Impaired collateral recruitment and outward remodelling in experimental diabetes

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Objective. In this study the effect of chronic hyperglycemia on acute ligation induced collateral vasodilation, monocyte chemotaxis and on structural outward remodelling of collaterals was investigated.

Research design and Methods. Femoral artery ligation was performed 8 weeks after alloxan or saline treatment in New Zealand White rabbits. Angiography was performed directly, one week and three weeks after ligation. These angiographic recordings were used to quantify number of collaterals, lumen and blood volume index. Reactive hyperaemia response was tested by intramuscular Laser Doppler measurements. Subsequently blood was sampled from the aorta for monocyte chemotaxis.

Results. Ligation resulted in markedly lower acute collateral vasodilation in diabetic compared to control rabbits. Also hyperaemic vasodilatory response to local ischemia was impaired in diabetic rabbits. This difference persisted at one and three weeks after ligation, with lower number of visible collaterals. In addition the collateral lumen was markedly lower in diabetic rabbits after the maturation phase. Likewise a reduced blood volume index in the region of growing collaterals was observed in diabetic animals. The monocyte migration towards VEGF-A and MCP-1 was strongly reduced in diabetic rabbits.

Conclusions. This study demonstrates that chronic hyperglycemia negatively affects the different phases of arteriogenesis: a) impaired shear induced vasodilatation, b) impaired outward collateral growth, reflected in the number of collaterals and blood volume index, c) inhibition of monocyte chemotaxis. Impairments were most evident in the acute phase of arteriogenesis. Therapies aimed at restoring acute collateral recruitment such as vasodilators may be of interest to improve collateral function in diabetes.
Impaired arteriogenesis in diabetic animals

Individuals with diabetes have a substantially increased risk (2-4 fold) of developing ischemic cardiovascular events, with a poor prognosis following these events (1), as illustrated by an increased incidence of critical limb ischemia and lower extremity amputation in diabetic individuals (2; 3). This poor clinical outcome may be caused by impaired compensatory responses in the setting of acute or chronic ischemia in diabetes, such as reduced vasodilation and delayed collateral remodelling. Cardiovascular disease places a high burden on economic reserves, medical capacities and the quality of life in diabetic patients, stressing the importance of unravelling the underlying pathophysiological mechanisms in order to improve current therapies (4).

Arteriogenesis, i.e. the acute recruitment (acute phase) and subsequent outward remodelling (remodelling phase) of collateral arteries, plays an important role in the adaptation to flow obstruction and tissue ischemia (5; 6). During the occlusion of a conduit artery, often caused as a complication of atherosclerosis, blood flow is redirected through adjacent pre-existing collaterals. In contrast to angiogenesis, which is initiated by ischemia, arteriogenesis occurs in regions of high fluid shear stress. Prolonged elevated shear stress results in outward remodelling of a pre-existing collateral (7). Several studies have demonstrated the importance of increased levels of eNOS mRNA and protein in regions of increased shear stress (8; 9). The increased NO release in regions of increased shear stress leads to the acute vasodilatory response of pre-existing collaterals and is critical for arteriogenesis (10). In diabetic subjects this response may be impaired due to endothelial dysfunction reflected by impaired NO-release and/or vasodilatory responses (11-13). Since vasodilation is the initial step of outward remodelling, impairments in this phase might be fundamental for impaired outward remodelling. Under conditions of prolonged increased shear stress structural outward remodelling occurs and involves attraction and adherence of monocytes and the degradation of the extracellular matrix (14). In both diabetic animals and diabetic patients impaired attraction of circulating cells (15; 16) and impaired collagenolysis have been described (17; 18).

In healthy animal models therapeutic arteriogenesis by means of growth factors has been demonstrated to stimulate outward remodelling of collateral vessels (19-22). However, therapeutic application of these growth factors showed limited success in clinical phase II studies, which can in part be attributed to co-morbidities in the patient population such as diabetes (23). Although previous studies have demonstrated that outward remodelling may be impaired by hyperglycemia (18; 24-26), little is known about disturbances in the acute, vasodilation, phase of the arteriogenesis process. We hypothesize that the diabetic state may induce significant disturbances in collateral development by impairment of both the acute and structural remodelling phase of arteriogenesis. The rabbit ischemic hind limb model has been used extensively in arteriogenesis research. In recent years we have adapted this model in order to study collateral artery growth longitudinally in the same animal (27). The aim of the present study was to investigate the effect of experimental diabetes on both the acute and remodelling phases of collateral development in the ischemia hind limb model as well as the role of monocyte chemotaxis.

RESEARCH DESIGN AND METHODS

Animal model. The present study was performed with the approval of the Animal Experimental Committee of our institution. Thirty-one New Zealand White rabbits were included and randomly assigned to receive either alloxan or saline injection (same volume as alloxan). Alloxan (110 mg/kg) was injected into the lateral ear vein to induce type I-like diabetes in the rabbit. To prevent initial hypoglycemia, 10 ml of 5% glucose was injected (i.v.) after alloxan administration and drinking water with 10% glucose was supplemented for the first 24h. Weight and blood glucose levels were determined on a weekly basis. Rabbits with blood glucose
Impaired arteriogenesis in diabetic animals

levels below 10 mmol/l (n=9) were excluded for further investigation. In a subset of these alloxan treated rabbits (n=4) blood glucose levels did not change and served as controls for alloxan side effects. Eight weeks after saline or alloxan injection, unilateral femoral artery ligation was performed in both diabetic (n=10) and control rabbits (n=12). During the procedure the rabbits were ventilated with isoflurane (2-3%). The left femoral artery was ligated (day 0) under sterile conditions by placing 2 ligations (approximately 2 cm apart) distal to the branches of the circumflex artery and the deep femoral artery. The occlusion of a conductance artery causes blood flow redistribution through interconnecting (pre-existing) arterioles, which causes functional changes in the endothelium through activation of the shear stress responsive element (28). Buprenorphine was given (i.m.) as post-operative analgesia and was continued twice a day for 2 days. During the 3 weeks follow-up period no pressure sores or signs of gangrene were observed in the ligated limbs of both control and diabetic rabbits. Animals were sacrificed by lethal bleeding.

**X-ray angiography (XRA).** Angiograms were performed in the same animal immediately (within 30 minutes), 1 week and 3 weeks after femoral artery ligation, to monitor the remodelling of collaterals over time. Coronal XRA series (12 frames/s) were obtained using a portable X-ray system (BV Pulsera, Philips Medical Systems, Best, The Netherlands) (in-plane resolution 300 x 300 µm; field of view 220 x 220 mm; operated at tube voltage 72 kV). Bolus injections of a non-ionic iodine contrast agent (Omnipaque, Amersham Health, The Netherlands, 5 mL/s, 240 mg Iodine/mL, 1.6 mL/kg body weight) were given through a catheter (4F) inserted via the carotid artery and placed 2-3 cm proximal to the abdominal aorta bifurcation. XRA films were digitally stored for off-line analysis. The number of collaterals was counted by two independent observers as defined by Longland (29), which requires identification of the stem, midzone, and re-entry zone. Angiographically visible collaterals were derived from three main vessels, the circumflex artery, the deep femoral artery and the internal iliac artery. For the three week time point collaterals were categorized as smaller or larger 600 µm (pixel size 300 µm) in diameter.

**Quantitative subtraction angiography.** To address the importance of the luminal volume of collateral arteries we developed and applied quantitative subtraction angiography. This method enables automated and observer independent collateral artery growth quantification. To this end computational software was developed in MATLAB (The Math Works Inc, Natick, MA). Early precontrast frames of the angiographic time-series, frame numbers 3-12 before contrast injection, were averaged to provide a noise-suppressed precontrast mask image (I_{pre}) on which all anatomic structures were depicted except the blood vessels. The frame with maximum contrast intensity of the collateral arteries was defined. Five frames above and below this maximal intensity frame, frames were averaged to provide a noise suppressed maximal contrast image (I_{max}). For signal analysis the quantitative description by Bushberg et al. was used (30). On the pertaining logarithmic subtraction images (I_{sub}) the region of interest was manually drawn based on predefined landmarks in the adductor magnus muscle of the ligated limb in the direct surrounding of the occlusion. This is the site of collateral anastomoses derived from the deep femoral artery and the internal iliac artery, as depicted in figure 1. In this region of interest, the number of enhanced pixels (above noise level), due to collateral filling were quantified directly, 1 and 3 weeks after ligation. In addition, the signal intensities of the pixels in the subtraction angiogram were normalized to the maximal absolute signal intensity in the aorta, to provide a measure of the blood volume as function of signal intensity relative to the aorta enhancement. The blood volume index is then defined as the sum of pixel intensities (I_{sub} above noise level), normalized to the maximum aortic signal intensity in the subtraction images in the region of collateral growth.
Impaired arteriogenesis in diabetic animals

Reactive hyperaemia response was tested, three weeks after ligation, in a subset of healthy (n=5) and diabetic (n=5) rabbits with intra-muscular laser Doppler in the gastrocnemius muscle. An intramuscular Laser Doppler needle probe was positioned in m. gastrocnemius of the right limb, as described before (31). Temperature and blood pressure were kept constant during the measurement period. Baseline Laser Doppler measurements were started after a twenty minute stabilization period. Subsequently, a vascular clamp was placed on the iliac artery and the iliac vein. After ten minutes the clamp was released and the reactive hyperaemia response in terms of peak perfusion and time to peak, could be assessed.

Monocyte chemotaxis analysis was performed ex vivo as previously described (16), three weeks after ligation. Briefly, blood-derived monocytes were isolated from about 65 ml whole blood obtained in heparinized tubes by arterial puncture just above the bifurcation of the iliac artery. Blood was layered onto Histopaque-1077 (Sigma) and the mononuclear interface was collected. Subsequently, monocytes were isolated from mononuclear cell fraction using a further gradient centrifugation. The collected monocytes were washed in PBS and resuspended in DMEM (Biochrom). The number of isolated monocytes was counted by light microscopy using a Neubauer chamber. The vitality of the isolated monocytes was assessed by trypan blue exclusion; routinely, this was higher than 72%. Monocyte chemotaxis was quantified using a modified 48-well Boyden chamber (Nuclepore). The chemoattractants VEGF-A (1 ng/mL) (Reliatech), MCP-1 (30 ng/mL) (Reliatech) or fMLP (10^{-8} mol/L) (Sigma) were added to the lower chamber. The monocyte suspension (5x10^5 cells/mL) was added to the upper chamber, which was separated from the lower chamber by a 5-μm-pore-size polycarbonate membrane (Nuclepore). After incubation for 1.5 hours at 37°C in a 5% CO₂ atmosphere, adherent cells on the filter membrane were scraped to remove the non-migrated cells. The migrated cells adhering to the lower side of the membrane were counted in 5 high-power fields and in 3 different wells using light microscopy.

Capillary to fiber ratio. Immediately following the lethal bleeding, three weeks after ligation, the tibialis and soleus muscle were dissected from the lower limb, both from the ligated and the contra-lateral side. Cryosections (10 μm), cut perpendicular to the muscle fiber direction, were stained using nitroblue tetrazolium / 5 bromo-4-chloro-3-indolylphosphate-p-toluidine salt (NBT/BCZP; Gibco, Grand Island, NY) of alkaline phosphatase in endothelial cells. The ratio of capillary to fiber was scored in three randomly selected optic fields in each muscle section.

Statistical Analysis. All results were expressed as median and interquartile range, except data from subtraction angiography and the capillary to fiber ratio, which were expressed as mean and standard error of the mean. Differences in the glucose levels, total number of collaterals, collateral lumen, blood volume index and monocyte migration function of control and diabetic rabbits were compared by the Mann-Whitney 2-tailed test. The level of statistical significance was set at p < 0.05.

RESULTS

Animal model. Glucose levels in rabbits that received alloxan increased after 2 days, reached a steady state within one week, and remained elevated until sacrifice. Glucose levels were significantly increased in the diabetic rabbits compared to the controls, 23.2 [17.7-30.3] and 6.55 [6.2-7.6] mmol/l, respectively. Body weight at the end of the study was not different between the diabetic and control animals, respectively 3.2 [3.0-3.5] and 3.1 [3.0-3.2] kg. Rabbits treated with alloxan without any effects on glucose levels showed responses similar to the untreated rabbits (data not shown).

X-ray angiography
Number of collaterals. Immediately after ligation (0 weeks) no collateral recruitment in diabetic rabbits was observed (figure 2) whereas in healthy animals 6.5 (5-7.75; p=0.0001) collaterals were counted. One week after ligation the number of collaterals was 30% lower in diabetics than control rabbits, 10 (8.5-11.5) versus 13 (10.25-14.0; p=0.058) collaterals, respectively. Three weeks after ligation a significantly lower number of collaterals was observed in diabetic rabbits 10 (9.5-12.0) compared to controls 13.5 (11.25-14; p=0.026).

Size of collaterals. In diabetic animals, the size of the collaterals was smaller than in controls (figure 3, data are expressed as percentage of total number of collaterals). Three weeks after ligation only 12.5 (0-26)% of collaterals in diabetic animals was larger than 600 µm. In the control group this percentage was markedly higher, 43 (30-50%; p=0.002).

Quantitative subtraction angiography. Subtraction angiography in the region of remodelling collaterals showed less enhanced pixels in the tissues of diabetic rabbits than controls suggestion a reduction in blood volume (figure 4). In the control group the number of enhanced pixels increased significantly within 1 week, in contrast to the diabetic rabbits which showed only significant increase 3 weeks after ligation. Diabetic rabbits had a markedly lower blood volume index than controls, values were 57% lower directly after ligation (p=0.030), 61% after one week (p=0.004) and 45% after three weeks (p=0.045).

Reactive hyperaemia. Impaired vasodilatory response in diabetic rabbits was confirmed by reactive hyperaemia experiments, performed in a subset of rabbits (4 controls and 4 diabetic rabbits). The peak perfusion, based on microvascular vasodilation capacity (31), occurred within 2 seconds in control animals and was completely absent in diabetic rabbits.

Monocyte chemotaxis. In figure 5 the migratory response of monocytes towards two different growth factors (VEGF-A and MCP-1) and the chemoattractant peptide (fMLP) as a positive control are shown (data are expressed as a percentage of unstimulated monocytes). In control animals, VEGF-A and MCP-1 induced a strong chemotactic response in monocytes. VEGF-A-induced migration of monocytes was two-fold lower in diabetic rabbits compared to controls (p=0.019). The same was observed for MCP-1 stimulation (p=0.028). No difference between controls and diabetic rabbits was observed in the fMLP-induced migratory response.

Capillary to fiber ratio. In contra-lateral limb capillary to fiber ratios were higher in the soleus muscle than in the anterior tibialis muscle, respectively 2.57 ± 0.14 and 1.98 ± 0.13 (mean ratio ± sem). Hyperglycemia did not affect the capillaries to fiber ratios in the tibialis and soleus muscle in the contra-lateral limb. Three weeks after ligation the ratios were similar to baseline levels, indicating that neither ligation nor hyperglycemia had an effect on capillary to fiber ratio.

DISCUSSION
Pre-existing collaterals provide an alternative way of blood supply to a region distal to an arterial occlusion (32). Progressive occlusion of a conductance artery due to atherosclerosis results in sustained blood flow redistribution through these collaterals, thereby triggering these vessels to increase their lumen (acute vasodilation), express adhesion molecules and attracting factors that ultimately lead to structural outward remodelling of the pre-existing collateral artery (19; 33). This study demonstrates that chronic hyperglycemia negatively affects the acute phase of the arteriogenic process. Both shear induced vasodilation and monocyte migration were impaired in diabetic rabbits. In addition, we observed impaired outward collateral growth in diabetic rabbits, as reflected by the number of collaterals and the blood volume index in the region of remodelling collaterals compared to non-diabetic animals.

The most prominent differences between healthy and diabetic rabbits were observed in the acute phase of the arteriogenic process. Angiography showed a rapid recruitment of pre-existing collateral arteries directly after
Impaired arteriogenesis in diabetic animals

ligation in healthy rabbits in contrast to the diabetic rabbits. In addition, the post-occlusive reactive hyperemic vasodilatory response was impaired in our diabetic animals. In the contralateral limb pre-existing collaterals were not visible, in both the diabetic and the non-diabetic animals. These data concur with earlier studies that showed impaired flow mediated vasodilation or post occlusive reactive hyperemic vasodilatory response in diabetes (34; 35). The defect in collateral recruitment could also have been caused by an impaired run-off secondary to a decrease in capillary to fiber ratio. However, we did not observe an effect of chronic hyperglycemia on baseline capillary to fiber ratio nor were there any differences in this ratio three weeks after ligation. The current study is to our knowledge, one of the first to show an impaired immediate recruitment of pre-existing collaterals in diabetes. Both shear mediated vasodilation and reactive hyperaemia (in part) are mediated by nitric oxide. Because shear stress induced vasodilation is postulated to be the initiation step of arteriogenesis, loss of this vasodilatory response might contribute to the poorer outcome after occlusion of a conduit artery in case of diabetes. Our assumption that impaired recruitment has detrimental effects on collateral growth is confirmed by the work of Yu et al. (36) who demonstrated impaired contraction-stimulated hyperaemia and impaired arteriogenesis in an eNOS knockout mouse model. One of the main pathways responsible for vasodilation after high fluid shear stress is the PKA/Akt-eNOS pathway (37; 38). An explanation for the impaired vasodilation response in pre-existing collaterals to increased shear stress in diabetic rabbits, as observed in this study, might be the impaired eNOS activation and NO generation (39), by mechanisms such as inhibition of phosphorylation of PI3K and Akt, and peroxynitrite generation by hyperglycemia (40). Besides the adverse effects of diabetes on vasomotor tone regulation, mechanotransduction and expression of vasoactive proteins might also be affected by hyperglycemia. Further studies are necessary to elucidate the exact role and underlying defect in the impaired shear stress sensing in pre-existing collaterals resulting in impaired outward remodeling.

Sustained shear stress leads to activation of the collateral endothelial cells. Subsequently, monocyte recruitment and adhesion to activated endothelium occurs. The migrated monocytes mature into macrophages and release different growth factors important in outward remodelling of the collateral. In this study the impaired migratory response of monocytes towards VEGF-A and MCP-1 gradient in diabetic rabbits, confirms the results described in clinical studies (16). The inhibitory effects of hyperglycemia on monocyte function might also be explained by an impaired signalling downstream the VEGF receptor (41). Also for the migration towards VEGF, impaired eNOS signalling has been shown in endothelial progenitor cells derived from diabetic patients (15).

Previous studies on arteriogenesis focused on post mortem angiograms and/or hemodynamic measurements. We introduced the technique of serially obtained in vivo angiograms (27). Both number and lumen of collaterals were increased directly after ligation up to 21 days after ligation in control rabbits, but this process was significantly impaired in diabetic rabbits. These findings agree with a previous report showing a significant lower angiographic score in the diabetic ischemic mice model (18; 26).

The quantification of the collateral lumen and grading of collateral filling based on the commonly used Rentrop classification is subjective. We have applied subtraction angiography to quantify the blood volume index (BVI) in the region of collateral growth. Advantages of our quantitative subtraction angiography are the operator-independent analyses and the quantitative values. The disadvantage of this method is that no absolute blood volume or flow values are derived. For several reasons we preferred this method above other blood flow analyses. First, blood volume index is a measure of collateral dependent full thickness limb perfusion and unlike Laser Doppler Imaging is not limited to superficial tissues. It is
Impaired arteriogenesis in diabetic animals

assumed that superficial and deep perfusion are correlated and indeed recovery of skin-perfusion in diabetic ischemic mice is significantly impaired (18; 26). However, this correlation has never been tested. Secondly, the angiographic method allows longitudinal follow-up, which is a major advantage over the accurate but destructive methods required for microspheres or collateral conductance measurements. On our subtraction angiograms the BVI was derived from first-pass of the contrast medium, which is directly related to the blood flow. The subtraction analysis showed a significant difference in BVI between diabetics and controls directly after ligation (acute phase) as well as during the remodelling phase of arteriogenesis. In summary, we conclude that the number of collaterals and the blood volume index are important contributing factors to the blood perfusion recovery distal to the occlusion and are valuable measures to quantify the level of collateral growth.

The current study results emphasize the importance of shear induced vasodilation of pre-existing collaterals in arteriogenesis. If we seek to restore the impaired collateral remodelling in diabetic subjects we hypothesize that improvement of shear induced collateral recruitment by suitable vasodilators might show benefit. The importance of nitric oxide in the arteriogenic process has already been described by Yang et al. In addition, it has been described that NO is critical for effective therapeutic arteriogenesis achieved by delivery of exogenous growth factors (e.g. VEGF, FGF2) (42). Future studies should give us a better understanding of the impairment in the PKA/Akt-eNOS pathway in diabetic subjects. Selection of a vasodilator candidate that bypasses the impaired signalling level might open new methods of therapeutic arteriogenesis in diabetic patients by restoring the impaired recruitment of collaterals, but also monocyte chemotaxis and growth factor signalling.

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REFERENCES


Impaired arteriogenesis in diabetic animals


FIGURE LEGENDS

Figure 1: Example of an x-ray angiogram and the region of interest (circle) in the ligated limb (A) and post-subtraction angiograms (B), three weeks after ligation.

Figure 2: Number of collaterals in the left limb immediately, 1 week and 3 weeks after ligation. The p-values between diabetic rabbits (grey boxes) and controls (white boxes) are presented at different time points. Values are represented as median (bolded line), 25-75 percentiles (box) and 5-95 percentiles (whiskers).
Figure 3: Percentage of collaterals with a lumen larger than 600 µm in the left limb 3 weeks after ligation. The diabetic rabbits (grey boxes) had a significantly lower percentage of collaterals larger than 600 µm than the control rabbits (white boxes). Values are represented as median (bolded line), 25-75 percentiles (box) and 5-95 percentiles (whiskers).

Figure 4: The blood volume index (BVI), defined as the sum of pixel intensities in a predefined region of interest, in the left limb immediately, 1 week and 3 weeks after ligation. BVI was persistently lower in the diabetic rabbits compared to the controls. Data are presented as mean ± SEM, *P<0.05.
**Figure 5:** Chemotactic response of monocytes towards VEGF-A (10 ng/ml), MCP-1 (10 ng/ml) and fMLP (10^{-8} M) gradient. Monocytes were isolated from either diabetics (grey boxes) or control rabbits (white boxes). Data are presented as median (bolded line), 25-75 percentiles (box) and 5-95 percentiles (whiskers).