Effect of metformin on apoptosis induction in ovarian cancer cells

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Abstract

Introduction: Ovarian cancer is one of the most difficult problems in gynecologic oncology and the search for new drugs effective in the treatment of this kind of cancer, especially in cases resistant to current forms of therapy, remains a challenging priority.

The aim of the study was to analyze the effect of metformin on apoptosis and the *BIRC5* gene expression in ovarian cancer cell line SKOV-3. The *BIRC5* gene encodes survivin protein.

Material and methods: SKOV-3 cells were treated with metformin (10 mM). Apoptotic changes in studied cells were analyzed by double staining using a mixture of fluorochromes – Hoechst 33258/propidium iodide (PI). The expression of the *BIRC5* gene at the mRNA level was analyzed using the real-time PCR technique.

Results: Treatment of cells with metformin causes changes in the cell shape from oval to spindle and leads to the separation of the cells from the monolayer. Furthermore, metformin induces apoptosis and necrosis of ovarian cancer cells. A statistically significant increase in the number of apoptotic cells after 48 and 72 hours' treatment with metformin relative to a control cells seems to be correlated with a decrease in the expression of the *BIRC5* gene at the mRNA level.

Conclusions: Metformin seems to be a promising agent, whose use in ovarian cancer patients may contribute to improving the effectiveness of therapy.

Key words: metformin, ovarian cancer, SKOV-3, apoptosis, *BIRC5*, survivin.

Introduction

Metformin (1,1-dimethylbiguanide hydrochloride), a biguanide derivative, is a widely prescribed antihyperglycemic drug which is used as the first-line therapy for diabetes mellitus type 2. Metformin primary actions include inhibition of hepatic glucose production and reduction of insulin resistance in peripheral tissue, leading to enhanced glucose uptake and utilization in skeletal muscle [1]. Metformin has been shown to act as a tumor growth inhibitor, at least in part by upregulation of AMP-activated protein kinase (AMPK) activity and by downstreaming suppression of signaling through the mammalian target of rapamycin (mTOR) [2-5]. It has been shown that the anticancer effect of metformin has a close relationship with the apoptosis induction [6-9]. In studies concerning triple negative breast cancers (TNBC, triple-negative breast cancer) it was found that metformin caused the cell cycle arrest in the S phase and induction of apoptosis [10]. Similar results showing apoptosis induction by metformin in pancreatic cancer cell line ECC-1 were obtained by Wang *et al.* [11] in relation to the apoptosis induction in pancreatic cancer cell line ASPC-1, BxPC-3, PANC-1 and SW1990, exposed to metformin.

It was also found that metformin-induced apoptosis is associated with PARP (poly-ADP-ribose polymerase) cleavage and activation of caspase-3, -8, and -9 in a time- and dose-dependent manner [11]. In the case of endometrial cancer cell line ECC-1, metformin also enhances the effects of other anticancer drugs such as paclitaxel (PTX) [12].

Survivin is a protein acting as an apoptosis inhibitor and is involved in cell cycle regulation, especially at the transition from the G1 to S phase. This protein is encoded by the *BIRC5* gene (baculoviral IAP repeat containing 5), which is located on the long arm of chromosome 17 (17q25.3). Survivin expression in non-neoplastic tissues is limited to the normal thymocytes, hematopoietic CD34+ stem cells and colon epithelial cells while survivin overexpression is observed in many types of malignant cells [13].

This protein controls the caspase-dependent and caspase-independent apoptosis [14]. In the cells receiving the signal to apoptosis, survivin inhibits caspase 3, 7 and 9 [15].

Currently, in the literature there are no reports on the relationship between metformin, survivin and apoptosis. In view of the above, the aim of this study was to analyze

the effect of metformin on apoptosis and the *BIRC5* gene expression in ovarian cancer cell lines SKOV-3.

We used ovarian cancer cells for our studies because this kind of cancer is one of the most difficult problems in gynecologic oncology [16] and the search for new drugs effective in the ovarian cancer treatment, especially in cases resistant to current forms of therapy, remains a challenging priority.

Material and methods

Cell culture

The human SKOV-3 cell line was obtained from the American Type Culture Collection (ATCC) based in Rockville, MD, USA. Cells were grown as a monolayer in sterile polystyrene dishes (Nunc, Denmark). Cells in the logarithmic phase of growth were used in all experiments. The cells were routinely screened for the Mycoplasma contamination and were maintained as a monolayer in RPMI 1640 (Immuniq, Poland) supplemented with 10% heat inactivated FBS (Cambrex, Basel, Switzerland), penicillin (10 U/ml) and streptomycin (50 µg/ml). The cells were grown at 37°C in a 5% CO_2 atmosphere in a culture medium and were passaged after covering about 80-90% of the dish.

Spectrophotometric determination of cells survival after treatment with metformin

The SKOV-3 cell line survival after metformin treatment (Sigma-Aldrich, Corp., St. Louis, MO, USA) was determined by the spectrophotometric method (17) in 96-well plates. We determined IC_{50} parameter using a computer program ED50plus v1.0 (http://www.free-downloads-center.com/download/ed50plus-v1-0-2434.html).

Double staining with Hoechst 33258 and propidium iodide

For determination of apoptotic changes, by double staining, cells were incubated with a concentration of 10 mM metformin, for 24, 48 and 72 hours. Then, a suspension of cells having a density of 1×10^5 cells/ml was prepared and a mixture of fluorochromes – Hoechst 33258/propidium iodide (PI) (Sigma-Aldrich, Corp., St. Louis, MO, USA) was added. The concentration of the individual fluorochromes was as follows: Hoechst 33258 – 0.13 mM, PI – 0.23 mM and the cells were incubated at room temperature for 10 min in total darkness. The cells were then applied to glass slides and at least 300 cells were counted under the fluorescence microscope (Olympus IX 70, Japan), using UV filter (360-370 nm) [18]. The percentage of cells following fraction was calculated: live – blue-gray uniform color; early apoptosis – deep blue with a distinct chromatin condensation, cells in late apop-

tosis – pink, with a purple stained nucleus and chromatin segregation and necrotic cells were red. The total number of cells in the sample was taken as 100%. Representative areas of cells stained at 48 h of incubation with metformin were chosen for photographic documentation.

RNA isolation and cDNA synthesis

Total RNA was isolated using Trizol® Reagent (Sigma-Aldrich Corp., St. Louis, MO, USA) according to the manufacturer's protocol and quantified spectrophotometrically. First-strand cDNAs were obtained by reverse transcription of 1 µg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, USA) following the manufacturer's protocol.

Quantitative real-time RT-PCR (RT-qPCR)

Real-time PCR gene expression analysis of the target gene (*BIRC5*) was performed using TaqMan® Gene Expression Assays (Applied Biosystems) according to the manufacturer's instructions. The HPRT1 (hypoxanthine phosphoribosyltransferase 1) gene was used as an internal control. The assay numbers for these genes were as follows: *BIRC5* – Hs04194392_s1, HPRT1 – Hs02800695_m1. Each PCR reaction was performed in a 10 µl volume that included 5 µl of 2 × TaqMan Universal PCR Master-Mix (Applied Biosystems), 4.5 µl of water diluted cDNA template (50 ng) and 0.5 µl of TaqMan® Gene Expression Assay consisting of a pair of unlabeled PCR primers and TaqMan probe with a FAM™. The RT-qPCR reaction was carried out using the Mastercycler ep realplex (Eppendorf). Relative RNA quantification was performed using the ΔCt method. ΔCt ($Ct_{BIRC5} - Ct_{HPRT1}$) values were recalculated into relative copy number values (number of *BIRC5* mRNA copies per 1000 copies of HPRT1 mRNA).

Statistical analysis

Statistical analysis was performed using the STATISTICA version 11.0 (StatSoft, Poland). ΔCt values obtained from quantitative real-time PCR were recalculated into relative copy number values. Obtained results were not normally distributed (Kolmogorov-Smirnov test) and therefore, nonparametric statistical tests were used for analyzing the results. A value of $p < 0.05$ was considered statistically significant.

Results

Changes in the morphology of cell line SKOV-3 after treatment with metformin

The spectrophotometric method with MTT allowed to determine the IC50 value of metformin (14 mM).

For further experiments, the concentration of 10 mM was selected corresponding to the MET survival of more than 60% relative to control cells, not treated with the drug. This is a concentration corresponding to the physiological dose used in the women tumors treatment [19].

Figure 1 shows the morphology of cells treated with metformin (10 mM, 24-72 h) observed with the use of a phase contrast microscope (Olympus IX 70, Japan). Changes in the cell shape were dependent on the time of incubation. It was noted that in the case of control cells (not treated with metformin), over time, the number of cells in the visual field increased. After 72 hours, almost all the dish was covered with cells.

In contrast, in samples treated with metformin, the number of cells increased more slowly, which confirms the anti-proliferative properties of the tested compound. Metformin induced elongation and changes in the cell shape from oval to spindle and separation of the cells from the surface of the monolayer (Fig. 1).

Apoptotic changes induced by metformin

The double staining method with fluorochromes Hoechst 33258 and propidium iodide allowed to observe morphological changes in the nucleus, associated with the different phases of cell death. Four fractions of the cells were identified: live cells, in the early phase of
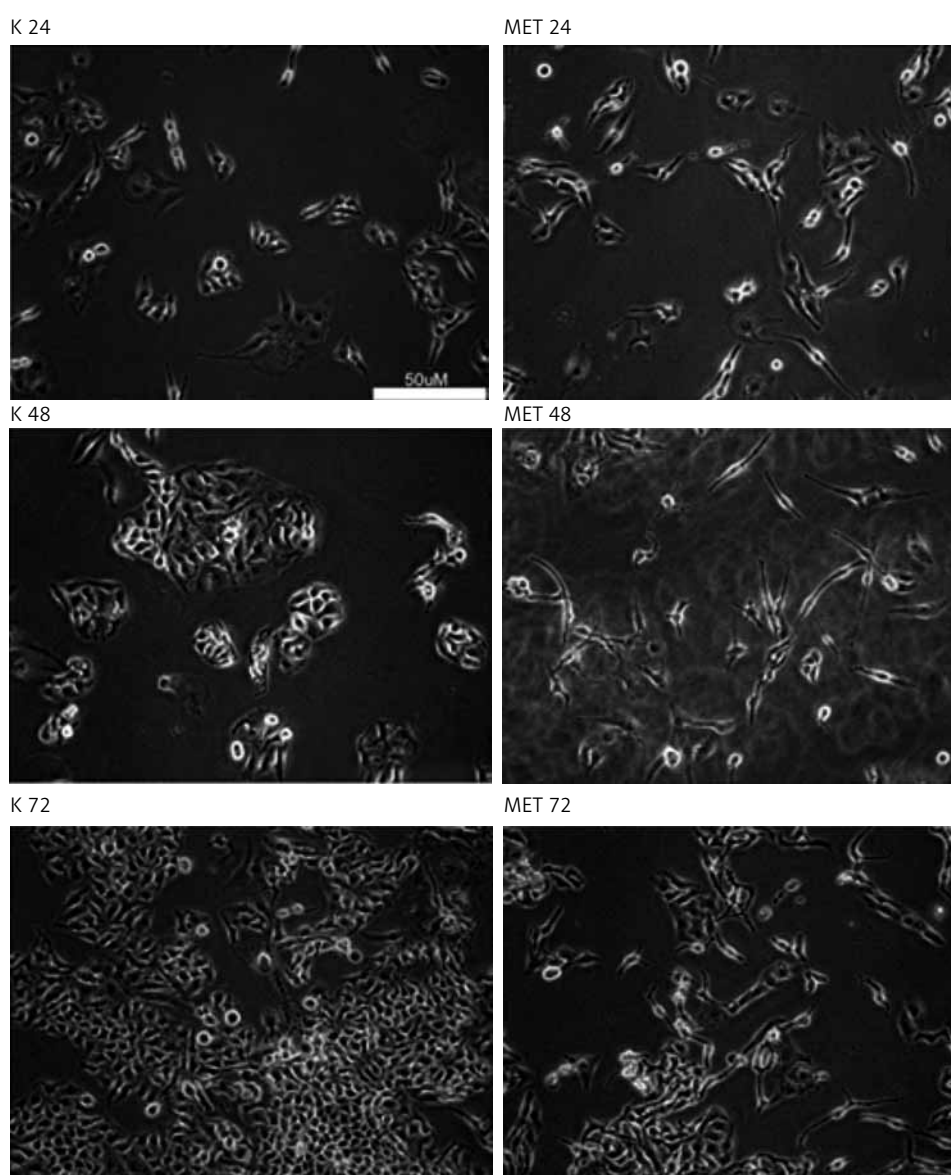

Fig. 1. The morphology of control cells and cells treated with metformin (10 mM, 24-72 hrs.). Pictures were taken using an inverted microscope equipped with a camera, in a phase contrast (Olympus IX70, Japan) (scale = 50 µm). K – control cells, not treated with metformin, MET 24, 48 and 72 – cells treated with metformin for 24, 48 and 72 hours

apoptosis, late stage apoptosis and necrotic cells. Sample images of control cells and incubated with metformin are shown in Figure 2. After treatment the cells with metformin changes in the structure, size and shape of the cell nucleus, such as chromatin condensation and marginalization accompanying early apoptosis and the appearance of apoptotic bodies, typical changes of late apoptosis were noted. There was also observed damage of the cell membrane, allowing propidium iodide to penetrate into the cell nucleus and color it red – a typi-

cal hallmark of necrosis. The quantitative analysis of individual fractions is presented in Figure 3. It was shown that metformin induced both apoptotic and necrotic cell death. After 24 h incubation with the drug, the number of apoptotic and necrotic cells was similar. However, after 48 and 72 hours, apoptosis was the predominant fraction. The percentage of apoptotic cells was 18.8% and 24.3%, respectively for 48 and 72 h and the percentage of necrotic cells was 10.2% and 14.2%, respectively. These differences were statistically significant (Fig. 3).
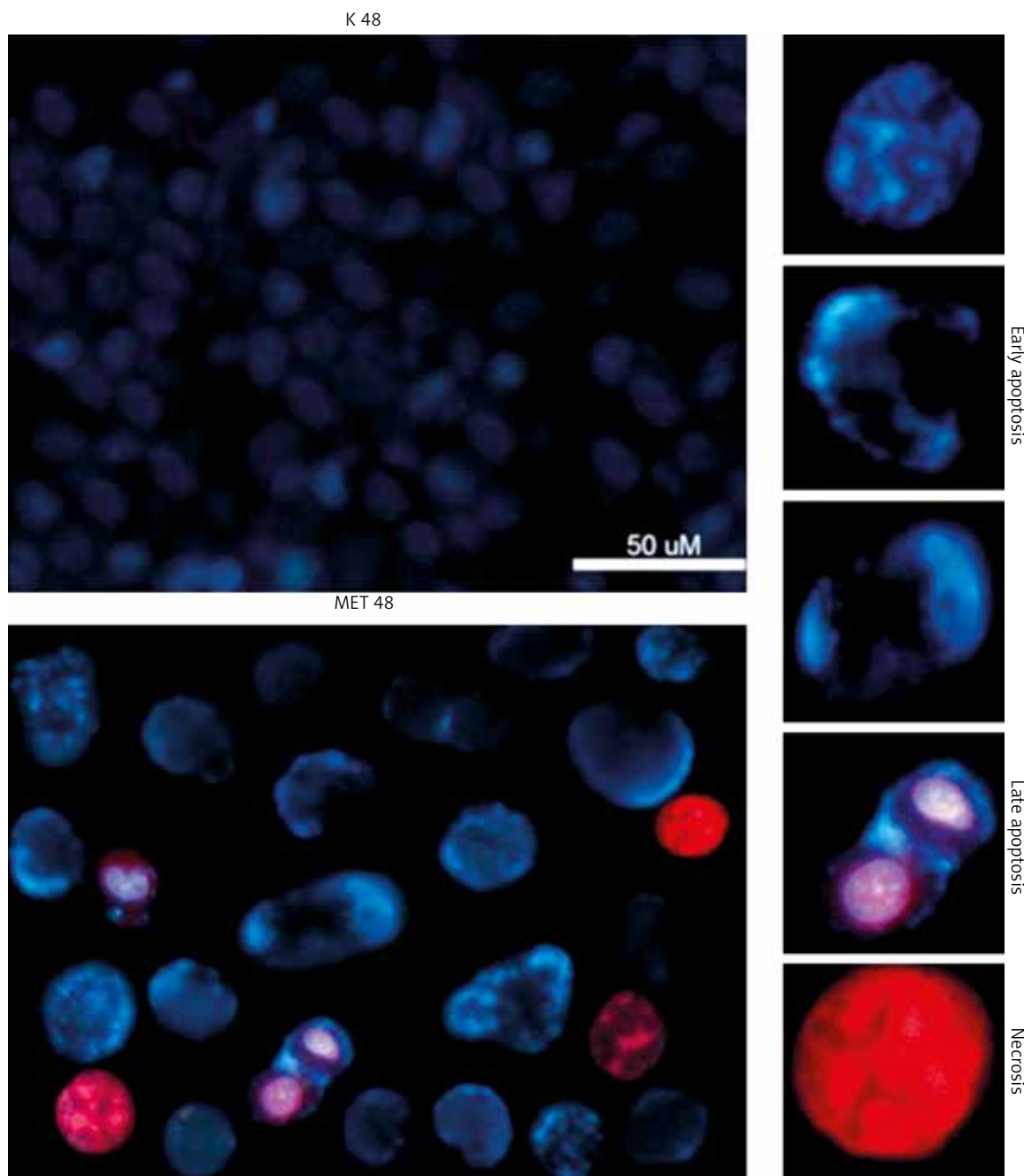

Fig. 2. Morphological changes, typical of apoptosis and necrosis observed after 48 hours of incubation of the SKOV-3 cell line with metformin (10 mM). Observation was carried out using a fluorescence microscope (Olympus IX70, Japan). The right panel shows the selected cells in the early and late stages of apoptosis and necrosis
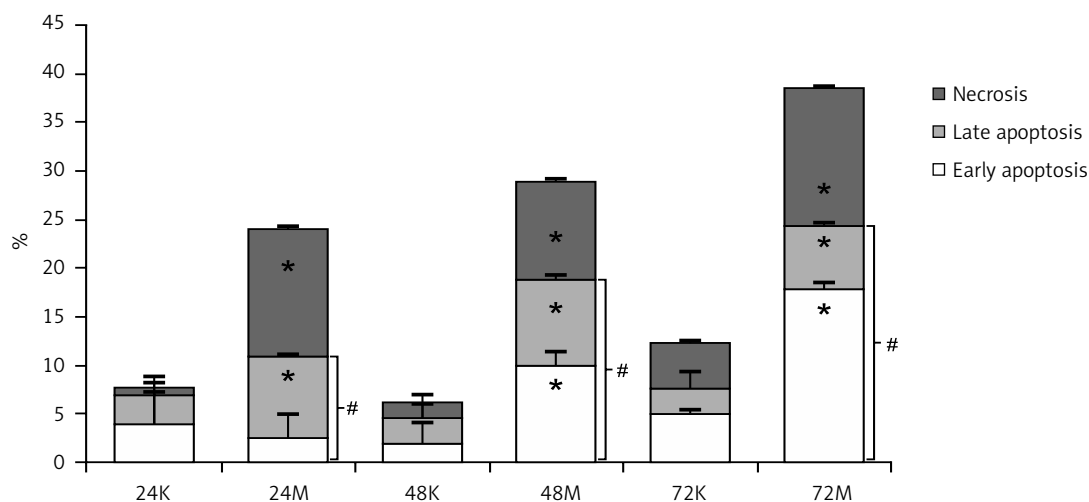
Fig. 3. Double staining of the SKOV-3 cell line with Hoechst 33258/PI mixture (metformin at a concentration of 10 mM). Results are presented as ± SD of four experiments. (*) $p < 0.05$ statistically significant differences between trials treated and not treated with metformin, (#) $p < 0.05$ statistically significant differences in the number of apoptotic cells in samples fraction incubated with metformin and control cells

Effect of metformin on the BIRC5 gene expression

The *BIRC5* mRNA expression was analyzed in ovarian cancer cell line SKOV-3 treated with 10 mM metformin solution. Gene expression was investigated in cells exposed to the compound for 24, 48 and 72 hours. Metformin untreated cells were used as a control. In the case of the *BIRC5* mRNA expression, a statistically significantly higher level was found in cells incubated with metformin for 24 and 48 hours as compared to cells incubated for 72 hours. In untreated cells, the *BIRC5* mRNA expression level was the highest after 48 hours from the start of the experiment, whereas the lowest value was reached at 72 hours of the experiment. It was also observed that after 48 and 72 hours of incubation, metformin decreased the *BIRC5* mRNA expression level, compared to the control.

A diagram illustrating the relative expression of *BIRC5* mRNA in metformin-treated and untreated ovarian cancer cell line SKOV-3 is shown in Figure 4.

Discussion

The maintenance of homeostasis in a multicellular organism is associated with the balance between such processes as proliferation, differentiation and cell death. Ovarian cancer is most frequently diagnosed at an advanced stage [20, 21]. In the diagnosis of changes in the differentiation of malignant cancer, it is very important to check the family history, biochemical markers and ultrasonography image (USG) [22]. Progression of the disease might be related to immune response to the developing cancer [23]. Moreover, recent studies show that diabetes is conducive not only to cardiovas-

cular diseases, nephropathy, retinopathy and neuropathy, but also to the development of many types of cancers. Recent data have shown that metformin, a drug commonly used in diabetic patients, can significantly decrease the incidence rate and death risk associated with malignant cancers [24]. However, the evidence of the effects of metformin on ovarian cancer is still limited and inconclusive. Metformin was introduced into the clinical practice in the 1950s for the treatment of type II diabetes mellitus but presently it is often used for the treatment of other conditions associated with insulin resistance, especially polycystic ovarian syndrome [25, 26]. Current studies report positive effects in
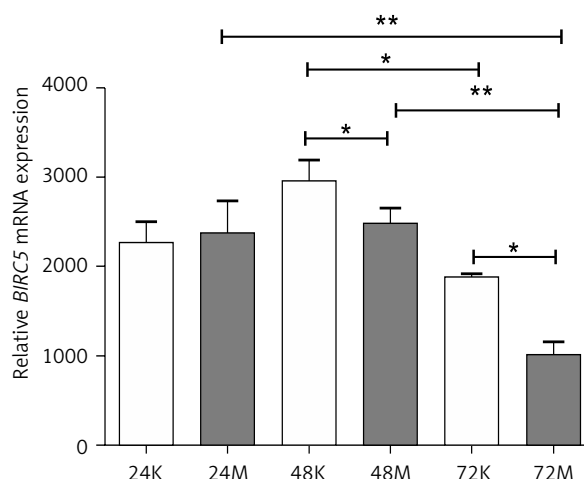

Fig. 4. Relative *BIRC5* mRNA expression in metformin-treated and untreated ovarian cancer cell lines SKOV-3 after 24, 48 and 72 hours. K – control cells not treated with 10 mM metformin, M – cells treated with metformin. (*) $p < 0.05$; (**) $p < 0.01$ – statistically significant differences

the treatment of gynecological cancers such as ovarian, endometrial and cervical cancer [27]. For ovarian cancer, preclinical studies have shown that metformin can inhibit the proliferation of cancer cell lines. It was found that approximately 50% of diabetic patients receiving metformin survived for 5 years longer than nondiabetic patients who did not receive metformin [28, 29].

Our study confirms cytotoxic and anti-proliferative properties of metformin on the SKOV-3 cells. Similar studies were performed on the OVCAR-3 and OVCAR-4 cell lines, where metformin induced significant proliferation inhibition in a dose- and time-dependent manner. The drug induces an increase in the AMPK activity. Moreover, upregulation of AMPK was associated with a reduced phosphorylation of p70S6K and S6 kinase. In turn, these downregulations are known to decrease mRNA translation and protein synthesis, that would explain the anti-neoplastic effect [3]. In the metformin treatment of mouse ovarian cancer, an increase in the AMPK level was observed. Inhibition of mTOR pathway by AMPK was also shown, and so was reduction of the expression of cyclin D1, which in turn inhibits the proliferation of ovarian cancer cells [30].

Necrosis and apoptosis are considered as two main mechanisms leading to cell death. Apoptosis, also called programmed, physiological, active, suicide or altruistic cell death, is a form of cell death in which a programmed sequence of events involving activation of specific genes leads to cell elimination. This process is essential for embryonic development, in the elimination of old cells having the mutated genetic material, as well as atrophy of the hormonal dependent tissues (e.g. peeling of the mucosa during the menstrual cycle). In normal conditions, cells are constantly being produced and apoptosis takes place to eliminate a similar number of older cells, so that the final number of cells is constant [6].

Excessive cells resistance to apoptosis may be associated with neoplastic transformation, while too extensive cell death due to apoptosis or necrosis may result in degenerative diseases and neurological disorders [31].

In this article we have evaluated the level of apoptosis in ovarian cancer cells treated by metformin. Our results were confirmed by other researchers. Metformin induced apoptosis in OVCAR-3 and OVCAR-4 cell lines in an AMPK-independent manner and provoked a cell cycle arrest in the S and G2/M phase. Apoptosis in OVCAR-3 and OVCAR-4 cells was induced by activating caspases 3/7, down-regulating Bcl-2 and Bcl-xL expression, and up-regulating Bax and Bad expression [32].

It has been shown that survivin plays a key role in the apoptosis modulation.

In the cell lines, an increased expression of this protein is associated with inhibition of apoptosis induced by factors stimulating external and internal apoptotic pathways [33]. The expression of survivin was not observed in the mitochondria of normal cervical, colorectal and kidney cells, whereas this protein was detected in mitochondria of breast cancer cells [34]. In response to cell death-inducing agents, the mitochondrial survivin level decreases because the protein is released into the cytoplasm, where it prevents the activation of caspases and thereby inhibits apoptosis [15].

According to a published study by Nair *et al.* [35], the molecular basis of the metformin and survivin relationships are associated with a decreased expression of Sp transcription factors. The pancreatic cancer cell lines Panc1, and Panc28 L3.6pL treated with metformin showed a reduced Sp1, Sp3 and SP4 protein expression and a decreased expression of proto-oncogenes, which are regulated by these transcription factors (e.g. Bcl-2, survivin, cyclin D1, vascular endothelial growth factor and its receptors, and fatty acid synthase).

In the liver cancer gene therapy, anti-apoptotic activity of survivin was inhibited by use of antisense RNA, which resulted in induction of apoptosis and an increase in cell sensitivity to chemotherapy [36, 37].

The present study confirmed that apoptosis caused by metformin treatment of ovarian cancer cells SKOV-3 is associated with alteration in the *BIRC5* mRNA expression. A statistically significant increase in the number of apoptotic cells after 48 and 72 hours' treatment with metformin relative to control cells seems to be correlated with a decreased expression of the *BIRC5* gene at the mRNA level. According to the literature, and on the basis of our results, metformin, a compound that reduces the *BIRC5* gene expression appears to be a promising agent that may increase the therapy effectiveness of ovarian cancer patients.

Disclosure

Authors report no conflicts of interest.

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