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Late-Gestation Ventricular Myocardial Reduction in Fetuses of Hyperglycemic CD1 Mice is Associated with Increased Apoptosis

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Abstract

Previous work in our laboratory showed reduced myocardium and dilated ventricular chambers in gestation day (GD) 17 hearts that were collected from hyperglycemic CD1 mouse dams. Pre-breeding maternal immune stimulation, using Freund's complete adjuvant (FCA), diminished the severity of these fetal heart lesions. The following experiments were performed to detect possible changes in fetal heart apoptotic cell death, under hyperglycemic conditions and with or without maternal immune stimulation. Female CD1 mice were injected with 200 mg/kg of streptozocin (STZ) to induce insulin-dependent diabetes mellitus. Half of these mice received prior FCA injection. Fetal hearts were collected on GD 17 and myocardial apoptotic cells were quantified using flow cytometry. A panel of apoptosis regulatory genes (Bcl2, p53, Casp3, Casp9, PkCe) was then examined in the fetal myocardium using RT-PCR. Early apoptotic cells and late apoptotic/necrotic cells were significantly increased in fetal hearts from STZ or STZ + FCA dams. Paradoxically in the face of such increased cell death, the expression of pro-apoptotic genes Casp3 and Casp9 was decreased by diabetes, while the anti-apoptotic gene Bcl2 was increased.

Keywords

Bcl2; apoptosis; fetal heart; mouse; hyperglycemia; diabetes

INTRODUCTION

Cardiovascular complication is one of the most common causes of morbidity and mortality in adult diabetic patients (Adeghate, 2004). Furthermore, maternal hyperglycemia is an inducer of birth defects that include a high incidence of cardiovascular malformations (Reece et al, 1996). Such heart defects in infants of diabetic mothers represent a preventable segment of anomalies (Becerra et al, 1990; Ferencz et al, 1990). These include major anomalies of cardiovisceral and atrioventricular concordance, as well as defects of the cardiac outflow tract and atrioventricular valves (Loffredo et al, 2001).

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Our laboratory detected ventricular chamber dilation and myocardial reduction in late gestation fetal hearts, collected from hyperglycemic pregnant CD-1 mouse dams (Gutierrez et al., 2007). Data are limited but suggest the rate of heart myocardial apoptosis may increase in adult mice under a hyperglycemic environment (Fiordaliso et al., 2001; Cai et al., 2002). Frustaci et al. (2000) also observed both increased apoptosis and necrosis in myocytes, endothelial cells and fibroblasts of the human adult diabetic heart. Under conditions of chronic hyperglycemic insult, however, positive effects have been reported on the viability of myocardial cells (Schaffer et al., 2000; Ricci et al., 2008).

Diverse forms of maternal immune stimulation in rodents offer protection against birth malformations caused by chemical teratogens or maternal diabetes mellitus (Punareewattana and Holladay, 2004; Prater et al., 2004, 2007; Hrubec et al., 2006, 2009;). This protection has been associated with improved regulation of apoptosis in fetal target tissues of the teratogens (Sharova et al., 2000; Holladay et al., 2002). In our laboratory, maternal treatment with the non-specific immune stimulant, Freund's complete adjuvant (FCA), decreased the fetal heart myocardial reduction caused by diabetes (Gutierrez et al., 2009). We hypothesized that dysregulation of apoptosis may be in part responsible for the myocardial reduction, and resultant increased ventricular chamber dimension, in late gestation fetal mouse hearts during the diabetic pregnancy. We also hypothesized that alleviation of such changes by maternal treatment with FCA may be in part related to normalization of fetal myocardial apoptosis.

MATERIALS AND METHODS

Animals

Six-to seven-week old CD1 female mice were purchased (Charles River) and housed 5 per cage for a 1-week acclimation period. Mice were fed a standard rodent diet (Harlan Teklad Global Diet 2018, Madison, WI). Tap water was provided ad libitum. Mice were maintained under controlled conditions of temperature (22 °C), humidity (40–60 %) and lighting (12/12 hour light/dark cycle). For breeding, males were housed overnight with females, and females checked for vaginal plugs the next morning, which was designated day 0 of gestation (GD 0). All procedures were approved prior to study initiation by the Virginia Tech Institutional Animal Care and Use Committee (IACUC). Mice were in all cases then humanely handled.

Study design

Mice were divided prior to breeding into three treatment groups, in a randomized complete block design. 1) Control females were injected intraperitoneally (IP) with citrate buffer (0.05 M, pH: 4.5). 2) Diabetic females were produced by streptozocin (STZ) injection. These mice were injected with 200 mg/kg STZ (Sigma, St. Louis, MO) dissolved in a citrate buffer (0.05 M, pH: 4.5), 7 days before breeding, as previously performed in our laboratory (Punareewattana and Holladay, 2004; Gutierrez et al., 2007). Blood glucose (BG) levels in tail vein blood were then determined every 3–5 days using Accucheck compact blood glucose monitoring kits (Roche Applied Sciences, Indianapolis, IN) following tail venipuncture. The STZ-injected mice were hyperglycemic at first bleeding, with BG values ≥ 500 mg/dl (Figure 1). BG levels in this range caused increased craniofacial and neural tube defects in previous studies (Gutierrez et al., 2007). BG levels in the STZ mice subsequently did not change appreciably over the duration of the experiments (data not shown). 3) Immune stimulated diabetic females were produced using Freund's complete adjuvant (FCA). Diabetic mice, again ≥ 500 mg/dl BG, were injected twice with FCA (30 μ l IP), first at 1 week and again at 1 day before the STZ administration. This identical procedure of immune stimulation of pregnant diabetic mice reduced externally-visible morphologic defects in the F₁ offspring (Punareewattana and Holladay, 2004). The latter study found no adverse

developmental effects from FCA alone, and for this reason an FCA-only group of pregnant mice was not included in the present experiments. The STZ and FCA injection schedule is summarized in the line drawing shown below.

Flow cytometric determination of myocardial apoptosis

Pregnant females from the control, STZ, and STZ + FCA groups were euthanized by CO₂ inhalation and fetal hearts (5 per litter) were collected from arbitrarily selected fetuses at GD 17 by micro-dissection, using an Olympus Zoom Stereo Microscope SZX7 (Olympus America Inc., Melville, NY). Hearts were placed in Dulbecco's minimal essential media (DMEM) with 10 % fetal bovine serum after removing left and right auricles, and gently swirled to remove blood in the ventricles. Hearts were then removed from the DMEM and placed, by litter, in 1ml solutions of enzyme dispase II (Roche Applied Sciences) for a 30 min incubation at 37 °C. The enzyme dispase II solution, which gently suspended myocardial cells, included annexin V (5 µl/ml) and propidium iodide (35 µl/ml) for detection of early apoptotic (EA) cells and late apoptotic/necrotic(LA/N) cells, respectively. Annexin V is a 35–36 kDa Ca²⁺ dependent phospholipid-binding protein that has a high affinity for phospholipid-like phosphatidylserine (PS) exposed on cell surfaces during apoptosis, and binds to cells with exposed PS. Propidium Iodide(PI) binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4–5 base pairs of DNA. PI is membrane impermeant and generally excluded from viable cells. Samples were gently pipetted after the incubation to further encourage suspension of cells, and then analyzed by flow cytometry (FACS Aria, BD Biosciences, Franklin Lakes, NJ).

Myocardial apoptosis/proliferation-related gene expression

Fetal hearts, at GD 17, were collected by micro-dissection under sterile conditions from a second group of pregnant female mice. These included control, STZ, and STZ + FCA pregnant mice, prepared identically to the above-described apoptosis studies. The left and right auricles and the origin of the great vessels were removed, to increase focus on the ventricular myocardium as a gene expression target tissue. The ventricles were placed in sterile saline for 1–2 min, and then transferred to RNA later and stored at –20 °C until later analysis. The RNeasy fibrous tissue mini kit was used to extract RNA (Qiagen, Valencia, CA). Hearts were removed from RNA later and weighed to achieve 20–30 mg of tissue, which equated to 6–7 hearts per litter. Tissue was placed in a collection tube and 400 µL of β-mercaptoethanol + RLT buffer were added. Hearts were immediately homogenized for 1 min in the collection tube using a tissue disruptor (Qiagen, Valencia, CA). RNase free water and 10 µl of proteinase K were added to the mix to facilitate disruption of the heart tissue. Samples were then incubated with DNase I for 15 min at room temperature for RNA extraction. RNA quality and quantity were read using a Biophotometer (Eppendorf, Westbury, NY), after which the script cDNA synthesis kit from Bio-rad (Hercules, CA) was used with 1 µg of RNA from each sample.

Primers were designed using software Beacon designer and sequences submitted to Invitrogen (Carlsbad, CA) for elaboration. Primer sequences are shown in Table 1. Samples were analyzed by real time polymerase chain reaction (RT-PCR) for quantification of a preliminary panel of apoptosis regulatory genes (Bcl2, p53, Casp3, Casp9 and PkCe) using the SYBR green supermix from Bio-rad (Hercules, CA).

Statistical analysis

JMP software from the SAS family was used to run an ANOVA, to detect differences among groups. When a significant difference was observed ($p < .05$), a Tukey's statistical test was used to further analyze gene expression differences among groups. Gene expression data were presented as mean $\pm$ standard deviation.

JMP software was also used to run principal component analysis (PCA) to detect cluster changes in gene expression. The PCA test is designed for condensation of raw data, generated from numerous genes, to a coordinately meaningful and more readily interpretable set of parameters. PCA of the correlation matrix of gene expression was used for visualization of gene expression change under the different treatments. Next, ANOVA of PCA scores was used to determine which of the principal components were affected by treatment (Sharova et al., 2000).

The raw data used to run ANOVA and PCA were the output obtained from the delta CT (Δ CT) equation of gene expression. All the charts showing expression of individual genes or ratio of gene expression were then created using the delta-delta CT ($\Delta\Delta$ CT) output to detect changes (up or down gene regulation) compared to the control group.

RESULTS

Maternal diabetes significantly increased the percentage of early apoptotic cells in the GD 17 fetal myocardium compared to control. This effect was not alleviated by maternal FCA injection (Figure 2). Maternal diabetes also significantly increased the percentage of late apoptotic/necrotic fetal myocardial cells. However, fetal heart cells from diabetic mice treated with FCA showed control-level late apoptotic/necrotic myocardial cells, suggesting some cell death protection was rendered by FCA (Figure 3). Representative histograms for the apoptosis quantification are shown in Figure 4.

The majority of apoptotic genes examined showed treatment-related expression differences. The level of Bcl2 expression was significantly up-regulated in fetal myocardial cells from both hyperglycemic groups (diabetic; diabetic + FCA, Figure 5). Expression of the pro-apoptotic gene Casp3 tended toward reduction in diabetic mice ($p=0.55$) and was significantly reduced in diabetic mice that received FCA, but did not differ from diabetic mice that did not receive FCA. The expression of pro-apoptotic gene Casp9 was generally similar to Casp3, and was significantly reduced in diabetic mice compared to control. Expression of anti-apoptotic gene PkCe, and pro-apoptotic gene p53, did not change among treatment groups.

Gene expression was also examined by PCA for evidence of clustered shifts in expression. The results of the PCA correlated well with the analysis of individual gene expression. Three principal components (PC1-3) explained 89% of the variation in gene expression due to the different treatments (Table 2). The first principal component (PC1) represents the average level of expression for all genes but was biased slightly towards p53, Casp3 and Casp9 and their ratio to Bcl-2. PC2 was biased toward Bcl-2 and PkC-e, with lower representation to Casp3 and 9. PC3 represented mostly gene p53. ANOVA indicated that the treatments affected PC1 and PC2, such that these PC explained 70% of the variation in gene expression due to the different treatments (Table 2). A scatter plot of PC1 and PC2 was therefore created to visualize the effects of the different treatments (Figure 6). The plot shows the profile of fetal apoptotic gene expression shifting due to maternal hyperglycemia in relation to PC1 and PC2 axis. The diabetic groups were shifted to the right along the PC1 axis, largely as a consequence of up-regulated Bcl2 in the diabetic groups. The diabetic groups also tended to be more shifted along the PC2 axis when compared to the control

group. Particularly, diabetic mice that received FCA were shifted to the bottom of the PC2 axis. This again was a reflection of up-regulation Bcl2 in the diabetic groups and a tendency for a higher gene ratio of PkCe in the STZ + FCA group.

DISCUSSION

We recently detected late gestation myocardial reduction in fetuses collected from hyperglycemic CD1 mouse dams (Gutierrez et al., 2007). Available data in the literature are limited but support the idea that hyperglycemia may increase adult heart myocardial apoptosis. Using an FVB diabetic mouse model and TUNEL assay, Cai et al. (2002) described increased myocardial apoptosis and cardiac structural changes at >12 mM/LBG. These authors also described increased apoptosis of cardiac myoblast H9c2 cells exposed *in vitro* to high levels of glucose (22–33 mM/L). Fiordaliso et al. (2001) also used *in vitro* murine myocardial cell exposures to hyperglycemic culture conditions, and reported activation of p53 and p53-regulated pro-apoptotic genes, as well as increased cell death. In humans, Frustaci et al. (2000) detected increased apoptosis and necrosis in myocytes, endothelial cells and fibroblasts of adult hearts of diabetic and diabetic/hypertensive patients. The latter authors proposed that local increases in angiotensin II with diabetes may enhance oxidative stress, activating cardiac cell apoptosis and necrosis.

Flow cytometric results supported a hypothesis for hyperglycemia-related dysregulated apoptosis in the fetal mouse heart, similar to the above-described adult mouse and human heart data. In particular, the present mice showed increased percentages of early apoptotic myocardial cells, and increased late apoptotic/necrotic cells compared to F₁ progeny of non-diabetic control mice. Use of flow cytometry and annexin V, including in fetal tissues, has become accepted as a reliable, stand-alone indicator of apoptosis that compares well to other probes, e.g., 7-aminoactinomycin D (7-AAD) and TUNEL (George et al., 2004; Besteman et al., 2005; Eckstein et al., 2005). The present results therefore suggest that maternal immune system stimulation with FCA may improve the harmful effects of hyperglycemia on the fetal myocardium, by reducing inappropriate cell death.

The present mice also showed altered fetal apoptosis-related gene expression after maternal injection with FCA. Previously, Sharova et al. (2000) examined gene expression in fetal palate tissue from ethyl carbamate-exposed pregnant mice, and reported decreased expression of anti-apoptotic genes and increased expression of pro-apoptotic genes. These authors suggested that immune protection against cleft palate formation may in part be mediated through maternal immune regulation of fetal gene expression, possibly via cytokines that cross the placenta. The present fetal hearts displayed up-regulation of the anti-apoptotic gene Bcl-2 in both hyperglycemic groups (STZ and STZ + FCA), and reduced expression of pro-apoptotic factors Casp3 (in the STZ + FCA group) and Casp9 (in the STZ group). These findings were in the opposite direction of what might be expected, considering the flow cytometric histograms that clearly showed increased fetal myocardial apoptosis/necrosis in hyperglycemic dams.

The literature does support up-regulation of Bcl2 under high hyperglycemic conditions. Schaffer et al. (2000) exposed neonatal Wistar rat cardiomyocytes for 3 days to 25 mM/L hyperglycemic media, and detected significantly increased Bcl2 gene expression. In those studies, two pro-apoptotic factors, Bax and Bad remained unaltered under the same hyperglycemic conditions. Ricci et al. (2008) similarly found that myocytes from newborn and adult rats over-expressed Bcl2 and Akt factor under chronic exposure to a highly hyperglycemic media. Bojunga et al. (2004) also showed increased levels Bcl2 in the hearts of adult diabetic rats (26 mM/LBG) compared to controls. In the same study pro-apoptotic genes Bax and Bak showed increased levels.

Possibly, increased GD 17Bcl2 and decreased Casp3 and Casp9, may represent an internal attempt by the fetal myocardial cells to overcome prior activation of external apoptotic pathways (e.g., FAS-or TNF α -mediated) after a window of earlier increased apoptosis. Bcl2 among other functions has a positive impact in mitochondrial cell death protection, preventing the release of cytochrome-c and interfering with protein Apaf1, blocking the formation of the apoptosome and in turn mitochondrial mediated apoptotic pathway activation (reviewed by Reed, 1998). Additional experiments will be required to analyze a broader panel of gene expression in fetal myocardial cells in the diabetic pregnancy, and to examine earlier windows in fetal heart development and gene expression.

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Figure 1. Maternal glucose levels at GD17. C: control group, STZ: diabetic group, STZ+FCA: diabetic group treated with FCA. Different letters above bars imply significant difference between groups, $p < 0.05$.

Figure 2.

Percentage of early apoptotic myocardial cells. Maternal diabetes caused a significant increase in early apoptotic fetal myocardial cells ($p < 0.01$), with (STZ group) or without (STZ+FCA group) maternal immune stimulation.

Figure 3. Percentage of late apoptotic-necrotic myocardial cells. Maternal diabetes caused a significant increase in late apoptotic/necrotic fetal myocardial cells (STZ group; $p < 0.01$).

Figure 4.

Fetal myocardial apoptosis determined by flow cytometry. Histograms from top to bottom are from fetal hearts of representative control, diabetes + FCA, and diabetes groups, respectively.

Figure 5. ANOVA results for gene expression. The data represent $\Delta\Delta\text{CT}$ output. GEL: gene expression level, C: control group, STZ: diabetic group, STZ+FCA: diabetic group treated with FCA. Different letters above bars imply significant difference between groups, $p < 0.05$.

Figure 6.

Principal component analysis (PCA) of the effect of maternal diabetes and treatment with FCA on fetal myocardial gene expression. PC1 and PC2 representing the first and second principal components are shown. C: control group, STZ: diabetic group, STZ+FCA: diabetic group treated with FCA.

Table 1

Target genes and primer sequences used for gene expression RT-PCR.

Gene	Gene function	Primer 5'-3' (forward and reverse)
Bcl2	Anti-apoptotic gene, prevents releasing of cytochrome c	CCT TGG CGT GTC TCT CTG TCC TGT GAT TCT CCC TTC TTC
P53	Pro-apoptotic gene	ATG AAC CGC CGA CCT ATC C CAC AAA CAC GAA CCT CAA AGC
Caspase3	Pro-apoptotic gene, effector caspase	GAC TTC CTG TAT GCT TAC TCT AC ATT CCG TTG CCA CCT TCC
Caspase9	Pro-apoptotic gene, activator caspase	GGT GGA GGT GAA GAA CGA C TCA ATG GAC ACG GAG CAT C
PkCe	Anti-apoptotic cardio-protector gene	ACC TCC CCT TGT GAC CAG TGC TCC TCT CCT CGG TTG
B-actin	Housekeeping gene	CGT GCG TGA CAT CAA AGA G TGC CAC AGG ATT CCA TAC C

Table 2

Principal components explaining variation in gene expression due to the different treatments.

Genes	PC1	PC2	PC3	PC4	PC5
Bcl2	-0.2030	0.8176	0.0817	0.0198	0.5321
P53	0.41631	0.2446	0.7022	0.3979	-0.3396
Casp3	0.54470	-0.0356	0.2431	-0.7606	0.2535
Casp9	0.57505	-0.1894	-0.3003	0.5039	0.5378
PrkCe	0.39758	0.4842	-0.5922	-0.0932	-0.4978
Eigenvalue	2.196	1.327	0.921	0.498	0.055
Cumulative % of variation explained	43.939	70.486	88.916	98.885	100
ANOVA probability that treatment had no effect (<i>p</i> value)	$P < 0.01^*$	$p < 0.05^*$	$p < 0.95$	$p < 0.059$	$p: 0.72$