Intimal Neovascularisation is a Prominent Feature of Atherosclerotic Plaques in Diabetic Patients with Critical Limb Ischaemia


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Introduction. Neovascularisation of atherosclerotic plaques correlates with increased plaque instability and subsequent risk of vascular complications. Diabetics have widespread atherosclerotic involvement of the arterial tree and a more aggressive form of the disease culminating in increased plaque instability. This results in a greater incidence of ischaemic sequelae than in non-diabetics. Previous studies have examined neovascularisation as a marker of plaque instability in both the carotid and coronary territories and revealed a greater degree in both symptomatic and diabetic patients. This is the first study to examine intimal neovascularisation in lower limb peripheral arterial disease.

Methods. Arterial specimens were taken from 20 patients, ten of whom were type 2 diabetics, undergoing major lower limb amputation for unreconstructable critical ischaemia. Sections were stained with H&E for morphological assessment and inflammatory cell characterisation. Additional sections underwent immunohistochemical staining for CD31 and von-Willebrand Factor (vWF) and the number of intimal vessels per four 40x magnification fields assessed.

Results. There was a more prominent inflammatory infiltrate in diabetic subjects compared to non-diabetic controls. Diabetic patients had a greater degree of intimal neovascularisation compared to controls with a median of 11.5 and 2.0 vessels per field respectively (P < 0.05). Sub-group analysis revealed that diabetic patients medicating with HMG-CoA Reductase inhibitors (Statins) had a greater degree of neovascularisation compared to those not taking this class of medication.

Conclusion. Diabetic patients with critical limb ischaemia requiring amputation demonstrate a greater degree of plaque intimal neovascularisation and inflammatory infiltrate compared to their non-diabetic counterparts. This may explain the greater plaque instability and subsequent cardiovascular complications seen in these patients.

Keywords: Plaque; Diabetes; Neovascularisation; Angiogenesis; Statins.

Atherosclerosis is the dominant cause of morbidity and mortality in patients with diabetes, being responsible for 80% of deaths and nearly three quarters of hospitalisations. The angiopathy seen in diabetes has distinct features separate from the more common atherosclerosis and has been extensively studied, especially in relation to coronary, carotid and retinal arterial disease. In particular, comparisons of coronary and carotid intimal neovascularisation between diabetics and non-diabetics demonstrate increased numbers of intimal new vessels in the former.

In atherosclerotic disease, intimal neovascularisation is a marker of the advanced plaque and is associated with extensive chronic inflammatory cell infiltrate and elevated levels of the pro-angiogenic cytokines such as basic Fibroblast Growth Factor (bFGF) and Vascular Endothelial Growth Factor (VEGF). Atherosclerosis is characterised by a chronic fibro-proliferative inflammatory infiltrate with a significant angiogenic component, most noticeable in advanced disease. In diabetic patients, the atherosclerotic process is more widespread, involving a greater portion of the arterial tree, with particular propensity for the smaller diameter vessels. Inflammation and plaque neovascularisation have both been identified as major characteristics of plaques at risk of rupture and are associated with increased potential to become symptomatic. Indeed, higher intimal vessel counts are seen in those with symptomatic carotid and coronary disease compared to patients who were symptom free. However, there remains a paucity.
of studies into intimal angiogenesis in the lower limb arteries.

The aetiology and origin of these intimally located new blood vessels is less clear. Increased depth of the atherosclerotic plaque results in the core cellular components being unable to survive solely on diffusion for their metabolic needs and so neovascularisation is required to meet demands for nutrients and oxygen. These new vessels are thought to develop from capillaries originating from the adventitial vasculature and vasa vasora penetrating the media to reach the intima and, to a lesser degree, from the lumen itself.

Although it was previously held that arterial events such as thrombosis, embolism or plaque haemorrhage were a consequence of luminal narrowing alone, it is now broadly accepted that both the activity and the morphology of individual plaques, precipitates infarction and worsening ischaemia following either atheroma rupture or endothelial erosion. The importance of intimal neovascularisation in acute disease of peripheral vascular beds is unknown. Intimal capillaries are more prevalent in the fibrous cap and shoulder. Here the accumulation of inflammatory cells contributes to plaque instability by cytokine activation and destruction by proteolytic enzymes causing the plaque to rupture where the fibrous cap is thinnest. Atheromatous rupture and haemorrhage arises in regions of fragile newly formed microvessels within advanced plaques, which is significant in the pathogenesis of acute coronary syndromes and carotid embolisation.

It was our hypothesis that atheroma in diabetic patients with critical lower limb ischaemia demonstrates a higher level of intimal angiogenesis. Given that intimal neovascularisation is associated with increased plaque fragility and risk of complications, this could explain the greater risk of arterial events seen in the diabetic population. This study aims to investigate intimal neovascularisation and the degree of inflammation of non diabetic and diabetic patients with advanced lower limb atherosclerosis.

Methods

Ethical approval was obtained from the local research ethics committee for Central Manchester Healthcare trust.

Patients undergoing major amputation for unrecognisable, chronic lower limb arterial disease were recruited to the study. Samples were obtained from ten patients with type 2 diabetes mellitus and ten non-diabetics for comparison. Patient data was collected for demographics, concurrent medications, serum lipid profile and level of disease on colour duplex scanning. Following completion of the amputation, arterial samples were harvested from the distal superficial femoral artery or distal popliteal vessels for above and below knee amputations respectively.

Samples were fixed in formaldehyde, paraffin wax embedded and cut into 5 μm sections. Sections were stained with haematoxylin and eosin (H&E) for morphological analysis and assessment of inflammatory infiltrate. Additional sections underwent staining with immunohistochemistry for CD31 and von Willebrand Factor (vWF) to clearly visualise the endothelium and hence delineate microvessel density within the intima. Slides were counterstained with elastica van Gieson to more clearly define the internal elastic lamina, facilitating visualisation of the intima.

Sections were graded according to American Heart Association (AHA) guidelines and then viewed at 40x magnification for assessment of the number of intimal vessels per field. The number of inflammatory cells within the intima and media were also assessed. Analysis of each slide was carried out separately by four independent investigators who were blinded to the patient’s diabetic status. In each slide, evaluation of the number of intimal vessels was carried out in four different quadrants of the arterial wall. Counts were correlated with patient demographics and concurrent medications to determine any further association.

Of the 20 samples obtained for the study, 18 were suitable for analysis. Twelve specimens were from the distal superficial femoral artery (SFA) while in six patients, samples were taken from the below knee popliteal artery. Six of the samples in each group were from the distal SFA.

The two patient groups were well matched for age, co-morbidity and anti-platelet use as shown in Table 1. Non-diabetic patients had a lower prevalence of statin use (50% versus 75%). Diabetic subjects had a greater incidence of anticoagulant usage; this was primarily due to warfarin prescriptions for atrial fibrillation. The diabetic cohort also had higher serum cholesterol, HDL and triglyceride concentrations.

Data was evaluated using the Mann-Whitney U-test using StatsDirect statistical software version 2.4.1 (StatsDirect ltd, Cheshire, UK). Significance was taken at a P value of 0.05 or less. Inter-observer variability was evaluated using the Fleiss-Nee-Landis test.

Results

Two of the samples from diabetic subjects were AHA class VI plaques and had delaminated after
processing and therefore were unsuitable for analysis. All remaining intimal plaques were classified AHA grade IV or V and so were relatively homogenous in terms of severity of plaque morphology. Inter-observer variability was evaluated using the Fleiss-Nee-Landis test (where $k = 0.03$).

In both groups, an infiltrate consisting of predominantly chronic inflammatory cells was found with the diabetic subjects having a median of 40.0 cells (range 12–82) per four 40x objectives compared to 28.5 cells (range 15–67) in the controls although this difference was not significant ($P = 0.26$).

CD31 staining revealed a median of 9.5 and 2.0 vessels in diabetics and controls respectively ($P < 0.05$). vWF staining identified a greater number of vessels within the arterial intima, with a median of 11.5 vessels per four 40x objectives in diabetic patients, compared to the control specimens which had a median of 2.0 vessels ($P < 0.05$) (Fig. 1). Representative images of non-diabetic sections with sparse intimal new vessel formation are shown in Fig. 2(a,b). Typical appearances of sections of the arterial wall of diabetic patients demonstrating more prominent intimal neovascularisation are shown in Fig. 3(a,b).

Although there was a difference in the overall counts between the two staining methods (see Fig. 1), a similar degree of difference in median counts between the diabetic and the non-diabetic controls was found, regardless of the staining method used.

While the numbers in this analysis are small nonetheless, sub group analysis with regard to diabetic status and statin therapy showed that, in the diabetic group, a trend towards a greater degree of neovascularisation was present if they were medicating with a statin although this failed to reach statistical significance. A median of 16.5 vessels were seen in the statin users compared to 5.5 in those diabetics who were not taking a statin ($P = 0.07$). In the non-diabetic group, no such difference was seen with a median of 2.0 vessels per field in both patients both taking and not taking a statin ($P = 0.34$).

**Discussion**

It has been over a century since the role of intra-plaque neovascularisation in atherosclerotic plaque progression and in the development of complications was first postulated. The normal human intima is devoid of blood vessels, the cells within it obtaining oxygen and nutrients via diffusion from the arterial lumen. Angiogenesis only becomes a feature when the intima has proliferated beyond a critical thickness with these new vessels originating from capillaries within the vasa vasorum and, to a lesser degree, from the lumen itself.

The mechanism by which this occurs is poorly understood but may represent a response by plaque tissue to hypoxia and inflammation with the production of angiogenic factors such as bFGF, VEGF and platelet derived growth factor. The original concept that neovascularisation is a late feature in plaque progression has recently been challenged as intimal neovascularisation has been demonstrated within plaques of atheroma even in less advanced stages of the disease with new vessels present in lesions as early as AHA class II plaques. Diabetes is known to promote the advancement of atherosclerosis.

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**Table 1. Patient demographics**

<table>
<thead>
<tr>
<th></th>
<th>Diabetics*</th>
<th>Controls (non-diabetic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>M:F</td>
<td>5:3</td>
<td>3:7</td>
</tr>
<tr>
<td>Mean age (range)</td>
<td>70.4 (56–89)</td>
<td>69.7 (53–86)</td>
</tr>
<tr>
<td>Anti-platelet agent (%)</td>
<td>6/8 (75)</td>
<td>10/10 (100)</td>
</tr>
<tr>
<td>Anticoagulants (%)</td>
<td>4/8 (50)</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>Statin use (%)</td>
<td>6/8 (75)</td>
<td>5/10 (50)</td>
</tr>
<tr>
<td>Insulin Use (%)</td>
<td>2 (25%)</td>
<td>0</td>
</tr>
<tr>
<td>Mean serum</td>
<td>4.4</td>
<td>4.0</td>
</tr>
<tr>
<td>Cholesterol mmol/L</td>
<td>3.1 (1.3–5.7)</td>
<td>2.8 (0.9–5.6)</td>
</tr>
<tr>
<td>Mean HDL mmol/L</td>
<td>0.98 (0.7–1.4)</td>
<td>1.20 (0.9–2.1)</td>
</tr>
<tr>
<td>Mean Triglycerides mmol/L (range)</td>
<td>2.1 (1.3–3.7)</td>
<td>1.5 (1.2–1.9)</td>
</tr>
<tr>
<td>Level of disease:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- aorto-iliac</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>- femoral</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>- distal</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>Sample location:</td>
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<td></td>
</tr>
<tr>
<td>- SFA</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>- Distal popliteal</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

* All diabetic subjects were either medicating with oral hypoglycaemic agents (87.5%) or insulin. One patient was taking both insulin and an oral hypoglycaemic agent.

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**Fig. 1.** Box and Whisker plot for median number of intimal vessels per four 40x objectives.
in coronary arteries. This is thought to be the result of several mechanisms such as apoprotein and lipoprotein distribution abnormalities, a procoagulant state, insulin resistance and hyperinsulinaemia. Additionally, glycation of proteins within the plasma and arterial wall, glycation and oxidation abnormalities and also hormone, growth factor and cytokine enhanced smooth muscle proliferation and foam cell formation also play a role.

Macrophages are the most abundant inflammatory cell present in atherosclerotic lesions and are responsible for the production of the multitude of cytokines central to atherogenesis. Endothelial cell activation results in leukocyte adhesion and migration into the atherosclerotic lesion resulting in destabilisation of the plaque through the release of proteases such as those of the matrix metalloproteinase family produced by plaque macrophages. Macrophages adherent to the endothelial lining of intra-plaque blood vessels have also been demonstrated and are more prevalent in advanced plaques. The proximity of these vessels to inflammatory infiltrates and the expression of adhesion molecules on the endothelium of vessels within the plaque both suggest that this microvasculature may aid recruitment of inflammatory cells into lesions thereby starting a positive feedback mechanism. It is not surprising therefore that an increase in macrophage infiltration was found in the diabetic subjects. The diabetic patients who were on statin therapy had a greater degree of neovascularisation when compared to those not.

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Statins are thought to work in two ways. First by a lipid lowering effect which has been shown to suppress proteolytic activity, decrease endothelial cell

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Fig. 2. Control specimens with immunohistochemical staining for CD31 (a) and vWF (b) at 40x magnification. The black arrows indicate areas of neovascularisation.

Fig. 3. Diabetic patient specimen stained for both CD31 (a) and vWF (b) at 40x magnification. The black arrows indicate areas of neovascularisation.
activation and reduce oxidative stress. Second by their direct anti-inflammatory properties on plaque macrophages with resultant plaque stabilisation. However, recent studies have questioned their role in plaque angiogenesis. Statins have been proposed to have an anti-apoptotic effect on endothelial progenitor cells thereby enhancing the pro-angiogenic effects of these drugs. Further work however has demonstrated a biphasic, dose dependant effect on angiogenesis. At low dose, 10 nM serum concentration, statins were shown to be pro-angiogenic due to decreased expression of plasminogen activator inhibitor-1 and thrombospondin-1. At higher doses, 1–10 μM, the statins proved to be anti-angiogenic, possibly by diminished production of interleukin (IL)-8 and by inhibition of urokinase plasminogen activator synthesis. This may imply that although the statins could stabilise the plaque at higher concentrations by their direct anti-inflammatory effect and anti-angiogenic effects, at low concentrations they may actually increase plaque fragility by encouraging neovascularisation.

The two most commonly used markers for neovascularisation are the endothelial markers CD31 and vWF. Interestingly, the vWF stain was found to be more sensitive when visualising the neovascularisation, but gave a higher level of background staining, hence the discrepancy in the number of vessels seen with the CD31 and vWF stains.

In this patient sample, we have demonstrated that diabetic patients with critical lower limb ischaemia have a greater degree of intimal neovascularisation when compared to non-diabetic controls highlighting the importance of inflammation in later stages of atherosclerosis. Inflammation is known to be an important factor in the development of the unstable atherosclerotic plaque. This may explain the increase in complications related to atherosclerosis seen in the diabetic population with diabetes itself acting as a pro-inflammatory condition. Further work into the subject is underway, with particular focus on the role of statins in plaque angiogenesis.

The numbers in this study were small, causing difficulties in making concrete conclusions. This is especially so with regard to statin usage and the degree of neovascularisation in the diabetic population. Only two out of the eight diabetics were not taking this class of medication. Accordingly, we cannot state with confidence the significance of these results. Further experiments are required to investigate this hypothesis further. This is surprising as statins have been shown to stabilise atherosclerotic plaques and therefore it would be expected that those subjects treated with these medications would show a lesser degree of intimal neovascularisation.

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References


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