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Soluble human interleukin-6-receptor modulates interleukin-6-dependent *N*-glycosylation of α_1-protease inhibitor secreted by HepG2 cells

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Interleukin-6 (IL-6) induces changes in gene expression and the *N*-glycosylation pattern of acute-phase proteins in hepatocytes. IL-6 exerts its action via a cell surface receptor complex consisting of an 80 kDa IL-6 binding protein (gp80) and a 130 kDa glycoprotein (gp130) involved in signal transduction. A genetically engineered gp80-derived soluble human IL-6-receptor (shIL-6-R) significantly enhanced the IL-6 effect on *N*-glycosylation changes (revealed by reactivity with the lectin-concanavalin A) of a_1-protease inhibitor (PI) secreted by human hepatoma cells (HepG2). Stable transfection of IL-6-cDNA into HepG2 cells (HepG2-IL-6) resulting in constitutive secretion of 2 μg of IL-6 per 10^6 cells in 24 h led to a down-regulation of surface-bound gp80 and subsequent homologous desensitization of HepG2-IL-6 cells towards IL-6. Soluble human IL-6-R functionally substituted membrane-bound gp80 resulting in a reconstitution of responsiveness of HepG2-IL-6 cells.

Acute-phase response; α_1-Protease inhibitor; Hepatoma cell; Interleukin-6; *N*-Glycosylation; Soluble interleukin-6-receptor

1. INTRODUCTION

IL-6 is a major hepatocyte stimulating factor [1,2]. It regulates the expression of a number of plasma proteins referred to as acute-phase proteins (APP) [1–3]. IL-6 is also involved in the regulation of *N*-glycosylation of APP in hepatocytes [4]. IL-6 exerts its action via a cell surface receptor complex consisting of two components, an 80 kDa glycoprotein (gp80) capable of IL-6 binding, and a 130 kDa glycoprotein (gp130) which does not bind IL-6, but is involved in initiation of signal transduction [5,6]. The cDNAs of both components have been cloned [5,7]. It turned out that the cDNA-sequence for gp80 in hepatocytes is identical to the one found in other cell types [8]. Studies on the mechanism regulating gp80 and gp130 expression in hepatocytes have demonstrated that gp80 mRNA and its functional protein is upregulated by glucocorticoids, but not by IL-6 [9]. However, gp130 mRNA was shown to be slightly increased by IL-6 and to a greater extend by the combination of IL-6 and glucocorticoids [10]. IL-6 triggers the association of gp80 and gp130 leading to a functional IL-6-receptor complex [6].

In hepatocytes IL-6 and gp80 are internalized after their interaction resulting in the down-regulation of surface gp80 [11]. It is presently not known whether gp130 is internalized together with IL-6 and gp80. A gp80 mutant lacking cytoplasmic and transmembrane parts could functionally substitute for gp80 [6,7]. Moreover, analogous fragments of the gp80 soluble human IL-6-receptor (shIL-6-R) were found in human urine [12].

APP secreted by the liver carry *N*-linked complex-type glycans, which may be formed as bi-, tri- or tetra-antennary structures [13]. Stimulation of hepatocytes may either increase or decrease the relative amounts of bi- versus multi-branched glycans on the APP polypeptide backbone [14,15]. These changes may be assessed using crossed affinity-immunoelectrophoresis (CAIE) with the lectin Con A as a ligand [16]. Con A immunoelectrophoresis separates glycoforms of plasma proteins bearing various amounts of bi-antennary *N*-glycans [17]. Forms possessing tri- and/or tetra-antennary *N*-glycans do not react with the lectin in CAIE, forms having one bi-antennary unit react weakly, forms having two bi-antennary structures react strongly. By assessing relative amounts of a particular Con A-glycoform of the APP studied it is possible to evaluate either relative increases or decreases of bi-antennary compared to tri- and/or tetra-antennary *N*-glycans.

The present studies were undertaken to elucidate the role played by the shIL-6-R in the regulation of *N*-glycosylation of APP by IL-6. The studies were carried

Abbreviations: APP, acute-phase proteins; Con A, concanavalin A; CAIE, crossed affinity-immunoelectrophoresis; GlcNAc, *N*-acetylglucosamine; GnT, GlcNAc-transferase; IL-6, interleukin-6; IFN, interferon; LIF, leukemia inhibitory factor; PI, a_1-protease inhibitor; rh, recombinant human; shIL-6-R, soluble human interleukin-6-receptor; TGF, transforming growth factor; TNF, tumor necrosis factor.

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out using the human hepatoma cell line HepG2 as target cell and PI as a typical hepatic APP. HepG2 cells were stably transfected with IL-6-cDNA (HepG2-IL-6) and used as a model for a 'chronically inflamed liver'. In these cells gp80 is constitutively down-regulated and the induction of APP is solely dependent on the addition of shIL-6-R.

2. MATERIALS AND METHODS

2.1. *Reagents*

Restriction enzymes, polynucleotide kinase, calf intestinal phosphatase, T4-DNA ligase were from Boehringer Mannheim (Germany). [α-^{32}P]dCTP (110 TBq/mmol) and [α-^{35}S]dATP (44 TBq/mmol) were from Amersham International (United Kingdom). Geniticin (G418-sulfate) and DMEM were from Gibco (Eggenstein, Germany). RhIL-6 was prepared as described in [18]. The specific activity was 1.5×10^6 B-cell stimulatory factor 2 units/mg protein [19]. Platelet-derived purified TGFβ1 was from R&D Systems Inc. (Mineapolis, MN, USA). Rh LIF was a generous gift of Dr. H. Baumann (Buffalo, NY, USA) [20]. Rh INFγ was from Bioferon (Laupheim, Germany). Con A type IV was from Sigma Chem. Co. (St. Louis, MO, USA). Polyclonal antiserum against human PI was from Dakopatts (Hamburg, Germany). Human IL-6-cDNA and gp80-cDNA were isolated as described [8,21]. Human PI-cDNA was supplied by Drs. G. Ciliberto and R. Cortese (IRBM, Rome, Italy).

2.2. *Cell cultures*

HepG2 cells (ATCC, Rockville, MD, USA) were cultured in DMEM/DMEM-F12 medium. NIH/3T3 cells (ATCC) were grown in DMEM. Culture medium was supplemented with 10% fetal calf serum, streptomycin (100 mg/l) and penicillin (61 mg/l).

2.3. *Stable transfection of cells*

Transfections were carried out as described in [22]. NIH/3T3 cells were stably transfected with the expression vector pBMGNeo [23] carrying the extracellular domain of the human gp80-cDNA fused to a translational stop codon (H. Schooltink et al., in preparation) under the transcriptional control of the mouse metallothionein I promoter. HepG2 cells were stably transfected with an IL-6-cDNA in the same expression vector.

2.4. *Generation of soluble human IL-6-R*

ShIL-6-R was obtained from the conditioned medium of NIH/3T3 cells transfected with the gp80-cDNA lacking the sequences coding for the transmembrane and cytoplasmic domains. Specific activity of shIL-6-R was estimated in a cell-free IL-6 binding assay. One unit was defined as the capacity of shIL-6-R to bind 100 ng of ^{125}I-rhIL-6 (H. Schooltink et al., in preparation). Medium from untransfected NIH/3T3 cells was used as a control.

2.5. *RNA-preparation and Northern blot analysis*

HepG2 and HepG2-IL-6 cells were cultured in the presence of cytokines and/or shIL-6-R for 21 h. Subsequently, total RNA was prepared [24] and subjected to Northern blot analysis. A 1.35 kb *Pst*I fragment of human PI-cDNA was labelled by random priming [25] and used as a hybridization probe.

2.6. *Determination of the production and N-glycosylation pattern of PI secreted by HepG2 and HepG2-IL-6 cells*

HepG2 and HepG2-IL-6 cells were incubated with cytokines and shIL-6-R for 48 h with replacement of medium after 24 h. Analyses were carried out in the media collected over the final 24 h. PI secreted by hepatoma cells and accumulated in the culture medium was measured by an electro-immunoassay [26]; accumulation of APP secreted by human hepatoma cells into the culture medium paralleled the rate of synthesis of [^{35}S]methionine-labelled APP [27]. The *N*-glycosylation pattern of PI was studied by CAIE with Con A [50 μM] as a ligand [17,28]. Three glycoforms were observed (Fig. 2): variant 0=non-reactive with Con A, composed of three tri-antennary glycans; variant 1=weakly reactive with Con A, composed of 1 bi- and 2 tri-antennary glycans; variant 2=reactive with Con A, composed of 2 bi- and 1 tri-antennary glycans. The area under the precipitates was determined by planimetry and relative amounts of PI-glycoforms were expressed as percentages of total. In order to express the degree of relative changes of *N*-glycans composition the PI-Con A reactivity coefficient (RC) was calculated according to the formula: (sum of Con A reactive variants) / (Con A non-reactive variants). An increase of the RC value reflects increased relative amounts of bi- versus multi-branched *N*-glycans.

2.7. *IL-6 assay*

IL-6 was measured using the IL-6-dependent murine plasmacytoma cell line B9, generously supplied by Dr. L. Aarden (Amsterdam, The Netherlands) [29].

2.8. *ShIL-6-R assay*

NIH/3T3 conditioned media (100 μl) were incubated in a total volume of 500 μl of PBS containing 0.01% Tween 20 with [^{125}I]rhIL-6 for 3 h at 4°C followed by incubation with a monospecific polyclonal antiserum against shIL-6-R (T. Stoyan et al., in preparation). The immunocomplexes were precipitated using protein(A)-Sepharose and counted in a γ-counter.

3. RESULTS

In HepG2 cells IL-6 moderately increased in a dose-dependent manner PI synthesis at mRNA (Fig. 1) and protein (Table I) levels. Addition of shIL-6-R to the incubation medium enhanced the effect of IL-6. IL-6 decreased Con A reactivity of PI secreted by HepG2 cells with a maximum at the dose of 100 U/ml (Fig. 2, Table I). ShIL-6-R augmented the IL-6 effect, especially at high doses of IL-6 (Table I). Increasing concentrations of shIL-6-R added to a constant amount of IL-6 amplified its effect in decreasing PI-Con A reactivity in a dose-dependent manner with a maximum at a dose of 5 U/ml (data not shown).

HepG2-IL-6 cells constitutively synthesized and secreted rhIL-6 in amounts of 2 μg/10^6 cells/24 h. These cells showed similar levels of PI mRNA and secreted comparable quantities of PI as non-transfected HepG2 cells in the absence of IL-6. Con A reactivity of PI was also comparable to that of PI from intact non-stimulated HepG2 cells (Fig. 2). Treatment of HepG2-IL-6 cells with $ZnCl_2$ or addition of exogenous IL-6 did not change PI-mRNA or PI-Con A reactivity (data not shown). Exposure of these cells to LIF, INFγ or TFGβ affected PI-Con A reactivity (Fig. 2, Table I) while it had only a marginal effect on PI mRNA levels (Fig. 1). Addition of shIL-6-R to HepG2-IL-6 cells resulted in a dose- and time-dependent induction of PI mRNA and protein (Fig. 1). ShIL-6-R significantly decreased Con A reactivity of PI secreted by these cells in a dose-dependent manner with a maximum at 5 U/ml (Fig. 2, Table I).

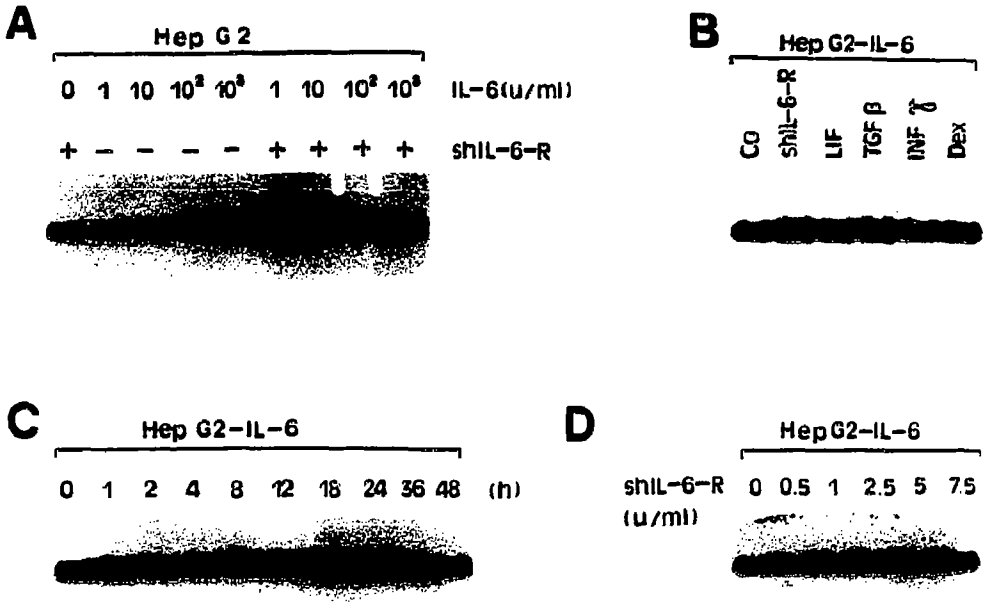

Fig. 1. Northern blot analysis of α_1-protease inhibitor: (A) Induction of PI-mRNA by IL-6 and combinations of IL-6 and shIL-6-R in HepG2 cells; (B) effect of shIL-6-R, LIF, TGFβ, IFNγ and dexamethasone on PI-mRNA expression in HepG2 cells stably transfected with IL-6-cDNA (HepG2-IL-6 cells). Time- (C) and dose- (D) dependent induction of PI-mRNA by shIL-6-R in HepG2-IL-6 cells.

4. DISCUSSION

There are three major findings of this study: (i) shIL-6-R enhances the IL-6 effect on the *N*-glycosylation of PI secreted by HepG2 cells; (ii) prolonged exposure of HepG2 cells to IL-6 leads to their homologous desensitization; (iii) shIL-6-R reconstitutes the responsiveness of IL-6-desensitized HepG2 cells as measured by changes in PI *N*-glycosylation.

Soluble cytokine receptors have been identified for a number of cytokines (reviewed in [30]). Their function, however, still remains unknown. They are generally believed to compete for the ligand with their membrane counterparts which finally leads to a decrease in the

Table I

Effect of shIL-6-R on synthesis and Con A-reactivity of PI secreted by HepG2 and HepG2-IL-6 cells

		PI-synthesis[a]		Con A-reactivity (RC[b])	
	shIL-6-R (5 U/ml)	–	+	–	+
HepG2	Control	100	100	3.5	3.5
	IL-6 1 (U/ml)	105	110	2.4	2.2
	IL-6 10 (U/ml)	120	140	1.5	1.0
	IL-6 100 (U/ml)	155	200	1.3	0.6
	IL-6 1000 (U/ml)	150	200	1.6	1.0
HepG2-IL-6	Control	100		4.0	
	shIL-6-R 1.0 (U/ml)	140		1.2	
	2.5 (U/ml)	180		0.75	
	5.0 (U/ml)	220		0.7	
	7.5 (U/ml)	200		1.0	
	10 (U/ml)	180		1.2	
	LIF 10 (U/ml)	130		1.2	
	TGFβ 2 (ng/ml)	120		5.6	
	INFγ 100 (ng/ml)	90		2.7	

[a] Analysis was carried out by means of electroimmunoassay. Results are expressed as percentages of the control and represent mean values of four experiments.

[b] Analysis was carried out by means of crossed affinity-immunoelectrophoresis with Con A as a ligand. RC = reactivity coefficient of PI-Con A calculated as described in Materials and Methods.

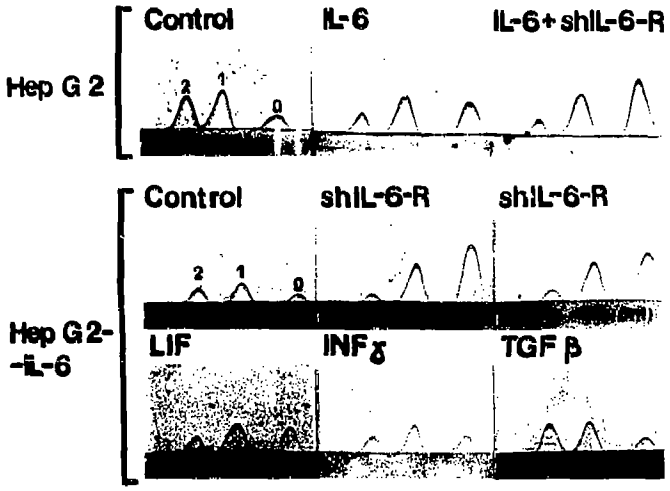

Fig. 2. Con A affinity electrophoresis of PI secreted by HepG2 (upper part) and HepG2-IL-6 cells (lower part) upon incubation with IL-6 (100 U/ml), shIL-6-R (5 U/ml, 7.5 U/ml), LIF (10 U/ml), IFNγ (100 U/ml) and TGFβ (4 ng/ml). Incubation of HepG2 cells with IL-6 and shIL-6-R was performed at concentrations of 100 U/ml and 5 U/ml of IL-6 and shIL-6-R, respectively. PI variant 0 – non reactive with Con A; variant 1 – weakly reactive; variant 2 – reactive with Con A.

biological activity of the cytokine. Our findings indicate that soluble IL-6-Rs display a unique activity resulting in the enhancement of IL-6 effects on hepatocytes. This is supported by the loss of responsiveness of HepG2 cells following continuous exposure to IL-6. The possible mechanism involved is probably related to the down-regulation of the gp80-IL-6 binding protein [11]. Interestingly, responsiveness of IL-6-desensitized cells could be reconstituted by shIL-6-R which functionally substitutes for membrane-bound gp80.

During various inflammatory processes changes in the *N*-glycosylation profiles of APPs in patient sera were observed [31]. In 'acute inflammation' increases (type I), in 'chronic inflammatory states' decreases (type II) in relative amounts of bi- versus multi-branched *N*-glycans were found [31]. In some inflammatory disorders such as systemic lupus erythematodes (SLE) [32] or AIDS (A. Mackiewicz et al., in preparation) no alterations in *N*-glycosylation were seen. However, intercurrent infection in the course of SLE leads to type I changes [32]. Alterations in APP *N*-glycosylation seem to be controlled by cytokines affecting hepatocytes [17,28]. IL-6 is a major inducer of both types of alterations in *N*-glycosylation [4]. Other cytokines such as TGFβ, LIF, INFγ, TNFα or IL-1 interact with IL-6 which finally leads to type I or type II changes [17,28]. Here we report that shIL-6-R may be an important component of the regulatory network governing *N*-glycosylation of APP.

In view of our findings and the recent report on high IL-6 levels in SLE patient sera [32] no alterations in *N*-glycosylation of APP could be associated with down-regulation of gp80 on the hepatocytes and subsequent desensitization to IL-6 in these patients. Furthermore, type I alterations seen in SLE patients with intercurrent infection [33] might be attributed to the effect of shIL-6-R.

Post-translational modification of oligosaccharide side chains of glycoproteins is a multi-step enzymatic process. Highly specific glycosidases and glycosyltransferases sequentially process an oligosaccharide precursor to yield various types of *N*-linked glycans [34]. The branches that occur on complex-type oligosaccharides are initiated by the incorporation of a *N*-acetyl-glucosamine (GlcNAc) residue. This reaction is catalyzed by GlcNAc-transferases (GnT). GnT III, GnT IV and GnT V catalyze the formation of bi-, tri- and tetra-antennary structures, respectively. Control of the level of relative activity of different GnTs is one of the regulating mechanisms of branching during the synthesis of complex-type *N*-linked oligosaccharides. Recently, IL-6 has been demonstrated to increase GnT IV and GnT V activity and decrease GnT III activity accompanied by an increase of tri- and tetra-antennary glycans on glycoproteins [35]. It should be noted that reduced GnT III activity might also contribute to the formation of multi-branched structures, since GnT III and GnT V compete for the same substrate [36]. Accordingly, the IL-6 effect on changes in the *N*- glycosylation profile of PI is most probably due to the regulation of gene expression of GnTs.

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