Phenotypic characterization of keratinocytes migrated from polymer support – *in vitro* study

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The keratinocytes are able to migrate from the poly (2- hydroxyethylmethacrylate) disc if it is transfered to the new Petri-dish colonized with irradiated 3T3 mouse fibroblasts, and form a ring-shaped colony around the disc. The phenotypic characterization of human keratinocytes migrated from these discs was studied using a group of monoclonal antibodies. The keratinocytes in the external periphery of the colony of cells which migrated from the disc express the proliferating cell nuclear antigen (PCNA), $\alpha 2$, $\alpha 3$ chains and $\alpha 5\beta 1$ integrin receptor. A protein of the desmosome complex, desmoplakin-1, was also expressed. Involucrin and cytokeratin-10 were expressed after prolonged cultivation. These results suggest that the migrated keratinocytes are able to proliferate, recognize extracellular matrix molecules important in the process of the re-epitelization of the wound, and terminally differentiate *in vitro*. They are encouraging for further experiments with respect to the development of a support for keratinocyte cultivation and for grafting in clinical practice.

1. Introduction

Extensive burn injuries of the skin represent a very serious damage of the organism, frequently with fatal prognosis for the patient. The grafting of cultured autologous keratinocytes (KC) based on Green *et al.*'s results [1], is one of technologies available for the treatment of burn injury in clinical practice [2]. The cell culture is trypsinized and a sheet of KC on the flat gauze is grafted on to the wound [3]. This procedure is quite complicated from a clinical point of view, in respect of handling the KC during the grafting. The cultivation of autologous KC on a support which could be used for direct grafting and to cover a wound, should lead to much easier use in clinical practice. In our previous paper, we showed that KC can be grown on poorly adhesive poly (2-hydroxyethylmethacrylate) (poly HEMA) after its preincubation with bovine serum and, moreover, that KC is able to migrate from the poly HEMA disc to the Petri-dish precolonized with irradiated fibroblasts [4]. This *in vitro* model mimics quite well the wound surface, and should be used for the study of KC migration and differentiation before application of cultured cells on the synthetic polymer support to a patient in clinical trials.

The phenotypic characterization of KC migrated from the synthetic polymer support is demonstrated *in vitro* by a group of monoclonal antibodies which characterize the level of their differentiation in a submersed *in vitro* system. The proliferation potential of migrated KC was estimated by the detection of the proliferating cell nuclear antigen (PCNA). The integrin receptors ($\alpha 2$, $\alpha 3$, $\alpha 5\beta 1$) were visualized to estimate their contact with the extracellular matrix of the wound bed, and their terminal differentiation ability was evaluated by the detection of cytokeratin 10 (CK 10) and involucrin (inv). The desmoplakin 1 (DP-1) expression was used as a marker for the formation of intercellular contacts.

2. Material and methods

2.1. Polymer preparation

The poly HEMA discs, 25 mm diameter, were prepared in our laboratory and preincubated with 25% bovine serum for 24 h as described elsewhere [4].

2.2. Cell culture assay

The human KC were cultured on the surface of a poly HEMA disc according to a slightly modified Green *et al.*'s method [1, 5–7]. Briefly, the lethally irradiated (6000 rad) 3T3 mouse fibroblasts, density

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2×10^4 cells/100 mm^2, were used as feeders. The human KC (3×10^4 cells/100 mm^2) of first subculture were seeded 1 day later. The discs with KC at the level of confluent or semiconfluent growth, were inverted (the KC were on the lower surface of the poly HEMA disc) and transferred to a new Petri-dish with irradiated 3-T-3 fibroblasts; this mimics an adhesive surface of a wound bed [4]. The phenotypic characteristics of KC migrated from the poly HEMA support were studied during the course of 1 month after inversion.

2.3. Immunohistochemical characterization of KC

The proliferation potential of KC was evaluated by the production of monoclonal antibodies against PCNA. The monoclonal antibodies against the α5β1 integrin receptor recognizing fibronectin and invasin [8], α2 chain (HAS-4 antibody) of the integrin receptor (receptor for collagen I, II, III, IV, laminin [8]) and α3 chain (VM-2 antibody) of the integrin receptor (receptor for laminin, collagen, fibronectin, invasin, epiligrin [8]), were used to estimate the ability of KC to interact with molecules of extracellular matrix, which is important in the course of re-epithelization [9]. Desmoplakin-1 was used as a marker for the formation of the intercellular contacts. The terminal differentiation of KC was evaluated by the visualization of CK 10 and involucrin (antibody Sy-5), which normally occur in suprabasal layers of the epidermis [10, 11]. The antibodies against PCNA, α5β1 integrin receptor and CK 10 were obtained from DAKO, and the antibody against desmoplakin 1 from PROGEN.

The standard immunoperoxidase procedure was used for the immunohistochemical reactions. The swine anti-mouse serum labelled with peroxidase (SWaM-Px) as a second-step antibody, was obtained from ÚSOL (Prague) and the diaminobenzidine from SIGMA. The specificity of the reaction was verified by the incubation of specimens with non-immune mouse serum instead of the specific antibodies.

3. Results and discussion

The KC migrated after the transfer of the inverted poly HEMA discs with these cells to a new Petri-dish with irradiated 3-T-3 fibroblasts (Fig. 1) and formed distinct colonies. The migration of KC from both the human/pig skin recombinants [12] and poly HEMA discs [4] was described previously. The KC migrate from the poly HEMA discs to more adhesive surfaces very well, because hydrogels, including poly HEMA, are generally known as poorly adhesive supports [13]. The KC on the periphery of zone of the migrating cells, as well as KC in small colonies, exhibited PCNA in the nuclei. The KC in the centre of the colonies were usually PCNA negative (Fig. 1). This finding suggests a good proliferation potential of KC migrated from poly HEMA discs, because the PCNA expression is generally accepted, including cell biology of the skin [14], as an excellent marker of cell proliferation.

The KC on the periphery of the zone of migrated cells were positive for all tested integrins, i.e. α2 and α3

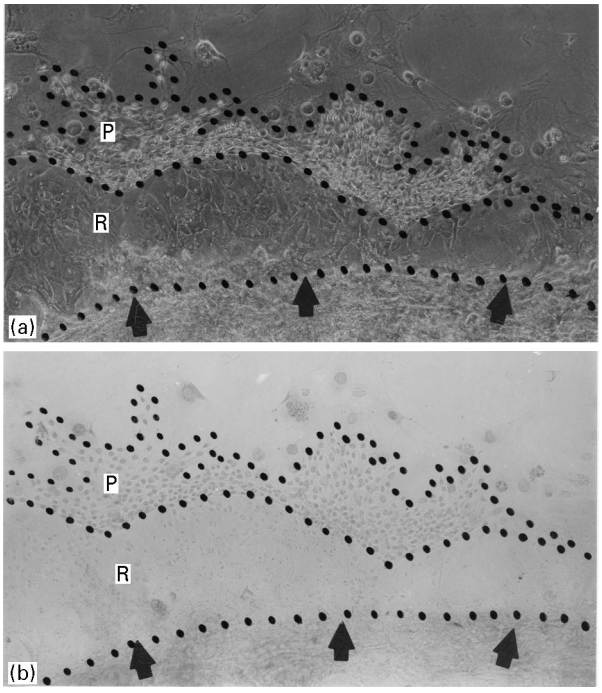

Figure 1 Migration of keratinocytes 3 d after the transfer of the inverted disc to the Petri-dish colonized with irradiated 3-T-3 cells. (a) Phase contrast microscopy, and (b) PCNA detection. The margin of the inverted disc is arrowed; R, zone of migrated but non-proliferating (resting) keratinocytes; P, zone of proliferating keratinocytes. Original magnification ×40.

chains or α5β1. The α2 chain of the integrin receptor was also highly expressed in mitotic KC. The cells in the centre of the zone of migration or the colony were negative, or almost negative, at an experimental time of 1 month (Fig. 2). The expression of the β1 chain of the integrin receptor is typical for KC of a basal cell layer [15] and β1 is highly expressed in KC with characteristics of the skin stem cells [16]. In native epidermis, the α2 and α3 chains of integrin receptors are usually expressed in the layer of basal cells [17]. The high expression of these integrin chains is typical for damaged skin, where highly positive KC of basal-layer cell type characteristics migrate from the periphery of the wound to its centre during the course of healing [9].

The desmoplakin-1 protein, which represents part of the desmosoms (important intercellular contacts of KC [18]), was expressed in colonies of KC. Although the positivity was observed intracytoplasmatically, the borders of the cells were only poorly positive or negative. The explanation of this phenomenon requires further observations, including immunoelectron microscopy.

The exceptional KC in the second layer were positive for involucrin, beginning in the second week of cultivation after the transfer of the inverted poly HEMA to a new Petri-dish with irradiated 3-T-3 (Fig. 4). At the beginning of the third week of inverted disc cultivation, multilayered colonies were characterized by moderate positivity of the majority of the cells for this protein. The cells on the surface of these colonies were strongly positive (Fig. 4). Involucrin is one of the markers of the terminal differentiation of KC [10, 19] and it is widely used in the cell biology of KC.

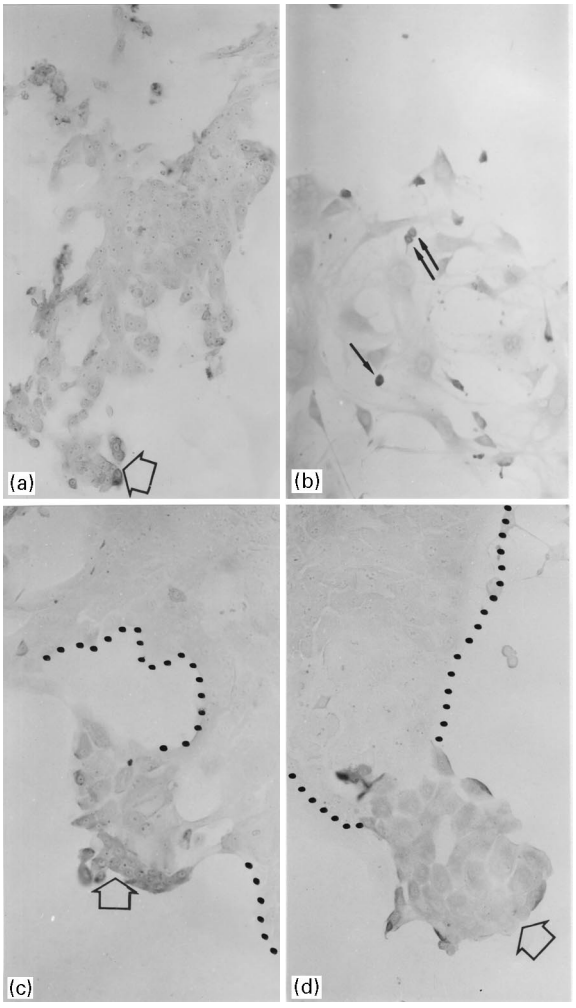

Figure 2 Expression of α2 integrin chain in the peripheral part of the zone of migrating keratinocytes (⇒); (b) highly α2 positive mitotic keratinocytes among the lethally irradiated 3-T-3 fibroblasts. The metaphasic cell is arrowed and telophasic cells are indicated by double arrows. Only the periphery of the zone of migrating keratinocytes is positive for (c) α3 integrin chain (⇒) and for (d) α5β1 integrin receptor (⇒). (…) The margin of negative or almost negative keratinocytes. Second week after the transfer of inverted disks. Original magnification × 90.

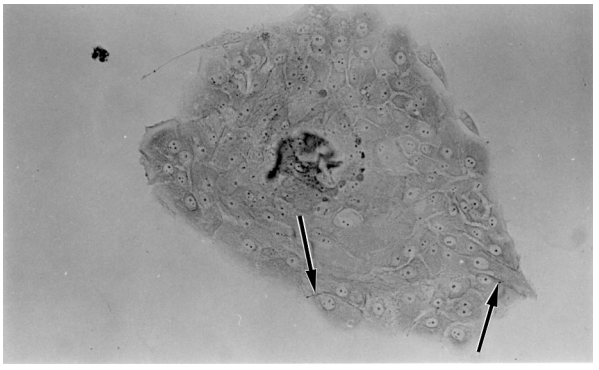

Figure 3 Desmoplakin-1 detection in a colony of keratinocytes. The cells express the cytoplasmic positivity. The exceptional accumulation of desmoplakin-1 in the intercellular contacts is indicated by arrows. Second week after the transfer of inverted discs. Original magnification × 90.

The KC positive for CK-10 were frequently observed from the third week of cultivation of inverted discs. The stratification of the expression of types of cytokeratins in the epidermis is good evidence of the

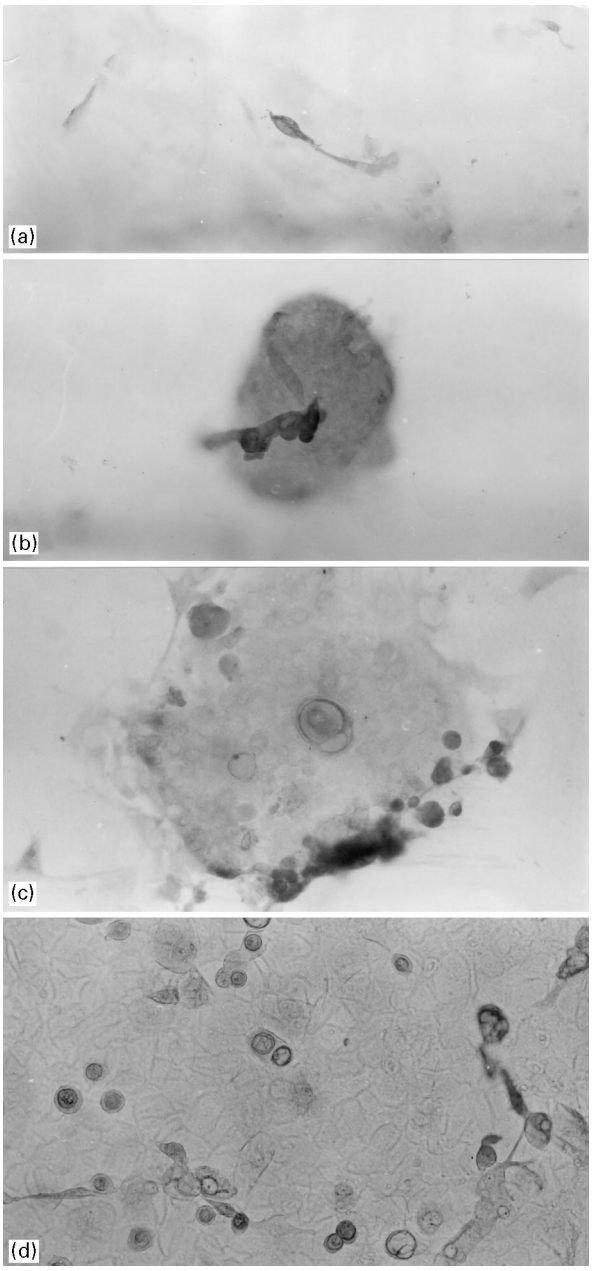

Figure 4 Involucrin detection in keratinocytes in (a) the second and (b) the third week after the transfer of inverted discs. The cytokeratin-10 positive keratinocytes are seen (c) in a small colony and (d) in a culture with subconfluent growth of keratinocytes, in the third week after the transfer of the inverted discs. Original magnifications: (a, b) × 40 and (c, d) × 90.

specific gene activity which is reflected in the functional phenotypic differentiation of KC in the skin [20]. The CK-10 is expressed strictly suprabasally and is a good marker of irreversible growth arrest in the programme of cellular terminal differentiation [11].

In conclusion, the KC which migrated from the inverted poly HEMA discs adhere to the adhesive surface and proliferate. They express the receptors for the interaction with an extracellular matrix and they are able to terminally differentiate. These results encourage the continuation of these experiments with the perspective of clinical application.

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