Serum Differentially Modulates the Clonal Growth and Differentiation of Cultured Limbal and Corneal Epithelium

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Purpose. The stem cell-containing limbal epithelium is in proximity with highly vascularized tissue, as opposed to the transient amplifying cell-containing corneal basal epithelium, which resides on top of avascular corneal stroma. We therefore speculate that limbal stem cells are preferentially under the modulation of serum-derived factors.

Methods. Using a previously reported serum-free, chemically defined culture system for ocular surface epithelium, a culture condition primarily supporting transient amplifying cells of both corneal and limbal epithelia, we compared the clonal growth measured by colony-forming efficiency (CFE), colony size, and BrdU labeling, as well as colony differentiation measured by colony morphology and immunofluorescence staining, with the monoclonal antibody AE-5 against keratin K3 when fetal bovine serum (FBS) was added at different concentrations.

Results. The addition of 1% FBS decreased CFE and colony size in peripheral corneal cultures but had no effect in limbal cultures. Both cultures showed no obvious difference in colony morphology or BrdU labeling and AE-5 staining. In contrast, at 10% or 20% FBS, CFE and colony size increased in limbal cultures, but dose dependently decreased in peripheral corneal cultures. The presence of a unique subpopulation of progenitor cells in limbal cultures different from transient amplifying cells in corneal cultures was further supported by the emergence of a higher proportion of a unique type (B) colonies in limbal cultures that had high BrdU labeling and heterogeneous or negative AE-5 staining, indicative of their being in a proliferating, undifferentiated state. These colonies showed continuous growth in late cultures and could be passaged into serum-free medium.

Conclusion. These results indicate that serum contains factors responsible for stimulating limbal progenitor cells into clonal proliferation. Invest Ophthalmol Vis Sci 1993;34:2976–2989.

For all self-renewing epithelial tissues, a constant cell pool size is achieved by maintaining a delicate balance between cellular proliferation and differentiation.[1,2] Cellular proliferation is performed by stem cells and transient amplifying cells.[3–5] Stem cells have a long life span, a high clonogenic potential, and a unique capacity for self renewal.[4–7] Under normal circumstances, stem cells are slow-cycling with a low mitotic index, but they can be activated by demand for cell regeneration. According to current stem cell models,[5–10] the stem cell mitosis generates stem cells in a process of self-renewal, transient amplifying cells, or both as a result of cell 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 cell pool size. After a certain number of mitoses, transient amplifying cells differentiate into postmitotic cells, which eventually terminally differentiate to perform specific tissue functions. This cascade of tissue genesis indicates that stem cells are the ultimate source of cellular proliferation and differentiation. Depletion of the stem cell population by disturbances of stem cell renewal or differentiation, or both, into transient amplifying cells can potentially lead to various disease states. Therefore, studies of the regulation of stem cells are crucial in understanding the pathogenesis of various epithelial diseases.

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The corneal epithelium is a self-renewing tissue. Recent studies of the expression pattern of a differentiation-related keratin K3[11] and the label-retaining property upon treatment with a tumor promoter and wounding,[12] have suggested that the stem cells of corneal epithelium are located in the limbal basal epithelium. This concept has further been supported by several of our studies, which demonstrated that abnormal corneal epithelial wound healing can occur after partial[13,14] or total[15,16] removal of the limbus, and that transplantation of the limbal tissue is effective in reconstructing the corneal surface that has been deprived of corneal and limbal epithelium.[17,18] The fact that corneal epithelial stem cells are anatomically separated from their transient amplifying cells makes the ocular surface epithelium an ideal and unique model to study the regulation of epithelial stem cells (for review, see Tseng[19]).

It should be recognized that the advances in the understanding of hematopoietic stem cells, the best studied stem cell system, are largely based on the development of single-cell clonal growth assays, such as the in vivo[20] and in vitro[21] spleen-colony forming assay, and the in vitro agarose assay.[22] These assays allow one to trace the proliferative and differentiative development of a single progenitor cell at different stages of the cascade. As a first step to study the progenitor cells of limbal and corneal epithelium, we recently developed a single-cell clonal growth assay using a serum-free, chemically defined culture medium and have shown that this culture condition primarily supports the proliferation of transient amplifying cells.[23]

Anatomically, the limbal epithelium is in close proximity to the limbal vascular system,[24] whereas the corneal epithelium is situated on the avascular stroma. This difference in vascular supply suggests that the concentrations of serum-derived factors may be different at these two locations. We thus speculate that limbal stem cells at the limbal basal epithelial layer might preferentially be under the regulation of serum-derived factors. In this report, we investigated the hypothesis that serum-derived factors might preferentially modulate a subpopulation of limbal epithelial progenitor cells.

MATERIALS AND METHODS

Animals

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 months of age, were used in all experiments. Before sacrifice 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 (DMEM), fetal bovine serum (FBS) (lot no. 338726 and 3390267), and amphotericin B were purchased from Gibco (Grand Island, NY). Eagle's modified essential medium (EMEM) (without Ca^{2+}) came from Whittaker (Walkersville, MD). Bromodeoxyuridine (BrdU) and Dispase II were purchased from Boehringer (Mannheim, Germany). A mouse monoclonal antibody against BrdU was obtained from Becton Dickinson (Mountain View, CA). A mouse monoclonal antibody, AE-5, against keratin K3 was kindly provided by T-T Sun (New York University, NY). An Elite ABC immunoperoxidase detection kit was purchased from Vector Lab (Burlingame, CA). Crystal violet was obtained from Difco Laboratories (Detroit, MI). All other chemicals, including powdered MCDB 151 medium, were from Sigma (St. Louis, MO). Sixty millimeter cell culture dishes with 2 mm grids were obtained from Corning (Corning, NY).

Medium MCDB 151 was buffered with sodium bicarbonate in tissue culture water and enriched with a supplement (S): insulin–transferrin–selenium (5 μg/ml, 5 μg/ml, 5 ng/ml, respectively), hydrocortisone (5 μg/ml), mouse epidermal growth factor (5 ng/ml), and phospho-ethanolamine/ethanolamine (0.1 mM each). An additional 0.27 mM calcium was added to the medium to reach a final concentration of 0.3 mM. During cell isolation, Dulbecco's modified essential medium containing 10% FBS, 50 μg/ml gentamicin, and 5 μg/ml amphotericin B was used to stop enzymatic digestion.

Single Cell Isolation and Cell Culture

The techniques of cell isolation and culture have previously been described.[23] In short, after sacrificing the rabbits, corneoscleral buttons were removed by a peritomy made 2 mm posterior to the corneoscleral junction and carefully denuded of endothelium and adherent iris. Limbus, peripheral, and central cornea were then separated by two trephines measuring 10 mm and 6 mm. After enzymatic digestion with Dispase II (1.2 U/ml) for 1 hour for the corneal epithelium and 3 hours for the limbal epithelium, the loosened epithelial sheets were removed and separated into single cells by a second digestion for 10 minutes with 0.1% trypsin and 0.02% EDTA in calcium-free Eagle's modified essential medium for 10 minutes, followed by aspiration through a 23-gauge needle. The completeness of basal cell removal was verified by frozen sections of the residual tissues. Five hundred viable cells from either limbal or peripheral corneal epithelium were seeded in a 60-mm cell culture dish containing 3 ml of the medium MCDB 151 + S, yielding a seeding density of approximately 18 cells/cm^2. All cultures were incu-

bated at 37°C, under 95% humidity and 5% CO_2, and media were changed every 2 days.

To test the modulating effect of serum, FBS in a concentration of 1%, 10%, or 20% was added to the medium. To investigate the effect of serum on the proliferating capacity of the cells upon subculture, 5000 single cells were seeded per 60-mm dish at the density of approximately 180 cells/cm^2 in serum-free or serum-containing MCDB 151 + S. On day 14, cells were subjected to Dispase II treatment for 15 minutes, and single cells were obtained by an additional incubation in trypsin/EDTA for 5 minutes and one-time aspiration through a 23-gauge needle (for enzyme concentrations, see above). After cell counting, single cells derived from each of the above media were seeded into medium with or without 20% FBS at a density of 500 cells per 60-mm dish.

Assay of Proliferation

The entire surface of the dishes was screened 24 hours after seeding to determine the number of attached cells. To determine the influence of serum on cell attachment, the total number of attached cells was determined in two separate experiments with four dishes per condition using a previously reported method.[23] To allow a comparison between experiments and between limbal and peripheral corneal cultures, the total number of the attached cells in the controls was set as 100%, and the absolute number of attached cells of serum-added cultures was expressed as a percentage. The attached cells were then monitored daily 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 originated 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. This index reflects the proportion of seeded cells able to form colonies and was calculated from a total of 3 experiments with a minimum of 4 dishes per condition. Colony size, or the number of cells per colony, was expressed as the mean value from 25 randomly selected colonies per dish in 2 dishes per condition, for a total of 3 experiments. This measurement reflects the proliferative capacity of the cells in each colony. The number of the colonies with a particulate morphology and their colony size in the serum-containing cultures were counted in 2 dishes per condition, for a total of 3 experiments. To demonstrate the relative mitotic activity in each given colony, the BrdU labeling index was measured using a technique modified from that reported by Staquet et al.[25] The cultures were incubated with fresh medium containing 10 μM BrdU for 4 hours and terminated with −20°C methanol. After incubation in 2 N HCl for 20 minutes, and in PBS containing 0.5 % Tween 20 and 0.1 % bovine serum albumin for 15 minutes, cultures were incubated with a monoclonal antibody against BrdU followed by a standard peroxidase–antiperoxidase technique. Randomly selected colonies were photographed, and the number of labeled nuclei divided by the total number of cells per colony from 30 colonies was designated as the labeling index.

Assay of Differentiation

Consecutive phase contrast photographs of randomly selected colonies in medium MCDB 151 + S with and without 1%, 10%, or 20% FBS were taken every second day starting on day 6 to document changes in cell morphology. On days 6, 14, and 21, cell culture dishes from both primary and secondary cultures were terminated either with 70% ethanol for crystal violet staining or with −20 C° methanol for immunofluorescence studies. The methanol-fixed dishes were incubated in PBS containing 0.8% bovine serum albumin and 0.1% sodium azide for 15 minutes. The dishes were incubated with AE-5, a monoclonal antibody against keratin K3, related to cornea-type epithelial differentiation.[11] After staining with an FITC-linked secondary antibody, the dishes were examined with a Zeiss Axiophot fluorescence microscope (Zeiss, Oberkochen, Germany).

Statistical Analysis

The influence of serum concentration on cell attachment, colony-forming efficiency, colony size, and fraction of BrdU labeled cells was studied separately within location (limbus or peripheral cornea) using one- and two-way analyses of variance (experimental runs were treated as blocks). Power transformations[26] and the log transform were used as necessary to affect homogeneity of variance and normality in these data. Correlations were studied with Pearson's correlation coefficient. Student-Neuman-Keuls multiple comparisons were employed to determine which differences between concentrations were significant after analysis of variance.

RESULTS

FBS Differentially Stimulated CFE in Early Limbal Cultures but Inhibited in a Dose-dependent Fashion That of the Peripheral and Central Corneal Cultures. On day 3, attached single cells began to form colonies. On day 6, the total number of colonies in each dish was counted, and the CFE was calculated. In the serum-free control cultures, CFEs of the peripheral and central corneal epithelium were not significantly different (P, not significant), but both were significantly higher than that of the limbal epithelium ($P < 0.05$, Fig. 1), a finding

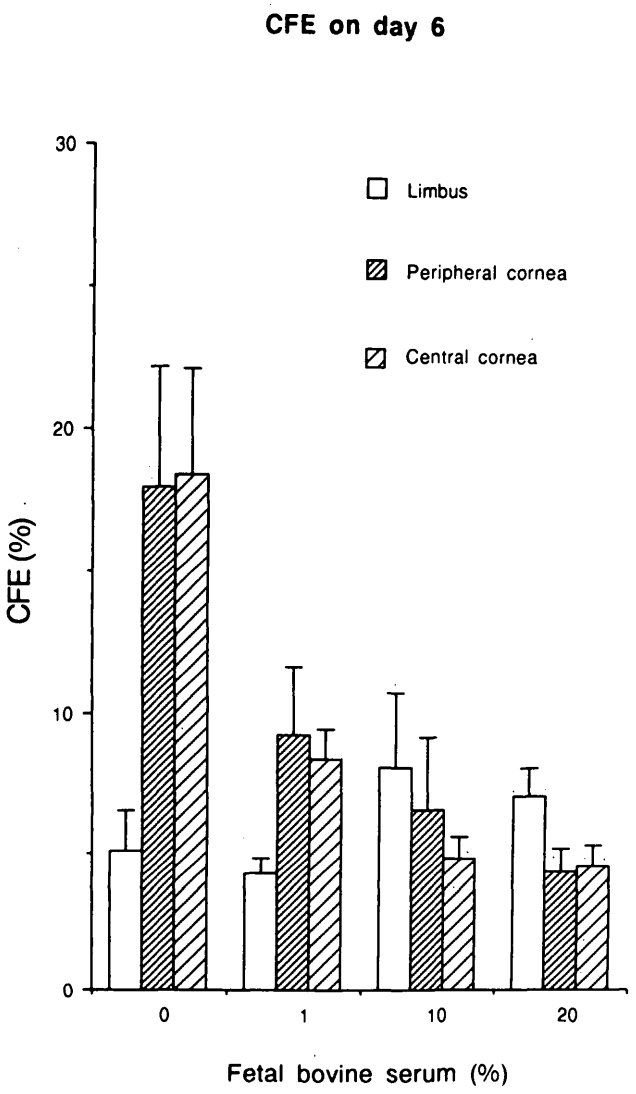

FIGURE 1. Colony-forming efficiency (CFE) of limbal (open bars), peripheral corneal (shaded bars), and central corneal (intermediate bars) cultures on day 6. CFE was determined as described in Materials and Methods. Compared to CFE of the control without fetal bovine serum (FBS), the absolute CFE of cultures containing 1% FBS induced a significant reduction of CFE in peripheral and central corneal cultures but no change in limbal cultures. Addition of 10% or 20% FBS induced an increase of CFE in limbal cultures but a decrease of CFE in peripheral and central corneal cultures.

consistent with our earlier report.[23] In the limbal cultures, the addition of 1% FBS significantly reduced CFE in two experiments ($P < 0.05$) and did not change CFE in one experiment (P, not significant). However, the addition of 10% and 20% FBS in the limbal cultures caused a significant increase of CFE in all three experiments ($P < 0.05$). In contrast to this stimulatory effect of FBS on CFE of limbal cultures, both peripheral and central corneal cultures showed a significant, dose-dependent decrease of CFE in response to 1%, 10%, or 20% FBS in all three experiments ($P < 0.05$, Fig. 1). Because the peripheral corneal cultures showed approximately the same response in CFE as the central corneal cultures, we elected to limit the remaining experiments to a comparison between limbal and peripheral corneal cultures.

To determine if the above changes of CFE were affected by the initial cell attachment, the number of attached cells was counted 24 hours after seeding. Both control and serum containing cultures showed single attached cells of similar morphology, as described before.[23] Cell mitosis did not occur before day 2, and there was no difference in the cell attachment rate between limbal and peripheral corneal cultures in serum-free controls, a finding consistent with our previous report.[23] As shown in Table 1, in comparison with the attached number of cells of the serum-free control set as 100%, the cell attachment rate for the limbal cultures was not changed by the addition of 1%, 10%, or 20% FBS (P, not significant). However, the cell attachment rate of the peripheral corneal cultures was significantly decreased by the addition of increasing concentrations of FBS ($P < 0.05$, Table 1). No significant correlation was found between cell attachment rate on day 1 and the CFE on day 6 in the limbal cultures (P, not significant), but there was a significant correlation in the peripheral cultures ($P < 0.044$), suggesting that CFE of the peripheral corneal cultures might be affected by the reduced initial cell attachment rate. Because the reduction of the cell attachment rate on day 1 was not quantitatively equivalent to the reduction of CFE on day 6 for the peripheral corneal cultures, we think that the effect of serum on the cell attachment on day 1 alone might not explain the decrease of CFE on day 6.

FBS Dose Dependently Stimulated the Colony Size of Limbal Cultures but Inhibited That of Peripheral and Central Corneal Cultures. To assess the proliferative capacity of those cells recruited into clonal proliferation, colony size was determined on day 6. In serum-free

TABLE 1. The Effect of Serum on Cell Attachment in Limbal and Peripheral Corneal Cultures

	MCDB 151 + S	*+1% FBS*	*+10% FBS*	*+20% FBS*
Limbus	100 ± 7	92 ± 12	98 ± 5	99 ± 7
Peripheral cornea	100 ± 5	78 ± 4	71 ± 16	63 ± 22

Cell attachment rate (%) was measured as described in "Methods." The data were expressed as mean ± SD (%) by setting the control in MCDB 151 + S as 100%. There was no statistically significant difference in the limbal cultures (P is not significant), but significant differences in the peripheral corneal cultures ($P < 0.05$) when increasing concentrations of FBS were added to the medium.

control cultures, the limbal epithelium exhibited a smaller colony size than the peripheral corneal epithelium (data not shown), a result consistent with our previous findings.[23] In both limbal and peripheral corneal cultures, addition of 1% FBS reduced the colony size significantly compared to the serum-free control ($P < 0.05$, Fig. 2). In limbal cultures, colony size was significantly increased by the addition of 10% FBS and 20% FBS, as compared to 1% FBS ($P < 0.05$), and reached a level similar to that of the serum-free control (Fig. 2). In contrast, both 10% and 20% FBS led to a significant reduction of the colony size in the peripheral corneal cultures ($P < 0.05$, Fig. 2). The analysis of CFE and colony size together suggests that the addition of 1% FBS inhibits clonal growth of both limbal and peripheral corneal cultures, but that the addition of 10% and 20% FBS stimulates clonal growth of the limbal cultures while it inhibits that of the peripheral corneal cultures.

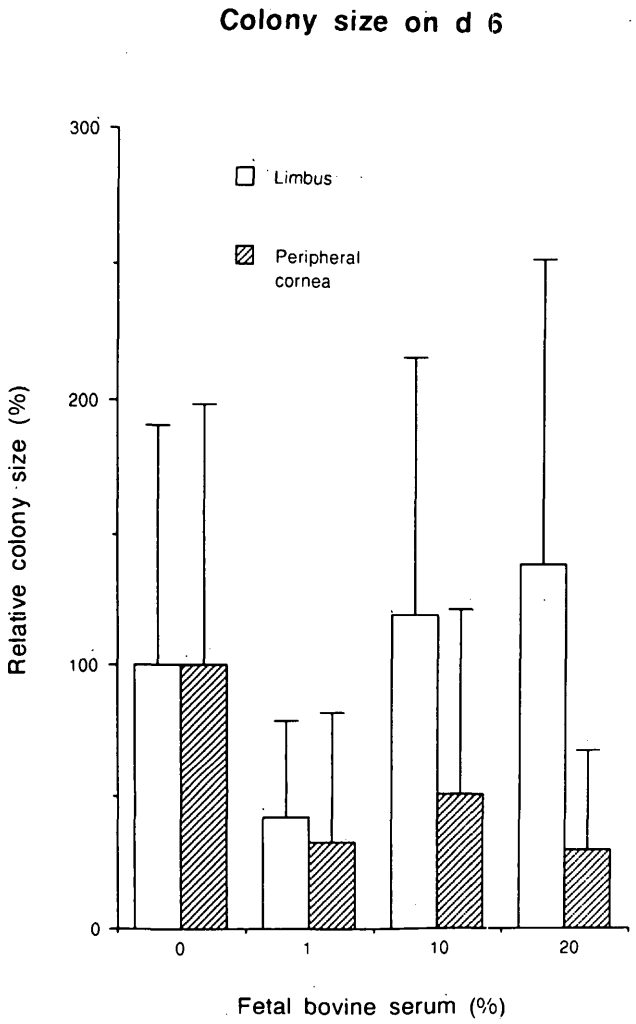

FIGURE 2. Colony size of limbal (open bars) and peripheral corneal (shaded bars) cultures on day 6. Colony size of each culture was determined as described in Materials and Methods. Compared to the control without fetal bovine serum (FBS), the relative colony size of cultures containing 1% FBS was reduced in both cultures. In contrast, the addition of 10% or 20% FBS increased colony size of limbal but decreased that of peripheral corneal cultures.

FBS Modified Colony Morphology in Early Limbal and Peripheral Corneal Cultures. On day 6, colony morphology of the serum-free control cultures was uniform and similar for both limbal and peripheral corneal epithelium, a finding consistent with our earlier findings.23 These colonies consisted of small oval to round cells that were more cohesive in the center but more migratory in the periphery of the colony (Figs. 3A, 3B). Among this cell monolayer were isolated areas of stratification (arrow in Fig. 3B). The addition of 1% FBS induced a more variable colony morphology in both limbal and peripheral corneal cultures. Approximately 70% of the colonies primarily consisted of medium to large squamous cells that were migratory and not cohesive (Fig. 3C). Serial follow-ups of these colonies confirmed that these cells, though largely separated, were indeed derived from a single attached cell and became separated because of migration. The remaining 30% of the colonies consisted of a mixture of medium to large squamous and small cells that were more cohesive with irregular profiles (Fig. 3D). In limbal cultures, the latter colonies contained 24 + 16 cells per colony and were larger than the former colonies, which contained 18 + 17 cells per colony. A similar result was obtained for peripheral corneal cultures with a colony size of 36 + 20 versus 11 + 7, respectively. Furthermore, the BrdU labeling index of the latter colony type was six times higher than that of the former ($P < 0.001$). These results indicate that 1% FBS induced a similar change of colony morphology in both limbal and peripheral corneal cultures, suggesting that the same subpopulation of cells in both cultures could have been affected by 1% FBS.

An increase of FBS to 10% or 20% further modified the colony morphology in both limbal and peripheral corneal cultures. There were two major types of colonies. The first, denoted as type A, was also similar to that observed in 1% FBS and consisted of a spectrum of colonies ranging from colonies containing large, noncohesive, squamous cells (Fig. 3E) to colonies containing more cohesive cells (Fig. 3F). The second, denoted as type B, was unique for these high serum-containing cultures and consisted of small, cohesive, nonmigratory cells. Among these type B colonies, some had irregular profiles (Fig. 3G) and others had regular round profiles with (Fig. 3H) or without (Fig. 3I) desquamating cells. The proportion and colony size of these two types of colonies were analyzed to determine if there was a difference between limbal and peripheral corneal cultures. As shown in Table 2, when 10% FBS was added, limbal cultures displayed a

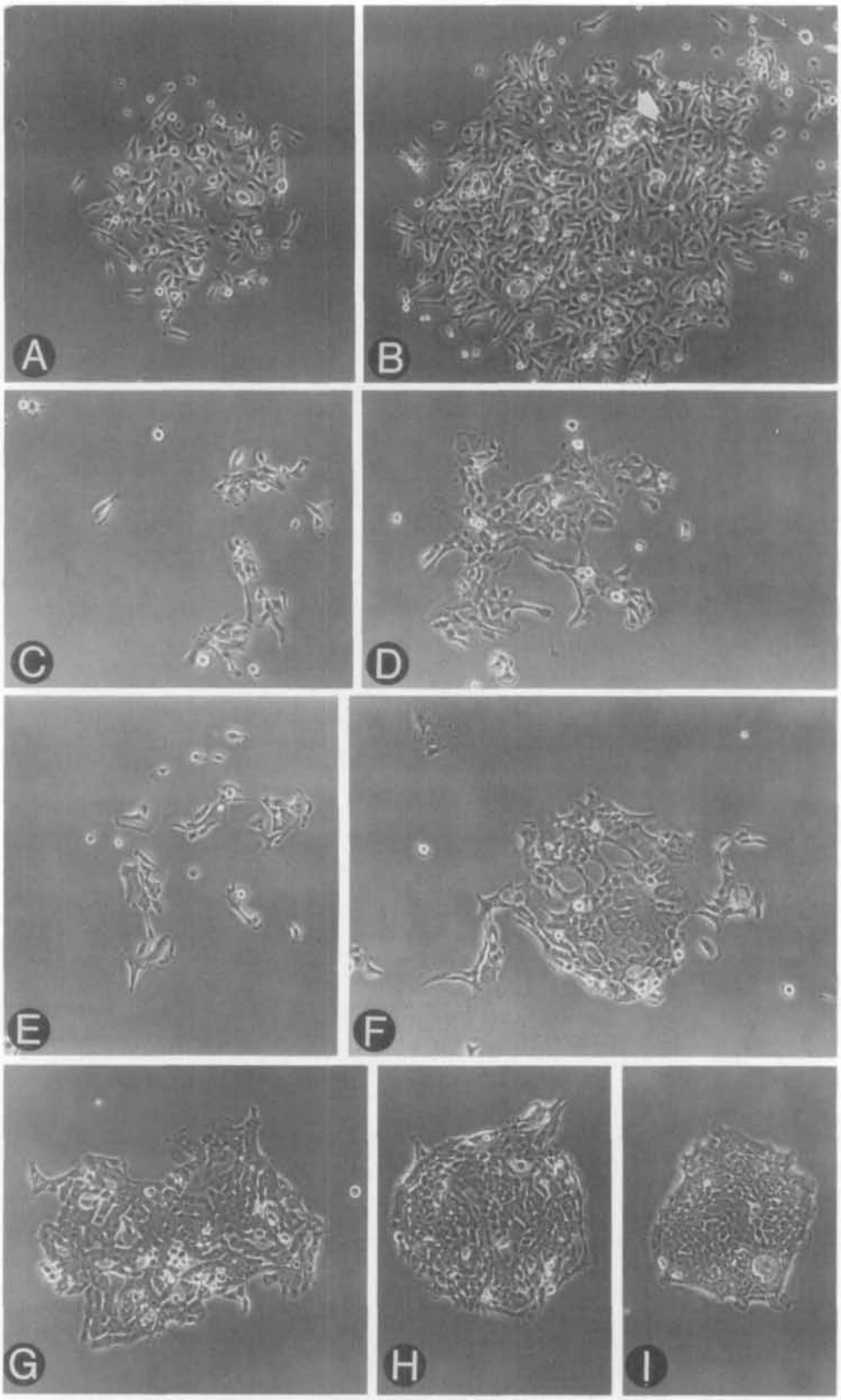

FIGURE 3. Representative phase-contrast micrographs of colony morphology on day 6. Compared to the control (**A,B**), which had a more uniform colony morphology with cells more cohesive in the center and more migratory in the periphery, the addition of 1% FBS resulted in more variable colony morphology, and the cells became more migratory and noncohesive (**C,D**). Addition of 10% or 20% FBS resulted in two major types of colonies, type A (**E,F**), similar to that of 1% FBS, and type B (G-I), consisting of small, cohesive, nonmigratory cells (arrow indicates the area of stratification).

TABLE 2. Proportion and Colony Size of the Two Colony Types in High Serum-Containing Limbal and Peripheral Corneal Cultures That Contain High Concentrations of Serum

	10% FBS		*20% FBS*	
Type	*A*	*B*	*A*	*B*
I. Number of colonies/dish				
Limbus	17 ± 5	29 ± 10	16 ± 4	27 ± 8
Peripheral cornea	14 ± 9	15 ± 9	13 ± 5	6 ± 5
II. Colony size				
Limbus	14 ± 9	80 ± 49	16 ± 9	91 ± 66
Peripheral cornea	15 ± 19	85 ± 85	21 ± 15	52 ± 43

Mean ± SD from three experiments with four dishes for each condition. Descriptions of the colony types A and B are given in the text and in the legend of Figure 3. For media and culture conditions, see "Methods."

higher proportion of the type B to the type A colonies than the peripheral corneal cultures. Primarily because of a selective decrease in the frequency of type B colonies in the peripheral corneal cultures, this proportion was even greater in the limbal cultures grown in 20% FBS (Table 2). Because the colony size of type B colonies was significantly larger than that of type A colonies in both cultures, this difference in the frequency of type B colonies inferred that the limbal cultures had higher numbers of cells. In addition, the limbal cultures contained 5 to 10 times more of the round form of type B colonies (Figs. 3H, 3I) than the peripheral corneal cultures. These data indicate that limbal cultures contain a subpopulation of progenitor cells that can be stimulated to form colonies with a distinctive morphology by higher concentrations of FBS.

To determine whether these changes of colony morphology also reflected changes in the proliferative capacity, the BrdU labeling index was measured on day 6. The labeling index makes an assertion about the percentage of labeled, presumably proliferating, cells from the total number of cells per colony and their distribution within the colonies. The type B colonies had a labeling index four times higher than the type A colonies ($P < 0.001$). Together with the colony size data, these results indicate that 10% and 20% FBS inhibit the proliferation of a subpopulation of cells but facilitate the proliferation of another subpopulation of cells in both limbal and peripheral corneal cultures. Furthermore, limbal cultures seem to contain a higher percentage of colonies with a high proliferative rate than cultures in low concentrations of FBS.

Serum Facilitated Continuous Clonal Proliferation of Limbal and Peripheral Corneal Cultures. On days 14 and 21, an increasing merger of neighboring colonies precluded a reliable measurement of CFE or colony size. To access clonal proliferation, dishes were stained with crystal violet, and the areas covered by macroscopically visible colonies were compared. On day 14, limbal cultures in serum-free medium showed significantly smaller cell-covered areas than peripheral corneal cultures (Fig. 4, first row, left two columns), a finding consistent with our earlier report.[23] Although the total number of colonies was lower in 1% FBS (Fig. 2), the total cell-covered area increased in limbal cultures but remained the same in peripheral corneal cultures (Fig. 4, second row, left two columns). As the concentration of FBS was further increased to 10% and 20% (Fig. 4, third and fourth rows, left two columns), colonies became larger and denser and also increased in number in the limbal cultures. Because the colonies of the peripheral corneal cultures also became larger in size but remained unchanged or smaller in number, the limbal cultures had a similar, if not larger, cell-covered area than the peripheral corneal cultures. This result indicates that colonies expanded faster in medium containing higher concentrations of FBS than in serum-free medium, and that the number of colonies increased in the limbal but decreased in the peripheral corneal cultures containing higher concentrations of FBS.

On day 21, limbal cultures in serum-free medium continued to proliferate and exhibited a macroscopic appearance similar to that on day 14. In contrast, peripheral corneal cultures started to degenerate and covered a smaller area of the dish than those on day 14 (Fig. 4, top row, right two columns). Both cultures in 1% FBS showed disrupted colonies, indicative of progressive degeneration (Fig. 4, second row, right two columns). On the contrary, colonies in both 10% and 20% FBS-containing cultures continued to proliferate into very large colonies (Fig. 4, bottom two rows, right two columns), indicating that factors present in FBS are crucial for continuous clonal proliferation.

To detect the progenitor colonies of these continuously proliferating colonies, we performed serial follow-ups of randomly selected colonies between day 6 and day 14 in cultures containing 20% FBS. Noncohe-

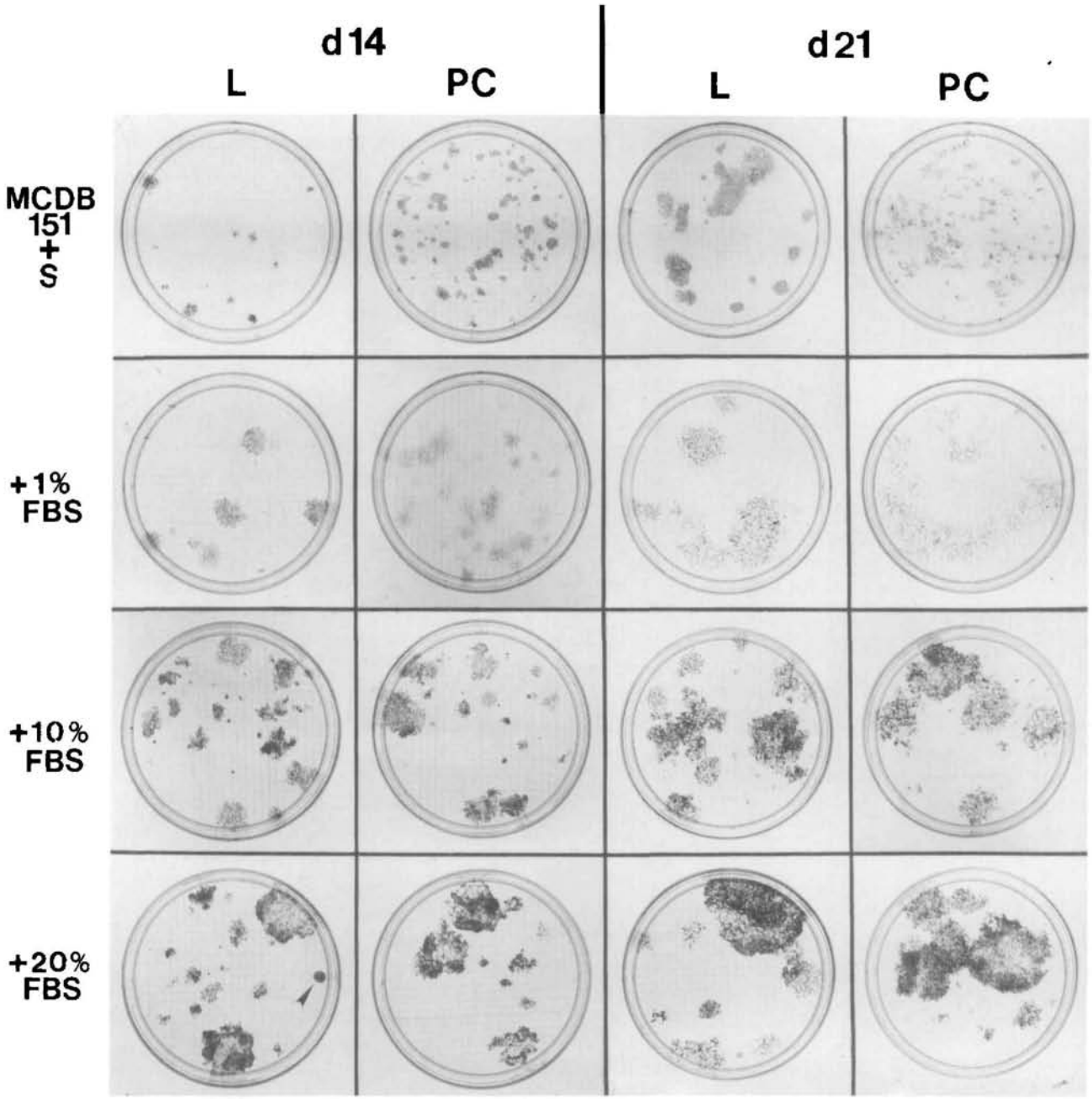

FIGURE 4. Composite of cultures containing no FBS (MCDB 151 + 9), or 1%, 10%, and 20% FBS, stained with crystal violet on day 14 (left panel) and on day 21 (right panel). In each panel, the left row represents limbal (L) cultures, and the right row represents peripheral corneal (PC) cultures (arrows indicate small type B colonies).

sive type A colonies (Fig. 3E) always degenerated and failed to proliferate, but cohesive type A (Fig. 3F) and all type B colonies (Figs. 3G, 3I), on the contrary, continued to proliferate. Because some of the latter types of colonies could also slow down their proliferation, resulting in small colonies on day 14 (Fig. 4, left lower corner, arrow), the capacity for continuous proliferation seemed not to be directly correlated to the colony morphology on day 6. This might explain why the limbal cultures, which on day 6 contained a higher proportion of type B colonies, on day 14 covered an area of the dish similar to that of the peripheral corneal cultures.

To investigate further how FBS modulated the number of proliferating cells that could be subcultured for clonal growth, cultures were grown in the

presence or absence of 20% FBS at a seeding density of 180 cells/cm^2. On day 14 of the primary culture, cells were passaged at a density of 18 cells/cm^2 into medium with or without 20% FBS. Six days after passage into the serum-free medium, the limbal cultures had a CFE of 0.5 + 0.5% and colony size of 6 + 4 cells per colony when they were initially grown in serum-free medium, but a CFE of 24 + 9% and colony size of 42 + 55 cells per colony when they were initially grown in 20% FBS. Such a difference could also be visualized on day 14 by crystal violet staining of the dishes. As shown in Figure 5, cultures initially grown in serum-free medium showed no macroscopically visible colonies (Fig. 5A), but cultures initially grown in 20% FBS showed some colonies (Fig. 5B) 14 days after passage into serum-free medium. Similar results were obtained from the peripheral corneal cultures (data not shown). This result indicates that initial exposure to 20% FBS had preserved the clonogenic potential during subculture in the serum-free medium. For a comparison, cultures initially grown in serum-free medium showed only a few visible dense colonies (Fig. 5C), but cultures initially grown in 20% FBS showed no visible colonies (Fig. 5D) 14 days after passage into 20% FBS-containing medium. This result indicates that subsequent exposure of 20% FBS during subculture stimulates the clonogenic potential that is originally preserved in the serum-free medium but inhibits that of progenitor cells preserved in 20% FBS.

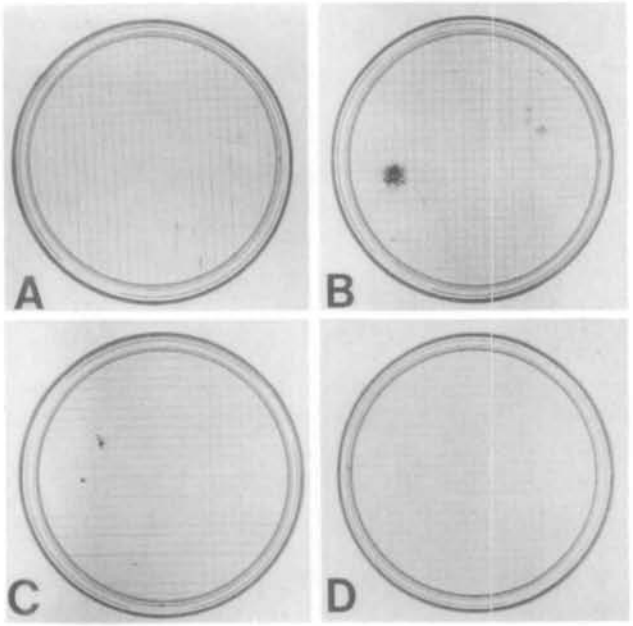

FIGURE 5. Composite of crystal violet-stained cultures on day 14 in serum-free (MCDB 151 + S) medium (**A,B**) and 20% FBS-containing medium (**C,D**). These cultures were passaged at a density of 18 cells/cm^2 from the primary culture seeded at 180 cells/cm^2 grown in serum-free (**A,C**) or 20% FBS-containing (**B,D**) medium.

Serum Modified Epithelial Differentiation in Limbal and Peripheral Corneal Cultures. To determine if the extent of cellular differentiation was affected by the addition of serum, we studied the expression of keratin K3, which is related to cornea-type differentiation,[11] by immunofluorescence staining with the monoclonal antibody AE-5. There was a spectrum of nonuniform staining patterns among colonies from the serum-free control cultures for both limbal and peripheral corneal epithelium. The majority of the colonies showed a similar heterogeneous staining pattern with predominantly weakly positive basal cells and occasional strongly positive cells that were often suprabasally located (Fig. 6A, arrow). The remaining colonies, however, were either entirely negative or stained strongly positive with AE-5. In contrast, colonies in cultures containing 1% FBS were more uniform and cells stained homogeneously were strongly positive, although they were not suprabasally located (Fig. 6B). Type A and B colonies, which were derived from 10% or 20% FBS-containing cultures, were nonuniform again and could be either homogeneously strongly positive (Fig. 6C), almost entirely negative (Fig. 6D), or heterogeneously positive (Fig. 6E). Although immunofluorescence is not quantitative, the increase of AE-5 staining intensity suggests that colonies in cultures containing 1% FBS were more differentiated and that 10% or 20% FBS also induces some less-differentiated colonies.

To examine whether the change in the keratin K3 expression could be correlated with the proliferative capacity, we performed double labeling with AE-5 and anti-BrdU antibody. The result shows that predominantly AE-5-negative areas of the colonies contained relatively more BrdU-positive nuclei than areas that stained strongly positive with AE-5 (see Figs. 6E, 6F). However, areas of strongly positive keratin staining were not completely devoid of BrdU-positive nuclei (Fig. 6F). Therefore, colonies with weak AE-5 staining tended to have a higher percentage of proliferating cells than those with strong staining.

DISCUSSION

We recently developed a serum-free chemically defined clonal culture system that allows us to investigate factors that can modulate the clonal growth and differentiation of single progenitor cells from limbal and peripheral corneal epithelium.[23] In this report, we noted that addition of FBS at low (1%) and high (10% or 20%) concentrations has different modulating effects on the proliferation and differentiation of the limbal and peripheral corneal epithelium. Addition of 1% FBS induced similar colony morphology (Figs. 3C, 3D), which showed a uniformly stronger AE-5 straining for both limbal and peripheral corneal cultures

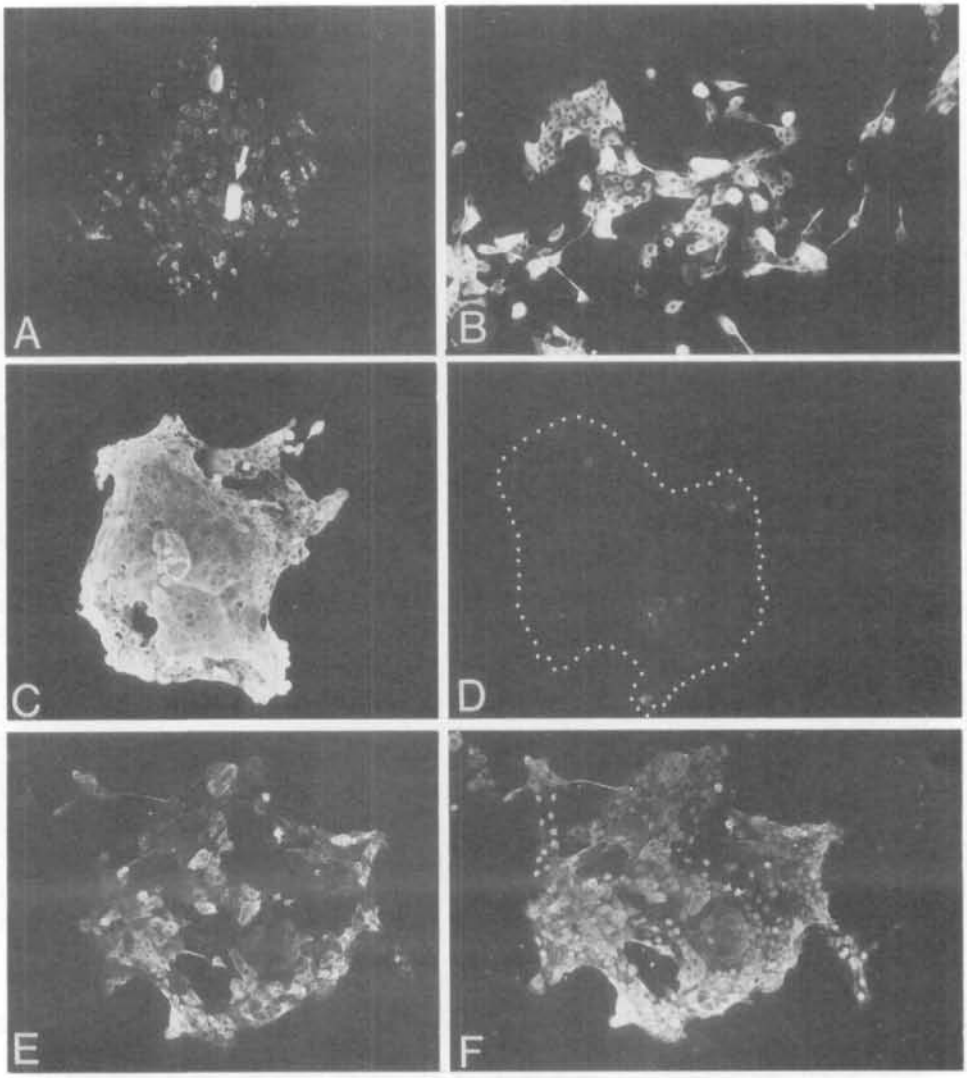

FIGURE 6. Immunofluorescence micrographs of serum-free control colonies (**A**) stained with AE-5 showed the characteristic weakly positive basal cells and some strongly positive suprabasal cells (arrow). In contrast, the addition of 1% FBS resulted in colonies with homogeneously strongly positive cells (**B**). The type B colonies derived from 10% or 20% FBS-containing cultures were either strongly positive (**C**), almost entirely negative (**D**), or heterogeneously positive (**E**). When AE-5 staining was double-labeled with anti-BrdU antibody, the predominantly negative AE-5 areas of the colonies contained relatively more BrdU-positive nuclei (cf. **E,F**) than predominantly AE-5 positive areas.

(Fig. 6B), indicating that the differentiation of a common subpopulation of progenitor cells in both cultures is promoted. At this low concentration, the efficiency of cellular recruitment for clonal proliferation (clonogenicity) was not affected in limbal cultures, as evidenced by the unchanged CFE (Fig. 1) and cell attachment rate (Table 1). In peripheral corneal cultures, the reduced CFE could be explained by the reduced cell attachment rate (Fig. 1 and Table 1). These results lead us to speculate that 1% FBS does not significantly alter the clonogenicity of both cultures. These data, however, differ from those of colony size, which were significantly reduced by the addition of 1% FBS in both limbal and peripheral corneal cultures (Fig. 2), indicating that the recruited progenitor cells cannot continuously proliferate at the same rate as those of the control cultures. These results suggest that a low (1%) concentration of FBS, in general, inhibits proliferation and stimulates differentiation.

In contrast, addition of high (10% or 20%) FBS stimulated the proliferation of progenitor cells from the limbal epithelium but dose-dependently inhibited the proliferation of those cells from the peripheral (and central) corneal epithelium. This was evidenced

by the increase of CFE and colony size in limbal cultures and their decrease in peripheral corneal cultures (Fig. 1 and 3). Furthermore, there was a higher proportion of type B colonies to type A colonies in limbal compared to peripheral corneal cultures (Table 2). In contrast to type A colonies, the type B colonies had a higher BrdU labeling index, indicative of a high proliferative capacity, and heterogeneous or negative AE-5 staining, indicative of a less differentiated state. The increased proliferative capability of limbal cultures could still be observed when the cultures were prolonged from an early (day 6) to a late (day 14 to 21) stage (Fig. 4). As reported previously,[23] this serum-free culture system primarily supports the clonal proliferation of transient amplifying cells. Therefore, stem cells, if present but not activated to be converted to transient amplifying cells, will not manifest their clonal proliferation. That is why the serum-free controls of peripheral corneal cultures showed a much higher clonal proliferation ([23], as well as this report). Because type B colonies were found in both limbal and peripheral corneal cultures, it is likely that they were derived from the proliferation of transient amplifying cells and not stem cells. The addition of high concentrations of FBS reversed this trend, with a higher proportion of proliferating type B colonies in limbal cultures, indicating that there is a unique subpopulation of progenitor cells (presumably stem cells) in limbal cultures that have differentiated into early transient amplifying cells that then exhibit type B colony growth.

From this study, it is also interesting to note that both limbal and peripheral corneal cultures contain similar type B colony growth. As stated above, type B colonies might represent the clonal growth of an early stage of transient amplifying cells. This result suggests that the peripheral corneal epithelium also contains these early transient amplifying cells, of which the number are less than that of the limbal epithelium. Therefore, such a distinction made by the addition of serum factors in the current culture system does not exhibit in an all-or-none fashion. This modified view on the concept of limbal stem cells is also revealed in our recent finding in their differential responses to a prolonged phorbol ester tumor promoter treatment (Kruse and Tseng, in press). Recently, using subconjunctival injection of 5-fluorouracil to eliminate short-cycling cells, we also observed that some long-cycling cells were present in the corneal epithelium though the number was less than that of the limbal epithelium.[27] Taken together, these data suggest that the distinction between stem cells and transient amplifying cells may not commence abruptly at the border between the limbus and the peripheral cornea.

Based on the current model for progenitor cells in the ocular surface epithelia (described in the introduction), our data further indicate that the clonal proliferation of some transient amplifying cells of corneal and limbal epithelium are inhibited by both low and high concentrations of serum factors, and that serum-derived factors promote cellular differentiation coupled with proliferation. Serum contains a number of complex factors that can inhibit proliferation,[28] promote differentiation,[29] and cause irreversible growth arrest.[30] Attempts to isolate these factors have shown that certain fractions of serum completely inhibit the growth of keratinocytes.[31] Among these are alpha-globulins,[32] e.g., fetuin; high-density lipoprotein;[33] factors released by platelets,[34] e.g., transforming growth factor beta (TGFβ);[33,35] and extracellular calcium concentration.[33] For normal epithelial cells, TGFβ inhibits proliferation and modulates differentiation.[36–39] In the limbal and peripheral corneal epithelia, we recently reported that TGFβ at a concentration between 1 and 5 ng/ml inhibits proliferation in the current culture system.[40] Addition of TGFβ to the medium inhibits EGF-stimulated proliferation in serum-free clonal cultures of human and murine keratinocytes[36,38,39,41] or human and rabbit tracheal epithelia,[37] and in serum-containing clonal cultures of murine keratinocytes,[42] or in rat lingual epithelium.[43] Because the mitogenic effect of EGF, which is also present in this culture system, can be modified by TGFβ[36–38,39,41–44] and because serum contains TGFβ[35] and vitamin A,[44] both of which can induce autocrine and paracrine production of TGFβ,[45,46] one can imagine that the addition of even a small amount of serum to culture medium can inhibit the proliferation, a finding that has also been observed by others.[47–49]

Addition of FBS decreased the cell attachment rate of peripheral corneal culture but did not change that of limbal cultures (Table 1). This finding is not contradictory to that reported by others showing that serum fibronectin promotes cell attachment because cell attachment is a complex process mediated by integrin and nonintegrin mechanisms. For stratified epithelia, the basal epithelial cells use integrin $\alpha6\beta4$ to attach laminin[50] and $\alpha3\beta1$ or $\alpha5\beta1$ to fibronectin. Because the latter fibronectin receptors are absent in freshly isolated keratinocytes and are only expressed after several days in culture,[51] it is unlikely that the addition of serum will promote the attachment of basal (progenitor) cells, especially in this low-density culture system. Because of the serum-derived differentiating influence (most likely via TGFβ), the rapidly differentiated progenitor cells actually lost the cell attachment capacity. This notion is supported by a recent report showing that the expression of $\alpha5\beta1$ integrin by keratinocytes decreased, together with the loss of cell attachment, when terminal differentiation is induced by a phorbol ester tumor promoter.[52] Our finding showing that cell attachment was preferentially de-

creased in peripheral corneal cultures supports the viewpoint that those transient amplifying cells initially isolated from the corneal epithelium are more differentiated than those from the limbal epithelium and thus are more susceptible to these putative serum-derived differentiating factors. Compared to these late transient amplifying cells, the cell attachment of early transient amplifying cells just converted from limbal stem cells is not as much affected.

In an effort to search for the putative factor(s) in serum responsible for the observed conversion of stem cells to transient amplifying cells and the progressive differentiation of transient amplifying cells, we have recently noted that the addition of retinoic acid to this serum-free culture system can mimic the effect of the addition of serum.[53] Specifically, the addition of 10^{-9} M to 10^{-7} M retinoic acid selectively stimulated CFE of the limbal epithelium but dose dependently inhibited clonal proliferation of transient amplifying cells from peripheral corneal epithelium. Furthermore, the addition of retinoic acid prevented abnormal terminal differentiation, as exemplified by the disappearance of cornified envelope expression, a finding that could also be noted by the addition of serum. We thus speculate that the vascular source of vitamin A might play a major role in modulating the proliferation of limbal basal epithelium, a region known to contain the stem cell population.

As described previously, serum contains a number of complex factors and, aside from an inhibitor such as TGFβ, serum also contains several stimulators (or mitogens) for epithelial growth. Therefore, it is conceivable that the modulating effect of serum noted in this study cannot be explained by TGFβ alone, especially when the serum concentration is raised from 1% to 10% or 20%. This notion is supported not only by the previously mentioned differential promotion of limbal stem cell proliferation but also by the change of AE-5 staining from a uniformly strong staining seen in cultures containing 1% FBS to a negative staining seen in some type B colonies in cultures containing 20% FBS (Fig. 6). This negative staining pattern is not consistent with the effect of increasing TGFβ concentrations. We have recently discovered that the addition of increasing concentrations of TGFβ to this culture system invariably stimulated AE-5 expression, indicative of enhanced epithelial differentiation. Possibly because cellular differentiation is relatively inhibited rather than promoted by high concentrations of FBS, clonal growth can thus be preserved upon subpassage into serum-free medium (Fig. 5). Future studies are needed to substantiate if retinoic acid (or vitamin A) is the sole factor for activating stem cell differentiation into transient amplifying cells, to resolve if the action of retinoic acid can also be exerted via other growth-modifying effects than to induce the autocrine production of TGFβ, and to investigate the interactions between TGFβ and other growth-promoting factors in modulating the clonal proliferation of limbal and corneal progenitor cells.

Key Words

clonal growth, corneal epithelium, limbus corneae, serum, stem cells

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