Phorbol Ester Preferentially Stimulates Mouse Fornical Conjunctival and Limbal Epithelial Cells to Proliferate In Vivo

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PURPOSE. The authors investigated whether fornical epithelium displays a differential in vivo response to acute and chronic stimulation when compared with bulbar and palpebral epithelia.

METHODS. To induce an increase in epithelial proliferation, 0.5% phorbol myristate (TPA) was topically applied in petrolatum daily to both eyes of SENCAR mice for 12 days. Control mice (three per group) received petrolatum only. After 6, 12, 18, and 24 hours (acute) and 2, 3, 4, 5, 7, 9, and 12 days (chronic) of TPA treatment, mice (three per group) were administered intraperitoneally 0.1 ml 40 μCi [3H]thymidine ([3H]Tdr) 1 hour before they were killed. Conjunctival epithelium was fixed and processed for autoradiography, and the labeling index (LI; number of [3H]Tdr-labeled nuclei per 1000 basal keratinocytes) was determined for each of the epithelial zones.

RESULTS. Under normal situations, the LI was lowest in fornical epithelium (1.9 ± 0.5) compared with bulbar (4.4 ± 0.9) and palpebral (5.5 ± 0.5) epithelia. Within 24 hours of TPA treatment, a 12-fold increase in fornical basal cell labeling was noted compared with a 2.5- and 5-fold increase in bulbar and palpebral basal cell labeling, respectively. Fornical epithelium maintained a significantly greater proliferative response (4.5-fold increase) during chronic stimulation than either bulbar or palpebral epithelia (0.5- and 1.5-fold increase, respectively).

CONCLUSIONS. The more vigorous response of the fornical epithelium to acute and chronic stimulation is strong evidence that this epithelium has a greater proliferative capacity than the other two epithelia, which is consistent with the authors’ hypothesis that conjunctival epithelial stem cells are primarily located in the fornical region. (Invest Ophthalmol Vis Sci. 1998;39:301-307)

Cell kinetic investigations of a number of epithelia, including palmar epithelium, trunk epidermis, corneal and conjunctival epithelium, hair follicle, and dorsal tongue epithelium, have revealed that basal keratinocytes are heterogeneous with respect to their proliferative capacity. Some basal cells cycle slowly (a feature of stem cells), some divide rapidly (a feature of transit amplifying cells), and some are postmitotic in the following scheme: stem cell → transit-amplifying cell → postmitotic cell. The transit-amplifying populations appear to be randomly distributed along the basement membrane, whereas subpopulations of basal cells displaying slow-cycling characteristics have a nonrandom distribution. The location of these slow-cycling basal cells is one of the principal means used to define stem cell-rich regions of epithelia. For example, in palmar epithelium and in trunk epidermis, the bottom of the deep rete ridges is thought to be the site of the stem cells (nonserrated cells). In murine epidermis, cells with stem cell characteristics are thought to be located at the center of an epidermal proliferative unit. In intestinal and colonic epithelium, the region just above the bottom of the crypts is considered to contain the stem cells. Within the hair follicle, the bulge region of the outer root sheath epithelium has been demonstrated to contain a subpopulation of cells with stem cell characteristics. Corneal epithelial stem cells have been localized exclusively to a region at the edge of the corneal epithelium known as the limbus. Similar studies have been performed in the conjunctival epithelium, and the fornical zone has been shown to be a site enriched by stem cells.

Another important property of stem cells is their great proliferative capacity, which usually outlasts the life span of the animal (reviewed in ref. 15). Cell culture has been widely used to assess proliferative capacity because the propagation of cells from stem and non-stem cell-enriched regions under identical conditions allows comparisons to be made of various proliferative properties (for example, colony-forming ability, growth rate, growth potential). When studied in this manner, limbal epithelial cells (stem cell-enriched region) grow better than central corneal epithelial cells (non-stem cell-enriched region) in human explant culture and in rabbit cell culture. Similarly, within the rabbit conjunctival epithelium, fornical epithelial cells proliferate more rapidly, remain relatively smaller in size, and reach confluence earlier than bulbar or palpebral cells. Furthermore, whereas rabbit fornical epithelial cells can be subcultured several times, bulbar or palpebral cells can be subcultured only once before senescing. In studies on the mouse hair follicle, the upper follicle (a region enriched by stem cells) contains cells that have a significantly greater proliferative capacity than those of the lower follicle and se-
baceous glands (non-stem-cell-enriched regions). One limitation of the above studies is that, once placed in an in vitro environment, stem and transit-amplifying cells proliferate rapidly, making it impossible to distinguish these two populations. To circumvent this problem, investigators have taken advantage of the fact that, after stimulation with a hyperplastic agent, regions rich in stem cells show preferential stimulation when compared with non-stem cell regions. One of the best demonstrations of this phenomenon is the response of the limbal epithelium to the application of the tumor promoter phorbol myristate (TPA). Under normal conditions, the proliferative rate of the limbal epithelium is lower than that of corneal epithelium. After 4 to 5 days of topical treatment with TPA, a 5- to 10-fold increase in the proliferative rate of the limbal epithelium was observed compared with a 3-fold increase in the proliferative rate of corneal epithelium. These results indicated that the limbal epithelium had a greater proliferative capacity than central corneal epithelium and was important in the formulation of the hypothesis that corneal epithelial stem cells are concentrated in the limbal epithelium.

As discussed above, there is evidence that the conjunctival epithelial stem cells are concentrated in the fornical zone. In the present work, we test this hypothesis by comparing the in vivo response of the bulbar, fornical, and palpebral conjunctival epithelia to acute and chronic growth stimulation. We demonstrate that the fornical epithelium responds more vigorously to acute and chronic TPA stimulation than do bulbar and palpebral epithelia. This constitutes evidence that the fornical epithelium has a greater proliferative capacity than the other two adjacent epithelia. These findings support the hypothesis that the fornical zone is enriched in conjunctival epithelial stem cells and has several implications for the behavior of stem and transit-amplifying cell populations.

MATERIALS AND METHODS

Acute Stimulation

To determine the acute response of the anterior surface-stratified ocular epithelia, SENCAR mice were administered a single topical application of 0.5% TPA in petrolatum in both eyes. Control mice were administered petrolatum only. After 6, 12, 18, and 24 hours of treatment, mice (three per group) were administered intraperitoneal 0.1 ml 40 μCi tritiated thymidine ([3H]TdR; specific activity, 80 Ci/mmol) 1 hour before they were killed. Eyes were surgically removed and processed for histology or autoradiography as described previously.

Chronic Stimulation

To determine the response of the ocular surface epithelia to chronic stimulation, 0.5% TPA in petrolatum was topically applied once daily to both eyes of SENCAR mice. After 1, 2, 3, 4, 5, 7, 9, and 12 days of TPA treatment, mice (three per group) received [3H]TdR and were processed as described above.

Based on its anatomic location, degree of stratification, and density of goblet cells, adult murine conjunctival epithelium can be divided into three zones. The bulbar zone is covered by a stratified squamous epithelium and is contiguous with the limbal zone of the cornea. Bulbar epithelium contains primarily single goblet cells and is easily distinguished from the adjacent limbal epithelium. The fornical epithelium contains one or two cells layers and is less stratified than either the bulbar or the palpebral region. It contains the greatest number of goblet cells, which frequently appear as clusters. The palpebral epithelium is the most highly stratified of the three zones, consists of four to seven cell layers, and is contiguous with the mucocutaneous junction of the eyelid. Palpebral goblet cells appear singly and increase in number toward the fornix. A labeling index was determined for each of the different conjunctival zones by counting at least 1000 nuclei from three different sections for each determination, and these results were expressed as the percentage of labeled cells per 1000 nuclei.

RESULTS

Exposure to Phorbol Myristate Increased Proliferation in Bulbar, Fornical, and Palpebral Conjunctival Epithelia

To determine the relative proliferative response of various anterior ocular surface epithelia, we exploited the ability of TPA to increase epithelial proliferation. We have previously shown that TPA induces cell proliferation in several murine epithelia, including those of cornea, limbus, epidermis, and hair follicle. Furthermore, this approach is superior to other physical means of inducing proliferation (for example, incision wounding), because it allows the direct comparison of the proliferative responses of intact bulbar, fornical, and palpebral epithelia and the corneal and limbal epithelia. Control experiments showed that daily application of petrolatum, the vehicle for TPA, had no effect on [3H]TdR labeling of any of the anterior ocular surface epithelia (Figs. 1a, 1b, 1c, 2a, 2b). The unperturbed fornical conjunctival epithelium had a lower proliferative rate (1.9 ± 0.3%) than bulbar (4.4 ± 0.5%) and palpebral (5.5 ± 0.5%) epithelia. It is also lower than corneal (6.8 ± 1.5%) or limbal (3.8 ± 0.5%) epithelia (Table 1, Fig. 3). The absolute values for the proliferative rates in the present study are somewhat higher than previously published values; however, the relative rates of proliferation in the different epithelia are maintained—that is, cornea has the highest rate and fornix has the lowest rate.

Within 12 hours of exposure to TPA, an increase in the number of [3H]TdR-labeled nuclei was observed in all conjunctival epithelial zones and in the limbal epithelium (Table 1). This increase in labeled nuclei reached a maximum 24 hours after exposure to TPA (Table 1, Figs. 1d, 1e, 1f, 2c, 2d). At this time, a 12-fold increase in fornical basal cell labeling was noted, compared with a 2.5- and 5-fold increase in bulbar and palpebral basal cell labeling, respectively (Fig. 4). Limbal epithelium also responded more dramatically compared with corneal epithelium (7.5-fold versus 3-fold increase; Fig. 4). These data indicate that the fornical and limbal epithelia (putative sites rich in conjunctival and corneal epithelial stem cells, respectively) had the greatest proliferative response to an acute stimulus.

Chronic Exposure to Phorbol Myristate Stimulates Cell Proliferation in Fornical and Limbal Epithelia to a Greater Degree Than Epithelia from Other Regions of the Anterior Segment

We next considered whether the fornical and limbal regions could sustain elevated proliferative rates after repeated TPA treatments. All five epithelia of the anterior segment showed a
Bulbar  Fornix  Palpebral

Figure 1. Autoradiograms of the response of the bulbar (a, d, g), fornical (b, e, h), and palpebral (c, f, i) epithelia to a single exposure (d, e, f) and a 2-day exposure (g, h, i) of phorbol ester. Response of bulbar, fornical, and palpebral epithelia to petrolatum treatment is shown in a, b, and c. Note the low level of \(^{3}H\)thymidine (\(^{3}HTdR\)) incorporation (arrows) in unperturbed fornical epithelium compared with bulbar and palpebral epithelia (a, b, c). A single exposure of phorbol myristate (TPA) (d, e, f) results in marked increases in \(^{3}HTdR\) incorporation in all three conjunctival epithelia, most notably in the fornical epithelium (e). Note the marked decrease in \(^{3}HTdR\) incorporation in bulbar and palpebral epithelia after 2 days of TPA treatment (g, i), whereas the fornical epithelium (h) has a higher proliferative profile.

marked decrease in proliferative activity by the second day of TPA treatment (Figs. 1g, 1h, 1i, 2e, 2f, 5, 6). Whereas the proliferative rate for bulbar and palpebral epithelial cells had nearly returned to control values after 2 days of TPA treatment, fornical epithelial proliferation remained higher than in controls. Similarly, limbal epithelial proliferation was greater than control values and was higher than corneal epithelium after 2 days of treatment. During the remaining 10 days of TPA treatment, fornical epithelium maintained a greater proliferative response (4.5- to 6-fold increase) than either bulbar or palpebral epithelium (0.5- and 1.5-fold increase, respectively; Fig. 5). Consistent with our previous study,\(^5\) limbal epithelium demonstrated a greater proliferative response (2.5- to 3-fold increase) than corneal epithelium (less than 1-fold increase) during continuous TPA stimulation (Fig. 6).

DISCUSSION

Fornical and Limbal Epithelial Cells Share Similar Kinetic Properties

A careful examination of the biochemical, cell kinetic, and clinical findings indicates that the limbal epithelium exhibits a great number of features consistent with a tissue rich in stem cells. Specifically, the limbal epithelium lacks the differentiation-dependent K3/K12 keratins\(^10\); is rich in slow-cycling cells\(^3\); has a large proliferative capacity\(^3,13,16,17\); is superior to conjunctival epithelium for repairing corneal epithelium\(^21-23\); when deficient or absent, is responsible for the pathogenesis of corneal diseases such as persistent epithelial defect\(^11\); and is the site of origin of most corneal epithelial dysplasias and neoplasms.\(^24-26\) Thus, the cell kinetic behavior of resting and perturbed limbal epithelium serves as a paradigm for a stem cell-enriched tissue.

A comparison of the limbal epithelium with the bulbar, fornical, and palpebral conjunctival epithelia has revealed that the fornical epithelium shared many kinetic properties with limbal epithelium. Under resting conditions, limbal and fornical epithelia incorporate low amounts of pulse-administered \(^{3}HTdR\). This phenomenon is thought to reflect the presence of slow-cycling cells that ordinarily do not incorporate pulse-administered \(^{3}HTdR\). A relatively low proliferative rate appears to be a generalized feature of stem cell-rich regions (that is, the nonserrated basal keratinocytes of deep rete ridges\(^1,2\) and the bulge keratinocytes of the hair follicle\(^3\)). Detection of
Cornea Limbus

Figure 2. Autoradiograms of corneal (a, c, e) and limbal (b, d, f) epithelia that have been exposed to a single (c, d) or a 2-day treatment (e, f) of phorbol ester. The response of corneal and limbal epithelia to petrolatum treatment is shown in a and b. Note the low level of \( [\text{H}] \)-thymidine (arrow) in unperturbed corneal epithelium (a) and limbal epithelium (b). A single exposure of phorbol myristate (TPA) results in marked increases in \( [\text{H}] \)-TdR incorporation in corneal (c) and limbal epithelia (d). After 2 days of TPA treatment, both regions show a decrease in \( [\text{H}] \)-TdR incorporation.

Label-retaining cells is a more direct confirmation of the presence of slow-cycling cells, and label-retaining cells are concentrated in limbal\(^5\) and fornical epithelia.\(^5\) Thus, under normal, unperturbed conditions, the cell kinetics of the fornical epithelium mirrors that of the limbal epithelium.

In the present study, we chronically stimulated the conjunctival and corneal-limbal epithelia with the rationale that regions with great proliferative capacity would respond selectively to continuous perturbation. This indeed was the case; continuous TPA treatment resulted in a higher proliferative rate for fornical epithelium than for bulbar or palpebral epithelium. This response of the fornical epithelium mimicked that of limbal epithelium; both sustained a higher proliferative rate throughout a course of chronic TPA stimulation when compared with adjacent epithelia. The present data provide in vivo confirmation of our earlier cell culture findings\(^1\) and show

Table 1. Proliferative Responses of the Anterior Surface Stratified Ocular Epithelia after the First 24 Hours of Phorbol Myristate Stimulation

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Corneal</th>
<th>Limbal</th>
<th>Bulbar</th>
<th>Fornical</th>
<th>Palpebral</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.8 ± 1.5</td>
<td>3.8 ± 0.5</td>
<td>4.4 ± 0.9</td>
<td>1.9 ± 0.5</td>
<td>5.5 ± 0.5</td>
</tr>
<tr>
<td>6</td>
<td>2.0 ± 0.7</td>
<td>3.2 ± 0.6</td>
<td>2.4 ± 0.6</td>
<td>4.3 ± 1.5</td>
<td>4.0 ± 1.3</td>
</tr>
<tr>
<td>12</td>
<td>2.3 ± 0.6</td>
<td>9.5 ± 3.9</td>
<td>9.3 ± 2.8</td>
<td>9.8 ± 3.5</td>
<td>10.8 ± 2.8</td>
</tr>
<tr>
<td>18</td>
<td>7.6 ± 2.3</td>
<td>16.3 ± 5.7</td>
<td>12.5 ± 6.0</td>
<td>24.5 ± 6.0</td>
<td>18.3 ± 1.9</td>
</tr>
<tr>
<td>24</td>
<td>21.1 ± 3.7</td>
<td>33.4 ± 5.0</td>
<td>15.3 ± 4.5</td>
<td>26.6 ± 7.1</td>
<td>34.0 ± 6.2</td>
</tr>
</tbody>
</table>

Values represent the percentage of \( [\text{H}] \)-thymidine-labeled nuclei per 1000 basal nuclei and data from at least eight eyes pooled from two independent experiments and arc presented as mean ± SD.
that the fornical epithelium has a greater proliferative capacity than the other two conjunctival zones. Taken together, the above findings support the hypothesis that the conjunctival epithelial stem cells are primarily located in the fornical region.

**FIGURE 3.** Proliferative rate of unperturbed corneal, limbal, and conjunctival epithelia. Mice (three per group) received \[^{3}\text{H}\]thymidine 1 hour before they were killed, and a labeling index was calculated (see Materials and Methods). Each value represents the average ± standard deviation of at least eight eyes pooled from two independent experiments. Limbal and fornical epithelia (putative sites rich in slow-cycling cells) display the lowest proliferative rate.

**FIGURE 4.** Proliferative response of corneal, limbal, and conjunctival epithelia to acute phorbol myristate (TPA) stimulation. Mice (three per group) received 0.5% TPA in petrolatum topically in both eyes, whereas control mice received petrolatum only. After 24 hours of treatment, mice received \[^{3}\text{H}\]thymidine 1 hour before they were killed, and a labeling index was calculated (see Materials and Methods). Each value represents the fold increase over control ± standard deviation and is derived from at least eight eyes pooled from two independent experiments. Fornical and limbal epithelia (putative sites rich in slow-cycling cells) displayed the greatest response to an acute stimulus.

**FIGURE 5.** The proliferative response of mouse bulbar, fornical, and palpebral epithelia to chronic phorbol myristate (TPA) stimulation. Mice (three per group) were administered 0.5% TPA in petrolatum topically in both eyes, whereas control mice were administered petrolatum only. After 1, 2, 3, 4, 5, 7, 9, and 12 days of treatment, mice were administered \[^{3}\text{H}\]thymidine 1 hour before they were killed, and a labeling index was calculated (see Materials and Methods). Each value represents the fold increase over control ± standard deviation and is derived from at least eight eyes pooled from two independent experiments. TPA treatment resulted in a marked increase in the proliferative activity of all three conjunctival epithelia at day 1, followed by a marked decrease after 2 days. Fornical epithelium maintained a significantly greater proliferative response than either bulbar or palpebral epithelia during the remaining 10 days of treatment.

**Phorbol Myristate-Sensitive Cells Are Preferentially Located in Fornical and Limbal Epithelia**

In the present study, the response of the corneal-limbal and conjunctival epithelia to a single exposure of TPA was identical; all regions had a sharp increase in proliferation that peaked by 24 hours. A similar temporal increase in \[^{3}\text{H}\]TdR incorporation into DNA, peaking between 18 and 30 hours, was reported for mouse skin after a single application of TPA. Continuous exposure to TPA resulted in a dramatic decline in the proliferative rate for all the epithelia. However, the decreases observed for fornical and limbal epithelium were significantly less than those in corneal, bulbar, and palpebral epithelium. It is generally believed that there is a hierarchy of proliferative capacity within the transit-amplifying cell population: Early transit-amplifying cells are capable of many rounds of cell division, whereas late transit-amplifying cells can undergo a few rounds of division before differentiation. Therefore, one possible explanation for the present findings is that the fornical and limbal epithelia are enriched by subpopulations of cells that are continuously stimulated to proliferate by TPA.
whereas the corneal, bulbar, and palpebral epithelium contain greater numbers of cells that cease dividing and begin to differentiate in response to TPA. We consider that these responses are the in vivo correlate of the well-described behavior of cultured keratinocytes to TPA. Specifically, TPA treatment of cultured murine keratinocytes revealed that some cells were stimulated to proliferate, whereas others were induced to differentiate. An explanation for this divergent response among basal cell populations to TPA is that the more differentiated cells exhibited accelerated differentiation, whereas the less mature cells exhibited greater proliferation in response to TPA. More recently, when cultured rabbit corneal and limbal keratinocytes were exposed to TPA, an acceleration of differentiation in a majority of cells from both regions was observed. However, the size of the proliferating subpopulation was greater in cultured limbal keratinocytes than in cultured corneal keratinocytes, leading to the suggestion that the limbal epithelium contains more slow-cycling progenitor cells. Studies that examined the effects of multiple exposures of TPA on murine skin reported 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. This led to the speculation that multiple exposures to TPA may select for a proliferative population of keratinocytes, which is consistent with our present findings.

References


32. Dunn JA, Jeng AY, Yuspa SH, Blumberg PM. Heterogeneity of \[^{3}H\]phorbol 12,13-Dibutyrate binding in primary mouse keratinocytes at different stages of maturation. *Cancer Res.* 1985;45:5540-5546.