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Preventive effect of non-mitogenic acidic fibroblast growth factor on diabetes-induced testicular cell death

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ABSTRACT

Fibroblast growth factor (FGF)-1 was found to protect the heart from oxidative damage, but clinically its long-term use was restricted for its undesirable proliferating activity on cells. Thus a cluster of amino acids responsible for the proliferation were deleted in the native FGF-1 to create a non-mitogenic FGF-1 (nmFGF-1). Whether the nmFGF-1 protects male germ cells from diabetes-induced apoptotic death was examined in diabetic mice induced with multiple low-doses of streptozotocin, followed by nmFGF-1 treatment for 6 months. Diabetic mice showed a decrease in testicular weight and an increase in apoptotic cell death. Treatment with nmFGF-1 alleviated the diabetic effects on testicular weight and apoptotic cell death. Mechanistically, nmFGF-1 may alleviate diabetes-induced germ cell death by decreasing the BAX/Bcl-2 ratio and endoplasmic reticulum stress as well as associated cell death, which is associated with Nrf-2 activation.

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1. Introduction

Diabetes is a global health concern and over the years, the number of those diagnosed with diabetes continues to increase. Male sexual dysfunction is a well-known complication in men diagnosed with type-1 diabetes [1], but there are only a few reports on how diabetes affects the male reproductive system. The apoptotic effect of diabetes on the testes has not been investigated extensively, and only a few apoptotic mechanisms have been identified thus far. The characterization of apoptosis in male germ cells can be uniquely identified by cellular swelling and decondensed-homogeneous chromatin, but the germ cells can also exhibit the classical morphology of apoptosis [2]. Age can be a key identifier in the extent of apoptosis. Extensive studies in mice have shown that young individuals have a higher incidence of apoptotic cell death in the testis compared to adults. This can be explained by the role of apoptosis to eliminate overproduced, abnormal, or damaged germ cells during the time spermatogonia undergo mitotic divisions [2]. Germ cells are unique in that different stress situations initiate distinct apoptotic cell death pathways. For example, temperature-related stress predominantly induces mitochondrial pathway-dependent apoptotic germ cell death, but hypothermia uses both the mitochondrial and endoplasmic reticulum (ER) pathways [3,4]. We have demonstrated that diabetes predominantly induces germ cell apoptotic death through the mitochondrial and ER stress-associated cell death pathways, which are dependent on many factors such as the pro-apoptotic factors expressed and glycated end-products to activate caspase and oxidative signaling [5–7]. Several fibroblast growth factors (FGFs) were also found to play certain roles in diabetes-induced testicular germ cell death [8].

The FGF family consists of 22 mammalian members that have been shown to be important in regulating tissue patterning and organogenesis of several organs, including the testes. Primarily, the FGFs that are important in organogenesis are the paracrine factors including FGF-1–10, 16–18, 20, and 22; however, the endocrinal members comprising of FGF19, 21, and 23 are also involved in maintaining normal spermatogenesis and provide protection from apoptosis [8]. FGF-1 and FGF-2 were the first two FGFs

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characterized and are identical in 55% of their amino acids [9]. Both are paracrine factors that have similar mitogenic activity and are expressed in embryogenesis during differentiation. FGF-1 and FGF-2 also were shown to have stimulatory effects on basal luteinizing hormone-stimulated testosterone production [10].

FGF-1 is distinctive from other FGF paracrine factors because it is considered "endocrine-like," in that it can act like a vasodilator, neuromodulator, and has been shown to protect the brain and heart from ischemic reperfusion [11–13]. FGF-1 also has the ability to stimulate blood vessel ingrowth for angiogenesis, useful in wound healing, which has been extensively supported by previous studies that have shown FGF-1 to reverse diabetic wound healing in rats with skin lesions through increased cell proliferation [14,15]. Diabetes is highly associated with neuropathy and ischemia in various organs throughout the body including the testes whereas FGF-1 is known as a potential treatment for ischemia and neuropathy in the heart and spinal cord [13], but has not yet been investigated to treat diabetic complications in the testes.

Therefore, the present study examined the effect of FGF-1 on the diabetic induction of testicular cell death. However, FGF-1 as an in vivo application was unsuccessful in that the excessive proliferative nature of native FGF-1 may lead a tumorigenic risk, thus a non-mitogenic form (nmFGF-1) was developed by different groups via deletion of the amino acids responsible for the mitogenic function [16,17]. To explore whether chronic application of nmFGF-1, without undesirable long-term mitogenic effects, can protect male germ cells from apoptosis caused by diabetes, we have used multiple low-dose injections of streptozotocin (STZ) in FVB mice to induce a type-1 diabetes model. Diabetic and age-matched control mice were treated with and without nmFGF-1, from Wu et al. [17] for 6 months. Cardiac functional and histopathological examination showed that diabetes induced an obvious cardiomyopathy, shown by cardiac dysfunction and histological remodeling at 6 months onset diabetes, which, however, was significantly attenuated by nmFGF-1 treatment [18]. Here we used the animal model to investigate the protective effect on diabetes-induced testicular cell death and potentially underlying mechanisms.

2. Materials and methods

2.1. Animals and diabetes model

All mice were housed in the University of Louisville Research Resources Center at 22 °C with a 12-h light/dark cycle with free access to food and tap water. All procedures were approved by Institutional Animal Care and Use Committee, which is certified by the American Association for Accreditation of Laboratory Animal Care. As described in our recent study [18], the type-1 diabetes mouse model was induced using five daily doses of 50 mg/kg body weight STZ. There were four groups: control (CON), diabetes (DM), nmFGF-1treated control (nmFGF-1), and nmFGF-1-treated diabetes (nmFGF-1 + DM). CON and nmFGF-1 mice were given the same volume of sodium citrate buffer during the time of STZ injections in the DM and nmFGF-1 + DM mice. Whole blood glucose was detected using the mouse tail vein blood 5 days after the last STZ injection. Mice with blood glucose level ≥ 250 mg/dl were considered diabetic. The nmFGF-1 was provided by Xiaoping Wu from Jinan University in which he amplified the plasmid pUC-nmFGF-1 by a standard polymerase chain reaction with the appropriate primers to eliminate the N terminal residues 1–27, purified (95%), and transformed into *E. coli* strain BL21 [17]. After injections of STZ, both diabetic and age-matched control mice were intraperitoneally injected with either nmFGF-1 at 10 μg/kg body weight in the nmFGF-1 and DM/FGF-1 groups or sodium citrate buffer with the same daily injection volume as the nmFGF-1 groups in the CON and DM groups until sacrificed. All mice were sacrificed 6 months after the start of nmFGF-1 treatment wherein the testes tissue and tibia were harvested.

2.2. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) Assay

One testis from each mouse was fixed in a 10% formalin for 24 h and embedded in paraffin. The paraffinized tissues were sectioned at 5 μm and embedded on slides with two groups each; CON (n = 5) and DM (n = 5); nmFGF-1 (n = 4) and nmFGF-1 + DM (n = 5). Each group was stained for TUNEL with the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA), as described in previous studies [5,7]. Briefly, each slide was deparaffinized and rehydrated, and treated with proteinase K (20 mg/l) for 15 min at room temperature. Slides were treated with 3% hydrogen peroxide to quench endogenous peroxidases for 5 min, and then were incubated with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and digoxigenin-11-dUTP at 37 °C for 1 h. Then 3,3-diaminobenzidine (DAB) chromogen was applied. Methyl green was used as counterstaining. For a negative control, TdT was omitted from the reaction mixture. Under the microscope, apoptotic cells exhibited brown nuclear staining as the TUNEL positive and were quantitatively counted manually. From each testis 30 seminiferous tubule's cross-sections from one slide were selected in a same pattern to avoid repetitive counting. The apoptotic cells were counted from the spermatogonia, primary spermatocytes, and secondary spermatocytes, but not the spermatid or spermatozoa. Results were presented as TUNEL positive cells divided by total number of seminiferous tubule cross sections to give an average per tubule and then multiplied by 100 for the total number of TUNEL positive cells in 100 tubules.

2.3. Immunohistochemical staining

Testicular tissues fixed in a 10% neutral-buffered formalin were embedded in paraffin and sectioned at 5 μm, as described for TUNEL staining. The sections were deparaffinized in xylene and rehydrated in graded alcohol solutions. After sections were incubated with a retrieval solution (Dako, Carpinteria, CA) for 15 min at 98 °C, the sections were then treated with 3% hydrogen peroxide for 15 min at room temperature and blocked with 5% BSA for 1 h. The sections were incubated with primary antibodies including anti-proliferating cell nuclear antigen (PCNA, 1:2000 dilution, Cell Signaling, Danvers, MA), Bax (1:200, Cell Signaling), Bcl-2 (1:200, Cell Signaling), and anti-4-hydroxy-2-nonenal (4-HNE, 1:200, Alpha Diagnostic International, San Antonio, TX) at 4 °C overnight. After washing with PBS, these sections were incubated with horseradish peroxidase conjugated secondary antibody for 1 h at room temperature. For the development of positive cells, a brown color was created when peroxidase substrate DAB was used (Vector Laboratories Inc., Burlingame, CA) and then hematoxylin was used as counterstaining. Quantification for PCNA, Bax, and Bcl-2 was achieved by counting the positive cells manually. Results were presented as positive cells divided by total number of seminiferous tubules to give an average per tubule and then multiplied by 100, as described above for TUNEL studies. Quantification for 4-HNE was performed using the Image-Pro Plus 6.0 software, and presented as the fold of control for the integrated optical density (IOD), reporting the average intensity/density of each DAB-stained region.

2.4. Western blot

Protein extraction was first performed by homogenizing the testicular tissue from each group, CON (n = 5), nmFGF-1 (n = 5), DM (n = 4) and nmFGF-1 + DM (n = 4), in RIPA lysis buffer (Santa Cruz

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