Thrombomodulin Deficiency in Human Diabetic Nerve Microvasculature

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Human diabetic neuropathy is multifactorial in etiology, with ischemia as a final common pathology. Although impaired vascular endothelial cell function in diabetic microvascular injury is established, the role of thrombomodulin (TM)-dependent protein C antithrombotic mechanism in the pathogenesis of neuropathy is unclear. This neuropathologic case-control study investigated whether vascular endothelial TM expression is deficient in peripheral nerve microvessels in diabetic neuropathy. Sural nerve biopsies from 7 patients with diabetic neuropathy and 10 with axonal neuropathy without vasculopathy were immunostained with anti-TM and anti–von Willebrand factor (vWF; an endothelial cell marker) antibodies. The proportion of TM-positive microvessels was expressed relative to total vWF-staining vessels, according to vessel caliber and regional distribution within the nerve. In diabetic nerves compared with reference controls, the proportion of TM-positive endoneurial microvessels was 15-fold lower (0.02 vs. 0.30 in diabetic nerves vs. controls, $P < 0.004$), and the proportion of small-caliber epineurial microvessels was 10-fold lower (0.04 vs. 0.43, $P < 0.001$). No TM expression was detected at the perineurium in diabetic or control nerves. We demonstrate a substantial reduction of vascular endothelial TM expression throughout human diabetic neuropathy. These findings suggest that an impaired native TM-dependent protein C antithrombotic mechanism may contribute to microvascular ischemia in the pathogenesis of diabetic neuropathy.
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An estimated 16 million individuals in the U.S. have diabetes (1). Peripheral neuropathy is the most prevalent neurological sequelae, present in ~60% of individuals with non–insulin-dependent diabetes (2). Although the precise mechanisms underlying human diabetic neuropathy remain controversial, a consensus of research indicates that microvascular ischemia is a final common pathological event (3,4). Despite the established importance of microangiopathy, no prior studies have investigated the role of impaired native thrombomodulin (TM)–protein C antithrombotic mechanisms in the pathogenesis of diabetic neuropathy.

TM is a vascular endothelial glycoprotein receptor that binds and terminates the prothrombotic actions of thrombin, yielding an enzyme cofactor complex that activates nascent protein C (5). Activated protein C (APC), an important antithrombotic enzyme, functions by inhibiting factors Va and VIIIa and has pro-fibrinolytic function by modifying clot formation and inhibiting plasminogen activator inhibitor-1 (PAI-1; the main inhibitor of tissue plasminogen activator) (6,7). Disturbances of the TM–protein C antithrombotic mechanism are the most important known etiology for clinical disorders of venous thrombosis (8). The TM–protein C antithrombotic mechanism also plays a crucial role in protecting the microcirculation during myocardial ischemia, disseminated intravascular coagulation, and stroke (9–14). Animal and human data suggest that regional differences in brain TM expression and microvessel protein C activation can determine the extent of microvascular thrombus formation and brain infarction (14–16). Moreover, this reduced TM expression in brain subcortical microvessels is postulated to predispose to lacunar infarction, the predominant stroke subtype in diabetic patients (15). Although the importance of the TM–protein C mechanism in the prevention of thrombosis and ischemic injury is well established, no prior studies have investigated a potential role for TM deficiency in peripheral nerve microvessels as a pathogenic mechanism in diabetic neuropathy.

There are several reasons to consider reduced microvascular TM in the pathophysiology of human diabetic neuropathy. First, exposure of cultured endothelial cells to advanced glycation end products (AGEs) downregulates the expression of TM (17). AGEs are deposited on the microvasculature of human nerve in diabetes (18). Second, vascular disease in diabetes is linked to upregulation of proinflammatory cytokines that are known to downregulate vascular endothelial TM expression and reduce TM-dependent protein C activation (16). Finally, clinical studies demonstrate that plasma TM levels are increased in individuals with diabetes, reflecting proteolytic injury to the vascular endothelium. These elevations in plasma TM fragments in diabetic patients are inversely related to protein C activity and positively related to increased markers of thrombin generation, hence constituting a

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AGE, advanced glycation end product; APC, activated protein C; IL-1β, interleukin-1β; PAI-1, plasminogen activator inhibitor-1; TM, thrombomodulin; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor.

marker of a procoagulant state (19). Furthermore, elevated plasma TM is strongly linked to the presence of microangiopathy and peripheral neuropathy (20).

To our knowledge, no prior studies have examined whether TM is present in the human nerve microvasculature and whether it is altered in diabetic neuropathy. This neuropathology case-control study investigates the hypothesis that TM is normally present in human nerve microvasculature, but that it is reduced in diabetic nerve microvasculature. Reduced TM in the diabetic peripheral nerve microvasculature could constitute elements of a regional procoagulant state, increasing susceptibility to microvascular thrombosis and subsequent ischemia.

RESEARCH DESIGN AND METHODS

Sural nerve tissue was obtained from patients with type 2 diabetes who underwent biopsy at the University of Maryland from 1998 to 2000 as part of a work-up for peripheral neuropathy. Sural nerve tissue from nondiabetic subjects was obtained from diagnostic biopsies performed on patients with axonal neuropathy who had no evidence of vasculopathy and no identified cause for the peripheral neuropathy. Subjects were excluded from the study if they had a history of peripheral vascular disease, vasculitis, collagen vascular disease, infection with human immunodeficiency virus, an active infection or inflammatory condition, or a medical history of a procoagulant state.

The neuropathologic assessment was based on analysis of paraffin-embedded tissue, teased fiber preparations, semithin epoxy sections, and electron microscopy. Evaluation included a determination of myelinated fiber size and density, the presence and relative number degenerating fibers, regeneration clusters and thinly myelinated axons, and segmental remyelination. The diabetic microangiopathy was characterized based on the severity of endothelial hyperplasia, capillary closure, thrombosis, reduplication of capillary wall basement membrane, and/or pericyte degeneration. Each pathological abnormality was graded according to its severity using an ordinal point scale (0 = normal, 1 = mild, 2 = moderate, and 3 = severe).

TM was detected on the surface of peripheral nerve microvessels with a modified biotin-avidin peroxidase immunohistochemical method using a specific monoclonal anti-TM antibody (Dako, Santa Barbara, CA). To identify nerve microvessels, serial sections were immunostained with a polyclonal anti–von Willebrand factor (vWF) antibody (Dako), a vascular endothelial cell marker. Target epitopes were unmasked on these formalin-fixed paraffin-embedded sections by microwaving sections in 10 mmol/l sodium citrate, pH 6, before immunohistochemistry. Nonimmune mouse and rabbit immunoglobulin served as negative controls for the staining specificity of the primary antibodies. The number of vWF-positive vessels within the epi-, peri-, and endoneurium was counted by light microscopy at 40× magnification on a Zeiss Photomicroscope III. The number of larger epineurial vWF-positive vessels with smooth muscle was counted, as was the number of small- and medium-caliber epineurial vWF-positive vessels. Each sural nerve biopsy cross-section typically contained 6–12 nerve fascicles with 40–100 total endoneurial and 100–200 epineurial microvessels visualized with the anti-vWF antibody. Multiple sections were obtained for each biopsy section. The proportion of TM-positive endo-, peri-, and epineurial microvessels relative to vWF-positive vessels was determined separately and expressed as follows: proportion of TM-positive vessels = total number TM-positive vessels/total number vWF-positive vessels.

The proportion of TM-positive vessels was separately determined for the epi-, peri-, and endoneurial regions of sural nerve sections from each subject and compared using a one-way ANOVA test with a P value <0.05 accepted as significant. In this analysis, the general estimating equations approach of Zeger et al. (21) was used to account for the serial autocorrelation of fractions from the same subject. The correlation structure was assumed to be exchangeable.

RESULTS

Clinical and demographic profiles and neuropathologic rating scores for the sural nerve biopsies from 7 type 2 diabetic patients and 10 controls without evidence of vasculopathy are listed in Table 1. All diabetic and control patients presented symptoms and signs of neuropathy, such as numbness, paresthesias, pain, weakness, and/or autonomic symptoms. Clinical examination and electrophysiological testing confirmed the presence of neuropathy. The duration of neuropathy was short for the individuals with diabetic neuropathy included in this study. The HbA_{1c} values were relatively low in the diabetic group and normal in the control subjects. The prevalence of microvascular (retinopathy and nephropathy) and macrovascular disease (coronary and cerebrovascular diseases) in both the diabetic and axonal control subjects is presented in Table 1.

The pathology in the diabetic subjects consisted predominantly of varying degrees of axonal degeneration and loss with minimal evidence of demyelination. In all of the diabetic subjects, microangiopathic changes consisted of moderately severe endoneurial microvessel thickening. In both diabetic and axonal neuropathy control nerves, the vascular endothelium was stained by immunohistochemistry using anti-vWF antibodies (Figs. 1 and 2). Vessels were stained with vWF independently of vessel size or anatomic location, within the endo- and epineurium, in both the diabetic and nondiabetic reference control nerves. Perineurial vessels were stained with vWF in most diabetic and some of the nondiabetic reference control nerves.

The proportion of TM-immunoreactive microvessels and level of immunoreactivity were reduced relative to vWF immunostaining within all nerve regional locations for both diabetic and reference control nerves. TM expression was restricted to the vascular endothelial cell surface facing the vascular lumen. The TM immunoreactivity on the endothelial cells of small- and medium-caliber epineurial microvessels and endoneurial vessels was lighter in comparison to the larger epineural vessels with a surrounding smooth muscle wall (Fig. 1). There was no evidence of immunostaining on axons, Schwann cells, or myelin sheaths. The connective tissue within the epi-, peri-, and endoneurium had no TM immunoreactivity. Rare inflammatory cells were TM immunopositive.

The proportion of microvessels expressing TM on the vascular endothelial surface was markedly reduced in diabetic nerves (Table 2 and Fig. 2). The proportion of endoneurial vessels with TM immunoreactivity present was 15-fold lower in diabetic compared with control nerves. The proportion of small- and medium-caliber epineurial vessels with detectable TM was 10-fold lower in diabetic compared with control nerves. The proportion of larger epineurial vessels with TM present at the vascular endothelial lumen was reduced in diabetic nerves compared with reference controls, but this finding did not reach statistical significance. TM immunoreactivity was virtually absent from the perineurial microvessels, which serves as a barrier against the interstitial space, in both diabetic and reference control nerves.

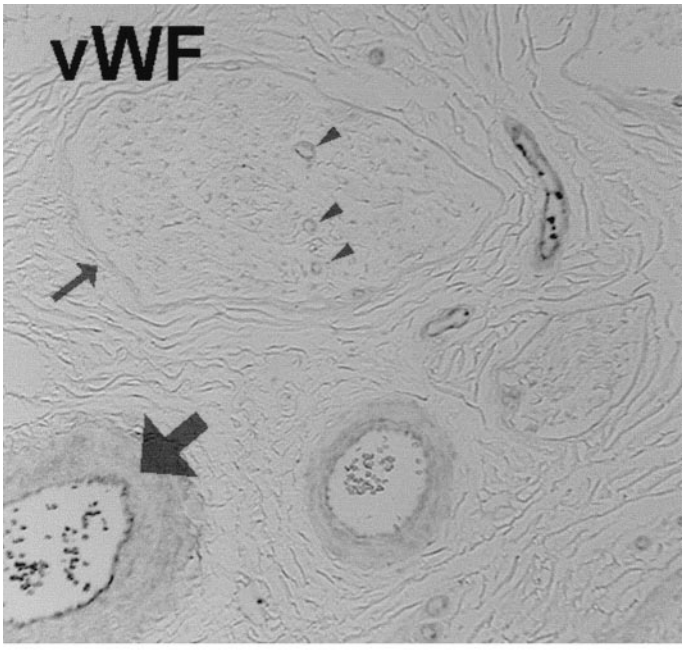
DISCUSSION

This report presents the first neuropathologic evidence of reduced TM in the microvessels of peripheral nerve in patients with diabetic neuropathy. We demonstrate a 15-fold reduction in the proportion of TM-immunoreactive endoneurial microvessels and a 10-fold reduction in the proportion of TM-positive smaller-caliber epineurial microvessels in human diabetic nerves compared with refer-

TABLE 1
Clinical and demographic characteristics of the patients

	Age	Sex	Race	Diabetes duration (years)	Neuropathy duration (years)	HbA_{1c}	Fasting glucose	Pathology and degree of severity	BMI	Retinopathy	Nephropathy	Coronary artery disease	Cerebrovascular disease	Hypertension	Dyslipidemia	Tobacco use
Diabetic subject no.																
1	45	F	AA	20	1	5.8	120	Axonal (3)	35	—	—	0	0	+	0	0
2	49	F	C	2	1	6.0	77	Axonal (2)	40	0	0	+	+	+	+	0
3	52	M	C	9	3	8.5	279	Axonal (3)	24	+	0	0	+	0	+	0
4	77	F	C	10	2	5.9	110	Axonal (1)	30	+	0	0	0	+	+	0
5	45	F	AA	7	2	6.0	77	Axonal (1)	26	0	0	0	0	0	0	0
6	67	M	C	20	1	5.7	191	Axonal (3)	25	0	+	0	0	0	0	+ (pipe)
7	56	F	C	0.5	0.5	6.1	137	Axonal (2)	22	0	0	0	0	0	0	0
Control subject no.																
1	44	F	I	—	5	5.8	90	Axonal (1)	40	0	0	0	0	0	0	0
2	74	F	C	—	5	5.5	88	Axonal (1)	29	0	0	0	+	+	0	0
3	66	M	C	—	1	4.8	76	Axonal (3)	40	0	0	0	0	+	0	0
4	43	M	C	—	2	—	92	Axonal (1)	—	—	—	—	—	—	—	—
5	49	M	C	—	2	—	87	Axonal (1)	—	—	—	—	—	—	—	—
6	41	F	AA	—	1	—	94	Axonal (1)	—	—	—	—	—	—	—	—
7	46	F	C	—	1	4.6	76	Axonal (2)	35	0	+	0	0	0	0	0
8	66	F	C	—	20	5.4	105	Axonal (1)	30	0	0	0	0	+	+	+
9	57	F	C	—	2	5.0	87	Axonal (1)	35	0	0	0	0	0	+	0
10	21	F	C	—	2	5.8	88	Axonal (1)	22	0	0	0	0	0	0	0

Severity of axonal neuropathy: 0 = none, 1 = mild, 2 = moderate, 3 = severe. AA, African-American; C, Caucasian; I, Indian; +, present; 0, not present.



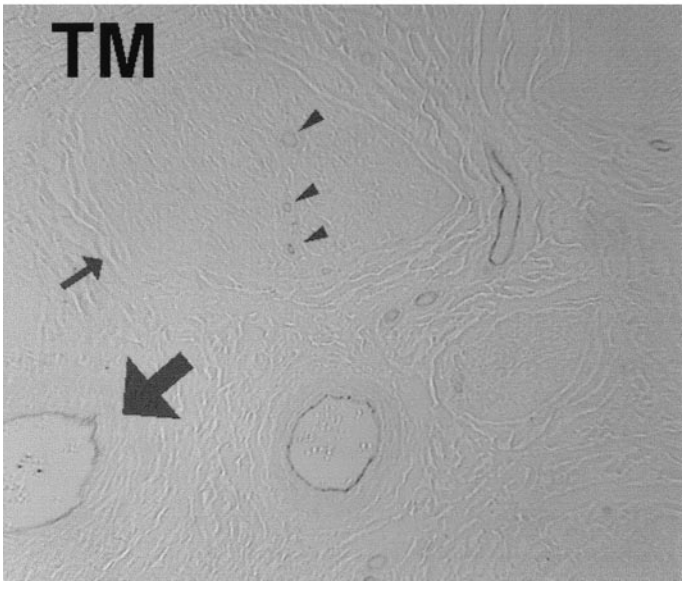
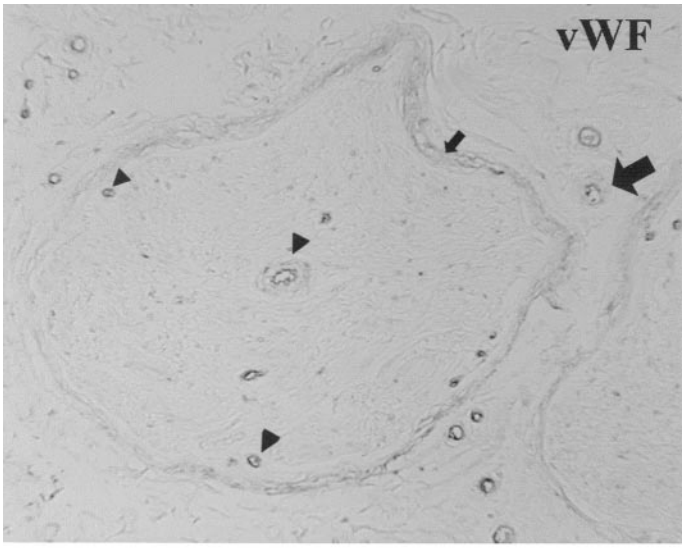


FIG. 1. This formalin-fixed paraffin-embedded sural nerve biopsy is from an individual with axonal neuropathy without diabetes, vasculitis, or inflammation. vWF immunoreactivity is detected on the epineurial (large arrow) and endoneurial (arrowheads) vessels, but not on the perineurial vessels (small arrow). The vWF-stained section is lightly counterstained with hematoxylin. No TM immunoreactivity is detected on the perineurial vessels (small arrow). The larger epineurial vessels with smooth muscular wall (large arrow) stain more intensely relative to the smaller-caliber epineurial and endoneurial vessels. No TM immunoreactivity is detected on the axons, myelin sheaths, or Schwann cells (magnification 180×).



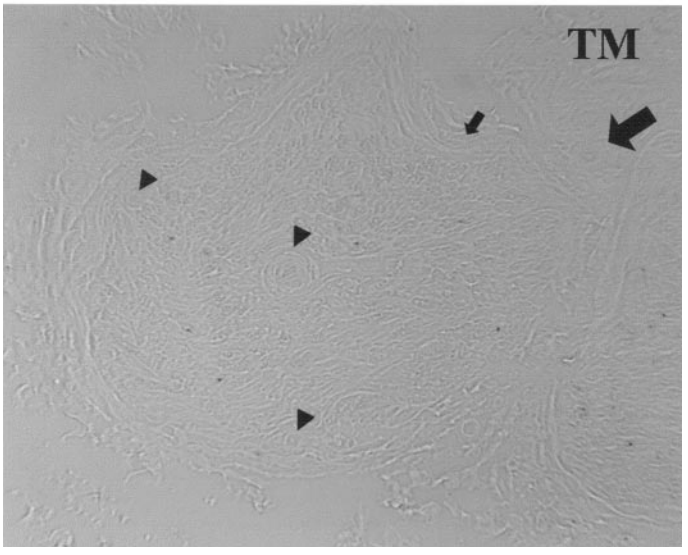


FIG. 2. No TM immunoreactivity was detected on the endo-, peri-, or epineurial vessels on this sural nerve biopsy from an individual with type 2 diabetes. In contrast, vWF immunoreactivity was intense on the epineurial (large arrow), perineurial (small arrow), and endoneurial (arrowheads) vessels (magnification 180×).

ence controls with axonal neuropathy. Furthermore, in both diabetic neuropathy and reference control nerves, we show that TM is absent from the perineurium, which serves as a diffusion barrier against the interstitial space. This pervasive reduction in TM throughout the peripheral nerve microvasculature suggests that impairments of the TM-dependent protein C antithrombotic mechanism may predispose the nerve to microvascular thrombosis and ischemia, the final common pathology in diabetic neuropathy.

APC, the product of TM-dependent protein C activation, protects the microcirculation by inhibiting clotting factors Va and VIIIa, modifying clot formation, promoting fibrinolysis by enhancing the action of t-PA, and inhibiting PAI-1 (6,7,22,23). APC also has distinct anti-inflammatory effects, including reducing leukocyte activation/infiltration and reducing nuclear translocation of nuclear factor κ-B (24), which upregulates expression of adhesion molecules and inflammatory cytokines. In experimental arterial occlusion models, the TM–protein C mechanism plays a crucial role in protecting the coronary and cerebral artery microcirculation (9,10,14,25). Augmenting TM–protein C antithrombotic function prevents thrombosis formation in arterial-venous graft models (26,27) and protects against organ failure and the lethal effects of disseminated coagulation in both animals (28) and humans (11). Studies in transgenic

TABLE 2
The proportion of nerve microvessels with TM immunoreactivity on the endothelial surface

	Type 2 diabetic subjects	Nondiabetic control subjects	*P*
n	7	10	
Microvessel location and caliber			
Endoneurium	0.02 (0.00–0.10)	0.30 (0.13–0.71)	<0.004
Perineurium	0	0	
Epineurium			
Small/medium	0.04 (0.01–0.12)	0.43 (0.24–0.78)	<0.001
Large	0.33 (0.04–0.61)	0.52 (0.14–0.91)	<0.15
Total nerve microvessels	0.04 (0.01–0.12)	0.32 (0.18–0.56)	<0.001

Data are means (95% CI). Results are from one-way ANOVA, assuming a negative binomial distribution.

mice specifically establish the fundamental importance of TM deficiency. Genetic deletion of the TM gene in mice causes embryonic lethality (29), whereas selective vascular endothelial cell TM ablation causes spontaneous and fatal arterial and venous thrombosis by 3 weeks of age (30). Mice homozygous for a single TM gene amino acid substitution (Glu404-Pro) have reduced capacity to generate APC, inhibit thrombin formation, and resist infection (31). These TM-homozygous mutant mice are more susceptible to endothelial cell injury and platelet thrombus deposition, and they have exaggerated cytokine production and mortality after exposure to low-dose endotoxin compared with littermate normal mice (31). Collectively, these studies establish the critical protective role of the TM-APC mechanism in inflammatory-prothrombotic vascular conditions.

Although the importance of TM-APC mechanisms in the prevention of thrombosis and ischemic injury in the systemic circulation and brain are established, the importance of regional vascular bed–specific TM expression by endothelial cells in human peripheral nerve microvasculature is unknown. Recent epidemiological studies and experimental animal models provide evidence that impaired TM–protein C antithrombotic mechanisms may contribute to diabetic microangiopathy. Elevation in soluble plasma TM fragments represents the product of proteolytic injury at the vascular endothelial surface, and it occurs in a number of disorders characterized by vascular endothelial injury. Cross-sectional studies reveal that diabetic patients have elevated plasma TM levels, and elevated plasma TM levels are related to the presence of diabetic microangiopathy and peripheral neuropathy (20,32). Elevated plasma TM is not simply a marker of microvascular endothelial injury. In diabetic patients, elevated plasma TM is inversely related to protein C activity and positively related to markers of thrombin generation, indicating a procoagulant state (19). In animal models, regional differences in brain TM expression determine the extent of protein C activation (16). Moreover, this reduced TM expression in human brain subcortical microvessels is postulated to predispose to lacunar infarction, the predominant stroke subtype in diabetic patients (15). Although the importance of the TM–protein C mechanism in the prevention of thrombosis and brain ischemia is established (25), only one prior study has investigated a potential role for TM in diabetic neuropathy.

Although plasma TM was related to neuropathy and improved with insulin treatment in streptozotocin-induced diabetes, weak TM immunoreactivity was similarly observed on some endoneurial microvessels in both treated and untreated diabetic rats. Hence, this animal model does not establish whether diabetes or its treatment is related to altered TM expression in nerve microvasculature (33). These findings must be interpreted with caution because streptozoticin-induced diabetes may differ from human diabetic neuropathy in its time course and biological mechanisms. No prior studies, to our knowledge, have examined the modulation of other hemostatic and fibrinolytic parameters in human or animal peripheral nerve microvasculature. Hence, this study presents the first evidence that microvascular TM is reduced in human diabetic neuropathy. Further studies are necessary to determine whether altered plasma levels of TM correlate with both regional disturbances of TM expression in the nerve and severity of neuropathy.

The mechanisms underlying the TM reduction in the microvasculature of human diabetic peripheral nerves cannot be determined in this case-control study. However, there are a number of well-recognized metabolic and inflammatory mechanisms associated with diabetes that may have an impact on microvascular endothelial cell expression of TM and antithrombotic function. AGEs accumulate in the vasculature in diabetes (18). In vitro exposure of vascular endothelial cells to AGEs directly downregulates TM expression (17), and AGEs may further decrease TM indirectly via their effects on inflammatory mediators.

Selected proinflammatory cytokines are strongly associated with diabetic microvascular disease and may further reduce vascular endothelial TM. In vitro and animal models show that interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) decrease TM mRNA and protein levels and decrease TM-dependent protein C activation (16,35). Vascular endothelial cells release proteolytic TM degradation product in the presence of TNF-α and neutrophils (36). Vascular endothelial growth factor (VEGF) gene transfer in experimental diabetic neuropathy improves nerve blood flow and restores peripheral nerve function (37). In vitro endothelial cell exposure to VEGF increases thrombin-dependent protein C activation by increasing TM transcription and translation and blocking IL-1β– and TGF-β–induced suppression of TM (38). Furthermore, APC has distinct anti-inflammatory actions, and TM has mitogenic responses, that are separate from their antithrombotic functions, which modulate local response to inflammatory stimuli (39,40). Hypoxia downregulates

TM transcription and expression of TM from cultured vascular endothelial cells (41). Further investigations are needed to both establish the mechanisms that lead to TM deficiency in the diabetic peripheral nerve microvasculature and determine whether TM–protein C antithrombotic mechanism impairments are linked to the development of diabetic neuropathy or its rate of progression.

The findings in this study are preliminary, and they are limited by the small sample size and the retrospective nature of the neuropathologic case-control design. Our results cannot distinguish whether the observed TM deficiency in diabetic nerve microvessels contributed to the clinical neuropathy or is a consequence of generalized vascular injury. In addition, this study was conducted in patients that were referred for diagnostic nerve biopsy, which may introduce a selection bias. These subjects with diabetic neuropathy represent a subset of patients with a relatively rapid presentation or multifocal pattern, rather than the more typical indolent diabetic polyneuropathy. Despite the fulminent presentation in these neuropathy patients, neuropathologic and microangiopathy findings were consistent with those routinely observed in human diabetic neuropathy. However, in the present study, we cannot exclude the possibility that the low TM expression is unique to this rapidly progressive group of patients. Moreover, the HbA_{1c} values were only modestly elevated in these diabetic neuropathy patients, suggesting that hyperglycemia may not be the sole factor leading to the observed reduction in microvascular TM.

In summary, this neuropathologic case-control study demonstrates a substantial reduction in TM expression throughout the nerve microvasculature in human diabetic neuropathy. TM expression is deficient on the microvessels throughout the endo- and perineurium, the region of the blood-nerve diffusion barrier, as well as in small-caliber epineurial microvessels in diabetic nerves compared with reference control nerves. These findings suggest that impairment of the TM–protein C antithrombotic mechanism may contribute to the pathogenesis of human diabetic neuropathy and raise the possibility that TM deficiency might play a similar role in microvascular angiopathy in other diabetic target organs, such as the kidney, retina, and brain.

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