Evidence for a Matrix Metalloproteinase Induction/Activation System in Arterial Vasculature and Decreased Synthesis and Activity in Diabetes

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Pathological remodeling characterized by extracellular matrix (ECM) deposition contributes to the diabetic vascular complications. Matrix metalloproteinases (MMPs) regulate ECM turnover. However, the expression profile of the MMP system in diabetic human tissue remains unknown. The objectives of this study were *1*) to identify a local MMP induction/activation system that exists in arterial vasculature and *2*) to determine how the MMP system may be altered in diabetes. Internal mammary artery specimens were obtained from patients who did (n = 14) and did not (n = 14) have diabetes and were undergoing coronary artery bypass grafting surgery. ECM inducer protein (EMMPRIN); membrane-type MMP (MT-MMP); and MMP-1, -2, and -9 were quantified by immunoblotting and densitometric scanning (pixels). Pro–MMP-1 and MMP-2 levels were decreased from 952 ± 120 and 1,081 ± 508 pixels, respectively, in nondiabetic tissue to 398 ± 62 and 249 ± 42 pixels in the diabetic tissue ($P < 0.05$). Both EMMPRIN and MT-MMP expression and total MMP activity were decreased by twofold in diabetic patients ($P < 0.05$). These results demonstrated for the first time that an MMP induction and activation system exists in human arterial vasculature and that it is downregulated in diabetes. Decreased MMP activity may contribute to increased collagen deposition and pathological remodeling in diabetes. *Diabetes* 51: 3063–3068, 2002

Cardiovascular complications are the leading cause of morbidity and mortality in patients with diabetes (1,2). Alterations in vascular structure contribute to the pathogenesis of vascular complications of diabetes. In animal models, pathological remodeling of the mesenteric arteries exhibits extracellular matrix (ECM) deposition, intimal proliferation, and increased media-to-lumen ratio (3–6). In the retinal and renal vasculature, thickening of the basement membrane characterized by collagen deposition contributes to diabetic retinopathy and nephropathy, respectively (7,8). Structural changes in the peripheral arterial vasculature of patients with diabetes are not completely understood.

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that degrade ECM proteins such as collagen and elastin and are essential for cellular migration and tissue remodeling under physiological and pathological conditions (9,10). MMPs are synthesized as latent proenzymes, which are later activated by serine proteases, including trypsin and plasmin, active MMP-2, and recently described membrane type (MT)-MMPs (10,11). The MT-MMPs possess a transmembrane domain that anchors the enzyme to the membrane and can exert local MMP activation. For example, MT1-MMP contributes to the activation of MMP-2, which then can activate other secreted pro-MMPs to their active forms (12). The major MMP species expressed in the vasculature include MMP-1, MMP-2, MMP-9, and MT1-MMP, and both endothelial and smooth muscle cells can synthesize these enzymes (10). In addition to growth factors and cytokines, recent studies have identified an ECM metalloproteinase inducer protein (EMMPRIN) that stimulates the expression of MMP-1, MMP-2, and MMP-3 in fibroblasts (13). Whether this protein exists in endothelial and smooth muscle cells remains to be determined. The activity of MMPs is tightly regulated by tissue inhibitors of MMPs (TIMPs), and the MMP/TIMP ratio is critical for coordinating matrix production and degradation (10). To date, four distinct TIMP proteins have been identified, and TIMP-1 and TIMP-2 are the best characterized isoforms (10). TIMP-1 forms a complex with several MMP species, including MMP-1 and MMP-9 (10,14,15). The expression profile of this protein in peripheral arterial vasculature is also unclear.

The expression and activity of MMPs in diabetes have been studied predominantly in renal tissue. Several studies have documented downregulation of MMP-2 and MMP-9, which may explain mesangial matrix expansion found in diabetic nephropathy (8,16). However, Romanic et al. (17) reported increased renal MT5-MMP expression in patients with diabetes. A recent study demonstrated increased MMP-9 expression in aortic tissue homogenates obtained from diabetic animals (18). Whether and to what degree diabetes affects the expression and activity of the MMP induction/activation system in patients with diabetes remains unknown. Therefore, we investigated the presence and activity of the components of the MMP system in internal mammary artery (IMA) specimens obtained from

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ECM, extracellular matrix; EMMPRIN, extracellular matrix metalloproteinase inducer protein; IMA, internal mammary artery; MMP, matrix metalloproteinase; MT-MMP, membrane-type matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinase.

TABLE 1
Patient demographics and list of medications

	Control	Diabetic
Age (mean ± SD)	60 ± 2.6	60 ± 2.5
Race (black/white)	5/13	7/15
Sex (female/male)	5/13	8/14
Blood glucose (mg/dl; mean ± SD)	108 ± 18	162 ± 24
Body surface area (m^2; mean ± SD)	1.9 ± 0.2	1.9 ± 0.2
Smoking history	5	4
Type 1/type 2 diabetes	—	4/18
Medications		
Ca^{2+} channel blockers	3	4
ACE inhibitors/all receptor blockers	9	6
β-Blockers	11	11
Cholesterol-lowering drugs	7	5
Diuretics	5	4
Antiaggregant therapy	13	6
Antidepressant therapy	1	3
Vasodilators	10	6
Oral agents/insulin	—	18/6

ACE, angiotensin-converting enzyme.

patients who did and did not have diabetes and were undergoing coronary artery bypass surgery.

RESEARCH DESIGN AND METHODS

Tissue collection. The study protocol was approved by the Human Assurance Committee at the Medical College of Georgia, and written informed consent was obtained from all of the participants before the surgery. IMA specimens were obtained from patients who were undergoing coronary artery bypass graft surgery. Artery specimens were placed in cold Dulbecco's modified Eagle's medium and kept on ice. After surrounding fat was carefully removed, arterial specimens were rinsed in sterile saline, cut into 3-mm segments, and immediately snap-frozen. Although all patients had coronary artery disease, the vessels used in this study were used for bypass conduits and considered relatively healthy. Therefore, specimens obtained from patients without diabetes were used for control. Patient demographics are summarized in Table 1. Medications of the control patients and patients with diabetes were similar with the exception of the hypoglycemic agents (Table 1).

Vascular MMP extraction. Frozen artery specimens were homogenized in extraction buffer (1:10 wt/vol) containing 0.15 mol/l NaCl, 20 mmol/l ZnCl, 1.5 mmol/l NaN_3, 10 mmol/l cacodylic acid, and 0.01% Triton X-100. After centrifugation at 4°C for 10 min at a speed of 800*g*, the supernatant was concentrated using a Centriplus concentrator. Samples were centrifuged at 3,000*g* for 4.5 h at 4°C, and the protein content was measured using Bio-Rad Protein Assay (Bio-Rad, Richmond, CA). Samples were stored at −80°C in small aliquots.

Western analysis. Protein levels of MMP species (MMP-1, MMP-2, MMP-9, MT1-MMP, EMMPRIN, and TIMP-1) were determined by immunoblotting using antibodies specific for each species. Vascular extracts (20 μg) were diluted to the appropriate loading concentration in sample buffer containing 0.1 mol/l Tris-HCl, 4% SDS, and 0.01% bromophenol blue and loaded onto a 10% SDS-polyacrylamide gel. Samples were then separated at 40 mA using a Tris-glycine running buffer (0.2 mol/l Tris-base, 0.2 mol/l glycine [pH 6.8], and 0.1% SDS). The separated samples were transferred onto a nitrocellulose membrane in Tris-glycine transfer buffer supplemented with 20% methanol. The immunoblots were blocked for 1 h in blocking grade powdered goat milk (5%) diluted in 0.2 mol/l Tris-base, 1.4 mol/l NaCl, 0.1% Tween 20, and 0.02% NaN_3. The membranes were then incubated overnight with the primary antibody for each MMP species at dilutions recommended by the manufacturer (Oncogene Research Products, Cambridge, MA). For collagen type 1, antibody was obtained from Abcam Limited (Cambridge, U.K.). After the immunoblots were washed, secondary antibody conjugated to horseradish peroxidase was added and developed using ECL detection kit from Amersham Life Sciences (Arlington Height, IL). Recombinant MMP proteins were used as positive controls.

Gelatin zymography. Basal MMP activity was detected using gelatin zymography. Vascular extracts (20 μg) were loaded on 10% gelatin zymography gels (Bio-Rad) and initially separated under nonreducing condition at 15 mA through the stacking gel, which was increased to 20 mA during the rest of the separation. The gels were then rinsed twice in 2.5% Triton X-100 and incubated overnight (16 h) in substrate buffer containing 50 mmol/l Tris-HCl, 5 mmol/l $CaCl_2$, and 1 μmol/l $ZnCl_2$. The activated gels were stained by Coomassie blue R-250 followed by destaining in 55% methanol and 7% acetic acid.

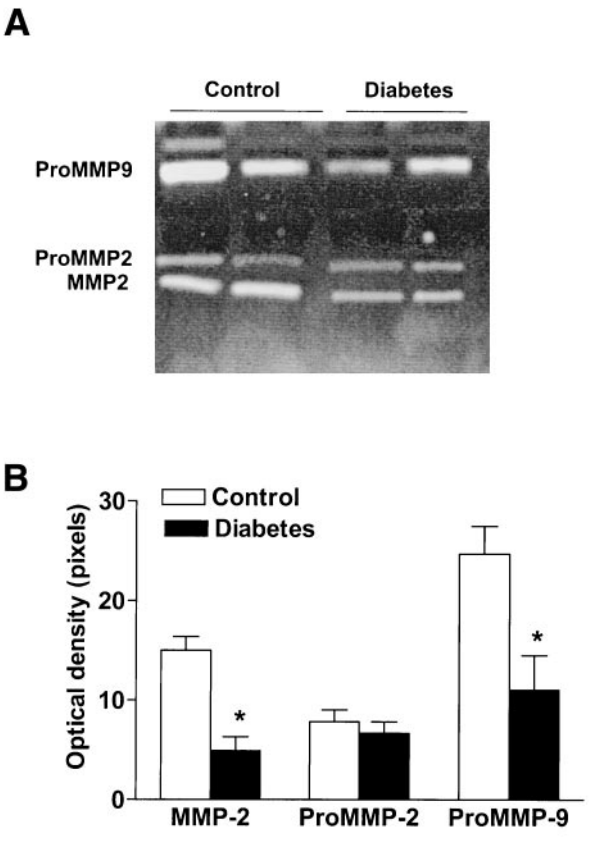

FIG. 1. *A*: A representative SDS-PAGE zymogram from control and diabetic IMA samples. MMP activity as determined by white lytic bands was detected at the 90- to 60-kDa region. *B*: Densitometric analysis of lytic bands detected in gelatin zymograms were plotted as mean ± SE of 14 subjects in each group. *$P < 0.05$ vs. control.

Data analysis. The zymograms and immunoblots were analyzed by densitometric scanning. In zymograms, lytic bands that demonstrate MMP activity at various molecular weights were analyzed by Gel Pro Image Analysis Program (Media Cybernetics, Silver Spring, MD) and expressed as optical density (pixels). In immunoblots, bands corresponding to the known molecular weight of each MMP species were analyzed in a similar manner. The images of zymograms and immunoblots were coded for the person who performed the image analysis.

The data obtained from densitometric analyses were compared by analysis of variance. The results are given as mean ± SE. An alpha level of $P < 0.05$ was considered to be statistically significant.

RESULTS

Arterial MMP activity in diabetes. Total MMP activity in the diabetic tissue was investigated by zymographic analysis of IMA specimens obtained from patients who were undergoing coronary artery bypass grafting surgery and compared with activity of control patients who do not have diabetes. Gelatinolytic activity as indicated by white lytic bands was detected in all samples. The molecular weight ranged between 40 and 90 kDa, which was consistent with latent and active forms of several MMP species, including MMP-2 and MMP-9. A representative gelatin zymogram of control and diabetic specimens is shown in Fig. 1*A*. Densitometric analysis of zymographic data demonstrated that MMP-2 and pro–MMP-9 activity detected at 62 kDa and 92 kDa, respectively, were decreased in the

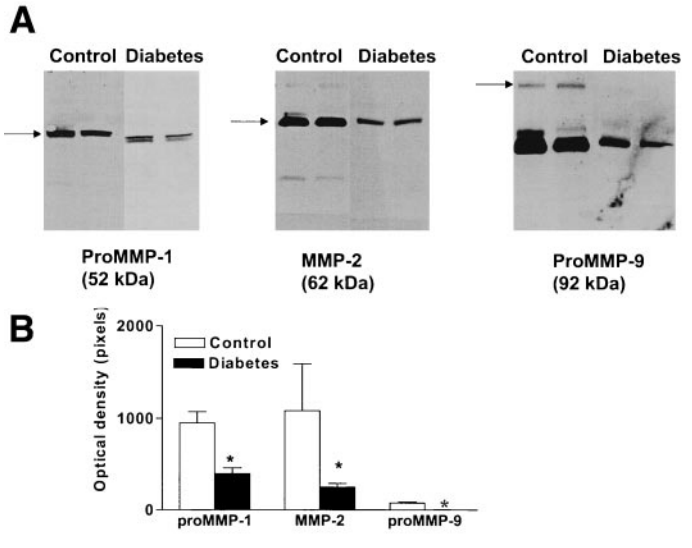

FIG. 2. A representative immunoblot demonstrating the presence of MMPs in vascular tissue. Control (n = 14) and diabetic (n = 14) IMA samples were subjected to immunoblotting for MMP-1, -2, and -9. *A*: Immunoreactive bands corresponding to the molecular weight of pro–MMP-1, MMP-2, and MMP-9 were detected and indicated by the arrows. A low molecular weight band (~60 kDa) was also observed with MMP-9 antibody and most likely represents a degradation product. *B*: Densitometric analysis of immunoreactive bands indicates that MMP proteins are decreased in diabetic tissue; n = 14. *P < 0.05 vs. control.

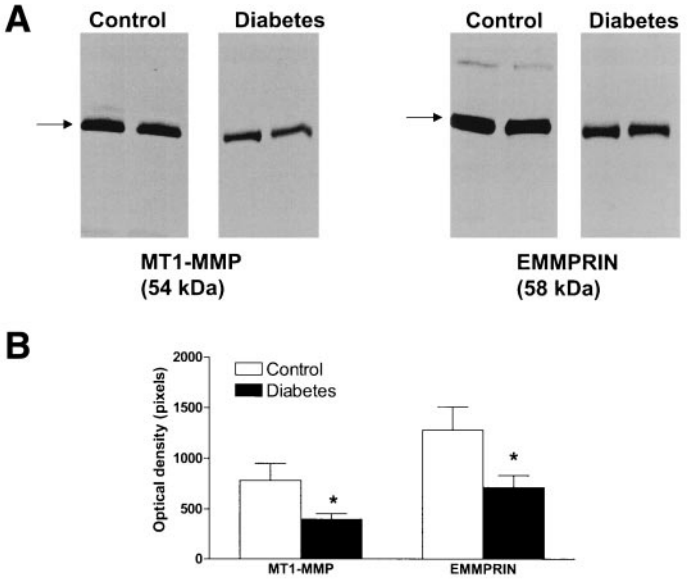

FIG. 3. Evidence for the MMP induction and activation system in vascular tissue. Control (n = 14) and diabetic (n = 14) IMA samples were subjected to immunoblotting for EMMPRIN and MT1-MMP. *A*: Immunoreactive bands corresponding to 54-kDa MT1-MMP and 58-kDa EMMPRIN were indicated by the arrows in a representative immunoblot. *B*: Densitometric analysis of immunoreactive bands indicates that MMP inducer and activator proteins are decreased in diabetic tissue; n = 14. *P < 0.05 vs. control.

diabetic tissue ($P < 0.05$; Fig. 1*B*). Latent MMP-2 was also decreased, but it was not statistically significant.

Expression profile of the arterial MMP system. For determining whether the gelatinolytic activity detected in zymograms correlates with MMP protein levels, immunoblots were performed on the zymography extracts. Bands corresponding to the molecular weight of latent MMP-1 (~52 kDa), active MMP-2 (~62 kDa), and latent MMP-9 (~92 kDa) were detected in all specimens. A representative immunoblot is shown in Fig. 2*A*. For MMP-9, we also detected a strong lower molecular weight signal around 60 kDa in all the specimens, which was decreased in the diabetic tissue. Densitometric comparisons of control and diabetic tissue summarized in Fig. 2*B* demonstrated that pro–MMP-1 and MMP-2 levels were decreased by two- and fourfold, respectively, in the diabetic arterial tissue. Active MMP-9 was not detectable in the diabetic tissue.

To determine whether proteins involved in the induction and activation of MMPs are present in the arterial peripheral tissue and to what extent, if any, the diabetic state alters this system, we also investigated the relative abundance of inducer protein EMMPRIN and MMP-2 activator MT1-MMP. As shown in Fig. 3*A*, both EMMPRIN and MT1-MMP were detected in IMA specimens and both were decreased in the diabetic tissue (Fig. 3*B*).

Another component of the MMP system is the TIMP proteins, which play an inhibitory role. Western analysis for TIMP-1 protein indicated the presence of a high molecular weight protein of ~65 kDa as well as a relatively faint band at the expected molecular weight of 29 kDa. The higher molecular weight band is likely to represent an MMP-1/TIMP-1 complex. Densitometric analysis of the 29-kDa bands demonstrated a significant decrease in the diabetic tissue (Fig. 4). These results suggested that decreased MMP activity cannot be attributed to increased TIMP-1 protein.

Expression of collagen type 1. To assess whether decreased MMP activity is associated with increased collagen deposition, we determined collagen type 1 levels in the same tissue extracts by immunoblotting. As shown in

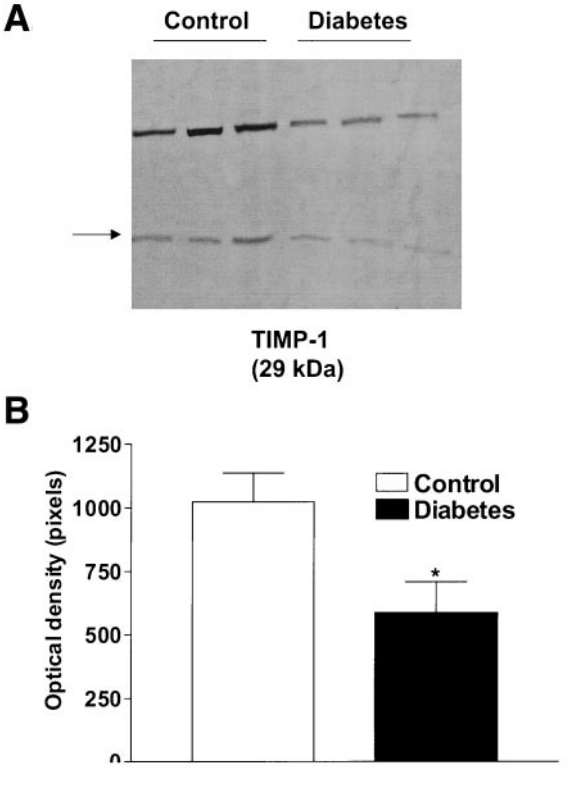

FIG. 4. Expression of TIMP-1 in control and diabetic vascular tissue. A representative immunoblot demonstrating decreased TIMP-1 (29 kDa) levels in the diabetic tissue (*A*) and densitometric analysis of all cases (n = 6 in each group; *B*). *P < 0.05 vs. control.

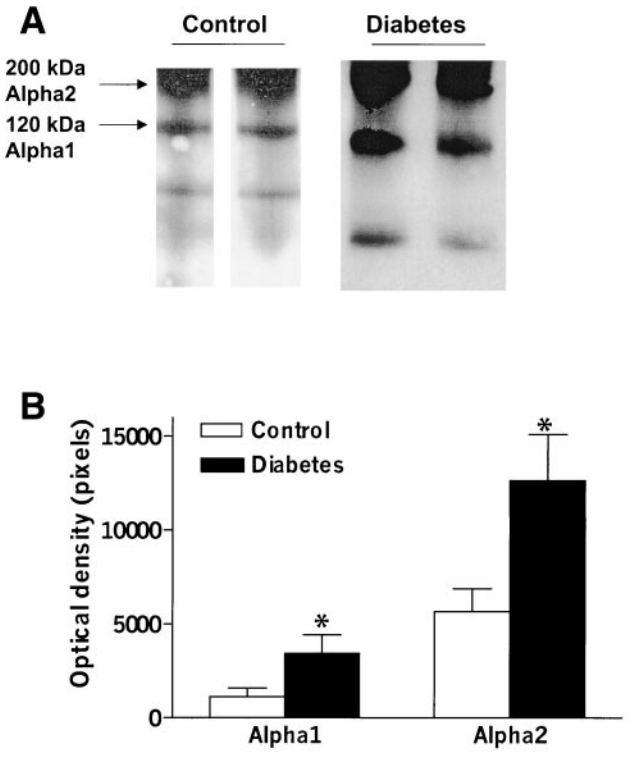

FIG. 5. Expression of collagen type 1 in control and diabetic vascular tissue. *A*: A representative immunoblot demonstrating increased α1 and α2 chains of collagen type 1 in the diabetic tissue. *B*: Densitometric analysis of all cases ($n = 5$ in each group); *$P < 0.05$ vs. control.

Fig. 5, bands corresponding to both the α1 (120 kDa) and α2 (200 kDa) chains of collagen type 1 were detected. Densitometric analysis of the immunoblots demonstrated a threefold increase in collagen type 1 in the diabetic tissue ($P < 0.05$ vs. control).

DISCUSSION

This study questioned whether all of the components of the MMP system are present in the peripheral arterial vasculature and to what degree diabetes alters the MMP synthesis and activity. The important findings of this study are threefold. First, the presence of inducer (EMMPRIN) and activator (MT1-MMP) proteins in the peripheral arterial vasculature demonstrated for the first time that there is an MMP induction and activation system at the vascular level. Second, this vascular induction and activation system is downregulated in diabetic tissues. Finally, MMP activity and MMP-1, -2, and -9 content are decreased in the diabetic tissue. These results provide important information regarding the molecular and cellular basis of enhanced vascular remodeling and collagen deposition in diabetes and contribute to our understanding of the potential mechanisms responsible for diabetic complications.

Diabetes causes a variety of changes in both the microvasculature and the macrovasculature. Microvascular complications manifest as diabetic retinopathy and nephropathy, whereas macrovascular complications are evidenced by atherosclerosis in larger vessels of heart, brain, and lower extremities (1). The vascular changes involve both cellular and extracellular components. For example, Vranes and colleagues (4,19) demonstrated that diabetes is associated with hypertrophy of the smooth muscle cells in both mesenteric and renal vasculature and that these changes are associated with ECM expansion, intimal proliferation, and medial enlargement (6). The most common extracellular pathology in diabetes is the thickening of the basement membrane as a result of the deposition of ECM proteins, including collagen and fibronectin (1). ECM is a dynamic structure that requires constant synthesis and degradation by MMPs (11). This is tightly controlled by TIMPs (11). Therefore, increased ECM protein synthesis, diminished MMP activity, and/or increased TIMP activity might contribute to vascular collagen deposition and fibrosis (10,11). The major sources of ECM proteins and MMPs in the vessel wall are endothelial and vascular smooth muscle cells (10). MMPs are a family of zinc-dependent enzymes, and several species are commonly expressed in the vasculature, including MMP-1 (10,11), MMP-2, and MMP-9 (20). All of these enzymes are secreted in zymogen forms, which are later activated by other proteinases (10). Therefore, the regulation of these enzymes occurs in a complex manner that requires protein–protein interactions at the synthesis, activation, and inhibition steps. A comprehensive analysis of the MMP system in diabetes has not been reported. In this study, several proteins that contribute to the expression, activation, and inactivation of MMPs were investigated in control and diabetic tissue.

The expression of MMP species is upregulated by a number of factors, including growth factors, cytokines, and physical stress. Recent studies have demonstrated that in addition to soluble factors, EMMPRIN, a 58-kDa membrane-bound protein, can also induce MMP-1, MMP-2, and MMP-3 expression in fibroblasts. This inducer protein was originally purified from tumor tissue and may contribute to increased MMP activity, resulting in angiogenesis and tumor cell invasion (13,14). Recently, Spinale and colleagues demonstrated the presence of this inducer protein in cardiac myocytes (15). Furthermore, myocardial EMMPRIN levels are increased in chronic heart failure that is associated with enhanced remodeling and ventricular dilation (15). However, the role of a local MMP induction system in the peripheral vasculature that undergoes extensive remodeling remains unclear. This study demonstrated the presence of EMMPRIN in the arterial tissue for the first time. Furthermore, evidence suggests that EMMPRIN levels are decreased in diabetes, indicating that reduced inducer protein levels may result in decreased MMP expression, which in turn contributes to vascular remodeling and ECM deposition in diabetes.

The majority of MMPs are activated by proteolytic cleavage with tissue and plasma proteinases such as trypsin and plasmin as well as active MMP-2. The activation of pro–MMP-2 occurs primarily on the cell surface by membrane-bound MT1-MMP, which can also degrade ECM proteins directly. Both smooth muscle and endothelial cells possess MT1-MMP and provide a localized MMP activation system (21,22). Rajavashisth et al. (21) reported that inflammatory cytokines and oxidized LDLs increase endothelial MT1-MMP expression and proposed that these factors might regulate the ECM degradation in human atheromas by inducing MT1-MMP expression. To date, five MT-MMP genes have been identified (17). MT1-MMP is the best described isoform; however, the regulation of the

expression and activity of this protein in patients with diabetes remains unclear. A recent study reported that MT5-MMP is upregulated in kidneys from patients with diabetes (17). The authors suggested that elevated expression of MT5-MMP may contribute to the pathogenesis of renal tubular atrophy and end-stage diabetic nephropathy. The current study investigated MT1-MMP levels in the peripheral vasculature. In contrast with previous findings of Romanic et al. (17), significant decreases in the MT1-MMP protein levels were observed in diabetic vascular tissues. The explanations for this difference might be twofold. First, we studied MT1-MMP, which may be regulated differently than MT5-MMP. Second, the gene expression might be tissue-specific because we investigated protein levels in peripheral vasculature as opposed to kidney tissue. The reduction of MT1-MMP, an MMP activator protein, in diabetic arteries provides a mechanistic explanation regarding the regulation of ECM turnover in the macrovascular complications of diabetes.

Several laboratories studied the expression and activity of the microvascular MMP system in the diabetic state. Del Prete et al. (16) reported that MMP-2 gene expression is downregulated in the glomeruli and tubulointerstitial tissue obtained from patients with diabetes. In experimental diabetes, significant decreases in latent and active forms of MMP-2 and MMP-9 in the renal tissue have been reported (8). Furthermore, mesangial cells maintained in high-glucose medium exhibited increased synthesis and accumulation of ECM components. A recent report by Singh et al. (23) demonstrated that high glucose stimulates angiotensin II synthesis, which in turn activates transforming growth factor-β1 and results in decreased MMP-2 secretion by the mesangial cells. These studies focused on the structural changes in the microvasculature, and our knowledge on the MMP expression in the macrovasculature remains limited. A recent study demonstrated that the activity and expression of latent MMP-9 but not MMP-2 are increased in the aortic extracts obtained from diabetic animals (18). The authors also reported that high glucose stimulated the synthesis of pro–MMP-9 at both the mRNA and protein levels in endothelial and not vascular smooth muscle cells in a redox-sensitive manner cells. On the basis of these observations, it has been proposed that augmented MMP activity might contribute to the advanced atherosclerosis in diabetes. In the current study, we focused on the expression and activity of MMP species that degrade interstitial collagen (MMP-1) and denatured collagen (MMP-2 and MMP-9) because they are expressed in human vessels and the substrates are abundant in the vascular tissue. We demonstrated that in vessels obtained from patients with diabetes, there is a significant decrease in MMP-9 and MMP-2 levels as compared with vessels obtained from patients who do not have diabetes. These results contrast with increased MMP levels reported by Uemara et al. (18) as discussed above. We speculate that this difference might occur for two reasons. First, we studied MMP expression and activity in patients with a long-standing history of diabetes, whereas Uemara et al. investigated MMP activity in animal models. Second, the changes in MMP synthesis and activity might be time-dependent. We speculate that in the early phase of diabetes, the MMP system may be upregulated to allow the smooth muscle cells to migrate and contribute to intimal hyperplasia. However, with the progression of diabetes, the MMP system is suppressed, causing ECM deposition and fibrosis.

There are two limitations to this study that must be recognized. First, the majority of patients in both control and diabetic groups were on combination therapy with lipid-lowering drugs, angiotensin-converting enzyme inhibitors, and/or calcium channel blockers. All of these medications have been reported to lower MMP activity (24–28). Although the use of lipid-lowering drugs and angiotensin II inhibition seems to be lower in the diabetic cohort, assessment of the effect of individual drugs on MMP activity would require a much larger patient size and cannot be evaluated in the present study. Second, as discussed above, duration of diabetes may affect MMP activity, and we do not have information regarding this variable for all of the study subjects. Nevertheless, this study demonstrated the presence of various components of the MMP system in the peripheral vasculature. Our results demonstrating that both MMP inducer/activator proteins and several members of the MMP family are decreased in diabetes provides insight with respect to the mechanism of diabetic complications. The vascular MMP induction/activation system may be a potential target for ECM regulation by cytokines and growth factors in diabetes.

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