The Influence of the Adventitia on the Presence of Smooth Muscle Cells and Macrophages in the Arterial Intima

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Objective: To test the hypothesis that atherosclerosis may be initiated by hypoperfusion or thrombotic occlusion of the adventitial vasa vasorum.

Design: In a new model of atherogenesis, an early atherosclerotic lesion may be initiated by removal of the adventitia from the carotid artery of the New Zealand White rabbit, wherein lie the vasa vasorum.

Setting: Animal laboratory, University Department of Surgery and Medicine.

Materials: 15 rabbits fed a normal diet and 32 fed a high cholesterol diet.

Chief Outcome Measures: Immunocytochemistry was undertaken to demonstrate the presence of smooth muscle cells and macrophages within the intimal lesions. Smooth muscle cells were labelled with a monoclonal antibody designated HHF35 and macrophages were labelled with a rabbit specific, macrophage specific antibody, RAMI1.

Chief Results: In rabbits fed a normal diet, at day 14, the intimal lesion was composed exclusively of smooth muscle cells. By day 28, such lesions had regressed. In rabbits fed a high cholesterol diet, at day 14, the intimal lesion was composed of a mixture of macrophages and smooth muscle cells. By day 42, the pattern of cellular distribution was such that macrophages (present as foam cells) were predominant. In the presence of persistent hypercholesterolaemia these lesions did not regress.

Conclusions: This new model can produce two different cellular responses that may mimic the intimal lesions seen with re-stenosis after angioplasty or in hypercholesterolaemic man and as such, might be useful in separating out these two different pathophysologies.

Key Words: Atherosclerosis; Adventitia; Vasa vasorum; Immunocytochemistry.

Introduction

In the pathogenesis of atherosclerosis, key roles are played by smooth muscle cells and macrophages. Therefore, their identification and patterns of distribution in animal models of the disease may provide important information about lesion development. However, the initiating circumstances that eventually lead to the formation of an atherosclerotic plaque remain obscure.

For almost 150 years it has been considered that atherosclerosis begins at the luminal surface of the artery, with the necessary antecedent of endothelial trauma. More recently, it has been appreciated that the adventitia may play a crucial role in the maintenance of the integrity of the inner layers of the arterial wall.

In a recently described model of atherogenesis, compression of the adventitial layer of a rabbit carotid artery, by placement of a soft silastic collar around the outside of the vessel, induces the formation of an intimal lesion in which smooth muscle cells and macrophages accumulate beneath an anatomically intact endothelium. It has been suggested that these observed changes may be mediated by obstruction of the adventitial vasa vasorum, with the subsequent creation of a localised region of ischaemia within the arterial wall.

Based upon this particular model, an hypothesis has been developed which proposes that in man, atherosclerosis may be initiated secondary to hypoperfusion or thrombotic occlusion of the adventitial vasa vasorum and that such a process, affecting the outer layers of the arterial wall, can proceed to cause the intimal changes typically seen in this disease.

In other studies performed by us, occlusion of the vasa vasorum in the pig femoral artery was associated
specifically with the formation of an intimal hyperplastic lesion.\textsuperscript{14} We have shown also, that such a lesion can develop following the removal of the adventitia from the rabbit carotid artery.\textsuperscript{16} However, in this study, immunocytochemical techniques are employed to assess the influence of removal of the adventitia on the presence of smooth muscle cells and macrophages within the intimal lesion. Variations in the pattern of cell distribution are noted with time and with diet. Such patterns of cell distribution may aid in the understanding of the pathophysiology of early atherosclerosis.

**Materials and Methods**

**General methodology**

New Zealand White male rabbits (2.25–2.75kg) were used exclusively in the study. All procedures undertaken were licensed by the Animals (Scientific Procedures) Act (United Kingdom) 1986. The rabbits were fed either a standard pelleted chow (SD-1, SDS Limited) or the standard chow adjusted by the addition of 1.5% cholesterol (w/w) plus 10% olive oil, regulated to provide 100–200g of feed per rabbit per day. Water was provided ad libitum.

Rabbits were pre-medicating with Fentanyl/Fluanisone (Hypnorm, 0.2ml/kg subcutaneously, Janssen Animal Health Limited) and later anaesthetised with Midazolam (Hypnoval, 0.2ml/kg intravenously, Roche Products Limited).

The carotid arteries were exposed. Both arteries were gently mobilised and held in vascular slings. The adventitia and surrounding fatty areolar tissues were then carefully removed by sharp surgical dissection, along a clearly defined plane from the underlying media of one of the vessels. The contralateral carotid artery within each individual rabbit acted as a control: the vessel was handled and mobilised in a similar fashion and for a similar length of time, but the adventitia was left intact. In survival experiments, layers were closed in continuous fashion with 3/0 Dexon (Davis and Geck Limited). Postoperative analgesia and topical antibiotics to the wound were used.

For non-survival procedures, the rabbits, whilst under anaesthesia, were given Pentobarbitone Sodium (Euthatal, 1ml/kg intravenously, RMB Animal Health Limited).

For later analysis, immediately following death, the rabbit carotid arteries were cannulated and pressure perfused (using a Watson-Marlow pump) at 100mmHg, first with physiological saline to flush out remaining blood and then with either neutral buffered formalin (for light microscopy and immunocytochemistry), or Karnovskys’ fixative (for electron microscopy).

**Histology and Immunocytochemistry**

For histology, samples were passed through a Shandon Hypercenter-XP enclosed tissue processor (Shandon Southern Products Limited). After dehydrating through graded alcohols, samples were cleared in CNP-30 and embedded in paraffin wax. Sections were cut at 4mm from several levels within each block and de-waxed in xylene prior to staining with Mayer’s haematoxylin and eosin (H&E) and with Van Gieson’s stain (VG).

For transmission electron microscopy (TEM), tissues were post-fixed in 1% osmium tetroxide and then dehydrated in graded alcohols and epoxy propane prior to embedding in araldite. Ultra-thin sections were taken using a diamond knife from suitable areas of the block face as seen previously on phase contrast sections and then stained with uranyl acetate and lead citrate, prior to examination under the electron microscope (Jeol-100C).

Immunocytochemistry was used to demonstrate the presence of smooth muscle cells and macrophages within the intimal lesions seen. Macrophages were shown using a rabbit specific, macrophage specific monoclonal antibody, RAM11.\textsuperscript{17} Sections were processed after endogenous peroxidase activity was first blocked using (3% aqueous) hydrogen peroxide. RAM11 antibody (diluted to 1:1000 in phosphate buffered saline plus 1% bovine serum albumin) was incubated with the tissue. After rinsing, biotinylated anti-mouse antibody (diluted to 1:20) was added. To localise the biotin-avidin binding sites, diamino-benzidine was added (together with a small quantity of 30% hydrogen peroxide) to produce a brown coloration. The slides were then stained with Mayers’ haematoxylin and sections were “blued” in running tap water.

Smooth muscle cells were revealed using a monoclonal anti-muscle antibody designated HHF35 that labels muscle actin only.\textsuperscript{17} Tissue processing followed the same protocol as for macrophage labelling. Controls in each case were samples incubated without the addition of the primary antibody.
Normal anatomy

Rabbits were sacrificed without any procedures undertaken. This allowed assessment of the normal anatomy of the rabbit carotid artery.

Removal of the adventitia

Two groups of rabbits were fed either a normal or a high cholesterol diet. Rabbits on a normal diet were sacrificed on days 7, 14 and 28. Those on a high cholesterol diet were sacrificed on days 7, 14, 28 and 42.

Results

Several rabbits were used to obtain samples of normal rabbit carotid arteries. The surgical exposure of the arteries proved straightforward in all cases. Over the dissected length in the neck, a single, constant side branch partly supplying the strap muscles was present. Vasa vasorum were in part visible to the naked eye and ran longitudinally along the adventitial surface of the artery, branching to run circumferentially in a plexus around the vessel wall.

Light and electron microscopy showed that the intima consisted simply of an endothelial monolayer resting directly upon a broad, fenestrated internal elastic lamina (IEL) (Fig. 1). The media consisted of 12–18 "lamellar units" composed of smooth muscle cells separated by elastin and collagen. No external elastic lamina (EEL) was evident. Vasa vasorum were found only in the adventitial layer and did not pass into the media.

Adventitial removal

In 15 rabbits fed a normal diet, an intimal lesion was always noted in those arteries from which the adventitia had been excised. No lesion was ever seen in the contralateral control vessels, which had been handled and mobilised in a similar fashion but which had the adventitia left intact. Light microscopy revealed that the lesion formed beneath an anatomically intact endothelium and was between 6–10 cell layers thick (Fig. 2) and maximal in size by day 14 (n = 8). However, by day 28 the lesion had regressed completely (n = 7).

TEM demonstrated these cells to have the appearance of smooth muscle cells. Serum cholesterol levels in this group were always less than 2mmol/l.

In 32 rabbits fed a high cholesterol diet, intimal lesions were also noted in those arteries from which the adventitia had been removed. Again, no lesions were ever seen in the contralateral control vessels. Such lesions were also maximal in size at day 14 (n = 8), and appeared 6–10 cell layers thick. In contrast to the rabbits fed a normal diet, these lesions did not regress. Rather, they were maintained at 4–6 cell layers thick up until day 42 (n = 8) when the experiment was terminated (as part of this study, eight

Fig. 1. Transmission electron micrograph to show the endothelium (E) of the rabbit carotid artery, resting upon a well defined, fenestrated internal elastic lamina (I). The media is composed of 12–18 "lamellar units" comprised of smooth muscle cells (S) separated by elastin and collagen. (TEM × 2000).

Fig. 2. Light micrograph showing the intimal hyperplastic lesion at day 14, in a rabbit fed a normal diet, following removal of the adventitial layer. The lesion forms beneath an anatomically intact endothelium (E) and above the internal elastic lamina (I). (H&E × 500).
rabbits had been terminated on day 7 and a further eight rabbits on day 21. TEM demonstrated these cells to have the appearance of lipid-laden "foam cells" (Fig. 3). Serum cholesterol levels in this group increased from a mean of 11.2 mmol/l at day 7 to a mean 63.9 mmol/l at day 42.

Immunocytochemistry confirmed that in rabbits fed a normal diet, the lesions at day 7 and at day 14 were composed almost exclusively of smooth muscle cells (HHF35 staining—Fig. 4). No staining was seen with RAM11. The cells were present beneath the anatomically intact endothelium and interestingly seemed to be aligned longitudinally in the direction of luminal blood flow, rather than circumferentially as in the media.

In rabbits fed a high cholesterol diet, a mixed distribution of smooth muscle cells and macrophages was found (Fig. 5). At day 14, when the lesion was maximal in size, smooth muscle cells comprised approximately 50% of cells present, with macrophages comprising the other 50%. This was confirmed by both HHF35 and RAM11 stains. The macrophages were present as the lipid-laden "foam cells" previously seen on TEM and were usually located touching the IEL, but not the endothelium.

At day 42, when the lesion was somewhat smaller, macrophages were predominant, comprising 70–80% of the lesion. Smooth muscle cells (20–30%) appeared as a band of cells immediately beneath the endothelium. Thus with time, the patterns of cellular distribution appeared to change, compared to the animals on normal diet.

**Discussion**

Controversy surrounds the origins and evolutionary inter-relationships between the early lesions of atherosclerosis, which are often separated into two major...
forms: the fatty streak and the fibrous plaque. Fatty streaks are composed almost exclusively of macrophages (with some lymphocytes) and have relatively few smooth muscle cells. The macrophages present are often seen as lipid-laden "foam cells". Fibrous plaques are composed predominantly of smooth muscle cells, some of which may become lipid-laden and also take on the appearance of "foam cells". Whether one, both or neither lesions eventually progress to form a mature, complex atherosclerotic plaque remains unclear. In this study, it would appear that in rabbits fed a normal diet, a lesion results that resembles the fibrous plaque. However, in rabbits fed a high cholesterol diet, the lesion seems more complex, not necessarily representative of either the fatty streak (as a high percentage of smooth muscle cells remain) or the fibrous plaque, but perhaps a more likely candidate for development into a mature atherosclerotic plaque than either.

The adventitia contains vasa vasorum, lymphatics and nerve plexuses that together maintain the integrity of the arterial wall. The vasa vasorum provide oxygen and other nutrients by diffusion inwards for the outer media. If the vessel wall thickness exceeds a "critical depth" then the supply of oxygen to the medial layer is maintained by vasa vasorum which penetrate into the media and run within it. Previously, it has been demonstrated that by manipulating the arterial adventitia, changes could be observed within the intima. Specifically, occlusion of the vasa vasorum has been shown to cause intimal hyperplasia in the pig, possibly associated with a lowered P02 within the media. It has been demonstrated also, that by occluding the vasa vasorum, the trans-arterial transport of macromolecules such as fibrinogen and low density lipoprotein is impeded, with the subsequent accumulation of these compounds within the vessel wall.

We have shown that removal of the adventitia induces the formation an intimal lesion and can be presumed therefore, to be causally related. In each case, the lesion developed beneath an anatomically intact endothelium. This questions the necessity for endothelial damage to promote atherogenesis as suggested by the response to injury theory. It has been shown that the patterns of cellular distribution vary depending upon diet. With a normal diet, presumably the smooth muscle cells within the media, following the loss of the adventitia (and in particular the vasa vasorum, supplying oxygen and other nutrients to the outer arterial wall), are stimulated to migrate towards the intima, where they align themselves beneath the endothelium in the direction of blood flow. Which chemical messages are released to promote smooth muscle cell migration and proliferation remains unknown at present.

However, it has been suggested that hypoxia may be the direct stimulus to promote smooth muscle cell proliferation. It may be that such cells, upon migration from a hypoxic media towards an area of improved oxygenation, i.e. the intima, undergo a phenotypic change from a contractile to a synthetic state such that they could then be responsible for secreting chemotactic factors that would draw macrophages and other cells to the area. The accumulation of cells beneath the endothelium may tend to precipitate endothelial trauma due to rheological factors and hence promote the cascade of events that would allow further atherosclerotic plaque development once the sub-endothelium was exposed. However, by day 28, these same cells forming the intimal lesion have regressed. They may return to the media from whence they came, or undergo cell death in situ (the former seems more likely to us). The mechanism(s) controlling the original migration and proliferation may therefore have been reversed.

Removal of the adventitia however, seems to be able to produce two different cellular responses, in that when a high cholesterol diet was used in addition to removal of the adventitia, the intimal lesions were composed predominantly of macrophage-derived foam cells. Such lesions did not regress before the experiment was terminated. Rather, it would appear that in the presence of hypercholesterolaemia, macrophage based lesions persist and may even be able to prevent complete smooth muscle cell regression as evidenced by the presence of smooth muscle cells within the lesion at day 42. Again, the mechanism(s) by which macrophages may mediate this process and indeed, why macrophages themselves remain, is unclear.

In conclusion, by use of the monoclonal antibodies HHF35 and RAM11 it has been possible to demonstrate that a smooth muscle cell predominant lesion may regress whereas a macrophage predominant lesion does not. The smooth muscle cell lesion may therefore, be analogous to the intimal lesion seen with re-stenosis after angioplasty, whereas the mixed lesion might be analogous to the more complex
plagues seen in hypercholesterolaemic man. Further understanding of the cell signalling in these models may help therapy, not only to prevent atherosclerosis, but also to promote regression of established disease.

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**References**


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