Diabetic conditions promote binding of monocytes to vascular smooth muscle cells and their subsequent differentiation

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Diabetic conditions promote binding of monocytes to vascular smooth muscle cells and their subsequent differentiation. Am J Physiol Heart Circ Physiol 298: H736–H745, 2010. First published December 18, 2009; doi:10.1152/ajpheart.00935.2009.—Diabetes is associated with significantly accelerated rates of atherosclerosis, key features of which include the presence of excessive macrophage-derived foam cells in the subendothelial space. We examined the hypothesis that enhanced monocyte-vascular smooth muscle cell (VSMC) interactions leading to subendothelial monocyte retention and differentiation to macrophages under diabetic conditions may be underlying mechanisms. Human aortic VSMCs (HVSVMCs) treated with diabetic stimuli high glucose (HG) or S100B, a ligand of the receptor for advanced glycation end products, exhibited significantly increased binding of THP-1 mononuclear cells. Diabetic stimuli increased the expression of the adhesive chemokine fractalkine (FKN) in HVSVMCs. Pretreatment of HVSVMCs with FKN or monocyte chemoattractant protein-1 (MCP-1) neutralizing antibodies significantly inhibited monocyte-VSMC binding, whereas monocytes treated with FKN showed enhanced binding to VSMC. Mouse aortic VSMCs (MVSMCs) derived from type 2 diabetic db/db mice exhibited significantly increased FKN levels and binding to mouse WEH178/24 monocyte cells relative to nondiabetic control db/+ cells. The enhanced monocyte binding in db/db cells was abolished by both FKN and MCP-1 antibodies. Endothelium-denuded aortas from db/db mice and streptozotocin-induced diabetic mice also exhibited enhanced FKN expression and monocyte binding, relative to respective controls. Coculture with HVSVMCs increased CD36 expression in THP-1 cells, and this was significantly augmented by treatment of HVSVMCs with S100B or HG. CD36 mRNA and protein levels were also significantly increased in WEH178/24 cells after coincubation with db/db MVSMCs relative to control MVSMCs. These results demonstrate that diabetic conditions may accelerate atherosclerosis by inducing key chemokines in the vasculature that promote VSMC-monocyte interactions, subendothelial monocyte retention, and differentiation.

macrophages; cell adhesion; monocyte chemoattractant protein-1; fractalkine; S100B

CARDIOVASCULAR COMPLICATIONS are the leading cause of morbidity and mortality in patients with diabetes mellitus. Diabetes is associated with significantly accelerated rates of atherosclerosis, which is hallmarkmed by the presence of considerable amounts of macrophage-derived foam cells in the subendothelial space (2, 4). Macrophages and foam cells express key scavenger receptors and play important roles in the uptake of oxidized lipids, lesion development, and, ultimately, plaque instability and disruption (37). Coronary atherectomy specimens from patients with diabetes mellitus exhibit larger areas of monocyte-macrophage infiltration compared with those from patients without diabetes (30). Although much is known about the mechanisms of monocyte recruitment to the endothelium in lesion-prone areas, the mechanisms by which monocytes/macrophages are retained within the vessel wall and lead to foam cell formation, especially under diabetic conditions, are less well documented.

Evidence suggests that vascular smooth muscle cells (VSMCs) migrating and proliferating from the media to the intima may play important roles in monocyte/macrophage retention (5, 10, 24, 26). The potential of VSMCs to interact with monocytes/macrophages has been demonstrated by the expression of adhesion molecules in VSMCs within injured arteries and atherosclerotic lesions relative to the normal vessel wall (12, 24, 26). In addition, a correlation has been demonstrated between adhesion molecule expression on intimal VSMCs and mononuclear cell infiltration (3). Furthermore, the expression of intercellular adhesion molecule (ICAM)-1 on VSMCs occurs before or coincident with mononuclear cell infiltration, suggesting a causative role of VSMCs in monocyte/macrophage accumulation in atherosclerosis (13, 32).

Chemokines are a large family of proteins that induce monocyte recruitment to regions of inflammation, and several types of chemokines have been identified. Among these, monocyte chemoattractant protein (MCP)-1 and fractalkine (FKN) have been reported to be important mediators of the interaction between monocytes and VSMCs or vascular endothelial cells, and they play distinct roles in the development of neointimal thickening and atherosclerosis (16, 26, 36, 38, 46, 50). However, the exact roles of MCP-1 and FKN in the binding of monocytes to VSMCs under diabetic conditions have not been clearly understood.

We previously reported that treatment of human aortic VSMCs with angiotensin II (ANG II) or platelet-derived growth factor-BB (PDGF-BB), two proatherogenic factors, significantly increased the binding of human monocytes at least in part by activating arachidonic acid metabolism in VSMCs (5). Interestingly, the binding of monocytes to VSMCs inhibited serum deprivation-induced monocyte apoptosis, whereas monocyte CD36 expression and oxidized low-density lipoprotein (ox-LDL) uptake were significantly increased under these conditions (6). These data indicate that the interaction between monocytes and VSMCs may contribute to monocyte survival as well as their differentiation to the macrophage phenotype and foam cell formation. However, it is not clear how diabetic conditions can accelerate these events.

Hyperglycemia and subsequent formation of advanced glycation end products (AGEs) are recognized as essential mediators in the pathogenesis of diabetic vasculopathy, since improvement in blood glucose control reduces the long-term complications of diabetes mellitus (4, 31). AGEs are formed

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from the nonenzymatic modification of proteins/lipids in vivo by glucose and related factors such as methylglyoxal that accumulate in diabetes and during aging. Increased levels of AGEs and expression of the cell surface receptor for AGEs (RAGE) are found in, and are correlated with, the severity of atherosclerotic lesions from diabetic subjects (15, 40, 51, 52). S100B, a member of the multigenic S100/calgranulin family of proteins, another physiological ligand for RAGE, is upregulated together with RAGE in the vessel wall on arterial injury, as well as in diabetes and inflammation (7, 9, 19, 20, 39). Interactions of these ligands with RAGE can lead to the perturbation of important vascular homeostatic functions, such as VSMC proliferation (18) and inflammatory gene expression (20, 35). We previously showed that S100B induces key signaling events and inflammatory gene expression in VSMCs and that RAGE expression was enhanced in cultured VSMCs from diabetic mice (35). Evidence also shows that blockade of RAGE signaling events and inflammatory gene expression in VSMCs (20, 35). We previously showed that S100B induces key monocyte adhesion and differentiation under diabetic conditions. We also examined key chemokines and factors triggering these adhesive interactions, whether such interactions occur ex vivo in cultured VSMCs and aortas derived from mouse models of diabetes, and whether they result in monocyte to macrophage phenotypic changes.

MATERIALS AND METHODS

Cell Culture

**Human aortic smooth muscle cells.** Human VSMCs (HVSMCs) were obtained from Clonetics (San Diego, CA) and grown in smooth muscle cell (SMC) basal medium supplemented with human epidermal growth factor (10 μg/ml), human fibroblast growth factor-2 (2 μg/ml), and 5% fetal bovine serum (FBS) as recommended by the manufacturer. Cells between passages 4 and 9 were used in the experiments.

**Mouse aortic VSMCs.** All animal studies were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee. Insulin-resistant and type 2 diabetic male db/db mice (strain BKS.Cg-m+/+lepr db/db, stock no. 000642) and nondiabetic age-matched male heterozygotes (strain BKS.Cg-m+/+lepr db/+), were obtained from Jackson Laboratories (Bar Harbor, ME). Mouse aortic VSMCs (MVSMSs) were isolated from 10- to 12-wk-old diabetic db/db and control db/+ mice under sterile conditions by enzymatic digestion as described earlier (25). MVSMSs were cultured in DME/F12 culture medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml). Cells between passages 4 and 7 were used for experiments. In addition, for a model of type 1 diabetes, normal male C57BL/6 mice obtained from Jackson Laboratories (Bar Harbor, ME). Mouse aortic VSMCs (MVSMSs) were isolated from 10- to 12-wk-old diabetic db/db and control db/+ mice under sterile conditions by enzymatic digestion as described earlier (25). MVSMSs were cultured in DME/F12 culture medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml). Cells between passages 4 and 7 were used for experiments. In addition, for a model of type 1 diabetes, normal male C57BL/6 mice obtained from Jackson Laboratories were injected with streptozotocin (STZ) as described previously (54) to induce diabetes and used 2 wk after established hyperglycemia. Mice injected with normal saline (NS) were used as controls. The blood glucose levels were 413.38 ± 15.08 mg/dl in diabetic db/db mice vs. 108.66 ± 1.53 mg/dl in control db/+ mice and 418 ± 26.4 mg/dl in STZ-induced diabetic mice vs. 99.83 ± 4.9 in NS-injected control mice.

**Monocytic cells.** Human THP-1 monocytic cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), streptomycin (100 mg/ml), and β-mercaptoethanol (50 μM). Mouse monocytic cells WEHI78/24 (29) (a gift from Dr. J. A. Berliner, University of California, Los Angeles) were grown in DME/F12 supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml).

Reagents

Fluorescent labels 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxyethyl ester (BCECF AM) and calcine O,O′-diacetaetoxymethylester (calcine AM) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). S100B protein (bovine brain) was purchased from Calbiochem (San Diego, CA). Enzyme-linked immunoassay (ELISA) kits for human and mouse FKN, mouse monoclonal anti-human MCP-1, and anti-human, anti-rat, or anti-mouse FKN antibodies were obtained from R&D Systems (Minneapolis, MN). Monoclonal anti-mouse MCP-1 neutralizing antibody was obtained from eBioscience (San Diego, CA). Alexa Fluor 488 conjugated anti-rabbit IgG antibody (A-11008) was obtained from Invitrogen (San Diego, CA). Mouse monoclonal antibody to CD36 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-RAGE antibody was obtained from Sigma-Aldrich Chemicals. Primers for β-actin gene (catalog no. 0809006) were obtained from Ambion (Austin, TX).

Monocyte-VSMC Binding Assays

Monocyte-VSMC binding assays were performed as described earlier (5, 42) with some modifications. Briefly, monocytes were fluorescently labeled by incubating with either BCECF AM (5 mg/l) in RPMI 1640 for THP-1 cells or calcine AM (5 μM) in DME/F-12 for mouse monocytes (WEHI78/24) in foil-covered tubes at 37°C for 30 min. Fluorescently labeled cells were washed twice to remove unincorporated label and resuspended in medium containing 0.5% FBS. Monocytes (5 × 10^6 cells/well) were added to 24 wells containing confluent VSMC monolayers that were serum-depleted for 48 h. After incubation for 30 min at 37°C, medium was removed and VSMC layers with attached monocytes were gently washed twice with DME/F12 containing 0.5% FBS. BCECF AM-labeled THP-1 cells were lysed by adding 0.2 ml of 0.1 M Tris containing 0.1% Triton X-100 in each well. Fluorescence was measured using an /Max Microplate reader (excitation, 485 nm; emission, 535 nm; Molecular Devices, Sunnyvale, CA). Data were analyzed using SoftMax Pro. Calcine AM-labeled WEHI78/24 cells bound to MVSMSs were fixed with 1% glutaraldehyde for 10 min, and images were collected using a fluorescence microscope. The bound WEHI78/24 cells were counted using Quantity One 4.3.1 software (Bio-Rad, Hercules, CA). The number of adherent cells per well was expressed as a percentage of control. In some binding assays, HVSMCs were preincubated with monoclonal antibodies against MCP-1 (10 mg/l), RAGE (10 mg/l), or FKN (2.5 mg/l) for 1 h before addition of THP-1 or WEHI78/24 cells. Normal mouse IgG was used as a negative control.

Flow Cytometry Analysis of CD36

After 24 h of coculture with HVSMCs, unbound THP-1 cells were collected by gentle aspiration, and bound THP-1 cells were collected by gently shaking the plates and carefully pipetting the medium. Monocyte surface CD36 expression was measured by flow cytometry after staining of THP-1 cells with R-phycocerythrin-conjugated human CD36 antibody (BD Pharmingen, San Diego, CA) according to the supplier’s protocols. Samples were analyzed on a MoFlo MLS flow cytometer (Dako-Cytomation, Fort Collins, CO), and results are expressed as the percentage of cells positive for CD36.

Reverse Transcription and Real-Time PCR

Total RNA was extracted from cells with RNA-STAT, and real-time quantitative RT-PCR with 18S internal control were performed using the SYBR green PCR master mix kit (Applied Biosystems, Foster City, CA) with an ABI 7300 real-time PCR thermal cycler.
(Applied Biosystems) as described previously (5, 42, 48). Reaction conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were performed in triplicate in a final volume of 20 μl. Standard curves were generated using Applied Biosystems software. Dissociation curves were run to detect nonspecific amplification and to confirm that single products were amplified in each reaction. The quantities of the test gene and internal control 18S mRNA were then determined from the standard curve using the Applied Biosystems software, and mRNA expression levels of test genes were normalized to 18S levels. PCR primers were obtained from Integrated DNA Technologies. Primers for human FKN were 5'-TCTGCACTCAGCTGCTCTG-3' (forward) and 5'-CATGATGCTGTTGTGTG-3' (reverse); for mouse FKN, 5'-GGCTAAGCCTCAGACATTG-3' (forward) and 5'-CTGTATGGAGGGGACTCA-3' (reverse); and for mouse CD36, 5'-GGAAACCACGCTTTCACAAAAC-3' (forward) and 5'-GCTACAGCCAGATTCGAA-3' (reverse).

**ELISA.**

For soluble FKN measurement, cell culture supernatants were collected and centrifuged at 3,000 rpm for 5 min to remove the cell debris. Medium was concentrated with an Amicon Ultra centrifugal filter unit (Millipore, Bedford, MA). Soluble FKN levels were assayed using an ELISA kit as described by the manufacturer and normalized to total cell protein. To examine cellular FKN expression, cells were washed twice with cold PBS and lysed. ELISAs were performed using 50 μg of cell lysate protein. Heparin-treated blood samples from db/db and db/db mice and normal saline- and STZ-treated mice were used to evaluate plasma levels of FKN. FKN concentrations in plasma were analyzed by sandwich-type ELISA using the manufacturer’s instructions.

**Immunofluorescence Staining**

MYSMCs from db/db and db/+ mice were grown on coverslips, and binding assays with mouse WEHI78/24 cells were performed as described earlier (6). After binding, cells were washed twice with PBS and fixed in 1% paraformaldehyde in PBS for 15 min at room temperature. Fixed cells were washed twice with PBS with 2 mM MgCl2, blocked by incubation with 1% BSA in PBS for 1 h at room temperature, and incubated with primary antibody (CD36, 1:50) in 1% BSA/PBS for 1 h at 37°C. After being washed four times with PBS containing 0.02% Triton X-100 (PBS/TX100) and once with 1% BSA/PBS, cells were incubated with Alexa Fluor 488-conjugated anti-rabbit IgG secondary antibodies in 1% BSA/PBS for 1 h at 37°C. Cells were then washed with PBS/TX100 once, incubated with PBS/TX100 containing 4–6-diamidino-2-phenylindole (0.2 mg/l) for 10 min, washed twice with PBS/TX100 and once with PBS, and rinsed with water. Cells were then examined by fluorescence microscopy (Olympus IX51; Olympus Optical, Hamburg, Germany), and fluorescent cells were directly counted in five different fields per slide in triplicate groups.

**Morphological Studies and Immunohistochemistry**

All histological studies were performed with frozen aortic sections. Mouse aorta were fixed in 10% neutral buffered formalin and stored frozen at −70°C. Immunohistochemistry was performed as described previously (22, 48). Briefly, 5-μm sections were incubated overnight at room temperature with goat anti-rat FKN antibody (1:25) diluted with 2% casein in BSA. After washing, sections were incubated with horseradish peroxidase-conjugated donkey anti-goat antibody for 20 min. Aortas were examined using light microscopy at ×400 magnification. Densities of FKN-positive staining areas and nuclear staining (hematoxylin) were measured using Image-Pro Plus 5.1 (Media Cybernetics, Silver Spring, MD), and FKN staining was normalized to nuclear staining density. For negative nonimmune control slides, the primary antibody was skipped.

**Statistical Analysis**

Data are means ± SE of multiple experiments. Student’s t-tests were used to compare two groups, or analysis of variance with Tukey’s post hoc tests for multiple groups, using PRISM software (GraphPad, San Diego, CA). Values of P < 0.05 were considered statistically significant.

**RESULTS**

**Ex Vivo Adhesion of Monocytes to Smooth Muscle Layer of Endothelium-Denuded Aortas From Mice**

Aortas from mouse models of type 1 and type 2 diabetes were rapidly excised under general anesthesia, carefully trimmed to remove fat and connective tissue, and washed twice with ice-cold PBS. They were placed in DMEM containing collagenase (1 g/l) and elastase (0.3 g/l) for 10 min at 37°C. The ends of aortas were threaded onto a 27-gauge needle, and digestion solution was allowed to flow through several times. The endothelium-denuded aortas were then opened longitudinally and pinned onto 8% agar in 35-mm plates with added 1 ml of DMEM containing 1% heat-inactivated FBS (HI-FBS). Each aorta was incubated for 30 min with 1.0 × 106 calcein AM-labeled WEHI78/24 monocytes per dish. After incubation, unbound monocytes were removed by washing three times with 1 ml of DMEM + 1% HI-FBS. Aortas were fixed with 1% glutaraldehyde for 10 min, and images were collected using a fluorescence microscope. The numbers of monocytes bound to aortas were counted in a minimum of three fields per aorta and aortas from at least four mice per group.

**Chronic HG Treatment Increases HVSMC/THP-1 Cell Binding**

We first examined whether treatment of VSMCs with HG can lead to increased binding of monocytes. HVSMCs were treated with 25 mM d-glucose (HG) for 7 days, and monocyte-VSMC binding assays were performed using the human THP-1 monocyctic cell line as described in MATERIALS AND METHODS. Results showed that monocyte binding to HVSMCs treated with HG was significantly increased in a time- and dose-dependent manner with optimal effects seen with 25 mM glucose treatment for 3–10 days (Fig. 1A and B). Mannitol treatment (5.5 mM glucose plus 19.5 mM mannitol), used as an osmolar control, showed no effect relative to normal glucose (NG). TNF-α treatment (20 μg/l, 4 h) of the HVSMCs was used as a positive control for the binding assays (Fig. 1C).

Increased HVSMC/THP-1 cell binding could be due to increased cell-surface expression of VCAM-1 and/or ICAM-1 on HVSMCs. However, no change on HVSMC surface VCAM-1 or ICAM-1 expression was found by 7 days of exposure to HG as demonstrated by flow cytometry (Cai Q and Natarajan R, unpublished results). Furthermore, we excluded the possibility of HVSMC proliferation as the cause for HG-induced binding because cell number was not significantly changed during this treatment period.

On the other hand, THP-1 cells stimulated with conditioned medium prepared from HVSMCs treated with HG for 7 days showed significantly increased binding to unstimulated HVSMCs (172.7%, P < 0.01, vs. NG conditioned medium) (Fig. 1D). These results suggest that soluble factors released from HVSMCs cultured in HG play a key role in enhancing monocyte-VSMC binding under HG conditions.
Increased Monocyte-VSMC Binding in HVSMCs Treated With RAGE Ligand S100B

Levels of RAGE and its ligands, such as AGEs and S100B, are increased in diabetes and contribute to vascular inflammation and atherosclerosis (15, 19, 20, 35, 39, 41, 42, 53). Therefore, we next investigated whether the RAGE ligand S100B could promote monocyte-VSMC interactions. Treatment of HVSMCs with S100B significantly increased the binding of THP-1 cells in a time (Fig. 2A) and dose-dependent manner (Fig. 2B) with maximal effects at 40 mg/l for 3 days (P < 0.001). This effect was sustained for at least 7 days (Fig. 2A). Furthermore, S100B-induced THP-1 cell binding was significantly inhibited by pretreatment of HVSMCs with a RAGE antibody (P < 0.001) but not with control IgG (Fig. 2C), suggesting that S100B-induced monocyte-VSMC binding is mediated via RAGE activation in HVSMCs.

Role of Chemokines MCP-1 and FKN in Monocyte-VSMC Binding

Chemokines such as MCP-1 and FKN (CX3CL1) play important roles in inflammation and atherosclerosis. MCP-1 is a strong chemotactic agent that is produced in VSMC by several factors, including HG, AGEs, and S100B (11, 18, 35). It is also induced by HG in THP-1 cells (42). FKN also has strong chemotactic effects, and its expression is increased in the aortas of patients with atherosclerosis or diabetes (50). Furthermore, the unique feature of FKN is its existence as both membrane-tethered and soluble forms. Therefore, it has dual activities, with the soluble form acting as a potent chemoattractant and the membrane-tethered form acting as an adhesion molecule via interactions with its receptor, CX3CR1, on monocytes (46). Therefore, we next examined involvement of both MCP-1 and FKN in HG- or S100B-induced HVSMC/THP-1 cell interactions. As shown in Fig. 3A, FKN mRNA was significantly induced by HG and S100B in HVSMCs. Furthermore, treatment of HVSMCs with HG or S100B, but not with mannitol, significantly increased the levels of FKN protein in both VSMC culture supernatants (Fig. 3B) and cell lysates (Fig. 3C).

Next, to examine the functional role of MCP-1 and FKN in monocyte-VSMC binding, we preincubated HG- or S100B-treated HVSMCs with neutralizing antibodies against MCP-1 (10 mg/l), FKN (2.5 mg/l), or normal IgG as a control before THP-1 cells were added to the binding assays. Results showed that pretreatment of HVSMCs with MCP-1 antibody significantly blocked S100B-induced THP-1 cell binding (P < 0.001) (Fig. 4A) but had no effect on HG-induced binding. On the other hand, pretreatment with FKN antibody significantly in-
hindered both HG- and S100B-induced THP-1 cell binding ($P < 0.001$) (Fig. 4A). Combined treatment of MCP-1 and FKN antibody did not have any synergistic effects (Fig. 4A). In addition, direct treatment of THP-1 cells with recombinant FKN significantly increased their binding to HVSMCs ($P < 0.001$) in a dose (Fig. 4B) and time-dependent manner (Fig. 4C) ($P < 0.001$). Together, these results demonstrate that MCP-1 and FKN play key roles in monocyte-VSMC interactions under diabetic conditions.

### Enhanced Monocyte Binding to VSMCs Derived From Diabetic db/db Mice Relative to Control db/+ Mice

To test the in vivo relevance of these findings, we next examined whether monocyte binding is enhanced in MVSMCs derived from db/db mice, a well-established model of obesity, insulin resistance, and type 2 diabetes model. Our previous studies showed that short-term ex vivo cultures of MVSMCs derived from db/db mice exhibit enhanced proinflammatory responses including MCP-1 expression and monocyte binding (25). In this study we examined whether FKN expression is also increased and whether both MCP-1 and FKN play a role in enhanced monocyte-VSMC interactions in db/db cells. We first performed monocyte-VSMC binding assays using fluorescently labeled WEHI78/24 monocytic cells. As shown in Fig. 5A and the bar graph quantification in Fig. 5B, there was significantly greater binding of mouse WEHI78/24 monocytes to MVSMCs derived from db/db mice relative to those from control db/+ mice ($P < 0.001$). These data further confirm our previous findings and illustrate the “preactivated” state of MVSMCs cultured from db/db mice. This sustained proinflammatory response could be due to a “metabolic memory” induced by the diabetic milieu (48).

Next, we analyzed FKN mRNA (by real-time quantitative RT-PCR) and protein levels (by ELISA) in db/db and db/+ MVSMCs. Results showed that FKN mRNA (Fig. 5C) levels and protein levels in both cell lysates (Fig. 5D) and culture supernatants (Fig. 5E) were significantly increased in db/db compared with db/+ MVSMCs ($P < 0.05$). Furthermore, pretreatment of db/db MVSMCs with MCP-1 or FKN neutralizing antibodies significantly attenuated the enhanced WEHI78/24 mouse monocyte binding to the db/db MVSMCs with no change in db/+ MVSMCs (Fig. 5F). In addition, pretreatment with a combination of MCP-1 and FKN neutralizing antibodies showed greater inhibitory effects on the monocyte-VSMC interactions in db/db MVSMCs than either alone (Fig. 5F). These results demonstrate that MCP-1 and FKN may operate through both similar and distinct mechanisms to promote monocyte-VSMC interactions under diabetic conditions.

### Increased Monocyte Adhesion to Endothelium-Denuded Aortas (VSMC Layer) From Mouse Models of Type 1 and Type 2 Diabetes

To further evaluate the in vivo significance, we tested monocyte binding to intact endothelium-denuded aortas with exposed VSMC layer from mouse models of both type 1 (STZ injected) and type 2 (db/db) diabetes as described in MATERIALS AND METHODS. Aortas with exposed VSMC layer from db/db mice bound significantly more WEHI78/24 cells than those
from their nondiabetic (db/+ controls (P < 0.001) (Fig. 6, A and B). Similarly, endothelium-free aortas derived from STZ-injected diabetic mice also exhibited increased monocyte adhesion relative to control NS-injected mice (P < 0.01) (Fig. 6, C and D).

Since FKN expression was increased in db/db MVSMCs and was induced by diabetogenic agents in HVSMCs, we next examined the expression levels of FKN in plasma and aortas from both db/db and STZ diabetic mice. Aortas from db/db (Fig. 7, A and B) as well as STZ-injected mice (Fig. 7, C and D) showed significantly increased FKN immunostaining compared with db/+ or NS-injected mice, respectively (P < 0.001). However, plasma concentration levels of FKN were not altered in both db/db and STZ-treated mice compared with corresponding controls (Park J and Natarajan R, unpublished results). Thus local increment of FKN expression in VSMCs may play an important role in recruiting monocytes under diabetic conditions.

Diabetic Conditions Enhance VSMC Binding-Induced Monocyte CD36 Expression

In the next step, we examined the functional consequence of the increased monocyte binding to VSMCs under diabetic conditions. We recently demonstrated that macrophage type B scavenger receptor (CD36) expression, an index of monocyte differentiation and foam cell formation, was upregulated in both THP-1 cells and human peripheral blood monocytes on binding to VSMCs, suggesting that monocytes bound to VSMCs undergo a proatherogenic differentiation program (6). In this study we tested whether VSMC binding-induced monocyte CD36 expression could be further enhanced under diabetic conditions.
enhanced expression of CD36 in THP-1 cells from multiple experiments is shown in Fig. 8B. However, CD36 expression in THP-1 cells cocultured with HVSMCs in the presence of osmotic control mannitol (5.5 mM glucose + 19.5 mM mannitol) did not show any difference compared with those cocultured with control HVSMCs in NG (Fig. 8B, Man/Coculture vs. Coculture). These results demonstrate that monocyte-HVSMC binding induced by HG and RAGE ligands enhances a proatherogenic differentiation program in monocytes that could be responsible for the accelerated atherosclerosis seen in diabetes.

Enhanced CD36 Expression in Mouse Monocytic Cells Bound to Diabetic MVSMCs

We next examined whether this phenotypic modulation of monocytes seen during binding to HG/S100B-treated HVSMCs also occurs after incubation with MVSMCs derived from diabetic db/db mice. Mouse WEHI78/24 monocytes were cocultured with db/+ or db/db MVSMCs, and total RNA from bound monocytes was used to determine CD36 mRNA levels by real-time quantitative PCR. There was a significant increase in CD36 mRNA expression in WEHI78/24 monocytes attached to MVSMCs from db/db mice relative to db/+ mice (Fig. 8C). In addition, evaluation of CD36 expression in bound WEHI78/24 cells by immunostaining with a CD36 antibody showed significantly enhanced CD36 staining in WEHI78/24 cells bound to db/db MVSMCs compared with db/+ MVSMCs (Fig. 8D). These results demonstrate that binding of monocytes to VSMCs under diabetic conditions has functional and pathological consequences by greatly accelerating a differentiation program in monocytes. Furthermore, the “metabolic memory” in the ex vivo cultured VSMCs from diabetic mice also is able to promote this monocyte differentiation in a sustained fashion.
DISCUSSION

Atherosclerotic lesions in patients with diabetes are characterized by excessive macrophage/foam cell infiltration compared with those from nondiabetic individuals, suggesting that monocyte recruitment to the vessel wall is augmented under diabetic conditions (30). However, this process alone cannot account for macrophage accumulation in the lesions, since monocyte recruitment is counterbalanced by egression mechanisms (27, 34). Our findings in this study indicate that diabetic conditions may counteract this egression by enhancing monocyte binding to VSMCs and subsequent subendothelial retention leading to accelerated monocyte differentiation associated with foam cell formation in the pathology of atherosclerosis.

Treatment of HVSMCs with HG or S100B, a RAGE ligand, could significantly promote binding of human THP-1 cells to VSMCs. Chronic, but not acute, treatment of HVSMCs with HG induced the binding of monocyctic THP-1 cells. Soluble factors seemed to play a major role in mediating these heterotypic interactions. Chronic hyperglycemia can promote diabetic complications via increased AGE formation within the arterial wall. Our results suggest an additional pathological role for AGE-RAGE interactions by promoting monocyte binding to VSMCs and retention within atherosclerotic plaques. This can be accomplished by soluble factors released from VSMCs after RAGE ligaton. MCP-1 seemed to be a key candidate soluble factor in this context, since S100B-induced binding was attenuated by an MCP-1 blocking antibody. This is supported by reports showing that AGEs and S100B can increase MCP-1 expression in cultured VSMCs (18, 35). MCP-1 is a CC-type chemoattractant cytokine that specifically induces transendothelial chemotaxis of monocyctic cells and has been detected in atherosclerotic lesions. Our earlier data showed that HVSMC/THP-1 cell binding induced by growth factors such as ANG II and PDGF-BB was partially mediated by MCP-1 released by HVSMCs (5). These reports coupled with our current results suggest that, apart from its well-known role in monocyte migration and chemotaxis, MCP-1 may further promote persistent subendothelial monocyte retention by enhancing active VSMC/monocyte interactions, and this may be augmented in the diabetic state. However, HG-induced monocyte-VSMC interaction was not blocked by MCP-1 antibody, suggesting that HG- and S100B-induced monocyte binding may require a different set of soluble factors in addition to common ones such as FKN, shown presently. It also suggests activation of both common and different signaling pathways by HG and S100B in VSMC. This could due to the fact that S100B signals via receptor-mediated mechanisms, unlike HG. Further studies are required to understand these divergent factors and signaling mechanisms.

Another finding was the important role of FKN in monocyte-VSMC interactions both in vitro under diabetic conditions and in vivo in diabetes animal models. FKN is a unique chemokine for VSMC-monocyte interactions because it has dual functions, acting as both chemoattractant and adhesion molecule (1, 46). In patients with atherosclerosis or diabetes, FKN expression was upregulated and concentrated in the medial area of the vessel wall (50). In the current study, inhibition of FKN effectively suppressed the increased binding of THP-1 cells to HVSMCs under diabetic conditions (HG and S100B). Furthermore, HVSMCs showed increased expression of FKN mRNA and protein under diabetic conditions. A recent study showed HG-induced FKN expression in VSMCs (11), but the role of RAGE ligands or in vivo relevance was not demonstrated.

Previous reports showed increased levels of S100B and other S100/calgranulin family proteins in aortic tissues of both human atherosclerotic plaques and animal models of diabetes. The S100 proteins induce diverse responses in various cell types including vascular cells as well as macrophages in a RAGE-dependent manner, demonstrating a clear role in the initiation and amplification of proinflammatory responses and vascular dysfunction (9, 17, 19, 28, 43, 53). In this study, we demonstrated a novel role for S100B in FKN expression and in promoting monocyte-VSMC interactions. It is not clear whether the S100B concentrations used in our in vitro studies are relevant to those present in the vascular compartment in vivo. The circulating S100B levels can vary substantially and become very high in some disease conditions (9, 19, 53), and it also is highly likely that local concentration of S100B in diabetic vessels or lesions are much higher than in serum. Furthermore, in vivo, S100B may be acting in concert with other S100 proteins, RAGE ligands, and proinflammatory cytokine/chemokines and thus may require much lower concentrations for cellular activation compared with in vitro studies. Our results showing that RAGE ligands promote monocyte VSMC interactions further support the “two hit” model of vascular complications in which vascular cells primed by the proinflammatory actions of RAGE ligands in diabetes may contribute to accelerated vascular disease on exposure to additional risk factors (41). Interestingly, MVSMCs cultured from the aortas of type 2 diabetic db/db mice exhibited greater capacity to bind to mouse monocyctic cells relative to MVSMCs from nondiabetic db/+ mice. Moreover, similar to the in vitro results with HVSMCs, this enhanced binding was inhibited by MCP-1 and FKN antibodies, further supporting key mediatory roles for these soluble factors in the increased VSMC-monocyte interactions under diabetic conditions. Antibodies to MCP-1 and FKN showed some synergistic effects in inhibiting WEHI78/24 cell adhesion to VSMCs from diabetic mice, suggesting that MCP-1 and FKN may have similar as well as independent functions in promoting monocyte adhesion under diabetic conditions. This is supported by a study showing greater reduction in macrophage accumulation and atherosclerosis in MCP-1 receptor knockout mice with FKN deficiency (38). We have obtained further evidence supporting the in vivo relevance of our results by showing that intact endothelium-denuded aortas (with exposed VSMC layer) from both db/db and STZ-injected type 1 diabetic mice exhibited enhanced monocyte binding relative to aortas from control non-diabetic animals. In db/db MVSMCs, cellular and soluble FKN were increased compared with db/+ cells. Furthermore, FKN expression in db/db or STZ-injected mice aortas was also higher than in db/+ or NS-treated mice aortas, respectively. However, plasma FKN levels did not show any changes in the diabetic mice, suggesting that increased local FKN may be an important mediator of accelerated atherosclerosis in diabetes. Although several reports have demonstrated the role or expression of FKN in atherosclerosis models including ApoE null mice (8, 23, 38, 45), ours is the first evidence of increased aortic expression of FKN in diabetic mice. In addition, we have observed increased RAGE immunostaining in aortas of db/db
and STZ-injected diabetic mice relative to control db/+ or saline-injected control mice, respectively (Meng L, Hang Y, Reddy MA, Natarajan R, unpublished observations), suggesting the in vivo relevance of RAGE-dependent monocyte-VSMC interactions.

The pathophysiological consequences of VSMC-monocyte adhesive events remain poorly characterized. The differentiation of monocytes to macrophages and subsequent transformation to lipid-laden foam cells constitute major events in the development of atherosclerotic lesions (14). One of the key upregulators of monocyte to macrophage differentiation is the upregulation of the macrophage type B scavenger receptor CD36, the expression of which correlates with the uptake of ox-LDL and foam cell formation (21). Our results show that the monocyte expression of CD36 was significantly increased following coincubation with VSMCs under diabetic conditions. These results suggest that key neighboring cell-cell heterotypic communication occurs in the subendothelial space that promotes retention, foam cell formation, and atherosclerosis, and these events are greatly augmented under diabetic conditions. Therefore, VSMC-monocyte interactions are key regulatory signals promoting vascular dysfunction during the pathogenesis of atherosclerosis. The precise molecular mechanisms and factors responsible for increased CD36 formation have not been elucidated in this study. Soluble and insoluble factors induced by HG, growth factors, S100B, fatty acids, and glucose-induced oxidation products could be key players in not only promoting the intercellular interactions but also subsequent monocyte CD36 expression and differentiation (44, 47).

Together, we have examined the relatively less studied mechanisms of monocyte retention in the vascular wall and demonstrate that VSMC-monocyte adhesive interactions and parameters of subsequent monocyte differentiation are significantly enhanced under diabetic conditions. Such interactions may facilitate foam cell formation and thereby accelerate the progression of atherosclerosis in diabetic patients.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES


