

# EXPRESSION OF CYTOKINES AND THEIR RECEPTORS BY PSORIATIC FIBROBLASTS.

## II. DECREASED TNF RECEPTOR EXPRESSION



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**Psoriatic fibroblasts produce enhanced amounts of IL-6 in vitro. This state of activation may reflect an altered expression of cytokine receptors, involved in auto/paracrine induction of IL-6. Cultures of dermal fibroblasts derived from lesional psoriatic (PP) and normal control (NN) skin were therefore analysed for their ability to bind biotinylated recombinant human cytokines using flow cytometry. PP and NN fibroblasts bound negligible amounts of IL-1 $\alpha$  and IL-1 $\beta$ , but clearly bound IL-4, IL-6 and TNF- $\alpha$ . Serum upregulated the number of NN fibroblasts which bound TNF- $\alpha$ , and to a lesser extent IL-6, but not the number of binding sites per cell. In contrast, this upregulation was significantly less in PP fibroblasts. This was not a result of differences in growth characteristics, receptor occupancy or an inability of stimulated PP fibroblasts to bind TNF- $\alpha$ . Immunocytochemistry of cells grown on slides showed that the TNF receptor type 1 (TNFR1, p55) was the predominant receptor in NN fibroblasts and was localized to the nucleus and cytoplasm. The expression of TNFR1 was clearly decreased in PP fibroblasts, which confirmed the binding studies. A slow and serum-induced shedding of TNFR1 was observed, but not of the TNFR2 (p75), in both types of fibroblasts. Confluent multi-passaged PP fibroblasts display both a decreased TNFR expression as well as an enhanced IL-6 production under serum conditions. These inherent abnormalities of PP fibroblasts imply the involvement of dermal fibroblasts in the maintenance of chronic inflammation in psoriasis.**

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Human dermal fibroblasts are actively involved in inflammatory processes. These resident cells are not only responsible for the production and maintenance of the connective tissue matrix, but are also able to activate the overlying epidermal cells and contiguous endothelial cells, as well as to attract and activate infiltrating leucocytes. Their ability to take part in cellular communication is, at least in part, determined by the expression of adhesion molecules, cytokines and cytokine receptors. Human dermal fibroblasts are able to express the major histocompatibility complex (MHC) class I and II molecules,<sup>1,2</sup> and  $\beta_1$  integrins and the intercellular adhesion molecule (ICAM-1).<sup>3,4</sup> It has furthermore been reported that fibroblasts produce interleukin(IL-1) isoforms, IL-6, IL-8, monocyte chemotactic protein (MCP-1), leukaemia inhibitory factor (LIF), platelet-derived growth factors (PDGF)

and the colony stimulating factors (CSF).<sup>5-11</sup> Human dermal fibroblasts also express receptors (R) for IL-1, tumour necrosis factor (TNF), the interferons (IFN), PDGF and the transforming growth factors (TGF).<sup>12-16</sup> It is important to note that cytokine receptors often reflect the activation state of cells and are involved in the regulation of cytokine activity. Most receptors occur, apart from membrane forms, in soluble forms.<sup>17</sup> Soluble receptors either antagonize cytokine activity by scavenging, neutralization and prevention of extravascular escape of cytokines, or agonize cytokine activity by increase of persistence and chaperoning of cytokines.<sup>18</sup> The activity of cytokines is therefore, at least in part, the result of both the expression of cytokines as well as their receptors.

Active participation of dermal fibroblasts in the inflammatory mechanisms of psoriasis is evidenced by their hyperactive state in vitro. Psoriatic fibroblasts produce elevated amounts of superoxide anion, PDGF-BB and IL-1 $\beta$ , express elevated levels of surface proteases (e.g. exoaminopeptidases and CD26), protein kinase C activity and PDGFR $\beta$ , and show an altered response to serum factors, growth factors and several therapeutics.<sup>19-25</sup> Fibroblasts are furthermore a potential source for the locally produced bioactive IL-6 in psoriasis.<sup>26-27</sup> We observed that psoriatic fibroblasts express an increased production of IL-6 in vitro (see previous paper, pp 70-79).

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IL-6 links cutaneous inflammation and keratinocyte hyperproliferation in psoriasis. Moreover, in a mouse model, a psoriasiform inflammatory reaction was preceded by an increase in the endogenous IL-6 production in the skin.<sup>28</sup> The present study was conducted to investigate whether the enhanced fibroblast-derived IL-6 activity in psoriasis was associated with an altered receptor expression. We therefore analysed the expression of binding sites for IL-1 $\alpha$ , IL-1 $\beta$ , IL-4, IL-6 and TNF- $\alpha$ , and the production of biologically active IL-6, as well as the IL-6 related cytokines IL-1 and TNF- $\alpha$ , of early passages of fibroblast cultures derived from lesional psoriatic (PP) and normal control (NN) skin. Experiments were performed using a low and high-calcium basal medium and stimuli such as cytokines and serum.

## RESULTS

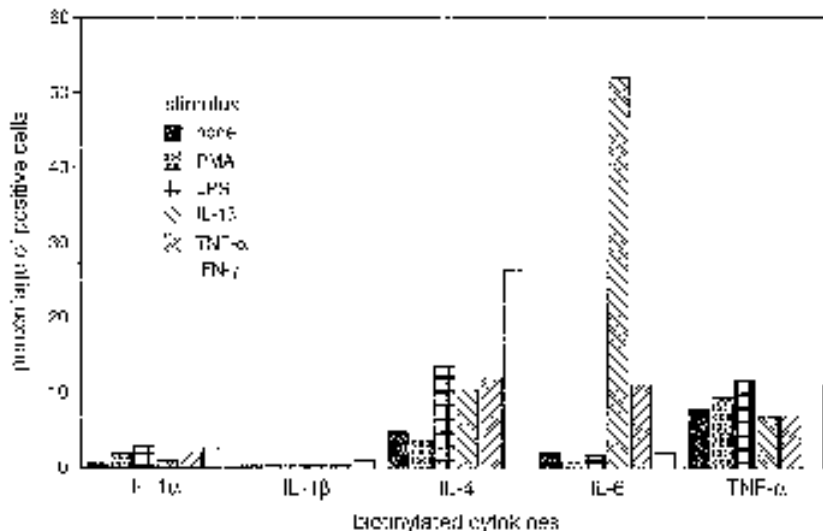
### *PP and NN fibroblasts clearly bind IL-4, IL-6 and TNF- $\alpha$ , but not IL-1 $\alpha$ or IL-1 $\beta$*

Cultures of NN fibroblasts hardly bound biotinylated recombinant human IL-1 $\alpha$  or IL-1 $\beta$ , even after stimulation with phorbol myristate acetate (PMA), lipopolysaccharide (LPS), or the recombinant human cytokines IL-1 $\beta$ , TNF- $\alpha$ , or IFN- $\gamma$ , as shown in Figure 1. Fibroblasts positive for binding with IL-4, IL-6 and TNF- $\alpha$  were clearly detectable and the binding of the former two cytokines was upregulated in stimulated fibroblasts. For instance, IL-1 $\beta$ -treated fibroblasts showed a substantially increased binding of IL-6, and

IFN- $\gamma$ -treated fibroblasts displayed increased binding of IL-4. PP fibroblasts also clearly bound IL-4, IL-6 and TNF- $\alpha$  (Fig. 2), but negligible amounts of IL-1 $\alpha$  and IL-1 $\beta$  (not shown). The binding of IL-4, IL-6 and TNF- $\alpha$  by both types of fibroblasts could be modulated by the use of different culture conditions, as shown in Figure 2. The binding of these cytokines was lower, although with a varying degree, using a high-calcium (1.05 mM), serum-free medium (a 1:1 ratio of F10:DMEM) when compared to a low-calcium (0.15 mM), serum-free medium (KBM/unsupplemented MCDB 153) (second versus first bars, Fig. 2). An increased binding was observed using fetal calf serum (FCS)-supplemented (5% v/v) medium (third versus second bars, Fig. 2). The observation that PP fibroblasts were able to bind more IL-4 than NN fibroblasts after a 24 h period under serum conditions was not statistically significant.

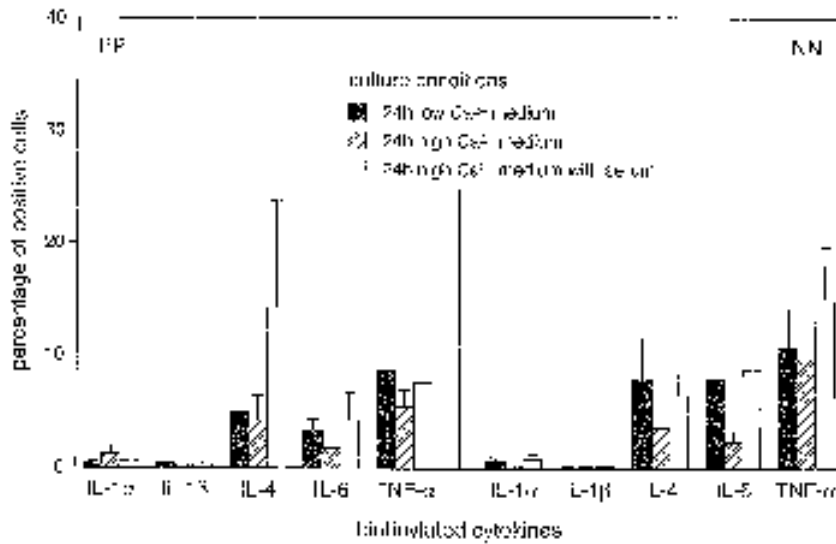
### *PP fibroblasts express a decreased binding of TNF- $\alpha$ under serum conditions*

We observed that the binding of IL-6 and TNF- $\alpha$  by NN fibroblasts, but not the other cytokines, increased when cells were cultured for up to 72 h in the presence of FCS. Serum upregulated the mean percentage of NN fibroblasts able to bind TNF- $\alpha$  (Fig. 3A), but not the number of TNF- $\alpha$  binding sites per cell, as indicated by the relative fluorescence intensities of the same cell samples (Fig. 3B). Culturing PP fibroblasts, however, for up to 72 h under serum conditions resulted in a



**Figure 1.** The binding of cytokines by fibroblasts.

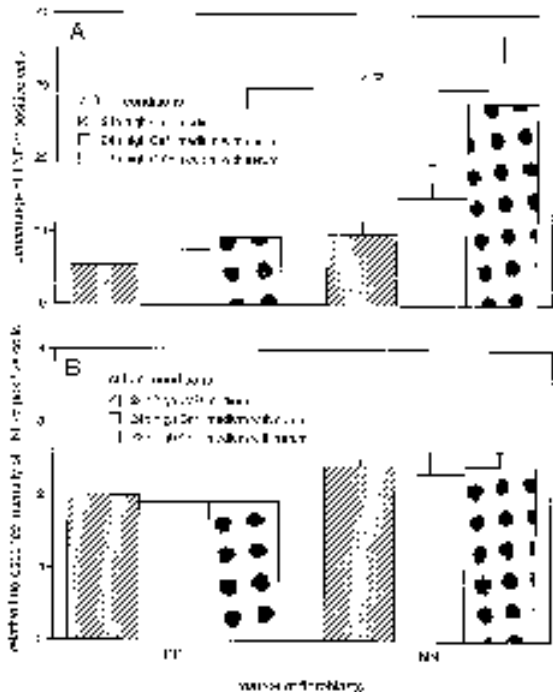
Subconfluent NN fibroblast cultures were incubated with a high-calcium medium containing 5% FCS for 24 h and analysed for their ability to bind the cytokines mentioned on the X-axis using flow cytometry, as described in Materials and Methods. The stimuli used were 50 ng/ml PMA, 10  $\mu$ g/ml LPS, 100 U/ml IL-1 $\beta$ , 100 U/ml TNF- $\alpha$  and 100 U/ml IFN- $\gamma$ , added the last 14 h of the 24 h incubation. Results are expressed as the percentage of positive cells after labelling with biotinylated recombinant human cytokines of a representative experiment. The experiment was performed in duplo.



**Figure 2. Calcium and serum modulate the binding of cytokines by PP and NN fibroblasts.** PP and NN fibroblasts (both  $n = 10$ ) were incubated with a low or high-calcium, serum-free medium, or a high-calcium medium containing 5% FCS for 24 h, and analysed for their ability to bind cytokines using flow cytometry. Results are expressed as the mean  $\pm$  SEM percentage of positive cells.

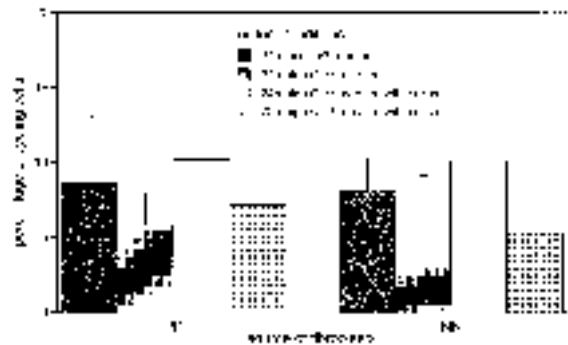
significantly lower number of fibroblasts that bound TNF- $\alpha$  when compared to NN fibroblasts ( $P < 0.03$ , Fig. 3A, and Fig. 5A and B). To a lesser extent the same was true for IL-6 (not shown). The increased binding of

TNF- $\alpha$  by NN fibroblasts under serum conditions correlated with a decrease in the percentage of cycling cells. The decreased binding of TNF- $\alpha$  by PP fibroblasts, as mentioned above, is, however, not a result of differences in growth characteristics between PP and NN fibroblasts, as checked by cell counts (data not shown) and the percentage of cycling cells ( $G_2/M$  phase) (Fig. 4). Furthermore, TNF- $\alpha$  binding sites of PP fibroblasts were not occupied with endogenously produced TNF molecules in vitro, as evidenced by the observation that fibroblasts did not produce TNF- $\alpha$  and by acid stripping of fibroblasts prior to flow cytometry (Fig. 5C). Stimulated PP fibroblasts were also still able to up-regulate binding of TNF- $\alpha$  (Fig. 5D).



**Figure 3. PP fibroblasts express decreased binding of TNF- $\alpha$  under serum conditions.**

PP and NN fibroblasts (both  $n = 10$ ) were analysed using flow cytometry for the percentage of cells which bind TNF- $\alpha$  (A) and the relative number of TNF- $\alpha$  binding sites per cell (B), after culture in a high-calcium, serum-free medium for 24 h, or a high-calcium medium containing 5% FCS for 24 or 72 h. Percentages of positive cells and the signal to noise ratios are presented as mean  $\pm$  SEM. The  $P$  value indicates a significant difference between PP and NN fibroblasts.



**Figure 4. Growth characteristics of PP and NN fibroblasts.**

Cell cycle analysis was performed on PP and NN fibroblasts (both  $n = 8$ ) cultured in a low or high-calcium, serum-free medium for 24 h, or a high-calcium medium containing 5% FCS for 24 or 72 h. Cells were fixed, the DNA was labelled with propidium iodide and analysed using flow cytometry, as described in Materials and Methods. Results are expressed as mean  $\pm$  SEM percentage of cycling cells ( $G_2/M$ -phase).

**Shedding of TNFR1 (p55) does not explain the decreased expression of this receptor in PP fibroblasts**

Immunocytochemical staining of cells grown on slides showed that NN fibroblasts cultured under serum conditions clearly express the TNFR1 (the p55 receptor form), but only low levels of the TNFR2 (the p75 form) (Fig. 6). The TNFR1 was predominantly localized in the nucleus and to a lesser extent in the cytoplasm. In contrast, PP fibroblasts expressed very low levels of immunoreactive TNFR1.

Fibroblast-conditioned media contained the soluble TNFR1 as well as the soluble TNFR2, as measured with specific sandwich ELISA (Table 1). The shedding of TNFR1 was enhanced by serum and was maximal at 72 h. The presence of soluble TNFR2 was, however, almost negligible, which confirmed the immunocytochemical stainings. The amounts of soluble TNFR present in the supernatants of PP and NN fibroblast cultures were similar and do not explain the differences found for membrane-bound TNFR between PP and NN fibroblasts.

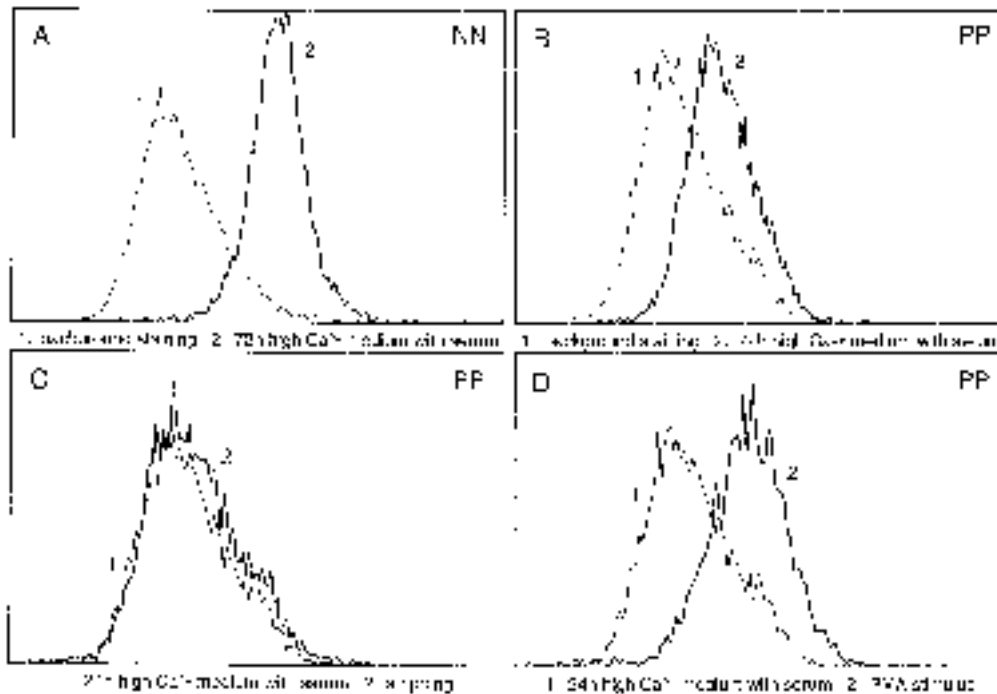
**PP fibroblasts co-express decreased TNFR levels and an enhanced IL-6 production**

The supernatants of the fibroblast cultures contained substantial amounts of biologically active IL-6

(Table 2). Both types of fibroblasts did not produce biologically active IL-1 or TNF- $\alpha$ . The serum-induced increase in IL-6 production correlated with the increase in binding of TNF- $\alpha$  in NN fibroblasts. PP fibroblasts, however, expressed an enhanced production of IL-6 together with a decreased binding of TNF- $\alpha$ .

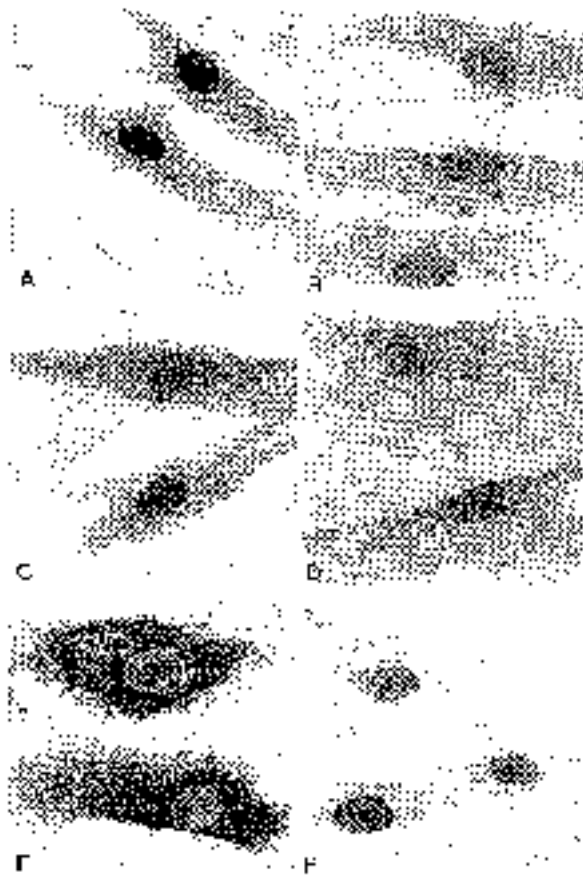
**DISCUSSION**

This is the first study in which the expression of cytokine receptors on lesional psoriatic dermal fibroblasts was investigated. We observed that PP and NN fibroblasts expressed negligible IL-1 binding sites (Figs 1 and 2). Epidermal cells, used as control cells, were positive for binding of IL-1 $\alpha$  and IL-1 $\beta$  which validated our methodology (not shown). Qwarnström and colleagues found that cultures of human gingival fibroblasts did express binding sites for both the IL-1 $\alpha$  and IL-1 $\beta$  isoform.<sup>12</sup> Apart from the possibility that biotinylation of IL-1 affects receptor binding, as Qwarnström and colleagues used iodinated IL-1, tissue-specific differences cannot be excluded. For example, the production of biologically active IL-1 can be triggered in gingival fibroblasts,<sup>29</sup> but not in dermal fibroblasts (see previous paper, pp 70–79). Our finding of negligible expression of IL-1 binding sites on dermal fibroblasts does not necessarily imply unresponsiveness of these cells to IL-1.



**Figure 5.** PP fibroblasts do not express occupied TNF- $\alpha$  binding sites and up-regulate the binding of TNF- $\alpha$  after stimulation.

Fluorescence intensities using biotinylated TNF- $\alpha$  are given of NN (A) and PP fibroblasts (B, C and D) (both  $n = 3$ ) cultured in a high-calcium medium containing 5% FCS for 24 or 72 h. A and B illustrate the decreased binding of TNF- $\alpha$  by PP fibroblasts. C shows the effect of acid stripping, and D the effect of a 50 ng/ml PMA stimulus for 14 h on PP fibroblasts. See Materials and Methods for details. Shown are a representative NN and PP fibroblast culture.



**Figure 6.** PP fibroblasts express decreased levels of TNFR1.

NN (A, C, E and F) and PP fibroblasts (B and D) were stained with the anti-TNFR1 antibody htr-9 (A and B) and the anti-TNFR2 antibody utr-1 (C and D). Staining of NN fibroblasts with mouse IgG<sub>1</sub> (E) and the antibody 5B5 (F) were used as controls. Cells were grown on slides in a high-calcium medium containing 5% FCS to confluence, fixed and stained, as described in Materials and Methods. Shown are a representative PP and NN fibroblast culture. The experiment was performed in duplo. Magnification: 315 $\times$ .

Only a few IL-1R type I molecules per cell are sufficient for an IL-1 mediated response.<sup>30</sup> This is confirmed by our novel finding that IL-1 $\beta$  stimulation of fibroblasts resulted in substantially upregulated binding of IL-6. The IL-4R has been demonstrated on gingival fibroblasts,<sup>31</sup> while dermal fibroblasts have been shown to give a biological response to exogenous recombinant human IL-4 and IL-6.<sup>32,33</sup> In this report we provide evidence that human dermal fibroblasts express binding sites for IL-4 and IL-6. Our observation that fibroblasts bind TNF- $\alpha$  confirms the findings by Berman and others.<sup>14</sup> The expression of cytokine binding sites by PP and NN fibroblasts was modulated by the use of different culture conditions. Calcium seemed to lower the expression of binding sites for IL-4, IL-6 and TNF- $\alpha$  (Fig. 2). However, extracellular calcium, using similar concentrations enhanced the IL-1R levels in cultured human keratinocytes.<sup>34</sup> This probably reflects cell-type specific differences. Addition of FCS resulted in

**TABLE 1.** Shedding of TNFR by PP and NN fibroblasts

	Psoriasis		Normal control	
	TNFR1 pg/ml	TNFR2 pg/ml	TNFR1 pg/ml	TNFR2 pg/ml
Culture condition				
24 h high Ca <sup>2+</sup> medium	8	<5	14	<5
24 h high Ca <sup>2+</sup> medium with serum	29	8	30	6
72 h high Ca <sup>2+</sup> medium with serum	120	10	100	7

Supernatants of PP and NN fibroblasts were analysed for soluble TNFR1 and TNFR2 by ELISA. Fibroblasts were cultured in a high-calcium (Ca<sup>2+</sup>), serum-free medium for 24 h, or a high Ca<sup>2+</sup> medium containing 5% FCS for 24 or 72 h. Results are given of a representative PP and NN fibroblast culture (both  $n = 3$ ), and expressed in pg/ml.

increased binding of IL-4, IL-6 and TNF- $\alpha$ . This increased binding initially paralleled the induction of fibroblast proliferation (third versus second bars, Fig. 4), implying enhanced responsiveness to cytokines of fibroblasts starting to proliferate.

Differences in cytokine binding between PP and NN fibroblasts became evident when confluent cultures were analysed. Culturing of PP fibroblasts for up to 72 h in the presence of serum did not affect the number of cells which bound TNF- $\alpha$ . This is in sharp contrast with the serum-upregulated binding of TNF- $\alpha$  by NN fibroblasts (Fig. 3A). The number of TNF- $\alpha$  binding sites per cell, however, did not increase under serum conditions in either NN or PP fibroblasts (Fig. 3B). As the expression of cytokine receptors may directly correlate with the fraction of cells in cycle,<sup>35</sup> we analysed the growth characteristics of PP and NN fibroblasts. It is important to note that studies on the proliferation rates of PP and NN fibroblasts in response to FCS are inconsistent.<sup>23,24</sup> Similar proliferation rates for both types of fibroblasts as well as a hyperproliferative state of PP fibroblasts have both been reported. In our study, however, there were no significant differences in cell counts and the percentage of cycling cells, although the percentage of proliferating cells was somewhat higher in confluent PP fibroblasts (72 h under serum conditions) when compared to NN fibroblasts (Fig. 4). Furthermore, the TNF- $\alpha$  binding sites of PP fibroblasts were not occupied with endogenously produced TNF- $\alpha$  (Fig. 5C), and PP fibroblasts did also not lose their ability to upregulate the binding of TNF- $\alpha$  (Fig. 5D).

TNF- $\alpha$  can bind to two types of TNFR, TNFR1 and TNFR2, which are both active in signal transduction and are responsible for nonredundant TNF activities.<sup>36</sup> Most immunomodulatory effects of TNF- $\alpha$  on fibroblasts are mediated by TNFR1. Immunocytochemistry showed that the expression of TNFR1 was clearly decreased in PP fibroblasts (Fig. 6), which is in line with the binding experiments (Figs 5A and B). These staining results imply that the decreased binding of TNF- $\alpha$  by PP fibroblasts is not due solely to an altered affinity

**TABLE 2. PP fibroblasts co-express decreased binding of TNF- $\alpha$  and increased IL-6 production**

Culture condition	Psoriasis		Normal control	
	Binding TNF- $\alpha$ %	IL-6 production U/10 <sup>5</sup> cells	Binding TNF- $\alpha$ %	IL-6 production U/10 <sup>5</sup> cells
24 h high Ca <sup>2+</sup> medium	1	33	16	11
24 h high Ca <sup>2+</sup> medium with serum	0	258	11	6
72 h high Ca <sup>2+</sup> medium with serum	1	300	40	13

The percentage of PP and NN fibroblasts which bind TNF- $\alpha$  and the corresponding production of IL-6 in U/10<sup>5</sup> cells were analysed using flow cytometry and the B9 bioassay, respectively. Fibroblasts were cultured in a high-calcium (Ca<sup>2+</sup>), serum-free medium for 24 h, or a high Ca<sup>2+</sup> medium containing 5% FCS for 24 or 72 h. The results are shown of a representative PP and NN fibroblast culture (both  $n = 5$ ).

of TNFRs for the TNF- $\alpha$ , but may merely reflect a decreased expression of TNFR 1. Both types of receptors are shed either by protein kinase C dependent proteolysis (induced by PMA) or by protein kinase C independent proteolysis (induced by IL-1 and IL-4).<sup>37,38</sup> We observed that serum enhanced shedding of TNFR1 by both types of fibroblasts, which may be due to serum-induced endogenous protein kinase C activity. The kinetics of the shedding was slow (Table 1). A similar slow process of release of soluble TNFR was also found using PBMC.<sup>39</sup> Amounts of soluble TNFR2 in the supernatants were very low but still detectable, being in line with the low expression of this receptor found with immunocytochemistry. Shedding, being similar for both types of fibroblasts, does not explain the decreased TNFR expression by PP fibroblasts. Furthermore, the observation that PP fibroblasts express negligible intracellular levels of immunoreactive TNFR does not support rapid TNFR upregulation and subsequent internalization of the receptors by these cells. Thus, at the moment, we speculate that PP fibroblasts exhibit a deficiency in serum-induced TNFR upregulation. This is in line with an elevated synthesis of extracellular matrix proteins and an increased expression of PDGFR $\beta$ , which have been considered as intrinsic phenotypic abnormalities of multi-passaged PP fibroblasts.<sup>20,40</sup> In vivo, PP fibroblasts also express elevated levels of PDGFR $\beta$ ,<sup>41</sup> and show an increased biological response to PDGF in vitro.<sup>42</sup> Although factors determining the responsiveness to TNF- $\alpha$  are not fully known yet, it has been shown that fibroblasts derived from affected skin from scleroderma patients do not respond to TNF- $\alpha$  in upregulating TNFR2 mRNA levels. Scleroderma and normal fibroblasts do not differ at the TNFR protein level, but scleroderma fibroblasts constitutively contain TNFR1 and TNFR2 mRNA, whereas normal fibroblasts constitutively express mainly TNFR 1 mRNA.<sup>14</sup>

TNF- $\alpha$  has mitogenic activity and many immunomodulatory activities on human dermal fibroblasts.<sup>43</sup> For example, TNF- $\alpha$  upregulates the expres-

sion of enzymes, cytokines (e.g. IL-1, IL-6 and IL-8), adhesion and MHC molecules, and stimulates chemotaxis. One would expect that the decreased TNFR expression by PP fibroblasts affects the inflammatory response of these cells to TNF- $\alpha$ . However, several arguments do not favour this hypothesis. First, we only observed a decreased TNFR expression in response to factors present in FCS, not to other stimulating agents (e.g. PMA, Fig. 5D). Second, PP fibroblasts co-expressed a decreased binding of TNF- $\alpha$  together with an increased production of IL-6 in contrast to NN fibroblasts (Table 2). Third, the degree of receptor density does not always correlate with TNF- $\alpha$  responsiveness,<sup>13</sup> and the biological actions of TNF- $\alpha$  are not always preceded by receptor internalization.<sup>44</sup> Moreover, using a murine model, subcutaneous injection of a combination of lithium chloride (a drug used to treat manic depressive disease and known to trigger or aggravate psoriasis as a side effect) and TNF- $\alpha$  induced a psoriasiform inflammatory reaction via an increase in the endogenous IL-6 production in the skin.<sup>28</sup> Immunohistochemistry of biopsies of human skin also showed that the in vivo expression of TNFR is altered in the dermal compartment of psoriatic lesions.<sup>45</sup> The decreased TNFR expression by PP fibroblasts demonstrated in vitro suggests that dermal fibroblasts are, at least in part, involved in maintaining the in vivo milieu characteristic of psoriatic lesions.

## MATERIALS AND METHODS

### *Patients and controls*

Ten patients with active plaque-type psoriasis and ten healthy volunteers, undergoing plastic surgery, were studied as described (see previous paper, p. 76).

### *Culturing of fibroblasts*

Split-skin specimens from PP and NN skin were used to establish fibroblast cultures (see previous paper, p. 76). For

experiments, fibroblasts of passage no. 2 to 6 were used. Cells were seeded in 175 cm<sup>2</sup> flasks and grown to subconfluence using Dulbecco's modification of Eagle's medium (DMEM: Gibco Ltd., Paisley, Scotland) supplemented with 10% FCS (Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were subsequently rinsed twice with PBS and incubated for 24 or 72 h with one of the following test media: (1) a low-calcium (0.15 mM), serum-free medium (keratinocyte basal medium, KBM/unsupplemented MCDB 153: Clonetics, San Diego, CA); (2) a high-calcium (1.05 mM), serum-free medium consisting of Ham's F10 (Gibco) and DMEM in a ratio of 1:1 supplemented with antibiotics; (3) the high-calcium medium supplemented with 5% FCS. The stimuli used were 50 ng/ml PMA (Sigma Chemie, Bornem, Belgium), 10 µg/ml LPS (*Escherichia coli* 026:B6, Difco Laboratories, Detroit, MI), 100 U/ml recombinant human IL-1β (UBI, Lake Placid, NY), 100 U/ml TNF-α (UBI) and 100 U/ml IFN-γ (Boehringer Ingelheim, Germany), added the last 14 h of the 24 h incubation with the serum-containing medium. Supernatants were collected by centrifugation and stored at -80°C.

### Quantitation of cytokine binding sites

Fibroblasts were collected (0.025% trypsin and 0.1% EDTA), trypsinization was stopped with 100 µg/ml trypsin inhibitor (Sigma), and cells were extensively washed with PBS. Viability was determined by trypan blue exclusion and always exceeded 95%. The mild trypsinization procedure did not affect the binding of cytokines, as checked by the use of cells collected with EDTA only. The labelling was performed according to the protocol supplied by the manufacturer. In short,  $5 \times 10^4$  cells were incubated for 1 h at 4°C with approximately 180 ng biotinylated recombinant human IL-1α, IL-1β, IL-4, IL-6 or TNF-α (all purchased from R&D Systems, Minneapolis, MN) using 12 × 75 mm round bottom, polystyrene tubes (Becton Dickinson). Unbound cytokines were removed by two rinses with PBS containing 0.1% bovine serum albumin (BSA) (washing buffer). Next, cells were incubated for 30 min at 4°C in the dark with avidin-FITC (R&D Systems), used in combination with IL-1 labelling, or streptavidin-phycoerythrin (Becton Dickinson) used in combination with the other cytokines. Subsequently, cells were rinsed twice, resuspended in washing buffer and used for flow-cytometry. Peripheral blood mononuclear cells and the U937 promonocytic cell line were used as positive controls for the expression of cytokine binding sites (not shown). Analysis was conducted using the FACScan<sup>c</sup> and Lysis<sup>c</sup> software programs (Becton Dickinson). The frequency of cells positive for the binding of cytokines was determined using single-parameter fluorescence histograms and corrected for aspecific staining of the second step (background). The results are presented either as histograms or as mean ± SEM percentage of positive cells. The relative number of binding sites per cell was estimated by the relative signal-to-noise values.<sup>46</sup>

To correct for the number of receptors already occupied with cytokines *in vitro*, the receptor-bound cytokines were stripped in some experiments before labelling. Briefly, cells were suspended in the first stripping buffer (10 mM Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub>·2H<sub>2</sub>O and 0.14 M NaCl, pH 4.0) and kept on ice for 20 sec.<sup>47</sup> The stripping was stopped by adding medium containing Hepes and BSA. Cells were rinsed twice with wash-

ing buffer before incubating the cells with the second stripping buffer (0.1 M glycine-HCl, pH 3.0) for 2 min on ice.<sup>12</sup> The stripping was stopped and cells were washed twice before labeling. The efficiency of the procedure was checked by stripping cells after incubation with biotinylated cytokines. This reduced the signal intensity to background levels.

### Cell cycle analysis

To analyse the cell cycle, cells were labelled with propidium iodide. Briefly,  $5 \times 10^4$  fibroblasts were fixed with ice-cold 70% ethanol on ice for 15 min. Subsequently, cells were collected by centrifugation ( $1000 \times g$ , 5 min) and treated with 1 U/ml DNase-free RNase A (Boehringer Mannheim, Germany) for 30 min at 37°C. Finally, cells were centrifuged and resuspended in 100 µl of 50 µg/ml propidium iodide (Becton Dickinson), after which flow cytometry was performed. As a negative control, cells were treated with 100 U/ml DNase (Sigma Chemical Co., St. Louis, MO) instead of RNase.

### ELISA for soluble TNFR

Soluble TNFR1 and TNFR2 were measured with a sandwich ELISA using the monoclonal antibodies MR1-1, MR2-2 and rabbit anti-soluble TNFR antiserum.<sup>39</sup>

### Bioassays for IL-1, IL-6 and TNF-α

IL-1, IL-6 and TNF-α activities were measured using the B9, D10 and WEHI assay, respectively, as described elsewhere.<sup>48–50</sup>

### Immunocytochemistry

PP and NN fibroblasts were grown on slides, fixed with a 1:1 methanol:acetone solution for 15 min at room temperature and stained with the mouse anti-human TNFR monoclonal IgG<sub>1</sub> antibodies htr-9 and utr-1 (Dr M. Brockhaus, Hoffmann-La Roche, Basel, Switzerland), at 10 µg/ml, using the supersensitive alkaline phosphatase kit (Biogenex, San Ramon, CA) to study the expression of TNFR1 and TNFR2, respectively.<sup>51</sup> Cells were counterstained with haematoxylin. The negative controls comprised staining with purified mouse IgG<sub>1</sub> (Becton Dickinson) at 10 µg/ml and omitting the first and second steps. Staining with 5B5 (Dako, Glostrup, Denmark), a purified mouse monoclonal IgG<sub>1</sub> antibody directed against human prolyl 4-hydroxylase, which is specifically expressed by fibroblasts, at 2 µg/ml, served as a positive control. Stainings were evaluated using a standard Zeiss 16 microscope (Carl Zeiss, Oberkochen, Germany).

### Statistical analysis

Flow cytometry, ELISA and bioassay results were analysed with the Wilcoxon Rank Sum Test using STATA<sup>TM</sup> (Computing Resource Center, Los Angeles, CA). *P* values <0.05 were considered statistically significant.

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