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Erythropoietin administration suppresses human monocyte function *in vitro* and during therapy-induced anemia in HCV patients

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ABSTRACT

Erythropoietin (EPO) is a hormone that controls red blood cell production. Binding of EPO to the EPO-receptor results in increased numbers of red blood cells in the circulation, which makes EPO a potent molecule to treat anemia in various groups of patients. Although numerous studies have examined the clinical effects of EPO, its immunological effects have received less attention.

In this study, we examined the immunological effects of EPO on human monocytes. We show that human monocytes express EPO receptor mRNA, and are responsive to EPO in cell culture. *In vitro* exposure of PBMC from individuals to EPO and the TLR4 ligand LPS showed a significant reduction of monocytes producing IL-6 and TNF, while the frequencies of IL-12p40, IL-10, MIP-1β and IL-8-producing cells did not change upon incubation with EPO. In addition, EPO did increase the phagocytic activity but did not affect the ability to produce ROS by monocytes. Moreover, we studied eight chronic HCV patients undergoing treatment with peg-IFN and ribavirin, who were administered EPO for treatment-induced anemia. Blood was collected before and 7 days after EPO injection. In 7 patients, we observed a significant decline at day 7 after EPO administration of the frequency of monocytes producing various pro-inflammatory cytokines following stimulation with the TLR4 ligand LPS and the TLR7/8 ligand R848, which is in line with our *in vitro* findings. Our findings demonstrate an inhibitory effect of EPO on the secretion of effector molecules by monocytes and a stimulatory effect on the phagocytic activity by monocytes.

1. Introduction

Erythropoietin (EPO) is a renally secreted hormone that promotes red blood cell production in bone marrow by binding to the EPO-receptor (EPO-R). This interaction results in an increased number of red blood cells in the circulation, which makes EPO a potent molecule to treat anemia in various groups of patients (Alavian et al., 2012). Although numerous studies have examined the clinical effects of EPO, its immunological effects have received less attention. Immune cells have been shown to bear the EPO-R, making them probable targets (Brines and Cerami, 2005; Jelkmann, 2007). Indeed, in polyclonally stimulated whole blood cell cultures from hemodialysis patients, EPO increased IL-2, IL-10 and IL-12 production, while IL-6 and TNF production was reduced (Bryl et al., 1998, 1999; Trzonkowski et al., 2002). Also granulocytes and neutrophils have been shown to be activated by EPO (Costa et al., 2008) and incubation of B cells with EPO led to increased IgM production (Kimata et al., 1991). Recently, it was shown that administration of EPO to mice reduced the production of IL-6 and TNF, as well as nitric oxide. Furthermore, systemic bacterial infection and impaired pathogen clearance was observed in these mice, which resulted in reduced survival (Nairz et al., 2011).

Eighty percent of patients infected with the hepatitis C virus (HCV) are unable to resolve the infection by their own immune system. It has been shown that natural killer (NK) cells as well as dendritic cells (DCs) are functionally impaired in chronic HCV patients compared to healthy individuals (Jinushi et al., 2004; Oliviero et al., 2009; Woltman et al., 2010). Besides innate immunity, adaptive immunity is affected as well. The continuous presence of high levels of viral antigens leads to a weaker effector function of HCV-specific T cells, which is a characteristic feature of immunity in chronic HCV patients (Shoukry et al., 2003; Spaan et al., 2012). Antiviral therapy consisting of pegylated interferon-alpha

Abbreviations: EPO, erythropoietin; EPO-R, EPO receptor; HCV, hepatitis C virus; NK, natural killer; DC, dendritic cell; PEG-IFN, pegylated interferon-alpha; Hb, hemoglobin; PBMCs, peripheral blood mononuclear cells; ROS, reactive oxidative species; PHA, phytohemagglutimin.

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(peg-IFN) and ribavirin has been the standard of care for chronic HCV patients for many years, with the recent addition of protease inhibitors to this treatment further improving the efficacy (Pawlotsky, 2011b). A major side effect of treatment with ribavirin and peg-IFN is anemia, which is even more pronounced by the addition of protease inhibitors (Pawlotsky, 2011a). Normalization of hemoglobin (Hb) levels can be achieved by ribavirin dose reductions, but this may lower the treatment efficacy. As an alternative to manage anemia, EPO can be administered to stimulate the generation of erythrocytes (Dieterich et al., 2003; Gergely et al., 2002; Pockros et al., 2004; Shiffman et al., 2007), while reducing the necessity of ribavirin dose adjustments, which may benefit the efficacy of therapy (Shiffman et al., 2007; Stickel et al., 2012). However, recent data showed no beneficial effect of EPO compared to ribavirin dose reductions on sustained viral response rates in HCV patients treated with boceprevir, peg-IFN and ribavirin (Lawitz et al., 2012; Poordad et al., 2012). To get more insight into the immunological effects of EPO in humans, we defined which human leukocyte subpopulations are potential targets for EPO, and explored the functional effects of EPO on these cells. We observed that EPO affected monocytes *in vitro*, which was in line with detectable EPO-R mRNA expression by monocytes. Moreover, similar to the *in vitro* effects, administration of EPO during antiviral therapy of chronic HCV patients resulted in reduced frequencies of monocytes producing cytokines.

2. Material and methods

2.1. Patients

Patients were treated at the Erasmus MC according to a study protocol and were seen at our outpatient clinic (EudraCT 2007-005344-25). Patients were treatment-naive, infected with HCV genotype 1 and were treated for 12–48 weeks with peg-IFN (Pegasys, 180 μg once weekly, Roche) and ribavirin (Copegus, 1200–2400 mg daily, Roche). Hb levels were monitored throughout therapy. Per protocol, at Hb levels below 6.8 mmol/l, EPO (NeoRecormon, Roche) was administered at a dose of 30,000 IU once weekly. When Hb levels increased above 7.5 mmol/l, EPO administration was discontinued. Eight patients were treated with EPO, heparinized blood was collected during antiviral therapy before administration of EPO and 7 days after the first injection of EPO. Furthermore, we included seven healthy volunteers outside the study protocol who were used as control(s). The institutional review board of the Erasmus MC approved the protocols, and informed consent was obtained from all individuals.

2.2. Cell culture

Peripheral blood mononuclear cells (PBMC) were isolated from venous blood by ficoll separation (Ficoll-Paque™ plus, Amersham). For all *in vitro* experiments, PBMC were suspended in serum-free X-VIVO15 medium (BioWhittaker) supplemented with L-glutamin (Cambrex), Pen-Strep (Invitrogen/Gibco) and HEPES (Cambrex) and used for the various assays.

2.3. EPO-R mRNA expression

For determination of the expression of EPO-R mRNA, PBMC from healthy volunteers were separated by cell sorting into $CD19^+$ B cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $CD3^-CD56^+$ NK cells, $CD14^+$ monocytes and granulocytes (FACS Aria SORP, BD). Granulocytes were sorted from full blood on the basis of the FSC-SSC profile. Cells were stored in RNAlater. Total RNA was extracted using the RNeasy kit (Qiagen) and cDNA was prepared using the iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR reactions were performed using a MyIQ5 detection system (Bio-Rad). Primers-probes for GAPDH (Hs00959427_m1) and EPO-R (Hs00959427) were obtained from Applied Biosystems. The expression of target genes was normalized to GAPDH using the formula: $2^{-\Delta Ct}$, $\Delta Ct = Ct_{EPOr} - Ct_{GAPDH}$.

2.4. ROS production and phagocytosis by monocytes

PBMC were rested for 1 h at room temperature, and DHR123 (0.1 μg/ml; Sigma) was added for 10 min, followed by pretreatment for 15 min with EPO (125 IU/ml, NeoRecormon). Next, cells were primed with fMLP (1 mM; F3506, Sigma) and incubated at 37 °C for 0, 5, 15 and 30 min. ROS was detected by flow cytometry (FACS Calibur 4, BD). EPO-pretreated PBMC were also used for the detection of phagocytosis. *Escherichia coli* FITC (2 μg/ml; Invitrogen) was added and incubated at 37 °C for 15 min. Cells were washed with trypan blue to remove unbound *E. coli* FITC. Phagocytosis was measured by flowcytometry (FACS Calibur 4, BD).

2.5. Expression of intracellular and cell surface molecules by flow cytometry

The frequencies of cytokine producing $CD14^+$ monocytes were determined by flow cytometry (Liu et al., 2011; Peng et al., 2011). PBMC from healthy individuals were first pretreated with or without EPO variant alpha (125 IU/ml, EPREX) or EPO (125 IU/ml, NeoRecormon) for 30 min. PBMC from HCV patients on therapy were not pretreated *in vitro* with EPO. For the expression of activation markers, cells were stained with CD80-FITC (MAB104, Beckman) CD86-APC (IT2.2, Biolegend) and HLA-DR-FITC (L243, BD Bioscience). For determining cytokine expression, cells were stimulated overnight with an optimal concentration of LPS (0.8 ng/ml, InvivoGen) or R848 (1 μg/ml, Alexis). Brefeldin A (10 μg/ml; Sigma) was added 2 h after the addition of TLR agonists. Samples were fixed, permeabilized and stained with MIP-1β-PE (D21-1351, BD Pharmingen), IL-6-FITC (MQ2-13A5, BD Pharmingen), TNF-PE-Cy7 (MAb11, eBioscience), MCP-1-APC (5D3-F7, eBioscience), IL-8-FITC (6217, R&D), IL-12p40-PE (C11.5, BD Pharmingen), IL-10-APC (JES3-19F7, Biolegend) and CD14-eFluor450 (61D3, eBioscience). Cytokine producing cells were detected by flowcytometry (Canto-II, BD).

2.6. Statistics

The Wilcoxon signed-rank test was used for paired non-parametric analyses. The level of significance for all tests was $P \leqslant 0.05$.

3. Results

3.1. The EPO-R is expressed by human monocytes

To determine if human monocytes are responsive to EPO, we first assessed the expression levels of EPO-R mRNA. As shown in Fig. 1, using highly purified monocytes, EPO-R mRNA was detected by real-time PCR. The EPO-R mRNA expression levels in monocytes were lower than observed in B cells and granulocytes, but higher than by other lymphocyte subtypes, like T cells and NK cells. These findings demonstrate that monocytes, next to B cells and granulocytes, are putative targets for EPO.

3.2. EPO increases phagocytosis ability of monocytes in vitro

To study the influence of EPO on monocyte function, we first investigated the ability of EPO-treated monocytes to perform phagocytosis of *E. coli* and to produce reactive oxidative species