

The Function of the Soluble Interleukin 6 (IL-6) Receptor In Vivo: Sensitization of Human Soluble IL-6 Receptor Transgenic Mice Towards IL-6 and Prolongation of the Plasma Half-life of IL-6

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Summary

Interleukin 6 (IL-6) is considered an important mediator of acute inflammatory responses. Moreover, IL-6 functions as a differentiation and growth factor of hematopoietic precursor cells, B cells, T cells, keratinocytes, neuronal cells, osteoclasts, and endothelial cells. IL-6 exhibits its action via a receptor complex consisting of a specific IL-6 receptor (IL-6R) and a signal transducing subunit (gp130). Soluble forms of both receptor components are generated by shedding and are found in patients with various diseases such as acquired immune deficiency syndrome, rheumatoid arthritis, and others. The function of the soluble (s)IL-6R in vivo is unknown. Since human (h)IL-6 acts on human and murine target cells, but murine IL-6 on murine cells only, we constructed transgenic mice expressing the hsIL-6R. We report here that in the presence of hsIL-6R, mice are hypersensitized towards hIL-6, mounting an acute phase protein gene induction at significantly lower IL-6 dosages compared to control animals. Furthermore, in hsIL-6R transgenic mice, the detected acute phase response persists for a longer period of time. The IL-6/IL-6R complex prolongs markedly the IL-6 plasma half-life. Our results reinforce the role of the hsIL-6R as an agonistic protein, help to understand the function of the hsIL-6R in vivo, and highlight the significance of the receptor in the induction of the acute phase response.

IL-6 is a multifunctional cytokine and plays a central role as a differentiation and growth factor of hematopoietic precursor cells, B cells, T cells, keratinocytes, neuronal cells, osteoclasts, and endothelial cells (for reviews see references 1 and 2). Moreover, IL-6 modulates the transcription of several liver-specific genes during acute inflammatory states (3). Altered IL-6 serum levels have been described in several hematological diseases such as plasmacytoma or Castleman's disease (4, 5), mesangial glomerulonephritis (6), osteoporosis (7), cachexia (8), rheumatoid arthritis (9), sepsis (10, 11), and AIDS (12, 13).

IL-6 exhibits its action on target cells by acting through a receptor complex consisting of a specific IL-6-binding protein (IL-6R) and a signal-transducing subunit (gp130). The IL-6/IL-6R complex induces the homodimerization of two gp130 molecules leading to a number of intracellular signaling events including activation of the transcription factor NF-IL-6, probably via the Ras-microtubulus-associated protein kinase cascade (14), and activation of the Janus ki-

nase/signal transducer and activator of transcription signaling pathway (15–17).

Soluble forms of the IL-6R (sIL-6R)¹ and gp130 (sgp130) are found in different body fluids and their concentrations may be elevated in patients with various inflammatory diseases such as AIDS and rheumatoid arthritis (13, 18–20). They are generated by limited proteolysis and are released from the cell surface (21–23). It has also been demonstrated that an IL-6R mRNA lacking the coding sequence for a transmembrane domain exists. This mRNA presumably derives from a differential splicing event and can lead to the synthesis of a sIL-6R protein (24). The sIL-6R retains its ligand-binding capacity (25, 26) and associates with sgp130 in the presence of IL-6 (23, 27). In vitro experiments have demonstrated that in contrast to other soluble receptors, such as the receptors for IL-1 or TNF, which are known to

¹Abbreviations used in this paper: hsIL-6R, human soluble IL-6R; PEPCK, phosphoenolpyruvate carboxykinase; sIL-6R, soluble IL-6R.

inhibit the effects of their ligands (28), sIL-6R acts agonistically on cells that express gp130. Most recently, it was shown that heart muscle cells respond to IL-6 only in the presence of the sIL-6R (29). By this transsignaling pathway, sIL-6R is able to activate target cells that express only gp130 molecules on their cell surface but lack membrane-bound IL-6R (30).

Despite the *in vitro* studies cited above and the finding that soluble forms of IL-6R and gp130 are found in various diseases, the function of these soluble proteins *in vivo* is not understood. The species specificity of IL-6, which acts on human and murine cells, whereas murine IL-6 is only active on murine cells, was the basis to construct transgenic mice expressing the human sIL-6R (hsIL-6R) to study the role of the hsIL-6R *in vivo* without the confounding effect of endogenous murine IL-6. Here we demonstrate that upon injection of human IL-6, transgenic mice expressing the hsIL-6R are hypersensitized towards the IL-6-mediated acute phase response. Moreover, hsIL-6R functions as a carrier protein for its ligand, thereby markedly prolonging the plasma half-life of IL-6.

Materials and Methods

Chemicals. Protein A-Sepharose CL-4B was obtained from Pharmacia (Freiburg, Germany). DMEM, MEM, and penicillin/streptomycin were from Gibco (Eggenstein, Germany). FCS was from Seromed (Berlin, Germany). The preparation of the monospecific antiserum against IL-6R was described previously (26). Recombinant human and recombinant murine IL-6 were prepared as described (31, 32). α -[32 P]dATP (110 TBq/mmol) was from Amersham International (Amersham, Bucks, UK). The anti-IL-6R mAb MT18 was kindly supplied by Dr. K. Yasukawa (TOSOH Corp., Tokyo, Japan). The rabbit IgG anti-mouse antibody was obtained from Dako (Hamburg, Germany). The hIL-6 ELISA kit was obtained from CLB (Amsterdam, The Netherlands). The hIL-6R ELISA kit was from Seromed (Gießen, Germany).

Generation of Phosphoenolpyruvate Carboxykinase (PEPCK)/hIL-6 Transgenic Mice. A 1.1-kb cDNA fragment coding for the extracellular part of hIL-6R was amplified by PCR and cloned into the Sall site of the pTZ18R vector. A 530-bp EcoRI-BglII DNA fragment of the mouse PEPCK promoter (33) was cloned upstream from the hIL-6R cDNA. A BamHI site was inserted upstream the PEPCK promoter. A 1.2-kb DNA fragment corresponding to the 3' portion of the human β -globin gene (34) followed by a poly-A site was inserted downstream from the hIL-6R cDNA between the Sall and the HindIII sites of the vector. The BamHI-BamHI fragment containing the entire construct (see Fig. 1 A) was gel purified and microinjected into the pronuclei of fertilized eggs of NMRI and B602F₁ mice. Mice were screened for the presence of hIL-6R by Southern blot of EcoRI-HindIII-digested tail DNA.

Animal Treatment. Mice were maintained in a 12-h light-dark cycle under standard conditions and were provided with food and water *ad libitum*. Procedures involving animals and their care were conducted in conformity with national and international laws and policies. rhIL-6 was injected intraperitoneally at doses indicated in the figures.

RNA Expression. Mice were killed by cervical dislocation and RNA was isolated from different organs by the phenol/chloroform method (35) and subjected to Northern blot analysis. 5 μ g

of heat-denatured RNA per sample was fractionated on a 1% agarose gel with 7% formaldehyde. The separated RNA was transferred to GeneScreen Plus membranes (DuPont-New England Nuclear, Dreieich, Germany) according to the supplier's instructions. The filters were prehybridized at 68°C for 2 h in 10% dextran sulfate, 1 M NaCl, 1% SDS, and hybridized in the same solution with 32 P-cDNA fragments labeled by random priming (36). The following probes were used: a 0.9-kb HindIII restriction fragment of human haptoglobin cDNA and a 1.2 PstI-XhoI restriction fragment of hIL-6R cDNA.

Serum IL-6 Measurements and Cell Growth. Blood was drawn by cardiac puncture under general anesthesia of the mice. A hIL-6 ELISA was performed according to the supplier's instructions. For the murine B9 proliferation assay, murine serum samples were serially diluted. The assay was performed as described (37). One B9 unit corresponding to \sim 1 pg hIL-6/ml led to half-maximal proliferation of B9 cells. Cells were grown in DMEM at 5% CO₂ in a water-saturated atmosphere. All cell culture media were supplemented with 10% FCS, 100 mg/liter streptomycin, and 60 mg/liter penicillin.

Serum IL-6/hsIL-6R Complex and IL-6R Immunoprecipitation. 100 μ l of murine serum was preincubated with 100 μ l of protein A-Sepharose (20 mg/ml) and 800 μ l of PBS/0.01% Tween for 2 h at 4°C. After centrifugation, the supernatant was incubated with the monoclonal anti-IL-6R antibody MT18 for 4 h at 4°C. Immune complexes were precipitated with 100 μ l protein A-Sepharose for 2 h at 4°C, separated by 12.5% SDS-PAGE, and subjected to Western blot analysis using a polyclonal specific antiserum against hIL-6R as previously described (26).

Results

Generation of PEPCK/hsIL-6R Transgenic Mice. To drive expression of the hsIL-6R in the liver of transgenic mice, we used the promoter of the mouse PEPCK gene, which is neonatal and thereby permits one to evaluate the influence of gene products after birth (33). The promoter has been shown to be specific for liver and kidney (38). DNA (Fig. 1 A) was microinjected into the pronuclei of fertilized eggs from NMRI mice. Four PEPCK/hsIL-6R transgenic founders were obtained and verified by Southern blot analysis. Mice were interbred to obtain homozygous and heterozygous transgenic PEPCK/hsIL-6R mice.

Characterization of PEPCK/hsIL-6R Transgenic Mice. Tail DNA was digested with EcoRI/HindIII and used for Southern blot analysis (Fig. 1 B). In homozygous mice, the level of the transgene was twofold of that observed in heterozygous mice. As can be seen in Fig. 1 C, the hsIL-6R-mRNA levels in homozygous mice were significantly higher than those observed in heterozygous mice. Accordingly, in immunoprecipitation experiments, the highest levels of hsIL-6R were detected in homozygous mice (Fig. 1 D). Transgenic mice expressed the hsIL-6R in the low microgram range as determined by ELISA (data not shown). When Northern blot analysis was performed with RNA from different mouse tissues, hsIL-6R-mRNA was detected in the liver and kidney, but not in heart, muscle, or brain (data not shown).

hIL-6 Does Not Influence the Expression of hsIL-6R. To exclude the possibility that hIL-6 induces the murine PEPCK

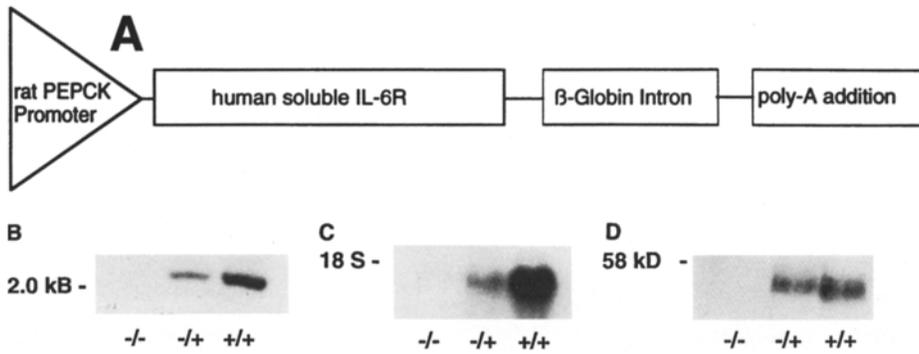


Figure 1. Characterization of PEPCK/hIL-6R transgenic mice. (A) PEPCK/hIL-6R construct injected into fertilized mouse eggs. (B) Southern blot analysis. Genomic tail DNA from control mice and heterozygous (-/+) and homozygous (+/+) transgenic animals was digested with EcoRI and HindIII. A ³²P-labeled 1.2-kb XhoI-PstI-cDNA fragment was used for hybridization. (C) Expression of hIL-6R mRNA in PEPCK/hIL-6R transgenic mice. Total RNA (5 µg), prepared from the liver of 8-wk-old control mice and heterozygous and homozygous transgenic mice

were subjected to Northern blot analysis and hybridized with a ³²P-labeled 1.2-kb XhoI-PstI-cDNA fragment. (D) hIL-6R was immunoprecipitated from mouse serum with the MT-18 antibody and subjected to Western blot analysis using a monospecific antiserum against hIL-6R.

promoter and drives the transcription of hsIL-6R, RNA prepared from liver tissue from homozygous transgenic mice 4 h after receiving different dosages of human IL-6 was subjected to Northern blot analysis and was hybridized with a hIL-6R probe. Contemporaneously, blood was drawn from the mice, and the hsIL-6R was immunoprecipitated with the monoclonal anti-IL-6R antibody MT18 and subjected to Western blot analysis. As can be seen from Fig. 2, different dosages of hIL-6 did not lead to changes of hsIL-6R mRNA or protein expression.

hsIL-6R Transgenic Mice Are Hypersensitized towards the hIL-6-induced Acute Phase Protein Expression. One major activity of IL-6 is the induction of the hepatic acute phase response (39). We therefore analyzed the induction of the acute phase protein gene expression by hIL-6 in the liver of hsIL-6R transgenic mice. Various dosages of hIL-6 were administered to the mice intraperitoneally, and mice were killed after 4 h (Fig. 3). In the top panel of Fig. 3, the haptoglobin mRNA expression in control animals is demonstrated. The strongest haptoglobin expression was seen when 40 µg hIL-6 was injected. In heterozygous hsIL-6R transgenic mice (Fig. 3, middle), the strongest haptoglobin expression was seen at a 10-fold lower dose (4 µg) compared to control animals. In homozygous hsIL-6R mice (Fig. 3, bottom), a hIL-6 dose of as low as 0.4 µg induced a significant haptoglobin mRNA expression. These experiments

clearly demonstrate that the hsIL-6R renders transgenic mice more sensitive to hIL-6 in a dose-dependent fashion. In contrast, when control, heterozygous, and homozygous hsIL-6R transgenic animals were injected with 8 µg of murine IL-6 and analyzed 4 h after injection, an equal induction of the acute phase response was seen in all animals (data not shown).

In hsIL-6R Transgenic Mice the IL-6-induced Acute Phase Protein Expression Is Markedly Prolonged. Time course experiments were performed with 8 µg hIL-6 injected intraperitoneally (Fig. 4 A). In control mice (Fig. 4 A, top), the strongest haptoglobin mRNA expression was detected after 4 h, declined after 8 h, and was hardly detectable after 12 h.

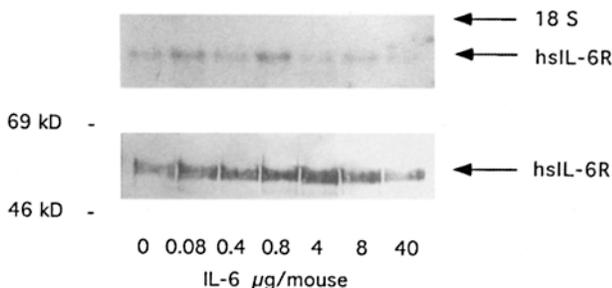


Figure 2. Analysis of hsIL-6R expression in response to IL-6. Total RNA prepared from the liver of homozygous transgenic mice that received various dosages of human IL-6 intraperitoneally, was subjected to Northern blot analysis (top). The hsIL-6R was immunoprecipitated with the MT-18 antibody and subjected to Western blot analysis (bottom).

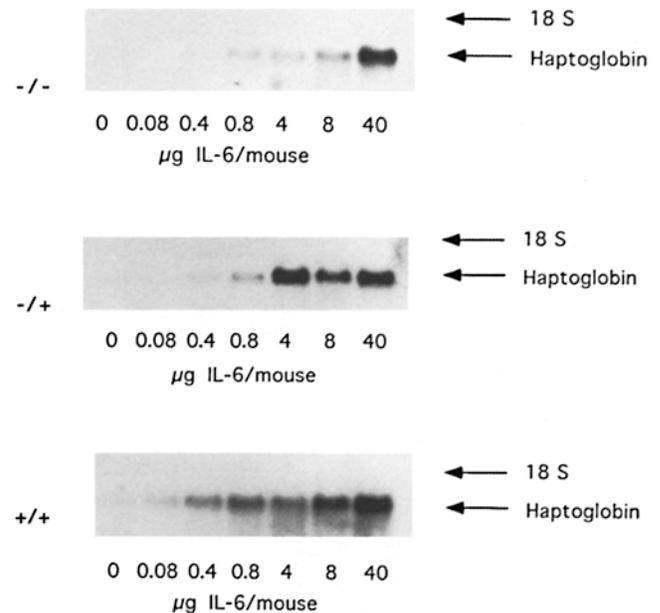


Figure 3. Dose-response of the hepatic acute phase protein expression. Haptoglobin expression in the liver of control mice (-/-), and heterozygous (-/+) and homozygous (+/+) transgenic mice was analyzed. Mice were injected intraperitoneally with various dosages of hIL-6 as indicated. Mice were killed 4 h after injection and RNA was prepared from the liver and subjected to Northern blot analysis. Filters were hybridized with a ³²P-labeled 0.9-kb HinfI restriction fragment of human haptoglobin cDNA.

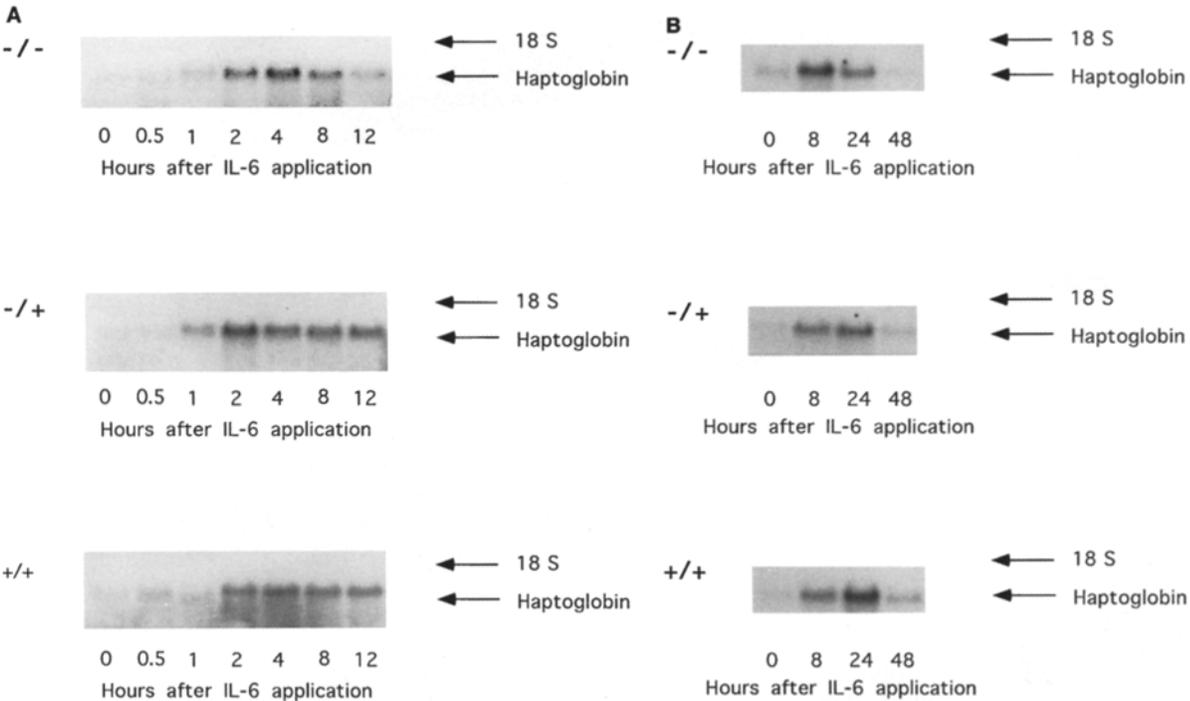
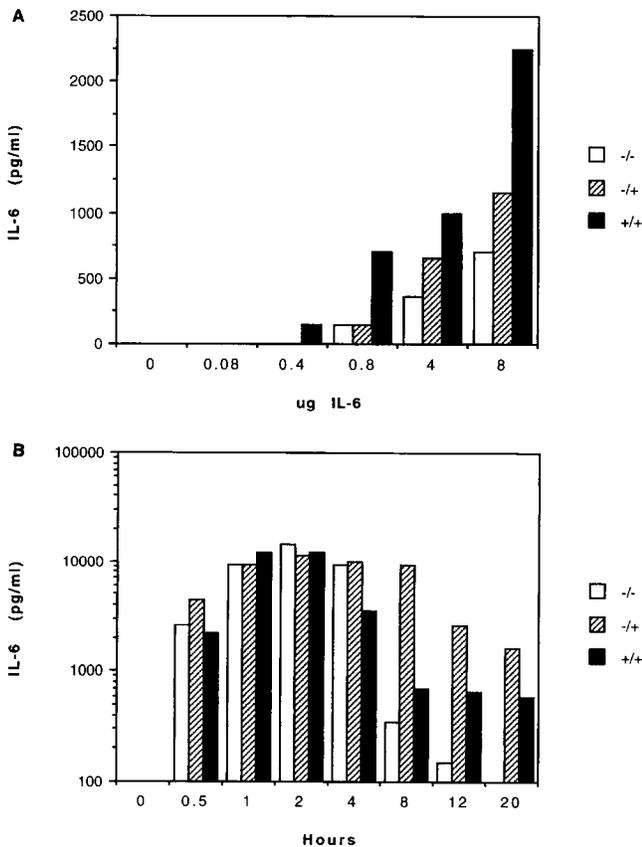


Figure 4. Time course of the hepatic acute phase protein expression. Haptoglobin expression in the liver of control mice (-/-), and heterozygous (-/+) and homozygous (+/+) transgenic mice was analyzed. Mice were injected intraperitoneally with 8 μg human IL-6 and killed after short- (A) or long-term (B) time periods as indicated. RNA was prepared from the liver and subjected to Northern blot analysis. Filters were hybridized with a ³²P-labeled 0.9-kb *Hinf*I restriction fragment of human haptoglobin cDNA.



In heterozygous *hsIL-6R* transgenic mice (Fig. 4 A, middle), the strongest haptoglobin mRNA expression was observed as soon as 2 h after human IL-6 application and was detectable throughout the experiment. In homozygous *hsIL-6R* mice (Fig. 4, bottom), an increase of haptoglobin expression was seen 0.5 h after IL-6 application and, as in heterozygous mice, was more persistent.

When 8 μg *hIL-6* was given for longer periods of time (Fig. 4 B) to control animals (top), the strongest expression of haptoglobin was seen after 8 h and declined thereafter. In heterozygous mice (Fig. 4 B, middle), the response was equally strong after 8 and 24 h; in homozygous *hsIL-6R* mice (Fig. 4, bottom), the expression of haptoglobin peaked at 24 h and was still pronounced after 48 h when compared to control animals (Fig. 4 B, top) and heterozygous transgenic mice (Fig. 4 B, middle).

sIL-6R Functions as a Carrier Protein for IL-6 Prolonging the Serum Half-life of IL-6. To investigate the fate of *hIL-6* injected into *IL-6R* transgenic mice, we determined IL-6 serum levels using the *hIL-6* ELISA (Fig. 5) and a murine B9

Figure 5. IL-6 serum levels. IL-6 serum levels in control mice (open bars), heterozygous (hatched bars), and homozygous (filled bars) transgenic mice were determined with a human IL-6 ELISA. (A) Dose-response experiments: mice were injected with various dosages of human IL-6 intraperitoneally as indicated and were analyzed 4 h after injection. (B) Time course experiments: mice were injected with 8 μg *hIL-6* intraperitoneally and were killed after indicated time periods.

assay. When dose–response experiments were performed, IL-6 levels were markedly elevated in heterozygous, and even higher in homozygous hsIL-6R transgenic mice when compared to control animals (Fig. 5 A). In the time course experiment, there were also markedly higher IL-6 levels in hsIL-6R transgenic mice compared to control mice (Fig. 5 B). IL-6 levels were undetectable 12 h after application in control animals, but were still significantly elevated 20 h after application in transgenic mice (Fig. 5 B). Similar results were obtained when IL-6 levels were determined using the B9 assay (data not shown).

Discussion

To determine the role of the sIL-6R *in vivo*, we made use of the species specificity of hIL-6, which acts on human and murine IL-6R, in contrast to murine IL-6 which acts only on murine IL-6R. Transgenic mice expressing the hsIL-6R were generated, and the function of the hsIL-6R was studied *in vivo* without the confounding action of endogenous murine IL-6.

There are three major findings of this study. First, the sIL-6R makes animals more sensitive to the cytokine IL-6. According to studies performed in cell culture, the sIL-6R renders target cells more sensitive and enables cells to respond to IL-6 which by themselves do not bind the cytokine because of lack of surface expression of the membrane-bound IL-6R (18, 30). The degree of sensitization we observed in the transgenic mice, however, exceeded by far the effect seen in cell culture: when hIL-6 was injected into hsIL-6R transgenic animals and the IL-6-mediated acute phase protein expression was measured, these mice were found to be 10–100-fold more sensitive towards hIL-6 as compared to control animals. In contrast, the injection of murine IL-6 did not result in an increased acute phase protein expression in hsIL-6R transgenic mice when compared to control animals. This finding confirms the species specificity of murine IL-6, which has no effect on human target cells and argues against any interference of endogenous murine IL-6 with the hsIL-6R. Since the acute phase response induced by murine IL-6 was equal in control, heterozygous, and homozygous hsIL-6R transgenic animals, one can conclude that the binding of hIL-6 to membrane-bound and soluble murine IL-6 receptor does not contribute to the dose-dependent, IL-6-induced hypersensitization of hsIL-6R transgenic mice demonstrated in our study. Second, time course experiments revealed that in hsIL-6R transgenic animals, the IL-6-mediated acute phase response persisted much longer as compared with control animals. In homozygous hsIL-6R transgenic mice, the acute phase response persisted as long as 48 h. Third, in hsIL-6R transgenic animals, the IL-6 serum levels are markedly elevated and the plasma half-life of IL-6 is significantly prolonged. In a dose-dependent fashion, hsIL-6R transgenic mice have two- to three-fold higher IL-6 serum levels as compared to control animals. Moreover, in time course experiments (Fig. 5) IL-6 levels are detectable for 20 h in hsIL-6R transgenic animals, whereas in control

mice, IL-6 levels could be measured for only 12 h. It is interesting that the IL-6 plasma levels are detectable longer in heterozygous hsIL-6R transgenic mice than in homozygous mice (Fig. 5 B). This effect becomes apparent after 4 h. One could argue that this represents a contradiction to the finding that in homozygous mice the acute phase response persists longer than in heterozygous mice (Fig. 4 B). It is likely that there is an increased retention of the IL-6/IL-6R complex in the circulation resulting in a prolongation of the half-life due to the increased size and stability of the complex. Moreover, it is well known that the IL-6/IL-6R complex is internalized after activation of membrane-bound gp130 (40, 41). It is possible that the clearance of IL-6 and thus the IL-6 levels *in vivo*, are determined by an equilibrium between retention and internalization of the IL-6/IL-6R complex, which in the case of higher IL-6R levels, as measured in homozygous hsIL-6R mice, is shifted towards internalization. This notion is supported by data from patients with sepsis, in whom highly increased IL-6 levels and significantly decreased sIL-6R levels were measured when compared with control patients (42).

Cytokine receptors are important in the regulation of cytokines and growth factors. There are multiple ways to regulate receptor function: (a) expression of receptors may be restricted to specific cell types; (b) the receptor number on the plasma membrane may vary because of receptor mRNA regulation or receptor internalization; (c) the receptor affinities may be modulated on some cells by additional membrane proteins that interact with the ligand-binding protein; or (d) the membrane-bound receptor may be transformed into a soluble form by limited proteolysis (28). Not only cytokines have soluble counterparts, but also other transmembrane proteins, such as adhesion molecules (intercellular adhesion molecule 1) (43), the LPS-binding protein CD14 (44), and the B and T cell membrane glycoproteins CD27 and CD40 (45). Soluble receptors for most cytokines have been detected in many human body fluids, the majority inhibiting the function of their respective ligands. The sIL-6R has been demonstrated to act agonistically on cells bearing gp130 molecules on their surface if IL-6 is present. IL-6 serum levels detected in normal individuals are very low. It is noteworthy that at such concentrations there is little or no responsiveness to this cytokine. In the presence of the sIL-6R, however, such concentrations could stimulate target cells.

We have recently shown that melanoma cells transfected with the murine sIL-6R, when injected into syngeneic mice, are much less effective in tumor induction or metastasis formation compared to untransfected melanoma cells (46, 47). In this situation the sensitivity towards the endogenous murine IL-6 seems to be amplified by the presence of the sIL-6R protein.

The biological importance of the shedding process as a major mechanism of generating soluble proteins is protean: (a) growth factors or cytokines are liberated such as TNF- α , TGF- α , and kit ligand; (b) soluble receptors are generated and act as competitors of the membrane-bound counterparts and thereby antagonize the cytokine response; and (c)

for receptors of the IL-6 family (formally shown for the IL-6R and the receptor for the ciliary neurotrophic factor) and for the LPS receptor CD14, it has been demonstrated that the soluble proteins act as agonists, i.e., together with their ligands they are able to induce signaling on cells which by themselves are unresponsive to the ligand. This process has recently been termed transsignaling (28). There is an increasing number of reports on cells being responsive to IL-6 only when IL-6R is present: bone resorption *in vivo* and in the osteoblast/osteoclast coculture (48–50), CD34⁺ stem cell proliferation (51), and the induction of alveolitis (52) are markedly enhanced in the presence of both IL-6 and IL-6R as compared to stimulation with IL-6 alone. In this context, generation of sIL-6R not only has the effect of sensitization and prolongation, but also of a change of quality of the response. We were able to demonstrate that the phorbol ester PMA induces quantitative

shedding of the sIL-6R, indicating that protein kinase C is involved in this process (22, 53). Most recently, we have shown that not only PMA, but also LPS markedly enhances shedding (54), indicating that increased generation of the sIL-6R might occur during bacterial infections. Based on these findings, it will be important to elucidate the mechanism responsible for the generation of soluble receptor proteins.

In the future, it will be interesting to study the status of the sIL-6R during inflammatory and infectious conditions. This might change our view of the function of IL-6 in various pathophysiological states. Moreover, the IL-6/IL-6R complex, and not IL-6 alone, might be regarded as the active cytokine *in vivo*. To further address this question, it will be interesting to generate double transgenic mice, expressing hIL-6 in conjunction with the shIL-6R.

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