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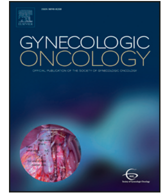

Hyperglycemia promotes insulin-independent ovarian tumor growth

Lisa D. Kellenberger, Jim Petrik *

Department of Biomedical Sciences, University of Guelph, Guelph, ON N1G 2W1, Canada

HIGHLIGHTS

- The role for elevated glucose in ovarian cancer proliferation and tumorigenesis is explored.
- Hyperglycemia, independent of insulin drives ovarian cancer progression
- Culturing cells in supraphysiological glucose significantly impacts cell function.

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ABSTRACT

Introduction. Epithelial ovarian cancer (EOC) is notoriously difficult to diagnose in its earlier and more treatable stages, making it one of the deadliest cancers in women. Comorbid diabetes is associated with poor prognosis in EOC and pro-growth insulin signalling is often considered to be the driving factor. However, EOC cells are also highly glycolytic and insulin-independent glucose uptake is essential to their metabolism.
Evidence of gluconeogenesis in cancer *in vivo* suggests that the normal concentration of circulating glucose does not meet the energy demands of the tumor and may therefore be a limiting factor in cancer cell metabolism. Diabetics have elevated blood glucose that has the potential to meet these energy demands and facilitate cancer progression.

Methods. To determine whether hyperglycemia is a potentially modifiable factor independent of insulin, orthotopic ovarian tumors were induced in mice with acute Type 1 (hypo-insulinemic) or Type 2 (hyper-insulinemic) diabetes.

Results. Hyperglycemia accelerated the growth of ovarian tumors in a glucose concentration-dependent manner and significantly shortened overall survival. Reciprocally, the presence of a tumor improved impaired glucose tolerance in both Type 1 and Type 2 diabetes. In mice with chronic Type 1 diabetes, hyperglycemia limited tumor growth without changing overall survival, indicating that systemic metabolic stress can accelerate time to death independent of primary tumor size. When modeled *in vitro*, long-term culture in 25 mM *vs* 6 mM glucose resulted in significantly different growth and metabolism.

Conclusions. Taken together, this study shows that systemic metabolic disturbances can have a profound impact on both the growth of ovarian tumors and on overall survival.

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1. Introduction

Comorbid diabetes affects 8–18% of all cancer patients [1,2]. In particular, women with Type 2 diabetes (T2DM) are at an increased risk of developing ovarian cancer [3] and epithelial ovarian cancer (EOC) patients with pre-existing diabetes have a much poorer overall survival rate than non-diabetic patients [4].

T2DM is characterized by hyperglycemia and hyperinsulinemia and is often related to obesity, which carries its own risk factors including high body mass index (BMI), sex steroid metabolism, and inflammation. Despite substantial epidemiological evidence, the underlying mechanisms of the cancer-diabetes association are still poorly understood. The mitogenic and pro-inflammatory effects of insulin are thought to drive tumor initiation and progression in diabetics [5]. However, the role of the underlying hyperglycemia has been largely overlooked, despite its pro-tumorigenic potential [6].

As in most malignancies, EOC cells import and metabolize considerably more glucose than their normal counterparts [7]. Elevated glycolytic activity in aerobic conditions is characteristic of the glucose metabolism in most cancer cells, and is accompanied by the conversion of pyruvate to lactate in large amounts (the Warburg Effect) [8]. The rate of insulin-independent glucose uptake through GLUT1 also increases with increasing extracellular glucose [9], suggesting that its total abundance in the environment is a rate-limiting factor for glucose metabolism in cancerous cells [10]. Furthermore, increased glucose

* Corresponding author.
E-mail address: jpetrik@uoguelph.ca (J. Petrik).

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uptake has been shown to coincide with the transition from pre-malignant lesions to invasive cancer and has been linked with overall tumor aggressiveness [11].

We hypothesized that diabetic hyperglycemia would accelerate the growth of ovarian tumors and EOC progression by providing an abundance of fuel needed for the maintenance of a cancerous phenotype. In this study, mouse models of Type 1 and Type 2 diabetes were used to investigate the development of surgically-induced orthotopic ovarian tumors in the presence of high glucose and/or high insulin.

2. Materials and methods

2.1. Animals

Wildtype (WT) C57bl/6 mice were purchased from Charles River Laboratories (Wilmington, MA, USA), and *Akt2* null C57bl/6 mice (https://www.jax.org/strain/006966) were purchased from Jackson Laboratories (Bar Harbour, ME, USA). An *Akt2* null mouse colony was established from breeder mice and animals were genotyped prior to beginning experiments. Animals were housed at the Central Animal Facility at the University of Guelph and all experiments were conducted per Canadian Council on Animal Care guidelines. Mice were kept on a 12 h light:dark cycle and had free access to food and water. For all experiments, adult mice were between 12 and 24 weeks old at the time of surgery.

2.1.1. Mouse model of epithelial ovarian cancer

Spontaneously transformed murine epithelial cells from C57Bl/6 mice (ID8) were diluted in 5 μl phosphate buffered saline (PBS) and injected under the ovarian bursa of WT or *Akt2* null mice. This orthotopic, syngeneic mouse model of EOC closely replicates ovarian serous adenocarcinoma in women. By 90 days post-tumor induction, mice develop large primary tumors, secondary peritoneal lesions, and significant abdominal ascites [12]. In survival experiments, mice were sacrificed when they became moribund due to the accumulation of ascites fluid.

2.1.2. Type 1 diabetes mellitus (T1DM)

Streptozotocin ([2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose]; STZ) is a pancreatic β-cell toxin commonly used to model T1DM [13,14]. Mice were treated with a standard streptozotocin dosing schedule (Animal Models of Diabetic Complications Consortium; AMDCC) two weeks prior to tumor induction surgery. Briefly, STZ (Sigma, Oakville, ON) was dissolved in 50 mM sodium citrate buffer (pH 4.5) made up fresh daily. Mice received 200 μl IP injections of 50 mg/kg STZ or vehicle control for six consecutive days. Blood glucose was measured ten days following the last injection and mice were considered hyperglycemic if non-fasted glucose was above 300 mg/dl (16.7 mM).

2.1.3. Type 2 diabetes mellitus (T2DM)

C57BL/6 mice homozygous for a mutant *Akt2* allele have poor glucose tolerance and reduced insulin sensitivity that result in hyperglycemia and hyperinsulinemia but are not obese [15,16]. Obesity often accompanies T2DM and is independently associated with diabetes and cancer so this model allowed us to isolate the contributions of high glucose and high insulin. Mice were genotyped using the following primers designed by Jackson Laboratories: *Akt2* wildtype reverse: TGCACAATCTGTCTTCATGCCAC; *Akt2* common forward: ACCAACCCCCTTTCAGCACTTG; *Akt2* mutant reverse: TACACTTCATTCTCAGTATTGTTTTGC. Products are 110 bp (wildtype), 277 bp (mutant), or 110 bp and 277 bp (heterozygote).

2.2. Food consumption

Food consumption at three weeks post tumor induction (PTI) was calculated by taking the initial weight of dry food and subtracting food weight after five days. This value was divided by the number of mice in the cage and the number of days in the measurement period to determine food consumed per mouse per day.

2.3. Intraperitoneal glucose tolerance tests

Mice were fasted for 4 h prior to intraperitoneal glucose tolerance tests (IPGTT). After recording baseline blood glucose, mice were given an intraperitoneal injection of glucose (1 mg/g body weight in saline solution at a volume of 10 μl/g body weight). Blood glucose measurements were taken at 15, 30, 60, and 120 min after glucose challenge using a handheld glucose monitor (Freestyle Lite, Abbott Laboratories). Values that exceeded the measurement range of the glucose monitor were considered 27.8 mM (maximum reading). Insulin levels were measured in trunk blood from fasted animals at sacrifice using an ultra-sensitive mouse insulin ELISA kit (Crystal Chem, Downers Grove, IL). Data are presented as the average integrated area under the curve (iAUC) ± SEM where iAUC is the area under the curve relative to fasting blood glucose (FBG) at time 0 (just before glucose injection). iAUC was calculated using the trapezoidal method. The glucose:insulin ratio was used as an indication of insulin sensitivity [17].

2.4. Tissue collection and preparation

Mice were fasted for 4–6 h prior to sacrifice by cervical dislocation. Following euthanasia, trunk blood was collected and fasting blood glucose was measured immediately using a handheld glucose monitor. The remaining blood was allowed to clot then centrifuged to obtain serum samples and stored at −80 °C. Ovarian tumors were removed, weighed, and divided into sections for subsequent analysis. Sections were either fixed in 10% neutral buffered formalin (Fisher Scientific, Whitby, ON) for wax embedding and tissue sectioning, or flash frozen in liquid nitrogen and stored at −80 °C for protein extraction. Secondary disease was evaluated at time of sacrifice by aspirating ascites and by visual assessment of the number of secondary lesions. Both ascites volume and secondary lesions were scored qualitatively. Metastatic tumor burden was scored on a relative 4-point scale with 0 indicating absence of secondary disease and 3 indicating the most widespread disease. Mice with scores of 0 (no observable peritoneal metastases) and 1 (1–2 observable metastases) were considered “low” and scores of 2 (3–10 observable metastases) and 3 (>10 observable metastases) were considered “high”. For Ascites fluid, volumes <5 ml were considered low, while volumes >5 ml were considered high.

2.5. Immunohistochemistry

To confirm the effects of streptozotocin, immunohistochemical detection of insulin was performed on 5 μm paraffin-embedded sections of pancreatic tissue from STZ-treated mice. Sections were deparaffinized in xylene and rehydrated in graded alcohol solutions. Endogenous peroxidase activity was quenched using 1% (vol/vol) hydrogen peroxide and antigen retrieval was performed by immersing slides in 10 mM citrate buffer at 90 °C for 12 min. Tissues were blocked in 5% (wt/vol) bovine serum albumin in PBS for 10 min and slides were then incubated overnight with anti-insulin primary antibody (Cell Signal Technologies, Danvers, MA) diluted in 0.01 M PBS (pH 7.5) containing 2% (wt/vol) BSA and 0.01% (wt/vol) sodium azide at 4 °C in a humidity chamber. All subsequent incubations were performed at room temperature. Anti-rabbit biotinylated secondary antibody (Sigma, Oakville, ON) was diluted in the same buffer and incubated for 2 h. Tissues were then washed in PBS and incubated with avidin and biotinylated horseradish peroxidase (ExtrAvidin, Sigma, Oakville,

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