Overexpression of transforming growth factor–β_1 correlates with increased synthesis of nitric oxide synthase in varicose veins

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Introduction: **Transforming growth factor-β_1 (TGF-β_1) is known to maintain a balance between apoptosis and cellular dysfunction and therefore may have a pivotal role in vessel remodeling during pathogenesis of vascular disorders. We previously demonstrated that inducible nitric oxide synthase (iNOS) mediates signal transduction in vascular wall during the development of varicose veins. Currently, we investigated the expression and correlation of TGF-β_1, iNOS, monocyte/macrophage infiltration, and loss of vascular smooth muscle cells (VSMCs), in a series of normal and varicose vein specimens.**

Methods: **Twenty varicose vein specimens were retrieved from 20 patients undergoing lower-extremity varicose vein excision, and 27 normal greater saphenous vein segments (controls) were obtained from 27 patients undergoing infrainguinal arterial bypass surgery. Principal risk factors (diabetes mellitus, hypertension, tobacco abuse) were also compared. Varicose vein segments were separated into tortuous and nontortuous regions based on their macroscopic and microscopic morphology. VSMC actin, CD68$^+$ monocytes/macrophages, iNOS, and TGF-β_1, were examined by immunohistochemistry, immunoblotting, and real-time reverse transcriptase polymerase chain reaction.**

Results: **According to the CEAP classification for chronic lower extremity venous disease, most of the patients were in class 2 for clinical signs of the disease (n = 11). Mean ages were 53.6 ± 4.7 years for the varicose vein group and 56.5 ± 4.4 years for the controls. The gender distribution was same in both groups. Immunoreactivity to TGF-β_1 and iNOS was significantly different in the tortuous regions of the varicose veins compared with nontortuous regions ($P < .01$). Not only was a significantly higher expression of iNOS noted in the varicose vein group ($P < .001$), but a differential expression of iNOS was also observed in the tortuous and nontortuous portions of the varicose veins. Significant overexpression of TGF-β_1 ($P < .01$) that correlated with overproduction of iNOS and with increased presence of CD68$^+$ monocytes/macrophages was observed in the varicose vein walls compared with normal veins.**

Conclusions: **This is the first evidence of TGF-β_1, as well as iNOS, being differentially upregulated in nontortuous and tortuous segments of varicose veins. The increased expression of TGF-β_1 and presence of macrophages, correlating with overproduction of iNOS, may be associated with varicosity development and deserves further study. (J Vasc Surg 2005; 41:523-30.)**

Clinical Relevance: **The pathogenesis of varicose veins, the most common manifestation of chronic venous disease, is debatable. Elucidation of mechanisms involved in the disease process is the first step to improved therapeutic modulations. Towards this goal, the relationship between NO production and TGF-β_1 in the molecular pathophysiology of chronic venous disease was investigated. The data identify for the first time, an important role for TGF-b1—iNOS—monocyte/macrophage signaling in the etiology of varicosities. Furthermore, we determine if there are any significant differences within the varicose vein group itself based on regional differences, by classifying the varicose tissues into tortuous and non-tortuous segments.**

Primary varicose veins, the most common manifestation of chronic venous insufficiency, affect up to 30% of people in Western countries.[1] The abnormal dilatation and tortuosity, hallmarks of varicose veins, provide evidence for venous wall remodeling.[2] Cell cycle dysfunction and deregulated turnover of the cells within the vein wall has been suggested to contribute to this remodeling in varicose veins.[3,4] The degree of cellularity in the vein wall is determined by the balance between the migration and proliferation of cells relative to their rate of egress and apoptosis. We observed significant differences in the expression and subcellular localization of the cell cycle regulatory protein, cyclin D1, in varix tissues compared with normal veins[5] and demonstrated that deregulated apoptosis plays a role in the pathogenesis of varicosities.[6]

Transforming growth factor-β_1 (TGF-β_1), an isoform of the fibrogenic cytokine TGF-β, is a multifunctional 25-kDa polypeptide that regulates diverse cellular functions such as proliferation, migration, differentiation, and extracellular matrix production. TGF-β_1 can act as a potent

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antiproliferative and apoptotic factor for proliferating vascular cells.[7] It has been shown to stimulate α-smooth muscle actin expression in fibroblasts both in vivo and in vitro[8,9] and also enhance the formation of the structural elements important for contractile force generation.[10] In vascular disease, TGF-β_1 is known to have a dualistic role and act as a bifunctional regulator of vascular smooth muscle cell (VSMC) proliferation, migration, and phenotype.[11] Hence, we hypothesized that this pleiotropic molecule can regulate the remodeling observed in varicosity development.

On the other hand, hypotheses that varicosities are caused by hydrostatic phenomena promoted by valvular incompetence lead to the thought that a significant factor in the pathogenesis of lower extremity varicose veins is vascular tone. Nitric oxide (NO), a potent intercellular molecular messenger, not only regulates vascular tone but also has diverse pathophysiologic functions such as inhibition of platelet adhesion/aggregation, mediation of the inflammatory cascade, neurotransmission, and cytotoxicity, among others.[12]

Additionally, NO is also a pleiotropic agent that has been linked to the signaling and execution of apoptotic cascades.[13] It either acts within the endothelium in which it is produced constitutively by endothelial NO synthase (eNOS), or it penetrates the endothelial layer affecting SMCs, which may also generate NO by inducible NO synthase (iNOS).[14] Whereas eNOS is calcium-dependent and produces small amounts of NO on stimulation, iNOS is a cytokine-inducible, calcium-independent, high-output enzyme that can be produced by almost any nucleated cell given the appropriate inflammatory stimulus.[15]

Several reports have referred to the modulation of cell proliferation and apoptosis by TGF and iNOS. Increased output of NO is also known to cause overexpression of TGF-β_1. However, this link between NO production and TGF-β_1 has not been investigated in the pathogenesis of varicose veins.

The current investigation examines the expression of TGF-β_1, iNOS, and eNOS in a series of varicose vein and normal vein specimens. The study determines if there are differences in the expression of these molecules in tortuous and nontortuous regions of varicose veins.

MATERIALS AND METHODS

This investigation was approved by the institutional review board of Maimonides Medical Center, and the patients gave written consent.

Patients. Twenty varicose vein specimens were atraumatically harvested from 20 patients undergoing lower-extremity varicose vein excision. The operative indications were pain, edema and venous stasis disease, or cosmetic purposes. All patients had symptoms ≥6 months. According to the CEAP classification of American Venous Forum[16] for chronic lower-extremity venous disease, 11 patients were in class 2 for clinical signs, 4 in class 3, 4 in class 4, and 1 in class 5. All patients were characterized as having primary varicosities except one patient who had secondary varicosities. Only the greater saphenous vein and its tributaries were studied. The greater saphenous vein specimens were collected from the thigh region and the tributaries from the calf region. A few tributaries were included to see if the results were similar.

The control group was 27 normal greater saphenous vein specimens obtained from 27 patients undergoing infrainguinal arterial bypass surgery who had no history of venous disease or evidence of reflux. None of these patients, histologically or by duplex examination, demonstrated venous thrombosis, and their vein segments were found to be free of acute or chronic inflammation by hematoxylin and eosin staining. All greater saphenous vein segments were from the thigh region, whereas the three greater saphenous vein normal tributaries included in the control group were from the distal calf region.

A preoperative lower-extremity venous duplex ultrasound assessment was performed on all patients. The superficial and the deep venous systems were studied by a standard method of examination as previously described.[6] Briefly, venous duplex sonography was performed on each patient beginning at the common femoral vein and continuing distally to the tibial and peroneal veins. Transverse and longitudinal views of the veins were obtained to determine venous flow with respiration and augmentation maneuvers and to document echogenic signals within the vein lumen to identify thrombus.

Tissue specimens. Upon atraumatic harvest, vein specimens were divided into two portions, of which one was immediately snap-frozen in liquid nitrogen. All specimens were transported to the lab. The varicose veins were classified as nontortuous or tortuous depending on their tortuosity, as evident upon macroscopic and microscopic examination by a pathologist and an investigator, after which they were processed.

The time taken to process all specimens was kept uniform, with no differences in the processing of varicose or normal vein samples. The frozen specimens were homogenized in denaturation/lysis buffer for RNA extraction and protein analyses. The remaining portion was fixed in 10% neutral buffered formalin solution containing ≈3.7% formaldehyde (W:V) for 6 to 12 hours and embedded in paraffin. Five segments of each vein specimen were embedded in each paraffin block. Transversal tissue sections of 5-μm thickness were mounted on poly-L-lysine coated slides. Each slide had five sections of one vein specimen.

Histology. Routine hematoxylin and eosin staining was performed for histologic evaluation of the specimens. Gomori's one-step trichrome staining was used to identify changes in collagenous connective fibers and to differentiate between collagen and smooth muscle fibers in both normal and varicose vein specimens. Verhoeff's elastic tissue stain (with van Gieson's stain to counterstain) was performed to assess the pathologic changes in the elastin network in all the veins included in the study.

Detection of TGF-β_1 transcripts. For RNA extraction, snap-frozen vein tissue homogenates underwent proteinase K digestion before total RNA was extracted using

Table I. Antibodies used for immunohistochemistry in varicose and normal veins

Antibody	*Clone/origin*	*Manufacturer*	*Titer*	*Antigen Retrieval*
Alpha actin	IA4	DAKO	1:50	pressure cooker, 0.25M Tris-base buffer, pH 9.0
CD68	KP-1	DAKO	1:6000	pressure cooker, 10mM Citrate buffer, pH 6.0
TGF-β1	Rabbit polyclonal	SantaCruz	1:200	pressure cooker, 10mM Citrate buffer, pH 6.0
eNOS	Rabbit polyclonal	Transduction Labs	1:1000	pressure cooker, 0.25M Tris base buffer, pH 9.0
iNOS	Rabbit polyclonal	Endogen	1:1000	pressure cooker, 10mM Citrate buffer, pH 6.0

TGF-β1, Transforming growth factor-β1; *eNOS,* endothelial nitric oxide synthase; *iNOS,* inducible nitric oxide synthase.

Table II. Patient demographics and clinical risk factors

	Normal vein (n = 27)	*Varicose vein (n = 20)*	P
Age ± SEM (years)	56.5 ± 4.4	53.6 ± 4.7	NS
Male:female (24:23)	14:13	10:10	.9
DM (n = 8)	6	2	.27
HTN (n = 8)	5	3	.75
Tobacco use (n = 5)	4	1	.28

SEM, Standard error of mean; *DM,* diabetes mellitus; *HTN,* hypertension. *P* values compare varicose vein to normal veins.

Ambion's total RNA isolation kit (Ambion, Austin, Tex) according to the manufacturer's standard protocol. mRNA expression of TGF-β_1 was determined by real-time reverse transcriptase polymerase chain reaction (RT-PCR) by using the GeneAmp 5700 SDS instrument (Applied Bio-Systems, Foster City, Calif) using reagents from QuantiTect Probe RT-PCR kit (Qiagen, Valencia, Calif), according to the manufacturer's instructions.

Total RNA (10 ng) from the vein tissues was used to reverse transcribe and PCR-amplify mRNA transcripts with TGF-β1 gene-specific primers: *forward primer*, 5'-CCT GGCGATACCTCAGCAA-3'; *reverse primer*, 5'-CCGGT GACATCAAAAGATAACCA-3'; and a fluorescent reporter probe, 5'-6FAM-CTGGCACCCAGCGACTCGC-BHQ1-3'. The amount of fluorescence detected is proportional to the amount of the PCR product formed and is calculated in real-time with GeneAmp 5700 SDS software.

The RT-PCR conditions were reverse transcription for 30 minutes at 50°C; initial denaturation at 95°C for 15 minutes, and 40 amplification cycles with denaturation at 94°C for 15 seconds, annealing, and extension at 60°C for 1 minute. Samples were analyzed in duplicate PCR reactions and data from two separate experiments were averaged for analysis. The sequence for glyceraldehyde phosphate dehydrogenase (GAPDH), a housekeeping gene, was used as an internal control.

Detection of TGF-β_1 and NOS expression. TGF-β_1, eNOS, and iNOS were detected in the vein walls by Western blotting, localized in vivo by immunohistochemistry as previously described,[6] and double staining was performed for co-localizing them with the various cell types.

Western blot analysis. Frozen venous tissue homogenates in a buffer containing 50mM Tris-HCl (pH 7.5) and a cocktail of protease inhibitors (Sigma Chemical, St. Louis, Mo) were used for immunoblotting. Equalized protein samples were electrophoresed using the Laemmli buffer system. Electrotransfer of the proteins was made to a polyvinyl diflouride membrane, and after blocking of nonspecific immunoglobulin (Ig) G binding, the membrane was incubated in primary antibodies overnight at 4°C. Bands were detected using horseradish-linked immunoassay by enhanced chemiluminescence system. Densitometric analysis of the immunoblots was performed using NIH ImageJ (Image-Pro Express v. 4.0) software. Blots were stripped and re-probed by standard protocols. Data are expressed as arbitrary units representative of three separate blotting experiments.

Immunohistochemistry. Localization of the expression of TGF-β_1, eNOS, and iNOS was by standard immunohistochemical techniques as previously described,[6] with primary antibodies and dilutions as shown in Table I. For double staining (10 varicose vein, 4 control specimens), anti-α-SM actin or $CD68^+$ was detected in the usual method with DAB substrate. TGF-β_1, eNOS, and iNOS were then localized by substituting peroxidase labeling with alkaline phosphatase-labeled avidin and detected using Fast Red.

Observation. The expression of proteins was evaluated according to staining intensity and immunocytochemical distribution-cytoplasmic and nuclear staining, based on the results of two independent blinded investigators. After the entire section was scanned at ×20 magnification and compared with the negative control, positive cellular expression was noted and the total number of immunoreactive cells determined at ×400 magnification. Ten random fields for each section and 5 sections for each specimen were evaluated.

Statistical analysis. The patient demographics data were analyzed by *t* test and χ^2. The Fisher exact test was used to compare the results obtained in the different groups. One-way analysis of variance (ANOVA) with post-hoc Bonferroni multiple comparison test was used to compare the TGF-β_1, eNOS, and iNOS expression in control, nontortuous varicose vein, and tortuous varicose vein groups. $P < .05$ was considered statistically significant. Statistical analyses were performed using StatView software (SAS Institute, Cary, NC). The Spearman nonparametric correlation of NOS versus TGF was calculated using Statistical Package for the Social Sciences (SPSS) software, version 8.0 (SPSS Inc.; Chicago, Ill).

Table III. Clinical, etiologic, anatomic distribution, and pathophysiology (CEAP) data

Class 2 (n = 11)	*Class 3 (n = 4)*	*Class 4 (n = 4)*	*Class 5 (n = 1)*
Male	*Male*	*Male*	*Male*
C_2 E_P $A_{S2\text{-}3}$ P_R	C_3 E_C $A_{S2,4}$ P_R	C_4 E_P $A_{S2\text{-}4;\ D9,11\text{-}14}$ P_R	C_5 E_P $A_{S4;\ D14;\ P18}$ P_R
C_2 E_P A_{S2} P_R	C_3 E_P $A_{S2\text{-}3;\ D11,13}$ P_R	C_4 E_S $A_{S2\text{-}3;\ D11,13,14}$ $P_{R,\ O}$	
C_2 E_P A_{S2} P_O			
C_2 E_P $A_{S2\text{-}3;\ D11,13}$ P_R	*Female*	*Female*	
C_2 E_P $A_{S2\text{-}3}$ P_R	C_3 E_P $A_{S2\text{-}3;\ D11,13,14}$ P_R	C_4 E_P $A_{S2\text{-}4;\ D11,13,14}$ P_R	
	C_3 E_P $A_{S2\text{-}3;\ D7,9,14}$ P_R	C_4 E_P $A_{S2\text{-}3;\ D11,13}$ P_R	
Female			
C_2 E_P $A_{S2\text{-}3}$ P_R			
C_2 E_P $A_{S1\text{-}4}$ P_R			
C_2 E_P $A_{S2\text{-}3}$ P_R			
C_2 E_P $A_{S2\text{-}3}$ P_R			
C_2 E_P $A_{S2\text{-}3}$ P_R			
C_2 E_P $A_{S2\text{-}3}$ P_R			

C (class): class 2, varicose vein; *class 3,* edema; *class 4,* hyperpigmentation, venous eczema, or lipodermatosclerosis; *class 5,* healed ulceration.
E (etiology): E_P, primary; E_C, congenital; E_S, secondary.
A (anatomy): A_S, superficial veins ($A_{S1\text{-}5}$); *S1*, telangiectasias/reticular veins; *S2*, great saphenous vein (GSV) above knee; *S3*, GSV below knee, *S4*, lesser saphenous vein; A_D, deep veins ($A_{D6\text{-}16}$); *D7,* common iliac; *D9*, external iliac; *D11*, common femoral; D12, deep femoral; D13, superficial femoral; *D14,* popliteal; A_P, perforating veins ($A_{P17,18}$); *P17,* thigh, *P18,* calf.
P (pathophysiology): P_R , reflux; P_O, obstructive.

RESULTS

Patient demographics and clinical characteristics. Demographics of patients in this study are shown in Table II. Table III gives the detailed information about each patients' disease status and the clinical, etiologic, anatomic, and physiologic classification. All patients from the varicose vein group exhibited reflux in the greater saphenous vein. In 7 patients, the deep venous system was also affected, 5 with reflux and 2 with reflux and evidence of concomitant nonocclusive deep venous thrombosis. Two patients in the varicose vein group had duplex evidence of greater saphenous vein thrombosis. Although in all patients the greater saphenous vein exhibited reflux, the lesser saphenous vein was also affected in 40%. The deep venous segments, common femoral, superficial femoral, and popliteal vein exhibited reflux in 20% of patients. Only two patients demonstrated reflux in the iliac vein and one in the deep femoral vein.

Histology. Consistent with our previous reports and those of other investigators, varicose vein specimens exhibited a more disorganized architecture compared with normal veins. Heterogeneity of the varicose vein wall was clear, and tortuous as well as nontortuous segments could be identified. The nontortuous segments bore considerable similarity in appearance to the normal vein segments. Hypertrophic regions and atrophic portions were distinct, but hypertrophic regions predominated atrophic areas in this study.

The medial layer was severely disorganized and exhibited a haphazard array of VSMCs (actin immunostaining), especially in tortuous varicose vein segments. The intimal layer exhibited cushion-like thickening in several areas. In the adventitia, the number of vasa vasorum was increased. Gomori's trichrome staining showed conspicuously decreased collagen matrix in varicose veins compared with normal veins. Verhoeff's elastin staining demonstrated increased degradation of elastin network. The wall layers of the varicose vein tissues, in addition to disruption of elastic lamellae, also revealed a significantly high degree of loss of VSMCs, although the VSMCs appeared enlarged. Monocytes/macrophages or cells immunopositive for antibody to CD68 were observed rarely in normal vein wall but were observed frequently in the varicose vein wall layers.

Table IV. Real-time reverse transcriptase polymerase chain reaction: gene transcripts in nanograms

	Normal vein (n = 10)	*Varicose vein nontortuous (n = 10)*	*Varicose vein tortuous (n = 10)*	P
TGF-β1	0.22	2.96	8.54	
GAPDH	0.571	0.258	0.362	
TGF-β1/ GAPDH	0.38 ± 0.5*	11.47 ± 3.6*	23.6 ± 5.8*	<.01

TGF-β1, Transforming growth factor-β1; *GAPDH,* glyceraldehyde phosphate dehydrogenase.
*Data are means ± SEM and significant.

Transcription of TGF-β_1. RT-PCR revealed an increased amount of TGF-β_1 gene transcripts in varicose vein tissues. The differences between the two varicose vein groups was significant, with the greater amount of mRNAs being present in the tortuous segments of the varix tissues (Table IV). The increased level of TGF-β_1 in varicose vein samples correlated with immunostaining data. Only a very small amount of expression of TGF-β_1 was detected in control vein samples ($P < .01$).

Differential expression of TGF-β_1. Normal venous tissues exhibited negligible immunopositivity to antibody for TGF-β_1. TGF-β_1 expression was both focal and pantropic in the mural layers of the varicose vessels. Cytoplasmic and nuclear localization of TGF-β_1 was observed in the

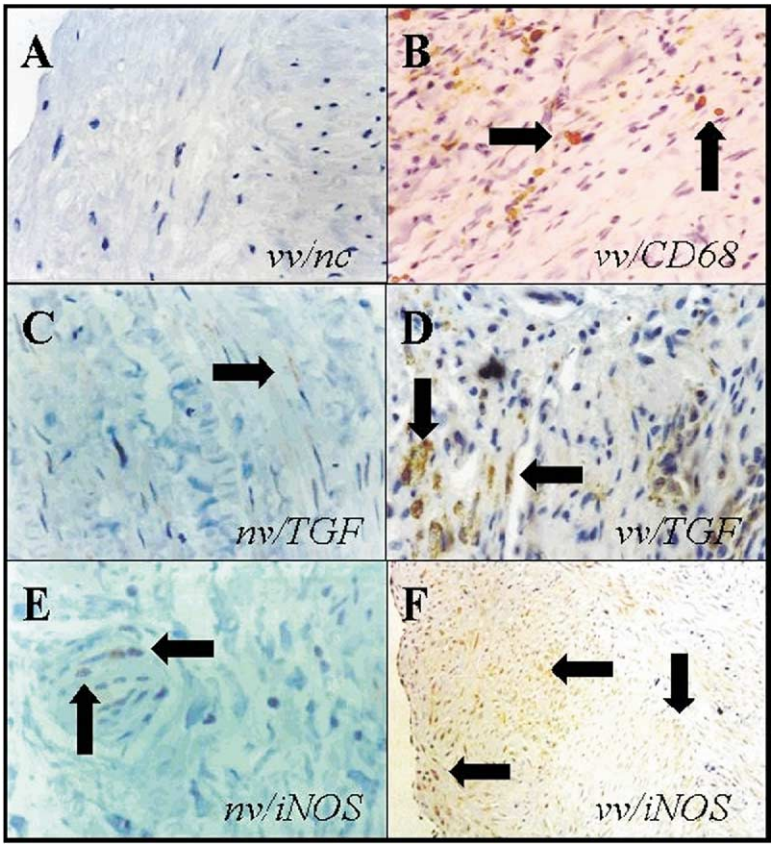

Fig 1. Representative photomicrographs of immunohistochemical analysis in vein wall (all at original magnification ×400 unless indicated otherwise). Formalin-fixed paraffin-embedded tissues were processed for immunolocalization of proteins as described under *Methods.* Brown staining (peroxidase) *(arrows)* indicates immunopositivity. Photomicrographs depict the staining pattern. **A,** Negative control *(nc)*. Nontortuous varicose vein (*vv*). **B,** $CD68^+$ cells in tortuous varicose vein. **C,** Transforming growth factor-β_1 (*TGF-*β_1) in control vein *(nv)* tissue. **D,** TGF-β_1 in non-tortuous varicose vein tissue. **E**, Inducible nitric oxide synthase (*iNOS*) expression in control vein. *F,* iNOS expression in tortuous varicose vein tissue (original magnification ×200).

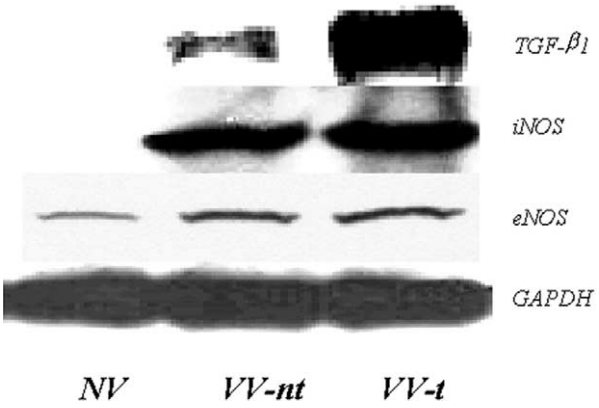

Fig 2. Western blot analysis for expression of transforming growth factor-β_1 *(TGF-*β_1*)* and inducible *(iNOS)* and endothelial *(eNOS)* nitric oxide synthases. Comparison of the normal vein *(NV)*, tortuous varicose vein *(VV-t)*, and nontortuous varicose vein *(VV-nt)* segments. *GAPDH,* glyceraldehyde phosphate dehydrogenase.

cells of the venous walls. Only varicose veins showed $CD68^+$ cells expressing TGF-β_1. Predominant expression of TGF-β_1 was in the adventitial layer and in regions of intimal fibrosis. However, the amount of protein for this relatively unique cytokine was diminished in the nontortuous segments compared with the tortuous segments of the varicose vein ($P < .05$). This was also confirmed in the Western blot analysis (Figs 1 and 2). Densitometric evaluation of the immunoblots revealed TGF-β_1 protein bands were of 2853 and 12280 units (arbitrary units, pixel density) in nontortuous and tortuous varicose vein segments respectively. Within tortuous varicose vein segments, in hypertrophic areas, there were 35.7% ± 4.8% cells positive for TGF-β_1; whereas in the nontortuous varix tissues, only 17.7% ± 1.14% cells expressed this protein.

Expression of eNOS. Expression of this isoform of NOS could be detected in the venous wall by immunologic techniques in the current study and was similar to observations in our previous study. There was no significant difference in expression of eNOS between the three vein groups.

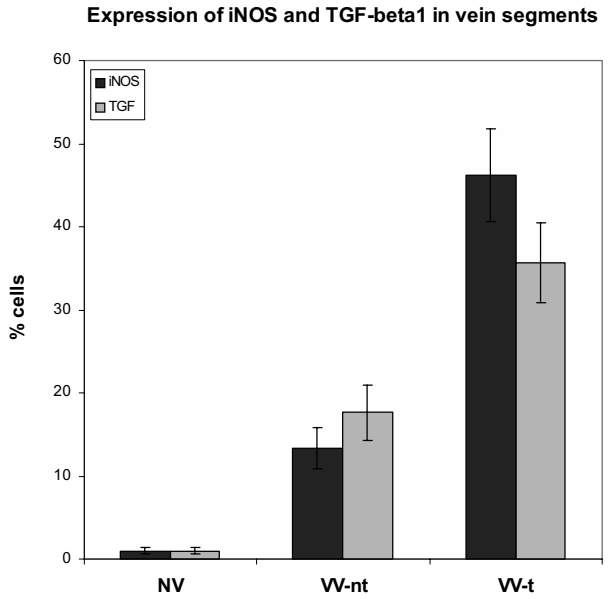

Fig 3. Immunohistochemical analysis for expression of transforming growth factor-β_1 *(TGF-β_1)* and inducible nitric oxide synthases *(iNOS)*. Comparison of the normal vein *(NV)*, tortuous varicose vein *(VV-t)* and non-tortuous varicose vein *(VV-nt)* segments. All values are expressed as mean ± SEM. Values for tortuous varicose vein and normal vein segments were significantly different ($P < .01$).

The pixel density units for eNOS protein bands were of 1975, 2297, and 2643 units in controls, nontortuous, and tortuous varicose vein segments respectively (Figs 1 and 2). Immunohistochemistry revealed its predominant localization to the endothelium and the endothelial cells of the vasa vasorum. Some smooth muscle cells also were positive for eNOS.

Differential expression of iNOS. Normal venous tissues exhibited negligible immunopositivity to the antibody for iNOS. Immunohistochemical staining demonstrated intense staining in several focal areas of the varicose vein wall and confirmed our previous results. This staining was absent from the negative controls. The varicose vein demonstrated a large number of cells expressing iNOS, with 46.28% ± 3.5% positive cells in tortuous varicose vein segments ($P < .001$). However, in the nontortuous segments, less than half that number of cells expressed iNOS ($P < .001$) and it was localized to infiltrating elements. Only varicose vein walls had $CD68^+$ cells expressing iNOS. We did not note any protein band corresponding to 130 kD for iNOS in the tissue homogenate of normal venous wall. In contrast, the protein profile of samples of varicose vein specimens demonstrated significant amount of protein (Figs 1, 2, and 3). Densitometric evaluation of the immunoblots revealed iNOS protein bands were of 6281 units in nontortuous and 7897 units in tortuous varicose vein segments.

The Spearman nonparametric correlation of NOS versus TGF was significant at the 0.01 level (2-tailed). Perhaps because of their small numbers, we did not observe any differences in the outcomes of tributaries compared with whole segments.

DISCUSSION

The present investigation demonstrates the expression of TGF-β_1 and NO synthases in the vessel wall of normal and varicose veins. Overexpression of TGF-β_1 that correlated with the upregulation of iNOS was observed in varicose vein specimens. Few studies have provided information at the molecular level based on regional histologic differences within the vein wall. Hence, to determine if there were any significant differences within the varicose vein group itself based on such regional differences, we classified the varicose tissues to tortuous and nontortuous segments. We demonstrated that the presence of TGF-β_1 and iNOS-positive cells was not only high in varix tissues compared with the controls but also that these molecules were differentially expressed in the tortuous and nontortuous segments of the varicose veins. The expression of TGF-β_1 and iNOS was also observed in the cells positive for smooth muscle α-actin and CD68.

Varicosis is a complex pathology characterized by venous hypertension, blood stagnation, and reflux leading to progressive venous wall remodeling evidenced by abnormal dilatation and tortuosity. There are several theories regarding the pathogenesis of varicosities. The primary cause is still unknown, although ample evidence implicates that the defect is in the wall of the lower limb veins. [17] It has also been suggested that increased expression of basic fibroblast growth factor and TGF-β_1 by varicose vein cells may have a pivotal role in the hypertrophy of the venous wall, although the exact mechanism leading to venous wall dilatations remains to be elucidated.[18] Our previous data on deregulated cell cycle and apoptosis and the hypertrophy observed in several regions of varicose veins lend credence to this observation.[5,6]

Resistance to apoptosis may have dual effects, increase the volume of the resistant cell subsets, and lead to the slow proliferation of these cells, thereby contributing to the focal progression of mural layer hypertrophy. Bujan et al[19] reported an increase in apoptosis and collagen synthesis in varicosities that contradicted our results and those of Urbanek et al,[3] who also found a down-regulation of VSMC apoptosis in varicose veins. However, their recent work demonstrates greater expression of TGF-β_1 and its latent polypeptide in varicose veins.[20] Both molecules were detected in the subendothelium and the media, particularly in areas of marked injury. Our findings and those of several others also suggest that the development of the varicose condition involves a restructuring of the elastic component of the vein wall, perhaps as a consequence of changes in the transcription mechanisms of muscle layer cells.

That TGF-β_1 has been found to be crucial in promoting connective tissue deposition underscores its role in the molecular events that lead to the remodeling and the acceleration of the final fibrosclerotic process characteristic of the varicose vein wall. However, the data from this study

and those from our previous investigations suggest a decrease and degradation of extracellular matrix in varicose veins. Perhaps vein cells respond differently to TGF-β_1 stimulation. Other studies have demonstrated an increase in metalloproteinases. It would be interesting to look at total collagen in the veins, but the current study did not make such an analysis.

NO is reported to enhance TGF-β_1 activity.[21] The role of NO in apoptosis is controversial, but attenuation of NO is known to have a key role in the activation of the caspase cascade. The iNOS upregulation observed here was significant as well as differentially upregulated in tortuous and nontortuous areas of the varicose veins. The expression of iNOS was very intense and implicates the induction of NO in these cells of the varicose vein tissues that can trigger a cascade of molecular events remaining to be elucidated. Both being pleiotropic, their effects are highly cell/tissue-specific and concentration-dependent. It is possible that multiple mechanisms are involved that may be partly mediated by TGF-β_1 induction.

It is noteworthy that an increased presence of $CD68^+$ cells, used for detection of monocytes/macrophages, was observed in both tortuous as well as nontortuous segments of the varicose vein wall. We had performed a battery of immunohistochemical assays for identifying the cells in the tissues of normal and varicose vein tissues. These included assays for pan T cells ($CD3^+$), cytotoxic T cells ($CD8^+$), B cells ($CD20^+$), and Ki 1 in T and B cells ($CD30^+$). However, only monocytes/macrophages were present in significantly high numbers in the diseased tissues compared with controls. Sayer et al[22] also recently reported that varicose veins display a greater inflammatory cell infiltrate than normal vein. The monocytes/macrophages increase in varix tissues correlates with increased TGF-β_1 and iNOS expression. $CD68^+$ cells were absent or rarely present in control veins tissues.

Other investigators have reported that monocytes/macrophages migrate into the venous walls and valves of patients with venous insufficiency, activating not only luminal venous endothelium but also endothelium in the vasa vasorum of refluxing saphenous veins.[23] The functional activation of monocyte/macrophages related to venous stasis as a consequence of venous hypertension has also been demonstrated.[24] Our results are further evidence of the role of the invading monocytes/macrophages in the etiology of varicose veins.

We speculate that NO released in the venous tissue by the upregulation of iNOS enhances the production of TGF-β_1 in varicose veins. This correlates with our previous results of downregulation of mediators of apoptosis. The endothelial isoform of NOS was expressed in all the samples studied. However, its expression in the various groups did not differ significantly. It is likely that the small amounts of NO produced by eNOS, although having a role in the regulation of the vascular tone, do not affect the pathogenesis of the varix development as much as the large amounts of NO produced by iNOS.

In conclusion, this study has demonstrated for the first time a correlation between TGF-β_1, iNOS, and monocyte/macrophage infiltration and loss of mural integrity in both tortuous and nontortuous varicose veins. The precise mechanisms underlying the effects of TGF-β_1 and iNOS upregulation and the molecular pathways leading to their differential expression in the venous wall layers are not clear at present. We are in the process of culturing cells from patient samples of varicose and normal veins, and it may be possible to study the expression and effects of these molecules in vitro to further explore the pathogenesis of primary venous dysfunction.

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