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Identification of Differentially Expressed Genes in Human Varicose Veins: Involvement of Matrix Gla Protein in Extracellular Matrix Remodeling

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Key Words
Gene expression · Suppressive subtractive hybridization · Extracellular matrix · Matrix Gla protein · Varicose vein · Vascular smooth muscle · Proliferation · Mineralization

Abstract
This study was designed to identify the global pattern of differentially expressed genes in human varicose veins. Using suppressive subtractive hybridization, we identified overexpression of genes known to be associated with extracellular matrix remodeling, including collagen III, tissue inhibitor of metalloproteinases I, dermatopontin, matrix Gla protein (MGP) and tenascin C. Real-time polymerase chain reaction analysis confirmed the differential expression of these genes. The overexpression of MGP transcript was associated with increased MGP level in varicose veins, in particular the undercarboxylated form of the protein. Smooth muscle cells from varicose veins showed increased proliferation rate and enhanced matrix mineralization. This observation correlated with the presence of ectopic mineralization areas in the varicose vein walls. The use of warfarin, to inhibit MGP activity, or siRNA targeting MGP transcript induced a reduction in the exacerbated proliferation of varicose vein smooth muscle cells. Our results suggest that high expression of MGP in varicose veins may contribute to venous wall remodeling by affecting proliferation and mineralization processes probably through impaired carboxylation of MGP. In addition, suppressive subtractive hybridization results also produce a profile of differentially expressed genes in varicose veins, in particular extracellular matrix components. Further study of these genes will provide insights into their specific roles in the etiology of venous disease.

Introduction

Superficial venous dysfunction of lower limbs leads to the development of varicose veins, the most common manifestation of chronic venous insufficiency (CVI). CVI is characterized by intraluminal venous hypertension with blood stagnation and venous reflux. The exact mechanisms by which varicosis occurs are still unclear although several risk factors are known to be involved, including genetic predispositions, increasing age, obesity, physical activity, standing occupations, multiple pregnancies and connective tissue abnormalities [1–3].

Dilatation, elongation and tortuosity of the varicose veins provide evidence for venous wall remodeling. Sev-

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eral experimental and clinical studies suggest that defects in the venous wall may be regarded as the primary cause of varicosis leading to secondary valvular incompetence [4, 5]. Modifications in the media layer of the venous wall appear as general features of varicose veins. These modifications include ultrastructural signs of smooth muscle transition from a contractile to a metabolic phenotype, disturbed smooth muscle cell (SMC)/extracellular matrix (ECM) balance and loss of contractile properties of the venous wall [6–11]. ECM in the vascular wall provides an essential structural framework necessary for the maintenance of vascular integrity and cellular regulations such as tissue-specific gene expression, movement, differentiation and cell shape [9]. Numerous studies have investigated involvement of ECM molecules and enzyme inhibitors in varicose vein development. Defects in elastin metabolism [4], dysregulation of collagen synthesis and expression [12–14], abnormal collagen to elastin ratio and loss of the regular collagen/elastin lattice of the vein wall [13, 15] have been shown to be involved in the pathogenesis of varicose veins. Imbalance between tissue inhibitors of metalloproteinases (TIMP) and matrix metalloproteinase (MMP) production, in favor of an antiproteolytic activity, has also been described as a possible pathway to facilitate ECM accumulation in the diseased venous wall [16].

This study aims at defining the global pattern of differentially expressed genes in human varicose vein SMC in order to identify new genes involved in vein wall dysfunction and remodeling. We used the suppressive subtractive hybridization (SSH) approach, a polymerase chain reaction (PCR)-based method of subtractive cloning, to select genes differentially expressed in varicose veins [17]. Although a large number of genes was isolated using this strategy, we focus on those known to be associated with ECM remodeling in vessels. Our results suggest that high expression of the calcification inhibitor matrix Gla protein (MGP) in varicose veins may contribute to venous wall remodeling by affecting proliferation and mineralization processes.

Materials and Methods

Tissue Samples

Lengths of normal human saphenous vein were removed from male patients (n = 36; age range 30–83 years) undergoing coronary artery bypass grafts.

Varicose human saphenous veins were obtained from male patients (n = 50; age range 40–81 years) undergoing surgical removal of varicose veins. In the operating room, tissues were placed in a cold physiological solution (see Solutions for its composition) and rapidly transported to the laboratory. All procedures followed were in accordance with institutional guidelines. Veins were then cleaned of adherent connective tissue and the endothelium was carefully removed by gently rubbing the intimal surface with the tip of small forceps. The vessels were then prepared for SMC primary culture preparation and/or rapidly frozen in nitrogen for RNA and protein extractions. For paraffin embedding, rings cut from each vein were fixed in formalin (3%).

SMC Culture

Venous media was cut into small pieces (1 × 1 mm) which were placed into collagen-precoated culture dishes in SMBM (Biowhittaker, Vallensbaeck Strand, Denmark). The medium was changed every 2 or 3 days. When cells reached confluence, subculture was obtained by harvesting the cells with 0.2% ethylenediaminetetraacetic acid and 0.25% trypsin. Cells were characterized using a monoclonal antibody against smooth muscle α-actin (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) and used at passages 2–4.

RNA Preparation

Total RNA was extracted separately from each vein using TRIzol reagent (Life Technologies SARL, Cergy-Pontoise, France) and genomic DNA contamination was eliminated with ribonuclease-free deoxyribonuclease I (Life Technologies). Absence of DNA contamination was verified by PCR using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers. For preparations of subtracted cDNA libraries, 2 pools of total RNA from nonvaricose (n = 27) or varicose veins (n = 25) were performed and poly(A)$^+$ RNA were isolated using an Oligotex™ mRNA midi kit (Qiagen, Courtaboeuf, France). RNA quantity was checked photometrically by absorption at 260 nm and RNA quality was assessed by migration on a 1% agarose gel and by capillary electrophoresis on an Agilent 2100 Bioanalyzer.

Generation of cDNA Libraries Using SSH

Subtracted cDNA libraries were prepared using the PCR-Select™ cDNA subtraction kit (Clontech, Saint-Quentin-en-Yvelines, France) according to the manufacturer's recommendations. We achieved the forward SSH to isolate genes upregulated in varicose veins with poly(A)$^+$ RNA (1 μg) from varicose and nonvaricose veins as tester and driver samples, respectively, and vice versa for the reverse SSH resulting in the isolation of genes downregulated. RsaI-digested tester cDNA was ligated to 2 different adaptators and hybridized twice to a 4-fold excess of driver cDNA to enrich for differentially expressed genes which were amplified by 2 rounds of PCR. The SSH cDNA libraries were cloned into the T/A cloning vector pCR®2.1-TOPO® (Invitrogen, Cergy-Pontoise, France) and subsequently transformed into TOP10 One Shot® Electrocomp™ cells (Invitrogen). Two libraries were created, referring to the SSH PCR products, each containing 1,170 clones. We amplified the cDNA insert by PCR (Gene Amp® PCR System 9700; Applied Biosystems, Foster City, Calif., USA) using M13 standard sequences flanking the vector cloning site. The PCR products (0.4–1.5 kb) were purified by filtration using MultiScreen® PCR plates (Millipore, Bedford, Mass., USA) and resuspended in 3× saline-sodium citrate. The 2,340 cDNA probes thus prepared were then used to construct a DNA microarray.

Table 1. The primer sequences used for the real-time RT-PCR analysis

Gene Name	Sense (5′–3′)	Antisense (5′–3′)	Product, bp
Collagen I	ACCAGCGATACCAGGCAGAC	CATGGAAACCGTGGTGAAACT	186
Collagen III	AGACCAGGAGTACCAGCAGCA	CAAAGGAGACACAGGACCCC	288
TIMP I	AGCCCTTTTCAGAGCCTT	TATCCATCCCCTGCAAAC	78
Dermatopontin	GGAAGCAGAGAATGGTCAGAGAA	AAGAACTCCTGAGCCACACACA	260
MGP	CGTTCGCAAAGTCTGTAGTCATC	CCTTCATATCCCCTCAGCAGA	129
Tenascin C	CACGCCAGGTATGAGTT	GCGATCCCAGACAGTCAG	230
GAPDH	AGTTAAAAGCAGCCCTGGTGA	GACAGTCAGCCGCATCTTCTT	118
Phosphoprotein P0	GAAGGGGGAGATGTTGAGCA	AAGTCACTGTGCCAGCCCA	191

Microarray Analysis

To test clones derived from the 2 subtracted cDNA libraries, we spotted the 2,340 cDNA probes on glass slides coated with poly-L-lysine (Sigma-Aldrich, Saint-Quentin-Fallavier, France) with the Eurogentec SDDS/C200 robot (Eurogentec SA, Seraing, Belgium). We hybridized 5 distinct pools of total RNA, each constituted with equal quantity of RNA extracted from varicose veins of 5 male patients (25 patients in total; age 51.7 ± 7.3 years), versus a reference pool of total RNA constituted with equal quantity of RNA from nonvaricose veins (11 patients; age 57.2 ± 11.3 years). We reverse transcribed and fluorescent labeled RNA (25 μg) from nonvaricose and varicose veins using the Cyscribe first-strand labeling kit (Amersham Pharmacia Biotech, Orsay, France) with Cy3- and Cy5-dUTP dyes, respectively. To avoid unspecific DNA binding, human cot-1 DNA (Invitrogen), yeast tRNA and poly(dT) were added to the mixed Cy3- and Cy5-labeled probes before hybridization. We performed hybridization during 14–16 h at 60°C in a hybridization cassette (Telechem International, Sunnyvale, Calif., USA).

The 5 microarrays were scanned using the ScanArray 3000 (GSI Lumonics). Measurements were obtained for each fluorochrome at a resolution of 5 μm/pixel. Fluorescent intensity values and ratios for each cDNA array were analyzed using GenepixPro 4.0 software (Axon Instruments, Union City, Calif., USA). Individual clones were classified as consistently expressed when spot labeling intensity was ≥1.5-fold the average background and all clones with defective signal (spot deformation, dust) were flagged and not further analyzed.

We submitted raw filtered data to a linear regression normalization on invariant genes (ratio between 0.66 and 1.5) present on the microarrays to compensate for moderate differences commonly observed between Cy3 and Cy5 signals. We considered genes as differentially expressed when their normalized ratio indicated a variation of at least 1.5-fold.

One hundred and ninety-two differentially expressed clones with at least 2-fold variations were sequenced from plasmid DNA using M13 reverse primer (Qiagen Sequencing Services, Hilden, Germany). Sequence files were screened against the GenBank nucleotide database using the BLASTN algorithm. The identified genes were classified into various biological process or molecular function groups using eGOn version 1.0, a tool for genomic data linked to gene ontology (http://nova2.idi.ntnu.no/egon/).

Validation of Differentially Expressed Genes by Real-Time Reverse Transcription PCR

The differentially expressed genes involved in the ECM functional group were selected and analyzed by real-time PCR on the iCycler iQ™ detection system (Bio-Rad SA, Marnes-la-Coquette, France) using SYBR green as fluorescent probe (Molecular Probes, Eugene, Oreg., USA). Gene-specific primers were designed to amplify fragments between 118 and 288 bp. PCR primers (listed in table 1) were synthesized by Sigma-Genosys Ltd. (Cambridge, UK). Total RNA samples (1 μg) from nonvaricose or varicose veins of 8 individual patients (minimum) were reverse transcribed into cDNA by the M-MLV reverse transcriptase (Life Technologies) and amplified as described previously [18]. A dilution range of the produced cDNA from an RNA pool of nonvaricose veins was added to 25 μl quantitative PCR reactions containing 0.5 μl Titanium™ Taq DNA polymerase (Clontech), 300 nM gene-specific primers or reference housekeeping gene primers, 300 nM of dNTPs and SYBR green (1:25,000). Amplifications were performed in triplicate and started with an initial step of 3 min at 95°C, followed by 40 cycles each consisting of 15 s at 95°C and 1 min at 60°C. The standard curve so obtained was used to check that PCR efficiencies were identical between the interest and reference genes. For data analysis, the fluorescent signals were normalized using 2 reference housekeeping genes, GAPDH and phosphoprotein P0, and the same results were obtained. Relative quantification, following the ΔΔCt method [19] was applied to compare amounts of mRNA in nonvaricose and varicose veins. Similar approaches were applied with total RNA extracted from nonvaricose or varicose SMC in primary culture (8 cultures, minimum). The average of gene expression ratios in nonvaricose conditions was normalized to 1. The average of gene expression ratios in varicose veins was expressed relatively to nonvaricose conditions.

Western Blot Analysis

Frozen pieces of saphenous vein were homogenized with a polytron in ice-cold RIPA buffer. The protein extracts were obtained and analyzed as described previously [10]. Nuclei and unlysed cells were removed by 15,000 *g* centrifugation for 20 min at 4°C. The protein concentration of the supernatent was determined using a Bio-Rad protein assay. Similar amounts of total proteins were diluted with Laemmli sample buffer and separated on 16.5 % polyacrylamide/sodium dodecyl sulfate gel for MGP detection. After transfer to nitrocellulose membrane, amounts of

proteins were checked by staining with Ponceau red. After blocking, the membrane was then incubated with α 3-15 MGP antibody (2 μg/ml) in blocking buffer [20, 21] and revealed with horseradish peroxidase-conjugated rabbit anti-mouse IgG (32 ng/ml; Immunotech, Marseille, France). Immunoreactive proteins were detected by enhanced chemiluminescence detection procedure (Amersham Pharmacia Biotech) and quantified using Quantity-One (Bio-Rad, Hercules, Calif.,USA). Equal loading was checked by reprobing the membrane with ERK 1,2 antibody (Santa Cruz Biotechnology).

Cell Proliferation Assay

Cell proliferation was determined by counting and 5-bromodeoxyuridine (BrdU) incorporation assay. Nonvaricose and varicose SMC were cultured as described above. Cells (5×10^{-4}/well, passage 4) were seeded in 6-well plates and made quiescent by incubation with serum-free media for 24 h. Quiescent cells were treated by warfarin to block MGP activity or by specific siRNA to block MGP translation. Inhibition of MGP activity was tested by incubating cells in DMEM containing 0.5% FCS (negative proliferation control) or 10% FCS (positive proliferation control) with or without warfarin (10 and 30 μM; Sigma-Aldrich). After 48 h, cell proliferation under each condition was determined either by counting after trypsinization or by incorporation of BrdU, determined after labeling and detection with alkaline phosphatase assay according to the manufacturer's instructions (Roche Applied Science, Indianapolis, Ind., USA).

Matrix Gla Protein siRNA

Knockdown of MGP was investigated by the siRNA approach. The sequences for siRNA were designed and synthesized by the manufacturer (Eurogentec SA). Human MGP-specific sequences of siRNA were compared with sequences in the human genome database to confirm that no other gene was targeted. The oligonucleotides encoding the MGP siRNA were 5′-GCCUGUGAUGACUACAGACdTdT-3′ and 5′-GUCUGUAGUCAUCACAGGCdTdT-3′. A universal scrambled siRNA was used as control (Eurogentec SA). Cells were incubated in DMEM containing 0.5% FCS (negative proliferation control) or 10% FCS (positive proliferation control) or 10% FCS and the polycation-based vector Jet-PEI™ (4.5 μg/ml; Qbiogene, Illkirch, France) with MGP siRNA or scrambled siRNA (4 μg/ml). After 48 h, the cells were trypsinized and counted or stained to revealed BrdU incorporation. For studies that examined mineralizing activity, siRNA treatments were performed when cells reached confluence and mineralization process was evaluated by von Kossa staining after 7 days of culture. All experiments were repeated at least 3 times and at least 4 different nonvaricose or varicose SMC primary cultures were tested.

Histochemistry and Immunohistochemistry of Venous Tissue and SMC Cultures

For histological analysis of venous tissue, 4-μm sections of paraffin-embedded veins were used. Hematoxylin and eosin staining was used for general histological evaluation and calcification was demonstrated by von Kossa staining. Positive or negative calcification staining was evaluated for 15 nonvaricose (patients aged 69.8 ± 1.8 years) and 12 varicose veins (patients aged 54.5 ± 4.5 years).

Immunostaining of the undercarboxylated MGP (GluMGP) with anti-GluMGP antibody or the carboxylated MGP (GlaMGP) with anti-GlaMGP was performed as described previously [22]. SMC primary cultures were seeded in 12-well plates at 25×10^{-4} cells/well in SMBM (Biowhittaker). For studies that examined mineralization activity, when confluence was reached, cells were incubated with or without warfarin (10 and 30 μM) to inhibit MGP activity, with or without vitamin K (3 and 10 μM) to induce MGP activity, and with or without β-glycerophosphate (10 mM) to induce mineralization process. After 7 days of culture, cells were fixed in 4% paraformaldehyde for 30 min at room temperature and von Kossa staining was performed as previously described [23].

Solutions

Physiological solution contained (in mmol/l) : 130 NaCl, 5.6 KCL, 1 $MgCl_2$, 2 $CaCl_2$, 11 glucose and 10 HEPES, brought to pH 7.4 with NaOH. RIPA buffer used in Western blot experiments had the following composition (in mol/l): 9.1 Na_2PO_4, 1.7 $NaHPO_4$, 150 NaCl and 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 10 g/l PMSF, adjusted to pH 7.4 with NaOH.

Statistical Analysis

All results are expressed as means ± SEM with n denoting the sample size. Significance of reverse transcription (RT)-PCR ratio, Western blot and cell proliferation studies was tested by the Student t test and the one-way ANOVA completed by the Fisher test when appropriate. Positive calcification staining between nonvaricose and varicose veins was statistically analyzed using the χ^2 test. Probabilities less than 5% ($p < 0.05$) were considered as significant. Statistical analysis of microarray data was used to assess significance. We used the significance analysis of microarrays method (http://www-stat.stanford.edu/~tibs/SAM) with a Δ value for cutoff significance corresponding to the lowest false-positive rate.

Results

Isolation and Identification of Differentially Expressed Genes

In order to isolate potential determinants of varicose disease, we applied SSH to mRNA extracted from nonvaricose and varicose veins. The subtraction was performed in both directions to generate both up- and down-regulated gene libraries in varicose veins. The screening of the subtracted clones (2,340) by hybridization of DNA arrays with 5 distinct pools of total RNA extracted from varicose veins led to the identification of 542 differentially expressed clones (≥1.5-fold, 23%), 283 clones from the upregulated library and 259 clones from the downregulated library. After sequencing of the 96 more differentially expressed clones (48 from each library) and elimination of redundant fragments of the same cDNA, 34 sequences were identified corresponding to 28 genes

Table 2. Differentially expressed genes after cDNA array analysis of varicose versus nonvaricose veins

Category and gene name	Accession No.	Mean ratio (n = 5)
Binding		
Cystein and glycin-rich protein 1	NM_004078	2.26 ± 0.15
Cell death		
Clusterin	BC010514	2.09 ± 0.18
Cell Growth		
N-Myc downstream-regulated gene 2	NM_201540	2.13 ± 0.13
C-fos	M16287	0.26 ± 0.03
Cell organization		
β-Actin	BC016045	2.32 ± 0.10
Gelsolin	BC026033	2.2 ± 0.28
Tropomyosin α-chain	AK057989	2.05 ± 0.23
Destrin	NM_006870	2.00 ± 0.14
γ-Actin	NM_001615	1,80 ± 0.04
ECM		
Cystatin C	NM_000099	2.4 ± 0.38
Dermatopontin	NM_001937	2.34 ± 0.14
Collagen III	NM_000090	2.3 ± 0.23
TIMP I	M59906	2.1 ± 0.12
MGP	M55270	2.1 ± 0.14
Tenascin C	X56160	2.09 ± 0.12
Metabolism		
NADH dehydrogenase subunit L4	AA934440	2.26 ± 0.16
Cytochrome b	NM_003001	2.25 ± 0.26
Cytochrome c oxidase subunit III	CK905578	2.24 ± 0.15
DC48 mRNA	AF271776	2.22 ± 0.20
ATP synthase 6	M73031	2.14 ± 0.11
Cytochrome c oxidase subunit I	BQ272451	2.13 ± 0.17
Pyruvate kinase	BC023592	1.93 ± 0.20
Protein phosphatase 1 regulatory subunit 12B	BC034430	0.51 ± 0.03
Monoamine oxydase B	BC022494	0.48 ± 0.05
Response to external stimuli		
CD74 antigen	BC024272	2.5 ± 0.30
Complement C1S component precursor	AK055183	2.30 ± 0.15
Interferon-induced transmembrane protein 2	BC009696	2.21 ± 0.3
Ferritin heavy chain	M11146	2.13 ± 0.22
Follistatin-like 1	NM_007085	2.13 ± 0.31
Selenoprotein P	BC040075	2.00 ± 0.05
Structural molecule activity		
Ribosomal protein S19	BC018616	0.50 ± 0.05
Ribosomal protein L13	BC020804	0.47 ± 0.05
Ribosomal protein L12	NM_000976	0.46 ± 0.06
Transport		
Glucocerebrosidase/propin 1	XM_375810	2.00 ± 0.20

from the upregulated library and 6 from the downregulated library (table 2). Four sequences were not found in the public databases.

Major functional classes of identified genes, transcriptionally regulated in varicose disease, included 9 metabolism genes (26.4%), 6 genes associated with ECM organization or regulation (17.6%), 6 genes involved in response to external stimuli (17.6%), 5 cell organization genes (14.7%), 3 genes with structural molecule activity (8.8%), 2 cell growth genes (5.8%), 1 gene related to binding function (2.9%), 1 cell death gene (2.9%) and 1 gene related to transport function (2.9%; table 2).

Vessel wall remodeling of varicose veins is characterized by disturbance of SMC/ECM organization. The

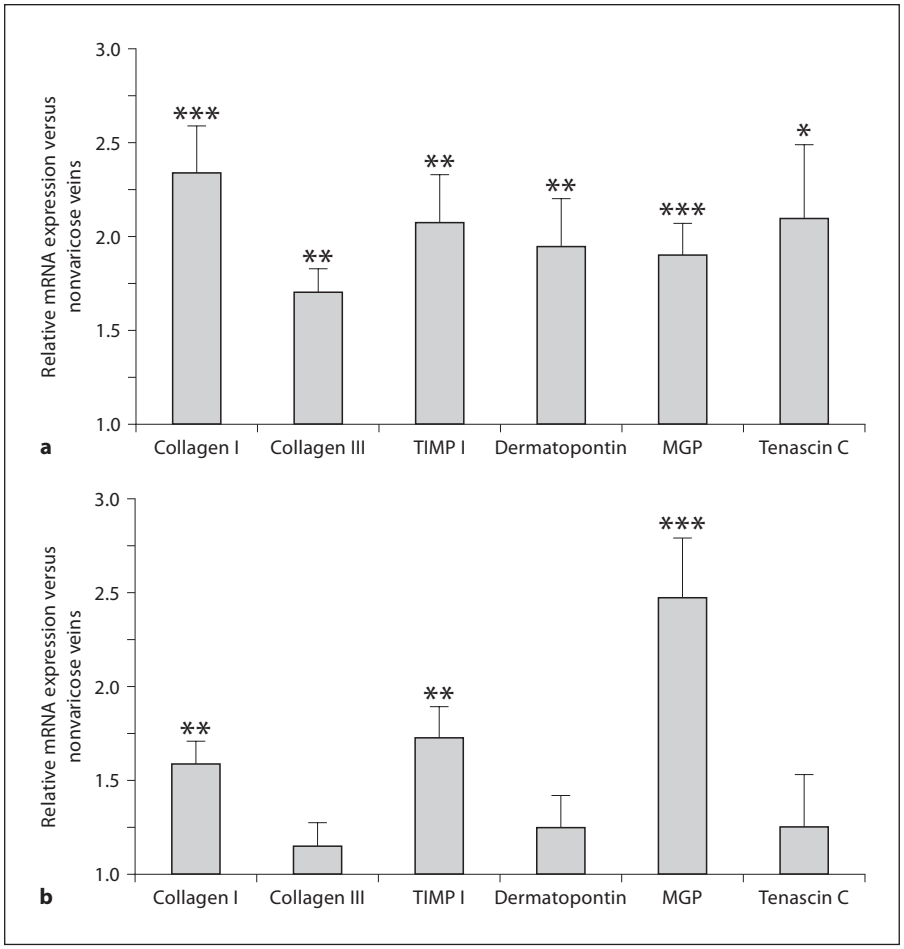

Fig. 1. Relative quantitative RT-PCR expression of selected genes involved in ECM organization or regulation in varicose vein (**a**) and SMC primary culture (**b**). The bar graphs show the percentage of variation in varicose versus nonvaricose conditions. Data are means ± SEM of 8–13 patients or 8–11 primary cultures. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus ribosomal phosphoprotein P0.

group of genes involved in ECM organization or regulation, found to be overexpressed by microarray analysis, contains genes previously characterized in varicose veins (collagen III, TIMP I) and genes for which no relationship to the CVI development was known (MGP, tenascin C, cystatin C and dermatopontin).

Analysis of Genes Involved in ECM Organization or Regulation by Real-Time PCR

The 6 upregulated genes associated with ECM organization or regulation were analyzed by real-time RT-PCR in individual total RNA extracts (n = 8–13) of nonvaricose and varicose veins. The genes encoding for human collagen III, TIMP I, MGP, tenascin C, cystatin C and dermatopontin were analyzed in nonvaricose and varicose tissue, and in SMC primary culture (fig. 1). The expression of collagen I gene, known to be upregulated in varicose disease was used as positive control [24]. In RNA samples from venous tissue (n = 8–13), overexpression of collagen I (2.33 ± 0.25), collagen III (1.70 ± 0.12), TIMP I (2.06 ± 0.26), dermatopontin (1.94 ± 0.26), MGP (1.9 ± 0.16) and tenascin C (2.08 ± 0.39) genes compared to RNA samples from nonvaricose veins was confirmed (fig. 1a). Expression of cystatin C gene appeared similar in nonvaricose and varicose RNA samples (1 ± 0.13 and 1 ± 0.15, respectively; $p = 0.91$; n = 11; data not shown). Other RT-PCR experiments confirmed the downregulation of monoamine oxydase B gene and overexpression of follistatin-1, β-actin and ferritin heavy chain genes detected by the microarray analysis (data not shown).

Primary cultures of SMC from varicose veins are often used as experimental model for the venous disease [10, 12, 25]. The expression of ECM genes upregulated in varicose veins was therefore also analyzed in primary cultures of SMC from varicose veins. Overexpression of collagen I (1.59 ± 0.12), TIMP I (1.73 ± 0.17) and MGP (2.47 ± 0.31) was confirmed ($p < 0.01$, n = 10). In contrast,

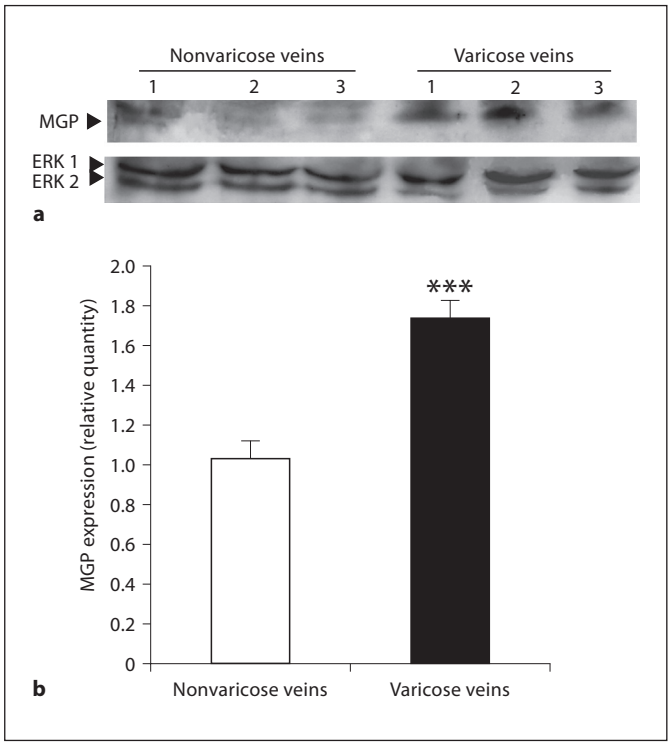

Fig. 2. Expression of MGP in human saphenous veins. **a** Western blot analysis of MGP in 3 representative samples of nonvaricose and varicose veins. ERK 1,2 expression was used to check the amount of protein in each lane. **b** MGP level was normalized to ERK 1,2 and expressed relative to that of nonvaricose veins as 1. Data are means ± SEM. *** $p < 0.001$; n = 9.

collagen III, dermatopontin and tenascin C gene expression (n = 8–11) did not significantly differ in SMC from nonvaricose and varicose veins (fig. 1b).

MGP Expression Is Upregulated in Varicose Veins

To investigate whether the change in MGP mRNA levels observed in microarray and real-time PCR experiments led to alterations in protein level, Western blot analysis was performed in nonvaricose and varicose veins (fig. 2). Western blots showed an immunoreactive band at the expected size for MGP (14 kDa; fig. 2a). The expression level of MGP was significantly higher in varicose veins than in nonvaricose veins (1.74 ± 0.09, $p < 0.0001$, n = 9; fig. 2b).

MGP Affects Venous SMC Number and Proliferation in vitro

To further examine the effect of MGP on the vasculature, experiments were performed in primary cultured SMC in vitro. First, we characterized the increase in nonvaricose and varicose SMC number in response to addition of 10% FCS medium. As shown in figure 3a, the increase in varicose SMC number was significantly higher than that in nonvaricose SMC. To assess a potential role of MGP overexpression in the increase in proliferation rate of varicose SMC, we then used warfarin to inhibit vitamin K-dependent γ-carboxylation of MGP, which is essential for its biological activities. Warfarin (10 μM) significantly decreased the number of nonvaricose SMC (48.1 ± 11.7%, n = 4, $p < 0.01$). Higher warfarin concentration had no significant further effect (fig. 3b). In contrast, treatment with 10 and 30 μM warfarin induced a dose-dependent reduction in the increase in varicose SMC numbers, reaching 90.1 ± 7.2% (n = 4, $p < 0.001$) in the presence of 30 μM warfarin (fig. 3b). Proliferation rate of nonvaricose and varicose SMC was investigated by BrdU incorporation analysis. In agreement with cell counting, proliferation rate of varicose SMC was significantly higher in varicose SMC (80.8 ± 0.4% of stained cells, n = 6, $p < 0.001$) than in nonvaricose SMC (49.5 ± 0.9% of stained cells, n = 6; fig. 3c). Warfarin (10 or 30 μM) significantly decreased the proliferation rate of nonvaricose SMC (35.3 ± 1.5%, n = 4, $p < 0.01$) and varicose SMC (70.2 ± 1.3%, n = 4, $p < 0.01$; fig. 3c).

This result suggests that although the warfarin-dependent component of serum-induced increase in SMC number was higher in varicose than in nonvaricose cultures, the warfarin-sensitive component of SMC proliferation was equal in varicose and nonvaricose SMC. Finally, we used MGP siRNA to selectively inhibit MGP transcript expression. Figure 3d shows a significant reduction in MGP mRNA expression level in both nonvaricose and varicose SMC following MGP siRNA treatment during 48 h (44.9 ± 6.7% and 69.2 ± 2.6%, respectively; n = 5; $p < 0.01$). MGP siRNA-mediated MGP knockdown was associated with a significant reduction in serum-induced increase in cell number in both nonvaricose and varicose SMC (20.2 ± 4.1% and 49.76 ± 3.8%, respectively; n = 5; $p < 0.01$; fig. 3e). This result was associated with a significant reduction in proliferation rate of nonvaricose and varicose SMC treated by MGP siRNA (fig. 3f).

The MGP siRNA-sensitive component of serum-induced increase in cell number was significantly higher in varicose than nonvaricose SMC ($p < 0.001$), but the MGP siRNA-dependent proliferation rates were similar between nonvaricose and varicose SMC.

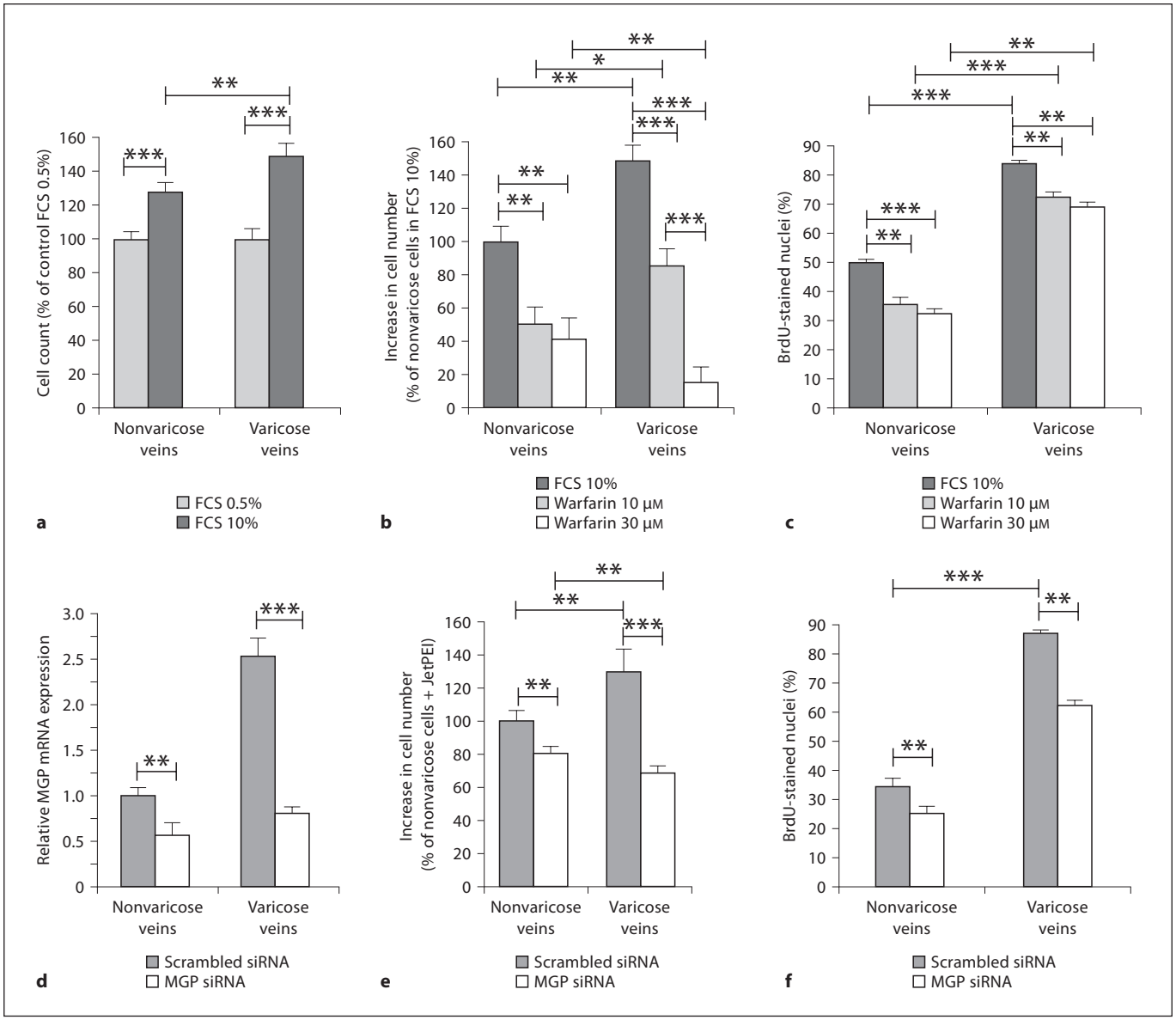

Fig. 3. Effect of warfarin and MGP siRNA on human saphenous SMC proliferation. **a** Cell number estimated in nonvaricose and varicose SMC maintained in 10% FCS medium for 4 days. **b** Increase in number of nonvaricose and varicose SMC maintained in 10% FCS medium for 4 days with or without 10 or 30 μM warfarin. **c** Percentages of BrdU-stained nuclei in nonvaricose and varicose SMC maintained in 10% FCS medium for 4 days with or without 10 or 30 μM warfarin. **d** Expression level of MGP mRNA in nonvaricose and varicose SMC cultured in 10% FCS medium with scrambled siRNA (control) or MGP siRNA. **e** Increase in number of nonvaricose and varicose SMC maintained in 10% FCS medium with scrambled siRNA (control) or MGP siRNA. **f** Percentages of BrdU-stained nuclei in nonvaricose and varicose SMC maintained in 10% FCS medium with scrambled siRNA (control) or MGP siRNA. Data are means ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n = 5.

ECM Mineralization Process in Varicose Veins and Differential Expression of Carboxylated and Undercarboxylated MGP Forms

Physiological functions of MGP have been previously associated with ECM calcification process in vessels [26–28]. We thus investigated whether a mineralization process was present in sections of 15 nonvaricose and 12 varicose veins stained with von Kossa. Representative photomicrographs shown in figure 4a demonstrate clear mineralization of ECM areas in the media of a varicose

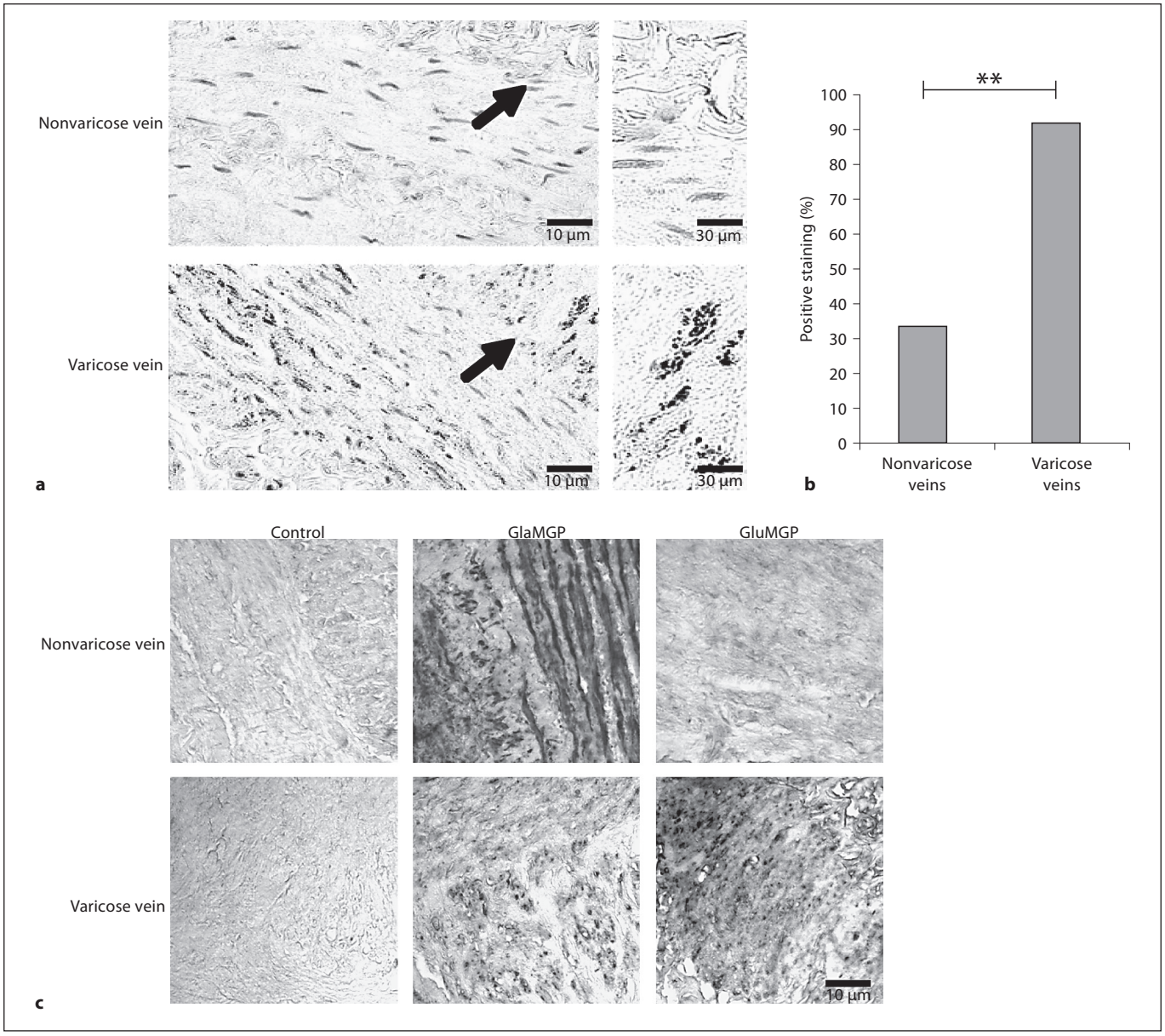

Fig. 4. Mineralization process and immunostaining of carboxylated and undercarboxylated MGP forms in varicose veins. **a** Representative histological sections of nonvaricose and varicose veins stained with von Kossa protocol. Mineralization deposits can be seen throughout the media of varicose vein. Black arrows show the localization of the enlarged areas displayed on the right. **b** Histogramm showing the percentage of positive von Kossa staining in nonvaricose (n = 15) and varicose veins (n = 12). ** $p < 0.01$. **c** Immunohistochemical staining of nonvaricose and varicose veins. Nonvaricose and varicose vein sections were stained with horse anti-mouse IgG (negative control), GlaMGP or GluMGP antibodies.

vein compared to the absence of such deposits in the media of a nonvaricose vein. The presence of mineral deposits was detected in the media of 92% of varicose veins and only in 33% of nonvaricose veins ($p < 0.01$) (fig. 4b).

Because only GlaMGP has calcification-inhibitory activity [29, 30], we used conformation-specific antibodies to localize GlaMGP and GluMGP species in nonvaricose and varicose vein sections. Immunohistochemical localization of GlaMGP was detected in the media of nonvar-

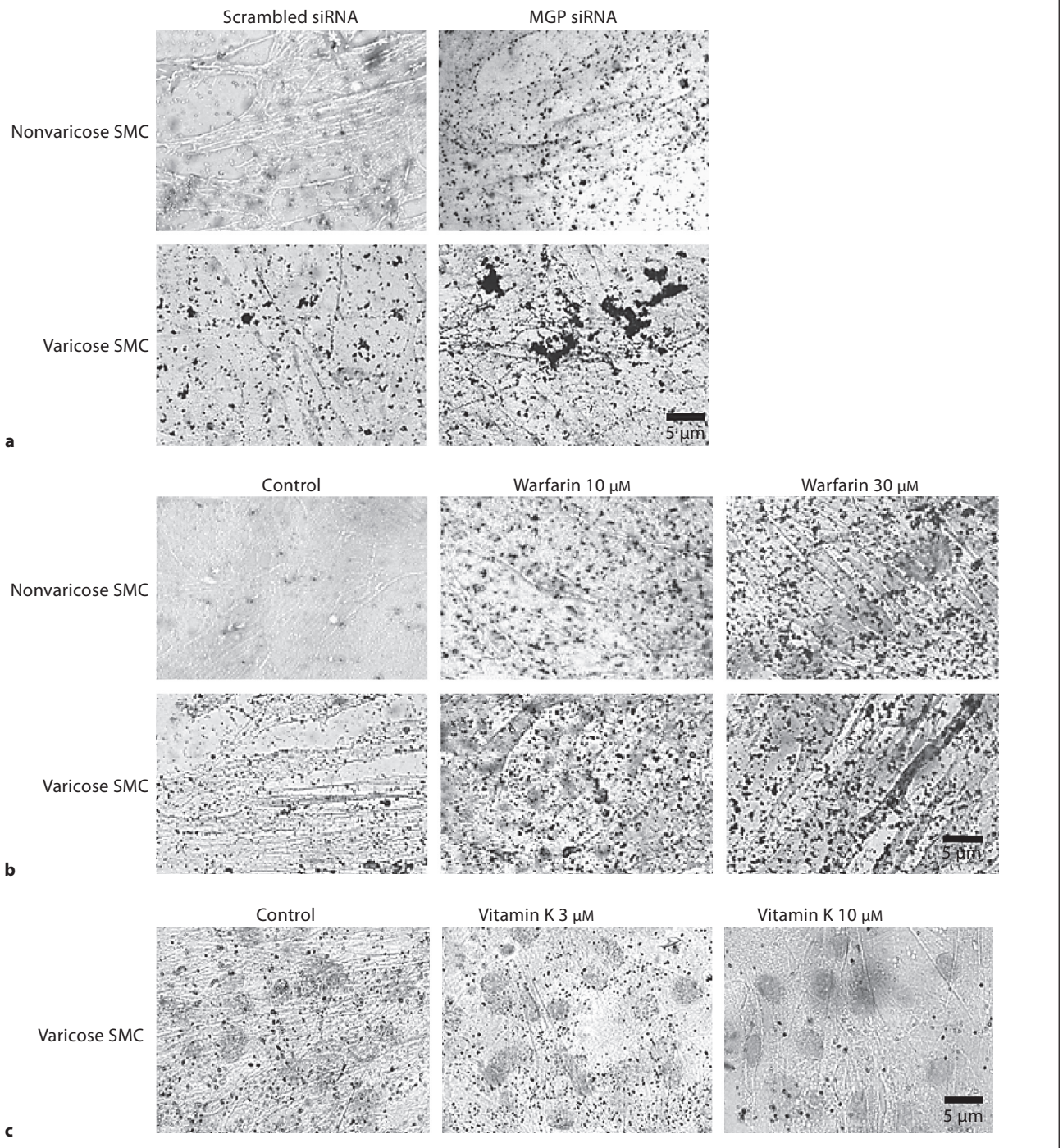

Fig. 5. Mineralization process in nonvaricose and varicose SMC cultures observed after von Kossa staining, 7 days after confluency. **a** Representative views of nonvaricose and varicose SMC cultures after scrambled siRNA transfection (control). Induction of mineralization by MGP siRNA was observed in nonvaricose and varicose SMC cultures. **b** Representative views of nonvaricose and varicose SMC mineralization process in control observations. MGP activity inhibition by 10 and 30 µM warfarin induced a dose-dependent increase in mineralization process in both nonvaricose and varicose SMC cultures. **c** The exacerbated mineralization process observed in varicose SMC cultures was sensitive to 3 and 10 µM of vitamin K.

icose and varicose tissues (fig. 4c). The most abundant GlaMGP staining was observed in the nonvaricose veins. In contrast, GluMGP was abundantly detected in the media of varicose veins, whereas poor staining was observed in nonvaricose veins (fig. 4c). No staining was observed in any of the negative controls (fig. 4c).

ECM Mineralization Process in Primary SMC Cultures

Because it has been shown that a calcification process occurs in cultured vascular SMC [31, 32], we examined the matrix mineralization potential of SMC after 7 days of culture, after MGP knockdown, or with or without inhibitor or stimulator of MGP activity. Varicose SMC cultures showed accumulation of mineral deposits localized to the ECM, whereas nonvaricose SMC cultures showed a nonmineralizing profile (fig. 5a, control). The mineralization process was induced in nonvaricose SMC cultures and increased in varicose SMC cultures by siRNA inhibition of MGP transcripts (fig. 5a). Similar observations were made in nonvaricose and varicose SMC cultures treated by increasing concentrations of warfarin. The mineralization process was induced by inhibition of MGP activity in nonvaricose SMC cultures and increased in varicose SMC cultures (fig. 5b).

The extensive mineralization process observed in varicose SMC cultures was partly sensitive to the MGP activator, vitamin K, as shown in fig. 5c. Vitamin K treatment of varicose SMC cultures induced a dose-dependent decrease in calcification spots.

Stimulation of MGP Expression by β-Glycerophosphate in Primary Venous SMC Cultures

As previously described, in vascular SMC, generation of inorganic phosphate by β-glycerophosphate correlates with mineralization [33]. To determine whether the induction of a mineralization process modulates MGP expression in varicose SMC, we tested the effect of 10 mM β-glycerophosphate on nonvaricose and varicose SMC cultures after 7 days of treatment. As shown in figure 6, β-glycerophosphate induced matrix mineralization in nonvaricose SMC cultures, whereas no additional mineralization was observed in varicose SMC cultures (fig. 6a). We then examined MGP expression by real-time PCR (fig. 6b). The results indicated that MGP expression was significantly increased by β-glycerophosphate treatment in nonvaricose SMC cultures (3.47 ± 0.18, $n = 3$, $p < 0.001$), whereas no significant variation was detected in varicose SMC cultures (fig. 6b).

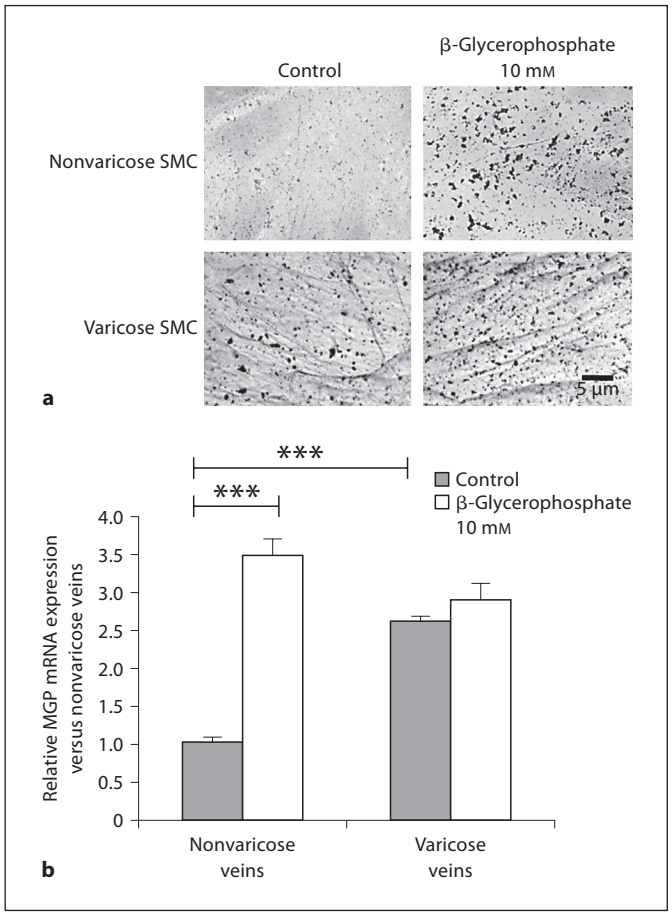

Fig. 6. Mineralization process and MGP mRNA expression in varicose SMC cultures after β-glycerophosphate treatment. **a** Representatives views of von Kossa staining showing that β-glycerophosphate (10 mM) induced mineralization process in nonvaricose SMC cultures compared to control. Varicose SMC cultures were not significantly sensitive to mineralization induction by β-glycerophosphate. **b** Relative quantitative RT-PCR expression of MGP mRNA in nonvaricose and varicose SMC cultures after β-glycerophosphate (10 mM) treatment. The bar graph shows the percentage of variation in varicose versus nonvaricose control conditions. Data are means ± SEM of 3 primary cultures. *** $p < 0.001$ versus ribosomal phosphoprotein P0.

Discussion

In this study, we report the identification of 34 differentially expressed genes related to CVI using cDNA subtractive hybridization and microarray technologies. Major functional classes of transcriptionally regulated genes in varicose disease corresponded to metabolism, ECM organization and regulation, response to external stimuli, and cell organization. Among the overexpressed genes involved in ECM organization and regulation, we identi-

fied the calcification inhibitor MGP. Functional analysis suggests that high expression of MGP in varicose veins may contribute to venous wall remodeling by affecting proliferation and mineralization processes. Furthermore, we demonstrated that GluMGP corresponds to the prevalent form present in varicose veins.

One of the major groups of differentially expressed genes was constituted by metabolism-associated genes suggesting a mitochondrial dysfunction in varicose SMC. Mitochondria are the main source of cell energy and are involved in the generation of reactive oxygen species in vascular SMC and other cell types [34]. The decrease in oxygen impairs energy production and generation of reactive oxygen metabolites by these organelles in varicose veins [35]. CVI is known to be associated with ischemic conditions [36]. Moreover, Bouaziz et al. [37] have shown that some veinotropic drugs are able to increase mitochondrial respiratory activity. Our results are in agreement with these data and identify genes that could be responsible for the mitochondrial dysfunction of varicose SMC, such as cytochrome b, cytochrome C oxidase subunits, pyruvate kinase and ATP synthase 6.

The present study reveals dysregulation of cell organization-related genes involved in actin-filament dynamic regulation. The remodeling of the actin cytoskeleton produces changes in cell shape and motility in response to external stimuli, and is therefore involved in signal transduction. These features of the actin cytoskeleton are regulated by a cohort of actin-binding proteins (ABP). These ABP were initially considered to be structural components that organize a stable actin cytoskeleton. Now, ABP are known to be regulators of cellular dynamics and key components of signaling processes [38]. We have previously demonstrated that contractile properties of human saphenous SMC were impaired. This dysfunction is due to a disturbance of signaling pathways that regulate SMC contraction and are associated with a reduction in actin cytoskeletal and ECM assembly [10, 25]. The present study suggests that altered expression in varicose SMC of β- and γ-actin isoforms, and the associated protein tropomyosin, could participate in structural changes of SMC occurring in varicose vein wall. Moreover, alteration of the actin network in varicose SMC could be increased by the upregulation of the actin-depolymerizing protein destrin and the actin-severing protein gelsolin.

Differentially expressed genes in varicose veins also included ECM-related genes. This group includes genes previously described in venous disease and genes for which no relationship has been identified to date. These results confirm previous studies, but also identify new genes that could participate in ECM remodeling of varicose veins. An increase in total collagen synthesis has been observed in the media and in SMC derived from varicose veins [39]. This augmentation has been ascribed to an overproduction of collagen I. Collagen I confers rigidity while collagen III is involved in the extensibility of tissue. Modification of the collagen I/III ratio might contribute to the weakness and decreased elasticity of varicose vein wall. Our study confirms the upregulation of collagen I mRNA in varicose veins and cultured SMC, while collagen III mRNA was only overexpressed in diseased tissues and not in cultured varicose SMC. These observations are in agreement with previous studies showing significant decrease in collagen III protein, whereas collagen III mRNA was not altered in cultured SMC and dermal fibroblasts derived from patients with varicose veins [14]. This decrease in collagen III content has been recently ascribed to an MMP3-dependent degradation in cultured varicose SMC [40]. The implication of TIMP/MMP balance in ECM degradation has been previously investigated in varicose vein [16]. Varicose veins are characterized by a higher than normal TIMP/MMP ratio. The present study shows the overexpression of TIMP I mRNA in varicose tissues and in cultured SMC derived from varicose vein in agreement with the high level of TIMP I protein previously found [16].

The present approach also identifies new ECM-related genes overexpressed in human varicose vein such as dermatopontin, tenascin C and MGP. These extracellular proteins have been shown to interact with other ECM components to regulate ECM formation. Dermatopontin interacts with decorin to regulate collagen fibrillogenesis [41]. Tenascin C is known as a regulator of cellular behavior by modulating the cell-matrix attachment and an inductor of MMPs [42]. However, we found that the overexpression of both genes in varicose tissue was lost in cultured SMC obtained from diseased veins, suggesting that factors involved in the activation of their transcription have disappeared in cultured condition. In contrast, MGP transcripts were upregulated in both tissues and SMC from varicose veins. This result revealed that factors involved in the activation of MGP transcription were maintained in culture condition and could not be ascribed to environmental parameters such as hyperpression, hypoxia or endothelial regulation. The overexpression of MGP in varicose veins is therefore related to intrinsic properties of varicose SMC.

We demonstrate for the first time that elevated expressions of MGP mRNA and protein were detected in dis-

eased vein. This result suggests that MGP could be involved in various mechanisms which contribute to varicose wall remodeling such as SMC proliferation, SMC differentiation and ECM disorganization. MGP is a mineral-binding ECM protein synthetized by vascular SMC and chondrocytes. The knockout of the MGP gene in mice causes lethal calcification and cartilaginous metaplasia of the media of all elastic arteries, indicating that MGP has an inhibitory effect on media calcification in vivo [43]. The use of warfarin, to inhibit MGP activity, or siRNA targeting MGP transcript induced a calcification process in venous SMC cultures and confirmed the role of MGP as a calcification inhibitor in venous vessels. Several studies have described that the growth arrest-specific gene 6, a secreted vitamin K-dependent protein, and its receptor, Axl, a membrane receptor tyrosine kinase, could modulate several cell functions in vessels, such as differentiation, adhesion, migration, proliferation, survival and calcification [44–46]. Warfarin could thus induce calcification in venous SMC cultures partly via the inhibition of growth arrest-specific gene 6 activity known to be involved in prevention of calcification. Nevertheless, the MGP signaling pathway has a crucial role in the inhibition of venous calcification as confirmed by the specific inhibition of MGP transcripts. High MGP mRNA expression and protein accumulation have been demonstrated in calcified arterial lesions and atherosclerotic plaques [26, 47–51]. Recently, Schürgers et al. [52] have shown that increased expression of MGP in atherosclerotic lesions was associated with a specific staining of GluMGP. The presence of GluMGP has also been reported in arterial calcification of aging rat and of rats treated with vitamin D [53, 54]. Our results show that in varicose veins MGP upregulation was associated with an increased calcification and a prevalent expression of GluMGP. The increased calcification process in varicose veins could thus be due to incomplete MGP carboxylation as observed in other vascular diseases. As vitamin K is an essential cofactor for MGP carboxylation, it might be expected that the local vascular vitamin K status is insufficient in varicose vein to mediate full carboxylation of all newly formed MGP. In varicose SMC cultures, our results showed that vitamin K supplementation inhibited the mineralization process, suggesting that in vitro, carboxylation of MGP could be partly induced and that the inhibitory effect of MGP could be restored. Future analysis of vitamin K levels in nonvaricose and varicose vein walls could contribute to understanding the MGP undercarboxylation mechanism. In venous nonvaricose SMC, we have shown that generation of inorganic phosphate by β-glycerophosphate correlates with calcification as observed in arterial SMC [55]. We showed that the β-glycerophosphate-induced mineralization was associated with an upregulation of MGP mRNA in nonvaricose veins. This result may be explained by a MGP-dependent negative feedback loop controlling mineralization as previously proposed in a chondrogenic cell line [56]. In varicose SMC cultures, the absence of β-glycerophosphate-induced increase in calcification process and upregulation of MGP suggested that this negative feedback should be dysregulated in venous disease.

As well as a role in matrix calcification, various studies have described that MGP has additional functions in the regulation of cellular mechanisms such as cell differentiation, apoptosis and gene expression [30, 57, 58]. We showed that inhibition of MGP activity or MGP expression significantly reduced serum-induced increase in cell number and proliferation of saphenous SMC, suggesting the involvement of MGP in the proliferation mechanism as previously demonstrated in the ATDC5 chondrocytes cell line [57]. Nevertheless, the MGP siRNA-sensitive component of serum-induced increase in cell number was significantly higher in varicose than nonvaricose SMC, whereas the MGP siRNA-dependent proliferation rates were similar in both conditions. The observed results in varicose veins could be due to a complex association of MGP-dependent effects, affecting proliferation processes and mechanisms influencing survival and/or apoptosis. A previous study has demonstrated that MGP regulated apoptosis at different stages of cell differentiation [57]. Decreased apoptosis in the medial layer of varicose veins has been described [59, 60]. Involvement of MGP in the decreased programmed varicose cell death will require further investigation. MGP has been shown to interact directly with bone morphogenetic protein 2 (BMP-2) in a concentration-dependent manner. BMP-2, a member of the transforming growth factor-β superfamily, was described to promote ectopic bone formation, gene expression, apotosis and to inhibit proliferation in vascular SMC [61]. Zebboudj et al. [62] have determined that the balance levels between MGP and BMP-2 was important to promote or inhibit BMP-2-dependent signaling pathways involved in the regulation of vascular cell differentiation and calcification. In saphenous veins, MGP could partly induce proliferation by decreasing BMP-2 activity. Furthermore, MGP might regulate vascular SMC migration or proliferation mediated by vitronectin receptor integrins of the $\alpha_V\beta$ family [63, 64]. Recently, Nishimoto and Nishimoto [65] have demonstrated that MGP binds to vitronectin and suggested that this

complex may have altered function for modulating BMP-2 and transforming growth factor-β activity.

CVI is partly characterized by remodeling of SMC of the media [9] and we show here that varicose SMC had a significantly higher proliferation rate than nonvaricose SMC. The inhibitory effect of MGP knockdown or inhibition on SMC proliferation provides evidence for a role of MGP in the increased proliferation observed in varicose vein wall.

In summary, the present study led to the identification of genes that are differentially expressed in a large panel of nonvaricose or varicose saphenous veins. A first analysis of differentially expressed clones revealed several interesting candidates that may be involved in CVI development, including MGP. We show that overexpression of MGP contributes to the high proliferative rate of SMC and that excessive ECM mineralization occurs in varicose wall. These alterations could be due to impaired carboxylation of MGP. Future studies should establish the mechanism underlying the role of MGP in these processes and should identify proteins associated with the MGP signaling pathway. These results reinforce the concept that molecules in the ECM can affect the behavior of surrounding SMC and may have important implications in the processes influencing the development of CVI.

Acknowledgments

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