

similar to those obtained with involucrin. The Y-chromosome visualization is demonstrated in Fig. 2. The healing effect of allo-RHPS is overviewed in Table 1.

Characterization of the course of epidermis regeneration

Day 4: As soon as four days after grafting, a multi-layered epithelium was developed on most of the grafted area (Fig. 1/A1, B1). The newly created epidermis was irregular and of variable thickness (Fig. 1/A, B). The healing process depended on local wound condition given by the presence or absence of adnexa remnants.

In the areas where RHPS adhered directly to the adnexa remnants, the allogeneic keratinocytes quickly stratified, as is visible in multilayered, already dried epithelium in the covering RHPS (Fig. 1/A2). The donor keratinocytes were in less than four days replaced by proliferating and migrating recipient cells originating from the adnexa remnants (Fig. 1/A1, A2). In these areas the new epidermis was fully developed and did not contain irregular cells, neither did this part of the section express any Y-chromosome signals. Like in normal skin, vimentin was negative (Fig. 1/A3) and keratin 10 (not shown) was expressed in suprabasal layers. Involucrin, which in normal skin is expressed in upper

spinous and granular layers, was also detected in deeper suprabasal layers (Fig. 1/A4). Keratohyaline granules of stratum granulosum were found only in two uppermost layers of the neoepidermis (Fig. 1/A2).

In areas where RHPS adhered to flat dermis without adnexa remnants the epidermis was highly irregular (Fig. 1/B1). Morphologically, basal cells were found to grow on the human (bottom) as well as on the pig (top) side (Fig. 1/B2). These cells expressed vimentin in the bottom layer and, especially strongly, in the top layer (Fig. 1/B3) adjacent to the pig dermis. Markers of terminally differentiated keratinocytes, i.e. involucrin and K10, were weakly expressed only in the central layer of regenerating epidermis (Fig. 1/B4). Granular and cornified layers were absent. In several places keratinocytes formed 'islands' of senescent (larger cells, often with vacuoles – Fig. 1/B2a) or dying cells (Fig. 1/B2b). These cells seemed to be of donor origin, although only some of them were positive for Y-chromosome signals (Table 1).

Day 6: On day 6, the wound under RHPS was fully epithelized, with a more flattened rete ridge pattern than in normal skin (Fig. 1/C1) and with 2–3 layers thick stratum granulosum (Fig. 1/C2). Moderate variable expression of vimentin was observed up to the granular layer (Fig. 1/C3). Keratin 10 and involucrin were

Fig. 1. Histology and immunohistochemistry of wound healing stimulated by RHPS

Healing of female donor sites treated with male keratinocytes grown to confluency on RHPS was followed by biopsy examination on days 4, 6 and 9 after grafting. It was found that on day 4 the healing process depended on local wound condition given by the presence or absence of adnexa remnants. Later, the difference disappeared. (A1) Intensive epithelization is seen in areas of close contact between the adnexa remnants and the covering RHPS. Note the island (i) of implanted keratinocytes not connected with the adnexa. (A2) In the dried RHPS above the newly formed epidermis there are still visible multilayering pycnotic nuclei (arrow). This indicates multiplication of donor keratinocytes immediately after grafting. Keratohyaline granules are present in only two uppermost layers of the neoepidermis. (A3) Vimentin-positive (brown) donor keratinocytes in the insert i (island i in A1) indicate their culture origin in contrast to vimentin-negative epidermis newly formed under the RHPS. *Phase contrast.* (A4) Involucrin is expressed in the majority of suprabasal layers, contrary to the normal skin where it is expressed mainly in the granular layer. The expression of K10 (not shown) was similar to involucrin. *Phase contrast.* (B1) Early regenerating epidermis is irregular in areas of close contact between the dermis with no adnexa remnants and the RHPS. Although irregular, the neoepithelium is already multilayered. Note variable thickness of the epidermis with islands of senescent cells (for details see B2). Dried RHPS peeled off during biopsy. (B2) Islands of senescent (a) or dying (b) cells; small cuboidal cells (arrows) surrounding the senescent, possibly donor keratinocytes (c). Stratum granulosum and stratum corneum are missing. (B3) Vimentin-positive cuboidal keratinocytes in bottom and top layers are surrounding the differentiated involucrin-positive (B4) cells. (B4) Differentiation marker involucrin in early regenerating epidermis is weakly expressed in the central layer only. The expression of K10 was nearly identical – not shown. *Phase contrast.* (C1) Fully stratified epidermis on day 6. Note dried RHPS peeling off. (C2) Developing stratum granulosum with keratohyaline granules in 2–3 uppermost layers prove full differentiation of the regenerating epidermis. (C3) Moderate variable expression of vimentin up to the granular layer. (C4) Involucrin is expressed in the majority of suprabasal layers. *Phase contrast.* (D1) Fully stratified epidermis on day 9. Note persisting connection (arrow) of the dried RHPS with the neoepidermis (see also D2). (D2) Detailed morphology shows prominent stratum granulosum of 5–6 keratinocyte layers. (D3) Nearly negative vimentin expression indicates replacement of cultured donor keratinocytes by the recipient ones. (D4) Involucrin is (like in A4 and C4) expressed in the majority of suprabasal layers. Expression of K10 was nearly identical (not shown). *Phase contrast.*

Explanations: R: dried RHPS. Bars: 50 μ m

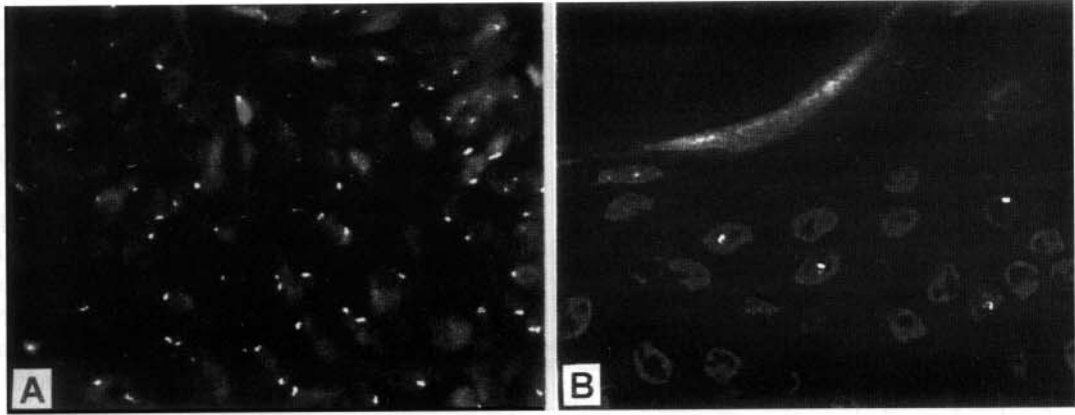


Fig. 2. Chromosome Y-positive keratinocytes are integrated into regenerated epidermis. (A) Control biopsy from a male - fluorescence *in situ* hybridization shows the presence of Y chromosomes. (B) Biopsy specimen from a female patient taken 6 days after grafting male-keratinocyte-RHPS. In the nuclei of some cells the Y chromosome was detected, proving the presence of donor cells. Magnification 1000x.

expressed in the majority of suprabasal layers (Fig. 1/C4). No more islands of senescent cells were found. The Y-chromosome signals were found either in individual keratinocytes or in small cell groups (Fig. 2.). The Y-chromosome signals were also detected under the epidermis and in the papillae of the corium, suggesting that some allogeneic fibroblasts also started to divide after transplantation of RHPS.

Day 9: On day 9 the epidermis was fully healed with a 5–6 layers thick stratum granulosum. The RHPS was still found attached to the upper layer of the neoepidermis (Fig. 1/D1). The boundary line between the dried RHPS and recipient stratum corneum was not always clearly recognizable (Fig. 1/D1, D2). Some residual expression of vimentin was observed in the basal layer

of the epidermis (Fig. 1/D3). Keratin 10 and involucrin were expressed in suprabasal layers (Fig. 1/D4). The epidermal cells did not show any Y-chromosome signals.

Discussion

The purpose of this study was to demonstrate that allogeneic keratinocytes cultured to confluency on xenodermis and grafted 'upside-down' (dermis up) can 'take' temporarily and positively influence the wound healing.

Previously, it was demonstrated that allogeneic keratinocytes grafted on deep dermal wounds in the form of cultured keratinocyte sheets may survive temporarily in the recipient epidermis (Burt et al., 1989; De Luca et al., 1989; Phillips et al., 1990; van der Merwe et al., 1990; Hudson et al., 1992; Zhao et al., 1992). However,

Table 1. Histological characterization of wound healing stimulated by RHPS (see Figs. 1 and 2)

Days after RHPS grafting	4 days		6 days	9 days	Normal skin
	RHPS adhered directly to adnexa remnants	RHPS adhered to flat dermis			
Morphology of epidermis	normal	irregular	normal	normal	normal
No. of keratinocyte-layers	8–14	0–20	8–18	8–17	9–16
Granular layer	+	–	++	+++	+++
Vimentin	No expression	Strong expression in the basal and top layers (interfaces with pig and human dermis)	Variable expression up to the granular layer	Weak expression in the basal layer	No expression in keratinocytes
Keratin 10	Suprabasal layers	Weak expression in the central layer	Suprabasal layers	Suprabasal layers	Suprabasal layers
Involucrin	Suprabasal layers	Weak expression in the central layer	Suprabasal layers	Suprabasal layers	Suprabasal layers
Y chromosome	–	+	++	–	+++