Role of Fibroblasts as the Principal Factor in Differentiation of the Epidermis

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The role of fibroblasts in the differentiation of the epidermis and its relationships with other somatic cells, specifically, keratinocytes, are studied using a skin equivalent formed from various types of fibroblasts separated from thoracic skin of a healthy donor, apparently healthy skin sites and plaques from a patient with psoriasis, keloids and upper eyelid skin of a normal subject, and keratinocytes separated from the umbilical skin of a newborn. Fibroblasts are shown to be active participants in the differentiation and formation of epidermis specificity. Various types of fibroblasts form a histologically different skin equivalent possessing the specific properties of the epidermis of the skin sites from which they were isolated.

Key Words: *fibroblasts; keratinocytes; skin equivalent; cell-to-cell cooperation*

Unraveling the mechanisms of cell-to-cell cooperation realized in the course of the immune response would definitely contribute toward solving problems in experimental and clinical immunology, dermatology, and transplantology. Disorders of this cooperation are interesting for pharmacology as well. Recent experimental and clinical findings have confirmed that fibroblasts (FB), which are both somatic and immunocompetent cells, play a key role in the mechanisms of interaction between immunocompetent cells and in the regulation of proliferation, differentiation, and migration of somatic cells [2-4].

However, these reports are often contradictory and superficial. This study was aimed at elucidating the role of FB as the principal factor in the specific differentiation of the epidermis.

MATERIALS AND METHODS

Human dermal FB and keratinocytes (KC) were used in the study. FB were separated from the thoracic skin of healthy donors and psoriasis patients and isolated from apparently healthy skin sites of patients with psoriasis. In addition, FB were isolated from keloids and upper eyelid skin of normal subjects. KC were separated from the umbilical skin of newborns or from the skin of adult donors.

Isolation of FB and KC from skin biopsy specimens. FB were isolated by two methods using 1) collagenase [5] and 2) slides: the derma was cut into 0.5 to 1.0 mm fragments, put in a 3-cm petri dish, covered with a slide, and incubated until FB started to migrate from the fragments onto the bottom of the dish. After 50% fusion was attained by trypsin treatment, the FB were transplanted to a flask.

In other experiments a preseparated (using the method described above) dermal component of the skin was placed in a culture flask with collagenase for 1-2 h of incubation. The cells were then repeatedly resuspended and washed in DMEM medium (analog of medium 199) with 10% bovine serum, and then centrifuged at 600 g/min (MLW) for 7 min.

In the next step the cells were transferred to special 75-ml plastic cuvettes, DMEM growth culture medium with 10% bovine serum was added, and the cells were incubated at 37°C with 5% CO_2 until they

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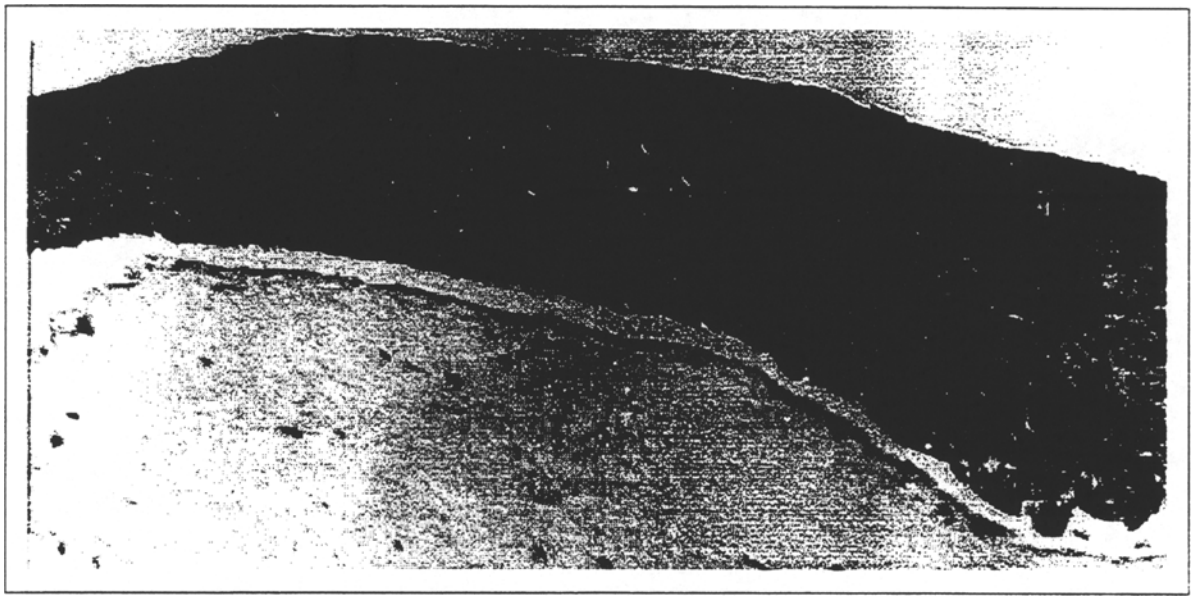

Fig. 1. Histology of SE formed of adult donor FB and neonatal KC. ×10.

covered 80-100% of the bottom of the cuvette. The cells were passaged by trypsin treatment.

For the skin equivalent (SE) the third or fourth passage of FB was used, when after trypsin neutralization the cells were resuspended and placed in Goryaev's chamber, counted, and then used for the preparation of artificial skin in a concentration of 200,000 cells per skin equivalent.

Keratinocytes were isolated from skin biopsy specimens with trypsin. The specimen was placed in 5% phosphate buffer saline with 2.5 mg/ml trypsin and refrigerated for 18 h. The separated epidermis was resuspended and neutralized with DMEM solution with 10% bovine serum, centrifuged at 600 g for 5 min, and the supernatant discarded. The cells resuspended to a solitary state were placed in special culture flasks with nutrient medium (Keratinocyte Growth Media, Clonetic) containing complement. The cells were incubated at 37°C and constant humidity in a 5% CO_2 incubator. The culture medium was replaced every three days. After a monolayer had formed, phosphate buffer saline containing 0.2% EDTA and 5% trypsin was added every day. The cells were then transplanted into a new flask. The first or second passage of KC was used.

Formation of SE. A skin equivalent is an organotypic tissue culture permitting the creation or modeling *in vitro* of tissue or an organ that is close to natural. SE makes it possible to study the differentiation, response reactions, and interactions between different cell types and the environment, making this

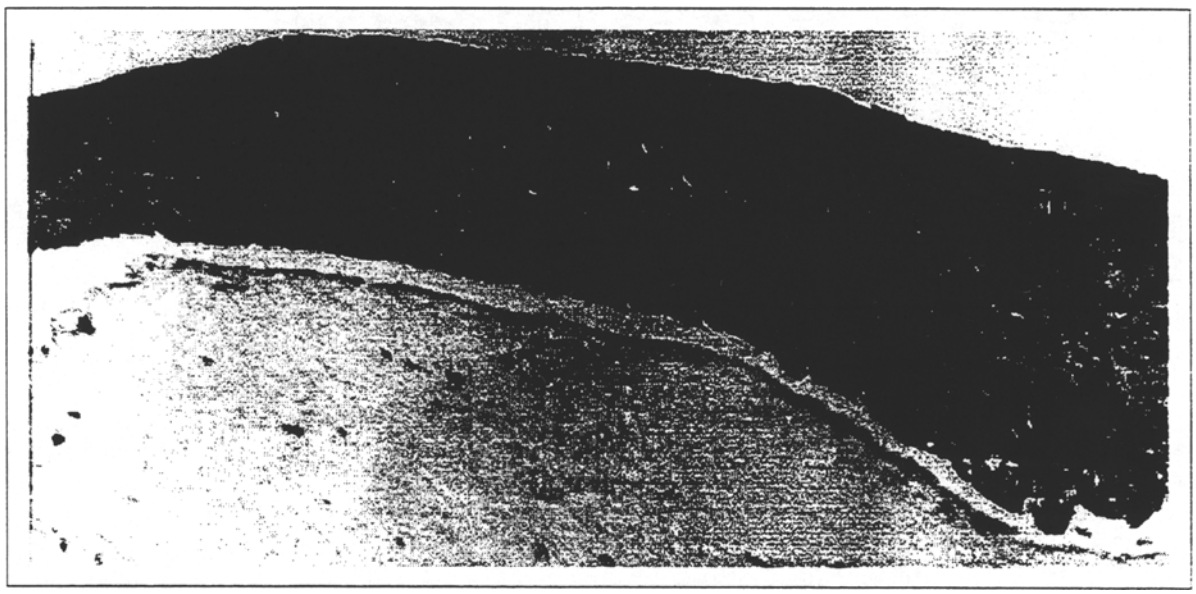

Fig. 2. Histology of SE including FB from keloids and neonatal KC (day 14). ×10.

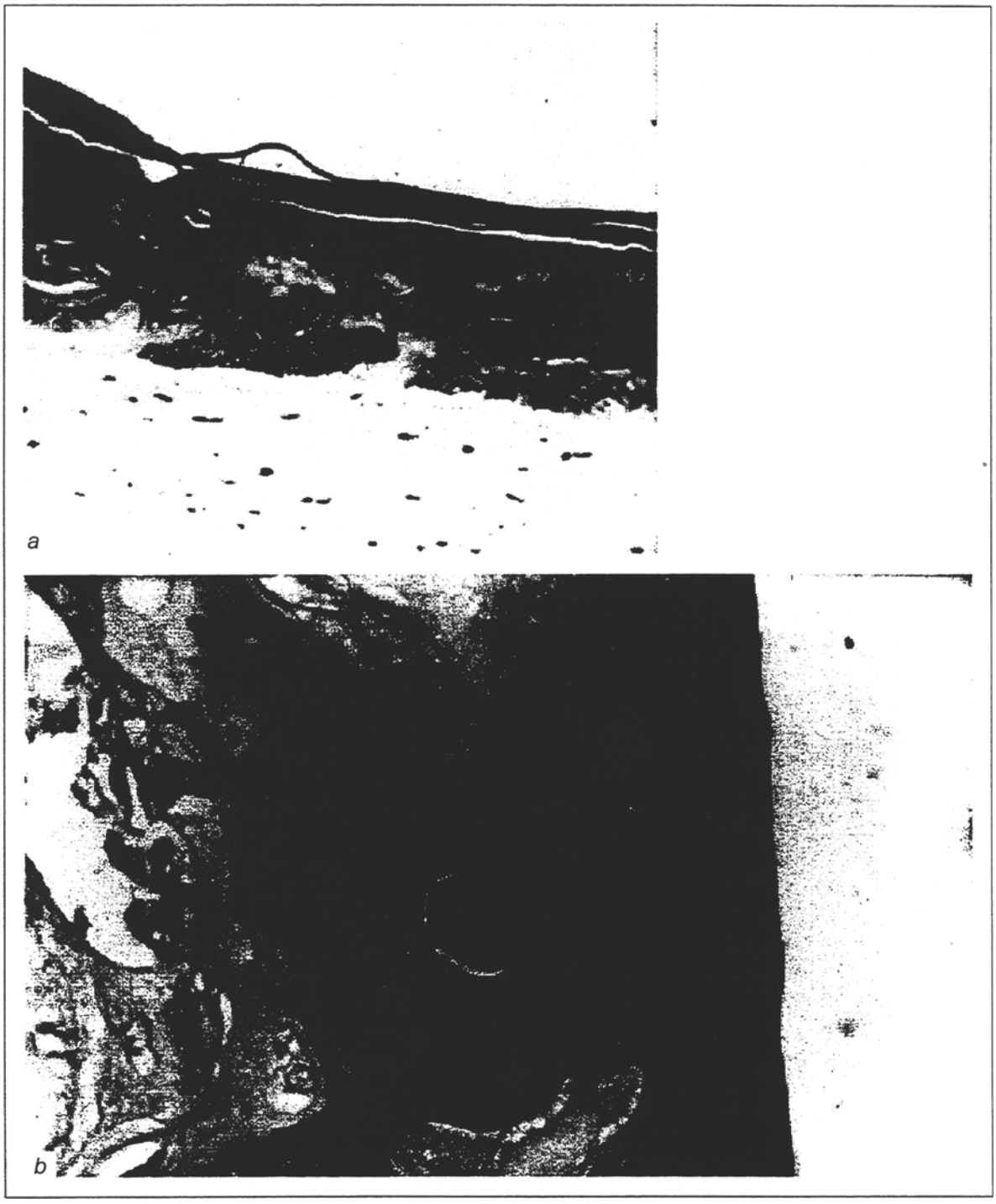

Fig. 3. Histology of SE including FB from normal upper eyelid skin of a donor and neonatal KC on days 14 (*a*) and 18 (*b*). ×10 (*a*), ×50 (*b*).

model similar to an *in vivo* tissue model. Dermal FB proliferating in type 1 collagen and surface-layered epidermal KC were used in one of the first organotypic experiments described for the skin, and this organotypic culture was dubbed "SE" [1]. Later other methods were employed, making use of different substrates and matrices for *in vitro* preparation of SE [4]. The principal difficulty arising in such experiments was and is the creation of conditions for the most favorable differentiation of the epidermis and selection of the dermal collagen matrix.

Maximal sterility of all manipulations and standardization of all the materials used, in our case FB and KC, are musts for the creation of SE.

We used the insert method representing our modification of the mesh method developed by M. Duvic *et al.* at the University of Texas. The preparation of SE starts with the preparation of an FB equivalent.

Fig. 4. Histology of SE formed of FB of involved skin sites from patients with psoriasis and neonatal KC (day 14). ×10.

To start out, a collagen solution was prepared, containing 10 ml type 1 collagen (Biocoat), 0.1 ml 10-fold DMEM, 0.011 ml penicillin-streptomycin sulfate, and 0.01 ml sodium bicarbonate. The resultant collagen solution was put on ice. Simultaneously, a suspension of dermal FB was prepared in a concentration of 1×10^6 cells/ml phosphate buffer saline. Resuspended cells were mixed with the collagen solution and poured into the inserts (1 ml) so that the final concentration of FB in the collagen was 200,000 cells/matrix. After collagen polymerization with FB, 1 ml FAD (Clonetic) was added for FB nutrition. The culture flasks with the cells were incubated at 37°C in 10% CO_2 for 24 h. The culture medium was not replaced over the entire course of formation of the FB equivalent.

For the formation of SE, 50,000 KC were applied to each FB component. One milliliter of medium was added to the lower part of the culture plaque every 24 h.

Histological analysis of SE. In order to ascertain the role of FB in the differentiation and rate of maturation of SE prepared by the two methods, histological analysis of SE was carried out on days 7, 10, 14, and 21 of its formation after preliminary staining with hematoxylin-eosin or methylene blue.

Before microtome slicing of SE, paraffin fixation was carried out, including special steps of SE exposure in 85, 90, and 100% alcohol, after which SE (in special holders) was placed in xeline for 2 h and fixed in paraffin. These paraffin-fixed blocks were used for subsequent histological analysis.

RESULTS

Figures 1-3 present 14-day SE formed using normal FB (Fig. 1), keloid FB (Fig. 2), and FB from the upper eyelid of a healthy donor (Fig. 3, *a*) and the same neonatal KC. The same KC are clearly seen to acquire different histological properties. When FB from the upper eyelid are used to prepare SE, keratinocytes form pseudoglandular formations (Fig. 3, *b*), which is not observed with keloid or normal SE. This difference is due not only to the capacity for the formation of individual SE layers, but to quantitative differences in the formed epidermis as well, including the basal, prickle-cell, granular, and horny layers.

Normal FB yield normal differentiation of KC, and the SE is represented by the dermal layer of the epidermis with clearly discernible basal layer, prickle-cell layer with stratum lucidum, and horny layer. The thickness of these layers varies for different types of FB and depends on the passage and the patients' age. Figure 1 shows epidermis with a well-expressed basal layer consisting of columnar cells forming a distinct line at the interface with the derma with dark oval or slightly elongate nuclei. These cells are closely connected to each other by their lateral surfaces. The horny layer is moderately developed.

Figure 2 shows an SE formed using FB isolated from keloids of adult patients and neonatal KC. The SE histology in this case exhibits signs of a keloid type of scar, which is represented by dyskeratosis or the inability of epidermal cells to undergo normal keratosis. The cells are disarranged and look like large, round formations segregated from one another; they have well-stained nuclei, a granular basophilic protoplasm, and small inclusions, such as remains of nuclei or pigment residues.

Figure 3 shows SE formed using FB separated from the upper eyelid of a healthy donor and neonatal KC. Histologic examination shows intensive invagination of the epidermis into the derma and glandular formations and a thin horny layer. This picture becomes more distinct by day 21.

When psoriatic FB are used (Fig. 4), the histologic picture of SE is highly abnormal. A specific feature of psoriasis is hyperkeratosis, or thickening of the horny layer [1], and in our case with an *in vitro* model it is difficult to determine the degree of hyperkeratosis, because the upper strata of the horny layer do not desquamate, as they normally do in real skin. However, hyperkeratosis is undoubtedly present in any case where psoriatic FB are used.

Another characteristic sign which we detected and which is presented in Fig. 4 is parakeratosis, a histologically typical feature of psoriasis. Parakeratosis is defined as the preservation of rod-shaped nuclei in the horny layer of the epidermis, which is seen in all SE created using psoriatic FB.

The third characteristic feature of psoriasis is acanthosis, or hyperplasia of the epidermis of the interpapillary space of the derma, which we detected during histological analysis of psoriatic SE. The fourth characteristic sign of psoriasis according to Lever is the absence or marked diminution of the granular layer, which is also seen in our model.

Hence, our histological findings permit us to assert that FB are active participants in the differentiation and formation of the specificity of the epidermis. FB taken from different body parts and proliferating in collagen matrix form on its surface an epidermis characteristic of the FB used in this matrix. In other words, the forming epidermis possesses the histologic signs characteristic of the FB used. Because the forming epidermis depends on the "specific" memory of FB and carries information characteristic of a particular disease, different FB form histologically different types of epidermis. Besides, SE, specifically prepared, can be a model for the study of wound healing.

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