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Human cytomegalovirus immediate early protein 2 enhances myocardin-mediated survival of rat aortic smooth muscle cells

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ABSTRACT

Human cytomegalovirus (HCMV) may increase the incidence of restenosis and predispose to atherosclerosis. The lesions of restenosis and atherosclerosis often contain smooth muscle cells (SMCs) with high rates of proliferation and apoptosis. One of the immediate early (IE) gene products of HCMV-IE2 affects transcriptional activities of some cellular factors in SMCs, including myocardin. In this study, we studied the effects of IE2 and myocardin on PI3K pathway inducer wortmannin induced apoptosis in rat aortic SMCs. We show that the transcriptional activity of myocardin on Mcl-1 promoter is enhanced by co-expression of HCMV IE2 in rat aortic SMCs; and the expressions of mRNA and protein of antiapoptotic genes-Mcl-1 and Bcl-2 are upregulated by IE2 alone and co-transfection of myocardin and IE2, but decreased by myocardin-specific shRNA in rat aortic SMCs. We further demonstrate that co-expression of myocardin and HCMV IE2 declines apoptotic cell numbers and caspase-3 activities induced by serum starvation plus wortmannin in rat aortic SMCs. The results suggest that HCMV IE2 enhances myocardin-mediated survival of rat aortic SMCs under serum deprivation and PI3-kinase inhibition, partly via activation of Mcl-1's antiapoptosis effect. Our study connects HCMV IE2 to myocardin-induced transcriptional program for rat aortic SMCs survival and proliferation, involving in HCMV related restenosis and atherosclerosis.

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1. Introduction

Human cytomegalovirus (HCMV) infection is endemic within human population but rarely causes symptomatic disease in healthy, immunocompetent individuals (Fowler and Pass, 1991). However, plethora of evidences implicates HCMV as a contributing agent in the pathogenesis of restenosis and atherosclerosis (Hendrix et al., 1990; Tanaka et al., 1999; Speir et al., 1994; Strandberg et al., 2009; Streblow et al., 2008). Persistent infection with HCMV has been associated with atherosclerosis, involving smooth muscle cells (SMCs) infection revealed by in vivo studies (Popovic et al., 2012). HCMV-infected SMCs within vascular lesions display enhanced proliferation and impaired apoptosis, which contributes to intima-media thickening, plaque formation and restenosis (Sinzger et al., 1995). One mechanism to account for these effects is through the known ability of HCMV to enhance cellular migration, accumulation and proliferation (Tanaka et al., 1999; Zhou et al., 1999; Reinhardt et al., 2005). Moreover, apoptosis occurs in restenotic and atherosclerotic lesions (Streblow et al., 2008), and related alterations in this process could also contribute to SMCs accumulation.

Studies suggest that HCMV-infected cells are protected from apoptosis through numerous mechanisms, including mediation of apoptosis inhibition regulated by HCMV-encoded immediate-early (IE) gene products. In normal permissive cells, HCMV IE1 and IE2 induce cell cycle arrest and prevent cell apoptosis to enable replication of viral DNA. Expression of HCMV IE1 and IE2 alters the cell cycle to generate an environment conducive to stimulating

Abbreviations: CMV IEs, cytomegalovirus immediate early proteins; SMCs, smooth muscle cells; SRF, serum response factor; PI3K, phosphoinositide 3-kinase.

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quiescent cells to enter G1/S phase, arresting cell cycle progression, inducing cellular macromolecule synthesis, and resisting apoptotic stimuli (Castillo and Kowalik, 2002; Castillo et al., 2000; Zhu et al., 1995). The proliferative signal in SMCs may relate to the ability of IE2 to bind some cellular death receptors and cell cycle proteins, as well as some cellular proapoptotic factors to inhibit their transcription activities (Castillo and Kowalik, 2002; Bonin and McDougall, 1997). Overexpression of IE2 prevents doxorubicin-induced apoptosis in SMCs (Tanaka et al., 1999) and p53-dependent apoptosis caused by nonpermissive temperature (Lukac and Alwine, 1999). IE2 is also shown to bind to p53 and suppress its transactivating function, which is important for induction of apoptosis (Speir et al., 1994; Casavant et al., 2006; Tsai et al., 1996). Numerous evidences support that HCMV IEs induce activation of the cellular signaling pathways including MEK/ERK pathway (Kim et al., 1999), which in turns contributes to apoptosis inhibition by HCMV. However, the specific regulation mechanism of apoptosis underlying interaction between host proteins and HCMV viral IEs needs to be further deciphered.

Myocardin is a cardiac and smooth muscle – specific transcriptional coactivator of serum response factor (SRF) that potently transactivates CArG box – containing cardiac and smooth muscle target genes (Zhou et al., 2011). It has been shown to be associated with cardiac myocytes apoptosis (Huang et al., 2009, 2012; Kobayashi et al., 2005) and cardiac hypertrophy (Zhou et al., 2011; Xing et al., 2006), and to transduce extracellular signals to the nucleus required for SMC differentiation and phenotype transition. Myocardin does not bind DNA alone, but forms a stable ternary complex with SRF bound to DNA (Zhou et al., 2011; Huang et al., 2009; Pipes et al., 2006). Researches have implicated that cardiocmyocytes with reduced myocardin function are more prone to apoptosis (Huang et al., 2009; Kobayashi et al., 2005; Seimi et al., 2004). Another study suggests that myocardin signal is related to embryonic survival (Huang et al., 2012). Ablation of the myocardin in the adult mouse heart leads to the rapid progression of heart failure and death, which is accompanied by the cell autonomous loss of cardiomyocytes via apoptosis (Huang et al., 2009). Moreover, Myocardin-related transcription factor family members are also found to transcriptionally induce proapoptosis pathway via MRTFs–SRF–CArG binding, which is independent of p53 (Shaposhnikov et al., 2012).

Our previous work has shown that HCMV IEs interact with myocardin to facilitate cardiac hypertrophy via transcriptional regulation on hypertrophic genes (Zhou et al., 2011). In the present study, we further investigate the associated role of HCMV IE2 and myocardin in modulating apoptosis induced by wortmannnin plus serum starvation in rat aortic SMCs, which involves Mcl-1, a potent antiapoptotic protein of the Bcl-2 family (Opferman et al., 2005). The finding suggests that the interaction between IE2 and myocardin might affect SMCs apoptosis via Mcl-1, thereby promoting cellular accumulation typical of proliferative diseases such as atherosclerosis and postangioplasty restenosis.

2. Materials and methods

2.1. Isolation of primary rat aortic SMCs and cell culture

Primary aortic vascular smooth muscle cells (SMCs) were isolated from the descending thoracic aorta of 3–4-week Sprague Dawley (SD) rats as described (modified by Methods in Cell Science 2002; 23:185–188). Briefly, segments of thoracic aorta (18–24 mm) rats were sacrificed under anesthesia with Nembutal (162.5 U/kg rat weight), after heparinizing rat (by injecting heparin, 1500 U/kg rat weight). Then rats were perfused with sterile saline. The thoracic aorta was isolated and washed in cold PBS three times in sterile petridish, and then incubated in DMEM containing 1 mg/ml collagenase II (Sigma) for 15 min at 37 °C. All external fat and connective tissue were detached, and the adventitia layer (outer white layer) was carefully removed. After the vessel was longitudinally opened, the endothelial cells were scraped off with a scraper or rub with sterile cue tip. After washed by sterile saline and serum free DMEM, the vessel parts were minced with scissors in DMEM with collagenase I and elastase III (Sigma) and digested by incubation of 80 min at 37 °C in a humified incubator under 5% CO_2, with repeated vigorous pipetting every 20 min. Then cells were collected and grown in DMEM-F12 containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco, USA). SMCs were confirmed by mouse monoclonal anti-α-SM-actin antibody (Abcam) via fluorescent immunostaining. SMCs at passage 3–6 were used for the experiments of this study. Cos-7 cells were cultured in DMEM containing 10% fetal calf serum (FCS, Hyclone), penicillin (100 U/ml) and streptomycin (100 U/ml).

Treatment of cells with wortmannin. Cells were seeded in 30-mm plates at a density of 5×10^5 cells/ml. They were then treated with the phosphoinositide 3-kinase (PI3 K)/Akt pathway inhibitor wortmannin (500 nM; Sigma–Aldrich Chemie, Steinheim, Germany) for 24 h in serum free medium. Control cells were incubated with the solvent for wortmannin–0.1% dimethylsulphoxide (DMSO), which does not affect SMCs.

2.2. Construction of plasmids

PcDNA3.1-myocardin encoding the full-length myocardin was provided by Dr. Olson of the University of Texas Southwestern Medical Center. Cytomegalovirus expression plasmids IEs (CMV IE1, CMV IE2 and CMV IE) were kindly provided by Dr. Julie A. Kerry of the Department of Microbiology and Molecular Cell Biology, Eastern Virginia Medical School. Mcl-1 luciferase reporter was generated by inserting 761 bp promoter sequence into XhoI/HindIII site of pGL3-basic vector, as previously described (Cao et al., 2011). The Mcl-1 CArG box mutation (ΔMcl-1) was introduced by changing the consensus sequences as follows: CArG- from $CC(AT)_6GG$ to $AA(AT)_6AA$. The shRNA construct pRI-GFP/Neo-myocardin was synthesized by Inovogen (Beijing, China), with pRI-GFP/Neo vector inserting small RNA interfering sequence of myocardin-CCGTGAAAGAGGCTATAAA.

2.3. Transient transfection and luciferase reporter assays

Cell transfection and luciferase assays were performed as described previously (Zhou et al., 2011) with minor modifications. Approximately 24 h before transfection, Cos-7 cells or rat aortic SMCs were seeded at 1.5×10^4 cells/cm^2 onto 24-well plates (corning). Transfections were performed with x-tremeGENE HP DNA Transfection Reagent (Roche Applied Science) according to manufacturer's instructions. Unless otherwise indicated, 100 ng of reporter and 400 ng of activator plasmids were used. The total amount of DNA per well was kept constant by adding the corresponding amount of expression vector without a cDNA insert. All the proteins were expressed at a very similar level as confirmed by Western Blotting. 36 h after transfection wortmannin treatment was conducted. Luciferase activity was measured by using a luciferase reporter assay system (Promega) on a Luminometer (Bioteck, USA). pRI-GFP/Neo-myocardin was transfected 24 h before the other expressing plasmids. Transfection efficiencies were normalized by total protein concentrations of each Luciferase Assay preparations. All experiments were performed at least three times with different preparations of plasmids and cells, producing qualitatively similar results. Data in each experiment were

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