Virchows Arch (1999) 434:221–226

ORIGINAL ARTICLE

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Epidermal participation in post-burn hypertrophic scar development

Received: 16 September 1998 / Accepted: 28 September 1998

Abstract The reconstruction of epidermal architecture over time in normotrophic and hypertrophic scars in untransplanted, spontaneously healed partial-thickness burns has scarcely been studied, unlike the regeneration of epidermal grafts used to cover burn wounds and the regeneration of the dermis during hypertrophic scarring. The expression of markers of epidermal proliferation, differentiation and activation in normotrophic and hypertrophic scars in spontaneously healed partial-thickness burns was assessed and compared with the expression of these markers in normal control skin of healthy persons to determine whether hypertrophic scarring is associated with abnormalities in the phenotype of keratinocytes. Punch biopsies were taken both of partial-thickness burns after re-epithelialisation and of matched unburned skin. At 4 and 7 months post-burn, biopsies were taken of normotrophic and hypertrophic scars that had developed in these wounds. The biopsies were analysed using immunostaining for markers of keratinocyte proliferation, differentiation and activation (keratins 5, 10, 16 and 17, filaggrin, transglutaminase and CD36). We observed a higher expression of markers for proliferation, differentiation and activation in the epidermis of scars at 1 month post-burn than in normal control skin of healthy persons. There was a striking difference between normotrophic and hypertrophic scars at 4 months post-burn. Keratinocytes in hypertrophic scars displayed a higher level of proliferation, differentiation and activation than did normotrophic scars. At 7 months post-burn all keratinocyte proliferation and differentiation markers showed normal expression, but the activation marker CD36 remained upregulated in both normotrophic and hypertrophic scars. Surprisingly, in matched unburned skin of burn patients, a state of hyperactivation was observed at 1 month. Our results suggest that keratinocytes may be involved in the pathogenesis of hypertrophic scarring.

Key words Keratinocyte · Hypertrophic scar · CD36 · Keratin 16

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Introduction

During wound healing, a sequence of inflammation, tissue regeneration and reorganisation results in the formation of mature scar tissue. Skin repair after burning is essentially similar to healing after simple trauma, yet burn-related ischaemia, hypoxia and oedema slow down wound healing ADDIN ENRf8 [24]. The end-result of burn wound healing can be normotrophic healing or abnormal wound healing, resulting, for example, in chronic ulcers or hypertrophic scars. Besides disabling contractures and disturbing cosmetic aspects, hypertrophic scars also cause a variable degree of discomfort, such as severe itching [24]. The aetiology of hypertrophic scarring is still not known, and until now research has been mainly focused on the dermal aspects of those scars (review: [24]), neglecting the epidermis. As hypertrophic scars most commonly occur after re-epithelialisation has been delayed [16], one could hypothesise that hypertrophic scarring results from abnormalities in the epidermal–dermal crosstalk rather than from isolated defects in the dermis. Keratinocytes can regulate the collagen synthesis of dermal fibroblasts [19], and conversely keratinocyte growth is supported by growth factors produced by fibroblasts [40]. In addition, activated keratinocytes are a source of specific pro-inflammatory cytokines and fibrogenic and growth factors, and as such have importance in

inflammation [6] and wound healing [27]. Further study of epidermal maturation in hypertrophic scarring is worthwhile.

Antibodies to specific markers, such as keratins, filaggrin, transglutaminase, are useful in study of the keratinocyte phenotype in tissue remodelling during burn wound healing, because they are expressed in different layers of the epidermis in a differentiation-specific manner [17, 23, 28]. Expression of CD36 in normal keratinocytes is absent, occurring only in response to specific immunological and nonimmunological stimuli [2, 5, 8, 22]. Research has previously been focused on regeneration of the epidermis in burns treated with grafts [9, 12, 30, 32, 41], and so we aimed to describe keratinocyte maturation in ungrafted, spontaneously healed partial-thickness burns. We focused on keratins, filaggrin, transglutaminase and CD36 as a starting point for further studies on keratinocyte maturation in hypertrophic burn wound scars. We wished to determine whether hypertrophic scarring is associated with abnormalities in the phenotype of keratinocytes.

We monitored the expression of epidermal maturation markers immunohistochemically in both normotrophic and hypertrophic burn wound scars during a 6-month follow-up.

Materials and methods

Twenty-two patients aged 19–74 years (mean age: 41 years) were treated for burns at the Burn Centre of the Red Cross Hospital in Beverwijk, The Netherlands. The extent of the total burn injury in individual cases varied from 5% to 62% (mean: 16%) of the total body surface area. After informed consent had been obtained from each patient, 3-mm punch biopsies were taken of two partial-thickness burns at three time points: 1 month, after re-epithelialisation had been completed [mean: 4.8 weeks, standard error (SEM): 0.32 weeks], 4 months (mean: 17.8 weeks, SEM: 0.77 weeks) and 7 months (mean: 30.6 weeks, SEM: 0.71 weeks) post-burn. Matched unburned skin of the same patients was obtained only at 1 month post-burn. At 4 and 7 months post-burn the scars were classified as normotrophic or hypertrophic using the Vancouver Scar Scale, a classification system based on consistency, elevation and colour [38].

Serial 5-µm cryostat sections were cut from Tissue-tek (Miles Elkhart, USA) embedded biopsies and attached to glass slides coated with poly-L-lysin and fixed in 100% acetone for 10 min. All incubations were performed at room temperature. An alkaline phosphatase anti-alkaline phosphatase (APAAP) staining method was used as described by Schaumburg-Lever [35]. In brief, sections were preincubated with 10% normal rabbit serum in phosphate-buffered saline (PBS), followed by incubation with antibodies against keratins (Keratin 5: Euro-Diagnostica, Malmö, Sweden; Keratin 10: Monosan, Uden, The Netherlands; Keratin 16: Sigma, Zwijndrecht, The Netherlands; Keratin 17: Dako, Glostrup, Denmark), Filaggrin (Biomedical Tech., Stoughton, Mass.), Transglutaminase (Biomedical Tech.) and CD36 (CLB, Amsterdam, The Netherlands). The sections were rinsed, incubated for 30 min with rabbit anti-mouse immunoglobulin antibodies (Dako) and incubated for 30 min with APAAP (Dako). The immunoreaction was visualised by using a solution containing new fuchsin (Chroma-Gesellschaft, Köngen, Germany), sodium nitrite, naphthol phosphate, dimethylformamide and levamisol in TRIS-HCl buffer (pH=8.0). Slides were counterstained with Mayer's haematoxylin and mounted in glycerol-gelatin (Merck, Darmstadt, Germany). The negative controls involved concentration-matched mouse IgG (Becton Dickinson, San Jose, Calif.) and omission of the first and second step.

Two investigators (T.E.H. and V.A.) assessed the staining intensity independently and compared it with expression in normal control skin from healthy persons undergoing plastic surgery. For each of the four epidermal layers (basal, spinous, granular and horny layers) the staining intensity of each marker was scored, using a semi-quantitative scale ranging from 0 to 3 as described previously (0=not detectable, 1=weak staining, 2=moderate staining and 3=strong staining) [15]. On most markers there was consensus, but in case of any discrepancy the mean value was calculated.

For all markers, the mean summary score (referred to from this point on as the mean score) of the four epidermal layers was used in the statistical analysis. Differences in the score between normal control skin from healthy persons with both burned and matched unburned skin, differences over time, and differences between normotrophic and hypertrophic scars were statistically analysed using the Mann-Whitney U-test, Wilcoxon matched-pairs signed-ranks test, a Chi-square test or an ANOVA test (SPSS version 5.0.2, SPSS, Chicago, Ill., 1993). A *P*-value equal to or lower than 0.05 was considered statistically significant.

Results

Biopsies from 22 burn patients were examined. Four patients who initially joined the study were lost to follow-up. This resulted in 38 biopsies taken at 1 month post-burn, 34 biopsies (23 normotrophic and 11 hypertrophic scars) taken at 4 months post-burn, and 36 biopsies (24 normotrophic and 12 hypertrophic scars) taken at 7 months post-burn.

In the haematoxylin-eosin-stained sections, the epidermis of burn scars showed a normal architecture except for the epidermal ridges and dermal papillae, which were not as deep or as numerous as in normal control skin. The epidermis of burned skin at 1 month post-burn contained only sporadically infiltrating cells, which had disappeared at 4 and 7 months post-burn. In the epidermis of matched unburned skin and in normal control skin, inflammatory cells were absent.

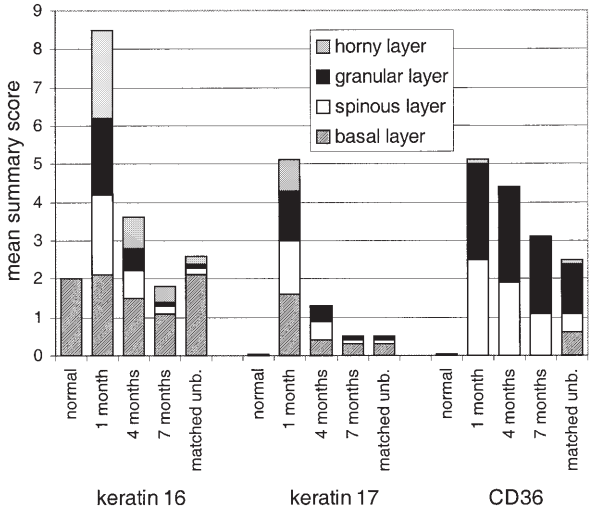

Fig. 1 Keratin 16 and 17 and CD36 expression in the epidermis in both normal control skin of healthy persons, in burn scars at 1, 4 and 7 months post-burn time, and in matched unburned skin of the patients

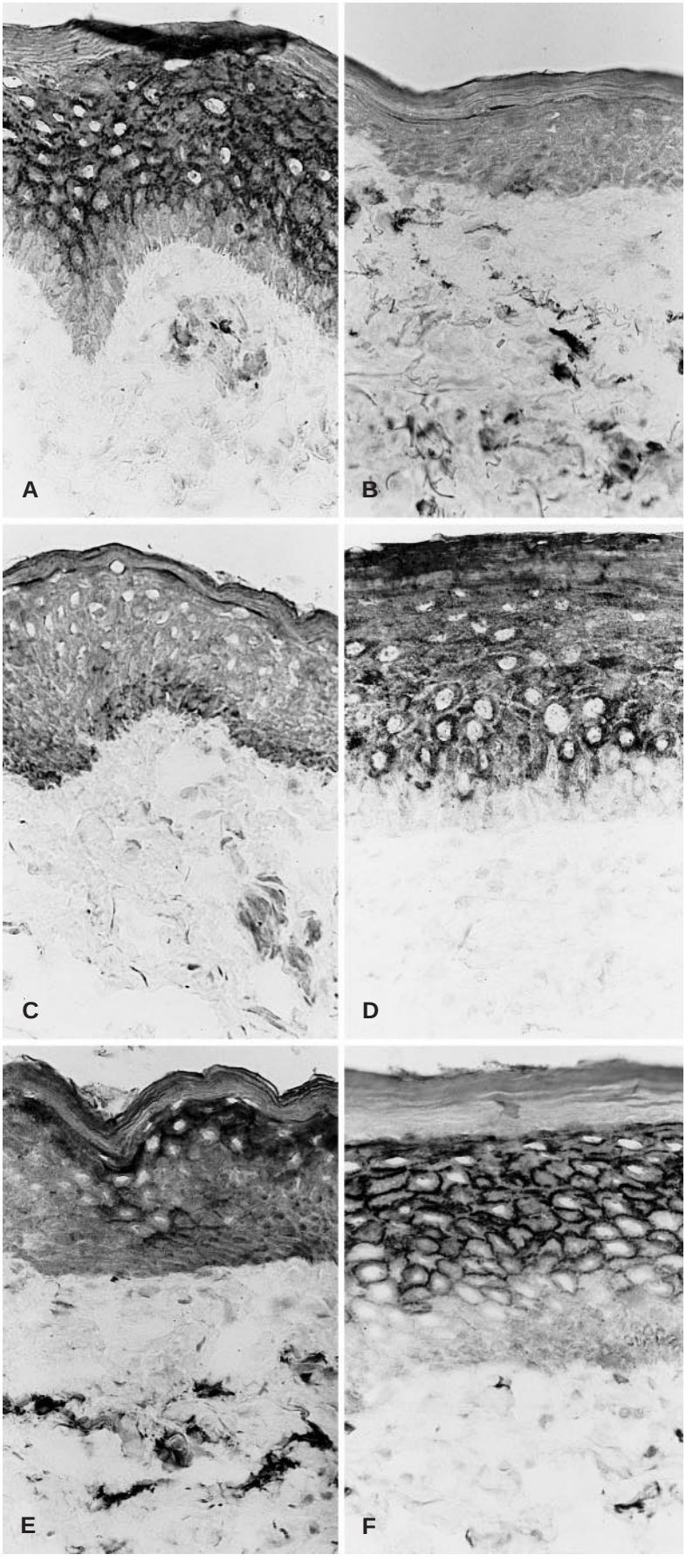

Fig. 2A–F Immunohistochemical localization of keratin 16 and CD36 in partial-thickness burn scars at 4 months post-burn time, matched unburned skin and normal control skin of healthy persons. CD36 **A** in matched unburned skin of a burn patient and **B** in normal control skin of a healthy person. **C** Keratin 16 in a normotrophic scar and **D** a hypertrophic scar from the same patient, and CD36 **E** in a normotrophic scar and **F** a hypertrophic scar from the same patient. Original magnification ×400

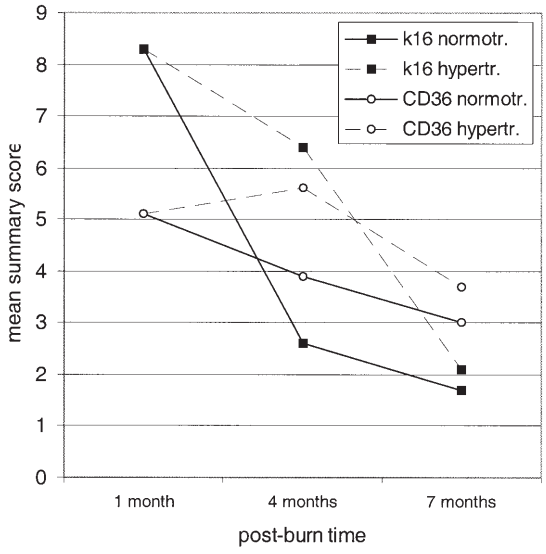

Fig. 3 Keratin 16 and CD36 expression in normotrophic and hypertrophic burn scars

The dermis of the normotrophic scars showed a normal architecture, whereas the hypertrophic scars showed the typical dermal collagen organisation of whorls and nodules.

At 1 month after burning, the expression of keratins 5, 16 and 17 (Fig. 1), filaggrin, transglutaminase and CD36 was clearly higher than in normal control skin of healthy persons (Mann-Whitney U test: $p<0.01$). No alterations were observed in keratin 10 expression.

The matched unburned skin of every patient taken at 1 month post-burn showed a normal expression pattern for all markers except CD36 (Fig. 1). 19 of the 22 showing an upregulation of this marker (Fig. 2A). The difference between this elevated expression in matched unburned skin and the absence of CD36 expression in the normal control skin of healthy persons (Fig. 2B) was highly significant (Wilcoxon test: $P<0.01$). There was no relation between the distance from the biopsy site of the matched unburned skin to the burned area and the CD36 expression (Chi-square test, $P=0.28$). No relation was observed between total burned body surface area and CD36 expression in the matched unburned skin biopsies (ANOVA test, $P=0.27$).

At 4 and 7 months post-burn, keratin 16 expression differed significantly between the epidermis of normotrophic (Fig. 2C) and of hypertrophic (Fig. 2D) scars. There was still an upregulation of keratin 16 in 9 of the 10 hypertrophic scars (mean score 6.4), as against 5 out of 21 normotrophic scars (mean score 2.6; Mann-Whitney U-test: $P<0.01$; Fig. 3). At 7 months post-burn the expression of keratin 16 had reverted to normal in both normotrophic (mean score 1.7) and hypertrophic scars (mean score 2.1; Mann-Whitney U-test: $P=0.83$).

Keratin 17 was still slightly upregulated in 11 of the 33 scars at 4 months, with a small, but statistically insignificant, difference between normotrophic (mean score 0.7) and hypertrophic scars (mean score 2.2; Mann-Whitney U-test: $P=0.24$). At 7 months no scars showed keratin 17 expression, as expected.

CD36 remained expressed by keratinocytes at both 4 and 7 months post-burn. At 7 months, 34 of the 36 scars still showed CD36 expression. This upregulation was significant compared with normal control skin of healthy persons (Mann-Whitney U-test: $P<0.01$). At 4 months post-burn the difference between normotrophic scars (mean score 3.9; Fig. 2E) and hypertrophic scars (mean score 5.6; Fig. 2F) was significant (Mann-Whitney U-test: $P=0.03$; Fig. 3), but there was no longer a significant difference at 7 months post-burn (mean scores normotrophic scars: 2.9, hypertrophic scars: 3.7; Mann-Whitney U-test: $P=0.38$).

The expression of keratins 5 and 10 and filaggrin and of transglutaminase in scars was no different from that in normal control skin at 4 and 7 months post-burn.

Discussion

Our data show that keratinocytes in burn wounds in which re-epithelialisation is just completed have entered an alternative pathway of differentiation and are expressing an activated phenotype compared with those in normal control skin from healthy persons. This is shown by the upregulation of keratins 5, 16 and 17, filaggrin, transglutaminase and CD36 in the 1-month-old burn scar. These results agree with those of previous studies on epidermal maturation in grafted burn wounds [9, 30, 33, 41] and show that the first aim of the newly formed epidermis is rapid migration and proliferation to cover the denuded area. This is followed by enhanced differentiation to restore the barrier function of the skin.

With regard to the chronic phase of spontaneously healed burn wounds, our study clearly demonstrates a time-dependent difference in keratinocyte phenotype between normotrophic and hypertrophic scars. This indicates that the distinction between normotrophic and hypertrophic scars is located in both dermis and epidermis.

A recent study has also revealed an upregulation of keratin 16 in the epidermis of hypertrophic scars [25]. However, the workers concerned did not observe time-dependent expression, probably owing to the unknown depth of the original wound and to dissimilar treatments, in particular grafting.

Epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) have been shown to upregulate suprabasal keratin 16 expression specifically in hyperproliferative epidermis [21, 26], and should be regarded as important growth factors in the pathogenesis of hypertrophic scarring. Potential sources of EGF or TGF-α in hypertrophic burn scars are the immunologically active cells present in the dermis or epidermis and the keratinocytes themselves. The latter play an important part in the expression of keratin 16, as it has been shown that keratinocytes can produce and be stimulated by TGF-α in an autocrine loop [11]. Alternatively, induction of keratin

16 in hypertrophic scars may be caused by the continuous upward mechanical forces to which the epidermis is exposed, because of the tumour-like growth of the reticular dermis. Keratin 16 is known to be involved in the reorganisation of the keratin filaments to facilitate mobility by keratinocytes [31].

The reasons for the prolonged CD36 expression by keratinocytes, the cause of its expression and its function in burn wound scars are all unclear. It is known that CD36 is upregulated by keratinocytes in response to some immunological and non-immunological stimuli [2, 5, 8, 22]. CD36 can be expressed by various other cell types, such as platelets, monocytes, macrophages, erythrocytes and endothelial cells [14]. It belongs to a family of integral membrane glycoproteins that bind a rather wide range of ligands [4, 13, 18, 39]. It may serve as a signal transduction molecule [29] and as a general adhesion molecule [34]. The biochemical features of the CD36 molecule expressed on keratinocytes of hypertrophic scars are identical to those on other cell types [1], suggesting that the function of CD36 on keratinocytes might resemble its role in other cell types.

In macrophages, CD36 has been identified as an adhesion molecule in the clearance of apoptotic neutrophilic granulocytes [34]. CD36 may serve as an adhesion molecule in keratinocytes, whether for inflammatory cells, or for adjacent keratinocytes to form a tight connection when the epidermis is mechanically challenged in scar formation. It may be that Langerhans cells upregulated in the burn scars (personal, unpublished, results) use the CD36 molecule as an adhesion factor to re-enter the epidermis after re-epithelialisation.

In platelets CD36 is a receptor for both thrombospondin [4] and collagen [39]. In erythrocytes it is implicated in the binding of malaria-infected cells to endothelium [13], and in macrophages in the binding and internalisation of oxidised LDL [18]. It is unlikely that CD36's main function in keratinocytes is to be a receptor for thrombospondin-1, as it has previously been shown that CD36-positive keratinocytes in a variety of skin diseases remained thrombospondin-1 negative [8].

In platelets and monocytes, CD36 serves as a signal transduction molecule [29], as it may in keratinocytes. After burn wound healing and during tissue remodelling, dermal–epidermal crosstalk is necessary for formation of a mature scar.

We do not have an explanation for the surprising observation that CD36 was expressed by the keratinocytes in the matched unburned skin. However, it suggests that this molecule is probably a marker for keratinocyte activation rather than for keratinocyte differentiation, as has previously been suggested [2, 20].

IFN-γ is one of the cytokines that have been reported to induce CD36 expression by keratinocytes [5, 7, 8, 37]. However, neither ICAM-1 nor HLA-DR, both of which also known to be directly upregulated by IFN-γ [10], was present on the keratinocytes of the burn scars (data not shown). This suggests that IFN-γ is not involved in the upregulation of CD36 in burn scars.

The upregulation of CD36 is likely to be influenced by the presence of an hormonally active peptide, as it is also upregulated in the matched unburned skin of the patients. No long-term follow-up data are available on cytokine levels that lead to activation of the noninvolved epidermis, so that there are no data to support this suggestion. Neuropeptides regulated by the nervous system and secreted by nerve endings are another possible source of centrally located messenger that might influence both the keratinocytes of the burn scars and the keratinocytes of the matched unburned skin. When the skin is injured signals elicited by the sensory nerves from this tissue reach the central nervous system, and in addition to this so-called orthodromic response, the sensory nerves are capable of a second, efferent, impulse to the skin, the antidromic response [3]. With this response, neuropeptides are released into the skin, which can influence keratinocyte activation in clinically unaffected skin. Neuropeptides can also modulate keratinocyte functions, such as cytokine production [36] and possibly CD36 expression. Nerves reach both the burned and the unburned skin and can thus influence keratinocytes of the whole skin.

We conclude that the development of hypertrophic scarring is not only an isolated dermal defect, but rather the result of a defect in the interaction between dermis and epidermis and also influenced by systemic neurohormonal systems.

Acknowledgements We thank Mr. T. M. van Os for preparing the figures. This work was supported by the Dutch Burns Foundation.

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