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Full Length Article

Conditional knockout of tissue factor pathway inhibitor 2 in vascular endothelial cells accelerates atherosclerotic plaque development in mice

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ABSTRACT

Background: Tissue factor pathway inhibitor-2 (TFPI-2) regulates matrix metalloproteinases activation and extracellular matrix degradation. Over-expression of TFPI-2 enhances atherosclerotic plaque stability. The aim of this study is to investigate the effect of conditional knockout (KO) of TFPI-2 in vascular endothelial cells on the initiation and development of atherosclerotic plaque.

Methods: A Cre/mloxP conditional KO system and Tek-Cre mice were used to generate offsprings with monoallelic deletion of the TFPI-2 gene in endothelial cells. TFPI-$2^{fl/+}$/Tek-Cre mice, TFPI-$2^{fl/+}$ mice and $ApoE^{-/-}$ mice (n = 6 for each group) were included. Arteries were obtained. HE, EVG and anti-α-SMA staining were used to examine the morphology of vessel and plaque. Protein expression and phosphorylation were detected by Western blot or immunohistochemistry.

Results: TFPI-$2^{fl/+}$/Tek-Cre mice were generated. TFPI-2 level decreased to 40.68% in TFPI-$2^{fl/+}$/Tek-Cre group. TFPI-$2^{fl/+}$/Tek-Cre developed plaques when no plaque was found in TFPI-$2^{fl/+}$ mice. Compared with $ApoE^{-/-}$ group, TFPI-$2^{fl/+}$/Tek-Cre group has smaller plaque area, decreased lipid content and less buried fibrous cap layers. MMP-2 and MMP-9 in TFPI-$2^{fl/+}$/Tek-Cre group was higher than in TFPI-$2^{fl/+}$ group. The phosphorylation of PPAR-α and PPAR-γ was decreased in TFPI-$2^{fl/+}$/Tek-Cre group.

Conclusions: A novel mouse model is presented and can be used to investigate the role of TFPI-2 in the process of atherosclerosis. Our findings suggest that monoallelic deletion of TFPI-2 gene in vascular endothelial cells leads to significant downregulation of TFPI-2. TFPI-2 deficiency may accelerate initiation of atherosclerotic lesion in mice. Elevated MMP-2 and 9 and decreased phosphorylation of PPAR-α and PPAR-γ may contribute to this phenotype.

1. Introduction

Atherosclerosis is a chronic disease affecting large- and medium-sized arteries and involving formation of plaques. Rupture of vulnerable atherosclerotic plaque is the leading cause of acute cardiocerebrovascular cevents [1], which triggers exposure of thrombogenic core to blood and formation of thrombus [2]. When the rupture happens in coronary artery, it leads to unstable angina and myocardial infarction [2]. Incidence of thrombus-mediated acute coronary events depends mainly on the vulnerability of plaques [3]. Generally, vulnerable plaques have thin fibrous caps, which reflects that matrix production and degradation are likely to influence the stability of plaques [3].

Tissue factor pathway inhibitor-2 (TFPI-2) is a Kunitz-type serine proteinase inhibitor, secreted by a wide variety of tissue and deposited in extracellular matrix (ECM) where it regulates ECM remodeling [4]. TFPI-2 regulates the activation of matrix metalloproteinases (MMPs) and plasminogen directly or indirectly [4], which plays a significant role in the regulation of ECM degradation [5]. It is documented that TFPI-2 strongly prevents ECM hydrolysis by inhibiting plasmin and MMPs [6,7]. Many studies have proved that TFPI-2 inhibits invasion and migration of tumor cells [8,9,10]. Rao found that TFPI-2 prohibited the plasmin and trypsin mediated activation of MMP-1 and MMP-3 [11]. We also discovered that TFPI-2 suppressed migration of vascular smooth muscle cells [12]. In addition, TFPI-2 and its first Kunitz-type domain, could inhibit the gelatinolytic activity of MMP-2 and MMP-9 [12,13]. Our recent study has suggested that over-expression of TFPI-2 could enhance atherosclerotic plaque stability in $ApoE^{-/-}$ mice,and this effect may be related to its inhibition of MMP-2, 9, and 14 [14].

With the purpose of gaining more convincing evidence about the impact of TFPI-2 deficiency on atherosclerosis, occurrence of atherosclerotic lesion, atherosclerotic plaque characteristics, changes in the expression and activity of the corresponding proteinases were examined in a mice model with conditional knockout of TFPI-2 in vascular endothelial cells.

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2. Methods

2.1. Mouse strains

Embryonic stem (ES) cells were obtained from 129Sv/Ev male mice. Blastocysts were prepared from C57BL/6 J mice. Flp transgenic mice and Tek-Cre transgenic mice were purchased from Model Animal Research Centre of Nanjing University, China. ApoE$^{-/-}$ mice originating from Johnson Laboratory of USA, were obtained from Nanjing Medical University, China. C57BL/6 J mice were provided by Shanghai Research Centre for Model Organisms, China. The mice were housed in a temperature-controlled (22–25 °C) environment with a 12-h light/dark cycle in the Animal Centre of Shanghai Medical College, Fudan University and had free access to water and food. All animals received humane care. All experiments on animals were conducted in accordance with the guidelines of the Standing Committee on Animals of Shanghai Medical College, Fudan University.

2.2. Plasmid construction and TFPI-2 gene targeting

A TFPI-2 conditional knockout (CKO) vector was constructed. A 2660 bp 5′ homologous arm (start from 7972, end at 10,631) and a 1404 bp 3′ homologous arm (15,117–16,520) were linked by an insertion cassette, which included a gene inactivation (GI) cassette and a downstream positive selection cassette (FRT-neo-FRT). The GI cassette, flanked by two oppositely oriented mutant loxP (mLoxP), was made up by a splice acceptor (SA), an internal ribosome entry site (IRES), a green fluorescent protein (GFP) reporter gene and a polyadenylation (pA) signal sequence. The IRES and GFP sequence reported the orientation of the GI cassette. Following the 3'homologous arm, the negative selection cassette PGK-TK-pA was added. The final TFPI-2 CKO plasmid was digested by EcoRI.

The targeting plasmids were linearized with PvuI and electroporated into ES cells. The GI cassette and the neo cassette were introduced into intron2 (8838–15,650) of murine TFPI-2 gene by homologous recombination (Fig. 1A). The targeted ES colonies were selected with G418 and GanC, and then the G418 and GanC resistant colonies were screened by polymerase chain reaction (PCR) using primers outside the homologous arms, paired with primers inside the insertion cassette (P1/P2 and P3/P4). The sequences of primers used for ES cell screening were listed in Table 1.

2.3. Generation of TFPI-2$^{fl/+}$/Tek-Cre mice and genotyping

Chimeric male mice were generated by injecting the identified ES cells into blastocysts, which were implanted into uteri of C57BL/6 J recipients. Chimeric male mice were crossbred with C57BL/6 J females for germline transmission and the offsprings were analyzed for mutated TFPI-2 allele via PCR (P1/P2, P3/P4) with isolated tail DNA. The neo cassette was removed by mating the chimeras with Flp mice. Heterozygous progeny carrying one floxed TFPI-2 allele (TFPI-2$^{fl/+}$) was backcrossed to C57BL/6 J mice for 6 generations to remove the 129S background. TFPI-2$^{fl/+}$ mice were validated via PCR screening with P5, P6, P7 (Table 1). TFPI-2$^{fl/+}$ mice were crossbred with Tek-Cre mice. Genotyping for the Tek-Cre mice were done with genomic PCR using Tek specific primers (P8/P9) (Table 1). Tek-Cre (Tie-2 Cre) [15] mice only express Cre-recombinase under vascular endothelial-specific receptor tyrosine kinase promoter, in vascular endothelial cells [16] and hematopoietic tissues [17]. TFPI-2$^{fl/+}$/Tek-Cre pups generated by mating TFPI-2$^{fl/+}$ mice with Tek-Cre mice were detected by genomic PCR with P8/P9, P5/P6/P7 and P5/IRES-R (P10) (Table 1).

2.4. Animal diet feeding and preparation of samples

Given TFPI-2$^{fl/+}$ mice have the same susceptibility to atherosclerosis as C57BL/6 J mice, which means no atherosclerotic plaque would develop [18], we included a group of ApoE$^{-/-}$ mice as positive controls. All mice were fed with a normal diet until 8 weeks of age, since when TFPI-2$^{fl/+}$/Tek-Cre mice, TFPI-2$^{fl/+}$ littermates and ApoE$^{-/-}$ mice (n = 6 in each group) were switched to a Western-type diet (WD) for up to 20 weeks of age. 200 μl blood was drawn through vena plantaris at the 8th week. Blood samples were treated with natrium citricum and centrifuged (3000 rpm, 20 min, 4 °C) to obtain plasma samples which were stored at −80 °C until final analysis of plasma TFPI-2 concentration with ELISA kit (Uscnlife). At the end of the 20th week, mice were sacrificed after a 4 h fast. Blood samples were taken from the retro-orbital plexus before thorough terminal exsanguination and frozen for measurement of total cholesterol (TC), triglyceride (TG), HDL cholesterol (HDL-c) and LDL cholesterol (LDL-c) with commercially available kits (Wako Pure Chemicals). After arterial perfusion, the major blood vessels were dissected carefully under a Nikon E4500 stereomicroscope (Nikon, Japan). Brachiocephalic arteries were removed with a piece of the aortic arch and the stump of the right subclavian artery still attached to aid orientation. Brachiocephalic arteries were embedded in paraffin. Sections were cut at 4 μm for the paraffin-embedded sections every 30 μm along the vessel. Six thoracic aortas from each group were mixed and homogenized for Western blot.

2.5. Brachiocephalic artery morphometrical and immunohistochemical analysis

The sections were stained with hematoxylin and eosin (HE), Verhoeff's elastin/van Gieson (EVG) (Genmed, Shanghai, China). Paraffin sections were subjected to immunohistochemical staining using primary antibodies against TFPI-2 (a rabbit anti-mouse TFPI-2 monoclonal antibody, Abcam), CD68 (a rabbit anti-mouse polyclonal antibody, Proteintech), MMP-2 (a rabbit anti-mouse polyclonal antibody, Santa Cruz) and MMP-9 (a rabbit anti-mouse polyclonal antibody, Santa Cruz), alpha-smooth muscle actin (α-SMA) (a rabbit anti-mouse monoclonal antibody, Abcam). For TFPI-2, CD68 and α-SMA staining, after quenching endogenous peroxidase activity, heat-mediated antigen retrieval was needed; for MMP-2 and MMP-9 staining, antigen retrieval by trypsinization was employed. After 10% goat serum blocking and primary antibody incubation, specimens were incubated with biotin-labeled goat anti-rabbit secondary antibody. Then, horseradish peroxidase (HRP) conjugated Streptavidin was added and positive staining area was detected by 3, 3-diaminobenzidine (DAB). Images were acquired with an Olympus DP71 microscope digital camera (Olympus, Japan).

Three vessel cross sections per mouse were quantified with computerized image-analysis software (Image-Pro Plus 6.0, Media Cybernetics). All morphometric analyses were made on HE, EVG and α-SMA stained sections. Lumen area, lesion area, lipid content of plaque [19], the number of buried fibrous caps and fibrous cap thickness were measured to evaluate morphometric characteristics of vessel and plaque. For TFPI-2, MMP-2 and MMP-9 immunohistochemical staining, the optical density (OD) of DAB positive area was measured with Image-Pro Plus 6.0 and the average OD was calculated as OD/total area [20].

2.6. Western blot

Primary antibodies against TFPI-2, MMP-2 and MMP-9, MMP-1 (a rabbit anti-mouse polyclonal antibody, Proteintech), MMP-13 (a rabbit anti-mouse polyclonal antibody, Proteintech), phospho-PPAR-α (a rabbit anti-mouse polyclonal antibody, Abcam), phospho-PPAR-γ (a rabbit anti-mouse monoclonal antibody, Santa Cruz), phospho-PI3Kγ/PKB (a rabbit anti-mouse monoclonal antibody, Santa Cruz) and phospho-NF-κB (a rabbit anti-mouse mono-clonal antibody, Santa Cruz) were used. 20 μg of protein from each sample was electrophoresed on pre-cast 12% Bis-Tris acrylamide gels (Bio-Rad). Proteins were electroblotted onto a nitrocellulose membrane, which was then blocked with 5% nonfat Milk in TBS-T for 1 h at room temperature with shaking. The

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