Mucocutaneous Junction As the Major Source of Replacement Palpebral Conjunctival Epithelial Cells

Jonathan D. Wirtschafter,1,2,3 Jeffrey M. Ketcham,1 Robert J. Weinstock,1 Tara Tabesh,1 and Linda K. McLoon1,4

PURPOSE. The conjunctival epithelium performs an important role in the homeostasis and integrity of the eye. To protect the integrity of the ocular surface, these cells must be replaced from locally concentrated or randomly distributed foci of stem cells. These slow-cycling stem cells produce transient amplifying cells that undergo further divisions before becoming mature conjunctival epithelial cells. In the current study, the source of palpebral conjunctival cells was determined.

METHODS. Adult rabbits were injected intraperitoneally with bromodeoxyuridine (BrdU) at a dose of 50 mg/kg body weight and killed after 1, 3, 5, and 7 days and 2 months. The orbital contents and eyelids were exenterated en bloc, frozen to maintain the orientation between the eyelids and globe, and sectioned in a parasagittal plane. Random midglobe sections were stained for the presence of proliferating cell nuclear antigen (PCNA). Additional sections were immunostained to detect BrdU-labeled conjunctival epithelial cells. BrdU-positive cells were counted in a series of 0.4-mm zones from the mucocutaneous junction of the eyelid, through the fornix and bulbar conjunctiva. A second set of rabbits received daily injections of BrdU for 2 or 4 weeks followed by a 2-month BrdU-free period before death and processing.

RESULTS. In all eyelid sections examined, there was a focus of PCNA-positive cells in the mucocutaneous junction and a few scattered PCNA-positive cells along the length of the palpebral conjunctiva toward the fornix. In both the upper and lower eyelids, the peak concentration of BrdU-labeled cells was found within 1 to 2 mm of the mucocutaneous junction at all postinjection intervals. These were always found within one cell height of the basement membrane in the basal layer of the epithelium. In the long-term studies, BrdU-labeled nuclei were retained at the mucocutaneous junction.

CONCLUSIONS. The mucocutaneous junction of the conjunctival epithelium is a source of actively dividing transient amplifying cells that migrate toward the fornix at a rate of approximately 1.7 mm/d with a transit time of approximately 6 days. Long-term retention of label at the mucocutaneous junction indicates that slow-cycling stem cells are present at this location. It appears that most palpebral conjunctival epithelial stem cells are located near the mucocutaneous junction. These results are not necessarily at variance with previous studies, but they diminish the relative importance of the forniceal region in palpebral conjunctival homeostasis. The mucocutaneous junction may provide a therapeutically significant source of replacement conjunctival cells.

The conjunctival epithelium is critical for maintaining homeostasis and integrity of the ocular surface. To protect the integrity of the ocular surface, these cells must be replaced from locally concentrated or randomly distributed foci of stem cells. A focal source of replacement cells for the conjunctiva has been ascribed to the fornix.1,2 However, it is possible that there are alternative focal concentrations or randomly distributed loci of stem cells for specific regions of the conjunctiva. The specific source of palpebral conjunctival cells has not been clearly demonstrated.

Epithelium is a constantly renewing tissue. In the skin, stem cells are generally diffusely positioned and not focally concentrated. These stem cells are slow-cycling cells that serve as progenitor cells for the tissue. In epithelium, a two-stage system has been described for proliferation.3 Epithelium contains a small population of stem cells that divide infrequently, and their daughter cells produce transient amplifying cells, which are a more actively proliferating population of cells.4 Two compartments, one slow-cycling and one proliferative, are present in the corneal limbus,5 the region responsible for corneal re-epithelialization after a corneal injury.6 In the present study, we identified a focus of proliferating cell nuclear antigen (PCNA)-positive cells at the mucocutaneous junction region of normal rabbit eyelid. We investigated...
the location and migration of transient amplifying cells in the palpebral conjunctiva in rabbits. Cohorts of palpebral conjunctival transient amplifying cells were identified and followed by using bromodeoxyuridine (BrdU), the thymidine analogue, by pulse-labeling of DNA during the S phase of the cell cycle. A preliminary report of the short-term labeling study has previously appeared. Long-term retention of BrdU label was examined along the length of the palpebral conjunctiva.

**MATERIALS AND METHODS**

All experimental procedures conformed to the National Institutes of Health guidelines for use of animals in research and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were purchased from Birchwood Farms (Red Wing, MN) and housed at the University of Minnesota.

A single injection of BrdU in sterile isotonic saline was administered intraperitoneally to adult rabbits at a dose of 50 mg/kg body weight. The rabbits were euthanized 1, 3, 5, and 7 days after the injections. Three to four eyelids were prepared at each experimental postinjection interval. The orbital contents including the lids were exenterated en bloc, and the orbital specimens 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 in as close to normal anatomic configuration as possible. Each half of the tissue specimen was frozen in a manner that maintained the orientation and continuity between the globe and the eyelids, and each specimen was sectioned in a parasagittal plane at 12 μm. The sections were identified according to their relationship to the midsagittal plane and whether each was in the lateral or medial half of each eye.

A second series of rabbits was injected daily with BrdU for 2 weeks or 4 weeks, and this was followed by a 2-month BrdU-free period.

The sections were processed for immunohistochemical localization of PCNA and BrdU. For PCNA immunostaining, after a 15-minute blocking incubation in normal serum, the sections were incubated with an antibody to PCNA (Chemicon, Temecula, CA) at a dilution of 1:40 for 1 hour. Sections were reacted using an alkaline phosphatase staining kit (Vectastain ABC; Vector, Burlingame, CA). For visualization of BrdU-positive nuclei, sections were incubated in 2 N HCl at 37°C for 1 hour, followed by several rinses in 0.1 M borate buffer. The sections were incubated in normal horse serum, followed by a 1-hour incubation with an antibody to BrdU (Boehringer-Mannheim, Indianapolis, IN) at a dilution of 1:100. The sections were processed using the ABC kit with avidin-biotin-peroxidase labeling. The sections were reacted with the heavy-metal-intensified diaminobenzidine procedure. Representative cross-sections were double immunostained for the presence of heparan sulfate proteoglycan (HSPG) with an antibody against HSPG (Chemicon), using the alkaline phosphatase kit and a substrate kit (Vector Red). All nuclei that incorporated BrdU were stained black. The HSPG was stained red. Additionally, some BrdU-immunostained sections were lightly counterstained with hematoxylin and eosin.

The mucocutaneous junction was defined as the point along the superficial edge of the lid margin at the transition point from keratinized stratified squamous epithelial cells of the eyelid skin to cuboidal epithelial cells of the palpebral conjunctiva. Often the openings of the tarsal glands could be seen at this junction.

The sections were examined using a light microscope interfaced with a computer. Morphometric analysis was done with the aid of image analysis software (Bioquant; R & M Biometrics, Nashville, TN). BrdU-labeled conjunctival epithelial cells were counted sequentially starting at the mucocutaneous junction of the eyelid continuously along the conjunctival surface to the fornix and onto the bulbar surface. Counts of BrdU-positive conjunctival epithelial cells were totaled for each full-thickness 0.4-mm zone along the eyelid cross-section. Counts were also made of BrdU-labeled cells that were within one cell of the basement membrane of the conjunctival epithelium for each of the 0.4-mm zones. Results were analyzed with computer software for statistical significance (Prism and Instat; Graphpad, San Diego, CA).

**RESULTS**

PCNA-positive cells were almost exclusively found in basal conjunctival epithelial cells within 0.4 mm of the mucocutaneous junction in rabbit eyelids (Fig. 1A). A few PCNA-positive cells were found randomly distributed along the length of the palpebral conjunctiva toward the fornix (Fig. 1B). This was true in both the upper and lower eyelids.

After pulse labeling with BrdU, BrdU-labeled nuclei were observed in almost all the 0.4-mm zones of the conjunctival epithelium including the palpebral, fornical, and bulbar regions. However, in the full-thickness counts including all cells from the basement membrane to the outermost cells, the position of heavily labeled areas varied as the distance from the eyelid margin increased after each of the postinjection intervals examined, that is 1, 3, 5, and 7 days after BrdU injection. One day after BrdU injection, there were many BrdU-positive cells found within the first 0.8 mm from the mucocutaneous junction (Figs. 2, 4). At 3 days after BrdU injection, the region with the highest concentration of labeled cells was located within approximately 4 to 5.8 mm from the mucocutaneous junction (Figs. 3, 4). Five days after the BrdU injection, the fornical region (approximately 8–10 mm from the mucocutaneous junction) had the highest concentration of labeled cells (Fig. 4). By 7 days, there was no identifiable focus of BrdU-labeled cells (Fig. 4). There appeared to be a migration of the peak number of labeled cells/0.4-mm zone at the lid margin toward the fornix (Fig. 4). In the lower lid conjunctiva the foci of labeling were located progressively greater distances from the mucocutaneous junction in the animals killed at 1, 3, and 5 days respectively. The peaks of label at 1, 3, and 5 days were significantly different from the values at the other zones along the eyelid conjunctiva. The same pattern of labeling was also found on the upper lid as the lead edge of labeling moved progressively away from the mucocutaneous junction over time. No focuses of labeled cells were seen 7 days after the BrdU injection (Fig. 4). Although not quantified, in the short-term BrdU-studies, there appeared to be heavier labeling of the palpebral conjunctiva than of the bulbar conjunctiva. The lymphoid tissue also stained heavily for the presence of BrdU at short post-BrdU survival intervals, indicating that the lymphoid tissue rapidly turns over within the fornical conjunctival submucosa.

In all specimens there was a relatively heavy area of BrdU labeling that consistently was found within 0.8 mm of the...
mucocutaneous junction after all postinjection intervals (Figs. 4, 5), although it varied quantitatively in animals killed 1, 3, 5, and 7 days after injection with BrdU, with the peak on day 1 (Fig. 5).

For approximate differentiation of stem cells from transient amplifying cells and other more mature cells by using location as a criterion, BrdU-positive cells were counted that were located on or one cell up from the epithelial layer basement membrane. There was always a focus of labeled cells at the mucocutaneous junction, but no other peak was seen along the basement membrane of the palpebral conjunctiva at any of the post-BrdU survival intervals (Fig. 6).

Long-term retention of BrdU label was demonstrated to reside primarily in the mucocutaneous junction after a BrdU-free period of 2 months (Figs. 7, 8) and in the fornix. Small numbers of BrdU-positive nuclei were found scattered randomly along the length of the palpebral conjunctiva basement membrane, but never in the quantity found at the mucocutaneous junction.

**DISCUSSION**

The mucocutaneous junction of the palpebral conjunctival epithelium in rabbits appears to be the source of actively dividing transient amplifying cells. Transient amplifying cells may go through several cycles of division, ultimately giving rise to terminally differentiated palpebral conjunctival epithelial cells. The transient amplifying cells and their daughter cells migrate toward the fornix to replace the mature cells that are continuously lost (Fig. 9). The long-term labeling study confirms that palpebral conjunctival stem cells must also be located in the mucocutaneous junction region where the BrdU-labeled transient amplifying cells are first identified.

The results of the present study are not necessarily at variance with the results of the previous studies, but they diminish the relative importance that they assign the fornical region in palpebral conjunctival epithelial homeostasis. This study did not completely exclude the possibility that some stem cells could be randomly and diffusely located throughout the palpebral conjunctiva, but it appears that this is not the predominant pattern. The present study differed from prior studies in that the absolute number of labeled cells was counted in a continuous sequence of zones measuring 0.4 mm from the mucocutaneous junction, whereas previous studies used a labeling index defined as the percentage of labeled cells per 1000 nuclei. The present strategy of counting labeled nuclei along the entire length of the palpebral conjunctiva in equally sized regions also differed from the previous analysis of...
three distinct regions, the bulbar, fornix, and palpebral conjunctiva, spatially separated from each other and not necessarily in a continuous sagittal plane. The morphometric analysis of the entire palpebral conjunctiva allowed the detection of movement of the foci of labeled cells from the mucocutaneous junction toward the fornix over several days. The change in location of the peak of BrdU-labeled cells strongly supports the hypothesis that large numbers of palpebral conjunctival cells are produced at the mucocutaneous junction at the eyelid margin and migrate toward the fornix. As in epithelium in general, the labeled cells from the basal layer of the epithelium move to the more superficial layers of the epithelial sheet. It is well accepted that epithelial cells migrate, both vertically within the epithelium and horizontally within the plane of the epithelial sheet. Tissue culture studies have demonstrated that this horizontal migration can be substantial. An overall movement of conjunctival cells of between 8.4 and 9.2 mm in 5 days would indicate a migration rate of between 1.68 and 1.84 mm/d. This is compatible with migration rates of approximately 1.5 mm/d determined by in vitro studies. The migration rate of proliferating cells from the corneal limbus in normal cornea is approximately 100 μm/d. After injury, progenitor cells, many of which may be resting at the limbus, can cover a denuded cornea within 24 hours, a rate of movement of approximately 6 mm/d.

Some BrdU-labeled epithelial cells remained near the mucocutaneous junction in all specimens, even 7 days after BrdU pulse labeling. It may be that some proportion of the transient amplifying cells mature or migrate more slowly than others or even may not migrate toward the fornix, and such processes would be reflected by the retention of labeled cells near the mucocutaneous junction. Stem cells, because they are slow-cycling cells compared with other cells, retain tritiated thymidine or BrdU labeling, so the observed stationary focus of BrdU-labeled cells near the mucocutaneous junction may include some stem cells. The presence of foci of PCNA labeling in this area suggests that there is a population of activated and transient amplifying cells in this region. Long-term retention of BrdU-labeling at the mucocutaneous junction supports the concept that this region, in fact, contains palpebral conjunctival stem cells.

It is interesting that 7 days after BrdU pulse labeling no specific focus of labeling was seen along the length of the palpebral conjunctiva. There are several possible explanations for the disappearance of the foci of labeled cells. First, cells may continue to divide, and this continued division could result in sufficient dilution of the BrdU label within the nuclei to levels below detection by antibody staining. However, the duration of the cell cycle in actively dividing epithelial cells has been demonstrated to be approximately 28.4 hours. This...
means that in 7 days approximately 6 cycles of division would have occurred. It is possible, however, that the duration of the cell cycle could be much shorter in this region of epithelium. The span of epithelial cell cycle duration determined in vitro or in tissue-stripping experiments has been postulated to be between 20 and 39 hours.\textsuperscript{15} A cell cycle duration of 20 hours

![Figure 3](image1.png)

**Figure 3.** Reconstruction of the entire length of the palpebral conjunctiva in a rabbit who received a single injection of BrdU 3 days before death. The black dots are BrdU-positive nuclei. Paired arrows indicate the region within which lies the focus of label in this eyelid. Bar, 0.5 mm.

![Figure 4](image2.png)

**Figure 4.** Location of BrdU-labeled conjunctival cells. The number of labeled cells found in full-thickness counts of palpebral conjunctiva at 0.4-mm increments from the mucocutaneous junction of the lower eyelids 1, 3, 5, and 7 days after a single injection of BrdU on day 0. Note the shift in peak concentration away from the mucocutaneous junction. To reduce graph complexity, each point and corresponding line represents the data from an individual eyelid at each of the postinjection intervals. *Significantly different from values at other days.*
would allow eight cycles of division within 7 days, which would certainly substantially dilute the BrdU label and result in its becoming undetectable. This assumes, however, that all epithelial cells continue to cycle at the same rate as in the basal layer. Other in vivo studies suggest that in adult epidermis, for example, the cell cycle time is on the order of 4.8 days.\(^3\) If this would allow eight cycles of division within 7 days, which would certainly substantially dilute the BrdU label and result in its becoming undetectable. This assumes, however, that all epithelial cells continue to cycle at the same rate as in the basal layer. Other in vivo studies suggest that in adult epidermis, for example, the cell cycle time is on the order of 4.8 days.\(^3\) If this

**FIGURE 5.** BrdU-labeled cells in relation to the eyelid margin. Comparison of the number of BrdU-labeled cells in the first 0.4-mm zone of the palpebral conjunctiva starting from the mucocutaneous junction at 1, 3, 5, and 7 days after a single injection of BrdU on day 0 with the corresponding number for the eighth zone, between 2.8 and 3.2 mm from the mucocutaneous junction. Note the first zone continues to retain a relatively high concentration of BrdU-labeled cells. Each point represents counts from three to four rabbit eyelids. Error bars indicate SEM. *, **; Pairs of data points with significant differences at P < 0.05.

**FIGURE 6.** BrdU-labeled nuclei found within one cell thickness of the basement membrane of the basal layer of the palpebral conjunctival epithelium 1, 3, 5, and 7 days after a pulse BrdU injection. Note there were always more labeled nuclei at the mucocutaneous junction at all post-BrdU time intervals. There was never a peak at any other point along the basal epithelial layer. Each data point represents the mean of four eyelid counts. Error bars indicate SEM. *Data are significantly different from values at other locations along the palpebral conjunctiva.
were the case in the palpebral conjunctiva, only a single cell cycle would have occurred during this same period. It is more likely that the loss of the migrating focus of labeled cells is due to their transit time—that is, their movement upward and diagonally through the thickness and along the surface of the conjunctival epithelium to the time when they are sloughed. This contention is supported by the fact that the focus of labeled cells at 3 and 5 days after a pulse BrdU injection is not found along the basement membrane (Fig. 6). Thus, the cells that had divided on day 0 had a transit time of between 6 and 7 days and could not be recognized beyond the completion of their transit time. This transit time is shorter than that of skin, which has been shown to have a transit time of between 10 and 14 days. However, in contrast to skin, the palpebral conjunctiva is exposed to a great deal of friction that occurs during repeated eye blinks. The average eye-blk rate is 8 to 12 per minute. This constant rubbing of two opposing epithelial layers is a relatively unique feature of the eyelids and could easily result in an increased sloughing rate compared with other epithelial sheets.

The mucocutaneous junction plays a vital role in the embryologic development of the eye and is a very active site during all stages of eyelid development. The leading edge of the developing eyelid in a mouse at embryonic day 15 forms from a loose aggregation of cells growing out of each future lid across the corneal surface. This is the future site of the mucocutaneous junction and supports the concept of cell migration from this area. Stem cells are usually located in relatively thick areas of epithelium in those regions where there is variation in thick-
ness. The mucocutaneous junction epithelium is relatively thick (approximately seven cell layers) compared with the rest of the conjunctiva. In some epithelial surfaces of the human body, it has been shown that the stem cells responsible for producing daughter cells that go on to replace lost epithelium reside in certain clusters or zones deep within that epithelium, rather than having random distribution throughout the epithelium. Examples of this architecture occur in the rete ridges of the palmar epithelium, the follicular epithelium at the bulge area of hair follicles, the epithelium of the corneal limbus, and the base of the crypts in the epithelium of the small intestines. Also, stem cells tend to be located in areas of increased pigmentation. Similar to the corneal limbus, the mucocutaneous junction is more highly pigmented than the remainder of the conjunctiva, and this pigmentation may help to protect the stem cells from the harmful effects of UV radiation, as has been postulated for other areas that contain epithelial stem cells.

The transient amplifying cells demonstrated in the present study point to the mucocutaneous junction as the location of the stem cells for the palpebral conjunctiva. Long-term retention of BrdU label after multiple injections followed by a 2-month BrdU-free chase period resulted in foci of BrdU-labeled nuclei in the mucocutaneous junction in these eyelids. This confirms the presence of slow-cycling cells at this location. The corneal limbus is thought to harbor stem cells for both the cornea and the perilimbal conjunctiva. Most squamous cell tumors of the conjunctiva arise presumably from a single clone of transformed stem cells in the perilimbal region corresponding to the interpalpebral fissure. Moreover, the much rarer squamous cell tumors (dysplasia, carcinoma in situ, and invasive squamous cell carcinoma) of the palpebral conjunctiva invariably involve the mucocutaneous junction, thus supporting the concept that the mucocutaneous junction is the predominant location of the stem cells for the palpebral conjunctiva.

The clinical significance of these observations requires further study. There are a number of disorders for which replacement conjunctiva is needed. Currently such replacement is obtained as autografts of healthy conjunctiva or other mucous membranes. New technologies make possible the in vitro production of artificial (skin) epithelial and fibroblast replacement materials. It is possible that the mucocutaneous junction might provide a therapeutically significant source of replacement conjunctival cells. It may be that these cells could be stimulated to be more productive in situ or used as a source of stem and transient amplifying cells for the in vitro production of artificial conjunctiva.

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References


