Keratinocyte Growth Regulation in Defined Organotypic Cultures Through IL-1-Induced Keratinocyte Growth Factor Expression in Resting Fibroblasts

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Balanced keratinocyte proliferation and differentiation resulting in regular tissue organization strictly depend on dermal support. Organotypic cultures represent biologically relevant *in vitro* models to study the molecular mechanism of the underlying dermal–epidermal interactions. To mimic the state of resting fibroblasts in the dermis, postmitotic (irradiated) fibroblasts were incorporated in the collagen matrix, where they typically support epidermal proliferation and tissue organization. In coculture with keratinocytes, fibroblasts exhibit an enhanced expression of keratinocyte growth factor and the interleukin-1 receptor (type I), which further increase with culture time. In cocultured keratinocytes, keratinocyte growth factor receptor as well as RNA expression and protein release of interleukin-1α and interleukin-1β are upregulated. We hypothesized that the modulated cytokine expression represents a basic mechanism for keratinocyte growth regulation. The functional significance of this double paracrine pathway, i.e., induction of keratinocyte growth factor expression in fibroblasts by keratinocytes via release of interleukin-1, was confirmed by interfering with both signaling elements: (i) interleukin-1-neutralizing antibodies and interleukin-1 receptor antagonist significantly inhibited keratinocyte growth factor release, keratinocyte proliferation, and tissue formation comparable to the effect produced by keratinocyte-growth-factor-blocking antibodies; (ii) addition of keratinocyte growth factor to cocultures with inactivated interleukin-1 pathway completely reverted growth inhibition; (iii) in organotypic cocultures with subthreshold fibroblast numbers both interleukin-1 and keratinocyte growth factor restored the impaired epidermal morphogenesis. Thus, epidermal tissue regeneration in organotypic cocultures is mainly regulated by keratinocyte-derived interleukin-1 signaling, which induces keratinocyte growth factor expression in cocultured fibroblasts. This demonstrates a novel role for interleukin-1 in skin homeostasis substantiating data from wound healing studies *in vivo*.* ***Key words: interleukin-1/keratinocyte growth regulation/KGF/organotypic cocultures. J Invest Dermatol 114:1075–1084, 2000***

To maintain normal epidermal integrity, keratinocytes proliferate and differentiate in a well-organized pattern, which is mainly controlled by autocrine- and paracrine-acting growth factors and cytokines (Luger and Schwarz, 1995; Schröder, 1995). Accumulating evidence indicates that mesenchymal–epithelial interactions are essential in regulating epidermal tissue homeostasis (for review see Fusenig, 1994). This interplay is largely mediated by soluble factors produced by dermal cells acting as paracrine regulators of keratinocyte growth and differentiation (Smola *et al*, 1993; Werner, 1998). The identity of these factors and their regulation, however, has only been partly understood so far.

Examination of the role of individual growth factors and cytokines *in vivo* is complicated by a multitude of epithelial and mesenchymal factors in the complex cutaneous microenvironment and by systemic influences from the circulation. Thus, keratinocyte culture systems have been used to study the role of cytokines in keratinocyte growth regulation. Many of these models failed to faithfully reproduce the *in vivo* epithelial architecture, however, and maintain epidermal–dermal interactions. Of considerable advantage for reproducing normal keratinocyte physiology *in vitro* has been the development of three-dimensional organotypic coculture models consisting of keratinocytes growing air-exposed on a collagen matrix populated with dermal fibroblasts (Bell *et al*, 1981; Asselineau *et al*, 1986; for review see Fusenig, 1994). Although these models yield excellent epidermal differentiation and morphology, the culture media used often contain serum or tissue extracts, both complex and undefined mixtures of compounds that may confound effects of individual cytokines on epidermal proliferation and differentiation (Contard *et al*, 1993; Fartasch and Ponec, 1994).

We have recently demonstrated that a defined culture medium without serum or tissue extract additives supports growth and differentiation of keratinocytes on a fibroblast-populated collagen gel comparable to that seen in serum-supplemented medium (Stark *et al*, 1999). This concerns both keratinocyte proliferation and differentiation as well as formation of a normal tissue architecture

Manuscript received January 14, 2000; revised March 2, 2000; accepted for publication March 5, 2000.

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Abbreviations: HDF, human dermal fibroblasts; HDFi, human dermal irradiated fibroblasts; IL, interleukin; IL-1RA, interleukin-1 receptor antagonist; KGF, keratinocyte growth factor.

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and a basement membrane at the light and electron microscopy level. Although fibroblast proliferation was reduced in the defined culture medium, the number of fibroblasts nevertheless increased 3- to 4-fold within 3 wk. This situation not only differs from the nonproliferative state of fibroblasts in the dermis *in vivo*, but the fluctuating number of fibroblasts and their proliferative state creates additional variables impeding the molecular examination of the dynamics of the epidermal-dermal cell interplay. Finally, with proliferative fibroblasts in the collagen gels, it has not been possible to determine exactly the dependence of keratinocyte growth on fibroblast cell numbers and its modulation by added cytokines (Coulomb *et al*, 1989; Stark *et al*, 1999).

It is well known from simpler coculture models that mouse or human fibroblasts rendered into an irreversible postmitotic state by X-ray or mitomycin C treatment are still capable (as feeder layers) of promoting keratinocyte growth (Rheinwald and Green, 1975; Limat *et al*, 1989). We have demonstrated that these cells, formerly called "lethally irradiated fibroblasts", are capable of expressing a large panel of cytokines at the RNA and protein level and respond to interleukin (IL) stimulation with enhanced growth factor expression (Maas-Szabowski and Fusenig, 1996). In such two-dimensional cocultures of irradiated fibroblasts and proliferating keratinocytes, we detected a novel type of keratinocyte-fibroblast interplay via a double paracrine pathway that involves IL-1 and keratinocyte growth factor (KGF) as the major players in controlling keratinocyte proliferation (Maas-Szabowski *et al*, 1999). It remained unanswered, however, whether this growth control mechanism was influenced by the artificial two-dimensional culture situation or would also function under the more physiologic conditions of organotypic cocultures and might thus be important for the situation in skin regeneration and homeostasis *in vivo*.

In this paper, we report on the regulation of keratinocyte proliferation in organotypic culture via KGF released by fibroblasts following induction by keratinocyte-derived IL-1. In order to standardize the system, we used postmitotic fibroblasts in the collagen gel and a defined serum-free medium. The results demonstrate the functional significance of this pathway as a major control mechanism for keratinocyte proliferation and epidermal tissue formation in this organotypic model. This strongly suggests that this mechanism plays a similar role in skin, representing an essential part of the complex epidermal-dermal interplay.

MATERIALS AND METHODS

Cell culture Normal human skin keratinocytes and human dermal fibroblasts (HDF) were derived from adult skin obtained from surgery (mostly breast reduction), as previously described (Smola *et al*, 1993; Stark *et al*, 1999). In brief, fibroblasts obtained from outgrowth of explant cultures were grown in Dulbecco's modified Eagle's medium (DMEM) (Bio Whittaker, Serva, Heidelberg, Germany) supplemented with 10% fetal bovine serum, and cells from passages 5–9 were used. For preparing fibroblast feeder cells (HDFi), trypsinized cell suspensions (0.05% trypsin/ 0.025% ethylenediamine tetraacetic acid, vol/vol) were γ-irradiated with 70 Gy. Normal epidermal keratinocytes obtained by trypsinization of split-thickness skin samples were isolated from the separated epidermis and plated with X-irradiated feeder cells in FAD medium (DMEM:Hams F12, 3:1) with 100 U penicillin per ml, 50 μg streptomycin per ml, and supplemented with 5% fetal bovine serum, 5 μg per ml insulin, 1 ng per ml recombinant human epidermal growth factor, 10^{-10} M cholera toxin, 10^{-4} M adenine, and 0.4 μg per ml hydrocortisone (Sigma, Deisenhofen, Germany) as described by Smola *et al* (1993). Absence of fibroblast contamination was routinely checked in cloning assays on fibroblast feeder layer, because this is the optimal culture condition to allow colony formation by single plated cells. Furthermore, absence of fibroblast contamination was proven by the lack of KGF expression (not expressed by keratinocytes) in keratinocyte monocultures by reverse-transcriptase polymerase chain reaction (RT-PCR) (Maas-Szabowski and Fusenig, 1996).

Organotypic cocultures Normal epidermal keratinocytes (passage 2) were seeded onto collagen gels (type I, rat tail tendon) with or without proliferating (HDF) or postmitotic (HDFi) fibroblasts cast in cell culture inserts (pore size 3.0 μm, polycarbonate; Falcon, Becton Dickinson, Heidelberg, Germany) as described in detail previously (Stark *et al*, 1999). After 24 h, medium was replaced by FAD medium additionally containing 50 μg per ml L-ascorbic acid (Sigma) or by defined medium (SKDM) and cultures were raised to the air–liquid interface. SKDM is based on the keratinocyte-defined medium KDM (Promocell, Heidelberg, Germany), supplemented with 5 μg per ml insulin, 0.5 μg per ml hydrocortisone, 0.1 μg recombinant human epidermal growth factor, 20 μg per ml transferrin, 50 μg per ml L-ascorbic acid (Promocell), 1 mg per ml bovine serum albumin (endotoxin- and fatty-acid-free, A-8806, Sigma) and adjusted to 1.3 mM Ca^{2+} (Stark *et al*, 1999). Medium with or without additives was replaced every 2 d. For cell counting HDF and HDFi were separated from the collagen by melting the gels in four times their volume of phosphate-buffered saline (PBS) at 60°C.

Functional analysis of cytokines Organotypic cocultures were prepared as described in serum-containing or defined culture medium (Stark *et al*, 1999). After 24 h, the medium was replaced by medium additionally containing growth factors or neutralizing antibodies: 10 ng per ml KGF (BTS, St. Leon, Germany), 10 ng per ml IL-1α, 1 μg per ml neutralizing antibodies against IL-1α or IL-1β, 100 ng per ml IL-1 receptor antagonist (IL-1RA) (all from Genzyme, Rüsselsheim, Germany), or 1 μg per ml KGF-neutralizing antibodies (R&D Systems, Wiesbaden, Germany). The antibody concentrations used were twice the amount for total blockade suggested in the data sheets. The additives were replaced with medium change every 2 d.

Fixation and embedding Before fixation organotypic cultures were incubated 16 h with 63 μM bromodeoxyuridine (BrdU) to label proliferating cells. After measuring the size of the gels, cultures were either fixed according to a standardized protocol for routine histology or embedded in Tissue Tec OCT Compound (Medim, Gießen, Germany) and frozen in liquid nitrogen vapor for cryosectioning.

Isolation of RNA Organotypic cocultures were washed with cold PBS and epithelia were mechanically detached from HDF/HDFi-containing collagen gels. Intact keratinocyte epithelia as well as pelleted HDF/HDFi from melted gels (see above) were lyzed 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, Germany).

Reverse transcription and PCR Twenty-mer primers from separate exons flanking the regions of the following locations on cDNA were chosen according to published sequences and amplified at the indicated annealing temperature: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 69–308 (62°C); IL-1α 84–504 (62°C); IL-1β 174–504 (62°C); IL-1 receptor type I 605–1090 (52°C); KGF 446–1027 (62°C); KGF receptor 1129–1281 (60°C) (for references see Maas-Szabowski *et al*, 1999). The RT-PCR was performed according to an established method with some modifications (Maas-Szabowski and Fusenig, 1996). The cDNA synthesis was carried out at 42°C for 80 min in 100 μl final volume containing 10 μg total RNA, 10 μl 10 × PCR buffer, 20 μl $MgCl_2$ (25 mM), 6 μl of each dNTP (10 mM), 2.5 μl RNasin (0.5 U per μl), 5 μl reverse transcriptase (50 U per μl), 2 μl oligo dT15 (50 μM), and 2 μl random hexamers (50 μM) (GeneAmp-PCR-Kit, Perkin Elmer, Weiterstadt, Germany). Four microliters of first-strand cDNA were added to the PCR mixture up to a volume of 50 μl following the product description. The mixture was transferred to a thermal cycler (Biometra, Göttingen, Germany) and amplified at the indicated annealing temperature, performing 24–30 cycles such that the product yield was in the exponential range. PCR fragments were separated on 1.5% agarose gels (Seakem; Biozym), ethidium-bromide-stained, and identified by their running positions on the gel and, additionally, by restriction mapping with two different enzymes.

Determination of PCR products and calculation of mRNA quantity Semiquantitative analysis of the PCR products was achieved by computerized image analysis of the stained bands (EASY plus; Herolab, Wiesloch, Germany) as described earlier (Maas-Szabowski and Fusenig, 1996; Maas-Szabowski, 1997). 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 GAPDH was used as internal standard.

Protein determination by enzyme-linked immunosorbent assay (ELISA) Selected cytokines were quantified by ELISA in aliquots of culture medium of organotypic normal epidermal keratinocytes and HDFi monocultures and cocultures. Medium was collected on days 2, 4, 6, 8, 10, i.e., always 48 h after medium change. ELISA kits for IL-1α were purchased from Endogen (Biozol, Eching, Germany), and for IL-1β and KGF from R&D Systems. Protein values in the supernatant of organotypic cultures were measured as pg per ml with standard deviations of data derived from duplicate measurements from two to three independent experiments.

Indirect immunofluorescence microscopy Cryosections at 4–6 µm were mounted on glass slides (Histobond, Medim) and fixed for 5 min in 80% methanol at 4°C followed by 2 min in acetone at –20°C, rehydrated in PBS, and blocked for 15 min in PBS with 1% bovine serum albumin. First antibodies were incubated overnight at 4°C in a moist chamber; for double labeling the two antibodies were applied simultaneously. After washing three times in PBS, the sections were incubated for 1 h at room temperature with species-specific, fluorochrome-conjugated secondary antibodies (Dianova, Hamburg, Germany) containing additionally 0.5 µg per ml bisbenzimide (Hoechst No. 33258) DNA dye for total nuclear staining. After three final washes with PBS, specimens were embedded in Mowiol (Medim) and examined and photographed using a Leica microscope (Leitz DMRBE, Bensheim, Germany) equipped with epifluorescence optics. To visualize proliferating cells, a monoclonal mouse anti-BrdU antibody (Dianova) was used. Proliferation was quantitated by counting the ratio of labeled cells within the basal keratinocyte layer of the epithelium. Differentiated keratinocytes were detected by mouse antihuman K1/K10 antibody (clone 8.60, Sigma) or mouse antihuman K2e antibody (Progen, Heidelberg, Germany), basement membrane components by rabbit antihuman collagen type IV antibody (generous gift of Dr. Jean-Michel, Foidart *et al*, 1980) or rabbit antihuman collagen type VII (generous gift of Dr. Leena Bruckner-Tudermann, Grassel *et al*, 1999).

RESULTS

Postmitotic fibroblasts support keratinocyte growth and differentiation comparable to their proliferating counterpart In organotypic cocultures, postmitotic (70 Gy X-irradiated) fibroblasts, at comparable numbers (2×10^5 cells per ml), had the same effect on keratinocyte growth promotion, differentiation, and structural organization as nonirradiated proliferating fibroblasts (**Fig 1**). The morphology of the reconstructed epidermis did not reveal significant differences throughout a culture period of 3 wk (**Fig 1*A*, *B*, *G*, *H***). Thus, the onset of expression and localization of epidermal differentiation markers, such as the early marker keratin 1/10 (**Fig 1*C*, *D*, *I*, *J***) or the late marker keratin 2e (**Fig 1*E*, *F*, *K*, *L***), as well as involucrin, transglutaminase, loricrin, and filaggrin (not shown), was virtually indistinguishable under the two conditions. Furthermore, this was true for cultures grown both in serum-containing (FAD) (**Fig 1**) and in defined (SKDM) culture medium (not shown), as demonstrated earlier for proliferating fibroblasts (Stark *et al*, 1999). Similar to this study, the expression of basement membrane components was slightly reduced in SKDM but not significantly different in cultures with HDFi, compared with proliferating fibroblasts (**Fig 1*C*–*F*, *I*–*L***).

In correlation with the unaltered morphogenesis, the percentage of proliferating basal cells in the reconstituted epidermis (immunolabeled after BrdU incorporation) does not manifest significant differences in the two culture types using proliferating or postmitotic fibroblasts (**Fig 2**). Again, nearly identical results were obtained in serum-containing (FAD) medium and in defined medium (SKDM).

Postmitotic fibroblasts are functionally active in the collagen matrix To demonstrate the viability and persistence of irradiated fibroblasts, their number was determined within a 12 d culture period and compared with the proliferating cells seeded on plastic and into collagen gels, respectively (**Fig 3**). Whereas the number of nonirradiated fibroblasts (HDF) increased with time, though to a lesser extent in collagen gels, the density of postmitotic fibroblasts (HDFi) initially decreased to 70%–80%, remaining rather constant thereafter. The persistent viability of irradiated fibroblasts inside the collagen gels was confirmed by vital staining over a 14 d culture period using the MTT test (not shown), and was comparable to cultures on plastic (see also Maas-Szabowski *et al*, 1999). The difference in cell density between HDF and postmitotic HDFi cultures became visible by histology of 3-wk-old cultures (see **Fig 1*G*, *H***). Presumably as a consequence of the lower final cell number, the rate of collagen contraction was considerably less with embedded HDFi starting with the same seeding density (2×10^5 cells per ml). Thus, in 10-d-old organotypic cocultures, proliferating fibroblasts had contracted the gels to 38.9% ± 3.9% of the original diameter, whereas the gel diameter was only reduced to 81.9% ± 7.8% by irradiated fibroblasts (both in FAD medium). This reduced collagen contraction by HDFi was even more obvious in defined culture medium (see also Stark *et al*, 1999).

Irradiated fibroblasts not only persisted over culture time but, for the most part, maintained their functional capacity to express characteristic paracrine acting growth factors (**Fig 4**). The RNA expression levels in postmitotic fibroblasts were generally lower than in proliferating cells but their relative ratios (compared to the internal GAPDH standard) were similar, thus confirming earlier data obtained in monolayer cultures (Maas-Szabowski and Fusenig, 1996).

Having achieved constant fibroblast numbers in organotypic cocultures throughout extended culture periods, the threshold number of mesenchymal cells required to support epidermal tissue organization could be determined. As shown earlier, keratinocytes are unable to reconstitute a normal epidermal tissue on collagen matrix in the absence of fibroblasts (in both FAD and SKDM). They lose their proliferative potential within the first two culture days (Smola *et al*, 1993, 1994), forming only a thin atrophic epithelium as seen in **Fig 5(*a*)**. A low density of postmitotic fibroblasts, up to 1×10^4 cells per ml, did not significantly augment keratinocyte proliferation and stratification (not shown), whereas this was clearly achieved with 5×10^4 cells per ml (**Fig 5*b***). Comparable to the effect of proliferating fibroblasts, 1×10^5 and 2×10^5 HDFi per ml gave rise to a multilayered keratinizing epithelium of similar thickness and differentiation within 1 wk (**Fig 5*c*, *d***). This clearly demonstrated that irradiated postmitotic fibroblasts are as potent as proliferating cells for supporting epidermal growth and differentiation in organotypic cultures and that a threshold number of 5×10^4–1×10^5 cells per ml is required.

Expression of cytokines and their receptors in organotypic cocultures The stimulating effect of fibroblasts on keratinocyte growth and differentiation was based on the expression and secretion of growth factors such as KGF (FGF-7), as observed recently by analysis of the separated keratinocyte and fibroblast compartments in two-dimensional (feeder-layer) cocultures (Maas-Szabowski *et al*, 1999). When an RNA analysis by RT-PCR was performed on the mechanically separated dermal equivalent, a strong enhancement of the constitutive KGF mRNA expression of HDFi was seen over 10 d of coculture compared with monocultures (**Fig 6*a***). Concomitantly, the expression of KGF receptor was increased in the cocultured keratinocytes compared with monocultures (**Fig 6*b***). Whereas IL-1α and IL-1β expression was higher in cocultured keratinocytes, though less strong than in two-dimensional cocultures on plastic, the IL-1 receptor type I was strongly upregulated in cocultured fibroblasts (**Fig 6*a***). These expression data were, for the most part, reflected in the protein levels of the culture supernatants collected at 2 d intervals in monocultures and cocultures, respectively (**Fig 7**). KGF protein was high already at day 2 and increased with time in coculture. Surprisingly, and in contrast to two-dimensional cultures, the overall KGF levels in the medium of HDFi monocultures in collagen gel were usually higher than in cocultures. Whereas IL-1α and IL-1β are barely or not at all detected in supernatants of HDF and HDFi monocultures in collagen, they are secreted in high amounts by keratinocytes in monocultures and cocultures. Comparable to two-dimensional cultures, initial high amounts of IL-1α were detected, which slightly declined over time, whereas

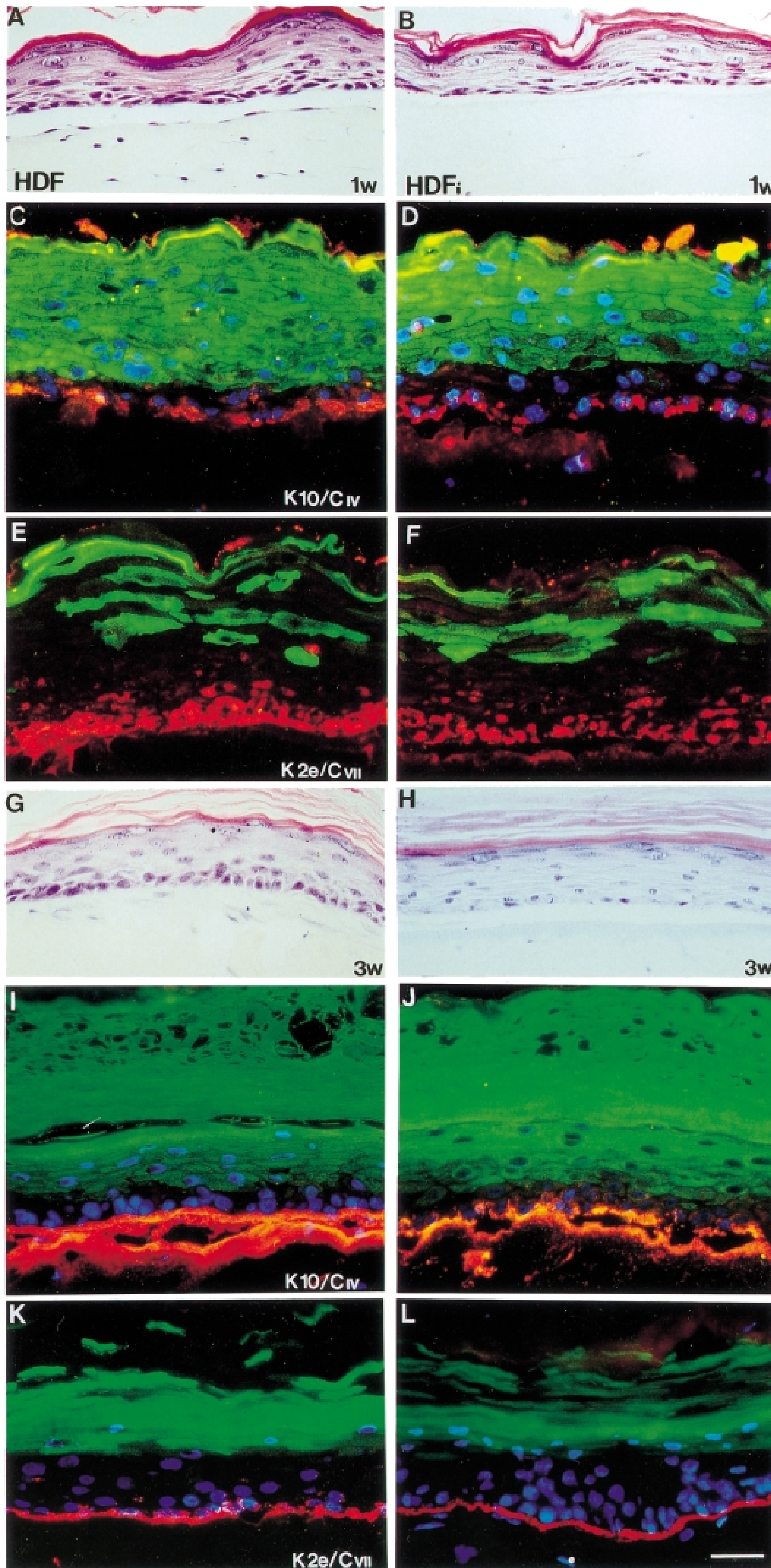

Figure 1. Epithelial growth and differentiation of keratinocytes is equally supported in organotypic cultures with proliferating or postmitotic fibroblasts embedded in the collagen matrix. Epidermal tissue morphology in cross-sections of organotypic cultures containing 2×10^5 proliferating (HDF, *A*, *G*) and postmitotic (70 Gy irradiated, HDFi, *B*, *H*) human dermal fibroblasts per ml collagen gel. Cultures were grown for 7 d (*A*, *B*) and 21 d (*G*, *H*) in serum-containing (FAD) medium prior to formalin fixation and processed for histology (hematoxylin and eosin staining). The cleft between epithelium and collagen gel represents a section artefact. By double immunofluorescent staining of cocultures with HDF (*C*, *E*, *I*, *K*) and HDFi (*D*, *F*, *J*, *L*) after 7 d (*C-F*) and 21 d (*I-L*) the early epidermal differentiation markers keratins 1/10 (in *green*) are visualized together with the basement membrane component collagen type IV (in *red*, *C*, *D*, *I*, *J*); in addition, the late differentiation marker K2e (in *green*) is labeled in combination with collagen type VII (in *red*, *E*, *F*, *K*, *L*). Nuclei were counterstained with bisbenzimide (in *blue*). *Scale bar*: 100 μm.

the values were rather constant, though lower, in cocultures. By comparison, the values of IL-1β in cocultures were higher than in keratinocyte monocultures. The discrepancies of the protein values with the RNA data as well as earlier findings in two-dimensional

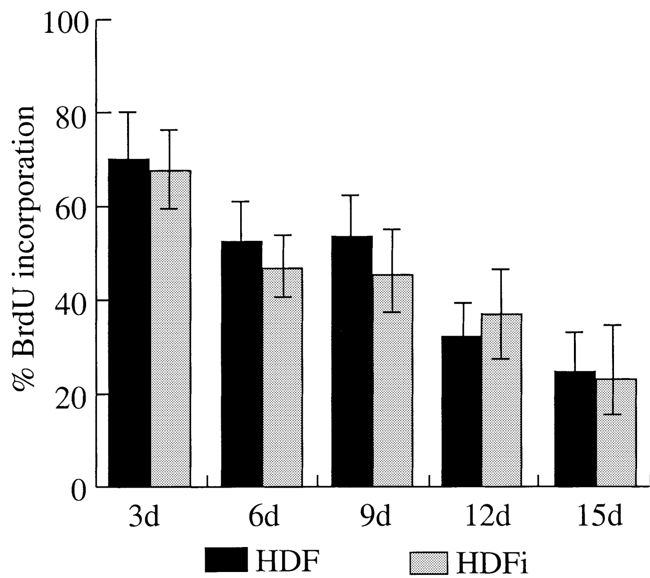

Figure 2. Kinetics of proliferative activity of basal keratinocytes in organotypic cocultures with HDF and HDFi, respectively. ■, HDF; ▒, HDFi. The percentage of basal cells with incorporated BrdU was analyzed after immunofluorescent staining and calculated as a percentage of BrdU-stained nuclei in total basal cell nuclei. *Bars* represent the means and standard deviations from three independent experiments counting nuclei in three vision fields per experiment.

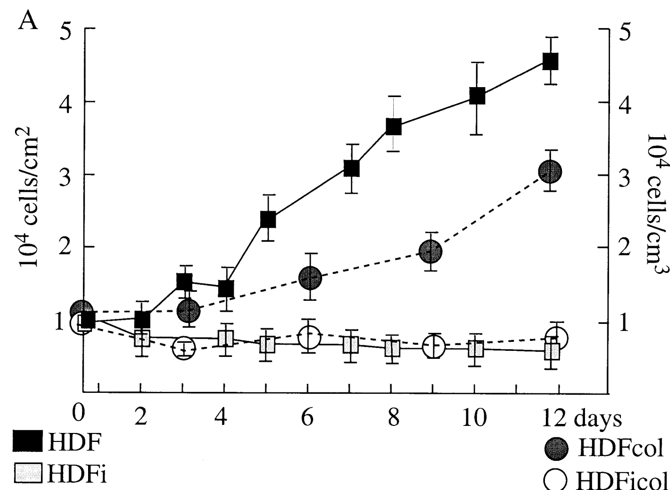

Figure 3. Postmitotic fibroblasts (HDFi) persist with a constant number on plastic and in collagen gels (col), whereas nonirradiated HDF continuously multiply. HDF and HDFi were seeded at a density of 1×10^5 per cm³ in collagen gel and 1×10^5 per cm² on plastic and grown in FAD medium. Every 2 d cells were harvested and counted in duplicate after trypsin detachment from plastic dishes and release from collagen gels, respectively. Data represent means with standard deviations from three independent experiments.

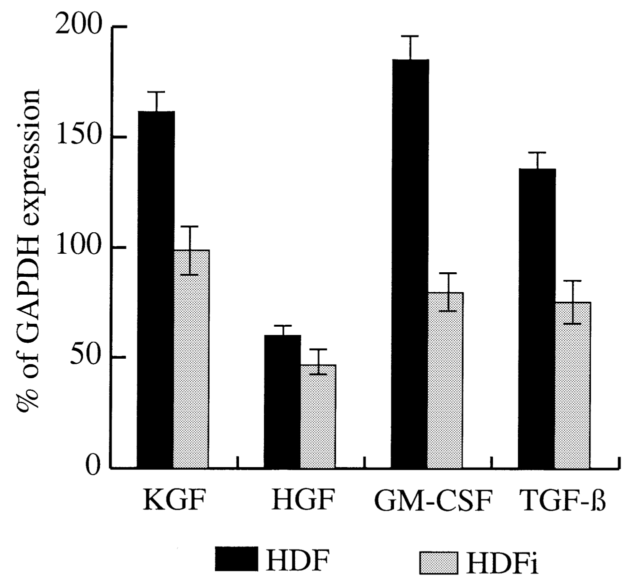

Figure 4. Expression levels of growth factors in monocultures of proliferating HDF and irradiated postmitotic HDFi embedded in collagen matrix. ■, HDF; ▒, HDFi. Two $\times 10^5$ cells per cm³ were seeded into collagen gels and RNA was extracted after 10 d of culture in FAD medium. For semiquantitative RT-PCR identical cycle numbers were used for GAPDH and each cytokine probe. Their expression rates were calculated as a percentage of GAPDH expression; *bars* represent means with standard deviations of duplicate measurements from two independent experiments.

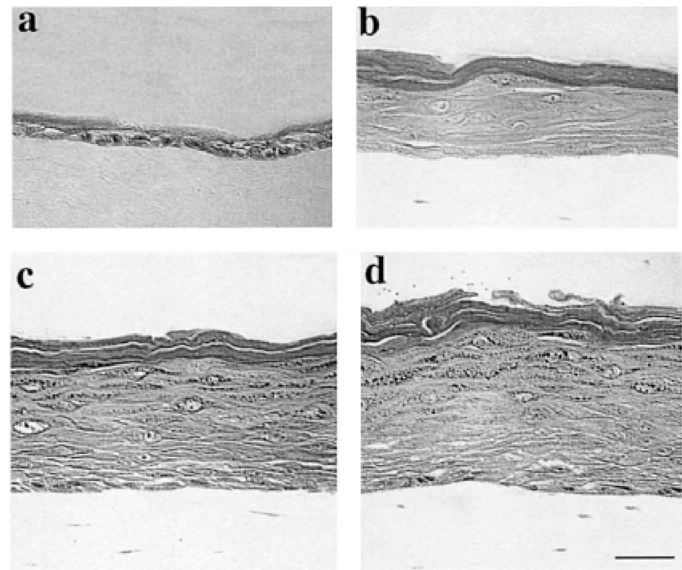

Figure 5. Effect of fibroblast numbers on epidermal tissue regeneration by keratinocytes. Histologic sections of 7-d-old organotypic cultures with no (*a*), 5×10^4 (*b*), 1×10^5 (*c*), and 2×10^5 (*d*) HDFi per ml in the collagen matrix. Hematoxylin and eosin; *scale bar*: 100 μm.

cultures may be due to the different geometry of the organotypic cultures as well as to absorption in the collagen matrix and will be discussed later.

Functional significance of IL-1 and KGF for keratinocyte growth regulation and tissue organization The implication of the postulated double paracrine signaling pathway between keratinocytes and fibroblasts involving IL-1 and KGF in keratinocyte growth regulation was documented both by addition of these factors and abrogation of their signaling. This was similarly confirmed in organotypic cultures under defined (**Fig 8**) as well as serum-containing (**Fig 9**) medium conditions. As neither IL-1 nor KGF had significant effects on keratinocyte growth and tissue organization in organotypic cultures in the absence of fibroblasts (not shown), the effects of factor addition on keratinocyte growth were tested in organotypic cocultures containing subthreshold postmitotic fibroblast numbers in the collagen gels (5×10^4 per ml) supporting only modest epidermal tissue formation within a 6 and 10 d culture period (**Fig 8*a*, *d***). KGF (10 ng per ml) significantly stimulated keratinocyte proliferation (measured by BrdU incorporation, not shown) and restored epidermal tissue formation to the extent seen in the presence of optimal (2×10^5

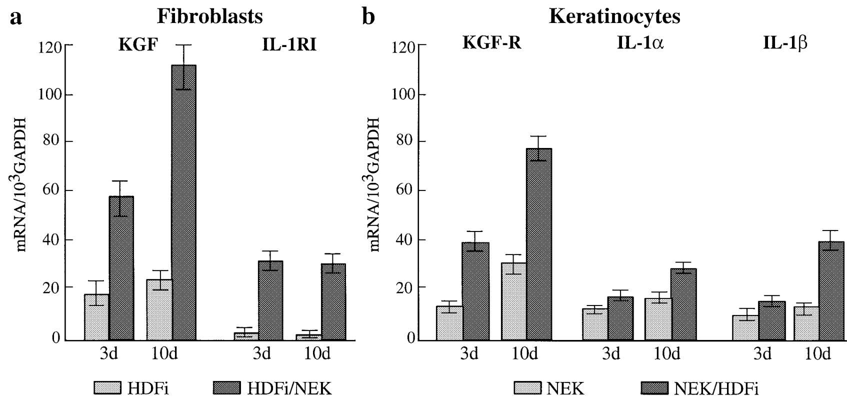

Figure 6. Cytokine and receptor expression in organotypic monocultures and cocultures by postmitotic fibroblasts (HDFi) and keratinocytes (NEK) grown for 3 and 10 d in FAD medium. ■, Cocultures; ■, monocultures. (*a*) HDFi; (*b*) NEK. Expression levels were determined by semiquantitative RT-PCR. mRNA values are calculated based on GAPDH expression. *Bars* represent the means with standard deviations of duplicate determinations from three independent experiments.

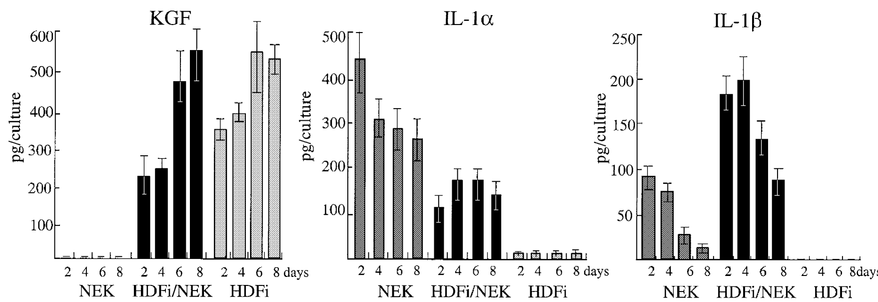

Figure 7. Kinetics of KGF, IL-1α, and IL-1β concentrations in the culture supernatants of postmitotic fibroblasts (HDFi) and normal epidermal keratinocyte (NEK) in organotypic monocultures and cocultures over an 8 d culture period. Cytokine levels were determined in aliquots of 2-d-conditioned media by ELISA and calculated as pg per organotypic culture. *Bars* represent means with standard deviations of duplicate measurements performed in three independent experiments.

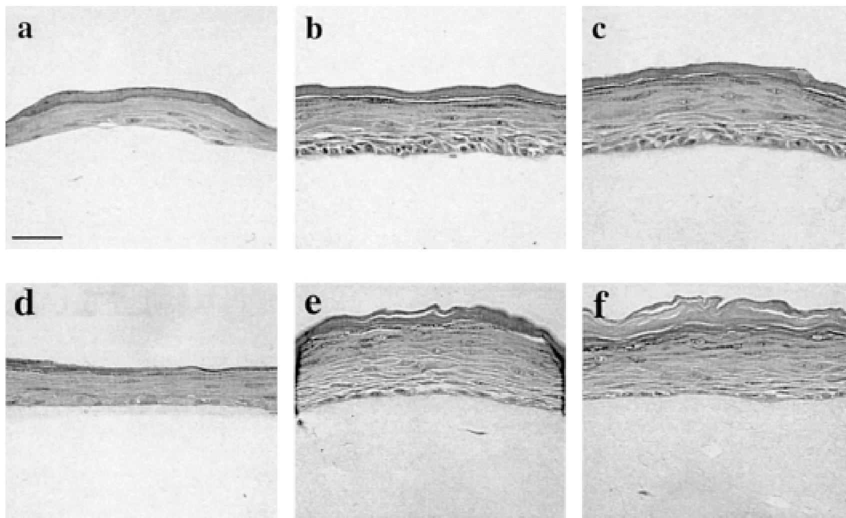

Figure 8. Keratinocyte growth stimulation by KGF and IL-1 in organotypic cocultures with suboptimal fibroblast numbers. Histology of organotypic cocultures containing 5×10^4 HDFi per ml collagen gel after growth for 6 d (*a-c*) and 10 d (*e-g*) in defined medium (SKDM). This subthreshold number of HDFi only supports poorly stratified epidermal tissues (*a*, *d*). Tissue thickness, organization, and differentiation are fully restored to control levels by the addition of KGF (10 ng per ml; *b*, *e*) and IL-1α (10 ng per ml; *c*, *f*), respectively. Factors were renewed with medium change every 2 d (hematoxylin and eosin staining). *Scale bar*: 100 μm.

per ml) fibroblast numbers (**Fig 8*b***, ***e***). Essentially the same effect was induced by addition of IL-1α (10 ng per ml) (**Fig 8*c*,*f***), although IL-1α and IL-1β have no direct stimulatory effect on keratinocyte proliferation in monocultures, as shown earlier (Maas-Szabowski *et al*, 1999). After the addition of either KGF or IL-1α, in cocultures with subthreshold fibroblast numbers epidermal differentiation exhibited features indistinguishable from those with optimal fibroblast numbers (not shown). These data demonstrate an essential regulatory role of KGF and IL-1, respectively, in keratinocyte proliferation and differentiation in an *in vivo*-like culture model.

To confirm the direct function of KGF and the indirect effect of IL-1 in this double paracrine pathway, both parts were individually inhibited in organotypic cocultures prepared with optimal fibroblast cell numbers. Under defined (not shown) as well as serum-containing culture conditions (**Fig 9**), blockade of either KGF, by a neutralizing antibody (**Fig 9*c***), or the IL-1 pathway, by IL-1α-neutralizing antibodies and IL-1RA (**Fig 9*b***), drastically suppressed epithelial tissue development, which resembled cocultures with subthreshold fibroblast numbers (see **Fig 8*a***). Moreover, addition of KGF to cocultures with blocked IL-1 signaling fully rescued epidermal growth and tissue development (**Fig 9*d***). This also included the typical expression and localization of differentiation markers (keratin K1/10, filaggrin, involucrin) as seen in control cultures (not shown). In agreement with these functional data, blockade of IL-1 signaling abrogated the coculture-induced increase in KGF expression as well as factor release into the culture medium (not shown; see also Maas-Szabowski *et al*, 1999).

Finally, the efficacy of this signaling blockade of keratinocyte proliferation became even more evident when the proliferative activity of basal cells was determined by BrdU incorporation (**Fig 10**). Both inhibition of IL-1 by IL-1RA (not shown) or IL-1α-neutralizing antibodies and neutralizing KGF antibodies resulted in a 40%–50% reduction of labeled cells. In turn, inhibition of keratinocyte proliferation by IL-1α antibodies was restored to normal levels by the addition of KGF (**Fig 10**). Comparable results

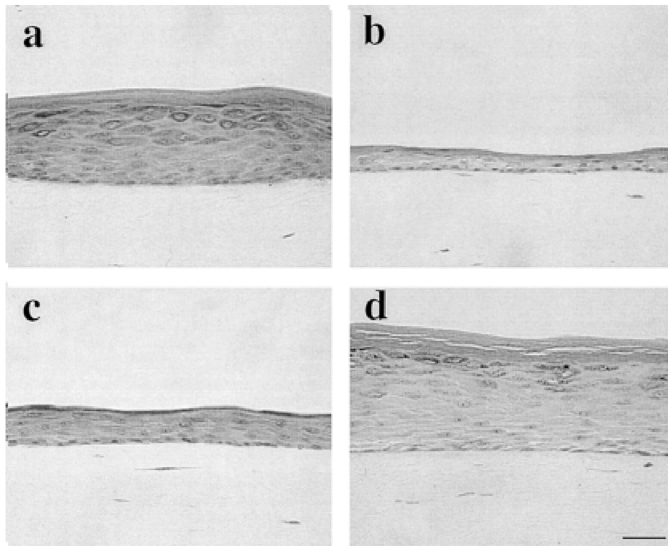

Figure 9. Inhibition of epidermal regeneration by blocking IL-1 and KGF, respectively. Histology of organotypic cocultures with optimal fibroblast numbers (2×10^5 HDFi per ml collagen gel) grown for 7 d in FAD medium (*a*, control) with IL-1α antibody (1 μg per ml) together with IL-1RA (100 ng per ml) (*b*) or with KGF neutralizing antibody (1 μg per ml) (*c*). Addition of KGF (10 ng per ml) fully restored growth inhibition caused by IL-1α antibody (1 μg per ml) and IL-1RA (100 ng per ml) (*d*). Hematoxylin and eosin; *scale bar*: 100 μm.

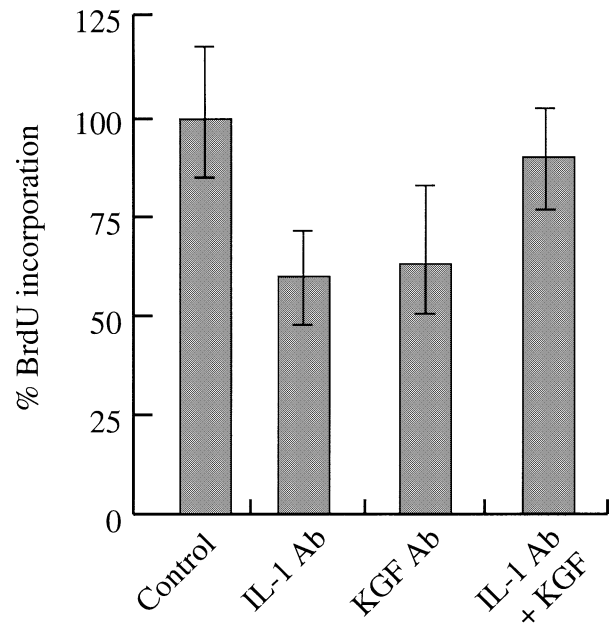

Figure 10. Decrease of keratinocyte proliferation in organotypic cocultures by blocking the IL-1α and KGF function. Proliferation was evaluated by BrdU incorporation in organotypic cocultures with 2×10^5 HDFi per ml collagen gel, grown for 7 d in FAD medium (control), in medium containing neutralizing antibodies (Ab) to IL-1α (1 μg per ml), KGF antibodies (1 μg per ml), or IL-1α antibody (1 μg per ml) together with KGF (10 ng per ml). *Bars* represent the percentage of BrdU-positive basal keratinocytes (means with standard deviations) detected on frozen sections with BrdU antibodies. Three vision fields per experiment were counted in four independent experiments.

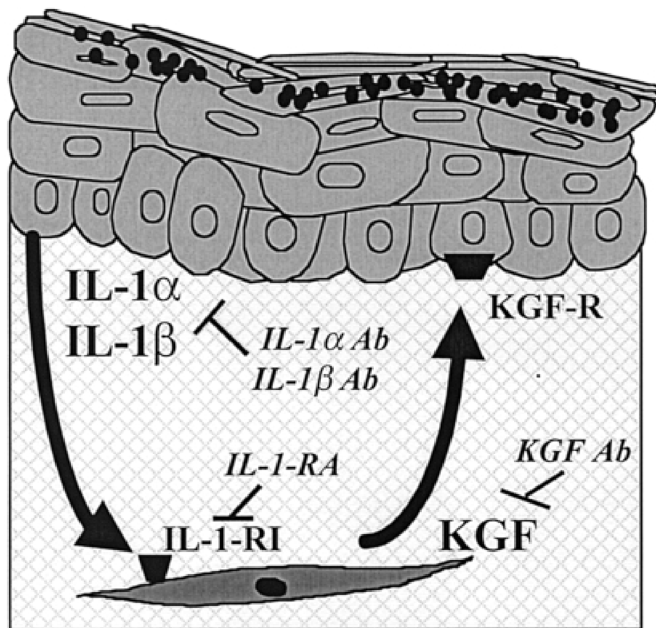

Figure 11. Schematic illustration of the double paracrine pathway of keratinocyte growth regulation in organotypic cocultures with fibroblasts involving IL-1 and KGF and their receptors.

were obtained in serum-supplemented and defined culture medium.

These data confirmed the functional significance of a double paracrine pathway between keratinocytes and fibroblasts involving IL-1 and KGF in organotypic cocultures, as identified earlier in two-dimensional feeder-layer cultures (Maas-Szabowski *et al*, 1999). This novel mechanism of keratinocyte-fibroblast interplay represents an essential regulatory mechanism in these *in vitro* skin equivalents and is potentially operative in skin regeneration *in vivo* as well (**Fig 11**).

DISCUSSION

Tissue regeneration as well as maintenance of homeostasis in rapidly renewing epithelia, such as the epidermis, require proper functioning of control mechanisms of growth and differentiation. In skin, this is mostly regulated by epithelial–mesenchymal interactions and mediated by diffusible factors (for review see Fusenig, 1994; Werner, 1998). In order to study the molecular mechanisms of this regulation under defined *in vitro* conditions while maintaining the major principles of skin biology, three-dimensional organotypic cocultures were developed with keratinocytes growing on a matrix populated with fibroblasts. By mimicking the natural tissue architecture, although in a simplified form, such conditions enable keratinocytes to recapitulate their tissue-specific growth and differentiation program *in vitro* (Bell *et al*, 1981; Asselineau *et al*, 1986; Parenteau *et al*, 1991; Contard *et al*, 1993; Fusenig, 1994). The function of diffusible factors mediating epithelial–mesenchymal interactions and the dynamics of basement membrane formation have been studied in such organotypic cocultures (Boxman *et al*, 1993; Smola *et al*, 1993, 1998). Still, the complex multifactorial interaction between keratinocytes and dermal fibroblasts in regulating tissue regeneration is poorly understood, partly due to the undefined culture conditions in such models. Quite recently, we have demonstrated that organotypic cocultures consisting of normal skin keratinocytes and fibroblasts equally well reconstitute a normal epidermal tissue architecture when grown in defined medium (Stark *et al*, 1999). Although slight differences in ultrastructure as well as basement membrane formation have previously been observed under these defined medium conditions, this culture model represents an essential step forward for standardizing culture conditions. Although this had no major effect on the analysis and neutralization of KGF and IL-1, factors produced in high amounts in the culture medium and which are present in low amounts or absent in serum, this may be different with other members of the fibroblast growth factor family or other

factors such as transforming growth factor β that are present in latent form in serum. Defined medium may also be advantageous when other cytokines, produced in low amounts and which may unspecifically bind to serum protein components, need to be analyzed.

In this report, we have further improved the organotypic coculture system by using fibroblasts rendered permanently postmitotic by high-dose X-irradiation and demonstrating that they are equally capable of maintaining normal keratinocyte proliferation and differentiation. As demonstrated earlier in two-dimensional cultures, these (heavily) irradiated fibroblasts are still vital, as far as cellular integrity, expression and secretion of growth factors and cytokines, and the response to specific inducers are concerned (Limat *et al*, 1989; Waelti *et al*, 1992; Smola *et al*, 1993, 1994; Bumann *et al*, 1995; Maas-Szabowski and Fusenig, 1996; Maas-Szabowski *et al*, 1999). The reduced level of expression, compared with nonirradiated, proliferating fibroblasts, may be attributed to disturbances in the expression and translation machinery of subpopulations due to X-ray-induced DNA damage. As these are most probably random effects damaging specific genes only in a certain number of cells, the cell population as a whole is still able to function properly, although quantitatively at a reduced rate (see also Maas-Szabowski and Fusenig, 1996, for further discussion).

For organotypic cocultures, the use of postmitotic cells has several advantages. (i) The fibroblast number is kept constant throughout extended culture periods, at least up to 3 wk. In contrast, proliferating fibroblasts, depending on the culture system and collagen concentration, multiply at different rates in the matrix, eventually populating the filter surface as well and thus causing considerable variation in cell numbers. (ii) Based on this maintenance of a stable fibroblast population, we could for the first time determine reliable cell numbers required for sustaining minimal or promoting optimal epidermal growth and differentiation. This also allowed us to establish organotypic cultures with subthreshold cell numbers in order to study the effect of added specific growth factors at given time points. With proliferating fibroblasts, different cell densities could not be maintained because of the different proliferative activity of fibroblasts depending on their seeding density (Coulomb *et al*, 1989). (iii) Importantly, the irradiated postmitotic fibroblasts resemble more closely the resting stage exhibited by the majority of fibroblasts in normal dermis. (iv) Finally, the resting fibroblasts also reduced the collagen gel contraction, a problem usually encountered with proliferating fibroblasts in collagen gels (Stark *et al*, 1999).

Using such postmitotic fibroblasts in two-dimensional cocultures (on plastic) with normal keratinocytes, we have recently elucidated a novel mechanism of epithelial–mesenchymal interactions based on a dynamic and reciprocal modulation of cytokine and growth factor production in both keratinocytes and fibroblasts (Smola *et al*, 1993; Maas-Szabowski *et al*, 1999). Here, we could provide functional evidence that this double paracrine mechanism of growth regulation is also operative in organotypic coculture systems. Via release of IL-1, keratinocytes elicit the enhanced expression of growth factors, particularly KGF, in fibroblasts and in this way promote their own proliferation. It had been shown earlier that KGF expression is strongly induced in proliferative and postmitotic fibroblasts by IL-1 (Chedid *et al*, 1994; Maas-Szabowski and Fusenig, 1996). Whereas the cytokines IL-1α and IL-1β themselves had no immediate effect on keratinocyte proliferation in monocultures (Maas-Szabowski *et al*, 1999, and this report), abrogation of their function in cocultures inhibited keratinocyte proliferation to an extent comparable with that obtained by neutralizing the potent KGF. Moreover, blocking IL-1 signaling abrogated the coculture-induced enhanced amount of KGF release into the culture medium (Maas-Szabowski *et al*, 1999, and this report).

There are several reports demonstrating a stimulatory effect of IL-1 on keratinocyte proliferation, but most data have been obtained in intact skin following irradiation (Blanton *et al*, 1989) or intradermal injection of IL-1 or IL-1 antibodies (Granstein *et al*, 1986; Oberyszyn *et al*, 1993). Obviously, under these *in vivo* conditions, discrimination between a direct and an indirect, fibroblast-mediated, proliferative stimulus of IL-1 on keratinocytes is not possible. Similarly, in the light of our studies, the reported IL-1 effects on keratinocyte proliferation in culture most probably represent an indirect action of IL-1, because most of these studies were performed in feeder-layer cultures of adult skin keratinocytes or on foreskin keratinocytes grown in serum-free medium (Pillai *et al*, 1988; Chen *et al*, 1995). The presence of functionally active fibroblasts has been demonstrated in the case of feeder-layer cultures (Maas-Szabowski and Fusenig, 1996; Maas-Szabowski *et al*, 1999). In the case of foreskin-derived keratinocyte cultures (even in low-calcium serum-free medium) variable numbers of fibroblasts are present due to the difficulty of properly separating the epidermis from the loose mesenchyme. Minor fibroblast levels, though virtually undetectable in keratinocyte mass cultures but apparent in cloning assays, were clearly at work expressing KGF (monitored by RT-PCR) and thus mimicked coculture effects (unpublished own data). In our hands, in keratinocyte monocultures, which were controlled for the absence of fibroblast contamination by RT-PCR-determined KGF expression (only expressed in fibroblasts), no significant stimulation of proliferation by recombinant IL-1α or IL-1β was observed (Maas-Szabowski *et al*, 1999). This is in agreement with an earlier study reporting no stimulation of proliferation by IL-1 in growth-arrested human keratinocytes (Ristow, 1990).

The data obtained in this study clearly document that a double paracrine pathway involving IL-1 and KGF is operative in the more *in vivo*-like organotypic cocultures. In addition to the reciprocal cytokine expression observed in two-dimensional cocultures, keratinocytes in organotypic cultures not only upregulate IL-1α and IL-1β, although less so than on plastic, but the KGF receptor as well. Similarly, the fibroblasts cocultured in the collagen gel exhibit enhanced expression not only of KGF but also of the IL-1 receptor type I. This combination of upregulation of the inducer in one cell type and its cognate receptor in the other will lead to increased sensitization of the signaling pathways. The upregulation of IL-1 receptor type I, not observed in two-dimensional cocultures, may explain the rather low increase in expression of IL-1, because low amounts of IL-1 may be sufficient to strongly stimulate KGF expression via an upregulated receptor level. Comparable to two-dimensional cultures, the amount of growth factors determined in the supernatant of cocultures and monocultures does not reflect the kinetics of mRNA expression except for the level of IL-1β, which is always higher in cocultures than in monocultures. The initial high levels of IL-1 in monocultures may be due to release from preformed pools caused by injury or culture stress, as also seen *in vivo* (Werner *et al*, 1992; Kupper and Groves, 1995; Wood *et al*, 1996; Maas-Szabowski *et al*, 1999). Similarly, release of IL-1 has been observed after mechanical stress of cultured keratinocytes (Lee *et al*, 1997; Takei *et al*, 1998). Presumably both IL-1α and IL-1β are active in these cocultures, because IL-1α is released in its active form and keratinocytes as well as fibroblasts are able to activate IL-1β (Maas-Szabowski *et al*, 1999, and further references therein). The IL-1α levels released into the culture medium are much higher in monocultures than in cocultures but this discrepancy with regard to the RNA data may be explained by capture of the released cytokine factor by its receptor on cocultured fibroblasts, as also suggested by others (Boxman *et al*, 1996).

Although this release of IL-1 induced strong upregulation of KGF expression at the mRNA level, the protein amount detected in conditioned medium was lower than that in monocultured fibroblasts in collagen gel. This may be due to an upregulation of basic KGF production in three-dimensional cultures in collagen, compared with cultures on plastic, and/or to binding of KGF to its strongly upregulated receptor on keratinocytes. Thus, the induction of KGF in fibroblasts, in addition to the upregulation of KGF

receptor in keratinocytes, is most probably the major, though not only, mediator of enhanced keratinocyte proliferation caused by the cocultured fibroblasts. This was most clearly demonstrated by the functional studies both by addition of the factors as well as inhibition of their function. In keratinocyte monocultures on collagen gel, i.e., in the absence of fibroblasts, the addition of even high amounts of KGF (or other growth factors) was not sufficient to induce sustained keratinocyte proliferation and formation of an epidermal tissue architecture (results not shown). Therefore, other fibroblast-released factors are additionally important for keratinocyte proliferation and differentiation. On the other hand, due to the ability to titrate the minimum numbers of postmitotic fibroblasts, organotypic cocultures with subthreshold fibroblast concentrations turned out to be excellent model systems to test the efficacy of added growth factors and cytokines. Thus, the addition of KGF and IL-1 completely replaced the function of the missing fibroblasts in restoring a normal epidermal tissue. By this, the essential function of both factors in keratinocyte growth regulation, but also their dependence on other components produced by fibroblasts, was clearly demonstrated. Moreover, blockade of the IL-1 pathway by either neutralizing antibodies or the soluble IL-1RA reduced epidermal regeneration to a level seen under suboptimal fibroblast support. Furthermore, KGF release into the medium, which was stimulated in cocultures, was abolished when the IL-1 signaling pathway was blocked, as also seen in two-dimensional cocultures (Maas-Szabowski *et al*, 1999). Even more convincingly, external addition of KGF could completely reverse the IL-1 blockade-mediated inhibition of epidermal tissue regeneration. We believe that by these studies the functional significance of a double paracrine pathway, by which keratinocytes through IL-1 release direct their own growth factor expression in fibroblasts, has also been proven in organotypic cultures. With these data derived from a more *in vivo*-like culture model, we were able to accumulate further evidence that this regulatory mechanism may also be relevant for the *in vivo* situation in skin as well as other organs, as may be postulated from wound healing studies (Werner *et al*, 1992; Marchese *et al*, 1995) and in hormone-dependent organ development of the prostate (Alarid *et al*, 1994).

The fact that blockade of this pathway did not lead to complete halting of keratinocyte proliferation may be explained by the inefficiency of the blocking antibody concentration or receptor antagonist used in these studies, as also seen in two-dimensional cultures (Maas-Szabowski *et al*, 1999). It is most likely, however, that the partial inhibition of keratinocyte proliferation by interfering with one growth factor signaling is due to the redundancy of factors involved in keratinocyte growth regulation, as also indicated by the expression (this paper) as well as induction (Maas-Szabowski and Fusenig, 1996; Maas-Szabowski *et al*, 1999; unpublished results) of other known growth factors by fibroblasts that may have a complementary or additional role in regulating keratinocyte growth and differentiation (Werner, 1998). With this more defined organotypic coculture system, the identification of further players in the complex epithelial–mesenchymal interaction is substantially facilitated, as is a thorough investigation of the intracellular signaling pathways involved using genetically modified fibroblasts from transgenic animals.

We thank Brigitte Nagel-Plagens and Dirk Breitkreutz for critically reading and Martina Kegel for expert typing of the manuscript. This work was supported by grants from the Deutsche Forschungsgemeinschaft (DFG Fu 91/4–1) and the European Commission (BIO4-CT 96–0036 and BE96–3524) as well as grants from Wella-Cosmital SA, Marly, Switzerland, and Beiersdorf AG, Hamburg, Germany.

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