THE CYTOKINE RESPONSE OF HUMAN CORONARY ARTERY ENDOTHELIAL CELLS TREATED WITH DOXORUBICIN: RESULTS OF AN *IN VITRO* EXPERIMENT

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The cytokine profile of primary coronary artery endothelial cells cultivated in the presence of doxorubicin (2 μg/ml and 6 μg/ml) was evaluated using enzyme-linked immunosorbent assay and qPCR. Cultivation of cells in the presence of these concentrations of doxorubicin for 24 h, upregulated expression of the following genes: *IL6* (by 2.30 and 2.66 times, respectively), *IL1B* (by 1.25 and 3.44 times), and *CXCL8* (by 6.47 times and 6.42 times), *MIF* (2.34 and 2.28 times), *CCL2* (4.22 and 3.98 times). Under these conditions the following genes were downregulated: *IL10*, *IL1R2*, *TNF*. Cultivation of cells in the presence of doxorubicin (2 μg/ml and 6 μg/ml) for 24 h also increased the secretion of IL-6.

Key words: primary human coronary artery endothelial cells; doxorubicin hydrochloride; qPCR; gene expression; cytokines

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INTRODUCTION

Doxorubicin is an anthracycline antibiotic used in the treatment of various types of malignant tumors in humans. However, despite its efficiency against various types of cancer, doxorubicin has cardiotoxic effects [1, 2]. The administration of doxorubicin leads to the accumulation of reactive oxygen species (ROS) because quinone, a part of the anthracycline moiety, is able to be reduced to semiquinone under the action of cellular oxidoreductases. Next, semiquinone is oxidized in the presence of molecular oxygen to form superoxide anion. Thus, a cycle of redox reactions is triggered; this results in accumulation of free radicals in the cell. In this context cardiomyocytes and vascular endothelial cells are sensitive to free radical damage [3]. The amount of ROS can be also increased by free divalent iron, which forms a complex with doxorubicin and resultant products are toxic radicals and reactive nitrogen species that induce nitrosative stress and mitochondrial dysfunction [4]. Reaching systemic circulation, doxorubicin affects vascular endothelial cells and this can lead to the development of chronic vascular diseases after the end of therapy [2]. The endothelium performs various important functions for maintaining vascular homeostasis; being the main regulator of substance transport and cell migration through the vascular wall it is especially important for the barrier function [5]. Regardless of the nature of the damaging agent, endothelial damage leads to disruption of the barrier function and release of pro- and anti-inflammatory cytokines; this promotes the destruction of adhesive and cellular contacts, initiating the development of endothelial-to-mesenchymal transition [6]. It is important to note that the balance of pro- and anti-inflammatory cytokines is important for eliminating the systemic side effects and toxicities of chemotherapy [7].

This study was aimed to evaluate the cytokine profile of primary human coronary artery endothelial cells cultivated in the presence of doxorubicin.

MATERIALS AND METHODS

Cultivation of Endothelial Cells in the Presence of Doxorubicin

A commercially available culture of primary human coronary artery endothelial cells obtained from healthy donor arteries (Cell Applications, USA) was used in this study. Endothelial cells were thawed and cultured using T-75 flasks (Techno Plastic Products, Switzerland) in Human MesoEndo Growth Medium (Cell Applications) according to the manufacturer's protocol. After reaching 80% confluence, the cells were seeded into T-75 and T-25 culture flasks (Techno Plastic Products). All cultural work was carried out under sterile conditions at 37°C, 5% CO_2 and high humidity in the cell incubator (Sanyo, Japan). Next, doxorubicin hydrochloride (Sigma Aldrich, USA) was added to the culture flasks to the final concentration of 2 μg/ml or 6 μg/ml and the flasks were incubated for 24 h. These doxorubicin concentrations were selected on the basis of literature data and OECD (Organization for Economic Co-operation and Development) recommendations for testing chemical compounds [8]. The same volume of deionized water was added to the cells used as control.

Isolation of RNA and Determination of Gene Expression Levels

To extract RNA, cells after incubation were washed with a cold solution of phosphate-buffered saline and then lysed with TRIzol (Thermo Fisher Scientific, USA). RNA isolation was performed using a commercial RNeasy Plus Universal Mini Kit (Qiagen, Germany) following the manufacturer's protocol. The quality and quantity of isolated RNA were determined using a Qubit 4 fluorometer (Invitrogen, USA) by assessing the RIQ (RNA Integrity and Quality) index and using the Qubit RNA IQ Assay Kit (Invitrogen). Gene expression was evaluated by qPCR with reverse transcription according to the protocol described previously [9]. The genes encoding the main pro-inflammatory cytokines (*IL1B*, *IL6*, *CXCL8*, *IL10*, *IL12A*, *IL12B*, *TNF*, *CCL2*, *MIF*), as well as some of their receptors (*IL1R1*, *IL1R2*), were selected as genes of interest. The gene expression results were normalized to the reference genes *GAPDH, ACTB*, and *B2M*.

Analysis of the Level of Cytokines Secreted by Primary Endothelial Cells

After incubation of cells with doxorubicin, the culture medium was aliquotted into 1.5 ml Eppendorf tubes, and stored at -40°C. The secretion of cytokines (IL-1β, IL-6, IL-8, TNFα) by endothelial cells cultivated the presence of doxorubicin was carried out by measuring their concentration in the culture medium by means of enzyme-linked immunosorbent assay and commercial kits (Invitrogen; Vector-best, Russia), according to the manufacturers' instructions. Optical density was measured using a Multiskan Sky microplate spectrophotometer (Thermo Fisher Scientific).

Statistical data processing was carried out using the GraphPad Prism 7 software (GraphPad Software, USA). Normality of distribution was assessed using the Shapiro-Wilk test; comparison between groups was assessed using the Mann-Whitney U test. The expression of the studied genes was calculated using the Pfaffl method [10] and expressed as a fold change relative to control endothelial cells.

RESULTS AND DISCUSSION

Cultivation of cells in the presence of 2 μg/ml doxorubicin for 24 h increased IL-6 secretion (p=0.045) compared to unexposed cells, while cultivation with 6 μg/ml doxorubicin led to a statistically significant decrease in the level of this cytokine (p=0.011), apparently due to increased cytotoxicity of this concentration of doxorubicin (Table 1). No statistically significant differences were found in secretion of tumor necrosis factor (TNFα), IL-1β, and IL-8. In addition, comparison of the secretion of the studied cytokines in the two study groups (incubated in the presence of 2 μg/ml doxorubicin or 6 μg/ml doxorubicin) showed differences only in the level of IL-6 (p=0.021).

A comparative analysis of the expression profile of genes encoding the studied cytokines revealed a significant upregulation of *IL6*, *IL1B*, *CXCL8*, *MIF*, *CCL2* and downregulation of *IL10*, *IL1R2*, and *TNF*; expression of other genes changed insignificantly (Table 2).

Our study has shown that human coronary artery primary endothelial cells are characterized by a specific response to exposure to 2 μg/ml and 6 μg/ml doxorubicin. The gene expression profile of endothelial cells cultivated with 2 μg/ml and 6 μg/ml doxorubicin did not differ from each other, but differences were detected in the level of IL-6 secretion into the culture medium.

It was previously demonstrated that cultivation of endothelial cells under conditions of the genotoxic load caused by mitomycin C increased both the level of *IL6* and *CXCL8* gene expression and concentration of their protein products in the culture medium [11]. It has also been shown that the addition of calcium phosphate bions to primary coronary artery endothelial cells induced secretion of pro-inflammatory cytokines (IL-6 and IL-8) into the medium due to increased levels of their mRNAs [12]. Our study revealed that exposure of endothelial cells to doxorubicin (2 μg/ml and 6 μg/ml) led to a marked increase in the expression of the *IL6* and *CXCL8* genes, as well as *IL1B*, compared to the control; however, at the protein level, these differences were shown only for IL-6. An increase in the level of pro-inflammatory cytokines may be one of the triggers for the development of endothelial dysfunction, which can subsequently lead to the development of serious cardiovascular catastrophes [13].

This study also revealed a statistically significant increase in the expression level of macrophage migration inhibitory factor (*MIF*) gene in the two study groups. Macrophage migration inhibitory factor (MIF)

Table 1. The level of pro-inflammatory cytokines secreted into the culture medium

Secreted cytokines	Level of cytokines secreted into the culture medium, Me (Q1;Q3)		
	Control	**Doxorubicin 2 μg/ml**	**Doxorubicin 6 μg/ml**
IL-1β	3.82 (3.61–4.13)	5.04 (4.27–6.04)	4.82 (4.68–5.15)
IL-6	8.21 (7.21–8.24)	24.69 (20.38–45.56)*	0.83 (0.74–2.86)**
IL-8	151.70 (136.90–174.80)	170.50 (134.60–179.20)	177.70 (175.40–194.30)
TNFα	10,48 (10,38–11,68)	11.13 (10.77–11.54)	10.50 (10.35–11.39)

*, statistically significant difference in IL-6 concentration between control and 2 μg/ml doroxubicin (p=0.045); **, statistically significant difference in IL-6 concentration between control and 6 μg/ml doroxubicin (p=0.011).

Table 2. Comparative analysis of cytokine gene expression profile

Gene	Protein	Primers	Fold change after treatment with doxorubicin	
			2 μg/ml	6 μg/ml
IL1B	Interleukin-1 beta	Forward: TGGCTTATTACAGTGGCAATG Reverse: GTGGTGGTCGGAGATTCG	1.25	3.44
IL6	Interleukin-6	Forward: GGCACTGGCAGAAAACAACC Reverse: GCAAGTCTCCTCATTGAATCC	2.30	2.66
CXCL8	Interleukin-8	Forward: CAGAGACAGCAGAGCACAC Reverse: AGTTCTTTAGCACTCCTTGGC	6.47	6.42
IL10	Interleukin-10	Forward: GGAGGACTTTAAGGGTTAC Reverse: TTCACAGGGAAGAAATCG	0.58	0.56
IL12A	Interleukin-12 subunit alpha	Forward: GCCTTCACCACTCCCAAAAC Reverse: TGTCTGGCCTTCTGGAGCAT	Lack of amplification	Lack of amplification
IL12B	Interleukin-12 subunit beta	Forward: GGACATCATCAAACCTGACC Reverse: AGGGAGAAGTAGGAATGTGG	Lack of amplification	Lack of amplification
IL1R1	Interleukin-1 receptor type 1	Forward: GGCTGAAAAGCATAGAGGGAAC Reverse: CTGGGCTCACAATCACAGG	1.35	1.71
IL1R2	Interleukin-1 receptor type 2	Forward: TGGCACCTACGTCTGCACTACT Reverse: TTGCGGGTATGAGATGAACG	0.38	0.50
TNF	Tumor necrosis factor	Forward: ATGAGCACTGAAAGCATGATCC Reverse: GAGGGCTGATTAGAGAGAGGTC	0.20	0.33
MIF	Macrophage migration inhibitory factor	Forward: GGTGTCCGAGAAGTCAGGCA Reverse: GGGGCACGTTGGTGTTTACG	2.34	2.28
CCL2	Monocyte chemoattractant protein 1	Forward: TTCTGTGCCCTGCTGCTCATAG Reverse: AGGTGACTGGGGCATTGATTG	4.22	3.98

is a homotrimeric protein that acts as a pleiotropic pro-inflammatory cytokine, which is involved in the processes of leukocyte recruitment, inflammation, and cell proliferation [14]. MIF is produced by various types of endothelial and epithelial cells. Increased MIF secretion promotes the recruitment of leukocytes by increasing the expression of E-selectin, ICAM-1, VCAM-1, IL-8, and MCP-1 [15]; this is a specific response to cell damage. Thus, it can be assumed that doxorubicin may contribute to the development of endothelial dysfunction. However, further *in vitro* and *in vivo* studies are needed to confirm this hypothesis. The presented research was performed at the first time: researchers are mainly focused on studies of the doxorubicin effects on cardiomyocytes. At the same time, there are a few reports on the doxorubicin effect on the vascular endothelium. For example, Ivanova has shown that a single administration of 4 μg/kg doxorubicin to rats had a significant impact on their body mass and vascular reactivity (an increase in the amplitude of vasoconstriction to phenylephrine by 18.5%, and a decrease in reactivity to exogenous NO) [16]. Another team of authors performed experiments in which they isolated the thoracic aorta of Wistar rats and incubated it in 10 μM doxorubicin. Results of this study have shown that incubation of isolated thoracic artery for an hour incubation leads to the increased vasoconstrictor reactions to phenylephrine [17].

A group of researchers from China demonstrated that the thoracic aorta of mice treated with 2.5 mg/kg doxorubicin for 3 weeks demonstrated signs of inflammatory infiltration as well as interstitial cell hypertrophy [18]. In addition, they showed that the addition of 100 μM doxorubicin to umbilical vein endothelial cells caused an increase in the concentration of lactate dehydrogenases in the culture medium and also decreased cell viability [18]. Besides studies describing the effects of doxorubicin on endothelial cells, there are reports on its effect on smooth muscle cells [19, 20]. For example, a single intraperitoneal administration of 4 mg/kg doxorubicin to mice changed functional activity of smooth muscle cells [21].

In summary it should be noted, that the available experimental data are limited to single studies aimed at studying molecular mechanisms of the doxorubicin action on endothelial and smooth muscle cells. The study we have conducted is a pilot study for subsequent analysis of the molecular-genetic mechanisms of the doxorubicin action on endothelial cells; it clearly requires additional experiments to be performed.

CONCLUSIONS

Results of this study indicate that primary endothelial cells of the human coronary artery, cultivated in the presence of doxorubicin

(2 μg/ml and 6 μg/ml) are characterized by changes in the level of cytokine expression, that is suggesting their pro-inflammatory activation.

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COMPLIANCE WITH ETHICAL STANDARDS

The work is not related to studies involving humans or animals and was performed on commercially available cultures of primary endothelial cells.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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ЦИТОКИНОВЫЙ ОТВЕТ ЭНДОТЕЛИАЛЬНЫХ КЛЕТОК КОРОНАРНОЙ АРТЕРИИ ЧЕЛОВЕКА НА ВОЗДЕЙСТВИЕ ДОКСОРУБИЦИНОМ: ЭКСПЕРИМЕНТ *IN VITRO*

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Методами иммуноферментного анализа и кПЦР оценен цитокиновый профиль первичных эндотелиальных клеток коронарной артерии, культивируемых в присутствии доксорубицина в концентрациях 2 мкг/мл и 6 мкг/мл. Показано, что при культивировании клеток в течение суток с этими концентрациями доксорубицина наблюдается повышение экспрессии генов *IL6* (в 2,30 и 2,66 раза соответственно), *IL1B* (в 1,25 и 3,44 раза), *CXCL8* (в 6,47 и 6,42 раза), а также *MIF* (в 2,34 и 2,28 раз), *CCL2* (в 4,22 и 3,98 раз); гипоэкспрессия показана для генов *IL10*, *IL1R2*, *TNF*. Культивирование клеток в присутствии доксорубицина (2 мкг/мл и 6 мкг/мл) в течение суток также повышало секрецию IL-6.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: первичные эндотелиальные клетки коронарной артерии человека; доксорубицина гидрохлорид; кПЦР; экспрессия генов; цитокины

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