Influence of Low Molecular Weight Heparin (Certoparin) and Unfractionated Heparin on the Release of Cytokines from Human Leukocytes

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Abstract—We analyzed the influence of heparins (unfractionated heparin, UFH and low molecular weight heparin certoparin) on the generation of IL-1ra, IL-6, IL-10, and IL-12p40 and from leukocyte fractions in vitro. Polymorphonuclear neutrophil leukocytes (PMN) and peripheral blood mononuclear cells (PBMC) from 16 different healthy donors were isolated and adjusted to $1 \times 10^6$ cells/ml supplemented RPMI 1640. Leukocyte fractions were differentially stimulated (PMN with 1 μg and 5 μg LPS, PBMC with 10 ng TSST-1 or 2 μg ConA) in the presence or absence of heparins (1 U/ml, 2 U/ml, and 4 U/ml) for 24 h at 37°C. Cytokine release was analyzed by ELISA. Certoparin but not UFH led to a dose-dependent increase in IL-6 from non-stimulated PBMC. In contrast, the release of IL-1ra, IL-10, and IL-12p40 was not modulated by heparins in a dose-dependent fashion. Increases in these cytokines occurred only as single incidents at intermediate heparin levels. An influence of the heparins on the apoptosis of PMN (measured as DNA-fragmentation in non-stimulated or LPS-stimulated cell-fractions) was not observed.

KEY WORDS: Heparin; cytokine; leukocytes; neutrophils.

INTRODUCTION

Anticoagulant therapy by heparin is extensively used in clinical settings for reduction of thromboembolic complications in surgery. Main target of heparin action is its ability to potentate the activity of the endogenous coagulation cofactor antithrombin-III (1). Beneath unfractionated heparin (UFH) low molecular weight heparins (LMWH, e.g. certoparin) became available for clinical use (2). Substitution of LMWH for UFH decreases the incidence of heparin-induced thrombocytopenia and may be related to a lower hemorrhagic risk (1, 2). In addition to its anticoagulant activities there is accumulating evidence that heparins significantly affect inflammatory processes. It was reported that heparins exert anti-inflammatory activities such as inhibition of leukocyte adhesion to endothelial cells, inhibition of L- and P-selectin expression or inhibition of reactive oxygen species generation (3, 4, 5). These anti-inflammatory activities of heparins may be related to the modulation of cytokine synthesis. The influence of heparins on cytokine generation was shown using isolated leukocyte fractions or whole blood assays (6, 7, 8, 9, 10). In these studies inconsistent results were reported, especially for the generation of proinflammatory TNF-α. Thus, it was the purpose of this study to analyze the influence of heparins (UFH and certoparin) on the generation of cytokines with known anti-inflammatory activities (IL-1ra, IL-6, IL-10), and of IL-12p40 from human leukocyte fractions.

MATERIALS AND METHODS

Materials

Histopaque 1119, Histopaque 1077, RPMI1640 medium supplemented with L-glutamine, toxic shock syn-
drome toxin-1 (TSST-1), lipopolysaccharide (LPS) from Escherichia coli (O55:B5), concanavalin-A (ConA), N-(2-hydroxyethyl)-piperazine-N′-(2-ethanesulfonic acid), and Tween 20 were obtained from Sigma (Deisenhofen, Germany). Protease-, peroxidase-, alkaline phosphatase-free bovine serum albumine (BSA, fraction V) was from Serva (Heidelberg, Germany). Propidium iodide was obtained from Molecular Probes Inc., Eugene, Oregon. Fetal calf serum (FCS) was from Life Technologies (Eggenstein, Germany). Phosphate-buffered saline (PBS, 10 mM Na$_2$PO$_4$/138 mM NaCl/2.7 mM KCl, pH 7.2) was from Sigma. Triton-X-100 was provided from Fluka BioChemika, Deisenhofen, Germany. Certoparin sodium (LMWH) was obtained from Novartis Pharma (Nürnberg, Germany) and heparin sodium (unfractionated heparin, UFH) was from Ratiopharm (Ulm, Germany).

**Isolation and Stimulation of Leukocyte Fractions**

Polymorphonuclear neutrophil leukocytes (PMN) and peripheral blood mononuclear cells (PBMC) were isolated by a one step procedure based on a discontinuous double Ficoll-gradient described by English and Andersen (11). Briefly, EDTA-anticoagulated peripheral blood (9 ml Monovette$^\text{TM}$, Sarstedt, Nürnberg, Germany) obtained from healthy volunteers were diluted with an equal volume of 0.9% NaCl and carefully overlain on a double-gradient formed by layering 10 ml of polysucrose/sodium diatrizoate adjusted to a density of 1.077 g/ml (Histopaque 1077) on 10 ml Histopaque 1119 in 50 ml Falcon tubes (Becton Dickinson, Heidelberg, Germany). The tubes were subsequently centrifugated at 700 × g for 30 min at room temperature. After centrifugation two distinct leukocyte cell layers (PBMC and PMN including eosinophil granulocytes) were obtained above the bottom sediment of erythrocytes. The cell layers were carefully aspirated and both PMN and PBMC were transferred to separate 50 ml-tubes which were subsequently filled with PBS. Centrifugation followed at 200 × g for 15 min at 4°C. After this first washing contaminating erythrocytes within the PMN-fraction were removed by hypotonic lysis using 0.3% NaCl for 2 min at room temperature. After reconstitution of physiological osmotic strength, cells were washed again with PBS. This method led to more than 95% pure and viable neutrophils including eosinophils.

Cell counting was performed using Tuerk staining solution (Sigma-Aldrich Chemie, Deisenhofen, Germany). Viability was measured by trypan blue (Sigma-Aldrich) exclusion test. Differential cell counts were performed by a modified Pappenheim staining (May-Grünwald solution and Giemsa solution, Sigma-Aldrich) of respective cell smears.

Isolated cells were adjusted to $1 \times 10^6$ cells/ml RPMI 1640 supplemented with L-glutamine (0.3 g/L), sodium bicarbonate (2.0 g/L), 10% fetal calf serum (FCS), and 20 mM N-(2-hydroxyethyl)-piperazine-N′-(2-ethanesulfonic acid).

Leukocyte fractions were differentially stimulated in the presence or absence of different concentrations of heparins (1 U/ml, 2 U/ml, and 4 U/ml). Final dilutions of heparins were made with RPMI1640. The concentrations of heparins were chosen according to a theoretical model of dilution of a therapeutic heparin dose in a distribution volume of 70 ml/kg. Incubations of cells without heparins were performed by the addition of an adjusted volume RPMI1640. PBMC were stimulated with TSST-1 (10 ng/ml) and ConA (2 μg/ml). PMN were stimulated with LPS (1 μg/ml and 5 μg/ml) for 24 h at 37°C in a humidified atmosphere (5% carbon dioxide) in 24-well tissue culture plates (Becton Dickinson). Due to the different numbers of isolated cells the experimental replications ranged from $n = 10$ (PMN) to $n = 16$ (PBMC). Supernatants were harvested and subsequently centrifugated at 2000 × g for 2 min at room temperature. Resulting supernatants were stored at −70°C.

**ELISA-System**

Mouse monoclonal antibodies (capture antibodies) and biotinylated goat polyclonal antibodies (detection antibodies) as well as recombinant human cytokines (standards) were supplied by R & D Systems (Wiesbaden, Germany). Volumina of 100 μl were used throughout ELISA procedures except 200 μl for blocking solution (PBS-containing 1% BSA) and washing buffer (PBS/Tween 0.05%). Cytokines were quantitated following the manufacturer’s ELISA-protocol. The capture antibodies were diluted in PBS and added to 96-well ELISA plates (MaxiSorp, Nunc, Roskilde, Denmark). The plates were incubated overnight at room temperature and then washed three times with wash buffer (AM-60 Plate Washer, Dynex Technologies, Denkendorf, Germany). After addition of samples and standards, the plates were left at 2 h at room temperature on a shaker (200 shakes/min), washed three times, and the biotinylated detection antibodies (diluted with PBS-Tween 0.05%) were added. After a subsequent three times washing step streptavidin-peroxidase (S5512, Sigma) was used as a 400-fold dilution in PBS of a stock