Structural and functional studies on the human hepatic interleukin-6 receptor

Molecular cloning and overexpression in HepG2 cells

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cDNAs coding for the human hepatic interleukin-6 receptor (IL-6-R) have been isolated from a library made from poly(A) RNA of dexamethasone-treated human hepatoma cells (HepG2). We found the hepatic IL-6-R to be identical to the one expressed by leucocytes. A polyclonal antiserum was raised in rabbits against the IL-6-R protein expressed in *Escherichia coli*. Although the entire IL-6-R protein was used for immunization, only antibodies to the cytoplasmic domain of the IL-6-R were obtained. It is demonstrated by affinity cross-linking and subsequent immunoprecipitation with antibodies against the ligand as well as against the receptor that the cloned cDNA codes for the functional IL-6-R on HepG2 cells. When the hepatic IL-6-R cDNA was overexpressed in HepG2 cells, these cells became more sensitive to low concentrations of IL-6 with respect to the induction of γ -fibrinogen mRNA.

INTRODUCTION

Interleukin-6 (IL-6) is a multifunctional cytokine which is synthesized by many different cells (for reviews see [1–4]). The major IL-6-producing cells are monocytes/macrophages, endothelial cells and fibroblasts. IL-6 acts on various cell types resulting in different biological responses. In B-cells IL-6 plays a role in the terminal differentiation into immunoglobulin-secreting cells [5,6]. In hybridoma/plasmacytoma/myeloma cells IL-6 induces proliferation [7–10]. In T-cells IL-6 acts as an activation and proliferation factor [11–13]. In haematopoietic stem cells IL-6 stimulates multipotent colony formation [14–16]. In cells of neuronal origin IL-6 has been found to change the morphology to neurite-extending cells [17]. In hepatocytes IL-6 has been described to be a major regulator of acute-phase protein synthesis [18–20].

Since IL-6 acts via specific cell surface receptors, the various biological responses of different cell types could be due to different IL-6 receptors (IL-6-Rs) or to different signal transduction pathways. We are interested in the elucidation of the molecular mechanisms involved in the IL-6-dependent regulation of acute-phase protein synthesis in liver. Thus far, little is known about the signal transduction of IL-6 after its interaction with the hepatic receptor. We and others have performed studies on the structure and regulation of the hepatic IL-6-R [21,22]. An IL-6-R cDNA from the human natural killer (NK)-like cell line YT has been cloned [23]. To find out whether hepatic cells express the same receptor as lymphatic cells, we used the IL-6-R cDNA of NK cells as a probe for screening several human cDNA libraries of hepatic origin.

In this paper we describe the isolation of hepatic IL-6-R cDNA clones, their expression in *Escherichia coli* and the production of a polyclonal antiserum against the recombinant protein. Furthermore, we present data on the regulation of the acute-phase protein γ -fibrinogen in HepG2 cells overexpressing the human hepatic IL-6-R.

MATERIALS AND METHODS

Materials

Restriction enzymes, polynucleotide kinase, calf intestinal phosphatase, T4 DNA-ligase, reverse transcriptase (Avian Myeloblastosis Virus), DNA polymerase I, Klenow enzyme, nuclease S1 and DNA-packaging mix were purchased from Boehringer (Mannheim, Germany). Exonuclease III was from AGS (Heidelberg, Germany). Carrier-free Na^{125}I (559 MBq/ μg), [α - ^{32}P]dCTP (110 TBq/mmol) and [α - ^{35}S]dATP (44 TBq/mmol) were obtained from Amersham International (Amersham, Bucks., U.K.). Geneticin (G418 sulphate), DNA methylase and Dulbecco's modified Eagle's medium (DMEM) were from Gibco (Eggenstein, Germany); fetal calf serum was from Seromed (Berlin, Germany). The cross-linker bis(sulphosuccinimidyl)-suberate was purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.). Recombinant human (rh)IL-6 (5×10^6 B-cell stimulatory factor-2 units/mg of protein) was prepared as described in [5,6]. RhIL-1 β (1.3×10^7 units/mg) was kindly supplied by Dr. A. Shaw (Glaxo, Institute for Molecular Biology, Geneva, Switzerland). RhTNF α (tumour necrosis factor) (lot no. K9129A1/70121; 6×10^7 units/mg) was a gift from Dr. V. Schwendemann, Knoll AG (Ludwigshafen, Germany). The isolation of an IL-6-R cDNA from NK cells has been described in [23].

Cell culture

HepG2 cells were cultured in DMEM containing 10% fetal calf serum. All cell culture media contained penicillin (61 mg/l) and streptomycin (100 mg/l).

RNA preparation and Northern blot analysis

Total RNA was prepared using the phenol extraction method [24] and as previously described [25]. RNA (5 μg) was heated to 65 °C for 10 min in 50% formamide/20 mM-Mops/5 mM-sodium acetate/1 mM-EDTA/2.2 M-formaldehyde prior to gel electro-

Abbreviations used: IL-6, interleukin-6; IL-6-R, interleukin-6 receptor; NK, natural killer; rh, recombinant human; DTT, dithiothreitol; PBS, phosphate-buffered saline (0.2 M-NaCl, 2.5 mM-KCl, 8 mM- Na_2HPO_4 , 1.5 mM- KH_2PO_4 , pH 7.4); TNF, tumour necrosis factor; DMEM, Dulbecco's modified Eagle's medium.

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phoresis in 1% agarose containing 2.2 M-formaldehyde, 20 mM-Mops, 5 mM-sodium acetate and 1 mM-EDTA. The separated RNA was transferred to GeneScreen Plus membranes (NEN, Dreieich, Germany) according to the supplier's instructions. The filters were prehybridized at 68 °C for 1 h in 10% dextran sulphate/1 M-NaCl/1% SDS and hybridized in the same solution with cDNA fragments labelled by random priming [26].

Preparation and screening of cDNA libraries

λ gt10 or λ gt11 cDNA libraries were prepared from poly(A) RNA isolated from either human liver or HepG2 cells treated with 1 μ M-dexamethasone for 18 h according to the method of Gubler & Hoffmann [27]. In short, 10 μ g of poly(A) RNA was used for first-strand synthesis primed with oligo(dT) or a combination of random hexamer primers and six specific oligonucleotides in 30 mM-Tris/HCl, pH 8.0, 10 mM-MgCl₂, 5 mM-dithiothreitol (DTT), 0.5 mM of each of dATP, dTTP, dCTP and dGTP, and 100 mM-KCl. Specific oligonucleotide primers were synthesized according to the cDNA sequence of the IL-6-R from NK cells: (1) AGCCAGCAGCGCGCAGCCGACGGCCAGCAT, (2) GGATGGGGTGCTCCGAGGACCCCACTCACA, (3) GTCTTGCCAGGTGACACTGAGCCAGCGGGG, (4) CCAAGGCGTGCCCATGGCCTCCGGGCTCC, (5) GAAG-AAGTAGTCTGTATTGCTGATGTCATA, and (6) GGGTC-CAGGCTGCTGGTG. After second-strand synthesis with DNA polymerase I, the double-stranded cDNA was blunt-ended using T4 DNA polymerase. The cDNA was methylated, 12-mer *Eco*RI linkers were added, the cDNA was ligated into *Eco*RI-cleaved λ DNA and packed *in vitro*.

Screening of the cDNA libraries was performed by using standard methods [28]: 1.7 kb *Xho*I and 1.2 kb *Xho*I/*Pst*I fragments from the NK IL-6-R cDNA were labelled by random priming [26] and used as probes.

DNA subcloning and nucleotide sequencing

Insert DNA from phages was subcloned into the plasmid pUC19 using standard techniques [28]. Sequencing was performed by the method of Sanger [29] from plasmid double-stranded DNA [30].

Expression of full-length and truncated IL-6-R cDNAs in *E. coli*

The 1.4 kb *Sal*I fragment from the plasmid pExIR 1 [31] containing the coding region of the IL-6-R was cloned into the bacterial expression plasmid pEx10 [32], which allows the expression of cDNA inserts fused to the first 98 amino acids of the bacteriophage MS2 polymerase under the control of the λ P_L promoter [33] (Fig. 1). The correct reading frame of the IL-6-R cDNA insert was confirmed by sequencing with a primer designed after the sequence upstream of the polylinker of the plasmid pEx10 (GACAAATCCTTGTCATGGG).

For the construction of 3'-deleted IL-6-R cDNAs we used stepwise unidirectional exonuclease III digestion of the full-length IL-6-R cDNA and subsequent nuclease S1 treatment followed by recircularization [28]. The 5'-deletion mutant Δ 67 lacks a *Pst*I fragment of 201 bp.

Purification of the full-length IL-6-R fusion protein and antiserum production

Expression of IL-6-R fusion protein was induced by a temperature shift and the fusion protein was purified by the separation of inclusion bodies from soluble proteins and by preparative SDS/PAGE [34]. The IL-6-R-containing fusion protein was excised from the gel and 1 mg was used for each subcutaneous injection into rabbits.

Fig. 1. Plasmid construct pBExIR1 for the expression of an IL-6-R fusion protein in *E. coli*

H, *Hind*III; P, *Pst*I; S, *Sal*I; X, *Xba*I; SP, signal peptide; TM, transmembrane domain; ori, origin.

Iodination of rhIL-6

RhIL-6 was iodinated according to the procedure of Markwell [35], with modifications as previously described [36]. A specific radioactivity of 250 kBq/ μ g was obtained. The biological activity of the iodinated IL-6 was essentially unchanged [36].

Affinity cross-linking, immunoprecipitation and electrophoresis

HepG2 cells (2×10^7) were washed twice with phosphate-buffered saline (PBS), mechanically removed from the culture dishes and suspended in a total volume of 500 μ l of PBS. After addition of 4 ng (1.2×10^5 d.p.m.) of ¹²⁵I-rhIL-6 in 10 μ l of PBS/1% BSA and incubation at 4 °C for 15 min, 10 μ l of the cross-linker bis(sulphosuccinimidyl)suberate (10 mM in PBS) was added. The cross-linking reaction was allowed to proceed at room temperature for 15 min. The reaction was stopped by addition of 500 μ l of PBS and centrifugation of the cells at 12000 *g* for 5 min. After washing with 2×1 ml of PBS, the sedimented cells were solubilized with 20 mM-Tris/HCl, pH 7.5, containing 5 mM-EDTA, 140 mM-NaCl and 1% Triton X-100. Free ¹²⁵I-rhIL-6 and ¹²⁵I-rhIL-6 cross-linked to plasma membrane proteins were immunoprecipitated with a specific antiserum against rhIL-6 or rhIL-6-R and analysed by SDS/PAGE (3–15% gels) [37].

Transfection of HepG2 cells

Approx. 5×10^6 HepG2 cells were transfected with 20 μ g of pExIR1 plasmid DNA as described in [31,38]. The transfected cells were incubated for 12 h, the medium was removed, and cells were washed twice with PBS and finally incubated for 24 h with fresh medium. The selection of the transformed HepG2 cells was performed in the presence of 500 μ g of G418/ml of medium. After 4 weeks G418-resistant colonies were isolated and probed for IL-6-R expression by Northern blot analysis. The clone with the highest IL-6-R expression was chosen for the experiments described in this paper. IL-6-R expression was maintained by these cells for > 20 passages.

RESULTS

Besides IL-6, glucocorticoids play an important role in the induction of acute-phase proteins in hepatocytes [3,20]. Therefore

Fig. 2. Regulation of expression of IL-6-R mRNA in HepG2 cells

HepG2 cells were incubated for 18 h in the presence or the absence of rhIL-1 β (1000 units/ml), rhIL-6 (100 units/ml), rTNF α (1000 units/ml), dexamethasone (DEX) (1 μ M), anti-rhIL-6 (rhIL-6 AS) and combinations thereof, as indicated in the Figure. Total RNA was prepared as described in Materials and methods section, and 5 μ g was separated on a formamide/formaldehyde gel and transferred to a nylon membrane. The Northern blot was hybridized with a 32 P-labelled 1.2 kb *Pst*I/*Xho*I fragment of the IL-6-R cDNA of NK cells.

Fig. 3. Map of human hepatic IL-6-R cDNA

(a) Restriction map and organization of IL-6-R cDNA. Arrows denote the positions of the primers used for cDNA synthesis. SP, signal peptide; TM, transmembrane domain. (b) Isolated clones coding for the IL-6-R. (c) Sequencing strategy.

we have studied the effect of dexamethasone in combination with IL-6, IL-1 β and TNF α on the expression of the hepatic IL-6-R in HepG2 cells (Fig. 2). It is evident from the Northern blot shown in Fig. 2 that dexamethasone treatment alone (lane 4) leads to an upregulation of the IL-6-R mRNA. The addition of IL-1 β or IL-6 did not influence the effect of dexamethasone (lanes 2 and 6). TNF α slightly enhanced the induction of IL-6-R mRNA by dexamethasone (lane 7). Treatment of HepG2 cells with the cytokines alone did not change IL-6-R expression (lane 3; and results not shown).

The cross-hybridization of the IL-6-R cDNA from NK cells with mRNA from hepatic cells cannot be regarded as a final proof of receptor identity, particularly in view of the fact that IL-6 elicits very different biological responses in lymphoid and hepatic cells. We therefore decided to carry out cDNA cloning

Fig. 4. Nucleotide and amino acid sequence of the human hepatic IL-6-R

At position 144 a silent point mutation was detected when the sequence was compared with the cDNA sequence of the IL-6-R from NK cells. The underlined sequence represents the signal peptide. *** designates consensus sequences of potential N-glycosylation sites. The putative transmembrane domain is boxed.

using the IL-6-R probe of NK cells to screen for the hepatic IL-6-R cDNA.

The screening of two human liver cDNA libraries did not result in the isolation of positive clones. Also, in an oligo(dT)₁₂₋₁₈-primed cDNA library constructed from poly(A)⁺ RNA of HepG2 cells stimulated with dexamethasone (see Fig. 2, lane 2), no IL-6-R cDNA clones could be found. The large size and the low abundance of the IL-6-R mRNA could explain the failure to isolate cDNA clones. We therefore used random primers and six specific primers (Fig. 3a, arrows) for the construction of a library from poly(A)⁺ RNA of dexamethasone-stimulated HepG2 cells. Screening of 2×10^8 plaques resulted in the isolation of 15 positive IL-6-R cDNA clones, seven of which were analysed and sequenced (Figs. 3b and 3c). As can be seen from Figs. 3(a) and 3(b), all of these clones started with a specific primer sequence

Fig. 4 shows the composite nucleotide sequence of the hepatic IL-6-R and the derived amino acid sequence. Comparison with the published sequence of the IL-6-R from NK cells [23] revealed complete identity, except for a silent point mutation at nucleotide position 144 (hepatic IL-6-R, G; NK cell IL-6-R, A).

For studies on the IL-6-R protein, specific antibodies are essential. Therefore the coding region of the IL-6-R cDNA was expressed as a fusion protein in *E. coli*, and the recombinant protein was used for the immunization of rabbits. The antiserum obtained recognized the fusion protein which had been used for

Fig. 5. Detection of C-terminally truncated IL-6-R fusion proteins by IL-6-R antiserum

(a) Coomassie-Blue-stained SDS/polyacrylamide gel (10%) with C-terminally truncated fusion proteins. Δ0–Δ67 denote the numbers of deleted amino acids in the respective constructs. (b) Western blot analysis of the same samples with IL-6-R antiserum (1:1000 dilution). The detected proteins are visualized using a peroxidase-conjugated second antibody.

Fig. 6. Cross-linking of ¹²⁵I-rhIL-6 to HepG2 cells

HepG2 cells (2×10^7) were incubated with 4 ng (2×10^5 d.p.m.) of ¹²⁵I-rhIL-6 at 4 °C for 15 min, the cross-linker bis(sulphosuccinimidyl)suberate was added to a final concentration of 1 mM, and incubation was continued for 15 min at room temperature. The cells were solubilized, and the cross-linked complexes were immunoprecipitated with different antisera (AS) (lanes 1–4). Complexes were separated on a denaturing SDS/polyacrylamide gradient gel and autoradiographed. Controls: ¹²⁵I-rhIL-6 with (lane 5) or without (lane 6) cross-linker; immunoprecipitation with pre-immune serum (lane 4); incubation of HepG2 cells with a 100-fold excess of unlabelled IL-6 (lane 2).

immunization (see Western blot, Fig. 5, lane 12). No cross-reactions with *E. coli* proteins were found (lane 11). To identify the part(s) of the IL-6-R molecule which is (are) recognized by our antiserum, we truncated the IL-6-R cDNA stepwise from the 3'-end and also at the 5'-end, expressed the deletion mutants in *E. coli* (Fig. 5a, arrows) and analysed their reactivity with the antiserum on a Western blot (Fig. 5b). In spite of the fact that in all cases large amounts of protein were synthesized, the antiserum only recognized the wild-type IL-6-R (lane 12) and the protein truncated at the N-terminus (lane 21). As soon as parts of the C-terminus, which forms the cytoplasmic domain of the receptor,

Fig. 7. Expression of IL-6-R mRNA in transfected HepG2 cells

Transfected HepG2 cells were incubated with increasing concentrations of ZnCl₂ for 18 h (lanes 3–6). Untransfected HepG2 cells incubated in the absence (lane 1) or presence (lane 2) of 1 μM-dexamethasone (DEX) were used as controls. Total RNA (5 μg) was used for the separation on agarose/formaldehyde gels. Northern blot analysis was carried out as described in the legend to Fig. 2 and in the Materials and methods section.

were deleted, the recognition by the antiserum ceased. It can be concluded that the antiserum exclusively recognizes the cytoplasmic domain of the IL-6-R. Accordingly, we were unable to stain intact IL-6-R-expressing human hepatoma cells with the antiserum (results not shown). However, the antiserum recognized the IL-6-R after solubilization of these cells (Fig. 6). ¹²⁵I-rhIL-6 was cross-linked to HepG2 cells and the cross-linked complexes were immunoprecipitated by an antiserum against IL-6 (lane 1) as well as by the anti-IL-6-R antiserum (lane 3). Both antisera immunoprecipitated three ¹²⁵I-rhIL-6-containing protein complexes of molecular masses of 100, 120 and 200 kDa, which have been described in previous studies [21]. A detailed analysis of the nature of these ¹²⁵I-rhIL-6-containing complexes has been carried out in our laboratory (D. Lenz, unpublished work). This observation is clear evidence for the fact that the IL-6-R cDNA cloned from HepG2 cells and used for the production of antibodies codes for the functional human hepatic IL-6-R.

It is known from previous experiments that HepG2 cells express only about 1000 IL-6-Rs/cell [21]. Since a detailed

Fig. 8. Cross-linking of ^{125}I -rhIL-6 to transfected HepG2 cells

Transfected HepG2 cells (2×10^7) were incubated with (lane 1) or without (lane 2) 0.1 mM- ZnCl_2 at 37 °C for 18 h. Cross-linking with ^{125}I -rhIL-6 was carried out as described in the legend to Fig. 6. Immunoprecipitation was done with an anti-rhIL-6 antiserum.

Fig. 9. Induction of γ -fibrinogen mRNA by rhIL-6 in transfected HepG2 cells

Transfected HepG2 cells were incubated with increasing concentrations of rhIL-6 in the absence (lanes 1–3) or presence (lanes 4–6) of 0.1 mM- ZnCl_2 for 18 h. Northern blot analysis was done as described in the legend to Fig. 2 and in the Materials and methods section. Hybridization was carried out with a ^{32}P -labelled 0.75 kb *Pst*I fragment of the human γ -fibrinogen cDNA.

coding region of the IL-6-R mRNA and lacking the non-coding region (2.5 kb at the 5'-end and 1.4 kb at the 3'-end). It should be noted that, in the transfected HepG2 cells also, low amounts of the endogenous IL-6-R mRNA can be seen. Having shown that transfected HepG2 cells overexpress IL-6-R mRNA, we asked whether these changes also occur at the protein level. Affinity cross-linking of ^{125}I -rhIL-6 to transfected HepG2 cells incubated in the presence (Fig. 8, lane 1) or absence (lane 2) of ZnCl_2 was performed. All three ^{125}I -rhIL-6-containing protein complexes were found to be upregulated after stimulation of the metallothionein promoter by ZnCl_2 .

The induction of mRNA for the acute-phase protein γ -fibrinogen by increasing concentrations of rhIL-6 was studied in transfected HepG2 cells in the presence and absence of ZnCl_2 (Fig. 9). We found that cells overexpressing the IL-6-R (lanes 4–6) responded to lower IL-6 concentrations than the control cells (lanes 1–3). It should be noted that at 100 units of rhIL-6/ml, overexpression of the IL-6-R did not lead to a further increase in fibrinogen induction (lanes 3 and 6), possibly reflecting a limitation in capacity.

DISCUSSION

It is known from the work of various investigators that different cell types show different biological responses to IL-6. In the cases of the cytokines IL-1 and TNF, different receptors have been found on different target cells [39–44]. The aim of our study has been the molecular characterization of the human hepatic IL-6-R. In this paper we show by cDNA cloning that the hepatic IL-6-R is identical with the 80 kDa receptor protein expressed in NK cells.

All of IL-6-R cDNA clones analysed were derived from specific primers used for the construction of the cDNA library. We are aware of the fact that such an approach may lead to the preferential isolation of clones corresponding to completely under-represented mRNAs. Therefore we performed cross-linking experiments to demonstrate that the cloned cDNA corresponds to the IL-6-R protein on the plasma membrane of hepatic cells (Fig. 6). The antiserum raised against our cloned hepatic IL-6-R expressed in *E. coli* recognized all three plasma membrane protein complexes to which IL-6 can be cross-linked and which can be immunoprecipitated by an anti-IL-6 antiserum. This finding clearly proves that the cloned cDNA indeed codes for the functional hepatic IL-6-R.

It is surprising that the antiserum against the recombinant human hepatic IL-6-R bound exclusively to the cytoplasmic domain of the receptor, although the entire protein had been used for immunization. At present we have no explanation for this phenomenon, but it may reflect particular features of the IL-6-R protein.

When the IL-6-R cDNA was stably transfected into HepG2 cells, the receptor could be induced by the addition of ZnCl_2 . Treatment with up to 50 μM - ZnCl_2 resulted in essentially no induction of IL-6-R mRNA (Fig. 7). HepG2 cells constitutively synthesize metallothionein protein [45], which chelates Zn^{2+} . This steep increase in receptor mRNA levels at 100 μM - ZnCl_2 may be explained by the fact that at this concentration the endogenous metallothionein protein is saturated, and enough free Zn^{2+} is available for the activation of the metallothionein promoter driving IL-6-R transcription.

As shown by cross-linking, all three ^{125}I -rhIL-6-containing complexes are upregulated in transfected HepG2 cells. This observation was unexpected, since we had transfected HepG2 cells with only one cDNA coding for the IL-6-R. There are two obvious explanations for this phenomenon. Firstly, the three bands could represent: (1) 80 kDa IL-6-R plus 20 kDa ^{125}I -rhIL-

receptor analysis with cells exhibiting such a low receptor number is difficult, we decided to stably express the coding region of the human IL-6-R cDNA in HepG2 cells. For the transfection we used a construct in which the mouse metallothionein I promoter drives transcription of the IL-6-R cDNA. Fig. 7 (lanes 3–6) shows a dramatic effect of ZnCl_2 on the expression of IL-6-R mRNA in the transfected HepG2 cells. The extent of overexpression can be estimated by comparison with the IL-6-R mRNA expressed by untransfected HepG2 cells (lane 1). In the presence of 1 μM -dexamethasone, an upregulation of IL-6-R mRNA was observed (lane 2). IL-6-R mRNA from HepG2 cells migrated above 28 S rRNA ($\blacktriangleright$), whereas IL-6-R mRNA from the transfected cDNA shows a much higher electrophoretic mobility ($\triangleright$). This size difference is due to the fact that HepG2 cells have been transfected with a construct carrying the entire

6 (= 100 kDa); (2) 80 kDa IL-6-R plus 2 molecules of 20 kDa 125 I-rhIL-6 (= 120 kDa), and (3) 2 molecules of 80 kDa IL-6-R plus 2 molecules of 20 kDa 125 I-rhIL-6 (= 200 kDa). An alternative to this homodimer model is a heterodimeric structure of the hepatic IL-6-R. In this case (an) additional abundant protein(s), which can be cross-linked either to 125 I-rhIL-6 or to a receptor polypeptide, must be postulated.

When γ -fibrinogen induction by rhIL-6 was studied in transfected HepG2 cells, it became evident that an upregulation of the IL-6-R made the cells more responsive to low IL-6 concentrations (1–10 B-cell stimulatory factor-2 units/ml). It should be noted that this concentration range corresponds to IL-6 levels in the plasma of patients with inflammatory diseases [46]. It remains to be established whether the number of IL-6-Rs on the surface of hepatocytes contributes to the regulation of acute-phase protein synthesis.

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