Synthesis of Collagen Is Dysregulated in Cultured Fibroblasts Derived From Skin of Subjects With Varicose Veins as It Is in Venous Smooth Muscle Cells

Patricia Sansilvestri-Morel, PhD; Alain Rupin, PhD; Stéphane Jaisson; Jean-Noël Fabiani, MD; Tony J. Verbeuren, PhD; Paul M. Vanhoutte, MD, PhD

Background—The dilatation and tortuosity observed in varicose veins provide evidence for progressive venous wall remodeling associated with abnormalities of smooth muscle cells and extracellular matrix. The present study was designed to examine if the phenotypic modulations observed in the venous smooth muscle cells of patients with varicose veins were also present in their dermal fibroblasts.

Methods and Results—Collagen type I (collagen I), type III (collagen III), and type V (collagen V) were compared in dermal fibroblasts derived from the skin of control subjects and patients with varicose veins. The synthesis of collagen I, the release of its metabolites, and the expression of its mRNA were increased in fibroblasts from patients with varicose veins, whereas the synthesis of collagen III was decreased but not correlated with a decrease in mRNA expression and in metabolite release. Matrix metalloproteinases (MMP1, 2, 7, 8, 9, and 13) and their inhibitors (TIMP1 and 2) were quantified in both cell types; only the production of proMMP2 was increased in cells derived from patients with varicose veins.

Conclusions—These findings suggest that the synthesis of collagen I and III is dysregulated in dermal fibroblasts derived from patients with varicose veins. These results are comparable with those observed in smooth muscle cells derived from varicose veins, thus suggesting a systemic alteration of tissue remodeling in subjects with varicose veins. (Circulation. 2002;106:479-483.)

Key Words: veins ■ collagen ■ metalloproteinases ■ remodeling

Varicose veins are a common disease in which a weakening of the vascular wall and incompetent malfunctioning valves allow venous reflux to occur. In this disease, the saphenous veins are dilated and tortuous, and intraluminal venous pressure is increased. The association between dilatation and increased severity of reflux is evident in that dilatation occurs first and induces the valvular incompetence.1 This may indicate that the primary defect leading to varicose veins resides in a dysfunction of the venous wall.2,3 Alterations in tissue remodeling occur in varicose veins. The content of elastin,4 laminin,5 fibronectin,5 and collagen6 are modified. Previous studies have shown that cultured smooth muscle cells derived from human varicose veins retain, at least in part, dysregulations of the synthesis of extracellular matrix proteins.7,8 Indeed, the expression and synthesis of collagen I was increased in cultured smooth muscle cells from varicose veins, whereas the synthesis of collagen III and fibronectin was decreased.7,8 These dysregulations could explain the alterations of the mechanical properties of the varicose veins.9 Indeed, smooth muscle cells and extracellular matrix are intimately associated through the integrins, and these interactions regulate venous contractility and integrity.10

Epidemiological studies have demonstrated an involvement of hereditary factors for the transmission of varicose veins.11,12 Most authors agree that heredity is the principal factor, with an aggravation of the symptoms due to environmental factors.11,12 The distensibility of arm veins in patients with varicose veins is increased abnormally, suggesting a systemic disease of the venous wall.2,11 Moreover, the elasticity of the venous wall in the lower limbs is reduced in patients with venous insufficiency, as well as in control patients with a high risk of developing varicose veins1 and in the children of patients with varicose veins.14 All these findings suggest a genetic basis for the disease.

Several studies have indicated that in certain hereditary pathological conditions, including Ehlers Danlos syndrome, Marfan syndrome, anetoderma, and pseudoxanthoma elasticum, the loss of integrity of matrix proteins in the skin reflects similar changes in major blood vessels such as the aorta.15,16 A correlation exists between the amount of elastic fibers from the skin and the temporal artery during aging.17 Thus, skin biopsies have been proposed to represent a valuable approach for predicting abnormalities of the extracellular matrix proteins in the arterial wall.18

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The present study was designed to test the hypothesis that cultured cells from nonvascular connective tissue from patients with varicose veins present dysregulations in the synthesis of collagens. Regulation of the synthesis of collagens and matrix metalloproteinases (MMPs) was therefore compared in cultured dermal fibroblasts derived from the skin of control subjects and patients with varicose veins.

Methods

Specimens

Twelve control skin biopsies were obtained from patients (8 men and 4 women; mean age, 71.9 years; range, 52 to 86 years) undergoing endarterectomy (recovered at the neck) or coronary bypass surgery (recovered at the thorax). Preoperative evaluation of these patients showed an absence of varicose abnormalities or retrograde flow by echo-Doppler studies. Ten skin biopsies recovered at the groin were collected from patients with varicose veins (4 men and 6 women; mean age, 64.3 years; range, 53 to 74 years) during saphenectomy (recovered at the thorax). Preoperative evaluation of these patients was stage III of venous disease with permanent retrograde flow and varicosities all along the venous axis and did not present with venous ulcers. All patients were informed and consented to the procedure.

Cell Cultures

Explants of the 2 groups of skins (control subjects and patients with varicose veins) were prepared according to the method described for smooth muscle cells5,6 by carefully putting the epidermis at the top. Cells were grown into collagen-precoated Petri dishes in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal calf serum (Dominique Dutscher), 10% horse serum (Boehringer-Mannheim), 2 mmol/L L-glutamine, 100 units/L penicillin, and 100 \( \mu \)g/mL streptomycin at 37°C in a 95% air, 5% CO\(_2\) atmosphere. Cell growth began within 3 to 5 days, and cells reached confluence after 2 weeks. Cells were then trypsinized, seeded at a density of 10,000 cells/cm\(^2\) (first passage), and subcultured to be used at passage 3 or 4 after 8 to 10 population doublings in the same culture medium described above without horse serum.

For determination of cell proliferation, both cell types were subcultured at passage 3 at a density of 8000 cells/cm\(^2\) and counted at different times with an automatic cell counter (Coulter Z1).

Metabolic Labeling and Synthesis of Collagen I, III, and V

Fibroblasts from controls and patients with varicose veins were subcultured at a density of 8000 cells/cm\(^2\) and used at confluence (day 10). Cells were then incubated for 15 hours in DMEM supplemented with 2 mmol/L L-glutamine, 100 \( \mu \)g/mL streptomycin, and 50 \( \mu \)g/mL of L-aspartic acid. Culture media were then collected. The metabolites of collagen I and III were measured using commercially available radioimmunoassays kits (Orion Diagnostica).

MMPs and Tissue Inhibitors of Metalloproteinases

Fibroblasts from controls and patients with varicose veins were cultured at a density of 8000 cells/cm\(^2\) and used at confluence (day 10). Cells were then incubated for 15 hours in DMEM supplemented with 2 mmol/L L-glutamine, 100 \( \mu \)g/mL penicillin, 100 \( \mu \)g/mL streptomycin, and 50 \( \mu \)g/mL L-aspartic acid. Culture media were then collected. Pro-MMP1, pro-MMP2, pro-MMP7, pro-MMP8, pro-MMP9, and pro-MMP13 and their inhibitors in free or complexed form (tissue inhibitor of metalloproteinase [TIMP] 1, MMPs-TIMP1, TIMP2, and MMPs-TIMP2) were quantified in supernatants using commercially available enzyme immunoassays kits (Amer sham Pharmacia Biotech). The active form of MMP2 was quantified in culture media using the MMP2 activity assay system kit (Amer sham Pharmacia Biotech).

Statistical Analysis

Data are presented as mean±SEM. Student’s \( t \) test for unequal observations was used to compare results obtained in the cultures of cells from control subjects and patients with varicose veins. Statistical significance was assumed at \( P<0.05 \).

Results

Rate of Proliferation and Morphology

The growth rates of fibroblasts derived from control subjects and patients with varicose veins were not significantly different (doubling time of 3.45±0.3 days and 2.57±0.46 days, respectively; \( n=8 \)). No difference in cell morphology was observed between the cell types during the phase of proliferation or at confluence (data not shown).

Total Synthesis of Proteins

The content of synthesized proteins during the tritiated proline incorporation was not significantly different between fibroblasts from controls and patients with varicose veins (7.0±2.1 disintegrations per minute [dpm] and 8.6±1.1 dpm per cell for control subjects and patients with varicose veins, respectively; \( n=8 \)).

Synthesis of Collagen I, III, and V

Fibroblasts from patients with varicose veins synthesized significantly less collagen III but more collagen I than fibroblasts derived from control subjects (Figure 1). No significant difference was observed in the synthesis of collagen V (Figure 1). Decreased amounts of collagen III were observed between the cell types during the phase of proliferation or at confluence (data not shown).

1.3 kb EcoRI fragment of human \( \alpha1(III) \) collagen cDNA (ATCC), and a 2 kb full human \( \beta \)-actin cDNA (Clontech). The results are expressed as relative height of the peak between collagen I or III and \( \beta \)-actin mRNA bands.

Telopeptides and Propeptides of Collagen I and III

Fibroblasts from control subjects and patients with varicose veins were subcultured at a density of 8000 cells/cm\(^2\) and used at confluence (day 10). Cells were then incubated for 15 hours in DMEM supplemented with 2 mmol/L L-glutamine, 100 \( \mu \)g/mL penicillin, 100 \( \mu \)g/mL streptomycin, and 50 \( \mu \)g/mL of L-aspartic acid. Culture media were then collected. The telopeptides of collagen I and III were measured using commercially available radioimmunoassays kits (Orion Diagnostica).

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Fibroblasts from patients with varicose veins synthesized significantly less collagen III but more collagen I than fibroblasts derived from control subjects (Figure 1). No significant difference was observed in the synthesis of collagen V (Figure 1). Decreased amounts of collagen III were found both in the culture medium and cell layer extract, whereas increased levels of collagen I were observed only in supernatants (data not shown). The percentage of total collagen production was significantly increased in cells from patients with varicose veins (27.5±2.7% and 34.5±1.4% of
total collagens/total proteins in cells from control subjects and patients with varicose veins respectively; \( P < 0.05; n = 8 \).

**Expression of Collagen I and III mRNA**
Northern blot experiments were performed to evaluate the expression of collagen I and III mRNA in fibroblasts from control subjects and patients with varicose veins. The increase in collagen I synthesis by cells from patients with varicose veins was correlated with a significant overexpression of the mRNA of collagen I (Figure 2A). A comparable expression of collagen III mRNA was found in fibroblasts from the 2 groups (Figure 2B).

**Release of Metabolites of Collagen I and III in Culture Media**
To estimate the level of secreted collagen I and III in the culture media, the N- and C-terminal propeptides of procollagen I, as well as the N-terminal propeptides of procollagen III were quantified. The augmentation of collagen I synthesis was confirmed by a significant increase of collagen I metabolites in the culture media of fibroblasts from patients with varicose veins (Figure 3A). The level of amino-terminal propeptides of procollagen III was similar in both cell types (Figure 3B).

**Production of MMPs and TIMPs**
Quantification of pro-MMP1, pro-MMP2, pro-MMP7, pro-MMP8, pro-MMP9, pro-MMP13, TIMP1, TIMP2, and the complex MMP1/TIMP1 was performed in the supernatants of fibroblasts from control subjects and patients with varicose veins (Table). The concentrations of pro-MMP7, pro-MMP8, pro-MMP9, and pro-MMP13 were lower than the cut off value of the assays for the 2 groups of fibroblasts, but pro-MMP1, pro-MMP2, TIMP1, TIMP2, and the complex MMP1/TIMP1 were produced by both cell types (Table). The concentrations of pro-MMP2 and TIMP1 were highest in both cell types, whereas the production of pro-MMP1 was weak (Table). The level of pro-MMP2 was higher than the concentration of its major inhibitor, TIMP2 (Table). No significant difference in the concentrations of pro-MMP1, TIMP1, TIMP2, and the complex MMP1/TIMP1 was observed between cells from the 2 groups, whereas a significant increase of pro-MMP2 was detected in fibroblasts from patients with varicose veins (Table). Quantification of the active form of MMP2 was therefore performed, but no significant difference was observed between cells derived from the 2 groups (9.78±1.17 ng/mL and 8.85±1.09 ng/mL, respectively; \( n = 8 \)).

**Discussion**
A dysregulation in collagen synthesis is described in varicose veins. \(^7,8\) Indeed, smooth muscle cells derived from varicose veins synthesized more collagen I, less collagen III, and similar quantities of collagen V. \(^7,8\) This imbalance might have consequences for the mechanical properties of the tissue. Because collagen I confers rigidity whereas collagen III is involved in the extensibility of a tissue, \(^9\) modification of the collagen I/III ratio might contribute to the weakness and the
pressed in fibroblasts derived from patients with varicose veins. With a reduction of mRNA expression, whereas collagen I and III synthesis was performed in dermal fibroblasts cultured from the skin of control subjects and patients with varicose veins (B). Cells from control subjects (white) and patients with varicose veins (black) were cultured at confluence, and supernatants were collected. The production of N-terminal (PINP) and C-terminal (PICP) propeptides, the C-terminal telopeptide (ICTP) of procollagen I (A), and the N-terminal propeptide (PINP) of procollagen III (B) was quantified by radioimmunoassays. Data are the mean±SEM of 8 experiments in each group. *P<0.05 for varicose veins vs control.

The reduction of collagen III synthesis without a variation of mRNA expression that was observed in both cell models for the patients with varicose veins could be due, at least in part, to an enzymatic degradation of the protein in the extracellular compartment. The similar level of aminoterminal propeptides of procollagen III found in both cell types argues in favor of this hypothesis. The production of pro-MMP1 and the inhibitors TIMP1 and TIMP2 was similar in the 2 groups, whereas the concentrations of pro-MMP7, pro-MMP8, pro-MMP9, and pro-MMP13 were under the threshold for detection with the assay used, so that it is difficult to make conclusions about their possible implications. These results are comparable with those previously described for venous smooth muscle cells.7 However, the concentration of pro-MMP2 was increased in fibroblasts from patients with varicose veins, whereas no difference was found in the venous smooth muscle cells derived from control and varicose veins.7 The observation that fibroblasts proliferate more rapidly than venous smooth muscle cells8 could be in relation to this result. Even if the level of active MMP2 was similar, such an overproduction of pro-MMP2 in the fibroblasts derived from patients with varicose veins could have consequences on matrix remodeling. Indeed, the concentration of TIMP2 (the preferential inhibitor of MMP2) was lower than that of pro-MMP2, which could create the appearance of active enzyme, especially in fibroblasts of patients with varicose veins that synthesized more pro-MMP2. Fibrillar collagens, and particularly collagen III, can be a substrate for MMP2,22 and thus the higher pro-MMP2 synthesis could be implicated in the degradation of collagen III in cells from patients with varicose veins. However, because an overactivity of MMP2 was not detected, the possibility exists that other MMPs are involved in collagen III degradation.

The decreased collagen III content in cells derived from patients with varicose veins could be responsible for the augmentation of collagen I synthesis and expression. Indeed, the levels of collagen I and III are coregulated in fibroblasts,23 and the addition of exogenous collagen III to cultured smooth muscle cells from varicose veins decreases the synthesis of collagen I. The decreased content of collagen III in cells derived from patients with varicose veins may have a different cause than that observed in patients with Ehlers Danlos type IV syndrome. In these patients, the procollagen III seems to accumulate in the intracellular compartment, is hardly present in a stable triple-helical conformation, and is only modestly secreted.24 By contrast, the content of collagen III is decreased in cell layers and the media of cultured cells from patients with varicose veins and seems to be degraded outside the cells because the level of metabolites is equivalent to control cells. Because Ehlers Danlos type IV syndrome is also

**Table 1: Quantification of MMPs and TIMPs in Culture Media of Fibroblasts From Control Subjects and Patients With Varicose Veins**

<table>
<thead>
<tr>
<th></th>
<th>Pro-MMP1</th>
<th>Pro-MMP2</th>
<th>Pro-MMP7</th>
<th>Pro-MMP8</th>
<th>Pro-MMP9</th>
<th>Pro-MMP13</th>
<th>MMP1/TIMP1</th>
<th>TIMP1</th>
<th>TIMP2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, ng/mL</td>
<td>8.3±1.1</td>
<td>4431±602</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>11.3±2.9</td>
<td>1284±345</td>
<td>24.0±4.2</td>
</tr>
<tr>
<td>Varicose veins, ng/mL</td>
<td>7.7±1.1</td>
<td>8092±1398*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>13.3±2.4</td>
<td>1593±257</td>
<td>23.6±4.4</td>
<td></td>
</tr>
</tbody>
</table>

Fibroblasts were cultured and supernatants were collected. Production of MMPs and TIMPs was quantified by enzyme immunoassays. Data are expressed as mean±SEM; n=8 in each group. ND indicates not detected (less than the threshold for detection with the assay).

*P<0.05 for varicose veins vs control.
characterized by molecular heterogeneity, it is difficult to compare the biochemical events with those observed in the present study.

Previous studies have described phenotypic alterations of dermal fibroblasts derived from venous ulcers of patients with varicose veins. Venous ulcer fibroblasts have an impaired ability to synthesize collagen in vitro, especially under hypoxic conditions. However, these ulcer-derived fibroblasts synthesized normal levels of fibronectin, whereas immunocytochemical analysis showed that venous ulcers were deficient in fibronectin. This deficiency in fibronectin may be due to the degradation of the protein by proteases present in the ulcers. Indeed, mRNA and the protein expression of MMP1, MMP2 (total and active form), and TIMP1 are significantly increased in venous ulcers.

These findings indicate that venous ulcers are characterized by elevated matrix turnover, and the phenotypic modulations observed in fibroblasts derived from these venous ulcers can be correlated with inflammatory processes. In the present study, the biopsies were obtained from patients who did not present with venous ulcers, and thus the inflammatory process may be limited. The increase in pro-MMP2 production in fibroblasts derived from the “healthy” skin of patients with varicose veins might constitute a predisposition for venous ulcers in these patients.

Several studies have demonstrated an involvement of hereditary factors for the transmission of the varicose vein pathology and a congenital weakness of the venous wall. A genetic defect in the regulation of the composition of the extracellular matrix might participate to the pathogenesis of varicose veins. Such a genetic defect should affect different connective tissues, because the smooth muscle cells of blood vessels and fibroblasts of the dermis both derive from embryonic mesenchyme. The dermal tissue of subjects affected by primary uncomplicated varicose veins presents some differences in comparison with control skin. The dermal tissue was found to be thinner than that of control subjects, and a modification of the content of collagen unassociated with changes of other components of the dermal connective tissue could be demonstrated. These findings add evidence for a systemic biochemical defect of the extracellular matrix affecting the entire body structure and not only the varicose veins of the lower limbs. Moreover, these results are in agreement with clinical observations suggesting a diffuse impairment of the collagenic matrix because acrocyanosis, blue discoloration of the sclerae, juvenile nose bleeding, and primary hand osteoarthritis are associated with varicose veins. Alterations in tissue remodeling occur in varicose veins and must also occur in the skin of patients with varicose veins. The present study demonstrates that the phenotypic modifications of cultured smooth muscle cells derived from varicose veins are also found in cultured dermal fibroblasts of these patients. Thus, the present results may suggest that varicose veins are the expression of a systemic pathology of the connective tissue with a genetic cause. This defect can explain the weakness of varicose veins and the propensity to develop ulcers.

References

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