Diabetes impairs arteriogenesis in the peripheral circulation: review of molecular mechanisms

Matthijs S. RUITER, Jolanda M. VAN GOLDE, Nicolaas C. SCHAPER, Coen D. STEHOUWER and Maya S. HUIJBERTS
Department of Internal Medicine, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University Medical Centre (MUMC+), 6200 MD Maastricht, The Netherlands

ABSTRACT

Patients suffering from both diabetes and PAD (peripheral arterial disease) are at risk of developing critical limb ischaemia and ulceration, and potentially requiring limb amputation. In addition, diabetes complicates surgical treatment of PAD and impairs arteriogenesis. Arteriogenesis is defined as the remodelling of pre-existing arterioles into conductance vessels to restore the perfusion distal to the occluded artery. Several strategies to promote arteriogenesis in the peripheral circulation have been devised, but the mechanisms through which diabetes impairs arteriogenesis are poorly understood. The present review provides an overview of the current literature on the deteriorating effects of diabetes on the key players in the arteriogenesis process. Diabetes affects arteriogenesis at a number of levels. First, it elevates vasomotor tone and attenuates sensing of shear stress and the response to vasodilatory stimuli, reducing the recruitment and dilatation of collateral arteries. Secondly, diabetes impairs the downstream signalling of monocytes, without decreasing monocyte attraction. In addition, EPC (endothelial progenitor cell) function is attenuated in diabetes. There is ample evidence that growth factor signalling is impaired in diabetic arteriogenesis. Although these defects could be restored in animal experiments, clinical results have been disappointing. Furthermore, the diabetes-induced impairment of eNOS (endothelial NO synthase) strongly affects outward remodelling, as NO signalling plays a key role in several remodelling processes. Finally, in the structural phase of arteriogenesis, diabetes impairs matrix turnover, smooth muscle cell proliferation and fibroblast migration. The review concludes with suggestions for new and more sophisticated therapeutic approaches for the diabetic population.

Key words: arteriogenesis, collateral circulation, diabetes mellitus, endothelium, peripheral arterial disease, vascular smooth muscle.

Abbreviations: ACh, acetylcholine; AGE, advanced glycosylation end-product; Ang, angiotensin; BH4, tetrahydrobiopterin; BMC, bone-marrow-derived cell; BMI, body mass index; CRP, C-reactive protein; DM, diabetes mellitus; EC, endothelial cell; EDHF, endothelium-derived hyperpolarizing factor; EPC, endothelial progenitor cell; ERK, extracellular-signal-regulated kinase; FGF, fibroblast growth factor; Fk-1, fms-like tyrosine kinase-1; FMD, flow-mediated dilatation; GM-CSF, granulocyte/macrophage colony-stimulating factor; HbA1c, glycated haemoglobin; HGF, hepatocyte growth factor; HIF, hypoxia-inducible factor; ICAM, intercellular adhesion molecule; KLF-2, Krüppel-like factor-2; MCP-1, monocyte chemotactic protein-1; MI, myocardial infarction; MMP, matrix metalloproteinase; NF-κB, nuclear factor κB; NOS, NO synthase; eNOS, endothelial NOS; NTR, neurotrophin receptor; PAD, peripheral arterial disease; PAI-1, plasminogen activator inhibitor-1; PKC, protein kinase C; RAGE, receptor for AGEs; ROS, reactive oxygen species; SMC, smooth muscle cell; SOD, superoxide dismutase; STZ, streptozotocin; TGF-β, transforming growth factor-β; TIMP, tissue inhibitor of metalloproteinases; TNF-α, tumour necrosis factor-α; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor; WT, wild-type.

Correspondence: Mr Matthijs S. Ruiter (email m.ruiter@intmed.unimaas.nl).
PAD (PERIPHERAL ARTERIAL DISEASE) IN DM (DIABETES MELLITUS)

DM is recognized as a major cardiovascular risk factor. PAD is a common vascular complication in the diabetic population, as diabetes increases the risk of developing PAD at least 2-fold [1–3]. Patients suffering from both DM and PAD exhibit poor lower extremity function and are at risk of developing critical limb ischaemia and ulceration, and potentially requiring limb amputation [4,5]. In Type 2 DM, PAD has a more distal and generalized manifestation [6,7]. PAD patients with DM are typically younger, with higher BMI (body mass index) and more neuropathy, and exhibit a greater number of cardiovascular co-morbidities compared with patients without diabetes. PAD impairs survival with a 2–3-fold increased risk of 5–10-year mortality [8]. Mortality for PAD patients is even higher in the presence of DM [9–11]. Diabetes is also known to complicate the treatment of PAD. DM patients have a less favourable outcome after leg bypass surgery, a higher incidence of restenosis, more surgical complications, longer hospitalization and a lower amputation-free survival [9,10,12,13].

A natural adaptive response to obstructed blood flow in a conducting artery is outward remodelling of pre-existing anastomoses. In this process, termed arteriogenesis, blood flow to the tissue distal to an occlusion can largely be restored. The sprouting of capillaries in response to tissue ischaemia, a process called angiogenesis, also occurs, but is not sufficient to restore flow to the distal part of the lower extremities [14]. However, although angiogenesis is involved in PAD and impaired wound healing in DM, it is beyond the scope of the present review. In arteriogenesis, the presence of DM limits the amount of collateral development and the adaptive response to blood flow obstruction [15]. Type 2 DM attenuates recruitment and functional outward remodelling of pre-existing collateral arterioles, demonstrated clinically in the coronary circulation [16,17] and experimentally in the lower extremities [18–20]. The impairment of arteriogenesis by Type 1 DM appears to be less severe [18].

Currently, several strategies to promote arteriogenesis in the peripheral circulation have been devised. Although some studies have targeted the diabetic collateral circulation, the mechanisms through which DM impairs arteriogenesis are poorly understood. The present review provides an overview of the current literature on the deteriorating effects of DM on the collateral circulation and on the different phases of arteriogenesis.

VASCULAR DYSFUNCTION IN DIABETES

Type 1 and 2 DM are two distinct conditions, but, in respect to vascular function, they share several mechanisms, which are addressed in a number of reviews [21–24]. The most important shared factors seem to be hyperglycaemia, oxidative stress, formation of AGEs (advanced glycosylation end-products) and PKC (protein kinase C) production. In addition, in Type 2 DM, the constant state of low-grade inflammation of the endothelium affects vascular function, and may play an important part in the aetiology of the disease [25,26]. Furthermore, Type 2 DM is associated with several imbalances, including dyslipidaemia and hypertension, which also affect vascular structure and function [24,27,28]. The diabetic artery displays a change in phenotype and function of the endothelium and smooth muscle, and an altered structure and composition of the extracellular matrix compared with the non-diabetic artery. As a result, the diabetic artery in general has a decreased wall/lumen ratio and a stiffer vessel wall compared with the non-diabetic artery [21,23,24,27]. Evidently, the effect of DM on vessels varies with size, region and function [24,29].

ARTERIOGENESIS IN THE PERIPHERAL CIRCULATION

The functional outward remodelling of pre-existing anastomoses starts after blood flow obstruction in an artery. In experimental models, the process takes 4 weeks, after which a number of pre-existing collateral arterioles are remodelled into conducting arteries [30]. When an arterial occlusion becomes manifest, blood takes the path of lowest resistance, through the pre-existing collateral anastomoses, increasing local blood flow in these vessels up to 200-fold [31]. The process is extensively described in a number of reviews [30,32–34]. After remodelling, the collateral artery is barely distinguishable from a normal artery, except for slightly higher collagen content between the SMC (smooth muscle cell) layers [31,35,36]. Collateral vessels grow from 30 to 50 μm in diameter to almost a 20-fold increase, and typically present a tortuous geometry [34,36]. Notably, this dramatic remodelling does not restore conductance to the initial level. Without intervention, the conductance of the collateral circulation reaches up to 50 % of the unobstructed artery [33,37].

EFFECTS OF DIABETES ON ARTERIOGENESIS

Impairment of arteriogenesis in the lower extremity by DM has been established by several studies, both in Type 1 DM [18] and Type 2 DM [15,18,20,38], but the exact mechanisms have not yet been clarified. In the following sections the effect of DM on the subsequent processes of recruitment and outward remodelling are addressed. A schematic overview of these findings is shown in Figure 1.
Acute phase

Several studies have investigated the acute phase of arteriogenesis, comprising sensing of shear stress, endothelial activation and subsequent vasodilatation.

Sensing of shear stress

The increase in flow through the collateral circulation following arterial obstruction induces hydrostatic pressure, cyclic strain and turbulent wall shear forces in the collateral vessels [31]. Shear stress on the endothelium has been identified as the main trigger for arteriogenesis [35,39,40] and, although sensing of fluid shear stress seems to be impaired in diabetes [41,42], research in this area is hampered by an incomplete understanding of the underlying mechanism. Several mechanisms have been proposed by which the vessel wall senses shear stress. Integrins, adhesion molecules, receptor tyrosine kinases, caveolae and ion channels link the internal cytoskeleton to the extracellular matrix and may serve as mechanoreceptors [43]. Common promoter elements responding to shear stress have been identified in several genes, including ICAM-1 (intercellular adhesion molecule-1), TGF-β (transforming growth factor-β) and eNOS (endothelial NOS (NO synthase)) [40,43,44].

Many pro-inflammatory stimuli inhibit KLF-2 (Krüppel-like factor-2), which plays a central role in the downstream signalling of shear stress [45]. As diabetic vessels are in a constant state of inflammation, KLF-2 inhibition may account at least in part for the decrease in shear sensing in DM. This state of inflammation also decreases eNOS steady-state mRNA [46], which is not only involved in shear signalling, but also in other stages in remodelling [47]. In addition, Woo et al. [41] have demonstrated that AGEs and ROS (reactive oxygen species) can lead to post-translational modification of ERK (extracellular-signal-regulated kinase)-5, resulting in reduced flow-induced activation of KLF-2. This may play a role in diabetes.

Another possible sensor for shear stress is the glyocalyx. This luminal lining of the endothelium, consisting of membranous glycoproteins, proteoglycans and associated plasma proteins, was proposed to serve as a
mechanosensing entity [42,48]. Hyperglycaemia reduced the glyocalyx content and decreased shear-induced dilatation, without affecting ACh (acetylcholine)-induced dilatation [42,48]. It is uncertain whether hyperglycaemia also plays a role in peripheral collaterals. Additionally, primary cilia on the endothelium, containing polycystin-1 for function and polaris for structure, play a role as antennae of the endothelial cell, sensing changes in shear stress and regulating vascular tone via NO production and Ca²⁺ signalling [49]. The effect of DM on primary cilia is not yet clear and the presence of cilia has to be confirmed in collateral arteries [32].

In addition to shear forces on the ECs (endothelial cells), changes in blood flow may exert cyclic stretch on the SMC, which induces vascular remodelling via several mechanisms involving eNOS, PKC and NF-κB [32]. The presence of cilia has to be confirmed in collateral arteries [32].

Endothelial activation

Prolonged exposure to shear stress leads to vasodilatation and activation of the endothelium [40,51]. Endothelial activation starts with the opening of Cl⁻ channels, increasing endothelial cell volume and permeability. In addition, eNOS expression and activation increase, mediating several processes in outward remodelling [52,53]. Within 12 h after ligation, expression of adhesion molecules [ICAM and VCAM (vascular cell adhesion molecule) and MCP-1 (monocyte chemotactic protein-1)] are up-regulated. Importantly, the diabetic vasculature is already in a state of inflammation, leading to elevated expression of ICAM, VCAM and E-selectin. Additionally, PKC, TNF-α (tumour necrosis factor-α) and NF-κB are present in higher concentrations as compared with the non-diabetic situation [21,54,55]. It is likely that this activated and inflammatory state affects the response to shear stress, but the extent to which this occurs is presently unclear. Although the activation of the endothelium and the onset of inflammation have not specifically been studied in diabetic arteriogenesis, production of MCP-1 has been investigated.

An important step in the activation of the collateral endothelium is the attraction of monocytes by MCP-1. Administration of MCP-1 increased post-ischaemic collateral conductance in healthy [35] and hyperlipidaemic [56] rabbits, but this has not yet been shown in diabetic animals. However, MCP-1 also plays a key role in the development of atherosclerosis [57]. Plasma MCP-1 levels are associated with traditional risk factors for atherosclerosis and with cardiovascular disease mortality [58,59]. In Type 2 DM patients, circulating MCP-1 levels are increased [60]. The MCP-1 levels correlate with blood glucose, HbA₁c (glycated haemoglobin), triacylglycerols (triglycerides), BMI and CRP (C-reactive protein) [54]. In cultured ECs, high glucose induces MCP-1 expression, a process mediated by ROS, NF-κB and PAI-1 (plasminogen activator inhibitor-1) [54]. Accordingly, experimental Type 1 DM elevates MCP-1 production from mast cells both under normoxic and hypoxic conditions [61]. In addition to the increase in MCP-1 levels, the expression of the MCP-1 receptor CCR2 (CC chemokine receptor 2) on monocytes is elevated in patients with DM [57]. It is therefore not likely that the attraction of monocytes is impaired in DM. Monocyte migration and receptor signal transduction are discussed later on in the present review.

Vasoreactivity

The influence of diabetes on endothelial function has been the topic of many studies, both clinical and experimental. However, the term endothelial function does not distinguish between ACh-mediated dilatation and FMD (flow-mediated dilatation). Although many clinical studies agree that ACh-induced dilatation is reduced in Type 2 DM [22,24,62], FMD appears to be either impaired [63] or unchanged [24,27,29,64]. Endothelial function in Type 2 DM patients is correlated with plasma CRP and TNF-α [64,65]. In addition, determination of the effect of DM itself in clinical studies of Type 2 DM is hampered, as endothelial function is affected by concomitant obesity, dyslipidaemia and hypertension [24,28]. In clinical research studying Type 1 DM, symptoms are less pronounced. The results vary from unchanged to decreased ACh-mediated dilatation. Furthermore, the majority of studies suggest unchanged FMD [24,27,66,67], although impaired FMD has also been observed [68]. Similar to the effects of DM on the endothelium, NO sensitivity in SMCs was reported to be decreased [62,69,70] or unchanged [24,27,66,67,69] in DM.

In animal models of diabetes, a decrease in endothelium-dependent vasodilatation is well established. The majority of these models represent Type 1 DM, in which insulin production is diminished by STZ (streptozotocin) or alloxan injection. In mesenteric arteries, abdominal aorta and thoracic aorta, chronic hyperglycaemia consistently reduced ACh-mediated vasorelaxation [24,71–73]. In femoral and mesenteric arteries, experimental DM impaired the NO/cGMP pathway of relaxation, but not the EDHF (endothelium-derived hyperpolarizing factor) pathway [74]. Wigg et al. [75], however, demonstrated that STZ-induced DM reduced the EDHF-dependent relaxation of mesenteric arteries, but not the NO-dependent relaxation of femoral arteries in rats. Overall, in these models of Type 1 DM, NO sensitivity remains unaffected, indicating the impairment is localized in the endothelium rather than the SMCs in these vascular beds [24]. It should be noted, however, that disease duration affects the outcome, as demonstrated in a study by Pieper [76] in which sensitivity to ACh increased shortly after chemical
induction of diabetes, but decreased after several weeks. Similar to ACh-induced relaxation, flow-induced vasorelaxation in mesenteric resistance arteries was attenuated in diabetic rats compared with non-diabetic littermates [77]. Vasodilatation in DM animal models was improved by L-arginine supplementation [78], eNOS gene transfer [79] and supplementation with the eNOS cofactor BH4 (tetrahydrobiopterin) [80]. Furthermore, in accordance with clinical studies, vasomotor function was related to HbA1c [81,82]. TNF-α, up-regulated in inflammation and DM, inhibits vasorelaxation both ex vivo [83] and in vivo [84].

In leptin-receptor-deficient Lepr<sup>db/db</sup> mice, an experimental model for Type 2 DM, ACh-induced vasorelaxation was decreased compared with WT (wild-type) or normoglycaemic Lepr<sup>Db/db</sup> control mice. This difference was established consistently in aorta rings, mesenteric arteries and coronary arterioles [85–90]. Administration of SOD (superoxide dismutase), anti-TNF-α antibodies or PKCβ inhibitors partially restored the impaired relaxation [85,86,88–90]. These results indicate roles for ROS, inflammation and PKC respectively. In addition, diabetes reduced FMD in coronary arterioles [86,88]. However, DM did not alter eNOS expression in the aorta and mesenteric arteries [87,90]. eNOS enzymatic activity requires several co-factors, including BH4, and is impaired in experimental insulin resistance [91]. In Lepr<sup>db/db</sup> mice, decreased BH4 availability resulted in uncoupling of eNOS, an impairment which could be restored by exogenous BH4 administration [89]. Uncoupling of eNOS results in production of superoxide rather than NO by NOS [92]. In clinical Type 2 DM, BH4 administration had the additional advantage of increasing insulin sensitivity [93]. Endothelium-independent vasorelaxation, in response to an NO donor, was unaltered [89] or decreased [87,88,90] in Lepr<sup>Db/db</sup> mice compared with control mice.

In addition to vasorelaxation, DM affects vasoconstriction. The production of vasoconstrictor prostanoids by the endothelium, causing the SMCs to contract, is enhanced by hyperglycaemia and by oxygen-derived free radicals in the endothelium [94,95]. In addition, increased levels of ET-1 (endothelin-1), a potent endothelium-borne vasoconstrictor, are found in DM, and even at the pre-diabetic insulin-resistant vasculature [96,97]. The increased tone was confirmed in several animal studies. In experimental Type 1 DM, α-adrenergic tone of the iliac artery was increased compared with non-diabetic controls [98]. In addition, noradrenaline-induced contraction was elevated by DM in skeletal muscle arterioles [72], and pressure-sensitive myogenic tone was enhanced in rat mesenteric and gracilis muscle by DM [72,99]. This may be due to increased activation of voltage-dependent Ca<sup>2+</sup> channels and/or PKC in SMCs. In Lepr<sup>db/db</sup> mice, vasomotor tone and sensitivity to constrictive stimuli were slightly increased [89,90].

The effects of DM on vasoreactivity in the lower extremities have been less extensively studied. In lower extremities of DM patients, ACh-induced and SNP (sodium nitroprusside)-induced vasodilatation was reduced compared with control subjects, a difference most pronounced in the presence of neuropathy [62]. In experimental DM, an impaired response to ACh was demonstrated in skeletal muscle arterioles of the STZ-treated rat hindlimb [82,100]. In addition, these arterioles demonstrated decreased NO sensitivity [82]. In iliac arteries, STZ-induced DM reduced NO sensitivity [98]. To summarize, although current literature is not conclusive about the exact mechanisms of impaired vasoreactivity in the diabetic vasculature, DM seems to increase constriction and decrease dilatation, reducing the recruitment and dilatation of collateral arteries. The locations and mechanisms of impairment are dependent on the type of DM, the presence of co-morbidities, the experimental model used and the vascular bed.

After activation and dilatation of the collateral arteries, the outward remodelling is directed further by circulating cells, growth factors and NO signalling.

**Circulating cells**

Attraction and invasion of monocytes into the vessel wall is the next important step in arteriogenesis [35,101]. In addition to monocytes, EPCs (endothelial progenitor cells) and other BMCs (bone-marrow-derived cells) have been investigated.

**Monocytes**

In a rabbit hindlimb ligation model, monocytes from alloxan-induced diabetic animals showed a reduced migratory response to both VEGF (vascular endothelial growth factor)-A and MCP-1, compared with monocytes from normoglycaemic animals [19]. In a coronary arteriogenesis study, monocyte chemotaxis by VEGF was shown to be reduced in Type 2 DM patients. As VEGF receptor-1 (Flt-1 (fms-like tyrosine kinase-1)) activity seemed unchanged, the authors suggest the defect is downstream in the VEGF signalling [102]. Monocyte chemotaxis in response to Flt-1 activation involves PI3K (phosphoinositide 3-kinase) and Akt, or the MAPKs (mitogen-activated kinases) p38 and ERK1/2. In monocytes of DM patients, phosphorylation of ERK1, Akt and p38 is higher than in controls [103]. In addition, monocytes from DM subjects express more RAGE (receptor for AGEs) protein, potentially making them more sensitive to AGEs [103]. These changes result in impaired, but not absent, receptor signalling in DM monocytes, which contributes to impaired remodelling [104].
EPCs

Similar to monocytes, EPCs are found in the remodelling vessel wall. EPCs, which are primarily involved in vasculogenesis and angiogenesis, are also believed to play a role in arteriogenesis by invading the remodelling wall and differentiating into ECs. However, the relevance of these cells in the remodelling vessel wall, and the extent to which they are able to assume endothelial characteristics in vivo, has been disputed [105], but, although the role of EPCs in arteriogenesis seems small, they may still provide therapeutic opportunities [51]. Both Type 1 and 2 DM are associated with decreased EPC number and function [106]. In a clinical study, a higher number of circulating EPCs was associated with more coronary collateral development [107]. In experimental research, the number of EPCs correlates with the severity of ischaemia and capillary density in the hindlimb [108]. More specifically, there is an inverse relationship between diabetes duration and number of EPCs in ischaemic hindlimb [108,109]. DM patients with PAD display a reduction in EPCs compared with DM patients without PAD. EPCs from diabetic PAD patients have a 35 % reduced capacity to adhere to mature ECs than EPCs from DM patients without PAD [109]. Moreover, CD34+ circulating cells produce fewer ECs in Type 1 DM patients [10]. In accordance with these clinical data, DM mice had suppressed EPC mobilization following hindlimb ischaemia [18,106,111–113]. More specifically, there is an inverse relationship between diabetes duration and number of EPCs in ischaemic tissue [108]. Furthermore, diabetic EPCs have a reduced angiogenic capacity, decreased eNOS expression, and more pro-inflammatory and have impaired integration [18,106,113,114]. In spite of these results, the mechanisms by which DM impairs EPC function remain largely unknown. In vitro, high glucose and TNF-α dose-dependently reduced the number of EPCs [115]. In cultures of CD34+ cells, more ECs were derived from non-diabetic subjects compared with Type 1 DM patients [110]. In vivo, reduced EPC performance can partially be explained by oxidative stress [116] and by eNOS uncoupling, which was shown to affect EPC function and mobilization [106,112]. To counter the impaired EPC response, non-diabetic EPCs were administered into DM hindlimbs following ischaemia, accelerating blood flow restoration [110]. Besides administration of high glucose and TNF-α, the vitamin B1 analogue benfotiamine or statins prevented the DM-induced decrease in circulating EPCs in mice subjected to limb ischaemia [113,117]. Similarly, insulin and G-CSF (granulocyte colony-stimulating factor) partially restored defective EPC mobilization in DM rats after ischaemia/reperfusion injury [118].

Other BMCs

Complementary to EPC and monocyte research, several studies have aimed at investigating BMCs. BMC implantation in experimental research improves post-ischaemic perfusion recovery, de-ambulatory impairment and ischaemic damage in both diabetic and control animals [119], unless the BMCs originate from DM patients. These diabetic BMCs increase arteriolar density and angiographic score to a lesser extent than non-diabetic BMCs [108]. In addition, BMCs from obese diabetic Zucker rats have less VEGF production, EC differentiation and EC colony-forming potential than BMCs from lean rats [120]. In contrast, VEGF production from BMCs was not affected by STZ-induced diabetes in rats [119]. Finally, DM decreased adhesion of BMCs to ECs and BMC-induced SMC recruitment in mice [108].

In summary, although the attraction of monocytes is not decreased in DM, downstream signalling seems to be impaired in monocytes from diabetic patients. A number of studies have demonstrated that both EPC and BMC function is attenuated by DM, but presently the extent to which these impairments affect arteriogenesis remains unclear.

Growth factors

After transformation to macrophages, monocytes produce growth factors. In addition, platelets adhere and produce IL-4 (interleukin-4), increasing adhesion molecule expression [51]. Numerous growth factors are involved in arteriogenesis, including MCP-1, VEGF, FGF (fibroblast growth factor), HIF (hypoxia-inducible factor), GM-CSF (granulocyte/macrophage colony-stimulating factor), HGF (hepatocyte growth factor), TNF-α, TGF-β and PDGF (platelet-derived growth factor) [34,51]. Administration of some of these growth factors was effective in improving arteriogenesis in a non-diabetic model [51,121].

In arteriogenesis, VEGF up-regulates adhesion molecules on the endothelium, produces MCP-1 and induces the proliferation of ECs and SMCs [16]. As described above, DM impairs VEGF-A signalling in monocytes. Serum VEGF-A levels in the patients from that study were increased [102]. This increase was confirmed in another study [122], but unchanged [123,124] or decreased [125] VEGF levels in DM have also been reported. In experimental DM, levels of Akt, eNOS and cGMP, downstream effectors of VEGF, were lower in the hindlimb of Type 2 DM animals [126]. In addition, Leprdb/db blunts the up-regulation of VEGF after femoral artery ligation [38]. In accordance, STZ-induced DM reduced VEGF production in the mouse ischaemic hindlimb [111,127]. Promotion of VEGF transcription restored this impaired post-ischaemic flow restoration by restoring Akt and eNOS levels, and by increasing EC proliferation and survival [127]. In the hindlimb, Type 2 DM reduces the VEGF receptor Flt-1. Following hindlimb ischaemia, DM animals exhibit higher Flt-1 expression than non-diabetic mice [126]; however, this does not lead to improved VEGF-induced arteriogenesis [126,128]. Notably, although DM reduces
VEGF-induced arteriogenesis, it promotes VEGF-mediated angiogenesis in capillary beds, as seen in retinopathy and plaque destabilization [128,129]. Waltenberger [104] provides an explanation for this paradox, stating that short-time stimulation of outward remodelling is decreased by a state of VEGF resistance, via the non-specific pre-activation of intracellular pathways. Long-time exposure to the angiogenic factor enhances neovascularization, despite the poor response.

The function of FGF-2, which stimulates EC and SMC proliferation, is impaired by STZ-induced DM in the remodelling hindlimb artery [130,131]. In vitro, hyperglycaemia decreased the mitogenic and chemotactic activity of FGF-2 in a time- and dose-dependent manner [132]. Glycation of FGF-2 decreased receptor binding, ERK phosphorylation and angiogenic activity. In vivo, this impairment of FGF-2 was demonstrated in diabetic mice [132]. Administration of FGF-2 in the murine DM ischaemic hindlimb increased perfusion, capillary density and mature vessel density [130,131]. Combination therapy of FGF-2 with a vasodilator magnified the improvement. This was demonstrated with both prostaglandin E1 and the 5-HT (5-hydroxytryptamine) receptor blocker sarpogrelate [130,131].

HIF-1α is an important factor in capillary sprouting in response to tissue ischaemia, but also plays a role in outward remodelling of arterioles. Levels of HIF-1α are decreased in Type 2 DM patients [133]. HIF-1α up-regulation in response to ischaemia/reperfusion injury was attenuated by experimental DM [118]. In agreement, remodelling induced by HIF-1α was shown to be impaired in experimental diabetic arteriogenesis [110]. Adenoviral HIF-1α administration restored impaired eNOS and Akt expression in Leprdb/db mice [134]. It was demonstrated that DM-induced methylglyoxal reduced HIF-1α activity, leading to decreased eNOS, VEGF and SDF-1 (stromal cell-derived factor-1) gene expression following ischaemia. This defect could be restored by the antioxidant SOD [135].

HGF stimulates EC growth without affecting the SMCs, restoring perfusion and angiographic score in experimental hindlimb ischaemia [136]. In vitro, incubation with high glucose reduced HGF mRNA and protein. Accordingly, HGF levels were lower in the ischaemic hindlimb of DM animals compared with normoglycaemic animals. The reduced perfusion restoration and angiographic score in Type 1 DM animals could be normalized by HGF gene transfer [136].

The up-regulation of GM-CSF mobilizes monocytes and their progenitors from bone marrow into the blood, and provides a stable inflammatory environment for the monocytes to adhere, invade and produce more factors. Locally, GM-CSF clearly increases collateral conductance by reducing monocyte apoptosis and extending the life cycle of monocytes and macrophages [51]. In Type 1 and Type 2 DM patients, intravenous injection of recombinant GM-CSF combined with local injections of peripheral blood mononuclear cells decreased lower limb pain and ulceration. Blood perfusion, angiographic score and ankle-brachial pressure increased compared with non-treated DM patients. Notably, heparin was administered during treatment to reduce the risk of embolism [137].

Overall, DM impairs the release and signalling of several growth factors involved in arteriogenesis. Both clinically and experimentally, this resulted in attenuated restoration of lower limb perfusion.

**NO signalling**

A protein that is activated early in the process of outward remodelling and continues to play a role during most of the processes is eNOS. The expression of eNOS is increased 6-fold in developing collateral arteries [138]. eNOS is essential in blood flow restoration and collateral outward remodelling [139], but not in formation of capillaries [140]. This is confirmed by a study in eNOS-knockout mice in which impaired arteriogenesis was restored by adenoviral eNOS administration [47]. In addition, inhibition of eNOS with L-NAME (N⁵-nitro-L-arginine methyl ester) 3 days after the onset of hindlimb ischaemia resulted in decreased blood flow recovery and smaller collateral artery diameter [139]. DM is known to affect eNOS. In experimental models, eNOS-mediated NO release was decreased in STZ-induced diabetic mice [18] and rats, but not until 12 weeks of hyperglycaemia [119]. Similarly, Leprdb/db mice had diminished eNOS expression [18] and phosphorylation [141]. In addition, up-regulation of eNOS and Akt in response to MI (myocardial infarction) was blunted by Leprdb/db [142]. It seems that hindlimb ischaemia reduced further eNOS expression in experimental Type 2 DM, but not in experimental Type 1 DM [18]. Clinically, it has been shown that DM patients with neuropathy exhibited decreased eNOS expression in the lower extremities compared with healthy subjects, regardless of the absence or presence of macrovascular disease [62]. Recently, another eNOS-related mechanism in the diabetic ischaemic hindlimb has been presented. p75NTR (p75 neurotrophin receptor), which is barely present in healthy ECs, becomes strongly expressed by capillary ECs after induction of peripheral ischaemia in STZ-induced diabetic mice. Expression of p75NTR impairs the survival, proliferation, migration and adhesion capacities of cultured ECs and EPCs in vitro and impairs blood flow recovery in vivo, via the Akt/eNOS pathway [143]. Antagonism of p75NTR in ischaemic muscle inhibited EC apoptosis, normalized EC proliferation and restored blood flow recovery in DM mice. As receptor antagonism had no effect on normoglycaemic ischaemic muscle, this appears to be a mechanism specific for the diabetic hindlimb [143]. In summary, DM impairs eNOS function,
which affects the process of arteriogenesis on numerous levels.

**Structural phase**

At 1 week after the onset of outward remodelling, the structural phase of arteriogenesis commences. After degradation of the extracellular matrix, the proliferation and migration of ECs and SMCs ultimately lead to maturation of the collateral artery.

**Matrix turnover**

MMPs (matrix metalloproteinases) and their inhibitors [TIMPs (tissue inhibitor of metalloproteinases)] regulate turnover and remodelling of the extracellular matrix. In arteriogenesis, the external elastic lamina and elastin are broken down by MMPs and plasmin, creating room for the expanding vessel [51]. In addition, MMPs promote SMC migration. During remodelling, MMP-2, MMP-9 and TIMP-1 are up-regulated in the intima. PAI-1 protects from excess proteolysis [144]. The balance between MMPs and TIMPs is essential in both the maintenance and remodelling of the vessel wall. Experimental DM impairs this balance during arteriogenesis [110]. In cell culture, hyperglycaemia inhibits expression and activity of MMP-1, MMP-2 and MMP-9 in ECs and SMCs [145]. In rats, STZ-induced DM amplifies the hindlimb ischaemia-induced up-regulation of MMP-2 and MMP-9, and suppresses the increase in TIMP-1 [111]. In contrast, Lepr<sup>db/db</sup> blunted the ischaemia-induced up-regulation of MMP-2, MMP-12 and MMP-16 in the murine hindlimb [38]. In another study, hyperglycaemia reduced the activation of MMP-1, MMP-2, MMP-3 and MMP-13 by promoting HGF and AGE accumulation. This process hampers remodelling of the vessel wall [144]. For clinical practice, it is important to realise that MMP transcription is strongly affected by glucose levels and oxidative stress. In well-controlled Type 2 DM patients, macrophage-derived MMP and TIMP levels were not affected [144].

**Proliferation, migration and maturation**

During the late phase of arteriogenesis, ECs and SMCs proliferate and migrate [35]. SMCs account for a large part of the production of new tissue, changing their phenotype from a contractile to a synthetic and proliferative one [32]. Not much is known about this process in the lower extremity circulation, in contrast with the field of MI. In remodelling coronary arteries, SMCs change not only to a synthetic phenotype, but exhibit an embryonic protein expression pattern [36]. In experimental MI, Lepr<sup>db/db</sup> diabetes blunted the up-regulation of Tie-2, the receptor for Ang (angiotensin) I, which promotes SMC recruitment and was shown to be pivotal in vessel maturation. Decreased capillary, but not arteriolar, density could be restored by adenoviral AngI administration [142]. Additionally, DM-enhanced AngII, identified as a vessel-distal promoter of vessel regression in MI [142]. Moreover, Lepr<sup>db/db</sup> mice had decreased SMC coverage in the infarcted myocardial area compared with WT mice, a difference which could be normalized by HIF-1α administration [134]. It is probable that these factors also play a role in peripheral arteriogenesis.

In the microcirculation, pericytes regulate EC survival, proliferation and migration by cell–cell and paracrine signalling. Although the relevance of pericytes in arteriogenesis has not yet been established, their presence in collateral arteries has already been confirmed [51]. It was suggested that pericytes may give rise to SMCs, but this is not entirely clear [51]. Pericytes can also originate from bone marrow [146]. Hyperglycaemia, ROS and AGEs promote pericyte apoptosis [106]. Therefore, if they play a role in lower extremity arteriogenesis, DM may impair pericyte function.

During the maturation phase, ECs and SMCs are arranged in an orderly manner and cell–cell contact is established. Elastin and collagen synthesis takes place, adding extra layers to the vessel [32,34]. FGF-2 stimulates fibroblast maturation [35]. Migration of fibroblasts is markedly impaired in Lepr<sup>db/db</sup> [147]. Cultured diabetic fibroblasts have elevated levels of pro-MMP-9 and MMP-9, but not of MMP-2 compared with WT fibroblasts. In both normoxic and hypoxic conditions, fibroblasts from Lepr<sup>db/db</sup> mice have decreased VEGF production compared with WT fibroblasts [147]. The final phase of arteriogenesis consists of the pruning of vessels that are eliminated in competition for flow [32].

Although DM seems to affect matrix turnover, fibroblast function and the proliferation and migration of ECs and SMCs in collateral arteries, it is questionable whether the factors involved in the late phases of arteriogenesis play a decisive role in the impairment by DM.

**THERAPEUTIC CONSIDERATIONS**

Arteriogenesis is a tightly orchestrated process. Although numerous studies have started to unravel the many pathways involved, it has become clear that stimulation or inhibition of a single factor may not be sufficient to influence the outcome. This was demonstrated in a number of studies concerning growth factor therapy. Levels of VEGF, FGF, HIF1α and HGF are decreased by DM in the ischaemic hindlimb, and administration of these factors improved arteriogenesis in experimental models [110,126,130,131,136]. In clinical practice, however, results of growth factor administration have been disappointing [148,149]. A possible explanation is the narrow therapeutic time frame and limited duration of the effect [150]. Additionally, in translating experimental research to clinical therapy, it should be considered that most experimental models are based on acute induction...
of ischaemia, whereas the progress in patients follows a more gradual course. Importantly, the molecules responsible for structural outward remodelling are involved in multiple processes throughout the body. Growth factor treatment can therefore lead to detrimental side effects, such as proliferative retinopathy, oedema, plaque destabilization and tumour growth [129,151,152].

In addition to growth factor signalling, DM affects monocytes. The impairment of monocyte function in DM appears to be in the downstream signalling rather than in the production and mobility. This may, however, not easily be corrected in a clinical setting. Other factors discussed in the present review include EPCs and other BMCs. Although many studies have investigated these factors, their role in arteriogenesis or the effect of DM has not yet been established to the extent that they can be translated to therapy. The same holds true for MMPs, fibroblasts and pericytes. Moreover, the latter factors and cells play a role in the structural phase of arteriogenesis. It is not likely that an intervention in the later stage greatly enhances outward remodelling.

Earlier in the remodelling process, eNOS may provide an interesting target for therapy. eNOS plays an important role both in the sensing of shear stress and in vasoreactivity. Impairments in eNOS levels or function in DM have been demonstrated [62,119,142]. The increase in eNOS potentiates arteriogenesis on a number of levels, but, as its function is not limited to arteriogenesis, this may affect other processes. Another interesting therapy may be provided by vasodilator therapy. Administration of a vasodilator may stimulate recruitment of collateral arteries, thereby enhancing shear stress on the vessel wall. Moreover, vasodilator therapy is not associated with the side effects found in growth factor therapy. In non-diabetic models, the potential of vasodilator therapy in revascularization has already been demonstrated [153]. A multi-level approach may be even more beneficial. Recent studies have demonstrated that combination therapy of FGF-2 with a vasodilator restored the impaired arteriogenesis in diabetic mice [130,131].

FUTURE PROSPECTS

In the development of therapy aimed at promoting arteriogenesis, the first challenge is to find a combination of factors that stimulate outward remodelling, without impairing arteriogenesis. On the basis of the present results, the combination of a vasodilator with one or possibly more growth factors may be effective. The appropriate method of administration may depend on the mechanism and possible side effects of the factor. Local administration, targeted delivery or gene transfer may prevent systemic adverse events; however, surgical interventions should be kept to a minimum, as DM is associated with surgical complications and longer hospitalization.

CONCLUSIONS

In conclusion, (i) DM increases the risk of developing PAD, complicates treatment and impairs arteriogenesis; (ii) DM elevates vasomotor tone and attenuates the sensing of shear stress, and increases the response to vasodilatory stimuli, reducing the recruitment and dilatation of collateral arteries; (iii) DM impairs the downstream signalling of monocytes, without decreasing attraction, and this could be detrimental in peripheral arteriogenesis; (iv) EPC and BMC function is attenuated in DM, but the extent to which this is relevant to arteriogenesis is presently unclear; (v) in diabetic arteriogenesis, growth factor signalling is impaired, and although these defects could be (partially) restored in animal experiments, clinical results were disappointing; (vi) NO signalling plays a key role throughout the remodelling process, and DM-induced eNOS impairment may therefore explain a large part of the attenuated outward remodelling in DM, making it an interesting therapeutic target; (vii) in the structural phase of arteriogenesis, DM impairs matrix turnover, SMC proliferation and fibroblast migration, but the extent to which these changes in the later phases of remodelling affect arteriogenesis remains uncertain; and (viii) therapy for improvement of arteriogenesis in DM should have a multi-level effect and aim at the early phase of remodelling.

REFERENCES


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