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Maternal diabetes and high glucose in vitro trigger Sca1$^+$ cardiac progenitor cell apoptosis through FoxO3a

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ABSTRACT

Recent controversies surrounding the authenticity of c-kit$^+$ cardiac progenitor cells significantly push back the advance in regenerative therapies for cardiovascular diseases. There is an urgent need for research in characterizing alternative types of cardiac progenitor cells. Towards this goal, in the present study, we determined the effect of maternal diabetes on Sca1$^+$ cardiac progenitor cells. Maternal diabetes induced caspase 3-dependent apoptosis in Sca1$^+$ cardiac progenitor cells derived from embryonic day 17.5 (E17.5). Similarly, high glucose in vitro but not the glucose osmotic control mannitol triggered Sca1$^+$ cardiac progenitor cell apoptosis in a dose- and time-dependent manner. Both maternal diabetes and high glucose in vitro activated the pro-apoptotic transcription factor, Forkhead O 3a (FoxO3a) via dephosphorylation at threonine 32 (Thr-32) residue. *foxo3a* gene deletion abolished maternal diabetes-induced Sca1$^+$ cardiac progenitor cell apoptosis. The dominant negative FoxO3a mutant without the transactivation domain from the C terminus blocked high glucose-induced Sca1$^+$ cardiac progenitor cell apoptosis, whereas the constitutively active FoxO3a mutant with the three phosphorylation sites, Thr-32, Ser-253, and Ser-315, being replaced by alanine residues mimicked the pro-apoptotic effect of high glucose. Thus, maternal diabetes and high glucose in vitro may limit the regenerative potential of Sca1$^+$ cardiac progenitor cells by inducing apoptosis through FoxO3a activation. These findings will serve as the guide in optimizing the autologous therapy using Sca1$^+$ cardiac progenitor cells in cardiac defect babies born exposed to maternal diabetes.

1. Introduction

After embryonic cardiogenesis is completed, the mammalian heart has very limited regenerative capacity, which prevents its recovery from ischemic injury and other cardiac damages. Cardiac progenitor/stem cells are ideal models for cardiac regenerative therapies. The journey in uncovering the true cardiac progenitors in the mammalian heart has been significantly hampered by the recent controversies surrounding the c-kit positive cardiac progenitors [1]. Previous efforts in cardiac regenerative medicine essentially focused on the c-kit positive progenitors, which are now considered for having a limited differentiation capability into cardiomyocytes [2]. Therefore, there is an urgent need in characterizing other types of cardiac progenitor cells.

Sca1$^+$ progenitors are a heart cell population displaying cell surface marker stem cell antigen-1 (Sca1). In the mouse model of myocardial ischemia, Sca1$^+$ cells harbor to the injury site and form new cardiomyocytes. In human, a population of cardiac progenitor cells isolated from adult or fetal biopsies using murine Sca1 antibody differentiates into cardiomyocytes. These evidence indicate that cardiac Sca1$^+$ progenitors have significant cardiac regeneration potential by differentiating into cardiomyocytes and other cells in the heart; however, the biological function of cardiac Sca1$^+$ progenitors is still elusive.

Presentational maternal diabetes induces congenital heart defects (CHDs). After reparative surgical procedures, a high percentage of CHD children still develop cardiac dysfunction leading to cardiomyopathy and heart failure [3]. Stem cell therapy using allogeneic mesenchymal stem cells has been shown beneficial effect in treating cardiac dysfunction in patients with cardiovascular

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diseases [4]. However, the ideal stem cell therapy is the one using autologous source of cardiac progenitor cells. Our previous studies have demonstrated that maternal diabetes induces heart cell dysfunction including enhanced apoptosis and impaired cell proliferation in the offspring [5–8]. Therefore, the first step towards designing an autologous stem cell therapy in treating CHD children of diabetic mothers is the characterization of cellular dysfunction in cardiac progenitors, which will eventually aid in optimization of these autologous progenitor functions. The main goal of the present study is to clarify the potential cellular dysfunction in offspring $Sca1^+$ cardiac progenitor induced by maternal diabetes *in vivo* and high glucose in vitro.

The Forkhead O (FoxO) transcription factor family consists of three members, FoxO1, FoxO3a and FoxO4, and they participates in an array of physiological and pathophysiological functions including cell cycle arrest, induction of cell apoptosis and differentiation [9]. FoxO factors trigger cell apoptosis by increasing pro-apoptotic gene expression including Bim, TRAIL and TRADD [9]. The active form of FoxO3a is non-phosphorylated and phosphorylation of FoxO3a at therinone (Thr) 32 residue by protein kinase suppresses the pro-apoptotic effect of FoxO3a by reducing its transcription activity. Maternal diabetes activates FoxO3a in the developing embryo through dephosphorylation at Thr-32 [9]. The present study aims to test whether FoxO3a activation mediates the pro-apoptotic effect of maternal diabetes and high glucose on $Sca1^+$ cardiac progenitor cells.

2. Materials and methods

2.1. Animals and reagents

Wild-type (WT) C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). FoxO3$\alpha^{+/-}$ mice on an FVB background were obtained from the Mutant Mouse Regional Resource Centers and crossed back to C57BL/6J background for 10 generations to generate FoxO3$\alpha^{-/-}$ mice on a C57BL/6J background, which were used before age 12 weeks. Streptozotocin (STZ) from Sigma (St. Louis, MO) was dissolved in sterile 0.1 M citrate buffer (pH4.5). The procedures for animal use were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee.

2.2. Mouse models of diabetic embryopathy

The mouse model of diabetic embryopathy has been extensively described previously [9–16]. Briefly, ten-week old WT or FoxO3$\alpha^{+/-}$ female mice were intravenously injected daily with 75 mg/kg STZ over two days to induce diabetes. Diabetes was defined as a 12 h fasting blood glucose level of $\geq$16.7 mM. Male (WT or FoxO3$\alpha^{+/-}$) and female (WT or FoxO3$\alpha^{+/-}$) mice were paired at 3:00 p.m., and pregnancy was established by the presence of the vaginal plug next morning, and noon of that day was designated as day 0.5 (E0.5). WT or FoxO3$\alpha^{+/-}$ female mice were treated with vehicle injections as nondiabetic controls. On E17.5, mice were euthanized and conceptuses were dissected out of the uteri, embryos with the yolk sacs were removed from the deciduas and then yolk sacs were removed from the embryos. The embryos were used for analyses.

2.3. Cell sorting, primary cell culture and transfection

Embryonic hearts were dissected from E17.5 embryos and cut into pieces and digested with trypsin (0.05%) and collagenase II (1 mg/ml) for 45 min. Digested cells were centrifuged for 5 min at 500 *g*. Cell pellets were re-suspended in IMDM culture medium (Invitrogen) with 10% fetal bovine serum (Invitrogen). Cell sorting for $Sca1^+$ cells were performed using the Sca1 Microbeads Kit (Miltenyi Biotec). $Sca1^+$ cells were maintained in IMDM (5 mM glucose) supplemented with 10% fetal bovine serum (Invitrogen), 0.1 mM β-mercaptoethanol (sigma), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen) at 37 °C in a humidified atmosphere of 5% CO_2. Lipofectamine 2000 (Invitrogen) was used for transfection of FoxO3a constitutive active (FoxO3a-CA) or FoxO3α domain negative (FoxO3a-DN) plasmid. FOXO3α-CA (Addgene plasmid # 1788) and FoxO3a-DN (Addgene plasmid # 1797) were gifts from Dr. Michael Greenberg. The constitutively active mutant of FOXO3a (Thr32, Ser253, and Ser315 were converted to alanine) cannot be phosphorylated by Akt [17]. The transactivation domain was deleted in dominant negative FoxO3a mutant [17]. $Sca1^+$ cells were cultured in IMEM with 90 mg/dl glucose as the normal glucose level. $Sca1^+$ cells were cultured in IMEM with 300, 450, 600 mg/dl glucose as high glucose levels.

2.4. Western blotting analysis

Equal amounts of protein from embryos or cells were resolved by the SDS-PAGE gel electrophoresis and transferred onto Immunobilon-P membranes (Millipore). Membranes were incubated in 5% nonfat milk for 45 min and then were incubated for 18 h at 4 °C with the following primary antibodies at dilutions of 1:1000 in 5% nonfat milk: FoxO3α (Cell Signaling Technology), p-FoxO3α (Cell Signaling Technology), and caspase 3 (Millipore). Membranes were then exposed to goat anti-rabbit or anti-mouse secondary antibodies. To confirm that equivalent amounts of protein were loaded among samples, membranes were stripped and probed with a mouse antibody against β-actin (Abcam). Signals were detected using the SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Scientific). All experiments were repeated three times with the use of independently prepared tissue lysates.

2.5. TUNEL staining

The TUNEL assay was performed using the In Situ Cell Death Detection Kit (Millipore). Cells were seeded on an 8-well Nunc Lab-Tek Chamber Slide system (Sigma). After transfection and high glucose treatment, cells were fixed with 1% paraformaldehyde in PBS, incubated with TUNEL reagent counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted with aqueous mounting medium (Sigma). TUNEL-positive cells in each well were counted. The percentage of apoptotic cells was calculated as the number of TUNEL-positive (apoptotic) cells divided by the total number of cells in a microscopic field from three separate experiments.

2.6. FACS apoptosis assay

$Sca1^+$ cells (1×10^6) were harvested from each group and washed by PBS once, and then suspended in 100 μl PBS. Five μl Annexin V (BD) was added into each sample, followed by incubation in dark at room temperature for 10–15 min. After incubation, cells were washed once using PBS, then 5 μl 7-AAD (Invitrogen) was added to each sample and incubated for 5 min. All samples were analyzed by FACS within 1 h.

2.7. Statistics

Data are presented as means ± SD (standard deviation). Three embryos from three separate dams were used for the *in vivo* studies and cell cultures experiments were repeated three times. One-way ANOVA was performed using the SigmaStat 3.5 software, a *Tukey* test was used to estimate the significance. Statistical significance

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