

Impact of Cell Source on Human Cornea Reconstructed by Tissue Engineering

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PURPOSE. To investigate the effect of the tissue origin of stromal fibroblasts and epithelial cells on reconstructed corneas in vitro.

METHODS. Four types of constructs were produced by the self-assembly approach using the following combinations of human cells: corneal fibroblasts/corneal epithelial cells, corneal fibroblasts/skin epithelial cells, skin fibroblasts/corneal epithelial cells, skin fibroblasts/skin epithelial cells. Fibroblasts were cultured with ascorbic acid to produce stromal sheets on which epithelial cells were cultured. After 2 weeks at the air-liquid interface, the reconstructed tissues were photographed, absorption spectra were measured, and tissues were fixed for histologic analysis. Cytokine expression in corneal- or skin-fibroblast-conditioned media was determined with the use of protein array membranes. The effect of culturing reconstructed tissues with conditioned media, or media supplemented with a cytokine secreted mainly by corneal fibroblasts, was determined.

RESULTS. The tissue source from which epithelial and mesenchymal cells were isolated had a great impact on the macroscopic and histologic features (epithelium thickness and differentiation) and the functional properties (transparency) of the reconstructed tissues. The reconstructed cornea had ultraviolet-absorption characteristics resembling those of native human cornea. The regulation of epithelial differentiation and thickness was mesenchyme-dependent and mediated by diffusible factors. IL-6, which is secreted in greater amounts by corneal fibroblasts than skin fibroblasts, decreased the expression of the differentiation marker DLK in the reconstructed epidermis.

CONCLUSIONS. The tissue origin of fibroblasts and epithelial cells plays a significant role in the properties of the reconstructed

tissues. These human models are promising tools for gaining a thorough understanding of epithelial-stromal interactions and regulation of epithelia homeostasis. (*Invest Ophthalmol Vis Sci.* 2009;50:2645-2652) DOI:10.1167/iovs.08-2001

Progress in the development of living substitutes has generated an increased clinical interest in the applications of tissue-engineered products. In ophthalmology, limbal epithelial progenitor cells expanded *ex vivo* through culture on various substrates have been used to treat stem cell deficiency in animal models and in humans.¹⁻⁵ More sophisticated substitutes made out of the stroma and the epithelium, and harboring the three-dimensional architecture of native tissues, have been developed for cornea and skin.⁶⁻¹³ In these substitutes, the stroma is reconstituted using fibroblasts embedded either in a collagen gel⁶ or in a sponge,⁷ or it results from the matrix secreted and organized by the fibroblasts themselves (self-assembly approach).¹³ The limited availability of limbal tissue and the poor outcome of allogeneic grafting has led to the question of whether other autologous tissue sources for fibroblasts and epithelial cells could be adequate for tissue engineering. One easily obtainable alternative cell source might be the skin. This organ is easily accessible, and its large surface could provide a great number of cells, thereby limiting the risk for creating a limbal stem cell deficit. Recently, the use of neonatal dermal fibroblasts for corneal stroma engineering was reported.¹⁴ However, the impact of dermal fibroblasts on epithelial thickness and function remains to be determined.

Skin and cornea share a common ectodermal embryonic origin. However, the skin is made up of a 15- to 20-cell layer-thick pluristratified squamous epithelium, whereas the central corneal epithelium is only six cell layers thick and does not contain the granular and cornified layers. Despite the morphologic similarities of their basal cells, the cutaneous and corneal epithelia might have diverged from each other, resulting in different programs of differentiation. Alternatively, basal cells may be equipotential; if they are, their specific *in vivo* differentiation would then be attributed to distinct local directives derived from the mesenchymal tissues.¹⁵ The role of the mesenchyme in the initial establishment of the histoarchitecture has been well established during embryonic development. Not only does it support differentiation, it controls the developmental fate of its overlying epithelium by exerting instructive influence on the developing epithelial structures.¹⁶⁻¹⁹ Thus, even if several lines of evidence indicate that the stroma contributes to the maintenance of the epithelial phenotype,²⁰⁻²³ little is known concerning the influence of stromal factors on epithelial cell differentiation and homeostasis during adulthood.

The study of stromal-epithelial interactions in specific situations such as wound healing has allowed the identification of cytokines involved in these cellular communication pathways.^{24,25} However, the potential influences of systemic factors and the presence of additional cell types, such as endothelial cells and circulating lymphocytic cells, complicate the analyses performed with the *in vivo* systems. In addition, though cell monolayers are simple, they lack structured extra-

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cellular matrix and normal histoarchitecture and are often unstable models.

The development of tissue-engineered, three-dimensional, reconstructed skin^{10,26} and cornea^{6,13,27} offers a valuable complementary *in vitro* approach. These models contain several layers of epithelial cells growing on top of tissue sheets comprising fibroblasts and extracellular matrix. They provide tissue substitutes that may be manipulated experimentally under more defined and reproducible *in vitro* conditions. Moreover, these completely biological cellular environments, which mimic the normal histology of the skin and the corneal epithelium, allow interactions between different cell types (i.e., fibroblasts and epithelial cells). We took advantage of these *in vitro* reconstructed tissues to assess the effects of epithelial-mesenchymal interactions on tissue formation.

The aim of the present study was to evaluate the contribution of epithelial and mesenchymal cells to determine the importance of the cell source in the production of corneal substitutes by tissue engineering. Their influence on the histologic features of the epithelium and the maintenance of homeostasis was investigated using three-dimensional *in vitro* models of cornea and skin reconstructed by the self-assembly approach of tissue engineering. Our results showed that the tissue origin of fibroblasts and epithelial cells impacted the thickness and differentiation of the epithelium and on the ultraviolet (UV)-absorption characteristics and light scatter of reconstructed tissues.

METHODS

This study was conducted in accordance with our institution's guidelines and the Declaration of Helsinki. The protocols were also approved by the institution's Committee for the Protection of Human Subjects.

Cell Extraction and Culture

Human limbal epithelial cells (HLECs) were isolated and cultured from two postmortem donor corneas unsuitable for transplantation (Banque Nationale d'Yeux du CHUQ, Québec, QC, Canada), as described previously.⁶ Briefly, the cornea was dissected from the ocular globe with curved scissors (Storz; St. Louis, MO), and the limbus was separated from the central cornea with a 7.5-mm diameter trephine (Pilling Weck; Markham, ON, Canada). The limbal ring, epithelium facing up, was incubated in a 2 mg/mL dispase (Dispase II; Roche Diagnostic, Laval, QC, Canada) in HEPES buffer (pH 7.4) for 18 hours at 4°C. The epithelium was mechanically removed from the stroma with forceps under a dissecting microscope (SMZ-2T; Nikon, Montréal, QC, Canada), cut into small pieces with a scalpel, and centrifuged for 10 minutes (200g) at room temperature. HLECs were then seeded in tissue culture flasks (BD Biosciences, Mississauga, ON, Canada) with feeder cells (irradiated [60 Gy] murine Swiss-3T3 fibroblasts; ATCC, Rockville, MD) and were subcultured up to the fourth passage.

Human corneal fibroblasts were obtained and cultured. The corneal epithelium and endothelium were removed from the stroma after digestion with dispase. Then the bare stroma was incubated for 3 hours at 37°C in a collagenase H solution (0.125 IU/mL; Roche Diagnostics). After 10-minute centrifugation at room temperature, the cells were seeded in 25-cm² tissue culture flasks (BD Biosciences) and were cultured as previously described.⁶ Corneal fibroblasts were used between their seventh and ninth passages.

Keratinocytes (skin epithelial cells) and dermal fibroblasts were obtained from human breast skin specimens and cultured as previously described.¹⁰ Briefly, the skin was incubated overnight at 4°C in a thermolysin (Sigma, Oakville, ON, Canada) solution (500 µg/mL in HEPES buffer, pH 7.4). The epidermis was peeled from the dermis and incubated for 30 minutes in a trypsin-EDTA solution (0.05% trypsin [MD Biomedicals, Montréal, QC, Canada] and 0.01% EDTA [JT Baker, Phillipsburg, NJ]) to dissociate epithelial cells. The dermis was incu-

bated for 3 hours in a collagenase H (Roche Diagnostics) solution (0.125 IU/mL in the fibroblast culture medium) to recover fibroblasts.

For each experiment, cells from two donors were analyzed. All cells cultured on plastic or reconstructed tissues were grown under 8% CO₂ at 37°C, and culture medium was changed three times a week.

Reconstruction of Tissues by Tissue Engineering

The self-assembly approach was used to produce four types of reconstructed tissues according to the following homotypic or heterotypic combinations: corneal fibroblasts with corneal epithelial cells (CC), corneal fibroblasts with skin epithelial cells (CS), skin fibroblasts with skin epithelial cells (SS), and skin fibroblasts with corneal epithelial cells (SC). To allow a fair comparison, all these reconstructed tissues were produced and cultured using identical experimental conditions. To produce the reconstructed stroma, 8000 fibroblasts/cm² were cultured for 40 days in Dulbecco modified Eagle medium (DMEM), supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 100 IU/mL penicillin G (Sigma), and 25 µg/mL gentamicin (Schering Canada, Pointe-Claire, QC, Canada) containing 50 µg/mL ascorbate (Sigma). Ascorbic acid induces extracellular matrix production and allows thick fibrous sheet formation in plastic culture flasks. After peeling from the flasks, two tissue sheets were superimposed to form a reconstructed dermis or stroma and were cultured for another week so that they could adhere. Then corneal or skin epithelial cells were seeded on the surface of the reconstructed stroma or dermis and cultured in submerged conditions in complete epithelial cell medium supplemented with ascorbate, as previously described.^{10,27} The complete epithelial cell medium consisted of a combination of Dulbecco-Vogt modification of Eagle medium (DME) with Ham F12 in a 3:1 ratio (Invitrogen, Burlington, ON, Canada), supplemented with 24.3 µg/mL adenine (Sigma), 5 µg/mL insulin (Sigma), 2 × 10⁻⁹ M 3,3',5'-triiodo-L-thyronine (Sigma), 5 µg/mL human transferrin (Sigma), 0.4 µg/mL hydrocortisone (Calbiochem, La Jolla, CA), 10⁻¹⁰ M cholera toxin (Sigma), 10% newborn calf serum (Fetal Clone II; Hyclone, Logan, UT), 10 ng/mL human epidermal growth factor (EGF; Austral Biological, San Ramon, CA), 100 IU/mL penicillin G (Sigma), and 25 µg/mL gentamicin (Schering Canada). After 7 days, reconstructed tissues were fed an EGF-free epithelial cell medium and were raised at the air-liquid interface for 2 additional weeks to induce epithelial differentiation. The experiment was repeated twice, each time with two different cell lines isolated from dermis and corneal stroma and from epidermis and corneal epithelium, respectively (*n* = 4 replicates per experiment for each reconstructed tissues).

Conditioned Media

Corneal and skin fibroblasts were cultured to subconfluence for 48 hours in complete DMEM supplemented with 10% fetal calf serum (Hyclone). The resultant culture media (conditioned media) were then harvested, filtrated through a 0.22-µm low protein-binding filter (Millex-GV; Millipore, Nepean, ON, Canada) after centrifugation, and stored at -80°C until use. When indicated, the conditioned media were further diluted 1:1 with fresh epithelial cell medium and added to the four reconstructed tissues for the entire period of epithelial cell culture. The media were changed every 48 hours. The experiment was repeated twice, each time with two different cell lines isolated from dermis and corneal stroma, respectively (*n* = 4).

Quantification and Influence of IL-6 on Reconstructed Tissues

The amount of IL-6 secreted by monolayers of subconfluent corneal fibroblasts into the conditioned culture media over a period of 48 hours was determined with a human IL-6 ELISA (Quantikine; R&D Systems Inc., Minneapolis, MN). One hundred microliters of each sample was diluted by 100-fold and tested according to the manufacturer's instructions. The mean value of four replicates from two different experiments was 1980.75 pg/mL ± 288.89 pg/mL. When indicated, 1000 pg/mL (half the quantity because the conditioned media were diluted 1:1 with fresh epithelial cell medium) of recombinant

human IL-6 (R&D Systems, Inc.) was added to the fresh epithelial cell medium and to the four reconstructed tissues for the entire period of epithelial cell culture. The media were changed every 48 hours. The experiment was repeated twice with duplicates in each experiment.

Measurement of Absorption Spectra

Absorbance (A) of the four reconstructed tissues was measured at room temperature with a scanning spectrophotometer (light beam, 1.5×4.0 mm; model Ultraspec 3000; Biochrom, Cambridge, UK) and with a double beam spectrophotometer (light beam, 5.0×5.0 mm; Varian UV-Vis-IR; Cary, Mulgrave, Australia). Indirect absorption spectra were measured using the Cary spectrophotometer with an integrating sphere (radius, 110 mm) of the internal diffuse reflectance accessory. The tissue was then placed in the transmission port, and the reflectance port was covered with a reference disc. Reconstructed tissues were immersed in a phosphate-buffered saline (PBS; 6.8 mM NaCl, 0.14 mM KCl, 1.61 mM Na_2HPO_4 , 0.08 KH_2PO_4) solution and placed in a quartz cuvette especially designed to measure absorbance from tissue samples. A fresh PBS solution was used after each measurement. Baseline absorbance was recorded using the cuvette filled with saline. Each spectrum obtained thereafter was corrected by subtracting the baseline absorbance. To normalize the different thicknesses of the tissues, the absorption coefficient (α) of the investigated sample was calculated using absorbance (A) and the equation $\alpha = A/d \times \log e$, where d is the thickness of the sample and $\log e = 0.434$.²⁸

The experiment was repeated twice, each time with two different cell lines (e.g., fibroblasts isolated from the dermis or the corneal stroma, or epithelial cells isolated from the corneal and skin epithelium; $n = 4$). Data are reported as mean \pm SEM. The absorption coefficient obtained with the corneal substitute was compared with that of Kolozsvari et al.²⁸

Histologic Analysis and Thickness Measurement

Biopsy samples from the reconstructed tissues (before or after the absorbance measurements) were fixed in a Bouin solution (Produits chimiques ACP, St. Léonard, QC, Canada) and embedded in paraffin. Five-micrometer-thick sections were stained with Masson trichrome. The thickness of the different tissues was also determined after fixation with a microscope using a micrometer with a resolution of $1 \mu\text{m}$.

Detection of Cytokines Expression in Fibroblast-Conditioned Media Using Protein Array Membranes

The presence of 79 different cytokines and growth factors was evaluated with an array kit produced by RayBiotech (Human Cytokine Array V kit; Cedarlane Laboratories, Hornby, ON, Canada). Briefly, 1 mL conditioned media were incubated with cytokine array membranes. To prepare this conditioned media, corneal and skin fibroblasts were plated at a density of 8000 cells/cm². Cells were cultured in DMEM containing 10% fetal calf serum (Hyclone), 100 IU/mL penicillin G (Sigma), and 25 $\mu\text{g}/\text{mL}$ gentamicin (Schering Canada). At 70% to 75% confluence, cells were washed twice in PBS (to remove residual serum proteins), and the complete culture medium was replaced with serum-free DMEM containing 0.5% bovine serum albumin (BSA; Sigma) for 48 hours. Supernatants were obtained as described and stored at 4°C overnight or at -80°C until analysis. After incubation of the conditioned media with the protein array, membranes were incubated with biotin-conjugated anti-cytokine antibodies. Membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated streptavidin. Signals were visualized using an enhanced chemiluminescence system (ECL Plus; Amersham Biosciences, Baie d'Urfé, QC, Canada). Five positive controls were included in membranes (three at the top left, two at the bottom right) to identify the orientation and to compare the relative expression levels among different membranes. The experiment was repeated twice ($n = 2$).

Immunofluorescence Staining

Biopsy samples from the reconstructed tissues were embedded in optimal cutting temperature compound (Somagen, Edmonton, AB,

Canada), frozen in liquid nitrogen, and stored at -70°C until used. An indirect immunofluorescence assay was performed on acetone-fixed (10 minutes at -20°C) cryosections ($5 \mu\text{m}$), as previously reported.²⁹ Sections were incubated for 45 minutes with rabbit polyclonal anti-human dual leucine zipper-bearing kinase (DLK; gift from Richard Blouin, Sherbrooke University)³⁰ antibody diluted in PBS containing 1% BSA. The secondary antibody, Alexa 594-conjugated chicken anti-rabbit IgG (Molecular Probes, Eugene, OR), was incubated for 30 minutes. Cell nuclei were counterstained with reagent (Hoechst 33258; Sigma). Tissue was observed under an epifluorescence microscope (Eclipse E600; Nikon) and photographed with a numeric charge-coupled device camera (Sensys; Roper Scientific, Trenton, NJ). Negligible background was observed for controls (primary antibodies omitted).

RESULTS

Macroscopic Analysis of the Reconstructed Tissues

Analysis of the four possible constructs revealed distinctive macroscopic features of the various reconstructed tissues, depending on the tissue source of fibroblasts and epithelial cells used. Macroscopic observations showed that the reconstructed cornea (CC) was uniformly transparent (Fig. 1A) but that the reconstructed skin (SS) was regularly whitish and opaque (Fig. 1B). Similar results were obtained after four independent experiments, including different cell lines (one typical result of four different experiments is provided). Thus, the apparent transparency/opacity exhibited by the reconstructed cornea and skin corresponded to that of their corresponding native tissues.

The macroscopic observation of the heterotypic tissues reconstructed with corneal epithelial cells and skin fibroblasts (SC; Fig. 1C) or skin epithelial cells and corneal fibroblasts (CS; Fig. 1D) revealed that tissue transparency was not entirely uniform within each construct, unlike CC (Fig. 1A) and SS (Fig. 1B) constructs, both of which exhibited uniform transparency and opacity. In addition, the two heterotypic reconstructions (Figs. 1C, 1D) were not as transparent as the reconstructed cornea (CC; Fig. 1A) or as opaque as the reconstructed skin (SS; Fig. 1B). Identical results were also obtained when different cell lines were used (data not shown). We therefore conclude from these results that the cell source of epithelial cells and fibroblasts influences the transparency of the reconstructed tissues.

Apparent Absorption Spectra of Reconstructed Tissues

To correlate these macroscopic observations with the transparency of the reconstructed tissues, we measured their attenuation in the range of 300 to 1000 nm by direct absorption measurement. The transparent reconstructed cornea (CC) presented a minimum of scatter; therefore, the curve shown in Figure 2A is a true absorption coefficient. However, the curves presented for the other substitutes include, in addition to the absorption coefficient, a coefficient of light scatter normalized for tissue thickness at each wavelength. This light scatter is highest in the case of the reconstructed skin (SS; Fig. 2A), indicating that the light scatter in the visible and near UV ranges within reconstructed tissues is highly dependent on the cell source.

We also studied corneal absorption in the middle UV region, focusing our attention toward the biologically significant range from 290 to 310 nm. The maximum UV absorption by the reconstructed cornea (CC) occurred at 265 nm, with a broad peak at 255 to 275 nm (Fig. 2B). A second method using indirect absorbance measurements (not shown) yielded an overall profile similar to the corresponding direct measure-

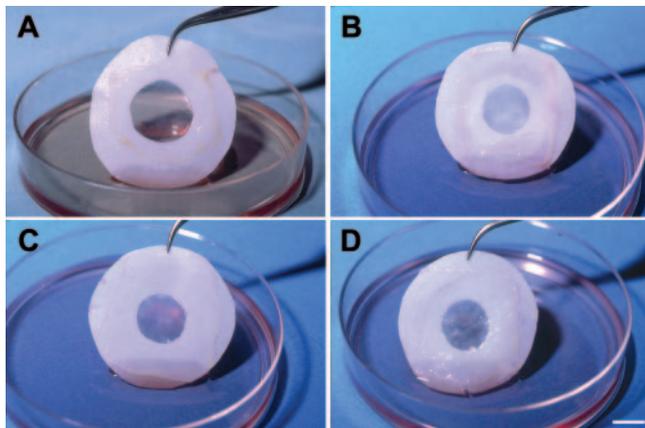


FIGURE 1. Transparency of the four types of substitutes reconstructed by the self-assembly approach of tissue engineering. (A) Reconstructed cornea (CC). (B) Reconstructed skin (SS). (C, D) Heterotypic tissues reconstructed with (C) skin fibroblasts and corneal epithelial cells (SC). (D) Corneal fibroblasts and skin epithelial cells (CS). Note that the macroscopic aspect of the reconstructed cornea was transparent (A), whereas that of the reconstructed skin (B) was whitish and opaque. Two heterotypic tissues (C, D) presented intermediate features that were neither as transparent as the reconstructed cornea (A) nor as opaque as the reconstructed skin (B). Scale bar, 1 cm.

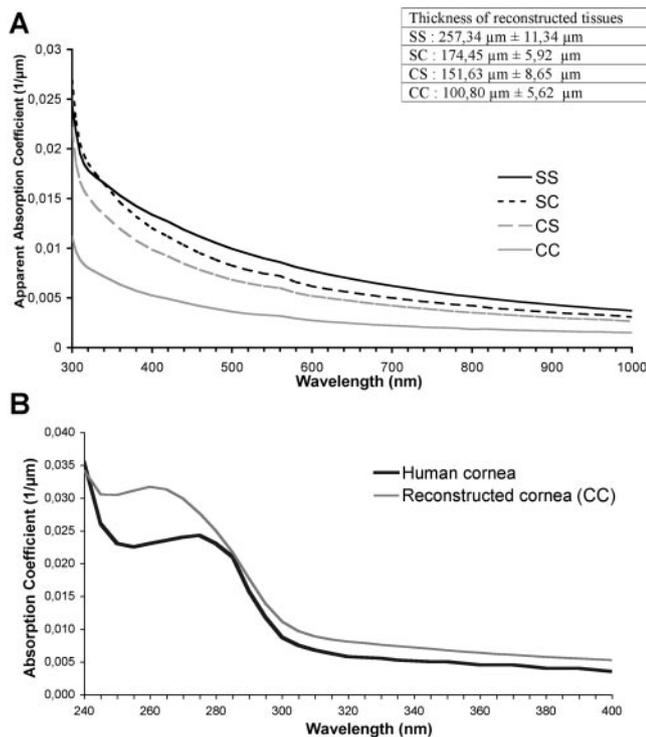


FIGURE 2. Apparent absorption coefficient for the four types of substitutes between 300 and 1000 nm (A) and comparison of the absorption coefficient for reconstructed cornea and native normal human cornea²⁸ between 240 and 400 nm (B). (A) In the reconstructed cornea (CC), there is almost no light scattering. This is, therefore, a true absorption spectrum. The other three curves include a light scatter coefficient, as suggested by their turbid appearances. Therefore, light scatter was largest with the reconstructed skin (SS), followed by the two heterotypic tissues (SC and CS) and the reconstructed cornea (CC), which was the lowest (significant difference). (B) Note that absorption coefficient of the reconstructed cornea was relatively high, from 240 to 310 nm. Curves represent the average measurements on four different samples (for all curves in A and reconstructed cornea in B). The SEM was no greater than 17.7% (A) and 16.1% (B).

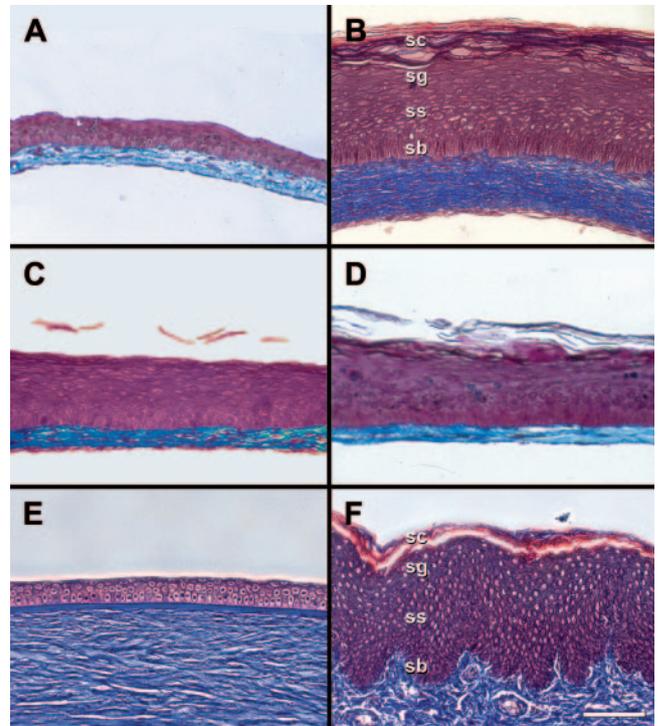


FIGURE 3. Histologic organization of the four reconstructed tissues. (A) Reconstructed cornea (CC). (B) Reconstructed skin (SS). (C) Corneal epithelial cells seeded on reconstructed stroma (SC). (D) Skin epithelial cells added on the reconstructed stroma (CS). (E) Human corneal epithelium. (F) Human skin epithelium. (A) A four- to six-cell layer-thick epithelium with cuboidal basal cells and no stratum corneum was observed for the reconstructed cornea (CC) (as observed for native cornea in situ). (B) A 15- to 20-cell layer-thick epithelium with the four characteristic cutaneous layers—stratum basale (sb), stratum spinosum (ss), stratum granulosum (sg), stratum corneum (sc)—was observed in the reconstructed skin (SS) (again as observed for native skin in situ). The thickness of the epithelium of the other two heterotypic reconstructed tissues was intermediate, with no or thinner stratum corneum. Indeed, when corneal epithelial cells were seeded on reconstructed dermis (SC), the number of cell layers was increased and the stratum corneum was absent (C). When skin epithelial cells were added on the reconstructed stroma (CS), the epithelium thickness was diminished and the stratum corneum was thinner (D) compared with reconstructed skin (B). Scale bar, 100 μm .

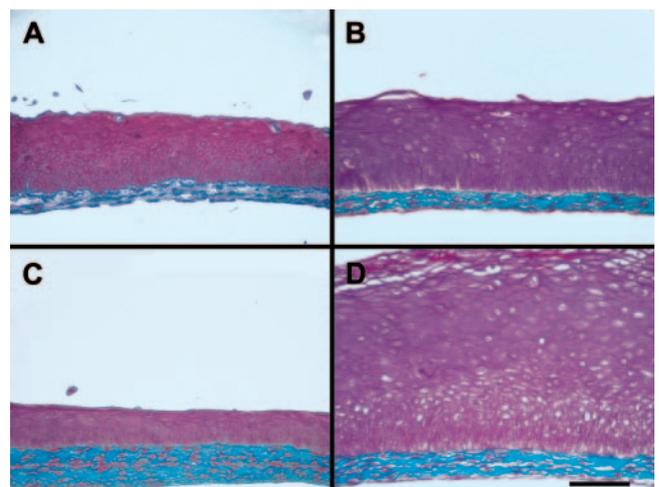


FIGURE 4.

ments. The measures were contained within the same range of values, and the curves of attenuation were in the same order. These results indicate that the reconstructed cornea presents absorption characteristics approaching those of cornea in situ and that light scattering is minimal when corneal cells are used.

Histologic Analyses of the Four Reconstructed Tissues

Histologic examination of the four reconstructed tissues showed that in all cases, the epithelial cells attached, proliferated, and differentiated to reform continuous and stratified epithelia. However, the thickness and differentiation (e.g., presence of the stratum corneum) of the reconstructed epithelia varied, depending on the tissue cell source.

Tissues reconstructed with homotypic cell types produced well-organized corneal and epidermal epithelia. Corneal epithelial cells stratified uniformly over the reconstructed stroma (CC), forming an epithelium five to six cell layers thick. The basal layer was composed of cuboidal cells with round nuclei. Superficial layers had spindle wing-like cells overlaid by flat squamous cells that did not cornify (Fig. 3A). These results are similar to normal human cornea in situ. In contrast, the epithelium of the reconstructed skin was thicker and more differentiated (Fig. 3B). Indeed, a stratified, well-differentiated epithelium with up to 15 to 20 cell layers constituting the four characteristic cutaneous layers was present. Cells of the basal layer were cuboidal. Typical stratum spinosum and stratum granulosum covered by several layers of cornified cells resembling a stratum corneum were distinguishable. This layer was sometimes thicker than normal, presumably because of the absence of desquamation under *in vitro* experimental conditions.

When corneal epithelial cells were combined with dermal fibroblasts (SC), they formed an epithelium thicker (10–11 cell layers; Fig. 3C) than when corneal fibroblasts were used (Fig. 3A). However, the strata were similar to those of the normal corneal epithelium, with elongated nucleated suprabasal cells and without granular or cornified anucleated layers. Although the thickness of the epithelium produced by skin epithelial cells cultured on corneal fibroblasts (CS) was similar (12 cell layers; Fig. 3D) to the other heterotypic combination, it was reduced compared with the reconstructed skin, and the differentiation was different. Indeed, the stratum granulosum and stratum corneum were thinner and sometimes completely absent. Thus, these results indicated that the source of mesenchymal and epithelial cell types influenced the thickness and histologic features of the reconstructed epithelium.

Soluble Factors Mediate Fibroblast-Epithelium Interactions in Reconstructed Tissues

To assess whether the effects of fibroblasts on the formation of the epithelium could be attributed to soluble factors, conditioned media collected from subconfluent monolayers of human corneal or skin fibroblasts were added to the four recon-

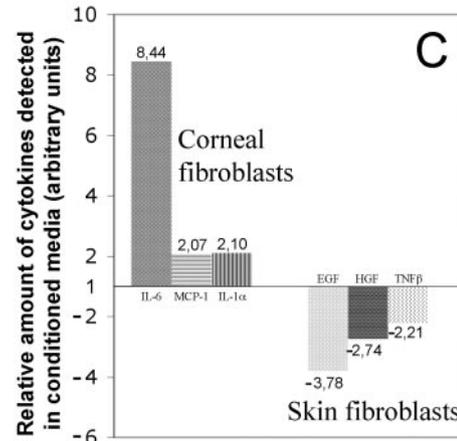
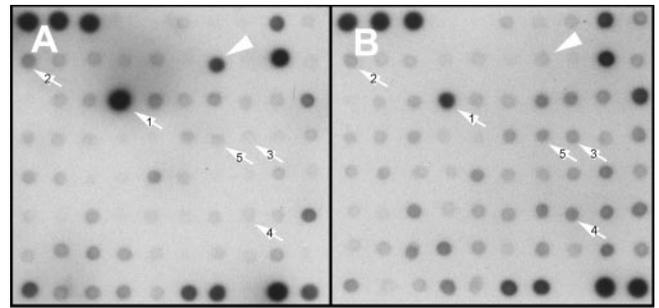


FIGURE 5. Detection of multiple cytokines secreted in conditioned media. Cytokine array membranes were incubated with conditioned media collected from human corneal (A) and skin (B) fibroblast cultures. *Arrowhead* indicates the detection of IL-6 in media conditioned by corneal and skin fibroblasts. Corneal fibroblasts highly expressed IL-6, whereas this factor was hardly detectable in media conditioned by dermal fibroblasts. *Arrows* indicate the detection of some factors that were produced in higher amounts either in media conditioned by corneal fibroblasts (MCP-1 [arrow 1], IL-1 α [arrow 2]) or in media conditioned by skin fibroblasts (EGF [arrow 3], HFG [arrow 4], TNF β [arrow 5]). (C) Ratio of the amount of growth factors detected in culture media conditioned by corneal fibroblasts over that of skin fibroblasts. A relative amount of 1 means that there was no difference in the secretion of a growth factor in both conditioned media. A positive number indicates greater secretion in medium conditioned by corneal fibroblasts, whereas a negative number indicates that skin fibroblasts secreted more. The membranes and graph represent 1 of 2 experiments that gave similar results.

structed tissues during the entire culture period of epithelial cells.

In reconstructed cornea (CC), medium conditioned by skin fibroblasts yielded an epithelium with increased thickness (Fig. 4A), resembling that of the reconstructed tissue composed of skin fibroblasts and corneal epithelial cells (SC; Fig. 3C). Moreover, this medium prevented the reduction in the number of epithelial cell layers observed in tissues reconstructed with corneal fibroblasts and skin epithelial cells (CS; Fig. 4D, compared with Fig. 3D) and allowed a partial correction of histologic properties of this epithelium. In addition, a much thicker anucleated stratum corneum was also restored (Fig. 4D).

An opposite effect was observed in tissues supplemented with media conditioned by corneal fibroblasts. In its presence, reconstructed skin presented a thinner epithelium characterized by a decrease in the thickness of the stratum corneum (Fig. 4B). Moreover, the addition of this conditioned medium to tissues reconstructed from skin fibroblasts and corneal epithelial cells reversed the increase in the number of epithelial cell layers and allowed the restoration of an essentially normal corneal epithelial architecture (Fig. 4C). Taken together, these observations indicated that corneal and skin fibroblasts in-

FIGURE 4. Histologic organization of the four tissues cultured with conditioned media. Conditioned media of subconfluent monolayers of corneal fibroblasts (B, C) or skin fibroblasts (A, D) was added to CC (A), SS (B), SC (C), and CS (D) for the entire culture period of the epithelium. Note that the corneal fibroblast-conditioned media gave rise to a lower number of cell layers in the epithelium of the reconstructed skin (SS) (B) and in the heterotypic tissue composed of skin fibroblasts and corneal epithelial cells (SC) (C). In contrast, media conditioned by skin fibroblasts was sufficient to increase the epithelium thickness of the reconstructed cornea (CC) (A) and the heterotypic tissue composed of corneal fibroblasts and skin epithelial cells (CS) (D). Scale bar, 100 μ m.

duced variations in the epithelial cell differentiation and stratification through soluble factors.

Analysis of Conditioned-Media Components

To seek these fibroblast-derived soluble factors transregulating epithelial cell growth and differentiation, we analyzed the conditioned media with cytokine array membranes. Several cytokines and growth factors were detected at various levels in media conditioned by corneal (Fig. 5A) and skin fibroblast monocultures (Fig. 5B). The most striking difference was the expression of IL-6 (Figs. 5A, 5B arrowhead). Corneal fibroblasts highly expressed IL-6, whereas this factor was hardly detectable in media conditioned by dermal fibroblasts. Furthermore, the signal for some factors (macrophage chemoattractant protein [MCP]-1, IL-1 α) was stronger in media conditioned by corneal fibroblasts. Skin fibroblasts produced higher amounts of other factors (epidermal growth factor [EGF], hepatocyte growth factor [HGF], TNF- β ; Figs. 5A, 5B, arrows 1-5). The keratinocyte growth factor (KGF) and TGF- β 1 signals were too weak to be quantified in both conditioned media. Thus, these results indicate that the patterns of secretion of soluble factors by skin fibroblasts and corneal fibroblasts are distinct.

Influence of IL-6 on Reconstructed Tissues

Cytokine array membranes revealed that IL-6 was the most differentially expressed factor secreted by corneal fibroblasts compared with dermal fibroblasts. To determine the role of IL-6 on epithelial cell differentiation, we exploited immunofluorescence staining to monitor the expression of DLK, a differentiation marker normally present in the granular layer (stratum granulosum) of the human epidermis (Fig. 6A). As shown on Figure 6B, expression of DLK was indeed present in the granular layer of the epidermis from the reconstructed skin (SS). It was not expressed in CS (data not shown). However, it was greatly diminished or absent when reconstructed skin (SS) was cultured in the presence of conditioned media from subconfluent monolayers of corneal fibroblasts (Fig. 6C) or in a medium containing 1000 pg/mL IL-6 (Fig. 6D). This latter

condition did not, however, reduce the epithelial thickness. These results, therefore, indicate that IL-6 clearly influences epidermal differentiation but also suggest that it is likely not the only cytokine involved in variations induced by the conditioned medium.

DISCUSSION

Tissue engineering, which allows the production of reconstructed tissues from a small biopsy specimen, may be an alternative to the shortage in donor cornea from corneal banks. Other cell sources could help to overcome the limited availability of corneal cells, but their suitability for corneal reconstruction should first be evaluated. The three-dimensional aspect of the tissues reconstructed *in vitro*, combined with their histologic features comparable to the native tissues, allowed us to use these models to examine the impact of the cell source on the homeostasis and functional properties of reconstructed skin and cornea. Our results showed that fibroblasts and epithelial cells were involved in the maintenance of adequate transparency and appropriate epithelium thickness. Thus, epithelial-mesenchymal interactions play crucial roles in the homeostasis of the mature epithelium during postnatal life. It is consistent with the mesenchymal influence taking place during embryonic development of the variety of epithelial phenotypes and during postnatal processes such as wound healing.³¹

Transparency is a functional parameter of utmost importance in corneal reconstruction and was shown to vary with the cellular composition of the reconstructed tissues. The best transparency was obtained when epithelial and mesenchymal cells originated from the cornea. The UV-absorption characteristics important for the protection of the highly sensitive structures of the eye were similar for the reconstructed cornea (CC) and the native normal human cornea,²⁸ with a peak of absorbance at 275 nm and a broad peak around 255 to 290 nm.

The present study also suggests that the best cell type for corneal reconstruction remains the limbal epithelium because clearly distinct epithelium morphologic properties were ob-

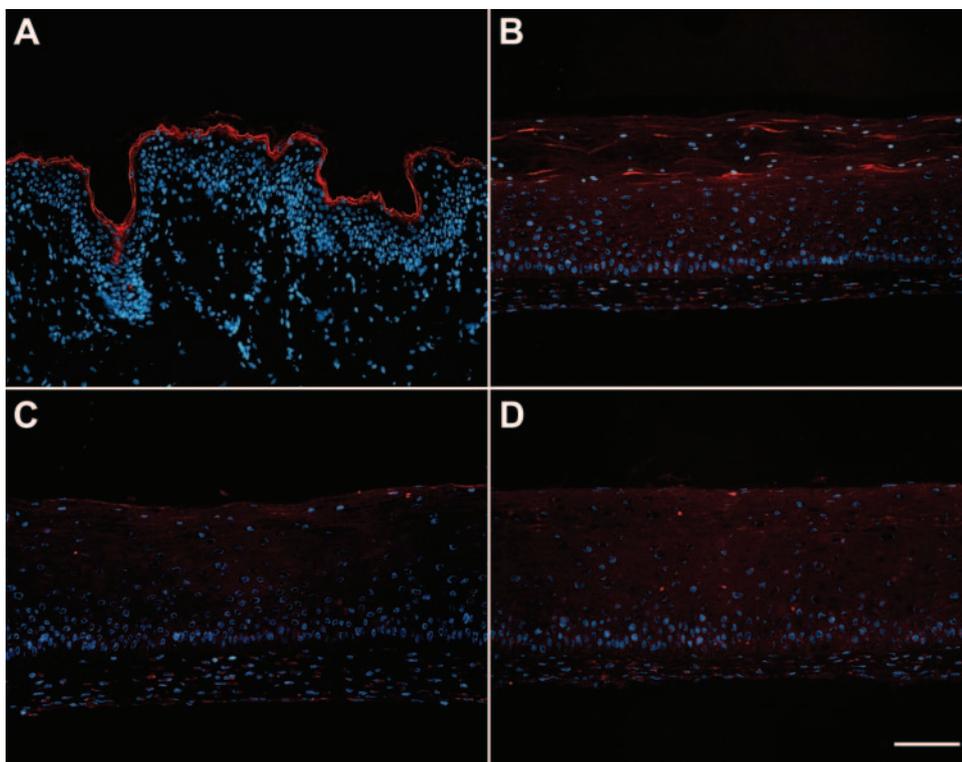


FIGURE 6. Indirect immunofluorescence of DLK in the human epidermis and reconstructed skin. Positive staining for DLK (red) was present in the granular layer of the human epidermis in native skin (A) and reconstructed epidermis (B). This differentiation marker was greatly diminished or not expressed when reconstructed skin (SS) was cultured in the presence of conditioned media from subconfluent monolayers of corneal fibroblasts (C) or in reconstructed skin (SS) cultured with 1000 pg/mL IL-6 (D). Nuclei were counterstained with reagent (Hoechst 33258; Sigma). Scale bar, 100 μ m.

tained in the tissues reconstructed with skin cells. This may appear to contrast with previous morphologic similarities reported for cultured epithelial sheets obtained from epidermis, cornea, conjunctiva, and oral mucosa.^{6,32-35} We attribute the variations to the poor differentiation status of the epithelial sheets used in the studies (cultured on plastic substratum, submerged in medium). The sheets consisted of only three to five stratified epithelial cell layers, in contrast to the number of layers (including differentiated layers) obtained in the present study after long-term culture of three-dimensional reconstructed tissues featuring a natural living stroma and epithelium and, therefore, much more like native tissues.

Although it is generally believed that epithelial differentiation is progressively and irreversibly determined during embryonic and early postnatal life,³⁶⁻³⁸ strong evidence supporting the concept that the stroma participates in the control of the plasticity of epithelial differentiation after birth is provided by the studies on uterus and vagina³⁹ and those on the preservation of stem cells within a niche.^{40,41} Our results suggest that postnatal human skin and corneal epithelial cells remain responsive to the influence of foreign stroma because the transparency, thickness, and histologic features were modified in heterotypic reconstructions. The partial preservation of the original epithelial specificity, such as the absence of a cornified layer in reconstructed SC, is consistent with results from epithelial-mesenchymal recombinations in vivo, which indicated that tissue-specific differentiation is probably determined by a combination of stroma-controlled mechanisms and intrinsic epithelial properties.^{15,21,42-46}

The variations obtained with our four types of tissue-engineered constructs show that changing the tissue origin of fibroblasts induced dramatic changes to the stratification and histologic features of the reconstructed epithelia. This is consistent with the observations that dermal fibroblasts modify the proliferation, basement membrane formation, and differentiation of skin epithelial cells cultured on plastic, de-epidermized dermis, or collagen gels.⁴⁷⁻⁵⁰ Therefore, our data further support the concept that the function of fibroblasts is not only to synthesize, degrade, and reorganize extracellular matrix components⁵¹ but also to modulate epithelial homeostasis in adult organs. Given that conditioned media from the appropriate fibroblast type were sufficient to normalize the epithelium of heterotypic constructs (SC, CS), we conclude that their differential influences on the epithelial cell properties was determined through diffusible factors and did not require direct cell-cell contacts. These paracrine factors may be involved in the regulation of epithelial cell turnover in healthy epithelium.

Comparison of the media conditioned by corneal and skin fibroblasts provided cytokine candidates as possible key regulators of the fine-tuned balance of epithelial cell proliferation and differentiation. In addition to factors secreted at similar levels by both types of fibroblasts (IL-1; HGF and KGF),^{48,52} IL-6 could have a differential role in homeostasis of the healthy corneal and skin epithelium because more IL-6 is secreted by corneal fibroblasts than by skin fibroblasts. In addition to specific immune responses⁵³ and stimulation of corneal epithelial cell migration,^{54,55} the abundant IL-6 synthesized by corneal fibroblasts may promote various activities, including regulation of the epithelial homeostasis, maintenance of corneal integrity,⁵⁶ and suppression of cornification of the corneal epithelium. Conversely, the small quantity secreted by skin fibroblasts and low levels of IL-6 in normal human skin⁵⁷⁻⁵⁹ may contribute to the high differentiation state (including cornification) of the skin epithelium. Furthermore, our observation that media conditioned by corneal fibroblasts decreased cornification in the SS and diminished the expression of a kinase involved in the last stages of epidermal differentiation^{29,30} (DLK in SS) are consistent with the decrease in keratinocyte differentiation associated with the addition of IL-6 to in vitro

models (present study and⁶⁰). However, it is likely that other factors are also involved because IL-6 does not induce the decreased thickness observed with the conditioned medium.

In conclusion, our investigation indicates that neither fibroblasts nor epithelial cells from skin would be an adequate source of cells for corneal substitutes. It also emphasizes that the influence of cell source on the histologic features and the functional properties of reconstructed tissues must be carefully studied in three-dimensional constructs allowing epithelial-mesenchymal interactions. The completely biological three-dimensional models are promising for gaining a thorough understanding of interactions between stromal and epithelial cells and regulation of epithelia homeostasis. In addition, these tissue-engineered corneas could eventually provide effective treatments for many corneal disorders.

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