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Downregulation of desmuslin in primary vein incompetence

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Objective: **Primary vein incompetence is one of the most common diseases of the peripheral veins, but its pathogenesis is unknown. These veins present obvious congenital defects, and examination of gene expression profiles of the incompetent vein specimens may provide important clues. The aim of this study was to screen for genes affecting the primary vein incompetence phenotype and test the differential expression of certain genes.**

Methods: **We compared gene expression profiles of valvular areas from incompetent and normal great saphenous veins at the saphenofemoral junctions by fluorescent differential display reverse-transcription polymerase chain reaction (FDD RT-PCR). Differentially expressed complimentary DNAs (cDNAs) were confirmed by Northern blotting and semi-quantitative RT-PCR. Similarity of the cDNAs sequences to GenBank sequences was determined. Gene expression status was then determined by Western blot analysis and immunohistochemical techniques.**

Results: **There were >30 differentially expressed cDNA bands. Sequence analysis revealed that a cDNA fragment obviously downregulated in incompetent great saphenous vein was a portion of the messenger RNA (mRNA) encoding desmuslin, a newly discovered intermittent filament protein. Northern blotting and semi-quantitative RT-PCR analysis revealed a similar mRNA expression profile of the desmuslin gene in other samples. Western blotting and immunohistochemical techniques localized the desmuslin protein mainly in the cytoplasm of venous smooth muscle cells. The amount of desmuslin was greatly decreased in the smooth muscle cells of incompetent veins.**

Conclusions: **The expression of many genes is altered in primary vein incompetence. Up- or downregulation of these genes may be involved in the pathogenesis of this disease. Desmuslin expression is downregulated in the abnormal veins. Its effect on the integrity of smooth muscle cells might be related to malformation of the vein wall. Further studies are needed to investigate other differentially expressed cDNAs and the exact role of desmuslin in this disease.**

Clinical Relevance: **Primary vein incompetence is a frequent and refractory disease of the peripheral veins. Exploring its pathogenesis may enhance our comprehension and management of this disease. We used reliable techniques to detect disease-related genes and confirmed downregulation of desmuslin in abnormal veins. Alteration of these genes might be used as disease markers or gene therapy targets. (J Vasc Surg 2006;43:372-8.)**

Kistner[1] first described primary vein incompetence in 1980. It is a frequent disease of peripheral veins and the most common cause of chronic venous insufficiency, ultimately results in refractory ulceration, accounts for considerable morbidity, and consumes a large part of health-care resources. Approximately 60% of patients may exhibit femoral and great saphenous vein incompetence at the same time. Some hypotheses have been proposed to explain the mechanism of primary vein incompetence, such as the vein valve hypothesis and the vascular remodeling hypothesis.[2,3] Abnormal cellular and molecular alterations of, for example, extracellular matrix, smooth muscle cells, and some cytokines, were found associated with malformation of the incompetent veins in many studies.[4-8]

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By epidemiologic research, Carpentier and Priollet[9] noticed a genetic predisposition in some patients that indicated these patients might have had hereditary defects before they presented with venous malformation. Travers et al[10] also regarded heredity as an important factor for more than half of the patients with a positive family history, suggesting a congenital weakness of the vein wall.

In this study, we used the fluorescent differential display reverse-transcription polymerase chain reaction technique (FDD RT-PCR) to look for genes involved in the pathogenesis of primary vein incompetence.

MATERIALS AND METHODS

Patient selection and sample preparation. Twelve patients who had normal vein valves but presented reflux in both the femoral and great saphenous vein, diagnosed by duplex sonography scanning and descending venography, were recruited to the patient group. None had a history of venous thrombosis. According to the CEAP classification, the patients were classified as $C_{2\text{-}4}E_PA_{S,D}P_R$. An additional nine patients who were going to receive coronary bypass and had neither femoral vein nor great saphenous vein reflux diagnosed by duplex sonography scanning were recruited to the control group. The first valvular areas of the great saphenous veins (away from the common femoral

Table I. Sequences of primers used in fluorescent differential display*

Anchored primers
TMR-$T_7(dT_{12})AP_1$-5′*ACGACTCACTATAGGGC*TTTTTTTTTTTTGA3′,
TMR-$T_7(dT_{12})AP_2$-5′*ACGACTCACTATAGGGC*TTTTTTTTTTTTGC3′
TMR-$T_7(dT_{12})AP_3$-5′*ACGACTCACTATAGGGC*TTTTTTTTTTTTGG3′
TMR-$T_7(dT_{12})AP_4$-5′*ACGACTCACTATAGGGC*TTTTTTTTTTTTGT3′
Randomized primers
M_{13}r-ARP_{18}-5’ *ACAATTTCACACAGGA*TGATGCTACC3′,
M_{13}r-ARP_{19}-5’ *ACAATTTCACACAGGA*TTTTGGCTCC3′,
M_{13}r-ARP_{20}-5’ *ACAATTTCACACAGGA*TCGATACAGG3′

*The italic sequences are primer T_7 and M_{13}

vein) in the two groups were harvested during saphenectomy or coronary bypass. Each sample was divided into two segments—one for histologic study, and the other was immediately frozen and conserved in liquid nitrogen.

Differential display and complimentary DNA retrieval. Total RNA was extracted in Trizol reagent (Gibco BRL, Gaithersburg, Md) following the manufacturer's instructions and further treated with RNase-free DNase I (Promega BRL, Madison, Wis) to remove residual genomic DNA. A260/A280 of the total RNA samples varied from 1.9 to 2.0. Concentrations of all the RNA samples were adjusted to 1.0 μg/μL.

Using a series of 2-base-anchored oligo(dT_{12}) primers (the Genomyx HIEROGLYPH mRNA Profile Kit and Genomyx HIEROGLYPH DD Kit, Genomyx Corporation, Foster City, Calif), we subdivided the messenger RNA (mRNA) population and synthesized the complimentary DNAs (cDNAs). Four anchored primers and three randomized primers were used (Table I).

After mixing 2.0 μL of total RNA (1.0 μg/μL) and 2.0 μL anchored primer (50 μM) and heating the mixture at 70°C for 5 minutes, 2.0 μL of 2'-deoxyribonucleoside 5'-triphosphate (dNTP) (200 μM, 1:1:1:1), 2.0 μL of dithiothreitol (DTT) (10 mM), 4.0 μL of 5× SuperScript II RT buffer, 0.1 of μL SuperScript II RT (20 U/μL), and 7.1 of μL distilled water were added. The mixture was incubated at 42°C for 10 minutes, at 50°C for 60 minutes, and at 70°C for 15 minutes.

The PCR system included 2.0 μL of cDNAs, 0.7 μL of the relevant anchored primer (5 μM), 1.75 μL of randomized primers (2 μM), 2.0 μL of dNTP (200 μM, 1:1:1:1), 1.5 μL of magnesium chloride (25 mM), 1 μL of 10× PCR buffer, 0.25 μL of AmpliTaq DNA polymerase (5 U/μL), and 0.8 μL of distilled water. Light mineral oil was overlaid.

After denaturation at 94°C for 5 min, PCR reaction was carried out for four cycles at 94°C for 30 seconds, 50°C for 40 seconds, and 72°C for 2 minutes, followed by 30 cycles at 94°C for 30 seconds, 55°C for 40 seconds, and 72°C for 2 minutes. PCR products then were separated on 6% DNA sequencing gel containing 7M urea. The gel was dried without fixing and screened in a GenomyxSC scanner (Beckman-Coulter, Fullerton, Calif).

Differentially expressed cDNAs were identified by Image Quant software (Molecular Dynamics, Sunnyvale, Calif). Corresponding gel bands were excised from the dried gel. Each gel slice was and incubated in 50 μL of distilled water, then boiled for 15 minutes to release the cDNAs. The cDNAs were precipitated by ethanol in the presence of 0.3M sodium acetate and 2.5 μL of 10 mg/mL glycogen, redissolved in 20 μL of distilled water, and then reamplified under PCR conditions as used in differential display; the anchored and randomized primers were replaced by T_7 and M_{13} universal primers. PCR products were purified using Qiagen PCR Purification Kit (Hilden, Germany).

Northern blotting confirmation. A portion of the purified cDNAs were labeled with [α-^{32}P]2'-deoxycytidine-5'-triphosphate (dCTP) to prepare probes according to the manufacturer's instructions. Thirty micrograms of total RNA of each sample, which had been used in differential display experiments, was electrophoresed on 1.5% agarose gel containing 6% formaldehyde and 1× morpholinopropanesulfonate (MOPS) buffer (20 mM of MOPS, 5 mM of sodium acetate, and 1 mM of ethylenediaminetetraacetic acid).

The RNAs were then transferred to a nylon membrane in 10× standard saline citrate (SSC) buffer and incubated at 80°C for 2 hours to crosslink the RNA and the membrane. cDNA probes then hybridized to the mRNAs on the membranes in buffer containing 50% formamide, 5× SSC, 5× Bernhardt's solution, 0.5% sodium dodecylsulfate (SDS), 10% dextran sulfate, and 100 μg/mL denatured salmon sperm at 50°C for 20 hours in a rotating hybridization oven (Tyler HI-16000, Edmonton, Alberta, Canada). After hybridization, the membranes were washed twice with 2× SSC and 0.1% SDS for 10 minutes, and twice with 0.1× SSC and 0.1% SDS for 30 minutes at room temperature.

The hybridization signals were visualized with chemiluminescence and recorded on x-ray film. The exposure time was 30 minutes. Hybridization signals were analyzed with a PhosphorImager scanning system (Molecular Dynamics/Amersham Biosciences/Amersham, Piscataway, NJ) and ImageQuant software (Molecular Dynamics, Sunnyvale, Calif).

Cloning and sequencing of differentially expressed cDNAs. Reamplified cDNAs were cloned into pGEM-Teasy vector (Promega BRL, Madison, Wis) after QiaexII gel (Qiagen) purification and transferred to *Escherichia coli* DH5α. After ampicillin selection and proliferation, the plasmids were extracted and digested by *Eco*RI enzyme. Positive clones were sequenced using SP_6 primer. cDNA sequences were analyzed for matches in the GenBank database via the BLAST program.

Semi-quantitative RT-PCR. To identify whether the differentially expressed cDNA that matched desmuslin mRNA in sequence presented the same expression profile in other vein samples, semi-quantitative RT-PCR was done with specific primers. The human β-actin gene was used for

Table II. Primer sequences used in semi-quantitative reverse-transcription polymerase chain reaction analysis

		Sequence	*Product length*
β-actin	forward	5′-GTGGGGCGCCCCAGGCACCA-3′	500 bp
	reverse	5′-CTTCCTTAATGTCACGCACGATTTC-3′	
Desmuslin	sense	5′-CTTGGCAGGATGAAATAG-3′	300 bp
	Antisense	5′-AACCAGGGTCACGGAACT-3′	

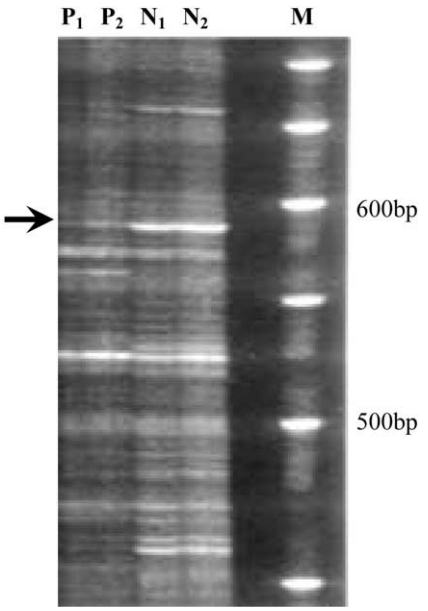

Fig 1. mRNA differential display (with primer pair AP_3 and ARP_{18}). P_1, P_2, N_1, N_2, and M represent two incompetent veins, two normal veins, and DNA marker, respectively. The P_{24} cDNA fragment is marked with the *black arrow* on the left.

the intrinsic reference. The sequences of these primers are listed in Table II.

Total RNA isolation was by the same procedure as previously described. Reverse transcriptions were performed by using oligo(dT_{17}) nucleotide. Exactly 2.0 μL of total RNA (1.0 μg/μL) and 1.0 μL of oligo(dT) primer (0.5 μg/μL) were incubated together at 70°C for 5 minutes. Then 2.0 μL of dNTP (200 μM, 1:1:1:1), 2.0 μL of DTT (10 mM), 4.0 μL of 5× SuperScript II RT buffer, 0.1 μL of SuperScript II reverse transcriptase (20 U/μL), and 8.9 μL of distilled water were added. The mixture was incubated at 37°C for 1 hour. The PCR system included 1.0 μL of cDNAs, 0.2 μL of sense and antisense β-actin primers, 4.0 μL of dNTP (200 μM, 1:1:1:1), 4.0 μL of magnesium chloride (25 mM), 5.0 μL of 10× PCR buffer, 0.25 μL of AmpliTaq DNA polymerase (5U/μL), and 33.35 μL of distilled water. Light mineral oil was overlaid.

After the PCR reaction was done for 20 cycles at 94°C for 30 seconds, 50°C for 40 seconds, and 72°C for 2 minutes, 1.0 μL of specific sense and antisense primers were added, and the reaction went on for 25 more cycles at 94°C for 30 seconds, 52°C for 40 seconds, and 72°C for 2 minutes. PCR products were then electrophoresed on 1.0% agarose gel and analyzed by using ImageQuant software.

Western blotting analysis. Equal amounts of vein samples from the two groups were homogenized in lysis buffer (20 mM of 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid [HEPES], pH 7.2; 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 150 mM of sodium chloride, 10 μg/mL of leupeptin, 10 μg/mL of aprotinin, and 1 of mM phenylmethylsulfonyl fluoride) and centrifuged. The supernatants were separated on 8% SDS polyacrylamide gel electrophoresis and blotted onto a nitrocellulose transfer membrane (Schleicher & Schuell Bioscience, Keene, NH). The membranes were probed with antidesmuslin monoclonal antibody (kindly provided by Yuji Mizuno) diluted 1:1000 in blocking solution.

The reaction was visualized by labeling with goat anti-rabbit immunoglobulin G secondary antibody (Sengon, Shanghai, China). The peroxidase reaction was developed with Amersham ECL reagents (Amersham Biosciences). After imaging, the membranes were stripped and reprobed by using monoclonal anti-β-actin peroxidase antibody as the primary antibody (Sigma, St. Louis, Mo).

Immunohistochemical study. Paraffin-embedded vein tissues were cut into 5 μm sections, deparaffinized in xylene, and rehydrated through a series of alcohol and water. The slides were placed in 10 mmol/L citrate buffer (pH 6.0) and microwaved for 15 minutes to enhance antigen exposure. The sections were first incubated in hydrogen peroxide (30 mL/L) for 10 minutes to quench endogenous peroxidase activity and then washed in phosphate-buffered saline (PBS) (pH 7.6). They were incubated with PBS containing normal goat serum for 30 minutes, followed by incubation with primary rabbit antihuman desmuslin antibody (1:200 dilution) for 30 minutes at 37°C. Sections were then incubated with goat anti-rabbit secondary antibody for 30 minutes before they were reacted with diaminobenzidine solution. On the basis of the intensity and the number of cells stained, *expression of desmuslin* was defined as moderate-to-strong staining affecting more than 30% of the slice.

Statistical analysis. The results were expressed as mean ± SD. The significance was determined by the *t* test or nonparametric test; $P<.05$ was considered statistically significant. All analyses were performed using SPSS 13.0 software (SPSS, Inc, Chicago, Ill).

RESULTS

Messenger RNA differential display. Screening the mRNA profile under primer AP_3 and ARP_{18} revealed an

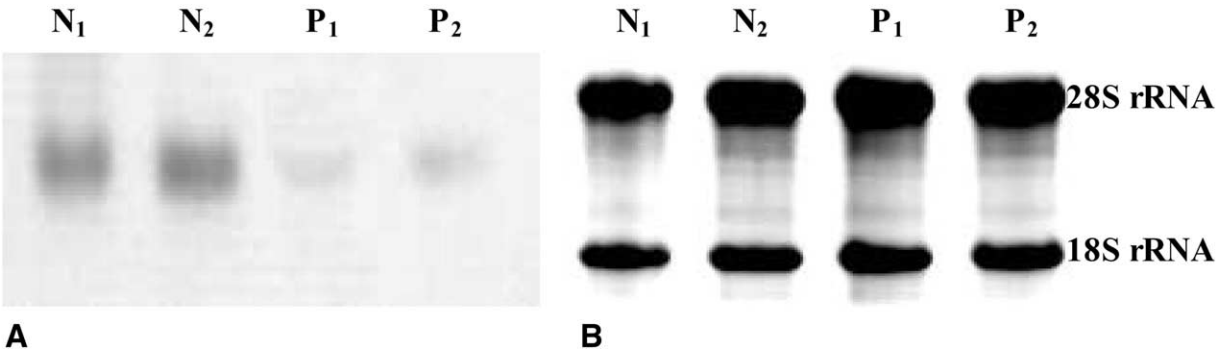

Fig 2. Northern blotting confirmation of differential expression of P_{24}. P_1, P_2, and N_1, N_2 represent two incompetent and two normal veins, respectively. A, Shows that the expression of P_{24} cDNA is much weaker in the incompetent veins than in the normal veins (normalized to the expression of the corresponding 28S rRNA as shown in B). This result is consistent with the result of differential display.

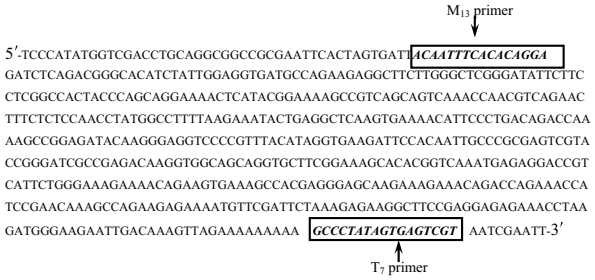

Fig 3. Sequence of P_{24} cDNA. T_7 and M_{13} universal primers at the two ends are indicated by *arrows*.

approximately 590-bp cDNA, which appeared much weaker in incompetent veins, as shown in Fig 1. The cDNA was named P_{24}.

Northern blotting. Differential expression of P_{24} cDNA was confirmed by Northern blotting analysis (Fig 2, *A*), which indicated that expression of the corresponding mRNA was at a very low level in the incompetent veins. Fig 2, *B* shows the abundance of 18S and 28S rRNA. The relative signal intensities (Fig 2, *A)*compared with those of the corresponding 28S rRNAs (Fig 2, *B)*were submitted to a *t* test; patient group, 0.06 ± 0.016 vs control group, 0.24 ± 0.039 ($P < .001$, 95% confidence interval [CI], 0.1478, 0.2089).

Sequence analysis. The nucleotide sequence analysis revealed that the P_{24} fragment consisted of 585 bases. As shown in Fig 3, the oblique bold bases near the 5′ and 3′ ends were the M_{13} and T_7 universal primers. Comparison with GenBank database sequences via the BLAST program revealed that P_{24} cDNA was a portion of the desmuslin mRNA.

Semi-quantitative RT-PCR. Results indicated that downregulation of desmuslin was also present in other patients with primary vein incompetence. This result agreed with those of the mRNA differential display and Northern blotting. The average relative integrated optical density values of desmuslin bands, normalized to the value of the corresponding β-actin (patient group, 0.19 ± 0.047; control group, 0.83 ± 0.082), were submitted to a *t* test ($P < .001$, 95% CI, 0.576, 0.710) (Fig 4).

Western blotting analysis. Desmuslin levels were significantly lower in the incompetent veins than the normal veins (the intensity of each desmuslin band was normalized to that of the corresponding β-actin band). Average intensities of the bands for the patient (0.25 ± 0.062) and control (1.03 ± 0.130) groups were submitted to a *t* test ($P < .001$, 95% CI, 0.687, 0.868) (Fig 5).

Immunohistochemical study. Moderate-to-strong expression of desmuslin was observed in 83.7% ± 6.2% smooth muscle cells of normal vein specimens, whereas only 21.2% ± 8.3% smooth muscle cells in incompetent veins presented a weak-to-moderate expression. The data were submitted to nonparametric test, $Z = -3.202$, $P = .001$. Desmuslin was localized in the cytoplasm of venous smooth muscle cells (Fig 6).

DISCUSSION

Using the FDD RT-PCR technique[11,12] in the present study, we discovered quite a few differentially expressed genes related with primary vein incompetence. The presence of differentially expressed cDNAs suggests that the expression of many genes is changed in response to the pathophysiologic circumstances of primary vein incompetence. Whether these differentially expressed genes are involved in the pathogenesis of this disease remains to be investigated.

In this study, we confirmed downregulation of the desmuslin gene in the incompetent vein walls. The desmuslin gene, located on chromosome 15q26.3,[13] encodes a newly discovered protein belonging to the intermediate filament protein (IF) family.[14] Sequence analysis suggests that desmuslin contains an IF signature, indicating that desmuslin may be a structural protein.[15] Of the various IF proteins, desmuslin is most similar to synemin, paranemin, tanabin, and nestin in terms of domain structure.[16-19] Desmuslin is classified as a type 6 IF protein. There are at least two distinct desmuslin subpopulations, one that inter-

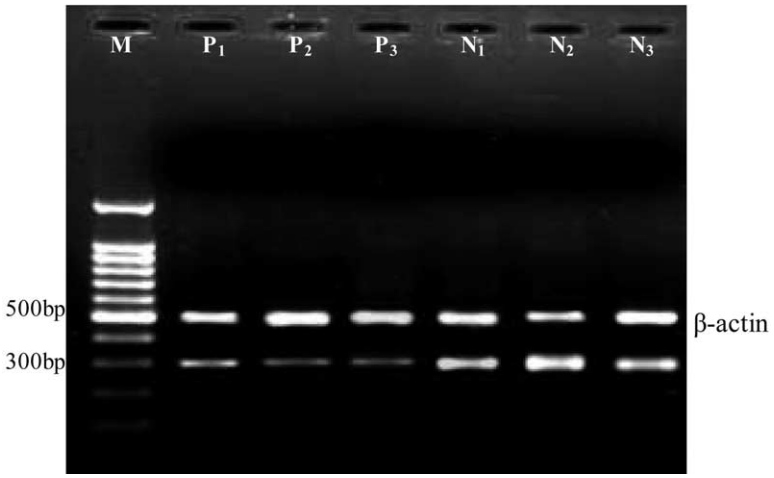

Fig 4. Semi-quantitative reverse-transcriptase polymerase chain reaction analysis of desmuslin mRNA in incompetent veins. *N* and *P* represent the normal and incompetent veins, respectively; *M* is the 100-bp DNA ladder. The figure shows that expression of desmuslin was also downregulated in other patients.

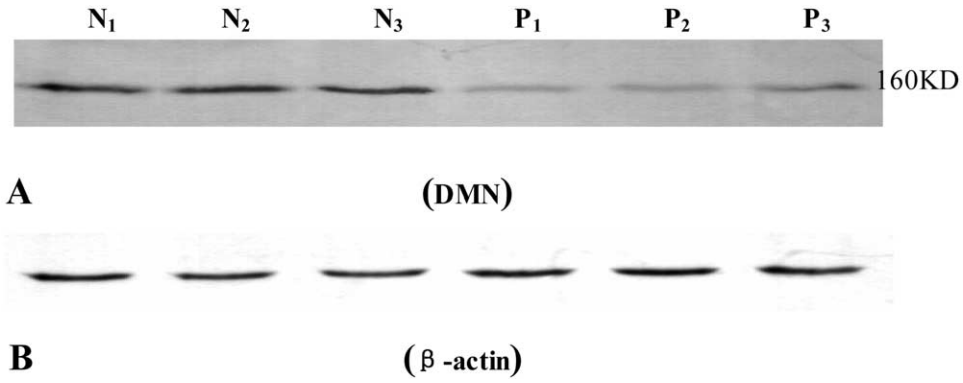

Fig 5. Western blotting analysis of desmuslin expression profile in normal and abnormal veins. A, Shows desmuslin expression. B, shows β-actin expression. *N* and *P* represent normal and abnormal veins, respectively. Desmuslin signals were much weaker in the patient group than in the control group.

acts exclusively with desmin within the Z-lines, and the other that interacts with both dystrobrevin and desmin at the costamere.

IF proteins such as desmuslin and desmin are thought to help maintain the structural integrity of tissues by mechanically reinforcing protein connections. Desmin encircles Z-lines and then makes longitudinal filamentous bridges between neighboring Z-lines. These bridges are supported by plectin and crystallin.[20] Plectin links desmin to the Z-discs and desmin to itself.[21]

Mutations in the desmin gene can cause desmin myopathies characterized by muscle weakness, cardiac impairment, and intracytoplasmic accumulation of desmin deposits.[22-25] Desmuslin forms a linkage between a component of dystrophin-associated protein complex (α-dystrobrevin) and a component of the Z-lines (desmin), thus connecting the extracellular matrix to the Z-discs. Laminin, the extracellular matrix protein laminin that interacts with α-dystroglycan, can extend this linkage to the extracellular matrix.[26] Disruption of any of the proteins in this linkage could potentially damage muscle cell integrity. We discovered that desmuslin expressed in venous smooth muscle cells is greatly downregulated. This decline in desmuslin may disturb the integrity of venous smooth muscle cells.

Many studies have confirmed that smooth muscle cells are important for the maintenance of venous tone. The "weak wall with secondary valvular incompetence" theory has been proposed as the most likely cause of primary vein incompetence.[27] Venturi et al[28] considered the primary defect could be in the smooth muscle cells of the vein wall, which are responsible for the synthesis and catabolism of both collagen and elastin. Rose and Ahmed[29] suggested that separation of muscle cells by fibrous infiltration prevented them from acting as a unified whole, with subsequent alterations in wall tone leading to pathologic dilatation. Wali and Eid[30] found that the smooth muscle fibers

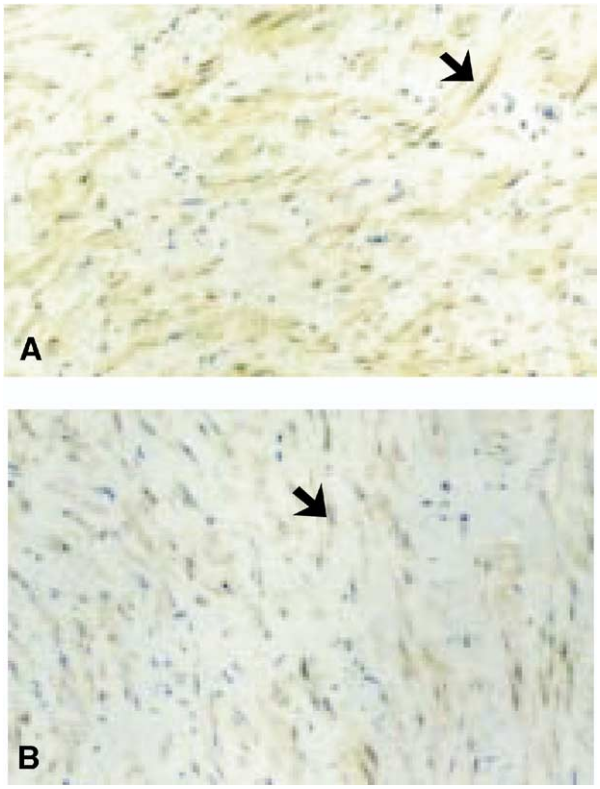

Fig 6. Immunohistochemical analysis of desmuslin in vein walls. A, Specimen of normal vein (×200 magnification); B, Specimen of incompetent vein (×200 magnification). Signals are abundant and intense in smooth muscle cell cytoplasm of normal veins but infrequent and weak in incompetent veins (as shown by *black arrows*).

degenerated, broke down into fragments, or else disappeared completely in incompetent vein wall. Disorganized muscular structure invaginated and separated into the layers containing increased amounts of collagen.

The separation and disruption of muscle cell fibers may cause diminished tone and vein wall dilatation, as "effective contraction cannot occur unless individual cells are in communication with each other."[30] Relaxation of the muscle cells, gradual decay of the muscle cells, and decreased tensile strength and elasticity of the connective tissue fibers are direct reasons for the dilatation and lateral blowouts of veins.[31] Taken together, these and our results suggest that the downregulation of desmuslin in the vein wall may be responsible for smooth muscle cell change and for weakness and dilatation of the vein wall.

CONCLUSION

We propose that downregulation of desmuslin, probably together with defects or deficits in other structural proteins, may result in venous smooth muscle cell malformation, which in turn, contributes to the dilatation of the vein wall and secondary valve incompetence. So far, we have no certain proof what the initial accident is, how these relative molecules interact with each other, and how they influence the function of the vein. The mechanism needs further investigation.

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AUTHOR CONTRIBUTIONS

Conception and design: JW, SW
Analysis and interpretation: XZ, HY
Data collection: HY
Writing the article: HY, XZ
Critical revision of the article: HY, XZ, JW, SW
Final approval of the article: HY, XZ, JW, SW
Statistical analysis: XZ
Obtained funding: SW
Overall responsibility: SW

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