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Interleukin-6 expression by fibroblasts grown in three-dimensional gel cultures

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We investigated the expression and biological activity of interleukin-6 (IL-6) by human fibroblasts cultured as monolayers and within three-dimensional type I collagen lattices. In the course of contracting the gel to a dense tissue-like structure, the cells upregulated their levels of IL-6 mRNA as well as IL-6 biological activity. While there was little mRNA and protein activity (6,500 U/ml) in monolayer cultures, fibroblasts in the 3D system showed a 13-fold increase in IL-6 mRNA on day 3. IL-6 protein was increased 6-fold (38,000 U/ml) on day 4. Stimulation of fibroblast cultures with IL-1α resulted in enhanced IL-6 production in both systems, but the fibroblasts embedded into the 3D network continued to exhibit higher levels.

Interleukin-6; Extracellular matrix; Three-dimensional collagen gel; Fibroblast

1. INTRODUCTION

Fibroblast metabolism plays an important role in the physiological functioning of the skin. Fibroblasts produce collagen and other structural components of connective tissue, as well as proteolytic enzymes responsible for the remodelling of deposited tissue. All of these functions need to be precisely regulated and compartmentalized in order to avoid pathological changes as may be observed in fibrosis and impaired wound healing.

Fibroblast functions may be regulated by cells of the immune system, like lymphocytes and monocytes. These cells infiltrate areas of tissue damage and support wound healing. The effect of those leucocytes may be mediated in part by cytokines like tumor necrosis factor (TNF) and interleukin-1 (IL-1) which can enhance, but also downregulate fibroblast activities [1,2].

IL-6 is a multifunctional cytokine with pronounced effects on host defense mechanisms (for review see [3,4]). Elevated IL-6 levels have been associated with autoimmune diseases, plasma cell neoplasias, and inflammatory states. Potent inducers are IL-1, TNF, platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) [5], as well as bacterial lipopolysaccharide (LPS) [6] and UV light [7]. Fibroblasts have been shown to synthesize IL-6 [8], its biological function, however, still needs to be elucidated.

We have studied the regulation of IL-6 by fibroblasts grown in 3D dermal equivalents, reconstituted mainly of type I collagen which are considered as a model system to study wound contraction [9]. This in vitro model has been extensively characterized with respect to mRNA steady-state levels and protein activity of collagen and collagenase as well as other proteins [10]. These studies revealed that the contact of the cells with the surrounding 3D matrix results in a complete reprogramming of the cellular metabolism. In the present report we demonstrate that in this system fibroblasts, even without stimulation by IL-1, show a pronounced upregulation of their IL-6 gene expression.

Abbreviations: BSA, bovine serum albumin; PBS, phosphate-buffered saline, pH 7.0; SDS, sodium dodecylsulphate; SSC, standard saline citrate: 0.15 M NaCl, 0.015 M Na citrate, pH 7.0; Denhardt's, BSA, polyvinylpyrrolidone, ficoll each at 0.02%.

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2. MATERIALS AND METHODS

2.1. *Cell culture*

Fibroblast cultures were established by outgrowth from skin biopsies of 2 healthy human volunteers. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS), 50 μg/ml sodium ascorbate, 300 μg/ml glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and grown in the moist atmosphere of a CO_2 incubator (5% CO_2, 95% air) at 37°C [11].

2.2. *Preparation of collagenous matrices*

Crude extracts of type I collagen from calf skin were further purified by dialysis and lyophilization as described [10], and dissolved in sterile

0.1% acetic acid at a concentration of 3.5 mg/ml. Gels of 150 mm diameter were cast in bacteriological Petri dishes by combining 13.8 ml of 1.76-fold concentrated DMEM, 9 ml collagen solution, neutralized with 1.5 ml of 0.1 N NaOH, 2.7 ml FCS and 3 ml DMEM (B. Coulomb, pers. comm.) equivalent to 1×10^7 fibroblasts (11th and 12th passage). Within 12 h the gels were contracted to 16% of their initial diameter, irrespective of the addition of IL-1. LPS was not detectable, when the collagen preparation was tested for stimulation of the monocytic cell line Mono Mac 6 [12] using TNF production as a read out (sensitivity 0.1 ng/ml).

2.3. *Stimulation by IL-1α*

Recombinant human (rh) IL-1α (specific activity 1×10^7 U/mg, Boehringer, Mannheim) was diluted into 0.1% BSA in PBS at a concentration of 50 U/ml. For continuous stimulation experiments, IL-1α was added to the cells prior to being seeded, as well as to the medium, at 10 U/ml. For late addition experiments, cultures were maintained in DMEM, 10% FCS with daily changes of media, and treated with IL-1 at 10 U/ml on day 3 for 24 h. Monolayers and gels were washed twice in cold PBS and lysed in 4 M guanidinium thiocyanate for subsequent isolation of RNA. Culture media were collected from IL-1 treated cultures and untreated controls, filtered (0.2 μm) and stored at −20°C until tested.

2.4. *RNA isolation and Northern analysis*

Monolayer cultures and collagen gels were lysed in 4 M guanidinium thiocyanate applying standard procedures [13]. DNA was sheared by drawing the lysate 6 times through a 22-gauge needle, followed by one extraction with phenol, chloroform, isoamylalcohol to remove the bulk of protein. RNA was purified by centrifugation through a 5.7 M CsCl cushion. Aliquots of 2 or 5 μg were electrophoresed in denaturing agarose gels containing 0.66 M formaldehyde, transferred to GeneScreen membranes (DuPont), fixed by UV-crosslinking and hybridized to cDNA probes labeled by random priming [14] using [α-^{32}P]dATP (sp.act. $2–5 \times 10^8$ cpm/μg). The following cDNA probes were used: IL-6, 0.56 kb *Eco*RI/*Pst*I fragment (gift from L.T. May, New York); PX7 (interstitial collagenase), 1.2 kb *Pst*I/*Eco*RI fragment (gift from B. Stein, Karlsruhe); pGAPDH (glyceraldehyde-3-phosphate dehydrogenase), 1.3 kb *Pst*I fragment (kindly provided by J. Uitto, Philadelphia), this sequence was used to assess equal loading of RNA per lane.

Filters were hybridized overnight at 42°C in 50% formamide, 5 × SSC, 100 μg/ml denatured salmon sperm DNA, 5 × Denhardt's, washed twice at room temperature in 2 × SSC, 0.1% SDS, followed by one or two washing steps at high stringency (60–65°C in 0.1 × SSC, 0.1% SDS). Autoradiography was from 4 h to 5 days at −80°C using intensifying screens (DuPont). Relative RNA amounts were assessed by densitometry (Hirschmann Elscript 400) of the specific hybridization signals.

2.5. *Evaluation of IL-6 activity*

For the detection of IL-6 activity, the murine hybridoma cell lines B9 [15] or 7TD1 [16] were used which proliferate only in the presence of externally applied IL-6. Briefly, the cells were incubated with dilutions of the IL-6-containing culture supernatants, and proliferation was monitored by incorporation of [^{3}H]thymidine into B9 cells [7], or by the conversion of dimethylthiazolydiphenyltetrazoliumbromide (Sigma) by live 7TD1 cells.

3. RESULTS

3.1. *Expression of IL-6 mRNA*

IL-6 production by fibroblasts grown as monolayers is low and visible only after prolonged exposure of the filter following hybridization (Fig. 1). Cells that have been seeded into and contract a collagenous matrix show a 13-fold increase in IL-6 mRNA over monolayers on day 3.

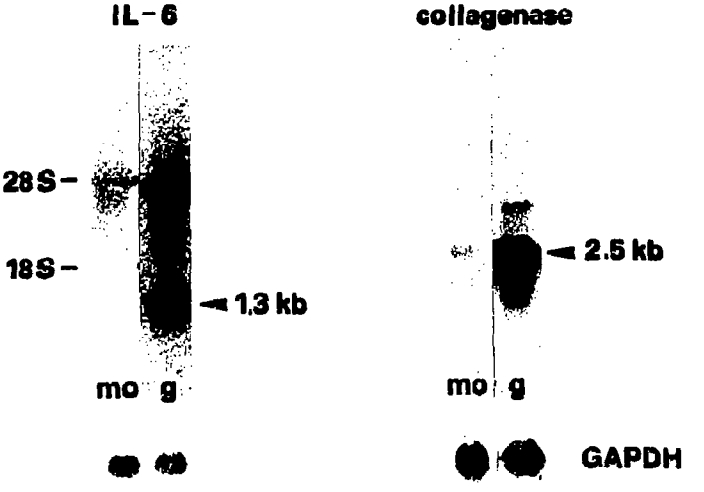


Fig. 1. Levels of mRNA for IL-6 and interstitial collagenase. Total RNA was extracted from normal human fibroblasts cultured for 3 days as monolayers (mo), or within 3D collagen gels (g). Expression of IL-6 mRNA (1.3 kb) and collagenase mRNA (2.5 kb) were determined by hybridization and densitometric evaluation of signal intensities. Comparable loading of RNA per lane was assessed by hybridizing a clone encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

As a control, levels of collagenase mRNA were assessed using the identical RNA preparations. Consistent with earlier observations [17] there is a dramatic rise in collagenase expression in the 3D cultures to 39-fold higher levels as compared to 3 day monolayer cultures.

3.2. *Expression of IL-6 protein*

We then asked whether the observed elevated levels of IL-6 mRNA stimulated synthesis and IL-6 activity. Using the hybridoma proliferation assay, we measured biologically active IL-6 protein in the culture supernatants of both monolayers and 3D matrices. Representative data of repeated experiments are summarized in Fig. 2. Paralleling the increase in IL-6 mRNA in 3D cultures, secreted IL-6 protein activity also rises. As compared to monolayer controls as a reference value, IL-6 protein activity in the supernatant of collagenous gels rises to almost twice this value within 6 h of gel culture, and after 2 days reaches more than 3-fold higher values compared to 2 day monolayer cultures.

Treatment of identical cultures with IL-1α at 10 U/ml, supplied at the onset of the cultures, stimulates further increased IL-6 secretion in both culture systems resulting in 6-fold higher production in the monolayer, and a time-dependent increase in the 3D cultures to 3-fold (6 h) and 9-fold (2 days) higher IL-6 production over the untreated monolayer.

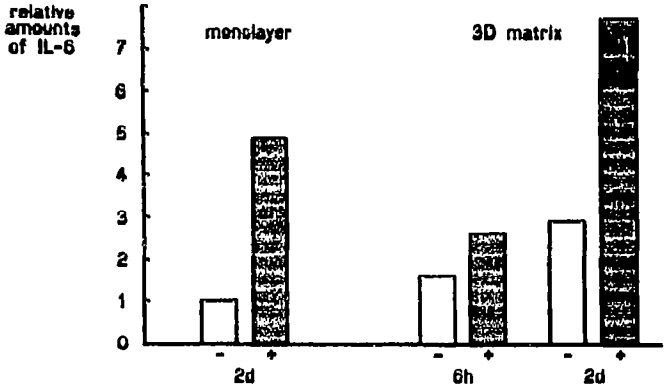
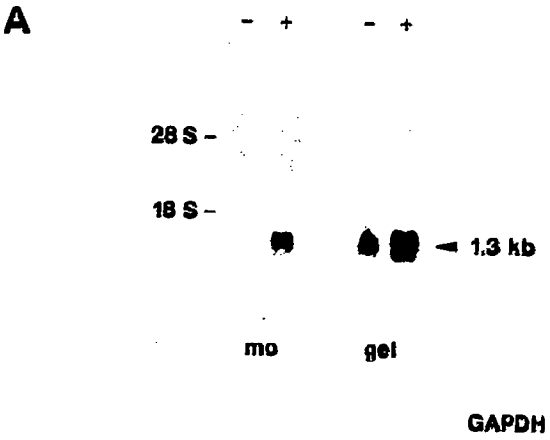


Fig. 2. Concentration of IL-6 secreted into the media of fibroblasts cultured in monolayer cultures and within 3D collagen I matrices. Fibroblasts were cultured for 2 days in regular cell culture Petri dishes (mo), and for 6 h or 2 days within 3D collagen lattices (3D matrix). Culture supernatants were assayed for IL-6 activity using a bioassay (see Section 2). Induction is expressed as multiples of the untreated control (1 = 3,500 U/ml IL-6). Open bars represent untreated cultures, shaded ones indicate cultures treated with IL-1α (10 U/ml). Each bar represents the average of duplicates.



B **IL-6 protein levels**

type of culture	IL-1 added (10u/ml)	secreted IL-6 (u/ml)	relative expression
monolayer	-	6500	1.0
	+	44000	6.8
3D matrix	-	38000	5.9
	+	46000	7.2

Fig. 3. IL-6 mRNA level (a) and protein concentration in culture supernatants (b) in response to IL-1α stimulus applied after varying times of culture in monolayers or collagenous gels. Fibroblasts were cultured as monolayers (mo) and within collagenous matrices (gel). On day 3 after culture initiation, one half of the cultures (+) was treated with IL-1α at 10 U/ml and incubated for 24 h. The other half was left untreated (−). Cells and supernatants were harvested on day 4 and analyzed for IL-6 mRNA and protein activity. Comparable loading of RNA per lane was assessed by hybridization with glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

3.3. *Responsiveness of fibroblasts embedded in three-dimensional lattices to IL-1α*

The increase in IL-6 mRNA and protein induced by IL-1 when added at initiation of the culture, led us to investigate whether fibroblasts are still able to respond to IL-1 stimuli by increasing their IL-6 synthesis when they have already been embedded within a contracted tissue-like matrix. Fig. 3 shows that they retain this capacity. When IL-1 is applied on day 3 after culture initiation, within 24 h IL-6 mRNA levels are significantly induced in comparison to untreated cultures. Concomitantly, there is a rise in IL-6 protein activity, with 46,000 U IL-6 per ml in the IL-1 treated gel culture versus 38,000 U in the untreated one.

4. DISCUSSION

In recent years, studies have been undertaken to design in vitro culture systems which enable us to investigate cell functions under more physiological conditions than monolayer cell culture [9,18]. We wish to characterize the metabolism of fibroblasts by embedding the cells into a 3D collagenous matrix which thus provides them with a physiological environment resembling their in vivo environment in the skin.

Synthesis [10] and degradation of connective tissue [17] have been extensively studied using this in vitro model. In this report, we demonstrate the expression of IL-6 by fibroblasts which have been cultured in 3D collagenous lattices, and compare these results to IL-6 expression by fibroblasts grown as monolayers. Seeding fibroblasts in contact with a 3D matrix leads to a time-dependent increase of IL-6. Within 3 days, steady-state levels of IL-6 mRNA increase 13-fold as compared to fibroblasts cultured as monolayers. Concomitantly, there is an increase in

IL-6 activity; however, induction of protein activity seems to be delayed as compared to the induction observed on the mRNA level. This delay may be attributed to the molecular processes involving translation and secretion of the mature product into the culture supernatant. Alternatively, lower levels of protein activity might result from regulatory mechanisms operating post-transcriptionally, possibly involving mRNA stability or alterations in translational efficiency.

A role for LPS in the IL-6 induction in collagen gels could be excluded, since LPS contamination of the purified collagen preparation was below the detection threshold of 0.1 ng/ml. Furthermore, IL-6 activity in 3D cultures was induced at LPS levels of 10 ng/ml or higher.

The mechanism leading to increased IL-6 mRNA levels in contracting collagen gels is unclear. Possible signal transduction pathways might involve cell/matrix interactions mediated by membrane-associated collagen receptors [19] that transmit the signals via the cytoskeletal network to the nucleus, resulting in altered gene expression. Recent evidence [20] showed that gel contraction depends upon the interaction of fibroblasts with the collagen fibrils via the $\alpha_2\beta_1$ integrin. mRNA as well as protein levels for both receptor subunits are selectively induced in 3D cultures, and gel contraction can be blocked by the respective antibodies. Future experiments will show in which way the expression of IL-6 is altered in 3D cultures that have been prevented from contracting by such antibodies. There is evidence, however, that the contact of fibroblasts with collagen fibrils, such as there is in cells grown on the surface of a collagen coat already suffices to lead to elevated IL-6 levels. This is in agreement with reports on cytokine induction by monocytes and macrophages cultured on fibronectin and collagen substrata which lead to induced levels of granulocyte-macrophage colony stimulating factor (GM-CSF), IL-1 and TNFα (reviewed in [21]).

While little is known about the modulatory action exerted by the matrix, the inducing effect of IL-1 on IL-6 gene expression involving a nuclear factor resembling or identical to NF-κB has been elucidated [22]. Our studies demonstrate that in addition to the inducing effect by cell/matrix interactions, IL-6 expression is further increased when IL-1 is added to the culture. The rise in IL-6 is more pronounced when the cells are stimulated from the beginning of the culture, as compared to those cells that have already constructed the tissue-like 3D network before they receive the IL-1 stimulus. This difference may reflect changes in the pattern of cell surface molecules, possibly involving the expression and/or distribution of the IL-1 receptor.

Results obtained in our own laboratory indicate that IL-1 under the conditions described has no significant effect on the levels of α_1(I) collagen mRNA (not shown). These observations are in good agreement with recent reports correlating increased collagen expression in monolayers stimulated with IL-1 [23] or with IL-6 [24] with low serum levels of 1%, whereas raising the FCS concentration to 10% abolishes this effect.

In contrast, a decrease in collagen content has been described in 3D gel cultures stimulated by IL-1 [18]. However, a direct comparison is questionable: even though both culture systems represent collagen I matrices supplemented with 10% FCS, the system described in [18] does not contract, and may thus feature different cell/matrix interactions than our system.

The data presented here, utilizing an in vitro model for connective tissue contractions which occur during wound healing, may thus reflect early events taking place during normal tissue repair. Further studies, applying in situ detection techniques to skin biopsies, are necessary to clarify whether elevated IL-6 levels play a role in this process, as well as in the inflammatory stage of fibrotic disease.

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