Evaluation of Transforming Growth Factor- β Signaling Pathway in the Wall of Normal and Varicose Veins

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Key Words

Chronic venous insufficiency · Varicose veins · Transforming growth factor- β_1 · Transforming growth factor- β receptor type II · Smad

Abstract

Objective(s): Extracellular matrix remodeling in the vein wall is involved in varicose vein pathogenesis, with transforming growth factor β_1 (TGF- β_1) playing a potential role. The aim of the study was to assess the TGF- β signaling pathway including its receptor (TGF- β RII) and phosphorylated receptor-regulated Smads (p-Smad2/3) in varicose veins.

Methods: Varicose veins from patients undergoing varicose vein surgery were the studied material, whereas normal greater saphenous veins from patients undergoing infringuinal arterial bypass surgery were the control material. Expression of TGF- β RII mRNA was assessed with RT-PCR, whereas expression of TGF- β RII and p-Smad2/3 proteins was assessed with Western blot. **Results:** A significantly increased TGF- β RII mRNA level was found in varicose veins ($287 \pm 24\%$), when compared with normal veins ($100 \pm 26\%$). The receptor protein expression reflected a changed mRNA level with significantly increased TGF- β RII protein in varicose veins ($290 \pm 21\%$), when compared with controls ($100 \pm 16\%$). Enhanced TGF- β RII expression was accompanied by increased p-Smad2/3 protein expression in varicose

veins ($257 \pm 19\%$) in comparison with normal veins ($100 \pm 9\%$). **Conclusion(s):** Increased TGF- β RII expression and activation in the wall of varicose veins may be involved in extracellular matrix remodeling related to TGF- β_1 and supports its role in the disease pathogenesis.

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Introduction

Varicose veins are among the most common features of chronic venous insufficiency (CVI), which exerts a high impact on patient quality of life [1]. The disease pathogenesis is still not clearly understood. Although valvular incompetence, alterations in smooth muscle cell arrangement and changes in extracellular matrix (ECM) composition are the most commonly noted factors affecting the development of varicose veins, the exact mechanisms through which they interact in the disease pathogenesis remain unknown [2, 3].

ECM is a complex supramolecular structure and its remodeling contributes to weakness and reduced elasticity of the varicose vein wall [2, 4]. Transforming growth factor β (TGF- β) has a profound effect on the synthesis and degradation of a large number of ECM molecules, including different types of collagen and proteoglycans. It also influences the synthesis of matrix metalloprotein-

ases and their inhibitors [4]. Among its 3 isoforms, TGF- β_1 plays a crucial role in vessel wall remodeling [5, 6]. Thus, the growth factor has been extensively studied in CVI pathogenesis, and increased TGF- β_1 mRNA levels, as well as its protein content, were found in the wall of varicose veins in comparison with normal veins [7–9].

TGF- β_1 is synthesized as an inactive molecule, and numerous proteases are involved in latent TGF- β_1 activation [10]. An active form of TGF- β_1 acts through membrane serine/threonine kinase receptors of type I and II [5]. The latter are expressed constitutively, whereas TGF- β RI is recruited after TGF- β binding to the type II receptor [5, 11]. Thus, the presence of TGF- β RII is essential to cellular responsiveness to TGF- β_1 [12]. Once the receptor complex is activated, it leads to intracellular Smad protein phosphorylation. Phosphorylated receptor-regulated Smads (p-Smad2 and p-Smad3) are transported into the nucleus and regulate gene transcription, including synthesis of collagen or tissue inhibitors of metalloproteinases [5]. However, in spite of the commonly accepted role of TGF- β_1 in varicose vein pathogenesis, the TGF- β signaling pathway has not been evaluated in the vein wall.

The aim of the study was to assess TGF- β RII mRNA and protein expression, as well as p-Smad2/3 protein expression in the wall of varicose veins and normal controls.

Materials and Methods

The investigation protocol conforms to the principles outlined in the Declaration of Helsinki and was approved by the Committee for Ethics and Supervision on Human and Animal Research of the Medical University of Białystok, Białystok, Poland. Patients on preoperative therapy with statins were excluded from the study [13]. All patients enrolled in the research provided informed consent after reviewing the study protocol.

Tissue Material

The varicose vein specimens were collected from patients undergoing varicose vein surgery. Patients with duplex evidence of greater saphenous vein (GSV) thrombosis, deep vein reflux, current or previous deep vein thrombosis, or a history of previous sclerotherapy were excluded from the study. Twenty-four patients with primary varicose veins were enrolled in the research (16 women and 8 men; mean age 52.9 ± 9.2 years, range 43–66). All of them were in class 2 according to the clinical signs of the CEAP classification. All patients exhibited saphenofemoral junction incompetence with reflux in the proximal femoral region of the GSV. The lesser saphenous vein was affected in 2 patients (8%). The control material consisted of GSV specimens harvested from the distal femoral region in 12 patients (8 women and 4 men; mean age 57 ± 5.1 years, range 48–65) with chronic limb isch-

emia undergoing infrainguinal arterial bypass surgery. Control patients had no history of venous disease or evidence of vein reflux.

RT-PCR Analysis

A total of 50 mg of harvested tissue was pulverized after immersion in liquid nitrogen. RNA was extracted from tissues via the AxyPrep Multisource Total RNA Miniprep Kit (catalogue number AP-MN-MS-RNA-50; Axygen Bioscience, Union City, Calif., USA). The total RNA concentration was determined by measuring the optical density at 260 nm. The ratio of A260/A280 was greater than 1.8. The RNA quality was checked by electrophoresis in a 1.2% agarose gel, followed by staining with ethidium bromide and examination of the 28S and 18S rRNA bands with an UV transilluminator. No significant degradation was observed in any RNA sample.

A total of 3 μ g of total RNA was reverse-transcribed into cDNA with the use of a RevertAid™ First Stand cDNA Synthesis Kit (catalogue number K1622; Fermentas, Vilnius, Lithuania) and oligo(dT)₁₈ primers.

For cDNA amplification, the following pairs of primers were used: (1) forward human TGF- β RII 5'-AGCAGAAGCTGAGTTCAACCTGGG-3'/reverse human TGF- β RII 5'-GGAGC-CATGTATCTTGCAGTTCC-3'; (2) forward human TGF- β RII 5'-AATATCCTCGTGAAGAACGA-3'/reverse human TGF- β RII 5'-CAGTCAACGTCTCACACACC-3', and (3) forward human β -actin 5'-TTGTAACCAACTGGGACGATATGG-3'/reverse human β -actin 5'-GATCTTGATCTTCATGGTGCTAGG-3'. Two pairs of TGF- β RII primers were used to eliminate the possibility that final RT-PCR products may originate from genomic DNA contaminations. All primers were synthesized by Sigma-Proligo (St. Louis, Mo., USA).

Aliquots of 5 μ l of the cDNA and 20 nM of each primer were subjected to PCR using Taq DNA polymerase (catalogue number EP0402; Fermentas). PCR thermocycling conditions for TGF- β RII and β -actin cDNA detection were set up as follows: 1 cycle of denaturation at 94°C for 4 min, 30 cycles of 94°C for 1 min/60°C for 1 min/72°C for 1 min, and a final primer sequence extension incubation at 72°C for 10 min. Ten microliters from each amplification reaction was analyzed with electrophoresis in 2% agarose gels in the presence of 0.5 μ g/ml ethidium bromide. DNA was visualized under UV light. Contamination was routinely checked by an RT-PCR assay of cDNA template-free samples (water control). The RT-PCR reaction products that were separated by agarose gel electrophoresis were photographed, scanned and analyzed using the QuantityOne software (Bio-Rad, Hercules, Calif., USA). The optical density of the TGF- β RII bands was normalized to the corresponding β -actin bands. The gene expression ratios were normalized to 100% in control veins, whereas in varicose veins, they were expressed relative to control veins.

Western Blot Analysis

Collected samples were washed with ice-cold 0.9% NaCl solution, cut, weighed and suspended in 0.15 M Tris-HCl buffer (pH 7.4) in the ratio 1:3 (w/v). The homogenates were prepared with a knife homogenizer (25,000 rpm for 45 s at 4°C) and sonicated (20 kHz, 4 $\times$ 15 s at 4°C). After centrifugation (10,000 g for 15 min at 4°C), supernatants were stored at -70°C until measurements were performed. The protein content was determined according to the method described by Bradford [14], and SDS/PAGE was

performed according to the method described by Laemmli [15]. Samples of tissue supernatants containing 20 µg of protein were subjected to electrophoresis. The following molecular mass standards were used: 208, 120, 99, 53, 43, 37 and 29 kDa (catalogue number 1610318; Bio-Rad). The gels were allowed to equilibrate in 25 mM Tris, 0.2 M L-glycine, 20% (v/v) methanol for 5 min, and the proteins were transferred to 0.2 µm pore diameter nitrocellulose membranes at 100 mA for 1 h. The membranes were then incubated in 5% dried defatted milk in TBST [20 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl, 0.05% (v/v) Tween 20] for 1 h with one of the following primary antibodies: (1) polyclonal anti-human TGF-β RII antibody (goat IgG; catalogue number AF-241-NA; R&D Systems Inc., Minneapolis, Minn., USA) at 1:500 dilution; (2) polyclonal anti-human p-Smad2/3 antibody (goat IgG; catalogue number sc-11769; Santa Cruz Biotechnology Inc., Santa Cruz, Calif., USA) at 1:200 dilution, and (3) monoclonal anti-human actin antibody (mouse IgG; catalogue number sc-8432; Santa Cruz Biotechnology Inc.) at 1:200 dilution. Species-specific secondary antibodies were then added at 1:7,500 dilution. Incubation was continued for 30 min with slow shaking. After that, nitrocellulose membranes were washed with TBS-T (5 times for 5 min) and treated with Sigma-Fast BCIP/NBT reagent (catalogue number B1911; Sigma Aldrich Chemie GmbH, Steinheim, Germany). Three measurements were performed in each tissue sample. The labeled membranes were photographed, scanned, and optical density was analyzed using an imaging QuantityOne software (Bio-Rad). The optical density of the TGF-β RII and p-Smad2/3 bands was normalized to the corresponding actin bands. The protein expression ratios were normalized to 100% in control veins, whereas in varicose veins, they were expressed relative to controls.

Statistical Analysis

Data are represented as the mean ± standard deviation. The normal distribution of the obtained results was confirmed by a Kolmogorov-Smirnov one-sample test of normality, and descriptive exploration and analysis of Skewness. Statistical analysis of the obtained results was carried out using one-way or Kruskal-Wallis analysis of variance (ANOVA). All tests were 2-tailed. $p < 0.05$ was considered statistically significant.

Results

RT-PCR analysis of the investigated tissues is shown in figure 1a. Only trace expression of TGF-β RII mRNA was found in the wall of normal veins (line 1), whereas strong expression of TGF-β RII mRNA was present in the wall of varicose veins (line 2). Densitometric analysis of TGF-β RII mRNA expression is shown in figure 1b. TGF-β RII mRNA expression was significantly increased in the wall of varicose veins, when compared with the wall of normal veins ($p < 0.001$). No significant difference in TGF-β RII mRNA expression was found between both sets of measurements performed with different pairs of primers. Furthermore, gender-based evaluation did not

Fig. 1. **a** RT-PCR analysis of TGF-β RII mRNA expression in the vein wall (lane 1, normal veins; lane 2, varicose veins). RNA (3 µg) was reverse-transcribed with the use of oligo(dT)₁₈ primers. cDNA amplification was performed with TGF-β RII and β-actin primers. **b** Densitometric analysis of TGF-β RII mRNA expression from 12 control samples and 24 varicose vein samples. The optical density of TGF-β RII bands was normalized to the corresponding β-actin band. The gene expression ratio was normalized to 100% in normal veins, whereas in varicose veins it was expressed relative to normal controls. Mean values ± standard deviations are presented. * $p < 0.001$, versus the normal vein wall.

reveal any significant difference in TGF-β RII mRNA expression between men and women with varicose veins ($p > 0.05$).

Western blot analysis of TGF-β RII protein expression in the investigated tissues is shown in figure 2a. TGF-β RII is a protein with a molecular mass of about 75–85 kDa. The presence of such a band was demonstrated in the wall of normal veins (line 1) and varicose veins (line 2). Densitometric analysis of TGF-β RII protein expression is shown in figure 2b. TGF-β RII protein expression was significantly increased in the wall of varicose veins,

Fig. 2. a Western blot analysis of TGF-β RII protein expression in the vein wall (lane 1, normal veins; lane 2, varicose veins). The same amount of protein extract (20 μg) was run in each lane. Actin was used as a loading control. The molecular mass standards are indicated on the left side. **b** Densitometric analysis of TGF-β RII protein expression from 12 control samples and 24 varicose vein samples. The optical density of TGF-β RII bands was normalized to the corresponding actin band. The protein expression ratio was normalized to 100% in normal veins, whereas in varicose veins, it was expressed relative to normal controls. Mean values ± standard deviations are presented. * $p < 0.001$, versus the normal vein wall.

when compared with the wall of controls ($p < 0.001$). Further analysis revealed no gender differences in TGF-β RII protein expression in the group of patients with varicose veins ($p > 0.05$).

Western blot analysis of p-Smad2/3 protein expression is shown in figure 3a. Bands corresponding to a molecular mass of 55 kDa were demonstrated in normal (line 1)

Fig. 3. a Western blot analysis of p-Smad2/3 protein expression in the vein wall (lane 1, normal veins; lane 2, varicose veins). The same amount of protein extract (20 μg) was run in each lane. Actin was used as a loading control. The molecular mass standards are indicated on the left side. **b** Densitometric analysis of p-Smad2/3 protein expression in 12 control samples and 24 varicose vein samples. The optical density of p-Smad2/3 bands was normalized to the corresponding actin band. The protein expression ratio was normalized to 100% in normal veins, whereas in varicose veins, it was expressed relative to normal controls. Mean values ± standard deviations are presented. * $p < 0.001$, versus the normal vein wall.

and varicose veins (line 2). Figure 3b presents densitometric analysis of p-Smad2/3 protein expression, which was significantly increased in the wall of varicose veins, when compared with the wall of normal veins ($p < 0.001$). There was no significant correlation between the level of p-Smad2/3 expression and gender in the group of patients with varicose veins ($p > 0.05$).

Discussion

The TGF family consists of numerous isoforms. Among them, TGF- β_1 and TGF- β_3 are present in the cardiovascular system with a very low expression of the latter one, whereas TGF- β_1 belongs to the most powerful cytokines involved in vessel wall remodeling [6]. The process is noted as one of the key factors in varicose vein pathogenesis, with a potential role of TGF- β_1 . Its increased mRNA level was demonstrated in varicose veins [8]. The growth factor protein content, as well as protein expression of TGF- β_1 latent and active forms, is also increased in varicose veins [7–9]. Furthermore, immunohistochemistry revealed increased staining for TGF- β_1 in tissue sections, as well as in cell cultures from varicose veins [8, 9, 16]. However, tissue responsiveness to TGF- β_1 depends on numerous factors, including TGF- β RII expression and activation [5, 12].

The results of our study demonstrated increased TGF- β RII mRNA expression in the wall of varicose veins. The increased TGF- β RII mRNA level was accompanied by the increased expression of TGF- β RII protein. The receptor expression is modified by TGF- β_1 activity. It has been hypothesized that high levels of active TGF- β_1 maintain a feedback loop in which TGF- β RII expression remains high [6]. If such a relation is present in the vein wall, increased TGF- β RII expression can result from enhanced TGF- β_1 activity in the wall of varicose veins [9].

Increased TGF- β RII expression may enhance TGF- β_1 binding. However, the growth factor-related metabolic response depends on receptor activation that leads to phosphorylation of receptor-related intracellular proteins Smad2 and Smad3 [5, 12]. The results of our study demonstrated increased protein expression of p-Smad2/3 in varicose veins. Thus, increased expression and activation of TGF- β RII may influence TGF- β_1 -related ECM remodeling in the wall of varicose veins. Increase in

mRNA expression and protein content of collagen type I, as well as increase in contents of some glycosaminoglycans, e.g., chondroitin and dermatan sulfates, belong to biological effects of TGF- β_1 activity [17–19]. Such changes in ECM composition were also shown in the wall of varicose veins [20, 21].

CVI is associated with increased expression of estrogen and progesterone receptors in the wall of varicose veins in females [22]. In contrast, we did not reveal any gender-related difference in the TGF- β signaling pathway in the vein wall. Different expression of sex hormone receptors may reflect different levels of estrogen and progesterone in females and males, whereas TGF- β_1 levels are not significantly different between men and women in healthy volunteers [23]. Furthermore, there is no correlation between TGF- β RII mRNA and gender in other diseases [24].

Assessment of varicose veins in humans gives information only at an established level of the disease. Also, varicose veins are usually compared with normal veins collected from patients with another vascular pathology, e.g., atherosclerosis [8, 9, 16] or CVI [21, 25]. However, such control material is accepted in studies on varicose veins, because collection of normal veins during autopsy is related to mRNA degradation in tissue samples, whereas from brain death organ donors to different expression of effector genes [26, 27]. Moreover, animal models of varicose vein development are far from perfect, because all of them are based on induction of venous hypertension, which does not exactly reflect the disease pathogenesis [28].

According to the results of our study, we can conclude that increased TGF- β RII expression and activation in the wall of varicose veins may be involved in ECM remodeling related to TGF- β_1 and supports its role in the disease pathogenesis.

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