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Original article

Inhibition effect of tacrolimus and platelet-derived growth factor-BB on restenosis after vascular intimal injury

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ABSTRACT

Excessive proliferation and migration of vascular smooth muscle cells(VSMCs) and delayed proliferation and migration of endothelial cells(ECs) were the main cause of restenosis after endovascular interventional therapy. Since tacrolimus has proved to be more sensitive to inhibiting VSMCs' proliferation,and platelet-derived growth factor-BB(PDGF-BB) benefitted ECs' and VSMCs' proliferation, this study was aimed to identify combined effect of tacrolimus and PDGF-BB, investigate any mechanisms underneath and demonstrate combined effect of two drugs in vivo. As the results showed we confirmed differential effect of PDGF-BB and tacrolimus on ECs and VSMCs. On the concentration level of 2–5 μg/ml tacrolimus plus 10 ng/ml PDGF-BB, combination of drugs could effectively promote ECs proliferation and migration, and meanwhile inhibit VSMCs proliferation and migration, and the inhibition of p-mTOR's expression within VSMCs played an important role in this differentiated effect. Raising concentration level of PDGF-BB would weaken inhibitory effect of tacrolimus on both kinds of cell. For injured intima, the mix solution of two drugs could promote intima healing and suppress excessive intimal hyperplasia.
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1. Introduction

Restenosis after interventional treatment of critical limb ischemia(CLI) was the major challenge for vascular intervention therapy [1,2]. No matter what method chosen—percutaneous transluminal angioplasty(PTA) [3], drug eluting balloon(DEB) [4], bare stent or drug eluting stent(DES) implantation [5,6], long term effect was unsatisfactory, especially for infrapopliteal arterial lesions caused by diabetes mellitus, on account of smaller arterial diameter and longer arterial lesions [7]. Incidence of vascular restenosis was up to 50%～70% for diabetic CLI within 3 months after the operation [7–10]. Operation impaired arterial intima, and then avulsion of arterial intima induced collagen exposure, plaque deposits, and persistent inflammation. It resulted in excessive proliferation and migration of VSMCs. It's generally accepted that over-proliferation and migration of VSMCs was the dominating reason of restenosis [11,12].

To address this problem, various drugs were applied to DES or DEB. Paclitaxel, rapamycin and its derivatives(everolimus, zotarolimus)were the most frequently-used drugs in clinical practice [13–15]. These drugs could strongly resist excessive proliferation of VSMCs. But they were more sensitive to ECs than to VSMCs, they would restrain ECs' proliferation, adhesion and migration, causing the delay of intimal restoration [16–18]. Delay of reendothelialization led to late thrombosis and restenosis [19,20]. Therefore, the key to reduce restenosis was how to accelerate early reendothelialization and suppress the excessive proliferation of VSMCs at the same time.

Platelet derived growth factor(PDGF) appeared in early wound healing stage, including PDGF-BB, PDGF-AB, PDGF-AA. They all had a variety of effects on many cell types by binding to PDGF α-receptor(PDGFRα) or PDGF β-receptor(PDGFRβ) [21,22]. Stimulation of receptors could activate several signaling pathways, and among these pathways, phosphatidylinositol 3-kinase(PI3 K) had an important role in intracellular signal transduction [21]. It could stimulate proliferation, migration, and differentiation of many cell types [23]. It could quit arrest at G0/G1 phase of the cell cycle [24]. PDGF could also stimulate VSMCs and ECs proliferation [25–27]. In the early stage of post-operation, proliferation and migration of ECs could accelerate the healing progress of intima. Tacrolimus (FK506) was a powerful immunosuppressive drug against transplant rejection [28]. As an inhibitor of lymphocyte activation,

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tacrolimus bound to intracellular FK506-binding protein 12(FKBP), and the FK506-FKBP compounds could restrain calcineurin's phosphorylation enzymatic activity. It would prevent dephosphorylation of nuclear factor of activated T cells(NFAT), and then reduce expression amount of cytokines(like IL-2,IFN-γ) and suppress T cells activation [17]. FK506 could restrain migration and proliferation of VSMCs and ECs, and was more sensitive to VSMCs than to ECs [17,29].

The aim of this study was to demonstrate the efficacy of two drugs in different concentrations. To see whether they would promote ECs proliferation and migration, and meanwhile suppress excessive proliferation and migration of VSMCs, and the effect was differential enough to tell. To investigate any mechanisms underneath and demonstrate combined effect of two drugs in vivo.

2. Methods

2.1. Cell cuture

Rat aortic endothelial cell(RAOEC) and rat aortic vascular smooth muscle cell(VSMC) were purchased from American Type Culture Collection(ATCC, America). They were cultured in Dulbecco's modified Eagle's medium (DMEM) plus 10% Fetal Bovine Serum(FBS)(both from Gibco, Carlsbad, USA). ECs and VSMCs were grown and passaged in CO_2 incubators(5%CO_2,37 °C), and passage 4–8 were used for cell experiments.

2.2. Proliferation assay

10,000 ECs or VSMCs were grown in 96-well plates in DMEM with 10% FBS for 24 h to get attachment. When cells got at least 70% confluence, both kinds of cells were cultured in DMEM plus 0.2% FBS for 0 h, 6 h, 12 h, 24 h, 48h, and then incubated with 10 μl Cell Counting Kit-8(CCK8)(Yeasen, Shanghai, China) working solution for 1 h to detect cell vitality(detection wavelength of 450 nm). After reaching 70% cell confluence and withdrawing serum(DMEM plus 0.2% FBS) for 24 h to maintain dormant, ECs and VSMCs were separately treated with PDGF-BB(Pepro Tech, Rocky Hill, USA) or tacrolimus(TRC, Toronto, CA) in different doses for 24 h. Then 10 μl CCK8 working solution was added into each sample and mix solutions were incubated for 1 h to detect optical density(OD). Since availability and safety of these two drugs had been tested in the experiment, Joint action of two drugs in different concentrations was also tested via CCK8.

2.3. Cytotoxicity test

After reaching 70% cells confluence in a 96-well plate and 24 h starving, each kind of cells was exposed to 10 ng/ml PDGF-BB and different concentrations of tacrolimus(0,10,20,30,40,50 μg/ml). After 24 h, 10 μl CCK8 working solution was added into each well and all wells were incubated for 1 h to get detected. In flow cytometry test, dormant ECs and VSMCs in 6-well plate were treated with different concentrations of tacrolimus plus 10 ng/ml

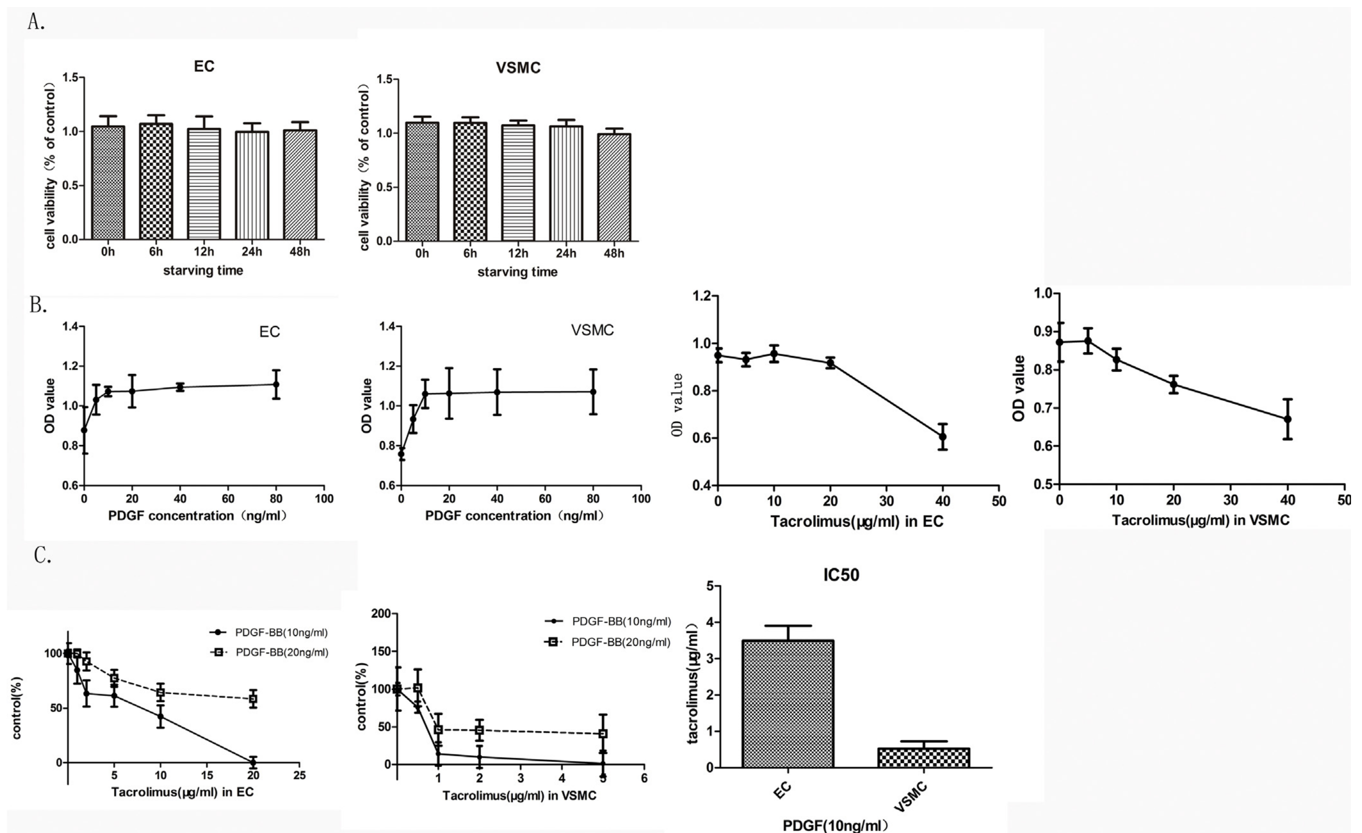

Fig. 1. (A)There were no significant difference among 0,6,12,24 and 48 h starving group. (EC:P = 0.259, VSMC:P = 0.3219; n = 12);(B)PDGF-BB could induce ECs and VSMCs proliferation in a dose-response manner(EC:$P < 0.0001$, VSMC:$P < 0.0001$;n = 12); Low dose tacrolimus didn't induce cell proliferation. For ECs, there were no significant difference among control, 5 μg/ml,10 μg/ml and 20 μg/ml tacrolimus treated EC group(P = 0.1302;n = 12), but 10 μg/ml tacrolimus was harmful to VSMCs vitality(P = 0.0058; n = 12); (C)Tacrolimus inhibited 10 ng/ml PDGF-stimulated ECs and VSMCs proliferation in a concentration-dependent manner (EC:$P < 0.0001$;VSMC:$P < 0.0001$;n = 12). In the same tacrolimus concentration level, raising concentration of PDGF-BB(20 ng/ml) would close the gap in proliferation inhibition between ECs and VSMCs. (EC:$P < 0.0001$; VSMC:$P < 0.0001$;n = 12).For VSMCs and ECs, IC50 of ECs and VSMCs were 0.53 μg/ml and 3.54 μg/ml respectively in 10 ng/ml PDGF treated group.

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