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Keratin expression in cultured skin substitutes suggests that the hyperproliferative phenotype observed in vitro is normalized after grafting

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Abstract

Cultured skin substitutes, consisting of fibroblasts and keratinocytes in a biopolymer matrix, are an adjunctive treatment for full thickness burn wounds. Previous studies revealed that cultured skin substitutes in vitro exhibit a gene expression profile similar to hyperproliferative skin or wounded normal skin. In the present study, we sought to determine whether this hyperproliferative phenotype is maintained after healing of grafted cultured skin in vivo. Immunohistochemistry was used to localize multiple keratin proteins in native human skin, and in cultured skin substitutes in vitro and after grafting to athymic mice. Keratin 6, keratin 16, and keratin 17, which are known to be upregulated during keratinocyte activation and in hyperproliferative epidermis, were highly expressed in cultured skin substitutes in vitro. These proteins were low or undetectable in native human skin, and were reduced in cultured skin after grafting. Conversely, keratin 15, which is downregulated in activated keratinocytes, was not detected in cultured skin substitutes in vitro but was upregulated after grafting to mice. The results confirm previous observations suggesting a hyperproliferative or activated phenotype in cultured skin substitutes in vitro, similar to wounded native skin. After grafting to athymic mice, the expression patterns suggest a normalization of cultured skin substitutes to a phenotype more closely resembling uninjured human skin.

Keywords: Tissue engineering; Cultured skin substitute; Keratin; Wound healing

1. Introduction

Cultured skin substitutes (CSS), comprised of autologous keratinocytes and fibroblasts with a collagen-based biopolymer matrix, are used clinically to facilitate wound closure in patients with massive burns [3,5,6]. Upon healing CSS provide permanent wound closure and can significantly increase donor site utilization [6]. However, because CSS contain only two cell types, limitations in anatomy remain that may contribute to decreased engraftment.

In a previous study, gene expression profiling of CSS revealed that in vitro CSS display a hyperproliferative phenotype, similar to wounded native skin and psoriatic epidermis [19]. This observation was supported by high levels of RNA expression, relative to native human skin, of multiple genes that are overexpressed in hyperproliferative keratinocytes, including keratin 16 (K16). Keratins, the major structural proteins of the epidermis, are formed from heterodimers of type I (K9–K23) and type II (K1–K8) proteins, which are often coexpressed as specific pairs in tissue-specific and differentiation-specific patterns [10]. Coexpression of K5 and K14 is a characteristic trait of proliferative keratinocytes, and these proteins are considered markers for epithelial cells [16]. The type I keratin K15 can also pair with K5 in basal keratinocytes [14]. Keratins K5, K14, and K15 are downregulated as basal cells differentiate [11], and suprabasal keratinocytes primarily express K1 and K10 [20]. Upon injury to the skin, a process termed keratinocyte activation is initiated to facilitate wound healing. Keratinocyte activation involves cellular changes

Abbreviations: CSS, cultured skin substitutes; EGF, epidermal growth factor; K6, keratin 6; K15, keratin 15; K16, keratin 16; K17, keratin 17

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at the wound edge, including cell elongation, reduced adhesion, and increased cell migration [8]. These changes are accompanied by a shift in expression of keratin genes in suprabasal keratinocytes, including a reduction in K1 and induction of K6, K16 and K17 [15,22]. Co-ordinately increased expression of K6, K16, and K17 is considered a hallmark of keratinocyte activation and is also seen in hyperproliferative skin disorders, such as psoriasis [13,15,17,20,22]. Basal K15 expression is known to be downregulated in activated keratinocytes [21,23].

In a previous study, keratins 5 and 10 were found to be similarly expressed in the epidermis of human skin and cultured skin in vitro [19]. The goal of the current study was to characterize expression of differentiation-specific keratins in CSS in vitro and after grafting to athymic mice, to determine if the hyperproliferative phenotype observed in vitro is maintained in vivo, and to better understand the phenotypic changes that occur during healing of grafted CSS.

2. Materials and methods

2.1. Preparation of CSS

Fibroblasts and keratinocytes were isolated from human breast skin, obtained with Institutional Review Board approval, from a healthy adult female undergoing elective plastic surgery, and were grown in selective cultures as previously described [1,4]. CSS were prepared as described in detail elsewhere [1,6,19]. Briefly, fibroblasts were inoculated onto collagen–glycosaminoglycan polymer substrates [2] at a density of 5×10^5 cm^{-2}; after 24 h, keratinocytes were harvested and inoculated at a density of 1×10^6 cm^{-2}. CSS ($n = 2$) were cultured at the air–liquid interface for 2 weeks in vitro, using medium described elsewhere [7]. Epidermal growth factor (EGF; 10 ng/ml) was present in the medium for only the first 3 culture days [7]. Biopsies of CSS were collected at the end of the culture period for immunohistochemistry. CSS were cut to make 2 cm × 2 cm grafts, which were transplanted to full thickness excisional wounds on athymic mice in accordance with NIH and institutional guidelines [1]. Mice were sacrificed at 1 through 8 weeks after grafting, and CSS biopsies were collected for immunohistochemistry.

2.2. Immunohistochemistry

Biopsies of the intact human skin, CSS in vitro, and CSS excised from mice were imbedded frozen using M1 embedding matrix (Lipshaw, Pittsburgh, PA). Immunohistochemistry on cryostat sections was performed using mouse monoclonal antibodies specific for human keratins K6, K16, K17 (US Biological, Swampscott, MA), or K15 (Novocastra Laboratories, UK), or non-immune mouse IgG (R&D Systems, Minneapolis, MN). Antibody detection utilized the Vector® M.O.M.™ (''Mouse on Mouse'') Kit with DAB substrate (Vector Laboratories, Burlingame, CA). The M.O.M. kit was used because the primary antibodies were of mouse origin, requiring the use of anti-mouse secondary antibody reagents for detection. The M.O.M. kit is intended to reduce non-specific background staining obtained in sections containing mouse tissue. However, non-specific staining was still observed in the dermal regions of CSS sections excised from mice. For this reason, the non-immune mouse IgG negative control antibody was used in parallel incubations, to demonstrate that the dermal staining was in fact non-specific. Non-specific background staining can be attributed to the presence of antigens derived from the host mouse circulation and wound bed. The absence of non-specific background in the epidermis suggests stable engraftment of the human keratinocytes. All sections were counterstained using Hematoxylin QS (Vector Laboratories).

3. Results

In sections of native human skin, K6, K16, and K17 proteins were very low or undetectable (Fig. 1A, E, and I). However, all three proteins were found at high levels in CSS in vitro (Fig. 1B, F, and J). K6 and K17 were found throughout the epidermis of CSS, including the basal cell layer. K16 staining was similarly high in the suprabasal cells of the epidermis but was not observed in basal keratinocytes. In contrast, K15 was localized to basal keratinocytes of native human skin (Fig. 1M), but was not found in CSS in vitro (Fig. 1N).

After grafting to full thickness wounds on athymic mice, changes were observed in expression and localization of these keratin proteins. At 1 week after grafting, expression of K6 and K17 was decreased in basal keratinocytes, and both proteins were essentially absent from the healed CSS by 4 weeks after grafting (Fig. 1C–D and K–L). K16 expression was reduced in vivo, and by 4 weeks after grafting the protein was found only in the uppermost levels of the epidermis (Fig. 1H). K16 protein was absent by 8 weeks after grafting (data not shown). In contrast to the downregulation of K6, K16, and K17 after grafting, K15 expression was induced, appearing in basal keratinocytes by 4 weeks after grafting (Fig. 1P).

4. Discussion

K6, K16, and K17 are considered markers of keratinocyte activation, and their expression is co-ordinately increased in wounded skin and in hyperproliferative skin disorders such as psoriasis [13,17,22]. Conversely, K15 is downregulated in activated keratinocytes of wounded skin and in psoriasis [21,23]. The expression patterns of K6, K16, K17 and K15 in CSS, reported here, suggest that the keratinocytes in CSS in vitro are in an activated state, consistent with previously

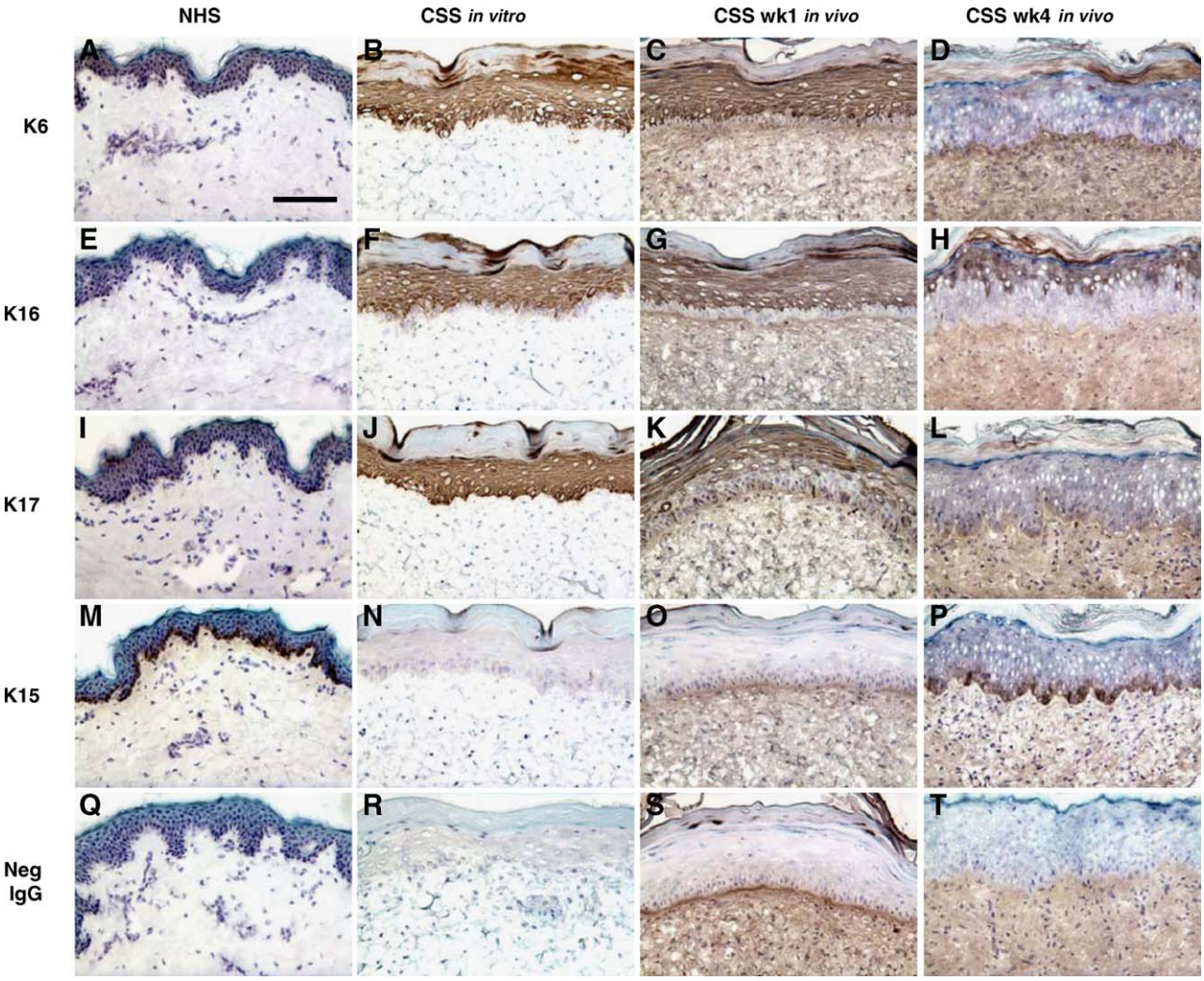

Fig. 1. Immunohistochemical localization of keratin proteins in native human skin and CSS. Shown are sections of native human skin (A, E, I, M, Q), CSS at 2 weeks incubation in vitro (B, F, J, N, R), and CSS at 1 week (C, G, K, O, S) and 4 weeks (D, H, L, P, T) after grafting to athymic mice (in vivo). For all sections, epidermis is at the top of the panel. (A–D) Keratin 6, (E–H) keratin 16, (I–L) keratin 17, (M–P) keratin 15. (Q–T) Immunohistochemistry using a non-immune mouse IgG antibody as a negative control. Note the non-specific background staining in the dermis of CSS sections in vivo, seen with the non-immune negative control primary antibody (S, T) as well as the keratin-specific antibodies (C, D, G, H, K, L, O, P). This background staining, seen only in sections of CSS after grafting, resulted from the detection procedure used for mouse monoclonal antibodies on tissues excised from mice. Scale bar in (A) is same for all panels (100 μm).

reported microarray expression data [19]. Thus, during the in vitro culture period the CSS exhibit a hyperproliferative phenotype similar to wounded skin. This observation contrasts with another report that examined expression of these keratins in reconstructed epidermis on de-epidermized human dermis [12]. In that study, expression of K6 and K16, but not K17, was induced by supplementation of the media with EGF (5 ng/ml) for the entire 2 week culture period. In the presence of keratinocyte growth factor (KGF), or in the absence of either growth factor, expression of K6, K16, and K17 was either weak or undetectable in the reconstructed epidermal cultures [12]. The high levels of expression of K6, K16, and K17 detected in CSS in the present study may be attributed to the presence of dermal fibroblasts in this organotypic skin model. The results suggest that factors secreted by the fibroblasts may act in a paracrine fashion to regulate differentiation of the epidermal keratinocytes, in the absence of exogenous EGF added to the culture medium. However, we can not rule out the possibility that differences in the culture conditions may contribute to the observed gene expression differences.

After grafting to mice, the coordinate decreases in K6, K16, and K17, and increase in K15, suggest that factors secreted from cells in the wound bed or circulating mediators regulate gene expression in CSS. The observed pattern of keratin expression in CSS after grafting indicates that the hyperproliferative phenotype is normalized in vivo. Therefore, in contrast to CSS in vitro, grafted CSS displays a phenotype resembling uninjured native human skin.

These observations offer possible mechanisms for improvements of the cultured skin model that may further

enhance wound healing with CSS. Studies in transgenic mice have shown that overexpression of K16 can cause abnormal keratinization and delayed skin maturation [9,18]. K16 has been proposed to play an active role in re-epithelialization following skin injury by modulating the keratin filament network, a process that is dependent on coexpression of other keratin genes [18,23]. Hypothetically, downregulation of K16 (and/or K6 and K17) in CSS in vitro may improve keratinization or accelerate maturation. Future studies aimed at regulating expression of these keratin proteins may be useful for enhancing the function of CSS.

Conflict of interest statement

The authors state that we do not have any conflicts of interest (any financial and personal relationships with other people or organizations that could inappropriately influence or bias our work).

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