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Anti-tumour effect of metformin in canine mammary gland tumour cells

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ABSTRACT

Metformin is an oral hypoglycaemic drug used in type 2 diabetes. Its pharmacological activity reportedly involves mitochondrial respiratory complex I, and mitochondrial respiratory complex inhibitors have a strong inhibitory effect on the growth of metastatic canine mammary gland tumour (CMGT) cell lines. It is hypothesised that metformin has selective anti-tumour effects on metastatic CMGT cells. The aim of this study was to investigate the in vitro effect of metformin on cell growth, production of ATP and reactive oxygen species (ROS), and the AMP-activated protein kinase (AMPK) mammalian target of rapamycin (mTOR) pathway in two CMGT clonal cell lines with different metastatic potential. In addition, transcriptome analysis was used to determine cellular processes disrupted by metformin and in vivo anti-tumour effects were examined in a mouse xenograft model.

Metformin inhibited CMGT cell growth in vitro, with the metastatic clone (CHMp-5b) displaying greater sensitivity. ATP depletion and ROS elevation were observed to a similar extent in the metastatic and non-metastatic (CHMp-13a) cell lines after metformin exposure. However, subsequent AMPK activation and mTOR pathway inhibition were prominent only in metformin-insensitive non-metastatic cells. Microarray analysis revealed inhibition of cell cycle progression by metformin treatment in CHMp-5b cells, which was further confirmed by Western blotting and cell cycle analysis. Additionally, metformin significantly suppressed tumour growth in xenografted metastatic CMGT cells. In conclusion, metformin exhibited an anti-tumour effect in metastatic CMGT cells through AMPK-independent cell cycle arrest. Its mechanism of action differed in the non-metastatic clone, where AMPK activation and mTOR inhibition were observed.

Introduction

Metformin (1,1-dimethylbiguanide) is a biguanide derivative widely used for the management of type 2 diabetes. The primary molecular target of metformin is the mitochondrial respiratory complex I, NADH dehydrogenase. Inhibiting mitochondrial oxidative phosphorylation reduces cellular synthesis of ATP, which in turn activates AMP-activated protein kinase (AMPK). Activated AMPK alters the cellular metabolism from anabolic to catabolic through multiple transcriptional regulatory elements, leading to inhibition of glucogenesis in the liver and enhanced uptake of glucose in muscle (Emami Riedmaier et al., 2013; Quinn et al., 2013). These mechanisms contribute to the decreased serum glucose levels observed in diabetic patients.

Epidemiology studies in diabetic patients have revealed a possible anti-tumour effect of metformin, as its use was associated with a 20–80% reduction in the incidence of various types of cancer, including hepatocellular, colorectal, pulmonary, and pancreatic carcinomas (Decensi et al., 2010; Noto et al., 2012; Franciosi et al., 2013). Other studies also reported a 35% reduction in tumour-related mortality with metformin (Noto et al., 2012; Franciosi et al., 2013).

Based on these observations, the anti-tumour effect of metformin has been studied in vitro and in vivo using various human cancer cell lines and animal tumour models. Human cell lines have included glioblastoma, breast, pancreatic, liver, and prostate cancer cells (Dowling et al., 2007; Ben Sahra et al., 2008; Song et al., 2012; Lonardo et al., 2013; Saito et al., 2013; Würth et al., 2013; Zhu et al., 2014). The anti-tumour effect of metformin is thought to be mediated

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through AMPK activation, similar to the anti-diabetic effects (Dowling et al., 2007; Song et al., 2012). Activation of AMPK inhibits mammalian target of rapamycin (mTOR) complex 1, a signalling molecule involved in cellular survival and protein synthesis through its effector targets, such as S6 kinase (S6K) and 4E-binding protein BP (4E-BP) (Mamane et al., 2006). Other possible anti-tumour mechanisms involve inhibition of insulin-like growth factor (IGF) signalling and induction of cell cycle arrest (Emami Riedmaier et al., 2013; Quinn et al., 2013).

Canine mammary gland tumours (CMGTs) are the most prevalent tumours in sexually intact female dogs (Withrow et al., 2012). In one study, 22/51 (43%) CMGT cases had regional lymph node involvement at the time of surgery (Szczubiał and Łopuszynski, 2011). Systemic chemotherapy is essential for improving prognosis because the metastatic rate of malignant CMGTs is high and metastasis is associated with a poor prognosis (Clemente et al., 2010; Withrow et al., 2012). We previously found that mitochondrial respiratory complex inhibitors (MRCIs) had an anti-tumour effect on metastatic CMGT cells in vitro (Saeki et al., 2015). However, to date, the MRCIs studied, namely rotenone, antimycin, and oligomycin, have been experimental drugs or pesticides. They are not available or have not been evaluated for clinical use in human and veterinary medicine (Xiong et al., 2012; Pagliarani et al., 2013; Seipke and Hutchings, 2013).

Because the long-term use of metformin is devoid of severe side effects (Ekström et al., 2012) and because metformin inhibits mitochondrial respiratory complex I in a similar manner to rotenone, we hypothesised that metformin has an anti-tumour effect on CMGT cells. The aim of our study was, therefore, to evaluate the anti-tumour effects of metformin on CMGT cells both in vitro and in vivo, and to determine its mechanism of action.

Materials and methods

Cell culture

Two clonal CMGT cell lines with different phenotypes, CHMp-5b (metastatic) and CHMp-13a (non-metastatic) cells, were used (Murai et al., 2012). The cell lines were maintained in RPMI 1640 medium (Wako Pure Chemical Industries) supplemented with 10% fetal bovine serum (FBS; Life Technologies) and 50 μg/mL gentamicin (Sigma-Aldrich) at 37 °C in a humid atmosphere with 5% CO_2. All other cell incubation steps used these conditions unless otherwise stated.

Cell viability assay

CMGT cells were seeded at a density of 2000 cells/well in 96-well flat-bottom plates and metformin (1,1-dimethylbiguanide hydrochloride; Sigma-Aldrich; catalogue number D150959) was added at the final concentrations indicated after 24 h. Cell viability was determined using Cell Counting Kit-8 (Dojindo Laboratories). The effect of AMPK activation on cell viability was examined using the AMPK inhibitor Compound C (InSolution Compound C, 10 mM in dimethyl sulfoxide or DMSO, Merck Millipore). Further details are provided in the supplementary materials (see Appendix: Supplementary material).

Immunodeficient mouse xenograft model

The study protocol was approved by The University of Tokyo Animal Care and Use Committee (accession number 14–857; date of approval, 18 Apr 2014). BALB/c-nu/nu mice were inoculated with 5×10^6 CHMp-5b cells. Then, mice were randomly assigned to control ($n = 5$) and metformin (300 mg/kg/day administered in drinking water; $n = 5$) groups. The mice were humanely sacrificed after three weeks of treatment. Further details are provided in the supplementary materials (see Appendix: Supplementary material).

Quantification of reactive oxygen species

Cellular reactive oxygen species (ROS) and superoxide anions were quantified using a detection kit (Total ROS/Superoxide Detection Kit, Enzo Life Sciences). Cells were seeded in normal culture flasks and allowed to settle for 24 h. Metformin (0, 1, 5, 10, and 20 mM) was added 6 h prior to analysis. After trypsinisation, cells were labelled according to the manufacturer's instructions. Dead cells were gated based on their forward scatter vs. side scatter area profiles. Fluorescence was measured and analysed using a flow cytometer and software (BD FACSCalibur and BD CellQuest Pro software; Becton-Dickinson).

Quantification of ATP production from oxidative phosphorylation

Cells were suspended at a density of 2×10^5 cells/mL in glucose-free RPMI (Sigma-Aldrich) supplemented with 2 g/L galactose (Sigma-Aldrich), and 100 μL of the suspension was added to 96-well plates (Nunc LumiNunc; Thermo Fisher Scientific), followed by incubation for 24 h. Metformin was added and ATP production was quantified after a 2-h incubation period (Mitochondrial ToxGlo Assay, Promega). Luminescence was measured using a plate reader (ARVO MX, Perkin Elmer).

Western blotting

Control and metformin-treated cells were lysed and 10 μg of protein resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Further details are provided in the supplementary materials (see Appendix: Supplementary material).

cDNA microarray

Total RNA was extracted from the two cell lines untreated or treated with 10 mM metformin for 24 h. Purified and labelled cRNA was hybridised to a microarray (Canine V2 [4 × 44K], Agilent Technologies). Detailed procedures and analysis methods are provided in the supplementary materials (see Appendix: Supplementary material).

Cell cycle analysis

Control and metformin-treated cells were trypsinised and fixed with 70% ice-cold ethanol. After fixation, the cells were washed with phosphate buffered saline (PBS) and stained with 50 μg/mL propidium iodide (Sigma-Aldrich), 0.1 mg/mL RNase A (Roche Diagnostics), and 0.05% Triton X-100 (Sigma-Aldrich) for 40 min at 37 °C. The stained cells were immediately analysed (BD FACSCalibur, Becton-Dickinson) and the data were then processed (FlowJo software, TreeStar).

Statistical analysis

All data are shown as means ± standard deviation (SD). The statistical methods and software used were as follows: two-sided Student's *t* test was performed using Excel 2013 (Microsoft), and Dunnett's test was performed using R software (R Development Core Team, 2013) and multcomp R package (Hothorn et al., 2008). The choice of the statistical methods was described in each figure legend. $P \leq 0.05$ was considered statistically significant.

Results

In vitro and in vivo anti-tumour effects of metformin on CMGT cells

Metformin decreased the in vitro viability of the cell lines in a time- and concentration-dependent manner (Figs. 1A, B). CHMp-5b was significantly more sensitive than CHMp-13a, with half-maximal inhibitory concentrations (IC50) of 2.2 mM and >20 mM (not determined), respectively, for a 48 h exposure (Figs. 1C, D).

Oral metformin administration in the xenograft mouse model resulted in a significant reduction in primary tumour growth for CHMp-5b (Figs. 1E, F). Comparison of tumour mass weight after removal of necrotic tissue also supported an anti-tumour effect of oral metformin treatment (Fig. 1G), whereas no significant difference was observed in the metastatic potential of the tumour (Fig. 1H).

Evaluation of ROS generation and AMPK pathway activation with metformin exposure

A significant increase in total ROS and superoxide production was observed in both cell lines following 6 h of exposure to metformin (Appendix: Supplementary Figs. 1A, B). However, ROS levels were higher in the less sensitive CHMp-13a cell line. Superoxide production was slightly higher in CHMp-5b cells (5, 10 and 20 mM metformin treatment); however, a significant change between the two cell lines was only observed after exposure to the highest concentration of metformin (20 mM). Therefore, both ROS and superoxide were not considered important parameters to explain the higher sensitivity of CHMp-5b to metformin.