Abstract

Introduction: Endometrial cancer is the most common gynecological tumor in peri- and postmenopausal women. The best known and best-described adipokines are: leptin, adiponectin, visfatin, resistin, and omentin.

Leptin (LEP) is primarily secreted by differentiated adipocytes, influencing neoplastic angiogenesis through the activation of the JAK/STAT pathway, which results in the increased proliferation of vascular endothelial cells as well as an increased expression of the vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and anti-apoptotic proteins, namely Bcl-2. In women whose BMI is higher than 25, the risk of endometrial cancer is doubled and in those whose BMI is above 30, this risk is tripled. A higher concentration of LEP in the serum of women with endometrial cancer compared to healthy volunteers was also confirmed.

In turn, adiponectin (ADP) demonstrates an insulin-sensitizing effect, increasing the expression of nitric oxide synthase, furthermore, it also has anti-inflammatory activity through inhibiting the expression of tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6). A 6-times higher risk of cancer development is noted in obese people, in whom the concentration of ADP was lower than in the group of healthy volunteers. It is highlighted that it is this adipokine that is most strongly associated with the risk of endometrial cancer.

In accordance with the recommendations of the Polish Association of Oncological Gynecology (PAOG) from 2017, a concentration of 60 mg/m2 is used for the treatment of endometrial cancer in adjuvant therapy. The mechanism through which cisplatin acts is related to the interaction of platinum compounds with the genetic material of the cell, which is deoxyribonucleic acid (DNA), together with which they cross-link within the DNA molecule and between the molecules. Consequently, the structure of the DNA is disturbed, cracks appear, DNA and ribonucleic acid (RNA) synthesis incorrectly, as well as the inhibition of cell division and apoptosis.

Aim: The overriding aim of this study was the assessment of changes in the expression pattern of genes coding chosen adipokines and pro-inflammatory factors in an Ishikawa line endometrial cancer cell culture exposed to the effects of cisplatin, compared to a control culture.

Materials and methods: Ishikawa line endometrial cancer cells were exposed to the effects of cisplatin at concentrations of 2.5 μM, 5 μM, and 10 μM for 12, 24, and 48 hours, and afterward compared to a control culture (C), which consisted of cells untreated using cisplatin. For each exposition period and cisplatin concentration, 3 technical repetitions were conducted.

To investigate the cytotoxic influence of cisplatin in regards to the Ishikawa endometrial cancer cell line, the Sulforhodamine B test was utilized, which is an anionic dye indicating the ability to bind itself to amino acid residues of cellular proteins. Determining the cytotoxicity in this test is based on the amount of cellular protein.

The extraction of whole ribonucleic acid (RNA) from the Ishikawa line endometrial cancer cell culture exposed to cisplatin and the control culture was carried out using the TRIzol reagent. In order to conduct the quality assessment of the isolated RNA extract, the electrophoresis technique with the addition of ethidium bromide was used.

Quantitative analysis of the obtained RNA extracts was conducted using spectrophotometric measurements.

The HG-U133_A2 oligonucleotide microarray technique was used to assess the expression profile of genes, selected pro-inflammatory factors, and adipokines in an Ishikawa line culture exposed to the effects of cisplatin, compared to a control. The genes used in the microarray analysis were selected based on the AffymetrixNetAffx™ Analysis Center database (http://www.affymetrix.com/analysis/index.affx).

The Real-Time Quantitative Reverse Transcription Reaction (RTqPCR) was used to confirm the observed changes selected using the microarray technique. This stage of analysis is significant, as the microarray experiment is a semi-quantitative, screening examination, therefore, the confirmation of the results using a quantitative technique is necessary.

The last stage of molecular analysis was the assessment of the changes in the concentration of the selected proteins in the Ishikawa line endometrial cancer culture medium, to which cisplatin was added, compared with a control culture, which consists of cells unexposed to the drug. To achieve this, a sandwich version of the Enzyme-Linked Immunosorbent Assay (ELISA) test in 96-well plates, in accordance with the recommendations of the manufacturer.

Statistical analysis was conducted using the Transcriptome Analysis Console program (Thermo Fisher Scientific) and STATISTICA 13.3 PL program (Cracow,

Polska).

Results: Based on the obtained results of the cytotoxicity test, it can be observed that, independently from the cisplatin concentration, which was added to the cell culture, a drop in the number of living cells, compared to the control culture, is noted.

Furthermore, on the basis of the dose-effect diagram, a 5 μM cisplatin concentration is the average inhibitory concentration (IC50) for the growth of Ishikawa line endometrial cancer cells.

From the results of the microarray experiment, it can be observed that under the influence of cisplatin, there is a decrease in the transcriptional activity of leptin and its' three receptors. A lower expression of the discussed genes was noted, the higher the concentration of the drug added to the culture and the higher the exposition time of the cells to the drug was ($p<0.05$).

In turn, for adiponectin and its' receptors, an opposite expression profile to leptin and its' receptors is noted, depending on the cisplatin concentration and treatment time of the endometrial cancer cells using it. The higher the drug concentration and cell exposition time, the higher the overexpression of adiponectin and its' receptors.

In the next stage of molecular analysis, changes in the expression profile of selected pro-inflammatory factors in the endometrial cancer cell culture under the influence of cisplatin were assessed, using the HG-U133_A2 oligonucleotide microarray technique ($p<0.05$). It can be observed that the addition of cisplatin to the cell culture resulted in overexpression of *TNF-α* and *IL-6* with a simultaneous decrease in *STAT3* and *JAK2* expression ($p<0.05$). Differences in the transcriptional activity of the assessed genes were more visible, the higher the utilized cisplatin concentration and incubation time were ($p<0.05$).

In turn, on the protein level, at a cisplatin concentration of 2.5 μM, statistically significant differences after 24 and 48 hour-long expositions to the drug were noted, compared to the control ($p<0.05$).

However, at higher cisplatin concentrations, to which endometrial cancer cells were exposed, statistically, significant differences were noted for higher concentrations regardless of incubation time ($p<0.05$). The conducted statistical analysis also indicated significant differences in the expression of leptin at the protein level between the individual exposition times. Significant changes in the adiponectin concentration and the proteome level were visible only when cisplatin at a 5 μM concentration was added

to the cell culture, regardless of the exposition time of the cells to the drug ($p<0.05$). Statistical analysis also indicated that at concentrations of 5 μM and 10 μM, the leptin concentration significantly changed between the exposition periods lasting 12 and 38 hours ($p<0.05$).

The final stage of molecular analysis was the calculation of the concentration for TNF-α, STAT3, JAK2, and IL-6 on the protein level in an Ishikawa line endometrial cancer cell culture exposed to cisplatin, compared to a control culture ($p<0.05$). Worth noting is the fact that in the case of TNF-α, its' expression profile on the transcriptome and proteome levels are opposite to each other. On the protein level, a lower concentration of this cytokine can be observed in the cell culture exposed to the drug, compared to the control ($p<0.05$). In the case of the remaining factors, on the mRNA and protein levels, the same trend of changes in expression was noted, whereas changes on the proteome level seem to be less clear compared to the transcriptome level ($p<0.05$).

Furthermore, in the final stage of analysis of the obtained results, positive relations between the expression of the assessed adipokines and pro-inflammatory factors on the mRNA and protein levels were searched for ($p<0.05$).

On the mRNA level, statistically significant positive relations of expression between *LEP* and *LEPROT* were found ($r=0.72$; $p<0.05$); LEP and LEPROTL1 ($r=0.97$; $p<0,05$); *LEP* and *LEPR* ($r=0.87$; $p<0.05$); LEPROT and LEPROTL1 ($r=0.76$; $p<0.05$); *LEPROT* and LEPR ($r=0.83$; $p<0.05$); *LEPROTL1* a *LEPRC* ($r=0.76$; $p<0.05$); *ADIPOR2* and *TNF-α* ($r=0.81$; $p<0.05$); *ADIPOR 2* and *IL-6* ($r=0.87$; $p<0.05$).

In turn, negative relations were noted between the following genes: *LEP* and *ADP* ($r= -0.91$; $p<0.05$); *LEP* and *ADIPOR2* ($r= -0.86$; $p<0.05$); *LEP* and *TNF-α* ($r = - 0.81$; $p<0.05$); *LEP* and *IL-6* ($r= -0.94$; $p<0.05$); *LEPROT* and *ADP* ($r= -0.83$; $p<0.05$); *LEPROT* and ADIPOR2 ($r= -0.92$; $p<0.05$); *LEPROT* and *IL-6* ($r=- 0.85$; $p<0.05$); *LEPROTL1* and *ADP* ($r= - 0.94$; $p<0.05$); *LEPROTL1* and *ADIPOR2* ($r= -0.87$; $p<0.05$); *LEPROTL1* and TNF-α ($r= -0.72$; $p<0.05$); *LEPROTL1* and *IL-6* ($r= -0.94$; $p<0.05$); *LEPR* and *ADP* ($r= - 0.96$; $p<0.05$); *LEPR* and *ADIPOR2* ($r= -0.91$; $p<0.05$); *LEPR* and *TNF-α* ($r= -0.74$; $p<0.05$); *LEPR* and *IL-6* ($r= - 0.85$; $p<0.05$).

On the protein level, a statistically significant strong positive relationship between expression was observed for: TNF-α and LEP ($r=0.83$; $p<0.05$); STAT3 and LEP ($r=0.98$; $p<0.05$); TNF-α and STAT3 ($r=0.87$; $p<0.05$) as well as one negative correlation between ADP and JAK2 ($r= -0.74$; $p<0.05$).

Conclusions: Cisplatin in an endometrial cancer cell culture of the Ishikawa line caused a change in the expression profile of genes and the proteins they coded: leptin and its' receptors; adiponectin; IL-6; TNF-α; and JAK-2. The results obtained by us suggest that cisplatin exerts a biological effect through the JAK/STAT pathway. Moreover, it is possible that in the regulation of TNF-α expression, microRNA particles are involved.

Changes in the expression profiles of leptin, adiponectin, IL-6, TNF-α, and JAK-2 can be explored in the context of utilizing them as supplementary diagnostic markers of endometrial cancer, monitoring the effectiveness of cisplatin therapy, and in the creation of new therapeutic strategies aimed toward the JAK/STAT signaling pathway.

Key words: endometrial cancer, Ishikawa lineage, cisplatin, adipokines, leptin, adiponectin, interleukin 6, tumor necrosis factor alpha, molecular marker, mRNA, protein, JAK/STAT, inflammatory process, carcinogenesis, Janus kinase 2, transcription induction factor 3