four to five layers of cells was also seen concomitantly. Also at 3 weeks, basal cells in the peripheral cornea showed similar changes to those seen in central cornea at 2 weeks. At 3 weeks, basal cells in the central cornea were no longer bound by 4G10.3, and began to change from cuboidal to tall columnar. Basal cells in the limbal epithelium never changed their intense binding (3-4+) and slender ovoid cellular shape. Note: the number of lines and the darkness of the shading in the basal cell indicates the binding intensity of 4G10.3.

To explain this phenomenon, it was hypothesized that there are two mechanisms for the "hyperproliferative" mode of corneal epithelial maturation and the influence of an immature corneal basement membrane. However, the exact nature of these mechanisms is unclear.

We also observed that there is a critical time point in epithelial maturation, between 1.5 and 2 wk of age, and that this is related closely to eyelid opening. After 2 wk of age, epithelial maturation that has just begun at the central cornea progresses expeditiously toward the peripheral cornea. This maturation process includes cellular shape changes, epithelial stratification, and decreased numbers of antibody-binding cells. Finally, the basal cells in the limbal region remain as the least differentiated primitive area and carry the 50-kD antigen characterized by strong binding to 4G10.3. This finding led to another important result; some part of the limbus always is occupied by intensely 4G10.3-bound basal cells that never change their binding intensity or cellular shape. This finding supports stem cell localization in the limbal basal cells. Some-
times basal cells in the peripheral cornea of adult rats show weak binding. This binding could indicate that basal cells in the peripheral cornea are composed of less differentiated transient amplifying cells.11

An unexpected result of our study was that 4G10.3 bound intensely to all conjunctival basal cells in rats younger than 1.5 wk of age. This contrasts with results of our previous studies using adult rats, where 4G10.3 bound weakly to occasional basal cells.14 These results suggest that conjunctival cells could be in a stem-like state during development, with a high potential for proliferation. It is unclear where the stem cells for conjunctival epithelium are or whether the monoclonal antibody 4G10.3 provides a marker for them. The stem cells may be the occasional cells we see binding 4G10.3 throughout the adult conjunctival epithelium. We also observed that many 4G10.3-binding cells are gathered in the fornix area, suggesting that this area might be one of the sources of the conjunctival epithelial proliferation (data not shown).

In summary, we studied stem cell localization in the developing rat cornea using the anti-50-kD monoclonal antibody that is a potential marker for the stem cells of the rat, rabbit, and human corneal epithelium. In the early developmental life of the rat, epithelial stem or stem-like cells are located throughout the basal layers of both the cornea and limbus, with 4G10.3-binding cells gradually becoming sequestered in the limbus as the corneal epithelium matures. Epithelial maturation of the cornea, including cellular shape changes, epithelial stratification, and loss of binding, occurs at the central cornea first, then progresses toward the periphery, but it does not involves the limbal basal cells. These basal cells located in a specific area in the limbus remain unchanged, with a constant cellular shape and strong binding to 4G10.3.

Key words: limbus, stem cells, developing rat, 50-kD protein, monoclonal antibody

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References