A New Type of Cytokine Receptor Antagonist
Directly Targeting gp130*

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Christoph Renne‡, Karl-Josef Kallen‡, Jürgen Müllberg, Thomas Jostock,
Joachim Grötzinger§, and Stefan Rose-John¶

From the I. Medizinische Klinik, Abteilung Pathophysiologie, Johannes Gutenberg Universität Mainz, Obere Zahlbacher Strasse 63, D-55101 Mainz, Germany and Institut für Biochemie, Klinikum der RWTH Aachen, Pauwelsstrasse 30, 52057 Aachen, Germany

The interleukin-6-type family of cytokines bind to receptor complexes that share gp130 as a common signal-transducing subunit. So far, receptor antagonists for interleukin-6-type cytokines have been constructed that still bind to the specific ligand binding subunit of the receptor complex, but have lost the ability to stimulate gp130. Such receptor antagonists compete for a specific receptor of a member of the cytokine family. Interleukin-6 only binds to gp130 when complexed with the interleukin-6 receptor that exists as a membrane bound and soluble molecule. Here we have constructed fusion proteins that consist of the soluble form of the human interleukin-6 receptor covalently linked to interleukin-6 receptor antagonists. These fusion proteins directly bind to gp130. Moreover, at concentrations of 10–50 nM they completely neutralize not only the biological activity of interleukin-6 but also of other cytokines of the interleukin-6-type family that act via gp130 homodimers, or gp130/LIF-R heterodimers. Therefore, these gp130 targeting cytokine antagonists might be useful therapeutic tools in disease states that are related to cytokines of the interleukin-6 family.

Interleukin-6-type cytokines (IL-2, IL-11, CT-1, CNTF, LIF, OSM) share the same folding topology of a four-helix bundle (1, 2). They all act via receptor complexes containing at least one molecule of gp130, the common signal-transducing protein of the IL-6-type family of cytokines (2). Whereas IL-6 and IL-11 use a homodimer of gp130, CT-1, CNTF, LIF, and OSM require a heterodimer consisting of gp130 and the LIF-receptor (LIF-R) (2). Recently it has been shown that, in the human system, OSM additionally binds and acts via a receptor complex consisting of gp130 and OSM-receptor, a protein related to gp130 and LIF-R (3). However, in mice, OSM only acts via the gp130 and OSM-receptor (4). Interestingly, IL-6, IL-11, CT-1, and CNTF bind first to specific receptor proteins and these complexes associate with the gp130 homodimer or the gp130/LIF-R heterodimer. In contrast, LIF and OSM bind directly to LIF-R and gp130, respectively, leading to the formation of heterodimeric gp130/LIF-R or gp130-OSM-R complexes (2).

An increased expression of IL-6 has been reported for several diseases like plasmacytoma/myeloma, Castleman’s disease, mesangial proliferative glomerulonephritis, osteoporosis, autoimmune diseases, and AIDS (5, 6). Antibody strategies have been exploited to neutralize the activity of IL-6. The administration of IL-6-neutralizing monoclonal antibodies to patients with rheumatoid arthritis and multiple myeloma has highly improved the conditions of the patients for several weeks (7, 8). Regrettably, the symptoms returned, because the high stability of antibodies in plasma increased the circulating levels of IL-6 that is normally cleared from the circulation within several minutes (9).

The contact sites between IL-6, the IL-6R, and gp130 have been mapped (10–12), and molecules were constructed that lost the ability to contact two gp130 molecules. These IL-6 mutants turned out to be IL-6 receptor antagonists (13–15). These proteins have been shown to completely abolish IL-6-induced growth of human myeloma cells (13–15). An animal model could, however, not be established, because the human IL-6 receptor antagonists did not inhibit the biological activity of murine IL-6 (13–15). Furthermore, it became clear that human myeloma cells were responsive not only to IL-6 but to most if not all cytokines of the IL-6 family (16). The constructed IL-6 receptor antagonists, however, only inhibited the growth of myeloma cells stimulated by IL-6 (13–15, 17).

The specific receptors for IL-6 cytokines exist in a membrane bound and a soluble form (18–21). The soluble receptors complexes with their ligand can elicit a biological signal on cells that only express the signaling subunits gp130 and LIF-R, a process that has been called transsignaling (22). Because virtually all cells of the body express gp130, the proteins LIF-R, IL-6R, CNTF-R and IL-11R are only expressed on some cell types (2). Recently it was shown that cell types that respond exclusively to IL-6/sIL-6R but not to IL-6 alone include hematopoietic progenitor cells (23), endothelial cells (24), osteoclasts (25), and neuronal cells (26). Direct stimulation of gp130 on such cells with the combination of IL-6/sIL-6R might prove to be of therapeutic value although the concentrations needed for effective stimulation make such an application difficult. We have recently constructed a designer cytokine (Hyper-IL-6) consisting of the bioactive parts of IL-6 and sIL-6R fused by a flexible protein linker. This cytokine was 100–1,000 times more active than the separate proteins IL-6 and sIL-6R (27). Because the designer cytokine Hyper-IL-6 directly binds to and stimulates gp130, we speculated that the fusion of sIL-6R to IL-6 receptor antagonists via a flexible peptide linker would...
FIG. 1. Schematic representation of mutants of IL-6 and IL-6/sIL-6R fusion proteins. Panel A, the structure of human IL-6 (43) with site I (IL-6R binding site), site II (gp130 contact site), and site III (gp130 contact site) indicated. A, B, C, and D denote the four helices of IL-6. Panel B, top, human IL-6. The four α-helices are denoted by shaded boxes with the first and last amino acid indicated on top of the boxes. Middle, IL-6 DFRD. In this IL-6 mutant, four-point mutations have been introduced: Y31D, G35F, S118R, and V121D resulting in a defective site II (contact site to gp130) (14). Bottom, IL-6 2a2/3C9. In this IL-6 mutant amino acids 50–58 are replaced by the corresponding mutant residues and the two point mutations, Q159E and T162P. IL-6 2a2/3C9 has a defective site III (contact site to gp130) (15). Panel C, the three fusion proteins of human soluble IL-6R with human IL-6, IL-6 2a2/3C9, and IL-6 DFRD are shown.
result in proteins that would bind to one gp130 protein without inducing its dimerization, thereby acting as an effective antagonist. Because gp130 is a constituent of all receptor complexes of the IL-6 family of cytokines, we analyzed whether these designer cytokine antagonists could inhibit receptor complexes consisting of gp130/gp130 homodimers or gp130/LIF-R heterodimers.

MATERIALS AND METHODS

Chemicals—Dulbecco’s modified Eagle’s medium with glutamax, minimum Eagle’s medium, and penicillin/streptomycin were purchased from Life Technologies, Inc. (Eggenstein, Germany). Fetal calf serum was obtained from Seromed (Berlin, Germany). The human sIL-6R ELISA was purchased from CLB (Hiss Diagnostics, Freiburg, Germany). DEAE-dextran and Nonidet P-40 were obtained from Sigma (Taufkirchen, Germany). Protein A-Sepharose CL-4B was obtained from Amersham Pharmacia Biotech (Freiburg, Germany). Restriction enzymes, T4-DNA-ligase and Vent polymerase were from New England Biolabs (Schwalbach, Germany). Tran35S-label (44 TBq/mmol) was from ICN (Meckenheim, Germany) and [3H]thymidine was purchased from Amersham Pharmacia Biotech (Aylesbury, U.K.). X-ray films (X-OMAT-AR) were from Eastman Kodak Co. The following antibodies have been used: MT18, recognizing the NH2-terminal immunoglobulin domain of the human IL-6R (28); IL-6-mAb-8, recognizing human IL-6 (29).

Cell Culture—Human hepatoma cells (HepG2), COS-7, and BAF/3 cells were grown in Dulbecco’s modified Eagle’s medium at 5% CO2 and 37 °C in a water-saturated atmosphere. The culture medium was supplemented with 10% fetal calf serum, 100 mg/liter streptomycin, and 60 mg/liter penicillin.

Construction of H-IL-6 Point Mutants—Standard cloning procedures were performed as described by Sambrook et al. (30). H-DFRD was constructed in a three-step process by PCR-ligation-PCR (31). As the first step three fragments of H-DFRD were made using the previous described H-IL-6 (27) as the common template. The PCR leading to the first fragment used the following oligonucleotides as primers 5’-CAGCATCAGTGTTCATCCAC-3’ (s1) sense and 5’-GAGGATATCCCGATTGTTTGTCAAT-3’ (as1) antisense for the second fragment the primers 5’-CGGGATATCCTCGACTTCATCTCAGCCCTGAGAAAG-3’ (s2) sense and 5’-TGTTCTCATCTGCACAGCTCTGGC-3’ (as2) antisense were used the third fragment was made with the primers 5’-AAGACCTGATCCAGTTCCTGCAG-3’ (s3) sense and a pCDM8 reverse primer (as3). The mutated nucleotides are underlined. As the second step the fragments were ligated by digesting the first and the second fragment with EcoRV and ligating them. After phosphorylation, the ligation product was amplified by a second PCR round using the primers s1 and as2. The product of this PCR was ligated via blunt ends to the third PCR fragment of step one, and a final PCR was made using the primers s1 and as3 for amplification. As the third step the last PCR product was cloned into the pCDM8 expression vector. Thereafter, the PCR DNA and pCDM8 containing H-IL-6 were digested with the unique sites XhoI and NotI and then ligated. For the construction of a H-AIL-6 cDNA, a EcoNI/NotI fragment of the previously constructed IL-6 receptor antagonist cDNA IL-6–2a2/3C9 in the vector pRSET (15) was cloned into the pCDM8-H-IL-6 vector (27) opened with EcoNI/NotI.

Transient Transfection of COS-7 Cells and Protein Expression—

FIG. 2. Expression of fusion proteins of sIL-6R and IL-6 receptor antagonists. A, ELISA measurements of supernatants of COS-7 cells transfected with expression vectors coding for H-IL-6 ( ), H-DFRD ( ), and H-AIL-6 ( ) fusion proteins. B, Western blot analysis of adjusted equal amounts of the fusion proteins H-IL-6, H-DFRD, and H-AIL-6 using an IL-6 specific mAb.

FIG. 3. Association of fusion proteins with soluble gp130. A, COS-7 cells were transfected with expression vectors coding for H-IL-6, H-DFRD, and H-AIL-6 fusion proteins. Cells were metabolically labeled with [35S]systeine/methionine. Supernatants were precipitated with the supernatants of the same cells. Protein complexes were precipitated with protein A-Sepharose.

FIG. 4. Biological activity of the fusion proteins H-IL-6, H-DFRD, and H-AIL-6. BAF/3 cells stably transfected with a cDNA coding for human gp130 were incubated (in the absence of IL-3) with increasing amounts of H-IL-6 ( ), H-DFRD ( ), and H-AIL-6 ( ) fusion proteins. Proliferation of the cells was assessed as incorporation of [3H]thymidine.
RESULTS

IL-6 is a four-helix bundle cytokine that interacts with a receptor complex consisting of IL-6R and a homodimer of gp130. As shown in Fig. 1A, the interaction sites of IL-6 with the receptor subunits have been named site I (contact site to gp130) (14), and site II (contact site to gp130) (11, 15, 34). A schematic representation of the IL-6 receptor antagonists (Fig. 1B) and the fusion proteins (Fig. 1C) used in this study is shown. H-IL-6 is a recently developed fusion protein with the sIL-6R covalently linked to human IL-6 by a 13-amino acid peptide linker (27). The sIL-6R is linked either to human IL-6 (H-IL-6) or to an IL-6 receptor antagonist with a defective site II (H-DFRD) (14) or a defective site III (H-AIL-6) (11, 15, 34).

The cDNAs coding for the fusion proteins were transfected into COS-7 cells, and the secreted proteins were quantified using a sIL-6R ELISA (Fig. 2A). The supernatants were adjusted to equal fusion protein concentrations. As shown in Fig. 2B, approximately equal amounts were detected on a Western blot using an anti-IL-6 monoclonal antibody. The somewhat weaker detection of the fusion protein H-AIL-6 might be because of reduced recognition of the mutated IL-6 within the fusion protein by the monoclonal antibody used for detection.

The fusion protein H-IL-6 has been shown to directly stimulate gp130 expressed on target cells (27). The fusion proteins H-DFRD and H-AIL-6 each have one defective contact site to gp130, either site II or site III, respectively (14, 15). They should, however, still be able to bind to gp130 via the second site. To test for the binding ability of the fusion proteins, we metabolically labeled COS-7 cells that had been transfected with H-IL-6, H-DFRD, and H-AIL-6 cDNAs. To confirm equal expression of the fusion proteins, the supernatants of the transfected COS-7 cells were immunoprecipitated with the mAb MT18, which recognized the NH₂-terminal immunoglobulin domain of the human IL-6R (28). The precipitated proteins were separated by SDS-polyacrylamide gel electrophoresis, and gels were subjected to fluorography. As shown in Fig. 3A, this procedure led to the precipitation of proteins of an apparent molecular mass of 70 kDa. The approximately equal intensities indicate equal protein concentrations in the supernatant of transfected cells. In a parallel experiment, a fusion protein consisting of the extracellular portion of human gp130 fused to the constant part of human immunoglobulin (Fc) was added to the supernatants of transfected COS-7 cells. Supernatants were treated with protein A-Sepharose, and precipitated proteins were separated by SDS-polyacrylamide gel electrophoresis and subjected to fluorography. As shown in Fig. 3B, radio-labeled H-IL-6, H-DFRD, and H-AIL-6 were all precipitated by fusion proteins.
the gp130-Fc protein albeit with slightly different efficiencies. H-IL-6 and H-DFRD were precipitated at nearly equal amounts compared with H-AIL-6 that was precipitated with reduced efficiency. From this experiment it can be concluded that the three fusion proteins directly bind to the extracellular portion of gp130.

We speculated that the fusion proteins H-DFRD and H-AIL-6, which both had only one intact contact site to gp130, would bind to and block gp130 by preventing homodimerization with gp130 or heterodimerization with LIF-R. We first tested whether the fusion proteins H-DFRD and H-AIL-6 exhibited biological activity on gp130-expressing cells. We used IL-3-dependent BAF/3 cells stably transfected with a human gp130 cDNA (27). As can be seen in Fig. 4, in the absence of IL-3 these cells proliferate when stimulated with 10 ng/ml H-IL-6. In contrast, stimulation of cells with H-DFRD and H-AIL-6 at concentrations up to 5 μg/ml led to no [3H]thymidine incorporation. This experiment clearly demonstrated that in contrast to H-IL-6, the fusion proteins H-DFRD and H-AIL-6 possessed no intrinsic biological activity.

We next asked whether the proteins H-DFRD and H-AIL-6 were able to inhibit the growth of BAF/3 cells stably transfected with gp130 and human IL-6R cDNAs. These cells can be stimulated by human IL-6 with half-maximal proliferation achieved at 0.5 ng/ml IL-6 (Fig. 5A). To cells stimulated with 0.5 ng/ml IL-6 we added increasing amounts of the two fusion proteins H-DFRD and H-AIL-6. The H-DFRD and H-AIL-6 fusion proteins completely inhibited the proliferation of the cells with an IC50 of 300–500 ng/ml as shown in Fig. 5B. An identical amount of COS-7 supernatants of mock transfected cells did not affect the IL-6-stimulated proliferation of gp130-transfected BAF/3 cells (data not shown). As an additional control we used the IL-6R specific IL-6 antagonists 2a2/3C9 (15) and DFRD (14) (Fig. 1B). As described previously (14, 15) these proteins inhibited the IL-6-induced proliferation of BAF/3 cells transfected with gp130 and IL-6R cDNAs. The LIF, OSM, or CNTF-induced proliferation of BAF/3 cells stably transfected with gp130, LIF-R, and CNTF-R remained unaffected by the two IL-6 antagonists 2a2/3C9 and DFRD (data not shown).

When BAF/3 cells stably transfected with human gp130 were used, proliferation can be stimulated with the fusion protein H-IL-6 (27). Cells grown in the presence of 10 ng/ml H-IL-6 were treated with increasing doses of H-DFRD and H-AIL-6. As shown in Fig. 6, the two fusion antagonists inhibited growth of the gp130-transfected BAF/3 cells with an IC50 of about 1,000 ng/ml.

Because the fusion antagonistic proteins were effective in blocking the activity of IL-6 and H-IL-6 due to the fact that the proteins directly bind to gp130, we tested whether these proteins might also block the biologic response of cytokines that induce heterodimerization of gp130 and LIF-R. We therefore tested the fusion proteins for their ability to inhibit proliferation of BAF/3 cells stably transfected with human gp130, human LIF-R and human CNTF-R cDNAs. These cells were stimulated with LIF, OSM, or CNTF. Fig. 7A shows that BAF/3 cells stably transfected with these cDNAs proliferated in response to the cytokines LIF and CNTF with EC50 values of about 1 ng/ml. The EC50 for OSM was slightly higher, about 5 ng/ml. The proliferation induced by LIF was inhibited by both fusion proteins with an IC50 of around 100 ng/ml (Fig. 7B). The inhibition of the biologic activity of OSM and CNTF, however, occurred with an IC50 dose of about 500 ng/ml (Fig. 7, C and D).

On hepatoma cells, cytokines of the IL-6 family have been shown to induce the synthesis and secretion of acute phase proteins (2, 35). Therefore we tested if the constructed fusion proteins were able to block the biological activity on HepG2 cells of gp130/LIF-R requiring cytokines. HepG2 cells treated

FIG. 7. Inhibition of LIF, OSM, and CNTF induced proliferation by H-DFRD and H-AIL-6 fusion proteins. A, proliferation of BAF/3 cells stably transfected with cDNAs coding for human gp130, human LIF-R, and human CNTF-R (in the absence of IL-3) in the presence of increasing amounts of LIF ( ), OSM ( ), and CNTF ( ). B, the same cells were stimulated with 0.5 ng/ml LIF ( ) in the presence of increasing amounts of the H-DFRD ( ) and H-AIL-6 ( ) fusion proteins. C, cells were stimulated with 1 ng/ml CNTF ( ) in the presence of increasing amounts of the H-DFRD ( ) and H-AIL-6 ( ) fusion proteins. D, cells were stimulated with 10 ng/ml OSM ( ) in the presence of increasing amounts of the H-DFRD ( ) and H-AIL-6 ( ) fusion proteins. Proliferation of the cells was assessed as incorporation of [3H]thymidine.
AIL-6 and more than 1,000 ng/ml for H-DFRD. HepG2 cells occurred with an IC50 dose of 200 ng/ml for H-

The inhibition of the biologic activity of LIF on fusion proteins, haptoglobin secretion was reduced to back-
treated with increasing amounts of the H-DFRD and H-AIL-6.

Fig. 8

Inhibition of the LIF-induced secretion of haptoglobin by H-DFRD and H-AIL-6 fusion proteins. A, HepG2 cells were incubated with increasing amounts of human LIF protein. B, HepG2 cells were incubated with 25 ng/ml human LIF in the presence of increasing concentrations of the fusion proteins H-DFRD and H-AIL-6. After 24 h of incubation, the haptoglobin concentration was measured by ELISA as described (33). ▼, LIF 25 ng/ml + H-DFRD; ■, LIF 25 ng/ml + H-AIL-6.

with increasing amounts of human LIF secreted the acute phase protein haptoglobin into the supernatant (36). Haptoglo-
bin concentration can be measured by ELISA (33). As shown in

Fig. 8A, HepG2 cells stimulated with LIF secreted haptoglobin into the medium with maximal stimulation at 25–50 ng/ml. When HepG2 cells stimulated with 25 ng/ml human LIF were treated with increasing amounts of the H-DFRD and H-AIL-6 fusion proteins, haptoglobin secretion was reduced to back-
ground levels. The inhibition of the biologic activity of LIF on HepG2 cells occurred with an IC50 dose of 200 ng/ml for H-
AIL-6 and more than 1,000 ng/ml for H-DFRD.

DISCUSSION

We have used the approach to covalently link IL-6 or IL-6
muteins with one defective contact site for gp130 (IL-6 receptor
antagonists) to the soluble form of the IL-6R to create fusion
proteins which directly bind to gp130. These fusion proteins
directly target the extracellular portion of gp130 and therefore
inhibit cytokines of the IL-6-type family, which interact with
either a homodimer of gp130 (like IL-6) or with a heterodimer
of gp130 and LIF-R (like CNTF, OSM and CTNF).

The coprecipitation studies with the extracellular portion
of gp130 linked to an Fc protein revealed that H-IL-6 and H-
DFRD proteins bind equally well to gp130, whereas H-AIL-6
seemed to bind with somewhat reduced efficiency. Experiments
with IL-6 receptor antagonists in the presence of soluble IL-6R
and a soluble form of gp130 with a C-terminal myc tag led to
the conclusion that site II and site III act independently from
each other as binding sites for gp130 (37). Although coprecipita-
tion with a gp130-Fc fusion protein does not necessarily
reflect the situation on the plasma membrane, these data
might indicate that site III of IL-6 is more important for gp130
contact than site II. This notion is, however, not supported by
the inhibition data presented in this manuscript. One would
expect that H-DFRD being a better binder of gp130 would be a
more effective antagonist of gp130. The fusion proteins H-
AIL-6 and H-DFRD, however, are not significantly different in
their inhibitory efficiency.

Disregulation of the expression of cytokines of the IL-6-type
family has been implicated in the pathophysiology of many
diseases including Castleman’s disease, multiple myeloma, os-
teoporosis, and the development of Kaposi’s sarcoma (6). Ef-
forts have been made to neutralize the activity of the IL-6-type
cytokines by mAbs or cytokine receptor antagonists (7, 8, 10,
11, 15, 17, 34, 39). Recently we reported on the development of
a specific targeting strategy of cytokine-secreting cells by a
bispecific diabody recognizing human IL-6 and CD3, which
induced T-cell-mediated killing of these cells (40). All these
strategies, however, act to neutralize or block one given cyto-
kine. IL-6 receptor antagonists or neutralizing mAbs do not
neutralize the biologic activity of IL-11, LIF, OSM, CNTF, or
CT-1. In the case of multiple myeloma cells, it has been shown
that these cells not only proliferate in response to IL-6 but also
in the presence of other if not all members of the IL-6 cytokine
family (16).

The gp130-interacting antagonist presented in this report
has the great advantage of blocking gp130 directly and thereby
blocking the biological activities all members of the IL-6-type
family of cytokines. In addition, it has recently been demon-
strated that IL-6 bound to soluble IL-6R has a longer plasma
half-life in vivo than IL-6 alone (41). Moreover, we have re-
cently shown that the fusion protein H-IL-6 when injected into
mice has a considerably longer bioavailability than IL-6 alone.2
We therefore anticipate that our newly developed gp130-tar-
geting fusion proteins will also show a more favorable pharma-
cokinetics than do IL-6 receptor antagonists (41). On the other
hand, one has to be aware that pleiotropic side effects upon
injection of the fusion proteins might occur, because gp130 is
present on all cells of the body and is involved in a wide
spectrum of cellular activities (2).

Recently, an inhibitor of LIF was constructed that exhibited
a lowered affinity for gp130 because of introduced point muta-
tions, whereas the affinity for LIF-R was unchanged (42). Con-
sequently, this LIF-R antagonist was shown to possess antag-
onistic activity not only for LIF but also for the LIF-R binding
cytokines CT-1, CNTF, and OSM (42).

Taken together we have provided evidence that fusion pro-
teins of IL-6 receptor antagonists and the soluble IL-6R can
be used to directly target the receptor subunit gp130, which is a
component of all receptor complexes of the IL-6 cytokine family.
These fusion proteins when given at a molar excess are able to
bind to the extracellular portion of gp130. Therefore we have
for the first time constructed an antagonist with the potential
to block all cytokines of the IL-6-type family. These novel
inhibitors of IL-6-type cytokines might have therapeutic impli-
cations for diseases that have been connected with disregula-
tion of the expression of these cytokines.

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