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Cultivation of human keratinocytes without feeder cells on polymer carriers containing ethoxyethyl methacrylate: in vitro study

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Abstract The cell/tissue engineering therapy of extensive or chronic skin wounds is a highly topical task of the contemporary medicine. One of possible therapeutic approaches is grafting of in vitro cultured keratinocytes directly to the wound bed, where the cells colonize the wound, proliferate and improve the re-epithelization process. Because the successful cultivation of keratinocytes needs an application of feeder cells, the exclusion of these cells from the cultivation system is highly required. In this study we show a positive influence of 2-ethoxyethyl methacrylate as a component of cultivation support on growth of keratinocytes without feeder cells. Keratinocytes cultured on these surfaces are able to migrate to the model wound bed in vitro, where they form distinct colonies and have a normal differentiation potential.

Introduction

The skin defects of traumatic and/or metabolic nature are a serious medical, social and economic problem [1]. Grafting of cultured keratinocytes on cultivation carriers of different structure and nature is a new therapeutic strategy consisting in transferring these elements to the skin defect [2, 3]. The cultured cells migrate from these supports to the wound and colonize the wound bed improving the re-epithelization. Unfortunately, keratinocytes usually need feeder cells for initial attachment and growth, which makes employment of cultured keratinocytes problematic from the point of view of clinical application. Exclusion of feeder cells from the system is possible using special media and growth supplements [4, 5] or by immobilization of bioactive ligands (for example mannose clusters) to cultivation surface [6]. However, these approaches are expensive and therefore not suitable for large-scale keratinocyte expansion in clinical practice. In the course of systematic study of biological properties of synthetic polymers as potential carriers for keratinocyte cultivation, we observed that poly(2-ethoxyethyl methacrylate) [poly(EOEMA)] stimulates the growth of human keratinocytes under in vitro conditions without feeder cells. Because keratinocytes must be able to colonize the surface of the wound bed [2, 7], we studied the process of cultured cell migration from a synthetic polymer carrier containing EOEMA units in model situation [8], including the ability of their differentiation by keratin immunocytochemistry.

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Materials and methods

Polymer preparation

The polymerization was carried out in a mould formed by polypropylene plate, a packing distance frame made from silicone rubber (0.5 mm) and a glass plate. A mixture of monomers consisted of 2-ethoxyethyl methacrylate

(EOEMA) (Sigma-Aldrich, Prague, Czech Republic) or 2-hydroxyethyl methacrylate (HEMA) (Degussa-Röhm, Darmstadt, Germany), crosslinker hexane-1,6-diol dimethacrylate (HDM) (Sigma-Aldrich, Prague, Czech Republic). The composition of polymer mixture is described in Table 1. The monomer mixture was bubbled with nitrogen for 2 min and the dissolved gases were subsequently removed in an ultrasound bath for 10 min.

The mould, firmly closed with screw clamps, was first filled with nitrogen and then with a monomer mixture. The UV-initiated polymerization (benzoin ethyl ether, BEE—concentration 0.5 wt.%) proceeded for a period of 1 h at room temperature using a mercury lamp Tesla RVK 125 W (Tesla, Prague, Czech Republic). The mould was located 15 cm from the UV lamp. The obtained samples were transparent films with an area of 8×8 cm and thickness corresponding to the thickness of used silicone seals.

Samples were swollen in distilled water to equilibrium and washed in fresh distilled water for 7 days. The efficiency of washing was checked by measurements of absorbance and pH of the aqueous extract against fresh washing water. Both values were in accordance with the requirements for polymer materials used in medicine. Gas chromatography showed monomer residues in concentrations of 0.4%, no initiator residues were found.

After washing, the samples were dried at room temperature for 3 days. Every sample was prepared in a clean room of class A.

Characterization of prepared (co)polymers was supplemented by methanol extraction (200 ml) at the boil for 48 h). The weight loss after methanol extraction was 11.8%. The extract was reduced to 1 mL and analyzed by SEC in THF on a PL MIX C column using an RI detector. In the extract were found only oligomers (M_w = 42,600, polydispersity index 1.79). In contrast, the weight loss after aqueous extraction was 0.3%. The facts mentioned above show that a part of hydrophobic EOEMA oligomers is not linked to the three-dimensional (co)polymer structure through chemical bonds, but it is impossible to extract it with water.

Table 1 Composition, water content and mechanical properties of copolymers

HEMA (%mol.)	EOEMA (%mol.)	Stress at break (Mpa)	Strain (%)	Water content (%)
0	100	1.34	344.55	1.6
10	90	1.57	308.62	2.4
20	80	1.74	273.02	3.7
40	60	1.58	195.27	8.6
60	40	1.29	182.35	14.8
80	20	0.71	115.88	24.3
100	0	0.43	94.63	33.0

(Co)polymers HEMA/EOEMA, 0.3 mol % of crosslinker HDM

Measurements of swelling in water

Polymer samples (1×0.5 cm) swollen to equilibrium in distilled water were freed of excess water and weighed on an analytical balance. Then the samples were dried at room temperature to constant weight. The water content was calculated as the ratio of the difference of weights of swollen and dry gel to the swollen gel weight in %.

Measurements of mechanical properties

Tensile stress at break and strain were measured according to ISO 37 standard with an Instron 5800 testing machine (Instron, High Wycombe, UK) at a speed of 10 mm/min and force 0–10 N. The tested samples were prepared from swollen films. Dog bone-shaped samples were 0.5 mm thick and with 4×30 mm working area.

Contact angle measurements

Contact angle measurements were carried out on a Tensiometer K 12 (Krüss GmbH, Hamburg, Germany) using the Wilhelmy plate method. Contact angles were measured with the following polymers: poly(HEMA), poly(HEMA-*co*-EOEMA) 40/60 and poly(EOEMA).

Biological properties

Keratinocytes were obtained from breast human skin from the Department of Aesthetic Surgery (Charles University, 3rd Faculty of Medicine, Prague) with informed consent of donors. Small pieces of skin were treated by 0.3% trypsin (Sigma-Aldrich, Prague, Czech Republic) solution at 4 °C overnight. First subculture of keratinocytes was provided in culture flasks (Nuncleon, Nunc, Denmark) in presence of mitomycin treated (Mitomycin C, Sigma-Aldrich, Prague Czech Republic) 3T3 cells (30,000 cells/cm^2). The cells were cultured in H-MEM (Academy of Sciences of the Czech Republic, Prague, Czech Republic) with 10% bovine (ZVOS, Hustopeče, Czech Republic) and with 2% foetal calf (Biochrom, Berlin, Germany) serum supplemented with epidermal growth factor, hydrocortisone, choleratoxin (Sigma-Aldrich, Prague, Czech Republic) and insulin (Novo Nordisk, Denmark) at 37 °C and 3.3% of CO_2 for 7 days. The residual feeder cells were enzymaticly removed and the keratinocytes were harvested again by trypsinization. Study of growth and migration of cells was performed with polymer discs (20 mm in diameter) prepared from poly(HEMA), poly(EOEMA) and

HEMA–EOEMA copolymers with the molar monomer ratios 80/20 and 60/40. Keratinocytes were seeded in the density 30 000 cells/cm^2 and cultured without feeder cells or (in the case of poly(HEMA)) in the presence of mitomycin-treated 3T3 feeder cells, for 7 days using the modified procedure of Green and co-workers as described above [2, 9, 10]. The growth of cells was estimated using the MTT test [8].

The wound repithelization was modelled in six-well plates (Nuncleon, Nunc, Denmark) by procedure schematically shown in Fig. 1. The wound bed was simulated by the wells with subconfluent net of feeder cells. The discs with cultured keratinocytes (in case of polyHEMA precolonized with feeder cells these were removed by mild trypsinization as described [2]) were transferred to these wells in the upside-down position. The control experiments were performed in wells without 3T3 cells. After 3 days, the discs were gently removed from the bottom of the wells and the attached cells were cultured for another 7 days. The tri- or quadruplicates of each polymer type were tested. The keratinocytes cultured on polymer discs or in model wound were evaluated by phase contrast microscopy (Nikon, Prague, Czech Republic) or after staining according to Giemsa-Romanowski. The affiliation of cells migrated from discs to keratinocyte lineage was estimated using immunocytochemical detection of panel of cytokeratins (except 1, 8, 19) by Ck1 = LP34 monoclonal antibody recognizing all differentiation types of keratinocytes. Normal differentiation was visualized by detection of cytokeratin 10 (CK10), a generally known marker of the terminally differentiated keratinocytes, using CK10 monoclonal antibody. Both antibodies were purchased from Dako (Brno, Czech Republic) and diluted as recommended by the supplier. The FITC-labelled swine anti-mouse immunoglobulins (SwAM-FITC, ALSEVA, Prague, Czech Republic) and peroxidase-labelled swine anti-mouse immunoglobulins (SwAM-Px, ALSEVA, Prague, Czech Republic) were employed as a second-step reagent in dilution 1:30. The Sigma-Fast^{TM} system containing diaminobenzidine tetrahydrochloride (Sigma, Prague, Czech Republic) was used to visualize the reaction product SwAM-Px treated samples. The FITC-labelled samples were observed in an Optiphot-2 microscope (Nikon, Prague, Czech Republic) equipped with a special filter block, CCD camera (COHU) and computer-assisted image analysis system LUCIA (Laboratory Imaging, Prague, Czech Republic). The control immunohistochemical reactions were performed with antibody anti-CD1a (Immunotech, Prague, Czech Republic) not occurring in human keratinocytes to exclude the non-specific binding of antibody to cells preferentially via Fc receptors [10].

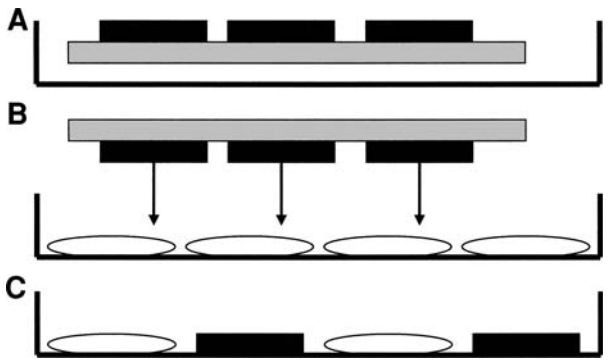

Fig. 1 Schematic presentation of study demonstrating migration of cultured keratinocytes (*small black quadrangles*) from polymer discs (*large grey quadrangle*) to Petri dish precolonized with 3T3 fibroblasts (*white ellipses*). Keratinocytes were cultured on experimental discs (**A**). They were transferred in upside-down position to dish with precultured 3T3 feeder cells (**B**). They migrated from discs and formed colonies in 3T3 precolonized wells (**C**)

Results and discussion

Swelling and mechanical properties of the studied materials in relation to their composition are shown in Table 1. Both the water content and mechanical properties were dependent on the HEMA content in tested materials. While the water swelling increased with increasing monomer content, the mechanical properties deteriorated with increasing HEMA content. Surface properties such as wettability of the tested polymers can be estimated from the contact angle measurements. Advancing and receding contact angles are shown in Table 2. The measured angles, in particular receding angles, correspond to the water content in each group of samples. Wettability decreases in the order: poly(HEMA) > poly(HEMA-*co*-EOEMA) 40/60 > poly(EOEMA).

Monitoring the possible influence of EOEMA on growth of keratinocytes we used a system where keratinocytes were cultured on poly(HEMA) with no EOEMA and with 3T3 feeder cells as a standard reference experiment, because it enables the good growth of keratinocytes [2, 6]. Surface of pure poly(HEMA) with no EOEMA and without feeder cells was colonized by a very low number of poorly spread keratinocytes (Fig. 2). The increasing concentration of EOEMA in the copolymers with HEMA positively influenced the number of keratinocytes although no feeder cells were present in the system. Content of 20% of EOEMA in copolymer induced the same growth of keratinocytes as was observed in poly(HEMA) without EOEMA but with feeder cells and all other higher concentrations of EOEMA in cultivation support significantly improved keratinocyte growth in comparison with polyHEMA precolonized with feeder cells used as standard. After 7 days the surface of poly(EOEMA) was colonized with a nearly

Table 2 Contact angle measurement

Time (min)	Poly(HEMA)		Poly(HEMA-*co*-EOEMA) 40/60		Poly(EOEMA)	
	Θ_a	Θ_r	Θ_a	Θ_r	Θ_a	Θ_r
0	46.80°	27.76°	79.01°	27.30°	80.41°	16.50°
30	54.30°	27.78°	79.54°	30.28°	82.26°	14.14°
60	55.23°	24.01°	82.42°	32.23°	85.60°	14.24°
90	55.96°	23.57°	82.14°	30.73°	85.79°	17.17°

Θ_a, advancing contact angle;

Θ_r, receding contact angle;

Surface tension of the used distilled water was 74.1 mN/m

confluent or confluent monolayer of cultured keratinocytes with extensive intercellular contacts (Fig. 2).

It is well known that the surface properties of a synthetic polymer material such as electric charge, hydrophobicity and surface energy significantly influence adsorption of plasma proteins and adhesion of cells that recognize their active epitopes by specific receptors such as integrins [for a review, see 11]. EOEMA is a hydrophobic monomer and, therefore, changes in surface properties of polymer carriers can be expected with increasing concentration of this monomer in cultivation support. This was verified by measurements of the wettability of tested samples. Interestingly, the keratinocyte growth without feeder cells on these supports containing EOEMA but without bioactive component was almost the same as on a polymer containing bioactive mannose clusters [6].

Cultured keratinocytes must be able to migrate from the cultivation carrier; where they colonize the wound bed and proliferate to improve the re-epithelization [7]. The same phenomenon was also observed in the case of keratinocytes cultured on polyHEMA with or without feeder cells, poly(HEMA-*co*-EOEMA) and poly(EOEMA). After transferring of experimental discs containing adherent keratinocytes (feeder cells were removed from discs by mild trypsinization when they were employed as feeder) in upside down position to the dishes precolonized with 3T3 cells, the keratinocytes migrated from the polymer discs and formed distinct colonies (Fig. 3). In comparison with all other tested discs, the number of colonies was very low when polyHEMA discs without feeder cells were used. The results obtained by conventional cytological staining of keratinocytes were verified by immunohistochemical detection of cytokeratins, cytoskeletal elements expressed by keratinocytes and never by fibroblasts (Fig. 3). This indicates that cells migrated from the discs to dish precolonized by fibroblasts are, really, keratinocytes. The expression of cytokeratin 10 as a specific marker of terminal differentiation of keratinocytes [12] confirmed the

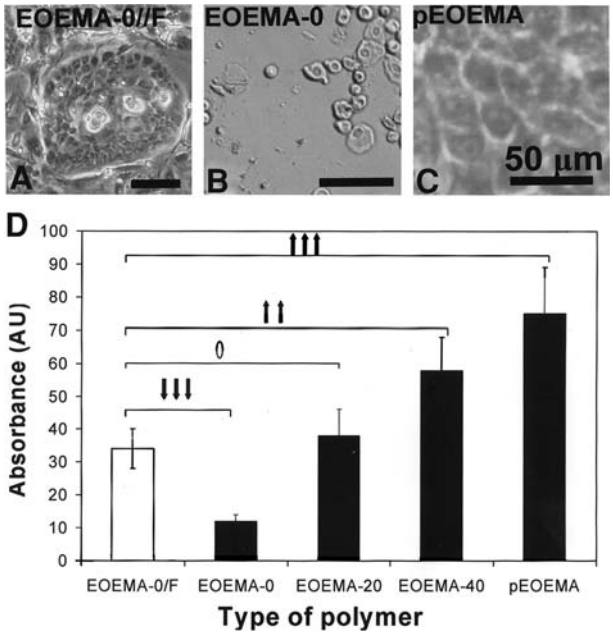

Fig. 2 Monolayer of human keratinocytes cultured on poly(HEMA) without EOEMA precolonized with 3T3 feeder cells (**A**, EOEMA-0/F), on poly(HEMA) without feeder cells and without EOEMA (**B**, EOEMA-0) and on poly(EOEMA) without feeder cells (**C**) on day 7 of cultivation in culture system (phase contrast microscopy). Comparison of cell growth on surface of poly(HEMA) without EOEMA and with feeder cells (EOEMA-0/F, white column), on the same material but without feeder cells (EOEMA-0), on supports with increasing concentration of EOEMA (EOEMA-20,-40) and on poly(EOEMA) (EOEMA-100) with no feeder cells on day 7 of cultivation (**D**). While keratinocyte growth was statistically significantly lower ($p = 0.03$, three arrows) in polyHEMA (EOEMA-0) without feeder cells than in co-culture containing these element, the cultivation support containing 20% of EOEMA with no feeder exhibited the similar cell growth properties as pure p(HEMA) precolonized with feeder cells (0, EOEMA-20). Increasing concentration of EOEMA in the support significantly stimulated the growth of keratinocytes even in comparison with p(HEMA) precolonized with feeder cells at $p = 0.03$ (three arrows) and $p = 0.05$ (two arrows). The unpaired Student *t*-test was used for evaluation. The number of cells was estimated from the light absorbance measured using the MTT test as described in Material and methods

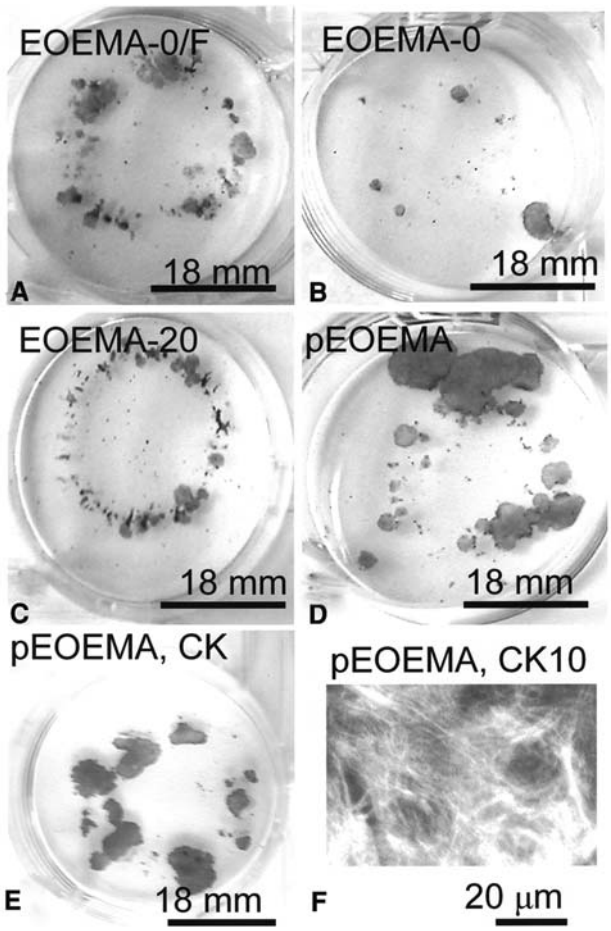

Fig. 3 Keratinocytes migrated from poly(HEMA) without EOEMA and with feeder cells (**A**, EOEMA-0/F), from the same material but without feeder cells (**B**, EOEMA-0), from copolymer of HEMA with 20% of EOEMA without feeder cells (**C**, EOEMA-20) and poly(EOEMA) without feeder cells (**D**) to 3T3-precolonized tissue culture dish. These cells migrated from all types of discs exhibit panel of cytokeratins (**E**) and cytokeratin 10 (**F**). Giemsa-Romanowski staining (**A**–**D**), bar lengths are 18 mm and 20 µm, respectively

normal differentiation potential of cultured elements (Fig. 3). This observation is not in contradiction with the observation of Ziegelaar and coworkers [13], who observed the positive influence of increasing EOEMA content in the HEMA copolymers on the collagenase production and activity by corneal cells in comparison with neat poly(HEMA), which suggests a normal cell differentiation. The in vitro procedure for study of cell migration seems to be important from the general point of view because it permits to reduce the number of experimental animals [14]. The possibility of exclusion of feeder cells from the cultivation system on the surface of poly(HEMA-*co*-EOEMA) or poly(EOEMA) cannot be explained on molecular level at this stage of investigation because other surfaces suitable for cell cultivation (for example tissue culture grade of polystyrene) do not support the cultivation of keratinocytes without feeder cells under described conditions. Moreover, it is known that hydrophobic surfaces (for example bacteriological grade of polystyrene) are not suitable for growth of cells because of the adsorption of biological bioactive molecules in inappropriate conformation for the interaction with cell surface receptors [for review see 11]. The hypothetical response can be obtained according to Vroman effect [15] on the adsorption adhesive molecules from protein cock-tail, their mutual interactions and interactions with the synthetic surface. However, the precise characterization of these needs further experiments in this field.

In conclusion, although the development of bioactive tailor-made biomaterials is one of the main tasks of contemporary medicine, this study indicates that materials facilitating adhesion and growth of distinct cell population by nonspecific mechanisms can be employed in medicine. Simplicity and a low charge of these systems are main advantages of these materials.

Conclusion

New support for cultivation of human keratinocytes without feeder cells using standard media was demonstrated in this study. Cultured keratinocytes normally differentiate and they are able to migrate from these supports to the model wound bed. This procedure is cheap and therefore it seems to be suitable for cell culture of these cells and perspectively for cell therapy of skin defects.

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