Atherosclerosis 221 (2012) 341–349

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

PTEN overexpression attenuates angiogenic processes of endothelial cells by blockade of endothelin-1/endothelin B receptor signaling

Hsiao-Mei Kuo[a,b,1], Chun-Yao Lin[c,1], Hing-Chung Lam[d], Pey-Ru Lin[b,e], Hoi-Hung Chan[a,f], Jui-Cheng Tseng[g], Cheuk-Kwan Sun[h], Te-Fa Hsu[i], Chia-Ching Wu[j], Chao-Yuh Yang[k], Ching-Mei Hsu[a,*], Ming-Hong Tai[a,b,l,**]

[a] Department of Biological Sciences, National Sun Yat-Sen University, 70 Lien-Hai Rd., Kaohsiung 804, Taiwan
[b] Department of Medical Education and Research, Kaohsiung Veterans General Hospital, Kaohsiung 813, Taiwan
[c] Division of Cardiosurgery, Kaohsiung Veterans General Hospital, Kaohsiung 813, Taiwan
[d] Division of Metabolism, Kaohsiung Veterans General Hospital, Kaohsiung 813, Taiwan
[e] Institute of Basic Medicine, National Cheng Kung University, Tainan 701, Taiwan
[f] Division of Gastroenterology, Kaohsiung Veterans General Hospital, Kaohsiung 813, Taiwan
[g] Division of Allergy, Immunology, Kaohsiung Veterans General Hospital, Kaohsiung 813, Taiwan
[h] Division of General Surgery, Department of Surgery, Chang Gung Memorial Hospital-Kaohsiung Medical Center, Kaohsiung, Taiwan
[i] Department of Cosmetic Applications & Management, Tung Fang Institute of Technology, Kaohsiung 829, Taiwan
[j] Department of Cell Biology and Anatomy, School of Medicine, National Cheng Kung University, Tainan 701, Taiwan
[k] Division of Cardiology, Department of Medicine, China Medical University Hospital, Taichung 40447, Taiwan
[l] Institute of Biomedical Sciences, National Sun Yat-Sen University, Kaohsiung 804, Taiwan

ARTICLE INFO

Article history:
Received 12 February 2010
Received in revised form 20 August 2010
Accepted 20 August 2010
Available online 31 August 2010

Keywords:
Thrombosis
PTEN
Endothelin-1
Endothelin B receptor
Vascular endothelial growth factor

ABSTRACT

Arteriovenous (AV) graft is frequently used as vascular access in hemodialysis patients. However, clotting or thrombosis of AV grafts often occurs and requires surgical removal. At present, the molecular pathogenesis underlying thrombosis of AV graft is not clear. The PTEN/Akt signaling has been implicated in the pathogenesis of vascular diseases. In this study, elevated PTEN expression and concomitant Akt inactivation was observed in endothelium of atherosclerotic brachial arteries from hemodialysis patients. To investigate whether PTEN upregulation affects endothelial function, adenovirus-mediated PTEN (Ad-PTEN) overexpression was performed in aorta rings and cultured endothelial cells. It was found that PTEN overexpression potently inhibited the microvessel sprouting in aorta rings and the angiogenic activities of endothelial cells including migration and tube formation. On the contrary, PTEN knockdown by RNA interference promoted the endothelial migration and reversed the Ad-PTEN-induced inhibition of endothelial migration. Expression analysis showed that PTEN overexpression attenuated the expression of endothelin-1 (ET-1) and endothelin B receptor (ETBR) in endothelial cells at transcriptional levels. However, exogenous ET-1 supply only partially reversed the PTEN-induced inhibition of migration and tube formation. This was delineated due to that PTEN overexpression also perturbed endothelial nitric oxide synthase (eNOS) activation and vascular endothelial growth factor (VEGF) release. In summary, PTEN upregulation induces endothelial dysfunction by attenuating the availability and signaling of multiple angiogenic pathways in endothelial cells, thereby may contribute to thrombosis of AV graft.

1. Introduction

When native vasculature dose not permit a fistula, arteriovenous bridge graft is often created in uremia patients by connecting brachial artery and vein with a synthetic vessel for vascular access. The graft usually is made of a synthetic material such as polytetrafluoroethylene (PTFE) and ready for use within weeks after formation. However, compared with AV fistula, AV bridge graft is prone to failure due to atherosclerosis in the arterial anastomosis, which leads to decreased hemodialysis flow and even graft or brachial artery thrombosis. At present, the molecular pathogenesis underlying thrombosis or atherosclerosis of AV graft is not clear.

Abbreviations: AV, arteriovenous; PTEN, phosphatase and tensin homolog deleted on chromosome ten; ET-1, endothelin-1; ETBR, endothelin B receptor; eNOS, endothelial nitric oxide synthase; VEGF, vascular endothelial growth factor.

* Corresponding author. Tel.: +886 7 252000x3619; fax: +886 7 5253609.
** Corresponding author at: Institute of Biomedical Sciences, National Sun Yat-Sen University, 70 Lien-Hai Rd., Kaohsiung 804, Taiwan. Tel.: +886 7 5252000x5816; fax: +886 7 5250197.
E-mail addresses: chingmei@mail.nsysu.edu.tw (C.-M. Hsu), minghongtai@gmail.com (M.-H. Tai).
[1] These authors contribute equally to this work.

0021-9150/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.atherosclerosis.2010.08.067

Endothelial dysfunction, an early marker of atherosclerosis, triggers the hemodynamic perturbations that lead to various inflammatory and vascular diseases such as thrombosis, atherosclerosis and diabetes. An imbalance of *de novo* endothelin-1 (ET-1) and nitric oxide (NO) synthesis is involved in the pathogenesis of endothelial dysfunction. ET-1, a potent vasoconstrictor peptide of 21-amino acids mainly secreted by endothelial cell, play important role in vascular physiology and cardiovascular diseases. Human ET-1 is derived from a 212 amino acids precursor, prepro-endothelin-1 (ppET-1), after the post-translational cleavage by specific membrane-bound endopeptidases, endothelin-converting enzymes. ET-1 acts via two main classes of G-protein-coupled receptors: endothelin A receptor (ETAR) and endothelin B receptor (ETBR) [1]. ET-1 is a potent angiogenesis activator [2] and enhances cell proliferation by reducing apoptosis in human umbilical vein endothelial cells via the ETB receptor [3], which in turn induce cell proliferation and migration [4,5].

Phosphoinositide 3-kinases (PI3Ks) and its downstream effector Akt are activated by various stimuli and involved in the physiology and diseases of the vascular system [6]. The PI3K/Akt pathway participates in prototypical endothelial functions such as the regulation of vascular tone, angiogenesis, control of adhesion, and recruitment of leucocytes to the vessel wall. The positive effect of PI3K on angiogenesis is counteracted by PTEN (phosphatase and tensin homolog deleted on chromosome ten), which plays an important role in many cellular processes including tumor suppression, embryonic development, cell migration and apoptosis [7]. Moreover, PTEN regulates various cellular processes in the pathogenesis of vascular diseases including, cardiac hypertrophy, heart failure, preconditioning and hypertension [8,9]. PTEN acts as a negative regulator of angiogenesis both *in vitro*, where it inhibits vascular sprouting and VEGF-induced tube formation, and *in vivo*, where PTEN overexpression or administration of PI3K inhibitors blocks tumor angiogenesis.

Endothelial cells, the primary cells responsible for production of ET-1 and NO, play a pivotal important role in angiogenesis. Angiogenesis is a process by which new vessels are formed from the pre-existing blood vessels via endothelial cells migration as well as proliferation [10]. It has been shown that ET-1 and NO has a direct angiogenic effect on endothelial and peri-vascular cells as well as an indirect action through the increased release of the potent angiogenic factor vascular endothelial growth factor (VEGF) [11]. The relationship between PTEN and tumor angiogenesis has been extensively studied. However, it remains unclear whether the cellular PTEN level modulates the physiological functions of endothelial cells. In this study, we observed a unique profile of endothelial PTEN accumulation in the atherosclerotic arteries from hemodialysis patients. Subsequently, we employed various laboratory approaches to elucidate how PTEN overexpression or knockdown affected endothelial function and the angiogenic pathways in endothelial cells particularly the ET-1 and its receptors pathway.

2. Materials and methods

2.1. Cell cultures and reagents

For production and propagation of Ad5 adenovirus, E1a-transformed human embryonic kidney 293 cells were purchased from Microbix Biosystems Inc. (Toronto, Canada) and maintain them at low-passage. Human umbilical vein endothelial cells (HUVEC; passage 3–6) were isolated from umbilical veins and cultured in M199 medium (Life Technologies, Gaithersburg, MD) containing 15% fetal calf serum, 20 U/ml porcine heparin (Sigma Chemical Co.), and 100 μg/ml endothelial cell growth supplement (Calbiochem; La Jolla, CA) as previously described [12]. Matrigel was from BD PharMingen (La Jolla, CA). For RNA interference studies, the control double-stranded, small interference RNA (siRNA) against PTEN and control siRNA were purchased from Santa Cruz Inc. (Santa Cruz; CA). ET-1 was from Peninsula Laboratories (Belmont, California).

2.2. Collection of explanted brachial arteries from hemodialysis patients and immunohistochemical analysis

The collection of surgical tissues during graft removal surgery was approved by the Institutional Reviewer Boards of Kaohsiung Veterans General Hospital (Kaohsiung, Taiwan). Due to grafts thrombosis with the brachial artery anastomosis involvement, the brachial arteries were excised from six hemodialysis patients within 2 months after grafts implantation. The specimens were segmented by 1 cm from the distal ends of the excised artery. By visual examination, the segments containing the atherosclerotic and non-atherosclerotic segments were used for subsequent analysis. Immunohistochemical analysis of PTEN expression in dissected arteries was performed as previously described [13].

2.3. Adenoviral vectors

Recombinant adenovirus vectors encoding human PTEN cDNA with hemagglutinin tag (Ad-PTEN) and green fluorescent protein (Ad-GFP) were generated as previously described [14]. The virus was amplified by two rounds of cesium chloride ultracentrifugation and desalted by G-25 gel-filtration chromatography. After titering by plaque-forming assay in 293 cells, the virus solution was aliquoted and stored at −80 °C before use.

2.4. Rat aortic ring assay

This *ex vivo* angiogenesis assay was performed as previously described [15,16]. Briefly, the thoracic aortas were excised from Sprague–Dawley rats (male; 8-week-old) and immediately placed into DMEM medium containing 10% FBS. The dissected aortas were cut into cross-sectional rings (1–1.5 mm in length) in a 24-well plate and infected with adenovirus vectors (5×10^9 pfu per ring) for 1 h. After infection, the rings were placed into wells of a 24-well plate coated with 0.5 ml of Matrigel in MCDB131 media and overlaid with 0.5 ml of MCDB131 media contain 5% fetal calf serum and bFGF (500 ng/ml; R&D, Minneapolis, MN). The rings were maintained at 37 °C for 7–10 days and the vascular sprouting was examined daily using microscope equipped with digital images system (Olympus; Tokyo, Japan). The greatest distance from the aortic ring body to the end of the vascular sprouts (sprout length) was measured by NIH Image program at 3 distinct points per ring.

2.5. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

RNA was isolated from endothelial cells using RNAzol (TEL-TEST, Inc., Friendswoods, TX). 5 μg of total RNA was used Superscriptase III (Invitrogene; Carlsbad, CA), real-time PCR in Lightcycler (Roche) using a SYBR green I assay. PCR reaction was performed in SYBR Green PCR Master Mix (Roche) following protocols provided by manufacturer. The primer sequences for ET-1: forward primer 5′-CTTCTGCCACCTGGACATCA-3′, reverse primer 5′-GGCTTCCAAGTCCATACGGA-3′. The primer sequences for ETAR: forward 5′-TCTGATGACCTC GGTCCC-3′, reverse 5′-GTTCATGCTGTCCTTATGGCT-3′. ETBR: forward 5′-AAT TACGATGGACTACAAAGGAAGTTA-3′, primer 5′-GCAAGCAGAAATAGA AACTGAATAGC-3′. VEGF: forward 5′-CCCTGATGAGATCGAGTACA-3′, reverse 5′-AGGAAGCTCATCTCTCCTAT-3. Expression was normalized to β-actin: forward