ORIGINAL ARTICLE _

Metformin induces apoptosis of melanoma B16 cells via PI3K/ Akt/mTOR signaling pathways

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Summary

Purpose: To investigate the influences of metformin on the proliferation and apoptosis of mouse melanoma B16 cells through regulating the phosphatidylinositol 3-hydroxy kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling pathway.

Methods: The mouse melanoma B16 cell strains were taken as the subjects of the study, and were randomly divided into the control group with RPMI-640 medium with the volume same with that in the metformin group (C group, n=6) and Q group [metformin:10 mmol/L (Q1 group, n=6), metformin:20 mmol/L (Q2 group, n=6), and metformin:40 mmol/L (Q3 group, n=6]. The cell counting kit-8 (CCK-8) method was employed to detect the proliferation of cells in each group at different culture times. The apoptosis of cells in each group was detected via flow cytometry and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL).

Results: The proliferation rate in *Q* group was lower than in C group, and the difference was statistically significant (p<0.05 or p<0.01). According to the results of flow cytometry, it was concluded that the number of apoptotic cells in Q group was higher than in C group (p<0.05 or p<0.01). TUNEL results revealed that compared with that in C group, the apoptosis rate in Q group was increased (p<0.05 or p<0.01). Moreover, western blotting results showed that the protein expression levels of PI3K, Akt and mTOR in Q group were higher than in C group.

Conclusions: Metformin can inhibit the proliferation of mouse melanoma B16 cells and induce their apoptosis probably through its regulation on the PI3K/AKT/mTOR signaling pathway in cells.

Key words: metformin, PI3K/Akt/mTOR, melanoma B16 cells, proliferation, apoptosis

Introduction

Melanoma is an infrequent skin carcinoma with extremely high grade of malignancy, characterized by early metastasis, poor curability, and high recurrence and mortality rates. With the gradual development of tumor cells and epithelial tumor cells invading adjacent tissues, the disease gradually worsens [1]. The depth of tumor cell invasion is closely related to poor prognosis and disease metastasis. Once tumor cells invade adjacent tissues, the 5-year survival rate of melanoma patients will be reduced from 91.3% to 16.0% [2]. It is urgent to improve the monitoring methods for metastatic melanoma, so as to facilitate the early

duct new clinical intervention researches in detecting the progression and deterioration of melanoma by using activated and specific markers.

Metformin is a biguanide oral hypoglycemic drug with multiple mechanisms of action, which has attracted much attention due to its significant hypoglycemic effect, low side effects, and excellent patient compliance. More and more scientific research has proved that, in addition to the hypoglycemic effect, metformin also has a variety of other functions including lowering blood lipids, controlling appetite, and improving the survival rate of ovarian cancer patients [3]. Metformin has diagnosis and treatment. It is also necessary to con- a certain therapeutic effect on a variety of diseases

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related to endocrine system disorders. At present, the pathogenesis of melanoma is not well understood. Some experts have confirmed that it may also be related to the endocrine hormone imbalance [4]. Therefore, in this work, the influence of metformin on the proliferation and apoptosis of mouse melanoma B16 cells and its possible mechanisms of action were studied, providing a new way to improve the survival rate and quality of life of melanoma patients in the future.

Methods

Materials

Mouse melanoma B16 cells were purchased from Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). Metformin with 99% purity was purchased from Sigma (St. Louis, MO, USA). Melanoma cell culture medium (RPIM-1640) and fetal bovine serum (FBS) were purchased from Gibco (Rockville, MD, USA). Cell counting kit-8 (CCK-8) was purchased from Dojindo (Kumamoto, Japan). The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) apoptosis kit was purchased from Roche (Basel, Switzerland). The diaminobenzidine (DAB) kit was purchased from Wuhan PhD Bioengineering Co., Ltd. (Wuhan, China). Phosphatidylinositol 3-hydroxy kinase (PI3K), protein kinase B (Akt) and mammalian target of rapamycin (mTOR) antibodies were purchased from Abcam (Cambridge, MA, USA).

Experimental groups

Mouse melanoma B16 cells were placed in 10% FBS + RPMI1640 and cultured in a cell incubator with 5% CO₂ and saturated humidity at 37°C for 2-3 days. Cells in the logarithmic growth phase were collected, and the cell concentration was adjusted to 2×10^5 /L. The control group (C group) was given an equal volume of RPIM-1640 medium. Based on previous experimental studies, the experimental group (Q group) was subdivided into Q1 group (100 mmol/L metformin), Q2 group (20 mmol/L metformin) and Q3 group (40 mmol/L metformin). Five replicates were made for each group.

Detection of proliferation of melanoma cells via CCK-8

After the cell concentration in Q group was adjusted to 2×10^5 cells/well, cells were seeded in 96-well plates, and cultured for 12 h. Subsequently, the corresponding con-

centration of metformin was added. The C group was only added with the same amount of medium. The cell proliferation was detected according to the instructions of CCK-8 after 3, 6, 12 and 24 h of culture. The optical density (OD) value at 450 nm was measured by a microplate reader.

Detection of cell apoptosis via flow cytometry

First, the cultured melanoma cells were placed into a 24-well plates. After incubation for 12 h, the final metformin concentration was adjusted to 10 mmol/L (Q1 group), 20 mmol/L (Q2 group) and 40 mmol/L (Q3 group), respectively, and the same amount of culture medium was added into the wells of C group, followed by 24-h of metformin intervention. The supernatant was aspirated using a pipette and washed 3 times with 3 mL of sterile phosphate buffered saline (PBS). The obtained samples were digested with trypsin, centrifuged in 1500 r/min, washed with PBS for 3 times (centrifuged in 1500 r/min for 5 min), and finally diluted to 5×10^8 cells/well. Then, 500 µL of buffer was added to suspend cells. Five µL of Annexin V-FITC/propidium iodide (PI) (fluorescein isothiocyanate) flow antibodies was quickly added and mixed evenly using an oscillator. The samples were packed in tin foil at room temperature to avoid light. Flow cytometry was performed as soon as possible after 20 min of antibody reaction.

Detection of cell apoptosis via TUNEL

Melanoma cells were cultured at a concentration of $1 \times 10^{\circ}$ cells/well for 24 h. Then, these cells were washed, rinsed, dripped with 50 µL of TUNEL detection solution, mixed, and incubated at 37°C in the dark for 60 min according to the operating instructions of TUNEL assay kit. Finally, the samples were sealed with anti-fluorescence decay sealers and observed under a green fluorescence microscope. TUNEL-positive (green) cell rate was calculated by selecting 10 fields of vision (magnification 200x).

Western blotting

The total proteins of each group were extracted and their concentrations were measured by bicinchoninic acid. In addition, the protein concentration in endothelial cells was measured. The prepared protein sodium dodecyl sulfate loading buffer was mixed and boiled at 95°C for 3 min. The same amount of proteins was separated by 10% polyacrylamide gel electrophoresis (Applygen Technologies Inc., Beijing, China), and transferred to the nitric acid films, followed by sealing with 10% skimmed milk. Then, the cells were incubated with

Table 1. Comparison of the effects of metformin on the proliferation of melanoma cells (n=6, x±s)

Groups	3 h	6 h	12 h	24 h
C Group	1.04±0.07	1.51±0.13	2.14±0.20	2.72±0.26
Q1 Group	1.01 ± 0.07^{a}	1.19±0.16 ^a	1.83 ± 0.19^{b}	2.21 ± 0.19^{b}
Q2 Group	0.81 ± 0.06^{b}	1.02 ± 0.11^{b}	1.28±0.16 ^c	1.71±0.16 ^c
Q3 Group	0.67 ± 0.03 ^{bd}	0.85 ± 0.07 ^{cd}	1.05±0.11 ^{ce}	1.18±0.19 ^{ce}

 ${}^{\rm a}p{>}0.05,\,{}^{\rm b}p{<}0.05,\,{}^{\rm c}p{<}0.01,\,vs.$ C group, ${}^{\rm d}p{<}0.05,\,{}^{\rm e}p{<}0.01,\,vs.$ Q1 group.

antibodies of PI3K (diluted at 1:2000), Akt (1:1000) and mTOR (1:1000) overnight at 4°C. After washing 3 times with tris-buffered saline with Tween-20 (TBST), the corresponding second antibodies were added for incubation at room temperature for 1 h. After washing 3 times with TBST, electrochemiluminescence (ECL) luminescent liquid was added for exposure and development, followed by processing using Image Lab 6 software. The gray values were calculated, and the relative expression levels of PI3K, Akt and mTOR proteins were expressed as the ratios of the gray values of the target bands to that of the corresponding internal reference glyceraldheyde 3-phosphate dehydrogenase (GAPDH).

Statistics

SPSS 23.0 (IBM, Armonk, NY, USA) was employed for statistical analyses of the experimental data obtained. The measurement data obtained were all expressed in the form of mean \pm standard deviation (x \boxtimes \pm s). T-test was used for data comparison between groups. P<0.05 showed that the difference was statistically significant.

Results

Effects of metformin on the proliferation of melanoma cells

Compared with that in C group, the OD value in Q1 and Q2 group was decreased (p<0.05), and that in Q3 group was reduced significantly (p<0.01) (Table 1).

Detection of apoptosis in each group via flow cytometry

With the increase of metformin concentration, apoptotic cells were increased significantly (Figure



Figure 1. Schematic of flow cytometry of apoptotic cells in each group. The bottom left quadrant shows living cells, the bottom right quadrant shows early apoptotic cells, and the top right quadrant shows dead or late apoptotic cells.



Figure 2. Comparisons of TUNEL staining results and apoptosis rate in each group. (magnification: 100×). ^ap<0.05, ^bp<0.01, *vs.* C group.



Figure 3. Comparisons of protein expression levels and protein band absorbance of PI3K, Akt and mTOR in melanoma cells of each group. ^ap<0.05, ^bp<0.01, *vs.* C group.

1). The apoptosis of melanoma cells could be promoted by metformin with different concentrations (Figure 1).

Comparison of apoptosis rate of melanoma cells in each group at 24 h

Compared with that in C group, the apoptosis rate in Q1 and Q2 group was increased (p<0.05), and the apoptosis rate in Q3 group was increased significantly (p<0.01) (Figure 2).

Comparison of expression levels of PI3K, Akt and mTOR in melanoma cells of each group at 24 h

Compared with those in C group, the expression levels of PI3K, Akt and mTOR in Q1 and Q2 group were decreased (p<0.05), and the expression levels of PI3K, Akt and mTOR in Q3 group were decreased significantly (p<0.01) (Figure 3).

Discussion

Melanoma, a highly invasive and refractory skin carcinoma, is mainly induced by genetic or environmental factors, such as sunlight and ultraviolet radiation, whose incidence rate and mortality rate have been increasing worldwide in the past 30 years [5,6]. Melanoma cells have the ability to invade other organs, such as lymph nodes, lungs, and the brain, which can significantly increase the melanoma-related mortality rate. The 5-year survival rate of melanoma patients is less than 20% due to drug resistance of metastatic melanoma to chemotherapy, radiotherapy and immunotherapy [7-9]. Melanoma has been widely concerned by the medical community because of rapid progress of the disease, lack of effective interventions, and poor prognosis. As a complex physiological phenomenon, metastasis consists of enhanced cancer cell movement, intravascular perfusion of blood vessels or lymphatic vessels, and degradation of extracellular matrix (ECM). In most cases, epithelial mesenchymal transformation (EMT) occurs in metastatic cancer cells, so that cancer cells are not bound by cell death mechanism, such as apoptosis, and migrate to the extracellular environment [10,11]. Cancer cells undergoing EMT spread from primary tumors to distant organs through blood or lymphatic vessels, thus becoming metastatic tumors. The metastasis of melanoma cells may be inhibited by inducing apoptosis, a complex process involving multiple genes, during cell cycle arrest.

Metformin, a biguanide drug widely used in the clinic, is currently the first-line drug in treating type 2 diabetes (T2D) due to its efficacy, safety and good compliance [12]. Interestingly, more and

more studies have confirmed that metformin has great potential in treating many other diseases besides T2D [13-15]. Many epidemiological analyses have shown that metformin may not only improve the prognosis of cancer patients, but also prevent the occurrence of tumors [16,17]. Moreover, some evidence has shown that as an anti-aging factor, metformin can regulate the microbial population in the body and promotes the healthy body development. At present, new applications of metformin in disease treatment are being explored. The exact mechanism by which metformin exerts a therapeutic effect has not been fully elucidated, and it may involve multiple pathways. In recent years, the scientific community has conducted extensive research on the protective effects of metformin on malignant diseases. The anticancer activity of metformin has a relationship with its indirect and direct effects. The indirect effect can be attributed to the general changes of blood glucose and insulin levels in the body, affecting the survival of cancer cells [18]. Thus, this tumor activity can be inhibited by lowering insulin levels. It is reported that metformin can also affect the inflammatory process which plays a key role in tumor progression. Metformin can inhibit the activity of nuclear factorκB and reduce the secretion of pro-inflammatory cytokines [19]. In addition, it is reported that metformin can activate immune responses of the body to cancer cells [20]. Therefore, in this study, the inhibitory effect of metformin on melanoma and its possible mechanism were explored.

In this study, it was concluded that metformin could inhibit the proliferation of mouse melanoma cells. The inhibition of metformin on melanoma cells was more pronounced as the metformin concentration was increased and the action time was prolonged. The results of flow cytometry and TUNEL assay showed that, compared with that of normal melanoma cells, the apoptosis rate of melanoma cells treated with metformin for 24 h was increased. Metformin (40 mmol/L) exhibited the most obvious effect on promoting apoptosis, based on which it was speculated that the higher the concentration of metformin, the more obvious the effect. However, the osmotic pressure is also increased with the elevated concentration of metformin, leading to difficulties in identifying whether it is a drug effect or an osmotic effect. In this study, it was investigated whether metformin represses the proliferation and facilitates the apoptosis of mouse melanoma cells through the PI3K/Akt/mTOR signaling pathway that plays an important role in the proliferation of a variety of tumor cells. Metformin at different concentrations could inhibit the expression levels of proteins in

the PI3K/Akt/mTOR pathway. Therefore, the PI3K/ Akt/mTOR pathway may be a potential target for future intervention in melanoma.

Conclusions

In this study, the inhibition and promotion of metformin on the proliferation and apoptosis of mouse melanoma cells were investigated at the cellular level. In the study, PI3K pathway inhibitors and gene editing techniques were not used, so that whether metformin works through the PI3K path-

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way was not confirmed. In addition, this study was still in the stage of *in vitro* experiment, so it was difficult to definitely reveal its effect and therefore *in vivo* experiments should be carried out. In the future, metformin and the PI3K/Akt/mTOR signaling pathway may be intervention points in treating melanoma.

Conflict of interests

The authors declare no conflict of interests.

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