

# DECREASED VASCULAR MATRIX METALLOPROTEINASE ABUNDANCE IN DIABETIC PATIENTS WITH SYMPTOMATIC MACROANGIOPATHY

The incidence of diabetic amputations is 2- to 3-fold higher in African-American patients compared to Caucasians. Vascular remodeling characterized by extracellular matrix (ECM) deposition occurs in diabetes and contributes to vascular complications. The matrix metalloproteinases (MMP) play important roles in the regulation of collagen turnover and vascular remodeling. However, the temporal expression profile of MMPs in diabetic vascular tissue during the disease process remained unknown. The objective of this study was to compare the vascular MMP system in African-American diabetic patients without symptoms to patients undergoing lower limb amputation due to severe vascular complications. Internal mammary artery (IMA,  $N=8$ ) and anterior/posterior tibial artery (AT/PT,  $N=8$ ) specimens were obtained from patients undergoing coronary artery bypass grafting and lower limb amputation, respectively. ECM inducer protein (EMMPRIN) and MMP activator membrane-type MMP (MT1-MMP), as well as MMP-1, -2, and -9, were quantified by immunoblotting and densitometry (pixels). MMP-1 and -9 levels were decreased from  $398 \pm 61$  and  $175 \pm 54$  pixels, respectively, in IMA tissue to  $287 \pm 31$  and  $51 \pm 36$  pixels in the AT/PT tissue ( $P<.05$ ). Both EMMPRIN and MT1-MMP expression was increased by 3-fold in AT/PT preparations ( $P<.05$ ). These results provided evidence that the molecular components required for the induction and activation of the MMP system exist in arterial vasculature and, MMP expression is downregulated in diabetic patients with severe complications despite elevated MMP inducer and activator proteins. Decreased MMP activity may contribute to pathological remodeling leading to increased incidence of amputations in African-American patients. (*Ethn Dis.* 2002;12[suppl3]:S3-18-S3-22)

**Key Words:** Diabetes, Amputation, African-American, Matrix Metalloproteinase

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## INTRODUCTION

The incidence of diabetes and related complications such as retinopathy, nephropathy, lower limb amputations, and stroke is higher in African Americans than in Caucasians.<sup>1,2</sup> 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 an increased media-to-lumen ratio.<sup>3-6</sup> Structural changes in the peripheral arterial vasculature during the disease progression, and especially in the African-American population, are poorly studied. 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.<sup>7,8</sup> 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.<sup>8,9</sup> 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.<sup>10</sup> 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.<sup>8</sup> Recent studies have identified an extracellular matrix metalloproteinase

inducer protein (EMMPRIN) that stimulates the expression of MMP-1, MMP-2, and MMP-3 in fibroblasts.<sup>11</sup> Whether the MMP induction/activation system exists in the peripheral vasculature, and to what degree diabetes affects the expression and activity of this system in African-American patients who are more prone to diabetic complications, remain unknown. Accordingly, we investigated the temporal expression of the MMP system in African-American diabetic patients with vascular disease.

## METHODS

### Patient Enrollment and 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 the participants prior to the surgery. Internal mammary artery specimens were obtained from patients undergoing coronary artery bypass graft surgery. Although all patients had coronary artery disease, the vessels used in this study were utilized for bypass conduits and considered relatively healthy. Therefore, internal mammary artery (IMA) specimens obtained from bypass patients were designated as asymptomatic diabetic patients ( $N=8$ ). Anterior and posterior tibial artery specimens of advanced disease state were obtained from diabetic patients undergoing lower limb amputation ( $N=8$ ). All patients were on combination therapy, which included calcium channel blockers, angiotensin converting enzyme inhibitors, diuretics, lipid lowering agents, and anti-

thrombotic therapy, as well as insulin and oral agents. The use of medications, mean age, and female/male ratio were similar in all groups. Artery specimens were placed in cold Dulbecco's Modified Eagle Medium (DMEM) 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 and stored at  $-80^{\circ}\text{C}$ .

### Vascular MMP Extraction

Frozen artery specimens were homogenized in an extraction buffer (1:10, wt/vol) containing 0.15 M NaCl, 20 mM ZnCl<sub>2</sub>, 1.5 mM NaN<sub>3</sub>, 10 mM cacodylic acid, and 0.01% Triton X-100. After centrifugation at  $4^{\circ}\text{C}$  for 10 minutes at a speed of 800 *g*, the supernatant was concentrated using a Centrifuplus concentrator. Samples were centrifuged at 3,000 *g* for 4.5 hours at  $4^{\circ}\text{C}$  and the protein content was measured using Bio-Rad Protein Assay (Bio-Rad, Richmond, Calif). Samples were stored at  $-80^{\circ}\text{C}$  in small aliquots.

### Western Analysis

Protein levels of MMP species (MMP-1, MMP-2, MMP-9, and MT1-MMP, and EMMPRIN) were determined by immunoblotting, using specific antibodies. Vascular extracts (20  $\mu\text{g}$ ) were diluted to the appropriate loading concentration in a sample buffer containing 0.1 M Tris-HCl, 4% SDS, and 0.01% bromophenol blue, and loaded onto a 10% SDS-polyacrylamide gel. Samples were then separated in a Tris-glycine running buffer (0.2 M Tris-base, 0.2 M glycine, pH 6.8, and 0.1% SDS) and transferred to nitrocellulose membranes. The immunoblots were blocked for one hour in blocking-grade powdered goat milk (5%) diluted in 0.2M Tris-base, 1.4 M NaCl, 0.1% Tween 20, and 0.02% NaN<sub>3</sub>. The membranes were then incubated overnight with the primary antibody for each MMP species at dilutions recommended by the manufacturer (Onco-

gene Research Products, Cambridge, Mass). After several washes, a secondary antibody conjugated to horseradish peroxidase was added and developed using the ECL detection kit from Amersham Life Sciences, Arlington Height, Ill. Recombinant MMP proteins were used as positive controls.

### Data Analysis

The immunoblots were analyzed by densitometric scanning. Bands corresponding to the known molecular weight of each MMP species were analyzed by Gel Pro Image Analysis Program (Media Cybernetics, Silver Spring, Md) and expressed as optical density (pixels). The images were coded for the person who performed the image analysis. The data obtained from densitometric analyses were compared by ANOVA. The results are given as mean  $\pm$  SEM. An alpha level of  $P < .05$  was considered to be statistically significant.

## RESULTS

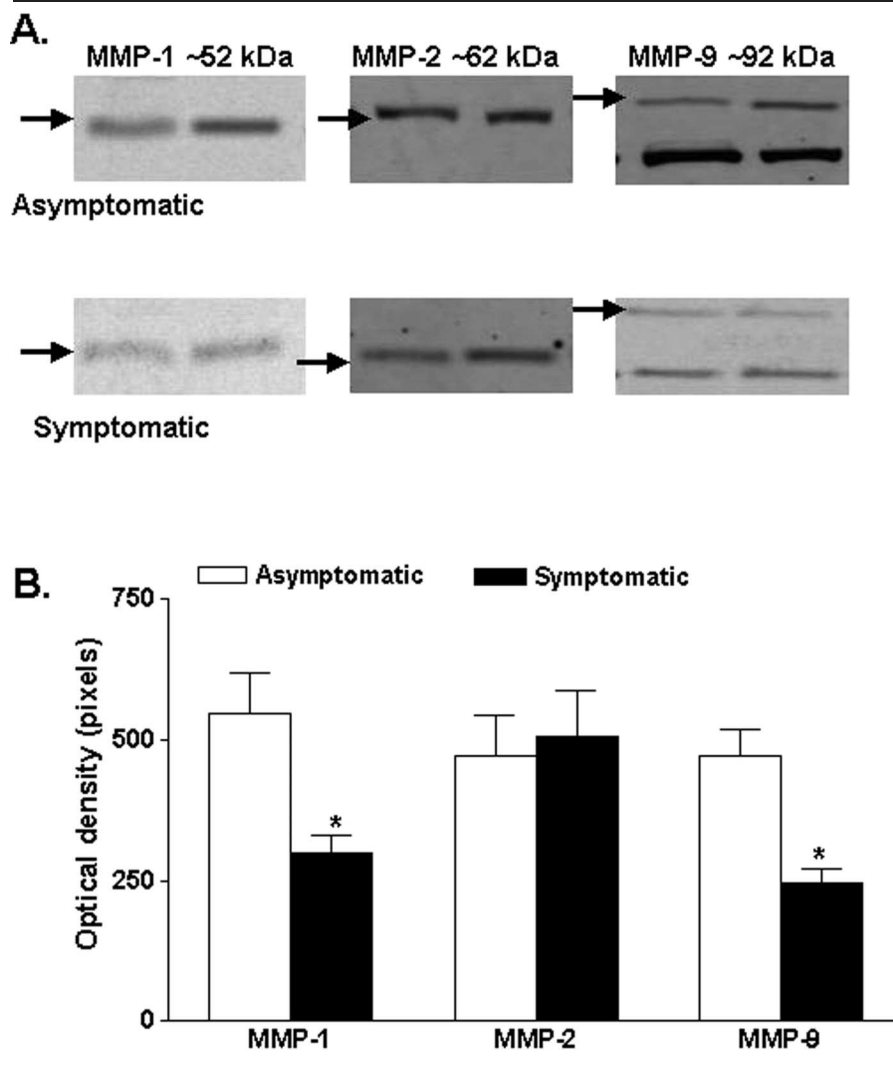
To determine the MMP expression profile, we investigated several members of the MMP family, which are commonly expressed in the vasculature. These included MMP-1, MMP-2, and MMP-9. Bands corresponding to the molecular weight of latent MMP-1 (~52 kDa), active MMP-2 (~62 kDa), and latent MMP-9 (~92 kDa) were detected in all specimens. For MMP-9, we also detected a strong lower molecular weight signal around 60 kDa. A representative immunoblot is shown in Figure 1A. Densitometric comparisons of asymptomatic and symptomatic patient groups summarized in Figure 2B demonstrated that proMMP-1 and proMMP-9 levels were decreased by 2-fold in the vascular tissue obtained from patients with advanced disease. Lower molecular weight MMP-9 was also decreased by 3-fold in this group. There was no significant difference in MMP-2 levels.

In order to determine whether proteins involved in the induction and activation of MMPs are present in the peripheral arterial tissue and to what extent, if any, the diabetic state alters this system, we also investigated the relative levels of inducer protein EMMPRIN and MMP-2 activator MT1-MMP. As shown in Figure 2A, both EMMPRIN and MT1-MMP were detected in internal mammary and tibial artery specimens and were increased in the advanced disease (Figure 2B).

## DISCUSSION

The present study compared the expression of the MMP system in the peripheral arterial vasculature of diabetic African-American patients with uncomplicated disease to patients with advanced vascular disease. The importance of the findings of this study are 3-fold. First, the presence of inducer (EMMPRIN) and activator (MT1-MMP) proteins demonstrated, for the first time, that there is an MMP induction and activation system at the arterial level. Second, this vascular induction and activation system is upregulated in advanced diabetic vascular disease. Third, MMP-1 and MMP-9 content are decreased in patients with advanced disease. 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 in this population.

Diabetes causes a variety of changes in both the microvasculature and 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.<sup>12</sup> The vascular changes involve both cellular and extracellular components. For example, Vra-



**Fig 1.** A representative immunoblot demonstrating the presence of MMPs in vascular tissue. IMA samples from asymptomatic and symptomatic diabetics were subjected to immunoblotting for MMP-1, -2, and -9. (A) Immunoreactive bands corresponding to the molecular weight of proMMP-1, MMP-2, and proMMP-9 were detected and indicated by the arrows. A low molecular weight band (~60 kDa) was also observed with MMP-9 antibody. (B) Densitometric analysis of immunoreactive bands indicates that MMP proteins are decreased in advanced diabetic vascular disease. \* $P < .05$  vs control

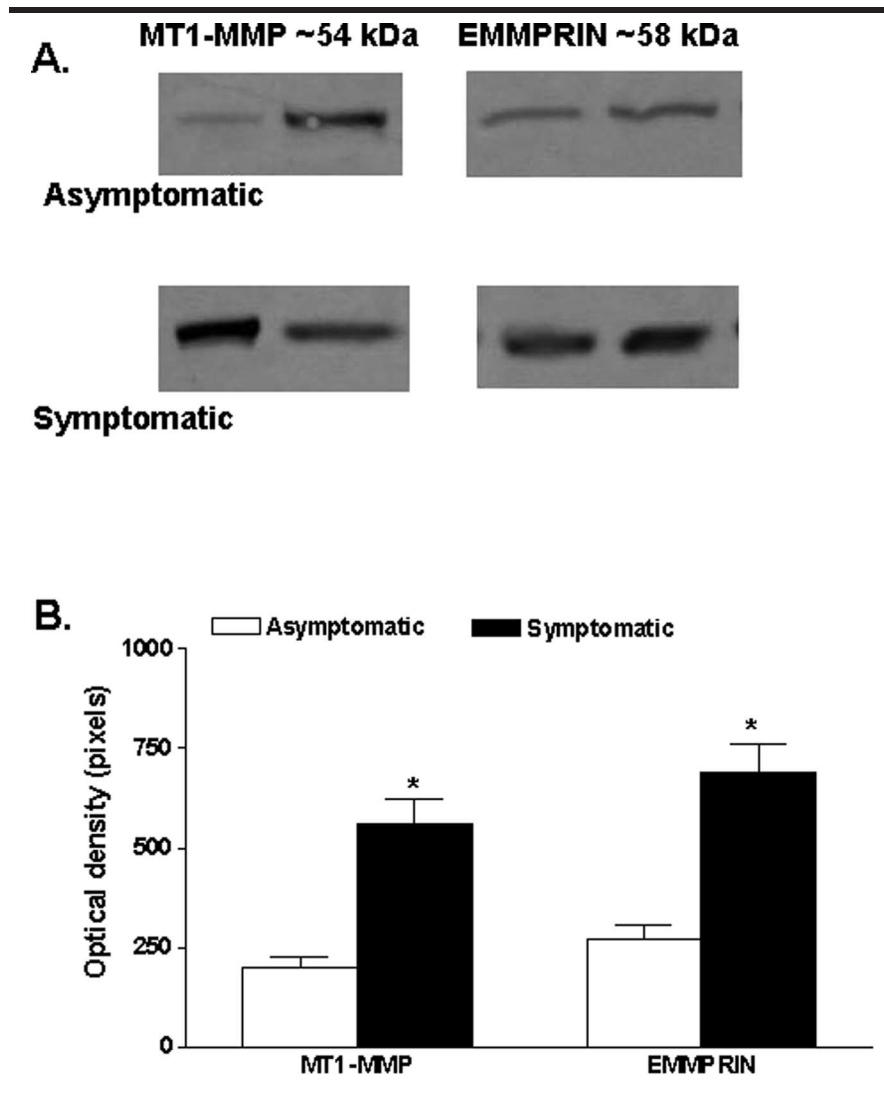
nes and colleagues demonstrated that diabetes is associated with hypertrophy of the smooth muscle cells in both mesenteric and renal vasculature<sup>4,13</sup> and these changes are associated with ECM expansion, intimal proliferation, and medial enlargement.<sup>6</sup> ECM is a dynamic structure that requires constant synthesis and degradation by MMPs,<sup>9</sup> which is tightly controlled by MMP tissue inhibitors (TIMPs).<sup>9</sup> Therefore, increased

ECM protein synthesis, diminished MMP activity, and/or increased TIMP activity might contribute to vascular collagen deposition and fibrosis.<sup>8,9</sup> The major sources of ECM proteins and MMPs in the vessel wall are endothelial and vascular smooth muscle cells.<sup>8</sup> MMPs are a family of zinc-dependent enzymes and several species are commonly expressed in the vasculature including MMP-1,<sup>8,9</sup> MMP-2, and

MMP-9.<sup>14</sup> All these enzymes are secreted in zymogen forms, which are later activated by other proteinases.<sup>8</sup> Therefore, the regulation of these enzymes occurs at the synthesis, activation, and inhibition steps. A comprehensive analysis of the MMP system during the progression of diabetes has not been reported. Therefore, in this study we examined the temporal profile of the MMP system.

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 proMMP-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.<sup>15,16</sup> Rajavashisth and colleagues reported that inflammatory cytokines and oxidized low density lipoproteins increase endothelial MT1-MMP expression and proposed that these factors might regulate the ECM degradation in human atherosclerosis by inducing MT1-MMP expression.<sup>15</sup> Our finding that with advanced disease, MT1-MMP levels increase, may be attributed to a positive feed-back mechanism to increase active MMP levels and decrease collagen deposition. An alternative explanation is that enhanced MT1-MMP expression may be involved in plaque rupture in advanced disease.

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.<sup>11</sup> However, the role of a local MMP induction system in the peripheral vasculature, which undergoes



**Fig 2.** Evidence for MMP induction and activation system in vascular tissue. 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 detected. (B) Densitometric analysis of immunoreactive bands indicates that MMP inducer and activator proteins are increased in advanced diabetic vascular disease. \* $P < .05$  vs control

extensive remodeling, remains unclear. This study demonstrated that EMMPRIN levels are increased in advanced diabetic vascular, indicating that inducer protein levels are upregulated in advanced vascular disease to stimulate MMP expression, which, in turn, contributes to vascular remodeling and extracellular matrix deposition in diabetes.

Several groups investigated the expression and activity of the microvascular MMP system in the diabetic state.

Del Prete et al reported that MMP-2 gene expression is downregulated in the glomeruli and tubulo-interstitial tissue obtained from diabetic patients.<sup>17</sup> In experimental diabetes, significant decreases in latent and active forms of MMP-2 and MMP-9 in the renal tissue have been reported.<sup>18</sup> Furthermore, mesangial cells maintained in high glucose medium exhibited increased synthesis and accumulation of ECM components. A recent report by Singh and colleagues

demonstrated that high glucose stimulates angiotensin II synthesis, which, in turn, activates TGF $\beta$ 1 and results in decreased MMP-2 secretion by the mesangial cells.<sup>19</sup> These studies focused on the structural changes in the microvasculature; however, 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, is increased in the aortic extracts obtained from diabetic animals.<sup>20</sup> The authors also reported that high glucose stimulated the synthesis of pro-MMP-9 both at the mRNA and protein levels in endothelial smooth muscle cells in a redox-sensitive manner, but not in the vascular smooth muscle cells. Based on 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 because the substrates are abundant in the vascular tissue. We demonstrated that in vessels obtained from diabetic patients undergoing lower limb amputation, there is a significant decrease in MMP-1 and MMP-9 levels as compared to vessels obtained from diabetic patients who do not present with vascular disease. These results contrast with increased MMP levels reported by Uemara (discussed above). We speculate that this difference might be due to 2 reasons. First, we studied MMP expression and activity in diabetic patients with a long-standing history of diabetes, whereas Uemara and colleagues 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 several limitations to this study that must be recognized. First, we investigated the MMP system in only African-American patients with coronary artery disease undergoing coronary artery bypass grafting surgery and lower limb amputations due to severe vascular disease. We focused on this population since the risk of diabetic complications is higher in African-American patients. Future studies comparing the MMP system in African-American and Caucasian study groups are required to determine whether changes in this system are related to diabetes and/or race. Second, we investigated only the protein levels of MMPs. MMP activity studies are warranted to determine whether enzyme activity correlates with enzyme expression levels. Finally, in this cohort of patients, we did not have information with regard to their long-term glucose control. Nevertheless, the results of this study provide evidence that despite increases in the MMP inducer and activator proteins, MMP levels decrease with disease progression in African-American diabetic patients and may contribute to increased risk of lower limb amputations in this population.

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