Reduced Expression of Vascular Endothelial Growth Factor Paralleled With the Increased Angiostatin Expression Resulting From the Upregulated Activities of Matrix Metalloproteinase-2 and -9 in Human Type 2 Diabetic Arterial Vasculature

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Abstract—Impaired angiogenesis could contribute to the increased incidence of coronary and peripheral artery disease in diabetic patients. Angiogenesis is initiated by vascular endothelial growth factor (VEGF), a potent angiogenic cytokine, and suppressed by angiostatin, which is generated by matrix metalloproteinase (MMP)-2 and -9 through proteolytic cleavage of plasminogen. We hypothesized that MMP-2 and -9 were upregulated in the diabetic vasculature, resulting in increased angiostatin production and reduced blood vessel formation. In diabetic internal mammary artery samples (n=32) collected from patients undergoing coronary artery bypass grafting surgery, capillary density was only 30% of that in the nondiabetic vessels (n=32), whereas VEGF expression was reduced by 48%. Diabetes upregulated the expression and the gelatinolytic activity of MMP-2 and -9. Active MMP-2 and -9 were released from diabetic arteries, but not from nondiabetic vessels, during phenylephrine-induced vasoconstriction. Diabetes enhanced transcription and protein expression of tissue inhibitor of MMP (TIMP)-1 but had an opposite effect on TIMP-2. In diabetic vessels angiostatin was increased by 62% and was positively correlated with the activities of MMP-2 and -9 (r²=0.806 and 0.742, respectively). This report indicated a strong correlation between the upregulation of MMP-2 and MMP-9 and the increased angiostatin expression in the human diabetic arterial vasculature. The enhanced angiostatin production with a reduced VEGF formation may explain the pathogenesis of impaired angiogenesis in diabetes mellitus. (Circ Res. 2006;99:140-148.)

Key Words: matrix metalloproteinase ■ angiostatin ■ angiogenesis ■ type 2 diabetes ■ vascular endothelial growth factor

The development of collateral vessels, angiogenesis, is a compensatory mechanism in response to the obstructive arterial diseases, which cause myocardial or lower-extremity ischemia.1,2 However, the angiogenic process is significantly reduced in diabetic patients with coronary and peripheral artery diseases. In fact, diabetes mellitus has been identified as a negative predictor of the development of coronary collaterals,3 and up to 80% of all deaths in diabetic patients is related to cardiovascular complications.4 Similarly, the impairment of peripheral neovascularization could also explain the high prevalence of limb ischemia and amputations in diabetic patients.1

Angiogenesis is initiated by proliferation of endothelial cells, which penetrate into the surrounding tissue, and is tightly regulated by growth factors and inhibitors.1 The potent angiogenic cytokine, vascular endothelial growth factor (VEGF), has a variety of effects, such as triggering proliferation, migration, survival of endothelial cells, and tube formation.1,5,6 Angiostatin, a potent antiangiogenic factor, antagonizes the angiogenic effect of VEGF7 and limits the growth and spread of new blood vessels.8 Matrix metalloproteinases (MMPs) regulate angiogenesis, on the one hand by facilitating extracellular matrix (ECM) degradation to allow new vessel expansion,9 and on the other hand, by interfering with angiogenesis through the production of angiostatin. Angiostatin is generated by the proteolytic cleavage of plasminogen by MMP-2, -7, -9, and -12.8,10 In the vasculature, MMP-2 and -9 are produced by smooth muscle and
endothelial cells, whereas MMP-7 and -12 are mainly secreted from macrophages. The activation of MMPs is regulated at the levels of transcription, proenzyme activation, and endogenous tissue inhibitors of MMP (TIMPs). Thus, a discordant regulation of the MMP/TIMP system may contribute to the defect in angiogenesis seen in diabetes with vascular diseases.

Regulation of MMPs in diabetes has been widely investigated. It has been shown that hyperglycemia increased activity and expression of MMP-2 and -9 in rat aortic smooth muscle cells and mouse vascular tissue and plasma. Chronic incubation with high glucose increased MMP-9 promoter activity, mRNA and protein expression, and gelatinase activity in bovine aortic endothelial cells. Chronic hyperglycemia has also been shown to attenuate coronary collateral development by enhancing the expression of MMP-9 and angiostatin in the myocardial interstitial fluid from a canine model of repetitive coronary occlusion. However, controversial data about the effect of diabetes on MMP activation still remain.

MMP-2 and -9 are known to be activated in a cellular environment under oxidant stress and with reduced NO bioavailability. Our previous study indicated that endogenous nitric oxide (NO) biosynthesis and NO-mediated signaling were compromised in the human diabetic arterial vasculature. Because of this inverse relationship between MMP activation and NO production, we hypothesized that MMP-2 and -9 were preferentially upregulated in the diabetic vasculature, resulting in increased angiostatin formation and thereby contributing to the impaired angiogenesis in diabetes.

Materials and Methods

Materials
Primary antibodies: mouse monoclonal anti-MMP-2, anti-MMP-9 (Oncogene, Boston, Mass), anti-TIMP-1 and anti-TIMP-2 (Calbiochem, La Jolla, Calif); rabbit polyclonal anti-angiostatin, anti-angiostatin, anti-VEGF, and anti-VEGF receptor-2 (anti-VEGFR2) (Calbiochem); and goat polyclonal anti-VEGFR1 (Calbiochem). Gene-specific primers were synthesized by NAPS Unit Oligonucleotide Synthesis Laboratory (Biotechnology Laboratory, The University of British Columbia). All other reagents were the highest purity grade purchased from Sigma (St Louis, Mo), unless specifically stated in the text.

Patient Samples
Internal mammary artery (IMA) samples were collected in total from randomly chosen 64 patients (nondiabetic, n = 32; diabetic, n = 32; aged 48 to 78 years) at St. Paul’s Hospital, Vancouver, Canada, undergoing coronary artery bypass grafting surgery with the approval of the institutional Research Ethics Board. Written informed consent was obtained from all of the patients. Type II diabetic status was accepted as diagnosed in patient medical records. The overall mean age was 66.1 ± 2.8 years, whereas the mean age of nondiabetic patients was 65.7 ± 3.0 and 66.4 ± 2.7 years, respectively. In this study, the nondiabetic group served as control. Summary of patient demographic is presented in the Table. The samples were flash frozen; 3-mm rings were also immersed in formalin and, within 24 hours, embedded in paraffin blocks.

Capillary Density
Paraffin blocks with embedded vessels were cut into 3-µm thick cross-sections, which were stained with antibodies against von Willebrand factor (dilution factor 1:400) for labeling endothelial cells. Images were obtained with a Nikon MicroPhot microscope and a SPOT digital camera. Only capillaries oriented perpendicular to the plane of sections were counted. Capillary density was calculated by dividing the total number of capillaries on the slides by the area of the stained sections (millimeter squared). Measurements were done with the ImageProPlus5 software.

Reverse-Transcription Polymerase Chain Reaction
Total RNA was extracted from the IMA specimen using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, Calif) following the instructions manufacturer. RNA (1 µg) was subjected to reverse transcription (RT) (Gene Amp PCR System 9700, Applied Biosystems) as previously described. RT reaction mixture (5 µL) was used in the PCR experiments with the addition of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, Calif) and gene-specific primers for MMP-2, MMP-9, TIMP1–4, VEGF165, and β-actin. PCR products were electrophoresed in ethidium bromide-stained agarose gel. Densitometry was analyzed by Quantity One Image software (Bio-Rad). Transcription of β-actin served as an internal standard for sample normalization.

Western Blotting
The procedure of Western blotting was previously described. IMA segments were ground with liquid nitrogen in a stainless-steel mortar and pestle. Tissue powder was mixed in 9 volumes of ice-cold lysis buffer (50 mmol/L Tris-HCl pH 7.4 with 3.1 mol/L sucrose, 1 mmol/L dithiothreitol, 10 µg/mL leupeptins, 10 µg/mL soybean trypsin inhibitor, 2 µg/mL aprotinin, and 0.1% Triton X-100). After 20 minutes incubation on ice, samples were homogenized by a glass homogenizer. Protein (10 µg) was separated on SDS-PAGE, then transferred to polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked overnight with blocking buffer and then incubated with primary antibodies. The immunoreactive proteins were visualized by chemiluminescence (Amersham Biosciences, Buckinghamshire, UK), and densitometric measurement was performed as described above. To ensure equal protein loading, membranes were stripped and reprobed with anti-β-actin antibody.

Gelatinolytic Zymography
The gelatinolytic activity was analyzed by separating protein (5 µg) on 8% SDS-PAGE gels copolymerized with gelatin (2 mg/mL). The gelatinolytic activity was identified as bands against the background of Coomassie blue–stained gelatin.

For detecting the release of gelatinolytic enzymes during vessel contraction, the incubation media of the arteries equilibrated at the conventional tension and stimulated with phenylephrine, as described, was collected and concentrated by spin concentrators with a molecular mass cutoff of 10 000 Da (Millipore, Centricon Plus-70 Centrifugal Filter Device). Briefly, the diluted washout (50 mL) was added to the spin concentrator and centrifuged in a swinging-bucket centrifuge at 3500g for 40 minutes at 4°C. Three hundred microliters of concentrated releasates were recovered, and 10 µL were immediately subjected to gelatinolytic zymography.

Reverse Zymography
Activities of TIMPs in tissue extract (50 µg) were determined by electrophoresis in 13% SDS-PAGE copolymerized with 1 mg/mL of gelatin and 50 ng/mL human recombinant MMP-2 or -9 (Calbiochem). Activity of TIMPs was visualized as dark bands against the clear background.

Statistics
Data were reported as mean ± SE. Parameters of diabetic and control groups were compared with unpaired Student’s t test (GraphPad Prism, San Diego, Calif), and a probability value of <0.05 was considered as significant. Correlation between angiostatin protein level and MMP activity was analyzed by linear regression analysis.
### Patient Demographics

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<th>Characteristics</th>
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<th>Control (n=32)</th>
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<td>Calcium-channel blockers</td>
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<tr>
<td>Alcoholic (%)</td>
<td>5.60</td>
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ACE indicates angiotensin-converting enzyme inhibitor; CABG, coronary artery bypass grafting.
Diabetic IMA. The capillary density in the medial area in the diabetic group was only 30% of that in the control group (Figure 1).

Diabetes significantly suppressed the number of capillaries in peripheral vascular disease (22.2%) and renal disease (27.8%), which was not the case for the nondiabetic group.

**Results**

**Patients Statistics**

The Table fails to show statistically significant differences between the diabetic and nondiabetic patients in terms of the parameters of glucose and lipid metabolism. The absence of a difference in the blood glucose levels is attributable to effective treatment by combinational glucose lowering therapy of diabetic patients. An initial diagnosis of hypertension was 2 times more frequent for diabetic patients than for nondiabetic patients, but effective antihypertensive therapy abolished this difference.

Diabetic patients, however, are often diagnosed with peripheral vascular disease (22.2%) and renal disease (27.8%), which was not the case for the nondiabetic group.

**Reduced Capillary Density and VEGF Expression in Diabetic IMA**

Diabetes significantly suppressed the number of capillaries in IMA. The capillary density in the medial area in the diabetic group was only 30% of that in the control group (Figure 1).

VEGF is a known potent proangiogenic factor; therefore, we investigated how diabetes affects the expression of this cytokine and its receptors in the arterial tissue. Although the expressions of VEGFR1 and VEGFR2 were not significantly different between the 2 groups (Figure 2A), both gene transcription and protein expression of VEGF165 in the diabetic vessels were decreased by 51.4±6.4% and 48.0±2.7%, respectively (Figure 2B and 2C).

**Regulation of MMPs and TIMPs in Diabetic IMA**

Vessels from the diabetic and control groups exhibited no differences in either gene transcription of MMP-2 and -9 or in the protein expression of the latent forms of these MMPs (Figure 3).

However, the expression of the active forms of these MMPs was significantly higher (by 43.4±6.9% and 68.1±8.8%, respectively) in diabetic arteries than in nondiabetic ones (Figure 3B).

Gelatinolytic activity of MMP-2 and -9 was also higher in diabetic IMA samples, as compared with the control IMA samples (Figure 4A), by: latent MMP-9, 36.4±3.5%; active MMP-9, 43.1±5.7%; latent MMP-2: 25.1±4.8%; active MMP-2, 30.2±3.8%.

Stimulation of diabetic arteries mounted in isometric conditions with the α-adrenergic agonist phenylephrine triggered a pronounced release of active forms of MMP-2 and -9, whereas they were only weakly detected in the media from the nondiabetic IMA samples (Figure 4B).

Diabetes also resulted in increased gene transcription and protein expression of TIMP-1 by 64.3±6.9% and 85.7±9.2%, respectively, whereas transcription and protein levels of TIMP-2 were markedly reduced by 66% and 30% (Figure 5A and 5B). Transcription of TIMP-3 and -4 was not different between the 2 groups (Figure 5A). Activity of TIMP-2 in diabetic IMA samples was 73% of that in the control, whereas activity of TIMP-1 was not significantly different between the two groups (Figure 5C).

**Increased Expression of Angiostatin in Diabetic IMA**

Proteolytic cleavage of plasminogen yields a variety of closely related molecular species of ~40 to 45 kDa containing Kringles 1-4 or 1-3 of plasminogen, collectively termed angiostatin.8,10 Diabetic tissue expressed elevated angiostatin protein level by 62%, compared with the nondiabetic vessels (Figure 6A). Expression of endostatin, another antiangiogenic factor generated by MMP proteolytic cleavage,21 did not differ between the 2 groups (Figure 6B).

When we pooled the gelatinolytic activities of MMP-2 and -9 of the latent and active forms, we observed the positive correlation (r²=0.806 and 0.742, respectively) between the total activities of these MMPs and the angiostatin expression in the diabetic IMA samples (Figure 7A). The similar correlation was not seen in the nondiabetic IMA samples (Figure 7B).

**Discussion**

We have shown a significant reduction in capillary density in diabetic arterial vasculature, which was accompanied by (1) decreased VEGF165 level; (2) upregulation of both MMP-2 and -9 protein expressions and enzymatic activities; (3) angiostatin expression, which was positively correlated with MMP-2 and -9 activities; and (4) differential regulation of TIMP-1 and -2. These findings present plausible molecular
mechanisms for impaired angiogenesis in diabetes, which could impact the perfusion of diabetic vascular tissue and thus contribute to the development of vascular disorders. These findings could be implicated in the enhanced levels of peripheral vascular disease (22.2%) and renal disease (27.8%) in the diabetic group in the present study. In addition, the higher prevalence of coronary arterial disease (33.3% versus 29.4%), chronic heart failure (16.7% versus 11.8%) and pre-myocardial infarction before surgery (55.6% versus 47.1%) in the diabetic groups may also suggest the reduced vascular perfusion.

Angiogenesis can be initiated by a potent proangiogenic mitogen, VEGF, and suppressed by angiostatin, which is generated by MMP-2 and -9. We observed that human diabetic IMA samples exhibited a significantly reduced capillary density (Figure 1), which could result from the impaired angiogenic process. We found a marked reduction of VEGF165 at the levels of both gene transcription and protein expression in the diabetic IMA samples, without a significant difference in its receptor levels as compared with the nondiabetic vessels (Figure 2). In addition, activity of MMP was elevated which strongly correlated with the augmented angiostatin formation in diabetic IMA samples. These findings indicate that diabetes affects both the mechanism of initiation of angiogenesis via VEGF, and the inhibitory mechanism that involves MMPs.

VEGF165 is the most abundant and best-characterized VEGF splice variant, whose signaling is mainly mediated through the VEGFR1 and VEGFR2, both of which are predominately expressed on endothelial cells. Decreased capillary density in patients with cardiomyopathy was associated with downregulation of VEGF165 and VEGFR. In ventricles from diabetic patients, the transcription of both VEGF and its receptor was decreased 2-fold. Therefore, VEGF downregulation could contribute to decreased capillary density in diabetic arterial vasculature and application of local VEGF gene transfer to diabetic patients could restore angiogenesis and improve mortality and morbidity of ischemic events in diabetes.

In vivo, the impact of defective VEGF signaling could be even greater. Insulin upregulates VEGF mRNA expression in smooth muscle cells via the phosphatidylinositol 3-kinase/Akt cascade, which is compromised in diabetic vascular tissue. Moreover, VEGF signaling through this cascade could be further downregulated in diabetic arteries. VEGF stimulates NO release and upregulates the expression of NO synthase, whereas endothelium-derived NO enhances the angiogenic response by inducing the synthesis of VEGF. NO production and cGMP elevation contribute to the angiogenic and mitogenic effects of VEGF. Therefore, down-
regulated endothelial NO synthase (eNOS) and decreased NO availability could, in part, explain the reduced VEGF effects.

In this study, we demonstrate significant activation of MMP-2 and -9 in the human diabetic vasculature. Increased protein levels and activities were detected in the protein homogenates of diabetic arteries. Besides, we detected the release of active forms of MMP-2 and -9 from the diabetic IMA samples during agonist-stimulated vasoconstriction. MMPs play a dual role in angiogenesis, on one hand, by facilitating endothelial cell migration and capillary growth via degradation of ECM components. For example, MMP-2 has been shown to bind to integrins on the tips of sprouting capillaries to facilitate new blood vessel expansion. On the other hand, MMPs suppress angiogenesis by generating a potent antiangiogenic factor, angiostatin, by the proteolytic cleavage of plasminogen. Both MMP-2 and -9 were found upregulated in the diabetic arteries, as compared with the nondiabetic vessels (Figures 3 and 4) and were strongly correlated with angiostatin expression (Figures 6 and 7). Diabetes is known to be associated with attenuated angiogenesis in the coronary and peripheral arterial vasculature, which is in agreement with our present work. Therefore, although MMP activation is essential for angiogenesis by allowing ECM remodeling and invasion, increased activity of MMP seems to have a strong inhibitory role in angiogenesis by increasing synthesis of angiostatin. Besides, the active MMPs released during agonist-stimulated vasoconstriction might have additional non-matrix-degradative roles, including vasoconstriction in endothelium-denuded arteries and during inhibition of NOS. Diabetes is associated with endothelial dysfunction and reduced NO production; therefore, the release of active MMPs may suggest the presence of an additional vasoconstriction mechanism in the diabetic arterial vasculature. Furthermore, the released MMPs could probably contribute to the accelerated development and progression of thrombotic and atherosclerotic events in diabetic vessels, as both MMP-2 and -9 regulate platelet activation as well as vascular remodeling.

Elevated MMP levels have been shown previously in diabetic tissue and cells under hyperglycemic conditions. Hyperglycemia and diabetes enhance expression and activity of MMPs via oxidative stress or advanced glycation end products. Aortic endothelial cells cultured in high glucose have been shown to produce more reactive oxygen species (ROS), which activate MMP-9. ROS are increased in human diabetic IMA via the activation of NAD(P)H oxidase. The increased levels of ROS in diabetes result in tyrosine nitration and decreased endothelial NO bioavailability, which further activates MMP.
Attenuated NO bioavailability has also been shown to promote MMP activation.\textsuperscript{15,16} We have previously observed reduced eNOS expression and activity, and reduced NO production in the human diabetic IMA samples.\textsuperscript{17} eNOS dysfunction, coupled with activation of NAD(P)H-dependent oxidase, is largely responsible for enhanced superoxide production in human diabetic vascular tissue.\textsuperscript{33} Therefore, oxidative stress and limited NO bioavailability could be 1 of the causes of MMP-2 and -9 activation that we observed in diabetic arteries in the present work. It should be noted that a downregulation of MMP-2 and -9 in human diabetic IMA has also been reported.\textsuperscript{14} The possible reasons for the discrepancy are: (1) this investigation did not segregate type 1 and 2 diabetic patients, which may complicate its interpretation. (2) The absence of any proteinase inhibitors in the protein extraction buffer likely allows MMP activation during the extraction process. (3) The diabetic and nondiabetic samples were run separately during electrophoresis, and it is difficult to juxtapose pieces from different gels to compare the levels of proteins. Besides, the authors did present a brief review of controversial data obtained in different studies on MMPs in diabetic tissue and cells in hyperglycemic conditions.\textsuperscript{14} 

Activation of MMPs is also regulated by TIMPs. Imbalance between MMPs and TIMPs could be a reason for vascular complications. However, the mechanisms of TIMP regulation in diabetes or their involvement in angioangiostatin production and angiogenesis are at present uncertain. The novelty of the present study is the demonstration of the differential regulation of TIMP-1 and -2 in the diabetic arterial vasculature, in which TIMP-1 was found increased in both transcription and protein levels, whereas TIMP-2 was markedly downregulated (Figure 5). In fact, TIMP-2 activity has been shown to inhibit endothelial cell migration via several mechanisms, including inhibition of MMPs.\textsuperscript{35}

We have also found an increased expression of angioangiostatin in diabetic IMA samples (Figure 6), which is well known to be antiangiogenic because of its inhibitory effects on endothelial cell proliferation\textsuperscript{36} and inhibition of smooth muscle cell proliferation and migration in the coronary artery.\textsuperscript{37} The positive correlation between the angioangiostatin expression and

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{A, Representative RT-PCR data showing the gene transcription of TIMP-1, -2, -3, and -4 in diabetic (n=26) and control (n=26) IMA samples. B, Representative immunoblots indicating the immunoreactivity of TIMP-1 and -2 (diabetic, n=20; control, n=20). Protein extract was separated on 13% SDS-PAGE, and the blots were probed with anti-TIMP-1 or -TIMP-2 antibodies (dilution, 1:200). C, Representative reverse zymogram showing activity of TIMP-1 and -2. Bar graphs are the densitometric analysis. *P<0.05 vs control. Densitometric results are normalized with the expression levels of \(\beta\)-actin shown in Figures 3B and 5A.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{Protein expression of antiangiogenic factors. Protein homogenate from diabetic and control IMA samples was subjected to 10% SDS-PAGE, and the membrane was probed with anti-angiostatin or anti-endostatin antibodies (dilution, 1:200). Representative Western immunoblots from 20 diabetic and 20 control IMA samples showing the expression of angiostatin (A) and endostatin (B). Bar graphs are the densitometric measurement. *P<0.05 vs control.}
\end{figure}
MMP activity in diabetic tissue suggests that MMP activation is the main cause of angiostatin upregulation in diabetic arteries (Figure 7). Angiostatin opposes the VEGF action, probably by attenuating endothelial cell proliferation and migration and inducing apoptosis, as well as reducing VEGF expression. Moreover, angiostatin negatively regulates endothelium-dependent vasodilation, as it decreases NOS by uncoupling eNOS activity. Pharmacological inhibition of NOS enhances angiostatin production and MMP activation. We therefore speculate that during compromised NO formation in such pathological conditions as diabetes, elevation of MMP and angiostatin result in impairment of angiogenesis in the large arteries.

In conclusion, the present study indicates that in human diabetes the observed impaired angiogenesis could be attributed to the reduced VEGF level, as well as the enhanced activation of MMPs, which are strongly correlated with the augmented generation of angiostatin. A more complete understanding of the molecular mechanisms involved could be beneficial for the development of therapeutic strategies to restore or improve angiogenesis in the diabetic vasculature.

Sources of Funding
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Disclosures
None.

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Reduced Expression of Vascular Endothelial Growth Factor Paralleled With the Increased Angiostatin Expression Resulting From the Upregulated Activities of Matrix Metalloproteinase-2 and -9 in Human Type 2 Diabetic Arterial Vasculature

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