Interleukin-6 in psoriasis: expression and mitogenicity studies

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Summary. Interleukin-6 (IL-6) is a multifunctional cytokine which has been suggested to function as an autocrine mitogen in psoriatic epidermis. We report here the results of several experiments designed to further examine this hypothesis. Blot hybridization was unable to detect 1.3 kb IL-6 transcripts in RNA extracted from normal or psoriatic epidermal (keratome) biopsies, suggesting that IL-6 expression is very low in normal and psoriatic epidermis. Therefore, qualitative and semiquantitative PCR/Southern blot analyses were performed on keratome-derived RNA, and revealed variable but significantly increased IL-6 mRNA levels in lesional psoriatic relative to normal tissue. To further examine the ability of normal human keratinocytes (NHK) to express IL-6, RNA was extracted from rapidly proliferating secondary NHK cultures. IL-6 transcripts were nearly undetectable by blotting in keratinocytes grown in low-calcium serum-free medium, but low levels could be induced by treatment with 1.8 mM $CaCl_2$. IL-6 transcripts were strongly superinduced after cycloheximide treatment, suggesting that a labile protein regulates IL-6 mRNA levels in these cells. Finally, the mitogenic activity of IL-6 was examined in NHK under varying conditions of cell density and added growth factors. IL-6 did not stimulate high density keratinocyte growth in the presence or absence of other growth factors, but did stimulate clonal growth in epidermal growth factor (EGF)-deficient media at high concentrations (≥ 10 ng/ml). The proliferative effects of IL-6, but not of basic fibroblast growth factor, were abrogated by monoclonal antibodies directed against the EGF receptor. Taken together, these results suggest that the proliferative effects of IL-6 are mediated indirectly via the EGF/TGF-α receptor, and that autocrine overexpression of IL-6 may be limited in psoriatic keratinocytes.

Key words: Interleukin-6 – Psoriasis – Polymerase chain reaction – Keratinocytes – Cell proliferation

Interleukin-6 (IL-6) is a multifunctional cytokine, originally cloned as an interferon (IFN)-β-like antiviral activity from human fibroblasts [for reviews, see references 20,23]. In human skin, cutaneous ultraviolet-B irradiation leads to a rapid (12 h) increase in circulating IL-6 [45], suggesting that the skin may be a prominent source of this cytokine. However, the cutaneous cell type(s) responsible for the production of IL-6 are presently unclear. Fibroblasts, endothelial cells, and vascular smooth muscle cells are generally considered to be prominent sources of IL-6 in tissues [27], whereas large, low density mononuclear cells appear to be the principal IL-6-producing cells in the circulation [1].

Recently, increased levels of biologically active IL-6 have been reported to be present in blister fluid derived from psoriatic lesions [37], although bioactivity could not be detected in aqueous extracts of psoriatic scale [17]. Increased expression of IL-6 has been reported in keratinocytes within psoriatic lesions by immunocytochemical and in situ hybridization techniques [19, 30–34]. However, significantly increased production of IL-6 by peripheral blood mononuclear cells has also been obsrved in psoriatic patients [19, 30]. Since these cells are capable of entering the epidermal compartment in psoriatic lesions [4], and since proteins as large as albumin are capable of entering the epidermis from the circulation [38], the question arises whether the keratinocyte is the predominant cellular source of IL-6 in psoriasis and other inflammatory skin diseases. Psoriasis is characterized by a marked increase in keratinocyte proliferation [47], and three reports have suggested that IL-6 is apatient positive growth regulator for human keratinocytes [19, 25, 50]. Taken together, these data have suggested the hypothesis that IL-6 is a major autocrine regulator of keratinocyte growth [19, 26].

Previous data suggesting increased IL-6 gene expression in psoriatic keratinocytes in vivo have been limited to results of in situ hybridization and immunocytochemical experiments [19, 30–34]. Therefore, we investigated alternative methods in an attempt to confirm abnormal expression of IL-6 mRNA and protein in psoriatic epidermis, including RNA blot hybridization, polymerase chain reaction (PCR), and immunoblotting. In addition,

the effects of seum, calcium, and cycloheximide on IL-6 mRNA levels were investigated in cultured keratinocytes. Finally, in an attempt to clarify the direct effects of IL-6 on keratinocyte growth, we tested the effects of cell density and other growth factors on IL-6-stimulated proliferation in secondary cultures of normal human keratinocytes.

Materials and methods

Skin biopsies

After obtaining informed consent with Institutional Review Board approval, keratome biopsies (0.2–0.4 mm thick) were obtained from the buttocks of normal volunteers or from lesional buttocks or upper thigh skin of psoriatic volunteers as previously described [46].

Organ culture of keratomed tissue

Keratome strips were obtained from normal individuals and immediately cut into 1.5 cm squares. Tissue fragments were either frozen immediately or incubated in keratinocyte basal medium (KBM, Clonetics San Diego CA, USA) at 37 °C in an atmosphere containing 5% CO_2 for 2–4 h followed by freezing in liquid nitrogen as previously described [11].

RNA and DNA isolation and blot hybridization

RNA was extracted from keratome biopsis and organ cultures by guanidinium isothiocyanate lysis and ultracentrifugation exactly as previously described [11, 12]. Total RNA was extracted from cell cultures using RNAzol (Tel-Test, Friendswood, TX, USA) as directed by the manufacturer. Polydenylated RNA was prepared from total biopsy RNA and from cultured keratinocytes by oligo-dT cellulose chromatography [11, 12]. RNA quantitation, blot hybridization using random-primed ^{32}P-labelled probes, and analysis of specific RNA transcripts by densitometry were performed as previously described [11, 12].

Plasmids and hybridization probes

Plasmid DNAs were prepared by alkaline lysis followed by polyethylene glycol precipitation [3]. Hybridization probes (specific activity $1–4 \times 10^9$ cpm/µg DNA) were prepared by random priming [15] of a 900 bp PstI fragment from the human IL-6 cDNA plasmid $p\beta_2.15$ [28] and a 1.4 kb Eco RI fragment from the human TGF-α cDNA plasmid psp65C17N3 [9].

Polymerase chain reaction (PCR)

For qualitative PCR analysis, total RNA (1 µg) was reverse transcribed using 20–30 U avian myelocytomatosis virus reverse transcriptase Seikagaku America, Rockville, MD, USA) and an oligo (dT) primer (Pharmacia, Piseataway, NJ, USA), as previously described [21]. Using Taq polymerase, buffers, and nucleotides provides in a commercial kit (Perkin elmer Cetus, Norwalk. Coun., USA), 30 cycles of amplification (92 °C, 30 s, 57 °C, 30 s, 72 °C, 100 s) were performed. The upstream IL-6 primer, 5'-TCC TTC TCC ACA AGC GCC TTC GGT CCA-3', was derived from the junction of the first and second exons of the human IL-6 gene [49]. The downstream primer, 5'-TGT TCC TCA CTA CTC TCA AAT CTG TTC T-3', was derived from the third exon. All RNA samples were also tested by PCR for c-abl sequences using the primers previously described [41]. DNA blots of the PCR products were prepared by alkaline capillary transfer from 1% agarose gels to derivatized nyon membranes (Zeta-Probe, Bio-Rad, Richmond, Calif., USA) as described by the manufacturer, and hybridized

against the IL-6 cDNA probe under the same conditions used from RNA blot hybridization.

Semiquantitative PCR analysis will be described in detail (G. Fisher, S. Benrazavi, and J. Voorhees, manuscript in preparation). Purified human β-globin mRNA (Gibco BRL, Santa Clara, Calif., USA) (1.25 pg) was added to 1 µg of normal or psoriatic total biopsy RNA, and cDNA was prepared using M-MLV reverse transcriptase (200 U, Gibco BRL) and random hexamers (25 pmol) as primers. Threefold serial dilutions of cDNA were mixed with primers (100 pmol each) for IL-6 (forward, 5'-ACA ACC TGA ACC TTC CAA, AGA TGG-3'; reverse, 5'-CTT AAA GCT GCG CAG AAT GAG ATG-3') and β-globin (forward, 5-'AAG GCT CAT GGC AAG AAG GTG-3'; reverse 5-'CTG CAC CTG AGG AGT GAA TTC-3'). Amplification was performed using 2 U *Taq* DNA polymerase (Perkin Elmer Cetus) in 40 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 125 mM dXTP. Amplification was carried out for 23 cycles so that the amount of DNA formed remained proportional to the amount of RNA input. Denaturation was for 30 s at 92 °C and annealing was for 30 s at 57 °C. Extension was for 100 s at 72 °C in the first cycle, and prolonged by 3 s/cycle. After amplification, the 335 bp IL-6 and 201 bp β-globin products were analyzed by electrophoresis and blot hybridization, using a mixture of ^{32}P-labelled IL-6 and β-globin PCR products as probes. After hybridization, band intensities were quantitated using a phosphorimager (Molecular Devices). IL-6 band intensities obtained from non-saturating dilutions of input RNA were normalized to β-globin. Statistical comparison were performed using Student's t-test and a two-tailed hypothesis with and without logarithmic transformation of the data.

Cell culture

Primary cultures of normal human kineratinocytes were prepared as reviously described [12, 36, 48] from keratome biopsis of adult volunteers less than 30-years-old or from neonatal foreskins. Subcultures were expanded in either keratinocyte growth medium (KGM, Clonetics, San Diego, CA, USA), or MCDB 153 medium modified for high-density growth as previously described [12]. Subcultures were used in the second to fourth passage. As previously defined [12], 'standard medium' is basal MCDB 153 medium supplemented with ethanolamine, phosphoethanolamine, and hydrocortisone. 'Complete medium' is standard medium supplemented with insulin, epidermal growth factor (EGF), and bovine pituitary extract (BPE). Human dermal fibroblast cultures were prepared from punch biopsies as previously described [12], propagated in modified McCoy's 5A medium (Sigma St. Louis, Mo., USA) containing 10% fetal calf serum and antibiotics, and used before the tenth passage.

Growth assays

Glonal growth assays were performed as previously described [31]. Briefly, 500 keratinocytes from primary culture were inoculated into 60-mm dishes containing standard MCDB153 medium and the designated growth factors or additives. Recombinant human bFGF was obtained from Upstate Biotechnology Inc. (UBI, Waltham, MA, USA). Carrier-free rhIL-6 (Biosource International, Camarillo, CA, USA) had a specific activity of 6×10^6 U/mg, and the ED_{50} for ^3H-TdR uptake in B9 cells was 0.15 ng/ml; rhIL-6 was dissolved in PBS at 1 mg/ml, and diluted into culture medium just to use. Monoclonal antibodies (IgG1 kappa isotype) against EGF receptor (LA-1, UBI) and growth hormone (gift of R. Derynck, Genentech, South San Francisco, CA, USA) were added to culture medium at a final concentration of 2.2 µg/ml IgG at the time of cell plating. Cultures were incubated without medium change for 10 days, fixed, stained with crystal violet and photographed. The additives used had no effect on plating efficiency, as determined by microscopic examination of the stained plates. Higher density proliferation assays were also performed [36, 48] using subcultures of keratinocytes seeded at $1–3 \times 10^3$ cells/cm^2 in T25 flasks containing complete MCDB 153 medium.

Results

IL-6 expression in vivo

Figure 1A displays sequential hybridizations of the same RNA blot, containing both total and polyadenylted RNA isolated from five normal and five psoriatic volunteers. In the *upper panel*, the blot was probed with IL-6 cDNA alone. The blot was then stripped and rehybridized against a mixture of IL-6 and TGF-α cDNAs of comparable length, specific acitivity, and total activity. The IL-6 probe hybridized to a band of approximately 2 kb in 40 μg of total RNA *(left upper panel)*. This band comigrated with the 18S ribosomal RNA band on the ethidium bromide-stained gel prior to blotting (not shown), and was not observed when 5 μg of polyadenylated RNA was blotted instead of total RNA *(Right upper panel)*. When the same blot was rehybridized against the mixture of IL-6 and TGF-α probes, TGF-α transcripts (5.0 and 1.6 kb) were readily detectable and clearly enhanced in lesional psoriatic vs. normal epidermis (Fig. 1A, *lower panel*). Hybridization of the same blot with TGF-α alone revealed the same 5.0 and 1.6 kb bands as seen when the mixed probe was used, demonstrating that the 1.6 kb band is derived from TGF-α and not IL-6 (data not shown). Although no IL-6 mRNA could be detected by RNA blot hybridization in keratome samples of normal or psoriatic skin frozen in liquid nitrogen immediately after harvesting, 1.3 kb IL-6 transcripts became detectable after 2–4 h of organ culture of normal biopsies (Fig. 1B).

In order to increase the sensitivity of detection of IL-6 transcripts in normal and psoriatic epidermis, qualitative PCR amplification studies were undertaken. For these analyses, all biopsies were frozen immediately after harvesting, and not subjected to organ culture. The results of one of three qualitative PCR comparisons of IL-6 mRNA levels in normal and psoriatic keratome biopsies are shown in Fig. 2. The results of all three qualitative PCR experiments are summarized in Table 1. There was no significant difference in the number of positive samples in normal and psoriatic biopsy RNA, as assessed by positive EtBr staining or by blot hybridization of the PCR products. However, a significant increase in the number of strongly-positive bands was evident (Table 1).

Because the qualitative PCR analyses did not allow a reliable comparison of relative IL-6 mRNA levels in normal and psoriatic biopsies, semiquantitative PCR analysis was performed. To verify the linearity of amplification and to provbide an internal standard for comparisons, equal amounts of purified β-globin RNA were added to each sample prior to reverse transcription. After reverse transcription, β-globin- and IL-6-specific primers were added in parallel to serial dilutions of cDNA prior to amplification. As shown in Table 2, this analysis revealed that, whereas IL-6 mRNA levels were uniformly low in keratome biopsies of normal skin, IL-6 expression was clearly increased but highly variable in psoriatic lesions (Table 2). These results confirmed the impression derived from qualitative PCR analysis (Table 1) that a strongly positive signal was derived from a much higher proportion of psoriatic samples (14/15) compared with normal samples (2/21).

IL-6 expression in vitro

Small amounts of papillary dermis and variable numbers of infiltrating immune/inflammatory cells are unavoidably present in psoriatic tissue specimens. Therefore, IL-6 mRNA levels were analyzed in keratinocyte cultures, which are relatively free of inflammatory and other cell types. Since the presence of hydrocortisone (HC) has been shown to inhibit IL-6 expression in keratinocytes [25], HC was excluded from the medium for one passage (usually 5–7 days) prior to assay (KGM–HC). Under these conditions, IL-6 transcripts could be detected as a very faint band in 5 μg of polyadenylated RNA (Fig. 3A). Switching cultures from KGM–HC (0.15 mM CaCl₂) to the same medium containing 1.8 mM CaCl₂ for 24 h induced a small increase in 1.3 kB IL-6 transcript levels (Fig. 3A, *left panel*). In KGM–HC, IL-6 transcripts were not detectably induced 6 h after treatment with the following agents: 12-O-tetradecanoyl phorbol-13-acetate (TPA, Sigma St. Louis, MO, USA, 20 nM), recombinant human interleukin-1α (rhIL-1α, Dainippon, Osaka, Japan, 5 or 100 U/ml), ultraviolet-B irradiation (100 or 1000 J/m², Sylvnia FS-2 bulbs, through phosphate buffered saline, without lid), bovine bone transforming growth factor-β, Collagen Corp., Palo Alto, CA, USA), 10 ng/ml), or *E. coli* lipopolysaccharide (LPS, Sigma, 100 μg/ml)

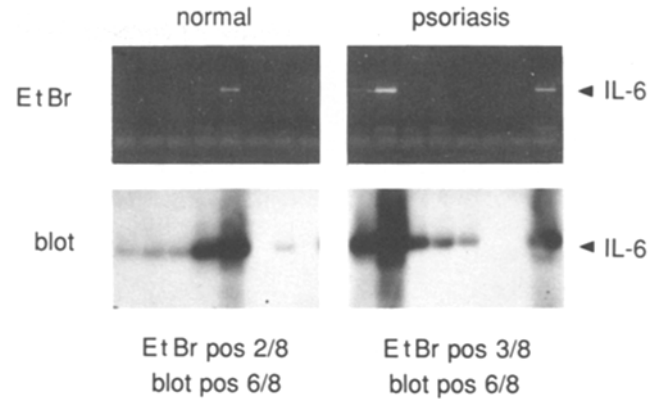**Table 1.** Qualitative PCR analysis of IL-6 mRNA levels in normal and psoriatic epidermis

	Normal (n = 25)	Psoriasis (n = 20)	P value
c-abl-positive (% of total)	21 (84%)	19 (95%)	–
EtBr-positive (% of abl-positive)	2 (9.5%)	6 (32%)	0.08
Blot-positive (% of abl-positive)	13 (62%)	15 (79%)	0.24
Strongly blot-positive[a] (% of abl-positive)	2 (9.5%)	14 (74%)	<0.0001

[a] Visual estimate of intensity at least that of positive control (cycloheximide-treated keratinocyte RNA)
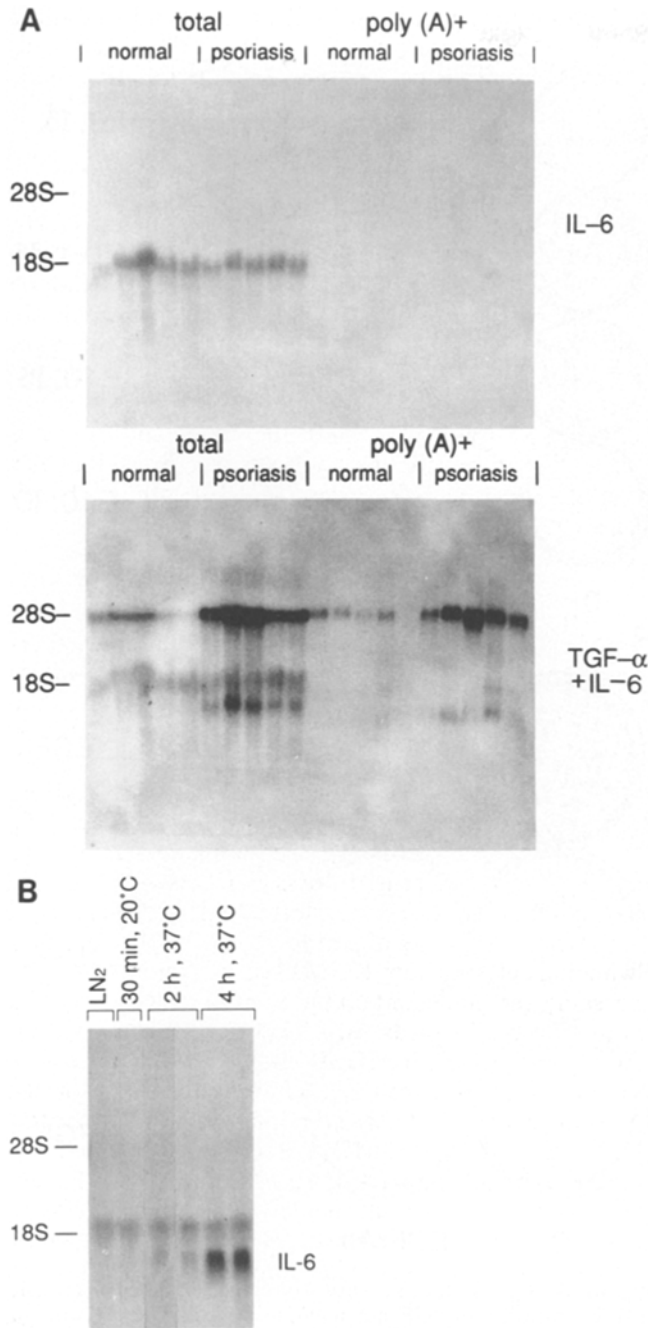
Fig. 1A, B. RNA blot analysis of IL-6 mRNA. **A** Comparison of IL-6 and TGF-α mRNA levels in normal vs. psoriatic epidermis. Total RNA (40 µg) or poly(A)$^+$ RNA (5 µg), isolated by a single cycle of oligo (dT) cellulose chromatography from the same individuals, were blotted and sequentially hybridized against an IL-6 probe *(upper panel)* or a mixture of IL-6 and TGF-α probes *(lower panel)*. Mobilities of ribosomal RNAs are shown to the left. **B** Keratome strips were incubated in KBM under the conditions shown above the figure. RNA was extracted and 50 µg total RNA from each condition was analyzed by blot hybridization. The expected mobility of IL-6 mRNA is indicated to the right, and the mobilities of ribosomal RNAs are shown to the left

(Fig. 3A, *right panel*). In experiments not shown, recombinant human tumor necrosis factor (rhTNF-α, Amgen Thousand Oaks, CA, USA, 20 ng/ml) either alone or in the presence of recombinant human interferon-gamma (rhIFN-gamma, Collaborative Research, Bedford, MA.,

Fig. 2. Qualitative PCR analysis of IL-6 mRNA levels in normal and psoriatic epidermis. *Upper panels* display ethidium bromide-stained gel, and *lower panels* display Southern blot of the same gel hybridized against the IL-6 cDNA probe. The results of one of three experiments are shown

USA, 100 U/ml) for 24 or 48 h also failed to induce detectable IL-6 mRNA. However, as shown in Fig. 3A, treatment with cycloheximide (CHX, 10–15 µg/ml, 2 to 4 h) consistently induced marked accumulation of IL-6 transcripts. No additional enhancement was observed when CHX was added 2 h prior to TPA, IL-1α, or UVB treatment (Fig. 3A). In marked contrast, IL-6 transcripts were detectable constitutively in adult human dermal fibroblasts, and could be strongly induced by treatment with the protein kinase C activators di-C8 and TPA (Fig. 3B). Similar results have been reported previously in the foreskin-derived fibroblast strain FS-4 [29].

Mitogenic assays

IL-6 demonstrated little or no effect on keratinocyte growth rate or maximum cell density when assayed under high-density conditions [48], either in the presence or absence of exogenous growth factors (data not shown). However, under these conditions, keratinocytes display marked cell proliferation in the absence of exogenous growth factors [8, 40, 44, 48]. Therefore, we analyzed the proliferative effects of IL-6 at clonal density to minimize

Table 2. Semiquantitative PCR analysis of IL-6 mRNA levels in normal and psoriatic epidermis

	Normal (n = 7)	Psoriasis (n = 6)	P value
PCR score[a]			
mean	0.46	5.69	0.05
±SEM	±0.08	±2.63	
range	0.23–0.87	1.52–18.52	
log PCR score			
mean	−0.37	0.59	0.0001
±SEM	±0.07	±0.16	

[a] Defined as IL-6 radioactivity (dpm)/β-globin radioactivity (dpm) detected by phosphorimager after blot hybridization of PCR products against IL-6 and β-globin probes

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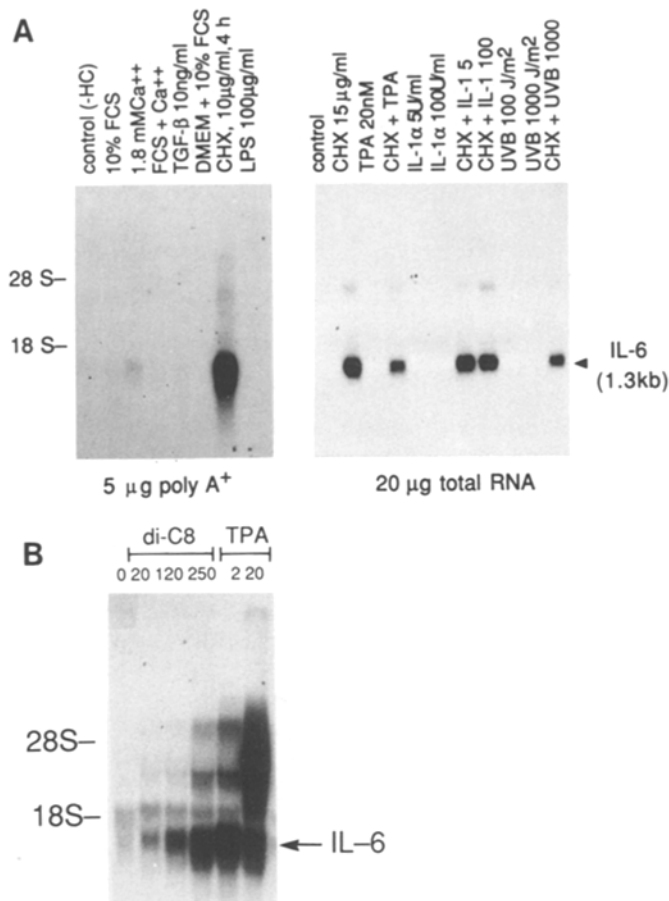
Fig. 3A, B. RNA blot analysis of IL-6 mRNA levels in cultured keratinocytes and fibroblasts. **A** Keratinocytes. Cells were grown in HC-deficient media for one passage prior to treatment. *Left panel:* neonatal keratinocytes were grown in MCDB 153 medium modified for high density growth [12]. With the exception of cycloheximide (CHX) treatment, which was for 4 h, cells were treated as shown at the top of the Figure for 24 h prior to isolation of polyadenylated RNA (5 µg/lane). *Right panel:* adult keratinocytes were treated for 2 h with CHX, then either with or without an additional 4 h of treatment with the agonists shown. Total RNA (20 µg) was then analyzed by RNA blot hybridization. Expected mobility of IL-6 mRNA is shown to thge right, and mobilities of ribosomal RNAs are shown to the left. **B** Fibroblasts. Confluent secondary cultures of human dermal fibroblasts were treated with the indicated concentrations of dioctanoylglycerol (di-C8, µM) or TPA (nM) for 4 h prior to RNA extraction. Expected mobility of IL-6 mRNA is shown to the *right*, and mobilities of ribosomal RNAs are shown to the *left*

potential autocrine effects of keratinocyte-derived growth factors. To test for EGF-like mitogenic effects, clonal growth assays were performed in the absence of EGF. Under these conditions, carrier-free, purified recombinant human IL-6 of known biological activity stimulated the clonal growth of keratinocytes at 10 ng/ml, albeit to a lesser extent than did EGF (Fig. 4A). However, in the absence of insulin, IL-6 had no growth-promoting effect (Fig. 4A). The growth-promoting effects of IL-6 were completely inhibited by anti-EGF receptor monoclonal antibodies, but not by isotype-matched antibodies against human growth hormone (Fig. 4B). In contrast, anti-EGF
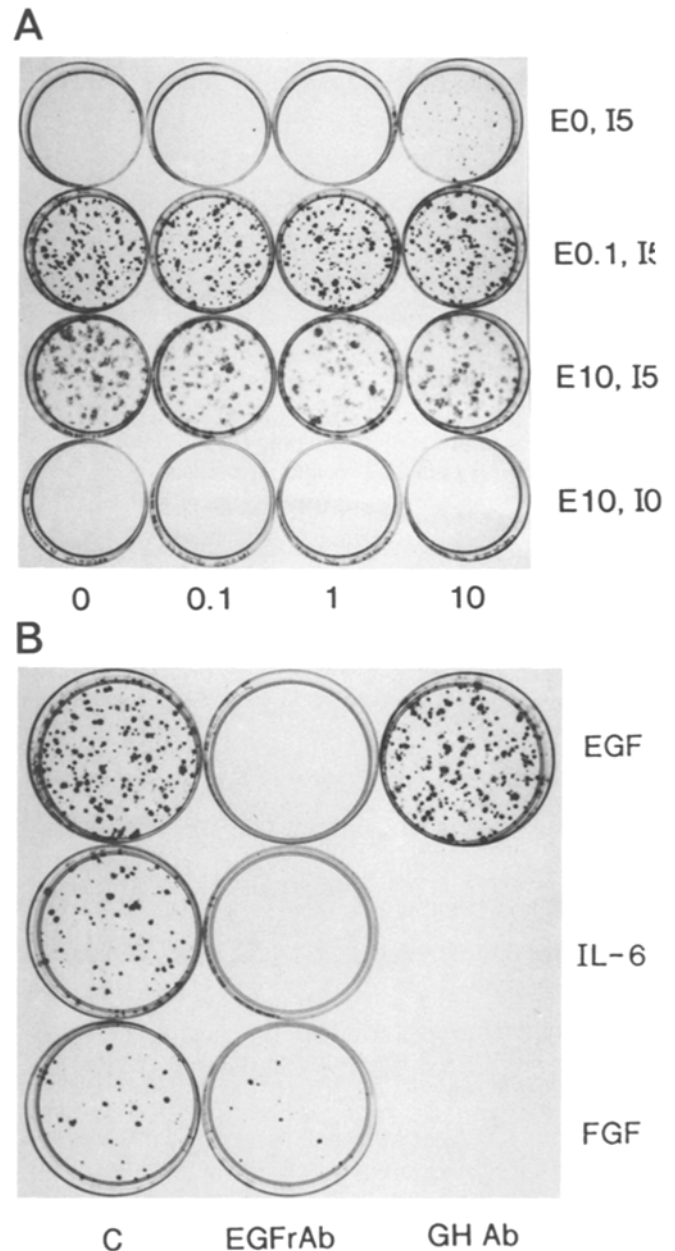
Fig. 4A, B. EGF-like effects of rhIL-6 on clonal keratinocyte growth. **A** IL-6 substitutes for EGF, but not for insulin. MCDB 153 (without BPE) was supplemented with EGF (E, in ng/ml) and insulin (I, in µg/ml) at the concentrations indicated to the right of the Figure, and IL-6 (in ng/ml) as indicated below the figure. **B** Inhibition of rhIL-6-stimulated keratinocyte growth by anti-EGF receptor monoclonal antibodies. Keratnocytes were plated in MCDB 153 without BPE in the presence of insulin (5 µg/ml) and the factors indicated to the left of the Figure: EGF (10 ng/ml), IL-6 (10 ng/ml), or bFGF (10 ng/ml). At the time of plating, mAbs were also added as indicated at the bottom of the Figure: none *(C)*, anti-EGF receptor mAb *(EGFRAb)*, or growth hormone mAB *(GH ab)*.

receptor antibodies had little or no effect on the stimulation of clonal keratinocyte growth by basic fibroblast growth factor (Fig. 4B).

In the course of these studies, we observed that certain preparations of IL-6 containing high amounts of total protein were able to substitute for insulin in stimulating
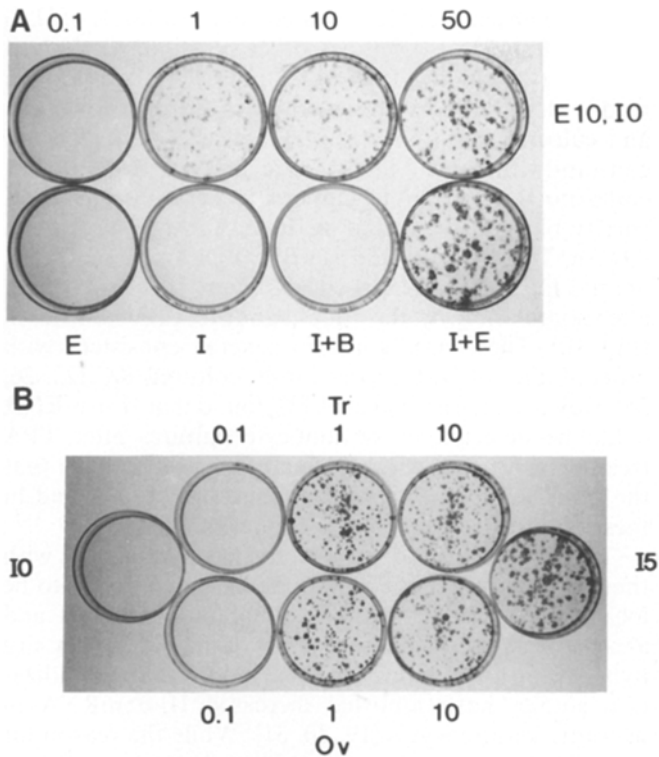
Fig. 5A, B. Insulin-like effects of various proteins on clnal keratinocyte growth. Cultures were fixed, stained, and photographed after 10 days of incubation. **A** Effects of BSA. *Top row:* cells were plated in MCDB 153 supplemented with EGF (*E*, 10 ng/ml) in the presence of the concentrations of BSA (in μg/ml) shown above the Figure. *Bottom row:* cells were plated in MCDB 153 supplemented with: EGF (*E*, 10 ng/ml); insulin (*I*, 5 μg/ml); insulin (*I*, 5 μg/ml) and BSA (*B*, 50 μg/ml); or insulin and EGF (*I + E*). **B** Effects of transferrin *(Tr)* and ovalbumin *(Ov)*. Cultures were plated in MCDB containing EGF (10 ng/ml) in the absence of insulin (except for the rightmost dish, *(I5)* which received 5 μg/ml insulin), in the presence of Tr or Ov at the concentrations (in μg/ml) indicated above and below the figure

clonal keratinocyte growth (data not shown). Therefore, we tested the effects of bovine serum albumin (BSA) as well as transferrin and ovalbumin on clonal keratinocyte growth in the absence of added insulin. Each of these proteins markedly stimulated keratinocyte growth at concentrations as low a 1 μg/ml, provided that EGF was present (Fig. 5A and B). In addition, Fig. 5A demonstrates that BSA substitutes for insulin, but not for EGF, as a growth-promoting additive.

Discussion

Studies involving transplantation of psoriatic skin to murine recipients suggest that the initiating and/or propagating mechanisms for psoriatic epidermal hyperplasia do not reside exclusively within the epidermis, but rather within an abnormal cutaneous microenvironment containing dermal cells, immune/inflammatory cells, and circulating factors [5, 16, 24]. Nevertheless, overexpression of a keratinocyte mitogen in an autocrine fashon might be an important final common pathway mediating psoriatic

epidermal hyperplasia. In these studies, we re-examined previous findings of IL-6 expression in psoriasis [9, 30–34] in order to investigate further the hypothesis that overexpression of IL-6 by keratinocytes might be important in the pathogenesis of psoriatic epidermal hyperplasia.

We initially used RNA blot hybridization for this purpose, because the results confirm that a transcript of the expected sze is being detected. Although limited sensitivity is the principal disadvantage of this technique, this problem is often overestimated. RNA blot hybridization is capable of detecting approximately 10^5 to 10^6 target molecules [21]. Given an estimate of approximately 10 pg of RNA per cell [42], 40 μg total RNA represents approximately 4×10^6 cells. Therefore, blots loaded with 40 μg total RNA should detect from less than one to a few transcripts per cell. Keratome biopsies contain approximately the same amount of RNA and DNA (unpublished data). The double-stranded probe will detect genomic sequences from both strands of each parental chromosome, and therefore 10 μg genomic DNA and 40 μg total RNA should yield approximately the same hybridization if the target RNA sequence is present at a level of one copy per cell. Consistent with these estimates, we routinely obtain strong positive hybridization to DNA blots containing 10 μg genomic DNA [11, 12].

Under these conditions, IL-6 transcripts were not detectable by RNA blot analysis in normal or psoriatic epidermal keratome biopsies (Fig. 1A, *upper panel*). Moreover, non-specific hybridization to 18S ribosomal RNA was demonstrated, and could be eliminated by selection of polyadenylated RNA. In contrast, 5.0 and 1.-6 kb TGF-α transcripts were clearly detectable under all conditions, and were markedly and uniformly elevated in psoriatic lesions relative to normal skin [10] (Fig. 1A, *lower panel*). Although its physiologic significance is unclear, the demonstration of IL-6 transcripts after organ culture of normal keratome biopsies (Fig. 1B) shows that it is possible to detect IL-6 transcripts under our conditions of RNA isolation from skin tissue. The nuclear protooncogenes *c-fos*, *c-jun*, and *c.myc* undergo similar, but much more marked, induction in organ culture [11], and similarities in the regulation of IL-6 and *c-fos* gene expression have been noted [39]. From these results, we conclude that IL-6 transcripts are expressed at a very low level in both normal and psoriatic keratome biopsies, at levels of less than one to at most a few copies per cell when averaged over the tissue sampled.

In order to explore IL-6 expression in keratome biopsies levels less than one copy per cell, we analyzed RNA samples by reverse transcription and PCR. Making use of conditions designed to optimize sensitivity, titration of linearized IL-6 plasmid DNA demonstrated that it was possible to detect 1.5 molecules of linearized IL-6 plasmid DNA per reaction (data not shown). Assuming only a 1% efficiency of cDNA synthesis, this assay would still detect approximately one IL-6 molecule per 1000 cells (1 μg/reaction $\times 10^5$ cells/μg RNA $\times 0.01$ efficiency factor = 10^3 cells). Although this analysis revealed no difference in the number of individuals in whom IL-6 was detectable, the number of psoriatics displaying a strongly positive signal after blot hybridization of the PCR was

clearly increased (Table 1). Therefore, semiquantitative PCR analysis was undertaken, using experimental conditions designed to maintain a linear response to input RNA. This analysis revealed uniformly low expression of IL-6 in normal biopsies, and highly variable but significantly elevated expression in psoriatic biopsies (Table 2).

The cellular source of the IL-6 transcripts detected in keratome biopsies by PCR is not clear, since tissue sampled by this method contains small numbers of dermal fibroblasts, endothelial cells, smooth muscle cells, and variable numbers of macrophages, all known to be robust sources of IL-6 [1, 27]. Even if techniques for dermal–epidermal separation were employed, other potential sources of IL-6, such as Langerhans cells, melanocytes and epidermal macrophages, could not be excluded. Moreover, the results shown in Fig. 1B indicate that the tissue manipulations required for such separations could lead to artifactual expression of IL-6 mRNA. Despite these limitations, several lines of evidence favor the concept that much, if not all, of the increased IL-6 expression in psoriatic lesions is derived from fibroblasts and/or infiltrating inflammatory cells rather than from keratinocytes.

Analysis of the in vivo blot hybridization data (Fig. 1) indicates that IL-6 transcripts are only present at less than one to a few copies per cell on average over the tissue. Since at least some of this signal is likely to be derived from infiltrating macrophages and contaminating dermal tissue, the amount derived from keratinocytes is likely to be even lower than this. Moreover, variability of lesional IL-6 mRNA levels was detected by semi-quantitative PCR (Table 2). This would be most consistent with the presence of variable numberrs of infiltrating mononuclear cells and/or variable dermal contamination. Certainly, if IL-6 expression is occurring in lesional keratinocytes, it must be quite heterogeneous within a lesion or from lesion to lesion. In this regard, Ohta et al. gave suggested that IL-6 expression is found at the advancing borders of psoriatic plaques [31].

Several studies have reported increased IL-6 immunoreactivity within the epidermal compartment [19, 30–34]. However, despite the demonstration that immunostaining can be blocked by preincubation with purified antigen, it can be difficult to prove that the observed staining actually reflects the presence of tissue antigen in the absence of corroborating biochemical evidence. Under conditions in which 10 ng of purified recombinant IL-6 was readily detectable, we were unable to detect any band which comigrated with it by immunoblot analysis of nonionic detergent lysates of normal or psoriatic keratome biopsies (data not shown). Moreover, this analysis failed to reveal any band which was increased in psoriatic lesions relative to normal skin (data not shown). Even if IL-6 protein is present in the epidermis at levels too low to be detected by immunoblotting, it is possible that it may be derived from the circulation, and not from keratinocytes [38]. In this regard, the skin is apparently a major site of binding and catabolism of systemically-administered IL-6 [7] and TNF-α [2].

Blot hybridization analysis of keratinocytes propaga-

ted in the absence of HC revealed very low levels of IL-6 mRNA (Fig. 3A). In contrast, other keratinocyte-derived transcripts, including TGF-α [10] and CRABP-II [13], are readily detectable by this method in normal epidermis and cultured keratinocytes [12], and are markedly and constantly increased in psoriatic lesions. IL-6 mRNA could not be detectably induced in keratinocytes by a variety of agonists, including IL1α, TPA, IFN-gamma, TNF-α, TGF-β, LPS, and UVB irradiation. In contrast, dermal fibroblasts expressed abundant IL-6 transcripts after stimulation by the PKC agonists TPA and di-C8 (Fig. 3B). These results are, in general, consistent with other studies of IL-6 expression in cultured KC [22, 26, 35]. However, Kirnbauer et al. [22] found that IL-6 mRNA could be detected in keratinocyte cultures after TPA treatment. Among other explanations, it is possible that the small number of fibroblasts that are often found in these cultures could explain this discrepancy.

Taken together, our results are most consistent with those of Gillitzer et al., who found IL-6 expression to be localized to the vicinity of dermal blood vessels and absent from the epidermis in psoriatic skin by in situ hybridization [18]. However, three other in situ hybridization studies have reported increased IL-6 mRNA in psoriatic keratinocytes [19, 30, 31]. While the reason for these discrepancies is not clear, our observaton of non-specific hybridization of IL-6 to 18S rRNA (Fig. 1A) indicates that in situ hybridization results should be interpreted with caution. Our results are also consistent with those of Gearing et al. [17], who were unable to detect IL-6 biological activity in psoriatic scale despite its reported presence n psoriatic suction blister fluid (37). Nevertheless, it remains possible that psoriatic keratinocytes express elevated, albeit very low, amounts of IL-6 mRNA.

Although unresponsive to cytokine treatment or PKC activators, IL-6 mRNA was strongly induced by cycloheximide (CHX) in cultured keratinocytes (Fig. 3). it is possible that an unstable protein (which would be rapidly depleted after CHX treatment) normally functions to destabilize IL-6 transcripts in keratinocytes, as has been observed for GM-CSF mRNA in other cell types [43]. Like other cytokine genes which appear to be regulated at the level of RNA stability [6], IL-6 contains an AT-rich domain in its 3′ untranslated region [49]. Recently, IL-6 mRNA stability has been shown to be increased in response to TNF-α in IL-1.stimulated fibroblasts [14]. Whether this mechanism contributes to the induction of IL-6 mRNA observed in keratome biopsy organ cultures (Fig. 1B) remains to be determined.

Whether produced by keratinocytes in an autocrine mode, by dermal and/or inflammatory cells in a paracrine mode, or by other cells in an endocrine mode, IL-6 could play an important role in the regulation of epidermal growth. To this end, we confirm previous reports [19, 25, 40] of the mitogenicity of IL-6 for human keratinocytes, albeit with reduced potency relative to EGF/TGF-α or bFGF. Demonstration of IL-6 mitogenicity required the use of clonal, rather than high-density conditions, and was observed only in the presence of insulin and the absence of EGF. Surprisingly, stimulation of keratinocyte

growth by IL-6 could be blocked by monoclonal antibodies directed against the EGF receptor (Fig. 4B), suggesting that the EGF receptor signal transduction system may be a dosnstream mediator of IL-6 action in keratinocytes. In addition, BSA, transferrin, and ovalbumin could all substitute for insulin in promoting keratinocyte growth at concentrations as low as 1 μg/ml, suggesting that the growth-promoting effects of insulin may not be mediated via specific insulin/IGF-1 receptors in keratinocytes. These results demonstrate that the mitogenic effects of cytokine preparations containing large amounts (≥1 μg/ml) of carrier proteins should be interpreted with caution when assayed under serum-free conditions.

Clearly, these results do not exclude the possibility that IL-6 is expressed by psoriatic keratinocytes in vivo. However, they do strongly suggest that lesional keratinocytes do not express high levels of this cytokine, despite being markedly hyperproliferative. Whatever its source, IL-6 is undoubtedly present in the psoriatic millieu, and we confirm that this cytokine can be mitogenic for keratinocytes under appropriate experimental conditions. However, on the basis of the results of the anti-EGF receptor blocking experiments presented here, it appears that IL-6 may act indirectly to modulate the effects of TGF-α and/or other EGF receptor ligands on epidermal proliferation. For all these reasons, we do not favor the notion that IL-6 is a major autocrine mitogen for psoriatic keratinocytes. It is to be hoped that future studies of the immunogenetic basis of psoriasis will eventually elucidate the roles of IL-6, as well as other cytokines and growth factors, in the pathogenesis of this complex disease.

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