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Short communication

Ureaplasma isolates differentially modulate growth factors and cell adhesion molecules in human neonatal and adult monocytes

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ABSTRACT

Generally regarded as commensal bacteria, the pathogenicity of *Ureaplasma* has often been considered low. Controversy remains concerning the clinical relevance of *Ureaplasma* infection in the pathogenesis of inflammation-related morbidities. Recently, we demonstrated *Ureaplasma*-driven pro-inflammatory cytokine responses in human monocytes *in vitro*. We hypothesized that *Ureaplasma* may induce further inflammatory mediators. Using qRT-PCR and multi-analyte immunoassay, we assessed the expression of granulocyte-colony stimulating factor (G-CSF), vascular endothelial growth factor (VEGF), intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) in term neonatal and adult monocytes exposed to *Ureaplasma urealyticum* serovar 8 (Uu8) and *U. parvum* serovar 3 (Up3). *Ureaplasma* significantly induced VEGF mRNA in neonatal (Up3: $p < 0.05$, versus broth control) and adult monocytes (Uu8: $p < 0.05$) as well as ICAM-1 mRNA in neonatal cells ($p < 0.05$ each). As far as protein expression was concerned, Up3 stimulated VEGF release in both monocyte subsets ($p < 0.01$) and enhanced secretion of ICAM-1 protein in neonatal monocytes ($p < 0.05$). In adult cells, ICAM-1 protein release was increased upon exposure to both isolates (Uu8: $p < 0.05$, Up3: $p < 0.01$). *Ureaplasma*-induced responses did not significantly differ from corresponding levels mediated by *E. coli* lipopolysaccharide (LPS). The stimulatory effects were dose-dependent. *Ureaplasma* infection, on the contrary, did not affect G-CSF and VCAM-1 expression. Of note, co-infection of LPS-primed neonatal monocytes with *Ureaplasma* enhanced LPS-induced ICAM-1 release (Uu8: $p < 0.05$). Our results confirm *Ureaplasma*-driven pro-inflammatory activation of human monocytes *in vitro*, demonstrating a differential modulation of growth factors and cell adhesion molecules, that might promote unbalanced monocyte responses and adverse immunomodulation.

1. Introduction

Ureaplasma (*U.*) *urealyticum* (serovars 2, 4, 5, 7–13) and the separate species *U. parvum* (serovars 1, 3, 6, 14) have been related to genital tract infection and infertility and have been associated with chorioamnionitis and preterm birth (PTB) (i.e., delivery < 37 weeks of gestation) [1,2]. Besides, epidemiologic and experimental data indicate a role of *Ureaplasma* infection in neonatal morbidities, such as bronchopulmonary dysplasia (BPD), intraventricular hemorrhage (IVH) and retinopathy of prematurity (ROP) in preterm infants [3,4] as well as pneumonia, sepsis and meningitis in preterm and term neonates [5,6]. However, being detected in the lower genital tract of 40–80% of women of reproductive age, the pro-inflammatory capacity of *Ureaplasma* and the relevance of detecting these organisms in clinical specimen remains subject of discussion [1,5,6]. A number of studies do not support a causal relationship between *Ureaplasma* genital tract colonization and PTB [7] or neonatal respiratory tract colonization and BPD [8]. Data from epidemiologic studies and animal models show heterogenous results on *Ureaplasma*-induced intra-uterine inflammation [1,2] and do not conclusively support a correlation of pulmonary or systemic inflammation and the presence of *Ureaplasma* in preterm infants [3,5,8].

In vitro data on *Ureaplasma*-induced inflammation is scarce. Using primary human monocytes, our group recently demonstrated *Ureaplasma*-driven pro-inflammatory cytokine responses and cytokine imbalances *in vitro* [9]. With inflammation being a pathological condition orchestrated by cytokines, chemokines and several other

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inflammatory mediators, the present study addressed the impact of *Ureaplasma* isolates on the expression of growth factors and cell adhesion molecules by human monocytes. Granulocyte-colony stimulating factor (G-CSF) controls neutrophil production and differentiation and may either reduce inflammatory immune responses or exacerbate inflammation [10]. Vascular endothelial growth factor (VEGF) is a key angiogenic and inflammatory mediator, that regulates growth, structure and function of tissue vasculature, and modulates chemotaxis, vascular permeability and endothelial barrier dysfunction [11]. Adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), promote attachment of inflammatory cells to the endothelial lining and consecutive invasion into adjacent tissues, linking systemic inflammation, endothelial cell activation and endothelial dysfunction [12].

Using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) and multi-analyte immunoassay, we analyzed the expression of G-CSF, VEGF, ICAM-1 and VCAM-1 in human term neonatal and adult monocytes stimulated with *U. urealyticum* serovar 8 (Uu8) and *U. parvum* serovar 3 (Up3) in the absence or presence of *Escherichia coli* (*E. coli*) lipopolysaccharide (LPS).

2. Materials and methods

2.1. Bacterial strains and culture conditions

U. urealyticum serovar 8 (Uu8) (American Tissue Culture Collection (ATCC) #27618) and *U. parvum* serovar 3 (Up3) (ATCC #27815) were propagated in a liquid in-house medium ("broth") containing 82% autoclaved PPLO medium (Becton, Dickinson & Company, USA), 10% heat-inactivated horse serum, 7% urea (20% aqueous solution) and 1% phenol red (0.2% solution) (Sigma-Aldrich, USA), adjusted to pH 6.5. For each experiment, aliquots of mid-logarithmic-phase broth cultures were inoculated 1:10 in 5 ml broth. 10-fold serial dilutions were incubated overnight to obtain titers of 5×10^8 color-changing units (CCU)/ml. CCUs and corresponding amounts of Uu8 and Up3 DNA were assessed as described before [9]. Re-culture using broth and selective agar plates (medco Diagnostika GmbH, Germany) confirmed viability of inoculated organisms.

2.2. Isolation of $CD14^+$ monocytes from cord blood and peripheral blood mononuclear cells

Cord blood samples were taken from healthy term newborns (n = 6) delivered by elective caesarean section after written parental consent. Exclusion criteria comprised clinical or laboratory evidence of chorioamnionitis and/or neonatal infection and congenital malformation. The study has been approved by the Ethic Committee of the Medical Faculty of Wuerzburg and was conducted in accordance with the World Medical Association Declaration of Helsinki. Cord blood was collected from the umbilical vein using a closed system (Macopharma International, France), and was processed within 2 h. Adult leukocyte concentrates were obtained from randomized and anonymized apheresis products from healthy donors (n = 6) at the Department of Immunohematology and Transfusion Medicine, University Hospital Wuerzburg. Cord blood and peripheral blood mononuclear cells were isolated by means of Ficoll-Paque gradient centrifugation (Linaris, Germany). Positive selection of $CD14^+$ monocytes was carried out using CD14 MicroBeads® (Miltenyi Biotec, Germany) according to the manufacturer's protocol. Purity was > 90%, as determined by flow cytometry. $CD14^+$ monocytes were re-suspended in RPMI 1640 medium (Sigma-Aldrich) containing 10% fetal bovine serum (Thermo Fisher Scientific, Germany).

2.3. Cell culture and stimulation assays

$CD14^+$ monocytes of each donor were transferred to 24-well culture plates (Greiner, Germany) at a density of 1×10^6 cells/well. Cells rested for 2 h before *Ureaplasma* isolates were inoculated at a concentration of 10^8 CCU/well as described previously [9]. For studies on LPS-primed monocytes, LPS (*E. coli* serotype 055:B5, Sigma-Aldrich) was added 90 min prior to the infection. Incubation periods, *Ureaplasma* concentration and LPS dose had been determined by preliminary experiments [9]. Flow cytometry confirmed cell viability ≥95% at 4 h and 24 h for native, *Ureaplasma*-stimulated and LPS-primed monocytes.

2.4. RNA extraction, reverse transcription (RT) and quantitative real-time RT-PCR (qRT-PCR)

For RNA extraction, monocytes were harvested after 4 h incubation and separated by centrifugation. Total RNA was extracted using the NucleoSpin® RNA Kit (Macherey-Nagel, Germany), eluted into 60 µl nuclease-free water (Sigma-Aldrich) and stored at −80 °C until reverse transcription. RNA quantitation was carried out by means of a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Germany). Amounts of 0.11–0.52 µg of total RNA of cord blood monocytes and 0.13–0.50 µg of total RNA of adult monocytes were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). First strand cDNA was stored at −80 °C. For quantitative detection of G-CSF, VEGF, ICAM-1 and VCAM-1 mRNA, cDNA was diluted 1:10 in deionized, nuclease-free water and analyzed in duplicates of 25 µl using 12.5 µl iTaq™ Universal SYBR Green Supermix (Bio-Rad Laboratories, USA) and a 7500 Real-Time PCR System (Applied Biosystems, USA) [9]. All primers applied are given in Table 1. Amplification was normalized to the reference gene *PPIA* (*peptidyl prolyl isomerase A*).

2.5. Multi-analyte immunoassay

Supernatants were collected after 24 h incubation and stored at −80 °C until analysis. Concentrations of human G-CSF, VEGF, ICAM-1 and VCAM-1 were assessed using Luminex® multiplex kits and the xPonent® software (Merck group, Germany). Samples of neonatal and adult monocytes were analyzed in duplicate. Concentration of each mediator was calculated from an individual standard curve. The lower detection limits of the assays were 3.24 pg/ml (G-CSF), 2.0 pg/ml (VEGF), 2.12 pg/ml (ICAM-1) and 13.84 pg/ml (VCAM-1).

2.6. Statistical analysis

Prism® 6 software (GraphPad Software, USA) was used for statistical analysis. Data are expressed as mean ± standard deviation (SD). Differences among groups were analyzed using the non-parametric Kruskal-Wallis test and Dunn's multiple comparison post hoc-test.

Table 1
Primer sequences used for qRT-PCR.

Gene symbol	Sequence accession #	Forward primer	Reverse primer
CSF3	NM_000759.3	5′-CTGCTTGAGCCAACTCCA-3′	5′-AGTTCTTCCATCTGCTGCC-3′
VEGFA	NM_001171623.1	5′-CCATCCAATCGAGACCCT-3′	5′-AGGTTTGATCCGCATAATCTG-3′
ICAM1	NM_000201.2	5′-CAGACCTTTGTCCTGCCA-3′	5′-AAGGAGTCGTTGCCATAGGT-3′
VCAM1	NM_001078.3	5′-GCAAGTCTACATATCACCCA-3′	5′-AGTTGCATTTCCAGAAAGGT-3′