EPIDERMAL KERATINOCYTES MAY HAVE AN IMPORTANT ROLE IN HYPER-TROPHIC SCARRING PATHOGENESIS:
AN IMMUNOHISTOCHEMICAL STUDY (USING P63 AND KI-67 STAINING)

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SUMMARY. The role of epidermal keratinocytes in the early phase of normal unimpaired wound healing has been extensively studied. However, little is known of the cell biological process in the epidermis and the role of keratinocytes in hypertrophic scar formation. This study investigated the possible role of p63 in the early phase of hypertrophic scarring pathogenesis. Nine skin samples were taken from nine patients during plastic surgery operations, as follows: 1. six samples from patients who on account of thyroid disease or other reasons presented risk factors (RFs) for hypertrophic scarring; 2. one sample from a healthy young person (as control); and 3. one sample from the upper eyelid during blepharoplasty and one sample from an elderly patient during breast reduction. All the patients were women, and were followed up clinically for 12 months. Skin specimens were cultured and sectioned, and analysed by histology and immunohistochemistry. In normal skin, nuclear p63 was abundantly expressed by the basal cells, but expressed by very low levels of transient amplifying (TA) keratinocytes covering the surface. TA keratinocytes, immediately after their withdrawal from the stem cell compartment, reduced p63, even though they possessed a proliferative capacity. In some skin samples with RFs possessed a high level of p63 expression - not only basal stem cells but also four to five rows of parabasal cells. Four of the six skin samples with RFs showed significant epidermal abnormalities through the expression of both p63 and ki-67. Staining for ki-67, a marker for cell proliferation, revealed more increase in the suprabasal than in the basal keratinocyte proliferation rate. These results suggest that the epidermal keratinocytes may have an important role in hypertrophic scarring pathogenesis, using paracrine or epithelial-mesenchymal signalling. At 3, 6, and 12 months post-operation this finding clini-
monitor the survival of epithelial progenitor cell compartments in skin epithelium. We also chose ki-67, which is a nuclear antigen expressed in all phases of the cell cycle except G0 and is an accepted marker for monitoring cells that are actively replicating. Accordingly, small specimens were cultured, sectioned, and analysed by histology and immunohistochemistry. We postulate that the identification of p63 as a keratinocyte stem cell marker will be of practical importance for the clinical application of epithelial cultures in cell therapy, as also for studies on epithelial tumorigenesis.

Materials and method

Six skin samples were obtained from six patients during mammoplasia, all of whom were having treatment for thyroid disease or other risk factors (RFs). We chose thyroid disease as an RF for hypertrophic scarring on the basis of our data, which showed a possible relationship between thyroid disease and hypertrophic scar formation. Other risk factors were allergy, sweating, smoking, etc. Three other samples were obtained from three patients (one sample from a healthy young donor during breast reduction, one sample from an elderly donor during breast reduction, and one sample during upper eyelid blepharoplasty). The age of the patients ranged from 24 to 67 yr. All the patients were white women. Table I summarizes the clinical data.

<table>
<thead>
<tr>
<th>Biopsy sex</th>
<th>Age</th>
<th>Operation or diagnosis</th>
<th>Risk factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal healthy skin from young donors (during breast reduction)</td>
<td>1 24 F</td>
<td>Breast reduction</td>
<td>Thryeopathy, allergy</td>
</tr>
<tr>
<td>Normal skin -RF's</td>
<td>2 44 F</td>
<td>Re-abdominoplasty (prior scar was normal scar)</td>
<td>Smoking, psychology</td>
</tr>
<tr>
<td></td>
<td>1 37 F</td>
<td>Breast reduction</td>
<td>Thryeopathy, smoking</td>
</tr>
<tr>
<td></td>
<td>2 24 F</td>
<td>Breast reduction</td>
<td>Thryeopathy, smoking</td>
</tr>
<tr>
<td></td>
<td>3 24 F</td>
<td>Mastectomy</td>
<td>Hipercholesterolemia, allergy</td>
</tr>
<tr>
<td></td>
<td>4 32 F</td>
<td>Breast reduction</td>
<td>Hipercholesterolemia, allergy</td>
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<td></td>
<td>5 24 F</td>
<td>Breast reduction</td>
<td>Hipercholesterolemia, allergy</td>
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<tr>
<td></td>
<td>6 24 F</td>
<td>Breast reduction</td>
<td>Hipercholesterolemia, allergy</td>
</tr>
<tr>
<td>Normal skin</td>
<td>1 67 F</td>
<td>Breast reduction</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 41 F</td>
<td>Blepharoplasty</td>
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</tr>
</tbody>
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Small fragments of all samples were cultured, sectioned, and analysed by histology and immunohistochemistry.

Cell culture

Primary cultures of skin samples were generated as described in lab.

Fresh tissue specimens were kept in cold Dulbecco’s modified Eagle medium (with 10% foetal bovine serum and 2% penicillin and streptomycin) and transferred to our lab within 2 h of excision. The tissues were cut into small pieces in a sterile culture dish (Petri dish). Three millilitres of growth medium consisted of Dulbecco’s modified Eagle medium containing 10% foetal calf serum, 100u/ml penicillin and 0.1 ml/ml streptomycin sulphate. Each specimen was divided into four sterile culture dishes (Petri dishes) - two dishes were kept in M1H and two in EMA. The cultures were incubated at 37 °C in a humidified atmosphere of 10% CO₂/90% air. The cultures were checked every day and the medium was changed every second day.

Immunohistochemical staining

1. Cultured cells were fixed as described previously (Tseng and Green, 1994) and stained with the mouse monoclonal antibody to p63 (Yang et al., 1994) and to human ki-67 (DAKO, Carpinteria, CA).

2. Paraffin sections. Briefly, 3-um thick sections were cut from paraffin blocks containing representative skin samples. Paraffin sections were dewaxed in xylene, rehydrated through a series of graded alcohols, placed in 10 mM citrate buffer, and submitted to heat retrieval using a vapour lock for 40 min. After heating, the slides were allowed to cool to room temperature and were briefly washed with tris-buffered saline and endogenous peroxide in methanol for 5 min. Immunohistochemical staining was performed as described by Pelligrini. Sections were stained with mouse mAb to p63 and to human ki-67.

Histology

Tissue samples were fixed in 3.7% formalin, then dehydrated in 75, 80, 96, and 100% ethanol, treated with noxyl, and embedded in paraffin wax. The tissues were cut into 8um slices and processed with haematoxylin-eosin for histology.

Results

1. Haematoxylin and eosin staining

Upper eyelid skin

The epidermis was of normal thickness in the basal layer, keratinocytes were columnar, and scattered melanocytes were visible at the dermis-epidermis junction. Eyelid skin lacks a distinct interface between the papillary, the reticular, and the subcutis.

Skin from elderly donor (Figs. 1,2).
Skin from healthy young donor

Figs. 1, 2 - Compared to skin from a healthy young donor, the integument was characterized by atrophy. The stratum corneum was a little altered in thickness, and there was a reduction in the dermoepidermal papillae. There was also a reduction in the number of melanocytes and Langerhans cells. The most significant cutaneous changes were seen in the dermis, especially in the superficial layers. The elastic fibres, which are responsible for maintaining collagen orientation, physiological recoil, and laxity, underwent intrinsic degenerative changes. Dermal collagen, which accounts for 70 to 80% of the skin’s dry weight, also decreases with ageing. Much of this loss occurs in the reticular dermis.

The skin epithelium showed all the features of a normal squamous stratified epithelium. First, the basal or germinative layer contained two types of cells (keratinocyte progenitors forming a row of cells with scant cytoplasm and nuclei often oriented perpendicular to the underlying basal lamina); and second, parabasal cells formed one or more layers of cells larger than basal cells and with more abundant cytoplasm. The dominant portion of the squamous epithelium represented the mid-zone with cells characterized by a gradual increase in size and amount of cytoplasm, often containing large vacuoles and the superficial zone formed of terminally differentiated flat cells located near the external surface.

Skin from young donors with RFs (Figs. 3, 4)

Figs. 3, 4 - Well-differentiated cells that gradually increase in size. Note increased acanthosis compared to previous samples.

2. P63 and ki-67 expression

Normal young healthy skin

P63 was consistently expressed in the nuclei of epidermal cells, cells of the germinative hair matrix, and the external root sheath of the hair follicles. No p63 staining was observed in the cells of Henle’s layer, Huxley’s layer, cuticle, or in the perifollicular connective tissue sheath. The nuclei of sebaceous gland basal cells were strongly stained by p63, and an uneven nuclear staining of the apocrine and eccrine sweat glands’ basal/myoepithelial cells was depicted. No dermal mesenchymal cell, endothelial cell, pericyte, smooth muscle cell, neural cell, or adipocyte showed any immunoreactivity for p63 (Figs. 5, 6 - Cell culture, paraffin section).

Ki-67 expression

This was detected in a few keratinocytes, mostly in the parabasal layers but rarely in the basal layer. This suggests that basal cells act as stem or reserve cells, whereas parabasal cells comprise the actively replicating compartment of this epithelium (Figs. 7, 8).

In normal skin from old donors, p63 was expressed in...
basal epithelial cells and in a small proportion of suprabasal cells (Fig. 10). In skin from the upper eyelid there were no big differences in comparison with normal healthy skin (Fig. 9).

**Ki-67 expression**

In old skin the proportion of cells expressing ki-67 antigen decreased, concomitantly with the progressive degeneration of the epithelium (Fig. 11). In upper eyelid skin there were no big differences compared to healthy skin (Fig. 12).

Skin obtained from patient with risk factors (potential for hypertrophic scarring)

It has been shown that the growth of megacolonies depends on outward migration of the rapidly proliferating cells located in a rim close to the colony perimeter (Barrandon and Green, 1987). The multiplication rate internal to this rim is much lower. Although the small cells located in the centre of the colonies are quiescent, they remain capable of growth (Barrandon and Green, 1987; Tseng and Green, 1994).

Ki-67 is a cell proliferation-associated nuclear antigen found in all stages of the cell cycle. Double staining for p63 and ki-67 revealed that the rapidly proliferating cells located close to the megacolony perimeter contained both p63 and ki-67.

Ki-67 was concentrated in the nucleoli, a site from which p63 seemed to be excluded. In the central part the colonies retain p63, but few cells contain ki-67. Similarly, in normal human epidermis, most cells in the basal layer of epidermis are not engaged in multiplication (Boezman et al., 1987; Potten and Morris, 1988; Clausen and Potten, 1990). Double staining for p63 and ki-67 revealed that only a small proportion of the cells containing p63 also contained ki-67, while all the cells containing ki-67 contained p63.

We have now shown that staining for p63 and ki-67 revealed that cells forming the basal layer expressed both p63 and ki-67. Although most cells expressing ki-67 also expressed p63, it was possible to observe cells expressing ki-67 but not p63. Most frequently, cells expressing high levels of p63 are expressed by keratinocytes that possess the ability to proliferate, and not simply by keratinocytes that are duplicating their DNA, as already shown in human epidermis.

These data confirm that p63 protein is principally restricted to keratinocyte stem cells; however, in some samples obtained from patients with RFs, the expression was not restricted only to stem cells but also to most of the parabasal cells.

**Discussion**

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Figs. 13, 14 - P63 expression is not restricted to keratinocyte stem cells but also to most of the suprabasal cells (4-5 rows).

Suprabasal cells expressed more ki-67 than basal cells, which suggests that most cells expressing ki-67 also expressed p63. These observations suggest that p63 was expressed by keratinocytes possessing the ability to proliferate, and not simply by keratinocytes that were duplicating their DNA (Figs. 15, 16).

Figs. 15, 16 - Suprabasal cells express more ki-67 than basal cells, suggesting that most cells expressing ki-67 also express p63. These observation suggest that p63 is expressed by keratinocytes that possess the ability to proliferate and not simply by keratinocytes that are duplicating their DNA.

Figs. 17, 18 - The proportion of cells expressing ki-67 antigen dramatically increased in the basal, parabasal, and intermediate zones.
Hypertrophic scarring in human skin is different from normal mature scar tissue at both biochemical and cellular levels. Previous histopathological and cell biological studies have mainly focused on the dermal process, and little is known of the involvement of the epidermis.

The physiological role of the uninjured epidermis is largely that of fluid conservation and as a barrier to microbial infection. This homeostatic function may require a limited gene product expression to maintain a steady state. After injury, however, the secretory role of the epidermis changes dramatically to stimulate local and systemic responses, allowing recruitment of immune effector cells from the circulation and promoting proliferation of adjacent parenchymal cells, including fibroblasts. The function of this epidermal response to injury is an attempt to restore both the skin and the body to their uninjured state. In the skin, this results in wound healing and the production of scar tissue. The result of the present study shows that the changes due to hypertrophy are not confined to the dermal compartment but also include the epidermis.

This report shows that in cultured keratinocytes p63 is principally confined to the small cells known to be capable of DNA synthesis and colony formation, and absent from the larger cells that are undergoing terminal differentiation.

We show here that keratinocytes obtained from elderly patients have greatly reduced p63 compared to keratinocytes from young healthy patients, even though they possess a very appreciable proliferative capacity. Ki-67 was expressed more in suprabasal than in basal cells. For instance, p63 was expressed by all basal and most suprabasal cells in four out of six cases of samples with RFs; in the other two samples the expression of p63 decreased, as witnessed by ki-67 expression.

We also observed that most cells expressing ki-67 also expressed p63. These observations suggest that p63 is expressed by keratinocytes that possess the ability to proliferate and not only by keratinocytes that are duplicating their DNA, as already shown in normal human epidermis. For these reasons, we postulate that the number of cells containing p63 and ki-67 and their distribution may in fact encompass all individuals who are susceptible to hypertrophic scar formation.

To our knowledge, these are the first preliminary data for investigating the relationship between the relative

**Clinical results (Table I)**

Figs. 19-21 - Patient no. 1, 9 months post-operation (breast reduction), with RFs (thyreopathy, allergy).

Fig. 22 - Patient no. 3, one year post-operation (mastopexy), with RFs (thyreopathy, psychology).

Fig. 23 - Patient no. 4, one year post-operation (breast red), RFs (thyreopathy, smoking).

Fig. 24 - Patient no. 5, 8 months post-operation (breast red), RFs (thyreopathy, hypercholesterolaemia).
numbers of p63-positive cells and of dividing cells expressing ki-67 and hypertrophic scarring. The data suggest that this protein may have a function in maintaining the proliferative potential of keratinocytes and the prevention of terminal differentiation - this may influence the growth and proliferation of fibroblasts through paracrine signalling.

Conclusion

Previous studies have focused largely on the role of fibroblasts in the formation of hypertrophic scars. The ever-increasing pool of information on epidermal-mesenchymal interactions now points to the role of feedback mechanisms that exist between the two cell types and the mutual control exerted by one over the other. The possibility of feedback from fibroblasts that subsequently changes the keratinocyte secretion profile is suggested, but not established.

The most striking epidermal abnormality is the intriguing finding that increased p63 expression and an increased proliferation rate appear to be associated with the clinical outcome after 6-12 months. Unfortunately there is no clinical opportunity for experimental manipulation of cell biological parameters such as altered gene expression and cellular proliferation. It is therefore impossible to conclude whether these are a consequence of hypertrophic scar formation or fortuitously involved in the pathogenic process. Our preliminary data suggest that the role of the keratinocytes now appears to be more important than was previously realized.

An aberration in this control over fibroblast production of collagen and ECM may play a role in the pathogenesis of hypertrophic scar formation, as one extreme in the spectrum of wound healing. Future studies will analyse fibroblast matrix and growth factor production in the co-culture model.

In summary, we have demonstrated that epidermal keratinocytes may play a pivotal role in the pathogenesis of hypertrophic scarring by increasing the growth and proliferation of fibroblasts through paracrine signalling. We tried to obtain new insights into the role of p63 as a possible candidate protein responsible for this interaction. Further studies to analyse and identify the candidate genes and proteins deserve investigation.

Clinical implications

The relative numbers of p63-positive cells and dividing cells expressing ki-67 and the pathogenic process of hypertrophic scar formation are still preliminary data. If our assumption proves to be correct, we can predict the early formation of hypertrophic scarring formation, and the treatment could some day be amenable to localized gene therapy. Intact genes could be introduced into the lesion, reintroducing normal cellular regulation into this disordered population of cells. This finding does not however eliminate other chance factors.
BIBLIOGRAPHY


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