

Y Chromosome and Vimentin Used to Trace the Fate of Allogeneic Keratinocytes Delivered to the Wound by the Recombined Human/Pig Skin

(allogeneic keratinocytes / xenodermis / RHPS / wound healing / Y chromosome / FISH / vimentin)

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Abstract. RHPS, composed of confluent allogeneic keratinocytes cultured on cell-free pig dermis, stimulates wound healing when applied with the keratinocyte layer facing the wound. So far it has not been clarified whether the confluent keratinocytes implanted 'upside-down' can 'take' or only stimulate healing by producing growth factors. Confluent male keratinocytes were grafted onto donor sites of three female patients. Biopsies were taken on days 4, 6 and 9 after grafting. The fate of donor cells was followed in paraffin sections by FISH for the Y chromosome and by persisting expression of vimentin taken as a marker of cultured keratinocytes. Histological evaluation was complemented by detection of keratin 10 and involucrin. All three donor sites healed within one week. On day 4 the early neoepidermis was multilayered but disordered after transplantation. A large proportion of cells were apparently of donor origin as indicated by the presence of Y chromosomes, irregular morphology, expression of vimentin in the bottom and upper layers of the neoepidermis, and by irregular expression of involucrin and keratin 10 only in the central layer of the neoepidermis. From day 6 onwards, the new epidermis acquired an ordered stratification. Involucrin and keratin 10 renewed normal distribution in suprabasal layers. Concomitantly, vimentin expression was decreasing. The Y chromosome was still found on day 6 but not on day 9. We concluded that confluent allogeneic keratinocytes temporarily 'take' to the wound and contribute to rapid wound closure, being replaced by the patient's epidermal cells after about one week.

Different types of tissue-engineered skin were among the first *in vitro* prepared tissues to be used in medicine. Cultured keratinocyte sheets (Rheinwald and Green, 1975; Green et al., 1979) have been used to heal burns (O'Connor et al., 1981) and other types of wounds (Hefton et al., 1986; Carter et al., 1987; Leigh et al., 1987; Gallico et al., 1989) since 1981. Autologous as well as allogeneic keratinocytes have a strong healing effect (Madden et al., 1986; Bolivar-Flores et al., 1990; Leigh, 1994). However, there are several problems preventing cultured keratinocyte sheets from routine usage. Epidermal sheets must be enzymatically released from the dish prior to application, they are very fragile and difficult to handle. Their 'take' had been highly variable, ranging from 0–100%.

A membrane delivery system was considered as a promising support for keratinocyte cultivation and their transport onto the wound (Barlow et al., 1992; Harris et al., 1998). Keratinocytes were grown on a membrane that could be peeled from the dish without enzymatic treatment and applied to the wound with the membrane up. Autologous keratinocytes cultured on a synthetic polymeric membrane in a serum-free medium formed distinct colonies from which the cells migrated to the wound surface, creating epithelium islands (Barlow et al., 1992).

For more efficient delivery of epidermal cells we developed recombined human/pig skin (RHPS). It is composed of allogeneic human keratinocytes cultured on the pig dermis (allo-RHPS) that is extracted, dried and free of pig cells and retroviruses (Matoušková et al., 1993). We prefer allo-RHPS, because allogeneic keratinocytes can be prepared in advance and kept in culture for immediate use. The 3T3 feeder layer technique was adopted in order to obtain large quantities of keratinocytes (Rheinwald and Green, 1975; Green et al., 1979). Such an approach made it possible to produce confluent outgrowth of keratinocytes on sizable pieces of xenodermis for wound treatment (Matoušková et al., 1993; Matoušková et al., 1997). The RHPS applied with keratinocytes facing the wound ('upside-down') remarkably stimulated healing of donor sites and deep dermal burns (Matoušková et al., 1997).

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Abbreviations: FISH – fluorescence *in situ* hybridization, RHPS – recombined human/pig skin.

It has been an open problem whether the confluent keratinocytes grafted 'upside-down' can change orientation and temporarily 'take', i.e. attach to the wound, proliferate and incorporate into the regenerating epidermis, or whether they stimulate epithelization solely by producing growth factors. Only pre-confluent proliferating keratinocytes were expected to be capable of successful 'take' (Barlow et al., 1992; Harris et al., 1998). The vertical reorientation of confluent differentiating cells from the inverted position was considered unlikely (Harris et al., 1998).

Several methods used sex-mismatched keratinocytes to determine the fate of cultured allografts. Brain et al. (1989) established by *in situ* hybridization for the Y chromosome that the allogeneic keratinocyte sheets did not survive application onto full-thickness wounds. In donor sites and other deep dermal beds allogeneic keratinocytes appeared to survive for less than a week when assessed by *in situ* hybridization (Burt et al., 1989; De Luca et al., 1989; Phillips et al., 1990) or DNA fingerprinting (van der Merwe et al., 1990), and for up to 3 months when assessed by the sensitive PCR technique (Zhao et al., 1992). The second marker of cultured keratinocyte 'take' is the expression of vimentin in the epidermis, as vimentin is not expressed in the normal healing skin, while it is expressed in cultured keratinocytes (Moll et al., 1998).

For elucidation of the RHPS healing mechanism, male keratinocytes grown to confluency on xenodermis were applied 'upside-down' onto donor sites of female patients. Early biopsies were evaluated by histology, immunohistochemistry for vimentin, keratin 10 and involucrin, and by fluorescence *in situ* hybridization (FISH) for the Y chromosome.

Material and Methods

Preparation of RHPS

Keratinocytes were obtained from the redundant skin of a 20-year-old male donor (screened for HIV, BWR and hepatitis A, B, C) after plastic operation. RHPS was prepared as described previously (Matoušková et al., 1993). Briefly, thin strips of pig skin (routinely used as a temporary cover of burns) were trypsinized to remove epidermis and fibroblasts. The dermis was thoroughly washed in water and by drying adhered to a 90-mm tissue culture dish. Male secondary keratinocytes were grown on xenodermis to confluence using the 3T3 feeder layer technique. The medium used was Eagle's MEM with Hanks' salts supplemented with all non-essential amino acids, 0.12 g/l sodium pyruvate, 1 g/l NaHCO₃, 0.5 µg/ml hydrocortisone (Sigma, St. Louis, MO), 5 µg/ml insulin (NOVO Nordisk, Bagsvaert, Denmark), 10⁻¹⁰ M cholera toxin (Sigma, St. Louis, MO), 5 ng/ml epidermal growth factor, 10% bovine serum (ZVOS Hustopeče, a.s., Uherčice, Czech

Republic) pretested for optimal growth of 3T3 cells, and 2% foetal bovine serum (Sigma, St. Louis, MO).

Patients, grafting with RHPS and biopsies

The Ethical Committee of the 3rd Medical Faculty of Charles University Hospital approved the study. Informed consent was obtained from all three patients. In three young women with full-thickness burns on 10–20% body surface area, freshly excised donor sites on the thigh (i.e. deep dermal wounds) were grafted with RHPS on an area of 50 cm². The recombined skin was peeled off the dish, placed 'upside-down' (keratinocytes facing the wound) on the wound bed and covered with vaseline gauze and dry gauze. The graft adhered to the wound and stopped bleeding immediately. From the first patient a punch biopsy (3-mm dia) was taken 4 days, from the second patient 6 days and from the third patient 9 days after grafting. Normal male skin was used as a control. The biopsies were fixed in 4% buffered formaldehyde (pH 7.4) for 24 h and then embedded in paraffin. Sections of 5-µm thickness were mounted on silanized glasses.

Fluorescence *in situ* hybridization

The paraffin sections were dried for 24 h at 57°C, deparaffinized by xylene 3 x 5 min, dehydrated by 99% ethanol 2 x 5 min and dried 2–5 min at 45–50°C. Forty min incubation at 95–99°C in the target retrieval solution DAKO® (DAKO A/S, Carpinteria, CA) was followed by 10 min postfixation in 4% formaldehyde. The CET Y-satellite III DNA SpectrumOrange probe (Vysis, Downers Grove, IL) was used for hybridization, according to the manufacturer's instructions. A Nikon microscope and the LUCIA programme (Laboratory Imaging, Prague, Czech Republic) were used for digital imaging.

Immunohistochemical staining

The paraffin sections were deparaffinized, dehydrated, dried, digested in DAKO® and fixed in formalin in the same way as applied for FISH. Immunostaining was performed using the Universal biotin-streptavidin peroxidase-AEC detection system (Immunotech, Marseilles, France). The primary antibodies used were Vik 10, 10 µg/ml (Exbio, Prague, Czech Republic) against keratin 10, VIM-13.2, 10 µg/ml (Sigma, St. Louis, MO) against vimentin, and anti-involucrin SY3 (involucrin is expressed in the granular and horny layer of the normal skin) (Hudson et al., 1992). The BH-2 Olympus microscope and SC 35 Olympus camera were used for taking photographs.

Results

Freshly excised donor sites of 3 female patients were grafted with confluent male keratinocytes grown on cell-free pig dermis. Re-epithelization of the wound, incorporation of donor keratinocytes, morphology and immunohistochemical characterization of the newly