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Erythropoietin acts as an anti-inflammatory signal on murine mast cells

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ABSTRACT

Recently it was found that the erythropoietin receptor (EpoR) is expressed on innate immune cells, such as dendritic cells and macrophages. We found that murine bone marrow-derived mast cells express the EpoR and that its expression is increased under hypoxic conditions. Interestingly, Epo stimulation of the cells did not activate signal transducer and activator of transcription molecules, nor did we find differences in the expression of typical STAT-dependent genes, the proliferation rate, and the ability to differentiate or to protect the cells from apoptosis. Instead, we demonstrate that stimulation of mast cells with Epo leads to phosphorylation of the receptor tyrosine kinase c-kit. We hypothesize that this is due to the formation of a receptor complex between the EpoR and c-kit. The common beta chain of the IL-3 receptor family, which was described as part of the tissue protective receptor (TPR) on other non-erythroid cells, however is not activated. To investigate whether the EpoR/c-kit complex has tissue protective properties, cells were treated with the Toll-like receptor ligand LPS. Combined Epo and LPS treatment downregulated the inflammatory response of the cells as detected by a decrease in IL-6 and TNF-α secretion.

1. Introduction

The cytokine Epo is the key regulator of erythropoiesis (Jelkmann, 2013; Krantz, 1991). The major sites of production are the kidney in the adult and hepatocytes in the fetal organism. The main function of Epo is the differentiation of late determined and differentiated erythroid progenitor cells by inhibiting apoptosis and by activating pro-survival signaling pathways (Lin et al., 1996; Nairz et al., 2012; Wu et al., 1995b). Genetic knock-out studies corroborated the central role of Epo for erythropoiesis, as the knock-outs for the cytokine or its cognate receptor EpoR generate mice that die at day 12.5 p.c. due to severe anemia (Wu et al., 1995a). The EpoR is a 66–78 kDa protein that acts as a homodimeric complex and upon binding of Epo to its extracellular domain transduces the signal into intracellular signaling events. This results in the induction of genes needed to successfully sustain erythropoiesis such as anti-apoptotic genes of the Bcl2 family (Motoyama et al., 1999).

However, in the recent years, additional functions of Epo outside the erythroid compartment have become apparent. Initial reports described a neuroprotective effect of Epo (Byts and Siren, 2009; Masuda et al., 1993). In addition, other tissue-protective effects, especially in the context of the innate immune response have been described (Nairz et al., 2012). This tissue protective function again relies on the ability of Epo to induce the transcription of genes needed for the protection of damaged cells from apoptosis (Ogunshola and Bogdanova, 2013). Epo has a protective role in events such as ischemia, blunt trauma and toxic or inflammatory injuries that themselves were shown to be triggers for the production of Epo in these cells (Brines and Cerami, 2013). However, the amount of Epo needed to exert these effects was found to be much higher than those for erythropoiesis and Epo seemed to act in a paracrine/autocrine manner (Ogunshola and Bogdanova,

Abbreviations: BMMC, bone marrow-derived mast cells; Epo, erythropoietin; JAK, Janus kinase; LPS, lipopolysaccharide; SCF, stem cell factor; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; TNF, tumor necrosis factor; TPR, tissue protective receptor.

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2013). Interestingly, an alternative form of the EpoR, the so-called tissue-protective receptor (TPR), seems to be responsible for the observed effects. This receptor was found to have reduced affinity for Epo (Masuda et al., 1993). Epo mimetics have been described that do not induce erythropoiesis but are still able to exert a tissue-protective effect (Brines et al., 2004), suggesting that a different binding site is used. In an effort to identify the TPR, Epo was shown to induce phosphorylation of the common beta chain (β_c) (Chin et al., 1997; Hanazono et al., 1995), the signal-transducing receptor subunit used by the IL-3R subfamily of cytokines. Subsequently it was shown that the β_c subunit and the EpoR can interact to form the TPR on cells of the innate immune system (Blake et al., 2002; Brines et al., 2004; Hanazono et al., 1995; Jubinsky et al., 1997), as bone marrow cells obtained from β_c-chain knock-out mice were unable to confer tissue protection after treatment with Epo (Brines et al., 2004), although erythropoiesis was unaffected (Scott et al., 2000). In this context, the EpoR TPR is thought to limit an expansion of tissue damage after activation of the innate immune system. As inflammatory processes are accompanied by hypoxia, Epo production is triggered directly at the site of inflammation where it can act locally to restrict the damaging effects of the inflammatory reaction (Brines and Cerami, 2012).

In neutrophils for example, Epo negatively regulates the production of reactive oxygen species (ROS) that protect against invading microbes (Shurtz-Swirski et al., 2002). In primary macrophages, NO, TNF-α and IL-6 are reduced by Epo treatment of the cells (Nairz et al., 2011). This suggests that Epo might have beneficial effects as an anti-inflammatory substance for the treatment of arthritis, colitis and encephalomyelitis (Nairz et al., 2012). However, others also describe a more activated phenotype, which is mostly connected to dendritic cells (Lifshitz et al., 2009, 2010; Prutchi Sagiv et al., 2008; Rocchetta et al., 2011).

Another receptor that is known to play a role in EpoR signaling is the type III receptor tyrosine kinase c-kit. In contrast to its ubiquitously expressed ligand stem cell factor (SCF), the expression of the receptor is restricted to hematopoietic progenitor cells, as well as differentiated mast cells, DCs and NK cells (Ray et al., 2010).

A number of studies show that c-kit is involved in the regulation of erythropoiesis (Munugalavadla and Kapur, 2005) and mutations of c-kit can result in decreased erythropoiesis, suggesting that there is a strong crosstalk between the two receptors (Wu et al., 1995a). Indeed, in the myeloid cell line HCD-57 SCF is able to induce phosphorylation of the EpoR through a direct interaction of c-kit with the cytoplasmic domain of the EpoR and SCF was able to replace Epo in the proliferation and survival of these cells (Wu et al., 1995a). However, others detected an increase in the number of apoptotic cells indicating that the signaling events downstream of SCF-induced EpoR activation are distinct from the Epo-induced signaling (Jacobs-Helber et al., 1997). While some studies did not find that Epo can activate c-kit (Wu et al., 1995a), others found this to be possible (Broudy et al., 1998), however, no physiological function has been assigned to this mode of activation yet.

The objective of the present study was to investigate the function of the EpoR on mast cells and our data show that EpoR signaling leads to anti-inflammatory events presumably via crosstalk with the receptor tyrosine kinase c-kit that can be activated by Epo in these cells.

2. Materials and methods

2.1. Reagents

Erypo FS® Epoetin alfa was purchased from Janssen–Cilag GmbH (Neuss, Germany) and LPS from *Salmonella abortus equi* was purchased from Sigma–Aldrich (Taufkirchen, Germany). Recombinant murine IL-3 and SCF were obtained from Immunotools (Friesoythe, Germany). Anti-pTyr as well as antibodies against β-actin, (p)c-Kit, (p)STAT3 and pSTAT5 as well as HRP-conjugated secondary antibodies were from New England Biolabs GmbH (Danvers, USA). Antibodies against IL-3β and STAT5 (C-17) were obtained from Santa Cruz Biotechnology (Dallas, USA).

2.2. Mice

For all experiments, 5- to 25-week-old female mice with a C57BL/6 background were used. Mice were maintained under specific pathogen-free conditions in accordance with the German policies on animal welfare.

2.3. Cell culture

Mice were dispatched by CO_2. Bone marrow from femoral and humeral bones was washed out. For the generation of mast cells (BMMCs), bone marrow cells were cultivated for 4 weeks in RPMI 1640 medium containing 20% FCS, 50 μM β-mercaptoethanol, 1% penicillin–streptomycin and 2% IL-3 containing supernatants, generated by the cell line X63Ag8-653 (Karasuyama and Melchers, 1988). Mast cells were isolated from the peritoneal cavity and cultured for 3 weeks in medium with 2% IL-3 containing supernatants and 10 ng/ml SCF. For generation of macrophages, bone marrow cells were seeded in 15 cm petri dishes in DMEM supplemented with 30% L929 supernatant, 10% FCS, and 1% penicillin-streptomycin for 8 days. Differentiated bone marrow-derived dendritic cells were obtained by cultivation in RPMI1640 medium containing supernatants of an X63 cell line producing murine GM-CSF for 8 days as described previously (Lutz et al., 1999).

2.4. Quantitative real-time PCR

RNA was extracted with the "High Pure RNA Isolation Kit" (Roche, Mannheim, Germany) following the manufacturer's protocol. cDNA was prepared using the "RevertAid First Strand cDNA Synthesis Kit" (Fermentas, St. Leon-Rot, Germany). Aliquots of the cDNA were used for quantitative real-time PCR analysis with the "SYBR Green Rox mix" (Thermo Scientific, Langenselbold, Germany) and the primers listed in Table 1. The results were analyzed using the Fast Real-time PCR System (AB Applied Biosystems, Darmstadt, Germany) and are presented as relative expression compared to *GAPDH* or *S29*.

2.5. Western blot analysis

For the preparation of cell lysates, cells were washed three times in PBS and starved overnight in RPMI 1640 with 0.1% BSA (Album solution from bovine serum, Fraction V, Sigma). Cells (5×10^6 cells per tube) were preincubated at 37 °C for 10 min prior to stimulation with Epo (50 U/ml), IL-3 or SCF (50 ng/ml) for 15 min. NP-40 lysis buffer (10% glycerol, 50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 1% NP-40, 2 mM $MgCl_2$), supplemented with protease- and phosphatase-inhibitors, was used to lyse cells. For immunoprecipitations of cell lysates 25 μl agarose beads (Protein A/G PLUS-Agarose, Santa Cruz Biotechnology) and 5 μl antibody were added to the lysates and incubated over night at 4 °C on a rocking platform. Beads were washed twice with NP-40 and once with TNE-buffer (10 mM Tris pH 8, 100 mM NaCl, 1 mM EDTA). Cell lysates or precipitated proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Whatman GmbH, Dassel, Germany). Membranes were incubated overnight at 4 °C with the primary antibody and the secondary, HRP-labeled antibody was added for 1 h at room temperature. After washing, proteins were