Original Investigation

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Does extracellular matrix of the varicose vein wall change according to clinical stage?

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ABSTRACT

Objective: The etiology and pathophysiology of chronic venous disease is not fully understood. This study aimed to determine the variation of the extracellular matrix proteins in varicose vein wall according to clinical stage.

Material and Methods: Forty varicose and 10 control veins were sampled from the saphenofemoral junction. The Clinical Etiologic Anatomic Pathophysiologic (CEAP) classification was used in patients with varicose veins. Samples were stained with hematoxylin-eosin, Masson's trichrome, EVG (Elastica-van Gieson) stain and with laminin, fibronectin, tenascin antibodies. Stained samples were examined immuno-histochemically. Changes in extracellular matrix were determined semi-quantitatively using light microscopy.

Results: It was observed that in the early stages (C2-C3) of chronic venous disease, fibrosis is increased in the intima and media layers, with fragmentation in lamina elastica interna, and increased tenascin expression in the intima layer. In advanced stages (C4-C6), the accumulation of tenascin in the intima continued along with fibrosis in the media layer, the thickness of the media layer increased and fibronectin deposition was observed.

Conclusion: This study showed that changes first occur in the intima during the early stages of the disease with addition of alterations in the media layer at later stages.

Key Words: Chronic venous disease, varicose veins, extracellular matrix proteins, CEAP

INTRODUCTION

The incidence of chronic venous disease is high among the population (1, 2). However, its etiology and pathophysiology are rather complicated and not entirely clear. There are two different theories that are advocated in terms of the pathophysiology of chronic venous disease. The first one accuses primarily the valvular incompetence and argues that the vessel dilation is due to hydrostatic forces. The other theory argues that a weakness initially develops in the vessel wall, and leads to vessel dilation as well as secondary valvular incompetence (3-5). Although we do not know which one comes first, we are aware of the fact that the vessel wall is involved in all circumstances.

There is a matrix around the cells in the vessel wall; actually, the matrix constitutes a large part of the vessel wall volume. The major macromolecules in the extracellular matrix are glucosaminoglicans and fibrous proteins.

Glucosaminoglicans ensure resistance to compressive powers. While collagen, one of the fibrous proteins, ensures resistance to tension, elastin provides flexibility, fibronectin the cell-matrix connection, laminin the basal lamina-matrix connection and tenascin the cellular migration (6). These proteins and glycoproteins were examined in patients with varicose veins and certain changes were identified. Although there are findings that show a decreased amount of collagen (7, 8), the amount of collagen generally increases (9-15) and this situation is even observed in cellular cultures (15-17). The increase in collagen occurs both in the intima (13) and in the media (12, 18). Furthermore, collagen fibrils enter the area between the muscle fibers in the media layer, thereby disturbing the circular order of muscles (19, 20). There is a decrease in the elastin fibers (10, 18, 21) and a fragmentation in the internal elastic lamina (13, 18, 20). However, there is an increase in other matrix proteins fibronectin, laminin and tenascin (18). The changes in the mentioned matrix proteins were examined as per the Hach stages; however, no statistical difference could be identified (22). Therefore, the exact chronological order of these changes has so far remained unknown.

The CEAP staging was created in 1994 for the purpose of staging patients with chronic venous disease. In this staging system, C0-C3 represent early stages of the disease, while C4-C6 represent the advanced stages (23, 24). We do not yet know the changes in the matrix proteins according to this staging system. Since knowledge of the alternations throughout these stages and the chronological order may aid in our understanding of varicose vein physiopathology, the changes in matrix proteins as per clinical stages were examined in our study in a single blind manner at the same anatomic point in patients with primary varicose veins.

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MATERIAL AND METHODS

Patients: Upon approval from the Ethics Committee of the School of Medicine, Ankara University, patients who presented to the Department for General Surgery, School of Medicine, Ankara University were included in the study. All patients were informed about the procedure and the handling of received samples, and informed consents were received. The patients who were to be operated on due to lower extremity varices were examined using Doppler ultrasonography after obtaining patient history. Patients without reflux of the saphenofemoral junction were excluded from the study. The patients were subjected to CEAP staging: Since there were no patients in the C1 group to be operated, the groups C2-C3 were classified as early stage and groups C4-C6 as advanced stage (Table 1). During the operation, great saphenous vein samples were received from the saphenofemoral junction. The control samples were collected from patients whose great saphenous vein was harvested for bypass surgery. The samples were stored at -80°C.

Staining: To prevent variations in staining, the staining procedures were performed on all the samples in the same session. Routine hematoxylene eosin staining for histological assessment, Masson trichrome staining for the demonstration of collagen and Verhoeff elastic tissue staining for the assessment of the elastin (with the Van Gieson staining as the counterstain) network were performed. Immuno-histochemical stainings were performed using the streptavidin-biothin-peroxidase method. The antibodies used were anti-laminin (Novacastra, 1/50), anti-fibronectin (Dako, 1/500) and anti-tenascin (Dako, 1/200). The preparations were stained using Mayer hematoxylene as the counterstain.

Evaluation: A pathologist who was blinded to the groups evaluated all specimens using the Olympus BX50 light microscope. The preparations were examined with respect to the presence of aneurysms, layer thickness, presence of fibrosis, fragmentation rate of the internal elastic lamina, muscle hypertrophy as well as the expressed amounts of laminin, fibronectin and tenascin (Tables 2 and 3).

Statistical Analysis

For the comparison of the groups, the chi-square test was used for categorical variables, while Student t test was used for continuous variables. ANOVA was used in cases where the distribution was normal for three and more groups, and the Kruskal-Wallis test was used in cases where it was not normal. A value of $p<0.05$ was considered statistically significant. The analyses were carried out using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA) 6.1 software program.

RESULTS

Patients: Forty varicose vein samples were collected from 32 patients (22 men, 10 women; mean age: 41.4±10.9; range: 16-64 years). Samples were received from both legs in 8 patients. Ten control samples were obtained from 10 patients who underwent infrainguinal bypass (5 patients), coronary bypass (4) and brachial artery aneurysm operations (7 men and 3 women; mean age: 55.0±9.8; range: 32-65 years). The average disease duration was found to be 12.2±8.3 (1-32 years) for the early stage and 15.8±10.6 (5-40 years) for the advanced stage.

Presentation of aneurysmal dilatations: Four aneurysmal dilatations were observed in the varicose vein group, while none was identified in the control group. No statistical difference was identified among groups.

Layer thickness: There were no differences between groups in terms of intima layer thickness. However, a significant difference was identified in the media layer thickness as compared to the control group ($p<0.05$). Furthermore, the thickness was high even in patients with a disease duration of less than 10 years ($p<0.05$).

Fibrosis (collagen content): There was increased fibrosis in the intimal layer in the early stage disease ($p<0.01$) (Figure 1). However, no differences were identified in the advanced stage. Fibrosis in the media layer was increased in both the early stage and the advanced stage ($p<0.005$), with no statistically significant difference between the early stage and the advanced stage.

Elastic fibers: No differences among the groups were identified in terms of elastic fiber content. While there was a difference in terms of the internal elastic lamina fragmentation rates between the early stage disease as compared to the control group, no differences were identified in the advanced stage ($p=0.01$) (Figure 2). Furthermore, the rate was found to be high in the group with a disease duration of more than 10 years, as well ($p<0.01$).

Muscle hypertrophy: No statistical differences were identified in comparison with the control group with respect to disease stage. However, hypertrophy was more prominent among those with a disease duration of less than 10 years ($p<0.05$).

Laminin: No significant differences were seen among groups with respect to laminin expression.

Fibronectin: No differences were seen among groups with respect to the intima layer. On the other hand, it was identified that fibronectin was increased in the media layer, with advanced disease ($p<0.05$) (Figure 3).

Table 1. Groups and patient numbers

Variable	Groups	Patient numbers
CEAP, clinical staging	(C_2, C_3)=early stage	27
	(C_4, C_5, C_6)=advanced stage	13
CEAP, severity score	(1-5)	12
	(6-9)	18
	(10 and above)	10
Age	<40 years	18
	>40 years	22
Disease duration	<10 years	22
	>10 years	18
Control group		10

Table 2. Fibrosis and muscle hypertrophy evaluation method

	Layer	0	1+	2+	3+
Fibrosis	All	None	Mild	Moderate	Severe
Muscle hypertrophy	Only media	None	Mild	Moderate	Severe

Table 3. Fibronectin, tenacin, laminin evaluation methods

		Intima	Media	Adventitia
Fibronectin	0	Thin subendothelial staining, only	Smooth muscle staining, only	Perivascular staining, only
	1	In addition to subendothelium, intimal connective tissue focal staining	In addition to smooth muscle, connective tissue focal staining	In addition to perivascular, connective tissue focal staining
	2	Intimal connective tissue diffuse staining	In addition to smooth muscle, connective tissue diffuse staining	In addition to perivascular, connective tissue diffuse staining
Laminin	0	Thin subendothelial staining, only	Smooth muscle staining, only	Perivascular staining, only
	1	In addition to subendothelium, intimal connective tissuefocal staining in thin strands	In addition to smooth muscle, connective tissue focal staining	In addition to perivascular, connective tissue focal staining
	2	Intimal diffuse staining in thin strands	In addition to smooth muscle, connective tissue diffuse staining	In addition to perivascular, connective tissue diffuse staining
Tenacin	0	No staining	Normal muscle staining	Perivascular staining, only
	1	Focal staining	Focal connective tissue staining within muscle	In addition to perivascular, connective tissue focal staining
	2	Diffuse staining	Diffuse connective tissue staining within muscle	In addition to perivascular, connective tissue diffuse staining

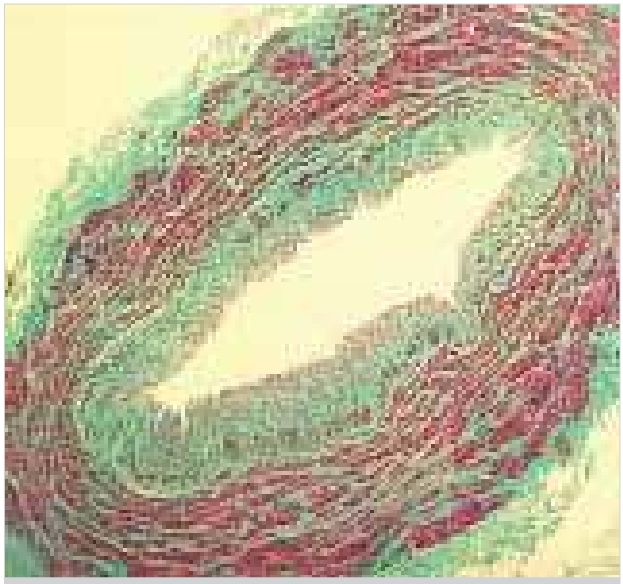

Figure 1. Diffuse and severe fibrosis in intima, mild fibrosis in the media. Diffuse muscle hypertrophy in the media. Masson trichrome x40

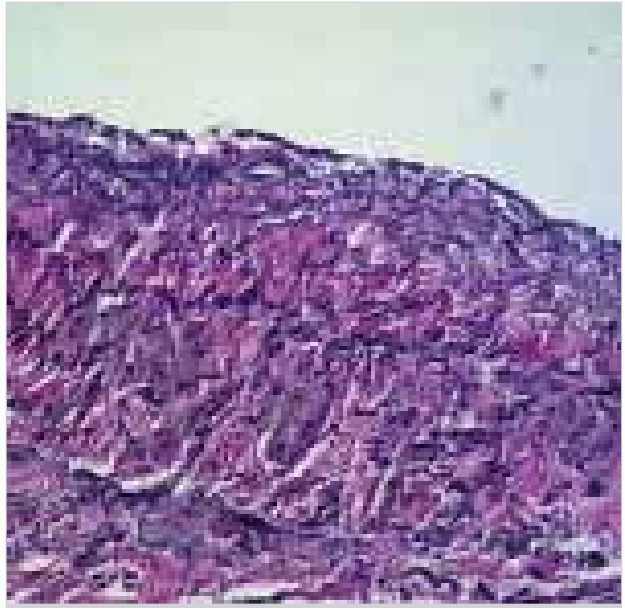

Figure 2. Internal elastic lamina fragmentation. Elastic van Gieson x400

Tenascin: It was increased in both the early stage and the advanced stages, in the intima layer ($p<0.05$) (Figure 4). As for the media layer, it was increased only in the advanced stage disease ($p<0.05$).

No differences were identified among groups for any parameters in the adventitia layer. The statistically important values are shown in Table 4. The results are summarized in Table 5.

DISCUSSION

Several epidemiological studies showed that varicose veins, the most frequent clinical form of chronic venous disease, are seen among women at a rate of 25-35% and of 10-20% among men, and the incidence increases with age.

Undoubtedly, a full understanding of the physiopathology of chronic venous disease would contribute to the development of an effective treatment. Since the changes in chronic venous

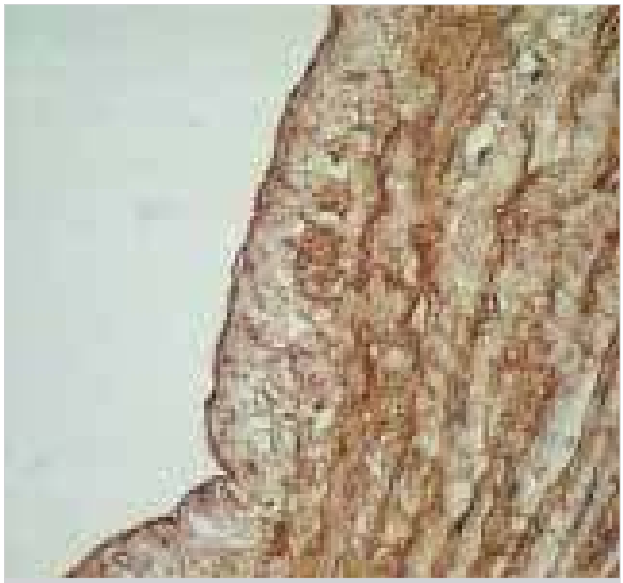

Figure 3. Increased fibronectin expression in intima and media. Fibronectin x400

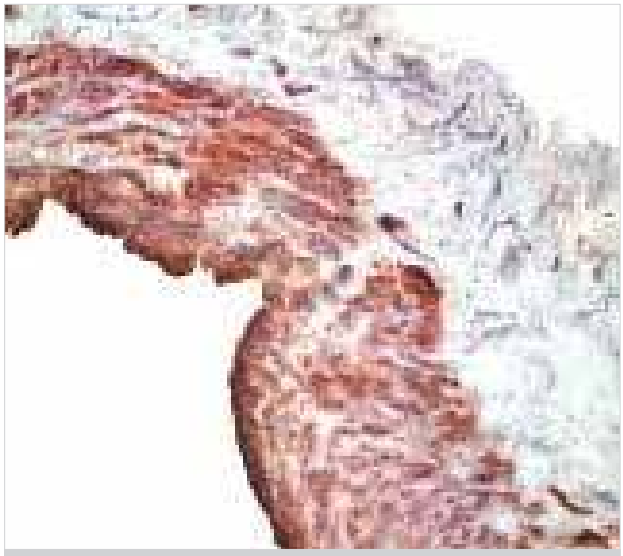

Figure 4. Increased tenacin expression in the intimal layer. Tenascin x200

disease physiopathology were mostly concentrated in the vein wall extracellular matrix, this component was examined in our study.

The vein wall morphology shows significant differences according to the anatomic position of the vein. Since these changes may influence the study results, all the examinations in this study were performed in a single point, the saphenofemoral joint, which is a physiopathologically important location. The presence of reflux in the saphenofemoral junction causes the varices to be more disseminated (25). Furthermore, reflux is more frequent at the saphenofemoral junction in advanced stage patients (26). The examinations were conducted at the saphenofemoral junction, with the assumption that early stage patients with reflux at the saphenofemoral junction could progress to the next stages. By this approach, it was attempted to create a correlation between clinical symptoms and morphological appearance.

Table 4. Statistically different values

Variable		Control	Early stage (C_2, C_3)	Advanced stage (C_4, C_5, C_6)
Media layer thickness (µm)	Mean±SD	336.5±55	471.7±30	553±269
Fibrosis, intima	Normal	7	5	4
layer	Increased	3	22	9
Fibrosis, media	Normal	10	15	4
layer	Increased	0	12	9
Fragmentation rate lamina	Normal	9	11	8
elastica interna	Increased	1	16	5
Fibronectin, intima	Normal	6	8	4
layer	Increased	4	19	9
Fibronectin, media	Normal	9	16	9
layer	Increased	0	4	5
Tenacin, intima	Normal	6	5	1
layer	Increased	4	22	12
Tanecin, media	Normal	10	23	8
layer	Increased	0	4	5

SD: standard deviation

Table 5. Outline of statictically significant results

	Intima	Media
Early stage (C_2, C_3)	Fibrosis, fragmented lamina elastica interna, tenacin accumulation	Fibrosis
Advanced stage (C_4, C_5, C_6)	Tenacin accumulation	Increased thickness, fibrosis, fibronectin accumulation, tenacin accumulation

According to the results of our study, it was seen that fibrosis was increased in the intima and media layers at the early stages, there was fragmentation in the internal elastic lamina and an increase in the tenascin expression in the intima layer. It was identified that tenascin continued to accumulate in the intima layer at the advanced stages; fibrosis continued in the media layer, the layer thickness increased and fibronectin and tenascin accumulated. These findings demonstrate that the pathological processes occurred mostly in the intima at the early stages, while the processes shifted to the media layer at the more advanced stages.

In various studies, the fragmentation occurring in the internal elastic lamina at the early stages was attributed to the smooth muscle cells that migrated from the media layer to the intima layer (27-30). The increase of tenascin, which has a role in migration, in the intima, supports this migration (31). The correction of the fragmentation in the internal elastic lamina at the advanced stages shows that there are no defects in elastin synthesis.

The increased thickness of the media layer and fibronectin accumulation in the advanced stages were attributed to collagen increase and muscle hypertrophy, although no statistical differences could be found.

It is expected that the thickness of muscle layers decreases with age, increases with disease duration (12). However, no significant correlations were found in this study between muscle thickness and age. It was identified that the muscle thickness increased when the disease duration was less than 10 years, and returned back to normal levels after 10 years. As for the media thickness, it was seen that it increased in disease duration of less than 10 years, while it returned to normal with disease duration of more than 10 years, parallel to muscle thickness.

When the stages of the patients and their disease durations were examined, it was seen that some patients with 20 years of disease were at stage C2, while some with 8 years of disease were at stage C5. This suggests that the disease does not progress if the changes in the early stage enable adequate vessel wall strengthening, while it progresses if the adequate resistance cannot be provided, thus leading to advanced symptoms.

Finally, the fact that significant statistical results have been obtained according to the CEAP stages is an indication that the CEAP staging is successful in showing disease progression of the disease.

CONCLUSION

In this study, it was revealed that alterations occurred in the intima layer and extended to the media layer as the disease progressed. This conclusion supports the view that the disease etiology is due to luminal pathology such as venous hypertension and thrombosis, rather than a defect on the vessel wall. A definitive conclusion can be reached once it is shown that such changes still occur in the presence of luminal etiologic factors. Further studies on this subject are required.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Ankara University Faculty of Medicine.

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

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Conflict of Interest: No conflict of interest was declared by the authors.

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