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Trans-signaling of interleukin-6 (IL-6) is mediated by the soluble IL-6 receptor, but not by soluble CD5

Samadhi Aparicio-Siegmund, Malte Deseke, Annett Lickert, Christoph Garbers*

Institute of Biochemistry, Kiel University, 24118 Kiel, Germany

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ABSTRACT

IL-6 exerts its pleiotropic activities on its target cells via the IL-6 alpha-receptor (IL-6R), which is expressed on a limited number of cell types. IL-6 can further signal via soluble forms of its receptor (sIL-6R), a process that has been termed trans-signaling. Recently, CD5 was described as an alternative alpha-receptor for IL-6 on B cells leading to the phosphorylation of the transcription factor STAT3 via the signal-transducing β-receptor gp130 in a Jak2-dependent manner. In this study, we sought to investigate whether IL-6 was also able to signal via soluble CD5 (sCD5) analogous to IL-6 trans-signaling. We show that IL-6 indeed binds to sCD5, but that this does not lead to the activation of signal transduction or cell proliferation. Furthermore, sCD5 did also not interfere with IL-6 classic signaling, suggesting that the affinity between the two proteins was too weak to provoke a biological effect. Thus, trans-signaling of IL-6 can only occur via sIL-6R, but not sCD5.

1. Introduction

Signaling via the ubiquitously expressed glycoprotein 130 (gp130) is a hallmark of all cytokines of the interleukin-6 (IL-6) family [1]. Specificity is gained through cell-dependent expression of other signaling and non-signaling receptors. Binding of IL-6 to its non-signaling IL-6 α-receptor (IL-6R), so-called classic signaling, leads to gp130 homodimerization and the activation of intracellular signaling cascades, including the Jak/STAT pathway [2]. Enhanced activities and increased serum levels of IL-6 are associated with inflammatory human diseases [3,4].

Most soluble cytokine receptors function as antagonistic decoy receptors, but the soluble IL-6R (sIL-6R) is a rare example of an agonist [5]. IL-6 binds to sIL-6R with the same affinity as to the membrane-bound IL-6R, and sIL-6R/IL-6 complexes can activate all cells of the human body via a gp130 homodimer. This mode of IL-6 signaling has been termed trans-signaling and represents an attractive therapeutic target [4,6]. The majority of the sIL-6R is generated by proteolytic cleavage of the membrane-bound precursor, and the two metalloproteases ADAM10 and ADAM17 have been described as efficient sheddases of the IL-6R [7–13]. Recently, a third modality of IL-6 signaling has been described, in which IL-6 couples to the IL-6R intracellularly and is then transported to the cell surface, where it stimulates gp130 on a neighboring cell. This pathway has been termed IL-6 cluster signaling/trans-presentation [14].

CD5 is a type I transmembrane protein that belongs to the scavenger receptor cysteine-rich (SRCR) superfamily [15,16]. It consists of three extracellular domains and was initially used as a cell surface marker for T cells; however, it is also expressed on B cells [16]. CD5 has an important role in T cell receptor signaling [17] and can be activated by a variety of different ligands, including zymosan, gp150, gp77-80, gp35-70 and CD5 itself [16,18]. Different forms of CD5 are generated by alternative processing of its mRNA [19], and naturally occurring soluble forms of CD5 (sCD5) appear to be generated by proteolytic cleavage of membrane-anchored CD5 [20]. Recently, IL-6 was described as another ligand for CD5 on human B cells, which leads to the activation of the transcription factor STAT3 via gp130 and Jak2, thereby promoting tumor growth [21].

In the present study, we analyzed a possible role for sCD5 in IL-6 trans-signaling. We show that although IL-6 is able to bind to sCD5, this interaction is much weaker compared to IL-6/sIL-6R interaction and therefore sCD5/IL-6 complexes are not able to provoke a biological response.

* Corresponding author. Institute of Biochemistry, Kiel University, Olshausenstrasse 40, 24118 Kiel, Germany.
E-mail address: cgarbers@biochem.uni-kiel.de (C. Garbers).

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2. Materials and Methods

Cells lines – Ba/F3-gp130 and Ba/F3-gp130-hIL-6R cells have been described previously [22]. HepG2 cells were obtained from DSMZ GmbH (Braunschweig, Germany). The cell lines were grown in DMEM high glucose culture medium (Gibco/Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum, penicillin (60 mg/l) and streptomycin (100 mg/l). Cells were cultured in a standard incubator with a water-saturated atmosphere at 37 °C and 5% CO_2. Ba/F3-gp130 cells were additionally supplemented with 10 ng/ml Hyper-IL-6, which is an IL-6/sIL-6R fusion protein. Ba/F3-gp130-hIL-6R cells were additionally supplemented with 10 ng/ml human IL-6 instead of Hyper-IL-6.

Antibodies and proteins – Monoclonal antibodies against signal transducer and activator of transcription-1 (STAT1), STAT3, pSTAT1 and pSTAT3 were purchased from New England Biolabs (Frankfurt am Main, Germany). The β-actin antibody was from Santa Cruz Biotechnology (Heidelberg, Germany). Secondary antibodies IRDye 800CW Goat anti-Mouse and IRDye 680RD Donkey anti-Rabbit were from LICOR Biosciences (Lincoln, NE, USA). Recombinant human soluble CD5 was from R&D Systems (Bio-Techne GmbH, Wiesbaden-Nordenstadt, Germany). Hyper-IL-6 was expressed and purified according to [23,24]. Expression and purification of human IL-6 and sIL-6R was described previously [22].

Stimulation and lysis of cells – For cytokine stimulation the individual Ba/F3 cell lines were washed twice in PBS and serum-starved for 2 h at 37 °C in DMEM. Subsequently the cells were counted and 2×10^6 Ba/F3-gp130 or Ba/F3-gp130-hIL-6R cells per well were seeded on a 12-well-plate in 400 μl DMEM. Indicated amounts of IL-6 and sCD5 for each well were mixed in 100 μl DMEM and after preincubation at 37 °C for 30 min were added to the cells for 15 min at 37 °C. The cell pellet was resuspended in 60 μl Laemmli buffer and boiled. 20 μl of the resulting cell lysates were loaded on a SDS-gel.

HepG2 cells were seeded for stimulation at a density of 1×10^6 cells per well in 6-well plates and after 16 h washed with PBS and serum-starved for 4 h at 37 °C in 900 μl DMEM. IL-6 and sCD5 were preincubated and stimulation was done as described for Ba/F3 cells. Cells were scraped on ice and the pellet was lysed in 100 μl lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM NaF, 1 mM Na3VO4, 1% IGEPAL [NP-40], 1% Triton X-100 and complete protease inhibitor cocktail tablets (Roche, Grenzach, Germany)).

Western blotting – The protein concentration of HepG2 lysates was determined using BCA protein assay kit (Thermo Fisher scientific, Waltham, MA, USA). 30 μg of total protein were separated on a 10% SDS gel and afterwards transferred onto a nitrocellulose membrane by semi-dry blotting. Membranes were blocked with Odyssey Blocking Buffer (LICOR Biosciences, Lincoln, NE, USA) washed in TBS and incubated overnight at 4 °C with antibodies against pSTAT1, STAT1, pSTAT3, STAT3 or β-actin diluted in TBS-T which contained 5% BSA. After three washing steps with TBS-T secondary antibodies IRDye 800CW Goat anti-Mouse and IRDye 680RD Donkey anti-Rabbit were diluted in TBS-T containing 5% BSA and incubated on the membranes for 1 h at room temperature. Following three washing steps fluorescence was detected on an Odyssey Fc Imaging System (LICOR Biosciences, Lincoln, NE, USA).

Proliferation assays – Ba/F3-gp130 cells were washed three times with sterile PBS. Afterwards, cells were resuspended in DMEM containing 10% FCS and 1% Penicillin/Streptomycin. After counting, 5×10^3 cells per well were cultured in a final volume of 100 μl in a 96 well plate with IL-6, sIL-6R and sCD5 as indicated. Cells were incubated for 48 h at 37 °C and cell viability was measured using the CellTiter-Blue Cell Viability Assay kit (Promega, Mannheim, Germany) following the manufacturer's instructions. Values were determined in triplicates per experiment, and relative light units (RLU) obtained after 60 min were normalized by subtraction of control values obtained after 0 min.

ELISA – 96 well ELISA plates were coated with either sIL-6R (2 μg/ml), sCD5 (2 μg/ml) or 5% BSA in PBS overnight. Wells were blocked for 1 h with 2% BSA/PBS at room temperature. After addition of IL-6 (0–2000 ng/ml) for 2 h, bound cytokine was determined with a biotinylated anti-IL-6 antibody (ImmunoTools, Friesoythe, Germany). Afterwards, streptavidin-coupled horseradish peroxidase (R&D Systems) was added, and the enzymatic reaction performed with BM blue POD (Roche Applied Science). Absorbance at 450 nm was determined on a Tecan infinite M200 PRO reader (Tecan, Maennedorf, Switzerland).

Presentations of experimental data – Data are expressed as mean values ± SD unless indicated otherwise. For Western blot experiments, one experiment from at least three with similar outcome is shown.

3. Results

3.1. IL-6 binds to sCD5

IL-6 binds with high affinity to the IL-6R. Recently, Zhang et al. identified CD5 as a second IL-6-binding receptor, which activates intracellular signaling on B cells in the absence of the IL-6R and constitutes a feed-forward loop to promote tumor growth [21]. In order to confirm this finding, we first coated ELISA plates with recombinant sIL-6R and added increasing amounts of IL-6 (0–2000 ng/ml) to the plates. We used an anti-IL-6 antibody to determine binding of IL-6 to its receptor in a concentration-dependent manner by this approach (Fig. 1A). In order to compare binding of IL-6 to sIL-6R and sCD5, we next coated ELISA plates with recombinant sCD5 and added the same amounts of IL-6 as before. Although IL-6 clearly bound to sCD5 in a concentration-dependent manner, we observed less and weaker binding compared to sIL-6R (Fig. 1B). Thus, we could reproduce binding of IL-6 to CD5 described before [21], but the affinity appeared to be significantly lower compared to IL-6/IL-6R binding.

3.2. IL-6/sCD5 does not induce trans-signaling on Ba/F3-gp130 cells

Having confirmed binding of sCD5/IL-6, we sought to investigate whether this complex would be able to activate cells via gp130 like sIL-6R/IL-6 complexes can do. Incubation of Ba/F3-gp130 cells with 10 ng/ml IL-6 and increasing amounts of sIL-6R (0–200 ng/ml) led to a dose-dependent proliferation of the cell line, as shown previously (Fig. 2A, [22,25]). When we incubated Ba/F3-gp130 cells with 10 ng/ml IL-6 and sCD5 (0–200 ng/ml), we did not observe any proliferation of the cell line, indicating that sCD5/IL-6 was not able to induce signaling (Fig. 2B). This was further confirmed by short-term treatment of Ba/F3-gp130 cells and subsequent analysis of phosphorylation of the downstream transcription factors STAT1 and STAT3. Whereas 10 ng/ml Hyper-IL-6, a designer cytokine where IL-6 is fused to the sIL-6R that mimics IL-6 trans-signaling, led to the phosphorylation of both STAT1 and STAT3, even very high amounts of sCD5 up to 1 μg/ml in combination with 100 ng/ml IL-6 did not induce any STAT phosphorylation (Fig. 2C, D).

3.3. sCD5 does not interfere with IL-6 classic signaling

As these first results indicate that sCD5 binds IL-6, but is unable to activate signal transduction in cells expressing gp130, we wanted to analyze whether sCD5 could act as an antagonistic decoy receptor. In IL-6 classic signaling, IL-6 binds to the membrane-bound IL-6R to initiate signaling, and the presence of sCD5 could thus

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