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Review

Human skin reconstruct models: A new application for studies of melanocyte and melanoma biology

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Summary. Studies of melanocyte and melanoma biology using monocultures of cells are limited because of culture-induced morphology changes and expression of genes related to growth, migration, and invasion, which do not reflect the *in situ* phenotype of normal melanocytes, nevus cells, or melanoma cells from biologically early progression stages. The development of organotypic cultures of human skin, in which culture artifacts are greatly diminished and cell-matrix and cell-cell interactions between different cell types can be investigated in a three-dimensional system, has opened a new era for melanoma research. Long-term *in vivo* studies, especially important for melanomagenesis and melanoma metastasis have become possible through grafting of skin reconstructs to immunodeficient laboratory animals. In this review, principles and different methods of skin reconstruction are introduced with focus on the application for pigment cell biology.

Key words: Organotypic culture, Melanocyte, Melanoma, Grafting, Skin reconstruct

Introduction

Through the easy access of melanocytic lesions on the body surface and marked advances in tissue culture techniques, hundreds of human melanocyte, nevus, and melanoma cell lines from all different progression stages have become available for experimental studies to a greater extent than for any other cancer type. The diverse and complex biology, immunology, and genetics of melanoma have put this tumor in the forefront of model systems for studying key issues of modern oncology, such as angiogenesis, apoptosis, drug resistance, immunosurveillance, invasion, and metastasis. While phenotypic and genetic characteristics of cells from biologically advanced lesions, such as vertical growth phase (VGP) primary and metastatic melanoma, are very similar *in vitro* and *in situ*, the culture-induced changes due to the two-dimensional growth conditions are significantly higher in cells from biologically early lesions, such as radial growth phase (RGP) primary melanoma, and from normal skin (Herlyn et al., 1987). Melanoma cells can gain genetic abnormalities over time in culture, for example, deletions of the p16 tumor suppressor gene, which are not found in the primary lesions *in situ* (Kamb et al., 1994; Guldberg et al., 1997). Cultured melanocytes and nevus cells are activated for expression of genes related to growth, migration, and invasion, which does not reflect the *in situ* phenotype of normal resting melanocytes or nevus cells, but rather of melanoma cells. The culture artifacts of melanocytes, i.e., non-dendritic but bi- to tripolar morphology, relatively high proliferation rate, and expression of melanoma-associated antigens, however, can be reverted when melanocytes are co-cultured with keratinocytes (Valyi-Nagy et al., 1993). The keratinocytes control the phenotypic characteristics of normal melanocytes, which is mediated through direct cell-cell contacts via the E-cadherin adhesion system (Hsu et al., 1996). The most advanced model to study melanocyte-keratinocyte interactions without culture artifacts is the three-dimensional skin reconstruction model. The melanocytes are located in the basal layer of the engineered epidermis in close contact to multiple keratinocytes through their dendritic extensions forming an epidermal melanin unit exactly as in the *in vivo* situation (Valyi-Nagy et al., 1990). Recently, different groups have started to characterize the biological behavior of melanocytes in skin reconstructs focusing on melanin transfer, response to ultraviolet light exposure, and DNA damage (Archambault et al., 1995; Bessou et al., 1996; Cario-Andre et al., 2000). Likewise, melanoma cells have recently been incorporated into skin reconstructs demonstrating a striking similarity to the growth and invasion properties of the original primary lesions from which they were established (Meier et al., 2000). Invasion of melanoma cells into the dermis of the respective human skin equivalent model has been observed by several groups (Bechetoille et al., 2000; Dekker et al., 2000; Eves et al., 2000) and more detailed

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Table 1. Dermis equivalents. Different materials and methods of dermis reconstruction have been established since the beginning of organotypic skin cultures.

DERMIS TYPE	PRODUCTION	CELL TYPE	REFERENCES
De-epidermized dermis	Sterilization of human skin without epidermis	None or repopulation with fibroblasts	Regnier et al., 1986; Ghosh et al., 1997
Collagen-glycosaminoglycan	Coprecipitation and homogenization	None or inoculation of fibroblasts	Boyce et al., 1988
Type I collagen	Isolation from rat tail or bovine dermis or tendon	Embedding of fibroblasts	Bell et al., 1981; Smola et al., 1998; Dekker et al., 2000
Polylactic acid, polyglycolic acid, polyglactin	Chemical synthesis of polymers	None or inoculation of fibroblasts	Cooper et al., 1991

studies about the mechanisms and molecules involved in tumor progression and invasion have just begun and are expected to increase in the near future (Hsu et al., 2000). Melanoma-skin reconstructs may particularly become an invaluable tool for investigations of tumor-stroma interactions, which have been increasingly recognized to be essential for tumor cell survival, growth, and invasion.

In this review, the different types and techniques of skin reconstruction are introduced with focus on the inclusion of melanocytes and melanoma cells for *in vitro* as well as *in vivo* studies after transplantation to experimental animals.

Nomenclature

The nomenclature of skin reconstruction is diverse. Organotypic culture of skin, skin equivalent, skin substitute, artificial skin, skin raft culture, skin reconstruct, epidermal-dermal composite, bioengineered skin, and three-dimensional skin culture are the most common terms.

Culture of skin cells

The isolation and cultivation of the three major cell types of normal human skin is well established and characterized. Stroma-supporting fibroblasts are isolated from the dermis, while stratified epithelium-forming keratinocytes and pigment-producing melanocytes are isolated from the epidermis. Keratinocytes are essential for skin reconstruction and fibroblasts are widely used for construction of the supporting dermis. The optional addition of melanocytes to keratinocytes in a ratio of 1:3 to 1:20 leads to pigmented skin reconstructs. Also Langerhans cells can be successfully integrated into the reconstruction model, when cord blood-derived $CD34^+$ hematopoietic progenitor cells are used (Regnier et al., 1997). Although other cells of the skin appendages, vasculature, and immune system can be maintained as monocultures, inclusion in organotypic culture systems has not been established yet.

Dermis equivalents

Different types of dermis equivalents have been developed (Table 1). They are the supporting structure beneath the stratified epithelium being formed by keratinocytes. Most widely used is de-epidermized dermis from normal human skin derived from surgery or *post mortem* (Regnier et al., 1986; Ghosh et al., 1997). After sterilization by treatment with glycerol, ethylene oxide, or gamma irradiation and after removal of the epidermis, the subcutaneous side of the dermis is seeded with fibroblasts, which re-populate the dermis, and the opposite, formerly epidermal side is seeded with keratinocytes.

Other groups, including ours, make dermis from type I collagen mixed with fibroblasts, which remodel the collagen before the seeding of keratinocytes on top. Figure 1 illustrates schematically the sequential reconstruction steps of this model. A combination of type I collagen and glycosaminoglycans was established by Boyce et al. (1988) and synthetic polymers have been employed as matrices as well (see Table 1).

In Fig. 2A, a histological example of a 2-week old skin reconstruct is presented. A composite of de-epidermized sterilized dermis cultured on top of a fibroblast-containing type I collagen matrix is shown in Fig. 2B. Fibroblasts migrate upwards and populate the de-epidermized dermis, which then is seeded on top with keratinocytes. A similar approach was recently described by Lee et al. (2000). Regardless of which dermis equivalent is used, it has become evident that fibroblasts play an important role both for the formation of a mature basement membrane (Smola et al., 1998) and for epidermal differentiation (Lee et al., 2000).

In skin reconstructed with de-epidermized dermis, an intact basement membrane and a papillary morphology of the epidermal-dermal junction zone is preserved, whereas in skin reconstructed with fibroblast-containing type I collagen matrix, a basement membrane is being produced by the fibroblasts and keratinocytes during the culture process (Smola et al., 1998) and the epidermal-dermal junction zone stays straight. It is unknown, what impact these differences have for experimental studies. The composition of de-epidermized dermis, which may contain different matrix-bound growth factors, proteases, and cytokines, is not clearly defined yet and differences between individual donors as well as in the preparation process need to be

considered.

Epidermis reconstruction

Undifferentiated keratinocytes are used for the reconstruction of the epidermis equivalent. Melanocytes are mixed with keratinocytes in a ratio 1:3 to 1:20 and seeded together onto the dermis equivalent. Alternatively, co-cultures of keratinocytes and melanocytes can be used. In general, approximately $5x10^5$ cells per cm^2 dermis equivalent are seeded and left under submerged culture conditions in low calcium, low serum medium for 3 to 6 days. Subsequent exposure to air induces a differentiation process in the keratinocytes leading to the formation of multiple layers piling up on top of each other. The different layers of a normal stratified epithelium are described in Fig. 2A. A macroscopic view of a pigmented skin reconstruct after 2 weeks of culture is demonstrated in Fig. 2C. The melanocytes are found in the basal layer as single cells surrounded by the neighboring keratinocytes (Fig. 2D). The melanocytic dendrites extend into the upper layers of the epidermis and pigment is transferred to the keratinocytes (Archambault et al., 1995). Recently, it has been shown that melanocytes in skin reconstructs protect epidermal basal cells from UVB-induced apoptosis (Cario-Andre et al., 2000).

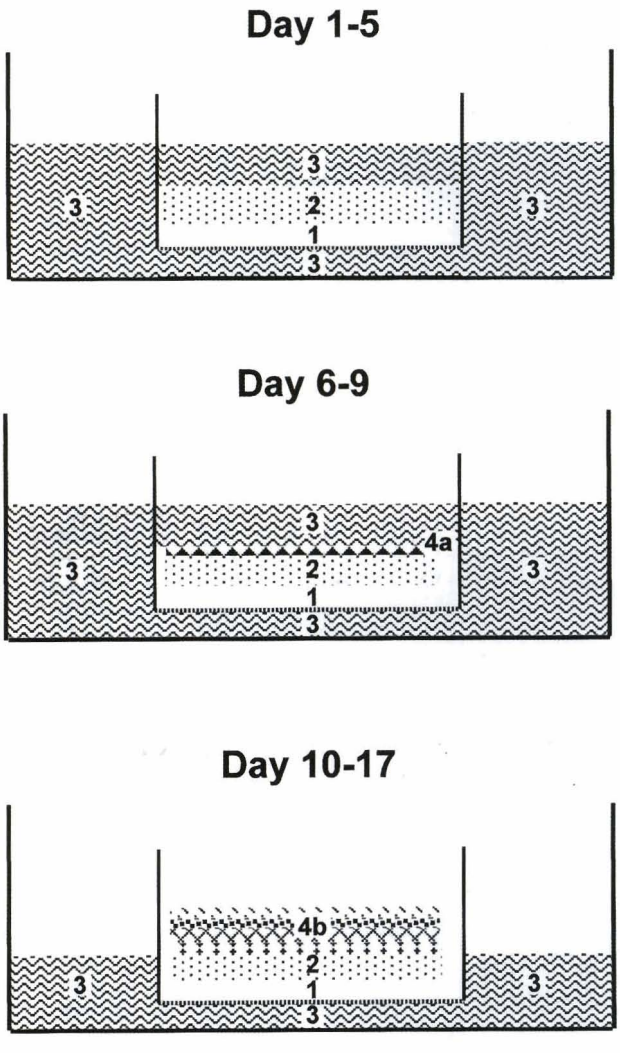

Fig. 1. Schematic of the sequential steps of skin reconstruction using type I collagen with embedded fibroblasts as dermis equivalent.

Grafting of skin reconstructs

Skin reconstructs are being used in medicine for covering wounds of patients (Kirsner, 1998). While skin reconstructs *in vitro* have a maximum life-span of approximately one month, survival is increased to several months or years when grafted to living hosts. Long-term studies have become possible, which is particularly of interest for carcinogenesis studies (Javaherian et al., 1998). Fig. 2E shows a pigmented human skin reconstruct on an immunodeficient albino mouse. Histological and immunohistochemical analyses revealed that: i) host vessels and immune cells infiltrated the dermis of the grafts, ii) a papillary morphology of the upper dermis developed, iii) the differentiation of the epidermis improved, and iv) the basement membrane matured (Fig. 2F). Obviously, not yet defined diffusible factors from the host's microenvironment support the morphology and viability of the grafted skin reconstructs.

Melanoma in skin reconstructs

When melanoma cells are incorporated into skin reconstructs, a progression stage-related growth pattern can be observed (Bechetoille et al., 2000; Eves et al., 2000; Meier et al., 2000). While cells derived from RGP primary melanomas or non-aggressive melanoma cell lines are found as single cells or clusters throughout the epidermis, cells derived from VGP primary melanomas or aggressive metastatic melanoma cell lines traverse the basement membrane and invade the dermis equivalent (Fig. 2G,H). Competence for metastasis and long-term growth properties may be tested when melanoma-containing skin reconstructs are grafted to living hosts, for example, to immunodeficient mice.

Induced expression of invasion-related adhesion molecules, such as melanoma cell adhesion molecule

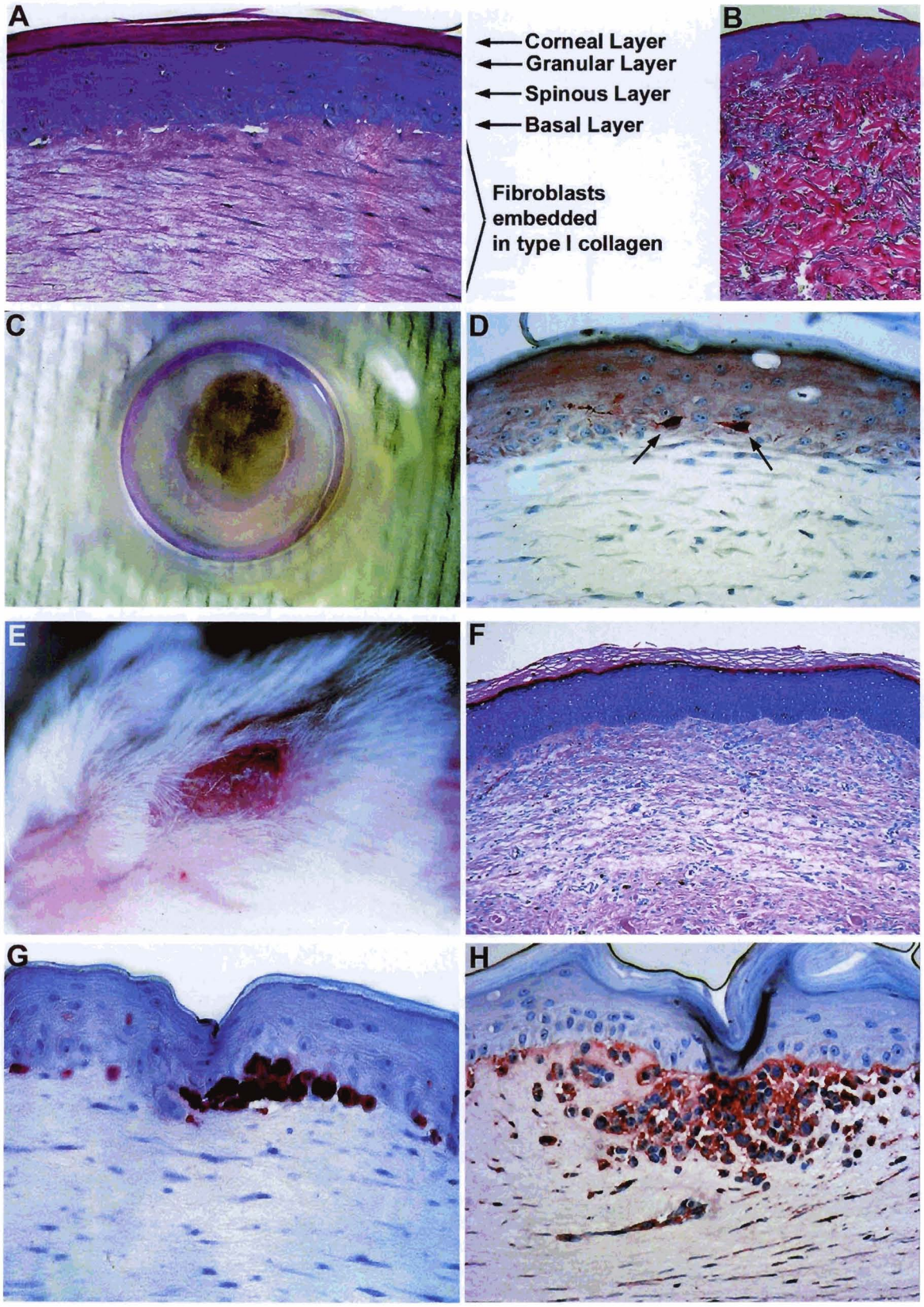

Fig. 2. Examples of human skin reconstructs. **A.** Normal human skin reconstruct after 13 days of culture. H&E, x 400. **B.** Normal human skin reconstruct made of de-epidermized sterilized human dermis cultured on top of type I collagen with embedded normal human skin fibroblasts, which migrated up into the dermis, after 16 days of culture. H&E, x 100. **C.** Macroscopic view of a pigmented normal human skin reconstruct; the dermis is made of type I collagen with embedded normal human skin fibroblasts. **D.** Normal human skin reconstruct with melanocytes (arrows) in the basal layer of the epidermis after 11 days of culture. S100 stain, x 200. **E.** Pigmented normal human skin reconstruct 20 days after grafting to a SCID mouse. **F.** Pigmented normal human skin reconstruct 39 days after grafting to a SCID mouse. H&E, x 100. **G.** Non-invasive human melanoma in a human skin reconstruct (S100 stain, x 400). **H.** Invasive human melanoma in a human skin reconstruct. Collagen IV stain, x 200

(Mel-CAM) or the beta 3 integrin subunit of the vitronectin receptor, can convert non-aggressive non-invading melanoma cells into aggressive invading ones as can be demonstrated in skin reconstructs (Satyamoorthy et al., unpublished data). Thus, a new model for mechanistic studies of human melanoma biology has become available and is expected to be of increasing use by various research groups.

Conclusions

Organotypic culture models as opposed to monolayer cultures mimic the three-dimensional structure of the respective reconstructed organ. They combine different organ-specific cell types and various differentiation stages of the same cell type with supportive matrix. Studies of cell-cell and cell-matrix interactions have become possible and culture artifacts, which are common in cells grown in monolayer are absent or diminished in organotypic cultures. The reconstruction of human skin is most advanced and has reached the stage of successful transplantation to patients to cover skin defects of ulcers or burns. More recently, melanocytes and melanoma cells have been introduced into the organotypic cultures and their biological behavior resembles the in vivo situation to a great extent. Long-term studies have become possible by grafting these skin reconstructs to immunodeficient laboratory animals. The time has now come to focus on more detailed studies of gene candidates and cell-cell as well as cell-matrix interactions of relevance to melanocyte transformation and melanoma biology.

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