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Metformin enhancing the antitumor efficacy of carboplatin against Ehrlich solid carcinoma grown in diabetic mice: Effect on IGF-1 and tumoral expression of IGF-1 receptors☆

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ABSTRACT

Diabetes has been listed as a risk factor for various types of cancer. Cancer cell development can be promoted by increased levels of IGF-1 and hyperinsulinemia that are associated with diabetes type II. Metformin is an anti-diabetic agent and its potential antitumor impact has become the objective of numerous studies. In this vein, we hypothesize that using metformin in diabetes type II mice may synergistic with carboplatin for reducing the risk of cancer. Therefore, the study aimed to evaluate the *in vivo* antitumor activity of metformin against solid EAC tumor growth in female diabetic mice and its potential pro-apoptotic and anti-proliferative effects with clarification of its inconclusive biological mechanisms. Mice were assigned into nine groups; normal control, diabetic control, diabetic plus EAC control, EAC control, and treated groups received carboplatin and/or metformin (100, 200 mg/kg). Metformin administration especially with high dose potentiated the antitumor activity of carboplatin displayed by increased pro-apoptotic activators "caspase-3 and bax" and reduced anti-apoptotic protein bcl-2. This was confirmed by the histopathological scores. Moreover, the combination therapy was effective in attenuating the expression of the pro-angiogenic mediator "VEGF" and the microvessel density as revealed by the CD_{34}. Additionally, this combination down-regulated the high levels of the mutagenic element "IGF-1" and its receptor expression, and attenuated the intensity of inflammatory mediators. In conclusion, it was found that metformin therapy could enhance apoptotic marker, and suppress the neovascularization and proliferation process. This clarified the ability of metformin to support carboplatin activity in reducing tumor progression in type II diabetes.

1. Introduction

With overloaded lifestyle, cancer remains the most frequent diagnosed disease and one of the primary reasons for mortality worldwide. In addition, diabetes mellitus type II is a substantial health problem. Furthermore, various meta-analysis studies reported that diabetes has been listed as a risk reason for various types of cancer, for instance, breast and colon cancer [1]. During the past 20 years, there was substantial dramatic evidence subsidize the association between both cancer risk and diabetes [2–4]. Moreover, there was plausibility that cancer cell development could be promoted by hyperinsulinemia, insulin resistance, and decreased levels of insulin-like-growth-factor-1 (IGF-1) [5] that were associated with diabetes type II patients [6,7].

Epidemiological and *in vivo* studies clarified that hyperinsulinemia increased the risk of cancer directly through induction of mutagenic effects and stimulation of DNA synthesis and/or indirectly through increased levels of IGF-1 signaling system [5,6]. IGF-1 is a potent mutagenic and anti-apoptotic element incorporating in the regulation process of cell proliferation in various epithelial cells [8,9]. IGF-1 receptors are reported in normal epithelial and carcinoma cells. After their activation, they inhibit apoptosis and allow the cell progression. Thus, IGF-1 can influence both premalignant and cancerous stages [8,9].

Growing evidence bolsters the probability that glucose-lowering treatments play a prospective value in potential future treatment of both diabetes types II and cancer by their dual effects in decreasing insulin resistance and IGF-1 levels [10,11]. In turn, it diminishes the risk of tumors development in diabetic patients [11].

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Metformin is widely used as a first-line anti-diabetic agent and its potential antitumor impact has become the objective of numerous observational studies [12,13]. Previous studies reported that metformin decreased the incidence of different types of cancer including ovarian cancer cells [14] and oesophageal cancer [15].

Diabetes mellitus is closely correlated with an increased risk of cancer development in diabetic subjects [16]. Therefore, the use of certain glucose-lowering medications based on cancer concerns has attracted the attention of many researchers. Moreover, these medications have become a subject of intensive investigation in diabetic patients. Nevertheless, the exact biological mechanisms are still vague and inconclusive, and studies are still restricted. In this vein, we hypothesize that using metformin in diabetes type II patients may have the ability to reduce the risk of cancer.

The Ehrlich tumor (EAC) is a mammary adenocarcinoma that can be grown in solid and ascitic forms relying upon its administration site. It is used as an experimental model to evaluate the influence of chemotherapeutics on tumor cells proliferation and host responses [17,18]. Therefore, the present study was conducted to elucidate the *in vivo* anticancer activity of metformin in EAC-bearing diabetic mice with hyperinsulinemia as well as a clarification to its pharmacological mechanism of action in delaying tumor occurrence. Besides, the study was focused on metformin anti-proliferative and apoptotic effects mediated by bax, and caspase-3 up-regulation with down-regulation of bcl2 and IGF-1 signaling system.

Angiogenesis is a prerequisite for the growth and progression of cancer in diabetic patients and its mechanism may be correlated with the increase in IGF-1 action [19]. As IGF-1 stimulate endothelial cell migration and the microvessel density, the present study was extended to evaluate the ameliorative effect of metformin on angiogenic markers in induced EAC solid tumor growth in diabetic mice.

2. Material and method

2.1. Chemicals and drugs

Streptozotocin (STZ) was used for induction of diabetes in mice (Sigma-Aldrich Co. Louis St., MO, USA). Metformin hydrochloride was purchased from Sigma Co. (Quesna, Egypt) as white powder dissolved with distilled water. Sucrose was procured from VACSERA (Cairo, Egypt); the holding company for biological products & vaccines. For tissue immunohistochemistry, primary mice antibodies against cleaved 17-kDa caspase-3 and IGF receptor type 1 (IGF-1R) were purchased from Biorbyt Co. (Cambridge, UK). The following antibodies were purchased: mice polyclonal antibodies against bax and bcl-2 from Abcam (Cambridge, UK), mouse monoclonal antibody against CD_{34} from Bio SB Co. (Santa Barbana, USA), mice polyclonal antibody against iNOS from Lab Vision Co. (Cairo, Egypt).

2.2. Experimental animal and housing condition

Seventy-two female albino mice (20–24 g in weight) were housed in polyethylene cages with normal light–dark cycle under controlled laboratory conditions (temperature, 25 ± 2 °C; humidity, 50 ± 10%) to acclimatize to the laboratory conditions. Mice were fed with high fat diet (HFD), prepared by mixing 20% sucrose and 10% lard with the basal diet. Standard food and water were kept *ad libitum*. Normal mice served as control and fed basal diet. The remaining diet was replaced daily. Mice' body weight was reported weekly. Experiment was performed in accordance with the guidelines of the institutional animal usage and research ethics committee of Suez Canal University following internationally accepted guidelines (NIH) for the Care and Use of Laboratory Animals. All efforts were made to alleviate animals suffering.

2.3. Experimental protocols

2.3.1. In vivo induction of diabetes in mice

For 4 weeks, the mice were fed with HFD. Blood glucose level was recorded every 3 days, recording its random and fasting rates. After that, mice were fasted overnight then received a single i.p. injection of STZ (30 mg/kg) dissolved in 0.1 M citrate buffer (pH 4.5) in a total volume of 1 ml/kg. Three days post STZ injection, the level of blood glucose was determined using One Touch Ultra Mini glucometer (USA).

In addition, orbital blood samples were collected and sera were separated to estimate insulin levels using ELISA kit (Biorbyt Co., UK). Homeostasis model assessment for insulin resistance (HOMA-IR) index was used according to Matthews et al. [20]. Using the following formula, (HOMA-IR index = [fasting glucose (mmol/L) × fasting insulin (μU/ml)]/22.5).

2.3.2. Induction of EAC solid form

A murine cell line of EAC was obtained from the department of Cell Biology, National Cancer Institute (Cairo, Egypt). It was used to make an *in vivo* model for EAC solid tumors. Trypan blue exclusion method was performed to check the viability and the count of EAC cells [21]. Mice were inoculated subcutaneously at two shaved sites on the lower ventral side with 100 μl EAC cells suspension (containing 2.5×10^6 cells/0.1 ml). The day of tumor implantation was considered the first day of the experiment.

2.4. Schedule of pharmacological treatments

Mice were divided into 9 main groups; each one consists of 8 mice. Therapeutic regimens with carboplatin and/or metformin were launched on the 8th day (one-week post inoculation of the tumor cells and assurance of their growth). These Therapeutic regimens continued for 14 days (ended at day 21st of tumor cells inoculation) (Fig. 1). The period of treatment was determined according to previous studies [22–24]. Metformin was administered orally using oral gavage, while carboplatin was given by i.p. injection. Mice were assigned into eight groups; group i: normal control received saline, group ii: diabetic control, group iii: diabetic plus EAC control. Groups from iv to viii were diabetic plus EAC positive and received the following therapeutic treatments; carboplatin (5 mg/kg), metformin (100 mg/kg·b·w), metformin (200 mg/kg·b·w), a combination of carboplatin and metformin (100 mg/kg·b·w) or carboplatin and metformin (200 mg/kg·b·w); respectively. One extra non-diabetic EAC group was added for confirmation the relation between the EAC in diabetic and non-diabetic mice. The doses were decided depending on equivalent typical doses of treatment according to Reagan-Shaw et al. [25].

2.5. Serum collection, solid tumor disks dissection and samples preparations

By the end of the protocol, mice were anaesthetized using i.p. thiopental sodium injection (50 mg/kg). Orbital blood samples were collected using heparinized microcapillaries. Then, mice were euthanized by decapitation and the solid tumor disks were carefully dissected.

Sera were separated from blood samples for analysis of insulin, IGF-1, tumor necrotic factor-alpha (TNF-α), and vascular endothelial growth factor (VEGF) using ELISA kits according to the manufacturer instructions of Ray Biotech (Norcross, GA, USA), Sun Red Biotechnology and Biorbyt Co (UK) respectively. The tumor disks were weighed and fixed in phosphate-buffered formalin 10% at pH 7.4 for 24 h for histopathological and immunohistochemistry staining.

2.6. Histopathological examination of EAC solid tumor

EAC solid tumors sections were stained using hematoxylin and eosin (H&E) stain to evaluate the histopathological characters of the tumor