

High-Mobility Group Box-1 Protein Promotes Angiogenesis After Peripheral Ischemia in Diabetic Mice Through a VEGF-Dependent Mechanism

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OBJECTIVE—High-mobility group box-1 (HMGB1) protein is a nuclear DNA-binding protein released from necrotic cells, inducing inflammatory responses and promoting tissue repair and angiogenesis. Diabetic human and mouse tissues contain lower levels of HMGB1 than their normoglycemic counterparts. Deficient angiogenesis after ischemia contributes to worse outcomes of peripheral arterial disease in patients with diabetes. To test the hypothesis that HMGB1 enhances ischemia-induced angiogenesis in diabetes, we administered HMGB1 protein in a mouse hind limb ischemia model using diabetic mice.

RESEARCH DESIGN AND METHODS—After the induction of diabetes by streptozotocin, we studied ischemia-induced neovascularization in the ischemic hind limb of normoglycemic, diabetic, and HMGB1-treated diabetic mice.

RESULTS—We found that the perfusion recovery was significantly attenuated in diabetic mice compared with normoglycemic control mice. Interestingly, HMGB1 protein expression was lower in the ischemic tissue of diabetic mice than in normoglycemic mice. Furthermore, we observed that HMGB1 administration restored the blood flow recovery and capillary density in the ischemic muscle of diabetic mice, that this process was associated with the increased expression of vascular endothelial growth factor (VEGF), and that HMGB1-induced angiogenesis was significantly reduced by inhibiting VEGF activity.

CONCLUSIONS—The results of this study show that endogenous HMGB1 is crucial for ischemia-induced angiogenesis in diabetic mice and that HMGB1 protein administration enhances collateral blood flow in the ischemic hind limbs of diabetic mice through a VEGF-dependent mechanism. *Diabetes* 59:1496–1505, 2010

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Several long-term complications of diabetes are characterized by vasculopathy associated with abnormal angiogenesis. Excessive angiogenesis plays a role in diabetic retinopathy, nephropathy, and neuropathy, whereas inhibited angiogenesis contributes to impaired wound healing and deficient coronary and peripheral collateral vessel development (1). The increased incidence of morbidity and mortality in diabetes, from coronary artery disease (CAD) and peripheral artery disease (PAD), can be because of the reduced ability for vessel neof ormation in the diabetic milieu (2). A diabetes-induced reduction in collateral vessel formation has been demonstrated in murine models: hind limb ischemia created by femoral artery ligation is associated with the reduced formation of capillaries and a reduction in blood flow to the ischemic hind limb in diabetic versus nondiabetic mice (3).

High-mobility group box-1 (HMGB1) is a nuclear protein that acts as a cytokine when released into the extracellular milieu by necrotic and inflammatory cells, and is involved in inflammatory responses and tissue repair (4). HMGB1 is released passively during cellular necrosis by almost all cells that have a nucleus (5), but is also actively secreted by immune cells such as monocytes and macrophages (6). The first identified cellular receptor for this nuclear protein was the receptor for advanced glycation end products (RAGE), which mediates the interactions between advanced glycation end product (AGE)-modified proteins and the endothelium and other cell types (7). HMGB1 function is altered in diabetes, and the signaling systems triggered by this protein are not fully understood. In fact, diabetic human and mouse skin show lower local levels of HMGB1 than their normoglycemic counterparts (8). Conversely, recent findings demonstrate that an increased serum HMGB1 level is associated with CAD in nondiabetic and type 2 diabetic patients and could contribute to the progression of atherosclerosis and other cardiovascular diseases (9). However, despite these apparently conflicting results, this cytokine occupies a central role in mediating the local and systemic responses to several stimuli and might have therapeutic relevance. Indeed, vessel-associated stem cells (mesoangioblasts), injected into the general circulation of dystrophic mice, migrate to sites of tissue damage in response to the HMGB1 signal, by a nuclear factor- κ B-dependent mechanism (10). Moreover, endogenous HMGB1 enhances angiogenesis and restores cardiac function in a murine model of myocardial infarction (11), and the exogenous administration of HMGB1

after myocardial infarction leads to the recovery of left ventricular function through the regeneration of cardiomyocytes (12). Importantly, HMGB1 is a chemotactic agent in vitro and in vivo for endothelial precursor cells (EPCs) (13), and recent findings demonstrate that HMGB1 administration significantly increases levels of growth factors including vascular endothelial growth factor (VEGF), basic fibroblast growth factor, and insulin-like growth factor-1 released by cultured human cardiac fibroblasts (14).

Given the preexisting data, this study examines whether HMGB1 plays a role in peripheral ischemia-induced angiogenesis in both normoglycemic and diabetic mice.

RESEARCH DESIGN AND METHODS

Mouse model of diabetes. All investigations were approved by the A. Gemelli University Hospital Institutional Animal Care and Use Committee. Male C57BL/6J mice (The Jackson Laboratory) aged 8–12 weeks old were used for experiments. All animals were allowed free access to food and water throughout the study. Diabetes was induced by administering 50 mg/kg body wt streptozotocin (STZ; Sigma) in citrate buffer (pH 4.5), intraperitoneally during the fasting state, consecutively for 5 days, as previously described (15). Hyperglycemia was verified, using blood obtained from the tail vein, 2 days after STZ injections, by an Accu-Check Active glucometer (Roche). We considered mice to be diabetic when blood glucose was at least 16 mmol/l (normal 5–8 mmol/l). Overall, 130 mice showed a blood glucose level of at least 16 mmol/l, both 1 and 2 weeks after the last STZ injection, and were included in the experimental diabetic group.

Experimental design and groups. To confirm the impaired ischemia-induced angiogenesis in diabetes, two groups of diabetic and age-matched C57BL/6J normoglycemic mice ($n = 10$ per group) were used. To investigate the role of HMGB1 in postischemic angiogenesis in nondiabetic mice, two more groups of normoglycemic mice ($n = 10$ per group) were studied. For HMGB1 treatment analysis, 50 diabetic mice were divided into five groups: mice treated with 200 ng HMGB1, mice treated with 400 ng HMGB1, mice treated with 600 ng HMGB1, mice treated with 800 ng HMGB1, and mice treated with PBS ($n = 10$ per group). To further define and clarify the HMGB1-VEGF interaction, 20 more normoglycemic mice and 60 more diabetic mice were used.

Mouse hind limb ischemia model. Unilateral hind limb ischemia was induced in both nondiabetic ($n = 50$) and diabetic (2 weeks after the onset of diabetes, $n = 130$) mice as previously described (16). Briefly, all animals were anesthetized with an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (8 mg/kg). The proximal and distal portions of the femoral artery and the distal portion of the saphenous artery were ligated. The arteries and all side branches were dissected free and excised. The skin was closed with 5–0 surgical sutures. A laser Doppler perfusion imager system (PeriScan PIM II; Perimed) was used to measure hind limb blood perfusion before and immediately after surgery and then at 7-day intervals, until the end of the study, for a total follow-up of 28 days after surgery. Before imaging, excess hairs were removed from the limbs using depilatory cream and mice were placed on a heating plate at 40°C. To avoid the influence of ambient light and temperature, results were expressed as the ratio between perfusion in the right (ischemic) and left (nonischemic) limb.

Exogenous HMGB1 protein administration. In 80 diabetic animals with unilateral hind limb ischemia, HMGB1 protein (HMGBiotech) was administered in a single dose by intramuscular injection, directly into the ischemic area, at a concentration of 200, 400, 600, and 800 ng per mouse in 0.1 ml of PBS, respectively ($n = 10$ per group). A separate group of 10 diabetic mice received an intramuscular injection of 0.1 ml of PBS in the ischemic area. Mice received HMGB1 or PBS at time 0 (that is, immediately after surgery).

In vivo inhibition of HMGB1 function. The activity of HMGB1 was locally inhibited in vivo in nondiabetic mice ($n = 10$) by an intramuscular injection of the HMGB1 inhibitor DNA binding A box (BoxA; HMGBiotech), directly into the ischemic area 1 h before the induction of the ischemic injury, at a concentration of 400 ng per mouse in 0.1 ml of PBS, as previously described (17).

In vivo inhibition of VEGF activity. In this study, we examined whether the blockade of VEGF signals by sFlt-1, a soluble form of the Flt-1 VEGF receptor (VEGFR), gene transfer into skeletal muscles can attenuate HMGB1-mediated vascular neof ormation in diabetic mice. Therefore, we used a selective and specific inhibitor of VEGF sFlt-1 (18). This isoform is expressed endogenously by vascular endothelial cells and can inhibit VEGF activity by directly sequestering VEGF and functioning as a dominant negative inhibitor against VEGFRs.

Either empty plasmid or sFlt-1 plasmid (100 μ g/30 μ l PBS) was injected into the right femoral muscle of 20 normoglycemic mice, 20 untreated diabetic mice, and 40 HMGB1-treated diabetic mice ($n = 10$ per group) using a 27-gauge needle 1 day before the induction of ischemic injury (19). To enhance transgene expression, all plasmid-injected animals received electroporation at the injection site immediately after injection with an electric pulse generator as previously described (20–22). To ensure VEGF inhibition, changes in VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1) phosphorylation were evaluated. A separate group of 10 HMGB1-treated (800 ng of HMGB1) diabetic mice received an equal amount of empty plasmid with an intramuscular injection on the same time schedule.

Histological assays. At 1 and 4 weeks after surgery, mice were killed by an intraperitoneal injection of an overdose of pentobarbital. The whole limbs were fixed in methanol overnight. The femora were carefully removed, and the ischemic thigh muscles were embedded in paraffin. All the specimens were routinely fixed overnight in 4% buffered formalin and embedded in paraffin. Four-micrometer sections of tissue samples were subjected to immunoperoxidase biotin–avidin reaction using the labeled streptavidin biotin method to determine CD31, VEGF, and HMGB1 expression. CD45 was used as a marker for inflammatory infiltrate. The sections for immunohistochemistry were cut and mounted on 3-aminopropyltriethoxysilane-coated (Sigma) slides, allowed to dry overnight at 37°C to ensure optimal adhesion, dewaxed, rehydrated, and treated with 0.3% H₂O₂ in methanol for 10 min to block endogenous peroxidase. For antigen retrieval (not necessary for HMGB1 and VEGF) the sections were microwave treated in 1 mmol/l EDTA at pH 8 (for CD31) and 10 mmol/l sodium citrate at pH 6 (for CD45) for 10 min, and then allowed to cool for 20 min. Endogenous biotin was saturated using a biotin blocking kit (Vector Laboratories). The sections were incubated at room temperature for 30 min with the following antibodies: purified rat anti-mouse CD31 (dilution 1:30; monoclonal [IgG2a]; BD Bioscience), rabbit anti-mouse HMGB1 (dilution 1:100, polyclonal; Santa Cruz Biotechnology), rabbit anti-mouse VEGF (dilution 1:100, polyclonal; Santa Cruz Biotechnology), and rabbit anti-mouse CD45 (dilution 1:50, polyclonal; AbCam). Binding was visualized using biotinylated secondary antibody (1 h of incubation) and the streptavidin–biotin peroxidase complex developed with diaminobenzidine. Finally, slides were counterstained with hematoxylin. Capillary density and leukocyte infiltration were measured by counting six random high-power (magnification $\times 200$) fields for a minimum of 200 fibers from each ischemic and nonischemic limb on an inverted light microscope, and were expressed by the number of CD31⁺ or CD45⁺ cells per square millimeter. Apoptosis was demonstrated in situ using the Mebstain Apoptosis kit II (Immunotech, Marseille, France), and the apoptotic index was determined by dividing the total number of myocytes showing nuclear positivity by the total number of cells in the fields examined (23). Necrosis was analyzed semiquantitatively with a 5 score for severity: 0 = none; 1 = necrosis of 1–5% of myocytes; 2 = necrosis of 6–25% of myocytes; 3 = necrosis of 26–50% of myocytes; and 4 = necrosis >50% of myocytes (24). The area was measured with a National Institutes of Health image analysis system (ImageJ 1.41). Two operators extracted the results independently.

Western blotting. Immunoblotting was performed on the homogenates of muscle tissues. The protein concentration of samples was carefully determined by the protein assay (Bio-Rad Laboratories). Equal amounts of protein were subjected to SDS-PAGE electrophoresis using 4–12% gradient gels under reducing conditions (Bio-Rad Laboratories) and transferred to nitrocellulose membranes (GE Healthcare). To ensure the equivalent protein loading and quantitative transfer efficiency of proteins, membranes were stained with Ponceau S before incubating with primary antibodies. Membranes were incubated with antibodies against HMGB1 (1:500; Santa Cruz Biotechnology), VEGF (1:500; Santa Cruz Biotechnology), Flt-1 (1:200; Santa Cruz Biotechnology), p-Flt-1 (1:200; Santa Cruz Biotechnology), Flk-1 (1:500; Santa Cruz Biotechnology), and p-Flk-1 (1:500; Santa Cruz Biotechnology). HMGB1, VEGF, Flt-1, p-Flt-1, Flk-1, and p-Flk-1 expression was normalized using a mouse monoclonal anti- α -tubulin antibody or anti- α -actin antibody.

Statistics. Statistical analysis was performed using STATA software (Version 10.0; STATA). Data are expressed as the mean \pm SEM. Comparison among groups was carried out using ANOVA followed by Fisher post hoc test. Repeated-measures ANOVA was used to assess the improvement in perfusion over time within groups. Significance was set at a probability value (P) of <0.05.

RESULTS

Impaired angiogenesis in diabetic mice after hind limb ischemia. Immediately after the femoral artery ligation, blood flow in the ischemic hind limb was equally reduced in both nondiabetic and diabetic mice (Fig. 1).

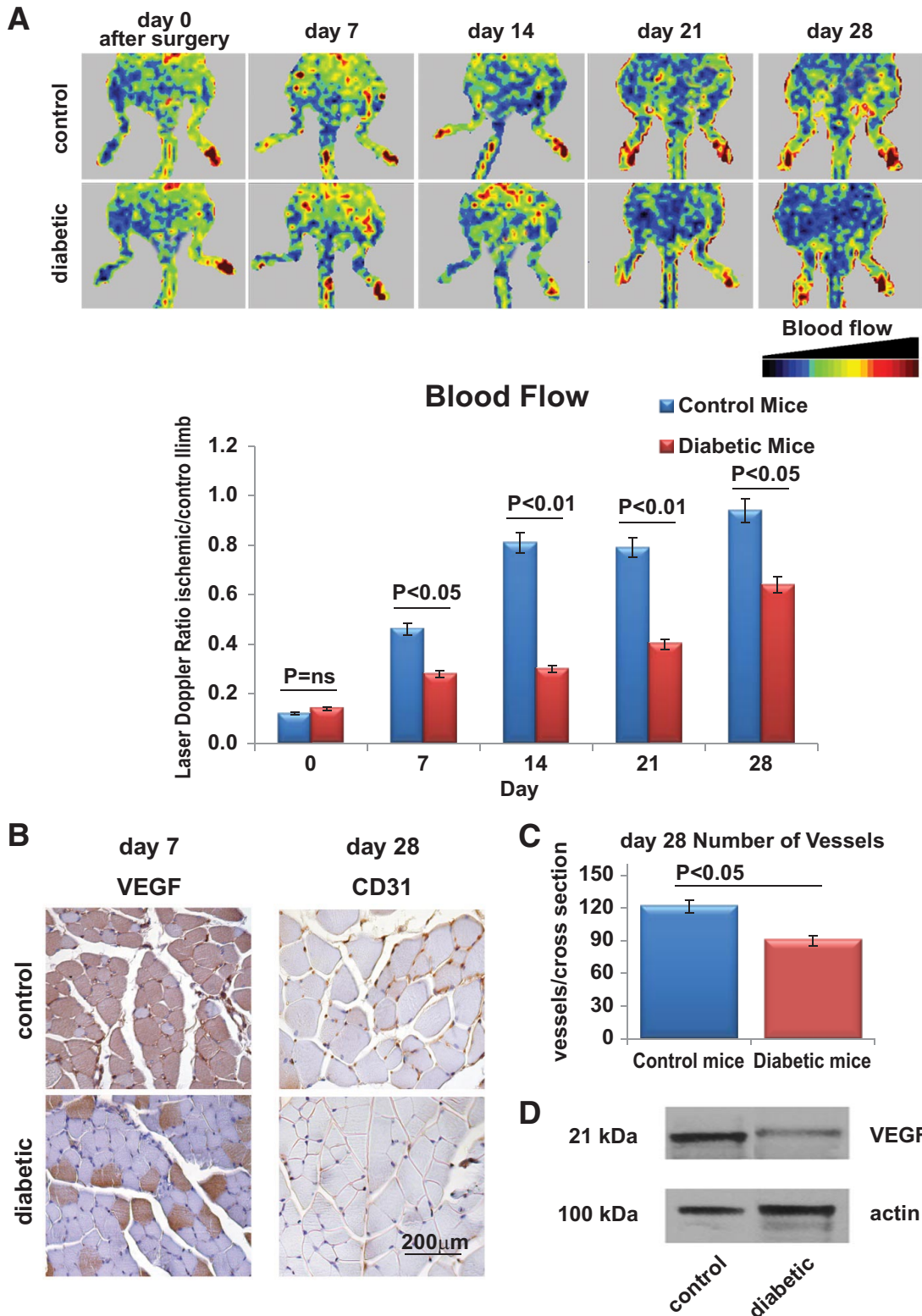


FIG. 1. A: Foot blood flow monitored in vivo by LDPI in control normoglycemic and diabetic mice. Representative evaluation of the ischemic (*right*) and nonischemic (*left*) hind limbs, immediately after, and on days 7, 14, 21, and 28 after surgery. In color-coded images, red indicates normal perfusion and blue indicates a marked reduction in blood flow in the ischemic hind limb. Blood flow recovery is impaired in diabetic mice compared with normoglycemic mice. The blood flow of the ischemic hind limb is expressed as the ratio between the perfusion of the ischemic limb and the uninjured limb. $P < 0.05$ and $P < 0.01$ vs. diabetic mice. **B:** Representative photomicrographs of ischemic muscle sections from control normoglycemic and diabetic mice stained with antibody directed against VEGF, 7 days after surgery, and against CD31, 28 days after surgery. Positive staining appears in brown. Magnification $\times 20$. **C:** Number of vessels per cross section is significantly reduced in diabetic mice with respect to normoglycemic mice. $P < 0.05$ vs. diabetic mice. **D:** Representative Western blot of VEGF protein content in the ischemic legs of control and diabetic mice on postoperative day 7. VEGF expression is reduced in the ischemic tissue of diabetic mice compared with control mice. ns, not significant. (A high-quality digital representation of this figure is available in the online issue.)

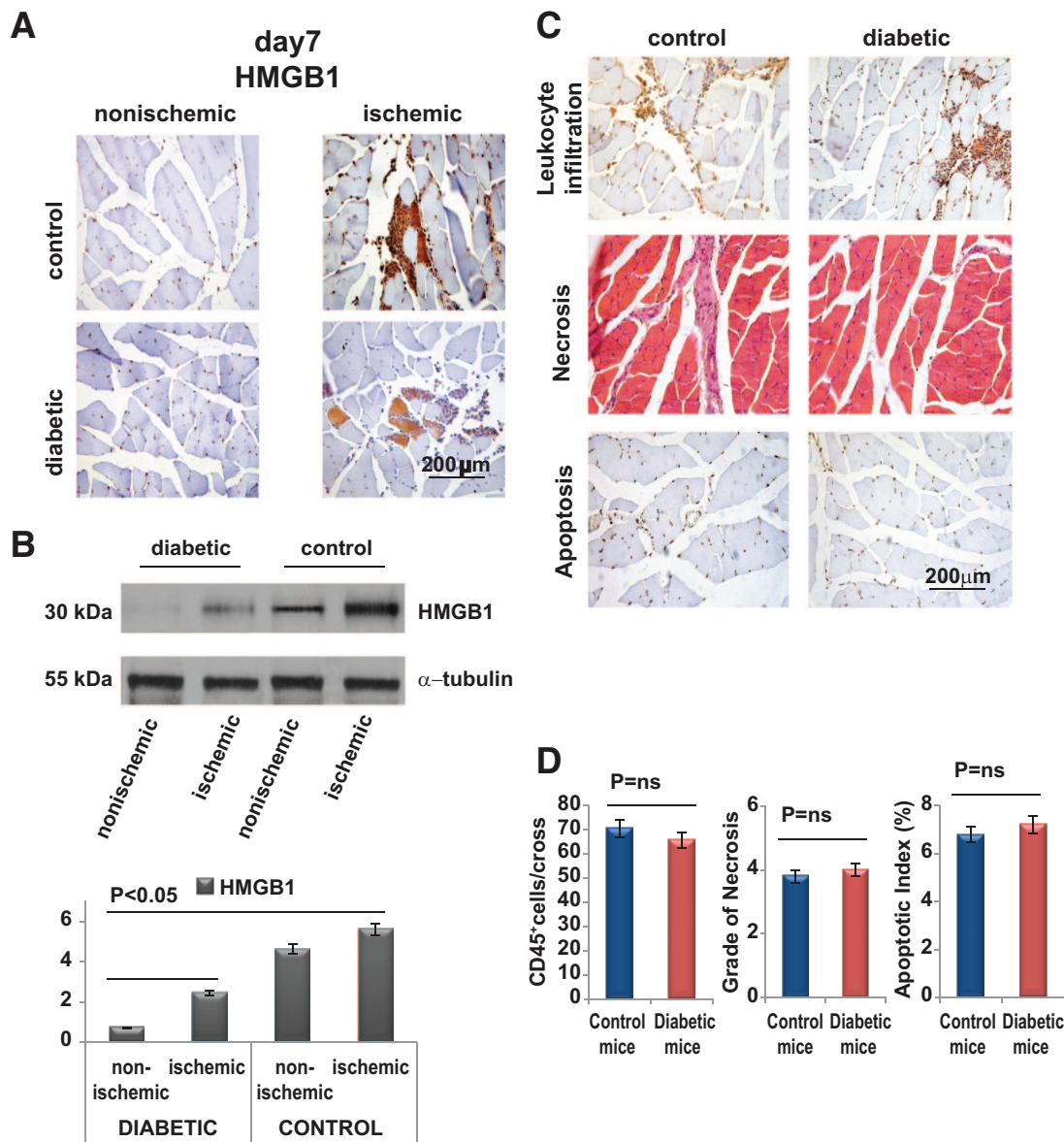


FIG. 2. *A*: Representative photomicrographs of nonischemic and ischemic muscle sections from control normoglycemic and diabetic mice stained with antibody directed against HMGB1 7 days after surgery. Magnification $\times 20$. Positive staining appears in brown. *B*: Representative Western blot of HMGB1 protein content in the ischemic legs of control and diabetic mice on postoperative day 7. HMGB1 expression is reduced in the ischemic tissue of diabetic mice compared with control mice. $P < 0.05$ vs. diabetic mice. *C*: Representative photomicrographs of ischemic muscle sections from control normoglycemic and diabetic mice stained with antibody directed against CD45, with hematoxylin-eosin, and with transferase-mediated dUTP nick-end labeling, 7 days after surgery. Magnification $\times 20$. For CD45 (leukocyte infiltration) and transferase-mediated dUTP nick-end labeling assay (apoptotic cells) positive staining appears in brown. *D*: Evaluation of leukocyte infiltration, apoptosis, and necrosis in the ischemic muscle sections from control normoglycemic and diabetic mice. There are no differences between control and diabetic mice according to all three aspects. ns vs. diabetic mice. (A high-quality digital representation of this figure is available in the online issue.)

Laser Doppler perfusion imaging (LDPI) was performed before, immediately after, and on days 7, 14, 21, and 28 after surgery. Perfusion recovery was significantly attenuated in diabetic mice compared with normoglycemic mice on postoperative days 7, 14, 21, and 28 (Fig. 1A). In addition, histological analysis revealed that the capillary density in the ischemic limb was significantly increased in nondiabetic mice, whereas no such increase was noted in diabetic mice at 4 weeks after the hind limb ischemia (Fig. 1B and C). Furthermore, immunostaining and immunoblot analyses showed increased VEGF expression in the ischemic tissue of normoglycemic mice compared with diabetic mice on postoperative day 7 (Fig. 1B and D). In agreement with previous data (3), these findings confirm

the relative inability of diabetes to mount a robust angiogenic response to ischemia after arterial occlusion (25).

HMGB1 in normoglycemic and diabetic ischemic hind limbs. To test whether HMGB1 is involved in impaired ischemia-induced angiogenesis, we first evaluated HMGB1 expression in both nondiabetic and diabetic mice by immunohistochemical and Western blot analysis 7 days after ischemic injury. In relation to the expression of HMGB1 in uninjured tissues, there was reduced nuclear positivity in diabetic hind limbs compared with normoglycemic mice (Fig. 2A). Interestingly, although operated normoglycemic mice showed strong expression of HMGB1 in infiltrating leukocytes (Fig. 2A), HMGB1-positive cells were reduced in ischemic hind limbs of diabetic mice compared with nondi-

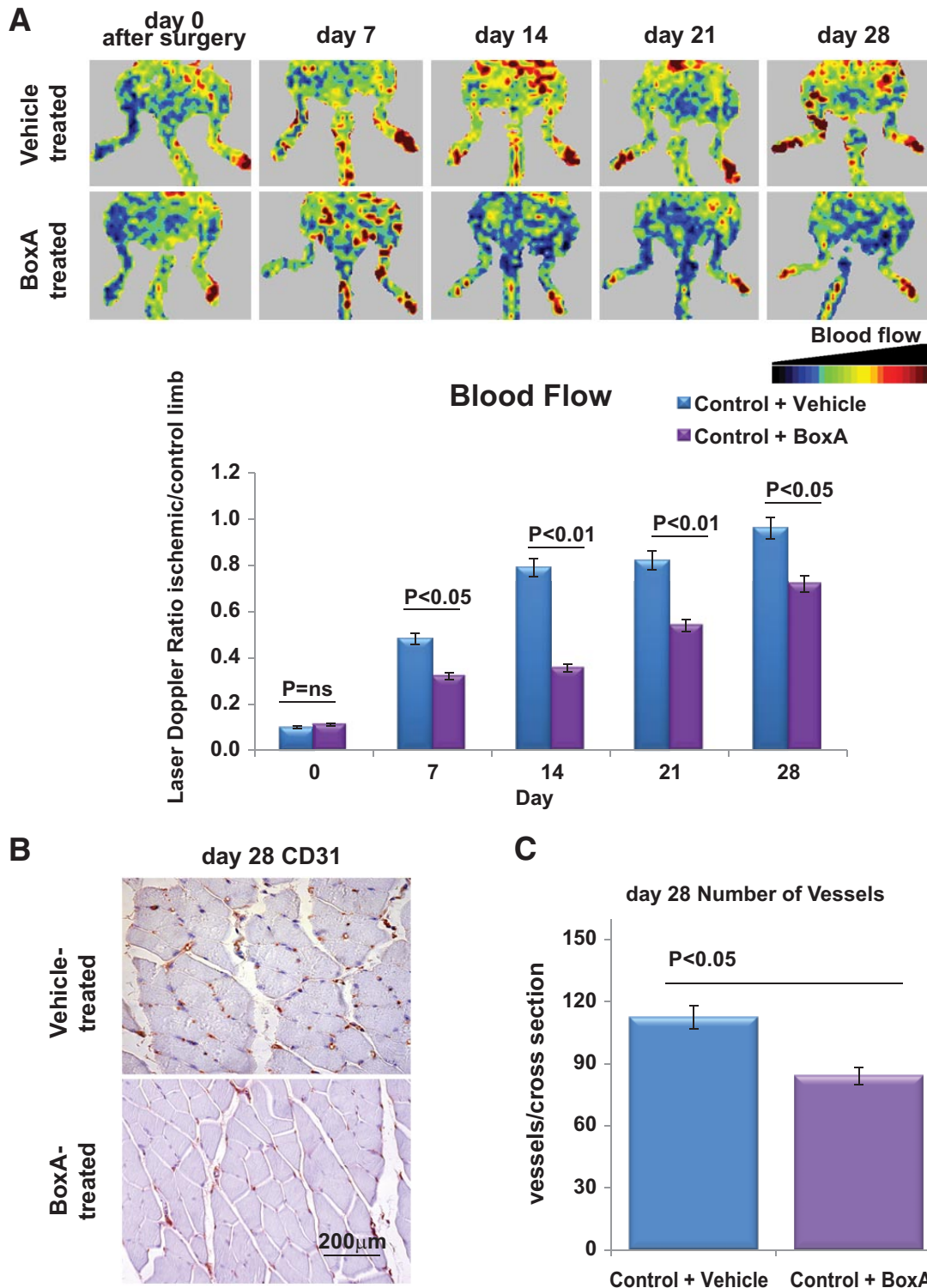


FIG. 3. A: LDPI ratio in control and BoxA-treated mice. Representative evaluation of the ischemic (*right*) and nonischemic (*left*) hind limbs immediately after and on days 7, 14, 21, and 28 after surgery. Blood flow recovery is impaired in BoxA-treated mice compared with vehicle-treated mice. The blood flow of the ischemic hind limb is expressed as the ratio between the perfusion of the ischemic limb and the uninjured limb. $P < 0.05$ and $P < 0.01$ vs. BoxA-treated mice. **B:** Representative photomicrographs of ischemic muscle sections from control and BoxA-treated mice stained with antibody directed against CD31 28 days after surgery. Positive staining appears in brown. Magnification $\times 20$. **C:** The number of vessels per cross section is significantly reduced in BoxA-treated mice compared with vehicle-treated mice. $P < 0.05$ vs. BoxA-treated mice. (A high-quality digital representation of this figure is available in the online issue.)

abetic mice at day 7 (Fig. 2A). Immunoblot analysis supported the evidence that HMGB1 protein expression was reduced in the ischemic tissue of diabetic mice (Fig. 2B). To test whether observed HMGB1 changes were dependent on

either different tissue damage between the two groups or an altered regulation per se, we analyzed leukocyte infiltration, apoptosis, and necrosis and noted there were no differences between control and diabetic mice according to all three

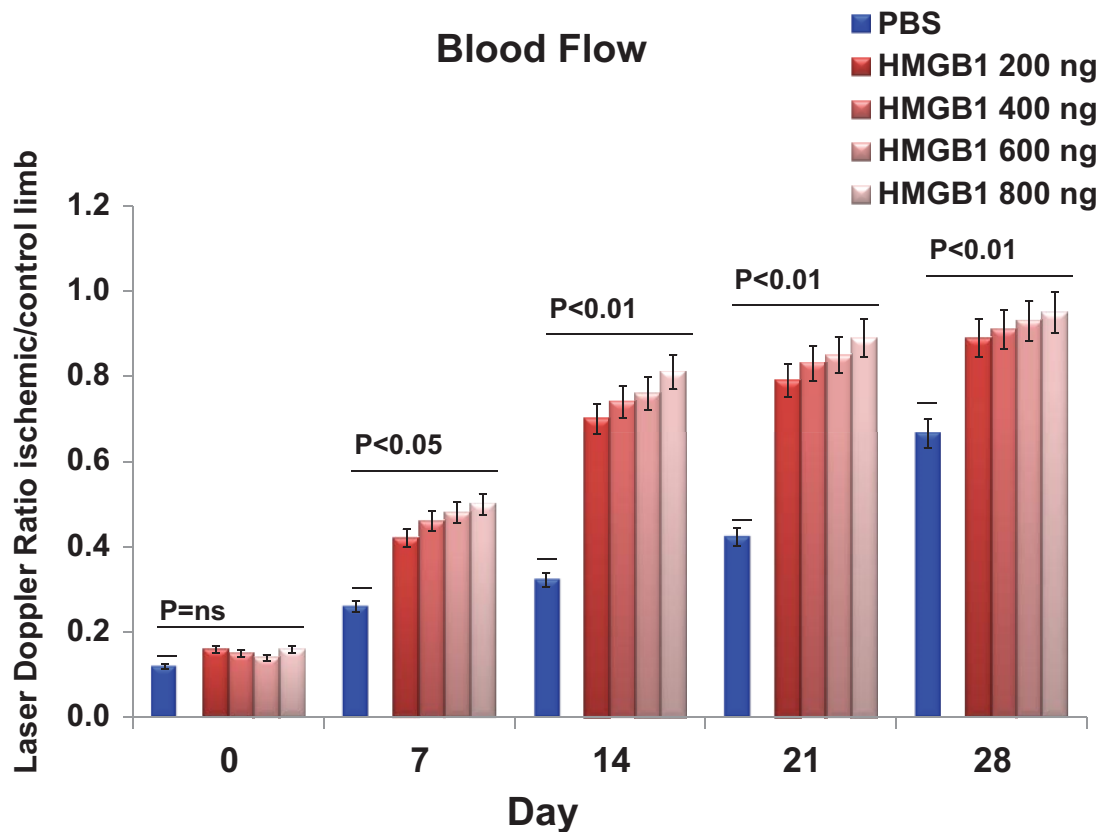


FIG. 4. LDPI ratio in diabetic mice treated with 200, 400, 600, and 800 ng of HMGB1 and with PBS (control group). Representative evaluation of LDPI ratio immediately after and on days 7, 14, 21, and 28 after surgery. HMGB1 administration restored blood flow recovery in diabetic mice compared with PBS-treated diabetic mice. The blood flow of the ischemic hind limb is expressed as the ratio between the perfusion of the ischemic limb and the uninjured limb. $P < 0.05$ and $P < 0.01$ vs. HMGB1-treated mice. (A high-quality digital color representation of this figure is available in the online issue.)

aspects (Fig. 2C and D). Therefore, it is possible to state that the observed difference in HMGB1 expression does not depend on a different response to ischemic injury between the two groups.

Inhibition of endogenous HMGB1 impairs ischemia-induced angiogenesis in normoglycemic mice. To further investigate the role of HMGB1 in postischemic angiogenesis in normoglycemic mice, we tested the effect of HMGB1 blockade in normoglycemic mice using the HMGB1 BoxA, a truncated form of the protein that acts as a competitive antagonist by inhibiting HMGB1 binding to its receptor RAGE (26), directly in the ischemic area. LDPI showed that perfusion recovery was significantly attenuated on postoperative days 7, 14, 21, and 28 in BoxA-treated mice compared with vehicle-treated mice (Fig. 3A). Consistent with the measurement of LDPI, anti-CD31 immunostaining at day 28 revealed that angiogenesis in the ischemic hind limb was impaired in mice treated with BoxA (Fig. 3B and C). To our knowledge, this is the first demonstration that HMGB1 plays an important role in ischemia-induced angiogenesis.

Exogenous HMGB1 administration enhances blood flow recovery in diabetic mice. The lower HMGB1 level in the ischemic hind limbs of diabetic mice and the impaired ischemia-induced angiogenesis observed in normoglycemic mice treated with competitive HMGB1-antagonist suggested that HMGB1 might have a function in postischemic vessel neof ormation in diabetic mice. Thus, we administered exogenous HMGB1 protein directly into the ischemic area of diabetic mice, by intramuscular

injection, at a concentration of 200, 400, 600, and 800 ng per mouse, respectively ($n = 10$ per group). Control diabetic mice ($n = 10$) received an equal amount of PBS on the same time schedule. In response to HMGB1 administration, perfusion recovery was significantly improved on postoperative days 7, 14, 21, and 28 compared with mice treated with PBS (Fig. 4). In accordance with LDPI data, HMGB1 administration significantly restored the number of detectable capillaries in the ischemic legs of diabetic mice to a normal level 28 days after surgery (Fig. 5A and B). Moreover, we evaluated whether VEGF is expressed in association with HMGB1-induced neovascularization. Immunostaining (data not shown) and Western blot analyses demonstrated that VEGF protein levels were significantly increased in the ischemic hind limbs of diabetic mice treated with 200, 400, 600, and 800 ng of HMGB1 compared with mice treated with PBS (Fig. 5C). These findings first demonstrate that exogenous HMGB1 administration enhances ischemia-induced angiogenesis in diabetic mice and that this angiogenic response occurs in association with VEGF production.

HMGB1 promotes angiogenesis in diabetic mice through a VEGF-dependent mechanism. Following the observation that HMGB1-induced postischemic neovascularization in diabetic mice occurs in association with VEGF generation, we tested the hypothesis that the angiogenic properties of HMGB1 might depend on VEGF activity. Therefore, we suppressed VEGF activity *in vivo* and evaluated whether HMGB1 was still able to improve postischemic angiogenesis in diabetic mice. The *in vivo*

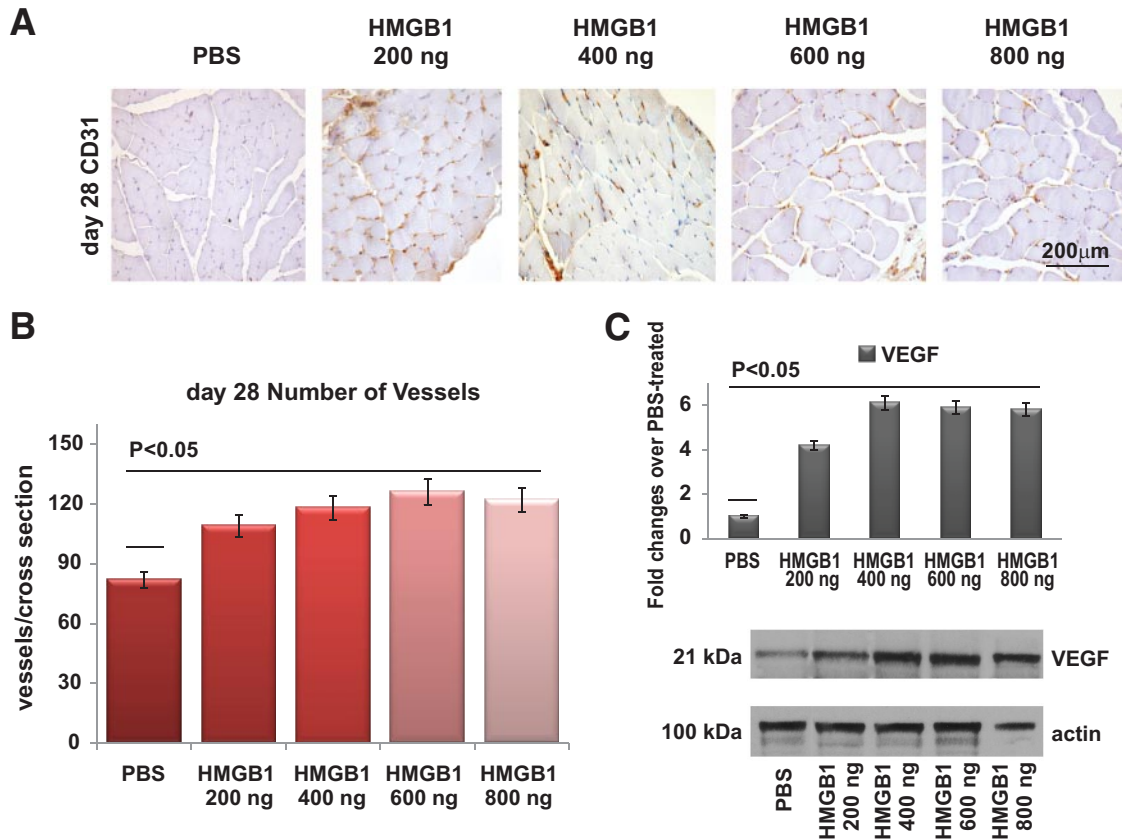


FIG. 5. A: Representative photomicrographs of ischemic muscle sections from diabetic mice treated with 200, 400, 600, and 800 ng of HMGB1 and PBS (control group), stained with antibody directed against CD31 28 days after surgery. Positive staining appears in brown. Magnification $\times 20$. **B:** The number of vessels per cross section is significantly increased in HMGB1-treated mice compared with untreated mice. $P < 0.05$ vs. PBS-treated control mice. **C:** Representative Western blot evaluation of VEGF protein content, 3 days after surgery in the ischemic legs of HMGB1-treated and PBS-treated mice. $P < 0.05$ vs. mice treated with PBS. (A high-quality digital color representation of this figure is available in the online issue.)

inhibition of VEGF was accomplished using the sFlt-1 plasmid, which suppresses VEGF activity both by sequestering VEGF and functioning as a dominant-negative inhibitor of VEGFRs (27). Changes in VEGFR (Flt-1 and Flk-1) phosphorylation were evaluated (Fig. 6A), confirming the inhibition of the VEGF pathway. Normoglycemic and diabetic mice transfected with the empty vector or sFlt-1 plasmid were used as controls (Fig. 6B). A significant reduction in HMGB1-induced neoangiogenesis was observed when VEGF activity was suppressed (Fig. 6C). LDPI demonstrated that the inhibition of VEGF activity resulted in a significant reduction of HMGB1-induced blood flow recovery on postoperative days 7, 14, 21, and 28. Consistent with these LDPI data, HMGB1 administration did not restore the number of detectable capillaries in the ischemic leg of diabetic mice 28 days after surgery, when VEGF activity was inhibited (Fig. 6C). These findings demonstrate that exogenous HMGB1 administration enhances ischemia-induced angiogenesis in diabetic mice via a VEGF-dependent mechanism.

DISCUSSION

The impaired angiogenic response to ischemia after arterial occlusion might contribute to the poor clinical outcomes observed in diabetic patients with CAD or PAD (25,28). Various hypotheses have been postulated to explain the impaired postischemic angiogenic response in diabetes, such as the vascular dysfunction characterized by both endothelial and vascular smooth muscle cell

impairments (29), the decreased release or defective function of EPCs from the bone marrow (30), or the presence of maladaptive dysregulation of vascular growth factor pathways (31). Although a number of factors are likely to contribute to reduced angiogenesis in diabetes, the results of our study are the first to describe alterations in the HMGB1 system as a potential contributor to this process.

Endothelial cells, which form the inner lining of blood vessels, express RAGE, the cell surface receptor that binds AGEs (32). One way in which AGEs might accelerate the development of macrovascular disease in diabetes is the induction of endothelial cell surface adhesion molecules resulting from the interaction of AGEs with their receptors RAGE (33), a phenomenon that might be a marker for the amount and progression of vascular disease in diabetes (34). But there is another important signaling system related to RAGE, that is, the HMGB1 pathway, involving a new cytokine that is released from certain cells in response to other cytokines and from necrotic cells (35). Upon binding to RAGE, HMGB1 activates key cell signaling pathways, for example, mitogen-activated protein kinases and nuclear factor- κ B (10). Through its secretion by activated macrophages HMGB1 again activates macrophages, resulting in the secretion of angiogenic factors such as VEGF, tumor necrosis factor- α , and interleukin-8 (36). Furthermore, several reports have suggested that HMGB1 plays a key role in angiogenesis through multiple mechanisms, including the upregulation of proangiogenic factors, promoting the homing of EPCs to ischemic tissues

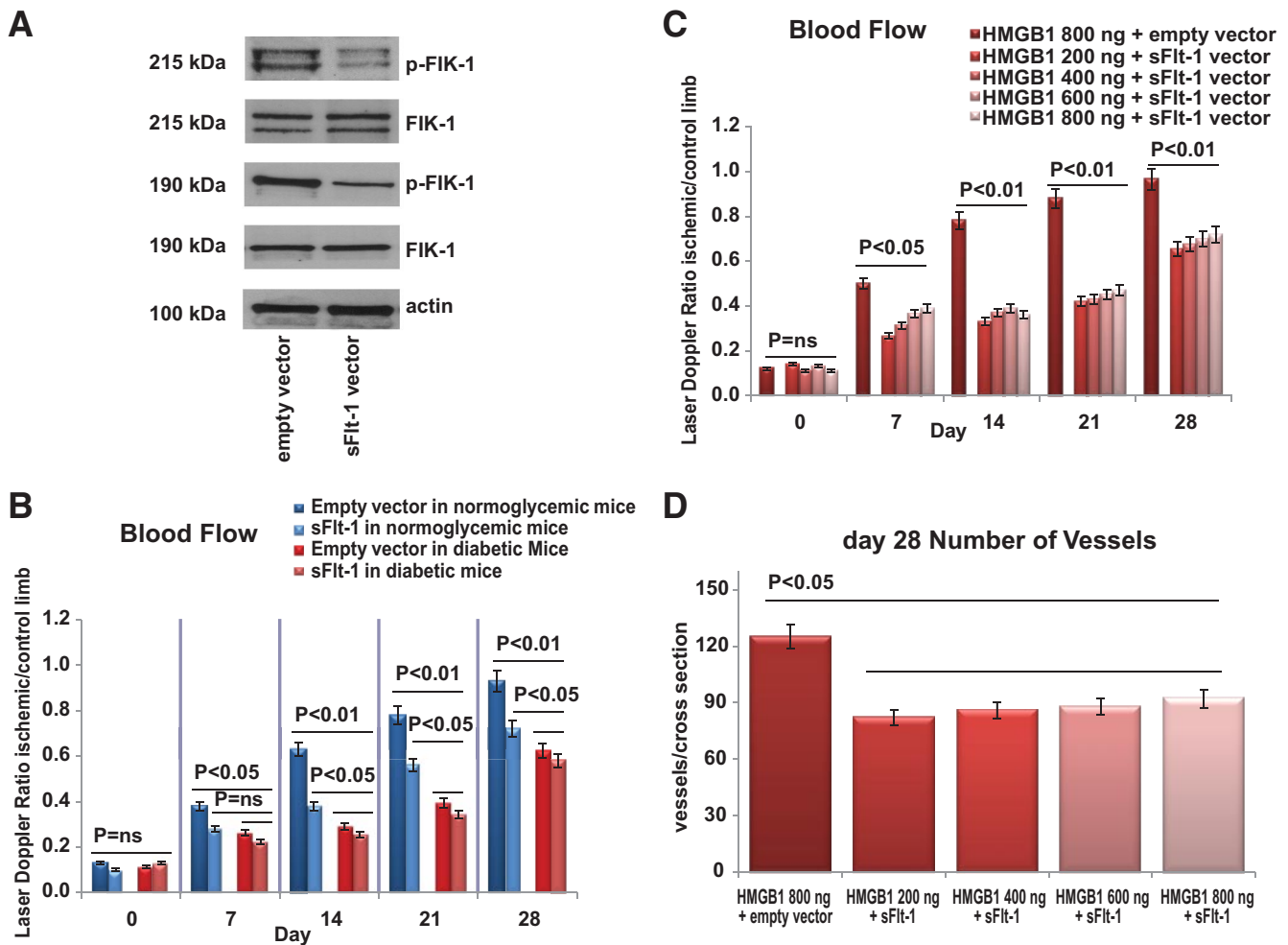


FIG. 6. A: Representative Western blot evaluation of VEGFR Flt-1 and Flk-1 protein content and their phosphorylated/activated isoforms (p-Flt-1 and p-Flk-1), 7 days after surgery, in the ischemic legs of diabetic mice previously treated with sFlt-1 or empty vector (control group). sFlt-1 treatment strongly reduced VEGFR phosphorylation, confirming the inhibition of the VEGF pathway. **B:** LDPI ratio in normoglycemic or diabetic mice previously treated with sFlt-1 or empty vector. Representative evaluation of LDPI ratio immediately after and on days 7, 14, 21, and 28 after surgery. VEGF inhibition attenuates postischemic angiogenesis in nondiabetic mice, but this group showed a better angiogenic response compared with diabetic animals. The blood flow of the ischemic hind limb is expressed as the ratio between the perfusion of the ischemic limb and the uninjured limb. $P < 0.01$ and $P < 0.05$ vs. sFlt-1-treated nondiabetic mice or vs. diabetic mice. **C:** LDPI ratio of the diabetic mice treated with 200, 400, 600, and 800 ng of HMGB1 previously treated with sFlt-1 or empty vector (control group). Representative evaluation of LDPI ratio immediately after and on days 7, 14, 21, and 28 after surgery. HMGB1-induced blood flow recovery in the diabetic mice is impaired when VEGF activity is inhibited. The blood flow of the ischemic hind limb is expressed as the ratio between the perfusion of the ischemic limb and the uninjured limb. $P < 0.05$ and $P < 0.01$ vs. sFlt-1-treated mice. **D:** The number of vessels per cross section is significantly reduced in HMGB1 + sFlt-1-treated mice compared with the HMGB1-treated mice that received the empty vector. $P < 0.05$ vs. sFlt-1-treated mice.

and inducing endothelial cell migration and sprouting (37). Other authors have demonstrated that the RAGE blockade inhibits HMGB1-induced neovascularization and endothelial cell proliferation in vitro (38) and that exogenous HMGB1 administration enhances angiogenesis and restores cardiac function in vivo (12). With regard to diabetes, a recent study showed that HMGB1 is underexpressed in the skin of diabetic mice and fibroblasts of patients affected by diabetes, that endogenous HMGB1 is crucial for skin tissue repair, that the reduced levels of HMGB1 in diabetic skin might impair wound healing, and that the exogenous topical administration of HMGB1 is able to correct this defect (8).

In our current study, we found that mice with diabetes have impaired perfusion recovery after femoral artery ligation and excision, in accordance with previous reports (39), and the preexisting evidence prompted us to investigate the role of HMGB1 in impaired ischemia-induced angiogenesis in diabetes. We observed that HMGB1 ex-

pression is reduced in the ischemic tissues of diabetic mice compared with normoglycemic mice. To our knowledge, this is the first demonstration that HMGB1 content is lower in the ischemic hind limb of diabetic mice. These findings are consistent with a previous report that showed that endogenous HMGB1 is reduced in other injured diabetic tissues (8). Thus, we further examined whether HMGB1 is crucial for postischemic angiogenesis. To test our hypothesis, we first inhibited the HMGB1 pathway using BoxA, which acts by inhibiting HMGB1 binding to its receptor RAGE, and we observed that when the local activity of this cytokine is reduced, ischemia-induced neovascularization is impaired in normoglycemic mice. This is another important result of our study because, in accordance with other authors, it seems reasonable to assume that HMGB1, through binding RAGE, can also act as an angiogenic switch molecule (37). Accordingly, we suggested that reduced HMGB1 in diabetic ischemic hind limbs might account, at least in part, for the impaired

ischemia-induced angiogenesis in diabetes. In agreement with this hypothesis, we noted that local HMGB1 administration enhanced postischemic neoangiogenesis in diabetic mice. These findings were evident 7 days after ischemia, when perfusion recovery and the inflammatory response were comparable in the ischemic tissue of control and diabetic mice, further suggesting that these changes in the HMGB1 system play a causative role in the impaired recovery seen at later time points. These data represent the third relevant discovery of our work because they indicate that the local administration of HMGB1 could be an attractive approach for treating PAD in patients with diabetes. There are several mechanisms by which HMGB1 can promote this process, but we focused our attention on the VEGF pathway. In this regard, we initially found that VEGF protein levels are significantly increased in the ischemic hind limbs of diabetic mice treated with HMGB1 relative to untreated mice. Thus, we tested the hypothesis that the angiogenic properties of HMGB1 might depend on VEGF activity. Therefore, we suppressed VEGF activity and found a substantial reduction of HMGB1-induced neoangiogenesis when VEGF activity is suppressed. These findings demonstrate that exogenous HMGB1 administration enhances ischemia-induced angiogenesis in diabetic mice via a VEGF-dependent mechanism.

Our observations are consistent with several studies that have shown that HMGB1 has the properties of an angiogenic cytokine in promoting endothelial cell sprouting and migration under hypoxic and necrotic conditions (37). Furthermore, studies on the transcriptional profiles of angiogenic endothelial cells have revealed HMGB1 as a potentially angiogenic factor (40). By contrast, other data have suggested a potential role for HMGB1 in atherosclerosis (41), demonstrating enhanced HMGB1 expression in atherosclerotic lesions compared with normal arteries. These considerations and our original results indicate that the HMGB1/RAGE system in cardiovascular diseases acts as a double-edged sword in a scenario, such as tissue ischemia, in which autocrine, paracrine, or the combined effects of the HMGB1/RAGE system on different cell types might lead either to injury or repair phenomena. However, an emerging function of HMGB1 in tissue repair is currently being actively investigated. For example, HMGB1 plays an important role in axonal regeneration (42) and myogenesis (43) in a RAGE-dependent manner. Furthermore, HMGB1 is implicated in stem cell homing and development (4,44). In fact, low doses of HMGB1 are capable of activating stem cells, which is expected to be useful in tissue regeneration, as demonstrated for cardiac (44) and neural (45) repair.

In conclusion, we have demonstrated that a disturbed tolerance against severe limb ischemia under hyperglycemia is, at least in part, attributable to the disturbance of the HMGB1 pathway, and that the local administration of the HMGB1 protein is sufficient to improve neoangiogenesis caused by limb ischemia in diabetic mice. We have also shown that this angiogenic response is dependent on VEGF. Therefore, the HMGB1 signaling system could be an attractive molecular target for treating PAD in patients with diabetic vascular complications.

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