Original Article

Phenotypic modulation of rat corpus cavernosum smooth muscle cells induced by platelet-derived growth factor-BB

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Abstract: Background: Phenotypic modulation within corpus cavernosum smooth muscle cells (CCSMCs) plays a critical role in the pathogenesis of erectile dysfunction (ED), yet little is known regarding the effects of platelet-derived growth factor-BB (PDGF-BB) on CCSMCs phenotype. Objective: The aim of the present study was to evaluate the effect of PDGF-BB on phenotypic modulation of CCSMCs. Method: Cell viability was measured by the MTS assay. For cell cycle analysis, cells were stained with propidium iodide and analyzed by cytometry. Wound healing assay were performed to detect the PDGF-BB-induced CCSMCs migration. The expressions of phenotype-associated genes were determined by immunoblotting and Western blotting. Result: PDGF-BB significantly promoted the cell growth and migration capability of CCSMCs. The levels of phenotype-associated genes showed a significant change in PDGF-BB-induced CCSMCs, which was in concordance with Western blot analysis. Conclusion: These findings indicated that CCSMCs switched their phenotype from a contractile to a proliferative state in response to the stimuli of PDGF-BB.

Keywords: Platelet-derived growth factor-BB, corpus cavernosum smooth muscle cells, phenotypic modulation

Introduction

Erectile dysfunction (ED) is defined as the persistent inability, for at least 3 months duration, to achieve or maintain penile erection sufficient for satisfactory sexual performance [1]. ED is a common problem in male over 40 years old and the number of men with ED is increasing as well. It is reported that the estimated number of men suffering from ED in the world is expected to achieve approximately 322 million in year 2025 [2]. ED may have a neurogenic, psychogenic or endocrine origin. However, the most common cause is thought to be vascular causes [3]. Normal physiologic penile erection needs interaction between corpus cavernosum smooth muscle cells (CCSMCs) and vascular endothelial cells in the corpus cavernosum. CCSMCs are surrounded by the endothelial-lined sinusoidal structures [4]. The importance of smooth muscle relaxation in penile erection has been demonstrated in both animal and human studies [5, 6]. More than 60% to 80% of patients with ED can be treated by oral phosphodiesterase (PDE)-5 inhibitors that induce smooth muscle relaxation of the penis [7, 8].

Unlike myocardium and skeletal muscle cells, which are terminally differentiated, vascular smooth muscle cells (VSMCs) and CCSMCs maintain plasticity in cellular phenotype and can change from a contractile state to a synthetic state in response to extracellular cues [9]. The synthetic state is characterized by a high level of proliferation, migration, extracellular matrix production, and vimentin over expression but low level expression of contractile cyto-
skeletal proteins such as alpha smooth muscle actin (α-SMA), smooth muscle myosin heavy chain (SMMHC) and desmin [10]. Emerging evidence has shown that the phenotypic modulation of smooth muscle cells (SMCs) plays an important role in the pathogenesis of various diseases of the cardiovascular and respiratory systems, such as atherosclerosis, hypertension, asthma, among others [11-13]. In addition, the phenotypic modulation of CCSMCs towards synthetic state plays a critical role in the development of ED [14].

The platelet-derived growth factor (PDGF) family consists of five different dimeric isoforms, PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and PDGF-DD [15]. Among the many growth factors that can contribute to such a phenotypic modulation of VSMCs, PDGF-BB possesses the most potent mitogenic and inflammatory effects. PDGF-BB is released primarily by vascular endothelial cells and platelets at the sites of vascular injury and plays a critical role in proliferative vascular diseases [16-18]. However, the exact underlying mechanism of these changes is not well understood yet. In the current study, we sought to assess the effects of PDGF-BB on proliferation, migration and phenotypic modulation of CCSMCs.

Material and methods

Cell isolation and culture

Adult male Sprague-Dawley rats with average body weight of 150-200 g were purchased from the Experimental Animal Center of Guangdong province, China. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the First Affiliated Hospital of Guangzhou Medical University (Guangzhou, China). All the surgical procedures were performed under anesthesia induced by sodium pentobarbital administration, and all efforts were made to minimize the suffering. CCSMCs were isolated and cultured according to the procedures described previously [19, 20]. Briefly, the corpus cavernosum tissues were removed and cut into 0.5-1 mm³ segments. The segments were placed uniformly at the bottom of a 25 ml culture flask containing 20% fetal bovine serum (FBS; Invitrogen). After 5 hours, the culture flasks were gently turned upright again when the tissues attached to the substrate. 2 mL of DMEM/F12 medium (Gibco, New York, USA) was poured into the bottle. The bottle were then put in an incubator at 37°C with 5% CO₂. Finally, the cells were identified using immunofluorescence staining, and the third or fourth passage of cells was used for subsequent experiments (Fig. 1).

MTS assay

CCSMCs were seeded into 96 well plates at a density of 5×10³ cells in each well. The cells were treated with PDGF-BB at different concentrations (5, 10, 20, 40 ng/ml) for 24 h. This assay measured the amount of formazan produced from [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, MTS]) by the dehy-
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dehydrogenase enzymes of metabolically active cells (Promega, Madison, WI, USA). Thus, the quantity of formazan produced (as measured by the absorbance at 490 nm) is directly proportional to the number of living cells. Absorbance values of the treated cells were compared with the untreated cells.

Cell cycle analysis

CCSMCs were stimulated with PDGF-BB (20 ng/ml) in serum-deprived medium for 48 h. The cells were harvested, washed with PBS, and fixed with ice-cold 70% (v/v) ethanol for 24 h. Washed with PBS, cells were re-suspended in propidium iodide (PI) solution containing 50 mg/ml PI, 50 mg/ml RNaseA and 0.1% Triton X-100 in PBS. Afterward, the cells were incubated for 30 min at 37°C in the dark. Percentages of cells in sub-G1, G0/G1, S, or G2/M phase were analyzed on a FACScan flow Cytometer (Beckman Coulter, CA, USA).

Wound healing assay

CCSMCs were cultivated in six-well culture plates (Corning, NY) at a density of 5×10^5 cells/well and grown to confluence overnight. The growth medium was removed, cells were washed and a wound was produced (0.5 mm). Cells were rinsed to remove non-adherent cells, and fresh medium with 1% fetal bovine serum was added. Digital images were taken at time points with an inverted microscope (CKX41; Olympus) equipped with digital camera.

Immunofluorescence staining

CCSMCs were cultured in a 12-well plate at a concentration of 1×10^5/well. Cells were fixed by 4% paraformaldehyde for 20 minutes at room temperature, followed by permeabilization with 0.5% Triton (lot#84704, Invitrogen, Carlsbad, CA, USA). Primary antibodies such as α-SMA (NOVUS), Osteopontin (OPN) (abcam) and Calponin (abcam) were incubated at 4°C overnight while corresponding secondary antibodies were incubated at room temperature for 45 minutes. Nucleus was stained by DAPI (4',6-diamidino-2-phenylindole). Finally, cells were observed and images were captured under fluorescence microscope (ix73, Olympus, Tokyo, Japan).

Western blotting

The starved cells were pretreated with PDGF-BB (5, 10, 20, and 40 ng/ml) for 24 h. The cells were homogenized in ice-cold lyses buffer (2 mmol Tris-HCl, pH 7.4, 50 mmol NaCl, 25 mmol EDTA, 50 mmol NaF, 1.5 mmol Na_3VO_4, 1% Triton X-100, 0.1% SDS, supplemented with protease inhibitors 1 mmol/L phenylmethylsulfonylfluoride, 10 mg/L pepstatin, 10 mg/L aprotinin and 5 mg/L leupeptin) at 4°C for 15 min. The concentration of protein was determined using the BCA protein assay regents (No. #23225, Thermo Pierce, Rockford, IL, USA). Equal amounts of total protein were separated and incubated with primary antibodies, including anti-SMA (1:500, Novus) and anti myocardin (1:500, Abcam). A 1:5000 dilution of horseradish peroxidase-labeled antimouse/rabbit IgG fragment was used as a secondary antibody (Abcam); GAPDH was used as an internal control (1:3000, Abcam). The
bounded secondary antibodies were reacted to the ECL detection reagents (#1856135, Thermo) and exposed to X-ray films (#47410, Fujifilm, Tokyo, Japan).

**Statistical analysis**

All data in this study were analyzed by SPSS 13.0 (SPSS, Chicago, IL, USA), and results were designated as mean ± standard deviation. T test was used to compare two groups of samples and One-Way ANOVA was employed to compare multiple groups of samples. All experiments were performed in triplicates.

**Results**

To investigate the influence on cell viability, CCSMCs were exposed to 0, 5, 10, 20, and 40 ng/ml of PDGF-BB. After the equivalent treatment, cells were measured by the MTS assay. The results demonstrated that CCSMCs proliferation was stimulated by PDGF-BB, with a peak at the 5th day. The proliferative effect was more significant at a concentration of 20 ng/ml compared to the other concentrations (P<0.05, for all) (Figure 2). Thus, 20 ng/ml PDGF-BB was chosen for the next studies.

To investigate the effects of PDGF-BB on cell cycle, CCSMCs were exposed to 20 ng/ml PDGF-BB. Untreated cells were measured as controls. Cell cycle analysis showed that PDGF-BB at a concentration of 20 ng/ml altered cell cycle progression, decreased G0/G1 populations and increased the number of cells in S phase and G2/M phases (Figure 3). The proportion of cells in G0/G1 decreased to 77.57% ± 5.72% from original 90.79% ± 3.46%, while proportion of cells in S phase increased from 7.85% ± 2.68% to 16.47% ± 4.42% and proportion of cells in G2/M increased from 2.46% ± 1.25% to 5.96% ± 2.18% as determined by counting cells using a haemocytometer.
Given that PDGF-BB was related to cell viability in CCSMCs, we studied whether PDGF-BB could affect CCSMCs migration. Wound-healing assay showed that the migration of CCSMCs was notably increased in comparison with control cells (Figure 4). These results suggested that PDGF-BB induced the proliferation and migration of CCSMCs. CCSMCs may switch their phenotype from a contractile and synthetic state in response to the stimuli of PDGF-BB.

To further assess whether CCSMCs switch their phenotype, we evaluated the expression of modulation-associated genes. As shown in Figure 5, our data manifested that compared with the control group, the expressions of contractility-associated genes α-SMA and calponin were markedly decreased in CCSMcs stimulated with PDGF-BB. However, the expression of proliferative gene OPN was elevated. Furthermore, Western Blot showed that PDGF-BB inhibited the expressions of α-SMA and Myocardin in a time and dose-dependent manner (Figure 6). This is consistent with the expression at the mRNA level in our previous study [21]. These results suggested that rat CCSM cells treated with PDGF-BB tend to possess the proliferative phenotype.

Discussion

Phenotypic modulation of CCSMCs is a dynamic process influenced by both profound and subtle changes in the microenvironment of the cell. Phenotypic modulation, one of the most important characteristics within CCSMCs, has a critical role in the pathogenesis of ED [14]. This study is the first to reveal the phenotypic changes in CCSMCs under the stimuli of growth factors and the role of PDGF-BB in their phenotypic transition. Although CCSMCs play a central role in the development of ED, only animal models were used to study ED for a long time.

Figure 4. The migration ability of CCSMCs was evaluated by wound-healing assays. The migration of CCSMC treated with PDGF-BB was notably increased in comparison with control cells. The wound of cells was visualized at 0 and 24 h.
Technical requirement in obtaining pure primary smooth muscle cells from corpus cavernosum tissue hinder the establishment of primary CCSMC line until recent breakthrough in methodology. In our previous studies, we obtained primary CCSMCs with purity over 95% [14, 22]. The CCSMCs were initially stimulated with the different concentrations of PDGF-BB (0, 5, 10, 20 and 40 ng/ml). The data demonstrated that the proliferation of CCSMCs were significantly changed in the cells that stimulated with PDGF-BB at a concentration of 20 ng/ml compared to the other concentrations. Therefore, we chose 20 ng/ml as the best concentration to investigate phenotype changes in CCSMCs. Our results showed that smooth muscle cells underwent a modulation from a contractile to a synthetic phenotype, which is associated with a shift in the expression of modulation-associated proteins.
Basic scientific research found that the pathogenesis and development of ED are associated with some key molecular mechanisms, such as PDGF-BB, tumor necrosis factor (TNF-α) and interleukin 1β (IL-1β) and other inflammatory cytokines [23, 24]. In aortic smooth muscle cells, the binding of PDGF-BB to PDGF-receptor leads to phosphorylation of the receptor and activation of subsequent downstream signaling molecules including focal adhesion kinase (FAK), c-Src and mitogen-activated protein kinases (MAPKs) [25, 26]. FAK is a non-receptor tyrosine kinase that normally activated by various growth factors stimuli and integrin signaling, its phosphorylation at Tyr397 induces Src-dependent phosphorylation of the remaining FAK tyrosine residue, to allow full activation of its kinase activity, which involved in the regulation of aortic smooth muscle cells motility and growth [27, 28]. Other reports revealed that PDGF-BB initiated a multitude of biological effects through activating extracellular signal-regulated kinase 1/2 (ERK1/2) MAP kinase. And ERK1/2 transduces mitogenic signals to the nucleus by phosphorylating and activating specific transcription factors for migration and proliferation [29]. Other pathways such as c-Jun NH2-terminal kinase (JNK), Akt and p38 MAPK were also reported to contribute to proliferation, migration, and collagen synthesis of smooth muscle cells [30, 31]. PDGF-BB also activates cell cycle regulators such as Cyclin D, Cyclin E, CDK2 and CDK4, which lead to moving out of cells from G0/G and increasing the fraction of cells in G2/M and S phase in vascular smooth muscle cells [32, 33]. This is consistent with our finding in CCSMCs [14, 22].

PDGF-BB exert synergism effect upon binding their receptors to activate PI3K, Akt, mTOR, p70S6K sequentially to promote phenotypic modulation from contractile toward synthetic state [34]. Calponin is major component of differentiated smooth muscle and stabilize actin cytoskeleton by forming the unique actin-binding interface [35]. Here we confirmed the down-regulation of α-SMA, SMMHC and calponin in CCSMCs. These SMC markers genes, such as α-SMA and SMMHC contain multiple CArG [CC(AT-rich)6GG] elements in the promoter-enhancer regions, and expression of these genes is controlled by the ubiquitously expressed trans-binding factor, serum response factor (SRF), and its co-activator, myocardin. It has been showed that myocardin was exclusively expressed in SMCs and cardiomyocytes, and markedly induced the transcription of multiple CArG-containing SMC marker genes in the presence of SRF [36, 37]. In consistent, PDGF-BB was up-regulated under hypoxia condition and involved in long term corpus cavernosum fibrosis therefore contributes to ED [38]. Phenotypic modulation towards synthetic state from contractile state in CCSMCs was known to be associated with lower erectile function [22]. When cavernous nerve was stimulated, penile erection was induced with increased length and diameter of the penis and increased ICP [39]. The major limitation of the present study is the absence of in vivo experiments. Therefore, the exact underlying molecular mechanism of this effect remains to be further elucidated in vivo.

The present study firstly showed that CCSMCs switched their phenotypes from a contractile to a proliferative state under the stimuli of PDGF-BB. Thus, PDGF-BB plays an important role in phenotypic modulation of CCSMCs.

Figure 6. The effects of PDGF-BB on the expression of contractility-associated genes. PDGF-BB inhibited the expressions of α-SMA and Myocardin in a time and dose-dependent manner.
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Disclosure of conflict of interest

None.

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