EXPRESSION OF CYTOKINES AND THEIR RECEPTORS BY PSORIATIC FIBROBLASTS I. ALTERED IL-6 SYNTHESIS

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Interleukin(lL-)6 is overproduced in psoriatic lesions. We investigated the contribution of dermal fibroblasts to the local IL-6 production. Fibroblasts (passageno. 2 to 6) derived from lesional psoriatic (PP) and normal human (NN) skin were used to analyse the secretion of IL-6, and the related cytokines IL-1, IL-8 and TNF-α, and the expression of their corresponding mRNA by bioassay, ELISA and Northern hybridization, respectively. PP fibroblasts cultured under serum-free conditions produced increased amounts of bioactive IL-6 when compared to NN fibroblasts. Differences were partially restored in the presence of growth factors or serum. The serum-induced IL-6 production reached a maximum within 24 h after seeding and remained unchanged in PP fibroblasts, whereas comparable amounts of IL-6 were produced only 6 days later in NN fibroblasts. There was a clear expression of IL-6 mRNA in both types of fibroblasts under serum-free conditions. Unexpectedly, fetal calf serum, inactivated fetal calf serum as well as human serum completely inhibited the expression of IL-6 mRNA in all the PP fibroblast cultures investigated. NN fibroblasts were clearly less sensitive to this inhibiting effect of serum. Furthermore, medium supplemented with serum-free component or calcium also repressed IL-6 mRNA expression in PP fibroblasts in contrast to NN fibroblasts. Cycloheximide fully restored the repressing effect of serum indicating that serum induced a labile repressor protein. PP and NN fibroblasts produced negligible amounts of IL-1 and TNF-α, but the production of IL-8, however, was comparable to that of IL-6. Our results show a differently regulated IL-6 synthesis in PP fibroblasts in vitro, suggesting an active contribution of dermal fibroblasts to the local IL-6 production in psoriasis.

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Psoriasis is a disease characterized by chronic cutaneous inflammation and increased proliferation of keratinocytes. Cutaneous homeostasis is clearly perturbed in psoriasis. Alterations observed in psoriatic skin include activation of resident and infiltrating cells, expression of inflammatory mediators, their receptors and adhesion molecules, and activation of intracellular signal transducing pathways.[1,2] Several lines of investigation favour a contribution of the dermal fibroblast to the pathophysiology of psoriasis. Experiments using athymic nude mice showed that skin biopsies from psoriatic lesions, following transplantation onto these mice, retained the major histologic features of psoriasis.[3] Later on, combined grafting studies showed that the classical characteristics of psoriasis only persisted when psoriatic epidermis was transplanted together with psoriatic dermis.[4] Moreover, the rates of proliferation of normal and psoriatic human keratinocytes differ in vivo, but not in monoculture.[5] It thus appeared that psoriatic keratinocytes need to be in contiguity with dermal fibroblasts to maintain epidermal hyperplasia in vivo. Several groups observed that the growth of human keratinocyte in vitro is supported by the use of fibroblasts or even connective tissue-derived small molecular weight material.[5,6] Furthermore, Saiag and colleagues found that upper dermal fibroblasts from psoriasis patients cultured in a collagen lattice induced hyperproliferation in normal epidermal biopsies.[7] Reproducibility of this finding depends on the anatomical location of the fibroblast (upper versus deeper dermis), the type of fibroblast culture (fibroblasts incorporated in collagen lattice versus monoculture) and epidermal cell culture (biopsy versus monolayer) used in the fibroblast-keratinocyte co-culture model.[5,8,9] All together, studies suggest the involvement of fibroblast-derived cytokines in the pathogenesis of psoriasis. For instance, human dermal fibroblasts are able to produce interleukin(IL-)1, IL-6 and IL-8,[10-12] which are not only

actively involved in inflammation but are also mitogenic for epidermal cells.[13–15] Espinoza and colleagues have indeed shown that psoriatic fibroblasts produce elevated amounts of IL-1β.[16]

We previously reported an elevated IL-6 bioactivity in psoriatic suction blister fluids but not in the corresponding serum samples.[17] Analysis of IL-6 mRNA of keratinocyte and fibroblast cultures indicated that fibroblasts were the most likely cellular source for this local IL-6 production. Reports on the altered epidermal IL-6 expression in psoriasis are conflicting. Increased epidermal expression of IL-6 protein was detected with immunochemistry and of IL-6 mRNA with in situ hybridization using cultures of keratinocytes as well as skin biopsies from active psoriatic plaques.[14–18] However, neither IL-6 protein nor IL-6 bioactivity could be detected in scales from psoriatic lesions and, using in situ hybridization, the increase of IL-6 mRNA in lesional psoriatic epidermis could not be reproduced by others.[19,20] Furthermore, the presence of IL-6 mRNA in epidermal keratome biopsies was only observed with the sensitive PCR technique.[22] Contaminating dermal fibroblasts were assumed to be the source of the increased IL-6 expression in lesional psoriatic keratome biopsies.[21]

In the present study we therefore investigated the secretion of IL-6 protein and expression of its RNA using early passages of fibroblasts derived from lesional psoriatic (PP) and normal control (NN) skin. The protein and mRNA expression of IL-1, IL-8 and tumor necrosis factor (TNF-)α were also studied. Experiments were performed with media supplemented with calcium, growth factors, cytokines and several types of serum.

RESULTS

PP fibroblasts secrete low but increased amounts of IL-6 compared to NN fibroblasts under serum-free culture conditions

With a low-calcium (0.15 mM), serum-free medium (medium 1: KBM/unsupplemented MCDB 153) PP and NN fibroblasts hardly survived (Fig. 1B). However, low but clearly detectable IL-6 levels were present in the corresponding fibroblast-conditioned media. PP fibroblasts secreted more bioactive IL-6 on each test day (5 consecutive days starting from day 3 after seeding), and on average about three times more IL-6 than NN fibroblasts, as illustrated in Figure 1B. Findings were reproduced with a high-calcium (1.05 mM), serum-free medium (medium 3: a 1:1 ratio of F10:DMEM), although, overall, the IL-6 levels were lower with the latter medium (Fig. 1C,D). Fibroma-derived fibroblasts used as a control expressed on average 5 U/ml IL-6, being similar to the values of the PP fibroblasts. It is important to realize that very low numbers of PP fibroblasts are thus able to produce several units of IL-6.
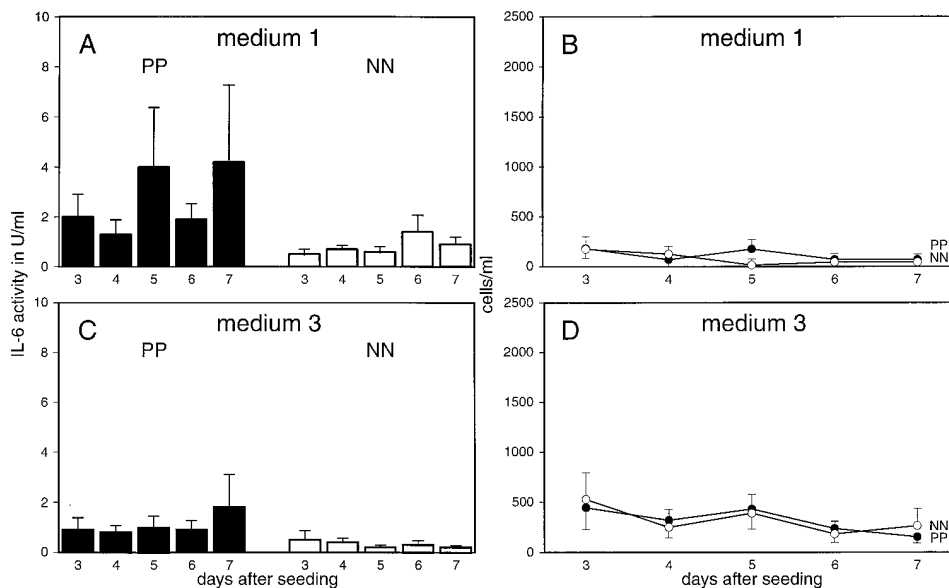

Figure 1.   PP fibroblasts secrete low but increased amounts of IL-6 compared to NN fibroblasts under serum-free culture conditions.

The IL-6 production (A and C) and cell number (B and D) of PP and NN fibroblasts (both $n = 6$) were monitored on 5 consecutive days starting from day 3 after seeding using the B9 assay and cell counts, respectively. Cells were seeded at 500/cm² in 12-well plates using medium 1 (A,B) and 3 (C,D). See Materials and Methods for a description of the media. Closed bars and circles represent PP fibroblasts and open bars and circles NN fibroblasts. Results are given in mean ± SEM.

Differences between PP and NN fibroblasts were more pronounced in the supernatants of larger numbers of fibroblasts (Table 3).

The IL-6 production by PP fibroblasts is rapid, high and persisting under serum-stimulated conditions

Using medium 1 supplemented with growth factors, such as insulin, epidermal growth factor and a pituitary extract (medium 2), PP and NN fibroblasts were rescued from death, but did not show a proliferative response (Fig. 2B). The IL-6 levels, although higher for PP fibroblasts, increased more substantially for NN fibroblasts (Fig. 2A versus Fig. 1A). Differences in IL-6 production between both types of fibroblasts were more marked when serum was used as a stimulus. Medium 4, which is medium 3 containing 5% fetal calf serum (FCS), was the only culture medium supporting the proliferation of fibroblasts (Fig. 2D). The number of both PP and NN fibroblasts doubled during the test-period of 5 days. The amounts of bioactive IL-6 present in the supernatants increased about six times using NN fibroblasts but only two times using PP fibroblasts during this period (Fig. 2C). The IL-6 production corrected for the corresponding cell numbers reached a plateau level at the beginning of the test (about 10 U/1000 cells) and persisted in PP fibroblasts throughout the test-period, whereas comparable levels of IL-6 were produced only after 1 week of culture in NN fibroblasts (Table 1). The IL-6 production by PP fibroblasts was already maximal at 24 h after seeding. The de novo production of IL-6 protein by confluent fibroblasts, how-

ever, checked by changing the medium after 6 days of culture, was not increased in PP fibroblasts when compared to NN fibroblasts (Table 2).

Serum represses the expression of IL-6 mRNA in PP fibroblasts

PP and NN fibroblasts cultured with medium 1 had a clear expression of steady state IL-6 mRNA levels, as shown in Figure 3. FCS added to medium 1 decreased the IL-6 message to undetectable levels in all the PP fibroblast cultures, but only in half of the NN fibroblast cultures investigated. When using medium 3, or medium 3 supplemented with FCS the expression of IL-6 mRNA was not detectable (not shown). To study
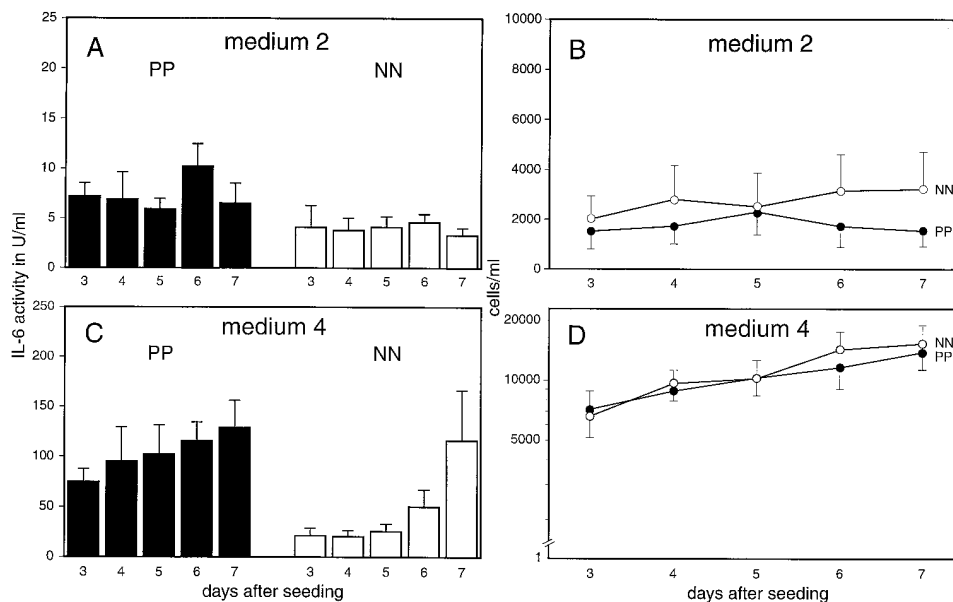
TABLE 1. IL-6 production by PP and NN fibroblasts using a serum-containing medium

	Psoriasis U/1000 cells	Normal control U/1000 cells	P value
Days in culture			
3	10.2 ± 1.8[a]	3.1 ± 0.7	0.007
4	10.7 ± 4.0	2.1 ± 0.5	0.004
5	11.7 ± 4.9	2.3 ± 0.4	0.010
6	10.4 ± 1.5	3.1 ± 0.7	0.007
7	8.9 ± 1.0	7.3 ± 3.3	0.080

[a] The IL-6 activity present in the supernatants of PP and NN fibroblasts (both $n = 6$) was monitored on 5 consecutive days starting from day 3 after seeding using the B9 assay. IL-6 activities were corrected for the corresponding cell number and expressed in U/1000 cells fibroblasts. Cells were seeded at 500/cm² in 12-well plates using medium 4. Results are given in mean ± SEM. P values < 0.05 were considered statistically significant.


Figure 2.   PP fibroblasts rapidly secrete high and persisting amounts of IL-6 under serum-stimulated conditions.

See legend to Figure 1 for details. Medium 2 is medium 1 supplemented with growth factors. Medium 4 is medium 3 supplemented with FCS, as described in Materials and Methods .
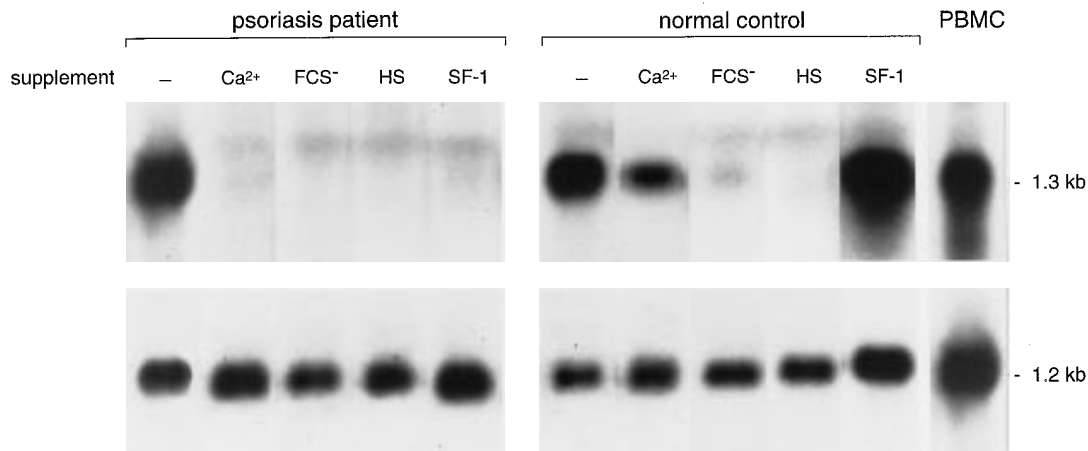
TABLE 2. The difference in IL-6 production between PP and NN fibroblsts is lost during culture

	Psoriasis U/ml	Normal control U/ml
Type of supernatant		
Day 1	30[a]	7
Accumulated IL-6 at day 7	38	36
Newly synthesized IL-6 at day 7	45	57

[a] The IL-6 production of PP and NN fibroblasts ($n = 3$) seeded at 500 cells/cm² and cultured in medium 4 using 12-well plates was measured with the B9 assay and expressed in U/ml. Supernatants were collected at day 1 and 7 after seeding. Medium was changed 24 h before collecting supernatants at day 7 to analyse the amount of newly synthesized IL-6. Results of representative cultures are given.
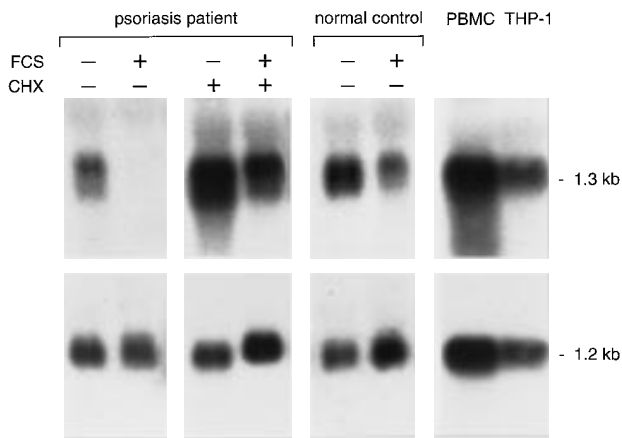
Figure 3. Serum represses the expression of IL-6 mRNA in PP fibroblasts.

The expression of steady-state IL-6 mRNA levels in PP and NN fibroblasts (both $n = 6$) were analysed by Northern hybridization after 2 days of culture in medium 1, or medium 1 supplemented with 5% FCS. Autoradiography, to detect the message for IL-6 (1.3 kb band) and GAPDH (1.2 kb band), was carried out at $-70°C$ for 3 and 1 days, respectively. Cycloheximide (CHX: 10 µg/ml) was added 3 h before harvesting. See text for details. PBMC and THP-1 cells were used as controls.

the mechanism of the inhibitory process we analysed the effect of cycloheximide. Results showed that the inhibitory effect of serum components required de novo protein synthesis.

Serum and calcium modulate the IL-6 mRNA expression differently in PP and NN fibroblasts

PP and NN fibroblasts responded differently to test-sera such as FCS, inactivated FCS, human serum and a serum-free component, as shown in Figure 4. In PP fibroblasts all types of serum repressed the IL-6 mRNA expression completely. Unexpectedly, the IL-6 message was also repressed when using the serum-free component as a supplement. However, in NN fibroblasts the repression of IL-6 mRNA expression was only complete in part of the cultures tested with FCS and inactivated FCS, but always complete with human serum. Moreover, the serum-free component even enhanced the IL-6 message in NN fibroblasts. Calcium, used as supplement, repressed the expression of IL-6 mRNA in PP fibroblasts, whereas the IL-6 message was not affected in NN fibroblasts.

Expression of IL-8 protein and mRNA levels are similar to IL-6

Apart from IL-6, fibroblasts also secrete IL-8, but only negligible amounts of IL-1 and TNF-α using conditions with or without serum. The production of IL-6 and IL-8 using different culture conditions are presented in Table 3, and correspond to the same cultures of which RNA data is presented in Figure 3. PP fibroblasts produced clearly increased amounts of IL-6 and IL-8 when cultured with both the low and high-calcium serum-free media. The serum-induced IL-6 production

Figure 4. Serum-dependent repression of IL-6 mRNA expression in PP fibroblasts is not restricted to FCS.

Steady-state IL-6 mRNA levels in PP and NN fibroblasts ($n = 3$) were analysed by Northern hybridization after 2 days of culture in medium 1 supplemented with calcium or different types of serum. The final calcium (Ca²⁺) concentration was 1.05 mM. FCS, inactivated FCS (FCS⁻), human serum (HS) and serum-free component (SF-1) were all added to a final concentration of 5% (v/v). See legend to Figure 3 for details. Results of representative cultures are given. PBMC were used as a control.

TABLE 3.   IL-6 and IL-8 production by fibroblasts under different culture conditions[a]

		Psoriasis		Normal control	
		IL-6[b] U/ml	IL-8[c] pg/ml	IL-6 U/ml	IL-8 pg/ml
Medium	Serum				
1	−	2961	1600	289	30
1	+	1932	940	74	690
3	−	222	310	21	13
3	+[b]	2171	1140	194	1650

[a] The amount of IL-6 and IL-8 present in 48 h supernatants of PP and NN fibroblasts ($n = 3$) were measured with the B9 assay and IL-8 ELISA, respectively. Media used were medium 1 and 3, and these media supplemented with 5% FCS. Cells were grown on 625 cm² culture plates to semi-confluence before test-media were added. Results are given of a representative PP and NN fibroblast culture.
[b] Medium 3 supplemented with serum is medium 4.

was also elevated in PP fibroblasts after 2 days of culture (Table 3). This is in line with Figures 1, 2 and Table 1. Differences in the IL-8 production, however, were completely restored by serum. In fact, the kinetics of the serum-induced IL-8 production by PP and NN fibroblasts were similar (not shown). Fibroblasts did not express IL-1α, IL-1β or TNF-α mRNA. The expression of IL-8 mRNA, however, was similar to that of IL-6 mRNA using the different culture conditions. The use
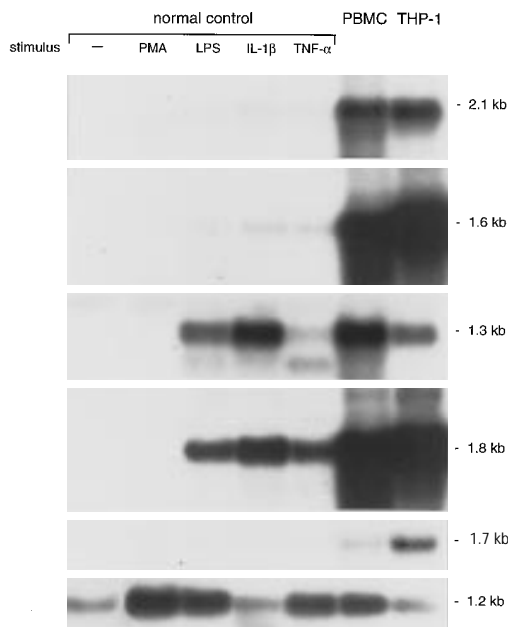
Figure 5.   Fibroblasts express IL-6 and IL-8 mRNA, but not IL-1α, IL-1β and TNF-α mRNA.
Steady-state levels of IL-1α, IL-1β , IL-6, IL-8 and TNF-α mRNA in NN fibroblasts were analysed by Northern hybridization after two days of culture in different media with and without stimuli. Autoradiography, to detect the message for IL-1α (2.1 kb band), IL-1β (1.6 kb band), IL-6 (1.3 kb band), IL-8 (1.8 kb band), TNF-α (1.7 kb band) and GAPDH (1.2 kb band), was carried out for 10, 2, 3, 4, 4 and 1 days, respectively. Results of a representative culture are given. Experiment was performed in duplo. PBMC and THP-1 cells were used as controls.

TABLE 4.   Cytokine production profile of fibroblasts

		Cytokines[a]			
		IL-1 U/ml	IL-6 U/ml	IL-8 pg/ml	TNF-α U/ml
Medium	Stimulus				
1	−	0	210	20	0
1	serum	0	169	45	0
3	−	0	15	2	0
3	serum[b]	0	258	40	0
4	PMA	0	294	53	0
4	LPS	0	13 796	7900	0
4	IL-1β	0	38 603	1050	0
4	TNF-α	0	4523	600	0

[a] The IL-1, IL-6 and TNF-α activities and IL-8 immunoreactivity present in 48 h supernatants of NN fibroblasts were measured with the D10, B9, WEHI bioassay and IL-8 ELISA, respectively. IL-1, IL-6 and TNF-α are expressed in U/ml, and IL-8 in pg/ml. Media used were medium 1 and 3, these two media supplemented with 5% FCS, and medium 4 with stimuli. PMA (50 ng/ml), LPS (10 μg/ml), IL-1β (100 U/ml) or TNF-α (100 U/ml) were added as stimuli to the cells 14 h before collecting supernatants. Results are given of a representative culture. The experiment was performed in duplo. Supernatant of LPS-stimulated PBMC contained 56 000 U/ml IL-1; 28 000 U/ml IL-6; 800 pg/ml IL-8 and 15 U/ml TNF-α.
[b] Medium 3 supplemented with serum is medium 4.

of stimuli like phorbol myristate acetate (PMA), lipopolysaccharide (LPS), recombinant human IL-1β and TNF-α enhanced the expression of IL-6 and IL-8 mRNA, but that of IL-1α, IL-1β and TNF-α mRNA only to a negligible extent (Fig. 5). The expression of IL-6 and IL-8 mRNA was most profoundly enhanced by IL-1β. The corresponding amounts of cytokines present in the conditioned media of the same fibroblast culture are given in Table 4. It is important to note that although fibroblast IL-8 synthesis is similar to that of IL-6, the differences between PP and NN fibroblasts were most marked for IL-6. In summary, an inventory of our findings on the altered IL-6 synthesis in PP fibroblasts is given in Table 5.

TABLE 5.   IL-6 synthesis is altered in PP fibroblasts[a]

		Psoriasis	Normal control
	Stimulus		
IL-6 protein	none	+[a]	−
	FCS	+ +	+
IL-6 mRNA	none	+ +	+ +
	FCS	−	±
	inactivated FCS	−	±
	human serum	−	−
	serum-free component	±	+ +
	calcium	±	+ +
	CHX	+ +	+ +

[a] Summary of the B9 assay and Northern hybridization results, with the expressions of IL-6 protein and mRNA given in symbols, −: absent or low in all experiments; ±: present in part of the experiments; +/++: present/clearly present in all experiments. CHX: cycloheximide.

DISCUSSION

The serum-free production of IL-6 and IL-8 by PP fibroblasts was increased when compared to NN fibroblasts (Fig. 1 and Table 3). The growth factors present in medium 2 enhanced the production of IL-6 (Fig. 2) and IL-8 (not shown) most markedly in NN fibroblasts, resulting in a reduced difference in cytokine production between the two types of fibroblasts. Kinetics showed that PP fibroblasts rapidly produced high and persisting amounts of IL-6 under serum-stimulating conditions. The production of IL-6, not of IL-8, was significantly enhanced in PP fibroblasts when compared to NN fibroblasts (Table 1). Using confluent PP fibroblasts (6 days after seeding), however, we observed that the serum-induced IL-6 (Table 2) was similar or even less than their normal counterparts. This may explain why the differences in IL-6 production were lost after a culture-period of 1 week. It remains possible that PP fibroblasts display an altered post-translational modification of IL-6 resulting, for example, in a decreased aggregation of IL-6 monomeres which express more activity in the B9 assay,[22] or in a prolonged half-life of the protein. However, our finding that differences in IL-6 levels are already present in supernatants collected rapidly (24 h) after seeding do not favour an altered half-life of PP fibroblast-derived IL-6 protein.

The IL-6 production of normal human dermal fibroblasts is highly serum-dependent, as already reported for normal human monocytes and keratinocytes.[23,24] Normal human cells typically do not express IL-6 in the absence of a stimulatory signal.[25] Fibroblasts derived from lesional psoriatic skin, however, are less dependent on external stimuli for their IL-6 production. The enhanced production of IL-6 is not unique for PP fibroblasts. Constitutive production of IL-6, without exogenous stimuli, has already been described for dermal fibroblasts derived from affected skin sites from patients with systemic sclerosis,[26] synovial fibroblasts from patients with rheumatic arthritis,[27] mesothelioma cell lines,[28] and peripheral blood monocytes from psoriasis patients.[18] This suggests that overproduction of IL-6 could represent a general feature of affected mesenchymal cells thereby contributing to the maintenance of inflammation.

IL-6 production is regulated at the level of transcription and mRNA stability.[29,30] Transcription of the human IL-6 gene is normally enhanced by serum as evidenced by the location of the c-fos serum-responsive element homology (c-fos SRE) within the IL6 promoter.[31] PP and NN fibroblasts had a clear but similar expression of steady-state IL-6 mRNA levels using medium 1 (0.15 mM calcium). This protein-deficient medium may be unable to support the production of a repressor protein of IL-6 mRNA transcription.

Unexpectedly, this protein is present in serum-containing media, as explained later on. One also cannot exclude the possibility that medium 1 has a stabilizing effect on IL-6 mRNA. The corresponding IL-6 protein levels, especially of PP fibroblasts (Table 3), do not favour, however, inhibition of IL-6 translation. With medium 3 (1.05 mM calcium) the message for IL-6 is not detectable. Normal human keratinocytes, grown in a serum-free medium containing 0.15 mM calcium, did not express detectable IL-6 mRNA levels using Northern hybridization. Low levels of IL-6 mRNA could be induced in these cells by treatment with 1.8 mM calcium.[21] This suggests cell-type specific differences in the induction of IL-6 gene expression. Serum reduced the IL-6 message completely in all the PP fibroblast cultures investigated. The expression of IL-6 mRNA is, however, reported to be induced in human foreskin, synovial and bone marrow stromal fibroblasts within several hours in response to serum when fibroblasts were made quiescent by serum deprivation and RNA levels were analysed using blot hybridization with poly(A+) RNA or PCR amplified products.[32-34] Apart from technical differences, our findings using human dermal fibroblasts may be attributed to tissue-specific serum-responsiveness of fibroblasts. The repressing effect of serum on the IL-6 mRNA expression in PP fibroblasts could be completely abolished by cycloheximide treatment. This suggests that at least with medium 1, serum induces a labile protein which represses the levels of IL-6 mRNA.

Experiments were performed to determine whether the inhibiting effect was specific for FCS. In PP fibroblasts the IL-6 mRNA expression was repressed by all test-sera. Complement factors, known to enhance the IL-6 production of stimulated human monocytes,[35] did not affect the IL-6 mRNA expression in our experiments, as shown by the use of inactivated serum. Furthermore, the use of human serum showed that a species-specific serum component did not seem to be involved. However, the repression of the IL-6 message in NN fibroblasts was more susceptible to human serum than FCS. Whether the presence of cytokines, their soluble receptors or anti-cytokine autoantibodies in serum contributes to differences in responsiveness between PP and NN fibroblasts remains to be resolved.[36,37] The use of a serum-free supplement or calcium repressed the expression of IL-6 mRNA in PP fibroblasts, whereas the levels of IL-6 mRNA were unaffected or even enhanced in NN fibroblasts. Components of serum-free supplement (e.g. albumin, ethanolamines, insulin, transferrin and free fatty acids) and calcium are reported to be inducers of IL-6 production.[21,38,39] Thus, on the one hand our findings confirm that serum factors and calcium induce production of IL-6 in normal human dermal fibroblasts and on the other hand indicate that the IL-6 production in PP fibroblasts is regu-

lated differently by serum factors and calcium. Factors present in FCS and human serum as well as several growth factors were reported to induce proliferation in PP fibroblasts to a higher extent when compared to NN fibroblasts.[40,41] However, we and others did not observe differences in cell growth between both types of fibroblasts.[5] Our study suggests that IL-6, being anti-mitotic for fibroblasts, is not primarily involved in the regulation of PP fibroblast growth in vitro, as is the case for PDGF.[16] The altered IL-6 synthesis of multi-passaged PP fibroblasts confirms early studies which demonstrated several inherent abnormalities such as increased production of extracellular matrix components, intracellular signalling and resistence to therapeutics resulting in an hyperactive state of these cells in vitro.[42–44]

Human fibroblasts are, apart from IL-6, potent producers of IL-8, but do not produce IL-1α, IL-1β or TNF-α even when using potent stimuli like PMA, LPS and the cytokines IL-1β and TNF-α. These cells express negligible amounts of the corresponding mRNA (Table 4 and Fig. 5). Fibroblasts derived from different tissues display some discrepancies with regard to the expression of IL-1.[45] Espinoza and colleagues showed that anti-IL-1β polyclonal antibody partly neutralized the fibroblast mitogenic activity of supernatants of PP fibroblasts. Their results indicate that the latter assay probably is more sensitive when compared to the D10 assay.[16] The production of IL-8 was similar to that of IL-6. However, differences in the serum-induced production of IL-6 protein between PP and NN fibroblasts were specific for this cytokine (Table 3). Taken together, our findings show that PP fibroblasts display an altered synthesis of IL-6 in vitro, which is regulated differently by serum factors and calcium. These alterations are summarized in Table 5. The in vivo relevance of IL-6 to skin physiology and pathology has previously been extensively reviewed.[46,47] During cutaneous inflammation IL-6 maintains the inflammatory response by attracting leucocytes and activating inflammatory and resident cells via the induction of cytokines and adhesion molecules. Moreover, fibroblast-derived IL-6 may even enhance antigen presentation as IL-6 can induce the expression of the HLA-B7 gene in human fibroblasts.[48] Cutaneous IL-6 production is drastically increased during injury, infections, neoplasia and autoimmune diseases. We provide evidence that dermal fibroblasts are an important source of the elevated IL-6 levels in psoriatic lesions. The contribution of IL-6 to the pathogenesis of psoriasis is still obscure. However, the findings that vitamin D3 analogs are an efficient anti-psoriatic therapy and that these substances decrease fibroblast-derived IL-6 and IL-8 levels in vitro imply a role of dermal IL-6 and IL-8 in psoriasis.[49,50] In our opinion dermal fibroblasts are actively involved in maintaining the inflammatory response in psoriatic lesions.

MATERIALS AND METHODS

Patients and controls

Skin samples were obtained from ten otherwise healthy patients (Outpatient Department of Dermatology, University Hospital Rotterdam-Dijkzigt) with active plaque-type psoriasis after informed consent. Patients remained untreated for at least 3 weeks before entering the study. Ten individuals without history or signs of skin disease, undergoing abdominal or breast plastic surgery (Sint Franciscus Hospital, Rotterdam) served as normal healthy controls. All skin samples were collected after approval of the institutional medical ethical committee (MEC 104.050/SPO/1990/30).

Culturing of fibroblasts

Split-skin specimens from PP and NN skin, including epidermis and superficial dermis, were obtained using a portable dermatome (Davol Inc., Cranston, Rl). Epidermis was separated from dermis via trypsinization. Primary cultures of fibroblasts were obtained by plating dermal parts in a 25 cm² culture flask (Becton Dickinson, Plymouth, UK). The dermal pieces were allowed to attach after which 2 ml of culture medium was added to the flask. The culture medium consisted of Dulbecco's modification of Eagle's medium (DMEM: Gibco Ltd., Paisley, Scotland) supplemented with 10% FCS (Gibco), 100 U/ml penicillin and 100 ug/ml streptomycin. At confluence, cells were subcultured via trypsinization. After the first passage fibroblast cultures were free of epidermal cells. Contamination with mycoplasma species was excluded by testing antibiotic-free culture supernatants for adenosine phosphorylase activity (Mycotest: Gibco). For experiments, fibroblasts of passageno. 2 to 6 were collected, trypsinization was stopped with 100 µg/ml trypsin inhibitor and cells were extensively rinsed with PBS. Viability was determined by trypan blue exclusion.

Fibroblast-conditioned medium

Fibroblasts were plated in 12-well plates (Costar, Cambridge, MA) at a density of 500 viable cells/cm², using 1 ml of one of the following media: (1) a low-calcium (0.15 mM), serum-free medium (medium 1: keratinocyte basal medium, KBM/unsupplemented MCDB 153: Clonetics, San Diego, CA); (2) medium 1 supplemented with 5 µg/ml insulin, 10 ng/ml human recombinant epidermal growth factor, 0.5 µg/ml hydrocortisone, 0.4% (v/v) bovine pituitary extract and 50 U/ml gentamicine sulphate (medium 2: keratinocyte growth medium, KGM/supplemented MCDB 153: Clonetics); (3) 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 (medium 3); and (4) medium 3 supplemented with 5% FCS (medium 4). The effects of different types of serum on the production of cytokines were investigated using another culture system, described below. For kinetic studies, cells and supernatants were collected on 5 consecutive days starting from day 3 after seeding. Each test-condition was tested in triplicates. Supernatants were collected by centrifugation, supplemented with 0.1% bovine serum albumin in case of serum-free supernatants and stored at −80°C. Cells

present in the same wells were collected via trypsinization and counted microscopically (Carl Zeiss, Germany).

Bioassays for IL-1, IL-6 and TNF-α

IL-1 bioactivity was measured using a subline of a cloned murine T cell line D10.G4.1, designated D10(N4)M (kindly provided by Dr S. Hopkins, Manchester, UK).[51] IL-6 bioactivity was measured using a murine hybridoma cell line B9 (kindly donated by Prof. Dr L. Aarden, Amsterdam, The Netherlands).[23] Proliferation of the cytokine dependent cell lines D10 and B9 was measured via [³H]deoxythymidine incorporation. TNF-α bioactivity was measured using the murine fibroblast cell line WEHI 164.13 (kindly provided by Dr W. Buurman, Maastricht, the Netherlands).[52] The MTT cytotoxicity assay was used to measure the viability of WEHI cells.[53] Recombinant human IL-1β (UBI, Lake Placid, NY), IL-6 (Prof. Dr L. Aarden) and TNF-α (UBI) served as positive controls for the D10, B9 and WEHI assay, respectively. Cytokine activities of the samples were corrected for background activity of the culture medium and expressed in U/ml or in U/1000 cells, with 1 U/ml corresponding with half-maximal response.

IL-8 ELISA

IL-8 immunoreactivity was measured by ELISA, as described previously.[54] Dr K. Matsushima (Tochigi-ken, Japan) kindly provided the monoclonal anti-IL-8 IgG₁ (WS4) and the rabbit polyclonal anti-IL-8 antibody.[55] Recombinant human IL-8 (Dainippon, Japan) served as a positive control.

Northern hybridization

For RNA analysis fibroblasts were grown on 625 cm² culture plates (Gibco) until 60% confluence was reached. Subsequently, cells were rinsed 2 times with PBS and cultured for 48 h under test-conditions. The test-conditions comprised media 1 to 4 and medium 1 supplemented with calcium and different types of serum. The different sera comprised FCS, FCS treated 30 min at 56°C (inactivated FCS or FCS⁻), pooled human AB serum (HS: Red Cross Blood Center, Rotterdam, The Netherlands) and serum-free component (SF-1: Costar) and were tested at 5% (v/v). Calcium (hydrated calcium-chloride) was added to a final concentration of 1.05 mM. Cycloheximide (Sigma) was added to some plates (10 µg/ml) 3 h before cell harvesting. Supernatants were collected and cells were harvested using a rubber policeman (Costar). Total cellular RNA was isolated using the guanidium thiocyanate extraction procedure.[56] RNA samples of 25 µg were size fractionated by electrophoresis through 1% agarose and 17% formaldehyde gels, transferred to Nytran N nylon membranes (Schleicher and Schuell, Dassel, Germany),[57] and hybridized according to Jeffreys *et al.* with ³²P-labelled cDNA probes.[58,59] Autoradiography was carried out with Kodak films at −70°C for 1 to 10 days. Hybridized probes were removed from the nylon membranes according to Sambrook *et al.*[57]

Probes

Probes were derived from cDNA: IL-1α, a 0.6 kb *Eco*RI-*Hin*dIII fragment (Biogen, Basel, Switzerland); IL-1β, a 1.3 kb *Pst*I fragment (Genetics Institute, Cambridge, MA); IL-6, a 0.3 kb *Eco*RI-*Hin*dIII fragment;[60] IL-8, a 1.3 kb *Eco*RI fragment;[61] TNF-α, a 0.6 kb *Eco*RI-*Cla*I fragment (Celltech, Berkshire, UK); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a 0.7 kb *Eco*RI-*Pst*I fragment,[62] serving as a control for RNA loading.

Controls

Several cellular controls were used for the expression of cytokines at the protein and mRNA level: a fibroma-derived fibroblast cell line; NN fibroblasts stimulated with 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 or 100 U/ml recombinant human TNF-α; and PBMC and the myelomonocytic cell line THP-1 stimulated with LPS. Stimuli were added 14 h before cell harvesting.

Statistical analysis

Bioassay and ELISA results were analysed with the Wilcoxon Rank Sum Test using STATA™ (Computing Resource Center, Los Angeles, CA). P values 0.05 were considered statistically significant.

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