

## INVESTIGATIVE REPORT

# Tumour Necrosis Factor- $\alpha$ Does Not Influence Proliferation and Differentiation of Healthy and Psoriatic Keratinocytes in a Skin-equivalent Model

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**Tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) has been implicated in the pathogenesis of psoriasis. Its effect on keratinocytes from healthy and psoriatic skin was investigated. The keratinocytes were co-cultured with healthy and psoriatic fibroblasts in skin equivalents and grown in a serum-free medium for 15 days. TNF- $\alpha$  was added, or not, on day 12. The expression of differentiation and proliferation markers was investigated with immunohistochemistry. The epidermal growth rate was assessed by the percentage of Ki-67-positive nuclei in the basal layers of the outgrowths, which were all multilayered and orthokeratotic. The expression of the epidermal growth factor receptor, cytokeratin 16, involucrin and filaggrin displayed a hyperproliferative, regenerative pattern. No statistically significant differences in growth rate were found between the groups. These findings indicate a lack of effect of TNF- $\alpha$  on proliferation and differentiation in healthy and psoriatic keratinocytes. Further studies are warranted to elucidate the pathophysiological role of TNF- $\alpha$  in psoriasis. **Key words:** Ki-67; fibroblasts; proliferation markers; differentiation markers.**

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Psoriasis is a common, genetic skin disease of unknown aetiology. Several features of the psoriatic lesion, such as hyperproliferation and disturbed maturation of epidermal keratinocytes and the inflammatory reaction, may be explained by an altered phenotype of epidermal and dermal cells leading to an altered expression of and/or an altered sensitivity to different cytokines and altered epidermal–dermal interactions (1–5).

Tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), is a multifunctional proinflammatory cytokine. It has been implicated as a key cytokine in the pathogenesis of psoriasis because of its ability, alone or in interactions with other mediators, to induce several cytokines and adhesion molecules involved in the development of the psoriatic lesion (6–8). The biological activity of TNF- $\alpha$  is elevated in psoriatic lesions (9), and strong staining of TNF- $\alpha$  on keratinocytes and endothelial cells from psoriatically involved skin has been reported (8). Recent genetic studies have suggested that a TNF- $\alpha$  promoter polymorphism is associated with psoriatic arthritis (10) and juvenile-onset psoriasis (10, 11). Further, peripheral blood monocytes from psoriatic subjects produce more TNF- $\alpha$  than those from healthy subjects (12). However, previous studies

on the influence of TNF- $\alpha$  on the proliferation of keratinocytes have produced conflicting results (13, 14).

As mentioned above, interactions between the epidermis and dermis are of importance in psoriasis. Using different combinations of healthy and psoriatic keratinocytes and fibroblasts in a skin-equivalent model, it was previously shown that the influence on epidermal differentiation and/or proliferation by interferon- $\psi$  (IFN- $\psi$ ), another proinflammatory cytokine, depends on the origin of the cells (5). In the present study, the skin-equivalent model is used to study whether TNF- $\alpha$  influences the proliferation and differentiation of healthy and psoriatic keratinocytes and, if so, whether the response to TNF- $\alpha$  depends on the origin of the cells.

## MATERIALS AND METHODS

### *Patients*

Skin samples for the skin equivalents were obtained from 10 healthy volunteers (7 women and 3 men, aged 30–61, median 45 years) and 11 patients (4 women and 7 men, aged 23–66, median 46 years) with plaque psoriasis. The patients had had no topical or ultraviolet radiation treatment for at least 2 weeks and no systemic treatment for several months prior to biopsy. Lidocaine (Xylocaine; Astra, Södertälje, Sweden) was used for analgesia. Permission to conduct the study was obtained from the Ethics Committee at Karolinska Hospital, Stockholm. Informed consent was given by all subjects.

### *Chemicals*

Earle's modified Eagle's medium (EMEM) and trypsin 0.05%/EDTA 0.02% were purchased from Flow Laboratories (Stockholm, Sweden), RPMI 1640, penicillin–streptomycin 100X and L-glutamine 200 mM 100X from Gibco (Grand Island, New York, USA), collagen R and epidermal growth factor (EGF) from Serva (Heidelberg, Germany), L-ascorbic acid, O-phosphorylethanolamine, ethanolamine, human transferrin, dimethyl sulphoxide grade I (DMSO), Trizma (Tris buffer) and recombinant human TNF- $\alpha$  expressed in yeast (specific activity  $1 \times 10^7$ /mg protein,  $1 \times 10^7$ – $1 \times 10^8$  IU/mg) from Sigma (St Louis, MO, USA), hydrocortisone (50 mg/ml, Solu-Cortef) from Upjohn (Kalamazoo, MI, USA) and insulin (100 U/ml Acrapid) from Novo nordisk (Bagsvaerd, Denmark). The monoclonal antibodies used are listed in Table I. All other chemicals were of analytical grade and were from Kebo (Stockholm, Sweden). Petri dishes and culture flasks were from Falcon (Becton Dickinson, Plymouth, UK). Millex-GS 0.22  $\mu$ m was from Millipore (Bedford, MA, USA) and Acrocap was supplied by German Sciences (Ann Arbor, MI, USA). Human B serum was obtained from healthy donors at the blood centre at Karolinska Hospital, Stockholm.

### *Skin equivalents*

Skin equivalents were produced by implanting skin biopsies in pre-produced dermal equivalents consisting of fibroblasts in a collagen

Table I. Antibodies: anti-involucrin was from rabbits and all other antibodies were from mice

Antibody	Antibody class	Specificity	Dilution	Source
Anti-involucrin	Polyclonal	Involucrin	1:10	Biomedical Technology
Anti-filaggrin	IgG1	Filaggrin	1:500	Biomedical Technology
EGFRI	IgG2b	EGF receptor	1:100	Amersham
8.12	IgG1	Cytokeratin 13 <sup>a</sup> , 16	1:100	Sigma
Ki-67	IgG1- $\kappa$	Proliferating cell	1:100	Becton Dickinson

<sup>a</sup>Not present in the skin.

Ig: immunoglobulin; EGF: epidermal growth factor.

matrix (4, 15). The fibroblasts were from 5 of the 10 healthy controls and from the lesional skin of 5 psoriatic subjects. The fibroblasts were propagated and frozen in liquid nitrogen (4, 16). Fibroblasts from passage IV were used for the production of dermal equivalents as described previously (16, 17). The fibroblasts were grown and the dermal equivalents fabricated in a serum-containing medium (4, 16). Two different batches of B serum were used. One of the batches was used in an experiment in which 11 dermal equivalents from 1 healthy and 12 dermal equivalents from 1 psoriatic donor were evaluated. The other batch was used in all other cases.

Keratinocytes were obtained from 4 mm punch biopsies, taken from both involved and uninvolved skin of 6 psoriatic patients and from 6 of the 10 healthy controls as described previously (4). Most of the dermis was trimmed off the biopsies, and then they were divided into 4 pieces, each with an approximate area of 3 mm<sup>2</sup>. In total, 128 skin equivalents were evaluated, 48 skin equivalents containing keratinocytes originating from healthy skin, 43 containing keratinocytes from involved skin and 37 from keratinocytes from uninvolved psoriatic skin. Half of the material was combined with dermal equivalents containing fibroblasts from healthy skin and the other half with dermal equivalents containing fibroblasts from involved psoriatic skin. In this way, 6 different combinations of cells were obtained. The skin equivalents were cultured on grids in the air-medium interphase in RPMI 1640 supplemented with 50  $\mu$ g/ml L-ascorbic acid, 10  $\mu$ g/ml iron-saturated transferrin (5.4 mg transferrin was dissolved in 900  $\mu$ l water and mixed with 10  $\mu$ l 13 mM FeSO<sub>4</sub> and 100  $\mu$ l bicarbonate, pH 7.4, for saturation overnight), 0.1 mM *O*-phosphorylethanolamine, 0.1 mM ethanolamine, 5  $\mu$ g/ml insulin, 0.4  $\mu$ g/ml hydrocortisone, 1 ng/ml EGF, 2 mM L-glutamine and antibiotics (4). The medium was changed every 3 days. TNF- $\alpha$  2.5 ng/ml was added to half of the cultures on day 12. On day 15, the skin equivalents were snap-frozen for immunohistochemistry and kept at -80 C until used. All incubations were carried out at 37 C in an atmosphere of CO<sub>2</sub>/95% air and at 100% humidity.

#### Immunohistochemical staining

Cryostat sections, 5  $\mu$ m thick, were kept at -80 C until processed. An indirect 2-step immunoperoxidase staining method was used (16). The antibodies and the dilutions used are listed in Table I. Frozen sections from healthy skin or psoriatic lesions were used as positive controls. Control sections prepared and run in parallel excluding the specific primary antibodies were all negative. Epidermal growth was assessed by counting the number of Ki-67-positive nuclei and the total number of nuclei in the basal layers of the multilayered outgrowths. The areas in the closest vicinity of the original biopsies, where a light epiboly occurred before the outgrowths reached the dermal equivalents, were excluded from counting. Epidermal growth rate was expressed as the percentage of Ki-67-positive nuclei in the basal layer of the outgrowth (4). Two sections from each skin equivalent were counted.

#### Statistical methods

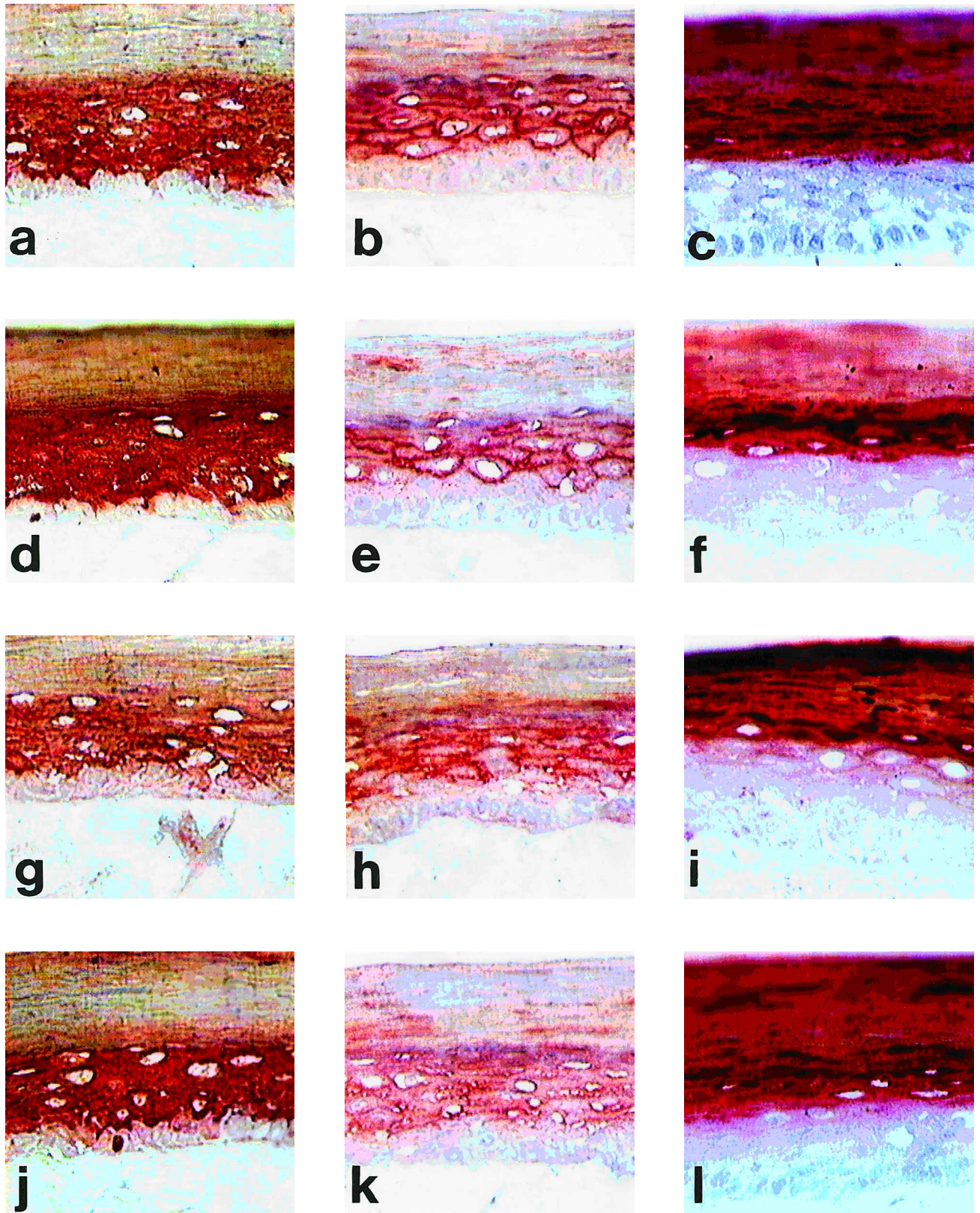
The data were normalized with logarithmic transformation and subjected to analysis of variance.  $p < 0.05$  was considered statistically significant.

## RESULTS

The initial outgrowth of the keratinocytes in this skin-equivalent model depends on migration, which is usually completed after 7 days (16). At this time-point the epidermal outgrowths usually cover the surfaces of the dermal equivalents. In the present study, the skin equivalents were cultured for 12 days in the serum-free medium to allow the formation of a multilayered epidermal outgrowth. The skin equivalents were then cultured for another 3 days in the presence or absence of TNF- $\alpha$  and thereafter evaluated by immunohistochemistry. In all skin equivalents the keratinocytes formed a multilayered outgrowth with a well-developed stratum granulosum and mainly with an orthokeratotic stratum corneum. The morphology of the epidermal outgrowths and the expression of the markers for proliferation and differentiation were similar in all cultures irrespective of whether the keratinocytes and the fibroblasts originated from healthy controls or from psoriatic patients, or whether TNF- $\alpha$  was present (Fig. 1). Involucrin was expressed from the stratum granulosum to the deeper spinous layers, filaggrin in the stratum granulosum and usually also in the stratum corneum, and the EGF receptor throughout the epidermal outgrowths. Cytokeratin 16 was seen suprabasally in a majority of the epidermal outgrowths, while a few outgrowths were also stained basally. The epidermal growth rate, assessed by the percentage of Ki-67-positive nuclei in the basal layer of the outgrowths, is illustrated in Fig. 2. Although there was a tendency towards an increased growth rate in keratinocytes from uninvolved psoriatic skin in skin equivalents containing psoriatic fibroblasts after the addition of TNF- $\alpha$ , no statistically significant differences were found between the groups.

## DISCUSSION

The skin-equivalent model offers an opportunity to study epidermal-dermal interactions. This study shows, in concordance with previous reports (4, 5), that the epidermal outgrowths of the skin equivalents are hyperproliferative and well differentiated irrespective of whether cells from healthy or psoriatic subjects are used. TNF- $\alpha$  did not influence either proliferation or differentiation of healthy or psoriatic



*Fig. 1.* Skin equivalents cultured for 12 days in a serum-free medium and thereafter in the absence (a–c, g–i) or presence of tumour necrosis factor- $\alpha$  2.5 ng/ml (d–f, j–l) for 3 days. The skin equivalents were fabricated from healthy keratinocytes (a–f) and from keratinocytes from psoriatic lesions (g–l). Healthy fibroblasts were used in b, e, h and l and psoriatic fibroblasts in a, c, d, f, g, i, j and k. Expression of markers associated with differentiation: cyokeratin 16 in the left column (a, d, g, j), involucrin in the middle column (b, e, h, k) and filaggrin in the right column (c, f, i, l). Cryostat sections, 5  $\mu$ m thick, were stained by a 2-step immunoperoxidase method. Counterstain haematoxylin, magnification  $\times$  370.

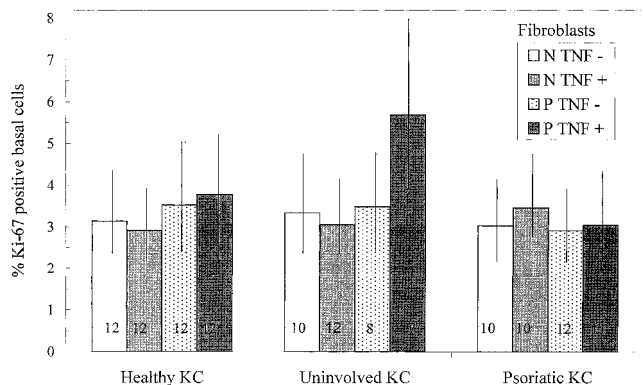


Fig. 2. Epidermal growth rate in the skin equivalents, assessed by the percentage of Ki-67-positive nuclei in the basal layer. Skin equivalents were cultured for 12 days in a serum-free medium, and then with or without 2.5 ng/ml TNF- $\alpha$  for 3 days. The results are expressed as geometric mean and 95% confidence limits. The number of skin equivalents in each group is given in the figure. N: normal; P: psoriatic; KC: keratinocytes.

keratinocytes in this model, although there was a tendency towards an increased growth rate in keratinocytes from uninvolved psoriatic skin co-cultured with psoriatic fibroblasts after the addition of TNF- $\alpha$ . The lack of effect of TNF- $\alpha$  in this study was not due to the absence of biological activity of the batch used, since the same batch successfully stimulated the production of IL-6 in healthy and psoriatic fibroblasts in dosages ranging from 0.01 to 10 ng/ml (18). These findings are diverge from previous reports on TNF- $\alpha$ . Kono et al. (19) reported that TNF- $\alpha$  markedly suppresses the growth of human keratinocytes, preponderantly in the late growth phase or preconfluent phase. Pillai et al. (13) found that TNF- $\alpha$  inhibits the proliferation of neonatal foreskin keratinocytes *in vitro* and that this effect is most marked in the preconfluent stages. They also reported that TNF- $\alpha$  induced differentiation in the confluent and preconfluent keratinocytes but not in postconfluent ones. In isolated cell cultures, the addition of TNF- $\alpha$  resulted in a significant dose- and time-dependent inhibition of growth, in both lesional psoriatic and normal epidermal cells (20). Inhibition was first seen 2 days after addition of the cytokine. Further, in a study in which involved psoriatic skin was grafted on to nude mice, subsequent treatment with TNF induced a marked decrease in epidermal thickness and labelling index of the psoriatic graft tissue (21). In contrast, subcutaneous perfusion of TNF- $\alpha$  in mice *in vivo* led to local proliferation of fibroblasts and blood vessels, and a hyperplastic reaction of the overlying epidermis (14). The discrepancies in results among different studies might be due to differences in experimental conditions. The response of a cell to a given cytokine is dependent on the local concentration of the cytokine, the cell type and other cell regulators to which it is concomitantly exposed (22). The overexpression of TNF- $\alpha$  in psoriasis led to suggestions that TNF- $\alpha$  antagonists could be used in the treatment of psoriasis. These drugs have been used successfully in other inflammatory diseases such as rheumatoid arthritis and inflammatory bowel diseases (23, 24). Surprisingly, however, systemic administration of TNF- $\alpha$  has been reported to clear psoriasis (25, 26). Hence, further studies are warranted to elucidate the role of TNF- $\alpha$  in the pathogenesis of psoriasis.

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