Arterial Intimal Hyperplasia After Occlusion of the Adventitial Vasa Vasorum in the Pig

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Oxygenation of the arterial wall is provided by diffusion of oxygen outward from the main vessel lumen and inward from the adventitial vasa vasorum. In a group of four Yucatan miniature pigs the oxygenation profiles across the superficial femoral arteries were recorded by polarographic oxygen microelectrodes. The profiles obtained suggested a relatively poorly oxygenated media (a trough value of approximately 25% that of the intimal oxygenation) with a progressive rise in oxygenation toward the intimal and adventitial surfaces. In four other survival experiments, occlusion of the adventitial vasa vasorum by flush ligation of the arterial branches that supply them resulted in the production of a focal, intimal hyperplastic lesion that was absent in control vessels (intimal to medial ratios [mean±SEM] of 0.053±0.008, n=8, p<0.001 and 0.013±0.001, n=8, respectively). By electron microscopy this lesion was seen to be composed mainly of smooth muscle cells. This evidence would support the hypothesis that arterial wall hypoxia may be involved in the initiation of intimal hyperplasia. It is proposed that human atherosclerosis may be initiated by occlusion of the vasa vasorum and concomitant hypoxia. (Arteriosclerosis and Thrombosis 1993;13:70–77)

KEY WORDS • atherosclerosis • vasa vasorum • oxygen microelectrodes • hypoxia

Atherosclerosis is a progressive disease of arteries that probably starts many years before its clinical manifestations by proliferation or migration of smooth muscle cells and foam cell formation between the endothelial cell layer and the internal elastic lamina (IEL). The initiating lesion in this process in humans is unknown. However, most authors during the last two centuries have presumed that the causative changes occur at the luminal surface of the artery.1–4

A new model of atherosclerosis has initiated intimal smooth muscle cell hyperplasia and foam cell formation by placement of a loose Silastic collar around the shape of the artery.5 It has been proposed that the collar might occlude the adventitial vasa vasorum, which would cause hypoxia to the smooth muscle cells of the outer media, thereby initiating their migration toward the luminal surface.5

However, it is also possible that changes to adventitial lymphatics or nerves might occur with adventitial manipulation. Therefore, we have studied the occurrence of intimal hyperplasia after ligation of the vasa vasorum in the pig femoral artery, with minimal interference with other structures in the adventitial layer.

Vasa vasorum run in the adventitial layer of arteries and supply oxygen and other nutrients to the outer media, whereas the inner media is supplied by diffusion from the main vessel lumen.9–11 Although the demand for oxygen by the normal arterial wall is relatively modest,12 evidence suggests that there may be a “water-shed” for oxygen in the center of the media of arteries.13 Likewise, oxygen microelectrode studies of vessels, even with normally functioning vasa vasorum, point to a media on the borderline of hypoxia.14–16

Our study demonstrates that adventitial changes can initiate intimal ones.

METHODS

Two groups of four female Yucatan miniature pigs (Charles Rivers, Maidstone, UK) (35.6–44.2 kg) were used in this study to follow the protocol shown in Table 1. All procedures were conducted under license and conformed to the regulations cited in the Animals (Scientific Procedures) Act (United Kingdom) of 1986. The first group of pigs was used to assess the normal anatomy of the superficial femoral artery and obtain normal oxygenation profiles. The second group was used to occlude vessels (vasa vasorum) in the adventitia of their eight femoral arteries.

The pigs were fed a standard pelleted chow and allowed water ad libitum. Premedication consisted of an intramuscular injection of 20 mg/kg body wt ketamine (Parke-Davis). After sedation the pigs were placed in a supine position onto a warming blanket on the operating table. Other exposed areas were covered by an aluminium foil blanket to maintain the pigs close to a temperature of 39°C. (In some cases infrared lamps were required.) The pigs were preoxygenated with 100% oxygen and given incremental halothane to 3% (RMB Animal Health Ltd.). An ear vein was cann-
At the time of recording, lights and other apparatuses, all wires shielded and taped flat to surrounding surfaces. To minimize electrical disturbance, all recording apparatuses were located within a Faraday cage and readings. To minimize electrical disturbance, all recordin-
ligation of the side branches and penetrating vessels, which was intended to occlude blood flow within the adventitial vasa vasorum. This was attempted in all pigs (eight vessels) in the group but was technically very difficult. The operative procedure to that point was approximately 3.5 hours, and at this time the pigs developed various cardiac arrhythmias (most notably atrial fibrillation) that precluded further measurements from being taken. Accordingly, only one recording from each of the four pigs (four vessels) was obtained after the occlusion of flow within the adventitial vasa vasorum. In this second group the pigs were allowed to recover. At the end of the procedure, the wounds were infiltrated with 0.5% lignocaine to a maximum dosage of 2 mg/kg. Tribiotic spray (3M Healthcare Ltd.) was applied to the wounds, which were then sutured with subcuticular Dexon (Cyanamid, Davis and Geek). Recovery after extubation was under an infrared heater. Analgesia was administered by means of 0.02 mg/kg i.m. buprenorphine (Reckitt & Coleman Ltd.), which was given twice daily thereafter. The pigs quickly recovered and again had free access to food and water.

At 3 weeks the pigs in group 2 underwent reoperation. The experimental segment of each artery and the control segments above and below were mobilized by sharp surgical dissection. The vessels were fixed and then removed, following the procedures previously described. For histological determination each artery was subdivided into its experimental and control segments. Sections were again cut at 4 µm from at least three separate regions of each segment and three different levels within each tissue block. Prepared transverse sections (hematoxylin and eosin) from these pigs were later assessed by a computerized morphometric analysis system (Sight Systems, Newbury, UK). The intimal width, which is the distance from the luminal surface of the endothelium to the inner aspect of the IEL, was measured, as was the width of the media, which is the distance from the outer surface of the IEL to the junction of the media and adventitia. The intimal-medial ratio was calculated for 12 sites around the circumference of each artery, which were equally spaced, and a mean ratio was obtained. This ratio was used to minimize distortion caused by nonperpendicular sectioning of the vessels and differences in arterial diameter based on the pig's weight. Statistical comparison of the control segments of arteries versus experimental segments was made by means of Student's unpaired t test.

Results

The pig superficial femoral artery originates in the femoral triangle and passes just below the skin on the inner aspect of the thigh. Below the knee it becomes very narrow (1–2 mm in diameter). However, at the sites of microelectrode puncture in both the experimental and control segments of the artery, its diameter varied from 3 to 4 mm. Vasa vasorum were clearly seen running in the adventitial layers. Resin casting demonstrated vasa vasorum that arose at branching points as expected but that also arose directly from the vessel lumen, which penetrated through the media to run in the adventitial layer (Figure 1).

In the first group of pigs and the control segments of the artery from the second group of pigs, light microscopi (hematoxylin and eosin and van Giesson's stain) showed that the endothelial cells were resting on a broad IEL. The media comprised two muscle layers, of which the inner one was oriented in a circumferential plane. There were 20–25 lamellar units that were visible. A lamellar unit is defined as a fibromuscular layer that consists of elastin and an adjacent compartment of circumferentially arranged collagen, elastin, and smooth muscle. The adventitial layer contained numerous vasa vasorum, although none were seen to penetrate into the media (Figure 2).

Transmission electron microscopy confirmed that the endothelial monolayer rested on a fenestrated IEL (Figure 3). The endothelial cells contained small vacuoles and pinocytotic vesicles. Collagen bundles were occasionally present in the subendothelial space, which would then appear to pass through the fenestrations in the IEL to mix with collagen in the media proper. The media comprised smooth muscle cells interspersed with collagen, elastin, and ground substance. Other cell types were not observed.

Three oxygen microelectrodes were used in the course of the experiments. One had an external tip diameter of 20 µm, and two had a tip diameter of 15 µm, both of which had cathodic recording surfaces of 5 µm. In each case, the polarization voltage after calibration was set at −0.75 V.

It was noticed that the output voltage drifted during the course of each experiment. Calibration of the probes before and after each procedure demonstrated that this drift varied from approximately 5% to 15% of the initial readings. Therefore, in these experiments the values for output voltage have been recorded and adjusted by a variable drift percentage, which is the average percentage drift in each experiment. Furthermore, because of the variation in the absolute values recorded by the different probes, absolute Po2 values have not been given; instead, the results have been expressed as percentages of luminal oxygen (i.e., the output voltage obtained with the probe tip in the blood of the main vessel lumen that equals 100%).
Barker et al
Adventitia and Atherogenesis
73

FIGURE 2. Transverse section across the superficial femoral artery of the Yucatan miniature pig showing the endothelium (E [arrow]), the two muscle layers of the media (M), and the adventitia (A). Located at the medial-adventitial junction are the vasa vasorum (arrows). Light microscopy with hematoxylin and eosin, ×350.

Oxygenation profiles that represented recordings across the left and right femoral arteries from pigs 1–3 and the right artery only of pig 4 were obtained, together with premanipulation recordings from the right artery only of pigs 5–8 (Figure 4). While the probe was withdrawn across the arterial wall in 20-μm intervals, there was a gradual fall in output voltage until a trough was reached in the middle to outer media. The mean trough oxygenation was approximately 25.9% of that recorded that was immediately adjacent to the intima (range, 18.2–39.9%, n = 11). While the probe was withdrawn further, the output voltage from the tissues rose as the probe approached the adventitial layer. When the probe was just out of the adventitial layer, the output voltage levels rose to levels above the original luminal value. The probe travel distance from the intima to the trough oxygenation level varied with the total vessel wall diameter but averaged 140–180 μm.

Because of the technically difficult circumstances described above, the measurement of postocclusion oxygenation profiles was not possible in all experimental arteries. However, it was possible to demonstrate in two arteries that there was an approximate 20% and 15% fall in the trough value after ligation of side branches and penetrating vessels. In two further arteries such large changes were not demonstrable, and measurements were abandoned in the remaining four vessels.

At 3 weeks after the ligation of arterial side branches and penetrating vessels, the eight vessels of pigs 5–8 were reexplored. Light microscopy of the samples taken from the experimental segments of these four pigs (eight vessels) showed significant intimal hyperplasia in six of the eight cases (Figure 5), with smooth muscle cells seen lying between an anatomically intact endothelium and the IEL. By transmission electron microscopy the smooth muscle cells were also seen in the fenestrations that were lying at right angles to the IEL (Figures 6A and 6B). In the other two cases no intimal hyperplasia was evident. Altogether the media appeared normal, with no areas of necrosis seen. Likewise, intimal hyperplasia was not seen in any of the control segments of arteries taken from the regions above or below the experimental segments. On comparison of the intimal-medial ratios, the mean ratio of the control segments was (mean±SEM) 0.013±0.001 (n = 8) and for the experimental segments 0.052±0.008 (n = 8) (p<0.001, Student’s unpaired t test).

Discussion
The superficial femoral artery of the Yucatan miniature pig has numerous vasa vasorum located at the medial-adventitial junction, which by their close proximity to the outer media supply it with oxygen and other nutrients by inward diffusion toward the main vessel lumen. As expected from the number of medial lamellar units, they were never seen to penetrate into the media.18 Resin casting demonstrated vasa vasorum that originated at arterial side branching points; importantly, they came directly from the main vessel lumen. Therefore, flush ligation of all the side branches and penetrating vessels would occlude flow in the adventitial vasa vasorum that were present.

The surgical exposure of the arteries to obtain oxygenation profiles did not involve their mobilization; therefore, this should have preserved an intact adventitia, vasa vasorum, and endothelium. In six of the eight arteries reexplored at 3 weeks, significant intimal hyperplasia was noted only from those experimental segments.
Figure 4. Line graphs showing oxygenation profiles across the superficial femoral arteries of Yucatan miniature pigs (recordings made at 20-um intervals). The oxygenation of luminal blood was defined as 100%.
FIGURE 5. Intimal hyperplasia seen at 3 weeks after the occlusion of the adventitial vasa vasorum. Light microscopy with hematoxylin and eosin, ×200.

in which side branch ligation had been performed. No control arterial segments taken from the same vessel showed any evidence of intimal hyperplasia.

Other investigators have conducted studies that involved interference of blood flow through the vasa vasorum. In the aorta of dogs, Wilens et al\textsuperscript{19} and Heistad et al\textsuperscript{20} ligated the vessels that supply the vasa vasorum and caused medial necrosis, a feature not seen in our experiments. Nakata and Shionoya\textsuperscript{21} occluded the vasa vasorum with a thrombin and gelatin mix and observed the production of an intimal proliferative lesion, which agrees with our findings.

The placement of a Silastic collar around the outside of a rabbit carotid artery is associated with the production of an intimal hyperplastic lesion beneath an intact vascular endothelium.\textsuperscript{5} It has been suggested that the collar, which touches the artery only at its ends, compresses the adventitial vasa vasorum, thereby creating a localized region of arterial wall hypoxia. On this basis, a new hypothesis has been formulated that suggests that arterial wall hypoxia, which is secondary to occlusion of the adventitial vasa vasorum by platelet and fibrin plugs, may be the initial lesion in atherosclerosis.\textsuperscript{8} Arterial wall hypoxia and atherosclerosis have been previously linked, although no specific mechanisms were proposed.\textsuperscript{22-26}

Hypoxia stimulates the proliferation of smooth muscle cells.\textsuperscript{27,28} Cultured endothelial cells (or monocytes) exposed to hypoxia show a significant increase in their production of mRNA for platelet-derived growth factor,\textsuperscript{29} which is a potent mitogen for smooth muscle cells and a potent chemoattractant for leukocytes.\textsuperscript{30} Therefore, obstruction of the vasa vasorum and the resulting medial ischemia may initiate the intimal hyperplasia seen in these studies.

We have shown the oxygenation profiles across the superficial femoral arteries of Yucatan miniature pigs. In each case a curve has been defined, with maximum wall oxygenation immediately adjacent to the vessel lumen, which diminished progressively to a trough level in the middle to outer media and rose progressively again toward the adventitia. This profile was consistent in its overall shape and is similar to profiles obtained in the dog by Heistad and Marcus\textsuperscript{10} and in the rabbit by Crawford et al\textsuperscript{15} and Crawford and Kramsch.\textsuperscript{14} Furthermore, we have limited evidence (two of four cases) to suggest that by occluding the flow of blood within the adventitial vasa vasorum, the oxygenation of the outer media may be lowered.

The introduction of the Whalen-Nair recessed gold oxygen microelectrode allowed more accurate determination of \( P_{O_2} \) profiles.\textsuperscript{31,32} Jurrus and Weiss\textsuperscript{33} performed one of the earliest in vitro studies with this electrode. A trough \( P_{O_2} \) was found in the media, with a relatively

FIGURE 6. Panel A: Transmission electron photomicrograph showing intimal hyperplasia beneath an intact endothelium (E) with smooth muscle cells (S) apparently migrating from the media through fenestrations in the internal elastic lamina (IEL [arrows]). \( \times2,500 \). Panel B: Seen at higher magnification within fenestrations in the IEL. \( \times4,000 \).
gradual and progressive increase in $P_O_2$ when approaching either the inner or outer surface of the vessel wall. They also reported that the oxygen profile was related to vessel wall thickness; as the thickness increased, the minimal oxygen tension in the media decreased. By showing the zone of low $P_O_2$ in the aorta of rabbits, they suggested that oxygen availability might be a critical factor in the progression of atherosclerosis.

Crawford et al. measured oxygen transport in vivo in the normal rabbit femoral arterial wall by means of the (modified) Whalen-Nair electrode. The profiles obtained were more in keeping with those of Jurrus and Weiss, in that once again an abrupt change in $P_O_2$ was not seen at the intimal surface. However, their study did not show a significant rise in $P_O_2$ toward the adventitial surface of the artery; consequently, they were unable to confirm that the vasa vasorum might provide a source of oxygenation to the outer layers of the vessel wall. The arterial wall in their experimental model (rabbit femoral artery) was very thin (a media of approximately 75 µm), and the vasa vasorum were probably not present to boost adventitial $P_O_2$ levels. No vasa vasorum were seen by them by light microscopy of the adventitial layers.

Some of the difficulties of using polarographic electrode measurements to assess oxygen tension in the arterial wall have been previously discussed. It has been suggested that electrodes consume oxygen, a process that results in the formation of hydrogen peroxide and hydroxyl ions, and because diffusion of the gas through the arterial tissue may be slower than the rate of consumption, the concentration of oxygen may become lowered, particularly toward the middle of the vessel wall. This criticism is considerably refuted when the Whalen-Nair type of recessed gold (membrane permeable to oxygen) microelectrode is used. Schneiderman and Goldstick examined the geometry of such microcathode tips and showed that oxygen diffusion produced by the very small oxygen consumption of the electrode itself was confined to the tip recess. Crawford and Cole have assessed a performance evaluation of recessed microcathodes and concluded that their use in $P_O_2$ measurement is entirely justified. In addition, the use of a membrane permeable to oxygen but impervious to protein is probably responsible for the near absence of cathodal poisoning. Cole et al. have also shown that the probe causes very little tissue damage on insertion into the vessel wall.

Crawford and Kramsch have assessed oxygen profiles in pathological states, namely early hypertension (a rabbit aortic coarctation model). They reported that oxygen tension was lowered throughout the media. Zemplenyi et al. examined oxygen profiles in balloon-deendothelialized rabbit iliofemoral arteries, which showed that the impaired oxygen supply of such vessels with substantial thickening was counteracted by increased oxygenation of the arterial wall because of proliferation of newly formed nutrient vessels in the adventitia. They commented that such an adaptation could be an important mechanism against hypoxia induced by arterial injury and may be a protective factor in atherogenesis.

We have outlined the normal anatomy of the superficial femoral artery of the Yucatan miniature pig and confirmed the presence of adventitial vasa vasorum. This study demonstrates that in large arteries there is a decrease in oxygenation toward the center of the media. This finding supports the view that the vasa vasorum are needed to supply the outer media with oxygen; in addition, the middle of the media may have an oxygen supply such that hypoxia of its cells may occur if it is reduced. Occlusion of the vasa vasorum in this experiment was associated with intimal hyperplasia of smooth muscle cells, which must have either all migrated from the media or be the product of some migration that results in proliferation. We suggest that hypoxia of the media that was caused by ligation of the vasa vasorum may have initiated the movement of smooth muscle cells. Whether this is done directly or by interaction with other cells or chemotactic agents will require further investigation. Furthermore, occlusion of the vasa vasorum in humans may be the initiating lesion of human atherosclerosis.

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