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LABORATORY INVESTIGATIONS

Wistar Rat Palpebral Conjunctiva Contains More Slow-cycling Stem Cells That Have Larger Proliferative Capacity: Implication for Conjunctival Epithelial Homeostasis

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Purpose: To determine the location of conjunctival epithelial stem cells.

Methods: Wistar rats received daily injection of 5-bromo-2-deoxyuridine (BrdU) at a dose of 5 mg/100 g for 2 weeks followed by a 1-month BrdU-free period before death. After the rats were sacrificed, the orbital contents and eyelids were exenterated en bloc, fixed in buffer formalin, and embedded in paraffin. To compare the proliferative capacity of ocular epithelial cells, 1.0% phorbol myristate (TPA) in petrolatum was topically applied once daily to both eyes of Wistar rats for 12 days. After 6, 12, 18, and 24 hours and 2, 4, 6, 8, 10, and 12 days of TPA treatment, rats were administered BrdU intraperitoneally 7 hours before they were sacrificed. The ocular epithelium was fixed and processed for immunochemistry, and the labeling index (LI) of every epithelial zone was determined.

Results: Slow-cycling cells, detected as label-retaining cells (LRCs), were found in bulbar, fornical, and palpebral epithelia and mucocutaneous junctions, as well as in limbal epithelia. The greatest numbers of LRCs were identified in palpebral epithelium. Under normal situations, in conjunctiva the LI was lowest in palpebral epithelium (2.1 ± 0.5) compared with bulbar (2.2 ± 0.5), fornical (2.3 ± 0.4) epithelia and mucocutaneous junction (3.4 ± 0.9), respectively. In cornea, the LI was lowest in limbal epithelium (1.8 ± 0.7) compared with central corneal epithelium (3.5 ± 0.6). Twenty-four hours after TPA treatment, an 8.2-fold increase in the palpebral epithelial basal cell labeling index was noted compared with 4.7-fold, 5.7-fold, and 3.8-fold increases in bulbar, fornical, and mucocutaneous junction epithelial basal cell labeling indices, and a sevenfold increase in the limbal basal cell labeling indices compared with a 2.1-fold increase in the corneal basal cell labeling index, respectively. Limbal and palpebral epithelia maintained a significantly greater proliferative response (5.5-to 6.3-fold increase, respectively) during chronic stimulation than corneal, bulbar, fornical epithelia, and mucocutaneous junction (0.6- to 2.3-fold increase, respectively).

Conclusions: In Wistar rat conjunctiva, slow-cycling cells are primarily located in palpebral epithelium, which has greater proliferative capacity than other conjunctival epithelia. This finding means that, in the Wistar rat, the conjunctival epithelial stem cells are mainly located in palpebral epithelium. These data open new perspectives in ocular epithelial development and are relevant in conjunctival wound repair. **Jpn J Ophthalmol 2003;47:119–128**

Key Words: 5-bromo-2-deoxyuridine, conjunctival epithelial cells, stem cells.

Introduction

The ocular surface is made up of conjunctival and corneal epithelial cells. Although anatomically continuous with each other at the corneoscleral limbus, the two cell phenotypes belong to two quite distinct subpopulations.[1] The epithelium comprising the

Received: February 14, 2002
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Jpn J Ophthalmol 47, 119–128 (2003)

Published by Elsevier Science Inc.

0021-5155/03/$–see front matter
doi:10.1016/S0021-5155(02)00687-1

Jpn J Ophthalmol
Vol 47: 119–128, 2003

conjunctiva can be divided in four morphologically distinct zones: bulbar, which covers the ocular globe from limbus to fornix; fornical, which is located in the folding region; palpebral, which is contiguous to the epidermis of the eyelid; and mucocutaneous junction, situated between the palpebral epithelium and the epidermis of the eyelid. The conjunctival epithelium forms a physical protective barrier and, through goblet cell secretions, contributes to the formation and maintenance of a "tear film," which produces a protective scaffolding over the ocular surface. Like other stratified epithelia, conjunctival epithelial cells are usually replaced by locally concentrated or randomly distributed foci of stem cells.

It has been determined that corneal epithelial stem cells are located in the limbal area.[2–5] In humans, the limbal palisades of Vogt and the interpalisade rete ridges are believed to be repositories of stem cells. It has been proven that in the mouse and the rabbit, the fornical epithelium is the focal source of replacement cells for the conjunctiva.[6,7] Recent studies about rabbit conjunctival stem cells have shown that most palpebral conjunctival epithelial stem cells are located near the mucocutaneous junction, suggesting that mucocutaneous junction basal cells are the major source of replacement palpebral conjunctival epithelial cells.[8,9]

Stem cells are considered to be cells endowed with a long life span, which might be equivalent to the life of the organism in which they reside. They possess biochemically and ultrastructurally undifferentiated characteristics and are responsible for long-cycling, presumably to conserve their proliferative potential and to minimize DNA error that could occur during replication.[10,11] Under normal conditions, they rarely divide, giving rise to more rapidly dividing "transit amplifying" cells, which are a more actively proliferative population of cells, but can only undergo a limited number of cell divisions before becoming postmitotic or terminally differentiated.[12]

Another basic and essential property of stem cells is their remarkable proliferative capacity, which usually outlasts the life span of the animal.[13] Therefore, the identification of stem cells can be based on the evaluation of proliferative capacity in vitro. Cells coming from stem and non-stem cell-enriched regions can be cultured under identical conditions for the comparison of various proliferative properties (e.g., colony-forming ability, growth rate, growth potential). When studied in this way, it has been reported that, within the rabbit conjunctival epithelium, fornical epithelial cells are able to proliferate more rapidly, remain relatively smaller in size, and reach confluence earlier than bulbar or palpebral epithelial cells. In cultivation of human conjunctival cells, however, it appears that the bulbar and fornical conjunctival epithelia have identical proliferative capacity.[14] In addition, limbal epithelial cells (stem cell-enriched region) grow better than central corneal epithelial cells (non-stem cell-enriched region) in human explant culture[15] and in rabbit cell culture.[16] Furthermore, human limbal epithelial cells can be subcultured several times, whereas central corneal epithelial cells can be subcultured no more than twice or not at all.[14] In an in vitro environment, however, stem and transit-amplifying cells will proliferate rapidly, and it is difficult to differentiate these two populations.

To overcome this problem, we have taken advantage of the fact that, after administration of a hyperplastic agent, regions rich in stem cells can be preferentially stimulated to proliferate when compared with non-stem cell regions. Using this method, it has been determined that, under normal conditions, the proliferative rate of limbal epithelium is lower than that of corneal epithelium. After 4 to 5 days of application of tumor-promoter phorbol myristate (TPA), however, the proliferative rate of limbal epithelium shows a 5- to 10-fold increase while that of corneal epithelium has only a 3-fold increase.[2] Similarly, mouse fornical epithelium has a greater proliferative capacity than other conjunctival epithelia.[7] These results are important in locating ocular epithelial stem cells.

As discussed above, there is evidence that the ocular epithelial stem cells may be concentrated in the mucocutaneous junction, fornical zone, and limbus. Although the hypothesis that the mucocutaneous junction is the major source of replacement for palpebral conjunctival epithelial cells is based on the long-term retention of BrdU labeling cells in the mucocutaneous junction, the proliferative capacity of mucocutaneous junction basal cells is unclear. In the present work, using immunochemistry with BrdU, we have identified subpopulations of ocular epithelial basal cells located in bulbar, fornical, and palpebral epithelia and in mucocutaneous junction as well as in limbal epithelium, that are normally slow-cycling, but can be preferentially stimulated to proliferate in response to the tumor promoter, TPA. No such cells can be labeled in corneal epithelium. In addition, the palpebral epithelium contains more slow-cycling cells that have larger proliferative capacity than other conjunctival cells. These cells may provide therapeutically significant sources of replacement rat conjunctival epithelial cells. Our findings, plus a reevaluation of

the literature, suggest that conjunctival stem cells mainly reside in a region where the density of goblet cells is the highest in the conjunctiva.

Materials and Methods

All animals were treated in accordance with the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Ophthalmic and Vision Research. The University of Akita Animal Care and Ethics Committee approved all experimental procedures.

Seventy-four Wistar rats weighing 200–250 g and of either sex were used in the study. The animals were housed in individual cages at constant room temperature (19–23°C) and humidity of 30–50%, and were maintained in a constant 12-hour light–dark cycle. Food and water were provided ad libitum.

Six animals received daily injection of BrdU (Zymed Laboratories, South San Francisco, CA, USA) at a dose of 5 mg/100 g for 2 weeks followed by a 1-month BrdU-free period before death and processing. Four other animals were intraperitoneally injected with BrdU 7 hours before death.

To assess the proliferative responses of ocular epithelia to daily application of phorbol myristate (TPA; Funakoshi, Tokyo), we applied 1.0% TPA in petrolatum once daily to both eyes of 30 Wistar rats for 12 days. Control rats (both eyes) received petrolatum only. Each group consisted of 4 animals, unless otherwise specified. After 12 and 24 hours (acute) and 2, 4, 6, 8, 10, and 12 days (chronic) of TPA treatment and petrolatum, rats were administered BrdU intraperitoneally 7 hours before they were sacrificed. After the rats were sacrificed by intraperitoneal injection of 50 mg/100 g pentobarbital sodium (Abbott Laboratories, North Chicago, IL, USA), both eyes (including lids) were surgically removed. The orbital contents and eyelids were cut vertically in the center (midglobe) of the specimen so that each cross-section would then include upper lid, globe, and lower lid, all as close to normal anatomic configuration as possible. At least 100 sections were cut from each animal. Some sections were stained with hematoxylin and eosin for histologic examination. Others were processed for immunochemistry, and the labeling index (LI; number of BrdU-labeled nuclei per 100 basal keratinocytes) was determined for each of the epithelial zones.

For BrdU labeling, a BrdU staining kit (Oncresprod, Boston, MA, USA) was used. Eyes including lids were cut into 3.0-mm–thick sections with a microtome. Sections were placed on previously subbed slides (poly-L-lysine coated; Matsunami Glass, Osaka). Slides were dried in a 60°C oven for 30–60 minutes. Sections were deparaffinized by treatment with xylene (three washes), 100% alcohol (one wash), 95% alcohol (one wash), and 85% alcohol (one wash). Slides were submerged for quenching in one part 30% H_2O_2 to nine parts absolute methanol, at room temperature for 10 minutes. After the slides were rinsed with phosphate-buffered saline (PBS; PH 7.4), 100 mL trypsin mixed solution was added to each section, slides were incubated in a moist chamber at 37°C for 5 minutes. Then slides were rinsed in distilled water (2 minutes, three times), sections were denatured with hydrochloric acid at room temperature for 30 minutes, rinsed with PBS, and incubated in blocking solution at room temperature for 10 minutes. Next, sections were incubated in biotinylated mouse anti-BrdU at room temperature for 30 minutes. Then slides were rinsed with PBS, and sections were incubated for 10 minutes in a moist chamber at room temperature with peroxidase-conjugated secondary antibody. Slides were rinsed three times in PBS (5 minutes per rinse), and then sections were reacted in a diaminobenzidine procedure. The slides were counterstained with two drops of hematoxylin, and then washed in tap water. They were immersed in PBS until the sections turned blue. Then rinsed in distilled water, and dehydrated in 95% ethanol (10 seconds, two times), 100% ethanol (10 seconds, two times) and xylene (10 seconds, two times). The sections were mounted under glass coverslips in histomount.

The LI was determined by counting at least 1000 nuclei for each determination, and the result was expressed as the number of labeled nuclei per 100 basal cells (percentage of labeled basal cells).

To analyze the differences between treated and control eyes, data (LI) from at least 400 sections were pooled from four independent experiments (8 eyes) and were presented as mean ± SD. The statistical significance of differences between groups was evaluated using the Student *t*-test. $P < .05$ was considered significant.

Results

Morphology of Wistar Rat Conjunctival Epithelium

Based on anatomic location, degree of stratification, and density of goblet cells, Wistar rat conjunctival epithelium can be divided into four zones (Figure 1): bulbar, fornical and palpebral epithelia, and mucocutaneous junction. Bulbar epithelium contains gob-

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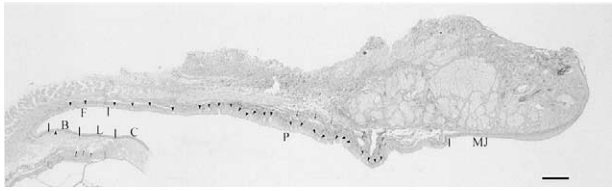

Figure 1. Morphology of Wistar rat conjunctival epithelium (hematoxylin and eosin stain). Arrowheads: Goblet cells. Arrows: Blood vessels. Note that palpebral epithelium contains more goblet cells than other conjunctival epithelia and limbal and palpebral epithelia are adjacent to a rich vascular network. MJ: mucocutaneous junction, P: palpebral, F: fornix, B: bulbar, L: limbus, C: cornea. Bar = 100 μm.

let cells and is easily distinguished from limbal epithelium. Fornical epithelium is located in the folding region. The palpebral epithelium is richly endowed with goblet cells and somewhat thicker than fornical epithelium. Furthermore, palpebral epithelium is adjacent to blood vessels. The mucocutaneous junction is situated between the palpebral epithelium and the epidermis of the eyelid. Limbal epithelium is thinner than corneal epithelium and overlies a rich vascular network.

Rat Palpebral Epithelium Contains More Slow-cycling Cells

When rats were injected intraperitoneally with BrdU at a dose of 5 mg/100 g body weight per day for 14 days, almost all the corneal and conjunctival epithelial basal cells were labeled. After a 4-week observation, however, all the labeled cells disappeared from the cornea; and small numbers of BrdU-positive cells were scattered in conjunctival epithelium as well as limbus. Long-term retention of BrdU labeling was demonstrated to reside primarily in palpebral and limbal epithelia. The greatest number of label-retaining cells (LRCs) was found in palpebral epithelium. Small numbers of LRCs were found scattered randomly in bulbar and fornical epithelia and mucocutaneous junction (Figure 2). In addition, a number of label-retaining goblet cells were found in palpebral epithelium (data not shown).

Exposure to Phorbol Myristate Preferentially Stimulates Rat Palpebral and Limbal Epithelial Cells to Proliferate

To compare the relative proliferative response of various anterior ocular surface epithelia, we applied 1% TPA in petrolatum ointment once daily in both eyes. TPA can induce cells to proliferate in several murine epithelia, including ocular epithelium, when the TPA concentration is 1%.[2] Furthermore, this approach is superior to other physical means of inducing proliferation (e.g., incision wounding); the direct comparison of the proliferative responses of the total epithelium is possible because the epithelium is not destroyed. Control experiments showed that a daily application of petrolatum, the vehicle for TPA, had no effect on BrdU labeling of the ocular surface epithelium. Under normal conditions, the palpebral epithelium had a lower proliferative rate (LI: 2.1 ± 0.5) than bulbar (LI: 2.2 ± 0.5), fornical (LI: 2.3 ± 0.4), and mucocutaneous junction (LI: 3.4 ± 0.9). Similarly, the limbal epithelium (LI: 1.8 ± 0.7) had a lower rate than corneal epithelium (LI: 3.5 ± 0.6) (Table 1). Within 12 hours of exposure to TPA, an increase in the number of BrdU-labeled cells was found in all ocular epithelia. This increase in BrdU-labeled cells reached a maximum 24 hours after exposure to TPA. At this time, an 8.2-fold increase in palpebral basal cells was noted, compared with a 4.7-fold, 5.7-fold, and 3.8-fold increase in bulbar and fornical epithelia and mucocutaneous junction basal cell labeling, respectively ($P < .05$). This indicates that, similar to limbal epithelium, palpebral epithelium had the greatest proliferative response to an acute stimulus in conjunctiva (Figures 3 and 4) (Table 1). Limbal epithelium also responded more dramatically compared with central corneal epithelium (7-fold vs. 2.1-fold increase, $P < .01$) (Figures 5 and 6).

After 2 days of TPA treatment, the epithelia of the anterior segment showed a marked decrease in proliferative activity. The proliferative rate in the bulbar, and fornical epithelia and mucocutaneous junction had nearly returned to control values, whereas the proliferation of palpebral epithelial remained higher than that of controls. Similarly, limbal epithelial proliferation was greater than control values and was higher than corneal epithelium. During the remaining 12 days of TPA treatment, limbal epithelium maintained a greater proliferative response (5.5- to 6.2-fold increase) than corneal epithelium (less than 2.0-fold increase; $P < .01$); the palpebral epithelium maintained a greater proliferative response than other conjunctival epithelia (5.9- to 6.3-fold versus 1.9-to 2.3-fold increase, respectively; $P < .01$) (Figures 5 and 6).

Discussion

Rat Palpebral and Limbal Epithelial Cells Share Similar Kinetic Properties

Davanger and Evensen proposed the concept that limbal epithelial cells are involved in the renewal of

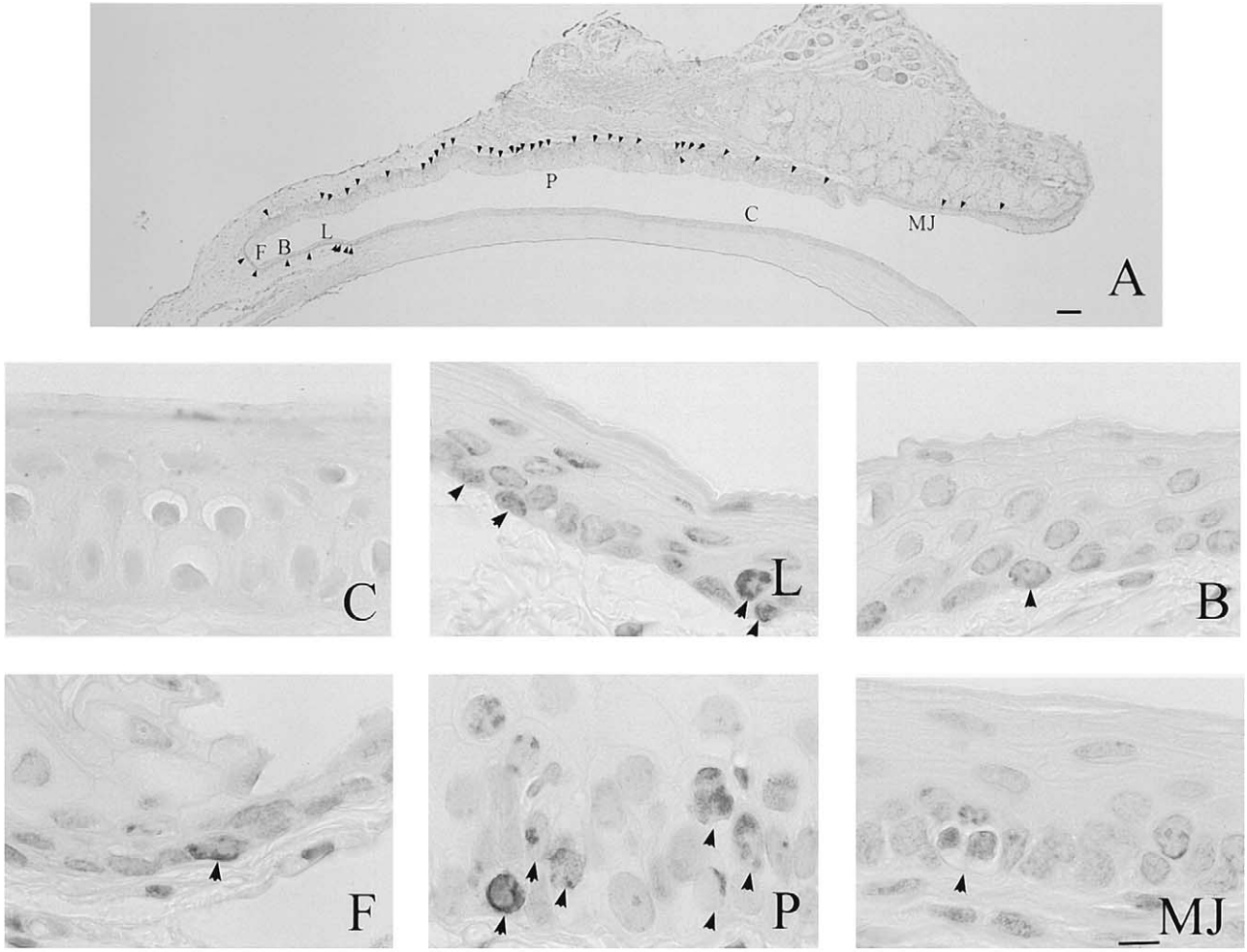

Figure 2. Wistar rat received daily injection of 5-bromo-2-deoxyuridine (BrdU) followed by a 1-month BrdU-free period before death and processing. (**A**) The label-retaining cells (LRC; arrowheads) of ocular surface. C: cornea, L: limbus, B: bulbar, F: fornix, P: palpebral, MJ: mucocutaneous junction. Note that palpebral and limbal epithelia contain more slow-cycling cells. No label-retaining cells can be found in corneal epithelium. Bar in (**A**) = 50 μm, in (**C**), (**L**), (**B**), (**F**), (**P**), (**MJ**) = 10 μm.

corneal epithelium in 1971.[17] Since then, much clinical and experimental evidence has been provided to support the theory that corneal stem cells are segregated in the limbus. Specifically, in comparison with the central cornea, the limbal basal epithelium and adjacent bulbar conjunctiva lack the differentiation-dependent K3/K12 keratins.[4] Limbal basal epithelium contains slow-cycling cells that have a larger proliferative capacity than corneal basal cells.[2,15] Limbal epithelial cells, rather than conjunctival epithelial cells, contribute to repairing corneal epithelial deficiency,[18–21] and are responsible for the pathogenesis of corneal epithelial dysplasias and neoplasms.[22] Although much has been learned about corneal stem cells, relatively little is known about conjunctival stem cells. Some studies have suggested that in mouse and rabbit conjunctiva, stem cells are primarily located in fornical epithelium. In the earlier studies of conjunctival stem cells, however, the mucocutaneous junction had attracted so little attention that its stem cells were rarely mentioned. Recently, using BrdU labeling, it has been proven that there are some slow-cycling cells in mucocutaneous junction, suggesting that these cells are palpebral epithelial stem cells. But their proliferative capacity was not compared with other conjunctival epithelial cells. Although in most investigations of mouse and rabbit conjunctiva, it appears that conjunctival stem cells are essentially concentrated in the fornix, in human eyes, conjunctival stem cells have been found to

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Table 1. Proliferative Response of Rat Ocular Epithelia after 24 Hours of Phorbol Myristate Stimulation*

Time (h)	Corneal	Limbal	Bulbar	Fornical	Palpebral	MJ
0	3.5 ± 0.6	1.8 ± 0.7	2.2 ± 0.5	2.3 ± 0.4	2.1 ± 0.5	3.4 ± 0.9
12	5.6 ± 0.2	8.6 ± 0.3	8.4 ± 0.2	7.6 ± 0.4	9.3 ± 0.1	5.3 ± 0.1
24	7.3 ± 0.5	12.6 ± 0.2	10.4 ± 0.1	13.3 ± 0.2	17.2 ± 0.3	13.6 ± 0.6

*Values represent the percentage of BrdU-Labeled nuclei per 100 basal nuclei and data from at least eight eyes (400 sections) pooled from four independent experiments and are presented as mean ± SD. MJ: mucocutaneous junction. Note that palpebral epithelium has larger proliferative capacity than other conjunctiva after 24-hour administration of TPA ($P < .05$).

be uniformly distributed in the bulbar and fornical conjunctiva.[14] This means that, in contrast to corneal stem cells that are solely located in limbus, differences between conjunctival stem cell locations should be taken into account. Up to now, the location of all conjunctival stem cells has not been clearly demonstrated. Although some markers for epithelial stem cells have been proposed,[23–25] their role in specifically identifying keratinocyte stem cells is still in question. To date, direct markers for ocular epithelial stem cells have not been found.[26] In the present study, using BrdU labeling, which can be used to la-

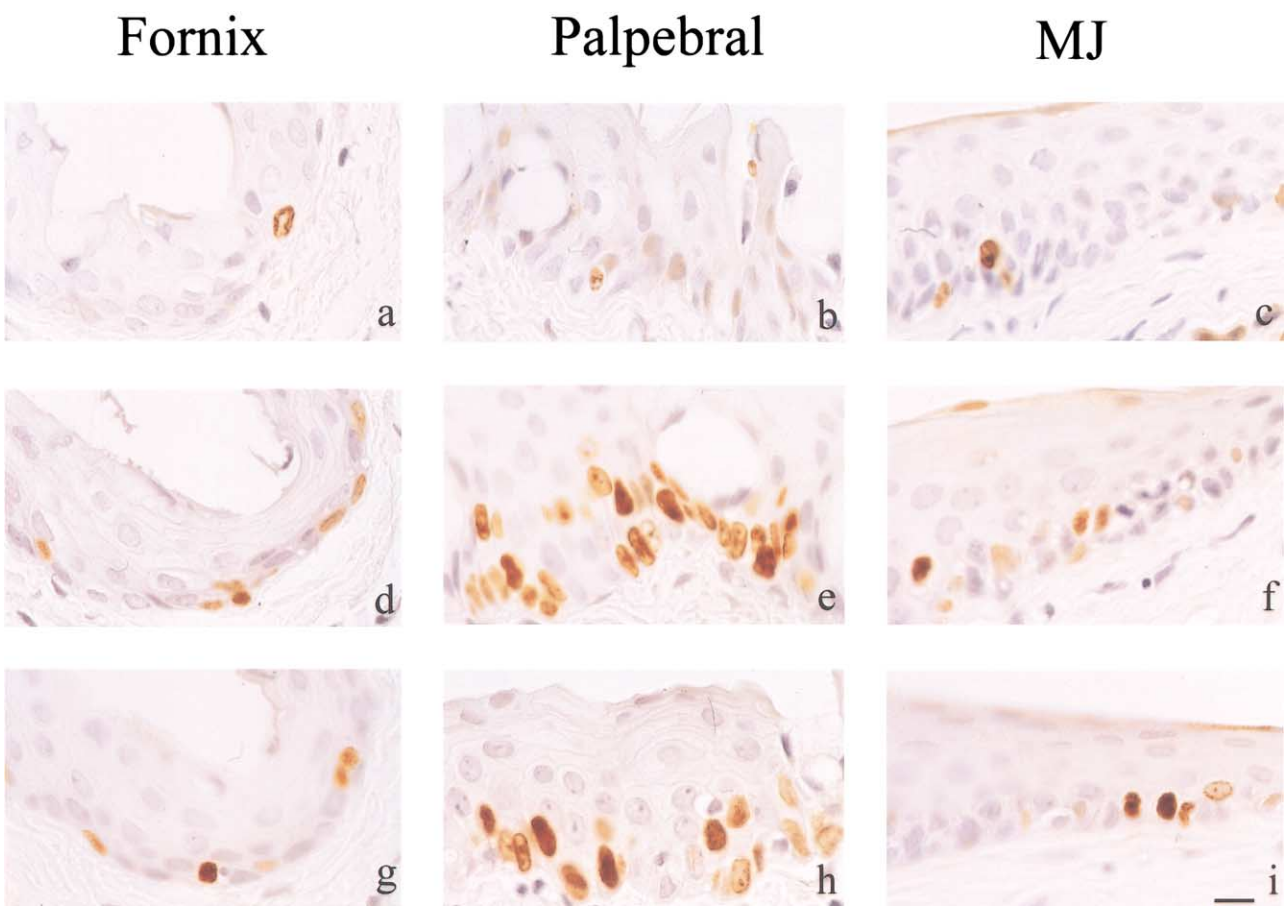

Figure 3. Immunochemistry of the response of fornical (**a**,**d**,**g**), palpebral (**b**,**e**,**h**) epithelia, and mucocutaneous junction (**c**,**f**,**i**) to a single 24-hour (**d**,**e**,**f**) exposure and a 2-day exposure (**g**,**h**,**i**) of TPA. Under unstimulated situation, 5-bromo-2-deoxyuridine (BrdU) labeling of fornical, palpebral epithelia and mucocutaneous junction are shown in (**a**), (**b**), and (**c**). Note the low level of BrdU incorporation in untreated palpebral epithelium. Twenty-four hours after single exposure of TAP there are marked increases in all three conjunctival epithelia, most notably in the palpebral epithelium (**e**). Note the marked decrease in BrdU incorporation in fornical epithelium and mucocutaneous junction after 2 days of TPA treatment (**g**,**i**), whereas the palpebral epithelium (**h**) maintains a higher proliferative profile. MJ: mucocutaneous junction. Bar = 10 μm.

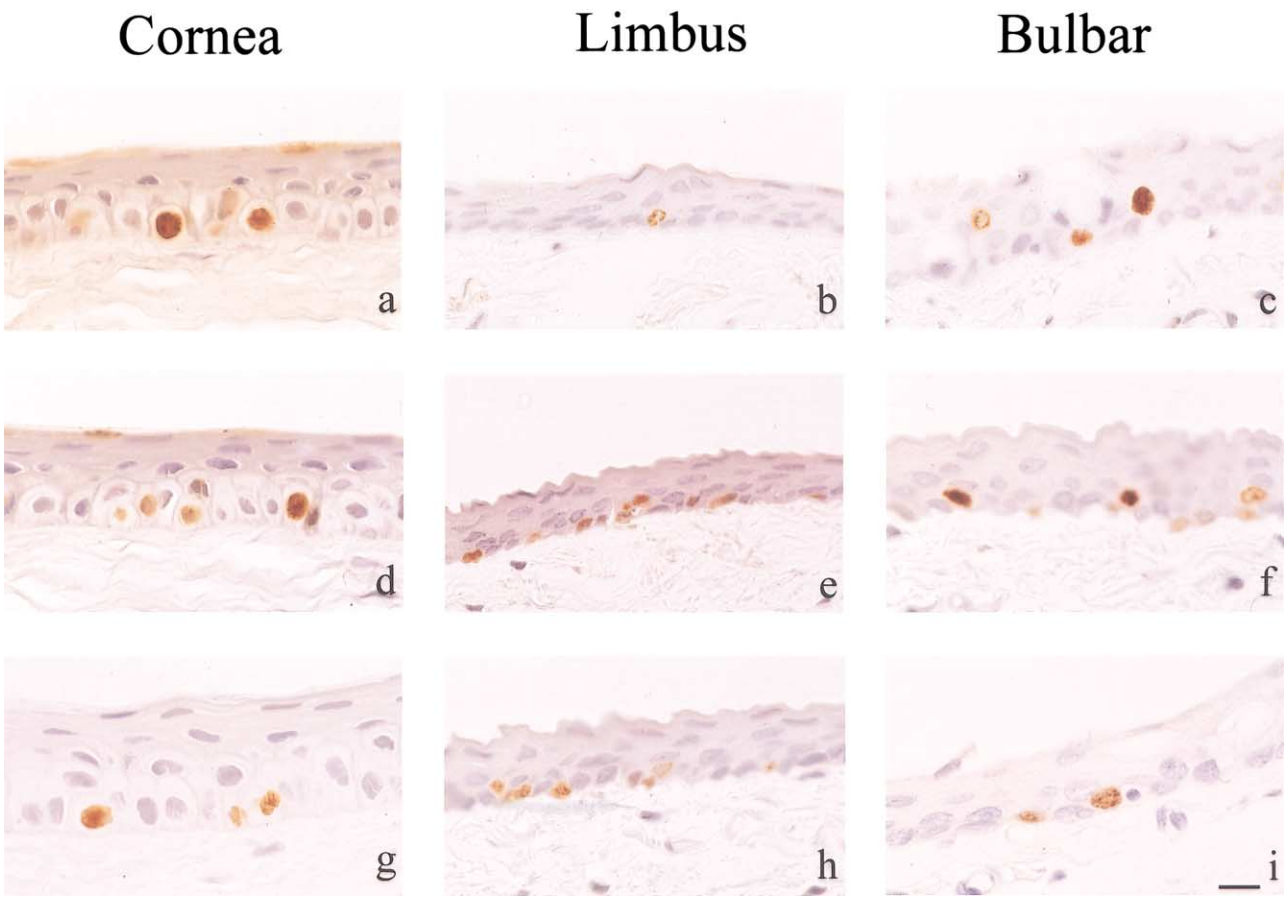

Figure 4. Immunochemistry of the response of corneal (**a**,**d**,**g**), limbal (**b**,**e**,**h**), and bulbar (**c**,**f**,**i**) of TPA. Under unstimulated situation, 5-bromo-2-deoxyuridine (BrdU) labeling of corneal, limbal, and bulbar are shown in (**a**,**b**,**c**). Note the low level of BrdU incorporation in untreated limbal epithelium. Twenty-four hours after single exposure of TPA there are marked increases in all three ocular epithelia, most notably in the limbal epithelium (**e**). Note the marked decrease in BrdU incorporation in corneal and bulbar epithelia after 2 days of TPA treatment (**g**,**i**), whereas the limbal epithelium (**h**) maintains a higher proliferative profile. Bar = 10 μm.

bel slow-cycling[8,9] and proliferating[27] cells of ocular epithelia, we have identified subpopulations of rat conjunctival epithelial basal cells that are normally slow-cycling, but have large proliferative capacity in response to a tumor promoter, TPA.

After rats were injected with BrdU daily for 2 weeks, over 90% of corneal epithelial basal cells, over 75% of limbal epithelial cells, and over 70% of conjunctival epithelial basal cells were labeled. After a 4-week observation, all of the labeled cells disappeared from cornea, and only a few BrdU-positive cells were found in limbus. Some BrdU-positive cells were found scattered along the length of the conjunctival basement membrane. Long-term retention of BrdU labeling was demonstrated to reside in the limbal, bulbar, palpebral, fornical epithelia, and mucocutaneous junction. The greatest number of LRCs was found in palpebral epithelium. It has been proven that limbal basal epithelium contains corneal stem cells, which are normally slow-cycling, but have larger proliferative capacity than corneal central epithelial cells. By comparison of the conjunctival epithelium with corneal epithelium, we found that rat palpebral epithelium shares many kinetic properties with limbal epithelium. Just as limbal epithelium overlies a vascular network, in the rat conjunctiva we found that the palpebral epithelium is adjacent to a rich vascular network (Figure 1). Under normal conditions, most limbal epithelial basal cells have a long cell cycle time. Therefore, they do not incorporate pulse-administered BrdU. A similar situation exists in rat conjunctiva where slow-cycling cells are primarily scattered in palpebral epithelium (Figure 2).

An important property of stem cells is their remarkable proliferative capacity; this means that stem cells

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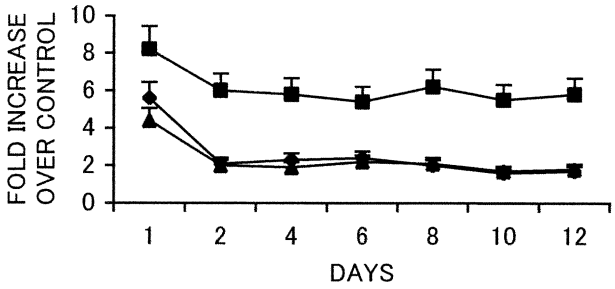

Figure 5. The proliferative response of fornical, palpebral epithelia and mucocutaneous junction to TPA stimulation. Rats (4 per group) were administered 1% TPA in petrolatum topically in both eyes, whereas control rats were administered 5-bromo-2-deoxyuridine (BrdU) 7 hours before they were sacrificed, and a labeling index was calculated (see Materials and Methods). Each value represents the fold increases over control ± SD and is derived from at least 8 eyes pooled from three independent experiments. TPA treatment resulted in a marked increase in the proliferative activity of fornical and palpebral epithelia and mucocutaneous junction at day 1, followed by a decrease after 2 days. Palpebral epithelium maintained a greater proliferative response than bulbar and fornical epithelia and mucocutaneous junction during the remaining 12 days of treatment ($P < .05$). ■: palpebral, ◆: fornix, ▲: mucocutaneous junction.

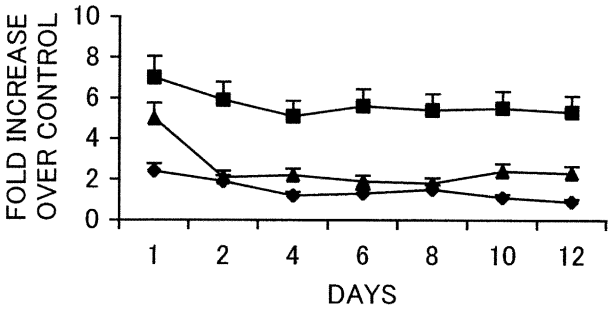

Figure 6. The proliferative response of corneal, limbal, and bulbar epithelia to TPA stimulation. Rats (4 per group) were administered 1% TPA in petrolatum topically in both eyes, whereas control rats were administered petrolatum only. After 1, 2, 4, 6, 8, 10, and 12 days of treatment, rats were administered 5-bromo-2-deoxyuridine (BrdU) 7 hours before they were sacrificed, and a labeling index was calculated (see Materials and Methods). Each value represents the fold increases over control ± SD and is derived from at least 8 eyes pooled from three independent experiments. TPA treatment resulted in a marked increase in the proliferative activity of corneal, limbal, and bulbar epithelia at day 1, followed by a decrease after 2 days. Limbal epithelium maintained a greater proliferative response than corneal epithelia during the remaining 12 days of treatment ($P < .05$). ■: limbus, ▲: bulbar; ◆: cornea.

can be preferentially stimulated to proliferate more than non-stem cells. Using this approach, we topically applied a tumor promoter, phorbol myristate (TPA), to the anterior surface of the eye. Control experiments showed that daily application of petrolatum, the vehicle for TPA, had no effect on BrdU labeling of ocular epithelia. After a single exposure of TPA, all regions had a sharp increase in proliferation that peaked by 24 hours; the LI of limbal and palpebral epithelia increased from a normal 1.8 ± 0.7 and 2.2 ± 0.5 to a 7- and 8.2-fold increase, respectively. Corneal and other conjunctival epithelia were also stimulated, but to a much lesser extent (4.7-, 5.7-, and 3.8-fold increase in bulbar, fornical epithelia, and mucocutaneous junction basal cell labeling, and a 2.1-fold increase in corneal basal cell labeling, respectively.). These data are in agreement with previous reports that in mouse skin after a single application of TPA, a temporal peaking increase in ^{3}H-TdR incorporations into DNA was found between 18 and 30 hours.[28]

Continuous exposure to TPA, however, led to a dramatic decline in the proliferative rate for all the epithelia. The decreases observed for palpebral and limbal epithelia were significantly less than those in other ocular epithelia. This proves that, in rat conjunctiva, palpebral epithelium contains more stem cells than other epithelia. It is generally believed that stem and early transit-amplifying cells are able to divide for many rounds, whereas late transit-amplifying cells are able to divide for only a few rounds before differentiation.[29,30]

In contrast to the greatest number of LRCs in palpebral epithelium, a few scattered label-retaining cells were found in mucocutaneous junction, fornical, and bulbar epithelia. This indicates that proliferative cells in conjunctival epithelia may include some cells coming from the mucocutaneous junction, bulbar and fornical epithelial stem cells. Therefore, one possible explanation for the present finding is that, in Wistar rat conjunctiva, the palpebral epithelium contains more stem cells than the mucocutaneous junction, bulbar and fornical epithelia. Stem cells coming from palpebral epithelium play a major role in conjunctival epithelial proliferation. Our data prove, at least in the Wistar rat, that the palpebral and limbal epithelia are enriched by subpopulations of cells that are continuously stimulated to proliferate, whereas the mucocutaneous junction, bulbar, and fornical epithelia contain a greater number of cells that cease dividing and begin to differentiate in response to TPA. Our results are consistent with those of other investigators who observed the behavior of cultured murine keratinocytes in response to TPA and found that some cultured murine keratinocytes were stimulated to proliferate, whereas others were induced to differentiate.[31,32] These data confirmed that the more differentiated cells exhibited acceler-

ated differentiation, and the less mature cells exhibited greater proliferation in response to TPA. Studies about the effects of multiple exposures of TPA on murine skin suggested that the induction of ornithine decarboxylase and the stimulation of DNA synthesis occurred earlier and was of a greater magnitude when compared with a single exposure.[33] Our results support these observations.

Rat Palpebral Epithelial Cells Play Major Role in Replacement of Conjunctival Epithelium

Conjunctival epithelium has a great regenerative capacity and can quickly reepithelialize new epithelium when corneal and limbal epithelia are destroyed.[34,35] Furthermore, conjunctival epithelial cells have shown increasing proliferation in response to wounds that concern only central corneal epithelium.[6] Conjunctival epithelial stem cells play an important role in these proliferative processes. Although some studies have proven that conjunctival stem cells are mainly located in fornix (in mouse and rabbit eye), in the human eye, however, it has been found that conjunctival keratinocyte and goblet cells derive from a common bipotent progenitor (stem cells), which are uniformly distributed in bulbar and fornical conjunctiva.[14] A recent study about rabbit conjunctival stem cells, using a single injection of BrdU, showing the migration of BrdU-labeled conjunctival epithelial cells from mucocutaneous junction to fornix, has led to the suggestion that mucocutaneous junction basal cells are the major source of replacement palpebral conjunctival epithelial cells.[9] One may argue, however, that none of these data show that the BrdU-labeled cells have come from mucocutaneous junction stem cells, and therefore the evidence is circumstantial.

In contrast with data obtained with [^{3}H] TdR-retaining experiments in mouse and rabbit, in the present study we found that the slow-cycling cells, within Wistar rat conjunctival epithelium, were primarily located in palpebral epithelium. Furthermore, palpebral epithelial cells can be preferentially stimulated to proliferate more than other conjunctival cells. Twenty-four hours after a single exposure to TPA, the LI of palpebral epithelial basal cells had an obvious increase in proliferation (from normal to 8.2-fold increase), whereas the LI of other conjunctival epithelia cells showed less proliferation (from normal to 3.8- to 5.7-fold increase). Moreover, during the remaining 12 days of TPA treatment, palpebral epithelium maintained a greater proliferative response (5.9- to 6.3-fold increase) than other conjunctival epithelia (1.9- to 2.3-fold increase). This indicates that the rat palpebral epithelial cells have a greater proliferative potential than other rat conjunctival cells. We consider, at least in the Wistar rat, that there are more stem cells in palpebral epithelium, and that these cells play a major role in the replacement of conjunctival epithelial cells.

Because central corneal epithelium is transparent and susceptible to trauma, its stem cells have to be located in the limbal region. By comparison, the conjunctival stem cells do not have this constraint. In a disturbed situation, corneal stem cells display protective action by proliferating new epithelial cells. On the other hand, conjunctival stem cells function not only to generate new epithelial cells, but also to synthesize and secrete more mucus, which serves as a protective barrier for the underlying epithelium.

Our findings are in disagreement with those studies reporting that conjunctival stem cells are mainly concentrated in fornix. This apparent discrepancy with the prior studies may be explained by species differences. In a study of mouse conjunctival stem cells, it has been found that goblet cells increase in number from palpebral epithelium to fornix (the highest density in the fornix). Ultimately, the hypothesis that stem cells of conjunctiva mainly reside in the fornix has been proven.[7] Moreover, it has been reported that, in rabbit conjunctiva, the distribution of goblet cells (the highest density in the fornix) coincides well with that of keratinocytes with a high in vitro proliferative potential.[6] In our study, however, we found that in Wistar rat conjunctiva, the goblet cells are primarily clustered in palpebral epithelium (Figure 1). This finding, in conjunction with the morphologic observation of rat conjunctiva that palpebral epithelium contains more goblet cells than other regions of the conjunctival epithelium,[36] raises the intriguing possibility that conjunctival stem cells primarily reside in a region where the density of goblet cells is the highest in the conjunctiva. It has been proven that, in conjunctival epithelium, there are two types of stem cells. One is keratinocytes, which can generate keratinocyte and goblet cells, and thus perform an important role in the replacement of conjunctival epithelium. The other is goblet cells, which display protective action through the secretion of mucus.[6,7,14] The distribution of goblet cells might be consistent with that of conjunctival stem cells. Based on these findings, we speculate that in conjunctiva the identification of stem cells (keratinocyte and goblet stem cells) can depend on the number of goblet cells. Major sources of conjunctival stem cells generally reside in a region where the density of goblet cells is the highest in conjunctiva. This finding may provide a new insight into the location of conjunctival stem cells.

Jpn J Ophthalmol
Vol 47: 119–128, 2003

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