IL-6 Receptor Independent Stimulation of Human gp130 by Viral IL-6

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The genome of human herpes virus 8, which is associated with Kaposi’s sarcoma, encodes proteins with similarities to cytokines and chemokines including a homologue of IL-6. Although the function of these viral proteins is unclear, they might have the potential to modulate the immune system. For viral IL-6 (vIL-6), it has been demonstrated that it stimulates IL-6-dependent cells, indicating that the IL-6R system is used. IL-6 binds to IL-6R, and the IL-6/IL-6R complex associates with gp130 which dimerizes and initiates intracellular signaling. Cells that only express gp130 but no IL-6R cannot be stimulated by IL-6 unless a soluble form of the IL-6R is present. This type of signaling has been shown for hematopoietic progenitor cells, endothelial cells, and smooth muscle cells. In this paper we show that purified recombinant vIL-6 binds to gp130 and stimulates primary human smooth muscle cells. IL-6R fails to bind vIL-6 and is not involved in its signaling. A Fc fusion protein of gp130 turned out to be a potent inhibitor of vIL-6. Our data demonstrate that vIL-6 is the first cytokine which directly binds and activates gp130. This property points to a possible role of this viral cytokine in the pathophysiology of human herpes virus 8. The Journal of Immunology, 2000, 164: 4672–4677.
Materials and Methods

Materials, Abs, and cell lines

The gp130 neutralizing Ab GPX7 was a kind gift from Dr. Yasukawa (Tosoh, Tokyo, Japan). BAF/3 cells stably transfected with human gp130 and IL-6R cDNAs have been previously described (26, 27). The preparation and characterization of the cell line HepG2-IL-6 has been described (18).

Expression and purification of recombinant vIL-6

vIL-6 DNA was amplified by PCR and inserted into the mammalian expression plasmid pBluescript II KS (Stratagene, La Jolla, CA). The DNA fragment was subcloned into the mammalian expression vector pCI-neo (Promega, Madison, WI). COS-7 cells were transfected using DEAE dextran. Supernatants were collected after 5 days, and purified at Ni-NTA agarose according to the manufactures instructions (Qiagen, Hilden, Germany). Column material was equilibrated with 50 mM phosphate buffer (pH 7.5), 500 mM NaCl, and 20 mM imidazole; after loading of culture supernatant the column was washed with equilibration buffer and eluted with 50 mM phosphate buffer (pH 7.5), 500 mM NaCl, and 100 mM imidazole. Eluted proteins were pooled and dialyzed against PBS at 4°C.

Purification of vIL-6 with Fc fusion proteins

COS-7 cells were transfected with the vIL-6 expression plasmid. Forty-eight hours after transfection cells were metabolically labeled for 8 h with 50 μCi/ml [35S]cysteine/methionine in cysteine/methionine-free medium (Life Technologies, Eggenstein, Germany). Supernatants were incubated for 2 h at 4°C with a rabbit antiserum raised against a bacterially expressed vIL-6 protein. Alternatively, supernatants were incubated with 2 μg/ml of human IL-6-Fc (28), human gp130-Fc, or a human IL-6R-Fc. The human IL-6R-Fc was constructed by fusing the cDNA coding for the extracellular portion of the human IL-6R to a CDNA coding for the human Fc portion of IgG1 (28) using the restriction sites Ssp I and Eco RI, respectively. The extracellular part of gp130 was fused to the Fc portion of human IgG1 (28) as a Novo-ECORI fragment. All Fc proteins were transiently expressed in COS-7 cells and purified at protein A-Sepharose (28). Immune complexes were precipitated with protein A-Sepharose, separated by SDS-PAGE, and visualized by fluorography.

BAF/3 cell proliferation assays

Proliferation of transfected BAF/3 cells was measured in 96-well microtiter plates. The cells were exposed to cytokines for 68 h and subsequently pulse-labeled with [3H]thymidine for 4 h. Proliferation rates were measured by harvesting the cells on glass filters, and the incorporated radioactivity was determined by scintillation counting. For each cytokine the proliferation assay was performed at least three times in triplicates. ABA Activity of the IL-6R system, the 35S-labeled vIL-6 protein was incubated with Fc fusion proteins containing IL-6, the extracellular portion of IL-6R, and the extracellular portion of gp130. Protein complexes were precipitated with protein A-Sepharose. As can be seen in Fig. 2A, neither IL-6-Fc nor IL-6R-Fc interacted with vIL-6. In contrast, gp130-Fc coprecipitated with the vIL-6 protein, indicating binding of vIL-6 to the extracellular portion of gp130. The specificity of the interaction of the Fc proteins with their cognate ligands is demonstrated in Fig. 2B. Human metabolically 35S-labeled IL-6 is precipitated with IL-6R-Fc as a broad band reflecting different glycosylation states of recombinantly expressed human IL-6 (31). No IL-6 was precipitated with IL-6-Fc and gp130-Fc (Fig. 2B, left panel). In contrast, a fusion protein of sIL-6R and IL-6 (HyperIL-6), which has been shown to directly stimulate IL-6R, was precipitated with protein A-Sepharose.

Analysis of phosphorylated STAT3 in hepatoma cells

HepG2-IL-6 cells were grown to confluency. Four hours before stimulation with cytokines, cells were serum starved. After 15 min of stimulation, cells were washed in PBS and lysed in Laemmli buffer. Proteins were separated by SDS-PAGE and visualized by silver staining.

Isolation and culture of human vascular SMC

SMC were obtained from pieces of human aortas obtained during aortic surgery (five male donors; mean age, 72 years) by courtesy of Dr. W. Schmiedt (Department of Heart and Thoracic Surgery, University of Mainz, Mainz, Germany). Isolated media fragments were prepared (29) and kept in medium containing 1 ng/ml human recombinant basic fibroblast growth factor-β, 5 ng/ml human recombinant epidermal growth factor, 25 mg/L gentamicin, and 1.25 mg/L amphotericin B at 37°C in 5% CO2 in a humidified atmosphere. The purity of SMC was evaluated by staining with trypan-blue exclusion.

RT-PCR of chemokine and cytokine mRNA and indirect immunofluorescence

Total cellular RNA was isolated from confluent SMC cultures and reverse transcription of 1 μg of total RNA was conducted followed by PCR which was performed as follows: initial denaturation for 5 min at 95°C, then denaturation for 40 s at 95°C, annealing for 1 min at 62°C, and extension for 3 min at 72°C. PCR primers for MCP-1, IL-6, gp130, and GAPDH were as described by Klouche et al. (24). PCR primers for the LIF-R have been described (30). The PCR products were run on 1% agarose gels in 1× TBE and stained with ethidium bromide. For immunocytochemical analysis, cells were fixed with 18.5% formaldehyde/12.5% glutaraldehyde for 2 h at room temperature and incubated with PE-labeled gp130-specific mAb (clone AM64, IgG1; PharMingen, San Diego, CA) overnight at 4°C followed by mounting and photography.

Results

To express vIL-6 in mammalian cells, the cDNA was cloned into an expression vector (28) which added a hexahistidine tag to the COOH terminus of the protein. The protein was purified to near homogeneity via Ni-affinity chromatography. As shown in Fig. 1, upon SDS-PAGE analysis of the purified protein a double band of 24–26 kDa is visible, which most likely reflects differentially glycosylated forms of vIL-6.

The metabolically 35S-labeled vIL-6 protein could be precipitated from the supernatant of transfected cells with a rabbit antiserum raised against a bacterially expressed vIL-6 protein (Fig. 2A). To test for physical interaction with receptor subunits of the IL-6R system, the 35S-labeled vIL-6 protein was incubated with Fc fusion proteins containing IL-6, the extracellular portion of IL-6R, and the extracellular portion of gp130. Protein complexes were precipitated with protein A-Sepharose. As can be seen in Fig. 2A, neither IL-6-Fc nor IL-6R-Fc interacted with vIL-6. In contrast, gp130-Fc coprecipitated with the vIL-6 protein, indicating binding of vIL-6 to the extracellular portion of gp130. The specificity of the interaction of the Fc proteins with their cognate ligands is demonstrated in Fig. 2B. Human metabolically 35S-labeled IL-6 is precipitated with IL-6R-Fc as a broad band reflecting different glycosylation states of recombinantly expressed human IL-6 (31). No IL-6 was precipitated with IL-6-Fc and gp130-Fc (Fig. 2B, left panel). In contrast, a fusion protein of sIL-6R and IL-6 (HyperIL-6), which has been shown to directly stimulate IL-6R, was precipitated with protein A-Sepharose.
Having shown a direct interaction of vIL-6 with gp130, we next asked whether the IL-6R was needed for biologic activity of vIL-6. We made use of the IL-3-dependent pre-B cells BAF/3 that do not express gp130 and are therefore not responsive to IL-6 or IL-6/sIL-6R. However, BAF/3 cells stably transfected with human gp130 cDNA or human gp130 and IL-6R cDNAs in the absence of IL-3 grow in response to IL-6/sIL-6R or IL-6, respectively (27, 28). BAF/3 cells transfected with gp130 cDNA (BAF-130 cells) grow in response to increasing amounts of HyperIL-6 (Fig. 3A). When the same cells were stimulated with IL-6, no growth of the cells was observed. However, cells stimulated with vIL-6 showed dose-dependent proliferation. In agreement with the lower efficiency of binding to gp130 (Fig. 2A), vIL-6 proved to have a 20- to 50-fold lower specific biologic activity than HyperIL-6 (Fig. 3A). BAF-130 cells stimulated with HyperIL-6 or vIL-6 led to a dose-dependent inhibition of cytokine-induced proliferation. In contrast, in BAF-130/IL-6R cells stimulated with human IL-6, gp130-Fc did not inhibit IL-6-induced proliferation (Fig. 4B). The lack of inhibition of human IL-6 may indicate that there is no free access of the gp130-Fc fusion protein to the membrane associated IL-6/IL-6R complex. Therefore, the gp130-Fc fusion protein specifically inhibits vIL-6 but not human IL-6 acting via the membrane-bound IL-6R.

Cytokine stimulation of gp130 leads to gp130 dimerization, subsequent phosphorylation of gp130, activation of Janus kinases and tyrosine phosphorylation of STAT3. We therefore asked whether cellular stimulation by vIL-6 was also mediated by gp130 activation. As shown in Fig. 4C, addition of a neutralizing mAb directed against gp130 completely blocked proliferation of BAF-130 cells induced by HyperIL-6 and by vIL-6. The same gp130 neutralizing mAb did not influence proliferation of untransfected BAF/3 cells induced by IL-3 (data not shown). These results indicate that both HyperIL-6 and vIL-6 acted via the membrane-bound IL-6R.

To demonstrate that stimulation of cells with vIL-6 led to intracellular phosphorylation and activation of STAT3 we employed HepG2-IL-6 cells. HepG2 cells upon stable transfection of a human IL-6 cDNA lost surface expression of IL-6R and therefore became completely unresponsive to IL-6. However, HepG2-IL-6 cells could be stimulated by the complex of IL-6/sIL-6R (18). As
shown in Fig. 5, in unstimulated and IL-6-stimulated HepG2-IL-6 cells, no phosphorylation of STAT3 was detectable. In contrast, incubation of cells in the presence of HyperIL-6 or vIL-6 resulted in phosphorylation of STAT3. This phosphorylation of STAT3 could completely be blocked by a neutralizing mAb directed against gp130. These experiments demonstrated that activation by vIL-6 was not mediated by the cell-bound IL-6R. Furthermore, our results suggest that vIL-6 and HyperIL-6 use the same cellular activation mechanism via gp130.

KS is characterized by new vessel formation and proliferation of spindle cells, “KS-cells,” which are associated with endothelial cells, fibroblasts, and inflammatory cells. Historically, proliferating spindle cells were considered to be derived from endothelial cells, but more recently, both types of mesenchymal cells, endothelial cells and SMC, have been proposed as potential progenitors. Immunohistochemical staining and Northern blot analysis revealed a coexpression of Ags specific for endothelial cells and SMC (32). In earlier studies we have observed that SMC could only be stimulated with IL-6 in the presence of sIL-6R (24). We therefore studied the stimulation of human primary SMC by vIL-6. In human SMC the mRNA expression of IL-6, of the monocyte-attracting chemokine MCP-1 and of gp130, are up-regulated in a time-dependent fashion upon stimulation by vIL-6 (Fig. 6). Both induction of MCP-1 as well as virally encoded chemokines may be
Cells surface expression of gp130 on human vascular SMC.**FIGURE 8.** The absence of sIL-6R had no effect on MCP-1, IL-6, and gp130 autocrine stimulation loop by vIL-6. Treatment with human IL-6 in gp130 preceded that of IL-6 (Fig. 6) indicating the induction of an proliferation of the cells (data not shown). Notably, expression of after treatment of the cells with vIL-6 (Fig. 8) and vIL-6-induced members LIF, CNTF, OSM, and CT-1.**FIGURE 7.** Induction of LIF-R mRNA expression in human vascular SMC by vIL-6. Human SMC were stimulated with 10 ng/ml HyperIL-6 or 500 ng/ml vIL-6, and the expression of LIF-R (A) mRNA was analyzed for 48 h. Expression of GAPDH (B) served as a control. M, m.w. marker; C, unstimulated control; 1–48, time of incubation (h).

involved in the characteristic attraction and presence of inflammatory cells in KS.

Besides gp130, the receptor subunit LIF-R is shared by cytokines of the IL-6 family such as LIF, CNTF, OSM, and CT-1 (15). We therefore asked whether the expression of LIF-R mRNA was also modulated by vIL-6 and HyperIL-6. As shown in Fig. 7, treatment of SMC with HyperIL-6 and vIL-6 led to an up-regulation of LIF-R mRNA as measured by the appearance of a 359-bp DNA band upon RT-PCR, indicating that stimulation of SMC by vIL-6 resulted in increased expression of the coreceptor for the IL-6 family members LIF, CNTF, OSM, and CT-1.

Furthermore, gp130 cell surface expression is strongly increased after treatment of the cells with vIL-6 (Fig. 8) and vIL-6-induced proliferation of the cells (data not shown). Notably, expression of gp130 preceded that of IL-6 (Fig. 6) indicating the induction of an autocrine stimulation loop by vIL-6. Treatment with human IL-6 in the absence of sIL-6R had no effect on MCP-1, IL-6, and gp130 expression and on proliferation (24). We conclude from these data that vIL-6, like the IL-6/sIL-6R complex (24), induces a proinflammatory state in human SMC.

**Discussion**

Our data show that vIL-6 directly interacts with gp130 and that the IL-6R is not needed for stimulation of target cells. Furthermore, we demonstrate that neutralization of gp130 abrogates vIL-6-induced STAT3 activation and cellular proliferation. These data are in contrast with recent data from Burger et al. (12), who showed that vIL-6-induced proliferation of IL-6 responsive human myeloma cells could be partially inhibited by an IL-6R antagonist that functions by occupying the IL-6R. The fact that in this study only partial inhibition of vIL-6 activity was found and that the bacterially expressed vIL-6 was about 40-fold less active than the material used in our study points to differences in the experimental systems used. Moreover, because correct folding of the bacterially expressed vIL-6 protein was not analyzed (12), it cannot be excluded that unfolded material affected the results and the estimation of specific activity.

Our results are in agreement with the studies by Molden et al. (13), who had shown that in gp130-expressing BAF/3 cells stimulation by vIL-6 led to the activation of STAT proteins. Moreover, because we showed that stimulation by vIL-6 led to IL-6R-independent proliferation, it can be assumed that in contrast to the situation with human IL-6, IL-6R is not required for the biologic activity of vIL-6. The fact that compared with IL-6/sIL-6R higher concentrations of vIL-6 are needed to stimulate cellular gp130 may reflect the lower affinity of vIL-6 to gp130. This is in agreement with the lower amount of vIL-6 precipitated with the gp130-Fc protein as compared with the vIL-6 antiserum (Fig. 2A). It is noteworthy that vIL-6 competes with IL-6/sIL-6R for gp130 interaction. These experiments suggest an interaction of vIL-6 and IL-6/sIL-6R at identical site(s) of gp130.

HHV8 has been associated with hematologic disorders like PEL, MCD, and MM (2–5, 7). Interestingly, a role for IL-6 has been demonstrated in these diseases (33). Moreover, it was shown that early hematopoietic progenitor cells require sIL-6R for their responsiveness to IL-6 (19, 21, 34). In a transgenic model, the presence of sIL-6R accelerated and increased the development of plasmacytomas (35). Because vIL-6 directly stimulates gp130, its mode of action can be compared with the IL-6/sIL-6R complex which does not require the IL-6R for activity. This view is strongly supported by the recent report of Aoki et al. (14) who showed that injection of vIL-6-expressing cells into mice induced strong extramedullary hematopoiesis, as had been seen as a consequence of the transgenic overexpression of IL-6 and sIL-6R (21). Interestingly, Aoki et al. (14) also observed increased angiogenesis in the animals harboring the vIL-6-expressing fibroblastic cells.

Taken together with our data of activation of human SMC by vIL-6, it may be speculated that secretion of vIL-6 by infected cells adds to the pathophysiology of HHV8-related diseases. In this respect it is of considerable interest that our gp130-Fc fusion protein led to complete inhibition of vIL-6 activity with no apparent effect on the biologic activity of IL-6.

The IL-6 family of cytokines comprises IL-6, IL-11, LIF, CNTF, CT-1, OSM, and NNT-1/BSF-3 (15, 36). While IL-6 and IL-11 act via specific receptors and together with these associate with a homodimer of gp130, LIF, CNTF, CT-1, OSM, and NNT-1/BSF-3 act via a heterodimer of gp130 and the related protein LIF-R. OSM can alternatively signal via a gp130 and OSM-R heterodimer (15, 36). Thus, there is no cytokine which stimulates
gp130 without the need for an additional protein. gp130 is expressed on all cells of the body, whereas the expression of LIF-R, OSM-R, IL-6R, IL-11R, and CNTF-R is limited (15). Therefore, the additional receptor component which, besides gp130, is needed for cellular activation determines the specificity of the biologic response. A protein such as vIL-6 could theoretically activate gp130 on all cells. Furthermore, stimulation of SMC by vIL-6 led not only to expression of IL-6 and MCP-1 but also to up-regulation of gp130 and LIF-R, which possibly increased the sensitivity of these cells to cytokines of the IL-6 family.

The demonstration that vIL-6 directly binds to and stimulates gp130 might have implications for HHV8 pathophysiology. vIL-6 has been shown to be expressed in KS, PEL, and MCD (2), and constitutive activation of STAT3 and resistance to apoptosis was shown to effectively promote development of plasmacytomas.

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