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ORIGINAL ARTICLE

Angiotensin II-accelerated vulnerability of carotid plaque in a cholesterol-fed rabbit model-assessed with magnetic resonance imaging comparing to histopathology

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Abstract This study sought to reveal the effect of angiotensin II (Ang II)-induced atherosclerotic vulnerability in rabbits and to determine whether *in vivo* magnetic resonance imaging (MRI) can determine the effect of Ang II on atherosclerotic development over time. In total, 24 elderly male New Zealand white rabbits underwent an intravascular balloon injury in the left common carotid artery (LCCA) and were subsequently fed a high cholesterol diet for 12 weeks. At 8 weeks, rabbits were randomly assigned to receive either Ang II (1.4 mg/kg/d, Ang II group) or vehicle (phosphate-buffered saline, control) via a subcutaneous osmotic minipump for 4 weeks. The rabbits were imaged three times: at baseline and at 8 and 12 weeks. After the 12-week MRI scanning, rabbits were euthanized to obtain pathological and histological data. Atherosclerotic plaques were identified in the 21 rabbits that survived the 12-week trial. Typical feature of vulnerable plaques (VP), intraplaque hemorrhage, were observed in 6 of 10 animals (60.0%) in the Ang II group. The Cohen *K* value of MR imaging between the AHA classifications was 0.82 (0.73–0.91; $P < 0.001$). MRI revealed that the change in carotid morphology were significantly different between the Ang II and control group plaques. Our results support an important role for Ang II in plaque vulnerability by promoting intraplaque neovascularization and hemorrhage as well as inflammation. The vulnerable features induced by Ang II in rabbit carotid plaques could be accurately monitored with MRI *in vivo* and confirmed with histomorphology.

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1. Introduction

Carotid vulnerable plaques (VP) may result in rapid worsening of stenosis and thrombus formation leading to stroke in patients with carotid atherosclerosis. Therefore, early detection of VP to allow early and effective interventions has become a major research interest. Pathologically, a typical VP often contains intraplaque hemorrhage (IPH) and a large lipid-rich necrotic core (LRNC) covered by a thin fibrous cap and is infiltrated by inflammatory cells, such as macrophages.

In recent years, a VP of the aorta can be successfully established through a balloon-induced vascular endothelium injury or by transfecting the abdominal aorta with the p53 gene followed by pharmacological triggering in rabbits given a high-fat diet (Qi et al., 2015; Phinikaridou et al., 2010). These types of models have been the most common models used for magnetic resonance imaging (MRI) studies, but few studies have been related to the rabbit carotid model, which is anatomically suitable for further investigation of the stroke caused by carotid atherosclerosis. In addition, some studies aimed at creating a carotid VP model, such as a high cholesterol diet combined with a balloon injury or cast placement, are both unable to create a human-like vulnerable plaque in a limited period (Ma et al., 2008; Den Dekker et al., 2014) because hepatic failure may develop in New Zealand white (NZW) rabbits after the long period of high cholesterol diet. So creating an accelerated VP plaque model in the carotid artery is our major research interest.

Angiotensin II (Ang II) is involved in various vascular events, such as endothelial activation and dysfunction (Pueyo et al., 2000; Laursen et al., 1997), cell proliferation (Kohno et al., 2000) as well as proinflammatory effects (Mervaala et al., 1999) of atherosclerotic lesions. Daiana et al. found that Ang II–induced hypertension can specifically increase the development of atherosclerosis in apoE knockout mice. Others have also reported that the carotid atherosclerotic lesions from Ang II–treated mice display a more pronounced vulnerable plaque phenotype, such as intralesional neovasculature and hemorrhage (Da Cunha et al., 2006; Cheng et al., 2006; Du et al., 2016). Based on these murine models, this study is aimed (1) to test whether Ang II act as an accelerating factor of the lesion by promoting plaque vulnerability in a rabbit atherosclerotic model and (2) to apply MRI measurements to monitor the effect of Ang II on atherosclerotic development over time and to see whether MRI is a useful modality for accurate assessment and follow-up evaluation of plaques.

2. Materials and methods

2.1. Animal model

This experimental protocol was approved by our institutional committee for animal use and care. In total, 24 male elderly NZW rabbits(Department of Laboratory Animal Science of Fudan University, Shanghai) weighing 3.0–3.5 kg were recruited and were fed an atherogenic diet containing 1% cholesterol (120–140 g/day) for 1 week and then subjected to a balloon-induced intimal injury of the left common carotid artery (LCCA). A balloon catheter with diameter of 2.5 mm and a length of 20 mm (Medtronic, AVE, Santa Rosa, CA) was inserted into the LCCA and inflated and deflated three times for 180 s after rabbits had been anesthetized with an intravenous injection of pentobarbital. After balloon injury of the LCCA, rabbits were maintained on a high cholesterol diet until sacrifice at the end of 12 weeks. Four weeks before the end of the trial, animals were randomly assigned to receive subcutaneous implantation of a minipump filled with either angiotensin II (1.44 mg/kg/d, AngII group) or phosphate-buffered saline (control). We choose an infusion rate of angiotensin II previously shown to accelerate atherosclerosis in apoE-KO mice (Daugherty et al., 2000).

2.2. Blood pressure and lipid determination

After anesthetization through the auricular vein but prior to sacrifice, catheters were placed in the right femoral artery for recording of the rabbit's arterial blood pressure. Arterial pressure was measured in a conscious rabbit using a pressure transducer and recorded on a thermal array recorder (RTA 1200 M; Nihon Kohden, Tokyo, Japan). Blood samples were collected from the ear artery at 0 week and the end of the 8-week cholesterol diet prior to the animal being sacrificed. Plasma total cholesterol (TC), triglyceride (TG), high density lipoprotein-cholesterol (HDL-C) and low density lipoprotein-cholesterol (LDL-C) were measured with enzymatic reaction kits from BioVision (Mountain View, Calif) and Wako Chemicals Co (Richmond, VA), respectively.

2.3. Histopathological analysis

Immediately after sacrifice, the injured LCCA tissues were removed from the rabbit and washed in saline. The distances from the surgical cut and the carotid bifurcation were used as internal reference points to co-localize between the MRI findings and the histological specimen. The carotid arteries were marked with suture ligatures on the left bifurcation over the total length imaged by MRI. The specimens were fixed in 10% formalin, sectioned in 3.0-mm transverse slices, decalcified, and embedded in paraffin. The paraffin-embedded specimens were sectioned at 10 μm thickness, stained with hematoxylin and eosin (HE), and subjected to histopathological and immunohistochemical analyses. HE staining was used for routine histopathological examination and compared with the MRI. Atherosclerosis formation was histomorphologically evaluated according to the AHA classifications (Stary et al., 1995).

2.4. Immunohistochemical (IHC) staining

All of the tissue samples were pre-treated as previously described (Torzewski et al., 1998). For Pentraxin3 (PTX3), RAM11 and CD31 staining, slides were first incubated with one of the following primary antibodies: PTX3 (1:200, code LS-B6679, Life span), RAM11 (1:100, code M0633, Dako), CD31 (1:200, code ab9498, Abcam) at 4 °C overnight. After a wash in PBS, sections were incubated with biotinylated anti-mouse secondary antibody (MaiXin Bio, Fuzhou, China) at room temperature for 30 min and then with the avidin–biotinylated horseradish peroxidase complex (ABC Elite kit, Vector, Burlingame, CA, USA) for 30 min. Peroxidase labeling was visualized using 0.2% (v/v) 3, 39-diaminobenzidine as a chromogen. When using the antibodies, the sections were