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Thrombospondin-4 ablation reduces macrophage recruitment in adipose tissue and neointima and suppresses injury-induced restenosis in mice

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ABSTRACT

Objective: Thrombospondin-4 (Thbs4) is a member of the extracellular calcium-binding protein family and is linked to cell adhesion and migration. Given the involvement of Thbs4 in vascular inflammation, we hypothesized that Thbs4 plays a role in restenosis.
Methods and results: Here we show evidence that Thbs4 is upregulated in wire-injured mouse arteries and correlated with CD68 expression. Macrophage infiltration is reduced in both adipose tissue (AT) and neointima of Thbs4/ApoE double knockout (DKO) mice after injury. Moreover, Thbs4 deficiency prevents restenosis in ApoE KO mice fed a Western-type diet (WTD). Lethally irradiated DKO mice that receive bone marrow from ApoE KO or DKO mice have reduced neointima development. While considering related mechanisms, we note decreased chemokine production in both AT and neointima of DKO mice. In addition, vascular smooth muscle cells (VSMCs) derived from DKO mice display suppressed proliferation and migration in comparison with controls. Thioglycollate (TG)-induced macrophages from DKO mice show retarded adhesion to VSMCs. Recombinant Thbs4 promoted macrophage adhesion to VSMCs, and enhanced VSMC proliferation and migration.
Conclusion: Collectively, these data highlight the significance of Thbs4 in regulating macrophage accumulation and treating restenosis.

1. Introduction

Restenosis, characterized by endothelial cell dysfunction, enhanced smooth muscle cell (SMC) migration into the intima and proliferation, as well as inflammatory macrophage activation, is a recurrent lumen narrowing occurring in 30–50% of patients subjected to percutaneous intervention of coronary, carotid and peripheral arteries [1,2]. The advent of new drugs and devices, including drug eluting balloons (DEB) and drug eluting stents (DES), have not eliminated the incidence of restenosis, which is still a significant clinical concern.

The contribution of obesity and metabolic syndrome to cardiovascular diseases, including restenosis after angioplasty, is well documented [3]. Adipose tissue (AT) inflammation has recently been identified as an early indicator of insulin resistance, type 2 diabetes and arteriosclerosis [4]. AT infiltration with inflammatory macrophages can lead to cytokine and chemokine production, thus contributes to inflammatory process, which participates in the initiation and progression of restenosis [1,5].

Thrombospondin (Thbs) proteins are induced in sites of tissue damage or active remodeling, suggesting their involvement in extracellular matrix structure and assembly. Thbs4, the fourth member of the Thbs gene family, has been identified in the *Xenopus laevis* genome [6,7]. Evidence has shown that it is important for neurogenesis [7], heart hypertrophy [8] and angiogenesis [9]. Indeed, Thbs4 has been reported to be expressed in VSMCs and influence the recruitment of macrophages and vascular inflammation in recent studies [10,11]. However, it is not as yet known if Thbs4 plays pivotal roles in restenosis.

Therefore, this study investigates the potential of Thbs4 in AT

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inflammation as well as neointima formation after vascular injury.

2. Materials and methods

2.1. Animals and diets

All animal studies were conducted with ethical approval from the local Ethical Review Committee of Ren Ji Hospital, Shanghai Jiao Tong University. Thbs4 knockout (KO) mice on a C57Bl/6 genetic background were purchased from the Jackson Laboratory. Genotyping was made by PCR following manufacturer's recommendations. Thbs4 KO mice were crossed onto an ApoE KO background (Jackson Laboratory) to generate matched litters of Thbs4/ApoE double KO (DKO) mice. Mice were kept on a Western-type diet (WTD: 42% calories from fat) starting at 6 weeks of age, and they were sacrificed at 16 weeks of age. All mice were housed, treated and cared for according to the National Institutes of Health (NIH) guidelines for the humane treatment of laboratory animals and the Animal Welfare Act.

2.2. Hematological analysis

At the time of sacrifice, the mice were anesthetized with overdose of isoflurane. Blood was collected by cardiac puncture using a 1-ml syringe with a 25-gauge 5/8 in. The monocytes in blood samples with EDTA anticoagulant were counted using an automatic blood cell analyser (Beckman Coulter, Inc., Brea, CA) Plasma glucose levels were measured according to manufacturer's instructions using a colorimetric method (BioVision, Inc., Milpitas, CA). Insulin levels were determined by ELISA using a commercially available kit (Mercodia, Winston Salem, NC, USA).

2.3. Femoral artery wire injury model

Each mouse was anesthetized by intraperitoneal injection of 50 mg/kg of pentobarbital diluted in 0.9% sodium chloride solution. The femoral artery wire injury was performed by three passages of a 0.014-inch guide wire (Radius X-TRa Support PTCA GUIDEWIRE; Radius Medical Technologies, Maynard, MA, USA) as previously described [12]. For subsequent neointimal hyperplasia analysis, mice were sacrificed 14 days later.

2.4. Bone marrow transplantation

Recipient male mice aged 8 weeks (ApoE KO or DKO mice) were exposed to a single dose of 9 Gy (0.19 Gy/min, 200 kV, 4 mA) x-ray total body irradiation, using an Andrex Smart 225 Röntgen source (Yxlon International, Hamburg) 1 day before transplantation. Bone marrow was isolated from donors aged 8 weeks by flushing the femurs and tibias of male ApoE KO or DKO mice with RPMI 10% serum. 5×10^6 bone marrow cells were injected intravenously into tail veins of irradiated recipients. Recipient mice were fed a standard chow diet for 4 weeks for recovery, and then they were fed a WTD for 8 weeks.

2.5. Histology and immunohistochemistry

Peri-gonadal (visceral) AT and femoral arteries were collected after circulation perfusion. For morphometry and immunohistochemistry, samples were fixed with 4% paraformaldehyde dissolved in PBS. For other analysis, samples were snap frozen in liquid nitrogen and stored at −80 °C. For morphometric analysis, the arterial sections were stained with Elastic Van Gieson (EVG) after deparaffinization and rehydration. A single observer who was blinded to the treatment protocols calculated intima-to-media (I/M) ratios using Image Pro Plus software (version 6.0, Media Cybernetics). The infiltration of macrophages into AT and arterial lesions was analysed using anti-CD68 (Santa Cruz Biotechnology, Santa Cruz, CA) immunostaining as previously described [12]. Percent positivity was calculated as number of positive cells/number of total nuclei in neointima. A mean value was generated from five independent sections of each sample.

2.6. Western blot analysis

Mouse tissue proteins were extracted using radioimmunoprecipitation assay (RIPA) lysis buffer and the supernatant was used for Western blot analysis as previously described [13]. Protein extracts (40–60 μg) were separated by electrophoresis on a polyacrylamide gel (10%) and transferred to nitrocellulose membranes. The primary antibodies against Thbs4 and Tubulin were purchased from abcam (abcam, Cambridge, UK). The expression level of Tubulin served as an internal control for protein loading. Protein expression was quantified using ImageJ 1.37v software (NIH).

2.7. Total RNA extraction and real-time RT-PCR

Total RNA was extracted from mouse tissues using TRIZOL reagent, reverse transcribed using qScript cDNA Supermix kit, and subjected to real-time PCR analysis. Primer pairs for mouse Thbs4 and β-actin are shown in Supplementary Table 1. Real-time PCR was performed using a standard TaqMan PCR kit protocol in an Applied Biosystems GraphPad PRISM 4.0 Sequence Detection System (Foster City, CA). Semi-log amplification curves were evaluated by the comparative quantification method (2-ΔΔCt), and the gene expression levels were normalized to β-actin for mouse.

2.8. Flow cytometry

Samples of visceral AT and femoral arteries were microdissected and digested with collagenase as described before with minor modifications [14,15]. We then passed the mixture through a nylon filter (pore size, 250 μm) to remove undigested material, and centrifuged the filtrate for 5 min at 200*g* at 4 °C to yield pellet for flow cytometry analysis. Counted cells were stained for 30 min, at room temperature with the following conjugated antibodies: FITC anti-mouse F4/80 and PE/Cy5 anti-mouse CD11b (Biolegend, San Diego, CA). After staining, cells were gently washed twice and resuspended in the fresh magnetic cell sorting (MACS) buffer (Miltenyi Biotec Inc. San Diego, CA). Data were acquired on FACS Calibur Flow Cytometer (BD Biosciences) and analysed with FlowJo (Tree Star Inc, USA).

2.9. Isolation and culture of cells

Mouse (8–12 weeks) peritoneal macrophages were collected by using a thioglycollate (TG) inflammation model. Sterile 1 ml 4% TG was injected intraperitoneally and after 4 days, mice were sacrificed and macrophages were harvested by lavage of the peritoneal cavity with sterile PBS. Isolated macrophages were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). For isolation of VSMCs, aortas were harvested from 8- to 12-week-old mice. VSMCs were isolated and grown as described previously [16], and confirmed by typical morphology and positive immunostaining with SM α-actin antibody.