In vitro differences between smooth muscle cells derived from varicose veins and normal veins

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Objective: The theory of primary venous dilatation leading to secondary valvular incompetence and varicose vein formation has received more attention nowadays. Although many studies have investigated the role of the main components of the venous wall in the development of varicose veins, the leading cause remains unknown. The present study was designed to establish the role of smooth muscle cells (SMCs) of the tunica media on the pathogenesis of varicose veins by analyzing the phenotypic and functional differences between SMCs derived from varicose veins and normal veins.

Methods: SMCs were isolated and cultured from saphenous veins of patients with varicose veins and normal veins. Cell proliferation and migration rates were compared. Expression of phenotype-dependent markers and matrix metalloproteinase-2 (MMP) production were analyzed by immunoblotting. Total collagen synthesis was evaluated by measuring the radioactivity of L-[3, 4-³H]proline in the media and the cell layer.

Results: SMCs derived from varicose veins demonstrated increased proliferation (2-fold, $P < .01$), migration (3-fold, $P < .001$), MMP-2 production (3-fold, $P < .01$), and collagen synthesis (>2-fold, $P < .001$), with decreased expression of phenotype-dependent markers compared with SMCs derived from normal veins ($P < .05$).

Conclusion: SMCs derived from varicose veins are more dedifferentiated and demonstrate increased proliferative and synthetic capacity than SMCs derived from normal veins. These properties may contribute to the remodeling of the venous wall and the weakening of its antipressure capacity. (J Vasc Surg 2009;50:1149-54.)

Clinical Relevance: Primary varicose veins are one of the most common diseases of periphery veins. We provide evidence that the alteration of biologic behaviors in cultured smooth muscle cells derived from varicose veins may contribute to the development of varicose veins. Therefore, medications that can help to maintain a nonmobile state of smooth muscle cells will prevent the progress of the disease.

Primary varicose veins are a frequent and refractory disease of the peripheral veins. The elusive nature of varicose vein formation and its wide prevalence throughout the world make it necessary to understand the mechanisms involved in the pathogenesis of the disease.

It has been well recognized that both primary incompetent valves and primary venous wall weakness can lead to the formation of varicose veins. However, the theory of primary venous wall dilation resulting from venous wall weakness has received more attention nowadays.[1] What is the cause of venous wall weakness? A lot of research on the components of the venous wall, including collagen and elastic content and smooth muscle cells (SMCs) of the

tunica media, has been initiated to find the reasons,[2-11] but the answer remains unclear.

Vascular SMCs (VSMCs) are mature cells that maintain great plasticity. Previous studies on the pathology of varicose veins have shown that SMCs phenotypically appear transformed from a contractile to a synthetic state.[5-11] These studies, however, draw their conclusions through only one aspect of SMC behavior or histologic examination. The present study attempted to make a detailed in vitro comparison of SMC behavior, including proliferation, migration, collagen synthesis, matrix metalloproteinase 2 (MMP-2) production and the phenotypic dependent markers, to identify differences in phenotype and function between SMCs derived from normal veins and those derived from varicose veins. The aim of this work was to verify that differences do exist in the biologic behaviors of SMCs derived from normal veins and varicose veins. These differences may contribute to venous wall remodeling and weakness.

MATERIALS AND METHODS

Specimens. SMCs were isolated from two groups of human saphenous veins. Thirteen control veins were obtained from patients (7 men, 6 women; 58.7 ± 1.2 years (range, 52-68 years) undergoing coronary artery bypass grafting. Patients had no history or symptoms of varicose veins and no retrograde flow proven by duplex ultrasound (DU) examinations. Twelve varicose veins were obtained

from patients (6 men, 6 women; mean age, 57.5 ± 1.6 years; range, 50-69 years) who were undergoing saphenectomy by stripping. All patients were diagnosed with primary varicose veins, with reflux confirmed by DU studies. By the CEAP classification, these patients were all type 2 (C_2). The study was approved by the local ethics committee, and informed consent was obtained from each patient.

Cell cultures. Explants from the medial layer of two groups of saphenous veins were prepared as previously described.[5] Cells were grown in 25 flasks in Dulbecco modified Eagle medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS, Hyclone), L-glutamine (2 mmol/L), penicillin (10^5 U/L), streptomycin (100 μg/mL), and nonessential amino acids (100 μg/mL) at 37°C in a 95% air and 5% carbon dioxide atmosphere. The culture medium was changed every 3 days. Cell growth began within 7 to 14 days, and cells reached confluence after 4 weeks.

Cells were trypsinized, seeded at a density of 10,000/cm^2 (first passage) in culture flasks without precoating, and subcultured. Only cells from passages 0 to 2 were used. Before each experiment, the phenotypes of the SMCs were confirmed by immunofluorescence with a monoclonal antibody against human α-actin (clone ASM-1; Chemicon). All experiments were repeated a minimum of three times from SMCs of at least three different patients.

Assessment of cell proliferation. [^{3}H]-thymidine incorporation and cell counting were used in the assessment of cell proliferation as previously described.[12] Briefly, 1×10^4 cells/well were seeded onto 24-well plates with complete media and allowed to adhere for 24 hours. The medium was replaced by serum-free medium for 48 hours to achieve synchronous growth arrest. Then 10% FBS was added as a stimulator of proliferation. At 48 hours after stimulation, [^{3}H]-thymidine (1 mCi/mL; specific activity, 20 Ci/mmol) was added to the cells and they were incubated 24 hours.

The cells were then washed, fixed with 10% trichloroacetic acid (TCA), and lysed with 0.3M NaOH and 1% sodium dodecyl sulfate (SDS) solution. Reactivity was counted in a Beckman scintillation counter. For cell counting, 5×10^3 cells/well were seeded in 96-well plates and the cells were trypsinized. Viable cells were counted using a hemocytometer for a period of 8 days. The experiments were done in triplicate.

Scratch wound migration assay. Cell migration was measured by using a monolayer scratch injury assay, as described previously.[13] Confluent cultures of SMCs grown in 6-well plates were wounded with a 200-μL pipette tip and incubated in standard culture medium. After 48 hours of incubation, the cells that had moved into the wound area were quantified. Four counts were made at various points along each wound that had been photographed initially and marked. Migrated cell numbers were derived from the average of these fields in triplicates.

Western blotting. Protein extracts were obtained by disrupting the cells in lysis buffer (0.05 mol/L HEPES, 0.15 mol/L NaCl, 1% NP-40, 1 nmol/L $MgCl_2$, 1
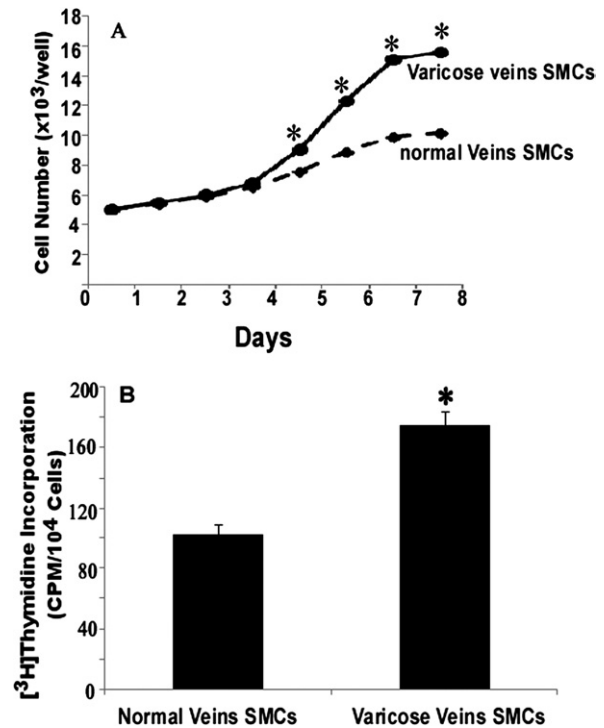

Fig 1. A, Cell proliferation was significantly higher (days 4 to 8) in smooth muscle cells *(SMCs)* derived from varicose veins. During days 7 to 8, cell numbers were 1.8-fold that of SMCs derived from normal veins. **B,** [^{3}H]-thymidine incorporation in SMCs derived from varicose veins was nearly twofold that of SMCs derived from normal veins. The *error bars* show the standard deviation. *$P < .01$.

mmol/L $CaCl_2$). Supernatants containing cytoplasmic proteins were collected and the protein concentration was determined by BCA method (Protein Assay Kit; Keygen Biotec, Nanjing, China). For each sample, 50-μg total protein was subjected to SDS-polyacrylamide gel electrophoresis on a 6% to 12% polyacrylamide gel. Separated proteins were electrophoretically transferred onto polyvinylidene fluoride membranes for immunodetection. After a blocking step with 5% non-fat milk in 0.02M Tris (0.05% Tween 20), membranes were incubated with primary antibodies diluted in blocking buffer, followed by horseradish peroxidase-conjugated appropriated secondary antibodies. Antigens were visualized by enhanced chemiluminescence. The bands on the film were quantified and normalized to β-actin.

Collagen synthesis assay. Because the major biosynthetic destination of proline is collagen, the incorporation of amino acid into newly synthesized proteins provides a reliable index of collagen synthesis. The amounts of radioactivity incorporated into soluble and insoluble protein fractions provide an assessment of collagen released into media (soluble protein) and that which was released but incorporated in and around the cell layer (insoluble protein).[14]
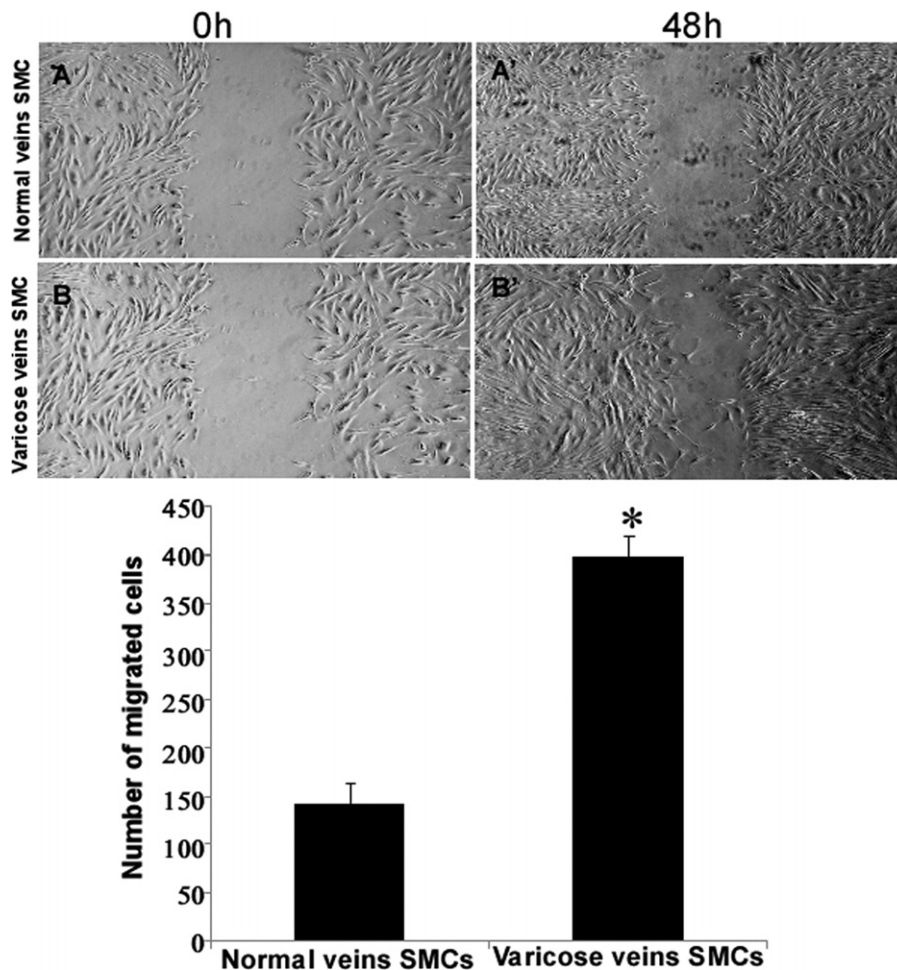
Fig 2. Representative result of wound healing assay shows a significantly higher migration in smooth muscle cells *(SMCs)* derived from varicose veins. The bar graph shows migration of SMCs derived from varicose veins was twofold higher than that of SMCs derived from normal veins. The *error bars* show the standard deviation. *$P < .001$.

For the L-[3, 4-^{3}H]proline experiments, culture media containing 50 μGi/mL L-[3,4-^{3}H]proline was added to each well. After 48 hours, the media were removed from the cells. TCA was added to the media to give a final concentration of 10% and left on ice for 1 hour. Precipitated protein was collected by centrifugation at $3000g$ for 30 minutes, washed with 4 mL ice-cold 10% TCA to remove any unincorporated labeled proline, and centrifuged again. The supernatant was carefully removed and the pellet suspended in 0.3 mL of 0.3M NaOH/0.3% SDS. The preparations were then warmed to 37°C until solubilized and added to 4 mL of liquid scintillant.

The cell layer was washed twice with PBS and removed from the plates by scraping into 1 mL of ice-cold 10% TCA. Precipitated protein was collected by centrifugation at $14,000g$ for 20 minutes. The cell layer precipitate was solubilized at 37°C for 1 hour in 0.3 mL of 0.3M NaOH/ 0.3% SDS and added to 4 mL of liquid scintillant. Radioactivity was counted in both the media and cell layer samples on a liquid scintillation analyzer.

Statistics. All experiments were performed at least three times, and values are expressed as mean ± standard deviation. Statistical significance ($P < .05$) was determined using independent samples t test.

RESULTS

Proliferation of SMC. Cell proliferation was significantly higher in varicose veins SMCs during days 4 to 7, with a cell number nearly 1.7-fold that of the number in SMCs derived from normal veins at the plateau phase (day 8; Fig 1, *A*). DNA synthesis was nearly twofold that of the value in varicose veins SMCs 48 hours after seeding compared with SMCs derived from normal veins (Fig 1, *B*).

Migration of SMC. Migration of SMCs derived from varicose veins was threefold that of SMCs derived from normal veins (Fig 2).

Expression of phenotype-dependent markers of vascular SMC. The expression level of contractile (differentiation) markers in VSMCs is a useful paradigm in the analysis of SMC phenotypic transition.[15] Varicose veins
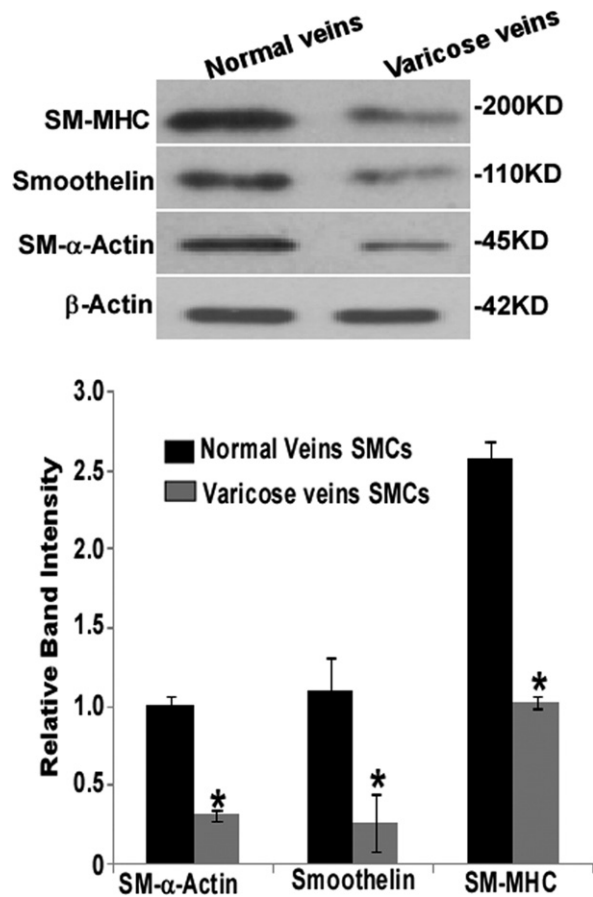
Fig 3. Expression of smooth muscle cell *(SMC)* phenotype-dependent markers in SMCs derived from normal veins and varicose veins. SMCs of the same passage were used for proteins extraction. Western blotting analysis of an equal amount of sample proteins showed decreased expression of α-actin, smoothelin, and smooth muscle myosin heavy chain *(SM-MHC)* in SMCs derived from varicose veins, which indicate a dedifferentiated phenotype alteration. Bar graphs indicate the relative ratios of the band intensity of detected proteins normalized to β-actin levels as loading control. *P < .01. The graphs are representative of three independent experiments. The results are means ± standard deviation *(error bars)*.

Fig 4. Collagen synthesis was significantly higher in smooth muscle cells (SMCs) derived from varicose veins both in the **(A)** cell layer and in the **(B)** media. *P < .001 compared with SMCs derived from normal veins. The *error bars* indicate the standard deviation.

derived SMCs demonstrated a more dedifferentiated phenotype with less α-smooth muscle (α-SM) actin, smooth muscle myosin heavy chain (SM-MHC), and smoothelin expression than SMCs derived from normal veins. Levels of α-SM actin, SM-MHC, and smoothelin were reduced to about 30%, 20%, 30%, respectively, of the values in cells derived from normal veins (Fig 3).

Collagen synthesis alteration in VSMC. The incorporation of L-[3,4-³H]proline into the cell layer and in the medium of varicose veins SMCs was significantly higher than into normal veins SMCs, more than twofold that of the SMCs derived from normal veins (Fig 4).

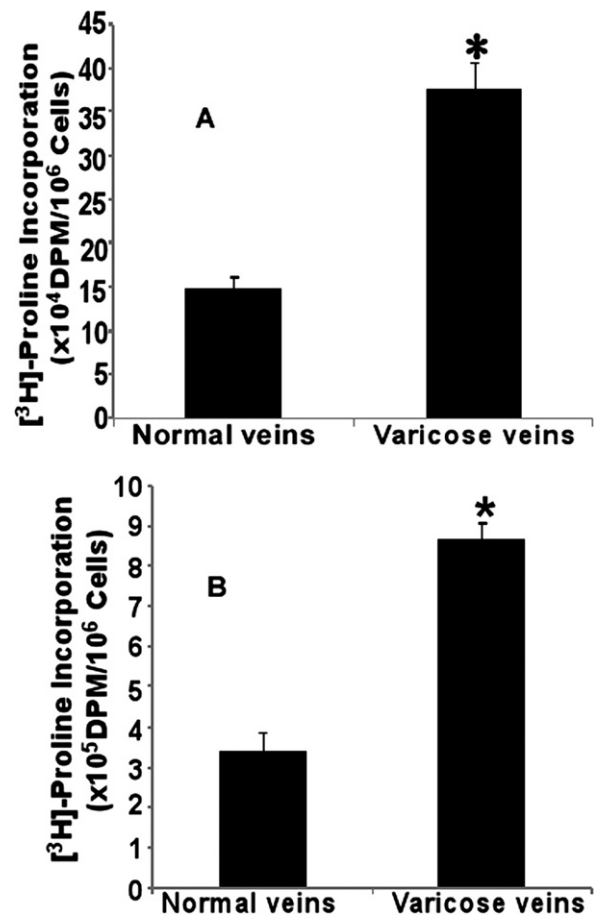**MMP-2 production in VSMCs.** Previous studies have shown that MMP-2 play a pivotal role in the regula-

tion and rearrangement of extracellular matrix components of the venous wall[16] and can precipitate varicose vein formation.[17,18] Consistent with a previous report,[8] we also found a significant higher production of MMP-2 in SMCs derived from varicose veins (Fig 5).

DISCUSSION

Varicose veins are a common vascular disorder of the lower extremity that affect 10% to 40% of people aged 30 to 70 years.[19] This study was designed to evaluate the role of SMCs in the pathogenesis of varicose veins.

The differentiation state of SMC has been implicated in various vascular pathologies such as atherosclerosis,[20,21] and intimal hyperplasia.[22,23] The principal function of mature SMCs is contraction. These cells have a high cytoplasmic volume fraction of myofilaments,[24,25] a low rate of proliferation,[26] and synthesize only small amounts of matrix proteins.[27]

Several studies have described phenotypic alterations of SMCs in varicose veins.[5-11] The electron microscopy find-
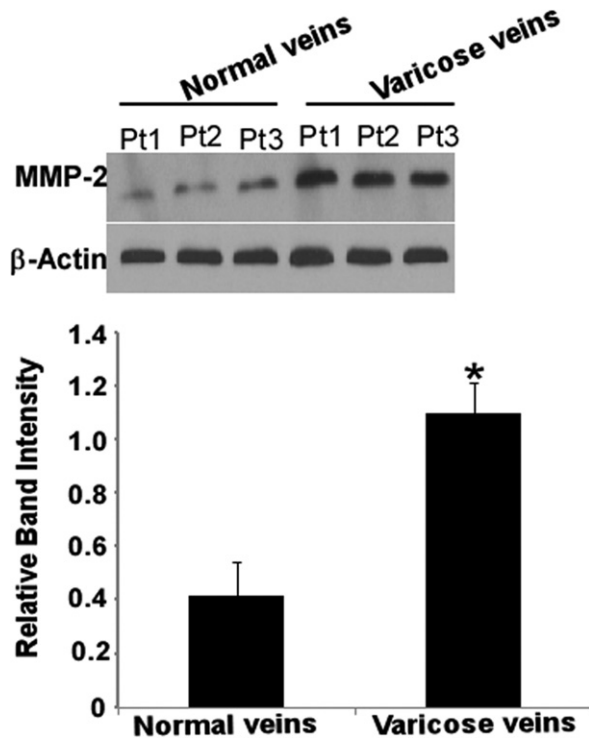
Fig 5. A, Representative Western blot for matrix metalloproteinase-2 *(MMP-2)* production shows MMP-2 production was significantly higher in SMCs derived from varicose veins than in SMCs derived from normal veins. **B,** The relative ratios of the band intensity of detected proteins normalized to β-actin are shown with the standard deviation *(error bars)*. *$P < .05$.

ings showed that SMCs in varicose veins were poorly differentiated, with an increase in secretory cytoplasmic organelles, which could reflect unusual possible synthetic and secretory roles of SMCs. The findings also showed a reduction in filament bundles, which could imply the decreased contractility of SMCs in varicose veins.[6] No available study has directly testified that there are phenotypic changes in varicose vein-derived SMCs, and these phenotypic alterations may contribute to the weakness of the venous wall.

In our study, we cultured the SMCs derived from varicose veins and normal veins and directly compared their biologic behavior. Our data showed that SMCs derived from varicose veins have significantly increased capabilities of proliferation, migration, and synthesis. This result can explain what we have seen in the hypertrophic portion of varicose veins, a high degree of cellularity both in the medial and in the thickened intima, which means SMCs have migrated to the intima and caused collagenization.

Our study also showed a decreased expression of SMC phenotype-dependent proteins, including α-SM actin, SM-MHC, and smoothelin. These are consistent with the characteristic of dedifferentiated phenotype of SMCs. Although we have not evaluated the contractility of SMC directly, the reduced levels of expression of VSMC contractile proteins can reflect the decreased contractility of SMCs derived from

varicose veins. Contractile properties of human saphenous smooth muscle are essential to maintain the required tone in vein wall.[28,29] Impaired contractility of SMCs with disturbance of extracellular matrix organization[5] may contribute to the primary weakness of venous wall.

Previous studies have investigated cultured SMCs derived from varicose veins to determine that the extracellular matrix modifications seen in varicose veins tissue are related to SMCs.[5,8] Increased expression of MMPs could induce degradation of extracellular matrix proteins and thus affect the mechanical properties of the vein wall. Recent evidence also suggests an effect of MMP-2 on SMCs of the vein wall, thereby causing changes in the venous constriction/relaxation properties.[17]

MMP-2 can be produced by SMCs, so it has raised an interesting question of whether SMCs derived from varicose veins can synthesize more MMP-2 than those from normal veins. Previous studies have shown that the plasma and venous tissue levels of MMP-2 are elevated in varicose veins.[8] Consistent with previous reports, our study found the SMCs derived from varicose veins demonstrated active synthetic capacity. They synthesized not only large amounts of collagen but also collagenase. The increased level of MMP-2 could induce not only degradation of collagen content[16] and migration of smooth muscle cells[30] but also venous relaxation by a mechanism involving hyperpolarization and activation of BKca,[17] which leads to progressive venous dilatation and formation of varicose veins.

Previous studies indicated that structural, biochemical, and functional changes in varicose tributaries and in nonvaricose veins from the same patient were similar,[31] which suggested that abnormalities within the vein wall exist before the varicosities develop and the defect of the vein wall may be the initial event of the disease. Our study results indicate that SMCs derived from varicose veins have particular intrinsic characteristics that may contribute to the primary defect of the vein wall. This study reinforced the notion that the imbalance in matrix production and degradation is intimately associated with the phenotype and function of SMCs. Our findings suggest that a genetic modification may have occurred in SMCs in patients with varicose veins.

CONCLUSIONS

Regardless of what caused these changes, our findings confirm the major role of SMCs, which could be ultimately responsible for the varicose process occurring in the vein wall because they are able to control both venous wall tone and extracellular matrix organization. Furthermore, this in vitro model can be used to explore the cellular and molecular mechanisms underlying the pathogenesis of varicose veins and to analyze the pathways involved in the SMCs phenotypic modulation, which may help develop new therapeutic strategies to prevent the progress of the disease.

AUTHOR CONTRIBUTIONS

Analysis and interpretation: YX, ZH
Data collection: YX, ZH, HY,YL

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