Granulocyte colony-stimulating factor (G-CSF) reduces not only gram-negative but also gram-positive infection-associated pro-inflammatory cytokine release by interaction between Kupffer cells and leukocytes

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Abstract. Objective and Design: An important principle for the beneficial effects of granulocyte colony-stimulating factor (G-CSF), a central mediator in the endogenous host response, is the reduction of systemic cytokine levels in various gram-negative models of sepsis and septic shock. There is debate, however, on whether G-CSF is protective also in gram-positive sepsis and acts directly or indirectly on macrophages and hepatic Kupffer cells (KC).

Methods: KC were harvested from either G-CSF-(200 µg/kg bw iv) or saline-pretreated Sprague-Dawley rats and stimulated in vitro for subsequent assessment of cytokine release over 24 h.

Results: Pretreatment with G-CSF led to a significant (p < 0.05) inhibition of lipopolysaccharide (LPS)-induced release of TNF-α (−81%), IL-6 (−82%) and IL-1β (−57%). Exposure of KC to heat-killed Staphylococcus aureus (S. aureus/SAC) caused a 2- to 3-fold higher TNF-α release, but similar IL-6 levels when compared with those after LPS stimulation. Still, G-CSF proved to significantly reduce the release of both TNF-α and IL-6 upon KC exposure with SAC for 24 h. Interestingly, in neutropenic animals (100 mg/kg cyclophosphamide), G-CSF was not capable to blunt the LPS-induced cytokine release, indicating that the action of G-CSF on KC is not direct in nature but targets cellular communication and function of neutrophils.

Conclusion: The present results demonstrate that pretreatment with G-CSF in vivo effectively prevents the overactivation of KC by both gram-negative and gram-positive bacterial substances, probably via modulation of neutrophil function. Thus, inhibition of proinflammatory cytokine response through G-CSF may represent a promising hepatoprotective approach during systemic inflammation.

Key words: Sepsis – Bioassay – TNF-α – Interleukin-1 – Interleukin-6 – Leukocytes – Neutropenia – Cyclophosphamide

Introduction

Despite enormous progress in antibiotic therapy and cardiovascular and pulmonary support, sepsis-related complications continue to represent a significant cause of mortality among patients in medical and surgical intensive care units [1]. The complex mechanisms of host response upon invading pathogens include the production and release of proinflammatory and immunomodulating cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) [2]. Moderate synthesis of proinflammatory cytokines seems to be necessary for an effective defense against infections and essential for survival. Thus, clinical studies simply encompassing anti-cytokine strategies failed to significantly improve outcome of septic patients [3]. However, an inappropriately triggered inflammatory response with excessive production and release of macrophage-derived proinflammatory cytokines can itself also lead to multiple organ dysfunction syndrome and patients’ death.

G-CSF is an endogenous glycosylated polypeptide best known for its important role in hemopoiesis, primarily of the neutrophil lineage [4]. G-CSF stimulates proliferation, differentiation, and maturation of neutrophil progenitors in the bone marrow as well as antibacterial function of neutrophils such as adhesion, migration, phagocytosis, superoxide anion production and antibody-dependent cell-mediated cytotoxicity [4, 5]. Despite some concern that G-CSF-associated neutrophil hyperactivity might aggravate organ dysfunction, G-CSF alone seems not to potentiate inflammatory lesions [6]. Meanwhile, a variety of animal studies have reported that...
G-CSF treatment is effective to ameliorate the outcome in different models of gram-negative infection [7–12]. Moreover, human trials indicate the anti-inflammatory action and the supportive role of G-CSF in the immune response to infection in the non-neutropenic surgical intensive care patient [13, 14].

An important, although most likely not the only mechanism of the beneficial effect of G-CSF in inflammatory disease states is the attenuation of LPS-inducible TNF-α concentrations, as shown both in vivo and in vitro [12, 15]. There is also evidence that G-CSF affects other proinflammatory cytokines, such as IL-1β and IL-6 [14, 16]. However, it is not known as to whether G-CSF-associated dampening of KC-dependent cytokine release is restricted to LPS and gram-negative stimulation. Moreover, little is known as to whether G-CSF acts directly on KC or whether its beneficial effect involves leukocyte function and leukocyte-KC communication.

Therefore, we herein studied the effect of G-CSF on production and release of different proinflammatory cytokines by KC upon stimulation with LPS but also with staphylococcus aureus (Cowan I strain) (SAC) as gram-positive antigen. In an additional series of experiments, neutropenic animals were used to evaluate as to whether the effect of G-CSF on KC is mediated by leukocytes. The approach chosen in this study combined the administration of G-CSF in vivo, the isolation of KC and their subsequent stimulation in vitro for evaluation of cytokine release.

Materials and methods

KC isolation

The experiments were conducted in accordance with the German legislation on protection of animals and the NIH Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council). After overnight fasting with free access to tap water adult male Sprague Dawley rats (body weight bw) 400–500 g purchased from Charles River Laboratories (Sulzfeld, Germany) were lightly anesthetized with ether and injected with either rG-CSF (Filgrastim, Neupogen; Amgen, Thousand Oaks, CA, USA; 200 µg/kg bw in 0.9% saline iv (n = 6) or identical volumes of 0.9% saline iv (n = 6). After 2 h, KC isolation was performed based on the method of Kawada et al. [17]. Briefly, after anesthesia with pentobarbital sodium (50 mg/kg bw ip) and iv heparinization (1 U/g bw), a blood sample was withdrawn (1 ml) and livers were perfused for 10 min in situ with oxygenated 37°C warm Krebs-Henseleit buffer (free of Ca2+ and Mg2+) via the portal vein at a flow rate of 12 ml/min, followed by 20 min ex situ 0.05% collagenase digestion (collagenase hepatoocyte qualified, biological activity 214 U/mg, prepared from Clostridium histolyticum; Gibco, Grand Island, NY, USA) at 37°C in a Ca2+-containing oxygenated recirculating system. Thereafter, the organ capsule was incised and the cells were gently liberated by careful raking with forceps. The cells were suspended in Click’s medium and incubated with 0.1% pronase (from Streptomyces griseus; Roche, Mannheim, Germany) and 0.003% DNAse I (from bovine pancreas, grade II; Roche) at 37°C and pH 7.3 for 30 min. The cell suspension obtained after this incubation step was filtered through a sterile nylon mesh (150 µm) and subsequently kept constantly at 4°C. After pelleting the cells for removal of dead cells, debris, and erythrocytes for 10 min at 450 × g and resuspension, a two layer Nycodenz (Nycomed, Oslo, Norway) gradient (11.5% and 17.5%) was used to further separate the non-parenchymal cell (NPC) fraction. After centrifugation at 1400 × g for 20 min, the hepatic stellate (Ito-) cell fraction was recovered from the top of the 11.5% layer and a combined fraction of KC and endothelial cells at the interface of the two Nycodenz layers. After resuspension of the obtained KC and endothelial cell fraction in an excess amount of cold phosphate-buffered saline (PBS; Seromed, Berlin, Germany), cells were pelleted at 450 × g for 10 min to remove Nycodenz. Viability of NPC, as determined with trypan blue exclusion, was >90%. 2 × 105 NPC/well were seeded into 24-well cell culture clusters (Costar, Cambridge, Massachusetts) and incubated for 4 h at 37°C in a 5% CO2-atmosphere. The nonadherent and dead cells were removed by vigorous washing with PBS, whereas KC adhered to the plastic surface.

KC cultures

KC cultures were incubated in Click’s medium with 10% FCS at 37°C in a 5% CO2-atmosphere with and without LPS (E. coli serotype 0128:B12, 1 µg/ml) (N = 14–19). Cell culture supernatants were harvested after 2, 4, 6, 8, 16 and 24 h of incubation, filtered through 0.2 µm sterile filters (Sartorius, Göttingen, Germany) and stored at –70°C until assayed for cytokines. Secondly, KC cultures were prepared as described above and incubated with and without SAC (10 µl 7.5% Staphylococcus aureus Cowan I strain 1 solution; Pansorbin Cells, Calbiochem, San Diego, CA, USA) (N = 7–12). Cell culture supernatants were harvested after 8 h and 24 h of incubation, filtered through 0.2 µm sterile filters and stored at –70°C until assayed for cytokines.

Cytokine assays

In KC supernatants, TNF-α was determined by its cytotoxic effect on the murine fibrosarcoma cell line WEHI 164 subclone 13 (kindly provided by Dr. S. Kunkel, Ann Arbor, Michigan), as previously described by our group [18]. In brief, 1 × 105 WEHI cells/well, incubated with serial dilutions of serum or KC supernatants, were grown for 24 h in 96-well microtiter plates. For the last 4 h of incubation, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (5 mg/mL, Sigma Chemical Co.) was added. Cells were lysed by addition of 10% sodium dodecylsulfate (Sigma). The amount of dark blue formazan crystals produced during this time was measured spectrophotometrically.

IL-6 was measured by the amount of proliferation of the murine hybridoma cell line 7TD1 (kindly provided by Dr. Van Snick, Brussels, Belgium) that only grows in the presence of IL-6. The proliferation of 7TD1, stimulated with serum or KC supernatants, was determined after an incubation period of 72 h, as described above, using the MTT assay. 7TD1 cells have been shown to react in a highly specific fashion to IL-6. Cytokine activity was determined by comparison with dilutions of recombinant human (rh) TNF-α standard (Genzyme, Boston, MA) and rhIL-6 standard (Roche, Mannheim, Germany). Concentrations of IL-1β were measured using a specific immunoenzymatic assay (EASIA; Medgenix/Biosource, Ratingen, Germany) according to the manufacturer’s instructions. The minimal detectable concentrations for IL-1 were 2 pg/ml.

Neutropenic animals

Animals received 100 mg/kg cyclophosphamide (Asta Medica AG, Frankfurt, Germany) in 0.3 ml 0.9% sodium chloride solution iv. When leukocyte counts were below 100 µl after 5 days, 200 µg/kg G-CSF was applied and KC were isolated 2 h later.

Statistical analysis

Data are reported as mean ± SEM. After approving the assumption of normality and equal variance across groups, differences were assessed using ANOVA followed by the Student Newman Keuls test. Values were considered significant if p < 0.05. Statistics were performed using the software package SigmaStat (Jandel Corporation, San Rafael, CA, USA).