Influence of PDGF-BB and PKGIα on Morphology and Cytoskeleton of Vascular Smooth Muscle Cell

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Abstract Primary rat aortic smooth muscle cells (RASMCs) were prepared by explant-attachment method. Both the primary cells and rat pulmonary artery smooth muscle cell line CS-54 were infected with recombinant adenovirus encoding gene cGMP-dependent protein kinases I α (pAd-PKGIα) and were treated with platelet-derived growth factor-BB (PDGF-BB) to study the effects on morphology and cytoskeleton organization. Cell morphology was observed by reverse microscope and stress fiber structure was analyzed by fluorescence staining. It was found that in both CS-54 cells and RASMCs, PDGF-BB not only caused obvious change of the morphology but also disrupted the cytoskeleton organization, which was indicated by the change of the amount and the distribution of stress fibers. Infection with pAd-PKGIα and treatment of 8-APT-cGMP resumed the structure of stress fibers. These results demonstrated that PDGF-BB has an influence on morphology and cytoskeleton organization of CS-54 cells and RASMCs, and increase of the expression and the activity of PKGIα can reverse the effect.

Key words PDGF-BB; PKGIα; rat vascular smooth muscle cell; stress fiber

Vascular smooth muscle cells (VSMCs) are highly specialized cells whose principal functions are contraction and regulation of blood vessel tone, blood pressure, and blood flow. VSMCs within adult blood vessels exhibit a low rate of proliferation, low synthetic activity, and express a unique repertoire of contractile proteins, ion channels, and signaling molecules required for contractile function [1]. However, VSMCs are able to modulate their phenotype in response to the change of local environment. There is clear evidence that phenotype alteration of the VSMC plays a critical role in the pathogenesis of atherosclerosis.

Platelet-derived growth factor (PDGF) is produced by activated platelets and vascular lesion macrophages. Increased PDGF activity has been linked with several diseases and pathological conditions. It increases VSMC's proliferation and migration and appears to be a key mediator of VSMC phenotypic modulation in atherosclerosis [2].

cGMP dependent protein kinases (PKG) are serine/threonine kinases presenting in a variety of eukaryotes. Two PKG genes, coding for PKG type I (PKGI) and type II (PKGII), have been identified in mammals. The N terminus (the first 90–100 residues) of PKGI is encoded by two alternatively spliced exons that produce the isoforms PKGIα and PKGIβ [3]. It has been acknowledged that PKGIα is a recognized anti-atherosclerosis factor [4].

Many researches have been carried out in this direction recently. But studying on the influence of PDGF on the change of VSMC morphology and cytoskeleton structure, which is an important part of phenotypic alternation, was rare. This experiment was designed to study the effect of PDGF on morphological and cytoskeletal changes of VSMCs and the reversal role of PKGIα in this process.

1 Materials and Methods

1.1 Explant culture and growth of rat RASMCs

Sprague-Dawley rats were provided by Experimental Animal Center of Jiangsu University. RASMCs

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were isolated from the thoracic aorta of the rats in reference to Lin's method [5]. Briefly, the rat was killed by cervical dislocation, and sterilized with 75% ethanol for 1–2 min. The thoracic aorta was taken out and the attached fat and connective tissue was cut off. Then the adventitia and intima of the vessel were removed carefully. The remaining thin, transparent, flexible membrane-like tissue was the required tunica media. The tissue was cut into small pieces around 1 mm^3. These explants were seeded onto bottom of culture flasks and cultured in DMEM containing 20% fetal bovine serum (Gibco), 0.1% penicillin and 0.1% streptomycin at 37 ℃ in a humidified atmosphere with 5% carbon dioxide. Cells growth was observed under reverse microscope.

1.2 Cell culture

CS-54 (a rat pulmonary artery smooth muscle cell line) cells were cultured in DMEM supplied with 10% neonatal bovine serum (Gibco) and maintained in a standard humidified 5% CO_2 tissue culture incubator. The medium was changed every second day and the cells were subcultured in routine procedure at confluence.

1.3 Virus Construction

The structure of recombinant adenovirus pAd-PKGIα and pAd-lacZ were kind gifts from Dr. Renate Pilz in University of California, San Diego, USA.

1.4 RNA isolation, reverse transcription and PCR

1.4.1 Total RNA isolation and reverse transcription

Cells were seeded in a 6-well plate at a final density of 1×10^6 cells/well, and were cultured for 24 h. Ad-PKGIα virus suspension was added at MOI=100, control group was replaced by equivalent PBS. Total RNA in the cells was isolated using BIOzol reagent (BIOER) and cDNA was synthesized in 20 μl of reaction volume containing 2 μg mRNA and 1 μl Reverse Transcriptase (Fermentas) according to the manufacturer's instructions.

1.4.2 Primers for PCR GAPDH: sense primer, 5'-GCTGGTCATCAACGGGAAA-3'; antisense primer, 5'-ACGCCAGTAGACTCCACGACA-3'; product length is 105 bp. PKGIα: sense primer, 5'-CTTCTTCGCCAACCTG-3'; antisense primer, 5'-TGAAATCGGAATGAGCC-3', product length is 369 bp.

1.4.3 PCR reaction system In 25 μl of reaction mixture, 1 μl cDNA, 0.5 μl of each primer, 2 μl of 25 mmol/L $MgCl_2$, 0.5 μl of 10 mmol/L dNTP Mix, and 1 unit of *Taq* DNA Polymerase (Fermantas) were included. The thermal cycle profile for PCR was 94 ℃ for 3 min followed by 35 cycles of 30 s at 94 ℃, 30 s at annealing temperature (49 ℃ for PKGIα and 55 ℃ for GAPDH), 45 s at 72 ℃ with an additional 7 min of incubation at 72 ℃ after completion of the last cycle. The PCR products were separated on 1% agarose gels, stained with EB and observed under UV-light.

1.5 Western blotting

Sample proteins were separated on SDS-PAGE gels and blotted onto polyvinyl difluoride (PVDF) membrane. The PVDF membrane was blocked with 3% (*W/V*) bovine serum albumin (BSA) in TBS-T for 1h at room temperature. The incubation with the primary antibody was at 4 ℃ overnight, and with the secondary antibody was 1 h at room temperature, with three washes after each incubation. Electrochemiluminescence reagents were used to show the positive bands on the membrane. The bands were detected by Typhoon 9400 (GE healthcare, USA).

1.6 Immunofluorescence assay

Cells were seeded on coverslips in 24-well plate and incubated at 37 ℃ in DMEM. According to the experimental design, recombinant virus was added at MOI= 100. Cells were treated with PDGF-BB at 100 ng/ml (Calbiochem) or 8-APT-cGMP at 1×10^{-4} mol/L (Biolog) respectively.

The cells grown on coverslips were fixed with freshly prepared 40 g/L paraformaldehyde in PBS at 4 ℃ overnight. After being penetrated with 0.3% Triton X-100 and blocked with 30 g/L BSA, the cells were incubated with 0.2 mmol/L Hoechst 33342 for 10 min to reveal nuclei. Then the cells were incubated with primary antibodies at 4 ℃ overnight, FITC- or TRITC-conjugated second antibodies for 1 h at room temperature, with three washes after each incubation. The morphologic changes of the cells were analyzed by fluorescence microscopy.

2 Results

2.1 Culture and identification of RASMCs

Cells sprout out from the explant edge in about 3 days by the vertical direction from the edge (Fig.1A).

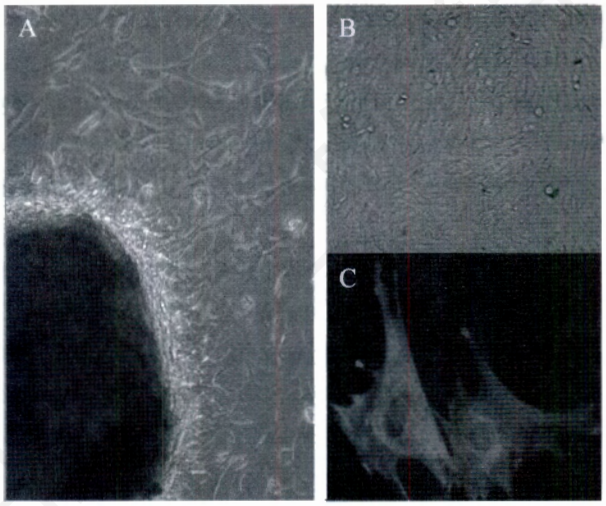

Fig.1 RASMCs generatated from rat aorta

A: results of reverse microscopic image (200×); B: a typical "hill-valley" growth pattern (200×); C: identification of RASMCS by immunofluorescence staining of SM-α-actin (400×).

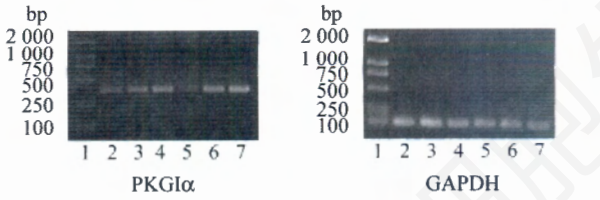

Fig.2 PKGIα expression in CS-54 cells and RASMCs

1: marker; 2, 5: CS-54 cells and RASMCs control; 3, 6: CS-54 cells and RASMCs infected by pAd-PKGIα; 4, 7: CS-54 cells and RASMCs infected by pAd-PKGIα and then stimulated by 8-APT-cGMP.

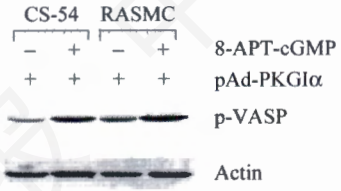

Fig.3 Increase of p-VASP level induced by 8-APT-cGMP treatment in CS-54 cells and RASMCs

The cell density varied around the identical explant and had slight differences in shape and size. Cells approached confluence approximately 3 weeks later. After being passaged, the cells overlapped in some regions but with less appearance in other regions, displaying a typical "hill-valley" growth pattern (Fig.1B).

For identification, the cells were immunofluorescently stained with anti-SM-α-actin antibody and observed by fluorescence microscope. The result showed that cultured cells were positive for SM-α-actin, confirming that they were RASMCs (Fig.1C).

2.2 Expression and activation of PKGIα in rat aortic smooth muscle cells

CS-54 cells and RASMCs were infected with recombinant adenoviral construct pAd-PKGIα encoding rat PKGIα and pAd-lacZ encoding β-galacotose at MOI=100. Total RNA of the cells was extracted and RT-PCR was performed to detect the expression of PKGIα mRNA.

RT-PCR results showed that the expression of PKGIα mRNA in both CS-54 cells and RASMCs infected with the pAd-PKGIα was significantly higher than that of control cells. 8-APT-cGMP (an isozyme-selective activator of PKGIα) treatment had no effect on the expression of PKGIα mRNA. GAPDH mRNA served as a control (Fig.2).

Vasodilator-stimulated phosphoprotein (VASP) is a member of the Ena-VASP protein family. VASP is regulated by PKG as its major substrate in human platelets and other cardiovascular cells. Therefore p-VASP is a reliable biochemical marker of vascular PKG activity. To observe the activation of PKGIα, CS-54 cells and RASMCs were infected with pAd-PKGIα virus at MOI=100 and then stimulated by 8-APT-cGMP. Total protein of the cells was extracted. Western blotting with antibody specific for Ser157 phosphorylated VASP (p-VASP) was performed to detect the phosphorylation of this protein. Equal loading was demonstrated by blotting with anti-actin antibody. The results showed that 8-APT-cGMP treatment can activate PKGIα. This suggested that the increase of PKGIα activity caused by 8-APT-cGMP was through affecting the activation but not the expression level of the protein (Fig.3).

2.3 PDGF-BB causes morphological change of CS-54 cells and RASMCs

Our live cell imaging results showed that cell morphology of both CS-54 cells and RASMCs were obviously changed after treatment with PDGF-BB. The cells changed their forms from spindle-shaped contractile smooth muscle cell to polygon or flat secretion smooth muscle cell. However, this morphological change disappeared after infection with pAd-PKGIα and treatment with 8-APT-cGMP (Fig.4). These results

provided morphological evidence that PDGF-BB could cause the alternation of phenotype in both CS-54 cells and VSMCs and PKGIα could reverse this procedure.

2.4 PDGF-BB induces cytoskeleton change in CS-54 cells and RASMCS and the change can be prevented by PKGIα

Fluorescent staining of CS-54 cells and RASMCs with phalloidin showed that control groups of both cell types had more stress fibers and stronger fluorescence intensity. The fibers presented a polar distribution. Longer microfilament paralleled the cell axis. In the groups treated with PDGF-BB, both CS-54 cells and RASMCs presented a disordered arrangement and non-polar distribution of the structure. Stress fibers were relatively shorter and distributed astatically. Some cells even lose their stress fibers. Fluorescence intensity was weak in these groups.

After being infected with pAd-PKGIα and then stimulated with 8-APT-cGMP before PDGF-BB treatment, fluorescent staining with phalloidin in CS-54 cells and RASMCs exhibited actin cables that extended the length of the cell, confirming that the original stress

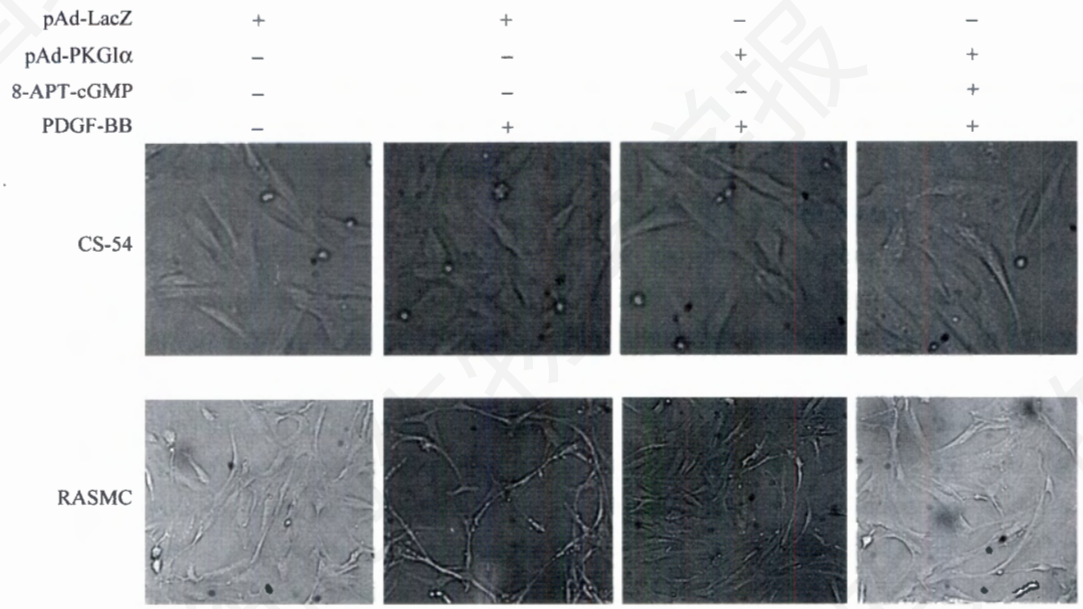

Fig.4 Morphology change of CS-54 cells and RASMCs (400×)

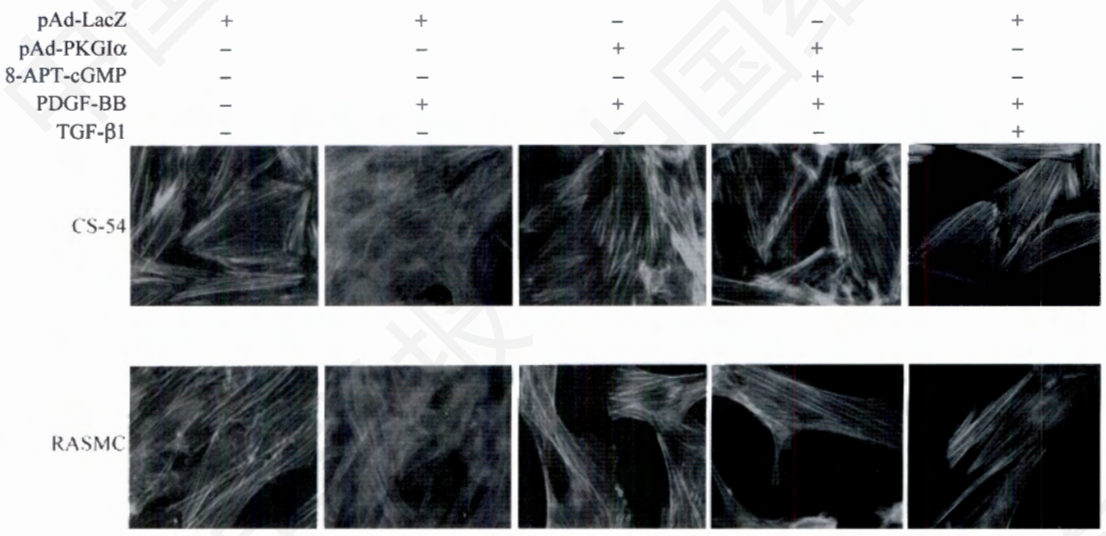

Fig.5 Stress fiber reduction and restoration of CS-54 cells and RASMCs (400×)

fibers were restored. The groups treated with both PDGF-BB and TGF-β1 (which could reverse the effect of PDGF-BB [6]) served as a positive control (Fig.5).

3 Discussion

Unlike terminally differentiated skeletal and cardiac myocytes, VSMCs retain remarkable plasticity and can undergo rather profound and reversible changes in phenotype in response to changes in local environmental cues [1]. Researchers have defined two phenotypes of VSMCs: contractile one and synthetic one [7]. Contractile phenotype is the mature type of VSMCs which is highly differentiated and characterized by spindle-like elongated morphology. On the other hand, however, synthetic phenotype is the immature and dedifferentiated status with hypertrophic appearance.

Local environmental change could cause the dediferrentiantion of VSMCs and this phenotype alternation plays important role in the process of vascular diseases, especially in atherosclerosis. It concluded that PDGF is the key factor in this alternation. However, morphological evidence for this conclusion was still lacking. Our results showed that with treatment of PDGF-BB, morphology and cytoskeleton of both CS-54 cells and RASMCs changed obviously compared with the control group. During this process, stress fibers of CS-54 cells and RASMCs were disarranged and reduced, and the cells transformed from contractile phenotype to synthetic one. This further confirmed that PDGF-BB played its role as mediator in modulation of VSMC phenotype through affecting the arrangement and amount of cytoskeleton of the cells.

Studies indicate that the NO/cGMP/ PKGI pathway inhibits proliferation and dedifferentiation of VSMCs, and thereby exhibits anti-atherogenic effect [8]. For example, neo-intima formation and stenosis after arterial injury in intact animals was reduced by restoring soluble guanylate cyclase or PKG to the injured blood vessels. Other genetic or pharmacological manipulations that enhance cGMP production in injured blood vessels yield similar results. Analyses of plaque composition suggest that PKG regulates VSMCs secreted factors which affect matrix remodeling and recruitment of other plaque cells, such as macrophages [9]. Our results showed that in CS-54 cells and RASMCs infected with pAd-PKGIα, stress fibers resumed both in arrangement and quantity, especially in the groups with high PKGIα activity by extra 8-APT-cGMP. This further confirmed that PKGIα played its anti-arthrosclerosis role through promoting the VSMCs to change their synthetic phenotype back to the contractile phenotype.

References

1 Zhou W, Negash S, Liu J, Raj JU. Modulation of pulmonary vascular smooth muscle cell phenotype in hypoxia: role of cGMP-dependent protein kinase and myocardin. Am J Physiol Lung Cell Mol Physiol 2009; 296(5): L780-9.

2 Andrae J, Gallini R, Betsholtz C. Role of platelet-derived growth factors in physiology and medicine. Genes Dev 2008; 22(10): 1276-312.

3 Hofmann F, Bernhard D, Lukowski R, Weinmeister P. cGMP regulated protein kinases (cGK). Handb Exp Pharmacol 2009; (191): 137-62.

4 Lincoln TM, Wu X, Sellak H, Dey N, Choi CS. Regulation of vascular smooth muscle cell phenotype by CYCLIC GMP and cyclic GMP-dependent protein kinase. Front Biosci 2006; 11: 356-67.

5 Lin J, Zhang ZL. A cell culture model *in vitro* for rat aortic vascular smooth muscle cells by modified tissue explants-attached method. Chin J Pathophysiol 2006; 22(4): 827-9.

6 Engel LC, Ryan U. TGF-β1 Reverses PDGF-stimulated migration of human aortic smooth muscle cells *in vitro*. In Vitro Cell Dev Biol Anim 1997; 33(6): 443-51.

7 Owens GK. Regulation of differentiation of vascular smooth muscle cells. Physiol Rev 1995; 75(3): 487-517.

8 Negash S, Narasimhan SR, Zhou W, Liu J, Wei FL, Tian J, *et al.* Role of cGMP-dependent protein kinase in regulation of pulmonary vascular smooth muscle cell adhesion and migration: effect of hypoxia. Am J Physiol Heart Circ Physiol 2009; 297 (1): H304-12.

9 Raghavan SA, Dikshit M. Vascular regulation by the L-arginine metabolites, nitric oxide and agmatine. Pharmacol Res 2004; 49(5): 397-414.

血小板源性生长因子-BB和cGMP依赖性蛋白激酶Iα对大鼠血管平滑肌细胞形态和骨架的影响

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摘要 原代大鼠主动脉平滑肌细胞采用植块法培养。用编码cGMP依赖性蛋白激酶Iα基因的重组腺病毒(pAd-cGMP dependent protein kinase I α, pAd-PKGIα)感染原代培养的大鼠主动脉平滑肌细胞和常规培养的大鼠肺动脉血管平滑肌细胞株CS-54, 并用血小板源性生长因子-BB (platelet-derived growth factor-BB, PDGF-BB)作用细胞, 研究PKGIα和PDGF-BB对形态学和细胞骨架的影响。倒置显微镜观察细胞形态, 免疫荧光观察细胞应激纤维结构。结果表明PDG-BB不仅可以使CS-54细胞和原代大鼠主动脉平滑肌细胞的形态发生明显的变化, 也使得细胞原有的应激纤维的数量减少, 分布紊乱; pAd-PKGIα感染并经8-APT-cGMP刺激后应激纤维又可以重新恢复。这些结果证明PDGF-BB对大鼠血管平滑肌细胞株CS-54和原代大鼠主动脉平滑肌细胞形态和细胞骨架结构有影响, 这些影响可以因PKGIα的表达和活性的增加而逆转。

关键词 PDGF-BB; PKGIα; 大鼠血管平滑肌细胞; 应激纤维

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