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What is the role of prolidase in pathogenesis of primary varicose veins?

Primer variköz ven patogenezinde prolidazın rolü nedir?

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ABSTRACT

Background: This study aims to investigate the venous staining of prolidase which is thought to be responsible for varicose vein formation.

Methods: The study included primary varicose vein samples of 20 patients (10 males, 10 females; mean age 51.6±6.3 years; range 32 to 62 years) who underwent C2-C4 varicose vein operation according to Clinical-Etiologic-Anatomic-Pathophysiologic classification and vein pathology samples removed for coronary bypass from 30 healthy controls (20 males, 10 females; mean age 55.5±6.8 years; range 38 to 70 years). Immunohistochemical staining was performed using prolidase antibody. Immunohistochemical staining of both groups was analyzed and compared with one another.

Results: There was no statistically significant difference between both groups in terms of demographic data (p>0.05). In immunohistochemical analysis of varicose samples, prolidase immunostaining was negative in four cases (20%) and positive in 16 cases (80%). In healthy venous tissue samples, prolidase immunostaining was negative in 26 cases (86.7%) and positive in four cases (13.3%). Statistical comparison of healthy veins removed for coronary bypass and varicose veins with respect to prolidase immunostaining showed significant difference (p<0.001).

Conclusion: When healthy veins were compared with varicose veins, the prolidase enzyme was stained more strongly in varicose veins. Prolidase enzyme may be playing an important role in the pathogenesis of varicose veins.

Keywords: Immunohistochemistry; prolidase; variscosis; vein.

ÖZ

Amaç: Bu çalışmada variköz ven gelişiminden sorumlu olduğu düşünülen prolidazın venlerdeki boyanması araştırıldı.

Çalışma planı: Çalışmaya Klinik-Etyolojik-Anatomik-Patofizyolojik sınıflamasına göre C2-C4 variköz ven ameliyatı uygulanan 20 hastanın (10 erkek, 10 kadın, ort yaş 51.6±6.3 yıl; dağılım 32-62 yıl) primer variköz ven örneği ile 30 sağlıklı kontrolden (20 erkek, 10 kadın; ort. yaş 55.5±6.8 yıl; dağılım 38-70 yıl) koroner baypas için çıkarılan damar patolojisi örnekleri alındı. İmmünohistokimyasal boyama prolidaz antikor kullanılarak yapıldı. Her iki grubun immünohistokimyasal boyanması incelendi ve birbiri ile karşılaştırıldı.

Bulgular: Her iki grup arasında demografik veriler açısından istatistiksel olarak anlamlı farklılık yoktu (p>0.05). Variköz örneklerin immünohistokimyasal analizinde prolidaz immün boyanması dört olguda (%20) negatif, 16 olguda (%80) pozitif idi. Sağlıklı venöz doku örneklerinde prolidaz immün boyanması 26 olguda (%86.7) negatif, dört olguda (%13.3) pozitif idi. Koroner baypas için çıkarılan sağlıklı venlerle variköz venlerin prolidaz immün boyanması açısından istatistiksel olarak karşılaştırılması anlamlı farklılık gösterdi (p<0.001).

Sonuç: Sağlıklı venlerle variköz venler karşılaştırıldığında, prolidaz enzimi variköz venlerde daha kuvvetli boyandı. Prolidaz enzimi variköz ven patogenezinde önemli rol oynuyor olabilir.

Anahtar sözcükler: İmmünohistokimya; prolidaz; varis; ven.

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Variscosis is a common health problem. The etiology of primary varicose veins remains unclear.[1] Positive family history, increased prevalence with age, female gender, and pregnancy are proven risk factors for the etiology of primary varicose veins (VVs). Obesity, decreased mobility, smoking, estrogen treatment, diabetes mellitus, and hypertension have been put forward to exacerbate VVs but their effects stay to be ascertained.[2,3] Although the risk factors are notorious, the pathogenesis and molecular mechanisms of VVs stay vague. Basic pathology of primary VV is degeneration of vein trunk and insufficiency of vein valves.[4] Disproportion in biosynthesis and breakdown of extracellular matrix (ECM) proteins give rise to reduction of venous tonus through structural infirmity of the vein wall. Then, it is thought to lead to venous insufficiency and VV.[3] Collagen is one of ECM elements. Different results have been reported about the collagen content in the wall of VVs in several studies.[4-6] Since the quantity of collagen is significant in terms of the elasticity and tonus of the vessel, the destruction of vessel collagen may take part in pathogenesis of VVs.

Prolidase, which is a member of the matrix metalloproteinase (MMP) family, plays an important role in the recycling of proline-containing proteins for collagen synthesis.[2-4] Since prolidase is the main regulatory enzyme in the metabolism of collagen, we believe that there might be changed prolidase activities in VV walls. Therefore, in this study, we aimed to investigate the venous staining of prolidase which is thought to be responsible for varicose vein formation.

PATIENTS AND METHODS

The study was conducted at Harran University Medical Faculty between January 2014 and June 2015. Primary varicose saphenous vein pathology specimens were removed from 20 patients (10 males, 10 females; mean age 51.6±6.3 years; range 32 to 62 years) who underwent C2-C4 varicose vein operation according to Clinical-Etiologic-Anatomic-Pathophysiologic classification. Healthy vein pathology specimens were removed for coronary bypass from 30 controls (20 males, 10 females; mean age 55.5±6.8 years; range 38 to 70 years). The variscosis diagnosis was defined with colored Doppler ultrasound in case of diameter ≥4 mm and >2 second retreats. Varicose vein pathological materials obtained from the middle part of the saphenous veins were removed by stripping method. Healthy vein samples obtained from proximal side of saphenous vein were removed for bypass. Detailed information about accompanying diseases and medications used were recorded. Exclusion criteria were recent deep vein thrombosis and thrombophlebitis.

The study protocol was approved by the Harran University Medical Faculty Ethics Committee. A written informed consent was obtained from each patient. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Immunohistochemical staining

The venous tissue samples of the cases were fixed in 10% formaldehyde solution and embedded in paraffin blocks. Sections with a thickness of 4 μm were collected from all blocks. The tissue sections were deparaffinized in xylene and then rehydrated in ethanol solutions of decreasing concentrations (100%-95%-75%). They were irrigated in phosphate buffered saline (PBS); then, they were incubated for 10 minutes in 3% hydrogen peroxide solution in order to allow inhibition of the endogenous peroxidase activity. The sections were boiled in 10 mmol/L of ethylenediaminetetraacetic acid buffer (pH 8.0) for antigen retrieval for five minutes at 850 watts and then for five minutes at 350 watts in a microwave. After that, the sections were treated with primary polyclonal rabbit antibody prolidase (GeneTex Biotechnology Inc., 1:100 dilution) for 24 hours at 4 °C. All the sections were irrigated in PBS solution and then incubated for 60 minutes in horse radish peroxidase conjugate of goat anti-rabbit immunoglobulin G. Then, chromogen diaminobenzidine was applied and counterstaining was performed using Mayer's hematoxylin.

Assessment of immunohistochemical expression

Two blinded pathologists evaluated and scored the specimens. In immunohistochemical staining, the cytoplasmic and nuclear staining in endothelial and muscular cells of varicose veins was considered immunohistochemically positive. Immunohistochemical expression of prolidase was assessed using a semi-quantitative scoring system for staining presence. Prolidase immunostaining was negatively scored as 0 and positively scored as 1.

Statistical analysis

Statistical data were analyzed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) software. In reporting statistical analyses, normally distributed continuous variables are given as the mean ± standard deviation, abnormally distributed continuous variables are given as the median values, and categorical variables are given as percent. The distribution of the data was tested by using a Kolmogorov-Smirnov test. In addition, comparisons between the two groups were

Table 1. Comparison of demographic characteristics and biochemical data between chronic venous insufficiency group and control group

	Control group (n=30)		Varicous vein group (n=20)		
	%	Mean±SD	%	Mean±SD	*p*
Age (year)		55.5±6.8		51.6±6.3	0.054
Male	66		50		0.239
Hypertension	10		20		0.318
Smoke	53		40		0.355
Diabetes mellitus	36.6		25		0.248
Prolidase immunstaining	13.3		80		0.0001
CEAP 0	96.6		0		-
CEAP 2	3.3		65		0.0001
CEAP 3	0		25		-
CEAP 4	0		10		-

SD: Standard deviation; CEAP: Clinical-Etiologic-Anatomic-Pathophysiologic classification.

performed with an unpaired two tailed t-test for the normally distributed continuous variables while the Mann-Whitney U test was used for those that were abnormally distributed. Pearson's chi-square test or Fischer's exact test was used for categorical variables. A value of $p<0.05$ was considered statistically significant with a 95% confidence interval.

RESULTS

Demographic characteristics of all cases are summarized in Table 1. No statistically significant differences were identified among the demographic data (age, gender, hypertension, smoke, diabetes mellitus) of cases with varicose veins and controls ($p>0.05$). Prolidase immunostaining was negative in four (20%) and positive in 16 (80%) cases (Figure 1a, b). In healthy venous tissue samples, prolidase immunostaining was negative in 26 cases (86.7%) and positive in four cases (13.3%) (Figure 2a, b). According to the Pearson chi-square test, a significant difference was identified between varicose vein and healthy vein tissues with respect to immunohistochemical expression for prolidase ($p<0.001$). The prolidase enzyme was expressed more strongly in varicose vein tissues as compared to normal vein tissues.

DISCUSSION

In this study, we intended to investigate the immunohistochemical staining of the prolidase enzyme, which is held responsible for variscosis. We observed that increased prolidase expression was associated with varicose dilatation.

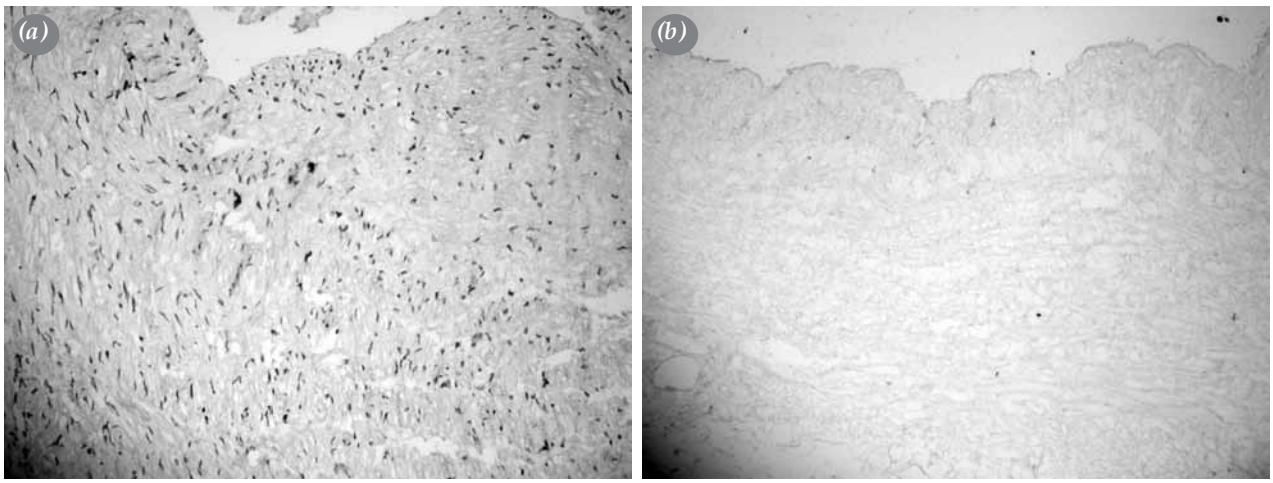

Figure 1. Prolidase staining in varicose veins. **(a)** Positive staining in varicose vein sample, **(b)** negative staining in varicose vein sample (original magnification, x200).

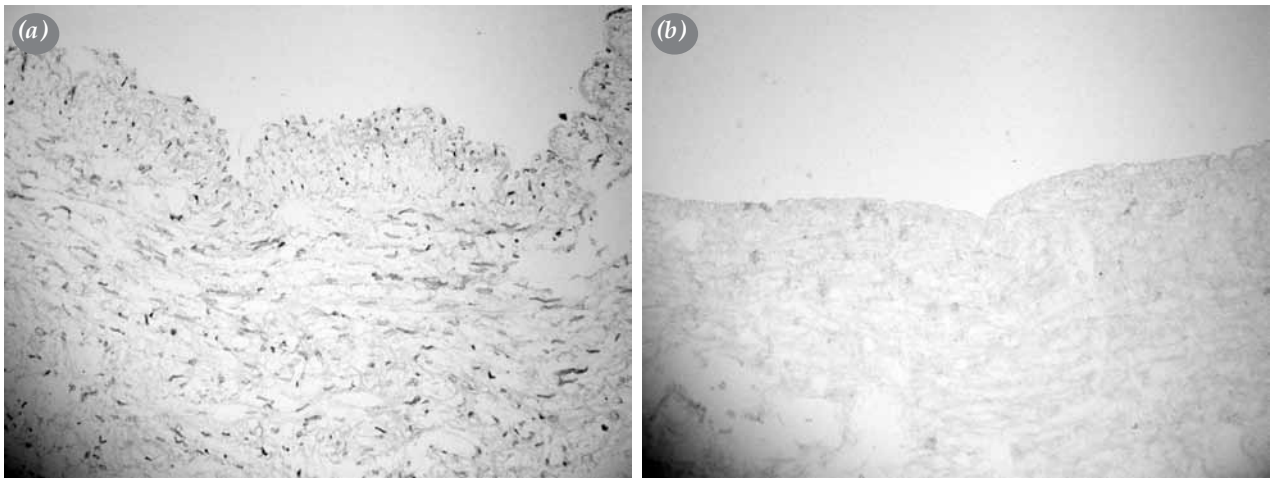

Figure 2. Prolidase staining in normal saphenous veins. **(a)** Positive staining in normal saphenous vein sample, **(b)** negative staining in normal saphenous vein sample (original magnification, x200).

Varicose veins are described by tortuosity, dilatation, and prolongation of the saphenous veins.[2] Recent studies suggest that this pattern is the consequence of impairment of the normal organization of the ECM and smooth muscle cells (SMCs) in veins. Venturi et al.[7] revealed a reduction in elastin-collagen ratio and isodesmosine and desmosine in varicose versus normal veins. Michiels et al.[8] demonstrated that hypoxia-induced leukocyte activation leads to free radical discharge, protease activation, and, as a result, ECM degradation in VVs. Also, they showed that hypoxia activates endothelial cells to secrete growth factors and stimulate SMC reproduction and ECM biosynthesis. Valvular insufficiency and the impact of increased hydrostatic pressure have been involved in the pathogenesis of VVs. Latest studies propose that the stability of vascular SMC proliferation and ECM deposition and degradation may be perturbed, resulting in loss of mechanical wall strength, venous expansion and prolongation.[9-12] Collagen and elastine are major proteins of ECM and provide mechanical strength to the vein wall. Decreased elastin substance has been incriminated in the pathogenesis of VVs.[7,12] But the effect of collagen content has not been frankly described. Researches propose raised,[6] reduced,[9] or unvaried[10] collagen substance in the varicose vein wall. Decreased collagen synthesis and increased collagen breakdown may lead to reduced collagen content and loss of mechanical wall strength in vascular walls. The final collagen quantity shows a stability in terms of its production and breakdown by MMP family. Various MMP activities have been described in diverse malignant, degenerative, and inflammatory vascular diseases. The serum and venous tissue levels of MMP-1, -2, -3, -9, and -13 are high in VVs with thrombophlebitis, proposing that MMPs may conduce to the variscosis pathogenesis.[13-16] Matrix metalloproteinases have been defined in all histologic layers of the venous wall, and over expression and activity have been shown in VVs with thrombophlebitis.[14] Increased activity of MMP in VVs with thrombophlebitis may be secondary to chronic inflammatory process. Raffetto et al.[16] have observed that long-term MMP-2 induced venous relaxation could cause increased venous expansion, chronic venous insufficiency, and VVs genesis. Kowalewski[14] and Sansilvestri-Morel[17] have reported increased MMP-2 protein expression in human VVs compared with normal veins. Whereas Badier-Commander[18] and Parra[19] have shown significantly decreased MMP-2 protein expression compared with normal veins. A likely clarification to these conflicting results may relate to various stages in varicose disease. Additionally, some studies demonstrated that the aortic wall expression and plasma level of MMPs is increased in abdominal aortic aneurysm.[20-24] Irwin et al.[25] have demonstrated increased expression of MMP-2, -9, and -13 in venous aneurysm and VVs compared with normal saphenous veins. Increased expression of MMP has been identified in the atherosclerotic plaque pathogenesis.[26,27] Matrix metalloproteinases are immediately concerned in atherosclerotic plaque destabilization and plainly exhibit that members of the MMP family have widely differing impact on atherogenesis.[28] These studies suggested a role of abnormal ECM metabolism in various vascular disorders by MMP family.

Prolidase is a manganese dependent cytosolic enzyme which is a member of the MMP family and the main regulatory enzyme in the metabolism of ECM. It plays an important role in the recycling of proline-containing proteins for collagen synthesis.[2-4] Some studies suggest that prolidase is a key enzyme in ECM construction and destruction.[4] Because majority of studies claim that perturbations in both synthesis and degradation of the structural elements occur in VVs segments, we sought to determine the prolidase expression in normal and varicose saphenous veins. We have demonstrated in our study that the prolidase enzyme was expressed in both normal saphenous venous and in VVs tissues; however, it was expressed more strongly in varicose cases. Bakuy et al.[29] have observed that reduced prolidase activity was highly paralleled with both the presence and the number of coronary artery aneurysm. Aoki et al.[30] have found that decreased collagen is one of the evident histopathological characteristics of cerebral artery aneurysms. Similarly, collagen was decreased and distorted in human dissections and aneurysms of the ascending aorta.[31] To our knowledge, the relationship between prolidase activity and varicose dilatation has not been investigated previously. We hypothesized that the venous tissue prolidase activity would be increased in VVs, as increased ECM turnover is a pathophysiologic mechanism in the progression to varicose dilatation.

Our study has some limitations. The study sample including 50 subjects provided a relatively low statistical power. Furthermore, saphenous vein diameters of coronary artery bypass grafting group were not measured with Doppler ultrasonography and not compared with VV group. Also, pathologic specimens were not obtained from different segments of saphenous vein and not compared with each other.

In conclusion, establishing the pathophysiology of VV genesis may help in identifying new treatment strategies. To the best of our knowledge, our study is the first to investigate the relationship between prolidase activity and varicose dilatation. In light of previous studies, we conclude that the prolidase enzyme plays an important role in the variscosis pathogenesis. We think that MMPs from the same family can show significant and different effects in vascular function and disease processes. We believe that the investigation of the family of MMP enzymes in wide series including more variscosis cases may shed light onto the pathogenesis of variscosis and offer novel approaches towards its treatment.

Declaration of conflicting interests

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