A Tumor Promoter-Resistant Subpopulation of Progenitor Cells Is Larger in Limbal Epithelium Than in Corneal Epithelium

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Purpose. In the epidermis, proliferative basal cells can be divided into two subpopulations according to their response to phorbol ester tumor promoters. The tumor promoter-sensitive subpopulation ceases mitosis and initiates terminal differentiation and, thus, represents more differentiated transient amplifying cells. In contrast, the tumor promoter-resistant subpopulation that continues to proliferate may be the target of neoplastic transformation by chemical carcinogens and may contain stem cells. Based on this concept, we examined the differential response of stem cell-containing limbal epithelium and transient amplifying cell-containing corneal epithelium to phorbol 12-myristate 13-acetate (PMA) treatment.

Methods. A reported serum-free clonal growth assay was used. The mitogenic response was measured by colony-forming efficiency (CFE), colony size, bromodeoxyuridine (BrdU) labeling index; the differentiation was assessed by colony morphology, AE-5 monoclonal antibody staining.

Results. The addition of PMA dose dependently inhibited the clonal proliferation of both limbal and corneal epithelial cultures with respect to CFE, colony size, and BrdU labeling index, suggesting that both cultures contain PMA-sensitive subpopulations. Nevertheless, the magnitudes of a decrease in CFE and colony size in peripheral corneal cultures were significantly greater than those in limbal cultures, indicating that the size of the PMA-resistant subpopulation is larger in the limbal epithelium. The inhibitory effect of PMA on clonal proliferation was partially reversible upon its early withdrawal, indicating that its inhibitory effect is continuous and coupled with progressive differentiation of progenitor cells in this culture system.

Conclusion. These results further suggest that the cell cycle length of progenitor cells correlates with the mitogenic pathway mediated via calcium- and phospholipid-dependent protein kinase C, the receptor inhibited by prolonged treatment of phorbol ester tumor promoters. Invest Ophthalmol Vis Sci. 1993;34:2501–2511.
cells in a process of self-renewal, transient amplifying cells, or both, as a result of differentiation. In contrast, transient amplifying cells have a short life span, are rapid cycling with a high mitotic index, and thus can effectively expand the size of a cell population. After a certain number of mitoses, transient amplifying cells differentiate into postmitotic cells, which eventually terminally differentiate to perform tissue-specific functions.

The corneal epithelium is a self-renewing tissue. Recent studies of the expression pattern of a differentiation-related K3 keratin, and the label-retaining property upon treatment with a tumor promoter or wounding, suggest that the stem cells of the corneal epithelium are located in the limbal basal epithelium and that the corneal basal epithelium primarily contains transient amplifying cells. This concept has further been supported by several of our studies that demonstrate that abnormal corneal epithelial wound healing can occur after partial or total removal of the limbus and that transplantation of limbal tissue is effective in reconstructing a corneal surface deprived of corneal and limbal epithelium. The concept that corneal epithelial stem cells are exclusively located at the limbus and are anatomically separated from the predominantly transient amplifying cell-containing cornea makes the ocular surface epithelium an ideal model to study the regulation of epithelial stem cells.

In the epidermis, proliferative basal cells can be separated into two subpopulations according to their differential response to phorbol ester tumor promoters. Upon exposure, the tumor promoter-sensitive subpopulation ceases mitosis and initiates terminal differentiation, suggesting that these cells are probably more differentiated transient amplifying cells. In contrast, the tumor promoter-resistant subpopulation consists of the smallest cells and can continue DNA synthesis with negligible differentiation. Because these resistant cells may be the target for neoplastic transformation by carcinogens, a phenomenon known to affect primarily stem cells, this subpopulation may well contain stem cells. Based on this concept, topical treatment of 12-0-tetradecanoyl phorbol 13-acetate, a tumor promoter, in mouse corneas causes a drastic increase of label (H-thymidine)-retaining cells in the limbal epithelium, further supporting the concept that the limbal epithelium contains a tumor promoter-resistant subpopulation of progenitor cells (ie, stem cells).

Recently, we have established a serum-free, chemically defined clonal growth assay for the ocular surface epithelium. This culture condition primarily supports the clonal proliferation of transient amplifying cells. Therefore, it is also feasible to assess the activation of limbal stem cells to transient amplifying cells when differentiation is stimulated. Using this culture assay, we sought to examine whether limbal and corneal epithelia can still be differentiated in culture by their differential response to a tumor promoter, phorbol 12-myristate 13-acetate (PMA).

**MATERIALS AND METHODS**

Animals were housed and treated according to the ARVO Resolution on the Use of Animals in Research. Male New Zealand white rabbits, between 4 and 6 mos of age, were used in all experiments. Before euthanasia with an intravenous overdose of pentobarbital, they received an intramuscular injection of 50 mg xylazine hydrochloride and 50 mg ketamine hydrochloride.

**Chemical Reagents and Cell Culture Media**

Dulbecco's modified essential medium, fetal bovine serum, and amphotericin B were purchased from Gibco (Grand Island, NY). Modified Eagle's minimal essential medium without Ca\(^{2+}\) came from Whittaker (Walkersville, MD). Bromodeoxyuridine (BrdU) and Dispase II were from Boehringer (Mannheim, Germany). A mouse monoclonal antibody against BrdU was obtained from Becton Dickinson (Mountainview, CA). The mouse monoclonal antibody AE-5 against K5 keratin was kindly provided by T-T Sun (New York University, NY). An Elite\(^{\circledast}\) ABC immunoperoxidase detection kit was from Vector Lab (Burlingame, CA). Crystal violet was from Difco Laboratories (Detroit, MI). All other chemicals including powdered MCDB 151 medium and phorbol 12-myristate 13-acetate (PMA) were from Sigma Chemical Co. (St. Louis, MO). Plastic cell culture dishes (60 mm) with 2 mm grids were obtained from Corning (Corning, NY).

Medium MCDB 151 was prepared by diluting its powder in tissue culture water buffered with sodium bicarbonate and enriched with the supplement (S) containing insulin-transferrin-selenium (5 \(\mu\)g/ml-5 \(\mu\)g/ml-5 \(\mu\)g/ml), hydrocortisone (5 \(\mu\)g/ml), mouse epidermal growth factor (5 ng/ml), and phosphoethanolamine/ethanolamine (0.1 mM each). To reach a final calcium concentration of 0.3 mM, 0.27 mM calcium was added to the medium. A stock solution of PMA was diluted in acetone or dimethylsulfoxide. After adding an appropriate dilution of this PMA stock solution to the medium, the final concentration of dimethylsulfoxide or acetone in the medium was 0.05%. Only during the cell isolation was Dulbecco's modified essential medium containing 10% fetal bovine serum, 50 \(\mu\)g/ml gentamicin, and 5 \(\mu\)g/ml amphotericin B used to stop the enzymatic digestion.
Single Cell Isolation and Cell Culture

The method of cell isolation has been previously described. In brief, the anterior segments of the rabbit eyes were excised at 2 mm posterior to the corneoscleral junction, and the adherent iris and the corneal endothelium were removed. The limbus, peripheral cornea, and central cornea were isolated by 6 and 10 mm trephines and subjected to digestion with 1.2 U/ml Dispase II for 1 h for peripheral and central cornea and for 3 h for limbus. The loosened epithelial sheets were then removed by pipetting. After a second brief digestion for 10 min with 0.1% trypsin and 0.02% EDTA in Eagle’s minimal essential medium, single cells were obtained by repeated aspiration through a 23-gauge needle.

For routine primary clonal cultures, 5000 viable cells were seeded in 60-mm cell culture dishes containing 3 ml MCDB 151 + S to yield a seeding density of approximately 180 cells/cm². Cultures were grown in this medium with or without the addition of 0.1 to 1.0 µg/ml PMA. When the entire dish was screened for attached cells 24 h later, 1.0 µg/ml PMA-added cultures had an attachment rate of 96 + 9% of the control cultures. In some experiments, cultures were first grown in PMA-containing medium for the first 3 days or for 24 h on day 3, and then switched to a PMA-free medium. All cultures were incubated at 37°C under humidified 5% CO₂, and media were changed twice a week.

Assay of Proliferation

The entire surface of each culture dish was screened for colony growth, and the total number of colonies was counted on day 6. A colony was defined as a group of four or more cells that had been derived from a single cell. Clonal proliferation was assayed on day 6 by the parameters of colony-forming efficiency (CFE), colony size, and BrdU labeling index. CFE (%) was calculated by dividing the total number of colonies on day 6 by the number of viable cells seeded on day 0. To determine CFE, we counted the entire surface of each dish in a total of 7 experiments with a minimum of 4 dishes per condition. This parameter reflects the proliferative capacity of those single cells that had initiated colony formation. The colony size, ie, the number of cells per colony, was calculated from a total of 3 experiments by counting 25 randomly selected colonies per dish with 4 dishes per condition per experiment. This parameter reflects the proliferative capacity of those single cells that had initiated colony formation. BrdU labeling was performed using a technique modified from a previous report. The cultures were incubated for 2 hr with a fresh medium containing 10 µM BrdU and terminated with -20°C methanol. After incubation for 15 min in 2 N HCl, and for 20 min in 0.5% Tween 20 and 0.1% bovine serum albumin, cultures were incubated with a monoclonal antibody against BrdU, followed by a standard peroxidase anti-peroxidase technique. Randomly selected colonies were photographed, and the number of labeled nuclei divided by the total number of cells per colony from 25 randomly selected colonies was designated as the labeling index. This parameter reflects the topographic distribution of the mitotic activity in each given colony.

Assay of Differentiation

Consecutive phase contrast photographs of randomly selected colonies were taken between days 3 and 6 to determine changes in cellular morphology. On days 6 and 14, culture dishes were either terminated with 70% methanol for crystal violet staining or with -20°C methanol for immunofluorescence studies. The methanol-fixed dishes were incubated for 15 min in PBS containing 0.8% bovine serum albumin and 0.1% sodium azide. The dishes were then incubated with the monoclonal antibody AE-5 against K3 keratin to assess the cornea-type differentiation. After staining with an FITC-linked secondary antibody, the dishes were examined and photographed under a Zeiss Axioshot fluorescence microscope (Zeiss, Oberkochen, Germany).

Statistical Analysis

Paired Student’s t-tests and Wilcoxon’s rank sum tests were used to examine the differences in corneal and limbal control cultures with respect to CFE, colony size, and BrdU labeling. Two-factor repeated measures analysis of variance and paired Student’s t-tests were used to test the interactions between location (limbal versus peripheral corneal) and dose (control versus TPA treatment).

RESULTS

PMA-Resistant Subpopulation Present in Both, But Larger in Limbal Than in Peripheral Corneal Epithelial Cultures

On day 3, some of the attached single epithelial cells began to form small colonies. In the control medium (MCDB 151 + S), these colonies continuously expanded, as previously reported. In contrast, after the addition of 1.0 µg/ml PMA on day 0, some colonies first noted on day 3 ceased proliferation by day 6 (Figs. 1A, 1B), whereas others continued to proliferate in a manner similar to the control (Figs. 1C, 1D). This result indicates that PMA selectively inhibits the clonal proliferation of a subpopulation of progenitor cells.

To quantify the inhibitory effect of PMA, the CFE was calculated on day 6. In the control medium, limbal cultures had a CFE significantly less than that of peripheral corneal cultures (P < 0.05, Fig. 2), a finding...
FIGURE 1. Typical changes of clonal growth in two different subpopulations of progenitor cells upon PMA treatment. Early colony formation was observed on day 3 of cultures (A, C). On day 6, with 1.0 μg/ml PMA treatment, one subpopulation degenerated (B); the other subpopulation continued to expand to a larger colony (D).

consistent with our earlier report. In contrast, the addition of 0.1 to 1.0 μg/ml PMA significantly reduced the CFE in both limbal and peripheral corneal cultures (P < 0.05). This is shown in one representative experiment (Fig. 2). To determine whether the size of the subpopulation of cells resistant to 1.0 μg/ml PMA in the limbal epithelium is larger than that in the peripheral corneal epithelium, we performed 7 separate experiments with a minimum of four dishes per experiment. The results show that the absolute number of PMA-resistant colonies varied between 1 + 1 and 83 ± 19 cells in limbal cultures and between 0 and 55 ± 16 cells in peripheral corneal cultures among these 7 experiments. Despite this variation, the absolute number of PMA-resistant colonies in limbal cultures was significantly larger than that in peripheral corneal cultures in all 7 experiments (P < 0.05). Furthermore, the magnitude of the decrease in CFE caused by 1.0 μg/ml PMA in peripheral corneal cultures was significantly greater than that in limbal cultures in all 7 experiments (P < 0.0024). These results show that though both limbal and peripheral corneal cultures contain colonies resistant to PMA, the size of this resistant subpopulation of progenitor cells is significantly larger in limbal than in peripheral corneal epithelia.

Although special care had been taken in surgically separating the limbus from the peripheral cornea, one cannot absolutely be certain that the preparations of peripheral corneal epithelial cells used in the above studies were not contaminated by adjacent limbal epithelial cells because of their close vicinity. To determine if the corneal epithelium also contains a genuine PMA-resistant subpopulation, we conducted four separate experiments using the central corneal epithelium. The mean CFE was 13.1 ± 3.6% in the control and 0.1 ± 0.16% in the 1.0 μg/ml PMA-added cultures (P < 0.001), indicating that the central corneal epithelium, which is anatomically well separated from the limbal epithelium by the peripheral corneal epithelium, also contains a subpopulation of PMA-resistant progenitor cells.

To determine whether the clonal proliferation of the PMA-resistant progenitor cells was also affected by the continuous PMA treatment, the mean colony size, i.e., number of cells per colony, was counted on day 6. Figure 3 shows the mean values of the combined data of 6 separate experiments comparing the control cultures with 1.0 μg/ml PMA-added cultures and of 3 separate experiments comparing control cultures with 0.1, 0.3, or 0.6 μg/ml PMA-added cultures. As previously reported, the colony size of control limbal cultures was significantly smaller than that of control peripheral corneal cultures (P < 0.026). The addition of 0.1 μg/ml PMA significantly reduced the colony size in both limbal or peripheral corneal cultures compared
Effect of Tumor Promoter on Limbal and Corneal Epithelium

CFE on day 6

![Graph](image)

**FIGURE 2.** Colony-forming efficiency (CFE) of limbal (open bars) and peripheral corneal (shaded bars) cultures on day 6. Compared to CFE of the control without PMA, CFEs of cultures containing 0.1 to 1.0 µg/ml PMA showed a significant decrease (P < 0.05).

with their respective control cultures (P < 0.05, respectively, Fig. 3). An increase to 0.3, 0.6, or 1.0 µg/ml PMA did not further significantly reduce the colony size (Fig. 3). For 1.0 µg/ml PMA, although the mean colony size of limbal cultures seemed to be larger than that of peripheral corneal cultures in 4 of 6 experiments, this difference was not statistically significant. Compared to the control, the magnitude of the reduction of the colony size in peripheral corneal cultures was significantly larger than that in limbal cultures (P < 0.017).

To ensure that the measurements of colony size reflected the cellular proliferation within each colony, we measured the BrdU labeling index on day 6. The addition of 1.0 µg/ml PMA significantly reduced the labeling index to 37 + 39% of the control in limbal cultures (P < 0.001) and to 41 + 48% of the control in peripheral corneal cultures (P < 0.001). The labeling index was not significantly different between limbal and peripheral corneal cultures containing 1.0 µg/ml PMA. Aside from the colonies with a low labeling index, we also noted that PMA-containing cultures included a subpopulation of colonies that had a labeling index similar to that of control cultures. These results indicate that PMA inhibits all three parameters of clonal proliferation, (i.e., CFE, colony size, and BrdU labeling) and confirm the existence of a subpopulation of colonies, of which proliferation is resistant to PMA treatment in both limbal and peripheral corneal cultures.

**Partially Reversible Inhibitory Effect of PMA on Clonal Proliferation**

When the culture life was extended to a later stage, PMA also dose dependently inhibited the clonal prolif-

Colony size on day 6

![Graph](image)

**FIGURE 3.** Colony size of limbal (open bars) and peripheral corneal (shaded bars) cultures on day 6. Compared to control cultures without PMA, colony sizes of cultures containing 0.1 to 1.0 µg/ml PMA showed a significant decrease (P < 0.05).
eration of both limbal and peripheral corneal cultures. On day 14, colonies in the control cultures had merged with one another, so the numeric assessment of CFE and colony size became unreliable. To circumvent this difficulty, dishes were stained with crystal violet and the cell-covered area was compared. As shown in Figure 4 for limbal cultures, the addition of 0.1 to 1.0 μg/ml PMA markedly reduced the cell-covered areas of the dish. Even in cultures containing 1.0 μg/ml PMA, there were still some PMA-resistant colonies (Fig. 4). The colony size of these PMA-resistant colonies was similar in all tested concentrations but smaller than that of the control. A similar result was noted in peripheral corneal cultures (data not shown). These results indicate that PMA continuously inhibits clonal proliferation in late cultures and that both limbal and peripheral corneal cultures contain some colonies resistant to PMA.

To determine if the inhibitory effect of PMA was reversible upon its early withdrawal, cultures were either short-term treated with 1.0 μg/ml PMA for 3 days starting from day 0, or only pulse-treated for 24 hr on day 3. On day 14, short-term exposure to 1.0 μg/ml PMA from day 0 to day 3 (Fig. 4A) resulted in a cell-covered area less than the control without PMA (control, Fig. 4) but more than those receiving the continuous treatment with 1.0 μg/ml PMA for 14 days. This result indicates that a subpopulation of progenitor cells that would otherwise be inhibited by continuous exposure to PMA could resume its clonal proliferation upon early withdrawal of PMA from the medium. The pulse treatment for 24 hr on day 3 (Fig. 4B) also exhibited a cell-covered area less than that of the control without PMA treatment but, interestingly, similar to that of the cultures that received short-term treatment for the first 3 days (Fig. 4A). Similar results were observed in peripheral corneal cultures (data not shown). These results further indicate that the susceptibility of clonogenic progenitor cells to PMA treatment varied during the early culture stage and reached a higher level on day 3, the time when colony formation begins (Fig. 1).

PMA-Modified Colony Morphology and Differentiation

On day 6, colony morphology was similar in both control limbal and peripheral corneal epithelial cultures. Control limbal cultures contained uniform colonies consisting of small round-to-elongated cells, which tended to be more cohesive in the center and more migratory in the periphery (Fig. 5A). As colonies became larger after day 6, they began to form suprabasal cells and desquamation (arrow, Fig. 5B). In contrast, colony morphology was less uniform in cultures continuously treated with 1.0 μg/ml PMA. Up to 80% of these colonies consisted of small cohesive polygonal cells (Fig. 5C). They contained fewer suprabasal cells (Fig. 5D) on day 14 than the controls if they continued to expand in size after day 6. The remaining colonies consisted of relatively large squamous cells (Fig. 5E). Similar results were observed in peripheral corneal cultures (data not shown). These results indicate that colony morphology can also be modified in the PMA-resistant subpopulation of both limbal and peripheral corneal cultures.

To determine if the change in colony morphology by PMA treatment was accompanied by a change in cellular differentiation, the expression of K3 keratin, a marker for cornea-type differentiation,7 was studied by immunofluorescence staining using the monoclonal antibody AE-5. On day 6, for control limbal and peripheral corneal cultures, the colonies, although uniform in morphology (Fig. 5A), showed a nonuniform staining pattern. The majority of the colonies (between 60% and 90%, depending on the experiments) was stained heterogeneously with positive and negative cells within the same colony (Fig. 6A). The remaining colonies could be either strongly positive (between 5% and 20%) (Fig. 6B) or predominantly negative (between 5% and 20%) (Fig. 6C). These results indicate that control cultures without PMA treatment exhibit a spectrum of epithelial differentiation with respect to K3 keratin expression. When the cultures were continuously treated with 1.0 μg/ml PMA, the smaller polygonal cohesive cells of the most prevalent
small colonies (up to 80%) (Fig. 5C) showed a heterogeneous staining pattern. About two-thirds of these colonies stained predominantly positive (Fig. 6D), and the rest stained predominantly negative (Fig. 6E). Both types of colonies could contain strongly positive cells that were not suprabasally located (arrow, Fig. 6E). Large colonies consisting of small cells as well as colonies that expanded after day 6 (Fig. 5D) were stained heterogeneously with a spectrum from negative to positive cells within the same colony (Fig. 6F), a pattern similar to that of the control (Fig. 6A). The colonies with large squamous cells, which made up about 20% of the total number of colonies (Fig. 5E), were also stained heterogeneously with a spectrum of negative to strongly positive cells within the same colony (Fig. 6G). Similar results were obtained for both limbal and corneal epithelial cultures. Like controls, PMA-treated cultures did not contain completely positive or completely negative colonies. These results indicate that colonies that continue to proliferate despite a continuous treatment with PMA retain a pattern of keratin expression similar to the majority (60–80%) of control cultures.

**DISCUSSION**

In this report, we demonstrate that prolonged PMA treatment markedly reduced the clonal proliferation...
FIGURE 6. Immunofluorescence staining with anti-K3 keratin monoclonal antibody AE-5 on day 6. In the control cultures without PMA, staining patterns were nonuniform, most were stained heterogeneously (A), and the remaining few could be either strongly positive (B) or strongly negative (C). Cultures treated with 1.0 μg/ml TPA resulted in those small colonies that were stained either predominantly positive (D) or negative (E), of which the latter had strongly positive cells (arrow, E). In those less frequently observed large colonies, staining was heterogeneous (F). The large squamous cells (E) were also heterogeneously stained (G).

of both limbal and corneal epithelium with respect to CFE, colony size, and BrdU labeling (Figs. 2 and 3), indicating that a subpopulation of progenitor cells is sensitive to PMA's antiproliferative effect. This subpopulation may be responsible for the development of those colonies that appeared on day 3 but subsequently ceased mitosis and degenerated on day 6 (Fig. 1). As a result of the inhibition of PMA proliferation, some colonies changed morphology and increased the expression of the differentiation-related K3 keratin, as evidenced by increased AE-5 immunofluorescence (Figs. 5 and 6). Because these PMA-sensitive progenitor cells are prone to differentiate into postmitotic cells, their differentiative status is within the spectrum of transient amplifying cells similar to that described for epidermal keratinocytes (see Introduction). Also, because clonal proliferation of both limbal and corneal epithelia was similarly affected, these PMA-sensitive transient amplifying cells are present throughout the entire corneal and limbal epithelia. That transient amplifying cells are also present in the limbal epithelium is consistent with several reports indicating that not all basal epithelial cells of the limbus are stem cells. Only 30% of mouse limbal basal epithelial cells can be activated and display the label-retaining property upon stimulation by a central corneal epithelial wounding. Using pulse labeling to detect rapid-cycling cells, 3H-thymidine-labeled nuclei have been noted in normal peripheral corneal and limbal epithelia. Recently, using intraperitoneal injection of BrdU, a thymidine analog, with a chase for 72 hrs in rabbits, we noted that the labeling index peaked at 24 hr in the corneal epithelium and 48 hr in the limbal epithelium, further suggesting that the transient amplifying cells of the limbus might have average cell cycles slightly longer than those of the cornea. The existence of transient amplifying cells with different cycling times has also been observed in our serum-free cultures in which limbal transient amplifying cells proliferate more slowly than those of the peripheral and central corneal epithelium. The relatively higher mitotic rate in the corneal epithelium, as opposed to the...
limbal epithelium, might generate the growth pressure that prevents conjunctival epithelial ingrowth with an intact limbal epithelium and explains why a removal of the limbal suprabasal epithelium can facilitate conjunctival epithelial ingrowth.10

Phorbol ester tumor promoters exert their effects by binding to specific receptors located primarily on cell surface membranes32 and activate the Ca325 and phospholipid-dependent protein kinase C (PKC). PKC serves as a receptor for phorbol ester tumor promoters and may account for most, if not all, of the specific binding of phorbol ester tumor promoters.33 Treatment of phorbol ester tumor promoters results in a dramatic decrease in cytosolic PKC activity accompanied by an increase of membrane-bound PKC activity, thereby phosphorylating several membrane-associated proteins that regulate growth and morphology, including receptors for growth factors (EGF, insulin, and IGF-I) and transferrin as well as vinculin, a cytoskeletal element. As reported previously, clonal proliferation in the present culture system is primarily derived from transient amplifying cells and modulated by such mitogens as EGF, insulin, and transferrin.26 For EGF, tumor promoter-induced PKC phosphorylation downregulates the EGF receptor, and thus explains the inhibitory effect of phorbol ester tumor promoters on EGF’s mitogenic effect.34 It is thus conceivable that treatment of tumor promoters can counteract the mitogenic effects exerted by these growth-promoting agents in this culture medium. Furthermore, prolonged PMA treatment can inhibit PKC activity.

Despite the fact that PMA, a kind of phorbol ester tumor promoter, inhibited clonal proliferation of both limbal and corneal epithelia, it is intriguing to observe that there exists a subpopulation of progenitor cells in which clonal growth was actually more resistant to PMA inhibition (Figs. 1–3). This PMA-resistant subpopulation is likely responsible for the existence of colonies that showed continuous growth in late cultures (Fig. 4) and displayed negative AE-5 staining, similar to the undifferentiated PMA-free controls (Fig. 6). Because the current culture system primarily promotes the proliferation of transient amplifying cells that have no capability of self-renewal,26,34 prolonged cultures of these cells result in progressive differentiation. It explains our recent finding that increasing concentrations of extracellular calcium enhance cellular proliferation coupled with progressive differentiation, as illustrated by a uniform increase of AE-5 staining.35 It explains why the prolonged PMA treatment caused a continuous inhibitory effect on clonal proliferation in late cultures (Fig. 4), indicating that more undifferentiated PMA-resistant cells had gradually become PMA-sensitive from differentiation. It also explains why a shorter exposure of PMA still allowed some PMA-resistant cells to preserve some proliferative capacity (Fig. 4). Because the pulse treatment with PMA for 24 hr on day 3 resulted in clonal growth comparable to the short-term treatment for 3 days (from day 0 to day 3) (Fig. 4), and because clonal proliferation started on day 3 (Fig. 1), the inhibitory effect of PMA seems to begin when progenitor cells were first actively engaged in mitosis. Because the resulting colony number and size were smaller than those of the control after an early withdrawal of PMA (Fig. 5), it suggests that the short-term PMA treatment had irrevocably stopped the colony growth of some progenitor cells and modified the growth behavior of the remaining resistant cells.

Because the present culture system facilitates differentiation of single progenitor cells along with proliferation, it is unlikely to preserve the “stemness” of the stem cells if they are indeed activated. Furthermore, significantly greater magnitudes of decrease in clonal proliferation with respect to CFE and colony size were observed in peripheral corneal cultures compared to limbal cultures (Fig. 2 and 3). This result indicates that the limbal epithelium contains a greater PMA-resistant subpopulation. This finding is consistent with that of Cotsarelis et al.,8 showing that topical treatment of 12-0-tetradecanoyl phorbol-13-acetate in mouse corneas causes a drastic increase of cells with a label (3H-thymidine)-retaining property (indicative of slow-cycling) in the limbal epithelium. This slow-cycling property of limbal epithelial cells was further demonstrated by our recent finding showing that the limbal epithelium was more resistant than the corneal epithelium to inhibition by 5-fluorouracil, an antimetabolite inhibiting DNA synthesis.31 Therefore, one can conclude that limbal epithelium contains more slow-cycling progenitor cells, a property suggestive of stem cells.

From this study, it is also interesting to note that both limbal and corneal epithelia contain similar PMA-resistant progenitor cells. Because this PMA-resistant subpopulation was also present in the central corneal epithelium, its existence in the peripheral corneal epithelium cannot be caused by contamination from the limbal epithelium as a result of regional heterogeneity of the limbo-corneal junction.36 The existence of PMA-resistant cells in the corneal epithelium suggests that these cells may represent an early stage of transient amplifying cells. If so, this further suggests that the limbal stem cells, if activated in this culture system, have rapidly differentiated into these early transient amplifying cells. The latter notion is supported by our recent finding that the clonal proliferation of a subpopulation of limbal progenitor cells is preferentially activated by the addition of high concentrations of bovine fetal serum or retinoic acid.37 If this interpretation is correct, separation of these two progenitor subpopulations by tumor promoter treatment resides within transient amplifying cells and not
at the level between stem cells and transient amplifying cells. Alternatively, it is also conceivable that there is actually a continuous spectrum of cell cycle length from limbal stem cells to corneal transient amplifying cells. Therefore, the corneal epithelium might also contain progenitor cells for which cell cycles are long enough to be in a similar range to that of the limbal stem cells. This interpretation is implied by the findings that over 95% of the mouse corneal basal cells and 80% of the limbal basal cells are labeled by a 7-day, continuous $^3$H-thymidine labeling (to label both rapidand slow-cycling cells), respectively, and that there is no regional difference in label-retaining cells between these two epithelia after a 4-week chase in the normal atraumatic state. Recently, using subconjunctival injection of 5-fluorouracil to eliminate short-cycling cells, we also observed that long-cycling cells were present in the corneal epithelium, although the number was less than in the limbal epithelium. Taken together, these data suggest that the distinction between stem cells and transient amplifying cells with respect to mitotic kinetics does not commence abruptly at the border between the limbus and the peripheral cornea.

Because differentiation of transient amplifying cells is progressive in this culture system and parallel with the increasing susceptibility to PMA, and because an increase of extracellular calcium concentrations promotes the clonal proliferation of transient amplifying cells, we speculate that among various signal transduction systems, the PKC-dependent mitogenic pathway gradually dominates during the differentiation of transient amplifying cells. In rabbit corneas, PKC activity in the epithelium has been found twice as high as that in the stroma-endothelial layer. Future studies are needed to determine if this speculation is correct and if it can be extrapolated to the in vivo situation.

Key Words

corneal epithelium, clonal growth, limbus, phorbol ester, stem cells

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