Role of fibroblasts in the regulation of proinflammatory interleukin IL-1, IL-6 and IL-8 levels induced by keratinocyte-derived IL-1

Ingeborg L. A. Boxman • Cindy Ruwhof
Otto C. Boerman • Clemens W. G. M. Löwik • Maria Ponec

Abstract In the epidermis, the keratinocytes are the first cells to be encountered by external stimuli and they are able to promote the inflammatory response by increased production and release of various cytokines. In their turn, these cytokines may directly affect the production of proinflammatory cytokines in human dermal fibroblasts. In addition, in both epithelial and mesenchymal cells cytokine production may be modulated by their mutual interaction, and thereby regulate the inflammatory response. The present study aimed to examine the role of fibroblasts in the regulation of proinflammatory IL-1, IL-6 and IL-8 levels induced by keratinocyte-derived IL-1. The data show that in fibroblasts exposed to conditioned media derived from cultures of normal human keratinocytes or squamous carcinoma cells (SCC-4), both the IL-8 and IL-6 mRNA expression as well as protein production were elevated. In addition, it was shown that these effects were induced by IL-1α. The IL-1α-induced increase in IL-8 and IL-6 production, both on the protein level as well as on the mRNA level, were concentration dependent and occurred almost simultaneously. While the induction of IL-6 and IL-8 occurred simultaneously, the IL-6 mRNA remained elevated for longer. In contrast to increased IL-6 and IL-8 production the IL-1α levels markedly decreased upon culturing of fibroblasts in keratinocyte-derived conditioned medium. From internalization experiments it could be concluded that binding of IL-1 to IL-1 receptors, and its subsequent internalization and intracellular degradation is the most likely mechanism involved in the reduction of IL-1 levels by fibroblasts. Comparing the rate of IL-1 reduction in the presence of various cell types indicated that the rate of IL-1 reduction is directly related to the number of IL-1 receptors found on these cell types. In conclusion, these results indicate that the release of IL-1α by activated keratinocytes may act as an inducer of IL-8 and IL-6 production in neighbouring fibroblasts. This may be an important pathway for the amplification of the inflammatory response. The amounts of both cytokines produced by fibroblasts were at least two to three orders of magnitude higher than those produced by keratinocytes, suggesting an important role of fibroblasts in the general inflammatory response. Furthermore, fibroblasts might be involved in turning off this inflammatory response by reducing IL-1 levels, most likely via IL-1 receptor-mediated uptake.

Key words Interleukin-8 • Interleukin-1 • IL-1 receptor • Keratinocyte – Fibroblast interaction

Introduction

There is increasing evidence that the release and production of cytokines, either preformed or newly synthesized, by keratinocytes and fibroblasts is altered after certain events, such as skin injury [1]. The keratinocytes become 'activated' and release, amongst other things, the proinflammatory cytokine interleukin-1 (IL-1) [2]. It has been shown that exposure of skin cells to IL-1 leads to an increased production of IL-6 and IL-8 [2–7]. Proinflammatory cytokines, such as IL-8 and IL-6 are pleiotropic cytokines and act on multiple cell types. In addition, both IL-8 and IL-6 are thought to play an important role in both the cellular and the humoral immune responses via different pathways [8]. At low concentrations IL-8 possesses neutrophil, lymphocyte and basophil chemotactic properties, while at higher concentrations IL-8 can activate neu-
trophils and T cells [9]. The major effects of IL-6 with respect to immune response are the stimulation of the final differentiation of B cells into antibody-producing plasma cells and the induction of acute phase proteins in hepatocytes [10]. Since the humoral and cellular pathways are complementary, the presence of both IL-6 and IL-8 will potentiate the immune response.

We have recently shown that in keratinocyte–fibroblast cocultures, IL-1α derived from epidermal cells increases IL-6 production in fibroblasts [11]. It has been shown that the transcription factors NF-IL-6 and NF-kB are needed for IL-1-induced IL-6 and IL-8 gene expression [12]. The present study aimed to examine the kinetics of induction of IL-6 and IL-8 production in human dermal fibroblasts by conditioned medium derived from keratinocyte cultures and by IL-1α. We have previously reported that IL-1 activity is strongly reduced in cocultures of keratinocytes and 3T3 fibroblasts [11]. Since this might represent a negative feedback mechanism we further investigated the processes involved in the reduction of IL-1 levels by fibroblasts.

### Materials and methods

#### Cell culture

Human fibroblasts (HFF), derived from surgical specimens of female breast skin, and 3T3 cells were cultured in Dulbecco’s Modification of Eagle’s Medium (DMEM) supplemented with 5% (v/v) fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml). Normal human keratinocytes (NHK) derived from juvenile foreskin and squamous carcinoma cells (SCC-4) [13] were cultured using the Rheinwald-Green feeder technique [14], as described previously [15].

#### Preparation of conditioned medium

HFF (1.25 x 10^5/cm^2) and SCC-4 were seeded as monocultures in DMEM/F12 (3:1) supplemented with 5% (v/v) FCS, and the medium was refreshed after 3 days. The supernatants (conditioned medium) of the 3–6-day culture period were collected and kept at −20°C until use. To prepare conditioned medium of 3T3-free NHK monocultures, 1.25 x 10^5/cm^2 NHK were seeded together with 1.8 x 10^5/cm^2 lethally irradiated 3T3 cells and grown to subconfluence in medium as described previously [15]. These cells were removed by 0.02% (w/v) ethylenediaminetetraacetate acid treatment [16] and NHK were then grown to confluence in DMEM/F12 (3:1) supplemented with 5% (v/v) FCS. NHK cultures were then refed with fresh medium and reincubated for 3 days. This supernatant was collected and kept at −20°C until use. For experiments, a mixture (1:1) of fresh and conditioned medium derived from HFF, SCC-4 and NHK was used. These media were termed CM-HFF, CM-SCC-4 and CM-NHK, respectively.

#### Stimulation of fibroblast cultures with either IL-1α

or with conditioned medium in the presence or absence of antibodies against IL-1

Fibroblasts, HFF or 3T3 cells were grown to confluence in DMEM/F12 supplemented with 5% (v/v) FCS. When HFF were exposed to conditioned medium the cells were incubated for 4, 8 and 24 h in CM-HFF, CM-SCC-4, CM-NHK or in control medium. For IL-1 stimulation, the HFF were refed with fresh medium. After 24 h, 0, 10 or 100 pg/ml IL-1α (Hoffman la Roche, Basel, Suisse) was added and the cells were reincubated for another 4, 8, 24 or 48 h. At various time points supernatants were harvested and stored at −20°C and the cells were lysed for RNA isolation. When antibodies against IL-1 were included in the experiment, HFF were incubated for 20 h in control medium or in a mixture of control medium and CM-SCC-4 or CM-NHK (4:1 v/v), the latter two media were preincubated for 1 h at 37°C either in the absence or the presence of anti-IL-1α and/or IL-1β (kindly provided by S. Poole, National Institute for Biological Standards and Control, Potters Bar, UK). Supernatants were harvested and stored at −20°C until use. Two independent experiments were performed for all conditions described above.

### Determination of IL-6 and IL-1 bioactivity

IL-6 and IL-1 levels were determined using the B9 hybridoma growth assay [17] and D10 growth assay [18], respectively, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) for the determination of cell numbers [19]. The levels of IL-6 and IL-1 are expressed as picograms per millilitre by reference to standard curves obtained with recombinant human IL-6 or recombinant human IL-1α, respectively. All assays were performed in biological duplicate (two culture dishes per experimental condition) (n = 2) and the cytokine levels in the supernatants were assayed in duplicate using five serial dilutions. Because the experiments were performed in duplicate no standard deviation is given. The internal variation of the mean values of the cytokine levels in the two independent cultures did not exceed 13%.

### Determination of IL-1 antagonist levels

IL-1α was measured using a radioimmunoassay (RIA) in which rabbit polyclonal antibodies to human recombinant IL-1α were used. The detection limit was 5 pg IL-1α/ml. This antibody did not crossreact with human IL-1β, IL-2, TNF, α-IFN, γ-IFN, GM-CSF or C5a. The RIA was carried out as described by Lommen et al. [20] with minor modifications.

### Determination of IL-8 levels

IL-8 was measured using an enzyme-linked immunosorbent assay (ELISA) in which monoclonal antibodies (MAbs) and polyclonal antibodies to human recombinant IL-8 were used, as described by Hack et al. [21]. The detection limit was 4 pg IL-8/ml. Standard and antibodies were a gift from Dr. L. A. Aarden (Red Cross Central Laboratory for Blood Transfusion, Amsterdam, The Netherlands).

### Analysis of mRNA

RNA was isolated according to the method of Chomczynski and Sacchi [22] and samples were analysed using the Northern blotting technique [23]. cDNA probes specific for human IL-1β (443 bp), IL-6 (819 bp) and IL-8 (585 bp) were prepared by reverse-transcription polymerase chain reaction [24] (kindly provided by Dr. R. A. de Paus, Department of Haematology, Leiden, the Netherlands). Hybridization was performed as described by Quax et al. [25]. The intensity of the bands of the autoradiograms was quantified photodensitometrically (Desaga CD60) and normalized for total mRNA using 28S rRNA as a control (kindly provided by Dr. C. Backendorf, Leiden, The Netherlands).

### IL-1 receptor binding assay

TriPLICATE confluent cultures of 3T3 cells and fibroblasts were washed twice with cold (0°C) ‘assay medium’ (DMEM containing 0.1% bovine serum albumin, buffered with 25 mM N-2-hydroxyethylpiperazine-N'-ethane sulfonic acid (HEPES), pH 7.4). The cells were then incubated in assay medium containing increasing amounts of 125I-IL-1α (0–4 ng/ml, specific activity 80 μCi/μg) in the presence or absence of a 500-fold excess of nonlabelled IL-1α. After a 5-h incubation at 4°C, the cells were washed twice with cold phosphate-buffered saline supplemented with 0.5% bovine.
serum albumin. The cells were then scraped and collected using a
cotton-wool stick and total cell-associated radioactivity was deter­
mined. The number of molecules specifically bound to the cell sur­
face was determined after subtraction of 125I-IL-1a bound in the
presence of an excess of nonlabelled IL-1a. Scatchard analysis
was performed in two independent experiments to determine the
maximum number of binding sites and the Kd values.

Internalization and degradation of IL-1 was studied as de­
scribed by Van Oosterhout et al. [26]. Briefly, cells were incubated
for various times at 37°C in the presence of 125I-IL-1a. The cells
were then treated or not treated with ice-cold isotonic glycine
buffer (pH 2) for 10 min to discriminate between intracellular ac­
tivity and the total amount of associated radioactivity, respectively.
Furthermore, 75 µl of culture supernatant was treated with 1 ml
25% trichloroacetic acid (TCA) and 75 µl 1% bovine serum albu­
m at 4°C for 30 min, centrifuged at 18000 g for 15 min and the
radioactivity in the supernatant and pellet determined. All experi­
ments (n = 2) were performed in triplicate and each sample
analysed in duplicate.

Results

Modulation of IL-8, IL-6 and IL-1 levels
and mRNA expression in HFF

Effects of conditioned media derived
from epidermal cell cultures

The effects of conditioned media derived from epidermal
cell cultures on the IL-8, IL-6 and IL-1 protein levels and
mRNA expression in HFF cultures were examined. Con­
fluent HFF cultures were incubated for various times in
control medium or in the conditioned media CM-HFF,
CM-NHK or CM-SCC-4, and the supernatants were
collected and RNA was isolated.

When HFF were grown in CM-NHK or CM-SCC-4,
levels of both IL-8 and IL-6 markedly increased with
time (Fig. 1a). The increase was more pronounced in
HFF cultures grown in CM-SCC-4 than in CM-NHK.
Exposure of HFF to CM-HFF did not affect either
IL-6 or IL-8 production (data not shown). In contrast
to increased IL-8 and IL-6 levels, IL-1 activity de­
creased within 24 h from 0.12 to 0.03 ng/ml in HFF
grown in CM-NHK and from 0.4 to 0.3 ng/ml in HFF
grown in CM-SCC-4. In HFF cultures grown for 24 h in
control medium or in CM-HFF no IL-1 activity was de­
tected.

Northern blot analysis indicated that exposure of HFF
to CM-SCC-4 and CM-NHK also resulted in an increase
in IL-8 and IL-6 mRNA levels (Fig. 1b). This increase
persisted for longer in cells grown in CM-SCC-4 than in
CM-NHK. Transiently elevated levels of IL-8 and IL-6
mRNA were also observed in HFF grown in control me­
dium, probably resulting from medium renewal at the be­
inning of the experiment. In addition, a transient increase
expression of IL-1β mRNA was seen upon exposure of HFF
to CM-SCC-4 and CM-NHK. No IL-1α mRNA could be

![Fig. 1a, b](image-url)
detected either in stimulated or in nonstimulated cells (data not shown).

IL-1α present in supernatants derived from SCC-4 or NHK was found to be responsible for the observed increase in IL-8 expression and production in HFF, since after preincubation of CM-NHK and CM-SCC-4 with antibodies directed against IL-1α the IL-8 production in fibroblasts was markedly decreased (Table 1). The IL-8 production was not affected by the neutralizing IL-1β antibody, indicating that there was hardly any IL-1β activity in CM-NHK or CM-SCC-4.

### Effects of IL-1α

We investigated whether the induction of the IL-8 and IL-6 production in HFF by supernatants derived from epidermal cultures was comparable to the effects induced by purified IL-1α. HFF were exposed for various times to increasing concentrations of IL-1α, and the IL-8 and IL-6 production was monitored. As shown in Fig. 2a, an increase in IL-8 and IL-6 levels was observed in culture supernatants derived from HFF exposed to IL-1α. Concomitantly with the increase in IL-8 and IL-6 levels, a reduction in IL-1 activity was observed in supernatants of HFF cultures exposed to IL-1α. IL-1 activity decreased within 48 h from the initial value of 100 pg/ml to 14 pg/ml, whereas at an initial concentration of 10 pg/ml, no IL-1 activity could be detected after 24 h.

Changes in IL-8 and IL-6 mRNA expression in HFF exposed to IL-1α were also time- and concentration-dependent. Detectable levels of IL-8 and IL-6 mRNA were measured as early as 4 h after stimulation of HFF with 10 pg/ml IL-1α (Fig. 2b). By prolongation of the incubation time to 24 h, the IL-8 mRNA expression decreased to un-
cells is more rapid than with HFF |ll|, 3T3 cells were
creased with time (data not shown).

bioactive IL-l remained low, while in SCC-4 monocul­

urates both IL-1 immunoreactivity and bioactivity in­

Fig. 3a–c Time course of the reduction in IL-1 protein, a Conflu­

I-IL-la at 37°C was found (■ intact molecule, TCA in­

were incubated with 125I-IL-1α (150 pg/ml) for the indented times

and in the supernatants collected. These supernatants were then

treated with TCA, as described in Materials and methods. In the

absence of fibroblasts (control) hardly any spontaneous degrada­
tion of 125I-IL-1α was observed (■ intact molecule, TCA insol­

bile; □ degradation product, TCA soluble). In the presence of

fibroblasts degradation of 125I-IL-1α was observed (■ intact mol­

ecule, TCA insoluble; • degradation product, TCA soluble). The

standard deviation is given in c, but was usually smaller than the

plot symbol.

detectable levels, indicating that transcription of the IL-8

gene was temporarily enhanced. In contrast, IL-6 mRNA

expression remained elevated during the 24 h period. Af­

ter stimulation with 100 pg/ml IL-1α, enhanced IL-8

mRNA levels were detected for a longer period. Elevation

in the IL-1β mRNA levels was observed only after stimu­

lation of HFF with 100 pg/ml IL-1α for 8 and 24 h. No

IL-1α mRNA could be detected either in stimulated or in

nonstimulated cells (data not shown).

Fibroblast-induced decrease in IL-1 protein levels

As described above, exposure of confluent cultures of hu­

dan dermal fibroblasts to CM-SCC-4 (Fig. 1a) or IL-1α

(Fig.2a) resulted in a decrease in IL-1 bioactivity. To ex­
amine whether this decrease was caused by the loss of IL-

1α protein, a RIA specific for human IL-1α was used.

Since the decrease in IL-1α levels in the presence of 3T3

cells is more rapid than with HFF [11], 3T3 cells were

used in these experiments. When 3T3 cells were exposed

to CM-SCC-4, the time course of the reduction in IL-1

immunoreactivity closely paralleled the reduction in

bioactivity (Fig.3a). Apparently the observed decrease in

IL-1 bioactivity was not caused by mere transformation of

IL-1 to its inactive form, but by a decrease in IL-1α pro­
tein levels. In addition, we found that in SCC-4/3T3 co-
cultures the extracellular levels of immunoreactive and

bioactive IL-1 remained low, while in SCC-4 monocul­
tures both IL-1 immunoreactivity and bioactivity in­
creased with time (data not shown).

To examine the reduction in IL-1 protein in the

presence of fibroblasts in more detail, confluent HFF or 3T3

cultures were incubated with 125I-IL-1α (150 pg/ml) for

various times at 37°C and the radioactivity in the super­

nats present in TCA-soluble (degraded IL-1) and TCA-

soluble (intact IL-1) fractions were determined. In the

presence of HFF the amount of degraded IL-1 increased

within 78 h to 22.3 ± 1.5% of total added IL-1. The rate of

IL-1 degradation was much higher in 3T3 cultures than in

HFF (61.8 ± 0.7% versus 9.2 ± 2.3% after 24 h). Hardly

any spontaneous degradation of 125I-IL-1α at 37°C was

found in the absence of fibroblasts (4.6 ± 0.5% after 78 h).

These findings were confirmed by running these super­
natants on an 18% SDS-PAGE gel followed by autoradi­
ography (data not shown).

It has been demonstrated that IL-1 can enhance the

production and secretion of proteolytic enzymes by fibro­

blasts [27]. Therefore, we further investigated whether

degradation of IL-1 was due to enhanced proteolytic ac­
tivity. To this end 125I-IL-1α (600 pg/ml) was added to

supernatants derived from 3T3 cell and HFF cultures stimu­

lated or not with 1 ng/ml IL-1 for 20 h and incubated for

20 h at 37°C. After TCA precipitation the distribution of

the radioactivity over the two fractions was not signifi­
cantly different from the control (unconditioned medium).

This indicates that there is no evidence for enhanced ex­

tracellular degradation. However, when 3T3 cells were

first incubated with 125I-IL-1α (5ng/ml) at 4°C, washed

and subsequently incubated at 37°C, it could be shown

that cell surface-bound IL-1α was rapidly internalized,
degraded and excreted. As early as 1 h after the start of

the experiment, detectable amounts of radioactivity were

present in the supernatants in the TCA-soluble fraction;

this amount increased to 85.7 ± 3.3% within 20 h (data not

shown).

IL-1 receptor binding

Since the reduction in extracellular IL-1 levels occurs

only in the presence of fibroblasts, presumably via IL-1

receptor-mediated uptake [28, 29], the number of IL-1

binding sites and Kd values were determined by Sca­

tcher analysis. The results of these experiments re­

vealed that the number of IL-1 binding sites on 3T3 cells

were significantly lower than those of HFF (Table 2).

However, the Kd values of both cell types were very

similar (3.9 ± 2.0 × 10^-10 M for HFF, 3.1 ± 1.5 × 10^-10 M

for 3T3). This indicates that the cellular receptor for IL-1

is not altered in these fibroblasts.
that the IL-1-induced stimulation of IL-8 mRNA expression was also inducible in HFF by IL-1α in a dose-dependent manner. The findings that total (added and secreted) bioactive IL-1 was reduced over time and that the IL-1-induced stimulation of IL-8 or IL-6 production in HFF was not affected by the presence of a neutralizing antibody against IL-1β, strongly suggest that the amount of IL-1β produced by fibroblasts was too low to exert an additional effect to exogenously administered IL-1α.

In contrast to increased levels of IL-6 and IL-8 measured during the exposure of HFF to IL-1α-containing media (conditioned media or IL-1α-supplemented medium), IL-1α bioactivity and immunoreactivity markedly decreased. The reduction in IL-1α levels can be fully ascribed to the action of fibroblasts, since in the absence of fibroblasts no spontaneous decrease in IL-1 protein was observed. Since this might represent a negative feedback mechanism we further investigated the processes involved in the reduction of IL-1 levels by fibroblasts. Various mechanisms may be responsible for the observed fibroblast-induced decrease in IL-1. The results of the present study show that it is unlikely that the decrease in IL-1 levels is due to binding of IL-1 to an IL-1 inhibitor [34], because the IL-1-inhibitor complex should have been detected in the RIA by the polyclonal antibody, which recognizes different epitopes on IL-1α [20] and such a complex should be TCA-precipitable. It is also unlikely that the decrease in IL-1 activity is caused by an increase in IL-1 receptor antagonist [35–37], since the reduction in the IL-1 bioactivity paralleled a decrease in IL-1 immunoreactivity. Furthermore, we can exclude the involvement of extracellular degradation of IL-1 by proteolytic enzymes, since 125I-IL-1 was not degraded when incubated in the presence of supernatants derived from IL-1-stimulated fibroblast cultures. We present evidence that 125I-IL-1 was only degraded in the presence of fibroblasts by receptor-mediated uptake. A higher rate of reduction in IL-1 was observed in 3T3 cells as compared to HFF. This observation is in line with the 15-fold higher number of IL-1 receptors on 3T3 cells than on HFF. The affinity of the IL-1 receptor for IL-1 was similar in both cell types. The finding that the number of IL-1 binding sites on 3T3 cells was higher than on HFF is in agreement with the results of Mizel et al. [28] and Akahoshi et al. [29]. In line with the hypothesis that the fibroblast-regulated decrease in IL-1 levels may function as a negative feedback mechanism, is the observation that the disappearance of extracellular IL-1 is paralleled by a decrease in IL-6 and IL-8 mRNA expression in fibroblasts.

It is generally accepted that in the epidermis the keratinocytes are the first cells to be encountered by external stimuli, initiating the immune response by increased production of various cytokines. However, in comparison with the amounts of IL-1 produced and secreted by keratinocytes, the production of IL-6 and IL-8 in these cells is very low [5, 11]. In contrast, fibroblasts produce 100 to 1000 times more IL-6 and IL-8 than keratinocytes. Therefore, the release of IL-1α by activated keratinocytes may act as an inducer of IL-8 and IL-6 production in neighbouring fibroblasts, which may be a potent pathway for the amplification of the signal and initiation of a more general inflammatory response. On the other hand, fibroblasts may also be involved in the overall regulation of
cytokine production by turning off this pathway by reducing the IL-1 activity via a receptor-mediated uptake and degradation (schematically presented in Fig. 5). It remains to be established whether or not interactive circuits in skin tissue involving signalling by diffusible cytokines such as IL-1, IL-6 and IL-8 also occur in vivo.

Acknowledgements We would like to thank Mrs. J. Kempenaar for excellent technical assistance, Prof. Dr. J. W. M. van der Meer for providing IL-1α and the IL-1 RIA, and Prof. Dr. L. A. Aarden for providing the IL-8 ELISA reagents.

References


37. Martel-Pelletier J, McCallum R, Pelletier JP (1993) The synthesis of IL-1 receptor antagonist (IL-1ra) by synovial fibroblasts is markedly increased by the cytokines TNFα and IL-1. Biochim Biophys Acta 1175:302-305