Proliferation and metastatic potential of endometrial cancer cells in response to metformin treatment in a high versus normal glucose environment

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Abstract. In order to improve our understanding of the potential preventive and therapeutic role of metformin, the present study aimed to investigate the capability of low-dose metformin in the efficient inhibition of cancer development and the reduction of the metastasis of endometrial adenocarcinoma type I and primary endometrial epithelial cells (eEPs), with the drug acting as a treatment in a hyperinsulinemic environment exposed to high and normal glucose conditions. The Ishikawa endometrial adenocarcinoma cell line and primary eEPs were exposed to an environment with high (17 mM) or normal glucose (5 mM) and treated with insulin, low-dose metformin (0.1 mM) or a combined treatment. Metastatic potential was assessed by migration and invasion assays, and relative cell proliferation was determined. Metformin at a low dose potently inhibited the insulin action, decreasing the ability of the endometrial cancer (EC) cell line to migrate and invade in a high and normal glucose environment, and decreasing the migration ability of the primary eEPs. In the EC cell line, the insulin treatment increased the proliferation, without any subsequent reduction of proliferation by the addition of 0.1 mM metformin; however, relative cell proliferation sensitivity to metformin was observed in the range between 1 and 5 mM regardless of the glucose concentration present. Overall, metformin at 0.1 mM is not efficient enough to decrease the proliferation in an EC cell line. However, at this concentration, metformin can inhibit the insulin action in endometrial epithelial cancer cells, demonstrating an anti-metastatic effect in high and normal glucose environments.

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Introduction

Endometrial cancer (EC) is one of the most common gynecological malignancies worldwide (1) and is classified into estrogen-dependent type I and estrogen-independent type II forms. The type I form is the most common, accounting for 75-85% of all cases of EC (2). Unopposed estrogen has been shown to increase the risk of EC development, as estrogens stimulate endometrial cell proliferation and inhibit apoptosis (3). Additionally, diseases associated with insulin resistance, such as obesity, type II diabetes mellitus and polycystic ovary syndrome (PCOS), are considered significant risk factors for the development and progression of type I EC (4-6). Insulin resistance is a condition in which target tissues have decreased sensitivity to insulin, and blood insulin levels consequently increase to maintain normal glucose levels. The chronic hyperinsulinemic state has been found to exert direct and indirect effects that contribute to EC development (7). A large body of evidence has suggested that women with diabetes possess a stronger association with neoplastic processes (5), and furthermore, diabetic patients with cancer experience increased mortality compared with normoglycemic individuals (8). Finally, a meta-analysis study has shown that women with diabetes have a two-fold risk for EC (9). Insulin resistance appears to play a central role in endometrial carcinogenesis. Therefore, treatment with insulin-sensitizing agents that act through reducing insulin levels could offer a general approach to prevent the development of cancer and reduce metastasis (10,11).

Metformin, an anti-hyperglycemic and insulin sensitizing agent, is the most commonly used drug for treating type II diabetes mellitus, as well as off-label insulin resistance

(as observed in women with PCOS) (12). In recent years, numerous studies have indicated that metformin could be effective as a cancer therapy, along with its traditional role in treating diabetes (13-15). Metformin use can prevent malignant transformation indirectly via systemic changes (improving hyperglycemia, hyperlipidemia and hyperinsulinemia) and via direct effects, such as suppression of mammalian target of rapamycin via 5' adenosine monophosphate-activated protein kinase activation, leading to reduced protein synthesis and cell proliferation (16,17). A meta-analysis of 18 observational studies showed that metformin therapy is associated with an overall 27% reduction in the risk of developing any type of cancer in patients with type II diabetes (18). A retrospective study showed that metformin use is additionally associated with less recurrence and improved overall survival in EC patients; although, when recurrence took occurred, it was not delayed compared with that in the control (19). However, the majority of these previous studies possessed methodological weaknesses and/or insufficient data. Additionally, experimental studies revealed that metformin is an effective antiestrogenic agent, inhibiting cell proliferation and leading to growth arrest, as well as inducing apoptosis in EC (17,20-22). Furthermore, recent findings have suggested that metformin is important in suppressing the migration and invasion of cancer cells, which could prevent metastasis (23-25). However, in these studies metformin action was observed at high supra-pharmacological concentrations and without estradiol addition, an important factor in endometrial proliferative disorders.

The present study investigated the capability of low-dose metformin (0.1 mM) to inhibit the development of cancer and reduce the metastatic potential of endometrial adenocarcinoma type I *in vitro* using the Ishikawa cell line in the high and normal glucose environments. Finally, the study evaluated the efficiency of low-dose metformin to prevent EC using normal uterine endometrial epithelial cells (eEPs) exposed to high glucose concentrations.

Materials and methods

Isolation and culture of primary eEPs. The primary eEPs were isolated from endometrial biopsies (between March and August 2015) taken from healthy, regularly cycling women (34.1±3.4 years old) undergoing laparoscopic surgery for benign reasons. Informed consent was obtained and the study protocol was approved by the the Clinical Hospital of Porto Alegre (Porto Alegre, Rio Grande do Sul, Brazil) and the University of Heidelberg (Heidelberg, Germany). Exclusion criteria were hormonal stimulation within the preceding 3 months, endocrinopathies, cancerous lesions and irregular menstrual bleeding. Human endometrial tissues were digested with collagenase type Ia (Gibco, Karlsruhe, Germany) for 2 h. Thereafter, the cells were washed, centrifuged (800 x g, room temperature) and separated using a 40-μm filter. The stromal cells passed through the filter and the epithelial cells were retained. Epithelial cells were recovered from the filter by backwashing with Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA).

Cell culture. The Ishikawa human endometrial adenocarcinoma cell line was purchased from Sigma-Aldrich (Merck

Millipore, Darmstadt, Germany; European Collection of Cell Culture authenticated). Ishikawa cells were grown in minimal essential medium (MEM; Sigma-Aldrich; Merck Millipore) supplemented with 5% fetal bovine serum (FBS; HyClone; Thermo Fisher Scientific Inc.), 1% penicilin-streptomycin (Gibco; Thermo Fisher Scientific Inc.), 1% non-essential amino acids (Sigma-Aldrich; Merck Millipore) and 5 μg/ml bovine insulin (Sigma-Aldrich; Merck Millipore), while the primary eEPs were maintained in DMEM/F12 supplemented with 5% FBS and 1% penicillin-streptomycin at 37˚C in a humidified atmosphere with 5% CO_2.

For the experiments in a high glucose environment, the cells were cultured in various conditions with DMEM/F12 medium containing 17 mM glucose, and for the experiments in a normal glucose environment, the cells were cultured with MEM containing 5.5 mM glucose. The media for all conditions were supplemented with 10^{-8} M estradiol. The different conditions included: a) Control group, medium only; b) insulin group, medium plus 100 ng/ml insulin; c) metformin group, medium plus 0.1 mM metformin; and d) insulin plus metformin group, medium plus 100 ng/ml insulin and 0.1 mM metformin. All chemicals were purchased from Sigma-Aldrich (Merck Millipore).

Migration and invasion analysis. To assess the ability of cellular motility in the different conditions, Transwell filters (6.5 mm in diameter; 5-μm pore size; Corning Inc., Corning, NY, USA) were used. The cells were harvested as aforementioned and suspended in serum-free medium. The Transwell filter was coated with 100 ml medium for the migration assay or with 100 μl Matrigel (1:1 dilution; Corning Matrigel Growth Factor Reduced; Corning Inc.) for the invasion assay. Thereafter, the primary eEPs (1x10^4 in 200 μl) or Ishikawa cells (2.5x10^5 in 200 μl) were added with the individual treatment conditions into the Transwell chamber. Medium containing 5% FBS was added to the lower well, then the Transwells were incubated for 16 h. Non-migrated/invading cells were removed with a cotton swab, and migrated/invading cells were fixed in 3.7% paraformaldehyde in phosphate-buffered saline, permeabilized with 100% methanol and stained with Giemsa. Cell migration/invasion, repeated five times, was quantified by blinded counting of the number of migrated/invaded cells in each insert in four different areas. Data are expressed as the percentage compared with the control group.

Cell proliferation assays. The CellTiter Glo Luminescent assay (Promega Corporation, Madison, WI, USA) was used to evaluate the relative cell proliferation of Ishikawa cells exposed to high and normal glucose conditions. The cells were seeded into 96-well plates at a density of 5,000 cells/well in 100 μl drug-free medium overnight. The cells were then treated with the indicated treatment (control, insulin, metformin or insulin plus metformin) for 72 h. After this period, the plates were equilibrated at room temperature for 30 min, and 100 μl of CellTiter Glo reagent was added in each well, mixed for 2 min and incubated at room temperature for 10 min. The luminescence was detected by a Centro LB 960 Microplate Luminometer (Berthold Technologies GmbH and Co. KG, Bad Wildbad, Germany).

In order to evaluate the sensitivity of the epithelial carcinoma cells to varying doses of metformin in high and
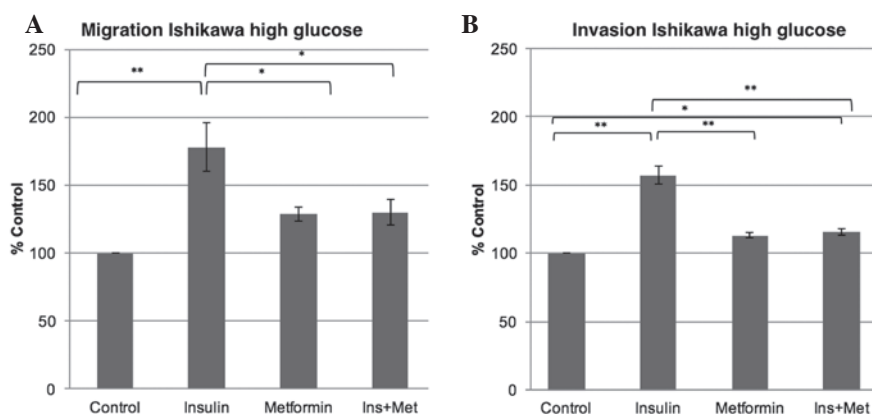
Figure 1. Migration and invasion of the Ishikawa cell line in a high glucose environment during 16 h of incubation. The insulin treatment increased the ability to migrate and invade. The metformin treatment was able to inhibit the insulin effect. (A) Migration assay in a high glucose environment; *P=0.023 and **P<0.001. (B) Invasion assay in a high glucose environment; *P=0.030 and **P<0.001. Mean ± standard error of the mean. Ins, insulin; Met, metformin.
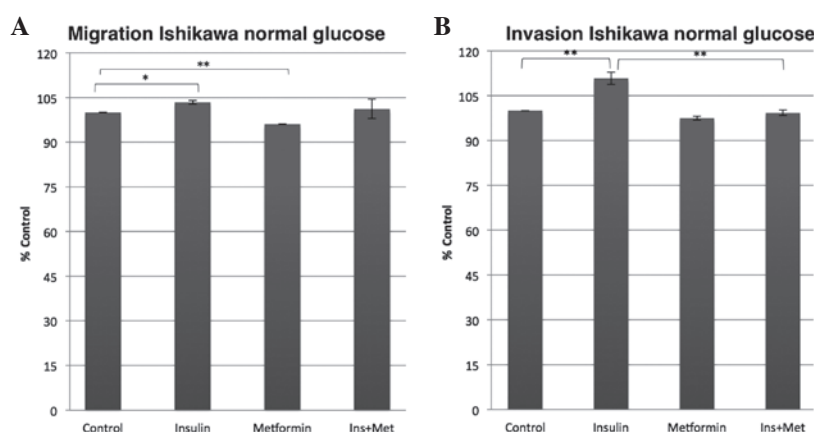

Figure 2. Migration and invasion of the Ishikawa cell line in a normal glucose environment during 16 h of incubation. The insulin treatment increased the ability to migrate and invade. The metformin treatment was able to inhibit the insulin effect. (A) Migration assay in a high glucose environment; *P=0.032 and **P=0.001. (B) Invasion assay in a high glucose environment; **P=0.006. Mean ± standard error of the mean. Ins, insulin; Met, metformin.

normal glucose conditions, the Ishikawa cells were seeded into 96-well plates at a density of 5,000 cells/well in 100 μl drug-free medium overnight. The cells were then treated with increasing doses of metformin (0, 0.1, 1 and 5 mM) for 72 h. A CellTiter Glo Luminescent assay was performed as aforementioned. The effect of the different treatments was calculated as a fold-change compared with the control group. Each experiment was performed in sextuplicate and repeated three times.

Statistical analysis. Quantitative data are represented as the mean ± standard error of the mean of at least three independent experiments. SPSS version 23 (IBM SPPS, Armonk, NY, USA) was used to perform a one-way analysis of variance and Turkey's post-hoc test, a generalized estimating equation test or Student's t-test, as appropriate. P<0.05 was considered to indicate a statistically significant difference.

Results

Low-dose metformin inhibits the insulin effect on the migration and invasion in the EC cell line. The present study examined the effect of a hyperinsulinemic environment with or without metformin treatment on the migration and

invasion ability of Ishikawa cells when exposed to a high or normal glucose environment. In a high glucose environment, insulin exposure increased the migration by 77% (P<0.001) and the invasion capability by 57% (P<0.001) in the Ishikawa cells compared with the control. While metformin alone did not confer any change in the migration and invasion capabilities of the Ishikawa cells in a high glucose environment when compared with the control, the metformin co-treatment (insulin plus 0.1 mM metformin) markedly attenuated the insulin effect, leading to a decrease in the migration ability by 47% (P=0.023) and in the invasion ability by 42% (P<0.001), therefore reducing the insulin effect (Fig. 1). In the normal glucose environment, however, the insulin and the metformin effects were less dominant compared with those in the high glucose environment. While the insulin exposure of the Ishikawa cells only slightly increased the migration and invasion ability by 3% (P=0.032) and 10% (P=0.006), respectively, compared with the control, the metformin co-treatment almost completely abolished the insulin effect on the invasion potential, as the metformin co-treatment inhibited the insulin effect on the invasion ability to a control rate (Fig. 2). Metformin alone on the other hand significantly decreased the capability of the Ishikawa cells to migrate by
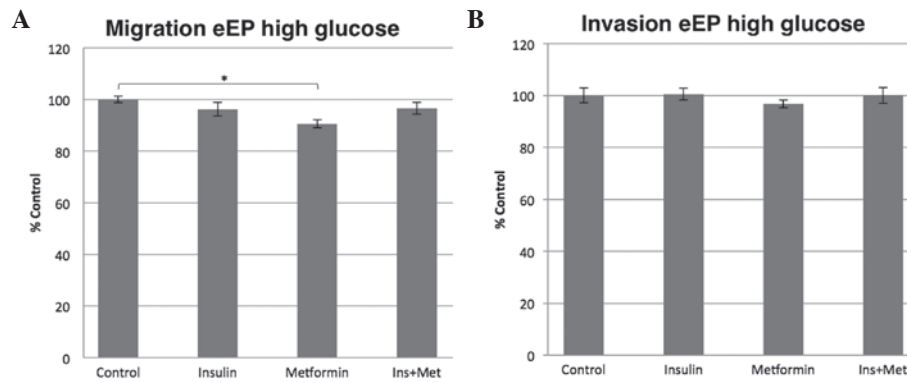
Figure 3. Migration and invasion of the primary eEPs in a high glucose environment during 16 h of incubation. The metformin treatment decreased the migration effect. (A) Migration assay in a high glucose environment. (B) Invasion assay in a high glucose environment. Mean ± standard error of the mean. *P<0.001. eEPs, endometrial epithelial cells; Ins, insulin; Met, metformin.
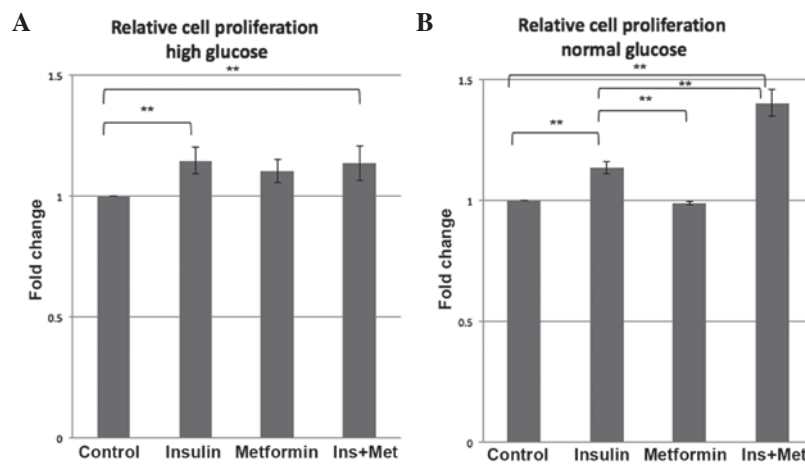

Figure 4. Relative cell proliferation of the Ishikawa cell line in a (A) high glucose and a (B) normal glucose environment. The insulin treatment increased the proliferation in the two glucose conditions and metformin at 0.1 mM was not able to reduce the proliferation. Mean ± standard error of the mean. **P=0.05. Ins, insulin; Met, metformin.

4% (P=0.001) and non-significantly decreased their ability to invade by 3% (P=0.069) compared with the control.

Low-dose metformin is effective in primary eEPs. To investigate the efficiency of metformin treatment, the effect of metformin on normal eEPs was analyzed. When the primary eEPs were cultured in a high glucose environment, insulin alone or in combination with metformin did not change the migration or invasion potential after 16 h. However, metformin alone inhibited the migration potential significantly by 10% (P<0.001) and the invasion capacity non-significantly by 4% (P=1.000) compared with the control (Fig. 3).

Low-dose metformin does not decrease the proliferation of the EC cell line. To determine if the glucose environment affects the proliferation potential of epithelial cancer cells, Ishikawa cells were treated using the four treatment conditions (control, insulin, metformin and insulin plus metformin) for 72 h in media containing high or normal glucose. As shown in Fig. 4, insulin treatment increased the proliferation of the Ishikawa cells by 1.15-fold in the high glucose condition (P=0.029) and by 1.13-fold in the normal glucose condition (P=0.003) compared to the control group. However, treatment

with 0.1 mM metformin did not have a significant effect on Ishikawa cell proliferation compared with the control group in the 72 h, either in a high or normal glucose environment. Furthermore, in this treatment period, 0.1 mM metformin in combination with insulin was not able to inhibit the stimulatory insulin effect on the proliferation potential in high glucose and even lead to an enhanced proliferation potential in the normal glucose condition compared with the control (1.40-fold; P<0.001).

Metformin decreases EC cell line proliferation in a dose-dependent manner. In order to test the sensitivity of the EC cell line to metformin at different concentrations in a high and normal glucose environment, the proliferation of Ishikawa cells treated with metformin (0, 0.1, 1 and 5 mM) in medium containing high and normal levels of glucose for 72 h was measured (Fig. 5). Independent of the glucose concentration in the medium, the proliferation potential was unchanged at 0.1 mM during 72 h (1.0-fold with high glucose and 0.97-fold with normal glucose relative to the control), while reduced proliferation was noted with 1 mM metformin (0.92-fold with high glucose; P=0.037), followed by a further reduction with increasing metformin concentration (0.81-fold at 5 mM
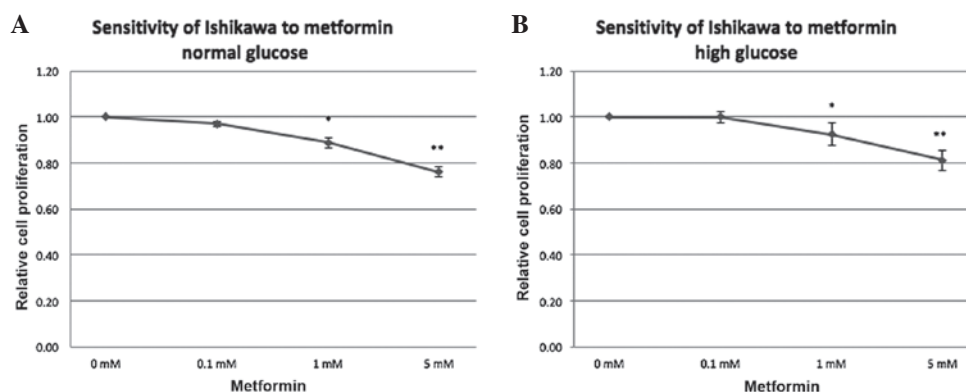
Figure 5. Sensitivity of the Ishikawa cell line to metformin in a (A) high glucose and in a (B) normal glucose environment. The Ishikawa cell line was cultured in the presence of varying concentrations of metformin for 72 h. (A) *P=0.037 and **P=0.002; (B) *P=0.027 and **P=0.002. The relative cell proliferation sensitivity of the Ishikawa cell line to metformin was in the range of 1 and 5 mM in the two glucose environments. Mean ± standard error of the mean.

with high glucose; P=0.002). In a normal glucose condition, a slightly more pronounced effect was noted compared with that in the high glucose environment (0.89-fold for 1 mM, P=0.027; and 0.79-fold for 5 mM, P=0.002).

Discussion

Considering the increasing level of metformin research in cancer therapy, as well as in patients with type II diabetes mellitus, thus extending its use to the non-diabetic population, the present study investigated the effectiveness of low-dose metformin in EC development and prevention. In order to analyze metformin as a cancer prevention agent and/or cancer progression treatment in endometrial adenocarcinoma type I eEPs and endometrial epithelial cancer cells were exposed to metformin in either a high glucose or normal glucose medium with or without insulin substitution.

Glucose is an essential nutrient that supports cellular energy homeostasis. The normal serum glucose level is usually maintained at ~5.5 mM. However, increased blood glucose levels and hyperglycemia contribute to growth and carcinogenesis in EC (26), and act as a critical link between the observed increased cancer risk in patients with type II diabetes (5). Furthermore, in conditions such as PCOS, insulin resistance can be obesity-independent. Despite a normoglycemic state, the associated hyperinsulinemia is believed to be a promoting factor not only for cancer initiation, but also for cancer progression (7,27).

Several studies have suggested that high glucose levels create an optimal environment for cancer cells to exhibit a resistance to metformin (28-30). The current study evaluated the insulin action and the metformin efficiency on the metastatic potential of EC cells when exposed to a high and normal glucose environment. The findings showed that insulin treatment in each glucose condition increased the migration and invasion ability of the endometrial epithelial cancer cells, however, this effect was more pronounced in the high glucose environment. In addition, low-dose metformin effectively attenuated the effects of insulin action in high and normal glucose conditions, leading to a less profound effect. Notably, the results in the normal glucose environment showed a decrease in the ability of the cells to migrate when treated with metformin compared with

the control group. However, a significant difference could not be found between the metformin and control groups in a high glucose environment, but compared with the normal glucose environmen, a tendency of cancer cell resistance was present when exposed to high glucose levels. By contrast, insulin alone or in combination with metformin showed no effect on primary eEPs exposed to high glucose conditions with regard to their migration or invasion potential after 16 h. We assume that primary eEPs do not have the metastatic potential observed in cancer cells. Furthermore the short incubation of 16 h may not be long enough for the insulin to activate the invasion potential of these cells. However, metformin alone was able to reduce the inherent migration capability of the eEPs. These results support the evidence that a high glucose environment interferes in the metformin effect on cancer cells.

To the best of our knowledge, the present study shows for the first time the ability of metformin to inhibit the metastatic potential of endometrial epithelial cancer cells, while the metformin efficiency in the inhibition of migration and invasion has already been reported in a dose-dependent manner in other types of cancer cells, including ovarian (23) and prostate (31) cancer cells, and osteosarcoma (32). Recently, Han et al reported an increased ability of adhesion and invasion in two EC cell lines, suggesting that targeting glucose metabolism may be a promising therapeutic strategy for the treatment of EC (33).

Besides the potential anti-metastatic effect of metformin, several studies have indicated that metformin may be a useful anti-proliferation agent for EC cells. Cantrell et al showed for the first time that metformin potently inhibits growth in two EC cell lines [half maximal inhibitory concentration (IC_{50}) of 1 mM metformin] after 72 h of treatment in a normal glucose environment (17). Takahashi et al demonstrated that after 48 h of treatment with increasing concentrations of metformin in normal glucose conditions, ≥5 mM metformin significantly reduce the number of viable cells (IC_{50} of 6.78 mM metformin) (21). Furthermore, the metformin inhibitory proliferation effect has been reported for certain other types of cancer. Wu et al showed that metformin inhibits the proliferation of ovarian cancer cells in a dose- and time-dependent manner in vitro (IC_{50} of 16.67 mM metformin) and in vivo in a nude mouse model (23). Kato et al examined the effects of metformin in prostate cancer cell proliferation using the MTS assay and cell counting.

The number of viable cells of three prostate cancer cell lines decreased significantly after incubation with metformin (IC_{50} of 5 mM metformin) for 48 h (31). However, in the present study, in the Ishikawa endometrial epithelial cancer cell line, it was observed that the insulin treatment increased cell proliferation, without any reduction in proliferation by the addition 0.1 mM metformin regardless of the glucose concentration present. This apparent discrepancy may result from the high micromolar metformin concentrations used in the previous studies, which are much greater than the steady-state levels in the patients' plasma. The present study observed the relative cell proliferation sensitivity to metformin in the EC cell line in the range between 1 and 5 mM in each glucose environment, although this was more pronounced in the normal glucose condition. Moreover, these findings were in the presence of estradiol, an important factor in endometrial proliferative disorders, but not used in the published endometrial studies.

In addition, the epidemiological and laboratory studies remain controversial. Numerous details of the action of metformin remain to be identified, and the risk of harm must be considered when designing novel metformin-based therapies. The knowledge gained from the combination of tumor genetics, patient metabolic profiles and the cellular environment will assist in determining the tumor preventative or treatment efficiency of metformin. We expect to improve the understanding of the mechanisms linking glucose metabolism, hyperinsulinemia and metformin treatment with EC development and progression. According to the present findings, it is indicated that metformin at 0.1 mM is not efficient enough to decrease the proliferation in 72 h of treatment in an EC cell line. However, in this concentration, metformin can inhibit the insulin effect by decreasing the high cellular invasion and migration potential in high and normal glucose environments, demonstrating an anti-metastatic effect of metformin in endometrial epithelial cancer cells. Nevertheless, further investigations are necessary to improve the knowledge of metformin as an effective targeted therapy in cancer, as well as a long term treatment to be efficient in the inhibition of target genes and proteins connected to the development and progression of cancer.

Acknowledgements

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