The hepatic interleukin-6 receptor

Down-regulation of the interleukin-6 binding subunit (gp80) by its ligand

Dietlind Zohlnhöfer, Lutz Graeve, Stefan Rose-John, Heidi Schooltink, Elke Dittrich and Peter Claus Heinrich

Institut für Biochemie der RWTH Aachen, Pauwelsstr. 30, 5100 Aachen, Germany

Interleukin-6 (IL6) exerts its action via a cell surface receptor composed of an 80 kDa IL6-binding protein (gp80) and a 130 kDa polypeptide involved in signal transduction (gp130). We studied the role of gp80 in binding, internalization and down-regulation of the hepatic IL6-receptor (IL6R) by its ligand in human hepatoma cells (HepG2). Comparison of transfected HepG2 cells overexpressing gp80 with parental cells indicate that gp80 is responsible for low affinity binding (K_d = 500 pM) of IL6. Furthermore, gp80 is rate-limiting in internalization and degradation of IL6. Internalization resulted in a rapid down-regulation ($t_{1/2} \approx$ 15–30 min) of IL6-binding sites at the cell surface. More than 80% of the internalized [^{125}I]rhIL6 was degraded. The reappearance of IL6-binding sites at the cell surface required >8 h and was sensitive to cycloheximide, suggesting that gp80 is not recycled after internalization. The down-regulation of the hepatic IL6R by its ligand might play an important role as a protection against overstimulation.

Hepatic IL6-receptor; Interleukin-6; Internalization; Down-regulation; Signal transduction

1. INTRODUCTION

Interleukin-6 (IL6) is a multifunctional cytokine synthesized by many different cells after appropriate stimulation. IL6 acts on a wide spectrum of target cells. It has been reported to be involved in: (i) the induction of immunoglobulin production in activated B-cells; (ii) the induction of proliferation of hybridoma/plasmacytoma/myeloma cells; (iii) the induction of interleukin-2 in T-cells, in cell growth and differentiation of T-cells to cytotoxic T-cells; (iv) the stimulation of multipotent colony formation in hematopoietic stem cells; and (v) the regulation of acute-phase proteins in liver (see reviews [1–5]).

IL6 exerts its action via cell-surface receptors composed of two subunits: an 80 kDa IL6-binding protein (gp 80) and a 130 kDa glycoprotein (gp 130) involved in signal transduction [6]. The cDNAs for both subunits have recently been cloned from natural killer cells and human placenta, respectively [7,8]. In liver, in hepatocytes in primary culture and in hepatoma cells, IL6 has been shown to be a major regulator of acute-phase protein synthesis [9–11].

As an approach to understand the interaction of IL6

with its target cells, we studied the binding, internalization and degradation of IL6 in HepG2 cells and in HepG2 cells overexpressing the 80 kDa IL6-receptor binding subunit. We demonstrate that IL6 down-regulates its surface receptor via endocytosis. The extent of internalization is determined by the number of 80 kDa IL6-receptor subunits.

2. MATERIALS AND METHODS

2.1. Chemicals

Carrier-free Na^{125}I (559 MBq/g) was obtained from Amersham International (Amersham, UK), iodobeads were from Pierce Chemical Co. (Rockford, IL, USA). Dulbecco's modified Eagle's medium was from Gibco (Eggenstein, Germany), fetal calf serum from Seromed (Berlin, Germany). Human recombinant interleukin-6 (5 × 10⁶ B-cell stimulatory factor-2 units/μg protein) was kindly provided by Drs. T. Hirano and T. Kishimoto (Osaka University, Japan).

2.2. Cell cultures

HepG2 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 61 mg/l penicillin and 100 mg/l streptomycin. HepG2-80kD cells were established after stable transfection of HepG2 cells with the coding region for the IL6R 80 kDa subunit as previously described [12]. To induce the expression of the IL6R 80 kDa subunit, HepG2-80kD cells were incubated with 100 μM ZnCl₂ for at least 8 h.

2.3. Iodination of rhIL6

RhIL6 was iodinated according to the procedure of Markwell [13] with modifications as previously described [14]. A specific radioactivity of 800 kBq/μg was obtained. The biological activity of the iodinated IL6 was essentially unchanged [14].

2.4. Binding studies

Cells were grown to confluency using 24-multi-well plates. After confluency was reached (5 × 10⁵ cells/well), cells were washed twice with ice-cold binding medium (Dulbecco's modified Eagle's medium without bicarbonate containing 0.2% (w/v) bovine serum albumin and 20 mM HEPES buffer, pH 7.0). Binding was initiated by the addition of [¹²⁵I]rhIL6 at different concentrations (1–2,048 pM) to duplicate wells. After incubation at 4°C for 2.5 h, medium was removed and cells were washed three times with phosphate-buffered saline containing 1 mM MgCl₂, 0.1 mM CaCl₂ and 0.2% bovine serum albumin. Cells were subsequently solubilized in 1 ml of 1 M NaOH and radio-activity was measured. Non-specific binding was determined by incubating the cells with [¹²⁵I]rhIL6 and a 100-fold excess of non-labelled ligand.

2.5. Internalization studies

In a total volume of 1 ml of binding medium, 2.5 × 10⁶ cells per 35 mm dish were preloaded with 1 nM of [¹²⁵I]rhIL6 at 4°C for 2 h. Internalization was initiated by warming up the cells to 37°C. At different times of incubation, cells were washed twice with binding medium. Surface-bound [¹²⁵I]rhIL6 was determined after subjecting the cells to 0.5 M NaCl/HCl, pH 1, for 3 min followed by an additional wash with phosphate-buffered saline containing 0.2% bovine serum albumin.

Internalized [¹²⁵I]rhIL6 was determined after lysis of the cells in 1 ml of 1 M NaOH. In some experiments the medium after exposure of the cells to [¹²⁵I]rhIL6 was analyzed for trichloroacetic acid-soluble and -precipitable radioactivity as described [15]. All measurements were performed in duplicate.

3. RESULTS

Liver and hepatoma cells are characterized by a low number of binding sites for IL6 [16,17]. Fig. 1 (upper panel) shows results of a binding study of [¹²⁵I]rhIL6 to human hepatoma cells (HepG2). From the Scatchard plot one type of high affinity binding sites ($K_d \approx 60$ pM) and about 2000 receptors per cell can be deduced. These data are in agreement with the findings of Sonne et al. [17]. In contrast, Baumann and coworkers [18] described two classes of IL6-binding sites on these cells: 5000 of low affinity ($K_{d2} = 500$ pM) and about 450 of high affinity ($K_{d1} = 15$ pM).

Since we were interested to study the role played by the 80 kD-IL6-receptor subunit (gp80) in binding and internalization, we stably transfected HepG2 cells with the respective cDNA under the transcriptional control of the mouse metallothionein I promoter (HepG2-80kD) [12]. These cells would allow us to experimentally manipulate the number of binding sites at the cell surface. Upon incubation of HepG2-80kD with 100 μM ZnCl₂ the gp80 mRNA was maximally induced after 4 h whereas maximal IL6-binding to the cell surface was observed after 6–8 h (data not shown).

Non-stimulated HepG2-80kD cells displayed essentially the same type of high-affinity binding sites as HepG2 cells (Fig. 1, middle panel). However, after stimulation with ZnCl₂, they exhibited additional low-affinity binding sites ($K_{d2} \approx 500$ pM, 12,000 receptors per cell), whereas the number of high affinity sites changed only slightly (Fig. 1, lower panel). We conclude from this that gp80 binds its ligand with low affinity, whereas
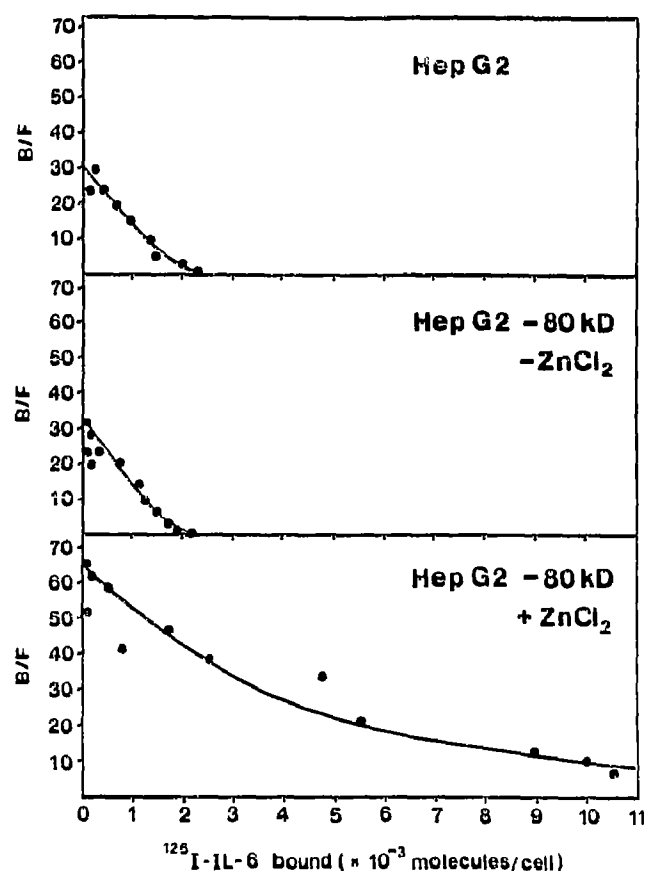

Fig. 1. Concentration dependence of binding of [¹²⁵I]rhIL6 to HepG2 cells and HepG2 cells overexpressing the 80 kDa IL6R subunit. Normal HepG2 cells (HepG2) and transfected HepG2 cells (HepG2-80kD) and transfected HepG2 cells treated with 0.1 mM ZnCl₂ (HepG2-80kD + ZnCl₂) were incubated with [¹²⁵I]rhIL6 at different concentrations at 4°C for 2.5 h. Specific binding was measured as detailed in Materials and Methods and the data were transformed using a Scatchard analysis. Data represent the results of at least 3 different experiments.

the high affinity binding is most likely due to complex formation of IL6, gp80 and the signal transducing gp130. Our finding complements the experiment performed by Hibi et al. [8] where the authors have shown that after transfection of human gp130-cDNA into Jurkat cells the number of high affinity binding sites had increased.

In order to learn more about the fate of IL6 and its receptor after their interaction, we performed internalization studies with HepG2 cells. Cells were preloaded with [¹²⁵I]rhIL6 at 4°C for 2 h. After a temperature shift to 37°C surface-bound and internalized [¹²⁵I]rhIL6 was determined at different times. [¹²⁵I]rhIL6 was present in the medium during the whole duration of the experiment. It can be seen in Fig. 2 (upper panel) that [¹²⁵I]rhIL6 was rapidly internalized (triangles). Concomitantly surface-binding sites for IL6 were lost (circles). We conclude from this observation that IL6 down-regulates its surface receptor.

Studies with HepG2 cells overexpressing gp80 revealed an increase in internalization of [^{125}I]rhIL6 proportional to the increased cell surface receptor numbers (Fig. 2, lower panel). Since the additional binding sites in HepG2-80 kD cells are mainly of low affinity type, we conclude that IL6-internalization can occur via gp80 alone.

We investigated whether the [^{125}I]rhIL6 taken up by these cells is degraded or recycled and secreted. We allowed the cells to internalize [^{125}I]rhIL6 for 1 h at 37°C, medium containing the radioactivity labelled ligand was replaced by normal binding medium and the cells were further incubated. Trichloroacetic acid-precipitable and trichloroacetic acid-soluble radioactivity in the culture medium was determined at different times. More than 80% of the [^{125}I]rhIL6 internalized within 1 h was degraded after 4 h.

In order to find out, whether the receptor is degraded along with its ligand or recycles to the plasma membrane, we incubated HepG2 cells with saturating amounts of unlabelled IL6 at 37°C for 2 h to downregulate the IL6-receptor. After removal of the ligand, cells were further incubated up to 24 h and the number of binding sites was determined with [^{125}I]rhIL6. It took >8 h to completely restore the IL6 binding capacity of the HepG2 cells (Fig. 3, circles). This very slow reappearance of IL6 binding sites is inhibited by cycloheximide (triangles) suggesting IL6-receptor de novo synthesis.

4. DISCUSSION

For several growth factors like insulin, epidermal growth factor or platelet-derived growth factor, it has been shown that, after binding, the respective receptors are internalized and down-regulated [19–23]. In the present study we have investigated the fate of the hepatic IL6-receptor and its ligand after their interaction. We show that in HepG2 cells and HepG2-80kD cells IL6 is rapidly internalized and that it concomitantly down-regulates its cell surface receptors with a $t_{1/2}$ of 15–30 min. Without IL6 gp80 in HepG2-80kD cells has a half-life of about 7 h (data not shown). The internalized ligand is degraded. There are two possible pathways for the internalized IL6-receptor. It can be degraded together with its ligand in the lysosomal compartment or it can be separated from its ligand and recycled to the plasma membrane. Separation of the receptor and the ligand in the endosomal compartment usually occurs at a pH around 5. Since it is not possible to dissociate [^{125}I]rhIL6 from its surface-bound receptor at pH ≈ 5 (unpublished data), it is unlikely that a separation of IL6 from its receptor occurs in the endosome. It is therefore more likely that the receptor is degraded together with its ligand. Our observation that the reappearance of IL6-binding sites after down-regulation requires >8 h and is cycloheximide sensitive, is in agree-
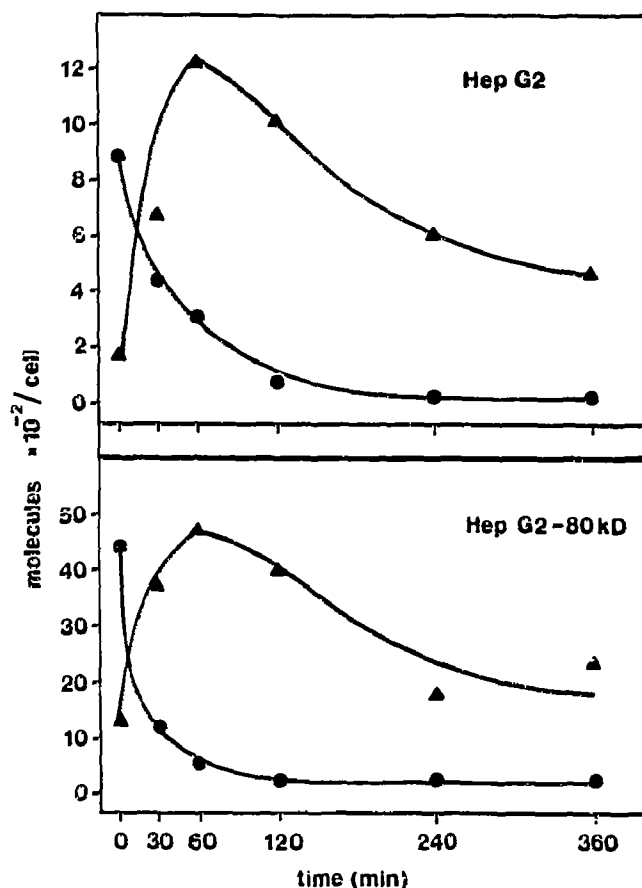

Fig. 2. Internalization of [^{125}I]rhIL6 by HepG2 and HepG2-80kD cells. 2.5×10^6 cells were preincubated with 1 nM [^{125}I]rhIL6 at 4°C for 2 h. Temperature was shifted to 37°C and surface-bound [^{125}I]rhIL6 (circles) and internalized [^{125}I]rhIL6 (triangles) was determined as described in Materials and Methods. The data represent the results of at least 2 separate experiments. Upper panel=normal HepG2 cells; lower panel=HepG2-80kD cells.

ment with this notion. Attempts to directly demonstrate the degradation of the 80 kDa IL6-receptor subunit failed, because we were unable to metabolically label this protein in parental hepatoma cells and also in cells overexpressing gp80.

In previous experiments with human hepatocytes in primary culture we could not detect internalization and degradation of [^{125}I]rhIL6 [24], possibly due to the insensitive methods used at this time. In a recent publication the internalization and degradation of IL6 was shown in primary rat hepatocytes with kinetics comparable with ours. However, these authors did not study the down-regulation of the IL6R by its ligand [25].

As mentioned before, the IL6R consists of the 80 kDa binding protein and in addition a 130 kDa glycoprotein involved in signal transduction. Therefore, the question arises whether gp130 is also internalized. We are currently exploring this question.

Three general functions for receptor-mediated endocytosis are known: (i) uptake of nutritional molecules
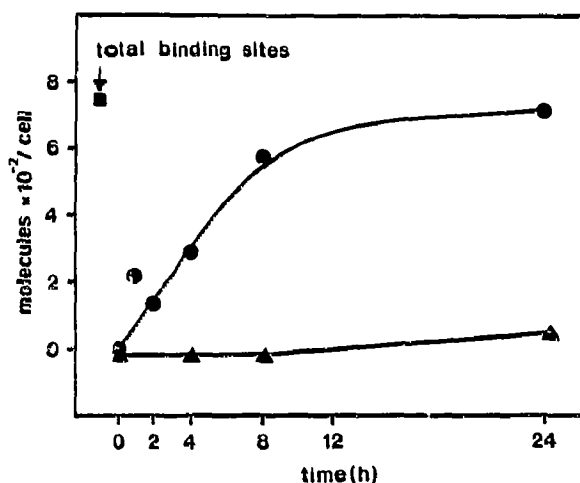
Fig. 3. Reappearance of IL6-binding sites at the cell surface after down-regulation of the IL6R in HepG2 cells. For the down-regulation of the IL6R, 2.5 × 10⁶ HepG2 cells were incubated with 1 nM of unlabelled rhIL6 at 37°C for 2 h. After removal of the ligand by 3 subsequent washes with binding medium, cells were further incubated at 37°C without (circles) or with 10 μg/ml of cycloheximide (triangles). At the times indicated in the figure, the number of binding sites per cell was determined as described in the legend to Fig. 1. The square represents the number of total binding sites before the start of the experiment. The data presented are the mean of 2 separate experiments performed in duplicate.

such as low density lipoprotein and transferrin; (ii) removal of injurious agents from the circulation such as galactose- or mannose-terminal glycoproteins; and (iii) internalization of signaling molecules such as hormones, growth factors and cytokines [26]. The receptors of the first two classes are usually recycled after endocytosis, whereas the signaling receptors in most cases do not get recycled. They are often degraded together with their ligands. We show here for the first time that this is also the case for the IL6R. The subsequent desensitization [26a] may be a protection of the cell against overstimulation. It is not known whether signal transduction and internalization are linked processes for cytokine receptors. For the interleukin-1 receptor and interleukin-2 receptor it was recently shown that receptor mutants incapable of signal transduction were still internalized [27,28]. On the other hand, it has been reported for the interleukin-2 receptor that impairment of internalization also inhibited signaling [29]. For the IL6R this will be the subject of future investigations.

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