INTRODUCTION

Epithelial-mesenchymal interactions control epidermal growth and differentiation and regulate tissue homeostasis in the epidermis as well as in other organs (for review see Alarid et al., 1994; Fusenig, 1994; Mackenzie, 1994). This interplay is based on three basic processes: production of soluble factors displaying autocrine and paracrine activities (Luger and Schwarz, 1990; Smola et al., 1993), cell-matrix interactions (Gailit and Clark, 1994; Ashkenas et al., 1996) and signalling by direct cell-cell contact (Waelti et al., 1992; Jahaverian et al., 1998). In skin, epidermal keratinocytes are separated from dermal mesenchymal cells by a complex extracellular matrix, the basement membrane, thus rendering cell-cell contact-mediated mechanisms less probable.

Epidermal keratinocytes are known to produce a series of soluble proteins in vivo and in vitro which modulate both their own growth and that of other cell types within the epidermis and dermis (Luger and Schwarz, 1990, 1996; Schröder, 1995). Most of these cytokines have been primarily interpreted as mediators of inflammatory and/or immunomodulatory reactions (Luger and Schwarz, 1995). Their functional significance in the dermal-epidermal interplay to regulate tissue repair and homeostasis is only poorly understood (Werner et al., 1992; Appleton, 1994; Gailit and Clark, 1994; Luger and Schwarz, 1996).

Based on earlier studies demonstrating a dynamic and reciprocal modulation of cytokine and growth factor expression in keratinocytes and fibroblasts in vitro (Smola et al., 1993, 1994), we postulated a double paracrine mechanism of keratinocyte growth regulation. In this study we wanted to provide evidence for the existence of such a mechanism and to substantiate its functional significance. In the first step we used the simplest version of coculture models of fibroblasts and keratinocytes, the feeder-layer system, where keratinocytes are seeded together with fibroblasts (feeder cells) on plastic dishes and grown submerged in culture medium. In order not to overgrow the keratinocytes, the mesenchymal feeder cells had to be converted into an irreversible postmitotic state, usually achieved by high-dose gamma-irradiation or mitomycin-C treatment. In these cocultures, keratinocyte proliferation is strongly stimulated keratinocyte proliferation, such as KGF. The functional significance of this reciprocal modulation was substantiated by blocking experiments. Both IL-1α- and -1β-neutralizing antibodies and IL-1 receptor antagonist significantly reduced keratinocyte proliferation supposedly through abrogation of KGF production, because IL-1 antibodies blocked the induced KGF production. These data indicate a regulation of keratinocyte growth by a double paracrine mechanism through release of IL-1 which induces KGF in cocultured fibroblasts. Thus IL-1, in addition to its proinflammatory function in skin, may play an essential role in regulating tissue homeostasis.

Key words: Fibroblast, Keratinocyte, Growth regulation, Coculture, Cytokine

SUMMARY

Epithelial-mesenchymal interactions play an important role in regulating tissue homeostasis and repair. For skin, the regulatory mechanisms of epidermal-dermal interactions were studied in cocultures of normal human epidermal keratinocytes (NEK) and dermal fibroblasts (HDF) rendered postmitotic by gamma-irradiation (HDFi).

The expression kinetics of different cytokines and their receptors with presumed signalling function in skin were determined at the RNA and protein level in mono- and cocultured NEK and HDFi. In cocultured HDFi, mRNA and protein synthesis of keratinocyte growth factor (KGF) (FGF-7) was strongly enhanced, whereas in cocultured keratinocytes interleukin (IL)-1α and -1β mRNA expression increased compared to monocultures. Thus we postulated that IL-1, which had no effect on keratinocyte proliferation, induced in fibroblasts the expression of factors stimulating keratinocyte proliferation, such as KGF. The functional significance of this reciprocal modulation was substantiated by blocking experiments. Both IL-1α- and -1β-neutralizing antibodies and IL-1 receptor antagonist significantly reduced keratinocyte proliferation supposedly through abrogation of KGF production, because IL-1 antibodies blocked the induced KGF production. These data indicate a regulation of keratinocyte growth by a double paracrine mechanism through release of IL-1 which induces KGF in cocultured fibroblasts. Thus IL-1, in addition to its proinflammatory function in skin, may play an essential role in regulating tissue homeostasis.

Key words: Fibroblast, Keratinocyte, Growth regulation, Coculture, Cytokine
Previous data suggested that direct cell-cell contact is required to promote keratinocyte growth, because fibroblast-conditioned medium could not substitute for feeder cells (Rheinwald and Green, 1975; Yaeger et al., 1991 and own unpublished studies). Recently we have demonstrated that stimulation of keratinocyte growth in such feeder-layer cultures is actually mediated by diffusible factors produced by the cocultured mesenchymal cells (Smola et al., 1993, 1994). We further showed that the constitutive expression level of some growth factors in fibroblasts is significantly upregulated by cocultured keratinocytes. Based on these studies, we postulated that keratinocytes do not simply and passively profit from factors constitutively expressed in mesenchymal cells but actively induce the expression of growth factors in fibroblasts, such as keratinocyte growth factor (KGF), a member of the fibroblast growth factor family (FGF-7). Similarly, increased RNA and protein levels for IL-6 and other cytokines have been reported in keratinocyte-fibroblast cocultures (Waelti et al., 1992; Boxman et al., 1993). More recently we have shown that postmitotic fibroblasts, though irreversibly blocked in proliferating by X-irradiation, are still functionally active to constitutively express cytokines and, even more so, react to external stimuli by modulating their mRNA and protein expression (Maas-Szabowski and Fusenig, 1996).

Several growth factors and interleukins have been detected in skin as well as in keratinocyte and fibroblast cultures, such as IL-1, IL-6, IL-8, granulocyte macrophage colony stimulating factor (GM-CSF), transforming growth factor (TGF)-α and -β, NGF, PDGF as well as several members of the FGF family (for review see Luger and Schwarz, 1995; Kupper and Groves, 1995; Schröder, 1995). They are discussed as potent mediators of immune reactions and inflammatory processes (Luger and Schwarz, 1990, 1995) but also of skin repair. In particular KGF, a typical paracrine acting growth factor, is considered to be a major regulator of keratinocyte growth and differentiation as demonstrated in cell culture studies (Rubin et al., 1995; Marchese et al., 1995). Comparably, KGF has been identified as a major mesenchymal-derived growth factor mediating hormone-induced epithelial proliferation in the prostate (Alarid et al., 1994). In vivo KGF is strongly upregulated in dermal cells of wounded skin (Werner et al., 1992) and targeted KGF overexpression in keratinocytes leads to epidermal hyperplasia (Guo et al., 1993). KGF receptor dominant negative transgenic mice exhibit delayed wound healing (Brauchle et al., 1994), whereas the absence of KGF in knock-out mice resulted in no abnormalities in epidermal growth or wound healing (Guo et al., 1996). This apparent discrepancy may be explained by redundancy in keratinocyte growth regulation on one side and/or existence of other ligands for the KGF receptor (e.g. FGF-10 (Beer et al., 1997; Igarashi et al., 1998).

By means of the feeder-layer coculture system of human skin keratinocytes and postmitotic dermal fibroblasts we demonstrate here a major role and indirect regulation of KGF/FGF-7 as a regulator of keratinocyte growth in epithelial-mesenchymal cell interactions. The studies essentially prove our hypothesis that, by a double paracrine mechanism, keratinocytes actively modulate the expression of their own growth factors in adjacent fibroblasts. These controlled interactions may also be functional in vivo and allow a rapid induction of cell proliferation in repair processes of skin, e.g. after wounding, irritation, and inflammation.

**MATERIALS AND METHODS**

**Cell culture**

Normal human skin keratinocytes (NEK) and dermal fibroblasts (HDFi) were derived from adult skin obtained from surgery, as previously described (Smola et al., 1993). Fibroblasts, obtained from outgrowth of explant cultures, were grown in Dulbecco’s modified Eagle’s medium (DMEM; Bio Whittaker, Serva, Heidelberg, FRG) supplemented with 10% FCS and cells from passages 5 to 8 were used. For preparing fibroblast feeder cells (HDFi), trypsinized cell suspensions (0.05% trypsin/0.025% EDTA, v/v) were gamma-irradiated with 70 Gray, plated at 2.8×10⁴ cells/cm² and 1×10⁴ cells/cm² as mono- and cocultures, respectively, and grown in FAD medium (DMEM:HamsF12/3:1) with 100 U/ml penicillin and 50 µg/ml streptomycin and supplemented with 5% FCS, 5 µg/ml insulin, 1 ng/ml EGF, 10⁻¹⁰ M cholera toxin and 24 ng/ml adenine (Sigma, Deideshofen, FRG). NEK were obtained by trypsinization of split thickness skin samples, isolated from the separated epidermis and plated on X-irradiated feeder cells in FAD medium as described (Smola et al., 1993). Absence of fibroblast contamination was routinely checked in cloning assays on fibroblast feeder-layer and NEK from passage 2 to 3 were used for these studies. For cocultures, NEK were seeded at 1.1×10⁴ cells/cm² on dishes where HDFi had been pre-cultured for two days and further grown in FAD medium with supplements. NEK for monocultures were seeded in FAD medium at a higher density (4×10⁴ cells/cm²) due to lower attachment and growth rates when seeded alone on plastic (Boukamp et al., 1990). Growth curves were drawn up with cell numbers determined by electronic counting (Casy, Reutlingen, FRG) of trypsinized cell suspensions at the indicated time points.

**Isolation of RNA**

Cocultures were washed with cold PBS and HDFi selectively detached by brief (3-5 minutes) incubation in 0.05% EDTA followed by gentle pipetting. With this procedure keratinocyte colonies remain attached to the culture dish, while fibroblasts are quantitatively eliminated as demonstrated earlier (Smola et al., 1993). The detached HDFi were suspended in serum-containing medium and pelleted at 1000 g. The feeder-cell pellet and the keratinocyte colonies, respectively, as well as monocultures were lysed in guanidinium-isothiocyanate solution and total RNA was extracted according to the method of Chomczynski and Sacchi (1987). Concentration and purity of RNA were determined by optical density at 260 and 280 nm, and electrophoretically by separation in denaturing agarose gels (1%, Seakem; Biozym, Oldendorf, FRG).

**Reverse transcription and PCR**

 Primer sequences were chosen from separate exons of the studied genes thus allowing us to distinguish cDNA products from those of contaminating genomic DNA and amplified at the optimized annealing temperature as described by Maas-Szabowski and Fusenig (1996). The RT-PCR was performed according to an established method (Singer-Sam et al., 1990) with some modifications: the cDNA synthesis was carried out at 42°C for 80 minutes in 100 µl final volume containing 10 µg total RNA, 10 µl 10x PCR-buffer, 20 µl MgCl₂ (25 mM), 6 µl of each dNTP (10 mM), 2.5 µl RNasin (0.5 U/µl), 5 µl reverse transcriptase (50 U/µl), 2 µl oligo dT15 (50 µM) and 2 µl random hexamers (50 µM) (GeneAmp-PCR-Kit, Perkin Elmer, Weiterstadt, FRG). Four microliters of first-strand cDNA were added to the PCR-mix up to a volume of 50 µl following the product description. The mixture was transferred to a thermal cycler (Biometa, Göttingen, FRG) and amplified with settings at 94°C for 1 minute, at the indicated annealing temperature (see above) for 1 minute, and at 72°C for 1 minute, performing 24-30 cycles. The number of cycles used for amplification of each cDNA was carefully optimized for each primer pair so that the product yields for the tested cytokines were in the exponential range. PCR fragments were separated on 1.5% agarose gels (Seakem; Biozym, Oldendorf, FRG), ethidiumbromide-stained,
and identified by their running positions on the gel and, in addition, by restriction mapping with two different enzymes.

**Determination of PCR products and calculation of mRNA quantity**
Quantification of the PCR products was achieved by computerized image analysis of the stained bands (EASY plus; Herolab, Wiesloch, FRG; see also Maas-Szabowski and Fusseneg, 1996). Photographs of ethidium bromide-stained PCR products were saved as digital images at the highest possible resolution, calibrated from black (0) to white (255) on a grey scale of 256 channels per pixel. The size and intensity of each band was assessed and integrated calculating the relative amount of PCR products (band area × band-specific intensity). The actual amount of DNA in each band was determined by running DNA standards of similar size on the same gel. The initial amount of mRNA molecules in the sample was calculated by taking product length, cycle number, and amplification efficiency as described previously (Wiesner, 1992). Values are presented as means with standard deviations of duplicate RT-PCR analyses of at least two culture assays. The mRNA amount of the house-keeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) was used as internal standard.

**Protein determination by ELISA**
Protein quantities of selected cytokines were determined by enzyme-linked immuno-sorbent assays (ELISA) in aliquots of culture medium of NEK and HDF/HDFi mono- and cocultures. Medium was collected at day 2, 4, 6, 8, 10, i.e. always 48 hours after medium change, and the cell number was determined. ELISA kits for IL-1α were purchased from Endogen (Biozol, Eching, FRG) and for IL-1β from Predicta (Genzyme, Rüsselsheim, FRG). Protein values in the culture supernatant were measured as pg/ml and calculated as pg/10⁶ cells with standard deviations of data derived from duplicate measurements and parallel cell counts from two to three independent experiments.

**Protein determination by western blot**
To separate heparin binding factors (in particular KGF) 48 hour-conditioned culture media were gently swirled with heparin Sepharose beads (Sigma, München, FRG) overnight at 4°C. Beads were pelleted by centrifugation (5 minutes, 300 g), washed 3 times with 20 mM Tris-HCI, pH 7.2, 200 mM NaCl and proteins eluted by boiling for 5 minutes in sample buffer (100 mM Tris-HCI, pH 6.8, 20% glycerin, 4% SDS, 100 mM 2-mercaptoethanol, 0.1% Bromphenol Blue) and cleared by centrifugation. Aliquots of the protein sample were left unaltered or deglycosylated with N-glycosidase F (Boehringer, Mannheim, FRG). IL-1β was identified by immunoblotting cell extracts with a mouse monoclonal antibody (Calbiochem, München, FRG) recognizing Ki67 (Catoretti et al., 1992) was added and left overnight at 4°C). After washing in PBS, cultures were incubated for 24 hours to 72 hours, cells labeled with 10 µM bromodeoxyuridine (BrdU) for 24 hours, and BrdU incorporation was measured by a cell proliferation ELISA-kit (Boehringer, Mannheim, FRG). Data presented are means with standard deviations of duplicate measurements of at least two different culture assays.

**Functional blocking of cytokines**
HDF/HDFi cocultures were grown on glass slides in FAD medium with 5% FCS. After 24 hours, medium was replaced with FAD medium additionally containing either 1 µg/ml neutralizing antibodies against IL-1α or IL-1β (Genzyme, Rüsselsheim, FRG), 10 ng/ml IL-1-receptor antagonist (Genzyme, Rüsselsheim, FRG), or 1 µg/ml KGF neutralizing antibodies (R&D Systems, Wiesbaden, FRG). These antibody concentrations exceeded at least twice the amount suggested in the data sheets for total blocking of the factor concentrations as measured in supernatants. The additives were replaced with medium change every two days. After 5 and 7 days, cultures were fixed for 5 minutes in 80% methanol and 2 minutes in acetone and further processed for immunohistochemistry.

**Immunohistochemistry**
Proliferating keratinocytes were visualized by a Mib1 antibody and quantitated by counting the ratio of labeled cells. Fixed cells were washed in PBS and pre-incubated for 15 minutes in PBS with 1% BSA. As first antibody a monoclonal mouse anti-human Mib1 antibody (Calbiochem, München, FRG) recognizing Ki67 (Catorretti et al., 1992) was added and left overnight at 4°C). After washing in PBS, cultures were incubated with a rabbit polyclonal antibody (Dianova, Hamburg, FRG) as described previously (Brauchle et al., 1994) using recombinant KGF (BTS, St. Leon, FRG) as standard. IL-1β was identified by immunoblotting cell extracts with a mouse monoclonal antibody (Genzyme, Rüsselsheim, FRG) as well as a rabbit polyclonal antibody (Genzyme, Rüsselsheim, FRG) and secondary peroxidase-bound anti-mouse and anti-rabbit IgG, respectively (Dianova, Hamburg, FRG), and detected by the ECL system (Amersham, Braunischweig, FRG) using recombinant IL-1β (Genzyme, Rüsselsheim, FRG) as standard.

**Spectrophotometric assay of IL-1β activation**
IL-1β is synthesized as propeptide and activated by cleavage through a converting enzyme (ICE). To detect IL-1β-activating enzyme activity, the cleavage of a colorimetric IL-1β homologous substrate was measured spectrometrically in keratinocyte cell lysate as described (Thornberry, 1994). NEK mono- and cocultures were washed with cold PBS and lysed with 400 µl hypotonic buffer (25 mM Hepes, pH 7.5, 5 mM MgCl₂, 1 mM EGTA, 1 mM PMFS, 10 µg/ml pepstatin, 10 µg/ml leupeptin). 100 µl cell lysate were mixed with 400 µl reaction buffer (100 mM Hepes, pH 7.5, 10% sucrose, 0.1% CHAPS, 10 mM EDTA) and 5 µl of the colorimetric ICE substrate IV (10 mM, stock solution in DMSO; Calbiochem, Bad Soden, FRG). The shift in optical density at 400 nm was measured over a time period of 25 minutes (Thornberry, 1994).

**Cell proliferation assay**
HDF were seeded at 5,000 and 8,000 cells, respectively, per well of 96-well multititer plates and preincubated in DMEM with 10% FCS for 24 hours, whereas NEK were seeded at 5,000 and 10,000 cells per well, respectively, and preincubated in complete keratinocyte growth medium (KGM, 0.15 mM Ca²⁺, Promocell, Heidelberg, FRG) for 48 hours. Then HDF or NEK cultures were washed once with serum- and supplement-free DMEM and keratinocyte basal medium (KBM), respectively. Thereafter the following growth factors at different concentrations were added in DMEM (fibroblasts) or KGM (keratinocytes): recombinant human IL-1α (Biozol, Eching, FRG), IL-1β (Innogenetics, München, FRG) and KGF (BTS, St. Leon, FRG). Cultures were incubated for 24 hours to 72 hours, cells labeled with 10 µM bromodeoxyuridine (BrdU) for 24 hours, and BrdU incorporation was measured by a cell proliferation ELISA-kit (Boehringer, Mannheim, FRG). Data presented are means with standard deviations of duplicate measurements of at least two different culture assays.

**RESULTS**

**Cytokine expression is maintained in irradiated fibroblast monocultures**
In order to prove that 70-Gy-irradiated fibroblast feeder cells, often called ‘lethally irradiated’, survived in culture and were functionally active, their survival and cytokine expression was monitored in monocultures of proliferating and irradiated cells (Fig. 1). While untreated fibroblasts expand threefold in 10-12 days, irradiated cells, as expected, did not multiply but survived...
In cocultures of NEK and HDFi mRNA expression of fibroblasts and keratinocytes

Cytokine expression is modulated in cocultured over a period of 10 days in postmitotic fibroblast cultures. the mRNA levels of these cytokines remained rather constant of the individual mRNAs were similar (Fig. 1B). Remarkably, generally lower as compared to proliferating cells, but the ratios expression levels in postmitotic fibroblasts (HDFi) were Fusenig, 1996). When compared at culture day 5, the mRNA comparable for HDF and HDFi (see also Maas-Szabowski and cytokine expression over an extended culture period when fibroblast feeder cells (HDFi) maintained a remarkable level of maintained their metabolic activity for several weeks as judged from their sustained function as feeder-layers for keratinocytes plated at low density (data not shown). Accordingly, postmitotic fibroblast feeder cells (HDFi) maintained a remarkable level of cytokine expression over an extended culture period when calculated on the basis of GAPDH expression which was comparable for HDF and HDFi (see also Maas-Szabowski and Fusenig, 1996). When compared at culture day 5, the mRNA expression levels in postmitotic fibroblasts (HDFi) were generally lower as compared to proliferating cells, but the ratios of the individual mRNAs were similar (Fig. 1B). Remarkably, the mRNA levels of these cytokines remained rather constant over a period of 10 days in postmitotic fibroblast cultures.

**Cytokine expression is modulated in cocultured fibroblasts and keratinocytes**

In cocultures of NEK and HDFi mRNA expression of cytokines by the individual cell types, keratinocytes and fibroblasts, was analyzed separately, after selective detachment of HDFi from feeder-layer cultures leaving almost pure keratinocyte colonies in the dish (see Smola et al., 1993). A clear separation of both cell types was not feasible before NEK had formed compact colonies (day 5) which were resistant to detachment by short EDTA treatment. Thus, due to contamination of NEK in the separated HDFi population, selective data of HDFi could not be obtained before day 6. On the other hand, pure NEK populations could be obtained from day 2 on after plating. The quantitative separation of both cell types was proven by analyzing the expression of mRNA specific for the individual cell type such as keratin 14 (for keratinocyte contamination in feeder cells) and collagen type I and MMP-1 (for fibroblast contamination in keratinocyte populations) (not shown here but see Smola et al., 1993, 1994).

Within a 12-day culture period, expression levels of most factors differed significantly between mono- and cocultured fibroblasts and keratinocytes, respectively. Representative values obtained in several experiments are shown for day 6 after plating (Fig. 2A). In HDFi, the expression of KGF as well as of IL-1α and -1β was strongly increased, while IL-8 and TGF-β1 mRNA levels were reduced. Changes were also seen in cocultured versus monocultured keratinocytes, with a distinct increase of IL-1α, IL-8, basic fibroblast growth factor (bFGF) and GM-CSF while others showed only minor changes (Fig. 2B).

The individual kinetics of cytokine expression within a 12-day coculture period revealed further characteristics with a constant, though moderate, increase of IL-1β in keratinocytes, whereas IL-1α mRNA exhibited a strong, though delayed, induction and remained at this high level (Fig. 2C). Expression of IL-1 receptor type I remained low in both HDFi and NEK without any changes over time (data not shown).

Due to problems with cell separation (see above) a clear attribution of cytokine expression to HDFi was not possible in cocultures up to day 6, but this did not affect the analysis of KGF mRNA expression, because KGF is exclusively produced in fibroblasts (Chedid et al., 1994). Accordingly, keratinocytes were constantly negative for these growth factors both in mono- and cocultures (data not shown). In cocultured fibroblasts, however, modulations were seen already after 1 day with a strong induction of KGF mRNA expression which remained high for the observation period of 8 days (Fig. 4D).

**IL-1α and -1β production and release is modulated in coculture**

To establish the significance of the observed mRNA modulations for keratinocyte growth regulation, we determined by ELISA the cytokine proteins released into the culture medium conditioned for 48 hours. This way the origin of distinct factors from either keratinocytes or fibroblasts could not be distinguished.

The protein levels of IL-1α and -1β were extremely high at day 2 in NEK monocultures (Fig. 3A,B) but rapidly declined with culture time. Still much higher levels of IL-1α and -1β were determined in supernatants of NEK monocultures during the first 48 hours after plating (inset to Fig. 3A and B). These high initial amounts of IL-1α and -1β in keratinocyte cultures dropped rapidly within the first 24 hours followed by a further decrease. Surprisingly, in the supernatant of freshly trypsinized
NEK similar high levels were determined for IL-1β (3910±600 pg/1×10^6 cells) and about half of the 2-4 hours culture value for IL-1α (3970 ±200 pg/1×10^6 cells).

In contrast, cocultures contained significantly lower levels of IL-1 during this initial 48 hours culture period and these remained rather unchanged with time (Fig. 3A,B, and insets). During the following culture period, the levels of IL-1α steadily decreased in monocultures but remained always higher than in the medium of cocultures, whereas the IL-1b concentration was always higher in cocultures. In HDFi monocultures, although IL-1α and -1b mRNA were clearly detectable, supernatant protein amounts for both cytokines remained at the background level. This discrepancy could be due to a lack of cytokine release, since both proteins could be detected by western blot analysis in the cell lysate of proliferating fibroblast monocultures (data not shown). In addition, by western blot analysis of supernatants from proliferating fibroblast cultures no IL-1β bands could be detected (data not shown).

The functional significance of IL-1β in these cultures was unclear because it had been reported that IL-1β is not activated in keratinocytes due to the lack of interleukin-1β converting enzyme (ICE) (Mizutani et al., 1991). Quite recently it was reported, however, that epidermal cells express different types of ICE-enzymes (caspases; Takahashi et al., 1998), stimulated keratinocytes activate IL-1β by ICE in vitro (Zepter et al., 1997) and that activation can also occur via an alternative mechanism involving a stratum corneum chymotryptic enzyme (Nylander-Lundquist and Egelrud, 1997). By western blot analysis we found the processed active form of IL-1β in the cell lysate of 5-day keratinocyte mono- and cocultures (Fig. 3C). Furthermore, we detected a protease activity in keratinocytes with specificity for a substrate homologous to IL-1b as measured by a spectrophotometric assay. In the presence of NEK lysate a threefold reduction in optical density at 400 nm after 20 minutes (0.125) compared to control (0.301) was observed, indicating substrate metabolism by an ICE-like activity. All this indicated that keratinocytes in mono- and coculture produce active IL-1β.

**Striking stimulation of KGF in cocultured fibroblasts**

The concentration of KGF released into the culture medium was assayed on western blot because an appropriate ELISA was not available and showed a continuous increase in coculture supernatants (Fig. 4A). This increase in secreted KGF was clearly visible at day 2, reached a maximum with a...
5- to 6-fold increase after 6 days and remained at this high level throughout day 10. The constitutive KGF levels in supernatants of monocultured HDFi were rather low and did not change over the same culture period (Fig. 4B). The KGF antibody used recognized two proteins of approximately 24 and 26 kDa, two differently glycosylated forms; after deglycosylation both bands disappeared and instead a new one of about 21 kDa was seen, at a similar location as recombinant bacterial KGF (Fig. 4C). This strongly suggested that the products detected in the supernatant of fibroblast mono- and cocultures were glycosylated, active forms of KGF.

**IL-1α and -1β stimulate fibroblast but not keratinocyte proliferation**

To determine which of the cytokines identified in cocultures might function as regulators of cell growth, their effect on fibroblast and keratinocyte proliferation was assessed by measuring BrdU incorporation into DNA in serum- and growth-factor-free DMEM and keratinocyte basal medium (KBM), respectively. In fibroblasts, both IL-1α and -1β induced a concentration- and time-dependent stimulation of HDF proliferation with a 7- and 10-fold higher BrdU incorporation by 1 ng/ml of IL-1α and -1β, respectively (Fig. 5A). In NEK, however, IL-1α or -1β had no effect on cell proliferation (Fig. 5B). This was not due to an altered functional response, because NEK cultured for up to three days in KBM can be fully restimulated by complete culture medium (KGM) indicating their preserved functional integrity (data not shown). Furthermore, recombinant KGF stimulated NEK proliferation with maximal effects at 0.1 ng/ml (Fig. 5B). These data indicated that the elevated levels of IL-1 in cocultures did not act as direct keratinocyte growth stimulators but might act as inducers of KGF expression in fibroblasts (as demonstrated earlier Chedid et al., 1994; Maas-Szabowski and Fusenig, 1996).

**Neutralization of IL-1 function abrogates keratinocyte proliferation**

To confirm the hypothesis of a double paracrine keratinocyte growth stimulation and the functional significance of IL-1 as inducer of KGF expression in cocultures, we inhibited the IL-1 signalling pathway via specific neutralizing antibodies and blocked the IL-1 receptor type 1 by an IL-1 receptor-antagonist (IL-1RA). As control, the effect of KGF neutralizing antibodies
Cytokine modulation in skin culture was determined in parallel cultures. The inhibitors were added to 2-day-old cocultures and further at 2-day intervals with medium change and cells immunostained with the Mib1 antibody (Cattoretti et al., 1992). Compared to untreated controls, where most keratinocyte nuclei were stained, cocultures treated with IL-1 antibodies, IL-1RA, or combinations of both as well as antibodies to KGF showed a drastic reduction in the number of proliferating cells (Fig. 6A-F). When this was quantitated by counting the number of labeled nuclei and calculating their frequency as a percentage of all nuclei (stained with bisbenzimide), a strong inhibition of proliferation ranging from 35% to 65% was observed (Fig. 6G). The decrease in proliferative cells was clearly visible after 3 days (data not shown), further increased at 5 days and stayed at this level at 7 days. The level of inhibition was similar for all IL-1 neutralizing antibodies but could not be further reduced by their combination with IL-1RA (Fig. 6G) nor by combining IL-1α and IL-1β neutralizing antibodies (not shown).

In addition to the Mib1 data, cell counts at different time points showed a nearly 2-fold increase in control cocultures as compared to a 1.2-fold increase in the treated ones at 6 days. This IL-1-blockade-mediated inhibition was fully reversible, because proliferation occurred at the same rate as in controls after removal of the antibodies (data not shown). IL-1α neutralizing antibodies clearly reduced the level of detectable IL-1α in the supernatant by more than 50%, while there was no effect on the IL-1β level, whereas IL-1RA was slightly elevated (Fig. 7A). This demonstrated the efficiency and specificity of the IL-1α neutralizing antibodies.

Most interestingly, the degree of inhibition of keratinocyte proliferation in cocultures treated with inhibitors to IL-1α and -1β was similar to that observed after addition of 1 and 2 μg/ml neutralizing KGF antibodies, respectively (Fig. 6F,G). Since both interleukins had no direct effect on NEK proliferation (see Fig. 5B), the reduced IL-1 levels supposedly reduced proliferation by lowering KGF production in fibroblasts. To prove this assumption, KGF protein levels were measured in the supernatants of cocultures treated with neutralizing antibodies to IL-1α and -1β (1 μg/ml, each). Strikingly, while KGF levels constantly increased in the supernatants of control cocultures (Fig. 7B; compare also Fig. 4B), this induction was completely abolished by IL-1α and -1β neutralizing antibodies.

These data provide strong evidence that the postulated double paracrine loop involving IL-1 signalling by keratinocytes to induce KGF production in fibroblasts, which then in turn enhances keratinocyte proliferation, is operative in these cocultures and plays an important role in regulating keratinocyte proliferation.

**DISCUSSION**

It is well established that tissue homeostasis of epithelial
organs, such as the epidermis, strictly depends on interactions with the adjacent mesenchyme (Guo et al., 1993; Ashkenas et al., 1996; for review see Fusenig, 1994; Mackenzie, 1994). The study of the molecular mechanisms of these interactions in vivo is complicated by too many variables involved in intact tissues and the lack of properly controlled experimental conditions. Thus, in vitro models have been developed representing different coculture systems of epithelial and mesenchymal cells; the most simple one, the feeder-layer culture, was pioneered by Rheinwald and Green (1975). The substantial stimulation of keratinocyte proliferation by coculture with postmitotic fibroblasts has been known since then and utilized in many studies, but the molecular basis of this keratinocyte growth regulation had been rather obscure (for review see Fusenig, 1994).

Recently, we proposed a novel mechanism of epithelial-mesenchymal interactions based on a dynamic and reciprocal modulation of cytokine and growth factor production in keratinocytes and fibroblasts (Smola et al., 1993, 1994). Here we now provide functional evidence that a double paracrine mechanism of growth regulation is operative in such a coculture system. Through release of IL-1, keratinocytes elicit the enhanced expression of growth factors, particularly KGF, in fibroblasts and in this way direct their own proliferation. Whereas the cytokines IL-1α and -1β themselves had no immediate effect on keratinocyte growth in monocultures, abrogation of their function in cocultures inhibited keratinocyte proliferation to an extent comparable to that obtained by neutralization of the potent keratinocyte growth factor KGF. Strong induction of KGF expression in proliferative and postmitotic fibroblasts by IL-1 (concentrations as low as 0.2 and 2 ng/ml) had been reported (Chedid et al., 1994; Maas-Szabowski and Fusenig, 1996). Accordingly, KGF production in cocultured fibroblasts was completely abrogated when IL-1 function was inhibited. Thus, by selectively inhibiting the first and the second part, respectively, of this double paracrine

![Fig. 6](image)

Assessment of keratinocyte proliferation in feeder-layer cocultures after Mib1 (red) staining of proliferating and counterstaining of all nuclei with bisbenzimide (blue) in 7-day control cultures (A) and after blocking the IL-1 pathway by neutralizing antibodies to IL-1α (B), IL-1β (C), IL-1RA (D), IL-1α neutralizing antibodies and IL-1RA (E) as well as after addition of KGF antibodies (F). Bar 100 μm, same magnification in A-F. (G) Quantitative evaluation of the inhibition of keratinocyte proliferation. Cocultures were analyzed 5 and 7 days after growth in normal culture medium (C), or after addition of neutralizing antibodies (Ab) to IL-1α and IL-1β (1 μg/ml each), IL-1RA (10 ng/ml), IL-1RA and IL-1α neutralizing antibodies, as well as KGF neutralizing antibodies (1 μg/ml). Mib1 positive cells were counted and results are given as percent of all nuclei (stained with bisbenzimide) counted in 6 different vision fields at a 160-fold magnification performed in two different experiments. Data are given as a mean with standard deviation.

![Fig. 7](image)

(A) Protein levels of IL-1α, IL-1β and IL-1RA in supernatants of cocultures treated with IL-1α neutralizing antibodies (1 μg/ml) as determined by ELISA in 2-, 4-, 6-day-old cultures. Data are given as a mean with standard deviation of duplicate measurements from 2 independent experiments. (B) Levels of KGF in heparin-affinity-enriched supernatants of control cocultures and those treated with neutralizing antibodies for IL-1α and IL-1β (IL-1Ab) (1 μg/ml each) in 2-, 4- and 6-day-old cultures as determined by western blot using a polyclonal rabbit anti-KGF antibody for detection.
pathway, its functional significance as a major component of the epithelial-mesenchymal interplay has been substantiated. The fact that these interventions did not completely abolish keratinocyte proliferation might be due to incomplete IL-1 neutralization (see Fig. 7) but also indicates that additional pathways are operative in the complex crosstalk between keratinocytes and fibroblasts. The latter is also indicated by the apparent contradictory results in KGF knock-out mice (Guo et al., 1996) and KGF receptor-deficient animals (Werner et al., 1994).

This novel type of mutually induced signalling circuits for growth regulation may have functional significance also in vivo as a fast regulatory mechanism in tissue repair processes. IL-1 release in the epidermis is known as an early consequence of skin irritation and injury (for review see Luger and Schwarz, 1996) and KGF is rapidly induced in dermal cells upon skin wounding (Werner et al., 1992). Furthermore, IL-1α and -1β are known as strong inducers of KGF production in fibroblasts in vitro (Brauchle et al., 1994; Chedid et al., 1994; Maas-Szabowski and Fusenig, 1996). Keratinocytes produce IL-1 constitutively and the epidermis is a vast reservoir of sequestered IL-1 with levels 100 to 1000 times greater than in most normal tissues (Kupper and Groves, 1995). IL-1 is stored in a cell-associated fraction either bound to the cell membrane or in the cytoplasm (Blanton et al., 1989; Cork and Duff, 1996), and is rapidly released by many stimuli including injury (Appleton, 1994; Dinarello, 1997). This release of stored IL-1 is considered as a primary defense mechanism in skin and has been mainly interpreted as a process leading to inflammatory reactions in the dermis (Kupper and Groves, 1995). IL-1 stimulates its own production (Kumar et al., 1992; Maas-Szabowski and Fusenig, 1996) and also that of other cytokines such as IL-8, IL-6, and GM-CSF both in keratinocytes and mesenchymal cells (Boxman et al., 1996; Maas-Szabowski and Fusenig, 1996) thus creating a cascade of pro-inflammatory stimuli (for review see Dinarello, 1997). In addition to these known interactions with endothelial cells and its chemotactic action on leucocytes, we here demonstrate a new function of IL-1 release and induction in keratinocytes initiating tissue repair mechanisms by stimulating keratinocyte growth factor production in mesenchymal cells.

It has been suggested that IL-1 directly stimulates keratinocyte proliferation (Kupper et al., 1986; Hashimoto and Yoshihawa, 1992) but most data have been obtained in intact skin following irradiation (Blanton et al., 1989), and intradermal injection of IL-1 or IL-1 antibodies (Granstein et al., 1986; Oberszyn et al., 1993). Obviously, under these in vivo conditions, the discrimination between a direct and an indirect, fibroblast-mediated, proliferative stimulus of IL-1 on keratinocytes is not feasible. Similarly, in the light of the here described double paracrine pathway, the reported IL-1 effects on keratinocytes in culture may as well represent an indirect action of IL-1 because they were performed in feeder-layer cultures of neonatal foreskin (Kupper et al., 1986) or on foreskin keratinocytes grown in serum-free medium (Pillai et al., 1988; Chen et al., 1995). While in the first case the presence of functionally active fibroblasts is obvious (Maas-Szabowski and Fusenig, 1996, and this paper), foreskin-derived keratinocyte cultures, even in low calcium serum-free medium, contain variable amounts of fibroblasts due to the difficulty of properly separating the epidermis from the loose mesenchyme. Minor fibroblast contaminations, though not microscopically visible in keratinocyte mass cultures, were clearly effective in causing KGF mRNA expression, and thus mimicking coculture effects (unpublished own data). In our keratinocyte monocultures with cell preparations thoroughly controlled for fibroblast contamination we could never see KGF expression by RT-PCR nor a significant stimulation of proliferation by recombinant IL-1α or -1β. This is in agreement with an earlier study reporting no stimulation of proliferation by IL-1 in growth-arrested human keratinocytes (Ristow, 1990).

We hypothesize that in a first step IL-1 is released from preformed pools in keratinocytes as also seen in vivo (Wood et al., 1996), which would explain the rapid induction of KGF both in vivo upon injury (Werner et al., 1992) and in culture as shown here. Both RNA and protein levels of KGF rise quickly in cocultured fibroblasts, while RNA expression of IL-1α and -1β starts much later in keratinocytes (Fig. 2C and D). The enormous IL-1 protein levels detected in the supernatant of early plated and even freshly trypsinized cells further argue in that direction. Similarly, release of IL-1 has been observed in keratinocytes in other stress situations such as after mechanical deformation (Lee et al., 1997; Takei et al., 1998) and in transfection processes (Komine et al., 1994).

Which of the two IL-1 classes, IL-1α or -1β, are the major players in the observed interplay or whether they are both similarly operative, cannot be fully answered at present. Both are synthesized as 31 kDa precursors that are cleaved to the mature 17 kDa factors. While IL-1α is biologically active in both precursor and mature forms, IL-1β requires proteolytic cleavage by IL-1 converting enzyme (ICE) for full activation (Dinarello, 1997; Lee et al., 1997; Zepter et al., 1997). It has been reported that human keratinocytes under physiologic conditions do not produce ICE and therefore produce only the immature 31 kDa IL-1β (Mizutani et al., 1991). More recently, however, constitutive expression (Takahashi et al., 1998) as well as induction and functional activity of ICE in keratinocyte cultures by nontoxic concentrations of inflammatory and immunologic stimuli has been described (Zepter et al., 1997). Our data document that keratinocytes produce active IL-1β by demonstrating that: (i) on western blots of cell lysates from mono- and cocultures active IL-1β is identified by its molecular mass; (ii) keratinocyte extracts enzymatically process an appropriate substrate; and (iii) IL-1β neutralizing antibodies lead to inhibition of keratinocyte proliferation in coculture comparable to that of IL-1α antibodies.

Both IL-1 classes are released at high levels in the supernatant of keratinocyte monocultures, whereas their levels are much lower in cocultures although their RNA expression is increased in the latter. This discrepancy may be explained by capture of the factors by the IL-1 receptor on fibroblasts as suggested by Boxman et al. (1996). This is particularly obvious during the first 24-48 hours after plating of keratinocytes on the precultured feeder cells (see Fig. 4).

Among the growth factors differentially expressed in cocultures, the paracrine acting KGF is an important stimulator of keratinocyte proliferation (Rubin et al., 1995). KGF expression is rapidly stimulated in fibroblasts and secreted at high (5 times over background) levels and, moreover, in its active glycosylated form is known to be more active than the recombinant version from E. coli (Sato et al., 1995; Hines and Allen-Hoffmann, 1996). Thus, the induced production of
KGF/FGF-7 is most probably the major, though not only, mediator of enhanced keratinocyte proliferation caused by cocultured fibroblasts. This function of KGF as mediator in epithelial-mesenchymal interactions may be relevant for the in vivo situation in skin as well as other organs as documented in wound healing studies (Werner et al., 1992; Marchese et al., 1995) and in hormone-dependent organ development (Alarid et al., 1994).

The use of permanently postmitotic (irradiated) fibroblasts for these studies (a prerequisite for feeder-layer cultures) is not considered a major deficiency for the biologic relevance of our data, because:
(i) In contrast to earlier notions classifying the high-dose-X-irradiated fibroblasts as ‘lethally’ irradiated cells, it has been clearly documented that these cells, though irreversibly postmitotic, survive in culture for several weeks acquiring a state of terminal differentiation (Limat et al., 1989; Bumann et al., 1995). (ii) In mono- and coculture, irradiated fibroblasts maintain a high level of constitutive cytokine expression and secretion (Waelti et al., 1992; Smola et al., 1993, 1994) and react to external signals with modulated expression and secretion of cytokines (Maas-Szabowski and Fusenig, 1996, and this paper). The reduced level of expression, as compared to non-irradiated, proliferating cells, may be attributed to disturbances in the expression and translation machinery of a fraction of cells due to X-ray-induced DNA damage (see also Maas-Szabowski and Fusenig, 1996, for further discussion).

Thus, we have good reason for assuming that these feeder cells, resembling in their postmitotic state the mostly non-proliferating cells in the dermis, are functionally competent cell populations. Therefore, we believe that the novel double paracrine mechanism of keratinocyte proliferation by cocultured fibroblasts documented in this report represents a first step in unravelling the complex epithelial-mesenchymal crosstalk in skin as well as other organs regulating tissue homeostasis.

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The cytokine modulating in skin culture


