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Original article

A new form of amphotericin B – the complex with copper (II) ions – downregulates sTNFR1 shedding and changes the activity of genes involved in TNF-induced pathways

AmB–Cu^{2+} downregulates sTNFR1 shedding and changes the activity of genes involved in TNF-induced pathways

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ABSTRACT

Background: A new form of amphotericin B (AmB)– complex with copper (II) ions (AmB–Cu^{2+}) – is less toxic to human renal cells. Cytokines, including Tumor Necrosis Factor (TNF), are responsible for nephrotoxicity observed in patients treated with AmB. Another problem during therapy is the occurrence of oxidized forms of AmB (AmB-ox) in patients' circulation. To elucidate the molecular mechanism responsible for the reduction of the toxicity of AmB–Cu^{2+}, we evaluated the expression of genes encoding TNF and its receptors alongside encoding proteins involved in TNF-induced signalization.
Methods: Renal cells (RPTECs) were treated with AmB, AmB–Cu^{2+} or AmB-ox. The expression of TNF and its receptors was evaluated by ELISA tests and real-time RT-qPCR. The expression of TNF-related genes was appointed using oligonucleotide microarrays.
Results: Only sTNFR1 was detected, and its level was lower in AmB–Cu^{2+}- and AmB-ox-treated cells. *TNFR1* mRNA was downregulated in AmB-ox, while *TNFR2* mRNA was upregulated in AmB and AmB–Cu^{2+}. Several changes in the expression of TNF-related genes coincided with changes in the expression of TNF receptors.
Conclusions: The lower toxicity of AmB–Cu^{2+} could result from the changes in the expression of TNF receptors, which coincided with the changes in the expression of genes encoding proteins involved in TNF-induced pathways. This situation might subsequently result in a changes in intracellular signalization and influence the toxicity of tested forms of AmB on renal cells.

Introduction

Antifungal antibiotic amphotericin B (AmB) is one of the most effective and commonly used polyene antibiotics against invasive fungal diseases (IFDs) [1]. Unfortunately, AmB can induce severe renal injury [2] and other acute and chronic side effects with severe courses that in worst cases can be associated with death risk [3]. To reduce AmB toxicity, new forms of the drug have been developed, such as lipid formulations [4], the NanoDisk [5], AmB in liquid crystals [6], chitosan-coated AmB-loaded solid lipid nanoparticles [7] and AmB complexes with specific ions. One such modification, which has been tested by the authors of this study, involves the generation of AmB complex with copper (II) ions. Results revealed that the AmB–Cu^{2+} complex is stable at physiological pH, causes AmB disaggregation and can exhibit high antifungal activity against *Candida albicans* [8–10]. A cytotoxicity test on normal human renal proximal tubule endothelial cells (RPTECs) showed that, at high concentration, the percentage of growth inhibition of cells treated with the AmB–Cu^{2+} complex was two times lower than in the case of AmB-treated cells [11]. Oxidized forms of AmB (AmB-ox), which can occur in a patient's circulation during therapy [12], were as toxic to the RPTEC as AmB [11]. Transcriptome

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analysis showed that the AmB–Cu^{2+} complex probably stimulated intrinsic "self-protection" mechanisms against oxidative stress [11]. Beside the generation of oxidative stress, one postulated mechanism of renal injury during AmB treatment is the stimulation of TNF (Tumor Necrosis Factor) expression, which subsequently causes changes in intracellular signalization, leading to the induction of inflammation [13,14]. TNF production is induced during several nephropathies, including glomerulonephritis, interstitial tubular nephritis and diabetic and drug-induced nephropathy [15]. This pleiotropic cytokine exerts its action through two receptors present in many cell types: TNFR1 (Tumor Necrosis Factor Receptor 1) and TNFR2 (Tumor Necrosis Factor Receptor 2) and activates several intrinsic pathways, leading to apoptosis, necroptosis, survival, proliferation and inflammation [16]. Numerous studies reported that AmB induces an increase of TNF serum levels [17,18] and its expression in immune cells [19,7] as well as in kidneys and tubular cells [20,14]. However, little is known about the induction of the expression of TNF and its receptors in renal cells without infiltrating immune cells, which are the main source of TNF and responsible for the induction of inflammation in the kidney. In this study, we examined if AmB–Cu^{2+} complex changes the expression of TNF and its receptors (at mRNA and protein level) in RPTEC cells comparing to controls and cells treated with AmB and AmB-ox. To test if changes in the expression of TNF and its receptors are concomitant with changes in the expression of genes encoding proteins involved in TNF-induced signalization, we performed a transcriptome analysis using the oligonucleotide microarray technique.

Materials and methods

Cell culture conditions

RPTECs (CC-2553, Lonza, Basel, Switzerland) were maintained as previously described [11]. Cells were treated with amphotericin B (Sigma-Aldrich, St. Louis, MO, USA), a complex of amphotericin B with copper ions (II) and oxidized forms prepared as previously [8,11]. The control cells were left untreated. After 6 h of incubation culture medium was collected for protein tests and cells were immediately proceeded with RNA extraction.

RNA extraction

Total RNA extraction was performed with the use of TRIzol® reagent (Invitrogen Life Technologies, CA, USA) according to producer's instructions. RNA extracts purification, integrity and quantitative assessment was performed as previously [11].

Oligonucleotide microarray procedure

Biotinylated aRNA was synthesized, fragmented, hybridized with the HGU133A 2.0 (Affymetrix, CA, USA) and subsequently stained with streptavidin-FITC with the use of a GeneChip 3′ IVT Express Kit and GeneChip Hybridization, Wash, and Stain Kit (Affymetrix), according to the manufacturer's instructions. Fluorescence intensity was measured with Gene Chip Scanner 3000 7G and GeneChip Command Console Software (Affymetrix).

Real-time RT-qPCR

The levels of *TNF*, *TNFR1* and *TNFR2* transcripts were evaluated with the use of the real-time RT-qPCR with a SYBR Green I chemistry (SYBR Green Quantitect RT-PCR Kit, Qiagen). The quantitative analysis was carried out using an Opticon™ DNA Engine Sequence Detector (MJ Research Inc., MA, USA). Amplification was performed using previously described oligonucleotide primers and thermal profile [21]. The mRNA copy numbers of the gene examined were recalculated per 1 μg of the total RNA.

Proteins' concentration in culture medium

The concentration of TNF, sTNFR1 and sTNFR2 proteins was appointed with the use of immnunoenzymatic tests, respectively: Quantikine® Human TNF-α Immunoassay, Quantikine® Human sTNFRI/TNFRSF1A Immunoassay, Quantikine® Human sTNFRII/TNFRSF1 B Immunoassay (R&D Systems, MN, USA), according to manufacturer protocol. Optical density was read at 450 nm using Wallac 1420 VICTOR2™ (PerkinElmer Inc., MA, USA).

Statistical analysis

The statistical analysis of the real-time RT-qPCR and ELISA results was performed using Statistica v. 12.0 software (StatSoft Inc., OK, USA). The one-way ANOVA followed by *post-hoc* Newman-Keuls test were used to assess differences in the expression of the studied genes between analyzed groups of cells. All of results were expressed as means ± SD. The significance level was set at $p < 0.05$. Microarray data analysis was performed using the GeneSpring 13.0 platform (Agilent Technologies, Inc., CA, USA) and PL-Grid Infrastructure. Significant genes were appointed with the use of one-way ANOVA with Benjamini–Hochberg multiple testing correction followed by the Tukey HSD *post-hoc* test ($p < 0.05$ and FC ≥ 1.1 – fold change).

Results

TNF, sTNFR1 and sTNFR2 proteins' concentration in culture medium

AmB–Cu^{2+} and AmB-ox caused a significant decrease in sTNFR1 concentration compared to AmB ($p = 0.0053$ and $p = 0.0061$, respectively) and control (untreated) cells ($p = 0.0001$ and $p = 0.0002$, respectively) (Fig. 1). The soluble form of TNF and TNFR2 proteins were not detectable in the culture medium in all cases.

TNF, TNFR1 and TNFR2 mRNA level in cells

mRNA of *TNF*, *TNFR1* and *TNFR2* genes was detectable in all cases. The level of *TNF* mRNA was comparable, and no statistically significant difference was found between the analyzed groups. In cells treated with AmB-ox, the level of *TNFR1* was lower than in those treated with AmB and the controls ($p = 0.0467$ and $p = 0.0389$, respectively) (Fig. 2). The level of *TNFR2* mRNA was higher in cells treated with AmB and AmB–Cu^{2+}, than in the controls ($p = 0.0031$ and $p = 0.0413$, respectively). The *TNFR2* mRNA level also was significantly higher in cells treated with AmB than those with AmB-ox ($p = 0.0024$) (Fig. 3).

Changes in the transcriptome of TNF-induced pathways

A microarray analysis of mRNA levels of genes encoding proteins involved in TNF-induced pathways revealed numerous changes coincided with changes observed in the expression of TNF receptors (Table 1). Simultaneously with the downregulation of sTNFR1 release from the cell surface, both in AmB–Cu^{2+} and AmB-ox, two genes, *MKNK2* (*MAP kinase interacting serine/threonine kinase 2*) and *CDC25 B* (*cell division cycle 25B*), were downregulated (Table 1). Coinciding with the increase in *TNFR2* mRNA level, both in AmB and AmB–Cu^{2+}, was the upregulation of *DUSP10* (*dual specificity phosphatase 10*) and downregulation of *ZFAND5 (zinc finger AN1-type containing 5). HSPA1A/HSPA1 B* (*heat shock protein*