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Glucagon-induced angiogenesis and tumor growth through the HIF-1-VEGF-dependent pathway in hyperglycemic nude mice

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ABSTRACT. In this study, we examined the effect glucagon-induced hyperglycemia on tumor growth as well as the role of the hypoxia-inducible factor 1 (HIF-1)-vascular endothelial growth factor (VEGF) pathway in this condition. A high concentration of glucose (HG) was utilized to treat HeLa cells under hypoxic or normoxic conditions, and transcriptional levels of HIF-1, VEGF, and basic fibroblast growth factor (bFGF) were evaluated. Moreover, the ability of an HIF-1 inhibitor to block the effect induced by HG was examined. By contrast, hyperglycemia was induced in nude mice by glucagon released from an osmotic pump, and microvessel density was determined with CD31 staining. Thus, the relationship among hyperglycemia, microvessel density, tumor growth, and the HIF-1 inhibitor were analyzed. We

found that HG increased transcription of the *VEGF* gene, which is downstream of *HIF-1*. Moreover, HG impaired the function of HIF-1 inhibitors [HIF-1 small interfering RNA (siRNA) and berberine] to affect the VEGF transcription level in tumor cells. By contrast, hyperglycemia increased tumor microvessel density and promoted tumor growth, which was inhibited by the HIF-1 inhibitor. However, hyperglycemia attenuated the effect of the HIF-1 inhibitor. Glucagon-induced hyperglycemia influenced tumor microenvironments through the HIF-1-VEGF-dependent pathway and promoted tumor growth and resistance to HIF-1 inhibition treatments.

Key words: Berberine; Hyperglycemia; Hypoxia-inducible factor-1; Glucagon; Tumor microenvironment

INTRODUCTION

The number of aging patients who suffer from diabetes and malignant tumors is increasing (van de Poll-Franse et al., 2007; Brower, 2012). Recent reports have shown that approximately 60% of all new cancer patients over 65 years old suffer from at least one other serious disease. Diabetes is common among these secondary diseases, affecting 16% of aging cancer patients. The positive association between diabetes and pancreatic, kidney, and uterine cancers has been well established (Brower, 2012). Hyperglycemia has also been observed in patients with paraneoplastic syndromes (van de Poll-Franse et al., 2007).

Glucagon is one of the main causes of diabetes and hyperglycemia, which reportedly cause difficulties in treatment (Villarreal-Garza et al., 2012). Hyperglycemia causes damage to endothelial cells and induces angiogenesis through genes such as basic fibroblast growth factor (*bFGF*) and vascular endothelial growth factor (*VEGF*) (Stenina, 2005). Han et al. (2005, 2007) found that insulin, heparin, and bFGF could inhibit endothelial cell injury caused by high glucose (HG) concentration, but the data related to the effect of bFGF are inconsistent. Furthermore, data related to the molecular mechanisms underlying hyperglycemia functions in tumors are insufficient. Thus, the relationships among tumor microenvironments, hyperglycemia, and angiogenesis-related genes must be further examined.

Tumor microenvironments are not homologous, and they can significantly alter glucose metabolism. Studies have revealed the existence of hypoxic regions in solid tumors showing a lack of oxygen and other nutrients, such as glucose, in regions far from tumor blood vessels (Harada et al., 2008; Semenza, 2012). These changes influence the tumor microenvironment, resulting in a specific cellular response to hypoxic and low glucose (LG) conditions in these regions. A key factor regulating the cellular response to hypoxia is hypoxia-inducible factor-1 (HIF-1), a heterodimeric factor composed of a hypoxia-regulated α-subunit and a constitutively expressed β-subunit (also known as aryl hydrocarbon receptor nuclear translocator). Although HIF-1α is consistently transcribed and translated, it is degraded in an oxygen-dependent manner. In the presence of oxygen, HIF-1α undergoes posttranslational modification by prolyl hydroxylases and the Von Hippel-Lindau tumor suppressor protein, after which it is degraded through the proteasome pathway. By contrast, low oxygen concentration impairs prolyl hydroxylation, resulting in the stabilization of HIF-1α. Accumulated HIF-1α

interacts with HIF-1β to form HIF-1, which activates several genes involved in glycolysis, recurrence, angiogenesis, and metastasis (Harada et al., 2008; Semenza, 2010a). HIF-1α has been suggested to be a novel target in cancer therapy. Berberine, a primary pharmacologically active component of *Rhizoma coptidis* used to treat metabolic syndromes, was recently found to repress HIF-1 at the posttranslational level (Lin et al., 2004). Berberine reportedly inhibits angiogenesis through the HIF-1-dependent pathway (Hamsa and Kuttan, 2012). Thus, we utilized berberine as an HIF-1 inhibitor to analyze the relationship between hyperglycemia and angiogenesis.

In our previous study, we confirmed that HG concentration under hypoxic conditions could elevate HIF-1 expression both *in vitro* and *in vivo*, which increased radiosensitivity by regulating the cell cycle of the HeLa cell line (Zhu et al., 2013). In the present study, we confirmed the reproducibility of increased HIF-1 expression caused by HG under hypoxic conditions in the A549 cell line. We investigated the mechanism by which high HIF-1 expression patterns led to upregulation of VEGF transcription using real-time quantitative polymerase chain reaction, then, investigated the relationship among microenvironments, hyperglycemia, tumor growth, and the influence of HIF-1inhibitor.

MATERIAL AND METHODS

Cell culture

Human A549 lung carcinoma cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum with a relatively HG concentration (4.5 g/L) or relatively LG concentration (0.45 g/L). The cells for normoxic cultures were incubated in a well-humidified incubator with 5% CO_2 and 95% air at 37°C. The cells for hypoxic cultures were incubated at <0.02% O_2 in a Bactron Anaerobic Chamber, BACLITE-2 (Sheldon Manufacturing Inc.; Cornelius, OR, USA).

Real-time reverse transcription-PCR

A549 cells were collected for real-time reverse transcription-PCR. Both transfected and untreated cells were collected and washed with phosphate-buffered saline (PBS). Total RNA from cells was purified using TRIzol reagent (Invitrogen; Carlsbad, CA, USA), and subjected to a quantitative real-time PCR assay using Thermal Cycler DICE and SYBR Premix Ex Taq (Takara Bio; Shiga, Japan) to quantify the mRNA levels of bFGF and VEGF. The results were normalized to the transcription levels of actin. In addition to siRNA, 50 mM HIF-1 inhibitor, berberine hydrochloride (Nacalai Tesque; Kyoto, Japan), was utilized to treat cells.

Western blot analysis

A549 cells were seeded on 6-well plates for Western blot analysis using 2 x 10^5 cells/well. The cells were cultured in a medium containing either HG or LG concentration for 20 h under normoxic or 0.02% O_2 conditions. Cell lysate prepared With CelLytic M cell lysis reagent (Sigma-Aldrich; St. Louis, MO, USA) was subjected to Western blot analysis. For *HIF-*

1 gene knockdown, A549 cells were transfected with 10 nM siRNA using the CodeBreaker siRNA Transfection Reagent (Promega; Madison, WI, USA) according to manufacturer instructions. After 24 h of culture, the cells were subjected to hypoxic treatment for an additional 20 h. Next, the cell lysate was harvested for Western blot analysis. Prior to sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot analysis, the protein concentration of the cell lysate was adjusted to 20 mg protein/20 L by performing a protein assay using the Quick Start Bradford Dye Reagent (Bio-Rad Laboratories; Hercules, CA, USA). HIF-1 and actin were detected using an anti-human HIF-1 mouse monoclonal antibody (BD Biosciences; Franklin Lakes, NJ, USA) and an anti-human actin mouse monoclonal antibody (BioVision Research Products; Mountain View, CA, USA), respectively, as described previously (Zhu et al., 2013). Each protein was detected using an anti-mouse horseradish peroxidase-linked whole antibody (GE Healthcare Bio-Science Corp.; Piscataway, NJ, USA) and an ECL-PLUS system (GE Healthcare Bio-Science Corp.) according to manufacturer instructions.

Tumor-bearing mice, glucagon treatment, and glycemia measurement

The A549 cell suspension (2 x 10^6 in PBS) was subcutaneously inoculated into the right hind leg of 7-week-old female nude mice (BALB/c nu/nu mice; the Animal Center of the Third Military Medical University, Chongqing, China) (Semenza, 2012). An osmotic pump (1007D; Alzet Osmotic Pump; Cupertino, CA, USA) loaded with various glucagon solution concentrations (0, 1, 2, 4, and 8 mg/mL in 3.44 mM cetrimid solution) was intraperitoneally applied to the tumor-bearing mice for continuous glucagon administration (Webb et al., 2002; Zhu et al., 2013). Glycemia in the plasma of nude mice was assessed at different times using a glucometer (Terumo GR102 Glucometer; Shibuya, Japan). The animal study protocol was approved by the Animal Care and Use Committee Guidelines of the Third Military Medical University.

Tumor xenograft growth assay

The sizes of solid tumors were measured with calipers. Tumor volume was calculated using the formula: 0.5 x length x $width^2$. The relative tumor volume was obtained by comparing the tumor volume each day with the initial value. The mice were treated with glucagon at a release rate of 5 g/h and/or berberine at 100 mg/kg.

Immunohistochemical analyses

Tumor xenografts were surgically excised on day 10, placed in formalin, and embedded in paraffin. Tissue sections of 3-5 μm thickness were used for immunohistochemistry. For antigen retrieval, treatment conditions for deparaffinized sections were adapted for the anti-mouse CD31 rat monoclonal antibody (BD Biosciences). The primary antibody was incubated at room temperature for 60 min at a dilution of 1:500. Secondary staining was performed using the alkaline phosphatase-anti-alkaline phosphatase method. The slides were counterstained with hemalaun solution. The reproducibility of each stain was confirmed in at least 3 independent tumors, and representative results are shown. To determine microvessel density (MVD), the areas with the highest numbers of capillaries and small venules were identified by scanning the tumor sections at a magnification of 40X. Individual vessels were counted in 9 random fields at a magnification of 100X.

Statistical analysis

The statistical significance of the differences was determined using the Student *t* test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Effect of HG on HIF-1 expression in A549 cells and transcriptional levels of VEGF and bFGF

Induction of elevated HIF-1 expression under hypoxic and HG conditions in HeLa cells was determined by Western blot analysis (Zhu et al., 2013). This phenomenon could be reproduced in A549 cells. Under hypoxic conditions, HIF-1α expression increased more under HG conditions than under LG conditions (Figure 1A and B). HG also increased transcription of *VEGF*, the gene downstream of *HIF-1*, under hypoxic conditions. However, the results differed for *bFGF*, another important gene related to angiogenesis in tumors. By contrast, these phenomena were not observed under normoxic conditions (Figure 1C), indicating that HG further induced VEGF expression under hypoxic conditions through the HIF-1-dependent pathway. Moreover, hyperglycemia may have induced angiogenesis.

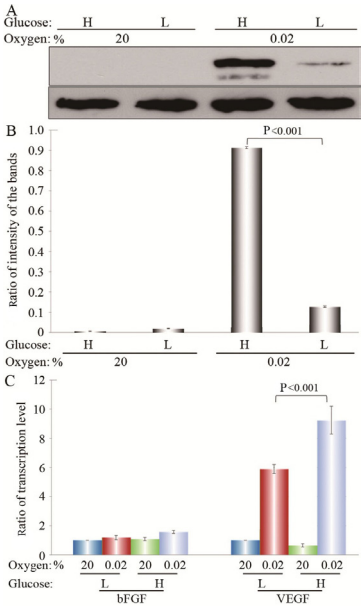

Figure 1. Relationship between HG, HIF-1 and its downstream genes under hypoxic conditions. **A.** Increased glucose concentration up regluated the expression of HIF-1 under hypoxic conditions in A549 cells. A549 cells were cultured for 24 h, and then cultured in fresh medium containing HG or LG under normoxic (20% oxygen) or hypoxic (0.02% oxygen) conditions for 20 h. Cell lysate was subjected to Western blotting for HIF-1 and -actin. **B.** The ratio of intensity of the bands, HIF-1 divided by actin, was quantified using the Image J software. Results are reported as the means ± SD, N = 3, **P < 0.001. **C.** The ratio of transcription level of bFGF and VEGF normalized by actin under HG and hypoxic conditions were checked by RT-PCR. Only the transcriptional level of VEGF dramatically increased after HG and hypoxic conditions. Results are reported as means ± SD, N = 3, **P < 0.001.

Effect of HG on the function of HIF-1α inhibitors *in vitro*

To explore the influence of HG on *HIF-1* regulation, we examined HIF-1 inhibitors with either HG or LG concentration under hypoxic conditions. A549 cells were transfected with 2 siRNAs (H1 and H2) that specifically targeted *HIF-1*α and a scrambled negative control, respectively. The knockdown effect of *HIF-1*α siRNA was examined by Western blot analysis. The HIF-1α protein could only be detected in the negative control samples under hypoxic conditions (Figure 2A). To determine whether HG impairs the knockdown effect of *HIF-1* siRNAs, we investigated VEGF mRNA levels after the hypoxic A549 cells were transfected with siRNAs in the presence of either HG or LG concentration under hypoxic conditions. Compared with the negative control, *VEGF* transcription was largely inhibited by *HIF-1* siRNAs under hypoxic conditions, but the VEGF mRNA level was significantly upregulated by HG and hypoxia (Figure 2B). Berberine, another HIF-1 inhibitor, was also utilized to determine whether its effects could be impaired by HG concentration. A549 cells were treated with berberine at HG or LG concentration under hypoxic conditions; the results were similar to those of *HIF-1*α siRNA (Figure 2C). Thus, HG impaired the inhibitory effects of various HIF-1 inhibitors *in vitro*.

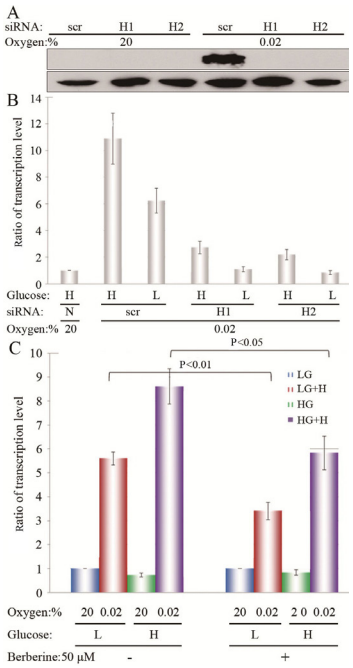

Figure 2. Transcriptional level of VEGF after HIF-1 inhibitor treatment. **A.** A549 cells were transfected with HIF-1 siRNA for 24 h, and then cultured under normoxic (20% oxygen) or hypoxic (0.02% oxygen) conditions for 20 h. Cell lysate was subjected to Western blotting for HIF-1 and actin. The expression of HIF-1 protein was inhibited after HIF-1 siRNA treatment. **B.** The ratio of transcription level of VEGF normalized by actin under HG and hypoxic conditions after HIF-1 siRNA transfection was detected by RT-PCR. **C.** The ratio transcriptional level of VEGF normalized by -actin under HG and hypoxic conditions after berberine (50 M) treatment was checked by RT-PCR.

Glucagon-induced hyperglycemia in nude mice

In our previous study, we induced hyperglycemia in nude mice (BALB/c nu/nu mice) using an osmotic pump that continuously released glucagon (Zhu et al., 2013). This method was based on a study by Webb et al. (2002), who reported that hyperglycemia in prohormone convertase 2-negative mice was successfully induced using glucagon. Hypoglycemia occurs when the conversion of proglucagon into mature active glucagon in prohormone convertase 2 (-/-) mice is inhibited. Although other methods induced hyperglycemia in other types of mice, the use of nude mice to establish tumor xenografts is convenient. Relationships among glucagon, hyperglycemia, and tumor growth were further examined by applying various glucagon concentrations to mice and observing them for 10 days. Blood glucose concentration was assessed prior to pump implantation and on days 5 and 10 post-implantation (Figure 3A). Cetrimide, a negative control, had no effect on blood glucose concentration. Glucagon delivered at a rate of 0.25 g/h produced a very weak effect. However, at delivery rates of 0.5, 1, and 2 g/h, blood glucose concentrations significantly increased. Differences in blood glucose concentrations among the 0.5, 1, and 2 g/h groups were not significant. Thus, we selected a delivery rate of 0.5 g/h for glucagon to induce hyperglycemia in nude mice in further experiments.

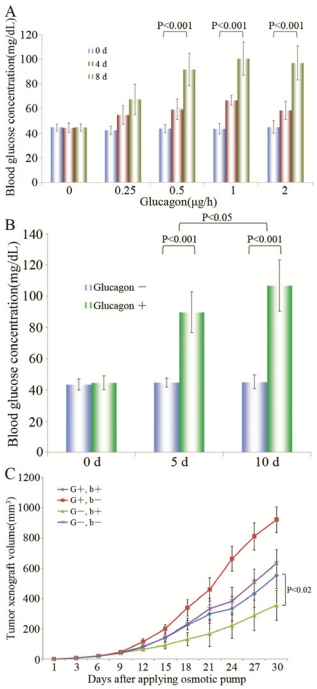

Figure 3. Glucagon treatment induced hyperglycemia in nude mice, and the growth of xenografts after glucagon and/or berberine treatment. **A.** Blood glucose concentration of nude mice after glucagon treatment from osmotic pump. The osmotic pump released glucagon at a rate of 0, 0.25, 0.5, 1, 2 g/h. Blood glucose concentration was checked by the Glucometer. **B.** Blood glucose concentration of nude mice after 5 days glucagon 0.5 g/h treatment from osmotic pump. **C.** Tumor growth assay was checked by cliper every 3 days. Mice were treated by glucagon, and/or bererine. The concentration of berberine was: 100 mg/kg, and the releasing rate of glucagon was 5 g/h.

Glucagon treatment promoted tumor xenograft growth

The influence of glucagon treatment on the growth of A549 cell xenografts in nude mice was examined. A total of 16 7-week-old nude mice were divided into 2 groups, including negative control and glucagon treatment groups. Approximately 2 x 10^6 A549 cells were subcutaneously inoculated into the right hind leg of nude mice, as described in the Material and Methods section. Ten days after transplantation, glucagon-containing osmotic pumps were intraperitoneally applied to the tumor-bearing mice, and glucagon was released at 0.5 g/h. Blood glucose concentration was measured to confirm hyperglycemia during tumor growth after glucagon treatment. Following the application of glucagon, blood glucose concentration significantly increased by 2.00-fold on day 5 and by 2.36-fold on day 10 (Figure 3B).

Nude mice treated with glucagon and/or berberine were separated into 4 groups. These mice were observed every 3 days following osmotic pump implantation to examine the growth rate of tumor xenografts. HG concentration induced by glucagon significantly promoted tumor xenograft growth. By contrast, berberine significantly inhibited the growth rate. However, when glucagon and berberine treatments were concurrently applied, the growth rate increased (Figure 3C), indicating that glucagon-induced hyperglycemia partially impaired the function of berberine *in vivo*. Thus, the potential mechanisms of this phenomenon were investigated.

Induction of MVD in the tumor microenvironment

We hypothesized that hyperglycemia influences the tumor microenvironments and increases tumor growth. To determine the influence of the glucagon-hyperglycemia axis on the tumor microenvironment *in vivo*, we treated mice with or without glucagon, and immunohistochemical staining of the hypoxic markers HIF-1α and pimonidazole was performed as described previously (Zhu et al., 2013). Without glucagon treatment, HIF-1 protein expression mainly occurred in regions that were relatively far from the tumor blood vessel but were still within the pimonidazole-stained regions. This result was consistent with that of a previous report (Sobhanifar et al., 2005). However, the glucagon-hyperglycemia axis increased HIF-1 expression even in pimonidazole regions (Zhu et al., 2013), indicating that the glucagon-hyperglycemia axis induced greater HIF-1 expression in the tumor xenograft. Thus, in this study, we explored the mechanism by which increased HIF-1 expression influenced tumor microenvironments and promoted tumor growth. Through immunohistochemical staining, MVD showed a large increase after glucagon treatment but decreased after berberine treatment compared with the negative control. Glucagon treatment significantly increased MVD in the berberine treatment group (Figure 4A-E).

DISCUSSION

The morbidity of both diabetes and cancer increases with age, and these diseases show positive feedback (Brower, 2012; Villarreal-Garza et al., 2012). Diabetes and hyperglycemia may be associated with an elevated risk of developing cancers of the pancreas, liver, and colon, among others (van de Poll-Franse et al., 2007). Thus, it is important to understand the relationship between cancer and diabetes, as well as the underlying mechanisms.

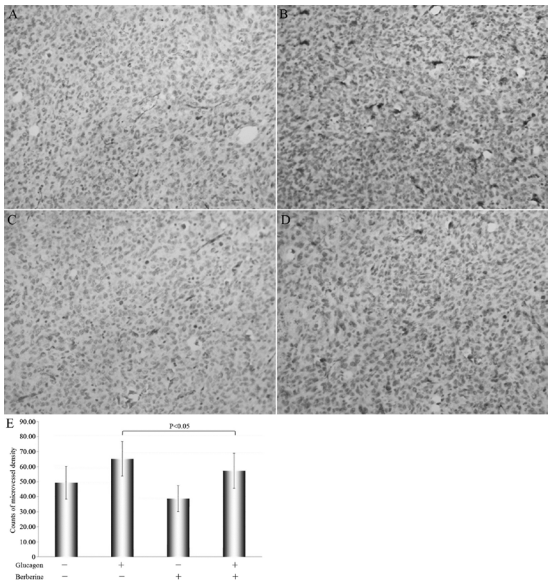

Figure 4. Microvessel density (MVD) measured by CD31 staining after glucagon and berberine treatment. (A-D) Representative images of MVD on day 10 after treatment. (E) Immunohistochemical staining of MVD of each combination of treatment on day 10. Results are reported as the means of nine random field ± SD.

In our previous study, we found that glucagon induces hyperglycemia *in vivo* and upregulates HIF-1 expression and activity even in severely hypoxic regions. Thus, we focused on tumor microenvironments and HIF-1 in this study. In most cases, tumor vascularization and tumor nutrition supply cannot meet the needs of tumor cells (Semenza, 2010a). These tumor cells form tumor-specific microenvironments known as tumor cords, which are composed of tumor blood vessels, normoxic regions, hypoxic regions, and necrotic regions (Semenza, 2010a, 2012). In addition to the low oxygen availability in hypoxic regions, the glucose concentration should also be low because glucose is consumed by cells in normoxic regions. HIF-1 is important for regulating the adaptability of tumor cells to hypoxia and can upregulate a large quantity of downstream genes associated with angiogenesis, glycolysis, and resistance to chemotherapy and radiotherapy (Semenza, 2010b).

To further analyze the function of HIF-1 on the relationship between the glucagon-hyperglycemia axis and tumors, we first performed *in vitro* experiments. We found that HG treatment elevated HIF-1 expression in A549 cells under hypoxic conditions. The transcriptional level of VEGF was also elevated. HG also impaired the effects of HIF-1 inhibitors. Glucagon-hyperglycemia-BALB/c nu/nu nude mice inoculated with tumor xenografts were utilized for *in vivo* experiments. Compared with the negative control groups, both the growth rate of tumor xenografts and tumor MVD were upregulated in the hyperglycemia group. Similarly to the *in vitro* results, hyperglycemia impaired the function of berberine, delayed tumor growth, and decreased the tumor MVD.

The relationship between glucagon and diabetes has been recently investigated. Glucagon is released from alpha cells and increases blood glucose levels. Excessive glucagon release can result in insulin resistance, which may cause diabetes (Cho et al., 2012). The results

of our studies, both previous and present, have revealed that glucagon-induced hyperglycemia upregulated HIF-1 expression and activity both *in vitro* and *in vivo*. HIF-1 is also thought to function in carcinogenesis (Michaylira and Nakagawa, 2006; Nys et al., 2011). Clinical studies indicate that elevated levels of the HIF-1α protein are significantly correlated with poor patient survival rates (Lu and Kang, 2010; Al-Salam et al., 2012). Nuclear accumulation of HIF-1α protein is also correlated with poor tumor differentiation in primary pancreatic cancers (Kim et al., 2009), explaining the high morbidity of tumors in diabetic patients.

HIF-1 is an important mediator of the tumor cell response to hypoxia, and controls the upregulation of several genes associated with solid tumor expansion, including *VEGF* (Semenza, 2010a). Although the *bFGF* gene is also associated with angiogenesis, HG treatment could only upregulate *VEGF* transcription under hypoxic conditions. Thus, HG treatment may have increased VEGF expression through the HIF-1-dependent pathway.

In our *in vivo* experiments, both the MVD of tumor xenografts and tumor growth rate were upregulated by glucagon treatment. In animal experiments, the function of HIF-1 in tumor growth was confirmed. High HIF-1 expression levels increase tumor xenograft growth, whereas knockdown of HIF-1α reduces tumor sphere formation and tumor xenograft growth (Ke et al., 2012). Thus, the glucagon-hyperglycemia (insulin-resistant)-HIF-1-VEGF pathway may have an important function in promoting tumor growth.

We inferred that this pathway impairs the effects of HIF-1 inhibitors because of the function of glucagon-hyperglycemia. Preclinical and clinical experiments have shown that HIF-1 inhibitors, such as siRNAs and berberine, can delay tumor growth and metastasis *in vivo* (Harada et al., 2009; Semenza, 2009; Bohonowych et al., 2011; Harada et al., 2012). However, our results indicate that HG abrogated the effect of *HIF-1* siRNA under hypoxic conditions *in vitro*. Glucagon-induced hyperglycemia impaired the effect of berberine by upregulating both MVD and growth rate, suggesting that hyperglycemia partially abrogated the function of HIF-1 inhibitors to resist chemotherapy or radiotherapy. Thus, hyperglycemic cancer patients treated with HIF-1 inhibitors may have a worse prognosis than those with normal blood glucose levels.

Our results reveal an important function of hyperglycemia in tumor microenvironments, which can promote tumor growth and cause resistance to tumor treatment. Thus, the pathway from glucagon to therapy resistance in diabetic patients can be expressed as glucagon-hyperglycemia (insulin-resistant)-HIF-1-VEGF-MVD resistance to therapy.

To overcome the effects of hyperglycemia on tumor microenvironments and tumor therapy, the blood glucose level of diabetic patients should be normalized. Excessive glucagon should also be inhibited to overcome insulin resistance.

Finally, other factors involved in tumor growth should also be explored in future studies. Berberine can partially reverse the effect of hyperglycemia on tumors. Because berberine can also inhibit glucagon and its function in diabetes treatments, the mechanism by which berberine and insulin resistance can influence this pathway should be further analyzed. Further investigation may reveal complications of treatment in patients with both diabetes and cancer, as well as provide solutions for overcoming these challenges.

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