Interleukin-1–Induced Growth Factor Expression in Postmitotic and Resting Fibroblasts

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Tissue homeostasis in skin is regulated by epithelial-mesenchymal interactions, mostly operating via diffusible factors. To study the underlying regulatory mechanisms, *in vitro* systems have been established to mimic the *in vivo* situation in skin. In co-cultures, keratinocytes grow either adjacent to irradiated fibroblasts on plastic or on top of collagen gels containing fibroblasts, thus forming 3-dimensional organotypic structures. Keratinocyte growth is supported in part by fibroblast-produced factors induced by keratinocyte mediators such as interleukin-1 (IL-1). To better understand this cellular interaction and its modulation by fibroblast proliferation and extracellular matrix (ECM), we examined the effect of IL-1 on growth factor expression in proliferating and growth-arrested x-irradiated human dermal fibroblasts on plastic and in resting cells embedded in collagen gels. By semiquantitative reverse transcriptase PCR, we demonstrated that IL-1α and IL-1β stimulated the expression of KGF, HGF, IL-1α, IL-1β, IL-1RI, and IL-8 in fibroblasts regardless of their physiologic condition, whereas that of TGF-β remained unaffected. The constitutive mRNA levels were usually lower in irradiated postmitotic and ECM-embedded cells than in proliferating fibroblasts. Cells responded to stimulation with IL-1 under all three culture conditions, although to different degrees depending on the growth factor. As demonstrated for HGF, IL-8, and IL-1β, the IL-1α–induced mRNA expression was followed by production and secretion of protein in irradiated fibroblasts. Thus, our findings show that resting and growth-inhibited fibroblasts, reflecting more closely the situation in dermis, exhibit lower constitutive growth factor expression levels but characteristically respond to IL-1 stimulation. ***Key words: cytokines/irradiation/collagen matrix/reverse transcriptase PCR. J Invest Dermatol 107:849–855, 1996***

Epithelial-mesenchymal interactions control epidermal growth and differentiation and thus regulate tissue homeostasis in the epidermis (Boukamp *et al*, 1990; Fusenig, 1994; Schröder, 1995). Hence, the function of fibroblasts is, in addition to producing extracellular matrix as a structural framework, to act as a cellular communication bridge between epidermis and dermis by synthesizing various mediators, such as growth factors and cytokines (Luger and Schwarz, 1995).

To study the regulation of epidermal cell proliferation and differentiation by fibroblasts, *in vitro* model systems have been developed to mimic epidermal-dermal interactions. In its simplest version, the feeder-layer system, keratinocytes grow in co-culture with postmitotic fibroblasts, originally the transformed mouse 3T3 cell line, submerged in culture medium and attached to plastic dishes (Rheinwald and Green, 1975). Homologous human fibroblast- and capillary endothelial-feeder cells have been demonstrated to function similarly and may provide a better tool for studying interactions between normal human mesenchymal and epithelial cells (Limat *et al*, 1989; Smola *et al*, 1993). In such co-cultures, it has been demonstrated that keratinocyte growth depends strongly on mesenchymal cells, particularly at low seeding densities. It has been postulated that direct cell-cell contact is required to promote keratinocyte growth, because fibroblast-conditioned medium cannot substitute for feeder cells (Rheinwald and Green, 1975; Yaeger *et al*, 1991).

More recently, however, it has been demonstrated that fibroblast-mediated keratinocyte growth stimulation does operate via diffusible factors and that their expression is specifically induced by co-cultured keratinocytes (Smola *et al*, 1993; Fusenig, 1994). Likewise, increased interleukin-6 (IL-6) concentrations have been detected in the conditioned medium of keratinocyte-fibroblast co-cultures as compared with monocultured cells (Waelti *et al*, 1992). Furthermore, stimulation of keratinocyte growth factor (KGF) and IL-6 mRNA expression was observed in fibroblast feeder cells induced by co-cultured keratinocytes, and IL-1 has been postulated as one possible inducer (Smola *et al*, 1993).

IL-1 is a potent mediator for stimulating growth factor expression in human dermal fibroblasts, and the induction of IL-1β (Kumar *et al*, 1992), IL-6 (Boxman *et al*, 1993), IL-8 (Schröder *et al*, 1990), hepatocyte growth factor (HGF) (Matsumoto *et al*, 1992), and KGF (Chedid *et al*, 1994) has been demonstrated in cultured fibroblasts. IL-1β is able to induce KGF (Brauchle *et al*, 1994) and IL-8 (Schröder *et al*, 1990) in human fibroblasts as well. IL-1 is present in normal human epidermis and is released in large amounts after injury, and likewise affects proliferation and chemotaxis of mesenchymal cells, as demonstrated *in vitro* (Schröder, 1995). In particular, it has been shown that IL-1, secreted by keratinocytes, is

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Abbreviations: HGF, hepatocyte growth factor; IL-1RI, interleukin-1 receptor type I; KGF, keratinocyte growth factor.

responsible for the increased IL-6 and IL-8 production by proliferating mesenchymal cells (Boxman *et al*, 1993; Morita *et al*, 1993).

It was not clear how the IL-1 response of rapidly proliferating fibroblasts, demonstrated in conventional culture, reflected the interactions *in situ*, where dermal fibroblasts are mostly postmitotic. More *in vivo*–like conditions are obtained in 3-dimensional organotypic cultures, in which dermal fibroblasts are embedded in a collagen matrix on which skin keratinocytes are cultured with their uppermost layers exposed to the air (Bell *et al*, 1979; Mackenzie and Fusenig, 1983; Asselineau *et al*, 1986). Under these conditions, keratinocytes produce a well-differentiated epithelium that resembles normal epidermis, while being dystrophic in the absence of fibroblasts (Smola *et al*, 1994). It was unclear whether in these organotypic co-culture systems, similar mechanisms are operating as in feeder-layer cultures. In particular, it was not known whether inducers, such as IL-1 produced by keratinocytes, would have similar effects on fibroblasts regardless of their proliferative activity and the presence of extracellular matrix.

In this study, we examined the modulation of constitutive and IL-1α– as well as IL-1β–stimulated growth factor expression by irradiated postmitotic fibroblasts (feeder cells) and fibroblasts embedded in collagen gels in comparison with proliferating fibroblasts in monolayer cultures. This was meant to determine whether irreversibly postmitotic fibroblasts in the 2-dimensional co-culture system or resting fibroblasts embedded in a 3-dimensional collagen gel was a more suitable model to mimic the mesenchymal reaction to signals transduced by keratinocytes in skin. Because of the low expression level of growth factor and cytokine genes, reverse transcriptase polymerase chain reaction (PCR) was used to determine RNA expression of several cytokine genes that are considered to be major contributors in the mesenchymal-epithelial interplay. These expression data were confirmed by determining concentrations of secreted growth factors in the culture medium.

MATERIALS AND METHODS

Cell Culture Normal human dermal fibroblasts were derived from explant cultures of human adult skin obtained at surgery, as described previously (Smola *et al*, 1993). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Bio Whittaker, Serva, Heidelberg, Germany) supplemented with 10% fetal bovine serum, and pre-confluent cultures between passages 5 and 8 were used in these studies. For preparing feeder cells, trypsinized single-cell suspensions (0.1% trypsin/0.05% ethylenediamine tetraacetic acid) were x-irradiated with 70 Gy, plated at 2.8×10^4 cells/cm^2, and grown in FAD medium (DMEM:Ham's F12, 3:1) with 100 U penicillin per ml and 50 μg streptomycin per ml, and supplemented with 5% fetal bovine serum, 5 μg insulin per ml, 1 ng epidermal growth factor per ml, 10^{-10} M cholera toxin, and 24 ng adenine per ml (Sigma, Deideshofen, Germany). Collagen type I gels were prepared from rat tail tendons, as described previously (Smola *et al*, 1993). Ten milliliters of the collagen gel solution (final collagen concentration of 3.2 mg per ml) containing 3×10^5 cells per ml was poured into a 10-cm dish and covered with DMEM supplemented with 10% fetal bovine serum.

Induction Assay Normal human fibroblasts were seeded at 2×10^4 cells/cm^2 on tissue culture dishes or at 3×10^5 cells per ml into collagen gels in DMEM with 10% fetal bovine serum. Irradiated fibroblasts were plated at 2.8×10^4 cells/cm^2 on tissue culture dishes or at 3×10^5 cells per ml into collagen gels in FAD with supplements. Medium was changed after 24 h. After 48 h in culture, cells were washed once with serum-free medium, and serum-free medium was added for another 24 h. Thereafter, fresh serum- and growth factor–free medium with or without recombinant human IL-1α (Biozol, Eching, Germany) or IL-1β (Sigma) was added, and the cultures were incubated at 37°C for periods from 1 h to 24 h. Where indicated, 1 μg actinomycin per ml was added to cultures to block RNA synthesis.

Isolation of RNA At indicated time points, monolayers or collagen gels were washed once with cold phosphate-buffered saline. Cells were lysed in guanidinium thiocyanate solution, and total RNA was prepared according to Chomczynski and Sacchi (1987). The concentration and purity of total RNA were determined by optical density at 260 and 280 nm and electrophoretically by separation in a denaturing agarose gel (1%, Seakem; Biozym, Oldendorf, Germany).

Reverse Transcriptase PCR Primer sequences were chosen from separate exons of the studied genes so that products from cDNA could be distinguished from those derived from any contaminating genomic DNA **(Table I)**.

Table I. Primer Sets Used for PCR to Determine Growth Factor Gene Expression[a]

Factor	Amplified Region	Ann. Temp.	Size (bp)	Reference
KGF	446–1027	62°C	582	Finch *et al*, 1989
HGF	1110–1287	64°C	177	Nakamura *et al*, 1989
IL-1α	84–504	62°C	421	March *et al*, 1985
IL-1β	174–504	62°C	391	Nishida *et al*, 1987
IL-1RI	605–1090	52°C	486	Sims *et al*, 1989
IL-6	51–678	64°C	628	Wong *et al*, 1988
IL-8	1584–1845	64°C	272	Mukaida *et al*, 1989
TGF-β1	1769–2013	62°C	245	Derynck *et al*, 1985
GAPDH	69–308	62°C	240	Tokumaga *et al*, 1987

[a] Positions of amplified regions of published sequences, annealing temperature (ann. temp.) of the primer set, and size of the PCR product are given.

Reverse transcriptase PCR was performed according to an established method (Singer-Sam *et al*, 1990) with some modifications. Synthesis of cDNA was carried out at 42°C for 80 min in 100 μl final volume containing 10 μg total RNA, 10 μl 10x PCR buffer, 20 μl 25 mM $MgCl_2$, 24 μl dNTP mix (1.25 mM each), 2.5 μl RNasin (20 U per ml), 5 μl 50-U/μl reverse transcriptase, 2 μl 50-μM oligo dT_{15}, and 2 μl 50-μM random hexamers (GeneAmp-PCR-Kit; Perkin-Elmer, Weiterstadt, Germany). Comparative studies demonstrated that the use of both oligo dT and random primers guaranteed a representative amplification of cDNA regions with different distances to the polyA tail. Four microliters of first-strand cDNA was added to the PCR mix up to a volume of 50 μl, following the product description. The mixture was transferred to a thermal cycler (Biometra, Göttingen, Germany) and amplified with settings at 94°C for 1 min, at the indicated annealing temperature **(Table I)** for 1 min, and at 72°C for 1 min, performing 24-30 cycles. The number of cycles was chosen individually for each primer pair, thus ensuring that results for both internal standard and test cytokines lay within the exponential phase of the detection curve. Once optimized, the PCR conditions were kept constant for each product throughout the different experiments. PCR fragments were separated and stained by ethidium bromide on 1.5% agarose gels (Seakem; Biozym), identified by running position on the gel, and further characterized by digestion with two different restriction enzymes.

Determination of PCR Products and Calculation of mRNA Amount Quantitative determination of the PCR products was performed by computerized image analysis of the stained bands (EASY plus; Herolab, Wiesloch, Germany). Photographs of ethidium bromide–stained PCR products were saved as digital images at the highest possible resolution of the camera. The image intensity was calibrated from black (0) to white (255) on a gray scale of 256 channels per pixel. The size and intensity of each band were assessed, and the results were integrated as follows: Relative amount of PCR products = band area × band specific intensity. The amount of DNA in each band was determined based on a DNA standard of similar size and of known concentration run on the same gel. Considering product length, cycle number, and amplification efficiency, the original amount of mRNA in the sample was calculated as described previously (Wiesner, 1992). Values are presented as means and SDs of duplicate reverse transcriptase PCR analyses of two different culture assays.

Protein Determination Fibroblasts cultured under the different conditions as described were stimulated with 20 ng IL-1α per ml (PeproTech, Rocky Hill, NJ), and protein of selected cytokines was determined by enzyme-linked immunosorbent assays (ELISA) in aliquots of culture medium collected after 6 h, 10 h, and 24 h. The IL-8 ELISA was purchased from Endogen (Biozol, Eching, Germany), IL-1β ELISA from Predicta (Genzyme, Rüsselsheim, Germany), and HGF ELISA from R&D systems (Wiesbaden, Germany). Protein data were measured as pg per ml supernatant and given as pg/10^6 cells, with the SD from duplicate measurements of two independent experiments.

RESULTS

Growth factor gene expression was analyzed in proliferating normal and x-irradiated human dermal fibroblasts growing on plastic dishes as well as in fibroblasts seeded in type I collagen gels. Cells were derived from different individuals and used at comparable passage levels (5-8). After plating, cells were cultured for 2 d in serum-

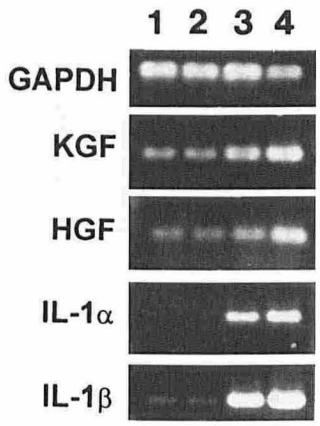

Figure 1. Concentration-dependent induction of growth factor mRNA expression by IL-1α. Untreated control *(lane 1)* and cells stimulated for 4 h with recombinant human IL-1α at 0.2 ng per ml *(lane 2)*, 2 ng per ml *(lane 3)*, or 20 ng per ml *(lane 4)* in serum-free medium. Total RNA was extracted, and the mRNA levels for GAPDH, KGF, HGF, IL-1α, and IL-1β were determined by reverse transcriptase PCR as described in *Materials and Methods* and visualized on ethidium bromide–stained agarose gels.

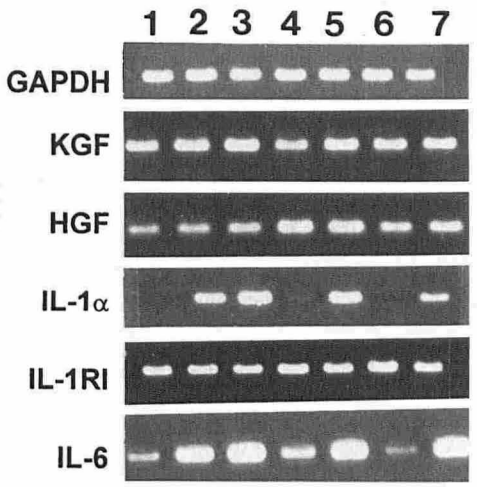

Figure 2. IL-1β–induced growth factor gene expression in proliferating and resting fibroblasts. Proliferating human dermal fibroblasts (HDF) on plastic and normal (HDF_{col}) as well as irradiated (HDF_{icol}) fibroblasts embedded in a collagen matrix were stimulated for 4 h with recombinant human IL-1β in serum-free medium. *Lane 1*, untreated HDF on plastic; *lane 2*, HDF treated with 5 ng IL-1β per ml; *lane 3*, HDF treated with 10 ng IL-1β per ml; *lane 4*, untreated HDF_{col}; *lane 5*, HDF_{col} treated with 10 ng IL-1β per ml; *lane 6*, untreated HDF_{icol}; *lane 7*, HDF_{icol} treated with 10 ng IL-1β per ml. Reverse transcriptase PCR products for KGF, HGF, IL-1α, IL-1RI, and IL-6 were visualized on ethidium bromide–stained agarose gels and compared with the expression of GAPDH.

containing and for 24 h in serum-free culture medium. Subsequently they were treated with different concentrations (0.2 ng per ml to 20 ng per ml) of recombinant human IL-1α and IL-1β, respectively. At this time, untreated fibroblasts had formed rapidly proliferating subconfluent cultures, whereas the collagen-embedded cells exhibited a reduced growth rate, in that cell numbers had increased only slightly compared with the initial seeding density. Fibroblasts irradiated by 70 Gy had formed monolayers of extremely large postmitotic cells. When embedded in the collagen matrix, they exhibited stellate morphology like that of proliferating fibroblasts, whereas cell numbers after 3 d were about 90% of the seeding density. Irradiated fibroblasts were analyzed in FAD, the medium used in keratinocyte-feeder cell co-cultures, but comparable values of mRNA expression were obtained using DMEM, the medium used for the other assays (data not shown).

Induction of Cytokine mRNA by IL-1α and IL-1β After treatment with IL-1α or IL-1β, a time- and concentration-dependent induction of growth factor and cytokine mRNA was detected by reverse transcriptase PCR in all cultures regardless of their proliferative states. With IL-1α, the concentration-dependent stimulation was particularly evident after 4 h, i.e., the time of maximal expression for most factors, as demonstrated in proliferating fibroblasts on plastic **(Figs 1, 3)**. Whereas the expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as the internal control for RNA expression, remained largely unchanged, the expression levels of KGF, HGF, IL-1α, and IL-1β were clearly stimulated with 2 and 20 ng per ml. Because 20 ng of IL-1α per ml gave positive and consistent results for all factors, we used this concentration for analyzing the response kinetics of fibroblasts under the different culture conditions. Higher concentrations of IL-1α (40 and 80 ng per ml) did not further increase, but often lowered, the effect (data not shown).

IL-1β exhibited a different specificity of stimulating cytokine expression, regardless of the culture and growth conditions, with consistent induction at a concentration of 10 ng per ml **(Fig 2)**. The expression levels of IL-1α and IL-6 were clearly enhanced at a comparable rate in proliferating fibroblasts on plastic as well as in fibroblasts embedded in collagen matrix, regardless of x-irradiation. On the other hand, KGF, HGF, and IL-1 receptor type I (IL-1RI) mRNA expression was essentially unchanged. There was an increased expression of HGF observed in collagen-embedded cells, which was, however, not regulated by IL-1β. GAPDH expression was unchanged whether cells were treated with IL-1β or not and whether they were proliferating, resting (collagen embedded), or postmitotic. This indicated that GAPDH mRNA expression was fairly constant under the different experimental conditions and that this rather stable marker was a reliable control for mRNA expression and equal RNA amounts in the individual reverse transcriptase assays.

Kinetics of Growth Factor Modulation by IL-1α To clarify the response of fibroblasts to IL-1 under different growth conditions, we determined the time kinetics of RNA expression of selected growth factors over a period of 24 h after treatment with 20 ng IL-1α per ml **(Fig 3)**. In irradiated fibroblasts, the constitutive levels of KGF mRNA were lower than those in proliferating cells, but the kinetics of induction were similar, exhibiting maximal stimulation after 4 h **(Fig 3*a*)**. Although the basal KGF mRNA levels were reduced further in fibroblasts growing for 3 d in collagen type I gels, the kinetics and relative degrees of induction were comparable to the other two situations.

A time-dependent increase in HGF mRNA expression was observed in all fibroblast cultures, with a maximum at 4 h after IL-1α induction **(Fig 3*b*)**. Whereas the constitutive levels were highest in irradiated cells, the relative induction was strongest in proliferating cells and lower, but very similar, in irradiated and collagen-embedded fibroblasts. IL-1α mRNA expression was constitutively very low under all culture conditions, but showed drastic autostimulation in proliferating fibroblasts (about 30-fold), with a maximum after 4 h **(Fig 3*c*)**. A comparable profile, though at a lower level, was obtained with collagen-embedded fibroblasts, whereas the induction in postmitotic feeder cells was very low. In contrast to irradiated and collagen-embedded cells, constitutive IL-1RI expression was high in proliferating fibroblasts and was further induced by IL-1α **(Fig 3*d*)**. Furthermore, IL-1RI levels showed a different time course, remaining stimulated for up to 24 h. As shown by others, IL-8 production in proliferating fibroblasts is inducible by IL-1α (Schröder *et al*, 1990). Here we demonstrate that this occurs similarly under different culture and proliferation conditions. The basal IL-8 values were highest in proliferating fibroblasts, but the induction levels were similar in irradiated and

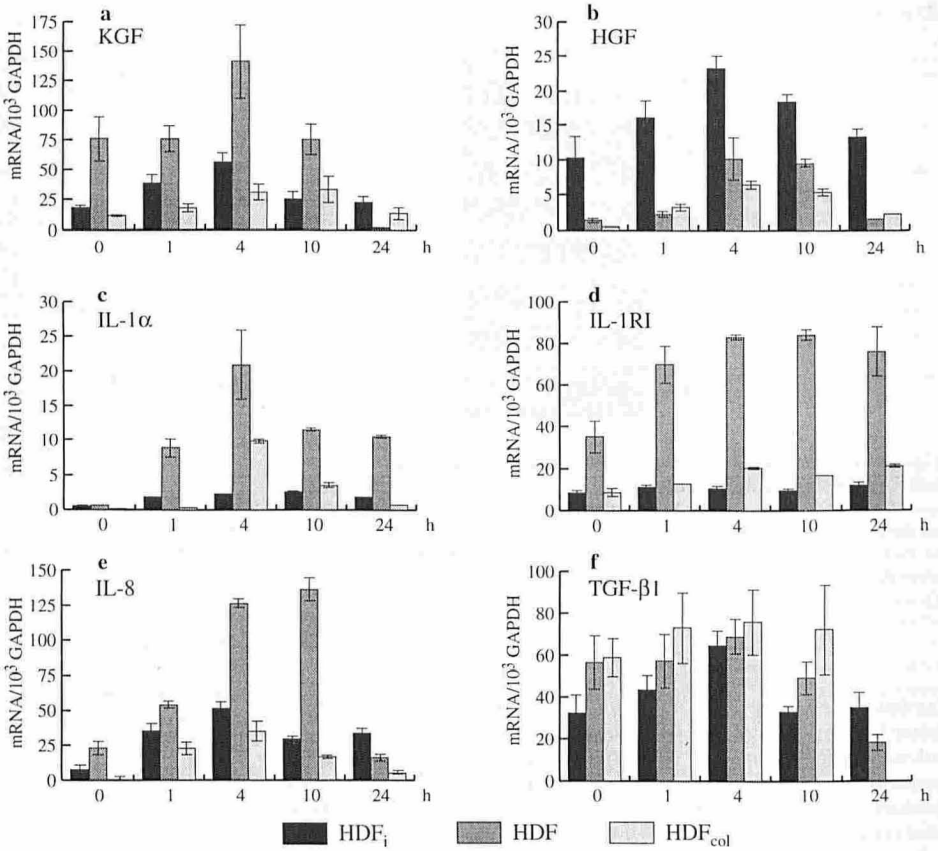

Figure 3. Time kinetics of IL-1α–induced growth factor gene expression in irradiated (HDF$_i$), proliferating (HDF), and collagen matrix–embedded (HDF$_{col}$) human dermal fibroblasts. Cells were stimulated with 20 ng recombinant human IL-1α per ml in serum-free medium. At 1, 4, 10, or 24 h later, the mRNA levels for KGF *(a)*, HGF *(b)*, IL-1α *(c)*, IL-1RI *(d)*, IL-8 *(e)*, and TGF-β1 *(f)* were calculated on the basis of band densities of the reverse transcriptase PCR products on ethidium bromide–stained agarose gels. Values were normalized against the expression of GAPDH and calculated as molecules of cytokine mRNA per 10^3 molecules of GAPDH mRNA. Data are expressed as the mean ± SD of four experiments.

collagen-embedded cells (about 10-fold) **(Fig 3*e*)**. Constitutive transforming growth factor (TGF)-β1 expression was relatively high and similar under all culture conditions, noticeably stimulated by IL-1α in irradiated cells only **(Fig 3*f*)**. A remarkable decline in TGF-β1 expression occurred under all conditions after 10 h, and even more so after 24 h.

To demonstrate that the IL-1α–induced elevated levels of cytokine mRNA were due to increased transcription and not to RNA stabilization, we blocked transcription by simultaneous treatment with 1 μg actinomycin D per ml **(Fig 4)**. This concentration was effective in blocking transcription without inducing cytotoxic effects in proliferating fibroblast cultures. Except for TGF-β1, which was only moderately affected, the expression of all other cytokines and IL-1RI was reduced to below 20% of the value induced by IL-1 in the absence of the inhibitor. Similar results were obtained with irradiated fibroblasts (data not shown), confirming that the elevated mRNA levels were due to enhanced transcription. Thus, our data clearly demonstrate that IL-1α is able to stimulate cytokine mRNA expression not only in rapidly proliferating dermal skin fibroblasts grown on plastic, but also in matrix-embedded resting fibroblasts and even in heavily irradiated postmitotic cells.

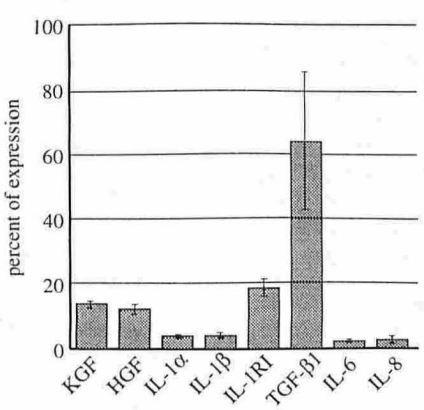

Figure 4. Actinomycin D inhibits IL-1 induction of growth factor gene expression. Subconfluent fibroblast cultures were treated with 20 ng IL-1α per ml and 1 μg actinomycin D per ml for 4 h. Cells were harvested, RNA was extracted, and growth factor mRNA levels were analyzed by reverse transcriptase PCR as described in *Materials and Methods* (see also **Fig 3**). Data are given as percent values of control cultures treated with IL-1α only (100%).

IL-1α–Induced Protein Secretion in Postmitotic Fibroblasts

To prove that the observed IL-1–induced elevated RNA levels of the cytokines analyzed were biologically relevant and reflected in higher protein levels, we determined the amounts of HGF, IL-8, and IL-1β in the conditioned medium by ELISA. Cultures of normal and irradiated fibroblasts were treated with 20 ng of IL-1α per ml, and medium was analyzed at 6 and 10 h after treatment.

HGF was produced and secreted by normal and irradiated fibroblasts in substantial amounts, with maximal values reached 6 h after stimulation **(Fig 5*a*)**. Although constitutive and stimulated mRNA levels were higher in irradiated fibroblasts, protein concentrations determined in the medium were always lower, but clearly demonstrated a significant increase, after IL-1 induction. On the other hand, the amount of IL-8 secreted into the medium was comparable in proliferating and postmitotic fibroblasts, both constitutively and at 6 and 10 h after IL-1α stimulation **(Fig 5*b*)**.

Another cytokine sensitive to IL-1α stimulation is IL-1β, which is also secreted by fibroblasts after stimulation (Kumar *et al*, 1992). Although the endogenous mRNA levels were similarly low in proliferating and postmitotic fibroblasts, IL-1β expression was upregulated by IL-1α in proliferating cells only **(Fig 6*a*)**. In both proliferating and irradiated cells, the constitutive medium levels

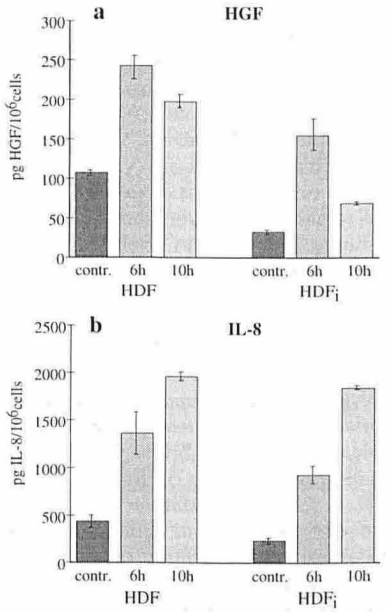

Figure 5. IL-1α induction of cytokine production in proliferating human dermal fibroblasts (HDF) and irradiated (HDF_i) fibroblasts on plastic. Subconfluent fibroblast cultures were treated with 20 ng IL-1α per ml in serum-free medium, and aliquots of medium were analyzed for HGF *(a)* or IL-8 *(b)* secretion at 6 h and 10 h after treatment. Data determined by ELISA are calculated as pg protein per 10^6 cells and given as mean ± SD of two experiments.

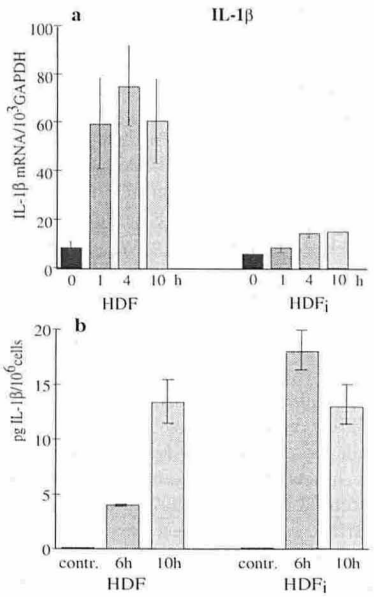

Figure 6. Protein and mRNA induction of IL-1β by IL-1α in proliferating and postmitotic fibroblasts. Subconfluent normal human dermal fibroblasts (HDF) and irradiated (HDF_i) fibroblasts in serum-free medium were treated with 20 ng IL-1α per ml. RNA was analyzed by reverse transcriptase PCR at 1 h, 4 h, and 10 h after induction *(a)*. Protein production was measured by ELISA in medium aliquots 6 h and 10 h after induction *(b)* and was calculated as pg/10^6 cells. RNA data are expressed as mean ± SD of four experiments, and protein data of two experiments.

were barely measurable by ELISA. After IL-1 treatment, a higher and earlier stimulation was observed in irradiated fibroblasts, although normal cells reached a similar end point after 10 h **(Fig 6*b*)**. The discrepancy between mRNA and protein expression in irradiated cells may be due to the upregulation of convertase activity after DNA damage (Brugarolas *et al*, 1995; Vasilakos *et al*, 1995).

Thus, although each of the selected factors exhibited individual levels of constitutive and induced secretion, in all cases a substantial induction was apparent in both proliferating and irreversibly postmitotic (irradiated) fibroblasts, as well as in resting fibroblasts embedded in collagen gel (not shown).

DISCUSSION

The growth-promoting effect on keratinocytes of co-cultured fibroblasts has been known for some time, and diffusible growth factors as well as cell-cell contact mechanisms have been postulated to be involved in this effect (Limat *et al*, 1989; Yaeger *et al*, 1991; Fusenig, 1994; Smola *et al*, 1994). Recently we were able to demonstrate that fibroblast-keratinocyte interactions occur via diffusible factors and that expression of growth factors in fibroblasts is stimulated by keratinocyte-produced inducers (Smola *et al*, 1993). We had postulated that IL-1 is a possible candidate for such a keratinocyte messenger (Fusenig, 1994).

Here we add further evidence to this hypothesis in demonstrating that x-irradiated postmitotic (feeder) fibroblasts, used in the simplest version of co-cultures, indeed respond to IL-1 stimuli by enhanced RNA and protein expression. This clearly documents that these heavily irradiated feeder fibroblasts maintained an operative signaling system and a correct transcription, translation, and secretion mechanism of several growth factors and cytokines. They behaved similarly to growth-retarded fibroblasts embedded in collagen gels.

To measure both the usually low constitutive and the induced mRNA levels in resting and proliferating fibroblasts, we used the sensitive technique of reverse transcriptase PCR. The semiquantitative reverse transcriptase PCR method allows determination of relative differences in mRNA levels between different samples, as the result obtained after reverse transcription and PCR is related to an equal original RNA content per sample. The RNA used in each sample was standardized by measuring the expression of the housekeeping gene GAPDH, because the expression of GAPDH was not noticeably altered by culture conditions or the proliferation state. This furthermore revealed losses or degradation of RNA as well as inefficiencies during the reverse transcriptase reaction. Because of its minimal variations of activation, GAPDH was chosen as internal standard, and the resulting amplified cDNAs could be calculated in a semiquantitative manner (Murphy *et al*, 1993). In the PCR step, GAPDH and an experimental gene product are amplified in separate tubes containing aliquots of the same cDNA sample. By keeping the amount of cDNA input constant and the number of cycles of PCR in the linear range of amplification, signals obtained were linearly related to specific RNA levels, with adequate reproducibility for every experiment (Singer-Sam *et al*, 1990). Analysis of parameters in the quantitative reverse transcriptase PCR, with specific internal standards, clearly showed that the relative and quantitative reverse transcriptase PCR assays yielded comparable results (Chen and Klebe, 1993). Although the method used here does not allow determination of quantitative absolute mRNA values, it does indicate major relative changes occurring in cells under different culture conditions and proliferation states, and after induction, with sufficient accuracy and good reproducibility.

The most important information in this study is that feeder fibroblasts, often named "lethally irradiated cells," are not only capable of maintaining a basal level of metabolic activity (Van der Schans *et al*, 1983), but also can still respond to specific stimuli by upregulating growth factor mRNA expression. This further indicates that DNA damage (predominantly single-strand breaks) caused by high-dose (70 Gy) x-ray irradiation was sufficient for blocking DNA replication and cell division but did not prevent RNA transcription. Whether the reduced levels of baseline expression of most factors in irradiated cells are a consequence of radiation

damage or are related to the postmitotic state of cells cannot be answered by these data. The comparably low constitutive levels of cytokine RNAs in untreated fibroblasts embedded for 3 d in collagen gels, a condition in which proliferation is reduced (Coulomb *et al*, 1989; Kono *et al*, 1990), suggest that such low levels are characteristic of resting fibroblasts. Moreover, the responses of both irradiated and collagen-embedded fibroblasts to IL-1 stimulation are comparable and show kinetics similar to those in proliferating fibroblasts. This demonstrates that under all these conditions, fibroblasts specifically respond to an external signal by upregulating cytokine expression.

In addition, protein determination of selected cytokines revealed a similar response in both irradiated and proliferating fibroblasts. Moreover, in both cases the amount of protein determined in the culture supernatant was similar in irradiated cells.

Whereas the protein concentrations of IL-8 roughly paralleled the RNA data, this was not the case for HGF. For HGF, secretion by irradiated cells was clearly lower than in normal fibroblasts, whereas mRNA levels were higher. In general, the data clearly indicate that irradiated cells had maintained their potential not only to provide constitutively produced growth factors, but also to respond in a typical way to exogenous stimulation by protein synthesis and secretion, comparable to proliferating cells.

It has already been shown that adult human skin fibroblasts express increased amounts of growth factors after stimulation with IL-1α, e.g., IL-1β (Kumar *et al*, 1992), IL-6 (Boxman *et al*, 1993), IL-8 (Schröder *et al*, 1990), HGF (Matsumoto *et al*, 1992), and KGF (Chedid *et al*, 1994). We could confirm the induction of KGF, HGF, IL-1α and -β, and IL-6 and -8, by IL-1α as well as IL-1β in proliferating fibroblasts. Furthermore, we observed increased expression of these growth factors not only in irradiated, but also in matrix-embedded cells after stimulation with IL-1. Elevation of IL-1α to 40 ng per ml and 80 ng per ml further enhanced some growth factor expression levels in collagen-embedded fibroblasts, in contrast to monolayer cells, in which the levels were decreasing at these doses (data not shown). This may indicate reduced IL-1 diffusion, increased absorption to collagen, or an altered response of fibroblasts embedded in extracellular matrix.

Pronounced synthesis of IL-6 in co-cultures of keratinocytes with postmitotic feeder cells had been reported, and putatively originated in the feeder cells (Waelti *et al*, 1992). We confirmed here the induction of IL-6 and in addition IL-8 mRNA expression, regardless of the physical condition of the cells. IL-6 mRNA expression was enhanced even in irradiated fibroblasts, comparable to that found in proliferating cells (data not shown). We could prove that IL-6 expression in such feeder-layer co-cultures occurs in irradiated fibroblasts, most probably via IL-1 released by keratinocytes.

Expression of TGF-β1 and IL-1RI in fibroblasts seemed to remain unaffected by IL-1 under all culture conditions. It has been reported that the level of IL-IRI expression can regulate the responsiveness of cells to exogenous IL-1 (Groves *et al*, 1994). Considering that the IL-1RI promotor resembles that of housekeeping genes (Kupper and Groves, 1995), a rapid and strong change in IL-1RI gene expression was not anticipated.

From these data, we conclude that under both keratinocyte-fibroblast co-culture conditions, i.e., in 2-dimensional (feeder layer) and organotypic cultures, the observed growth-stimulatory effect on keratinocytes by co-cultured fibroblasts is due to the upregulated production of several paracrine-acting growth factors and not primarily by constitutive baseline expression in fibroblasts, as suggested in previous studies (Smola *et al*, 1993, 1994). It is well known that keratinocytes produce IL-1α upon irritation in skin (Kupper and Groves, 1995; Luger and Schwarz, 1995) and also in culture (Boxman *et al*, 1993). We provide further evidence herein for the earlier assumption that IL-1α is one possible mediator by which keratinocytes induce the expression of several growth factors in co-cultured fibroblasts, such as KGF, HGF, and IL-1 itself, which in turn stimulate keratinocyte growth (Matsumoto *et al*, 1992; Chedid *et al*, 1994; Kupper and Groves, 1995). This stimulatory effect on keratinocyte proliferation is most probably the result of the combined action of several factors with known growth-enhancing activity on keratinocytes. The higher RNA expression level of HGF in postmitotic cells, initially interpreted as the major growth-stimulatory component in feeder cells, could be partly due to the FAD medium containing cholera toxin, a component that has been shown recently to stimulate HGF mRNA synthesis (Matsunaga *et al*, 1994). Indeed, constitutive HGF mRNA levels of irradiated fibroblasts cultured in DMEM were only 50% of those in FAD medium. Accordingly, HGF protein levels in the culture medium of irradiated cells were lower than those of proliferating cells.

In general, these data strongly indicate that "lethal irradiation" does not kill feeder cells, but rather induces terminal differentiation with irreversible growth arrest but largely preserved physiologic functions (Bumann *et al*, 1995). The overall similarity of constitutive cytokine expression and IL-1α response between irradiated and collagen-embedded fibroblasts further indicates that proliferation-inhibited fibroblasts are comparably regulated. Although the response to cytokine stimulation, in contrast to constitutive levels of cytokine mRNA, is similar in proliferating fibroblasts and resting cells, the relevance of conventional, rapidly proliferating fibroblast cultures as a model for the mostly nonproliferating fibroblasts in the dermis may be questioned. As demonstrated earlier for the production and metabolism of extracellular matrix (Eckes *et al*, 1992), fibroblasts in collagen gels more closely resemble dermal "fibrocytes" than the rapidly proliferating fibroblasts in conventional monolayer cultures. The data presented here suggest that the irradiation-induced postmitotic "feeder" fibroblasts may represent a similar model system for resting dermal fibrocytes to study their reaction on keratinocyte-released signal peptides.

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REFERENCES

Asselineau D, Bernard BA, Bailly C, Darmaon M, Prunières M: Human epidermis reconstructed by culture: is it normal? *J Invest Dermatol* 86:181–186, 1986

Bell E, Ivarson B, Merrill C: Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential *in vitro*. *Proc Natl Acad Sci USA* 76:1274–1278, 1979

Boukamp P, Breitkreutz D, Stark HJ, Fusenig NE: Mesenchyme-mediated and endogenous regulation of growth and differentiation of human skin keratinocytes derived from different body sites. *Differentiation* 44:150–161, 1990

Boxman I, Löwik C, Aarden L, Ponec M: Modulation of IL-6 production and IL-1 activity by keratinocyte-fibroblast interaction. *J Invest Dermatol* 101:316–324, 1993

Brauchle M, Angermeyer K, Werner S: Large induction of keratinocyte growth factor expression by serum growth factors and pro-inflammatory cytokines in cultured fibroblasts. *Oncogene* 9:3199–3204, 1994

Brugarolas J, Chandrasekara C, Gordon JI, Beach D, Jacks T, Hannon GJ: Radiation-induced cell cycle arrest compromised by p21 deficiency. *Nature* 377:552–557, 1995

Bumann J, Santo-Holtje L, Loffler H, Bamberg M, Rodemann HP: Radiation-induced alterations of the proliferation dynamics of human skin fibroblasts after repeated irradiation in the subtherapeutic dose range. *Strahlenther Onkol* 171:35–41, 1995

Chedid M, Rubin JS, Csaky KG, Aaronson SA: Regulation of keratinocyte growth factor gene expression by interleukin 1. *J Biol Chem* 269:10753–10757, 1994

Chen D, Klebe RJ: Controls for validation of relative reverse transcription-polymerase chain reaction. *PCR Meth Applic* 3:127–129, 1993

Chomczynski P, Sacchi N: Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987

Coulomb B, Lebreton C, Dubret L: Influence of human dermal fibroblasts on epidermalization. *J Invest Dermatol* 92:122–125, 1989

Derynck R, Jarrett JA, Chen EY, Eaton DH: Human transforming growth factor beta complementary DNA sequence and expression in normal and transformed cells. *Nature* 316:701–704, 1985

Eckes B, Hunzelmann N, Ziegler-Heidbrock HWL, Urbanski A, Luger T, Krieg T, Mauch C: Interleukin-6 expression by fibroblasts grown in 3-dimensional gel cultures. *FEBS Lett* 298:229–232, 1992

Finch PW, Rubin JS, Miki T, Ron D, Aaronson SA: Human KGF is FGF-related with properties of a paracrine effector of epithelial cell growth. *Science* 245:752–755, 1989

Fusenig NE: Epithelial-mesenchymal interactions regulate keratinocyte growth and

differentiation *in vitro*. In: Leigh I, Lane B, Watt F (eds.). *The Keratinocyte Handbook*. Cambridge University Press, Cambridge, UK, 1994, pp 71–94

Groves RW, Sherman L, Mizutani H, Dower SK, Kupper TS: Detection of interleukin-1 receptors in human epidermis. *Am J Pathol* 145:1048–1059, 1994

Kono T, Tanii T, Furukawa M, Mizuno N, Kitajima J, Ishii M, Hamada T, Yoshizato K: Cell cycle analysis of human dermal fibroblasts cultured on or in hydrated type I collagen lattices. *Arch Dermatol Res* 282:258–262, 1990

Kumar S, Millis AJT, Baglioni C: Expression of interleukin 1-inducible genes and production of interleukin 1 by aging human fibroblasts. *Proc Natl Acad Sci USA* 89:4683–4687, 1992

Kupper TS, Groves RW: The interleukin-1 axis in cutaneous inflammation. *J Invest Dermatol* 105:62S–66S, 1995

Limat A, Hunziker T, Boillat C, Bayreuther K, Noser F: Post-mitotic human dermal fibroblasts efficiently support the growth of human follicular keratinocytes. *J Invest Dermatol* 92:758–762, 1989

Luger TA, Schwarz T: The role of cytokines and neuroendocrine hormones in cutaneous immunity and inflammation. *Allergy* 50:292–302, 1995

Mackenzie IC, Fusenig NE: Regeneration of organized epithelial structure. *J Invest Dermatol* 81:189S–194S, 1983

March CJ, Mosley B, Larsen A, Cerretti DP, Braedt G, Price V, Gillis S, Henney CS: Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. *Nature* 315:641–646, 1985

Matsumoto K, Okazaki H, Nakamura T: Up-regulation of hepatocyte growth factor gene expression by interleukin-1 in human skin fibroblasts. *Biochem Biophys Res Commun* 188:235–243, 1992

Matsunaga T, Ghoda E, Takebe T, Wu L, Iwao M, Kataoka H, Yamamoto I: Expression of hepatocyte growth factor is up-regulated through activation of a cAMP-mediated pathway. *Exp Cell Res* 210:326–335, 1994

Morita E, Yamada S, Kimura I, Nakamura K, Sugita Y, Yamamoto S: Interleukin-8 production from cultured human dermal fibroblasts by stimulation with supernatants of cultured human epidermal cells. *Skin Pharmacol* 6:161–169, 1993

Mukaida N, Shiroo M, Matsushima K: Genomic structure of the monocyte-derived neutrophil chemotactic factor IL-8. *J Immunol* 143:1366–1371, 1989

Murphy E, Hieny S, Sher A, O'Garra A: Detection of *in vivo* expression of interleukin-10 using a semi-quantitative polymerase chain reaction method in Schistosoma mansoni infected mice. *J Immunol Methods* 162:211–223, 1993

Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, Sugimura A, Tashiro K, Shimizu S: Molecular cloning and expression of human hepatocyte growth factor. *Nature* 342:440–443, 1989

Nishida T, Nishino N, Takano M, Kawai K, Bando K, Masui Y, Hirai Y: cDNA cloning of IL-1alpha and IL-1beta from mRNA of U937 cell line. *Biochem Biophys Res Commun* 143:345–352, 1987

Rheinwald JG, Green H: Feeder layer system: serial cultivation of stains of human epidermal keratinocytes. *Cell* 6:331–344, 1975

Schröder JM: Cytokine networks in the skin. *J Invest Dermatol* 105:20S–24S, 1995

Schröder JM, Sticherling M, Henneicke HH, Preissner WC, Christoffers E: IL-1 or tumor necrosis factor stimulate release of three NAP-1/IL-8-related neutrophil chemotactic proteins in human dermal fibroblasts. *J Immunol* 144:2223–2232, 1990

Sims JE, Acres RB, Grubin CE, McMahan CJ: Cloning the interleukin 1 receptor from human T cells. *Proc Natl Acad Sci USA* 86:8946–8950, 1989

Singer-Sam J, Robinson MO, Bellvé AR, Simon MI, Riggs AD: Measurement by quantitative PCR of changes in HPRT, PGK-1, PGK-2, APRT, MTase and Zfy gene transcripts during mouse spermatogenesis. *Nucleic Acids Res* 18:1255–1259, 1990

Smola H, Thiekötter G, Baur M, Stark HJ, Breitkreutz D, Fusenig NE: Organotypic and epidermal-dermal co-cultures of normal human keratinocytes and dermal cells: regulations of transforming growth factor alpha, beta1 and beta2 mRNA levels. *Toxic In Vitro* 8:641–650, 1994

Smola H, Thiekötter G, Fusenig NE: Mutual induction of growth factor gene expression by epidermal-dermal cell interaction. *J Cell Biol* 122:417–429, 1993

Tokunaga K, Nakamura Y, Sakata K, Fujimori K, Ohkubo M, Sawada K, Sakiyama S: Enhanced expression of a glyceraldehyde-3-phosphate dehydrogenase gene in human lung cancers. *Cancer Res* 47:5616–5619, 1987

Van der Schans GP, Paterson MC, Cross WG: DNA strand break and rejoining in cultured human fibroblasts exposed to fast neutrons or gamma rays. *Int J Radiat Biol* 44:75–85, 1983

Vasilakos JP, Ghayur T, Carroll RT, Giegel DA, Saunders JM, Quintal L, Keane KM, Shivers BD: IL-1beta converting enzyme is not required for apoptosis induced by lymphokine deprivation. *J Immunol* 155:3433–3442, 1995

Waelti ER, Inaebnit SP, Rast HP, Hunziker T, Limat A, Braathen L, Wiesmann U: Co-culture of human keratinocytes on post-mitotic human dermal fibroblast feeder cells: production of large amounts of interleukin 6. *J Invest Dermatol* 98:805–808, 1992

Wiesner RJ: Direct quantification of picomolar concentrations of mRNAs by mathematical analysis of a reveRse transcription/exponential polymerase chain reaction assay. *Nucleic Acids Res* 20:5863–5864, 1992

Wong G, Witek-Giannotti J, Hewick R, Clark S, Ogawa M: Interleukin 6: identification as a hematopoietic colony-stimulating factor. *Behring Inst Mitt* 83:40–47, 1988

Yaeger PC, Stiles CD, Rollins BJ: Human keratinocyte growth-promoting activity on the surface of fibroblasts. *J Cell Physiol* 149:110–116, 1991