Modulation of IL-6 Production and IL-1 Activity by Keratinocyte-Fibroblast Interaction

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The present study was undertaken to investigate whether modulation of interleukin-6 and interleukin-1 production occurs owing to keratinocyte-fibroblast interaction. Normal human keratinocytes or squamous carcinoma cells were cultured either alone or in the presence of human foreskin fibroblasts or murine 3T3 cells. All cells tested produced interleukin-6, and interleukin-6 levels were markedly increased when normal or malignant keratinocytes were co-cultured with fibroblasts. The bioassay (species independent) and enzyme-linked immunosorbent assay (specific for human interleukin-6) together with use of complementary DNA probes specific for human or murine interleukin-6 revealed that fibroblasts are responsible for increased interleukin-6 levels. Moreover, interleukin-6 levels were increased when fibroblasts were cultured in conditioned media derived from normal human keratinocytes and squamous carcinoma cells-4 cultures. Interleukin-1α secreted by normal human keratinocytes and squamous carcinoma cells-4 cells was mainly responsible for the increased interleukin-6 production in fibroblasts. Although interleukin-1 activity increased linearly with the incubation time in squamous carcinoma cells-4 monocultures, interleukin-1 activity was low and remained unchanged in squamous carcinoma cells-4/3T3 co-cultures. Low interleukin-1 activity was most probably not due to inhibition of interleukin-1α production in squamous carcinoma cells-4/3T3 co-cultures because interleukin-1α messenger RNA expression in squamous carcinoma cells-4 cells remained unchanged in the presence of 3T3 cells. Furthermore, when 3T3 cells were incubated in conditioned medium derived from squamous carcinoma cells-4 cells, high interleukin-1 activity decreased to an undetectable level, suggesting that fibroblasts are involved in the suppression of interleukin-1 activity. The remaining interleukin-1 activity, however, was sufficient for maximal induction of interleukin-6 production in fibroblasts. These results suggest that the interaction between epithelial and mesenchymal cells is at least partly initiated by interleukin-1α secreted by the activated epithelial cell during skin injury or tumor invasion.

IL-1 in turn can induce modulation of the synthesis of various pro-inflammatory mediators and proteases in surrounding fibroblasts. An enhanced proteolytic activity and/or a possible induced production of an interleukin-1 inhibitor in fibroblasts and/or a receptor-mediated interleukin-1 consumption by fibroblasts will cause a decrease in interleukin-1 activity and thus exert a negative feedback. Key words: interleukin-6/interleukin-1/normal human keratinocytes/squamous carcinoma cells/fibroblast co-cultures.

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Cell to cell interaction plays an important role in a number of pathologic conditions in which cells normally located in different compartments come in close vicinity to each other. This occurs, for example, during wound healing or tumor invasion. Under these circumstances, the interaction of epithelial and mesenchymal cells may lead to the modulation of the production and secretion of various proteins, such as proteases, cytokines, growth factors, and hormones.

One of the cytokines that is known to play an important role in immune response and tissue repair is interleukin-6 (IL-6) [1-4]. IL-6 is produced by a variety of cells and acts on a wide range of tissues. The effects exerted are growth-inducing, growth-inhibiting, and differentiation-inducing effects, depending on the nature of the target cells. Macrophages and fibroblasts were originally identified as the predominant cellular source of IL-6 [5]. Recently, it has been demonstrated that this cytokine is produced by many cells throughout the body, including those of the epidermis. Both normal human epidermal keratinocytes and squamous carcinoma cells have also been found to secrete IL-6 [6,7]. The IL-6 production by keratinocytes can be increased by treatment with various stimuli, including IL-1α, IL-1β, tumor necrosis factor-α, granulocyte-macrophage colony-stimulating factor, tumor promoters, bacterial endotoxins, and ultraviolet B irradiation [4,8]. Although epidermal keratinocytes and dermal fibroblasts were clearly shown to produce IL-6, the information on the modulation of IL-6 production as a...
result of mutual interaction of these cells is scarce. Waelti et al. [9] published a paper in which they showed elevated levels of IL-6 in co-cultures of human keratinocytes and post-mitotic human fibroblasts. They attributed the increase in IL-6 production in fibroblasts to IL-1α derived from keratinocytes. Furthermore, they suggested that direct contact between keratinocytes and fibroblasts was necessary for the observed effect. In the present investigation, the modulation of IL-6 production and IL-1 activity in keratinocyte-fibroblast co-cultures was studied in more detail. We also investigated whether or not the modulation of IL-6 levels during epithelial—mesenchymal interaction is a more common phenomenon. For this purpose, normal human keratinocytes (NHK) or squamous carcinoma cells (SCC) were cultured either alone or in the presence of human foreskin fibroblasts (HFF) or murine 3T3 cells. Cells of murine and human origin used in combination with a bioassay (species independent) or enzyme-linked immunosorbent assay (ELISA) (detecting only human IL-6) together with Northern blot analysis [using complementary DNA (cDNA) probes specific for either human or murine IL-6] revealed that IL-6 levels were markedly increased in keratinocyte—fibroblast co-cultures, and that the fibroblasts were responsible for the observed effect. Furthermore, we established that IL-1α, originating from the keratinocytes, was responsible for the increase in IL-6 production. The results of the present study led us to propose a model for the interaction between epithelial and mesenchymal cells that involves signaling by diffusible cytokines such as IL-6 and IL-1α.

MATERIALS AND METHODS

Cell Culture

**Serial Cultures:** Normal human keratinocytes derived from juvenile foreskin, squamous carcinoma cells (SCC-4, SCC-9, SCC-12B2, SCC-12F2, SCC-13, and SCC-15) (kindly provided by Dr. J. Rheinwald [10]) were cultured using the Rheinwald-Green feeder technique [11], as described earlier [12]. The cells were grown in a mixture of Dulbecco's modified Eagle's medium/Ham's F12 media (3:1) supplemented with 5% fetal calf serum, 1 μM hydrocortisone. Medium used for NHK was further supplemented with 1 μM isoproterenol and 10 ng epidermal growth factor (EGF)/ml; 3T3 cells and HFF were cultured in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum.

**Mono- and Co-cultures:** For cultures, 1.25 × 10⁶ NHK, HFF, 3T3, or SCC cells were seeded/well. In some experiments, NHK or SCCs were seeded together with 1.80 × 10⁶ irradiated 3T3 cells or HFF/well (6-well plates, Costar). In all experiments the cells were cultured in a mixture of Dulbecco's modified Eagle's medium and Ham's F12 (3:1) media supplemented with 5% fetal calf serum and with or without 1 μM hydrocortisone. The medium was changed on days 3 and 6 and the supernatants were collected on days 3, 6, and 9.

**Two-Chamber System:** In experiments in which direct contact of SCC and fibroblasts was prevented, the cells were cultured in a two-chamber system. Under these conditions, the fibroblasts were plated onto a porous membrane (Nucleopore; pore size 3.0 μm; Transwell Costar), and SCC cells on the bottom of the well (6-well plates, Costar). Both cell types were first allowed to attach for 4 h and subsequently the fibroblast-populated inserts were transferred into a 6-well cluster containing the SCC cells on the bottom. At indicated intervals, the supernatants were collected and cells harvested by trypsinization.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>IL-6 Production (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCC-9</td>
<td>19,400 120,000 8,240</td>
</tr>
<tr>
<td>SCC-13</td>
<td>21,540 120,000 27,100</td>
</tr>
<tr>
<td>SCC-12F2</td>
<td>7,500 20,000 1,600</td>
</tr>
<tr>
<td>SCC-12B2</td>
<td>23,770 29,370 1,200</td>
</tr>
<tr>
<td>SCC-15</td>
<td>38,200 27,800 11,405</td>
</tr>
<tr>
<td>3T3</td>
<td>1,800 1,100 400</td>
</tr>
</tbody>
</table>

*1.25 × 10⁶ SCC were seeded in the presence (+) or absence (−) of 1.80 × 10⁶ lethally irradiated 3T3 cells and grown in culture medium supplemented with 5% fetal calf serum and 1 μM hydrocortisone. The medium was changed on days 3 and 6 and the supernatants were collected on day 3, 6, and 9. IL-6 levels in the supernatants were measured using the bioassay. The results are expressed as pg/ml total production.*
**Figure 2.** Effect of prevention of direct cell to cell contact on IL-6 production in co-cultures of SCC-4 cells with fibroblasts. For co-cultures grown on the same surface, 1.25 X 10^5 SCC-4 cells were plated together with 1.8 X 10^5 fibroblast-populated inserts. For co-cultures in which direct cell to cell contact was prevented, 1.25 X 10^5 HFF or 3T3 cells were plated on a Nucleopore filter and 1.25 X 10^5 SCC-4 cells were plated on the bottom of the dish (6-well plates, Costar). Both cell types were allowed to attach for 4 h and subsequently the fibroblast-populated inserts were transferred into a 6-well cluster containing the fibroblasts. Subsequently, the fibroblast-populated inserts were transferred into 54-cm^2 dishes and cultured in separate wells. At indicated intervals the cells were trypsinized and aliquots of cell suspensions were either frozen at -20 °C or lethally irradiated (. ) and DNA determination was performed. For this purpose, the cells were washed with phosphate-buffered saline and kept frozen at -20 °C until use. After thawing, the cells were harvested and the DNA content was determined using a fluorometric method [14].

**Table II.** Comparison of IL-6 Production by Fibroblasts and Keratinocytes as Assayed by Bioassay and by ELISA

<table>
<thead>
<tr>
<th>Cell</th>
<th>Bioassay</th>
<th>ELISA</th>
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<tbody>
<tr>
<td>HFF</td>
<td>400</td>
<td>260</td>
</tr>
<tr>
<td>HFF*</td>
<td>300</td>
<td>262</td>
</tr>
<tr>
<td>NHK</td>
<td>160</td>
<td>&lt;100</td>
</tr>
<tr>
<td>SCC-4</td>
<td>172</td>
<td>&lt;100</td>
</tr>
<tr>
<td>NHK (HFF*)</td>
<td>30,000</td>
<td>&gt;35,000</td>
</tr>
<tr>
<td>SCC-4 (HFF*)</td>
<td>38,600</td>
<td>&gt;35,000</td>
</tr>
<tr>
<td>3T3</td>
<td>1,500</td>
<td>&lt;100</td>
</tr>
<tr>
<td>3T3*</td>
<td>1,800</td>
<td>&lt;100</td>
</tr>
<tr>
<td>NHK (3T3*)</td>
<td>20,000</td>
<td>&lt;100</td>
</tr>
<tr>
<td>SCC-4 (3T3*)</td>
<td>17,000</td>
<td>260</td>
</tr>
</tbody>
</table>

* Monocultures were started by seeding 1.25 X 10^5 HFF, NHK, SCC-4, and 3T3 cells. When irradiated cells were used, 1.8 X 10^5 cells were plated. Co-cultures were started by seeding 1.25 X 10^5 SCC-4 or NHK together with 1.8 X 10^5 lethally irradiated ( ) HFF and 3T3 cells. The culture medium used was supplemented with 5% fetal calf serum and 1 μM hydrocortisone. IL-6 levels were measured in supernatants collected on days 3 of culture using the IL-6 bioassay or the human IL-6 specific ELISA (detection limit > 100 pg/ml).

**Table III.** Effect of Culture Conditions on IL-6 Levels in Supernatants of SCC-4 and 3T3 Cell Cultures

<table>
<thead>
<tr>
<th>Cell</th>
<th>Medium</th>
<th>Hydrocortisone</th>
<th>IL-6 (pg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCC-4</td>
<td>Control M</td>
<td>-</td>
<td>255</td>
</tr>
<tr>
<td>SCC-4</td>
<td>Control M</td>
<td>+</td>
<td>106</td>
</tr>
<tr>
<td>SCC-4</td>
<td>CM-3T3</td>
<td>-</td>
<td>258</td>
</tr>
<tr>
<td>SCC-4</td>
<td>CM-3T3</td>
<td>+</td>
<td>13</td>
</tr>
<tr>
<td>SCC-4</td>
<td>Control M</td>
<td>-</td>
<td>60,142</td>
</tr>
<tr>
<td>3T3</td>
<td>Control M</td>
<td>+</td>
<td>14,394</td>
</tr>
<tr>
<td>3T3</td>
<td>CM – SCC-4</td>
<td>-</td>
<td>897,821</td>
</tr>
<tr>
<td>3T3</td>
<td>CM – SCC-4</td>
<td>+</td>
<td>73,253</td>
</tr>
</tbody>
</table>

* SCC-4 and 3T3 monocultures were cultured in control medium supplemented with 5% fetal calf serum (control M) or in CM-3T3 and CM – SCC-4, respectively, in the presence or absence of 1 μM hydrocortisone. Supernatants were collected (day 3) and the cells were lysed for protein determination. IL-6 was assayed in the supernatants using the ELISA and the results are expressed as pg/mg protein. Data are given for one representative experiment.

**Mono-Cultures Grown in Conditioned Media:** In these experiments, the cells were grown in a mixture (1:1) of fresh and conditioned media (CM) derived from 3T3, HFF, or SCC-4 cells (CM-3T3, CM-HFF, and CM – SCC-4, respectively). Conditioned media were collected after incubation of confluent cell cultures with fresh culture medium for 48 h.

**Kinetic Studies:** For kinetic studies, SCC-4 cells were grown to confluency on the bottom of a 54-cm^2 dish and 3T3 cells on a porous membrane (Nucleopore; pore size 3.0 μm; 45 cm², Transwell Costar) in separate dishes. Subsequently, the fibroblast-populated inserts were transferred into 54-cm^2 dishes containing the SCC-4 cells on the bottom, fresh culture medium was added, and supernatants were collected after 4, 8, 24, and 48 h. At indicated intervals the cells were trypsinized and aliquots of cell suspensions were either frozen at -20 °C in a small volume of phosphate-buffered saline for DNA determination or kept frozen in control medium for preparation of cell lysates. The latter were prepared by three cycles of freeze-thawing. Monocultures of SCC-4 and 3T3 cells, which served as controls, were treated in the same manner.

**Protein and DNA Determination:** The protein content was assayed for normalization of the data. For this purpose, the cells were washed with phosphate-buffered saline and lyzed in 1 N NaOH, after which the protein content was determined [13]. For DNA determination, the cells were washed with phosphate-buffered saline and kept frozen at -20 °C until use. After thawing, the cells were harvested and the DNA content was determined using a fluorometric method [14].

**IL-6 Assay Bioassay:** IL-6 levels were determined by the hybridoma growth assay [15]. The levels of IL-6 are expressed as ng/ml by reference to a standard curve obtained with recombinant human IL-6. All assays were performed in triplicate. All experiments were performed in biologic duplicates (two culture dishes per experimental condition) and the cytokine levels in supernatants and lysates were assayed in triplicate using three serial dilutions. The internal variation in the mean values of cytokine levels in the two cultures did not exceed 15%.

**Elisa:** The amount of immunodetectable IL-6 was determined using a commercially available kit specific for human IL-6 (Immuno Technology Service Production BV, Wychen, The Netherlands). The detection limit of IL-6 determined by this assay was 100 pg/ml.

**IL-1 Bio assay** Interleukin-1 levels were determined by the D10 growth assay [16]. The levels of IL-1 are expressed as ng/ml by reference to a standard curve obtained with recombinant human IL-1α. All assays were performed in triplicate. To establish whether the IL-1 measured was IL-1α or IL-1β, the growth assay was also performed in the presence or absence of antibodies directed against human IL-1α or IL-1β (kindly provided by S. Poole, National Institute for Biological Standards and Control, Potters Bar, UK). Both antibodies had been raised in sheep using recombinant human IL-1α and IL-1β. The growth of D10 cells was completely inhibited at antibody concentration of 100 pg/ml. To establish the presence or absence of IL-1α or IL-1β, the antibodies diluted 1 to 10,000 were added to serially diluted supernatants of NHK and SCC-4 cells. All experiments were performed in biologic duplicates (two culture dish/experimental condition) and the cytokine levels in supernatants and lysates were assayed in triplicate using three serial dilutions. The internal variation in the mean values of cytokine levels in the two cultures did not exceed 15%.
RNA Isolation and Northern blot Analysis
Total cellular RNA was isolated by scraping cells into and grinding (1 min, Polytron, Kinematica GmbH, Switzerland) in ice-cold lithium chloride (3.5 M)/ureum (6.6 M). After overnight incubation at -20°C, the homogenate was centrifuged at 10,000 X g for 30 min at 4°C and the pellet was resuspended in 10 mM Tris/0.5% sodium dodecyl sulfate (SDS), pH 7.7. Total RNA was extracted with three cycles of phenol:chloroform:isoamylalcohol = 25:24:1, the upper phase collected, and RNA isolated by overnight precipitation at -20°C in 70% ethanol/0.1 M Na-acetate pH 5.2. After subsequent centrifugation, the pellet was resuspended in 10 mM Tris/1 mM ethylenediamine tetaacetic acid (EDTA), pH 7.6. Total RNA was quantified spectrophotometrically at 260 nm.

For analysis of murine IL-6 (m-IL-6), human IL-6 (h-IL-6), and glycerolaldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA expression, RNA samples (15 µg) were electrophoresed on 1% agarose/2.2 M formaldehyde gels and transferred to nitrocellulose membranes (Cellulose aldehyde-3-phosphate dehydrogenase blots were washed with 3 X SSC, 0.1% SDS). The membranes were hybridized with 32P-labeled probes specific for both h-IL-6 and m-IL-6 cDNA. The blots were washed with 3 X SSC, 0.1% SDS for 20 min at 55°C (1 X SSC = 0.15 NaCl, 0.015 M sodium citrate) followed by a 10-min wash with 1 X SSC, 0.1% SDS for 10 min at 60°C. The intensity of the bands of the autoradiograms obtained was quantified photodensitometrically (DASA, CDS60). A 597-bp cDNA probe for m-IL-6 and a 819-bp h-IL-6 cDNA probe were prepared with reverse transcriptase-polymerase chain reaction technique according to Kaaskoet al[18](kindly provided by R.A. de Paus, Department of Haematology, Leiden). The GAPDH probe [19] was kindly provided by Dr. R. Orringer.

For analysis of h-IL-1α and human-IL-1β and human-IL-1β cells were lysed in 4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.5, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol and subsequently total cellular RNA was isolated according to Chomczynski and Sacchi [20]. The RNA samples were analyzed using electrophoresis on a 1% agarose gel containing 7.5% formaldehyde and hybridization with 32P-labeled cDNA fragments at 60°C according to Quax et al [21]. A cDNA probe for h-IL-1α was kindly provided by Van Oostveen, Institute of Pathology, Free University, Amsterdam and h-IL-1β cDNA probe was kindly provided by R.A. de Paus, Department of Haematology, Leiden.

RESULTS

Production of IL-6 by Keratinocytes, Fibroblasts, and SCC Cells
Superantigens of cultures of NHK, HFF, SCC-4, and 3T3 cells were collected on day 3 and 6 after plating and the amount of IL-6 determined using the bioassay. All cells produce IL-6 (Fig 1A); the amount secreted into the medium was comparable for HFF and 3T3 fibroblasts and for SCC-4 cells, whereas that secreted by NHK was clearly lower. Irradiation of both human and murine fibroblasts did not significantly affect the IL-6 production.

Production of IL-6 in Co-Cultures of Keratinocytes and Fibroblasts
When SCC-4 cells or NHK were cultured in the presence of feeder cells (lethally irradiated 3T3 or HFF), IL-6 production was markedly increased (~10 to 30 times) (compare Fig 1A and B). Similarly, as in SCC-4 cells, in co-cultures of other SCC cells (SCC-9, SCC-12B2, SCC-12F2, SCC-13, and SCC-15) with lethally irradiated 3T3 cells, a marked increase in IL-6 production occurred (Table I).

In co-cultures of keratinocytes with lethally irradiated fibroblasts, the number of fibroblasts decreases in the course of the culture owing to expanding epithelial cells. To study the effect of irradiation of fibroblasts and to prevent the decrease of fibroblast number during culture, the cells were cultured in a two-chamber system. For this purpose, fibroblasts were seeded on the Nucleopore filter, and the SCC-4 cells on the bottom of the culture dish. These experimental conditions also allowed the examination of whether or not direct cell to cell contact was necessary for the stimulation of IL-6 production. IL-6 levels in mononucleocytes of SCC-4, 3T3, or HFF cells were less than 10 ng/ml for all three periods tested (Fig 2). IL-6 levels in co-cultures increased dramatically (10 to 100 times) during the second period (day 3-6) and remained high only in the two-chamber system in the third period (day 6-9), which suggests that fibroblasts are responsible for the observed increase in IL-6 production in fibroblast-keratinocyte co-cultures. The increase in IL-6 production was similar with both 3T3 cells and HFF. The increase in IL-6 production was observed irrespective of whether or not irradiated or living 3T3 cells were used.

Superantigens derived from mono- and co-cultures were assayed in both the bioassay (total IL-6 activity) and an ELISA specific for...
human IL-6 (Table II). When cells of human origin (HFF, SCC-4, NHK) were grown as mono- or co-culture, comparable amounts of IL-6 were detected by both methods (Table II). It should be noted that in the case of NHK and SCC-4 cells, levels of IL-6 were below the detection level of the ELISA and could be measured only by the more sensitive bioassay. Murine IL-6 could be measured in supernatants of 3T3 cells with the bioassay but not with the ELISA. High IL-6 levels measured in supernatants from NHK/3T3 cells could be detected with the bioassay but not with ELISA. These results confirm that fibroblasts are responsible for the observed increase in IL-6 production in fibroblast-keratinocyte co-cultures.

Effect of Conditioned Medium on IL-6 Production

As shown above, IL-6 production was markedly increased when fibroblasts were grown in the presence of keratinocytes. The finding that direct cell to cell contact is not necessary for the stimulation of IL-6 production (Fig 2) suggests that a soluble factor released by keratinocytes into the culture medium might be responsible for the observed effect. To study whether or not this factor is secreted by keratinocyte monolayers, conditioned media (CM) were harvested from these cultures. Subsequently, 3T3 cells were grown in a 1:1 mixture of control media and CM derived from SCC-4 cultures (CM-SCC-4). For comparison, SCC-4 cells were cultured in CM derived from 3T3 cultures (CM-3T3). Culture of 3T3 cells in CM-SCC-4 resulted in a marked stimulation of IL-6 production (Table III). The increase in IL-6 levels was observed both in supernatants and in cell lysates (data not shown). When SCC-4 cells were grown in CM-3T3, both extracellular and intracellular IL-6 levels remained unchanged. It should be noted that IL-6 is predominantly present extracellularly and under all conditions tested the ratio of extra- to intracellular IL-6 was about 500.

Keratinocytes are routinely cultured in the presence of hydrocortisone [11], which is known to inhibit IL-6 production [8]. Omission of hydrocortisone led to an increase in IL-6 production in SCC-4 and 3T3 cells regardless of whether or not the cells were grown in control or conditioned medium (Table III).

IL-6 mRNA Expression in SCC-4, 3T3, and HFF

To study whether or not elevated IL-6 levels induced by an interaction of keratinocytes with fibroblasts occurred as a result of an enhanced IL-6 production, we examined IL-6 mRNA expression in these cells. For this purpose, the cells were grown for 7 d in control medium or CM-SCC-4, CM-3T3, or CM-HFF. When a cDNA probe specific for m-IL-6 was used, m-IL-6 mRNA could be detected only in 3T3 cultures. Barely detectable levels of m-IL-6 mRNA were found when 3T3 cells were grown in control media (Fig 3A, lane 5), or in CM-3T3 (data not shown). The m-IL-6 mRNA expression was increased in 3T3 cultures grown in CM-SCC-4 (Fig 3A, lane 6). This increase was about 3 to 5 times after normalization on the basis of GADPH mRNA expression. Detectable amounts of m-IL-6 mRNA were found in co-cultures of SCC-4 cells and lethally irradiated 3T3 cells (Fig 3A, lane 1), although the number of irradiated 3T3 cells was markedly diminished on the day of harvest (7 d after the initiation of the culture).

When a cDNA probe specific for h-IL-6 was used, detectable amounts of h-IL-6 mRNA were found in SCC-4 grown in the absence of 3T3 (Fig 3B, lane 2), and no change was observed when SCC-4 were grown in CM-3T3 (Fig 3B, lane 3) or CM-HFF (Fig 3B, lane 4). h-IL-6 mRNA was undetectable in 3T3 cells (Fig 3B, lanes 5 and 6). The h-IL-6 mRNA expression in HFF was low (Fig 3B, lane 7) and did not increase when HFF were cultured in CM-HFF (data not shown). Culture of HFF in CM-SCC-4 led to a marked increase of h-IL-6 mRNA expression (Fig 3B, lane 8). This increase was about eightfold after normalization on the basis of for GADPH mRNA expression.

Table V. Effect of IL-1α and IL-1β Antibodies on IL-6 Production in Fibroblasts Induced by Conditioned Media Derived from SCC-4 and NHK Cultures

<table>
<thead>
<tr>
<th>Medium Condition</th>
<th>3T3 Cells</th>
<th>HFF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-6 Production (ng/ml)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-IL-1α</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-IL-1β</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-IL-1α/β</td>
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<td></td>
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</tbody>
</table>

* Both HFF and 3T3 cells were cultured in medium supplemented with 5% fetal calf serum to confluence. Subsequently, the latter two media were pre-incubated for 1 h at 37°C either in the absence or in the presence of anti-IL-1α and/or anti-IL-1β. Subsequently, the supernatants were collected and IL-6 assayed using the bioassay. Results of three independent experiments are given in mean and SEM.

Figure 4. Identification of IL-1α in supernatants of NHK and SCC-4 monocultures. To serially diluted supernatants of NHK (A) and SCC-4 (B) cultures neutralizing human IL-1α and/or IL-1β antibodies (1:10,000) were added and the remaining IL-1 activity analyzed using the IL-1 bioassay.
IL-1 Activity in Supernatants of SCC-4 and 3T3 Cultures

To establish the role of IL-1 in the induction of IL-6 production, IL-1 levels were measured in supernatants collected from SCC-4 or 3T3 cell cultures. Supernatants of SCC-4 cultures contained significant amounts of IL-1 (Table IV) and were similar, irrespective of whether or not the cells were grown in control or in CM-3T3 medium. IL-1 levels were undetectable in supernatants of 3T3 cultures when the cells were cultured in the control medium. No IL-1 could be detected, however, when 3T3 cells were cultured in CM-S CC-4, which contained approximately 4 ng IL-1/ml at the start of the experiment.

To identify whether or not IL-1 assayed in supernatants and cell lysates was IL-1α or IL-1β, neutralizing IL-1 antibodies were added to serially diluted samples. The growth of IL-1-dependent D10 cells in the presence of supernatants derived from SCC-4 cells and NHK could almost be completely blocked by antibody directed against IL-1α but not IL-1β (Fig 4). Thus IL-1 was identified as IL-1α. Similar observations were made with lysates derived from SCC-4 and NHK (data not shown). Moreover, the neutralizing IL-1α antibody decreased the IL-6 production in fibroblasts induced by supernatants derived from SCC-4 or NHK (Table V). The IL-6 production, however, was not reduced to control levels, probably because the IL-1 activity was also not totally inhibited as shown in Fig 4. The neutralizing IL-1β antibody had no effect on the IL-6 production. These results indicate that IL-1α present in CM of SCC-4 and NHK is mainly responsible for the IL-6 induction in fibroblasts.

Time Course of Changes in IL-1 and IL-6 Levels in SCC-4/3T3 Co-Cultures

To study the kinetics of modulation of IL-6 and IL-1 production in SCC-4/3T3 co-cultures in more detail, the following experiment was performed. Both cell types were grown
induced increase in IL-6 production was higher in HFF (about 0.1).

Methods.

Confluent 3T3 and SCC-4 monocultures were incubated with recombinant human IL-6 for 8 h (Fig 5A). In SCC-4/3T3 co-cultures, however, IL-6 levels increased during the culture period. At the 24-h interval, the intracellular IL-6 levels in SCC-4 cells were low, regardless of whether or not the cells were grown as a mono- or co-culture. In contrast, the intracellular IL-6 levels increased approximately 100 times in 3T3 cells when these were grown in co-culture with SCC-4 cells.

The situation was different when IL-1 levels were assayed (Fig 5B). An increase in IL-1 levels (with a lag time of about 8 h) was seen only in supernatants derived from SCC-4 monocultures, but not in those derived from the SCC-4/3T3 co-cultures. In 3T3 monocultures and in SCC-4/3T3 co-cultures IL-1 levels remained low throughout the whole period tested. Although extracellular IL-1 levels differed markedly in SCC-4 cells grown as monocultures as compared to co-cultures, no significant differences in intracellular IL-1 levels were observed among these cultures. Intracellular IL-1 concentration was always higher than extracellular IL-1; this latter finding is in agreement with data published by Kupper et al [22]. The intracellular IL-1 level was much lower in 3T3 cells than in SCC-4 cells and was increased (about 100 times) in SCC-4/3T3 co-cultures. Human neutralizing antibodies directed against IL-1 could not block the stimulation of D10 cell proliferation by 3T3 cell lysates isolated from SCC-4/3T3 two-chamber co-cultures, indicating that IL-1 detected in 3T3 cells was of murine origin only.

To check whether or not the presence of 3T3 cells led to a decrease in IL-1 synthesis in SCC-4 cells, IL-1α and IL-1β mRNA expression was analyzed in confluent SCC-4 monocultures and SCC-4/3T3 co-cultures (Fig 6). Similar IL-1α mRNA expression was found in SCC-4 monocultures (Fig 6A, lane 1), in SCC-4 monocultures that had been exposed to a human recombinant IL-6 (200 ng/ml) for 8 h (Fig 6A, lane 2), and in SCC-4/3T3 co-cultures (Fig 6A, lane 3). Although IL-1β activity in supernatants of SCC-4 cultures was not detected (Fig 4), IL-1β mRNA was expressed in SCC-4 monocultures (Fig 6B, lane 1) and the expression remained unchanged regardless of whether or not SCC-4 cells were grown in the presence of irradiated 3T3 cells (Fig 6B, lane 3) or exposed to recombinant human IL-6 for 8 h (Fig 6B, lane 2). Irradiated 3T3 cells (Fig 6A and 6B, lane 4) served as negative control.

Effect of IL-1 on IL-6 Production in HFF and 3T3 Cells

IL-6 levels increased linearly with time, whereas IL-1 levels remained low (±0.15 IL-1 ng/ml) and unchanged in supernatants derived from co-cultures during the whole two-chamber experiment (Fig 7A); this IL-1 concentration was sufficient, however, to induce maximal IL-6 production in 3T3 cells or HFF (Fig 7B). The IL-1α−induced increase in IL-6 production was higher in HFF (about 29 times) as compared with 3T3 cells (about 15 times). These dif-
ferences may be as a result of differences in the response of human and murine cells to human IL-1.

DISCUSSION
Mesenchymal-epithelial interaction plays an important role in normal development as well as in neoplasia. The proliferation and differentiation of normal epidermis has been shown to be regulated inductively or permissively by neighboring mesenchymal components [11,23,24]. In addition, we have recently shown that fibroblasts can modulate parathyroid hormone like protein production in both normal and malignant human keratinocytes [25-27].

In the present study, we confirm the recent findings of Waelti et al [9] that there is a marked increase in IL-6 production when keratinocytes are co-cultured with fibroblasts. In contrast to Waelti et al, we find that IL-6 production in co-cultures was also enhanced when direct cell to cell contact was prevented. We have further established that fibroblasts are responsible for the increased IL-6 production and that the observed effect seems to be species-independent because it was found for both human and mouse fibroblasts. We also show that the keratinocyte-induced IL-6 production in fibroblasts is a common phenomenon for epithelial cells because it has also been demonstrated with a number of malignant cells. The finding that the increase in IL-6 production also occurs when fibroblasts are cultured in CM derived from keratinocytes, suggests that one or more factors synthesized by, and released from, keratinocytes may be responsible for the observed effects. One of the possible candidates is IL-1, an interleukin known to be synthesized by normal [28] and transformed keratinocytes [29] and shown to exert various effects on cultured fibroblasts [30-33]. We could also establish that NHK and SCC-4 indeed secrete high amounts of IL-1α, which was mainly responsible for the increased IL-6 production in fibroblasts. The observation that the type of IL-1 excreted by keratinocytes is IL-1α is in agreement with previously published data [28,34,35], but at odds with other findings describing IL-1β as the most abundant form of IL-1 in supernatants derived from NHK (Waelti et al [9]), whose results were obtained using an ELISA specific for IL-1β, whereas we used an IL-1 bioassay for the detection of both bioactive IL-1α and IL-1β. The results of the present investigation suggest that SCC-4 cells express IL-1β at mRNA level, but that functionally active IL-1β protein is absent in the culture supernatants because it was not detected by bioassay. This observation is in agreement with that reported by Mizutani et al [36], who showed that keratinocytes cannot produce pro-IL-1/β and, furthermore, in agreement with findings of Cooper et al [9], who reported an accomodation of large, inactive IL-1β precursors in keratinocyte cultures. Therefore, Waelti et al [9], using an ELISA, most probably detected IL-1β prohormone in the supernatants of their cultures.

In SCC-4 monocultures, IL-1α production increased linearly with the incubation time, but IL-1 levels were low and remained unchanged in SCC-4/3T3 co-cultures incubated for 48 h in a two-chamber system. The low IL-1 activity in supernatants of SCC-4/3T3 co-cultures, however, was sufficient for maximal induction of IL-6 production in both HFF and 3T3 cells. Because intracellular IL-1 activity and IL-1α mRNA expression in SCC-4 cells remained unchanged in SCC-4/3T3 co-cultures, and furthermore IL-1 levels were not decreased in supernatants of SCC-4 monocultures when SCC-4 monocultures were cultured in CM-3T3, we assume that in SCC-4/3T3 co-cultures IL-1 production by SCC-4 is not inhibited by a factor released from the 3T3 cells. In contrast to the case in SCC-4 monocultures, IL-1 levels did not increase in SCC-4/3T3 co-cultures during the experimental period tested, suggesting that 3T3 cells are responsible for the observed effect. In addition, when 3T3 cells were incubated for 3 d in CM-SCC-4 (containing 4 ng IL-1/µl), IL-1 levels became undetectable by bioassay (limit of detection 1 pg IL-1/µl), which may be explained by the inactivation of IL-1 due either to its binding to an IL-1 inhibitor [38] or to competition of IL-1 and an IL-1 receptor antagonist for the IL-1 receptor [39,40] and/or to the action of proteolytic enzymes, the production of which is strongly enhanced by IL-1 in fibroblasts [30-33] and/or a receptor-mediated IL-1 consumption by fibroblast.

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