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Soluble IL-6 receptor induces calcium flux and selectively modulates chemokine expression in human dermal fibroblasts

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Abstract

Truncated forms of cytokine receptors have been regarded as modulators of the activity of their cognate ligands. In addition to inhibiting effects of their respective ligands, soluble receptors can also facilitate ligand-mediated signaling. Several studies have demonstrated that exogenous IL-6 in association with the soluble IL-6 receptor α (sIL-6Rα) can activate cells expressing the gp130 signal transducer lacking the specific, membrane-bound IL-6Rα. Since cell cultures of human dermal fibroblasts express high amounts of IL-6, we examined whether the addition of sIL-6Rα in association with endogenous IL-6 would be sufficient to stimulate these cells via gp130. As an early rapid signal we analyzed changes in intracellular free calcium concentrations ($[Ca^{2+}]_i$). Addition of sIL-6Rα induced an acute and transient increase in cytosolic free calcium concentrations in a dose-dependent fashion. This Ca^{2+}-signal was abolished when cells were pretreated with anti-IL-6 or anti-gp130 antibodies. Using flow cytometric analysis we could demonstrate membrane-associated IL-6 and gp130, but not IL-6Rα on fibroblasts. We also analyzed MCP-1 and IL-8 expression as a response involved in the more recently recognized chemoattractant functions of fibroblasts, and found MCP-1 to be up-regulated, but not IL-8. These data suggest that sIL-6Rα binds to cell-associated, endogenous IL-6 produced by fibroblasts and this complex then activates the cells via gp130. This pathway of fibroblast activation by sIL-6Rα adds another dimension to the role of fibroblasts in the cytokine network.

Introduction

IL-6 is a multifunctional cytokine that acts on a wide variety of cells. It was originally identified as a factor that induces Ig production in B lymphocytes (1). IL-6 also stimulates myeloma and plasmacytoma cells to proliferate, hematopoietic progenitors to expand, megakariocyte progenitors to proliferate and differentiate, and T cells to proliferate or differentiate into cytotoxic T cells (2). The effects of IL-6 are mediated by a functional IL-6 receptor (IL-6R), which consists in a heterodimer formed by an IL-6-binding α chain (IL-6Rα) and a signal-transducing subunit, gp130 or IL-6Rβ (3). Binding of IL-6 to the IL-6Rα induces homodimerization of two gp130 molecules (4), leading to intracellular signals such as, for example, a transient increase of 1,4,5-inositoltrisphosphate followed by a transient increase of cytosolic free calcium $[Ca^{2+}]_i$ (5). Cytosolic free calcium acts as a second messenger to control cellular processes such as cell growth, transformation, cytokine gene induction or secretion (6).

Soluble IL-6Rα (sIL-6Rα) can be generated through proteolytic cleavage of the IL-6Rα (7,8) or by alternatively spliced messages encoding a secreted form of the IL-6Rα (9). Soluble IL-6Rα is present in normal serum (76 ng/ml) and urine (>350 ng/ml) (10,11). Elevated concentrations have been reported in a variety of clinical conditions including inflammatory diseases and HIV infection (10,12). Other clinical conditions, e.g. severe septicemia, are characterized by decreased concentrations of sIL-6Rα (13).

Soluble IL-6Rα binds IL-6 with low affinity and prolongs the plasma half-life of IL-6 (14,15). The sIL-6Rα–IL-6 complex is

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able to interact with the signal transducing subunit gp130 and renders cells sensitive to IL-6 even when they do not express specific IL-6Rα on their surface (16–18).

Over the past few years fibroblasts have been recognized as important cellular sources of cytokines and chemokines. IL-8, for example, which is known for its ability to act as a very potent chemoattractant for neutrophils (19), is secreted in large amount by dermal fibroblasts when co-cultured with T cells or stimulated with IL-1 or lipopolysaccharide (20). Fibroblasts are also known to secrete MCP-1, which is a specific chemoattractant for monocytes (21) in response to tumor necrosis factor (TNF)-α and IL-1 (22).

The importance of the role of fibroblasts and soluble cytokine receptors in the cytokine network is illustrated by the present findings. Fibroblasts treated with sIL-6Rα reacted rapidly with an acute and transient increase of intracellular calcium concentrations and by expressing IL-6 and MCP-1, but not IL-8.

Methods

Materials

Recombinant human sIL-6Rα, mAb against gp130 and mAb against human IL-6 were from R & D Systems (Minneapolis, MN). Bradykinin (BK) was from Sigma (St Louis, MO). Antibodies used for flow cytometric analysis are described in the corresponding section (see below).

Cell cultures and preparation

Human dermal foreskin fibroblasts (Children's Hospital, University of Bern) at passage 5–12 were cultured and grown to confluence in MEM supplemented with 10% FBS (Seromed, Basel, Switzerland), 200 U/ml penicillin (Hoechst, Frankfurt, Germany) and 10 μg/ml chlortetracycline–HCl (Hoechst). Confluent fibroblast cultures were incubated for 24 h in MEM without FBS before stimulation with sIL-6Rα (R & D Systems).

Ca^{2+} measurements

Cytosolic free Ca^{2+} was determined in fura-2-loaded single cells with a calibrated video imaging system as described in detail by Reber and Reuter (23). In short, fibroblasts grown on glass-coverslides were incubated with 3 μM fura-2 acetoxymethylester (Molecular Probes, Eugene, OR) for 45 min at 37°C, and were then washed 3 times with a buffer consisting of 140 mM NaCl, 5 mM KCl, 1.5 mM $MgCl_2$, 2 mM $CaCl_2$ and 10 mM HEPES–NaOH (pH 7.4). Coverslides were glued to a Petri dish with four recording chambers by means of Vaseline. Changes in the intensity of fura-2 fluorescence were obtained by dual wavelength excitation (340/380 nm) with emission at 510 nm. Calibration of fluorescence in terms of $[Ca^{2+}]_i$ was calculated from the ratio 340/380 excitation fluorescent values.

Flow cytometry

After washing twice with PBS, confluent high-density fibroblasts were detached by incubation with 50 mM EDTA in PBS (pH 7.4) for 1 h on ice followed by gentle scraping with a rubber policeman. The surface expression of IL-6 and related molecules and the capacity to bind sIL-6Rα were assessed by labeling with specific antibodies followed by flow cytometry analysis on a FACScan II calibrated with fluorescent CaliBRITE beads using AutoCOMP software (Becton Dickinson, San Jose, CA). All incubations were carried out on ice for ⩾1 h; mAb and sIL-6Rα were used at 5 μg/ml, and rhIL-6 at 25 μg/ml (the highest concentration available). Reagents used included mAb to IL-6 (clone 6708.111; cat. no. MAB206), to IL-6Rα (clone 17506.1; cat. no. MAB227), to gp130 (clone 28126.111; cat. no. MAB228), recombinant human sIL-6Rα (cat. no. 227-SR-025), recombinant human IL-6 (cat. no. 206-IL-010) (all from R & D Systems); mAb to mucin [B72.3 (24); kindly provided by Dr E. R. Waelti, Department of Immunopathology, University of Bern, Switzerland], and fluorescein-conjugated polyclonal goat anti-mouse IgG (Sigma). Data were obtained with CellQuest software (Becton Dickinson) run on a Macintosh computer and analyzed with Repro-PowerMac software (Truefacts Software, Seattle, WA). Staining of intact fibroblasts is shown after gating on forward and side scatter as fluorescence emission intensity at 520 nm upon excitation at 488 nm, on a logarithmic scale covering four orders of magnitude.

Cytokine measurement (RT-PCR analysis and ELISA)

Total cellular RNA was extracted and purified using a single-step acid guanidinium thiocyanate–phenol–chloroform extraction method with Trizol reagent (Life Technologies, Paisley, UK) from 0.5×10^6 cells that had been treated with sIL-6Rα (2 nM) for 1, 7 or 24 h, or left untreated. RNA was resuspended in H_2O and quantitated by measuring absorbance at 260 nm. Semi-quantitative RT-PCR was carried out on mixtures of 1 ng total cellular RNA and 2.5 fg *in vitro* synthesized standard RNA as described (25). Poly(A)-tailed RNA was primed with oligo-$d(T)_{16}$ (2.5 μM) and reverse transcribed with 50 U of MMLV reverse transcriptase (Perkin-Elmer Cetus, Norwalk, CT) at 42°C for 15 min. MCP-1, IL-8, IL-6 and β-actin cDNAs were generated in a standard PCR reaction. Thirty amplification cycles of 94°C for 60 s and 60°C for 30 s each were performed with appropriate primers (25). Amplicons obtained from standard RNA were 370 bp in length and were separated from smaller cellular RNA-derived amplicons by 2% agarose gel electrophoresis and visualized under UV light after staining with ethidium bromide. Digitized images are presented in Fig. 3.

The levels of immunoreactive MCP-1 and IL-8 in cell culture supernatants were quantitated by a conventional sandwich ELISA, using mAb 2805.31 (capture) and biotinylated goat anti-human MCP-1 polyclonal antibody or mAb 6217.11 (capture) and biotinylated goat anti-human IL-8 polyclonal antibody respectively (all from R&D Systems) for detection with horseradish peroxidase-conjugated streptavidin (Southern Biotechnology Associates, Birmingham, AL).

Results

Soluble IL-6Rα induces a transient increase of cytosolic free calcium

Addition of increasing amounts of sIL-6Rα to fibroblasts resulted in a dose-dependent acute and transient rise in $[Ca^{2+}]_i$ (Fig. 1A). Low doses (0.2 nM) of sIL-6Rα induced a slight but delayed increase in $[Ca^{2+}]_i$. At higher concentrations

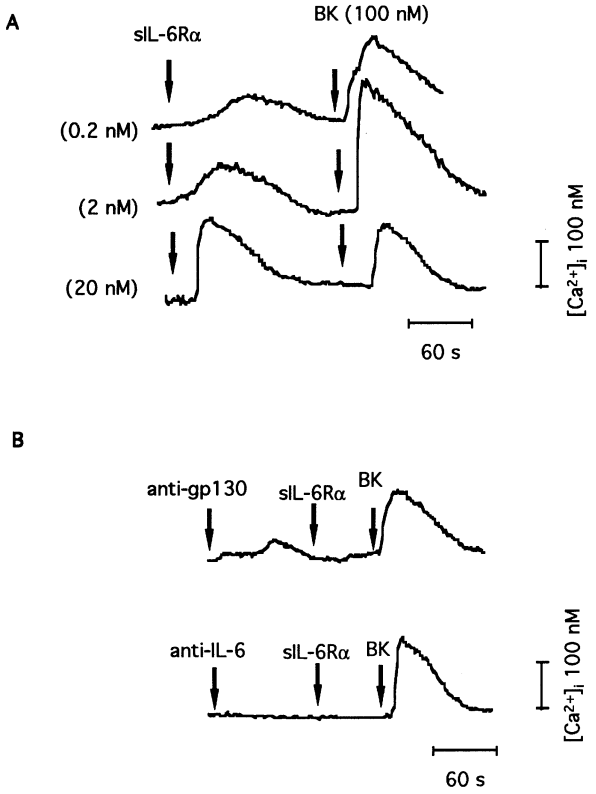

Fig. 1. sIL-6Rα mobilizes cytosolic free calcium in fibroblasts; antibodies against gp130 and IL-6 block mobilization of cytosolic free calcium. Ratio fluorometry of fura-2-loaded fibroblasts was used to measure $[Ca^{2+}]_i$. Basal $[Ca^{2+}]_i$ values in untreated cells were between 90 and 110 nM. (A) Increasing concentrations of sIL-6Rα were added to fibroblasts grown on glass coverslides and changes in $[Ca^{2+}]_i$ were measured. BK (100 nM) was used as a positive control, added after signals returned to baseline values. Traces represent mean values of an experiment with 10 cells measured in real time. (B) Fibroblasts were treated with 100 μg/ml of neutralizing mAb against gp130 (top trace) or with 100 μg/ml of neutralizing mAb against IL-6 (bottom trace) and were subsequently stimulated with sIL-6Rα (20 nM). After a delay of 60 s, cells were stimulated with BK (100 nM) as a control. Panels (A) and (B) are from the same experiment; experiments were repeated at least 3 times.

(20 nM), the $[Ca^{2+}]_i$ rose rapidly and showed an acute increase of ~170 nM $[Ca^{2+}]_i$ followed by a slow decrease. Human dermal fibroblasts express high amounts of functional BK B2 receptors. When activated with BK they respond with an acute and transient increase in $[Ca^{2+}]_i$ (26). Addition of BK (100 nm) was therefore used as a positive control.

To analyze the mechanism of calcium induction by sIL-6Rα, the role of gp130 and IL-6 was studied by exposing cells to anti-gp130 and anti-IL-6 antibodies prior to stimulation. Addition of anti-gp130 resulted in a small delayed transient increase in $[Ca^{2+}]_i$. (Fig. 1B) These cells could no longer be stimulated with sIL-6Rα (20 nM) while BK could still elicit a rapid rise in $[Ca^{2+}]_i$. Antibodies against IL-6 did not elicit a calcium response by themselves in the fibroblasts and completely blocked the effect of sIL-6α, but cells still responded to a subsequent stimulation with BK. Thus both gp130 and IL-6 play a role in the signal pathway of sIL-6Rα.

IL-6 and gp130 are expressed on the cell surface of fibroblasts, IL-6Rα is not

Since IL-6 and gp130 appeared to be involved in the signaling mechanism of sIL-6Rα, flow cytometry was used to visualize these molecules on the fibroblast cell surface. Confluent fibroblasts were detached by incubation with EDTA and labeled with specific antibodies followed by a FITC-conjugated antibody. Figure 2A shows that anti-IL-6 antibodies labeled nearly all cells as compared to irrelevant isotype (mouse IgG1) control antibodies; pre-incubation of the anti-IL-6 antibodies with saturating amounts of exogenous, recombinant IL-6 (25 μg/ml for 5 μg/ml) prior to its addition to the cells totally abrogated the signal, demonstrating the specificity of the binding and strongly indicating that fibroblasts constitutively express IL-6 on their surface. Antibodies to the IL-6Rα chain did not react with untreated fibroblasts (Fig. 2B), while anti-gp130 antibodies weakly but distinctly stained a majority of the cells (Fig. 2C) (note that the control pattern in panel C is at a lower level compared to those of panels A and B since it is from another experiment with different machine settings). Fibroblasts thus do not constitutively express all components of functional IL-6 receptors, but only the β chain (gp130). Most interestingly, sequential incubation of fibroblasts with sIL-6Rα followed by anti-IL-6Rα elicited a strong labeling of the majority of the cells (Fig. 2B), indicating that (i) sIL-6Rα actively bound to the fibroblasts and (ii) it could still be recognized by antibodies directed against it; pre-incubation of sIL-6Rα with 25 μg/ml soluble IL-6 before addition to fibroblasts and subsequent staining with anti-IL-6Rα followed by FITC-conjugated anti-mouse IgG noticeably reduced the extent of labeling. The incomplete inhibition of the labeling may reflect the binding of the complex formed by sIL-6Rα and exogenous IL-6 to gp130.

Differential expression of IL-6, MCP-1 and IL-8 after stimulation with sIL-6Rα

To investigate a late response upon sIL-6Rα stimulation, we determined IL-6, MCP-1 and IL-8 mRNA expression. Confluent cultures of human dermal fibroblasts were starved for 24 h in FBS-free medium and then stimulated with sIL-6Rα (2 nM) for various incubation periods. IL-6, MCP-1 and IL-8 mRNA were constitutively expressed at low levels in untreated fibroblasts (Fig. 3A). IL-6 mRNA steady-state levels increased after 1 h of stimulation with sIL-6Rα (2 nM). The mRNA steady-state levels of MCP-1 reached high concentrations after 1 h of stimulation with sIL-6Rα and decreased after 7 h to reach basal concentrations at 24 h. Interestingly, mRNA steady-state levels of IL-8 were not affected at all. Expression of β-actin mRNA steady-state levels was also unchanged. In order to substantiate this observation, we used increasing amounts of total RNA (from 10^{-2} to 10^2 ng) from untreated and sIL-6Rα (2 nM)-stimulated (1 h) fibroblasts, and added to each sample a fixed quantity of standard RNA (1 ng) (Fig. 3B). Amplicons derived from standard RNA could be competed with increasing concentrations of cellular RNA. We noted that in untreated fibroblasts, an at least 10-fold higher RNA concentration was required to obtain cellular RNA-derived MCP-1 amplicons.

To determine whether the mRNA steady-state levels of the two chemokines MCP-1 and IL-8 correlated with the protein

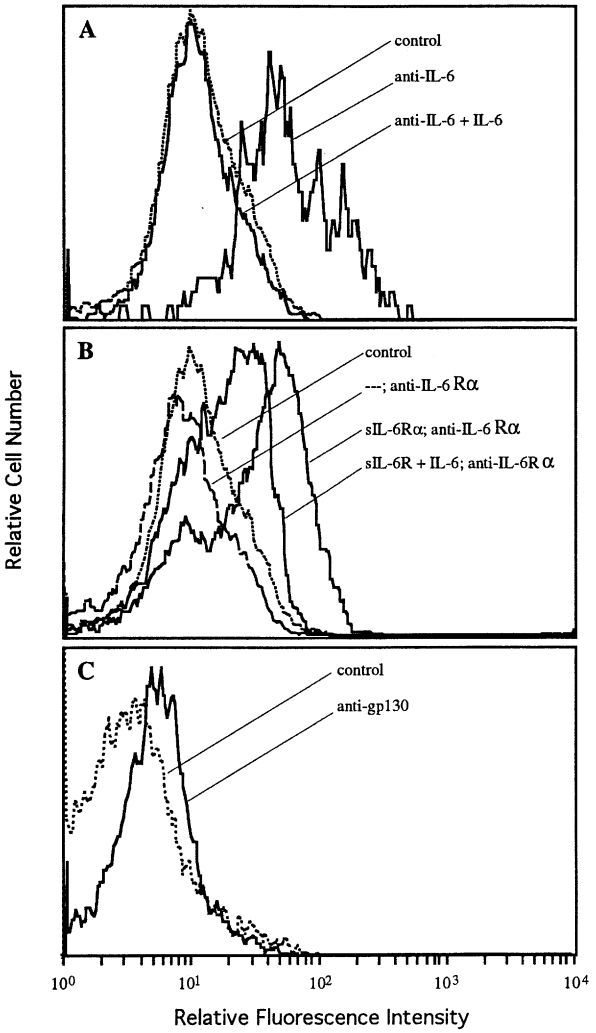

Fig. 2. Demonstration by flow cytometry of the expression of IL-6 and related molecules on the surface of fibroblasts. Confluent, adherent high-density fibroblasts were detached by incubation with EDTA, labeled as indicated below and analyzed by conventional flow cytometry as described in Methods. (A) Cells were labeled with isotype control antibody B72.3 (dotted line), antibody to IL-6 (solid line) or antibody to IL-6 that had been pre-incubated previously with recombinant human IL-6 (gray line), followed by FITC-conjugated goat anti-mouse IgG. (B) Cells were labeled with B72.3 (dotted line), anti-IL-6Rα antibody (stippled line), sIL-6Rα followed by anti-IL-6Rα antibody (solid line) or sIL-6Rα previously pre-incubated with IL-6, followed by anti-IL-6Rα (gray line). The last step reagent in all cases was FITC-conjugated goat anti-mouse IgG. (C) Cells were labeled with B72.3 (dotted line) or antibody to gp130 (solid line), followed by FITC-conjugated goat anti-mouse IgG. Panels (A) and (B) are from the same experiment; panel (C) is from another experiment with different machine settings.

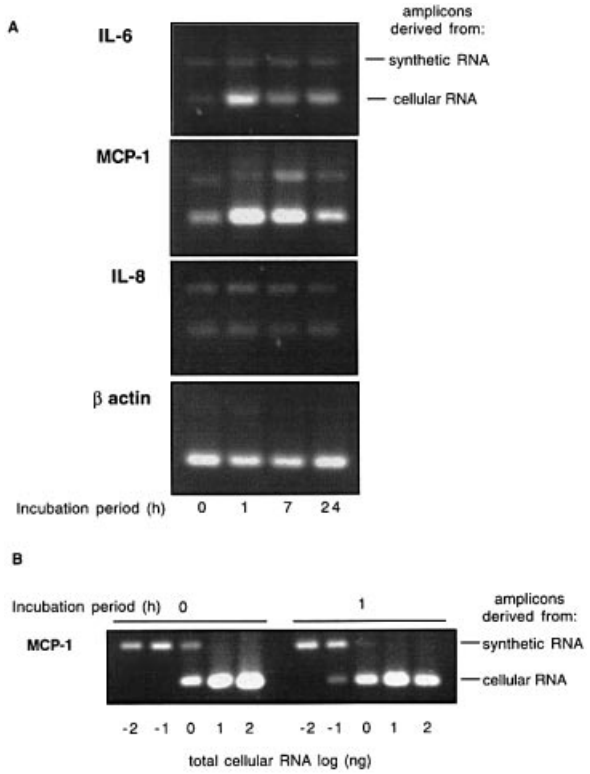

Fig. 3. Differential expression of IL-6, MCP-1 and IL-8 mRNA after stimulation with sIL-6Rα. (A) Fibroblasts were treated for 1, 7 and 24 h with sIL-6Rα (2 nM) or left untreated before RNA was extracted. PCR reactions were performed with a mixture of cDNA reverse transcribed from cellular RNA and *in vitro* transcribed polycompetitive RNA. Amplicons were resolved on 2% agarose gels and stained with ethidium bromide. Experiments were repeated 3 times. (B) Increasing amounts of total RNA (from 10^{-2} to 10^{2} ng) from untreated and sIL-6Rα-treated (1 h) fibroblasts were added to a fixed (1 ng) amount of standard RNA and RT-PCR reactions were performed as described above.

concentrations, cell culture supernatants were tested by ELISA specific for MCP-1 and IL-8 (Fig. 4). As expected both MCP-1 and IL-8 were constitutively produced and secreted at low levels (1.9 ± 0.1 ng/ml for MCP-1 and 1.1 ± 0.2 ng/ml for IL-8, mean ± SD). When fibroblasts were stimulated with either 2 or 20 nM sIL-6Rα for 24 h, MCP-1 increased to 4.5 ± 0.5 and 11.5 ± 1.4 ng/ml respectively, while IL-8 remained unchanged. Antibodies (10 μg/ml) against IL-6 reduced sIL-6Rα-stimulated MCP-1 concentrations by 66%.

Discussion

Fibroblasts are an important source as well as a target of cytokines, a feature that explains their impact on diverse disease processes. These cells not only respond to well-known inducers of inflammation, such as IL-1 or TNF-α, but as we reported in the present and in an earlier study (27), also to soluble cytokine receptors. Soluble forms of cytokine receptors are thought to modify ligand concentration by stabilizing the ligands or by specifically inhibiting interactions of ligands with their membrane-bound receptors (10).

In the present study we demonstrate that human dermal fibroblasts responded to sIL-6Rα with a transient increase in cytosolic free calcium concentration, and enhanced IL-6 and MCP-1 expression. Both of these findings point to an additional role of sIL-6Rα as an agonist stimulating fibroblasts. Interestingly, IL-8 was not concomitantly up-regulated, pointing to a

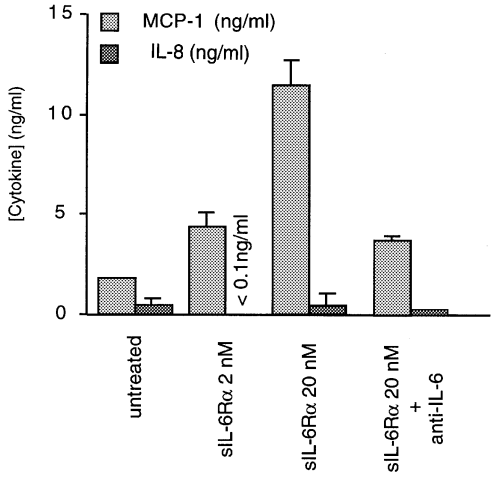

Fig. 4. Increased secretion of MCP-1 but not IL-8 after stimulation with sIL-6Rα. MCP-1 and IL-8 were measured in the cell culture supernatants by ELISA. Fibroblasts were either left untreated or stimulated with 2 or 20 nM for 24 h. In some cases fibroblasts were treated with 10 μg/ml antibodies against human IL-6 for 1 h prior to addition of sIL-6Rα. Results are expressed as mean ± SD of three experiments.

mechanism of activation different from that observed with IL-1 stimulation.

It is known that sIL-6Rα together with its ligand (IL-6) interacts with gp130 leading to a variety of biological effects (16,28). This has been demonstrated in studies in which cells lacking the IL-6Rα subunit were treated with sIL-6Rα together with recombinant exogenous IL-6 (18,29,30). In our experiments sIL-6Rα addition to fibroblasts was sufficient to stimulate fibroblasts. Since sIL-6Rα do not bind directly to gp130, we suggest that it first binds to endogenous, cell-surface associated IL-6. Using flow cytometry we could indeed demonstrate that sIL-6Rα binds to the fibroblasts and this binding could be reduced by adding exogenous rhIL-6. Moreover, the sIL-6Rα-induced calcium signal could be blocked by preincubation of the cells with antibodies against IL-6. Together these data support the hypothesis that endogenous IL-6 expressed on the cell surface of fibroblasts may act as the binding site for sIL-6Rα. The complex formed by the two molecules, e.g. sIL-6Rα and IL-6, could then bind to gp130. The latter is expressed on the fibroblast cell surface and, since antibodies against gp130 inhibited the sIL-6Rα-induced calcium signal, also seems to participate in this signal transduction pathway.

The ability to induce chemokines was suggested to be a common feature among cytokines using gp130 as the common transducer for signaling (31). We found that MCP-1 was significantly enhanced in fibroblasts treated with sIL-6Rα. Interestingly, this activation did not up-regulate IL-8, much in contrast to other stimuli such as IL-1. The same differential up-regulation of MCP-1 was found in IL-6-treated blood mononuclear cells (32). MCP-1 exerts a crucial function in the regulation of leukocyte trafficking by eliciting directional migration of mononuclear phagocytes (21). In diseases where elevated levels of sIL-6Rα are found, such as in HIV infection, skin fibroblasts may be important in regulating the extravasation of these cells by producing high amounts of MCP-1. Several reports point to the importance of IL-6 and IL-6-induced MCP-1 in Kaposi's sarcoma, which is characterized by a prominent leukocyte infiltrate (33,34). We observed an increased expression of IL-6 upon stimulation of the fibroblasts with sIL-6Rα. It is likely that the presence of sIL-6Rα could induce an autocrine growth loop and therefore be important in enhancing pathological processes such as Kaposi's sarcoma.

In conclusion, we show that soluble IL-6Rα is capable to induce signaling in human dermal fibroblasts. IL-6 is constitutively produced by fibroblasts, where it remains in part cell-associated, allowing it to serve as a binding site for sIL-6Rα. The signaling effect of this sIL-6Rα–IL-6 complex leads to expression of IL-6 and MCP-1. These *in vitro* findings support the concept that soluble cytokine receptors play a rather unexpected role acting directly as agonists on dermal fibroblasts by stimulating important cell functions, such as calcium release and chemokine expression.

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Abbreviations

BK	bradykinin
IL-6Rα	IL-6 receptor α-subunit
sIL-6Rα	soluble IL-6Rα
TNF	tumor necrosis factor

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