High-Glucose Conditions Promote Anchorage-Independent Colony Growth in Human Breast Cancer MCF-7 Cells

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Received March 2, 2018; accepted June 11, 2018

Previous studies have shown that hyperglycemia is connected to the malignant progression of breast cancer; however, the effects of hyperglycemia on tumorigenic potential in breast cancer cells are largely unknown. Here, we demonstrated that the ability of the human breast cancer cell line MCF-7 to undertake anchorage-independent colony growth was significantly enhanced when cultured under high-glucose conditions compared with that under physiological glucose conditions. The high-glucose conditions also promoted phosphorylation of Akt, suggesting that MCF-7 cells cultured in these conditions acquired an increased ability to undergo anchorage-independent growth at least in part through Akt activation, which has been linked to the development of breast cancer. These results raise the possibility that regulation of Akt activity contributes to the tumorigenesis of breast cancer under high-glucose conditions, and we propose that additional analyses of high glucose-induced tumor formation would provide novel strategies for the diagnosis and therapy of breast cancer with hyperglycemia.

Key words hyperglycemia; breast cancer; anchorage-independent colony growth; MCF-7; Akt

Diabetes and breast cancer are chronic diseases with an increasing incidence worldwide. Recent epidemiological research has shown that diabetes is strongly related to malignancy and poor prognosis of breast cancer.1,2) The sensitivity of breast cancer to therapy also varies between women with and without diabetes, and women with breast cancer and diabetes have been reported to be more likely to need hormonal therapy than nondiabetic women with breast cancer, suggesting that breast cancer with diabetes has a strong tendency to be the estrogen receptor-positive form of this disease.3–5) A growing body of evidence regarding major risk factors of breast cancer with diabetes has shown that a high glucose level corresponding to hyperglycemia modulates the functions of breast cancer cells, including their proliferation,6,7) survival,8–10) and migration.11–14) Our previous results indicated that the migratory capacity of the human estrogen receptor-positive breast cancer cell line MCF-7, but not the human estrogen receptor-negative breast cancer cell line MDA-MB-231, which has been linked with invasion and metastasis, is dependent on the concentration of extracellular glucose.11,12) Moreover, continuous culture under high-glucose conditions has been shown to promote MCF-7 cell survival under a hypoxic environment, which has been correlated with therapeutic resistance and a poor prognosis.15) These findings suggest that the glucose concentration and exposure time are critical for the progression of estrogen receptor-positive breast cancer; however, the major effects of glucose on tumorigenic potential are largely unknown. Therefore, studies aimed at understanding the biological phenomena that mediate the progression of breast cancer when combined with diabetes are necessary for the development of potential therapeutic targets for breast cancer.

In this study, to clarify the tumorigenic potential of MCF-7 under high-glucose conditions and the molecules involved in this process, we investigated anchorage-independent cell growth using an in vitro soft agar colony formation assay, which is a powerful tool for measuring in vivo tumorigenic potential. Our findings show for the first time that high glucose levels promote the anchorage-independent growth of single cells, at least in part through Akt activation, which has been linked to the development of estrogen receptor-positive breast cancer.

MATERIALS AND METHODS

Cell Culture and Cell Treatments Human MCF-7 breast carcinoma cell lines were maintained in minimum essential medium (MEM; Invitrogen, Carlsbad, CA, U.S.A.) containing 5.5 mM D-glucose, 10% heat-inactivated fetal bovine serum (FBS; GE Healthcare Life Sciences HyClone Laboratories, South Logan, UT, U.S.A.), and antibiotics in a humidified atmosphere supplemented with 5% CO2 at 37°C, as described previously.11,12,15) Cell treatments were as follows: MCF-7 cells were cultured in medium with a normal (5.5 mM) or high (25 mM) concentration of D-glucose or in an osmotic control medium (5.5 mM D-glucose plus 19.5 mM D-mannitol) with 10% FBS. The media were routinely changed every 2 or 3 d, and the cells were passaged every week when subconfluent. After culturing for 7 to 28 d, various assays were performed.

Soft Agar Colony Formation Assay for Anchorage-Independent Cell Growth MCF-7 cells were harvested by trypsinization into single-cell suspensions. Cell suspensions were mixed 1:1 (v/v) with medium containing 0.5% agarose for a final concentration of 0.25% agarose. The cell mixture (6000 cells/well) was plated on top of a solidified layer of medium containing 0.5% agarose in six-well tissue culture plates and incubated for 14 d at 37°C in a 5% CO2, 95% air incubator. To investigate colony formation under high-glucose conditions, normal-glucose (5.5 mM), high-glucose (25 mM), and osmotic control (5.5 mM D-glucose plus 19.5 mM D-mannitol) media were used. To investigate the effect of pretreatment under high-glucose conditions on colony formation, a monolayer of MCF-7 cells was cultured with normal-glucose, high-glucose, or osmotic control media for 7–28 d,
and single-cell suspensions of pretreated cells were plated for a colony formation assay using growth medium (normal-glucose medium). Images of colonies were captured using a phase-contrast microscopy system (Nikon, Tokyo, Japan). The assay was performed in triplicate and a total of 61–367 colonies were counted in each sample. The diameter (length and width) of each colony was measured and assigned a rank determined by the average of its length and width as follows: +, <90µm; ++, 90–150µm; ++++, 150–210µm; and +++++, >210µm. The colony volume was calculated using the following formula: length×(width)²/2.

**Phospho-Akt Measurement** MCF-7 cells were cultured with normal-glucose, high-glucose, or osmotic control media for 7–28 d, and the cells were measured for phospho-Akt using a specific antibody, as described previously. The treated MCF-7 cells were harvested by trypsinization into single-cell suspensions. Cell suspensions were fixed in 4% paraformaldehyde for 10 min and permeabilized with 90% methanol on ice for 30 min. After blocking with 0.5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, U.S.A.) in phosphate-buffered saline (PBS) for 10 min at room temperature, the cell suspensions were sequentially incubated with pAkt (Ser⁴⁷³) (193H12, Cell Signaling Technology, Danvers, MA, U.S.A.) for 1 h at room temperature and goat anti-rabbit Alexa 488 (Invitrogen) for 30 min at room temperature. After washing with PBS, fluorescence intensities were determined using a Tali Image-Based Cytometer (Invitrogen).

**Cell Viability Assay** MCF-7 cells were cultured with normal-glucose, high-glucose, or osmotic control medium for 7–28 d, and the cells (4×10⁴ cells/well) were seeded into 96-well plates for 24 h, followed by incubation with tamoxifen. After 24 h of treatment with tamoxifen, the cell viability was determined by methylthiazole tetrazolium (MTT) reduction using the CellTiter 96® Non-Radioactive Cell Proliferation Assay, according to the manufacturer’s instructions (Promega, Madison, WI, U.S.A.). The cell viability experiments were performed in triplicate and repeated at least three times.

**Statistical Analysis** All statistical analyses were performed using GraphPad Prism (ver. 5.00; GraphPad, San Diego, CA, U.S.A.). For the data of rank of colony, the Kruskal–Wallis test followed by Dunn’s multiple post hoc test was used. For multiple-group comparisons, either one-way ANOVA followed by Tukey’s post hoc test or two-way ANOVA followed by Bonferroni’s post hoc test was used. Differences were considered significant when the calculated p-value was <0.05.

**RESULTS**

**High Glucose Enhances Anchorage-Independent MCF-7 Cell Growth** To determine the effect of high glucose levels on anchorage-independent MCF-7 cell growth, we examined the soft agar colony-forming ability of a single MCF-7 cell under normal physiological conditions (5.5 mM glucose) or hyperglycemic conditions (25 mM glucose) for 14 d. Figure 1A shows representative photomicrographs of cell colonies in soft agar under each of the experimental conditions. The colony cultured with 25 mM glucose exhibited growth at a markedly higher rate than MCF-7 cells cultured with 5.5 mM glucose or osmotic control medium (5.5 mM glucose with 19.5 mM mannitol) for 14 d. The results of the quantitative analyses of the size and number of colonies (Fig. 1B) and colony volume (Fig. 1C) induced by MCF-7 cells under the indicated experimental conditions are shown. Findings similar to those presented above revealed that high glucose levels significantly promote...
anchorage-independent MCF-7 cell growth compared with 5.5 mM glucose or osmotic control medium.

Next, we investigated the effects of preculture with high glucose on anchorage-independent MCF-7 cell growth because it has been reported that breast cancer patients who suffered any hyperglycemia at all prior to the development of breast cancer.

Fig. 2. MCF-7 Cells Precultured under High-Glucose Conditions Promote Anchorage-Independent Growth

MCF-7 cells were cultured with normal-glucose (5.5 mM), high-glucose (25 mM), or osmotic control (5.5 mM D-glucose plus 19.5 mM D-mannitol) media for 7 or 28 d, and subjected to a colony formation assay for 14 d, as described in Materials and Methods. (A) Images of colonies produced by MCF-7 cells in each experimental condition. The colonies were observed and photographed under a microscope. Scale bars represent 100 µm. (B) Quantitation of the size and number of colonies in each experimental condition. The number of colonies grown on soft agar was counted and the distribution of colony size was examined. (C) Relative colony growth ratio among the different durations of preculture. The colony volume was measured as described in Materials and Methods and the ratio of the volumes of colonies produced by MCF-7 cells with pretreatment for 28 d relative to 7 d was estimated. Data are presented as means±S.E. ***p<0.001, n=61–162.

Fig. 3. High-Glucose Conditions Increase Akt Activation

MCF-7 cells were cultured with normal-glucose (5.5 mM, blue), high-glucose (25 mM, green), or osmotic control (5.5 mM D-glucose plus 19.5 mM D-mannitol, red) media for 7 or 28 d, as described in Materials and Methods. (A) The levels of Ser473-phosphorylated Akt in MCF-7 cells were assayed by flow cytometry at 7 or 28 d of the indicated cultures. Background fluorescence (black) was determined using a no-primary antibody control. (B) After background subtraction, quantitative analysis of fluorescence intensity was performed. Data are presented as means±S.E. **p<0.01, n=3–5. (Color figure can be accessed in the online version.)
MCF-7 cells precultured with 5.5 mM glucose or 5.5 mM glucose for 7 d prior to the colony formation assay did not cause a significant increase in the size of colonies compared with those cultured with conditions of 25 mM glucose for 28 d, compared with those cultured with 5.5 mM glucose. Moreover, the colony growth of cells precultured with 25 mM glucose for 28 d, compared with those precultured under the same glucose conditions for 7 d, was significantly enhanced in the number of colonies compared with those cultured with 5.5 mM glucose. The colony formation assay acquired an increased ability to undergo anchorage-independent MCF-7 cell growth.

Akt Activation under High-Glucose Conditions Akt (or protein kinase B, PKB) is a member of a family of serine/threonine kinases that play an important role in tumor formation and malignancy in various cancers including breast cancer. Akt activation has also been demonstrated to increase the potency of anchorage-independent MCF-7 cell growth. To examine the activation status of Akt under high-glucose conditions, MCF-7 cells were immunostained for the expression of phospho-Akt (Fig. 3A). Culture in high-glucose conditions for 7 and 28 d increased the expression of phospho-Akt by approximately 110 and 230%, respectively, compared with the level in normal physiological glucose or the osmotic control conditions (Fig. 3B). The total levels of Akt in each experimental condition were similar (Supplementary Fig. 1). To further understand Akt activation under prolonged high-glucose conditions, we investigated the effect of high glucose on Akt activation-dependent anti-cell death by tamoxifen because tamoxifen-induced cell death has been shown to be negatively correlated with Akt activation. Figures 4A and B show that high-glucose exposure for 28 d suppressed the cell death induced by tamoxifen. These results indicate that high-glucose conditions for 28 d promoted a key mediator of hyperglycemic history and hyperglycemic memory effects in MCF-7.

DISCUSSION

In this study, we demonstrated that prolonged high glucose levels promoted the ability of MCF-7 cells to undergo anchorage-independent colony growth at least in part through Akt activation, which is involved in the progression of estrogen receptor-positive breast cancer. Thus, we proposed that Akt activation is a key mediator of hyperglycemic history and hyperglycemic memory effects in MCF-7.

Cancer cells exposed to hyperglycemia have been shown to permanently acquire the aggressive characteristics of malignant progression, even after a return to normal conditions. We also observed promotion of anchorage-independent colony growth not only under prolonged high-glucose conditions, but also thereafter. Moreover, MCF-7 cultured under prolonged high glucose levels suppressed tamoxifen-induced cell death, which is negatively correlated with Akt activation, suggesting that a high-glucose environment imprinted Akt signaling onto MCF-7 and contributed to the malignant characteristics. A previous study reported Neuregulin 1-human epidermal growth factor receptor 3 (NRG1-HER3) signaling, leading to activation of the downstream phosphoinositide 3-kinase (PI3K)-Akt pathway, in mammary cancer cells with a hyperglycemic history and memory for tumor growth using a genomic approach, i.e., microarray. It is possible that Akt activation in MCF-7 under prolonged high glucose conditions is mediated by the NRG1-HER3 signaling pathway, although further studies are needed to validate this.

Akt activity is known to be directly regulated by glucose transporters in a high-glucose environment. Glucose transporter type 4 (GLUT4) has been reported to activate Akt by recruiting it to the cell surface and the mechanism of Akt activation by high glucose is thought to involve glucose transport via GLUT4. We also demonstrated that GLUT12-specific expression in estrogen receptor-positive cells is required for the promotion of MCF-7 cell motility in a model of breast cancer progression under high-glucose conditions, indicating that GLUT12 plays a crucial role in glucose sensing. Similarly, GLUT12 may regulate glucose sensing and mediation through Akt for the acquisition of high tumor formation ability. Estrogen receptor α was recently shown to bind p85α, the regulatory subunit of PI3K, leading to the activation of Akt by the...
phosphorylation of Ser473, resulting in tumor progression and chemotherapeutic resistance.\textsuperscript{24,25} Thus, it is suggested that Akt activation in estrogen receptor-positive breast cancer plays an important role in malignant progression, and Akt shows promise as a potential therapeutic targeting molecule for tumorigenesis in the hyperglycemia characteristic of diabetes.

The present study demonstrates that the ability of MCF-7 cells to undergo anchorage-independent colony growth is promoted by high glucose levels at least in part through Akt activation. We suggest that Akt activity regulates the progression of estrogen receptor-positive breast cancer through hyperglycemia, and we propose that additional analyses of high glucose-induced tumor formation would provide novel strategies for the diagnosis and therapy of breast cancer with hyperglycemia.

Acknowledgments This work was supported by a Nagai Memorial Research Scholarship from the Pharmaceutical Society of Japan (to Chihiro Matsui) and a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (Grant Number 15K07955 to Tomoka Takatani-Nakase). We thank Yoshiko Maeda, Ayaka Ueda, Kasumi Ueta, Keiko Matsumoto, Rina Tsuboi, Yukari Nishikawa, Natsumi Hisatsune, Yufu Hasegawa, Atsuko Nishijima, Yuka Shimura, and Akane Hishida (Department of Pharmaceutics, School of Pharmacy and Pharmaceutical Sciences, Mukogawa Women’s University, Hyogo, Japan) for their support.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES