Rapid Quantification of Aortic Lesions in ApoE^{-/-} Mice

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Key Words

Atherosclerosis · Atherosclerotic lesions · apoE^{-/-} mice ·
Plaque quantification · Lysochrome · Oil Red O

Abstract

The quantification of aortic lesions is an important end-point analysis for evaluating atherogenesis in mouse models of atherosclerosis. Morphometric methods involving the staining of aorta with a Sudan lysochrome followed by image analysis of the stained lesion area are commonly used. We have developed a more rapid method involving solubilisation of the stain retained by aortic lesions. In 2 separate studies, 5-week-old male apoE^{-/-} and C57BL/6 wild-type (apoE^{+/+}) mice were given a high fat (21%), Western-type diet for 13, 15 or 25 weeks. At study termination, the descending thoracic aorta (DA) and/or aortic arch (AA) were stained with Oil Red O (ORO). The incorporated stain was extracted using chloroform/methanol (2:1) solvent and quantified by spectrophotometry at 520 nm. In study 1 (13 weeks), ORO stain in the AA and DA of apoE^{-/-} mice was 1.9 and 1.4 times higher than background staining of apoE^{+/+} aorta tissue, respectively. At 15 and 25 weeks (study 2), ORO stain in the AA of

apoE^{-/-} mice was 1.9 and 2.5 times higher than the background, respectively. We conclude that the ORO solubilisation technique applied to AA samples is a very useful and rapid method for atherosclerotic lesion quantification.

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Introduction

Mouse models of heart disease have proved very useful for studying the influence of risk factors or therapeutic treatments on the pathogenesis of atherosclerosis [1–3]. The 2 models that are widely in use are apoE^{-/-} mice and LDL receptor^{-/-} mice [2]. Both mice show quite distinct plasma lipid profiles with a predominance of pro-atherogenic VLDL and chylomicron remnants in apoE^{-/-} mice and LDL in LDL receptor^{-/-} mice [3–5]. Both develop atherosclerotic lesions on high fat, high cholesterol diets, but lesion development in apoE^{-/-} mice is more rapid and extensive than in LDL receptor^{-/-} mice and arterial plaque development shows strong similarities to the human pathology [3].

Atherosclerotic lesion development in mouse models of heart disease has been studied at locations critical for the manifestation of human heart disease, namely the coronary arteries [6]. However, due to the small animal size, lesion development is more conveniently observed in the aortic root [7] and in the aorta [8]. The location of lesion development is influenced by haemodynamic forces on the arterial walls [9]. In the aorta, prominent lesions develop within the aortic arch and at points of arterial bifurcation [8, 9]. Lesion development is initially less extensive in the descending thoracic and abdominal aorta.

Quantification of atherosclerotic lesion development in mouse models is of primary interest as an end-point analysis for evaluating the impact of risk factors and treatments on disease development. Although lesions are microscopically visible through the translucent aorta after removal of adventitial fat, their presence is best demonstrated by staining using a Sudan lysochrome. These dyes are lipophilic and therefore target the fatty deposits in arterial plaque. The method of quantifying lesion staining conventionally involves capturing images and comparing the stained area with the total area. However, 2-dimensional analysis of a 3-dimensional structure, albeit flattened by various methods, is unsatisfactory, particularly considering that lesions may extend from the aorta into branching arteries. Preparation of hearts and sectioning for quantification of lesions at the aortic root is time consuming and labour intensive.

Our objective was to evaluate a rapid and simple method for quantifying lesion development in apoE^{-/-} mice by staining with the neutral lipid-targeting lysochrome Oil Red O (ORO; Sigma, Poole, UK) and then solubilising and quantifying the dye retained by the aorta tissue. A method using ORO solubilisation was previously described for quantification of lesion development in rat aorta [10], but it has not been adapted for use with aorta from mouse models of heart disease. We have developed a method using a multiwell plate format that is suitable for rapid analysis of mouse aorta samples. We have also measured lesion development in different sections of the thoracic aorta.

Methods and Materials

Animals and Diets

Study 1. The objective of this study was to develop the methodology and to quantitatively determine the distribution of lesion staining between the aortic arch and descending thoracic aorta. Twenty-one male apoE^{-/-} mice, congenic with C57BL/6J mice, were obtained from Charles River UK (Margate, UK) at age 4

weeks and were given a high fat (21%), high cholesterol semisynthetic diet similar in composition to a standard commercial Western-type diet widely used to accelerate atherosclerotic lesion development in mouse models of heart disease (Diet TD.88137, Harlan-Teklad, USA). As controls, 21 male wild-type C57BL/6J mice (apoE^{+/+}) were obtained at the same age, from the same supplier and given the same diet. All mice were fed ad libitum and had free access to deionised water. The mice were individually housed and maintained at 23°C under a 12-hour light-dark cycle for 13 weeks. Each mouse was provided with wood shavings, bedding and a hiding tunnel.

Study 2. Having established the methodology, study 2 was designed to quantitatively investigate aortic arch lesion staining at 2 different timepoints. In this study, 87 apoE^{-/-} and 68 apoE^{+/+} male weanling mice were given the same diet with the same conditions as described for study 1 and approximately half of the animals of each genotype were sampled at 15 weeks, with the remaining mice being sampled at 25 weeks.

Both studies were carried out in accordance with all UK Home Office animal welfare regulations and were approved by the Rowett Institute ethical committee. Animal care was provided by trained animal technicians with welfare supervision by qualified veterinary personnel.

Tissue Sampling and Processing

Mice were killed by i.p. injection of a lethal dose of sodium pentobarbital followed by exsanguination under deep terminal anaesthesia. Thoracic aortas, with the heart attached, were removed as follows: the front and left side of the rib cage was removed, followed by the left lung. The diaphragm was then severed to obtain better access to the aorta, which was carefully removed with fine curved scissors by gripping a piece of diaphragm attached to the end of the aorta and cutting up the connective tissue attaching the aorta to the muscle wall of the thoracic cavity. In order to obtain generous lengths of innominate, left common carotid and left subclavian arteries, which are attached to the aortic arch, the trachea was exposed at the neck and an incision was made well anterior to the heart, which was then removed with the aorta by cutting down under the heart. The aorta attached to the heart was placed in a balanced salt solution and, under a dissecting microscope, the aorta was cut at the point where it exits from the heart. The aorta was flushed with salt solution using a 25-gauge needle and syringe and was placed, with adventitial fat attached, into 10% neutral buffered formalin (Sigma). After 24 h, the fixed tissue was removed, blotted, frozen in liquid nitrogen and stored at -80°C. Samples were defrosted and dissected under a microscope to remove all visible adventitial fat.

Oil Red O Staining and Analysis

A 3% solution (w/v) of ORO in 2-propanol was prepared by heating the reagents to 56°C for 1 h and then cooling before filtering through a number 1 filter paper (Whatman, UK). A working solution was prepared by diluting the stock solution 6:4 with deionised water. Before use, the working solution was passed through a 0.2-µm filter.

Each aorta was rinsed in distilled water, quickly rinsed in 70% 2-propanol and stained for 30 min in the ORO working solution, then rinsed again for 10 s in 70% 2-propanol then returned to distilled water. During staining of study 2 aortic arches, ORO solution was gently flushed through each arch lumen once using a

syringe with needle. Aortas were inspected under the microscope and any small pieces of stained adventitial fat that remained attached were removed very carefully without disturbing the plaques. Aortas were then cut into 2 sections: the aortic arch including lengths of the innominate, left common carotid and left subclavian arteries, which were trimmed anterior to the presence of stained plaque and the descending thoracic aorta. Stained aortic arches and descending thoracic aortas were gently blotted and then transferred into 96 well polypropylene plates (Greiner Bio-One Ltd., Stonehouse, UK) containing 100 μ l chloroform/methanol (2:1 v/v). The plate was placed on an orbital shaker for 4 min, until all the stain was dissolved. Aorta samples were then removed, rinsed in PBS and homogenised in 200 μ l of PBS using a Precellys homogeniser (Bertin, Montigny-le Bretonneux, France) and 1-mm zirconia beads (5.5 g/cm³, BioSpec Products Inc., Bartlesville, Okla., USA). The homogenate was centrifuged on a bench-top microcentrifuge at 13,000 rpm for 5 min and protein was measured in the supernatant using the BCA protein Assay (Pierce, Rockford, Ill., USA). ORO was measured using a μ Quant plate reader (Bio-Tec Instruments Inc, Winooski, Vt., USA) at 520 nm and the quantity (μ mol) of ORO retained and released from the tissue was calculated using a standard curve of ORO (3.12–100 μ M), in chloroform/methanol 2:1. Results were expressed as μ mol ORO/ μ g protein.

Results

Mouse Growth

ApoE^{-/-} mice gained body weight at a lower rate than the wild-type (apoE^{+/+}) mice. Therefore, at termination of study 1, for example, body weight was significantly lower in the apoE^{-/-} group (apoE^{+/+} 31.1 $\pm$ 0.9, apoE^{-/-} 27.8 $\pm$ 0.5, mean $\pm$ SEM, p < 0.05).

Aortic Lesion Development

In study 1, the ORO staining of aortas from apoE^{-/-} mice revealed large atherosclerotic plaques in the arch region, whereas small lesion development in the descending aorta was limited to the points of intercostal artery branching. The staining in aortas from apoE^{+/+} mice was diffuse and there was no evidence of plaque formation.

In study 2, lesion staining in the aortic arch was similar to that in study 1 at the 15-week timepoint, but was much more pronounced by 25 weeks (fig. 1). There was also more evidence of lesion formation in the descending aorta, but this was not stained and quantified. There was no evidence of plaque formation in apoE^{+/+} mouse aortas at either timepoint (fig. 1).

Aortic Lesion Quantification

Study 1. Extraction and quantification of ORO from aorta sections demonstrated that lesion development in the aortic arch of apoE^{-/-} mice almost doubled the amount

Fig. 1. ORO-stained aortic arches from apoE^{+/+} mice fed a high fat diet for 25 weeks (a) and apoE^{-/-} mice after consuming the same diet for 15 (b) and 25 (c) weeks. At 15 weeks, lesions staining intensely are focused at the aortic arch and at branch points of the aorta, whereas by 25 weeks plaque occupies most of the luminal surface of the arch.

of ORO retained in this section compared with that of apoE^{+/+} mice (fig. 2), a difference that was highly statistically significant (p < 0.001). ORO retained by the descending thoracic aorta was also significantly (p < 0.001) greater in apoE^{-/-} mice compared to apoE^{+/+} mice, but was significantly (p < 0.01) lower than that for the aortic arch of apoE^{-/-} mice. There was no significant difference in the ORO retained by the arch compared to the descending aorta sections of apoE^{+/+} mice. The relative standard deviation for ORO analysis was about 15%, which increased slightly to 18% when correcting for protein. There was no significant difference in the protein content of aortic arch or descending thoracic aorta from apoE^{+/+} and apoE^{-/-} mice.

On the premise that lesser or greater lesion development in the aortic arch would be reflected by a similar trend in descending aorta from the same mouse, ORO data for aortic arch were correlated with ORO data for the descending aorta. Although the correlation was positive,

Fig. 2. Quantification of ORO staining in the aortic arch and descending thoracic aorta of apoE^{+/+} and apoE^{-/-} mice (aged 18 weeks) after 13 weeks consuming a high fat (21%), high cholesterol Western-type diet. Data are the mean $\pm$ SEM and dissimilar letters above the error bars indicate statistical significance ($p < 0.05$).

Fig. 3. Quantification of ORO staining in the aortic arch of apoE^{-/-} mice after consuming a high fat diet for 15 ($n = 43$) and 25 ($n = 44$) weeks. Staining in aortic arch of apoE^{+/+} mice fed the same diet for 15 ($n = 35$) and 25 weeks ($n = 33$) is shown for comparison. Data are the mean $\pm$ SEM and dissimilar letters above the error bars indicate statistical significance ($p < 0.05$).

as expected, it was very weak ($R = 0.342$) and was only just significant ($p = 0.042$).

Study 2. Quantification of ORO stain in the aortic arch of apoE^{-/-} mice showed a very significant time-related increase (fig. 3), as would be expected from the visually evident staining within the aorta tissue (fig. 1). In contrast, arches from apoE^{+/+} mice showed only background staining, and there was no significant difference between staining at 15 and 25 weeks (fig. 3).

Discussion

This study demonstrates that atherosclerotic lesion development in a mouse model of heart disease can be quantified by staining of aorta with ORO and subsequent solubilisation of the dye for quantification by spectrophotometry. The multiwell plate method is rapid and does not require time-consuming sample preparation or analysis. In study 1 at 13 weeks and study 2 at 15 weeks, the ORO extracted from the apoE^{-/-} aortic arch was almost 2-fold greater than that obtained from the apoE^{+/+} aortic arch, demonstrating that the aortic lesions accumulate a significant proportion of the ORO dye and that the results generated by the ORO quantification method

Table 1. Predicted group size (number of mice) required for changes in staining, due to aortic arch lesion formation, to reach statistical significance ($p < 0.05$)

Change in stain, %	10	20	30	40	50
Group size, n	105	23	10	7	5

show good inter-study reproducibility. The clear presence of aortic lesions in the apoE^{-/-} mice confirms several previous reports where these mice were given a high fat diet for approximately the same period of time [11, 12]. At 25 weeks, the staining in apoE^{-/-} arches was 2.5-fold greater than that in the apoE^{+/+} arches, confirming that this method can be used to quantitatively monitor increased lesion prevalence over time.

The between-replicate aorta ORO error, including analytical and biological variation, was found to be less than 20% relative standard deviation for both apoE^{-/-} and apoE^{+/+} mice, which compares favourably with the traditional morphometric method of quantification using image analysis [12, 13]. ORO stains neutral lipid in the aorta and, as a consequence, the level of staining in apoE^{+/+} aortas (background staining) was relatively high. How-

ever, the between-replicate staining variability was low and both the aortic arch and descending aorta for apoE^{+/+} mice gave similar results. Likewise, ORO staining of the aortic arch from apoE^{+/+} mice was the same at 15 and 25 weeks. This indicates that background variability in ORO staining would not adversely contribute error to the quantification of lesion staining. It would, however, be very desirable to include a group of appropriate apoE^{+/+} mice in any study design using apoE^{-/-} mice. Subtraction of this background staining from apoE^{-/-} data would then permit calculation of fold differences in lesion development due to a particular treatment. In the case of study 2, the increase in staining between 15 and 25 weeks was 1.7-fold when background staining was subtracted.

On the basis of power calculations, table 1 presents the predicted minimum number of animals per group that would be required to detect a statistically significant difference at 95% probability for various percentage increases in ORO staining, based on the between-replicate biological and analytical error. The distribution of staining in the aortic arch at 13–15 weeks was similar to that previously reported in apoE^{-/-} mice [14], with lesion focal points at bifurcations of the innominate, left common carotid and left subclavian arteries and also on the under surface of the arch. However, at 25 weeks, lesions covered most of the luminal surface of the aortic arch. A great advantage of the method described in this paper is that the composition of the plaque can be evaluated prior to stain elution and quantification. Dissection of the aorta and end face examination is entirely compatible with the described methodology and so information on plaque localisation, composition and even area measurements can be supplemented by a truly quantitative analysis of neutral lipid content. However, assumptions that plaque lipid content is always proportional to plaque volume may not be appropriate at all stages of plaque development, particularly at later stages when fibrous atheromas may develop. Nevertheless, comparative assessment of treatment effects on plaque development in relation to control values may be meaningful regardless of the linearity of the relationship between plaque volume and stain accumulation.

The descending aorta from apoE^{-/-} mice showed significantly less ORO staining than the aortic arch, which was consistent with the qualitative observation of stain distribution using light microscopy (fig. 1) and with previous reports [8]. Nevertheless, staining in the descending aorta of apoE^{-/-} mice was greater than that found in apoE^{+/+} mice, and inspection of aorta indicated that the major sites of lesion formation were at branches of the

intercostal arteries. The between-replicate variation for descending aorta ORO staining was similar to that for the aortic arch, but due to the better lesion/background ratio, quantification of lesion formation in the aortic arch is preferable. The positive correlation between lesion formation in aortic arch and in descending aorta of individual apoE^{-/-} mice was weaker than had been anticipated, which may indicate that lesion development along the length of the thoracic aorta shows some variability.

In conclusion, we have demonstrated a simple and rapid technique for the quantification of aortic lesion formation in apoE^{-/-} mice. The aortic arch, including short lengths of branching innominate, carotid and subclavian arteries, is most suited for this technique because it yields the best lesion/background staining ratio. Relative standard deviations that compare favourably with those obtained elsewhere for lesion area measurement allow statistically significant detection of modest changes in lesion development due to treatments of interest. While this technique does not indicate the localisation or composition of lesion formation, this can be ascertained, and if necessary morphometrically quantified, prior to solubilisation of the ORO stain. Added advantages of the solubilisation method include the ability to quantify staining in 3-dimensional structures such as branching arteries and within the volume of the plaque structure. This technique simplifies and facilitates a key end-point analysis in atherogenesis.

Acknowledgements

J.H.B., S.J.D. and M.-J.G. received funding from the Rural and Environment Research and Analysis Directorate of the Scottish Executive. I.-S.K. received funding from the Korea Science and Engineering Foundation (F01-2006-000-10172-0) and the Korea Research Foundation (KRF-2007-313-C00810). T.-Y.H. was funded by a grant from the Korean Food Research Institute. The authors would like to thank Donna Wallace, Anja Petrie, Hannah Orr and the staff of the Biological Resources Unit at the Rowett Research Institute for animal maintenance, care and welfare.

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