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Immunomodulatory effects of testosterone evaluated in all-trans retinoic acid differentiated HL-60 cells, granulocytes, and monocytes

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ABSTRACT

The sex hormones are known to affect innate immunity in humans. In this study we evaluated the immunomodulatory effects of testosterone in a model system comprising of all-trans retinoic acid differentiated HL-60 cells, and confirmed the results in human granulocytes and monocytes. Results showed that testosterone at pharmacological doses reduced the production of interleukin-8 and reactive oxygen species from differentiated HL-60 cells in a concentration dependent manner without affecting phagocytosis. The cells were stimulated with zymosan, lipopolysaccharide, or *Bacillus subtilis*. At the highest concentration of testosterone (120 μM), interleukin-8 secretion was reduced 42-80%, and production of reactive oxygen species was reduced 32-46%. Flutamide, an antagonist of the classical intracellular androgen receptor, was unable to antagonize the immunosuppressive effect of testosterone. We further demonstrated that the suppressive effect of testosterone has a short onset time. Our results suggest that testosterone affects the fast operating membrane bound androgen receptor or a rapid acting enzyme system. Testosterone, at pharmacological doses, was also shown to suppress generation of reactive oxygen species and interleukin-8 in human granulocytes and monocytes, respectively, to a similar extent as observed in differentiated HL-60 cells.

1. Introduction

A gender dimorphism in the human immune system is known to exist as females are more resistant to bacterial infections, suffer a higher incidence of autoimmune diseases and produce a more vigorous cellular and humoral immune response than males [1–4]. This suggests that the sex hormones (estrogen, progesterone, and testosterone) modulate the immune system.

Neutrophils and monocytes are key players as a first line of defense against invading microorganisms. During an infection, neutrophils and monocytes will phagocytose invading microorganisms and produce reactive oxygen species (ROS) and cytokines to combat the infection. It has been shown that neutrophils and monocytes are subject to immunomodulation by the sex hormones [5–9]. Estrogen is reported to decrease neutrophil chemotactic activity while progesterone has the opposite effect [10]. Neutrophilic production of ROS has also been reported to be affected by estrogen and progesterone; however, conflicting results predominate this research area [11–13]. In general, testosterone is accepted to be immunosuppressive and to increase susceptibility to various infectious diseases [4,7]. This has been illustrated in *in-vitro* experiments where testosterone inhibits monocyte and macrophage derived interleukin-6 (IL-6) [14,15] and IL-1 [16,17] as well as neutrophil generation of ROS [11,18]. To our knowledge, there have been no reports of testosterone effects on monocytic secretion of the chemoattractant cytokine IL-8.

Testosterone is utilized in replacement therapy where it is injected intramuscularly in high doses (200–500 mg) to males with reduced or absent endogenous testosterone; a condition termed primary- or secondary hypogonadism [19]. Testosterone is also administered to female-to-male transsexual people and is abused by athletes to promote muscle growth and performance [20,21]. The usage of testosterone is associated with adverse skin reactions like erythema, vesicles, burning, and rashes at the site of application [22–25].

To evaluate the immunomodulatory effects of testosterone, we utilize a model system comprising of the human promyelocytic leukemia cell line HL-60 [26,27], which has been terminal differentiated into granulocyte-like cells by all-trans retinoic acid (ATRA) [28]. The differentiated HL-60 cells posses multiple granulocytic characteristics, including expression and repression of key proteins and surface markers as well as the morphology and the ability to generate ROS [28–32]. However, ATRA differentiated HL-60 cells also possess monocytic functional characteristics as the cells are able to secrete a considerable amount of IL-8 [33,34]. This duality is exploited in the present study. To simulate a microbial challenge, zymosan, lipopolysaccharide (LPS), and *Bacillus subtilis* (*B. subtilis*) is used to induce ROS generation and IL-8 release. The effect of testosterone on phagocytosis is assessed as well. Furthermore, flutamide, an androgen receptor antagonist, is used to investigate the drug-receptor mechanism of testosterone. Supporting experiments are conducted on isolated human granulocytes to confirm results on ROS production obtained in differentiated HL-60 cells. Results

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on IL-8 secretion by HL-60 cells are confirmed in peripheral blood mononuclear cells (PBMC). Preparations of PBMC contain monocytes and lymphocytes but only the monocytes secrete IL-8 [35–38] and consequently the PBMC in this context are described as monocytes.

2. Material and methods

The content of LPS in all media, solvents, and solutions were below the detection limit in our kinetic-turbidimetric limulus amebocyte lysate (LAL) assay (<5 pg/ml). All cell work was performed in a laminar air flow unit.

2.1. Reagents

Testosterone (17β-Hydroxy-4-androstene-3-one) (Riedel-de Haën, Sigma-Aldrich, St. Louis, USA), flutamide (2-Methyl-*N*-[4-nitro-3-(trifluoromethyl)phenyl]propanamide) (Sigma-Aldrich, St. Louis, USA), and luminol (5-Amino-2,3-dihydro-1,4-phthalazinedione) (Across Organics, USA) were dissolved in dimethyl sulphoxide (DMSO). The final DMSO concentration was 1% in all experiments.

Human plasma was obtained from a healthy male volunteer by collecting venous blood in a sodium heparinised BD Vacutainer® (BD, Plymouth, UK) and isolating the plasma by centrifugation (600 x g, 10 min). Plasma was stored at −80 °C until use.

2.2. Microbial substances

B. subtilis (ATCC No: 6633; ATCC, Manassas, USA) was prepared as described previously [39]. LPS prepared from *Escherichia coli* O55: B5, 3.8 EU/ng (Lonza, Walkersville, USA), zymosan prepared from *Saccharomyces cerevisiae* (Sigma-Aldrich, St. Louis, USA) and fluorescein conjugated zymosan A (FITC-zymosan) (BioParticles®, Invitrogen, Carlsbad, USA) were reconstituted in Hanks' Balanced Salt Solution with $CaCl_2$ and $MgCl_2$ (HBSS) (GIBCO™, Invitrogen, Carlsbad, USA).

2.3. Cells

2.3.1. HL-60 cells

Cultivation and differentiation of the human promyelocytic leukemia cell line HL-60 (ATCC No: CCL-240; ATCC, Manassas, USA) was performed as described previously [40]. The HL-60 cells were differentiated for 7 days in the presence of 1 μM ATRA (Fluka, Sigma-Aldrich, St. Louis, USA). RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel), 1% GlutaMAX™ (GIBCO™, Invitrogen, Carlsbad, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, USA) was used as growth medium.

2.3.2. Isolation of granulocytes and monocytes

Granulocytes and monocytes were obtained from five healthy male volunteers (age: 25–35). Venous blood was collected in a sodium heparinised vacutainer. Granulocytes were isolated using Lympholyte®-poly isolation mixture (Cederlane Laboratories Limited, Burlington, Canada) and monocytes, as a constituent of PBMC, were obtained using Ficol-Paque™ PLUS (Amersham Biosciences, Uppsala, Sweden) according to the manufactures descriptions. Contamination of monocytes in the granulocytic suspension, and vice versa, was below 0.5% examined by flow cytometry.

2.4. Assay procedures

2.4.1. Measurement of phagocytosis

Challenge of differentiated HL-60 cells with FITC-zymosan was performed in eppendorf tubes with perforated lids to allow air access during incubation. Each tube containing differentiated HL-60 cells ($2{\cdot}10^5$ cells/tube), plasma (2.5%), and testosterone (120 or 0 μM (control)) were placed in a humidified atmosphere (37 °C, 5% CO_2) for 15 min to equilibrate. Next, FITC-zymosan particles ($4{\cdot}10^5$ particles/tube) were added and the tubes were incubated 0–120 min before examination by flow cytometry. Phagocytosis was stopped by placing the tubes on ice. After centrifugation (400 x g, 10 min) the cells were reconstituted in BD FACSFlow™ buffer (BD, Franklin Lakes, USA) and analyzed using a FACScan Cytometer and CellQuest™ Pro software v6.0.2 (BD, Franklin Lakes, USA). Differentiation of HL-60 cells results in two distinct cell populations determined in a forward-/side scatter dotplot. One fraction was stainable by 7-ADD indicating apoptotic cells. Only the viable cell fraction was gated and used for analysis (>5.000 cells in the gate). The software's histogram statistics calculated the percentage of FITC positive differentiated HL-60 cells in the gate as well as the geometric mean of the fluorescence intensity. The fluorescence intensity is in arbitrary units and describes the phagocytic capacity of the differentiated HL-60 cells. The background autofluorescence from differentiated HL-60 cells, measured by adding HBSS instead of FITC-zymosan, was less than 0.2%. Testosterone did not alter the morphology of the differentiated HL-60 cells compared to control cells during 120 min of incubation, as evaluated by examining the distribution of cells in the forward-/side scatter dotplots.

2.4.2. Measurement of ROS production

Stimulation of differentiated HL-60 cells and granulocytes was performed in white polystyrene LumiNunc™ 96-well microplates (Nunc, Roskilde, Denmark) with a final volume of 200 μl. Each well contained differentiated HL-60 cells or isolated granulocytes ($5{\cdot}10^5$ cells/well) as well as plasma (2.5%). Next, the microplate was placed in a humidified atmosphere (37 °C, 5% CO_2) for 15 min to equilibrate. Luminol (283 μM) and testosterone (120, 60, 30, or 0 μM (control)) was added and cells were challenged with LPS 1 ng/ml, zymosan 10 μg/ml, *B. subtilis* 10^6 cfu/ml, or HBSS (background). The concentrations of the microbial substances were known to induce a sub maximal response in the cells. All components were diluted in HBSS.

ROS were quantified by luminol enhanced chemiluminescence as previously described [40]. Luminescence was recorded for 180 min and the area under the curve (AUC) was calculated.

The chemically antioxidizing ability of testosterone and flutamide was assessed by examining reduction of DPPH (1,1-diphenyl-2-picrylhydrazyl) (Sigma-Aldrich, St. Louis, USA). DPPH produces stable radicals in methanolic solutions, but if an antioxidative agent is present the absorbance of DPPH at 517 nm will decline [41]. Testosterone (15–240 μM) and flutamide (10–160 μM) showed no antioxidative effects in this assay.

2.4.3. Measurement of IL-8 secretion

Stimulation of cells was performed in a 24-well microplate (Nunc, Roskilde, Denmark) with a final volume of 500 μl. Differentiated HL-60 cells or PBMC ($5{\cdot}10^5$ cells/well) were added to each well before addition of testosterone (120, 60, 30, or 0 μM (control)) and/or flutamide (80 μM). Cells were challenged with a microbial stimulus at a concentration known to induce a sub maximal cellular response. Differentiated HL-60 cells were challenged with LPS 10 pg/ml, zymosan 0.5 μg/ml, or *B. subtilis* 10^4 cfu/ml, whereas PBMC were challenged with LPS 50 pg/ml or zymosan 0.5 μg/ml. Supplemented RPMI 1640 was used as background. All components were diluted in supplemented RPMI 1640. After 20–24 h of incubation in a humidified atmosphere (37 °C, 5% CO_2) the supernatants were diluted and examined for the content of IL-8. Monocytes secrete IL-8 within 6–12 hours after a microbial challenge [42,43].

2.4.3.1. IL-8 immunoassay. IL-8 was measured in a time resolved dissociation enhanced lanthanide fluoro immunoassay (DELFIA) as previously described [44] with the following modifications: The microplate was coated with IL-8 monoclonal anti-human antibody (Cat. No: MAB208;