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Nuclear microprobe investigation into the trace elemental contents of carotid artery walls of apolipoprotein E deficient mice

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Abstract

Atherosclerosis is a progressive disease that causes lesions in large and medium-sized arteries. There is increasing evidence that the function of vascular endothelial cells is impaired by oxidation reactions, and that metal ions may participate in these processes. The nuclear microscopy facility in NUS, which has the ability to focus a 2 MeV proton beam down to sub micron spot sizes, was used to investigate the trace elemental changes (e.g. Zn and Fe) in atherosclerotic lesions in the common carotid artery of apolipoprotein E deficient mice fed a high fat diet. In this preliminary study, which is part of a larger study to investigate the effects of probucol on carotid artery atherosclerosis, two sets of mice were used; a test set fed a high fat diet +1% probucol, and a control set which was fed a high fat diet only.

The results show that the Zn/Fe ratio was significantly higher in the media of arteries of probucol treated animals without overlying lesion (4.3) compared to the media with overlying lesion (1.3) ($p = 0.004$) for test mice. For the control mice, the arterial Zn/Fe ratio was 1.8 for media without overlying lesion, compared with 1.0 for media with overlying lesion ($p = 0.1$). Thus, for media without overlying lesion, the Zn/Fe ratio was significantly higher ($p = 0.009$) in probucol-treated (4.3) than control mice (1.8), whereas there was little difference in the ratios between the two groups in media with overlying lesion (1.3 compared with 1.0).

These preliminary results are consistent with the idea that the levels of iron and zinc concentrations within the artery wall may influence the formation of atherosclerotic plaque in the carotid artery.

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1. Introduction

Atherosclerosis is characterized by the accumulation of cholesterol deposits in macrophages in large- and medium-sized arteries. This deposition leads to a proliferation of certain cell types within the arterial wall that gradually impinge upon the vessel lumen and impede blood flow. There is now also a consensus that atherosclerosis represents a state of heightened oxidative stress characterized by lipid and protein oxidation in the vascular wall [1]. The administration of hypolipidemic drugs and antioxidants is, therefore, a rational means of trying to prevent the development of atherosclerosis.

Apolipoprotein E-deficient (apo$E^{-/-}$) mice are commonly used as a model of hypercholesterolemia-induced atherosclerosis [2]. Apolipoprotein E is a component of three plasma lipoproteins – chylomicron remnants (CMr) very low-density lipoprotein (VLDL) and high-density lipoprotein (HDL). It is a ligand for many receptors –

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the LDL receptors, the VLDL receptor and the LDL receptor related protein (LRP) and megalin (gp 330). It is thus closely involved in the clearance of many lipoproteins most notably CMr and VLDL. The genetic removal of the apoE gene in mice results in profound hyperlipoproteinemia, affecting most remnants and VLDL. ApoE deficient mice spontaneously develop severe atherosclerosis that shows many features of the disease in humans [3,4].

Probucol is a lipid soluble antioxidant and cholesterol-lowering drug that attenuates atherogenesis in animals and humans and that protects human coronary arteries from restenosis. Several protective functions likely contribute to the protection offered by probucol, including anti-inflammatory activity, the promotion of re-endothelialization, inhibition of smooth muscle cell proliferation, and the protection of endothelial cell integrity [5].

Endothelial dysfunction is accepted as a surrogate marker of vascular pathology leading to atherosclerosis. Studies also indicate that zinc is vital to vascular endothelial cell integrity and zinc deficiency causes severe impairment of the endothelial barrier function [6–9]. Our previous study in rabbits showed that iron concentrations appear to correlate positively with the extent of lesion development and that zinc is inversely correlated with the atherosclerotic lesion formation [10]. It is possible that iron is pro-oxidative whereas zinc may antagonize any early lesion formation accelerated by free radical production caused by increased iron levels. The ratio of zinc to iron concentrations therefore may be a useful indicator of atherosclerosis progression.

As part of a larger study carried out on the protective property of probucol on atherosclerosis, we have used nuclear microscopy to investigate the trace elemental contents of the carotid artery walls of apoE-deficient mice.

2. Materials and methods

2.1. Materials

Male apoE$^{-/-}$ mice purchased from Animal Resources Centre (Perth, Australia) were used at 8–10 weeks of age, and then fed for 24 weeks ad libitum a high-fat diet containing 21.2 (w/w) fat and 0.15% (w/w) cholesterol (specifications of the Harlan Teklad diet TD88137) prior to culling. Seven carotid arteries from the control animals on high fat diet and animals fed a high fat diet supplemented with 1% (w/w) probucol were used.

Common carotid arteries were resected from animals following perfusion under near physiological pressure (~100 mm Hg) with Dulbecco's phosphate-buffered saline containing 20 μM butylated hydroxytoluene and 1 mM ethylenediaminetetraacetic acid as described previously [11]. Immediately after resection, arteries were placed in phosphate-buffered saline containing 30% sucrose and stored overnight at 4 °C. The next day, cold arteries were placed into plastic moulds containing Tissue-tek (ProSciTech, Thuringowa, Qld, Australia) and the moulds then transferred into a beaker containing 2-methyl-butane previously cooled in liquid nitrogen. Once the Tissue-tek solution appeared frozen, moulds containing the arteries were transferred onto dry ice, wrapped in aluminium foil and then stored at −80 °C before sectioning.

This study was approved by the local Animal Care and Use Committee.

2.2. Histochemistry and nuclear microscopy analysis

Carotid artery samples were sectioned (14 μm thick sections) using a Leica CM3050S cryostat. The sectioning started at the distal end gradually moving downwards in 10-μm steps, and as soon as the start of bifurcation was reached (see the reference point in Fig. 1), 14-μm sections were cut and picked up on pioloform coated aluminium target holders for nuclear microscopy analysis and imaging [10]. Serial sections were also cut and mounted on gelatin-coated slides for hematoxylin and eosin staining in order to monitor the lesion development and measure the lesion area. This analysis was carried out using the Carl Zeiss Axiophot 2 image analyzer utilizing the KS400 (version 3.18) analysis software. Subsequently sections at 100 μm, 200 μm, 300 μm, 400 μm and to 500 μm (below the bifurcation point) were taken in order to investigate thoroughly how the lesion develops along the common carotid artery (the results will be presented elsewhere).

Sections taken at the bifurcation reference point were scanned using the National University of Singapore nuclear microscopy facility operating with a 2.1 MeV proton beam focused to a spot size of approximately 1 μm^2 and with a beam current of 400–500 pA on average [12]. Techniques combining scanning transmission ion microscopy (STIM), Particle induced X-ray emission (PIXE) and Rutherford backscattering spectrometry (RBS) were employed simultaneously in the analysis. The area of interest was positioned using the structure and density information provided by STIM. Quantitative results were determined by RBS and PIXE: RBS provides information on matrix composition and incident charge; PIXE was used for measuring the concentrations of the elements from sodium upwards in the periodic table. X-rays of different elements were detected simultaneously using a lithium-drifted silicon X-ray detector placed at 90° to the beam axis and fitted with a filter designed for optimal detection of trace elements such as iron and zinc in biological specimens.

3. Results and discussion

The bifurcation of the common carotid artery is the point at which the artery divides into external and internal carotid arteries, and a region highly susceptible to atherosclerosis because of much higher wall stress compared to other regions [13]. The sections taken from the bifurcation of all carotid artery samples were analyzed. Eight out of 13 samples had lesions of size ranging from 21% to 78%