Abstract. Objective and design: Non-anticoagulant biological activities, such as anti-inflammatory and anti-apoptotic mechanisms of action, have been suggested for recombinant human activated protein C (rhAPC; drotrecogin alfa (activated)). However, these mechanisms are much less characterized and understood than rhAPC’s anticoagulant activity. Aim of the study was to determine the effect of rhAPC on the activity of the pro-inflammatory transcription factor nuclear factor kappa B (NF-kB) in mononuclear cells isolated from septic patients and to characterize an effect downstream from NF-kB activation, such as the release of the NF-kB-controlled chemokine Macrophage Inflammatory Protein-1-alpha (MIP-1-α).

Subjects: Peripheral blood was obtained from 13 septic patients and from 8 healthy controls.

Methods: Mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation and were incubated with or without rhAPC (10 μg/ml) for 2 h for the measurement of NF-kB activity in cell lysates or alternatively for 6 h for the determination of MIP-1-α levels in supernatants. NF-kB activity was measured by an ELISA-based assay directed against the p50 and the p65 subunit of NF-kB.

Results: RhAPC, at supra-pharmacological concentration (10 μg/ml), significantly inhibited NF-kB activity and the release of MIP-1-α in supernatants. NF-kB activity was measured by an ELISA-based assay directed against the p50 and the p65 subunit of NF-kB.

Conclusions: RhAPC at supra-pharmacological concentration (10 μg/ml) inhibits the activity of NF-kB in ex vivo isolated mononuclear cells of septic patients as well as the release of MIP-1-α, a proinflammatory chemokine regulated by NF-kB. These findings may represent immunomodulatory pathways by which rhAPC exerts specific anti-inflammatory activity in vitro in addition to its known anticoagulant and profibrinolytic activity and should be further investigated in an in vivo setting.

Key words: Drotrecogin alfa (activated) – rhAPC – Nuclear factor kappa B – Mononuclear cells – Sepsis

Abbreviations: APACHE, Acute physiology and chronic health evaluation; CRP, C-reactive protein; ELISA, Enzyme-linked immunosorbent assay; IL-1β, Interleukin-1β; IL-6, Interleukin-6; IL-8, Interleukin-8; LPS, Lipopolysaccharide; MIP-1-α, Macrophage inflammatory protein-1 alpha; NF-kB, Nuclear factor kappa B; p50/p65, subunits of nuclear factor kappa B; PROWESS, Protein C worldwide evaluation in severe sepsis; TNF-α, Tumor necrosis factor-alpha; RhAPC, Recombinant human activated protein C.

Introduction

The activated protein C (APC) pathway represents one of the major regulatory systems of hemostasis[1] and has been suggested to be a common link between coagulation and inflammation [2]. In addition to its well-defined role in coagulation, there is evidence from in vitro examinations that APC exhibits direct anti-inflammatory properties independent of its antithrombotic effects as indicated by the reduction of cytokine release from lipopolysaccharide (LPS)-stimulated monocytes [3, 4]. Results from the PROWESS-Trial (Protein C Worldwide Evaluation in Severe Sepsis; n = 1690 patients) demonstrated that the recombinant version of human APC (rhAPC, drotrecogin alfa (activated)) decreased inflammation, as indicated by a reduction of interleukin-6 (IL-6) levels in patients with severe sepsis [5]. It remained unclear whether this reduction of IL-6 levels was based on a direct anti-inflammatory effect of rhAPC or if this was a consequence of a bet-
ter organ-perfusion. Levels of several other pro-inflammatory biomarkers, however, remained unchanged in the PROWESS-Trial leading to the conclusion that a strong basis for systemic anti-inflammatory effects for rhAPC is still missing [6].

In vitro examinations suggest that inhibition of the nuclear transcription factor-kB (NF-kB) may be responsible for anti-inflammatory properties of APC: In the monocytic cell line THP-1 human APC in doses of 100–200 µg/ml significantly inhibited the LPS-induced nuclear translocation of NF-kB and the production of tumor necrosis factor-α [7]. Moreover, human APC (5–20 µg/ml) significantly inhibited the activation of both NF-kB and activator protein-1 (AP-1) in cultured human monocytes [8]. In transcript profiling experiments performed on cultured human umbilical vein endothelial cells (HUVEC) rhAPC at concentrations of 10–15 µg/ml was found to down-regulate mRNA expression of a cluster of proinflammatory genes and to inhibit the NF-kB pathway as well as genes regulated by NF-kB [9]. However, concentrations of APC used in these in vitro studies (up to 200 µg/ml) were much higher than plasma levels of rhAPC which may be reached under clinical conditions: The median blood levels achieved with rhAPC treatment (24 µg/kg/h) in patients with severe sepsis were about 45 ng/ml [10]. Thus, it is unclear whether results obtained from in vitro studies using supra-physiologic concentrations are of clinical relevance and further in vivo examinations are needed to address this question.

NF-kB is an important inflammatory signal as it activates pathways related to oxidation, adhesion, cytokine release, apoptosis, and nitric oxide production [11]. The most abundant form of NF-kB is a heterodimer composed of p50 and p65 subunits [12]. In unstimulated cells, NF-kB is localized in the cytosol [13]. Upon stimulation with e.g. lipopolysaccharide (LPS), cytokines and viruses, NF-kB translocates to the nucleus and initiates transcription of target genes responsible for the generation of a variety of proinflammatory molecules [11–13]. One of the target genes of NF-kB is the pro-inflammatory chemokine Macrophage Inflammatory Protein 1-alpha (MIP-1-α) [14]. MIP-1-α is produced by monocytes, neutrophils [15], activated lymphocytes [16], and fibroblasts [17] and is chemotactic for macrophages and T lymphocytes. In addition to its chemotactic activity, it potently activates macrophages to secrete TNF-α, IL-1 and IL-6 [18]. MIP-1-α is an important mediator for the local interaction of monocytes at the endothelium during infection and sepsis.

Inhibition of NF-kB activity levels by rhAPC has not been fully elucidated in septic patients. To further clarify this question we examined the effect of rhAPC on NF-kB activity in ex vivo isolated mononuclear cells of patients suffering from severe sepsis. To define regulatory effects of rhAPC on mediators downstream from NF-kB activation we set out to examine the impact of rhAPC on the release of MIP-1-α from human mononuclear cells isolated from septic patients.

Materials and methods

Reagents

RhAPC (drotrecogin alfa (activated)), analytical grade, was provided by Eli Lilly, Indianapolis, USA as part of an unrestricted grant.

Study patients

We studied 13 patients, admitted from home, presenting with severe sepsis and septic shock, defined according to the criteria of the American College of Chest Physicians/Society of Critical Care Medicine (ACCP/SCCM) consensus conference [19]. Septic patients received hydrocortisone continuously in a dose of 100–200 mg/day [20]. None of the septic patients was treated with rhAPC (Xigris®) at the time of blood sampling or before. Where indicated (>2 septic induced organ failures), treatment with Xigris® was started after having obtained blood samples. Eight age- and gender-matched healthy subjects without signs of intercurrent infective or inflammatory disorders served as controls. The research protocol was approved by the local ethics committee. Written informed consent of healthy subjects, patients or their legal representatives was obtained prior the investigation.

Blood sampling and isolation of mononuclear cells

Whole blood (60 ml) from septic patients and controls was sampled from a cubital vein into citrate-containing tubes very slowly and carefully to avoid artificial NF-kB activation. In septic patients blood sampling was performed within the first 48 h of severe sepsis. Peripheral blood mononuclear cells were obtained by Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation using LeucoSep® cell separating tubes (Greiner, Frickenhausen, Germany). Whole blood was poured into an LeucoSep® tube containing Ficoll-Paque Plus solution below the filter disc of the tube. The tube was then centrifuged at 400 × g for 40 min at 20°C. As a result, a distinct layer of mononuclear cells was found in the upper part of the tube.

In vitro stimulation with rhAPC

Mononuclear cells were resuspended in RPMI 1640 culture medium with L-glutamine in the absence of growth factors, antibiotics and fetal calf serum (FCS) and were distributed to 6-well cell culture plates at a cell density of 4 × 10⁶ cells/well. Cells were treated with rhAPC in a final concentration of 10 µg/ml for 2 h at 37°C with gentle agitation. For the measurement of MIP-1-α in cell culture supernatants another set of mononuclear cells was incubated with rhAPC (10 µg/ml) for 6 h. These supra-physiological doses of rhAPC was chosen according to the most recently published in vitro data on monocytes [4, 7, 8]. Treatment with rhAPC at the indicated concentration for up to 6 h did not affect mononuclear cell viability, as assessed by trypan blue exclusion (>90% viable cells).

Experimental protocol for determination of NF-kB activation

After an incubation time of 2 h whole-cell extracts from mononuclear cells were prepared as previously described [21] using a commercial kit (Nuclear Extract Kit, Active Motif, Carlsbad, CA, USA). Mononuclear cells were collected from the 6-well plates, washed with ice-cold PBS/phosphatase inhibitor solution and were directly lysed with complete lysis buffer. The solubilized proteins were separated from the cell debris by centrifugation (14000 × g for 20 min at 4°C), transferred to pre-chilled tubes and stored at ~80°C until NF-kB analysis. An ELISA-based assay was used for the determination of the activated p50 and p65 subunits of NF-kB (Trans AM™ kit from Active Motif, Carlsbad, CA, USA) consisting of a 96-well plate to which oligonucleotide containing the NF-kB consensus binding site (5'-GGGACCTTTCC-3') has been immobilized. Whole-cell extracts (containing 3 µg of total protein) were added to the wells and the activated NF-kB bound specifically to the oligonucleotide. By using a primary antibody directed against the active form of the p50 or the p65 subunit of NF-kB, the NF-kB complex bound to the oligonucleotide was detected. Addition of a secondary antibody conjugated to horseradish peroxidase provided colorimetric readout that