Vascular Pharmacology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Vascular Pharmacology

journal homepage: www.elsevier.com/locate/vph

Axitinib attenuates intraplaque angiogenesis, haemorrhages and plaque destabilization in mice

Bieke Van der Veken, Guido R.Y. De Meyer, Wim Martinet*

Laboratory of Physiopharmacology, University of Antwerp, Belgium

ARTICLE INFO

Keywords:
Atherosclerosis
Plaque stability
Intraplaque neovascularization
Angiogenesis
Mouse model

ABSTRACT

Aim: An increased density of intraplaque (IP) microvessels in ruptured versus nonruptured human plaques suggests that IP neovascularization has a major causative effect on plaque development and instability. Possibly, vascular endothelial growth factor (VEGF) or other angiogenic factors mediate IP microvessel growth and plaque destabilization. Because apolipoprotein deficient mice with a heterozygous mutation (C1039G +/−) in the fibrillin-1 gene ($ApoE^{-/-}Fbn1^{C1039G+/-}$) manifest substantial IP neovascularization, they represent a unique tool to further investigate angiogenesis and its role in atherosclerosis. Here, we examined whether administration of axitinib (inhibitor of VEGF receptor-1,-2 and -3) inhibits IP neovascularization and stabilizes atherosclerotic plaques.

Methods: $ApoE^{-/-}Fbn1^{C1039G+/-}$ mice were fed a western diet (WD) for 20 weeks. After 14 weeks WD, mice received axitinib (35 μg/g) or solvent i.p. 4 ×/week for 6 weeks. Cardiac function was monitored to evaluate the effect of axitinib on atherosclerosis-driven complications such as myocardial infarction.

Results: Axitinib significantly reduced IP neovascularization, with subsequent less prevalence of IP haemorrhages. The smooth muscle cell content doubled, whereas the amount of macrophages decreased. Overall cardiac function was improved in axitinib-treated animals. Moreover, the number of animals with myocardial infarction was decreased by 40%. Coronary plaque formation was observed in almost all control animals whereas treated animals showed a 30% reduction in the occurrence of coronary plaques.

Conclusions: Inhibition of VEGF receptor signalling by axitinib attenuates intraplaque angiogenesis and plaque destabilization in mice.

1. Introduction

Rupture of atherosclerotic plaques and the subsequent formation of thrombi are the most prevalent causes of clinical complications in atherosclerosis and lead to a tremendously high mortality rate [1]. A growing body of evidence indicates that intraplaque (IP) neovascularization is a critical factor stimulating plaque rupture [2,3]. Indeed, human vulnerable lesions are characterized by a structured web of IP microvessels that originate from the vasa vasorum and that grow through the media into the plaque. In rare cases, IP microvessels are also sprouting from the luminal side of the vessel wall. Hypoxia is the primary stimulus of IP angiogenesis and may arise due to plaque expansion and increased plaque inflammation [4]. The development of a network of IP microvessels is essential for a continuous supply of oxygen and nutrients into the growing plaque, with inflammatory mediators claiming the majority of the increased energy demand [5,6]. Importantly, due to an imbalance in pro-angiogenic factors (e.g. vascular endothelial growth factor (VEGF), angiopoietin [Ang] 2) and pro-maturation factors (e.g. platelet-derived growth factor (PDGF), angiopoietin [Ang] 1) inside the plaque, IP microvessels are characterized by open tight junctions, detachment of the basement membrane and poor coverage with pericytes, indicating that IP microvessels are extremely fragile and immature [7]. The immaturity of IP microvessels promotes extravasation of lipids, inflammatory mediators and erythrocytes in the plaque, and thus may advance plaque progression and rupture [8–10].

Throughout the years, scientific knowledge on the significance of IP neovascularization in plaque stability was mainly acquired through human specimens. The absence of a decent animal model of plaque rupture hampered the development of therapeutic strategies with IP microvessels as a valuable target. Recently, a unique model of spontaneous plaque rupture has been introduced, namely the $ApoE^{-/-}$ Fibrillin $(Fbn)1^{C1039G+/-}$ mouse. The heterozygous mutation $C1039G^{+/-}$ in the Fbn1 gene results in fragmentation of elastic fibres in the media of the vessel wall [11]. Combined with a western-type diet, degradation of elastic fibres leads to enhanced plaque formation in $ApoE^{-/-}$ mice with typical features of human unstable lesions

* Corresponding author.
E-mail address: Wim.Martinet@uantwerpen.be (W. Martinet).

http://dx.doi.org/10.1016/j.vph.2017.10.004
Received 9 June 2017; Received in revised form 4 October 2017; Accepted 19 October 2017

including IP neovascularization and haemorrhages. This mouse model also reveals sporadic plaque rupture and myocardial infarction without any mechanical intervention [12]. Accordingly, ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice are an ideal tool to investigate whether inhibition of IP neovascularization and thus a reduction in the amount of microvessels promotes plaque stability.

VEGF (with VEGFR-2 as its main receptor) is a main challenger during IP neovascularization [13]. VEGFRs are tyrosine kinases that autophosphorylate their intracellular tyrosine residues upon activation. Low levels of VEGF-A are important for vascular homeostasis, though at higher levels, VEGF-A initiates vascular endothelial cell (EC) proliferation/migration and increases vascular permeability, thereby inducing neovascularization [14]. These insights offer new possibilities in plaque stabilization through the inhibition of VEGF signalling [15]. In cancer research, VEGF-inhibiting therapies have proven their potential, however, in atherosclerosis research the success of this approach remains to be elucidated. Patients receiving VEGF-inhibiting medication can experience cardiovascular side effects such as high blood pressure and a slight increase (1%) in the occurrence of myocardial infarctions [16].

In this study, ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice were treated with axitinib, a drug that selectively inhibits VEGFR-1, -2 and -3 at subnanomolar concentrations, in order to unravel the impact of diminished IP neovascularisation on plaque destabilization and subsequent clinical complications.

2. Materials and methods

2.1. Mice

Female ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice were fed a Western diet (WD) (AB diets, Woerden, the Netherlands) starting at an age of 8 weeks [11,12]. The animals were housed in a temperature-controlled room with a 12 h light/dark cycle and had free access to water and food. Cases of sudden death were documented. Because IP microvessels are already developing at 14WD in ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice, this time point was chosen to start the treatment. Mice were treated with axitinib (35 mg/kg, i.p., 4 ×/week) for 6 weeks. Axitinib (kindly provided by Pfizer Inc., New York, USA) was dissolved in 3 parts of polyethylene glycol 400 and 7 parts of water with pH 2–3 to prevent precipitation. Control mice received PEG400/H_2O injections in the same frequency as the treated group. At the end of the experiment (20 weeks WD), blood samples were obtained from the retro-orbital plexus of anesthetised mice (sodium pentobarbital 75 mg/kg, i.p.). Subsequently, mice were sacrificed with sodium pentobarbital (250 mg/kg, i.p.). Analysis of total plasma cholesterol was performed via a commercially available kit (Randox laboratories, Crumlin, UK). All animal procedures were conducted according to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Experiments were approved by the ethics committee of the University of Antwerp.

2.2. Histology

After sacrifice of ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice, the proximal aorta, aortic arch, carotid artery and heart were collected. Tissues were fixed in 4% formalin for 24 h, dehydrated overnight in 60% isopropanol and subsequently embedded in paraffin. Serial longitudinal sections (4 μm) of the carotid arteries and aortic arch were cut and prepared for histological analysis. Cross-sections (4 μm) were cut from the proximal aorta and heart. Haematoxylin-eosin (HE) staining was performed to analyse the plaque size, plaque thickness and necrotic core. The plaque thickness was assessed by taking the mean value of 10 random measurements in the respective area. The plaque formation index was calculated on longitudinal sections by using the following formula [(Σ total plaque length / Σ total vessel length) × 100]. Necrosis was defined as acellular areas filled with necrotic clefts and necrotic debris. Immunohistochemical stainings for CD31 (anti-CD31, PC054, Binding Site, Birmingham, UK) and Ter-119 (anti-Ter-119, 550,565, BD Biosciences, USA) were performed to detect plaque ECs and erythrocytes, respectively. The analysis of IP microvessels and IP haemorrhages was done as follows. Fifteen consecutive slices were cut from the paraffin-embedded tissue. In every tissue slice, the amount of microvessels and haemorrhages in the plaques were counted per area. A mean value was calculated as a representative value for the amount of IP microvessels and haemorrhages per area (per vessel). Plaque composition was analysed with a Sirius red and anti-α-SM actin (A2547, Sigma, UK) staining to detect collagen and smooth muscle cells. Collagen type I and III was quantified under polarized light. Macrophages were detected by immunohistochemistry using an anti-Monocyte/Macrophage (553,322, Pharmingen, San Diego, CA) or anti-MAC3 (550,292, Pharmingen, San Diego, CA) staining. The hypoxic area was measured using pimonidazole (HP1-1000kit, hypoxyprobe, Massachussets, USA) [5]. The occurrence of myocardial infarctions (defined as large fibrotic areas) and coronary plaques was analysed on Masson's trichrome staining (transversal sections).

2.3. Echocardiography

Transthoracic echocardiograms were performed on anesthetized mice (isoflurane, 4% for induction and 2.5% for maintenance) at the end of the experiment using a VEVO2100 (VisualSonics, Toronto, Canada), equipped with a 25 MHz transducer. The left ventricular internal diameter during diastole (LVIDd) and left ventricular internal diameter during systole (LVIDs) were measured and fractional shortening [FS = (LVIDd − LVIDs) / LVIDd × 100] was calculated.

2.4. Blood pressure measurements

Peripheral blood pressure was measured at week 20 before sacrifice in conscious mice via a tail cuff. Mice were placed in plexiglas restrainers in a heating chamber (37 °C) and kept in the dark. Ten minutes of acclimatization was allowed before measurements were initiated. At the distal end of the tail, a pulse sensor and occluding cuff controlled by a programmed Electro-Sphygmomanometer (Narco Bio-systems, Austin, TX) were placed. Voltage output from the sensor and cuff were recorded and analysed by a PowerLab signal transduction unit and associated Chart software (ADInstruments, Colorado Springs, CO). To be accustomed to the procedure, mice were trained for 4 weeks before the first measurement.

2.5. Elisa

An enzyme-linked immunosorbent assay for quantitative detection of mouse VEGF-A (BMS619/2, eBioscience, San Diego, USA) was performed to detect VEGF-A in the plasma of control and treated mice.

2.6. Cell culture

Human umbilical vein endothelial cells (HUVECs) were cultured in M199 medium supplemented with 20% fetal bovine serum, 1% non-essential amino-acids and antibiotics. To investigate the expression of adhesion molecules or VE-cadherin, HUVECs were treated with 10 ng/ml human TNF-α (hTNF-α) in the presence or absence of axitinib (100 nM). After 24 h, cells were lysed to perform qPCR. Total RNA was isolated using an Isolation II RNA mini kit (BIO-52073, Bioline, Taunton, USA) according to the manufacturer's instructions. Reverse transcription was performed with a sensifast™ cDNA Synthesis Kit (BIO-65053, Bioline, Taunton, USA). Thereafter, Taqman gene expressions assays for VCAM-1 (Hs01003372_m1), ICAM-1 (Hs00164932_m1), *E*-selectin (Hs00204397_m1) and VE-cadherin (Hs00170986_m1) were performed in duplicate on an ABI-prism 7300 sequence detector system