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Endothelin receptor antagonist macitentan or deletion of mouse mast cell protease 4 delays lesion development in atherosclerotic mice

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ABSTRACT

Aims: To determine the impact of mixed endothelin receptor antagonist and mouse mast cell protease-4 (mMCP-4) in the development of atherosclerosis in the mouse model.
Materials and methods: Apolipoprotein E (ApoE) KO mice were crossed with mMCP-4 KO mice to generate ApoE/mMCP-4 double KO mice. Atherosclerosis was induced with a normal- or high-fat diet for 12, 27 or 52 weeks. Macitentan (30 mg/kg/day), a dual ET_A/ET_B receptor antagonist, was given orally for 6 weeks (27 week protocol). At sacrifice, aortas and brachiocephalic arteries (BCAs) were collected. *En face* Sudan IV staining was performed on aortas and BCA sections were subjected to Masson's trichrome stain and α-smooth muscle actin labeling.
Key findings: Under normal diet, both macitentan treatment and the absence of mMCP-4 reduced the development of aortic atherosclerotic lesions in 27-week old ApoE KO mice, but mMCP-4 deletion failed to maintain this effect on 52-week old mice. Under high-fat diet (WD), macitentan, but not the absence of mMCP-4, reduced aortic lesion development in ApoE KO mice. On BCA lesions of 27-week old WD mice, macitentan treatment had a small impact while mMCP-4 deletion showed improved features of plaque stability.
Significance: These results suggest that the inhibition of mMCP-4 reduces lesion spreading in the earlier phases of atherosclerosis development and can help stabilise the more advanced plaque. Macitentan treatment was more effective to prevent lesion spreading but did not improve plaque features to the same extent.

1. Introduction

Atherosclerosis is a major factor in the development of cardiovascular complications, from cardiac disease to stroke. It is characterized by the inflammatory accumulation of oxidised lipoproteins in the intima of large vessels, forming lesions populated with lipid-laden macrophages, lipid deposits, and matrix-secreting smooth muscle cells. These lesions develop asymptomatically over decades before serious complications occur, which can be through rupture of the lesion's fibrous cap and leakage of the pro-thrombotic content in the circulation.

Endothelin-1 (ET-1) is a 21 amino acid peptide that has been implicated in the development of the atherosclerotic plaque. It can be derived from its 38-residue precursor, Big ET-1, in two ways: through endothelin-converting enzyme or the two-step process of chymase (to produce the inactive ET-1 (1—31)) and neprilysin [1,2]. It is a highly potent vasoconstrictor and inducer of vascular oxidative stress, vascular smooth muscle cell proliferation and secretory activity. In the vasculature, both its receptors contract the vascular smooth muscle (VSM) with very high potency, with the ET_A receptor being the predominant isoform. The ET_B receptor is also present on the endothelium where it can counteract the VSM action as an inducer of endothelium-derived relaxing factors (EDRFs) such as nitric oxide and prostacyclin. The ET_B receptor is upregulated in VSM cells in atherosclerosis, and it is also present on plaque macrophages, thus participating in the inflammation processes of the lesion [3].

Chymase is a mast cell chymotrypsin-like serine protease that has a wide variety of substrates. It can degrade high-density lipoproteins [4,5], extracellular matrix proteins such as fibronectin [6] and can activate matrix metalloproteinases-2 and -9 [7] and signalling proteins such as transforming growth factor-β, thrombin, angiotensin-II and ET-1 [2,8,9]. In the murine model, the mouse mast cell protease-4 (mMCP-4) is the closest relative to human chymase, and importantly it does not break down Ang-II like most rodent β-chymases [8].

While the role of mast cells in atherosclerosis has been investigated [10], the effect of mMCP-4 selective inhibition remained to be explored. Inoue and colleagues have shown that chymase inhibition reduces abdominal aortic formation in an Ang-II infusion atherosclerotic model [11]. Bot and colleagues showed reduced plaque progression in ApoE KO mice with chymase inhibitor treatment. They also showed that chymase inhibition improves plaque stability when mice underwent

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mast cell activation [12]. However, these studies used chymase inhibitors that are not selective for mMCP-4, as they can inhibit other mast cell proteases with chymase-like activity (such as mMCP-1 or cathepsin G).

Few studies have examined the effect of ET receptor antagonists in the treatment of atherosclerosis. The ET-1 system is upregulated in the atherosclerotic lesion. The ET-1 and ECE-1 gene expression is increased, leading to higher Big ET-1, ET-1 and ECE levels in the lesion [3]. Interestingly, ET-1 (1–31) is also increased in the atheromas of atherosclerotic hamsters [13], which may be in concordance with increased chymase expression in lesions [14]. In clinical trial settings, the ET_A selective blocker atrasentan reduced plaque formation in early atherosclerosis [15] and the selective ET_A antagonist BQ-123, alone or combined with ET_B antagonist BQ-788, improved endothelial function in atherosclerotic patients [16,17]. Macitentan is a tissue-targeting orally available mixed ET_A/ET_B antagonist with a highly lipophilic profile [18], which can arguably help it target lipid-rich lesions. To our knowledge, this drug, indicated for pulmonary arterial hypertension [19], has never been studied in atherosclerotic settings.

Therefore, our aim was to determine the impact of either macitentan or the selective deletion of the mMCP-4 gene, in the ApoE KO atherosclerotic mouse model.

2. Materials and methods

2.1. Animals

Apolipoprotein E knock-out (ApoE KO) genitor mice on the C57Bl/6J background [20] were purchased from Jackson Mice (Bar Harbor, ME, USA) and bred in our facilities. Mouse mast cell protease-4 knock-out mice (mMCP-4 KO) [6], on the same background, were bred in our facilities. ApoE/mMCP-4 double KO (dKO) mice were generated by crossing both genotypes in our animal care unit. All animals were housed under controlled temperature 22 °C and humidity with a 12 h light-dark cycle (6h00–18h00), with food and water *ad libitum*. All protocols were approved by the Animal Protection Institutional Committee of the Université de Sherbrooke and complied with Canadian Council on Animal Care guidelines.

2.2. Study design

As shown in Fig. 1, male ApoE KO and ApoE/mMCP-4 dKO mice randomised on three protocols: 12, 27 or 52 weeks. Mice on the 12- and 27-week protocols received normal chow diet (ND) or high-fat, high-cholesterol diet (Teklad Western diet (WD), TD.88137, Envigo, Indianapolis, IN, USA). 27-week ApoE KO mice were also randomised between vehicle (PBS) or macitentan oral treatment (30 mg/kg/day) for their last 6 weeks. Mice on the 52-week protocol were all fed the ND without treatment.

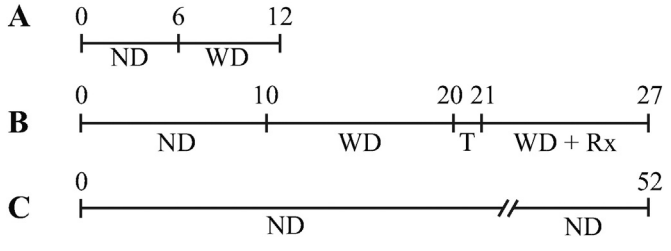

Fig. 1. Study timeline. ApoE KO and ApoE/mMCP-4 dKO mice on the 12-week protocol (A) were all fed ND for their first 6 weeks, then the WD group was switched to its new diet at 6 weeks old. The 27-week protocol mice (B) were all fed ND until they reached 10 weeks, then the WD mice were transferred to the high-fat diet for the remainder of the protocol. At 20 weeks old, the mice underwent training (T) to eat the vehicle-containing jelly. At 21 weeks, mice destined for the macitentan group were switched to the treatment (Rx) jelly, while control mice kept the vehicle. Mice on the 52-week protocol (C) were kept on ND for their whole lives.

Mice were all weaned at 3 weeks from ND-fed genitors. As shown in Fig. 1, 12-week mice were switched from ND to WD at 6 weeks. For their part, WD mice on the 27-week protocol were switched to WD at 10 weeks. They were then afforded 10 weeks on WD, after which they were trained to eat the treatment jelly with vehicle for 1 week, followed by either PBS or macitentan for the last 6 weeks.

Macitentan was suspended in a mix of polysorbate 80 and methylcellulose cp15 (0.05% each in water). This mix was solidified in a jelly of 20% sucralose and 14% gelatin with added artificial fruit flavour (banana). Before the first training day, the individually caged mice were deprived of food overnight, then the vehicle-containing jelly was placed in their cage and food was provided after they ate the jelly. The procedure was repeated (without food deprivation) every day in the morning (i.e. a once-a-day voluntary oral administration). After a week, mice destined for the macitentan group were switched to treatment. The jelly colour changed from transparent yellow to opaque white, but this did not modify compliance for the treatment.

Twenty-four hours after the last dose of macitentan, the mice were anaesthetised with ketamine/xylazine (87/13 mg/kg, intra-muscular). The mice were sacrificed by right ventricular blood collection and then perfused through the left ventricle first with saline then with phosphate-buffered formalin. The aorta, from the heart to the iliac arteries as well as the complete brachiocephalic artery (BCA), were collected and fixed in formalin for 24 h.

2.3. Aortic en face atherosclerosis imaging

The aortas and BCA were carefully dissected and cleaned to remove all adventitial fat, the BCA removed and stored in PBS. The aortas were then longitudinally cut to produce a Y-shape aorta. A first cut was started at the aortic root on its exterior curve and stopped after the arch. A second cut was then performed, starting on the interior curve from the aortic root, and going all the length of the aorta.

The aortas were then coloured using the lipid stain Sudan IV (in 70% ethanol). First, the aortas were washed in PBS and rinsed in 70% ethanol, then stained in Sudan IV for 15 min, followed by discolouration in 80% ethanol for 3 min. The aortas were then rinsed and stored in PBS.

For imaging, the aortas were pinned open on a black rubber pad covered with Parafilm, interior facing up. The aortas were then photographed using a Canon EOS500D (Rebel T1i) camera with a Sigma 70 mm macroscopic lens. The images were recorded in the RAW format and then digitally enhanced (white balance and contrast), cropped precisely along the shape of the aorta and then analysed using ImagePro 5.1 software to determine the lesion area as a percentage of total aortic area.

2.4. Histochemistry

The BCA were rinsed in ethanol 70%, blotted in paraffin and 4 μm sections were cut. A set of slides was stained with a rabbit anti-α-smooth muscle actin (α-SMA) monoclonal antibody (ab124964, Abcam, San Francisco, CA, USA) according to previously described methods [21]. Briefly, after toluene dewaxing and rehydration with ethanol and water, the antigen was retrieved by incubating the slides in a sodium citrate (10 mM with polysorbate 20 0.05%, pH 6.0) for 1 h at 90 °C. The slides were then cooled and incubated in 2% hydrogen peroxide. The sections were then permeabilized in 0.2% Triton. Sections were then incubated in 10% normal goat serum after which they were incubated overnight at 4 °C with the α-SMA antibody (1:500). The sections were incubated with biotinylated goat-anti rabbit-IgG (1:200), then incubated with the avidin-biotin-peroxidase complex (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA, USA). Immunostaining was visualized by developing sections in 0.025% diaminobenzidine (Sigma-Aldrich) and 0.03% peroxide.

Another set of slides was stained with Masson's trichrome stain (Sigma-Aldrich) according to the manufacturer's instructions. Briefly,

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