The Effects of Fibroblasts on the Growth and Differentiation of Human Bulbar Conjunctival Epithelial Cells in an In Vitro Conjunctival Equivalent

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Purpose. To study the effects of collagen matrix and fibroblasts on the growth and development of human bulbar conjunctival epithelial cells.

Method. Human bulbar conjunctival epithelial cells were cultured on three-dimensional collagen gels containing either normal human conjunctival fibroblasts (HCF), Swiss 3T3 cells, or no cells. After 1 week of culturing, half of the cultures were raised to the air–liquid interface and the rest of the cultures remained submerged. On day 14, cultures were fixed and sectioned for light and electron microscopic studies.

Results. Conjunctival epithelial cells cultured on fibroblast-contracted collagen lattice developed into a multicell-layer epithelium with characteristic epithelial structural features including microvilli, desmosomes, early hemidesmosomes, and basement membrane-like structures. Formation of all or some of the above features appeared to be influenced by the type of fibroblasts in the collagen lattices. Structures such as hemidesmosomes and basement membrane were only observed in epithelium developed on 3T3- but not on conjunctival fibroblast-condensed collagen lattices. In contrast, goblet cell differentiation was only observed in epithelia developed on normal HCF-supported collagen matrix. Epithelial cells cultured on acellular collagen gels did not develop into multicell-layer epithelium, and no differentiated characteristics were observed.

Conclusions. These results indicate that the type of fibroblasts dispersed in the collagen matrix plays an important role in the development and differentiation of conjunctival epithelial cells. Normal HCF-dispersed collagen matrix was less growth stimulating to epithelial cells and allowed them to undergo goblet cell differentiation. In contrast, 3T3-dispersed collagen matrix was more growth stimulating, resulting in thicker epithelium with a higher degree of stratification. Invest Ophthalmol Vis Sci. 1994;35:2865–2875.

Understanding the growth, development, differentiation, and maturation of the conjunctival epithelium is critical to understanding the pathogenesis of ocular surface disorders, such as ocular cicatricial pemphigoid, Stevens-Johnson syndrome, alkaline burn, and drug-induced pseudoepitheliomatous.

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odacyl acid-Schiff (PAS)-positive goblet cells were present in cytospin preparations of the dispase II-digested conjunctival epithelium but were lost upon subsequent culturing on plastic. We further showed that replating of such PAS-negative cells on natural substrates resulted in the reappearance of mucin-expressing cells, indicating that the precursor cells were actually present in conjunctival epithelial cells cultured on the plastic surface.10

Light and electron microscopic studies of the cultured rabbit conjunctival epithelia8 indicated that the cells were squamoid on plastic or glass but were changed to a monolayer sheet on collagen gel and adopted a well-organized globular growth pattern on matrigel. Furthermore, the latter two culture systems allowed the differentiation of cell polarity, tight junction formation, and early development of the basement membrane.8 On matrigel, the cells underwent a different morphogenesis to form a multicellular globule instead of a simple monolayer sheet. Due to the globule formation, the apical surface was not in contact with the medium directly, instead, it projected into the center of the multicellular globule. Obviously, the formation of globular structure on matrigel does not represent a close mimicry of the in vivo development. Although these models have provided much useful information in the study of the growth and differentiation of conjunctival epithelial cells, they were still limited by their two dimensional characteristics.

Critical to the continued existence of all multicellular "tissues" is their ability to continue inter-regulations by their constituent cells. Cells in tissues exist in association with a three-dimensional meshwork of extracellular matrix. Thus, fibroblasts and epithelial cells cultured in and on collagen gels, respectively, exhibited a morphology that differed from those cultured on plastic culture surface.11,12 To understand the effects of the chemical composition of the collagen matrix and the fibroblasts on the growth and differentiation of the conjunctival epithelial cells, it is necessary to develop an in vitro living conjunctival equivalent. By plating isolated human conjunctival epithelial cells on collagen gels containing either normal HCF, Swiss 3T3 cells, or no cells, we were able to reconstruct such living equivalents. Using this methodology, one would be able to alter the collagen matrix composition and the type of fibroblasts incorporated in the collagen matrix and to compare the epithelia thus developed. In this study, we showed that fibroblasts in the collagen matrix portion of the living conjunctival equivalents play important roles in the regulation of the growth and differentiation of the conjunctival epithelial cells and that PAS- and Alcian blue-positive cells were only observed in epithelia developed on normal conjunctival, fibroblast-dispersed collagen gels but not on 3T3 cell-dispersed and acellular collagen gels.

**MATERIALS AND METHODS**

Dulbecco's modified Eagles medium (DMEM), Ham's F12, trypsin-EDTA, calcium- and magnesium-free saline (CMFS), and fetal bovine serum were obtained from Gibco (Grand Island, NY). Dimethylsulfoxide (DMSO) (95%), bovine insulin, hyaluronic acid (HA), and cholera toxin were from Sigma Chemical Company (St. Louis, MO). Mouse epidermal growth factor (EGF, receptor grade) was from Collaborative Research (Boston, MA). Dispase II was a product of Boehringer-Mannheim (Indianapolis, IN). Modified M-K medium was from Chiron Ophthalmics (Irvine, CA). Tissue culture plastic plates (24-well) were from Linbro Flow Laboratories, Inc. (McLean, VA). Other tissue culture wares were obtained from Corning (Corning, NY). Type I collagen was prepared from rat tail tendons by acid extraction and salt precipitation and was finally dissolved in 0.1% acetic acid at concentrations ranging from 1 to 3 mg/ml.

Methods for securing human cadaver conjunctival tissue were humane, included proper consent and approval, and complied with the ARVO Statement on the Use of Animals in Research and the guidelines of the Declaration of Helsinki in Biomedical Research Involving Human Subjects.

**Isolation of Epithelial Cells**

Human conjunctival epithelial sheets were procured from cadaver eyes through the arrangement of the regional organ–tissue procurement organization. Procedures for conjunctival sheet removal from the cadaver and the isolation of conjunctival epithelial cells were as previously described with minor modifications.5 Briefly, the lid and surrounding eyelashes were sterilized with betadine. The two bulbar conjunctival sheets of both eyes were separated from 3 o'clock and 9 o'clock positions and cut from the area 2 mm from the limbus to the upper and lower fornices under sterile conditions. The conjunctival sheet was stored in the modified M–K medium in 4°C until experiment. During the experiment, the conjunctival sheet was rinsed 3 times with CMFS, secured onto a sterile paraffin-layered 60 mm dish, and subjected to dispase II (1.0 U/ml in CMFS) treatment at 37°C for 3 hours under 5% CO2 and 95% air. The conjunctival epithelial cells were dispersed from the surface by gentle pipetting several times with CMFS. Cells were finally collected by centrifugation at 800g for 5 minutes.

**Cell Culture**

Human bulbar conjunctival epithelial (HCE) cells were routinely cultured in DMEM/Ham’s F12 (1:1 by volume, 20 mM Hepes buffered), supplemented with 0.5% DMSO, 2 ng/ml mouse EGF, 1 µg/ml bovine...
insulin, 0.1 \( \mu \text{g/ml} \) cholera toxin, and 5% fetal bovine serum (SHEM 5% FBS). For primary culture, human conjunctival epithelial cells were plated at a density of \( 1.25 \times 10^6 \) per \( \text{cm}^2 \). Twenty-four hours after plating, the medium was replaced and was thereafter replaced every 4 to 5 days. Cultures were incubated in a humidified incubator under 95% air/5% \( \text{CO}_2 \). Cells from donor subjects younger than 35 years of age and same-cell strains were used in each set of the comparative studies presented. Human conjunctival fibroblasts (HCF) were isolated from conjunctival tissue explants on type 1 collagen-coated dishes in medium DMEM/F12 (1:1 by vol) supplemented with 10% FBS. HCF at passages 3 to 10 were used for the experiments. Swiss 3T3 cells were obtained from American Type Culture Collection and were cultured in the same medium as that of conjunctival fibroblasts.

**Construction of the Conjunctival Equivalent**

The collagen matrix portion of the conjunctival equivalent was constructed with type 1 collagen either acellularly or dispersed with conjunctival fibroblasts or Swiss 3T3 cells. Unless otherwise stated, all the solutions used were prechilled on ice. Construction of the collagen matrix was performed by mixing 3 volumes of type 1 collagen solution (1.5 mg/ml in 0.1% acetic acid, neutralized with the appropriate amount of 5N NaOH right before use) with 1 volume of 4X DMEM medium containing HA and 1 volume of IX DMEM medium alone or 1 volume of a single-cell suspension (fibroblasts or 3T3 cells) in the same medium. The final concentration of HA was 0.05% and the fibroblasts were 1.5 \( \times 10^6 \) per ml of collagen gel. The mixture was poured into 35 mm culture dishes at 3 ml/dish or 24-well tissue culture plates at 0.5 ml/well. Cultures were incubated at 37°C in a humidified incubator under 95% air/5% \( \text{CO}_2 \), where they gelled in a few minutes. The 4X DMEM medium used was supplemented with appropriate concentrations of DMSO, EGF, insulin, and cholera toxin so that the concentration of each supplement in the final mixture equaled to those in the SHEM medium.

**Preparation of Conjunctival Equivalent**

Single-cell suspension of the conjunctival epithelial cells were prepared in SHEM medium as described. The cells were seeded at 1.25 \( \times 10^4/\text{cm}^2 \) on the fibroblast-dispersed collagen matrix in 2 ml of medium per 35 mm dish and 1 ml of medium per well. Immediately after seeding, FBS was added to a final concentration of 5%. Seven days after epithelial cell seeding, half the conjunctival equivalents from each experimental group were removed from the submerged condition and were raised to the air–liquid interface on plastic grids. The remaining cultures stayed submerged throughout the entire experimental period. Cultures were observed daily with an Olympus (Olympus, Tokyo, Japan) inverted microscope and were photographed when necessary.

**Histology**

The specimens were subjected to both light microscopic and electron microscopic studies. For light microscopic studies, specimens were fixed in PBS buffered formalin (4%, pH 7.4) for 24 hours at room temperature and were then embedded in paraffin. Sections of 4 \( \mu \text{m} \) were cut and stained with hematoxylin and eosin HE, PAS, or Alcian blue. The sections were observed and photographed with a Zeiss Axioshot (Carl Zeiss, Oberkochen, Germany).

For electron microscopic study, the specimens were fixed in 4% glutaraldehyde, postfixed in 2% osmium tetroxide, dehydrated in graded alcohols, and embedded in epon. Ultrathin sections were examined with the Jeol-1200 CX transmission electron microscope (Jeol, Tokyo, Japan) for the presence of characteristic structures of a conjunctival tissue.

**RESULTS**

**Cell Isolation and Yield**

HCE cells used for the present study were isolated from the cadavers of persons 2 to 61 years old (29 ± 17.23 [mean ± SD], N = 21) with a mean cell yield per conjunctiva of 6.83 \( \times 10^6 \) cells (±SD 2.46 \( \times 10^6 \)). The interval of cadaver death to experiment (D-E interval) ranged from 12 hours to 7 days, with a mean interval of 2.02 days (±SD 1.64). The cell yield and viability from per case was not related to age, nor was it to the D-E interval when the preservation period in modified M-K medium was within 7 days.

**Attachment and Growth of HCE Plated on Collagen Gels With or Without Fibroblasts**

When the HCE cells were cultivated on acellular collagen gels, their attachment was noted between 2 to 4 hours after plating, which was similar to the rabbit conjunctival epithelial cells. The next day, the cells became flattened and were epitheloid. At the seeding density of 1.25 \( \times 10^6 \) per \( \text{cm}^2 \), the HCE cells required 2 weeks or longer to become confluent. The proliferative ability of the HCE cells appeared to be inversely correlated with the ages of the donor subjects (not shown).

Two to four hours after plating on the fibroblast-suspended collagen gels, the HCE cells started attachment and some became spindle shaped. After 24 hours of culture, cells were well spread and formed many colonies. Due to the dispersed fibroblasts, the lattices were contracted and detached from the edge of culture dish on day 3. On days 4 to 5, the fibroblasts were
FIGURE 1. Phase-contrast micrographs of human conjunctival epithelial cell cultures. Cells were grown on 3T3-dispersed collagen gels (a), human conjunctival fibroblast-dispersed collagen gels (b), and acellular collagen gels (c). Epithelial sheet formed on stroma containing 3T3 cells (a) appeared to be thicker and more widely spread than that formed on stroma containing normal human conjunctival fibroblasts (b). HCE cells on acellular collagen gels grew poorly and formed few patch-like colonies (c). Cultures were photographed on day 3.

observed in the collagen lattice with very high density. HCE cells formed organized sheets over the fibroblast-contracted collagen lattice, but the epithelial cell layer was not yet confluent. The epithelial layer formed on collagen matrix containing 3T3 cells (Fig. 1a) appeared to be thicker and larger than that formed on collagen matrix containing normal HCF (Fig. 1b). HCE cells on acellular collagen gels grew poorly and formed few patch-like colonies. (Fig. 1c).

During the first week of the experiment, the cultures were submerged in the medium to allow HCE to form a confluent monolayer sheet. The diameter of the fibroblast-dispersed collagen gels contracted from 5 cm to about 2.5 cm during this period. Of note, the extent of the collagen gel contraction was not obviously influenced by the type of cells dispersed, whether normal fibroblasts or 3T3. Instead, the number of cells originally dispersed appeared to be the major determinant for gel contraction. On day 7, half the cultures were raised to the air—liquid interface on plastic grids and were photographed. The culture conditions of the remaining half were not changed.

FIGURE 2. (top). Photomicrograph of a flat-mount preparation of the equivalent. A 15-day-old conjunctival equivalent was stained with PAS and Papanicolaou's procedures. Areas of fibroblasts suspended collagen-lattice not covered by HCE cells was stained in green. In these areas, fibroblasts in the reorganized collagen lattices were clearly seen (arrows). Areas of collagen lattice that were covered by HCE sheet was stained in purple.

FIGURE 4. (bottom right). Conjunctival epithelia developed on HCF-dispersed collagen-matrix with air—liquid interface exposure were sectioned and stained with PAS (top panel) and Alcian blue (bottom panel) to look for the possible presence of the goblet cell. There were PAS and Alcian blue positive cells (arrows) in our in vitro developed conjunctival epithelia, suggesting the presence of goblet cell differentiation.
A flat-mount preparation of a 15-day-old conjunctival equivalent stained with PAS and Papanicolaou's procedures is shown in Figure 2. Areas of fibroblast-suspended collagen matrix not covered by HCE cells was stained in green. In these areas, fibro-
blasts in the reorganized collagen lattice were clearly seen. Areas of collagen lattice that were covered by HCE sheet was stained in purple. Phase-contrast microscopic observations of the air–liquid interface-exposed samples indicated that 5 to 7 days were required for the stratification of the epithelium to occur and HCE sheet developed on 3T3-suspended collagen matrix appeared to stratify to a greater extent than those developed on conjunctival fibroblast-suspended collagen matrix.

Effect of Fibroblasts and Air–Liquid Interface Exposure on the Development of Epithelium

That the development and differentiation of HCE cells were influenced by the type of fibroblasts dispersed in the collagen matrix portion of the reconstruction was further confirmed by microscopic examination of the cross-sections (Fig. 3). Examination of the cross-sections prepared from 2-week-old submerged specimens showed that the epithelium developed on conjunctival fibroblast-dispersed collagen matrix was mostly a single-cell sheet with occasional 2-cell layer patches (Fig. 3a). In contrast, HCE cells on 3T3-dispersed collagen matrix developed into a 7- to 10-cell layer epithelium and stratification was evident (Fig. 3c). Epithelia formed under air–liquid interface exposure conditions (1 week submerged followed by 1 week air–liquid interface) were 3- to 4-cell layers (Fig. 3b) and 10- to 15-cell layers (Fig. 3d) thick for HCF-dispersed collagen matrix and 3T3-dispersed collagen matrix, respectively. Stratification of the 3T3-dispersed collagen matrix-supported epithelia with and without air–liquid interface exposure was not obviously different.

Goblet Cell Differentiation

 Conjunctival epithelia developed on HCF-dispersed collagen gels with air–liquid interface exposure were sectioned and stained with PAS and Alcian blue to look for the possible presence of goblet cell differentiation. Figure 4 shows that there were PAS and Alcian blue positive cells in our in vitro developed conjunctival epithelia, suggesting the presence of such differentiation. However, immunohistostaining with anti-mucin antibody is required to unequivocally confirm this result. In contrast, epithelia developed on 3T3-dispersed collagen matrix contained no PAS- and Alcian blue-positive cells.

Ultrastructural Features of the Epithelium

 The ultrastructures of the epithelia developed on HCF- and Swiss 3T3-suspended collagen matrix with and without exposure to air–liquid interface were compared. HCF-dispersed collagen matrix supported epithelium maintained in the submerged conditions is characterized by poorly developed superficial cell stratification, occasional microvilli, and large intercellular spaces. There were no cell–cell membrane interdigitations; however, few desmosomes were present (Fig. 5a). In air–liquid interface exposed samples, the surface cell layers were better stratified, and microvilli were more abundant. Although intercellular spaces were large, complex cell–cell membrane interdigitations and desmosomes were evident (Figs. 5b and 5c). However, hemidesmosomes and basement membranes at epithelium–collagen matrix junctions were not developed.

 Better-developed epithelial structure features were seen in epithelium developed on 3T3-dispersed collagen matrix under submerged conditions (Fig. 6). The epithelium may be divided into three different strata. In the surface layer, composed of 3- to 4-cell layers, cell stratification, microvilli, and desmosomes were all present (Fig. 6a). Cells in the basal layer were characterized with the presence of distinctive intercellular membrane interdigitations, desmosomes, and well-defined intracellular organelles. The four to five suprabasal cell layers appeared to be metabolically less active than the cells of the basal layers, as judged from the absence of Golgi apparatus and vesicles. The intercellular space of this layer is tighter than superficial layers. Rudimentary forms of basement membrane were present at the basal cell–collagen matrix junction, which is indicated by the presence of focal condensation of electron-dense ground substances (Fig. 6b). The best-developed epithelial structural features in our culture system were seen in epithelium developed on 3T3-dispersed collagen matrix with air–liquid interface exposure. It is characterized by abundant microvilli (Fig. 7a) and complex cell–cell membrane interdigitations with abundant, well-developed desmosomes (Fig. 7b). A relatively well-developed and discontinuous basal lamina-like structure with hemidesmosomes was present at basal cell–collagen matrix junction (Figs. 7c and 7d).

DISCUSSION

In this study, we isolated bulbal conjunctival epithelial cells from 21 donor subjects, and we found that cell yield and viability per case did not relate to age of the donor subjects, nor did it relate to the D-E interval when the preservation period in modified M-K medium was within 7 days. However, the proliferative ability of the HCE was inversely correlated with the ages of the donor subjects. We, therefore, used cells isolated from donor subjects aged 35 years or younger for this study.

In the in vitro conjunctival equivalents reconstructed in this study, the epithelial cell growth and/or
cause direct migration of cells. Previous work demonstrated that in the adult mouse, the subepithelial connective tissue can modulate the pattern of histodifferentiation of the overlying epithelium. During embryogenesis, the proliferation and histodifferentiation of epithelia are dependent on a suitable permissive mesenchyme. Under appropriate conditions, pure preparations of epithelial cells proliferate and stratify but typically lack normal patterns of histogenesis and macromolecular synthesis. Thus, an important role of the subepithelial connective tissue is to support epithelial proliferation and differentiation.

**FIGURE 5.** Transmission electron micrographs of the epithelium developed on HCF-suspended collagen stroma without (a) and with (b) exposure to air-liquid interface. HCF-stroma supported epithelium maintained in the submerged conditions is characterized by poorly developed cell stratification, cell-cell membrane interdigitations, and desmosomes (a). In air-lifted samples, the surface cell layers were better stratified, and microvilli were abundant. Although intercellular spaces were large, complex cell-cell membrane interdigitations and desmosomes were evident. However, hemidesmosomes and basement membranes at epithelium-stroma junctions were not developed (b). (c) An enlarged view of the outlined area in (b) showing membrane interdigitations with desmosomes.

differentiation are presumably influenced by the chemical composition of the collagen matrix and the type of cells dispersed in the matrix. Type I collagen is chemotactic for fibroblasts in culture and appears to

**FIGURE 6.** Transmission electron micrographs of the epithelium developed on 3T3-suspended collagen stroma without exposure to air-liquid interface. Surface microvilli, distinctive intercellular interdigitation, and desmosomes were present (a). Early developed basement membrane with focal condensation of electron-dense ground substance are present at the basal cell-stroma junction (b) and are indicated by arrows.
Studies on skin and mammary gland development also showed the importance of mesenchyme on epithelial proliferation and differentiation, and vice versa. Dermal stroma enhances attachment of epithelial cells and can induce the cells to synthesize their own extracellular matrix, which in turn can influence differentiation in vitro. The surrounding matrix can affect the expression of the genes of the matrix molecules, thereby contributing to the balance of matrix formation and degradation.

In our experimental model, the reorganized collagen gels containing living fibroblasts took the place of living mesenchymal tissue. The fibroblasts suspended in the collagen gels contracted the gels into a tissue-like fabric. The contraction changed the physicochemical properties of the collagen gels and, together with the embedded fibroblasts, served as a mesenchyme equivalent for HCE cells to grow and develop. HCE cells plated on collagen matrix containing no fibroblasts grew poorly and formed few patches of colonies (Fig. 1c). In contrast, HCE cells plated on fibroblast-suspended collagen matrix grew at a faster pace and formed bigger colonies during the same experimental period (Fig. 1b). Moreover, the growth of HCE cells on Swiss 3T3-suspended collagen matrix was further promoted (Fig. 1a). The results indicate that fibroblasts in the collagen matrix play an important role in influencing epithelial cell growth. The fibroblasts may affect epithelial cell growth and development by producing some paracrine growth factors to influence the epithelial cells. To test this hypothesis, we plated conjunctival epithelial cells on acellular collagen gels in the presence of fibroblast-conditioned medium. We found that epithelial cell growth was promoted by the conditioned medium; however, even 100% conditioned medium could not replace the promoting effect of the fibroblast-containing collagen gels. These observations suggest that some short half-life factor(s) produced by the fibroblasts may be involved or that bidirectional interactions between epithelial cells and fibroblasts are required. We do not know why 3T3 cells served as a better matrix support than normal conjunctival fibroblasts in epithelial cell growth. It is possible that 3T3 cells, an established embryonal cell line, may release a repertoire of autocrine factors that may be quantitatively and/or qualitatively differed.
from those released by HCF, and one or more of these putative factors may be required for the observed better growth of the conjunctival epithelial cells.

Exposure to air–liquid interface has been shown to improve the differentiation of cultured keratinocytes and dermal equivalents, a condition thought to be more physiological. Keratinocytes grown on collagen rafts and kept at the air–liquid interface were shown to induce differentiation-associated keratin polypeptide synthesis. The effect of air–liquid interface exposure was more obvious when a dermal equivalent or lattice consisting of collagen and fibroblasts was used as substrate. In our studies, more differentiated characteristics were expressed by HCE cells when maintained at the air–liquid interface. HCE cells cultured on the HCF-suspended collagen matrix would not stratify when maintained in a submerged condition and stratified when raised to the air–liquid interface (Figs. 3a and 3b). However, stratification of HCE cells was not affected by submersion or air–liquid interface exposure when they were cultured on the 3T3-suspended collagen matrix. In this system, HCE cells stratified regardless of whether they were air–liquid interface exposed or not. In this regard, 3T3 fibroblasts have been used to improve the culture of human keratinocytes to allow the development of a single cultured cell into a stratified colony.

Although 3T3 cell-dispersed collagen matrix supported a better epithelial cell growth and structural differentiation, it was unable to support the differentiation of goblet cells. In contrast, normal conjunctival fibroblast-dispersed collagen matrix was not as good in supporting epithelial cell growth, but it supported goblet cell differentiation. Thus, 3T3 cells may direct conjunctival epithelial cells into a proliferation and stratification pathway that resembles the pathologic states of the conjunctival squamous metaplasia. In contrast, normal fibroblasts appeared to direct them into a proliferation–differentiation coupled pathway, resulting in the formation of thinner epithelium and the differentiation of goblet cells characteristic of normal conjunctiva.

There have been reports on the formation of basement membrane structures by epithelial cells growing on collagen gels. Their epithelial culture systems all contained fibroblasts. Our previous studies with rabbit conjunctival epithelial cells cultured on collagen gels showed early formation of the basement membrane with focal condensation of electron-dense ground substance. It was shown that air–liquid interface culture is not crucial for basement membrane formation. In the present study, we demonstrated that the formation of basement membrane structures is influenced by the type of fibroblasts dispersed in the collagen matrix. Epithelium developed on HCF-suspended collagen matrix did not form hemidesmosomes and basement membrane. However, HCE cells cultured on the 3T3-suspended collagen matrix expressed a discontinuous basement membrane-like structure. Moreover, air–liquid interface exposure further promoted the differentiation of basement membrane structure. Under these conditions, hemidesmosomes are formed along the electron-dense basement membrane-like structure (Fig. 7).

In summary, our results show that type I collagen mixed with fibroblasts or 3T3 cells is a much better substrate for human conjunctival epithelial cell growth and differentiation than acellular collagen matrix. This system allows intercellular interactions via cell–cell contact and/or humoral factors in a paracrine–autocrine fashion. By manipulating the medium composition, reformation of a conjunctival epithelium with differentiated function is now possible under our culture conditions. Such a "living conjunctival equivalent" will allow us to analyze conjunctival epithelial cell growth regulation and goblet cell differentiation process; analyze the humoral factors involved in cell–cell interactions between epithelium and tenon fibroblasts and the possible roles of these factors in the development and maintenance of a functional conjunctival epithelium; develop a medium formulation that will allow long-term maintenance of our in vitro reconstructed living conjunctival equivalents; and examine the potential use of living conjunctival equivalents to promote wound healing of severe burns in animal models.

Key Words

human conjunctival equivalent, bulbar conjunctival epithelium, epithelial cell differentiation, conjunctival fibroblast, cell culture

References

6. Milstone LM, McGuire J, La Vigne JF. Retinoic acid


