

Impaired Collateral Recruitment and Outward Remodeling in Experimental Diabetes

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OBJECTIVE—In this study, the effect of chronic hyperglycemia on acute ligation-induced collateral vasodilation, on monocyte chemotaxis, and on structural outward remodeling 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, 1 and 3 weeks after ligation. These angiographic recordings were used to quantify number of collaterals, lumen, and blood volume index. Reactive hyperemic 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 with control rabbits. Also, hyperemic vasodilatory response to local ischemia was impaired in diabetic rabbits. This difference persisted at 1 and 3 weeks after ligation, with a 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 toward vascular endothelial growth factor-A and monocyte chemoattractant protein-1 was strongly reduced in diabetic rabbits.

CONCLUSIONS—This study demonstrates that chronic hyperglycemia negatively affects the different phases of arteriogenesis: 1) impaired shear induced vasodilatation; 2) impaired outward collateral growth, reflected in the number of collaterals and blood volume index; and 3) 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. *Diabetes* 57:2818–2823, 2008

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Received 18 February 2008 and accepted 1 July 2008.

Published ahead of print at <http://diabetes.diabetesjournals.org> on 15 July 2008. DOI: 10.2337/db08-0229.

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Individuals with diabetes have a substantially increased risk (two- to fourfold) of developing ischemic cardiovascular events, with a poor prognosis after 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 remodeling. Cardiovascular disease places a high burden on economic reserves, medical capacities, and the quality of life in diabetic patients, stressing the importance of unraveling the underlying pathophysiological mechanisms to improve current therapies (4).

Arteriogenesis, i.e., the acute recruitment (acute phase) and subsequent outward remodeling (remodeling 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 preexisting 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 remodeling of a preexisting collateral (7). Several studies have demonstrated the importance of increased levels of endothelial nitric oxide (NO) synthase (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 preexisting collaterals and is critical for arteriogenesis (10). In diabetic subjects, this response may be impaired because of endothelial dysfunction reflected by impaired NO release and/or vasodilatory responses (11–13). Because vasodilation is the initial step of outward remodeling, impairments in this phase might be fundamental for impaired outward remodeling. Under conditions of prolonged increased shear stress, structural outward remodeling 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 previously (17,18).

In healthy animal models, therapeutic arteriogenesis by means of growth factors has been demonstrated to stimulate outward remodeling 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 comorbidities in the patient population, such as diabetes (23). Although previous studies have demonstrated that outward remodeling may be impaired by hyperglycemia (18,24–26), little is known

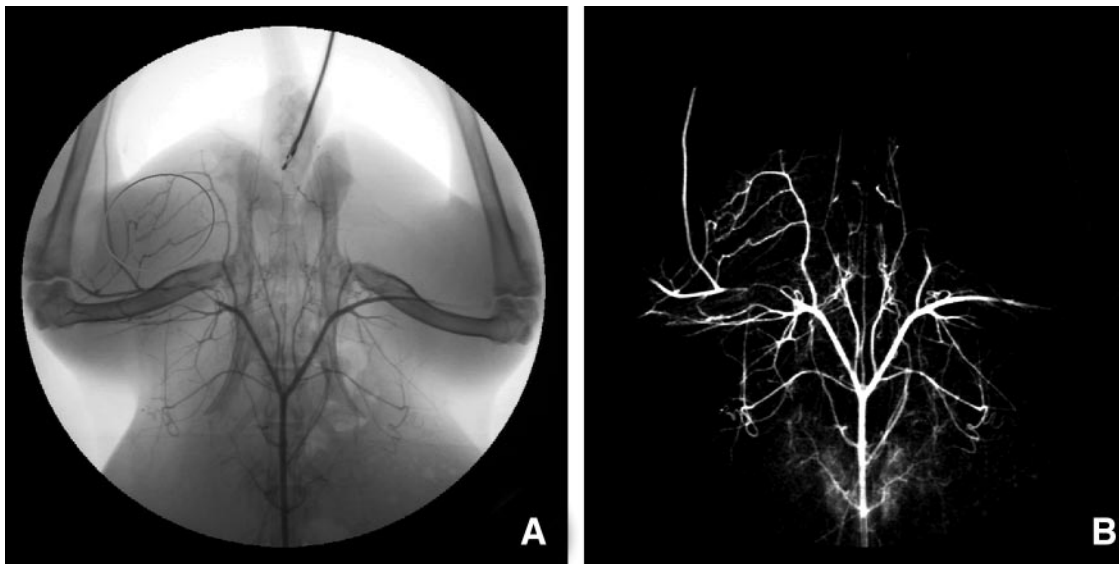


FIG. 1. Example of an X-ray angiogram and the region of interest (circle) in the ligated limb (A) and post-subtraction angiograms (B) 3 weeks after ligation.

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 remodeling phase of arteriogenesis. The rabbit ischemic hind limb model has been used extensively in arteriogenesis research. In recent years, we have adapted this model 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 remodeling phases of collateral development in the ischemia hind limb model and the role of monocyte chemotaxis.

RESEARCH DESIGN AND METHODS

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 1-like diabetes in the rabbit. To prevent initial hypoglycemia, 10 ml 5% glucose i.v. was injected after alloxan administration, and drinking water with 10% glucose was supplemented for the first 24 h. Weight and blood glucose levels were determined on a weekly basis. Rabbits with blood glucose levels <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 two ligations (~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 (preexisting) arterioles, which causes functional changes in the endothelium through activation of the shear stress-responsive element (28). Buprenorphine was given intramuscularly as postoperative analgesia and was continued twice a day for 2 days. During the 3-week follow-up period, no pressure sores or signs of gangrene were observed in the ligated limbs of either control or diabetic rabbits. Animals were killed by lethal bleeding.

X-ray angiography. Angiograms were performed in the same animal immediately (within 30 min), 1 week, and 3 weeks after femoral artery ligation to monitor the remodeling of collaterals over time. Coronal X-ray angiography (XRA) series (12 frames per second) were obtained using a portable X-ray system (BV Pulsera; Philips Medical Systems, Best, the Netherlands) (in-plane resolution $300 \times 300 \mu\text{m}$; field of view 220×220 mm; operated at tube voltage 72 kV). Bolus injections of a nonionic iodine contrast agent (Omnipaque; Amersham Health, Eindhoven, the Netherlands) (5 ml/s; 240 mg iodine/ml; 1.6

ml/kg body wt) 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 offline analysis. The number of collaterals was counted by two independent observers as defined by Longland (29), which requires identification of the stem, midzone, and reentry zone. Angiographically visible collaterals were derived from three main vessels: the circumflex artery, the deep femoral artery, and the internal iliac artery. For the 3-week time point, collaterals were categorized as smaller or larger than $600 \mu\text{m}$ (pixel size $300 \mu\text{m}$) 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, 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 maximal contrast intensity of the collateral arteries was defined. Five frames above and below this maximal intensity frame were averaged to provide a noise-suppressed maximal contrast image (I_{max}). For signal analysis, the quantitative description by Bushberg et al. (30) was used. 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 Fig. 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 maximal aortic signal intensity in the subtraction images in the region of collateral growth.

Reactive hyperemia response was tested 3 weeks after ligation in a subset of healthy ($n = 5$) and diabetic ($n = 5$) rabbits with intramuscular laser Doppler in the gastrocnemius muscle. An intramuscular laser Doppler probe was positioned in m. gastrocnemius of the right limb, as described previously (31). Temperature and blood pressure were kept constant during the measurement period. Baseline laser Doppler measurements were started after a 20-min stabilization period. Subsequently, a vascular clamp was placed on the iliac artery and the iliac vein. After 10 min, the clamp was released, and the reactive hyperemia response in terms of peak perfusion and time to peak could be assessed.

Monocyte chemotaxis analysis was performed ex vivo as previously described (16) 3 weeks after ligation. Briefly, blood-derived monocytes were isolated from ~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.

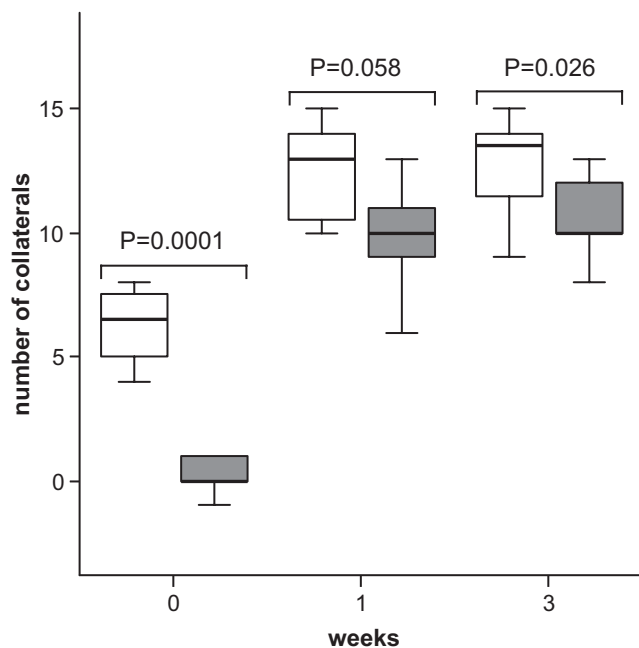


FIG. 2. Number of collaterals in the left limb immediately, 1 week, and 3 weeks after ligation. The *P* values between diabetic rabbits (■) and controls (□) are presented at different time points. Values are represented as median (bold line), 25–75 percentiles (box), and 5–95 percentiles (whiskers).

Subsequently, monocytes were isolated from mononuclear cell fraction using a further gradient centrifugation. The collected monocytes were washed in PBS and resuspended in Dulbecco's modified Eagle's medium (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 >72%. Monocyte chemotaxis was quantified using a modified 48-well Boyden chamber (Nuclepore). The chemoattractants vascular endothelial growth factor-A (VEGF-A; 1 ng/ml) (Reliatech), monocyte chemoattractant protein-1 (MCP-1; 30 ng/ml) (Reliatech), or formylMetLeuPhe (fMLP) (10^{-8} mol/l) (Sigma) were added to the lower chamber. The monocyte suspension (5×10^5 cells/ml) was added to the upper chamber, which was separated from the lower chamber by a 5- μ m-pore size polycarbonate membrane (Nuclepore). After incubation for 1.5 h at 37°C in a 5% CO₂ atmosphere, adherent cells on the filter membrane were fixed in 99% ethanol for 10 min and stained using Giemsa dye. The upper side of the polycarbonate membrane was scraped to remove the nonmigrated cells. The migrated cells adhering to the lower side of the membrane were counted in five high-power fields and in three different wells using light microscopy.

Capillary-to-fiber ratio. Immediately after the lethal bleeding, 3 weeks after ligation, the tibialis and soleus muscle were dissected from the lower limb, from both the ligated and the contralateral side. Cryosections (10 μ m), cut perpendicular to the muscle fiber direction, were stained using nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate-*p*-toluidine salt (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 are expressed as median and interquartile range, except data from subtraction angiography and the capillary-to-fiber ratio, which are expressed as mean and SE. 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 two-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 1 week, and remained elevated until the rabbits were killed. Glucose levels were significantly increased in the diabetic rabbits compared with the controls: 23.2 (17.7–30.3) and 6.55 (6.2–7.6) mmol/l, respectively. Body weight at the end of

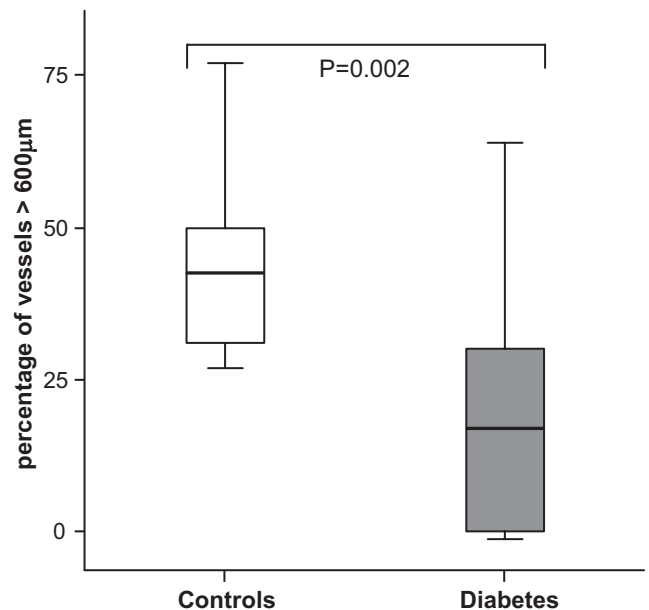


FIG. 3. Percentage of collaterals with a lumen >600 μ m in the left limb 3 weeks after ligation. The diabetic rabbits (■) had a significantly lower percentage of collaterals >600 μ m than the control rabbits (□). Values are represented as median (bold line), 25–75 percentiles (box), and 5–95 percentiles (whiskers).

the study was not different between the diabetic and control animals: 3.2 (3–3.5) and 3.1 (3.0–3.2) kg, respectively. Rabbits treated with alloxan without any effects on glucose levels showed responses similar to the untreated rabbits (data not shown).

XRA

Number of collaterals. Immediately after ligation (0 weeks), no collateral recruitment in diabetic rabbits was observed (Fig. 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 diabetic 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 with 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 (Fig. 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 >600 μ m. In the control group, this percentage was markedly higher, 43% (30–50) ($P = 0.002$).

Quantitative subtraction angiography. Subtraction angiography in the region of remodeling collaterals showed less enhanced pixels in the tissues of diabetic rabbits than controls, suggesting a reduction in blood volume (Fig. 4). In the control group, the number of enhanced pixels increased significantly within 1 week, in contrast to the diabetic rabbits, which showed a significant increase only at 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 1 week ($P = 0.004$), and 45% after 3 weeks ($P = 0.045$).

Reactive hyperemia. Impaired vasodilatory response in diabetic rabbits was confirmed by reactive hyperemia experiments, performed in a subset of rabbits (four controls and four diabetic rabbits). The peak perfusion, based

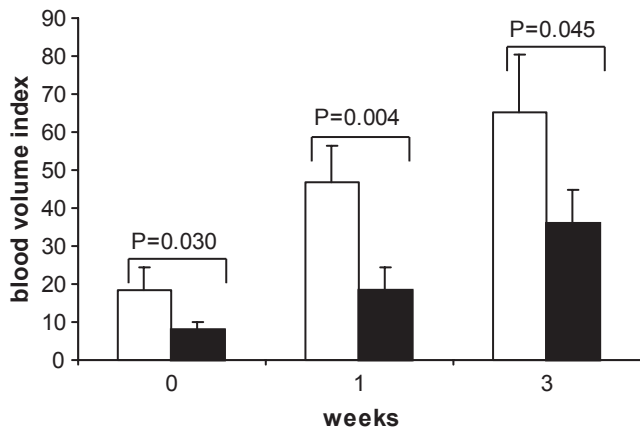


FIG. 4. The blood volume index, defined as the sum of pixel intensities in a predefined region of interest, in the left limb immediately, 1 week, and 3 weeks after ligation. Blood volume index was persistently lower in the diabetic rabbits (■) compared with the controls (□). Data are presented as means \pm SE.

on microvascular vasodilation capacity (31), occurred within 2 s in control animals and was completely absent in diabetic rabbits.

Monocyte chemotaxis. In Fig. 5, the migratory response of monocytes toward 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 twofold lower in diabetic rabbits compared with 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 the contralateral limb, capillary-to-fiber ratios were higher in the soleus muscle than in the anterior tibialis muscle: 2.57 ± 0.14 and 1.98 ± 0.13 (mean ratio \pm SE), respectively. Hyperglycemia did not affect the capillary-to-fiber ratios in the tibialis and soleus

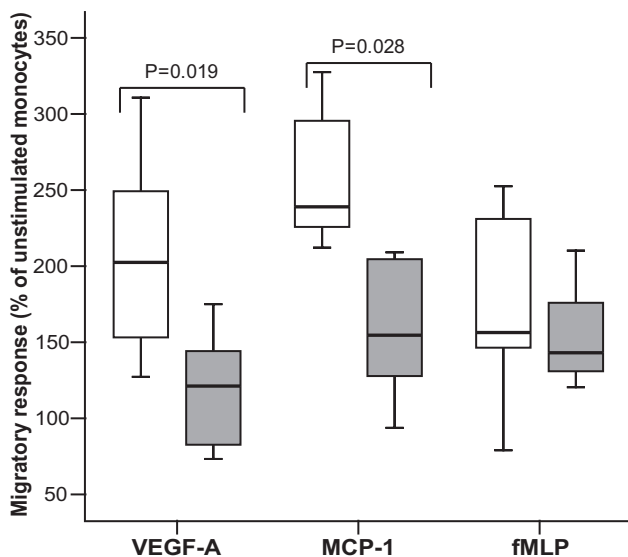


FIG. 5. Chemotactic response of monocytes toward 10 ng/ml VEGF-A, 10 ng/ml MCP-1, and 10^{-8} mol/l fMLP gradient. Monocytes were isolated from either diabetic (■) or control (□) rabbits. Data are presented as median (bold line), 25–75 percentiles (box), and 5–95 percentiles (whiskers).

muscle in the contralateral 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

Preexisting 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) and express adhesion molecules and attracting factors that ultimately lead to structural outward remodeling of the preexisting 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 remodeling collaterals compared with nondiabetic 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 preexisting collateral arteries directly after ligation in healthy rabbits in contrast to the diabetic rabbits. In addition, the postocclusive reactive hyperemic vasodilatory response was impaired in our diabetic animals. In the contralateral limb, preexisting collaterals were not visible in either the diabetic or the nondiabetic animals. These data concur with earlier studies that showed that impaired flow mediated vasodilation or postocclusive reactive hyperemic vasodilatory response in diabetes (34,35). The defect in collateral recruitment could also have been caused by an impaired runoff 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 3 weeks after ligation. The current study is, to our knowledge, one of the first to show an impaired immediate recruitment of preexisting collaterals in diabetes. Both shear-mediated vasodilation and reactive hyperemia (in part) are mediated by NO. 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 the 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 hyperemia and impaired arteriogenesis in an eNOS knockout mouse model. One of the main pathways responsible for vasodilation after high-fluid shear stress is the Akt-eNOS pathway (37,38). An explanation for the impaired vasodilation response in preexisting 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 preexisting collaterals that results in impaired outward remodeling.

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

Previous studies on arteriogenesis focused on postmortem 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 significantly 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 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, it is not limited to superficial tissues. It is assumed that superficial and deep perfusion are correlated and recovery of skin perfusion in diabetic ischemic mice is significantly impaired (18,26). However, this correlation has never been tested. Second, 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 blood volume index was derived from the first pass of the contrast medium, which is directly related to the blood flow. The subtraction analysis showed a significant difference in blood volume index between diabetic and control rabbits directly after ligation (acute phase) and during the remodeling 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 preexisting collaterals in arteriogenesis. If we seek to restore the impaired collateral remodeling in diabetic subjects, we hypothesize that improvement of shear-induced collateral recruitment by suitable vasodilators might show benefit. The importance of NO in the arteriogenic process has already been described by Yang et al. (10). In addition, it has been

described that NO is critical for effective therapeutic arteriogenesis achieved by delivery of exogenous growth factors (e.g., VEGF and fibroblast growth factor-2) (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 signaling 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 signaling.

ACKNOWLEDGMENTS

This study was supported in part by the Cardiovascular Research Institute Maastricht.

We thank Eline van den Burg for technical assistance.

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