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Pleiotropic effects of survivin in vascular endothelial cells

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ABSTRACT

Objective: To assess the effects of survivin (SVV)in vascular endothelial cells.
Methods: In this study, we applied a gain-of-function approach and ectopically expressed SVV in rat aortic endothelial cells (RAECs) using a SVV-expressing adenovirus. The resulting SVV expression on the steady-state mRNA and protein level in RAECs was determined by reverse transcription quantitative PCR and Western blot, respectively. Cell viability, apoptosis, and migration were assessed *in vitro* by CCK-8 assay, flow cytometry, and transwell assay, respectively. The effect of SVV on *in vivo* angiogenesis was evaluated by immunohistochemistry in nude mice. Non-infected RAECs and those infected with GFP-expressing control adenovirus were used as controls.
Results: Compared to non-infected or control adenovirus-infected RAECs *in vitro*, SVV-expressing cells had increased viability and migratory capability, but reduced apoptosis. *In vivo*, SVV-expressing RAECs were associated with a higher level of angiogenesis.
Conclusion: SVV is a positive regulator of endothelial cell survival and migration, and thus, stabilizes endothelial cells and stimulates angiogenesis.

1. Introduction

Peripheral artery disease (PAD) refers to the chronic narrowing or atherosclerosis of vessels outside the cerebrovascular and coronary circulations, most commonly in the lower extremities (Hiatt et al., 2008). PAD shares similar risk factors with coronary artery disease, including male gender, increased age, diabetes, smoking, hypertension, hyperglycemia, high cholesterol, and renal insufficiency (Norgren et al., 2007). Clinically, PAD presents a spectrum of diseases with varying severity from asymptomatic to intermittent claudication (IC) to critical limb ischemia (CLI). In addition to functional limitations and reduced quality of life, PAD is associated with increased risk of myocardial infarction, stroke, and death (Criqui et al., 2008; Remes et al., 2010). In approximately 20% to 30% of PAD patients with CLI, limb amputation is the only treatment option, which leads to a much higher mortality at approximately 48% at 1 year and 71% at 3 years after limb amputation (Lawall et al., 2011; Swaminathan et al., 2014). Therefore, PAD therapy aims toprovide risk factor management, cardiovascular protection, symptom relief, functional preservation, and amputation prevention. For patients presenting with CLI, percutaneous transluminal angioplasty or surgical revascularization is the treatment of choice (Adam et al., 2005; Arain and White, 2008; Norgren et al., 2007). However, up to 30% of patients are not eligible for these interventions and thus require novel approaches to stimulate vascular regeneration.

Research over the past decades has identified cell therapy and gene therapy as two promising approaches for treatment of PAD (Losordo and Dimmeler, 2004a, 2004b). Cell therapy mainly involves transplantation of autologous endothelial progenitor cells (EPCs) or bone marrow-derived stem cells, which are expected to be incorporated into neo-vessels and simultaneously stimulate angiogenesis through the paracrine production of growth factors, cytokines or chemokines (Lawall et al., 2011). Although some animal experiments and small scale clinical trials have been promising in terms of therapeutic efficacy, many questions remain to be addressed for cell therapy, including isolation and management of injected cells, defined phenotypes of these cells, delivery dosage, route, frequency of injections, and tolerance and safety (Lawall et al., 2011). In contrast, gene therapy involves delivery of therapeutic genes (and sometimes recombinant proteins) to stimulate angiogenesis or arteriogenesis of endogenous endothelial cells or EPCs. Most clinical trials using gene therapy have focused on a limited number of growth factors, including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and hepatocyte growth factor (HGF), and the therapeutic efficacy is still limited (Germani et al.,

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2009; Suzuki and Iso, 2013). Identifying new factors that can boost endothelial cell activity and *in vivo* angiogenesis or arteriogenesis may provide promising therapy for PAD.

Survivin (SVV), also known as *Birc*5, is a member in the inhibitor of apoptosis (IAP) gene family. SVV plays a dual role in controlling mitosis and apoptosis: it forms the chromosomal passenger complex (CPC) together with aurora-B, borealin, and INCENP, and is essential for chromosome movement and stabilization of microtubules during mitosis. Furthermore, SVV binds to and blocks the activity of caspase 3 and caspase 9 to inhibit apoptosis (Vagnarelli and Earnshaw, 2004; Wheatley and McNeish, 2005). Although minimally expressed in normal adult tissues, SVV is highly expressed in embryonic tissues and various types of human cancers, and its expression is correlated with poor cancer prognosis (Altieri, 2001; Balakier et al., 2013; Hoffman et al., 2002). In vascular endothelial cells, SVV expression is significantly boosted in response to platelet-derived growth factor (PDGF), VEGF, angiopoietin, as well as vascular injury (Blanc-Brude et al., 2002; Papapetropoulos et al., 2000; Tran et al., 1999). Functionally, SVV stimulates endothelial cell proliferation while inhibiting apoptosisin endothelial cells (Blanc-Brude et al., 2002). Thus, we investigated SVV as a potential target to stabilize vascular endothelial cells and stimulate angiogenesis or arteriogenesis *in vivo*.

In this study, we used rat aortic endothelial cells (RAECs) as the model system, and applied a gain-of-function approach to examine SVV activities. We also investigated the underlying molecular mechanisms of proliferation, apoptosis, migration, and *in vivo* angiogenesis.

2. Materials and Methods

2.1. Cell lines and cell culture

RAECs were obtained from CHI Scientific, (Maynard, MA, USA) and maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Adenoviral transduction

SVV cDNA was cloned into the recombinant adenovirus vector expressing the green fluorescent protein gene (Ad-GFP). Adenovirus was generated by Sino Geno Max (Beijing China). To transduce the target cells, RAECs were seeded into6-well tissue culture plates at 5×10^4 cells/well. After overnight incubation, RAECs were infected with Ad-GFP/SVV at different multiplicity of infection (MOI). The optimal MOI was determined by monitoring GFP expression at 24 h and 48 h after transduction using a Zeiss fluorescence microscope (DM60000, Leica, Germany) equipped with a digital camera. All future experiments were performed in three groups of cells: non-infected RAECs (NI), cells infected with control adenovirus Ad-GFP, and cells infected with Ad-GPF/SVV.

2.3. Reverse transcription quantitative PCR (RT-qPCR) analysis

Total RNA was extracted from cells using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA was synthesized using ReverTra Ace qPCR RT Kit (TOYOBO, Japan). The RT-qPCR was performed using the following primers: SVV-forward, 5'TTTTGTGGCTTTGCTCTATTGT 3′, SVV-reverse, 5'GGTAG GAGGACTCATCAGAAGGA 3′; Caspase3-forward, 5′ACGGGACTTGGA AAGCATC 3′, Caspase3-reverse, 5′-TAAGGAAGCCTGGAGCACAG 3′; BCL-2-forward, 5'GAGGATTGTGGCCTTCTTTG 3′, BCL-2-reverse, 5'GTTCC ACAAAGGCATCCCAG 3′; GAPDH (internal control)-forward, 5′ CAGTGCCAGCCTCGTCTCAT 3′, GAPDH-reverse, 5′AGGGGCCATCCAC AGTCTTC-3′. The PCR conditions included an initial denaturation at 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 60 s. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to analyze the steady-state mRNA level of a target gene relative to that of the internal control (GAPDH).

2.4. Western blot analysis

Total proteins were extracted from cells using lysis buffer (pH 7.4, 150mMNaCl, 50 mL Tris–HCl, 2 mM EDTA, 1% NP-40), and the protein concentration was measured using the Bradford assay. Equal amounts of total proteins from different samples were separated on SDS-PAGE gel and transferred onto nitrocellulose membranes. The membranes were then incubated with primary polyclonal antibodies targeting SVV, phosphorylated aurora B, aurora B, INCENP, cyclin D1, caspase-3, bcl-2 (Cell Signaling, Danvers, MA, USA) at 4 °C overnight (1:1000). Following incubation with secondary antibody (goat *anti*-rabbit IgG, Santa Cruz Biotech., Santa Cruz, CA, USA) at room temperature for 2 h, the signals were visualized by chemiluminescence (UVP, Upland, USA) according to the manufacturer's instructions.

2.5. Cell Counting Kit-8 (CCK-8) cell viability assay

To quantify cell viability, the CCK-8 assay was performed using the commercial kit (Beyotime, Jiangsu, China) following the manufacturer's instructions. Briefly, cells were seeded into 96-well plates at 2×10^3per well. After 12 h, 10 μL of CCK-8 was added into each well and cells were incubated at 37 °C for 4 h. Absorbance was measured at 450 nm for each well.

2.6. Flow cytometry

Apoptosis was induced in RAECs by H_2O_2 (1.0 μM) for 8 h. The cells were then stained with Annexin V: PE Apoptosis Detection Kit I (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instruction. Positively stained cells were detected by flow cytometry (BD, Franklin Lakes, NJ, USA).

2.7. In vitro cell migration assay

Cell migration was evaluated in 24-well transwell chambers (8-μm pore size; Corning, Lowell, MA, USA). The cells were seeded into the upper chamber at 5×10^5 cells/well. The bottom chamber was filled with RPMI-1640 medium supplemented with 10% FBS as a chemoattractant. Cells were incubated at 37 °C for 24 h in a humidified incubator containing 5% CO_2. The cells remaining on top of the upper membrane were removed with a cotton swab. Cells that migrated through the pores were fixed with methanol and stained with crystal violet. Cell numbers were counted and averaged from five random fields using light microscopy at 200 × magnification.

2.8. Enzyme-linked immunosorbent assay (ELISA)

RAECs (2×10^5) were cultured on 6-well plates for 48 h and the culture media was collected for the measurement of matrix metalloproteinase 2 (MMP-2), MMP-9, and angiotensin 2 (Ang-2) levels using ELISA Kits from USCN Life Science (Wuhan, China) following the manufacturer's instructions.

2.9. Animal studies and immunohistochemistry

All animal procedures were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by Chongqing Medical University (Chongqing, China). Five-week-old, female BALB/c nude mice were obtained from the Laboratory Animal Center of Chongqing Medical University (Chongqing, China) and maintained in a specific pathogen-free facility at room temperature (22 ± 1)°C on a 12/12-hrlight/dark cycle, with access to food and water *ad libitum*. To assess *in vivo* angiogenesis, 1×10^5