Expression Levels of Elastin and Related Genes in Human Varicose Veins

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Abstract. Among the suspected reasons for varicose vein formation are changes in the quantity and content of the elastin protein; however, comprehensive investigations about elastin assembly in varicose vein formation are yet lacking. In this study, we aimed to determine the changes in mRNA levels of elastin and some of its functionally related proteins, fibulin 5, LOX-L-1, MMP-2 and MMP-9 in varicose vein formation. We analysed the mRNA levels of elastin, fibulin-5, LOX-L1, MMP2 and MMP9 in samples of 35 healthy and 35 varicose great saphenous vein tissues. mRNA levels of these genes were determined by using real-time PCR and normalized with HPRT1. When we compared the patient and control groups, elastin mRNA levels were significantly higher in the patient group than in the control group (P = 0.047), although there were no significant differences in fibulin 5, LOX-L1, MMP2 and MMP9 mRNA levels between the patient and control groups. We showed that up-regulation of MMP2 mRNA expression was significantly correlated with hyperlipidaemia (P = 0.029). The up-regulation of elastin expression may play an important role in the pathogenesis of primary varicose veins. Additionally, the up-regulation of MMP2 expression was strongly correlated with hyperlipidaemia in varicose veins.

Introduction

Chronic venous insufficiency has a high morbidity rate causing various problems such as mild expansion of veins, endurations, ulcerations and more frequently primary varicose veins (Naoum et al., 2007). Varicose veins are usually described as abnormal expansions and tortuous texture of veins (Bidier-Commander et al., 2000). These kinds of varicose structures are frequently seen in the lower extremities. In the normal venous texture, the elasticity and distensibility of the veins are provided by the balance between elastin, collagen and proteoglycan contents of the extracellular matrix (Somers and Knaapen, 2006).

Elastin, one of the most important components of vascular extracellular structures that has to be regular, was found decreased and/or displaying structural changes in varicose vein walls (Venturi et al., 1996). Elastin assembly is a tender process that can be affected by several other proteins and factors. Both fibulin 5 and lysyl oxidase like 1 (LOXL-1) are important in binding of elastin fibres through the endothelial basal membrane (De Vega et al., 2009). LOXL-1 is an amine oxidase having important roles in the assembly of elastin fibres (Pascual et al., 2008). The high degree of cross-linking of the elastin structure is known to be provided by one or more members of the lysyl oxidase (LOX) family (Wagnes and Mecham, 2007). LOX and LOXL-1 play roles in cross-linking of elastin and collagen (Wagnes and Mecham, 2007). Previous studies have shown that decreased LOXL-1 expression causes serious defects in the elastin structure (Kagan and Li, 2003; Liu et al., 2004).
Fibulin 5 is an important elastin-associated protein bound to integrins, thus connecting the cell surface and elastin fibres (Yanagisawa and Davis, 2010). Although fibulin 5 expression is known to be down-regulated after birth, it is known to be reactivated after vascular injury (Yanagisawa and Davis, 2010). Fibulin 5 preferentially binds LOXL-1 to assist its activation, and their mutual interaction enables proper elastin assembly (Liu et al., 2006; Choi et al., 2009; Yanagisawa and Davis, 2010).

In a previous study, it was found that fibulin knockout mice had expanded vascular structures and vascular layout disturbances (Yanagisawa et al., 2002). Not only these structural parameters, but those degrading elastin fibres are also important in elastin remodelling. MMP-2 and MMP-9 are known to be the main enzymes in elastin assembly (Allan et al., 1995; Katsuda and Kaji, 2003). We have therefore also focused on these main enzymes having roles in degradation of elastin fibres, whether their expression were activated or not. MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) were found to degrade several collagen types and elastin (Allan et al., 1995). Previous studies suggested that MMP-2 and MMP-9 can be used as useful markers of blood stasis in varicose veins (Jacob et al., 2002; Kowalewski et al., 2004).

As more information will be supplied about elastin and collagen assembly, the gaps in our current understandings about various diseases will be filled. Investigation of some possible contributing parameters together, as in our study, will help us to evaluate the mechanism of varicose vein formation.

**Material and Methods**

**Subjects and specimens**

Patients (N = 35) consisted of those undergoing surgery for primary varicose veins at Marmara University, Department of Cardiovascular Surgery, Istanbul, Turkey, and the control group (N = 35) consisted of those undergoing elective coronary bypass surgery at the same department. Preoperative evaluation of greater saphenous vein (GSV) status was obtained by duplex ultrasonography both for control and patient groups. Table 1 shows the patient characteristics based on the classification of chronic venous disease. None of the control patients had clinical, ultrasonographic or intraoperative signs of varicosis (CO-C1). In the patient group high ligation with stripping of GSV with or without additional individual ligation and excision were performed. In the control group, approximately 5 cm of samples of GSV were obtained during the surgery. Both proximal and distal parts of the samples were used for the analysis. Patients were characterized as diabetes mellitus based on the fasting blood glucose concentration (≥ 126 mg/dl) (American Diabetes Association, 2004). Systemic arterial hypertension was considered to be present if the systolic blood pressure (SBP) was > 130 mmHg and/or DBP was > 90 mmHg. Obesity was defined as the body mass index (BMI) ≥ 30 kg/m². Hyperlipidaemia was considered as total cholesterol level ≥ 200 mg/dl, triglyceride level ≥ 150 mg/dl and/or low-density lipoprotein (LDL) level ≥ 130 mg/dl (Fischer et al., 2013).

The specimens were immediately taken and protected with fluid nitrogen to prevent any kind of reaction degenerating the RNA content (Air Liquide GT9). Materials were immediately taken to the laboratory in fluid nitrogen and homogenized by using the following procedures. Tissue samples were dissected to determine optimal lysis conditions with MagnaLyser (Roche, Indianapolis, IN) by using the commercial procedure.

**RNA isolation and quantitative PCR**

Total RNA was extracted by using a commercial Magnapure Compact RNA isolation kit (Roche). Synthesis of cDNAs was performed in a thermal cycler device (Applied Biosystems GeneAmp PCR System 9700, Foster City, CA) using a Transcriptor First Strand cDNA Synthesis kit (Roche). The resultant cDNA was subjected to fluorescence quantitative PCR (FQ-PCR). The PCR assays were carried out in a LightCycler 1.5 (Roche) device by using a LightCycler TaqMan master kit (Roche) and specific primer and probe sequences.

The primers and probes were designed by using the software, www.universalprobibrary.com. The genespecific primers and probes for elastin, fibulin 5, LOXL1, MMP2 and MMP9 used in quantitative PCR were as follows: forward primer for elastin 5’CAGCTAAATACGGTGCTGTG3’; reverse primer 5’ATCCCCAGGCGCGCCTTGG3’; and probe 5’-FAM-TGGAGGAG-3’-dark quencher; forward primer for fibulin 5 5’CTGCCCTTCCAGGCTCATAC3’; reverse primer 5’ CCTGTGCTCATTCTGTGA3’; and probe 5’-FAM-TGGAGGAG-3’-dark quencher; forward primer for MMP2 5’ ATACCTGGATGCCGTCGT3’; and probe 5’-FAM-GGCGGCGG-3’-dark quencher; forward primer for MMP9 5’ GACCAATCTCAGCACAG3’; and probe 5’-FAM-GGCGGCGG-3’-dark quencher; reverse primer 5’GCCACCCGAGTGTAACCATA3’; and probe 5’-FAM-CAGAGGAA-3’-dark quencher.

**Table 1. Demographic characteristics of the study groups**

<table>
<thead>
<tr>
<th></th>
<th>Patients (N: 35)</th>
<th>Controls (N: 35)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (year; mean ± SD)</strong></td>
<td>60.32 ± 9.88</td>
<td>62.06 ± 9.98</td>
</tr>
<tr>
<td><strong>Gender (M/F)</strong></td>
<td>17/18</td>
<td>8/27</td>
</tr>
<tr>
<td><strong>Smoking (%)</strong></td>
<td>8.6</td>
<td>28.6</td>
</tr>
<tr>
<td><strong>Obesity (%)</strong></td>
<td>20</td>
<td>34.3</td>
</tr>
<tr>
<td><strong>Hypertension (%)</strong></td>
<td>45.7</td>
<td>54.3</td>
</tr>
<tr>
<td><strong>Diabetes mellitus (%)</strong></td>
<td>25.7</td>
<td>40</td>
</tr>
<tr>
<td><strong>Hyperlipidaemia (%)</strong></td>
<td>28.6</td>
<td>37.1</td>
</tr>
</tbody>
</table>

N – number of individuals. Values are reported as mean ± standard deviation (SD) or number of patients (percentage of the total group).
scription, all samples were subjected to PCR amplification with oligonucleotide primers and probes specific for the constitutively expressed gene hypoxanthine-guanine phosphoribosyltransferase (HPRT1) and normalized. HPRT1 primers and probe were as follows: forward primer, 5'TGACCTTGATTTATTTTGACA-CC3', and reverse primer, 5'CGACAAGACGTCC-GTCCCT3'; and probe 5'-FAM-GCTGAGGA-3'-dark quencher.

The cycle number was determined as being with the linear amplification range from a linear amplification curve. The values of the sample copies were obtained after quantitative amplification and target gene Ct values were normalized to the respective Ct values obtained for HPRT1 by a previously designed relative quantification method (Livak and Schmittgen, 2001).

Statistical analysis

Values are given as mean ± standard deviation (SD). Statistical analyses were performed using SPSS version 18 software (SPSS Inc., Chicago, IL). Student's t-test was used to examine the significance of differences between the two groups and χ2 and Fisher's exact tests were used to compare demographic information to expression. P values lower than 0.05 denoted statistical significance.

Results

Vein tissue materials of 35 lower extremity varix patients and 35 control cases were examined in our study. Demographic data is shown in Table 1.

We evaluated elastin, fibulin 5, LOX1, MMP2 and MMP9 mRNA levels in the patient group with varicose veins compared to distal saphenous veins as control. When we compared the patient and control groups, the elastin mRNA level was significantly higher in the patient group (-0.222 ± 0.803) than in the control group (-0.898 ± 1.785), (P = 0.047). However, there were no significant differences in fibulin 5, LOX1, MMP2 and MMP9 mRNA levels between varicose GSV and control groups (fibulin 5, P = 0.472; LOX1, P = 0.501; MMP2, P = 0.444; MMP9, P = 0.633).

Real-time PCR was used in the molecular analysis to quantitatively measure the MMP2 relative gene expression levels (as fold change) and it confirmed its underexpression in 100 % varicose veins compared to the distal saphenous vein pool used as a control (Fig. 1). At the same time, RT-PCR analysis of MMP9 expression showed that MMP9 was 100 % up-regulated in the patient group compared to the control group (Fig. 1). For this reason, patients with varicose veins were separated into low and high levels of MMP2 and MMP9 mRNA according to the cut-off value of -6.8 and 4.8, respectively.

Chi-square (χ2) test was used to determine the effects of possible risk factors on the elastin and related gene expression. Expression levels of elastin, fibulin 5, LOX1 and MMP9 genes in the patient group were not significantly associated with gender, smoking, obesity, hypertension, diabetes, cardiovascular disease and hyperlipidaemia (Tables 2, 3). However, the up-regulation of MMP2 expression was positively correlated with hyperlipidaemia in the patient group (P = 0.029) (Table 3).

Discussion

The preceding morphologic and histochemical studies have emphasized the altered contents of elastin, collagen, and smooth muscle in varicose veins (Lowell et al., 1992). However, the gene expression alterations in varicose veins are not exactly known yet.

It was previously thought that prolonged venous hypertension causing increased pressure on the valvular area was responsible for the varicose vein formation, but recent findings showed that the venous dilatations were not located in the distal part of valvuli but in their proximal part, thus showing other still unknown factors such as the effects of structural deteriorations of the vein walls (Pascarella and Schmid Schönbein, 2005; Burkitt et al., 1976; Lee et al., 2003). The functional consistency of venous wall mainly depends on the balance be-

![Relative Gene Expression Levels](image_url)

Fig. 1. Relative gene expression levels of elastin, fibulin 5, LOX1, MMP2 and MMP9 in human varicose veins compared to the distal saphenous veins pool used as a control.
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Table 2. Correlation of elastin, fibulin 5 and LOX1 gene expression levels with clinical parameters of patients with varicose veins

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Elastin expression (N: 35)</th>
<th>Fibulin 5 expression (N: 35)</th>
<th>LOX1 expression (N: 35)</th>
<th>1P value</th>
<th>2P value</th>
<th>3P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low (N: 23)</td>
<td>High (N: 12)</td>
<td>Low (N: 17)</td>
<td>High (N: 18)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male gender (%)</td>
<td>77.8 %</td>
<td>22.2 %</td>
<td>50 %</td>
<td>50 %</td>
<td>77.8 %</td>
<td>22.2 %</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>33.3 %</td>
<td>66.7 %</td>
<td>-</td>
<td>100 %</td>
<td>66.7 %</td>
<td>33.3 %</td>
</tr>
<tr>
<td>Obesity (%)</td>
<td>71.4 %</td>
<td>28.6 %</td>
<td>41.9 %</td>
<td>57.1 %</td>
<td>71.4 %</td>
<td>28.6 %</td>
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<td>Hypertension (%)</td>
<td>75 %</td>
<td>25 %</td>
<td>50 %</td>
<td>50 %</td>
<td>68.8 %</td>
<td>31.3 %</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>88.9 %</td>
<td>11.1 %</td>
<td>77.8 %</td>
<td>22.2 %</td>
<td>66.7 %</td>
<td>33.3 %</td>
</tr>
<tr>
<td>Cardiovascular disease (%)</td>
<td>64.7 %</td>
<td>35.3 %</td>
<td>47.1 %</td>
<td>52.9 %</td>
<td>76.5 %</td>
<td>23.5 %</td>
</tr>
<tr>
<td>Hyperlipidaemia (%)</td>
<td>70 %</td>
<td>30 %</td>
<td>60 %</td>
<td>40 %</td>
<td>90 %</td>
<td>10 %</td>
</tr>
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</table>

Demographics and co-morbidities

<table>
<thead>
<tr>
<th>Parameters</th>
<th>MMP2 expression cut-off value (N: 35)</th>
<th>MMP9 expression cut-off value (N: 35)</th>
<th>1P value</th>
<th>2P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low &gt; -6.8 (N: 14)</td>
<td>High ≤ -6.8 (N: 21)</td>
<td>Low &lt; 4.8 (N: 15)</td>
<td>High &gt; 4.8 (N: 20)</td>
<td></td>
</tr>
<tr>
<td>Male gender (%)</td>
<td>44.4 %</td>
<td>5.6 %</td>
<td>33.3 %</td>
<td>66.7 %</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>33.3 %</td>
<td>66.7 %</td>
<td>57.1 %</td>
<td>42.9 %</td>
</tr>
<tr>
<td>Obesity (%)</td>
<td>14.3 %</td>
<td>85.7 %</td>
<td>50 %</td>
<td>50 %</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>43.8 %</td>
<td>56.3 %</td>
<td>55.6 %</td>
<td>44.4 %</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>33.3 %</td>
<td>66.7 %</td>
<td>41.2 %</td>
<td>58.8 %</td>
</tr>
<tr>
<td>Cardiovascular disease (%)</td>
<td>41.2 %</td>
<td>58.8 %</td>
<td>10 %</td>
<td>90 %</td>
</tr>
<tr>
<td>Hyperlipidaemia (%)</td>
<td>10 %</td>
<td>90 %</td>
<td>60 %</td>
<td>40 %</td>
</tr>
</tbody>
</table>

N – number of individuals. Values are reported as numbers with percentages, *P values lower than 0.05 denoted statistical significance. 1P value: MMP2 expression; 2P value: MMP9 expression.

tween formation and degradation of the extracellular matrix components. In our study, it seemed that we obtained opposite results to those of Venturi et al. (1996), who detected a strong relationship between venous wall dilatation and decreased elastin content; we found that elastin mRNA expression was higher in varix tissues, but this difference may be due to the posttranslational modifications of the elastin protein. There may be changes in the level of mature elastin proteins during translation and posttranslational stages; therefore, it will be useful to design further studies to measure the protein levels and mRNA expression together to evaluate the effects of posttranslational factors.

A decrease in elastin has been shown in chronic venous insufficiency, but few studies have been focused on the effects of some factors involved in elastin synthesis such as lysyl oxidases and fibulins (Pascual et al., 2008; Yanagisawa and Davis, 2010). Fibulin 5, an important elastin-associated protein, was previously reported to be activated after vascular injury (Yanagisawa and Davis, 2010), but it did not statistically differ between our study groups. LOXL-1, to which fibulin was detected to be preferentially bound, was shown to be significantly decreased in the varicose vein areas (Pascual et al., 2008). Therefore, in our study, the LOXL1 mRNA level reduction was not statistically significant, either. As in a previous study, fibulin knock-out mice had expanded vascular structures and vascular lay-out disturbances (Yanagisawa et al., 2002), and this mechanism can be investigated in larger study groups with detailed analyses of both mRNA and protein levels.

Some researchers have recently focused on the role of MMPs in chronic venous disease and varicose vein formation in the lower extremities (Raffetto and Khalil, 2008). Various studies have elucidated the pathophysiological mechanisms underlying the relationship between MMPs and the formation and complications of varicose veins (Raffetto and Khalil, 2008; Lim et al., 2010). We also determined mRNA levels of two elastin-degrading enzymes, MMP-2 and MMP-9, to evaluate their possible expression changes. Although previous studies suggested that MMP expression may decrease during the varicose vein formation and MMP2 and MMP9 can thus be used as markers for blood stasis in...
varicose veins (Parra et al., 1998; Jacob et al., 2002; Katsuda and Kaji, 2003), we could not detect any difference in these parameters for varicose vein patients compared to the healthy cases. Our findings supported some previous studies suggesting no difference in MMP-2 and MMP-9 levels in varicose veins (Sansilvestri-Morel et al., 1998; Ishikawa et al., 2000). Chang et al. detected increases in MMP2 expression in varicose vein tissues (Chang et al., 2011), but we detected the same pattern only in cases with both varicose veins and hyperlipidaemia. We concluded that up-regulation of MMP2 expression was associated with hyperlipidaemia in the patient group, suggesting the role of hyperlipidaemia in degenerative processes of the vein tissue. This finding should be proved by larger studies combined with other parameters in remodelling of the vein tissues.

In conclusion, we suggested that elastin may play an important role in the pathogenesis of primary lower extremity varicose veins. Additionally, the up-regulation of MMP2 expression may play a role in the development of varicose veins in patients with hyperlipidaemia. Further studies are required to elucidate the potential relationship between these proteins and primary varicose veins.

References
