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Treatment with sulphated galactan inhibits macrophage chemotaxis and reduces intraplaque macrophage content in atherosclerotic mice

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ABSTRACT

Experimental data from animal models and clinical studies support connections between the haemostasis and inflammation in atherogenesis. These interfaces among inflammation and thrombogenesis have been suggested as targets for pharmacological intervention to reduce disease progression. We hypothesize that the recently discovered antithrombotic drug Sulphated Galactan (SG) (isolated from the red marine alga *Acanthophora muscoides*) might reduce atherosclerotic plaque vulnerability and inflammatory gene expression in 10-week aged apolipoprotein E deficient (ApoE−/−) mice under high-cholesterol diet for additional 11 weeks. Then, the underlying cellular mechanisms were investigated in vitro. SG (10 mg/kg) or Vehicle was subcutaneously injected from week 6 until week 11 of the diet. Treatment with SG reduced intraplaque macrophage and Tissue Factor (TF) content as compared to Vehicle-treated animals. Intraplaque TF co-localized and positively correlated with macrophage rich-areas. No changes on atherosclerotic plaque size, and other intraplaque features of vulnerability (such as lipid, neutrophil, MMP-9 and collagen contents) were observed. Moreover, mRNA expression of MMPs, chemokines and genetic markers of Th1/2/reg/17 lymphocyte polarization within mouse aortic arches and spleens was not affected by SG treatment. In vitro, treatment with SG dose-dependently reduced macrophage chemotaxis without affecting TF production. Overall, the chronic SG treatment was well tolerated. In conclusion, our results indicate that SG treatment reduced intraplaque macrophage content (by impacting on cell recruitment) and, concomitantly, intraplaque TF content of potential macrophage origin in atherosclerotic mice.
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1. Introduction

Atherosclerosis progresses silently for years before becoming clinically evident. By causing acute thrombosis on terminal arteries, the disease leads to myocardial infarction and ischemic stroke, which dominates death and disability statistics for all regions of the world [1,2]. Atherogenesis results from an inflammatory process initiated by the intramural retention of cholesterol-containing low-density lipoprotein cholesterol (LDL) that activates vascular cells. Endothelial cells secrete chemokines that promote monocytes recruitment into the subendothelial space, where they differentiate into macrophages [2,3]. Macrophages are major contributors to the inflammatory response through secretion of pro-inflammatory mediators, such as chemokines, cytokines and reactive oxygen and nitrogen species, and matrix-degrading proteases [3]. In addition, macrophages present pathogen-associated antigens to T cells thereby stimulating T cell activation [4] and ingest modified and native lipoproteins, leading to massive accumulation of intracellular cholesterol, which transforms them into foam cells [5]. Intraplaque macrophages can proliferate [6] and also die, releasing their lipid contents and Tissue Factor (TF) with the final formation of a pro-thrombotic necrotic core, a key component of unstable

Abbreviations: CCL, C–C motif ligand; CXCL, C–X–C motif ligand; CV, coefficients of variation; HDL-c, High-density lipoprotein cholesterol; LDL, Low-density lipoprotein; LDL-c, Low-density lipoprotein cholesterol; MMP, Matrix MetalloProteinase; NMR, Nuclear Magnetic Resonance; PAR, Protease-Activated Receptor; SG, Sulphated Galactan; TF, Tissue Factor.

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plaques [3]. During plaque rupture, large amounts of TF are released, leading to thrombus formation [7] and elevated TF plasma levels in patients with unstable angina and acute coronary syndromes, which are predictive of cardiovascular mortality [8]. In atherosclerotic plaques, the expression of TF, a key initiator of coagulation cascade, has been detected in macrophages, foam cells, endothelial cells, smooth muscle cells and within the necrotic core [9–11]. Furthermore, the expression and activity of TF can be induced by various stimuli such as cytokines, growth factors, and biogenic amines [7]. Treatment strategies selectively reducing TF within mouse atherosclerotic plaques have been poorly investigated. In particular, some antithrombotic drugs might beneficially affect coagulation, intraplaque parameters of vulnerability, as well as inflammation. Among these drugs, the recently discovered antithrombotic drug Sulphated Galactan ([SG], derived from the red alga *Acanthophora muscoides*) might improve atherogenesis [12,13]. Therefore, we hypothesized that SG could be able to reduce intraplaque vulnerability (primary outcome, assessed by histological parameters) in ApoE −/− mice under high-cholesterol diet. Then, we assessed if SG treatment might affect inflammatory gene expression (secondary outcomes) within serum, aortic arches and spleen of atherosclerotic mice. Finally, the cellular mechanisms underlying SG-mediated effects were tested in vitro.

2. Materials and methods

2.1. Sulphated Galactan (SG) purification

The SG was extracted from the red alga *A. muscoides* by protease digestion and purified by anion-exchange chromatography, as previously described [12]. The purity of SG was checked by nuclear magnetic resonance (NMR) spectroscopy to exclude the presence of protein or other impurities [13]. The compound was verified as endotoxin-free (<0.25 EU/ml, using the limulus amoebocyte lysate Endochrome assay).

2.2. Mouse model of atherogenesis

10-week old ApoE −/− mice in C57Bl/6 background were submitted to high-cholesterol diet (20.1% fat, 1.25% cholesterol, Research Diets, Inc., New Brunswick, NJ) for 11 weeks [14]. Mice were randomly assigned to receive subcutaneous treatments either with Vehicle (NaCl 0.9%) or with SG. This drug (10 mg/kg, diluted in 200 μl NaCl 0.9% per injection) and Vehicle (equal volume of 200 μl NaCl 0.9%) were subcutaneously injected five times per week in the last six weeks before animal euthanasia. Mice well tolerated the treatment in the atherosclerosis protocol and no clinical adverse event was reported. This mouse protocol was approved by local ethics committee and Swiss authorities and conformed to the "position of the American Heart Association on Research Animal Use" and to Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

2.3. Detection of inflammatory mediators in mouse serum

Mouse serum levels of C–C motif ligand (CCL) 2 and C–X–C motif ligand (CXCL) 1 were measured by colorimetric enzyme-linked immunosorbent assay (ELISA, R&D Systems, Minneapolis, MN), following manufacturer's instructions. The limits of detection for ELISA were 3.9 pg/ml for CCL2 and 15.625 pg/ml for CXCL1. Mean intra- and inter-assay coefficients of variation (CV) were below 8% for both markers. Haematological parameters, serum triglycerides, total cholesterol, low-density lipoprotein cholesterol (LDL-c) and high-density lipoprotein cholesterol (HDL-c), free fatty acids and glucose were routinely measured and expressed in mmol/l.

2.4. Immunohistochemistry in atherosclerotic plaques

Aortic sinuses from all mice were serially cut in 5 μm transversal sections, as described previously [15]. Sections from mouse specimens were fixed in acetone and immunostained with specific antibodies anti-mouse CD68 (macrophages, ABD Serotec, Dusseldorf, Germany), anti-mouse Ly-6G (neutrophils, BD Pharmingen™, San Jose, CA), anti-mouse Matrix MetalloProteinase (MMP)-9 (R&D Systems) and anti-mouse TF (American diagnostic GmbH, Pfungstadt, Germany). Quantifications were performed using the MetaMorph software. Results were calculated as percentages of stained area on total lesion area or number of infiltrating cells per mm^2 of lesion area.

2.5. Oil Red O staining for lipid content

Five sections per mouse aortic sinus and thoraco-abdominal aortas were stained with Oil Red O, as previously described [15]. Sections were counterstained with Mayer's hemalun and rinsed in distilled water. Quantifications were performed using the MetaMorph software. Data were calculated as ratios of stained area on total lesion area.

2.6. Sirius red staining for collagen content

Five sections per mouse aortic sinus were rinsed with water and incubated with 0.1% Sirius red (Sigma Chemical Co, St Louis, MO) in saturated picric acid for 90 min. Sections were rinsed twice with 0.01 N HCl for 1 min and then immersed in water. After dehydration with ethanol for 30 s and cover-slipping, the sections were photographed with identical exposure settings under ordinary polychromatic or polarized light microscopy. Total collagen content was evaluated under polychromatic light. Interstitial collagen subtypes were evaluated using polarized light illumination; under this condition thicker type I collagen fibres appeared orange or red, whereas thinner type III collagen fibres were yellow or green [15]. Quantifications were performed with MetaMorph software. Data were calculated as percentages of stained area on total lesion area.

2.7. Real time RT-PCR

Total mRNA was isolated with Tri-reagent (MRC Inc.) from spleens and aortic arches of ApoE −/− mice. Reverse transcription was performed using the ImProm-II Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's instructions. Real-time PCR (StepOne Plus, Applied Biosystems) was performed with the ABsolute™ QPCR Mix (ABgene). Specific mouse primers and probes (Table 1) were used to determine the mRNA expression of TF, chemokines (Ccl2, Ccl3, Ccl5, Cxcl1), MMPs (Mmp-3, Mmp-8, Mmp-9, Mmp-10, Mmp-12), and markers of different T helper (Th) lymphocyte subsets (Th1: Tim3, Ifng; Th2: Gata-3, Il4; Treg: Foxp3, Il10; Th17: Rorc, Il17), and Hprt (reference gene) [14,16,17]. The fold change of mRNA levels was calculated by the comparative C_t method. The resultant C_t values were first normalized to the internal control. This was achieved by calculating a delta C_t (ΔC_t) by subtracting the internal control C_t values from the Hprt C_t value. A delta delta C_t ($\Delta\Delta C_t$) was calculated by subtracting the designated control ΔC_t value from the other ΔC_t values. The $\Delta\Delta C_t$ was then plotted as a relative fold change with the following formula: $2 - \Delta\Delta C_t$.

2.8. Human macrophage isolation, differentiation and tissue factor functional assays

Human macrophages were obtained as previously described [18]. Human monocytes were isolated from peripheral blood mononuclear cells of 3 healthy blood donors, after written informed consent. Monocytes were differentiated into macrophages using 24 h incubation with 500 U/ml interferon (IFN)-gamma in culture medium (10% FCS

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