Reconstruction of Epidermis on a Chitosan Cross-linked Collagen-GAG Lattice: Effect of Fibroblasts

G. SAINTIGNY, M. BONNARD, O. DAMOUR and C. COLLOMBEL

Laboratoire des Substituts Cutanés, Hôpital Edouard Herriot, Lyon, France

Reconstruction of epidermis on a fibroblast containing chitosan cross-linked collagen-GAG lattice at the air-liquid interface gives rise to a multilayered stratified epithelium, covered with a compact stratum corneum. Immunohistological studies reveal that the markers of epidermal differentiation are essentially distributed as in normal human skin and that the major proteins of the dermal-epidermal junction are present. Reconstruction of epidermis under identical culture conditions on a dermal equivalent that does not contain fibroblasts gives rise to an epithelium consisting of disorganized cell layers where the markers of differentiation are either displaced or not at all expressed, as is the case with filaggrin. In contrast, expression of the major constituents of the dermal-epidermal junction by the keratinocytes is completely independent of the presence of fibroblasts, even though it seems that the presence of fibroblasts is essential for the organization of the basement membrane proteins, creating a tight junction between dermal equivalent and the reconstructed epidermis.

The breakthrough for the serial cultivation of normal human keratinocytes, reported in 1975 by Rheinwald & Green (1), was achieved by co-culturing keratinocytes with mitotically inactivated fibroblasts which served as a "feeder layer". Studies of keratinocyte dependence on products from fibroblasts (2–4) indicated that epithelial cells in general may not be independent cell types.

Today, the molecular basis for the mitogenic activity that fibroblasts exert on keratinocytes in culture is only poorly understood. The presence of an endothelial cell specific growth factor in fibroblast-conditioned medium (5), and the identification of the keratinocyte growth-promoting activity of fibroblast-derived insuline-like growth factors (6), favour a classical paracrine mechanism. The fact, however, that fibroblast-conditioned culture medium has only little or no keratinocyte growth-promoting activity and does not substitute for a living feeder layer (1) questions a pure paracrine interaction. Yeager et al. (7) report that at least part of the fibroblast "feeder" activity involves a keratinocyte growth-promoting factor which is bound to the outer surface of the fibroblast plasma membrane.

In culture, fibroblasts do not only affect the proliferating activity of keratinocytes, but also their ability to undergo, at least in part, terminal differentiation (1, 4, 8). Kamalati et al.

(9) show that even the SV 40-transformed human keratinocyte line SV-K14, which is almost completely unable to differentiate under normal culture conditions (10), re-expresses markers of differentiation when co-cultured with fibroblasts.

The recently developed three-dimensional culture techniques of human keratinocytes on various dermal equivalents at the air-liquid interface give rise to a multilayered, stratified epidermis, covered with a compact stratum corneum (11). Unlike constructs in conventional immersed cultures, most of these constructs comprise two compartments: a dermal equivalent populated with fibroblasts and the reconstructed epidermis.

In vivo, epithelial differentiation is controlled by the supporting mesenchyme, and the effect of different components of the dermis on epidermal differentiation has been demonstrated during embryonic development (12). The different organotypic skin models provide an excellent means of studying dermal-epidermal interactions in vitro (7, 13–17).

Here we have used a chitosan cross-linked collagen-glycosaminoglycan matrix as dermal equivalent (18) to study in vitro the effect of normal human fibroblasts on the reconstruction of the epidermis and the composition and structure of the basement membrane zone.

MATERIAL AND METHODS

Preparation of the dermal substrate

Bovine collagen type I and III (SADUC, Lyon, France) was solubilized in 0.05 M acetic acid and mixed with chitosan (85% (w/v) deacetylated chitin extracted from shrimp shell. After homogenization, GAGs chondroitin-4 and chondroitin-6 sulfate (extracted from bovine cartilage) were added dropwise to the mixture. The final preparation was cast into sheets and lyophilized overnight and stored in 70% ethanol until use. The final composition was: 72% (w/v) collagen, 20% (w/v) chitosan and 8% (w/v) GAGs at 1.25% (w/v). Chitosan and GAGs were prepared by Dr. C. Gagnieu, INSA, Lyon, France.

Lamination of the dermal substrate (DS) was performed by using the same mixture as described above at a final concentration of 0.625% (w/v) containing 3% (v/v) dimethylsulfoxide.

Cells and culture medium

Foreskin fibroblasts (FF) derived from explants were subcultured in fibroblast tissue culture medium: Dulbecco's modified Eagle's medium (DMEM) (Flow Laboratories, UK) supplemented with 10% (v/v) bovine serum (PAA, Linz, Austria), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.5 mg/ml fungizone (Gibco Laboratories, Grand Island, N.Y., USA).

Normal human keratinocytes (NHK) were isolated from adult skin specimens (plastic surgery) by standard trypsinization overnight at 4°C in 0.25% (v/v) trypsin. Cells were cultured in keratinocyte culture
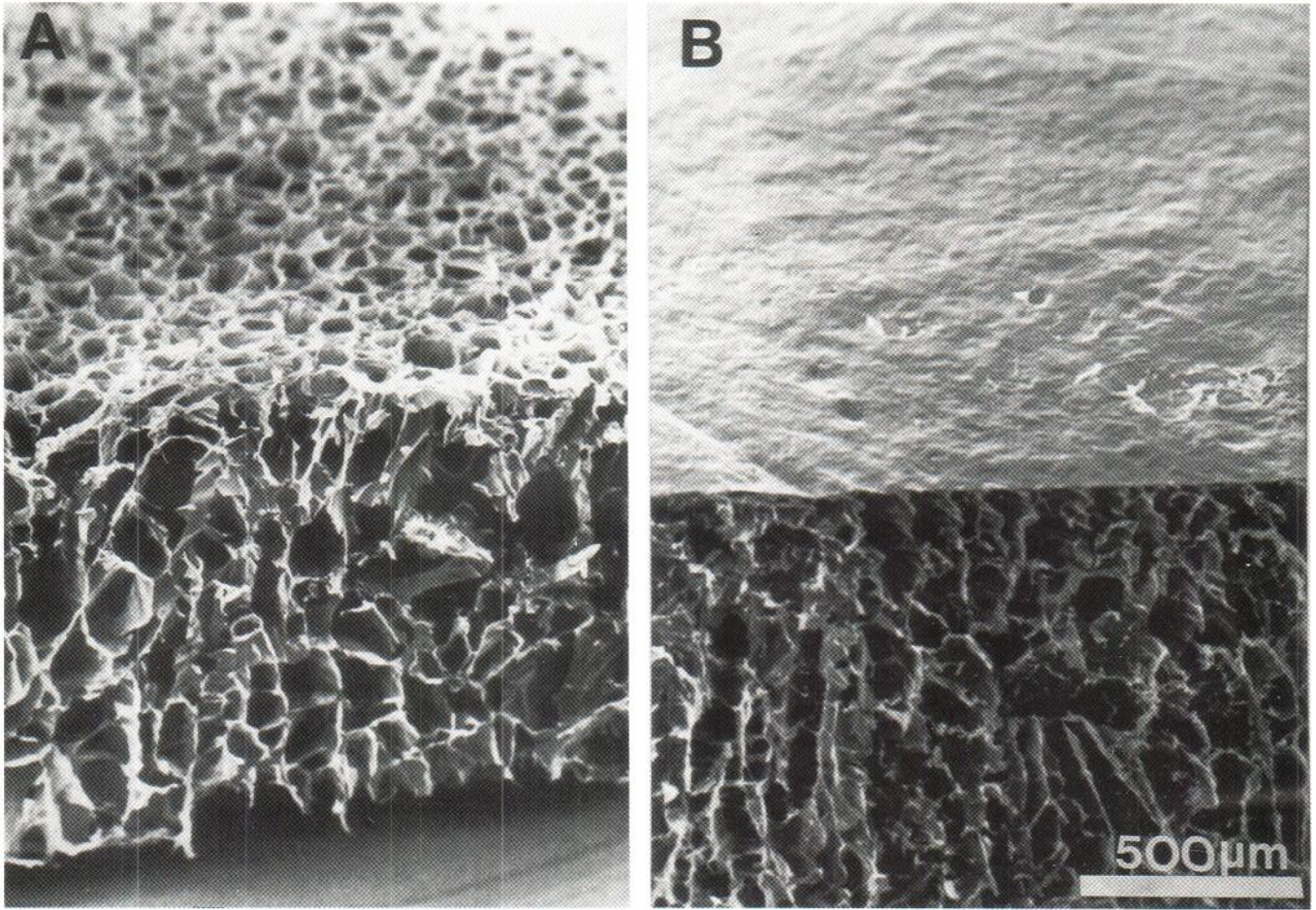
Fig. 1. Scanning electron-microscopic image of the collagen-GAG lattice before (A) and after lamination (B).

medium: DMEM/F12 (3:1) medium supplemented with 10% (v/v) fetal calf serum (Gibco) containing EGF (10 ng/ml), hydrocortisone (0.4 µg/ml), choleratoxine (10^{-10} M), transferrine (5 µg/ml) and tri-iodo-thyronine (2×10^{-9} M), adenine (1.8×10^{-4} M), insuline (5 µg/ml) and antibiotics as described above for FF. Cultures were incubated at 37°C, in a humidified atmosphere of 5% CO_2. The medium was changed 3 times a week. NHK were always used after the second passage for reconstruction of the epidermis.

Forty-eight hours after the keratinocytes had been seeded on the lattice, the cultures were lifted at the air-liquid interface and kept for 15 days in the above-described medium without choleratoxine, adenine, transferrine, and tri-iodo-thyronine. The medium was changed 3 times a week.

Electron microscopy

For scanning electron microscopy, dry samples of DS were sputter-coated with gold-palladium and observed under a Hitachi S 800 microscope to determine the pore size.

For transmission electron microscopy, samples were fixed in 2% (w/v) glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4) for 4 h at room temperature, rinsed overnight at 4°C in cacodylate buffer containing 0.2 M (w/v) glucose and then post-fixed in 2% (v/v) osmium tetroxide for 1 h, dehydrated in alcohol and embedded in epoxy resin. Ultra-thin sections were stained with uranyl acetate and lead citrate before examination with a Hitachi HU 120 electron microscope.

Histological studies

Samples were fixed in 10% formalin and embedded in paraffin. Sections of 6 µm were stained with hematoxylin-eosin-saffron and examined under a Zeiss IM 35 microscope.

Immunofluorescence studies

Unfixed samples were rinsed in phosphate-buffered saline, embedded in Tissue-Tec OCT compound (Miles, Naperville, Ill., USA), frozen in liquid nitrogen, and stored at −80°C until used.

Vertical frozen sections (5 µm) of tissue samples were processed for immunological studies. The mouse monoclonal antibody directed against the protein BM 600 (Nicein) was a generous gift of Dr. Ortonne (Nice, France). The other mouse monoclonal antibodies are commercially available: against human collagen IV (Serotec), fibronectin (Immunotech), and filaggrin (BTI). The monoclonal rabbit antibody against involucrin was obtained from Dakopatt.

For the immunofluorescence studies, Evans Blue (Sigma) staining was performed after reaction with antibodies. The fluorescein conjugate was purchased from Cappel Laboratories.

RESULTS

Dermal substrate

Fig. 1 shows the scanning electron-microscopic image of the collagen-GAG lattice before (Fig. 1A) and after lamination (Fig. 1B). After colonization of the dermal equivalent by fibroblasts, keratinocytes are seeded on the laminated surface of the lattice to prevent penetration of the cells into the dermal equivalent. The average pore size of the lattice is between 50 and 150 µm.

Histology

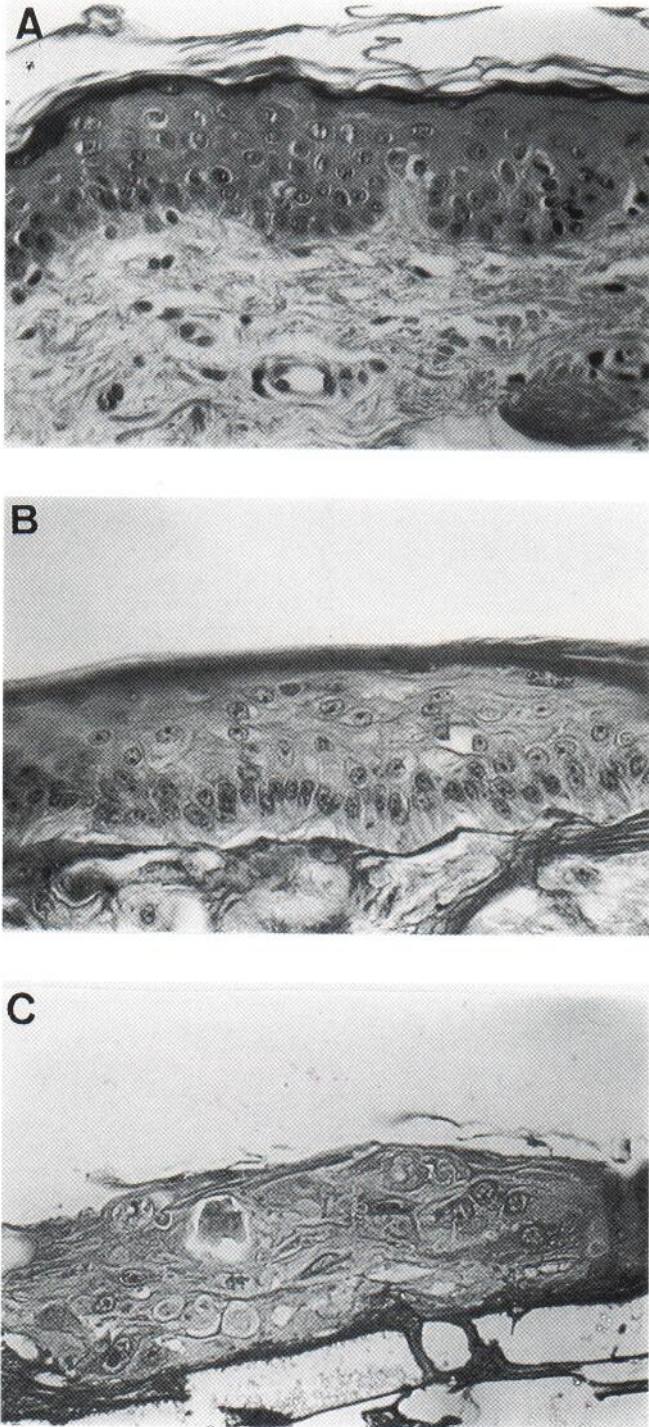Like normal human skin (Fig. 2A), reconstructed epidermis

Fig. 2. Histology of normal human skin (A) and epidermis reconstructed in the presence (B) and absence of fibroblasts (C).

cultured in the presence of fibroblasts (Fig. 2B) gives rise to a stratified epithelium with a basal cell layer and differentiated suprabasal layers covered with a stratum corneum. However, identical cultures performed in the absence of fibroblasts (Fig. 2C) exhibit a completely disorganized, non-differentiated epidermis without the characteristic cell layers. The stratum corneum is not existent.

Immunofluorescent studies

To characterize the architecture of the reconstructed epidermis, the degree of differentiation and the expression of constituents of the dermal-epidermal junction, cryostat sections of the epidermis reconstructed in the presence and absence of fibroblasts were stained with specific antibodies.

Fig. 3 reveals the effect of fibroblasts on the expression of two characteristic markers of epidermal differentiation, involucrin and filaggrin. In the presence of fibroblasts (Fig. 3C), involucrin expression starts in the suprabasal cell layers and reaches a maximum at the level of the stratum granulosum, the point where it becomes cross-linked into the cornified envelope. In the absence of fibroblasts (Fig. 3D), involucrin expression is observed throughout the multilayered, non-differentiated epidermal cell layers.

The expression of filaggrin seems to depend completely on the presence of fibroblasts in the dermal equivalent. Fig. 3E shows a distinct band of filaggrin underneath the stratum corneum of the differentiated epidermis cultured in the presence of fibroblasts, whereas in their absence (Fig. 3F) filaggrin expression seems to be completely suppressed.

Unlike their effect on the expression of epidermal differentiation markers, fibroblasts do not interfere with the expression of proteins characteristic of the dermal-epidermal junction. Fig. 4 shows the expression of collagen IV (Fig. 4A,B), fibronectin (Fig. 4C,D) and BM600 (Fig. 4E,F) in cultures with fibroblasts (Fig. 4A,C,E) and without fibroblasts. The same holds true for BP antigen (bullous pemphigoid), laminin and collagen VII (results not shown).

Electron microscopy

Even though fibroblasts do not interfere with the expression of the constituents of the dermal-epidermal junction, they seem to have a profound effect on their organization. Fig. 5 shows the electron-microscopic image of the dermal-epidermal junction in the presence of fibroblasts. It reveals the presence of numerous hemidesmosomes (*bold arrows*) along the dermal-epidermal junction with the formation of a lamina densa underneath. These structures as well as a tight adherence between the dermal equivalent and the reconstructed epidermis were only observed in the presence of fibroblasts.

DISCUSSION

The dermal equivalent, composed of chitosan cross-linked collagen-GAG with an average pore size of about 100 μm, provides an excellent environment for the growth and proliferation of fibroblasts. Seeded on the porous side, fibroblasts migrate into the lattice where they synthesize their own extracellular matrix. A minimum pore size is required, since a too tight texture would inhibit growth, proliferation and metabolic activity of the fibroblasts. The lamination of the lattice is essential to prevent the penetration of keratinocytes into the dermal equivalent.

Seeded on the laminated surface of the fibroblast-populated dermal equivalent, exposed to the air-liquid interface, the keratinocytes proliferate, differentiate and form a multi-
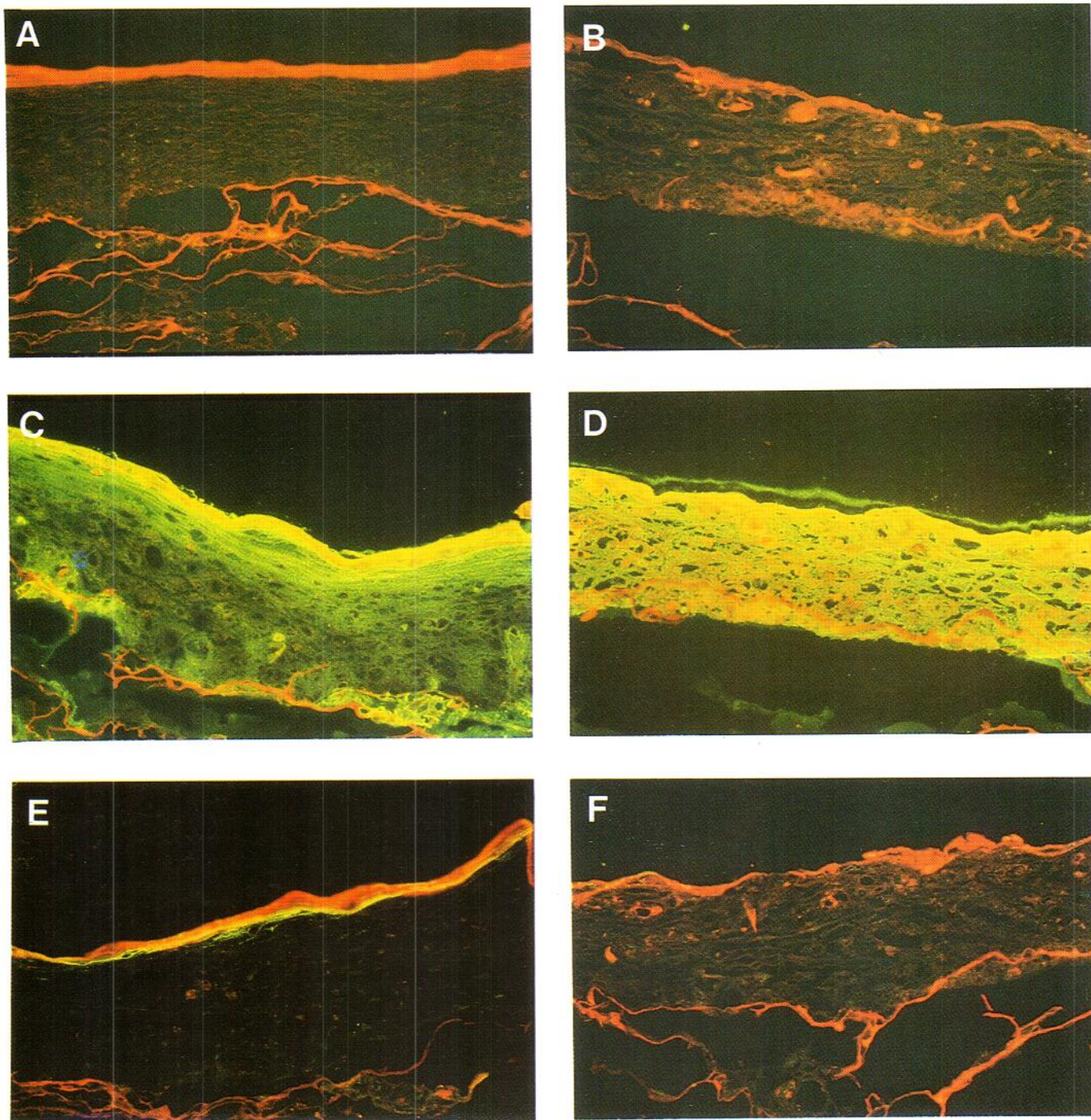
Fig. 3. Immunofluorescent staining of involucrin (C, D) and filaggrin (E, F) of epidermis reconstructed in the presence (A, C, E) and absence of fibroblasts (B, D, F). A and B are the controls obtained with non-immune serum.

layered stratified epithelium with a dense stratum corneum. Immunohistochemical studies reveal that the markers of epidermal differentiation are expressed and essentially distributed as in vivo. Beside filaggrin (Fig. 3E), this also holds true for the suprabasal keratin K10 (not shown). Expression of involucrin (Fig. 3C) and its cross-linking enzyme, the plasma membrane-associated transglutaminase TG_k (not shown), is detected already in the first suprabasal layers, whereas in vivo, expression starts in the upper spinous layers (19).

How strongly fibroblasts affect and possibly participate in

the epidermal differentiation becomes evident by analyzing the "epidermis" reconstructed under identical culture conditions but in the absence of fibroblasts. In this case the "epidermis" consists of several unorganized, keratinocyte layers without any stratum corneum. The markers of epidermal differentiation are either not at all expressed, as is the case with filaggrin, or their expression is not co-ordinated, as seen for involucrin.

These results provide further evidence for the important role of fibroblasts in the dermal-epidermal interaction. They
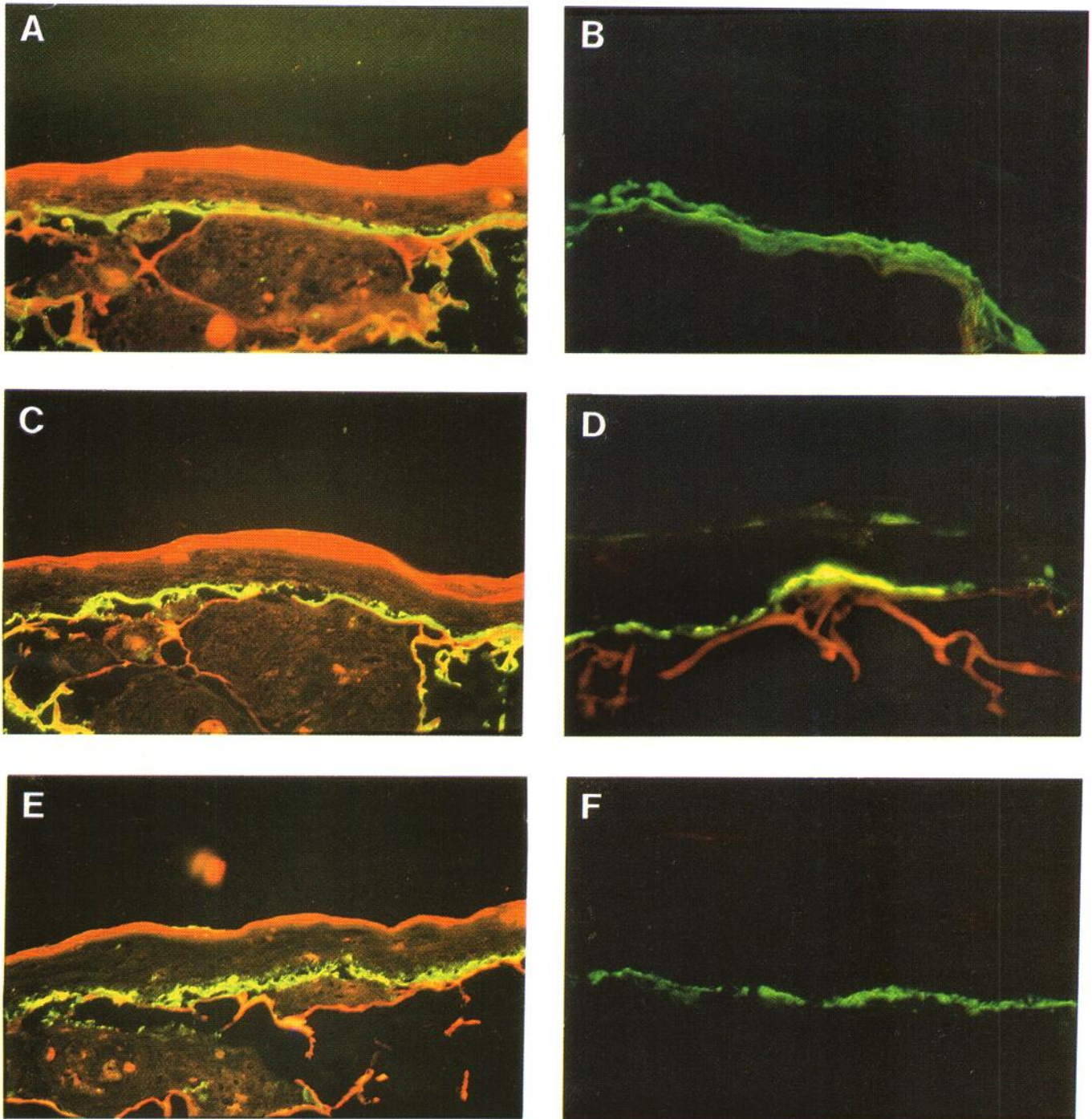
Fig. 4. Immunofluorescent staining of collagen IV (A, B), fibronectin (C, D), and protein BM6OO (E, F) in the dermal-epidermal junction of epidermis reconstructed in the presence (A, C, E) and absence (B, D, F) of fibroblasts.

are also in favour of a classical paracrine mode of interaction (5, 6), since fibroblasts and keratinocytes are not in direct contact but separated by the laminated layer.

These findings seem to be in contradiction with the fact that de-epidermized dead human dermis is an excellent, if not the best, support to reconstruct a fully differentiated epidermis in vitro (20). A possible explanation could be that after killing the fibroblasts by successive freezing and thawing, the human dermis still contains enough of the essential fibroblast-derived

factors which diffuse into the culture medium and assure the epidermal differentiation.

REFERENCES

1. Rheinwald JG, Green H. Serial cultivation of strains of normal human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell 1975; 6: 331–344.
2. Green H, Rheinwald JG, Sun TT. Properties of an epidermal cell type in culture: the epidermal keratinocyte and its dependence on products from fibroblasts. Prog Clin Biol Res 1977; 17: 493–500.

Fig. 5. Electron-microscopic image of the dermal-epidermal junction of skin reconstructed in the presence of fibroblasts. (*Arrows* indicate the formation of a lamina densa and *arrowheads* the structure of hemodesmosomes.)

3. Limat A, Hunziker T, Boillat C, Bayreuther K, Noser F. Post-mitotic human dermal fibroblasts efficiently support the growth of human follicular keratinocytes. J Invest Dermatol 1989; 92: 758–762.

4. Luker J, Crane IJ, Scully C, Prime SS. The effect of 3T3 fibro-blasts on the expression of anchorage independence and cornifica-tion of oral keratinocytes. Virchows Arch Cell Mol Pathol 1989; 57: 19–26.

5. Rubin JS, Osada H, Finch PW, Taylor WG, Rudikoff S, Aaron-son SA. Purification and characterization of newly identified growth factor specific for epithelial cells. Proc Natl Acad Sci USA 1989; 86: 802–806.

6. Barreca A, Giordano G, Cancedde R, Minuto F. In vitro para-crine regulation of human keratinocyte growth by fibroblast-derived insulin-like growth-factors. J Cell Physiol 1992; 151: 262–268.

7. Yaeger PC, Stiles CD, Rollins BJ. Human keratinocyte growth-promoting activity on the surface of fibroblasts. J Cell Physiol 1991; 149: 110–116.

8. Bohnert A, Hornung J, Mackenzie IC, Fusenig NE. Epithelial-mesenchymal interaction control basement membrane production and differentiation in cultured and transplanted mouse keratino-cytes. Cell Tissue Res 1986; 244: 413–429.

9. Kamalati T, McIvor Z, Howard M, Green MR, Brooks RF. Expression of markers of differentiation in a transformed human keratinocyte line induced by coculture with a fibroblast line. Exp Cell Res 1989; 185: 453–463.

10. Michel S, Schmidt R, Shroot B, Reichert U. Morphological and biochemical characterization of cornified envelopes from human epidermal keratinocytes of different origin. J Invest Dermatol 1988; 91: 11–15.

11. Schmidt R, vol. ed. Reconstructed human skin. Skin Pharmacol 1990; 3: 65–148.

12. Sengel P. Morphogenesis of the skin. Cambridge, London, New York, Melbourne: Cambridge University Press, 1976.

13. Coulomb B, Lebreton C, Dubertret L. Influence of human der-mal fibroblasts on epidermalization. J Invest Dermatol 1989; 92: 122–125.

14. Krejci NC, Cuono CB, Langdon RC, McGuire J. In vitro recon-stitution of skin: fibroblasts facilitate keratinocyte growth and differentiation on a cellular reticular dermis. J Invest Dermatol 1991; 97: 843–848.

15. Krueger GG, Jorgensen CM. Experimental models for psoriasis. J Invest Dermatol 1990; 95: 56S–58S.

16. Saiag P, Coulomb B, Lebreton C, Bell E, Dubertret L. Psoriatic fibroblasts induce hyperproliferation of normal keratinocytes in a skin equivalent model in vitro. Science 1985; 230: 669–672.

17. Shahabeddin L, Berthod F, Damour O, Collombel C. Character-ization of skin reconstructed on a chitosan-cross-linked collagen-glycosaminoglycan matrix. Skin Pharmacol 1990; 3: 107–114.

18. Sanquer S, Coulomb B, Lebreton C, Dubertret L. Human dermal fibroblasts modulate the effect of retinoids on epidermal growth. J Invest Dermatol 1990; 95: 700–704.

19. Michel S, Démarchez M. Localization and in vivo activity of epidermal transglutaminase. J Invest Dermatol 1988; 90: 472–474.

20. Regnier M, Asselineau D, Lenoir MC. Human epidermis recon-structed on dermal substrates in vitro: an alternative to animals in skin pharmacology. Skin Pharmacol 1990; 3: 70–85.