

Influence of thrombophlebitis on TGF- β 1 and its signaling pathway in the vein wall

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Abstract: Extensive extracellular matrix remodeling of the vein wall is involved in varicose veins pathogenesis. This process is controlled by numerous factors, including peptide growth factors. The aim of the study was to evaluate influence of thrombophlebitis on TGF- β 1 and its signaling pathway in the vein wall. *TGF- β 1* mRNA levels, growth factor content and its expression were evaluated by RT-PCR, ELISA, and western blot methods, respectively, in the walls of normal veins, varicose veins and varicose veins complicated by thrombophlebitis. Western blot analysis was used to assess TGF- β receptor type II (TGF- β RII) and p-Smad2/3 protein expression in the investigated material. Unchanged mRNA levels of *TGF- β 1*, decreased TGF- β 1 content, as well as decreased expression of latent and active forms of TGF- β 1 were found in varicose veins. Increased expression of TGF- β RII and p-Smad2/3 were found in varicose veins. Thrombophlebitis led to increased protein expression of the TGF- β 1 active form and p-Smad2/3 in the vein wall compared to varicose veins. TGF- β 1 may play a role in the disease pathogenesis because of increased expression and activation of its receptor in the wall of varicose veins. Thrombophlebitis accelerates activation of TGF- β 1 and activity of its receptor in the varicose vein wall.

Key words: TGF- β 1, TGF- β RII, p-Smad2/3, varicose veins, thrombophlebitis

Introduction

The prevalence of varicose veins varies from 1% to 77% in different population groups, and the disease exerts a substantial effect on patient's quality of life [1,2]. Varicose veins are also associated with numerous complications, including thrombophlebitis. Its incidence is estimated at 1 case per 1000 inhabitants, and varicose veins account for 62-93% of cases [3,4]. Superficial thrombophlebitis results in a 3-fold increase in the risk of deep vein thrombosis (DVT) in patients with varicose veins, and the incidence of DVT in this population ranges from 6 to 30% [3]. Despite its great social impact, the primary cause of the disease has still not been clearly explained [2,5]. However, ample evidence implicates extensive extracellular matrix (ECM) remodeling and structural weakness of the vein wall as key factors in the pathogenesis of varicose veins [6-8].

Tissue remodeling is a complex processes that is controlled by numerous factors, including transforming growth factor β (TGF- β) [9]. Among its three isoforms, TGF- β 1 plays a crucial role in vessel wall remodeling [10,11]. It is released as a latent high-molecular-weight complex, and numerous proteases are involved in its activation [12]. An active form of TGF- β 1 acts through membrane type I and type II serine/threonine kinase receptors [10]. The latter are expressed constitutively, whereas TGF- β RI is recruited after TGF- β binding to the type II receptor [10,13]. Thus, the presence of TGF- β RII is essential for cellular responsiveness to TGF- β 1 [14]. 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, where they regulate gene transcription [10].

Due to its broad influence on ECM remodeling, TGF- β 1 was previously assessed in varicose veins, but the data reported on that topic were inconclusive. Unchanged TGF- β 1 levels in cell cultures from varicose veins and a comparable amount of the active form of TGF- β 1 in the walls of varicose veins were demonstrated in some studies [15,16]. By contrast, increases

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in *TGF- β 1* mRNA levels, protein expression, immunostaining, and total content in the walls of varicose veins were reported in others [15,17,18], and decreased protein expression of the TGF- β 1 latent form was demonstrated in the walls of varicose veins in an older group of patients [19].

It is noteworthy that inflammation induces the expression of TGF- β 1, which in turn suppresses the expression of proinflammatory cytokines [20]. Thus, it can be hypothesized that TGF- β 1 may be also involved in thrombophlebitis. However, data regarding TGF- β 1 in varicose veins complicated by thrombophlebitis are missing. Thus, the aim of this study was to evaluate influence of thrombophlebitis on TGF- β 1 and its signaling pathway in the vein wall.

Materials and methods

Tissue material. Tissue material was collected from patients undergoing surgery for varicose veins complicated by thrombophlebitis, which was the principal operative indication. Patients undergoing preoperative therapy with statins or angiotensin II type I receptor blockers were excluded from the study [21,22]. Preoperative lower extremity venous duplex ultrasound assessments were performed. Patients with duplex evidence of greater saphenous vein (GSV) thrombosis, its distal incompetence, deep vein reflux, current or previous deep vein thrombosis, as well as those with a history of previous sclerotherapy, were excluded from the study. Sixteen women with primary varicose veins complicated by thrombophlebitis were ultimately enrolled in the study (mean age 55.3 \pm 5.2; range 47-66). All patients were in class 2 according to the clinical signs of the CEAP classification. All patients exhibited sapheno-femoral junction incompetence with reflux in the femoral region of GSV and incompetence of perforator veins in the calf region. The lesser saphenous vein was affected in 2 patients (12.5%).

Segments of varicose veins and varicose veins complicated by thrombophlebitis were the studied materials; segments of normal veins served as controls. A set of the above-mentioned tissue samples was collected from each patient. Normal veins consisted of GSV fragments that were macroscopically unchanged and competent upon Duplex examination; they were harvested distally to their stripped incompetent parts in the thigh regions. For the studies of varicose veins, the sac-like dilatations of the vein were collected, whereas hard, cordlike segments with thickened, infiltrated walls and an inner surface covered by organized thrombi were obtained for the studies of thrombophlebitic veins. Before the examination, thrombi were precisely removed. All collected samples were washed with ice-cold 0.9% NaCl solution, cut and weighed.

RT-pCR analysis. A total of 50 mg of harvested tissue was pulverized after immersion in liquid nitrogen. RNA was extracted from the tissues using the AxyPrep Multisource Total RNA Miniprep Kit (catalog number AP-MN-MS-RNA-50; Axygen Bioscience, Union City, CA, 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 quality of RNA 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 RNA was reverse-transcribed into cDNA with the use of a RevertAidTM First Stand cDNA Synthesis Kit (catalog 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 1 (5'-GCCCTGGACACCAACTATTGCT-3') and reverse human TGF- β 1 (5'-AGGCTCCAAATGTAGGGGAGG-3'), (2) forward human β -actin (5'-TTGTAACCAACTGGGACGATATGG-3') and reverse human β -actin (5'-GATCTTGATCTTCATGGTGTAGG-3'). The primers were synthesized by Sigma-Proligo (St. Louis, MO, USA).

Aliquots of 5 μ l of cDNA and 20 pM of each primer were subjected to PCR using *Taq* DNA polymerase (catalog number EP0402; Fermentas). PCR thermocycling conditions for TGF- β 1 and β -actin cDNA detection were set up as follows: 1 cycle of denaturing at 94°C for 4 minutes, 30 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute, followed by a final primer sequence extension incubation at 72°C for 10 minutes. Ten microliters of each amplification reaction was analyzed by 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 a RT-PCR assay of RNA 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, CA, USA). The optical density of the *TGF- β 1* bands was normalized to the corresponding β -actin band. The gene expression ratios were normalized to 100% in normal veins, whereas in varicose veins and varicose veins complicated by thrombophlebitis, they were expressed relative to normal veins.

ELISA analysis. Tissue samples were suspended in a 0.05 M Tris-HCl buffer (pH 7.6) in a 1:3 (w/v) ratio. 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 \times g for 15 minutes at 4°C), supernatants were stored at -70°C. The latent form of TGF- β 1 was activated to immunoreactive TGF- β 1, which was detectable via the employed TGF- β 1 immunoassay. The TGF- β 1 total content was assessed in tissue homogenates with the commercially available Quantikine[®] Human TGF- β 1 Immunoassay kit (catalog number DB100B; R&D Systems Inc., Minneapolis, MN, USA). The obtained results were expressed per g of protein, and the protein content was determined according to the method described by Bradford [23].

Western blot analysis. SDS/PAGE was performed according to the method described by Laemmli [24]. Samples of tissue supernatants containing 20 μ g of protein were subjected to electrophoresis. The following molecular mass standards were used: 208-kDa, 120-kDa, 99-kDa, 53-kDa, 43-kDa, 37-kDa, 29-kDa, 19-kDa and 7-kDa proteins (catalog 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 minutes, and the proteins were transferred to 0.2- μ m pore diameter nitrocellulose membranes at 100 mA for 1 hour. The membranes were then incubated in 5% dried nonfat milk in TBS-T [20 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl, 0.05% (v/v) Tween 20] for 1 hour with one of the following primary antibodies: (1) monoclonal anti-TGF- β 1 antibody (mouse IgG; catalog number MAB240; R&D Systems Inc.) at a 1:500 dilution; (2) polyclonal TGF- β RII antibody (goat IgG; catalog number AF-241-NA; R&D Systems Inc.) at a 1:500 dilution; (3) polyclonal p-Smad2/3 antibody (goat IgG; catalog number sc-11769; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at a 1:200 dilution; (4) monoclonal anti-human actin antibody (mouse IgG; catalog number sc-8432; Santa Cruz Biotechnology Inc.) at a 1:200 dilution. A species-specific secondary antibody was then applied at a 1:7500 dilution. Incubation proceeded for 30 minutes with slow shaking. Subsequently, nitrocellulose membranes were washed with TBS-T (5 times for 5 minutes each) and treated with Sigma-Fast BCIP/NBT reagent (catalog number B1911; Sigma Aldrich Chemie GmbH, Steinheim, Germany). Three measurements were performed for each tissue sample. The labeled membranes

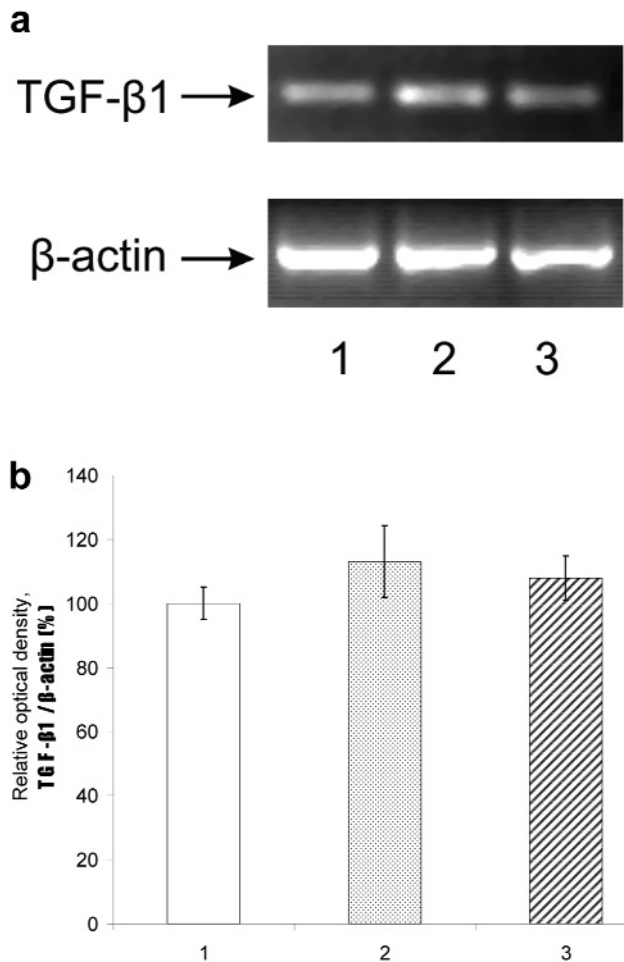


Fig. 1. (a) RT-PCR analysis of *TGF-β1* mRNA expression in vein walls (lane 1 – normal veins, lane 2 – varicose veins, lane 3 – varicose veins complicated by thrombophlebitis). RNA (3 μg) was reverse-transcribed using oligo(dT)₁₈ primers. cDNA amplification was performed with *TGF-β1* and β -actin primers. (b) Densitometric analysis of *TGF-β1* mRNA expression in 16 tissue samples from every examined group. The optical density of *TGF-β1* bands was normalized to the corresponding β -actin band. The gene expression ratio was normalized to 100% in normal veins, whereas in varicose veins and varicose veins complicated by thrombophlebitis, it was expressed relative to normal veins. Mean values \pm standard deviations are presented. No significant difference was demonstrated ($p > 0.05$).

were photographed, scanned, and the optical density was analyzed using imaging QuantityOne software (Bio-Rad). The optical density of the *TGF-β1*, *TGF-B RII* and *p-Smad2/3* bands was normalized to their corresponding actin bands. Protein expression ratios were normalized to 100% in normal veins, whereas in varicose veins and varicose veins complicated by thrombophlebitis, they were expressed relative to normal veins.

Ethical issues. 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 in Białystok, Poland. All patients enrolled in the study provided informed consent after reviewing the study protocol.

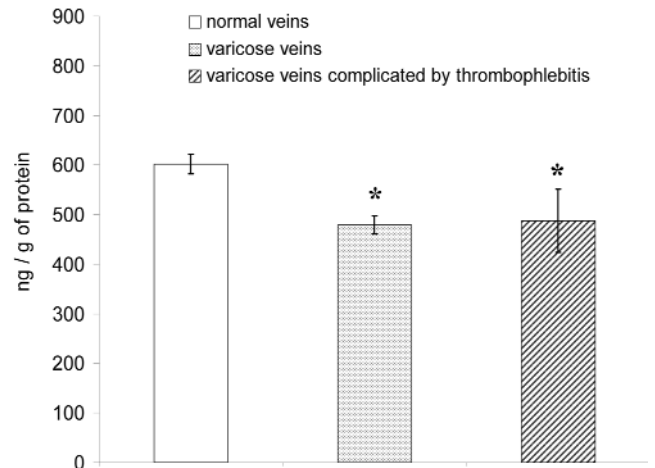


Fig. 2. *TGF-β1* content in vein walls, expressed as ng/g of protein. Mean values \pm standard deviations are presented. Statistically significant differences: *varicose veins versus control veins ($p < 0.05$). No significant difference was demonstrated between varicose veins and varicose veins complicated by thrombophlebitis ($p > 0.05$).

Statistical analysis. Statistical analysis of the obtained results was carried out by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. For all tests, a p value < 0.05 was considered as statistically significant. Mean values \pm standard deviations (SD) are presented.

Results

Expression of *TGF-β1* mRNA was demonstrated in normal veins (Fig. 1a; line 1), varicose veins (Fig. 1a; line 2), and varicose veins complicated by thrombophlebitis (Fig. 1a; line 3). However, densitometric analysis did not demonstrate any significant difference in *TGF-β1* mRNA levels ($p > 0.05$) between the groups (Fig. 1b).

Total *TGF-β1* content in vein walls is shown in Fig. 2. It was significantly decreased ($p < 0.05$) in varicose veins (479.28 ± 18.63 ng/g of protein) and in varicose veins complicated by thrombophlebitis (487.44 ± 64.24 ng/g of protein) compared to normal veins (601.23 ± 19.82 ng/g of protein). The total *TGF-β1* content was higher in varicose veins complicated by thrombophlebitis compared to other varicose veins. However, the difference was not statistically significant ($p > 0.05$).

Western blot analysis of *TGF-β1* protein expression is shown in Fig. 3a. Expression of active *TGF-β1* (single band with molecular mass of approx. 29 kDa) and latent *TGF-β1* (double band with molecular mass of approx. 55 kDa) was shown in normal veins (Fig. 3a; line 1), varicose veins (Fig. 3a; line 2), and varicose veins complicated by thrombophlebitis (Fig. 3a; line 3). The protein expression of latent *TGF-β1* was significantly decreased ($p < 0.001$) in varicose veins (Fig. 3b; bar 2) and varicose veins complicated by thrombophlebitis (Fig. 3b; bar 3) compared to normal veins

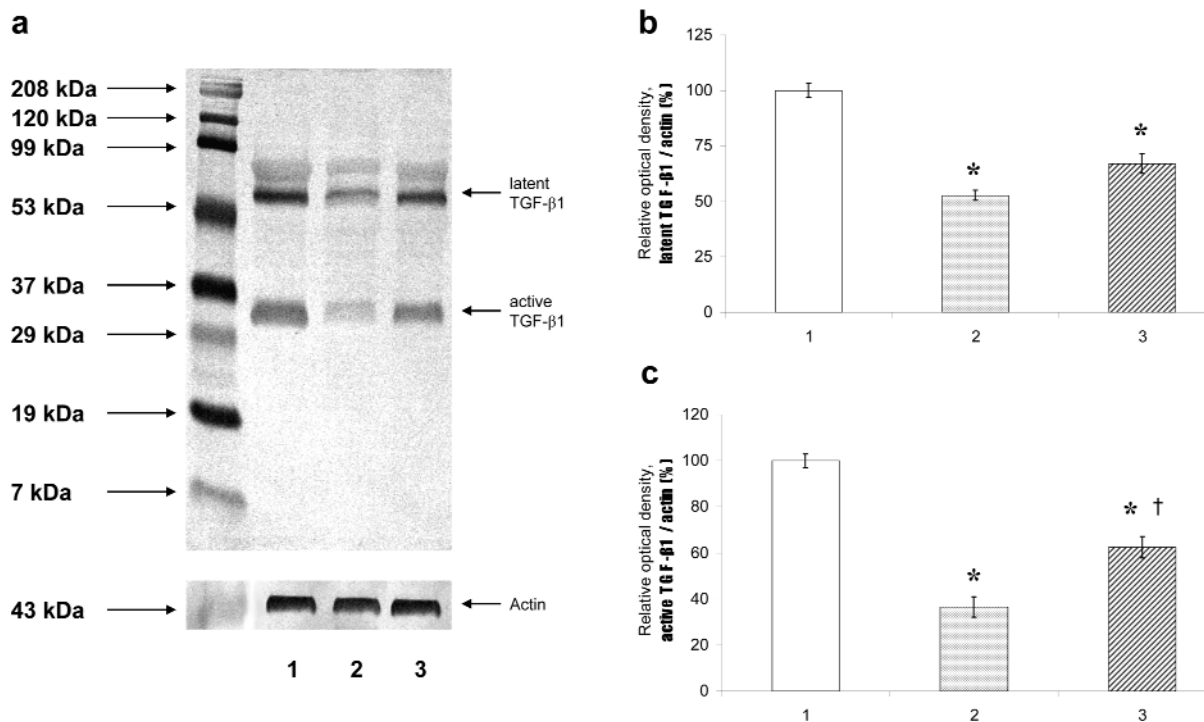


Fig. 3. (a) Western blot analysis of TGF- β 1 protein expression in the vein wall (lane 1 – normal veins, lane 2 – varicose veins, lane 3 – varicose veins complicated by thrombophlebitis). The same amount of protein extract (20 μ g) was run in each lane. Actin was used as a loading control. Molecular mass standards are indicated on the left side. (b) Densitometric analysis of latent TGF- β 1 protein expression in 16 tissue samples from every examined group. c. Densitometric analysis of active TGF- β 1 protein expression in 16 tissue samples from every examined group. The optical density of TGF- β 1 bands was normalized to the corresponding actin band. The protein expression ratio was normalized to 100% in normal veins, whereas in varicose veins and varicose veins complicated by thrombophlebitis, it was expressed relative to normal veins. Mean values \pm standard deviations are presented. Statistically significant differences: *varicose veins versus control veins, †varicose veins complicated by thrombophlebitis versus varicose veins ($p < 0.001$).

(Fig. 3b; bar 1). There was no significant difference in expression between varicose veins and varicose veins complicated by thrombophlebitis ($p > 0.05$). The protein expression of active TGF- β 1 was also significantly decreased ($p < 0.001$) in varicose veins (Fig. 3c; bar 2) and varicose veins complicated by thrombophlebitis (Fig. 3c; bar 3) compared to normal veins (Fig. 3c; bar 1). However, when both groups of varicose veins were compared, the protein expression of active TGF- β 1 was significantly higher in varicose veins complicated by thrombophlebitis ($p < 0.001$).

Western blot analysis of TGF- β RII protein expression is shown in Fig. 4a. The receptor is a protein with a molecular mass of 75-85 kDa. The presence of such a band was demonstrated in all tissue samples. Protein expression of TGF- β RII was significantly increased ($p < 0.05$) in varicose veins (Fig. 4b; bar 2) and varicose veins complicated by thrombophlebitis (Fig. 4b; bar 3) compared to normal veins (Fig. 4b; bar 1). There was no significant difference in expression between varicose veins and varicose veins complicated by thrombophlebitis ($p > 0.05$).

Western blot analysis of p-Smad2/3 protein expression is shown in Fig. 5a. Bands corresponding to a

molecular mass of 55 kDa were present in normal veins (Fig. 5a; line 1), varicose veins (Fig. 5a; line 2), and varicose veins complicated by thrombophlebitis (Fig. 5a; line 3). Fig. 5b presents a densitometric analysis of p-Smad2/3 protein expression, which was significantly increased in varicose veins and varicose veins complicated by thrombophlebitis compared to normal veins ($p < 0.001$). Furthermore, p-Smad2/3 protein expression was significantly increased in varicose veins complicated by thrombophlebitis compared to varicose veins ($p < 0.001$).

Discussion

The TGF- β family consists of numerous isoforms. Among them, TGF- β 1 and TGF- β 3 are present in the vascular system, although TGF- β 3 is expressed at very low levels [11]. TGF- β 1 exerts a profound effect on the synthesis and degradation of a large number of ECM components [10]. However, ECM undergoes physiological changes with age, including TGF- β 1 expression in vein walls [19,25]. Thus, to avoid age-related and individual differences between studied and control groups, we decided to assess TGF- β 1 in normal and

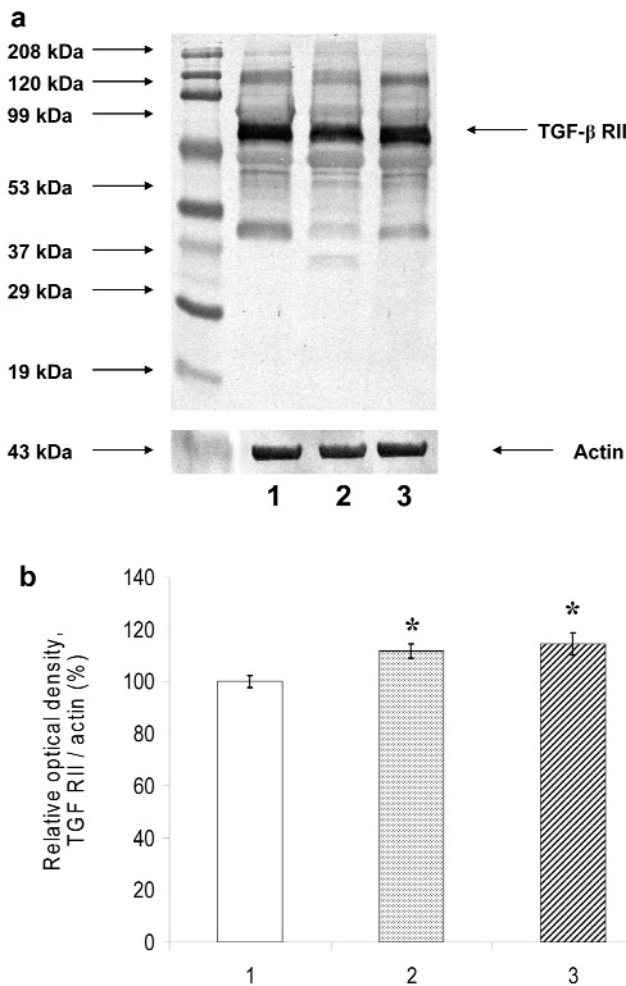


Fig. 4. (a) Western blot analysis of TGF- β RII protein expression in vein walls (lane 1 – normal veins, lane 2 – varicose veins, lane 3 – varicose veins complicated by thrombophlebitis). The same amount of protein extract (20 μ g) was run in each lane. Actin was used as a loading control. Molecular mass standards are indicated on the left side. **(b)** Densitometric analysis of TGF- β RII protein expression in 16 tissue samples from every examined group. 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 and varicose veins complicated by thrombophlebitis, it was expressed relative to normal veins. Mean values \pm standard deviations are presented. Statistically significant differences: *varicose veins versus control veins ($p < 0.05$). No significant difference was demonstrated between varicose veins and varicose veins complicated by thrombophlebitis ($p > 0.05$).

varicose veins harvested from the same patients with chronic venous insufficiency (CVI) symptoms.

Expression of *TGF- β 1* mRNA is induced by hypoxia, and varicose veins are closely associated with local ischemia. However, the stimulating effect of hypoxia on TGF- β 1 transcription is temporary and should not influence growth factor mRNA expression in chronic diseases [26,27]. We found comparable *TGF- β 1* mRNA levels in normal and varicose veins,

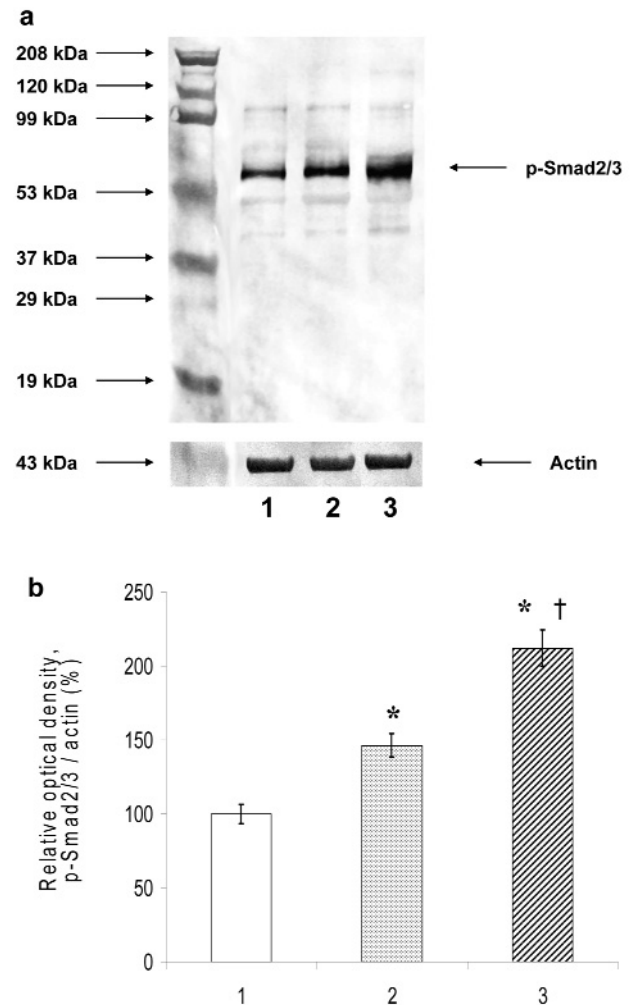


Fig. 5. (a) Western blot analysis of p-Smad2/3 protein expression in vein walls (lane 1 – normal veins, lane 2 – varicose veins, lane 3 – varicose veins complicated by thrombophlebitis). The same amount of protein extract (20 μ g) was run in each lane. Actin was used as a loading control. Molecular mass standards are indicated on the left side. **(b)** Densitometric analysis of p-Smad2/3 protein expression in 16 tissue samples from every examined group. 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 and varicose veins complicated by thrombophlebitis, it was expressed relative to normal veins. Mean values \pm standard deviations are presented. Statistically significant differences: *varicose veins versus control veins, †varicose veins complicated by thrombophlebitis versus varicose veins ($p < 0.001$).

in accordance with the above statement. However, our results are in contrast to the increased TGF- β 1 mRNA levels in varicose veins reported by others [18]. However, Jacob *et al.* studied varicose veins obtained from patients assigned to different classes (C2-C5) according to clinical signs of the CEAP classification, whereas varicose veins in the present study were obtained only from patients assigned to class C2. Thus, it can be hypothesized that the progression

of the disease to more advanced stages may lead to increased *TGF- β 1* mRNA expression.

In spite of the unchanged *TGF- β 1* mRNA levels, we found decreased TGF- β 1 content in varicose veins and varicose veins complicated by thrombophlebitis. In the present study, TGF- β 1 content was expressed per gram of protein, and increased protein content in varicose veins compared to normal veins can explain this divergence. By contrast, an increased total content of TGF- β 1, expressed per mg of DNA, has been reported in varicose veins [15]. We are convinced that the calculation of TGF- β 1 content per gram of protein is more appropriate for comparing results obtained using ELISA and western blot methods. Furthermore, Badier-Commander *et al.* compared normal veins and varicose veins from subjects 15 years younger than the control group. Due to the decrease of latent TGF- β 1 in walls of varicose veins with age, the age differences between studied and control groups should be considered when comparing these results [19].

An analysis of the protein expression of active and latent TGF- β 1 forms confirmed our results obtained by ELISA. Furthermore, these results are in accordance with those reported by Pascual *et al.* [19], who demonstrated decreased protein expression of latent TGF- β 1 in varicose veins of age-matched control and study groups with mean ages of 50 or over. However, when comparing varicose veins and varicose veins complicated by thrombophlebitis, a significant increase in active TGF- β 1 expression was demonstrated in the course of thrombophlebitis. A number of proteases, including matrix metalloproteinases (*e.g.*, MMP-9) and cathepsin D, are capable of activating latent TGF- β 1 [12,28-30]. It is noteworthy, that increased MMP-9, as well as cathepsin D activities have been demonstrated in varicose veins complicated by thrombophlebitis [31,32]. That may explain increased TGF- β 1 activation in the course of thrombophlebitis. But on the other hand, TGF- β 1 induces MMP-9 activation [33,34]. Thus, it still needs to be clarified if enhanced TGF- β 1 activation results from increased MMP-9 activity or leads to increased MMP-9 activation. Considering these doubts, cathepsin D may play a significant role in TGF- β 1 activation in the wall of varicose veins complicated by thrombophlebitis. Furthermore, proinflammatory cytokine IL-1 may activate TGF- β 1, which in turn plays a protective role in the inflammatory process by suppressing the expression of other proinflammatory cytokines, including IL-6 and IL-8 [20,35]. Thus, increased TGF- β 1 activation in the walls of varicose veins complicated by thrombophlebitis may be considered to be a regulatory mechanism involved in limiting the extent of inflammatory processes in the walls of varicose veins.

Increases in mRNA expression and the protein level of collagen type I, as well as increases in the levels of some glycosaminoglycans, *e.g.*, chondroitin and

dermatan sulfates, are attributed to the biological effects of TGF- β 1 activity [36-38]. Such changes in ECM composition have been shown in varicose veins [8,39]. However, it was not possible to formulate a hypothesis linking the above-mentioned TGF- β 1-related ECM changes and the growth factor mRNA and protein expression levels in varicose veins found in our study. However, tissue responsiveness to TGF- β 1 depends not only on its availability but on other factors as well, including TGF- β RII expression and activation [10,14]. For this reason, we decided to evaluate the expression of the growth factor receptor, which we found to be increased in varicose veins and varicose veins complicated by thrombophlebitis.

TGF- β 1 has a strong heparin binding affinity, and its activity is selectively potentiated by heparin [40]. By contrast, hyaluronic acid inhibits growth factor activity [41]. Increased heparin and decreased hyaluronic acid contents have been shown in varicose and varicose veins complicated by thrombophlebitis [8]. The above-mentioned factors should also accelerate tissue responsiveness to TGF- β 1 in varicose veins, which depends on receptor activation and phosphorylation of the receptor-related intracellular proteins Smad2 and 3 [10,14]. We demonstrated increased protein expression levels of p-Smad2/3 in varicose veins and particularly in varicose veins complicated by thrombophlebitis. Thus, the role of TGF- β 1 in ECM remodeling in varicose veins may be most strongly related to the increased expression and activation of its receptor.

The assessment of ECM remodeling in varicose veins in humans provides information at an established level of the disease, whereas animal models of varicose vein development do not precisely reflect human disease pathogenesis [42]. Nevertheless, according to the results of our study, we can conclude that varicose vein development does not influence *TGF- β 1* mRNA levels in vein walls and is further accompanied by a decrease in total TGF- β 1 levels and expression of its active/latent forms. For this reason, the role of TGF- β 1 in varicose vein pathogenesis should be considered in relation to the increased expression and activation of its receptor in the wall of varicose veins. Furthermore, thrombophlebitis accelerates activation of TGF- β 1 and activity of its receptor in the varicose vein wall.

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