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Pigment epithelium-derived factor promotes Fas-induced cardiomyocyte apoptosis via its receptor phospholipase A_2

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ABSTRACT

Aims: Cardiovascular diseases cause significant morbidity and mortality worldwide. Recently, our research team demonstrated that a multifunctional cytokine, pigment epithelium-derived factor (PEDF), plays a critical role in regulating myocardial infarction. However, few researchers have studied the molecular mechanisms by which PEDF and its receptors influence the pathophysiology of cardiovascular disease. We tested the hypothesis that PEDF affects cardiomyocyte apoptosis under hypoxic conditions and determined the role that its receptors phospholipase A_2 (PLA_2) and laminin receptor play in this process.
Main methods: Cardiomyocytes were isolated from neonatal mice and treated with PEDF under normoxic and hypoxic conditions; then, apoptosis was assessed using Annexin V/PI staining and flow cytometry. Western blotting and immunofluorescence staining were used to detect PEDF receptor expression, and siRNA knockdown of PEDF receptors was performed to determine which receptor was involved in mediating cardiomyocyte apoptosis.
Key findings: Our results demonstrated that PEDF increased cardiomyocyte apoptosis during hypoxia via Fas and that PEDF receptors were expressed on cardiomyocyte cell membranes. Furthermore, siRNA experiments indicated that the PEDF receptor PLA_2 was responsible for inducing cardiomyocyte apoptosis via the Fas pathway.
Significance: PEDF promoted Fas-induced cardiomyocyte apoptosis via its receptor PLA_2.

Introduction

Cardiovascular disease is responsible for significant morbidity and mortality worldwide (Miyares and Davis, 2012). Despite recent breakthroughs in cardiovascular medicine and advances in diagnostic procedures, ischemic cardiovascular disease, especially myocardial infarction (MI), remains one of the most serious health problems in modern society (Gjesing et al., 2013; Miyares and Davis, 2012). The biology of cardiac angiogenic and anti-apoptosis factors, including vascular endothelial growth factor (Albrecht-Schgoer et al., 2012), various fibroblast growth factors, hepatocyte growth factor and erythropoietin (Kawachi et al., 2012), have been extensively investigated. In contrast, few studies have examined the possible involvement of apoptosis mediators in the pathophysiology of cardiovascular disease.

Pigment epithelium-derived factor (PEDF), a member of the 50-kDa serpin-like peptide inhibitor family, is a multifunctional, pleiotropic protein (Liu et al., 2012; Tan et al., 2010). In recent studies, PEDF has been identified as a secreted protein that induces apoptosis (Zhang et al., 2007). Although PEDF was first identified in cultured pigment epithelial cells from fetal human retinas, other tissues also express PEDF (Bernard et al., 2009). PEDF is expressed most prominently in heart tissue (Liang et al., 2011; Rychli et al., 2010a), and experimental data demonstrate that it may contribute to heart failure (Rychli et al., 2010b). In explanted human ischemic heart tissue, PEDF levels are significantly lower than in healthy heart tissue; PEDF expression is also down-regulated by low oxygen levels (Rychli et al., 2010a), indicating that PEDF plays a more significant role in the pathological state than in the physiological state. Current evidence demonstrates that PEDF binds to at least two receptors: the 60-kDa laminin receptor (LR), which is present in endothelial cells, and the 80-kDa phospholipase A_2 (PLA_2) receptor, which is present in neuronal cells (Bernard et al., 2009). However, studies on the possible involvement of PEDF and its receptors in the pathophysiology of cardiovascular disease remain scarce. Recently, our research team demonstrated that the paracrine factor PEDF plays a critical role in the regulatory effects of stem cells against MI injury (Liang et al., 2011). However, little information is available regarding the possible pro-apoptotic action of PEDF in cardiomyocytes under pathological conditions; it is not known whether the heart even expresses PEDF receptors.

In this work, we tested the hypothesis that PEDF affects cardiomyocyte apoptosis under hypoxic conditions that mimic the ischemic heart disease microenvironment. We then sought to determine whether the PEDF receptors PLA_2 and/or LR were involved in mediating pro-apoptotic processes in cardiomyocytes.

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Materials and methods

Cardiomyocyte culture

Ventricular myocytes were isolated from C57B/6 neonatal mice as previously described (Rabkin, 2010). Briefly, hearts were rapidly excised from neonatal mice obtained from the Laboratory Animal Center of the Fourth Military Medical University. The ventricles were minced and placed in a dissociating solution. The harvested cells were centrifuged (1000 rpm, 5 min), and the sediment was resuspended in 10% newborn calf serum and incubated under 5% CO_2 at 37 °C. All animal experiments were performed with the approval of the Ethics Committee for Animal Experiments of The Fourth Military Medical University (Xi'an, China) (Permit Number 11006), and the experimental protocols strictly complied with the institutional guidelines and the criteria outlined in the "Guide for Care and Use of Laboratory Animals of The Fourth Military Medical University".

Apoptosis assay

Apoptotic cells were detected by Annexin V/PI double-staining flow cytometry. The apoptosis assay was used to study four groups: a control group, a PEDF-treated (Peprotech, NJ, USA) (200 ng/ml) group, a hypoxia group and a PEDF-treated (200 ng/ml) + hypoxia group. The cardiomyocytes in the control and PEDF groups were maintained under normoxic conditions (95% air–5% CO_2) at 37 °C for 6 h. Cardiomyocytes in the hypoxia and PEDF + hypoxia groups were exposed to hypoxic conditions (94% N_2–5% CO_2–1% O_2) in an anaerobic system (Thermo Forma) at 37 °C for equivalent periods. The cells were then harvested, washed twice with PBS and resuspended in binding buffer. Annexin V solution and PI were added to the cell suspensions, and Annexin V/PI-stained cell fluorescence intensities were analyzed using flow cytometry and Cell Quest software (BD Biosciences, CA, USA) within 1 h. Early apoptotic cells were Annexin V+/PI−, late apoptotic cells were Annexin V+/PI + and normal cells were Annexin V−/PI−. Early- and late-stage apoptotic cells were counted; the results are expressed as percentages of the total cell count (Wang et al., 2013).

Western blot analysis

For western immunoblot analysis, protein samples (40 μg/lane) from cardiomyocytes were separated on 10% SDS-PAGE gels, transferred to a nitrocellulose membrane (Immobilon-P, Millipore), incubated with anti-Fas, anti-β actin, anti-PLA_2 or anti-LR antibodies (rabbit anti-mouse, 1:1,000 dilution; Santa Cruz Biotechnology, USA) overnight at 4 °C and then incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG, 1:5000 dilution) for 1 h at room temperature. After washing, the protein bands were detected by chemiluminescence and quantified using the Image Lab software package (Bio-Rad Laboratories, Herts., UK).

Double immunofluorescence staining

Double-labeling immunofluorescence was performed to detect PLA_2 and LR localization in cardiomyocytes. Cardiomyocytes were cultured on coverslips in tissue culture dishes. After fixation in 4% paraformaldehyde for 20 min at room temperature, the cells were washed 3 times with PBS and blocked for 30 min. Goat anti-mouse antibodies raised against myosin heavy chain (MYH) (Santa Cruz, CA, USA) were used as a cardiomyocyte marker protein. Rabbit anti-mouse antibodies against PLA_2 (Santa Cruz) and rabbit anti-mouse antibodies against LR (Santa Cruz) were applied to cells in different dishes and incubated overnight at 4 °C. After washing, the PLA_2- and LR-stained cells were incubated with FITC-conjugated anti-rabbit (Santa Cruz) and Cy3-conjugated anti-goat (Santa Cruz) antibodies, respectively, for 1 h at room temperature. Nuclei were counterstained with DAPI, and the coverslips were mounted on slides using 50% glycerin. The stained samples were photographed and analyzed using an Olympus Fluoview FV100 (Olympus, Japan).

siRNA knockdown

Transient transfection of cardiomyocytes with siRNA targeting the PLA_2 and LR genes was performed using a siRNA Transfection Protocol (Santa Cruz). The cardiomyocytes were divided into four groups: a control group, a non-targeting (scrambled RNA, Santa Cruz) siRNA group, a PLA_2 siRNA group and an LR siRNA group. The cells were assessed 24 h after the addition of fresh media following transfection. Silencing was evaluated using RT-PCR and western blots, and levels of the apoptotic protein Fas were detected using western blots.

Statistical analysis

Statistical analysis was performed using Prism software (version 5.0, GraphPad, La Jolla, CA, USA). The results are represented as the means ± SD. The statistical significance of the results was assessed using one-way ANOVA and the LSD *t*-test. Differences during hypoxia with and without PEDF treatment were assessed using a two-way ANOVA followed by Tukey's test. Values of $P < 0.05$ were considered statistically significant.

Results

PEDF induces cardiomyocyte apoptosis under hypoxic conditions

To assess the pro-apoptotic effects of PEDF on cardiomyocytes, cardiomyocytes were treated with PEDF under hypoxic conditions. The number of apoptotic cardiomyocytes in each experimental group was determined using FACS after Annexin V/PI staining. As demonstrated in Fig. 1, PEDF treatment induced cardiomyocyte apoptosis under both hypoxic ($P < 0.01$) and normoxic ($P < 0.01$) conditions, and hypoxia increased cardiomyocyte apoptosis ($P < 0.05$).

PEDF induced cardiomyocyte apoptosis via Fas

To further elucidate the molecular signaling pathway by which PEDF induces cardiomyocyte apoptosis, we examined the expression levels of the apoptosis marker protein Fas in cardiomyocytes that had been treated with PEDF under hypoxic conditions. We observed increased Fas expression in PEDF-treated cardiomyocytes under both normoxic ($P < 0.01$) and hypoxic ($P < 0.01$) conditions (Fig. 2). Compared with control (normoxic conditions), hypoxia also activated Fas in cardiomyocytes ($P < 0.05$). These findings indicate that PEDF induced cardiomyocyte apoptosis under hypoxic conditions via Fas signaling pathway activation.

PEDF receptor expression in cardiomyocytes

To determine whether the PEDF receptors PLA_2 and LR are endogenously expressed in cardiomyocytes, western blotting experiments for PLA_2 and LR were performed with cardiomyocytes. Fig. 3 demonstrates that both PLA_2 and LR are expressed in mouse cardiomyocytes. The intensity of PLA_2 immunostaining was greater than that of LR immunostaining ($P < 0.01$), indicating that PLA_2 is the major endogenous PEDF receptor that is expressed in cardiomyocytes.

PEDF receptors are localized on the cardiomyocyte cell membrane

It has previously been reported that PEDF receptors are present on the cell membrane (Subramanian et al., 2012). To determine the subcellular localization of the cardiomyocyte PEDF receptors PLA_2 and LR, double immunofluorescence staining was performed with antibodies against MYH and PLA_2 or LR, and the results were investigated using fluorescence microscopy (Fig. 4A,B). The merged pictures revealed