Soluble IL-1 receptor type I binds to human dermal fibroblasts and induces calcium flux

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Abstract Soluble cytokine receptors appear to modify ligand concentrations by stabilizing ligands or by specifically inhibiting interactions of ligands with their membrane-bound receptors. Here we describe a new function of the soluble interleukin-1 receptor type I (IL-1sR I). This receptor induced a transient rise of intracellular free calcium concentration in human dermal fibroblasts in a dose-dependent fashion. Mobilization of calcium by IL-1sR I was abolished in the presence of an equimolar concentration of IL-1 receptor antagonist (IL-1ra). Neutralizing antibodies against IL-1β also abolished calcium mobilization stimulated with IL-1sR I indicating that IL-1β is involved. IL-1sR I bound with high affinity (K_d 1–2 nM) to the fibroblasts. In addition, IL-1sR I enhanced expression of IL-6 and IL-8 mRNA. The observation that IL-1sR I can act as a ligand and agonist for membrane IL-1 extends the concept of the ligand-receptor functions of both IL-1 and IL-1sR I and adds a new dimension to the cytokine network.

Key words: Interleukin-1; Soluble receptor; Calcium; Signal transduction; Fibroblast

1. Introduction

Interleukin-1 and its receptors control immunological and inflammatory processes [1]. Three members of the IL-1 family are known today: IL-1α, IL-1β, and IL-1 receptor antagonist (IL-1ra). These ligands bind to two distinct receptors that belong to the IgG superfamily: the type I and type II IL-1 receptors (IL-1R I/II). Both type I and type II receptors have been cloned from human and murine cell lines [2]. They possess a single membrane-spanning segment and an extracellular part displaying three immunoglobulin-like domains [3]. The type I IL-1 receptor mediates the biological effects of IL-1 while the type II receptor does not transduce signals and apparently serves as a decoy receptor for IL-1 [4]. IL-1 signaling is believed to result from the formation of a ternary complex consisting of an IL-1 agonist, IL-1R I and IL-1R accessory protein (IL-1R-AcP) [5]. Although much is known about IL-1 receptor heterogeneity and binding, there is little agreement as to how the signal transduction pathways are utilized and restricted. IL-1-induced receptor-mediated increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) has been reported in human fibroblasts and chondrocytes [6,7].

Soluble forms of both types of IL-1R are generated by proteolytic cleavage of the membrane anchored receptors [8]. Naturally occurring soluble forms of IL-1 receptor type I (IL-1sR I) and type II (IL-1sR II) exist physiologically in body fluids [9–11]. They are produced by mononuclear cells [12] and are considered natural 'buffers' capable of binding IL-1α, IL-1β, and IL-1ra in healthy and inflamed tissues [1].

IL-1α, IL-1β, and IL-1ra are synthesized by activated monocytes/macrophages as 31–35 kDa propeptides and are secreted after cleavage as 15–17 kDa mature bioactive peptides [1]. Blood mononuclear cells transiently express pro-IL-1α on their surface, through which they are capable of signaling to endothelial cells expressing IL-1R type I [13]. In fibroblasts and endothelial cells, pro-IL-1α and β remain cell-associated and usually are not secreted [14–16]. Membrane-associated IL-1 on fibroblasts may be used in a juxtacrine mechanism to activate T cells via their IL-1R [17].

We now show that signaling in the other direction may also occur. Soluble IL-1 type I receptor induced a rise in cytosolic free calcium in fibroblasts and this response was greatly enhanced when cells were pretreated with tumor necrosis factor α (TNF-α), which is well known to increase IL-1 expression in fibroblasts. Mobilization of cytosolic free calcium by IL-1sR I was abolished in the presence of an equimolar concentration of IL-1ra or by pretreatment of the cells with anti-IL-1β antibodies. IL-1sR I bound with high affinity to the fibroblasts (K_d 1–2 nM). In addition, fibroblasts responded to this interaction with an enhanced expression of IL-6 and IL-8 mRNA. These results suggest that soluble IL-1R I not only inhibits the activity of IL-1 in inflamed tissue by binding free IL-1, but also may act as an agonist by interacting with cell-associated IL-1.

2. Materials and methods

2.1. Materials

Recombinant human IL-1sR and recombinant human IL-1ra I were purchased from R&D Systems, Minneapolis, MN. Different batches

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Abbreviations: IL-1, interleukin-1; IL-1ra, interleukin-1 receptor antagonist; IL-1R I, type I IL-1 receptor; IL-1R-AcP, IL-1R accessory protein; IL-1sR I, soluble IL-1 receptor type I; BK, bradykinin; IL-6Rα, soluble IL-6 receptor α chain

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of IL-1sR I were used that gave comparable results. Recombinant human IL-1β was from Boehringer Mannheim Biochemica, Germany. Monoclonal antibodies (mAb) against IL-1β were from R&D Systems, and from Serotec Ltd Oxford, UK. Polyclonal antibodies against IL-1β and recombinant human TNF-α were from PeproTech, Rocky Hill, NJ. Lipopolysaccharides from *Escherichia coli* and bradykinin were from Sigma, St. Louis, MO.

2.2. Cell cultures

Human dermal foreskin fibroblasts from two different donors (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 mg/ml chlortetracycline-HCl (Hoechst). Where indicated, fibroblasts were treated with TNF-α (10 ng/ml) for 24 h. Mono-Mac-6 cells (kindly provided by Dr. Beda Stadler, Department of Immunology, University of Bern, Switzerland) were cultured in RPMI 1640 containing 10% FBS, 200 U/ml penicillin and 10 mg/ml chlortetracycline-HCl.

2.3. $[Ca^{2+}]_i$ 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 [18]. In short, fibroblasts grown on glass coverslides were incubated with 3 μM fura-2 acetoxymethylester (Molecular Probes) for 45 min at 37°C and were then washed three 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 nm/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.

2.4. Binding studies

IL-1sR I (0.5 nmol) was iodinated to a specific activity of 1838 Ci/mmol with Enzymobead reagent (Bio-Rad Laboratories, Richmond, CA) and 2 mCi Na ^{125}I (Amersham). Iodinated IL-1sR I was separated from free ^{125}I by gel filtration chromatography (Bio-Gel P-6 DG, Bio-Rad). 0.5×10^6 fibroblasts in binding buffer were incubated on ice with increasing concentrations of ^{125}I-IL-1sR I in the presence or absence of 500 nM unlabeled IL-1sR I. After centrifugation through 6% BSA in PBS, supernatants were removed and radioactivity of the cell pellets counted in a gamma counter.

2.5. Immunoprecipitation of IL-1β

Fibroblasts and Mono-Mac-6 cells were starved for 2 h in methionine free complete MEM or RPMI 1640, respectively. After 2 h, 50 μCi [^{35}S]methionine/cysteine (DuPont) was added and fibroblasts or Mono-Mac-6 cells were labeled for 24 h in the presence of TNF-α (10 ng/ml) or LPS (10 μg/ml), respectively. Cell culture supernatants were removed, sterile filtered and analyzed for secreted IL-1β by immunoprecipitation. Labeled cells were washed with cold PBS, and solubilized in 1 ml lysis buffer containing 25 mM Tris-HCl (pH 8), 50 mM NaCl, 1% DOC, 1% NP40 and protease inhibitors (1 μg/ml pepstatin, 1 μg/ml aprotinin, 5 μg/ml leupeptin, 17.4 μg/ml benzamide, 1.7 mM PMSF) for 30 min on ice. Cell debris and nuclei were separated from the cellular extracts by centrifugation at $10\,000\times g$. For immunoprecipitation, cellular extracts and cell culture supernatants were precleared twice with 50 μl protein A beads (Pharmacia) for 1 h and IL-1β immunoprecipitated by incubation with polyclonal anti-IL-1β antibodies coupled to protein A beads for 2 h. Precipitated proteins were separated by 10% SDS-PAGE and ^{35}S-labeled IL-1β was visualized by fluorography. Cell-associated IL-1β was also determined by ELISA (R&D Systems) in cellular extracts prepared from fibroblasts lysed by three cycles of freeze-thawing in 0.15 ml of 10 mM Tris-HCl (pH 7.5).

2.6. RT-PCR

Total cellular RNA was extracted and purified using a single step acid guanidinium thiocyanate-phenol-chloroform extraction method (Trizol, Life Technologies, Paisley, UK) from 0.5×10^6 cells that were incubated with or without IL-1sR (20 nM) for 5 h. RNA was resuspended in H_2O and quantitated by measuring absorbance at 260 nm. Semi-quantitative RT-PCR was carried out with mixtures of 1 ng total cellular RNA and 2.5 fg in vitro synthesized standard RNA as described [19]. PolyA tailed RNA was primed with oligo $d(T)_{16}$ (2.5 μM) and reverse transcribed with 50 U of M-MLV reverse transcrip-

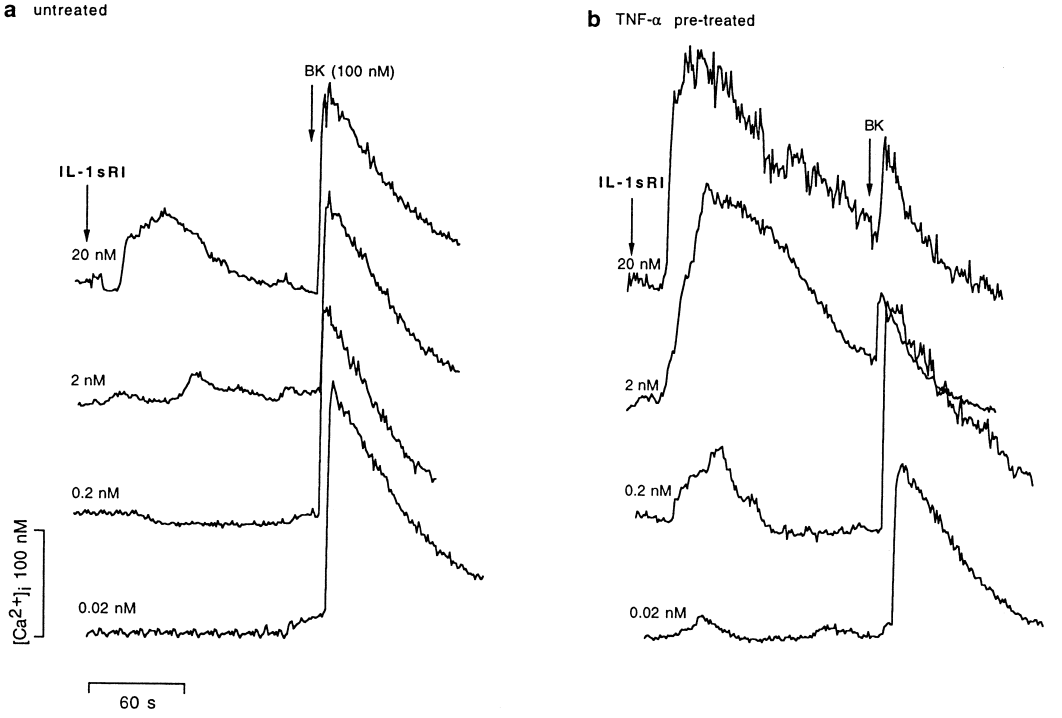

Fig. 1. IL-1sR I mobilizes cytosolic free calcium in fibroblasts. Ratio fluorometry of fura-2 loaded fibroblasts was used to measure $[Ca^{2+}]_i$. a: Increasing concentrations of IL-1sR I were added to fibroblasts grown on glass coverslides and changes in $[Ca^{2+}]_i$ were measured. Bradykinin (BK, 100 nM) was used as a positive control, added after signals returned to baseline values. b: The same experiment was performed with cells that had been pretreated with TNF-α (10 ng/ml) for 24 h. Traces represent mean values of an experiment with 10 cells measured in real time. The experiments were repeated at least three times with different batches of IL-1sR I and with fibroblasts from two different donors.

tase (Perkin-Elmer Cetus) at 42°C for 15 min. 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 [19]. Amplicons obtained from standard RNA are 370 bp in length and were separated from smaller cellular RNA-derived amplicons by 2% agarose gel electrophoresis and made visible by staining with ethidium bromide.

3. Results

3.1. IL-1sR type I causes mobilization of intracellular Ca^{2+} in fibroblasts

Addition of increasing amounts of IL-1sR I to fibroblasts resulted in a dose-dependent transient rise in $[Ca^{2+}]_i$ (Fig. 1a). This $[Ca^{2+}]_i$ rise was related to the IL-1 content of the fibroblasts. In cells in which IL-1 expression has been upregulated by pretreatment with TNF-α for 24 h, $[Ca^{2+}]_i$ flux was observed already at 0.02 nM of IL-1sR I (Fig. 1b). In native cells, a 100-fold higher concentration of IL-1sR I than in TNF-α pretreated cells was required to induce a similar Ca^{2+} response. All subsequent $[Ca^{2+}]_i$ measurements were therefore performed with TNF-α pretreated fibroblasts. When activated with bradykinin fibroblasts are known to respond with a transient increase in $[Ca^{2+}]_i$ [20]. Bradykinin stimulation was therefore used as a positive control. Cells which had been stimulated with IL-1sR I were still responsive to a second stimulation with bradykinin. The profile of the bradykinin-induced $[Ca^{2+}]_i$ changes by itself was similar to that elicited by IL-1sR I at the highest doses, i.e. a rapid initial rise was followed by a slower decrease in $[Ca^{2+}]_i$. Fibroblasts in Ca^{2+}-free buffer supplemented with 0.1 mM EGTA also responded to 20 nM IL-1sR I with a rapid, but short lasting increase in $[Ca^{2+}]_i$, (Fig. 2). Subsequent addition of extracellular Ca^{2+} (2 mM) caused a small transient rise in $[Ca^{2+}]_i$. However, depletion of intracellular calcium stores with thapsigargin, an inhibitor of endoplasmic reticular Ca^{2+}-ATPase [21], completely suppressed the Ca^{2+} signal after addition of either IL-1sR I or bradykinin. This result shows that the rapid rise in $[Ca^{2+}]_i$ was due to Ca^{2+} release from intracellular pools.

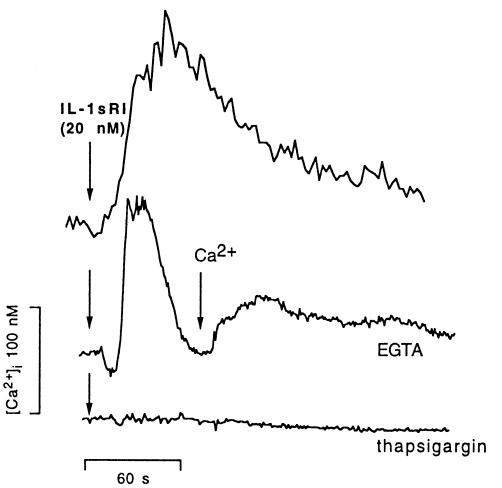

Fig. 2. IL-1sR I releases calcium from intracellular stores. Top trace: typical Ca^{2+} response to IL-1sR I of TNF-α pretreated fibroblasts. Middle trace: response to IL-1sR I in Ca^{2+}-free buffer containing 0.1 mM EGTA and effect of Ca^{2+} (2 mM) addition after the signal returned to baseline values. Bottom trace: lack of a signal in fibroblasts treated with thapsigargin (400 nM) to deplete internal Ca^{2+} stores. Experiments were repeated three times.

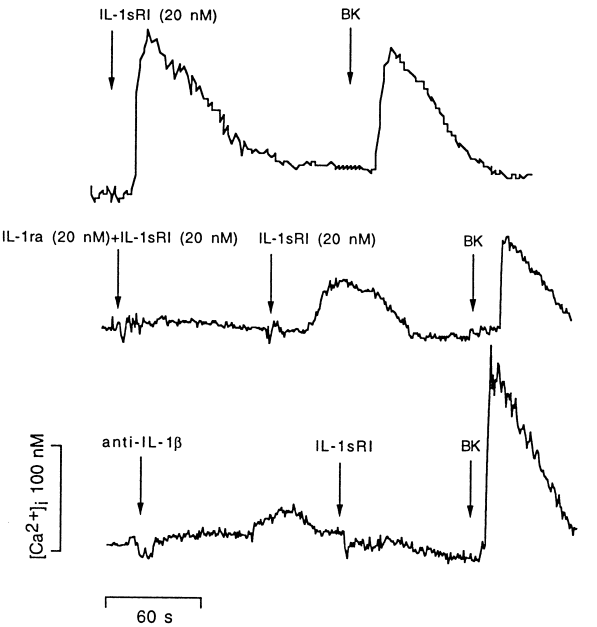

Fig. 3. IL-1ra and antibodies against IL-1β block mobilization of cytosolic free calcium. Top trace: typical Ca^{2+} response to IL-1sR I (20 nM) and bradykinin (100 nM). Middle trace: IL-1ra and IL-1sR I (20 nM each) were mixed and added to TNF-α pretreated fibroblasts. Excess IL-1sR I (20 nM) was added 90 s later. Bottom trace: fibroblasts were treated with neutralizing mAb against IL-1β (100 μg/ml) and subsequently stimulated with IL-1sR I (20 nM). Experiments were repeated at least three times. mAb against IL-1β from two different sources were used.

Receptor antagonist (IL-1ra) added in equimolar concentration together with IL-1sR I completely inhibited Ca^{2+} mobilization, but the cells still responded to 20 nM IL-1sR I added subsequently (Fig. 3). On the other hand, fibroblasts exposed to anti-IL-1β antibodies could no longer be stimulated with IL-1sR I indicating that IL-1 is involved in IL-1sR I induced signal transduction (Fig. 3). The response to bradykinin was unaffected under these conditions. In a separate experiment it was shown that IL-1β (0.1 nM) also induced a Ca^{2+} response in fibroblasts similar to that observed with IL-1sR I (data not shown), an observation that is consistent with recently published reports [6,7].

Collectively, the results obtained so far support the concept of a signaling function of IL-1sR I when interacting with membrane-associated pro-IL-1β.

3.2. IL-1sR type I binds to IL-1 on fibroblasts

Equilibrium binding assays with IL-1sR I showed that IL-1sR I indeed behaved like a ligand to membrane-bound IL-1. Specific binding of ^{125}I-IL-1sR I reached a saturation plateau at 7 nM (Fig. 4a) and Scatchard analysis yielded a K_d of 1–2 nM and approximately 3000 sites/cells (Fig. 4a, inset). In native fibroblasts, specific binding was significantly lower than in TNF-α pretreated cells (Fig. 4b). The increase of binding to TNF-α stimulated fibroblasts correlated with the higher intracellular Ca^{2+} mobilization (Fig. 1).

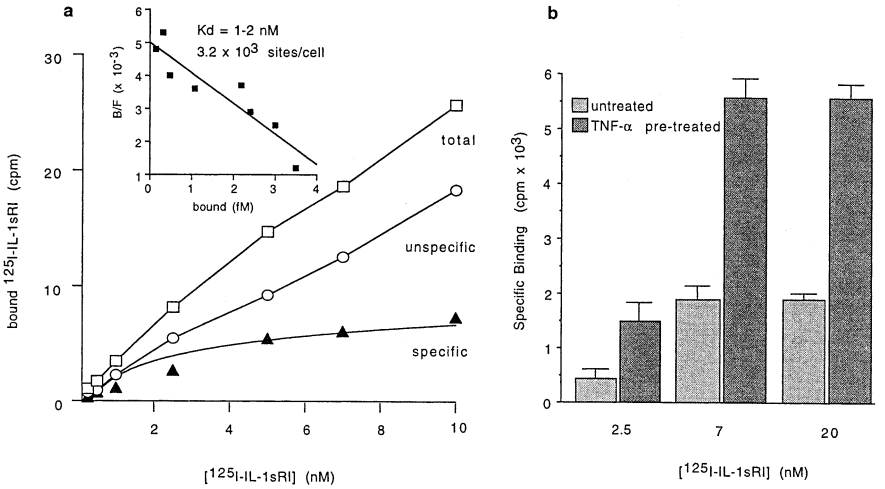

Fig. 4. IL-1sR I binds specifically to IL-1 on fibroblasts. a: Binding of ^{125}I-IL-1sR I to TNF-α pretreated fibroblasts (0.5×10^6 cells/measurement) was analyzed by an equilibrium binding assay. Total binding of labeled IL-1sR I (□), unspecific binding in the presence of an excess of unlabeled IL-1sR I (500 nM) (○), and specific binding (▲) is presented. The inset shows a Scatchard plot of the binding data, the number of binding sites, and the calculated K_d. b: Specific IL-1sR I binding to native and TNF-α pretreated fibroblasts at increasing concentrations of ^{125}I-IL-1sR I. Binding assays were repeated four times with two different batches of unlabeled IL-1sR I used for determination of unspecific binding.

3.3. *IL-1sR type I upregulates IL-8 and IL-6 mRNA expression*

Interleukin-8 and IL-6 mRNA are constitutively expressed at low levels in fibroblasts (Fig. 5). Stimulation with IL-1sR I (20 nM) for 5 h resulted in a significant increase of IL-8 and IL-6 mRNA. Expression of β actin mRNA was unchanged under the same conditions. The increase in IL-6 and IL-8 mRNA correlated with a 2–4-fold increase in cytokine secretion (data not shown).

3.4. *IL-1β is expressed, but not secreted by fibroblasts*

The apparent molecular mass of cell-associated IL-1β in fibroblasts was estimated by immunoprecipitation using anti-IL-1β antibody and SDS-PAGE. Cellular extracts of metabolically labeled and TNF-α stimulated fibroblasts were immunoprecipitated in the absence and presence of competing IL-1β (Fig. 6). Only one molecular species was observed at 33 kDa and as expected, the intensity of fluorescence decreased with increasing concentration of added unlabeled IL-1β. Neither secreted IL-1β nor pro-IL-1β was detected in the culture medium of TNF-α stimulated fibroblasts, although large amounts of pro-IL-1β were present in the cellular extracts. In contrast, pro-IL-1 was abundant in the supernatant as well as in the cellular extracts of LPS stimulated Mono-Mac-6 cells. Extracts from TNF-α stimulated fibroblasts contained significantly more IL-1β than extracts from untreated cells. This was shown by immunoprecipitation of metabolically labeled IL-1β (Fig. 7a) and by direct quantitation of IL-1β in the cellular extracts by ELISA (Fig. 7b).

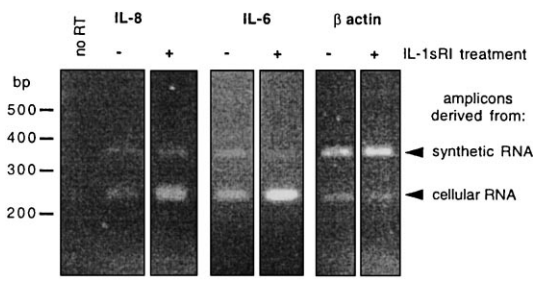

Fig. 5. Increased expression of IL-8 and IL-6 mRNA after stimulation with IL-1sR I. Fibroblasts were treated for 5 h with IL-1sR I (20 nM) before RNA was extracted. PCR reactions were performed with a mixture of cDNA reverse transcribed from cellular RNA and in vitro transcribed polycompetitive RNA. Amplicons were resolved on 2% agarose gels and stained with ethidium bromide. As a negative control reverse transcriptase was omitted in the RT reaction. Experiments were repeated at least three times.

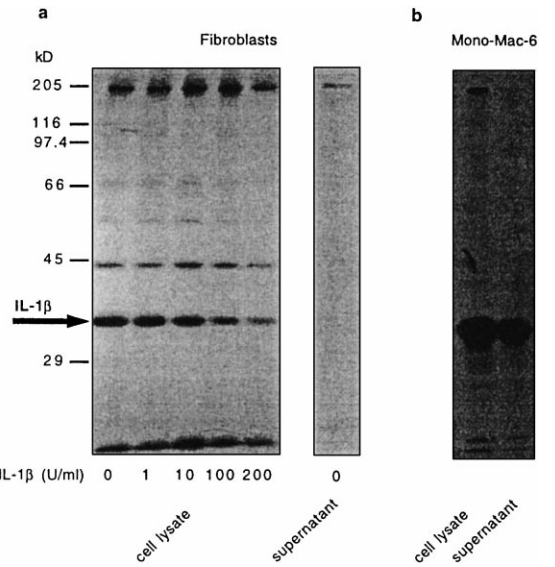

Fig. 6. Fibroblasts produce but do not secrete IL-1β. a: Fibroblasts were metabolically labeled with 50 μCi [^{35}S]methionine/cysteine for 24 h in the presence of 10 ng/ml TNF-α. Cellular extracts were prepared as described in Section 2. Increasing concentrations (0–200 U/ml) of unlabeled IL-1β added to aliquots to compete with ^{35}S-labeled IL-1β. IL-1β was immunoprecipitated with a polyclonal anti-IL-1β antibody and analyzed by SDS-PAGE and fluorography. Similarly IL-1β was immunoprecipitated from cell culture supernatants of stimulated fibroblasts. b: For comparison Mono-Mac-6 cells were labeled in the presence of LPS (10 μg/ml) and cell associated and secreted IL-1β was immunoprecipitated from cellular extracts and cell culture supernatants.

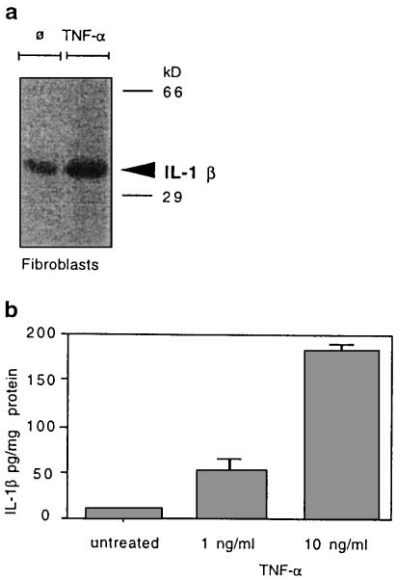

Fig. 7. TNF-α upregulates IL-1β expression in fibroblasts. a: Fibroblasts were metabolically labeled with 50 μCi [^{35}S]methionine/cysteine for 24 h in the presence or absence of 10 ng/ml TNF-α. Cell-associated IL-1β was then immunoprecipitated and analyzed by SDS-PAGE and fluorography. b: IL-1β was measured in the cellular extracts by ELISA. Fibroblasts were either left untreated or stimulated with 1 or 10 ng/ml TNF-α for 24 h.

4. Discussion

Our data demonstrate that soluble IL-1R type I binds to human dermal fibroblasts, induces in an agonistic way a transient increase of intracellular calcium concentration, and enhances IL-6 and IL-8 mRNA steady state levels. These results suggest that IL-1sR I interacts with IL-1 on fibroblasts and imply an inverted ligand-receptor relation. This is provocative and could add a new dimension to our understanding of ligand-receptor cross-talk and cell-to-cell communication.

The IL-1sR I induced increase in $[Ca^{2+}]_i$ could be blocked either by preincubation of IL-1sR I with IL-1ra or with antibodies against IL-1β. IL-1β thus appears to serve as a binding site for the soluble IL-1 receptor. Interestingly, the receptor binding characteristics of IL-1sR I were very similar to those of soluble IL-1β to membrane-integrated IL-1R I for which a K_d of 0.5–3.2 nM was reported [22]. The manner in which IL-1β could act as a receptor, however, remains unclear and needs further investigation. It is still controversial how it is anchored in the membrane. Both, pro-IL-1α and -β lack common signal sequences. Pro-IL-1α may be anchored by myristoylic acid [23] or by lectin-like interactions [24]. It is believed that it is transported to the cell surface where it is called membrane IL-1. Pro-IL-1β is also myristoylated, but no membrane form has been described so far [1]. In our experiments, we could identify a cell-associated form of IL-1β. Its apparent molecular mass was 33 kDa corresponding to that of the proform of IL-1β. In TNF-α stimulated fibroblasts the production of IL-1β was greatly upregulated, but neither the mature nor the proform of IL-1β was detectable in cell culture supernatants. Based on the specific binding of soluble IL-1R I, it can be assumed that at least its binding site is exposed on the cell surface.

IL-1 lacks a common signal sequence and does not reveal any domains that might explain a signal transducing capability. Such a function could, however, be acquired with help from accessory molecules. The IL-1 family member, IL-1 receptor accessory protein (IL-1R-AcP) [5], could provide assistance. IL-1 signaling in the traditional way is believed to result from formation of a ternary complex consisting of IL-1 agonist, IL-1R I and IL-1R-AcP. Association of IL-1R-AcP with the receptor/ligand (IL-1R I/IL-1α or -β) complex is thought to trigger the cellular response [25]. It is conceivable that IL-1sR I might also form a complex with membrane-bound pro-IL-1β which could associate with IL-1R-AcP and induce signal transduction via IL-1R-AcP. A similar hypothesis has been formulated for IL-6 and the IL-6 receptor family [26]. The soluble IL-6 receptor α chain (IL-6sRα) binds IL-6 with low affinity [27]. This IL-6sRα/IL-6 complex interacts with the signal transducing subunit gp 130 (also termed IL-6 receptor β chain) and renders cells sensitive to IL-6 even in the absence of membrane-bound IL-6Rα [28].

Soluble cytokine receptors have so far been considered as non-signaling receptors, serving as scavengers for excess cytokines [9,26]. We now show that type I soluble IL-1 receptor can also act as a ligand and transduce signals to fibroblasts. As a product of mononuclear cells, IL-1sRs are found in urine, plasma, serum and synovial exudate [12,29]. In patients with septicaemia [30] or with rheumatoid arthritis, elevated levels of receptor have been measured [10], pointing to a role of IL-1sRs in inflammatory reactions. Such new roles of IL-1 family members may enlarge and challenge our thinking about mechanisms governing inflammatory reactions and may even open new therapeutic approaches.

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