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Ultrasound-guided imaging of junctional adhesion molecule-A-targeted microbubbles identifies vulnerable plaque in rabbits

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ABSTRACT

Identification of vulnerable atherosclerotic plaques by imaging the molecular characteristics is intensively studied recently, in which verification of specific markers is the critical step. JAM-A, a junctional membrane protein, is involved in the plaque formation, while it is unknown whether it can serve as a marker for vulnerable plaques. Vulnerable and stable plaques were created in rabbits with high cholesterol diet with or without partial ligation of carotid artery respectively. Significant higher JAM-A expression was found in vulnerable plaques than that in stable plaques. Furthermore, JAM-A was not only expressed in the endothelium, but also abundantly expressed in CD68-positive area. Next, JAM-A antibody conjugated microbubbles (MB_{JAM-A}) or control IgG-conjugated microbubbles (MB_C) were developed by conjugating the biotinylated antibodies to the streptavidin modified microbubbles, and visualization by contrast-enhance ultrasound (CEUS). Signal intensity of MB_{JAM-A} was substantially enhanced and prolonged in the vulnerable plaque and some of the MB_{JAM-A} was found colocalized with CD68 positive macrophages. In addition, cell model revealed that MB_{JAM-A} were able to be phagocytized by activated macrophages. Taken together, we have found that increase of JAM-A serves as a marker for vulnerable plaques and targeted CEUS would be possibly a novel non-invasive molecular imaging method for plaque vulnerability.

1. Introduction

Atherosclerosis is currently one of the leading causes of death and disability in the world [1]. Enhanced lipid accumulation, inflammation, protease activity, and extracellular matrix reorganization are attributed to the formation of atherosclerotic lesions. Inflammatory monocyte recruitment has emerged as the crucial force driving the initiation and progression of atherosclerotic lesion formation, which in turn reflects the characteristics of the plaques [2,3]. Vulnerable plaque is characterized by a large lipid core and a thin, weakened fibrous cap infiltrated by macrophages in the vessel wall. Molecular imaging of these traits is of fundamental importance for vulnerable plaque identification.

Junctional adhesion molecule A (JAM-A, also known as F11R) is a member of the immunoglobulin superfamily adhesion molecules and expresses on a large variety of cell types, including platelets, leukocytes, epithelial and endothelial cells [4]. There is an increasing body of evidence that JAM-A participates in the process of leukocyte trafficking and recruitment [2,4,5]. Increase of JAM-A expression in inflamed and early atherosclerotic endothelium has been implicated in the recruitment of mononuclear cells, by interaction with VLA-4 [5]. In addition, impaired or downregulated JAM-A expression in endothelial cells reduced mononuclear cell recruitment into the arterial wall [6] and thus limited plaque size in

Abbreviations: AS, atherosclerosis; CCA, common carotid artery; CEUS, contrast-enhance ultrasound; HFD, high fat diet; JAM-A, junctional adhesion molecule-A; MB, microbubble; α-SMA, smooth muscle actin α.

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hyperlipidemic mice [7]. All of these data indicate that JAM-A is a critical molecule linking atherosclerosis and inflammation. Notably, JAM-A expression was functionally important in hematopoietic cells [8] and mesenchymal cells [9], both of which are involved in atherosclerosis. However, detailed JAM-A expression information in the vulnerable plaque is still undefined. Targeted contrast-enhanced ultrasound (CEUS) using microbubbles (MB) or nanobubbles has been shown to be a promising non-invasive imaging technique to evaluate atherosclerosis [10–12]. However, there are rare promising strategies to identify plaques at high risk to rupture, which are mainly due to the lack of molecular markers of vulnerable plaques. Given the role of JAM-A in initiation and progression of atherosclerosis, especially the vulnerable plaque formation [5,7,13,14], we were prompted to analyze the JAM-A expression in atherosclerosis and test the diagnostic values of JAM-A targeted microbubbles in identifying vulnerable plaques. Our study highlights that JAM-A is a stable marker for vulnerable plaques and ultrasound imaging with JAM-A targeted microbubbles would be valuable in distinguishing vulnerable plaques.

2. Materials and methods

2.1. Animal model

Thirty male New Zealand white rabbits (1.5–2.0 kg, 2–4 months old) from the experimental animal center of the Fourth Military Medical University (Xi'an, China) were housed and processed in accordance with the Institutional Animal Care and Use Committee in the Fourth Military Medical University. The model of vulnerable plaques was established as previously reported with minor modifications due to the anatomy differences between mice and rabbits. In our model, both the external and internal carotid arteries were partially ligated, leaving the occipital artery free of ligation [15]. For ligation of the artery, the branches of LCCA (left common carotid artery) were ligated while RCCA was left non-ligated in the same rabbit. Briefly, anesthesia was induced by intraperitoneal injection of 3% pentobarbital sodium (100 mg/kg). LCCA was exposed by blunt dissection and the two caudal branches of LCCA (left external and internal carotid artery) were ligated with 6–0 silk suture to induce vulnerable plaques. All rabbits were fed with a chow diet and water as libitum for 1 week before randomly divided into two groups. The control group (N = 10) was fed with chow diet throughout the entire experiments. In the atherosclerosis group (N = 20), the rabbits were fed with a high-fat diet (1.25% cholesterol, 120–140 g/d, Medicience Ltd, Jiang Su, China) for 24 weeks till killed.

2.2. En-face lipid staining

Carotid arteries were fixed in 4% paraformaldehyde and stained in Oil red O (Sigma, Shanghai, China) for 20 min. Plaque areas were measured with ImageJ (v1.48, Media Cybernetics, Bethesda, MD, USA) and calculated as percentage of total specimen area.

2.3. Histopathology and immunohistochemistry

For Oil red O staining on the slides (5 μm thickness), Oil O red (Sigma) at the final concentration of 0.5% was used for 10 min. The stained samples were washed with.

37 °C water for a few seconds. The lipid area and the total cross-sectional area of the carotid lesions were measured. Masson's trichrome was used for staining collagens. For analysis of the cellular components and interested molecules, immunostaining was included. The primary antibodies included macrophage-specific antibody anti-CD68 (diluted 1:100; Abcam), α-smooth muscle cell actin antibody (α-SMA) (diluted 1:100; Abcam) and Junctional Adhesion Molecule-A antibody anti-JAM-A (diluted 1:100; Abnova). All the slides were scanned by a Nanozoomer (HAMAMATSU, Japan) and histopathological images were analyzed by use of the computer-assisted morphometric analysis system ImageJ (v1.48, Media Cybernetics, Bethesda, MD, USA). The vulnerable index was calculated by (macrophage staining% + lipid staining %)/(VSMCs staining% + collagen staining%).

2.4. Western blot and real-time PCR analysis

The protein expression was assessed using specific antibodies (anti-JAM-A, 1:500, H00050848-M01, Abnova; anti-CD68, 1:1000, ab53444, Abcam; anti-α-SMA, 1:2000, ab5694, Abcam; anti-β-actin, 1:200, D110024, Sangon Shanghai). Secondary antibodies conjugated to IRDye TM 800 (1:20000, LI-COR, USA) were used for detection of the band signal intensity using the Odyssey infrared imaging system (LI-COR, USA). Real time-PCR was performed with SYBR Premix Ex Taq II (TaKaRa, Ohtsu, Japan) to determine the relative mRNA levels of JAM-A, CD68, α-SMA and β-actin (the forward and reverse sequences listed in the online Table S2). The specificity of the PCR amplification was verified by a dissociation curve analysis. Analyses of gene expression data were performed using the $2^{-\triangle\triangle Ct}$ method.

2.5. Immunofluorescence confocal microscopy

Briefly, tissue sections were incubated with anti-JAM-A antibody (mouse antigen, Abnova), anti-CD31 antibody (rat origin, ab7388, Abcam), anti-α-SMA antibody (rabbit origin, Abcam) and anti-CD68 antibody (rat origin, Abcam) in different combinations as indicated at a dilution of 1:200 overnight at 4 °C. Corresponding secondary antibodies recognizing each primary antigen (Alexa Fluor 555 goat anti-mouse, Alexa Fluor 488 goat anti-rabbit, and Alexa Fluor 633 goat anti-rat; Invitrogen, USA) was added for an additional 30 min. Sections were further counter stained with DAPI before visualization with FV-1000/ES confocal microscope (Olympus, Tokyo, Japan).

2.6. Characterization of JAM-A-targeted MBs and IgG-conjugated MBs

Biotinylation of JAM-A antibody or control IgG was performed with Biotin Labeling Kit-NH2 kit (Abnova, Taiwan) according to the manufacturer's instructions. Targestar-SA microbubbles (Targeson, Inc., USA), with an average of 2700 streptavidin molecules per μm^2 of microbubble surface, were dispersed by gentle shaking. About 200 μg biotinylated JAM-A antibody or control IgG was conjugated to 1.5×10^9/ml of Targestar-SA microbubbles by incubation at room temperature for 20 min, in which the antibody was estimated more than 20 fold in excess to saturate streptavidin-binding sites. Unbound JAM-A antibody or control IgG removed was by an additional purification. Briefly, purification of conjugated MBs was performed with a gradient centrifugation using a specific column, as instructed by the Targeson product protocol. To measure the conjugation efficiency, JAM-A antibody conjugated MBs and control IgG-conjugated MBs were further incubated with anti-mouse IgG secondary antibody at room temperature for 30 min. After rinse and removing the free secondary antibodies, the MBs were acquired for further flow cytometry analysis.

2.7. Measurement of targeted-MBs characteristics

Particle size, size distribution and concentration of MBs were measured with particle size analyzer (DelsaNano, Beckman Coulter,