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Cell kinetics in a model of artificial skin. An immunohistochemical and flow cytometric analysis

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SUMMARY

Bioengineered organs raised *in vitro* are candidate substitutes for natural organs in biological, pharmacological and clinical applications. We have studied cell kinetics in a human skin equivalent (HSE) using a combined immunohistochemical and flow cytometric approach. Morphological analysis has shown that, relative to unstimulated natural skin, cell proliferation mainly occurs in the basal layer of the epidermal equivalent. Immunohistochemical and flow cytometric measurements of the growth fraction suggested a cell turnover comparable to that of natural skin. Immunohistochemical labelling indices matched well with flow cytometric data. These observations are consistent with morphological and histochemical data demonstrating normal cell differentiation and tissue architecture in HSE and suggest that such HSE may be a usefull substitute for human skin.

INTRODUCTION

Long term subcultivation of keratinocytes provided the basis for the reconstruction of human epidermis *in vitro* (Rheinwald and Green, 1975). Further advances in culturing technology has permitted the production of different organotypic models that might be referred to as human skin equivalents (HSE; Bell *et al*., 1981; Asselineau *et al*., 1986; Dubertret, 1990; Parenteau *et al*., 1991, 1992). In such HSE the epidermis generated *in vitro* is combined with a connective tissue that may be considered a dermal equivalent, thus reconstructing the architecture of human natural skin. Nevertheless, the absence of several cell populations, including vascular cells, nervous cells, hair and secretory cells represents a major problem of these HSE.

Since bioengineered organs are designed to substitute natural organs in clinical, biological and pharmacological applications, it is important to clarify as much as possible the degree of similarity to corresponding natural tissues and organs. Although tissue architecture and cell differentiation in HSE appear similar to natural skin, little information is available concerning cell proliferation. Our aim was to study kinetic aspects in a model of HSE after completion of cell differentation.

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MATERIALS AND METHODS

Materials

Samples of human skin equivalent (HSE, Apligraf™, n=5) were kindly provided by Organogenesis Inc, MA, USA. HSE was constructed as described by Parenteau *et al*. (1991). Briefly, dermal fibroblasts and keratinocytes were derived from human neonatal foreskin. Passaged fibroblasts were mixed with bovine type I collagen to form a cellular lattice inside a culture insert. Keratinocytes were seeded on the surface of this lattice and grown to confluence while the culture remains submerged in the culture medium. The construct was then cultured at the air liquid interface to promote differentiation and cornification of the epidermis. After completion of epithelial cell differentiation, living HSE were incubated with bromodeoxyuridine (BrdU, 200 μM) for 2 h to label S phase traversing cells. Samples of normal human skin (n=3) were obtained from perilesional areas after skin punch biopsies, according to the ethical standards of the "San Matteo" Hospital, Pavia, Italy, where they were collected. These samples served as controls in our experiments.

Immunohistochemistry

Immunostaining. Specimens were fixed with 4% paraformaldehyde (w/v) in 0.1 M phosphate buffer, pH 7.4, for 6 h, dehydrated through graded concentrations of ethanol and routinely embedded in paraffin. Sections were obtained at 5-10 μm, rehydrated and stained with haematoxylin and eosin or processed for immunohistochemical staining according to the indirect streptavidin-biotin immunoperoxidase technique.

The sections were incubated serially with the following solutions: (1) 0.3% hydrogen peroxide for 30 min to remove endogenous peroxidase activity; (2) normal goat serum, diluted 1:20, for 30 min to reduce non-specific background staining; (3) mouse monoclonal antibodies to either Ki-67 antigen (BioGenex, San Ramon, USA) diluted 1:50, or BrdU (Becton Dickinson, Mountain View, CA, USA), diluted 1:30, overnight at 4°C; (4) biotinylated goat anti-mouse IgG (Super Sensitive kit, BioGenex, San Ramon, USA) for 1 h at room temperature; (5) streptavidin-biotinylated peroxidase complexes (Super Sensitive kit, BioGenex) for 1 h at room temperature; (6) 0.03%, 3,3'diaminobenzidine tetrahydrochloride, to which hydrogen peroxide (0.02%) was added just before use, for 5 min at room temperature. Each solution was prepared in 0.05 M *Tris* buffer, pH 7.4, containing 0.1 M NaCl (0.15M *Tris*-buffered saline) and between each step of the immunostaining procedure the sections were washed in the same buffer.

To allow the detection of BrdU by the corresponding antibody, the sections were immersed in 2.5 N HCl for 30 min at room temperature, thoroughly washed in borax buffer, pH 8.5, and treated with 0.5% pepsin in 0.01 N HCl for 30 min at 37°C.

To allow the detection of Ki-67 antigen by the corresponding antibody, the sections were treated with 0.2% trypsin, type III in Tris buffer, pH 7.8, containing 0.1% $CaCl_2$ for 10 min at 37°, and incubated in a microwave oven for antigen retrieval. Microwave heating was performed in an antigen retrieval solution (BioGenex) as irradiation fluid using a Panasonic microwave oven (model 4540, 900 W), as described previously (McCormick *et al*., 1993; von Wasielewski *et al*. 1994).

After immunostaining, some sections were lightly counterstained with haematoxylin. The sections were finally dehydrated, mounted and observed with a Zeiss Orthoplan microscope equipped with a Nomarski differential interference contrast device.

BrdU- and Ki-67-labelling indices were evaluated by two independent observers (AC, MC) by counting immunoreactive cells in three different sections of different samples (n=5).

Monoclonal antibodies and controls of the immunohistochemical reaction. Primary antibodies to Ki-67 antigen (clone MIB1, class IgG1) and BrdU (clone B44, class IgG1) have been previously characterized (Gratzner, 1982; McCormick *et al*., 1993). MIB1 antibody recognizes a wax resistant epitope of the Ki-67 nuclear antigen (345 and 395 kDa double band in Western blot analysis of proliferating cells) that is expressed in G1, S and G2-M phase traversing cells (Gerdes *et al*., 1984). In pulse-chase experiments, BrdU antibody recognizes DNA synthesizing, i.e. S phase traversing, cells.

Specificity controls included: 1) omission of the primary antibodies; 2) substitution of the primary antibodies with non-immune sera or monoclonal antibodies from the same immunoglobulin subclasses (Polak and Van Noorden, 1987). No immunostaining was observed after control procedures.

Flow cytometry

Samples of HSE were immediately trimmed in small pieces and immersed in either 80% ethanol at 4°C (10 min) followed by 0.1% Triton X-100 (15 min) or 4% paraformaldehyde for 5 min at room temperature.

Propidium iodide DNA staining. For single-parameter DNA measurements, cell suspensions were centrifuged and the pellet stained with a solution of propidium iodide (50 µg/ml, Calbiochem, San Diego, CA, USA), 0.1% Nonidet P40 (Calbiochem), and type 1A RNAse 50 Kunitz units/ml) in phosphate buffer for 30 min at room temperature. Finally, cells were filtered to remove aggregates prior to flow analysis.

Indirect immunofluorescence procedures. For BrdU-detection, single cell suspensions were pre-treated with 3 N HCl for 30 min at room temperature to denature DNA, and with 0.1 M sodium tetraborate for 15 min to neutralize HCl. Cells were incubated with BrdU-antibody B44, diluted 1:10 in phosphate buffer containing

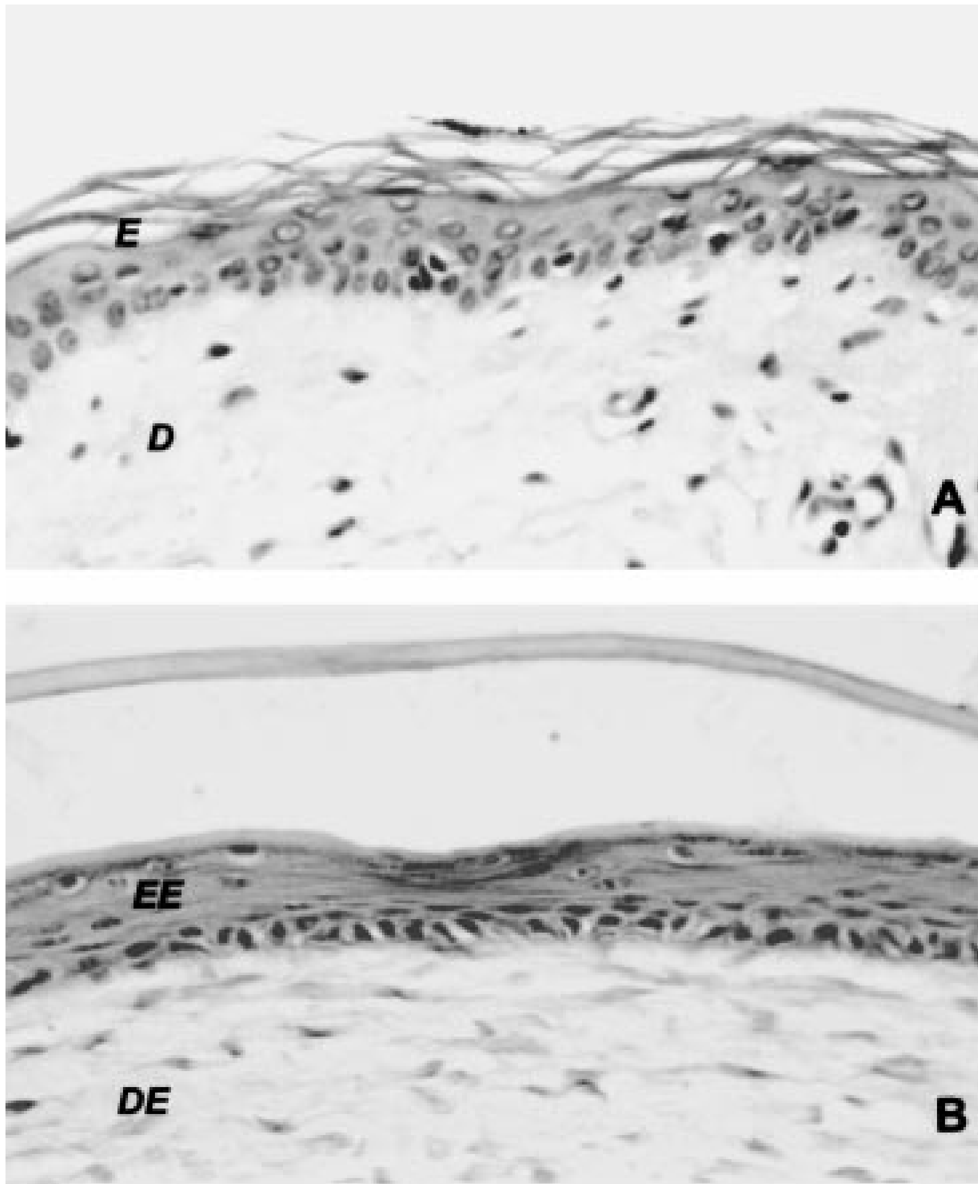

Fig. 1 - Tissue architecture of human natural skin (A) and human skin equivalent (B).
The stratified and keratinized epithelium in the skin equivalent (EE) is similar to natural epidermis (E).
The underlying dermal equivalent (DE) is composed of a loose connective tissue similar to natural dermis (D).
In the skin equivalent, glands, hairs and vessels and dermal papillae are lacking.
Haematoxylin-eosin staining. Magnification 300x.

0.5% normal goat serum for 30 min at room temperature. After two washes in phosphate buffer, the bound antibodies were labelled with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Dakopatts, Glostrup, Denmark), diluted 1:10 in phosphate buffer for 30 min. The samples were finally washed and counterstained with propidium iodide (5 µg/ml) for 30 min at room temperature.

Flow cytometry measurements. At least 40,000 cells were analyzed for each sample. Two parameter flow cytometry analysis (FITC-green vs. propidium iodide-red) of BrdU incorporation and of nuclear DNA content was performed with a FACStar Cell Sorter (Becton Dickinson). Data were collected with a Consort 30 software program running on a dedicated Hewlett Packard computer and displayed as dual parameter contour density plots. Single DNA analyses were performed with a Partec PAS II (Basel, Switzerland) arc lamp flow cytometer, with data displayed as frequency histograms.

RESULTS

HSE was composed by two distinct tissues, a stratified and keratinized epithelium and a loose connective tissue, i.e. the epidermal and the dermal equivalents, respectively (Fig. 1B). Within the epithelium, basal, spinous, granular and keratinized layers appeared similar to corresponding layers of natural epidermis (Fig. 1A,B). Within the dermal equivalent, only cells of the fibroblast lineage could be detected. No glands, hairs, vessels and nervous structures were recognizable (Fig. 1B).

The immunohistochemical detection of Ki-67 antigen and BrdU revealed positive cells only within the basal layer of the epidermal equivalent (Fig. 2 A,B). The immunolabelling was nuclear for both antigens. The labelling indices (+/- standard error) for BrdU- and Ki-67 antigen in the basal layer were 5.8 +/- 0.7 and 16.5 +/- 1.5, respectively. No positive cells could be detected in the dermal equivalent (Fig. 2 A,B).

The S phase dimension (+/- standard error) of HSE *in toto* (epidermal and dermal equivalents), as estimated by flow cytometric analysis of both DNA content and BrdU-incorporation, was 2.4 +/- 0.3 and 1.8 +/- 0.6, respectively (Fig. 3). The S phase dimension (+/- standard error), as estimated by immunohistochemical analysis of BrdU-positive cells, was 2.0 +/- 0.3.

No morphological evidence for cell damage, necrosis or apoptosis could be observed in any sample (Figs. 1B, 2A,B,C).

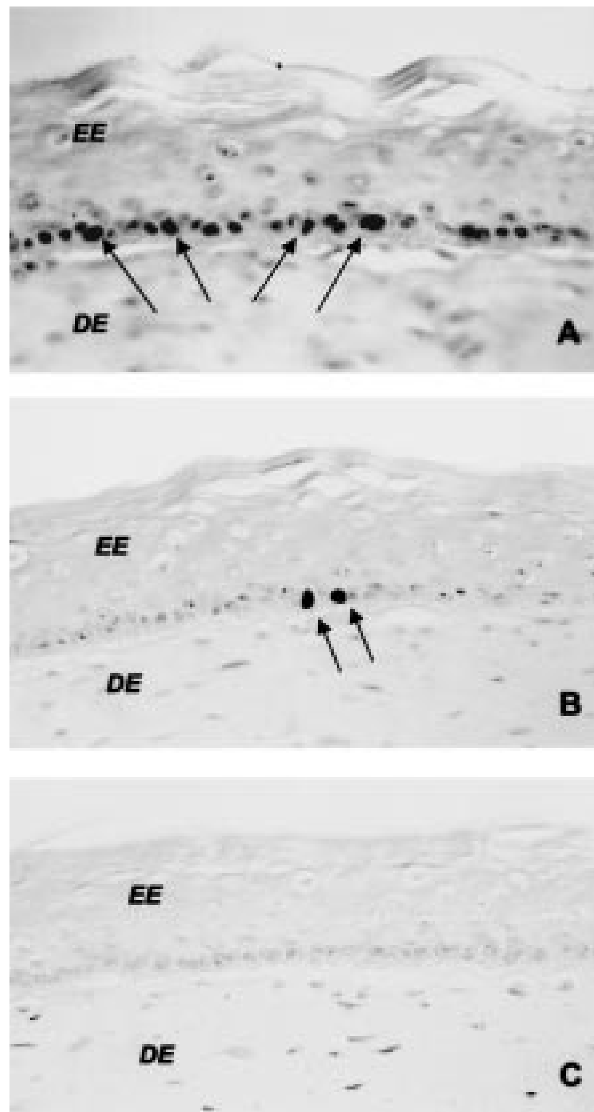

Fig. 2 - Immunohistochemical identification of replicating cells in human skin equivalent. Ki-67-antigen expression reveals G1, S and G2-M cells (arrows in A), whereas S phase cells are recognizable by BrdU-incorporation (arrows in B). Both proliferation antigens are expressed in the nuclei of some basal cells in the epidermal equivalent (EE). A control section of the immunohistochemical reactions is shown in C. DE, dermal equivalent. Magnification 400x.

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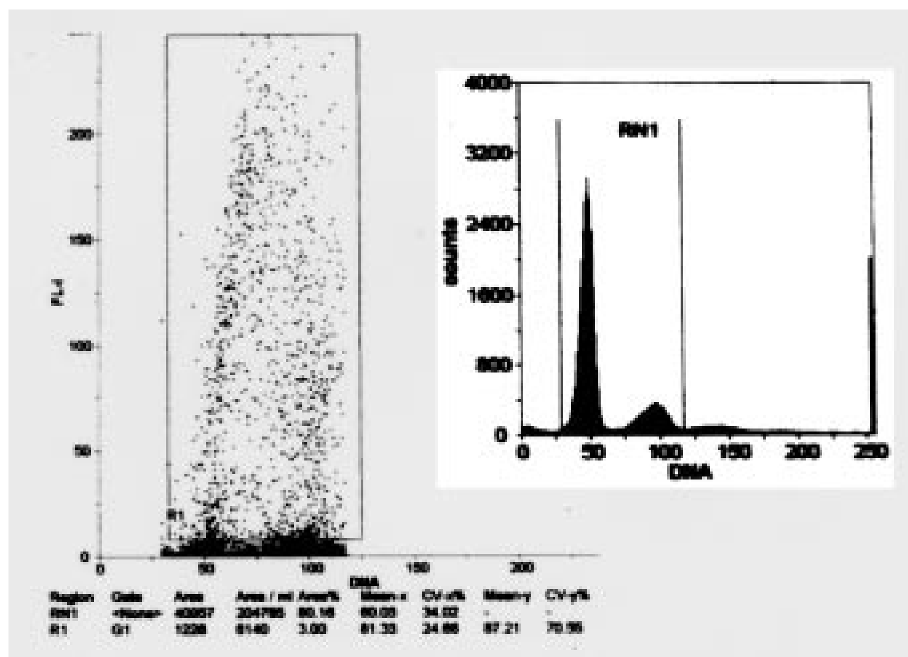

Fig. 3 - Evaluation of the growth fraction by flow cytometric analysis of S phase cells in one sample of human skin equivalent. The diagrams show the measurements of cell DNA content (right) and bromodeoxyuridine labelling (left).

DISCUSSION

Tissues and organs engineered *in vitro* may be interesting substitutes for natural counterparts in biological, medical and pharmacological applications. Since it is important to know the degree of similarity to corresponding natural organs, we have studied some aspects of cell proliferation in a model of human skin equivalent (HSE) composed of an epidermal equivalent and a dermal equivalent. It is important to point out that we have used a model of HSE after completion of cell differentiation, i.e. after epithelial stratification and keratinization. Previous morphological and histochemical studies have shown that cell differentiation in such HSE has occurred normally (Parenteau *et al*., 1992; Nolte *et al*., 1993). Moreover, successfull grafting in animals and humans has suggested that such HSE may be suitable for skin transplantation (Garlick and Taichman, 1994; Nolte *et al*., 1994; Falanga *et al*., 1998).

Our immunohistochemical analysis has revealed proliferating cells in the basal layer of the epidermal equivalent, whereas the cells of the dermal equivalent were found not proliferating. This is consistent with tritiated thymidine incorporation and proliferation antigen expression in natural skin under normal situations (Messier and Leblond, 1960; Clausen *et al*., 1980; McCormick *et al*., 1993; Andriessen *et al.*, 1995).

The growth fraction in the basal layer of the epidermal equivalent is comparable to the growth fraction in the basal layer of natural epidermis (Clausen *et al*., 1980; Potten, 1983). The number of Ki-67 antigen-positive cells exceeded the number of BrdU-positive cells. This observation agrees well with the distribution of these antigens in the cell cycle; in fact, in pulse labelling experiments, BrdU is incorporated almost exclusively in S phase cells, whereas Ki-67 antigen is expressed in all cycling cells, i.e. G1, S and G2-M-phase cells.

We have evaluated the S phase dimension in HSE equivalent *in toto*, i.e. epidermal and dermal equivalents. Flow cytometric values as determined by DNA propidium staining were consistent with those determined by BrdU incorporation and matched well with the immunohistochemical BrdU labelling index, despite the different number of cell counts obtained with the three techniques.

In conclusion, our histochemical data suggest that cell kinetics in a model of HSE are comparable to those observed in natural skin. These observations are consistent with morphological and histochemical data showing normal cell differentiation in this HSE, and support the view that engineered tissues may be candidate substitutes for natural organs.

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