ORIGINAL RESEARCH

Angiogenesis-Related Genes in Endothelial Progenitor Cells May Be Involved in Sickle Cell Stroke

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Background—The clinical aspects of sickle cell anemia (SCA) are heterogeneous, and different patients may present significantly different clinical evolutions. Almost all organs can be affected, particularly the central nervous system. Transient ischemic events, infarcts, and cerebral hemorrhage can be observed and affect ≈25% of the patients with SCA. Differences in the expression of molecules produced by endothelial cells may be associated with the clinical heterogeneity of patients affected by vascular diseases. In this study, we investigated the differential expression of genes involved in endothelial cell biology in SCA patients with and without stroke.

Methods and Results—Endothelial progenitor cells from 4 SCA patients with stroke and 6 SCA patients without stroke were evaluated through the polymerase chain reaction array technique. The analysis of gene expression profiling identified 29 differentially expressed genes. Eleven of these genes were upregulated, and most were associated with angiogenesis (55%), inflammatory response (18%), and coagulation (18%) pathways. Downregulated expression was observed in 18 genes, with the majority associated with angiogenesis (28%), apoptosis (28%), and cell adhesion (22%) pathways. Remarkable overexpression of the *MMP1* (matrix metalloproteinase 1) gene in the endothelial progenitor cells of all SCA patients with stroke (fold change: 204.64; P=0.0004) was observed.

Conclusions—Our results strongly suggest that angiogenesis is an important process in sickle cell stroke, and differences in the gene expression profile of endothelial cell biology, especially *MMP1*, may be related to stroke in SCA patients. **(*J Am Heart Assoc*. 2020;9:e014143. DOI: 10.1161/JAHA.119.014143.)**

Key Words: gene expression • *MMP1* • sickle cell anemia • stroke

Sickle cell anemia (SCA) is a monogenic disorder caused by the homozygous form of hemoglobin S (HbSS), which derives from a point mutation at the seventh codon of the β-globin gene (*HBB* [hemoglobin subunit β]), resulting in the substitution of glutamic acid for valine (p.Glu7Val) in the β-globin protein. SCA is a severe disease characterized by the polymerization of HbSS under hypoxic conditions, leading to the deformity and fragility of red blood cell membranes.[1–3]

Stroke is a catastrophic complication of SCA caused by stenosis and occlusion of large vessels.[4] The abnormal interaction between sickle red blood cells and the vascular endothelium is a critical factor in its pathogenesis.[5,6] Sickled red blood cells tend to adhere to the endothelium, resulting in endothelial cell activation in a hypercoagulable state, thrombus formation, and thus vascular occlusion. Three stroke syndromes may result from multifocal small vessel disease: clinical ischemic stroke (IS), hemorrhagic stroke, and clinically silent stroke (also referred as *silent cerebral infarcts*). IS is the classical stroke syndrome of SCA and is responsible for 20% of mortality in these patients.[7,8] Risk factors include prior transient ischemic attack, frequent acute chest syndrome, and increased systolic blood pressure.[9] IS affects ≈10% of pediatric patients with SCA, with the greatest prevalence during the first decade of life.[10] The risk of stroke is considered to be 300-fold higher in children

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Received August 5, 2019; accepted December 6, 2019.

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Clinical Perspective

What Is New?

- Genes related to endothelial cell biology were associated with stroke in patients with sickle cell anemia.
- The *MMP1* (matrix metalloproteinase 1) gene, which belongs to a family of proteins involved in inflammation, tissue reconstruction and repair, cell migration, and angiogenesis, presented remarkable overexpression in the endothelial colony-forming cells of patients with sickle cell anemia and stroke.

What Are the Clinical Implications?

- Overexpression of the *MMP1* gene may serve as a marker of stroke or the overall process of vasculopathy in patients with sickle cell anemia.

with SCA than in other children without sickle cell disease (SCD). Notably, at 45 years of age, 1 in 4 adults with the disease may be affected.[8] The standard method used to assess stroke risk in children with SCA is transcranial Doppler ultrasound. Children with elevated transcranial Doppler ultrasound velocity (defined as ≥200 cm/s) have a higher risk of developing primary stroke than those with normal transcranial Doppler ultrasound velocities (<170 cm/s).[10] For these patients, intervention with blood transfusions reduces the risk of stroke by >90%.[11]

Many studies have investigated genetic risk factors for stroke through gene association analysis and genome-wide association studies. This research has generated a list of putative polymorphisms in genes involved in different pathways, such as inflammatory and cellular adhesion, that may influence the risk of stroke.[12] These methodologies, however, are dependent on large sample amounts.[13] This difficulty, associated with differences in sample characteristics and experimental design, may explain much of the low reproducibility of results between different studies.[14]

On the basis of animal studies, several authors have hypothesized that circulating endothelial progenitor cells (EPCs) might have prognostic value for acute IS. Recruitment of EPCs toward infarcted brain areas and molecular mechanisms of postischemic angiogenesis have been described.[15–17] Endothelial colony-forming cells (ECFCs), adult EPCs that circulate in peripheral blood, have a high proliferative capacity and stable phenotype during in vitro culture and have been used as a model for the study of SCA endothelial function,[18] as biomaterial in gene therapy,[19,20] and as a pathophysiologic study tool for vascular disease.[21]

In this study, our approach was based on the use of ECFCs from SCA patients with and without stroke followed by cDNA microarray analysis of genes possibly related with endothelial cell dysfunction. This approach, applied in patients with precisely defined phenotypes, may allow the identification of differences in endothelial gene expression associated with clinical heterogeneity among patients affected by vascular diseases.[19]

Methods

The data that support the findings of this study are available from the corresponding author on reasonable request.

A total of 10 HbSS patients (SCA) were recruited from the Hematology and Hemotherapy Center of the University of Campinas (São Paulo, Brazil). Four patients (aged >18 years) had history of clinical primary stroke events in combination

Table 1. Demographics and Clinical Data of SCA Patients With and Without Stroke

Parameters	With Stroke	Without Stroke
No. of patients	4	6
Male/female	1/3	1/5
Age at blood collection, y	35 (26–44)	39.5 (29–55)
Age at time of stroke, y	28.5 (11–41)	…
RBC count (10^6/μL)	3.0 (2.8–3.6)	2.8 (2.3–3.7)
Hemoglobin, g/dL	9.4 (8.5–11.4)	8.3 (7.4–9.6)
Leukocytes, $\times 10^9$/L	9.6 (5.1–12.6)	8.2 (6.1–11.1)
Platelets, $\times 10^9$/L	396 (304–473)	352 (285–433)
Reticulocytes (absolute number)	9.0 (6.2–14.5)	11.9 (7.3–18.6)
Fetal hemoglobin (%)	3.3 (1.3–8.0)	6.0 (2.6–14.8)
Hemoglobin S (%)	34.0 (14.4–56.8)	74 (11.2–91.2)
Microalbuminuria, mg/g	39.4 (2.36–84.7)	64 (4.0–277.2)
Acute chest syndrome	0	0
Algic crisis	0	01 (16.6%)
Aseptic necrosis	0	1 (16.6%)
Cholecystectomy	4 (100%)	3 (50%)
Chronic kidney diseases	1 (25%)	1 (16.6%)
Leg ulcer	03 (75%)	0
Hepatopathies	0	2 (33.3%)
Systolic hypertension	01 (25%)	0
Osteoporosis	2 (50%)	02 (33.3%)
Priapism	0	0
Pulmonary hypertension	01 (25%)	03 (50%)
Retinopathy	2 (50%)	3 (50%)
Smoking	1 (25%)	1 (16.6%)
Alpha thalassemia	01 (25%)	02 (33.3%)
Moyamoya disease	0	0
RBC transfusion	04 (100%)	01 (16.6%)

RBC indicates red blood cells; SCA, sickle cell anemia.

Table 2. Hematologic Parameters of Patients With and Without Stroke

Patient	Sex	Age, y*	Age (y) at Time of Stroke	Smoking	RBC (10^6/μL)	Hemoglobin (g/dL)	WBC ($\times 10^9$/L)	Reticulocytes (Absolute No.)	Platelets ($\times 10^9$/L)	Fetal Hemoglobin (%)	Hemoglobin S (%)	α-Thalassemia
With stroke												
1	F	28	11	No	3.6	11.4	9.4	14.5	436	2.8	43.3	Normal
2	M	44	39	Yes	2.8	8.5	12.6	6.2	371	1.4	14.4	Normal
3	F	42	41	No	3.0	8.9	5.1	6.2	304	8.0	56.8	Heterozygous
4	F	26	23	No	2.8	8.9	11.2	16.1	473	1.3	21.3	Normal
Without stroke												
5	M	39	…	Yes	3.0	9.6	11.1	9.5	285	9.5	84.5	Normal
6	F	52	…	No	2.7	7.6	7.5	18.7	296	2.6	91.2	Normal
7	F	30	…	No	2.7	7.4	8.6	11.2	420	3.6	88.0	Heterozygous
8	F	56	…	No	2.4	8.2	7.8	7.3	299	2.8	11.2	Normal
9	F	29	…	No	2.3	7.9	8.2	12.4	433	14.8	79.2	Normal
10	F	29	…	No	3.7	9.2	6.1	16.9	383	3.1	90.3	Heterozygous

F indicates female; m, male; RBC, red blood cells; WBC, white blood cells.
*Age at sample collection.

with magnetic resonance imaging (MRI) evidence confirming IS. The other 6 patients (aged >20 years) had no previous history of clinical stroke or any evidence of silent infarcts, confirmed by MRI (control group). All patients underwent MRI, performed by a neurologist, at the Neuroimaging Laboratory of the Department of Neurology at the University of Campinas. In the control group, just after MRI, peripheral blood samples were collected and ECFC cultures were carried out immediately. Demographics and clinical data of SCA patients with and without stroke are shown in Table 1. Moreover, hematologic parameters, SCA-related complications, treatment, and MRI findings for each patient are shown in Tables 2 and 3.

Patients with MRI evidence of hemorrhagic stroke and patients with other hemoglobinopathies such as HbC, HbD, and β-thalassemia or hereditary persistence of fetal hemoglobin were not enrolled in this study. The only treatment for the patients in the stroke group was blood transfusion, aiming to keep the percentage of HbS <30%. Among the control group (patients without stroke), 1 patient was under regular blood transfusion because of low hemoglobin level and high frequency of vaso-occlusive crises (>3 crises per year). We emphasize that no patients were under hydroxycarbamide therapy. Written informed consent was obtained from all participants, and the study was approved by the university ethics committee, in accordance with national guidelines.

MRI Acquisition Parameters

MRIs were acquired in a 3-T Philips Intera Achieva scanner with the following sequences: (1) high-resolution volumetric T1-weighted image (180 slices, 1-mm thickness; angle flip, 8°; repetition time [TR], 7.1 ms; echo time [TE], 3.2 ms; matrix, 240×240; field of view, 240×240 mm); (2) 3-dimensional T2-weighted image (1.5-mm^3 isotropic voxels; TR, 1800 ms; TE, 340 ms; field of view, 230×230×180 mm^3); (3) 3-dimensional fluid-attenuated inversion recovery (voxel size, 1.2×1.2×0.6 mm^3; field of view, 250×250×190 mm^3; TE, shortest; TR, 4800 ms; inversion time [TI], 1650 ms); (4) 3-dimensional susceptibility-weighted imaging (SWI; 2-mm thickness; TR/TE, shortest); (5) diffusion-weighted images (thickness, 4 mm; TE, 70 ms; TR, 3500 ms); and (6) magnetic resonance angiography (TE, 3.5 ms; TR, 22 ms).

MRI Evaluation for Signs of Stroke and Cerebral Small Vessel Disease

All images were revised by a neurologist with experience in imaging and stroke (F.C.) in addition to routine radiological evaluation. All imaging sequences described earlier were used to evaluate signs of acute or remote stroke lesions and other major structural lesions. In addition, we evaluated signs of cerebral small vessel disease, also known as cerebral microangiopathy or microvascular disease, which results from damage to the cerebral microcirculation and mainly affects the blood supply and tissue of the deep white- and gray-matter areas of the brain.[22] These signs include white-matter hyperintensities in the periventricular and deep white and gray matter, enlarged perivascular spaces, lacunes, microbleeds, and cerebral atrophy that were not related to a specific macroscopic focal injury such as trauma or infarction.[22,23]

Table 3. Clinical Data, Treatment, and Neuroimaging Characteristics of SCA Patients

Patient	Clinical Data	Treatment	MRI Signs of Cerebral Small Vessel Disease* and Other Relevant Findings
1	Cholecystectomy Leg ulcer Retinopathy Stroke	Deferasirox Folic acid Transfusion	Lacunar infarcts and signs of small vessel disease
2	Cholecystectomy Leg ulcer Membranoproliferative Glomerulonephritis Osteoporosis Retinopathy Stroke	Acetyl-salicylic acid Alendronate sodium $CaCO_3$ Deferasirox Enalapril maleate Folic acid Transfusion	Signs of small vessel disease
3	Cholecystectomy Osteoporosis Pulmonary hipertension Retinopathy Systolic hypertension Heart valve replacement Stroke	Alendronate sodium $CaCO_3$+ vitamin D Enalapril maleate Folic acid Transfusion Warfarin	Lacunar infarcts and area of gliosis in the left frontal lobe. Signs of small vessel disease
4	Cholecystectomy Leg ulcer Stroke	Calcium Deferasirox Folic acid Transfusion	Lacunar infarcts. Signs of small vessel disease
5	Erectile dysfunction	Acetyl-salicylic acid	No evidence of small vessel disease
6	Cholecystectomy Hepatopathy Osteoporosis Pulmonary hipertension Retinopathy	$CaCO_3$+ Vitamin D Folic acid	MRA with tortuous vessels. No evidence of small vessel disease
7	Retinopathy	Folic acid	No evidence of small vessel disease
8	Algic crisis Avascular necrosis Chronic hepatopathy Chronic kidney disease III Cholecystectomy Osteoporosis Pulmonary hipertension	$CaCO_3$+ vitamin D Deferiprone Deferasirox Doxycyline Enalapril maleate Folic acid Transfusion	Signs of small vessel disease
9	No complication	Folic acid	No evidence of small vessel disease
10	Cholecystectomy Pulmonary hypertension Retinopathy Hypothyroidism	Enalapril maleate Folic acid Levothyroxine sodium	No evidence of small vessel disease

MRA indicates magnetic resonance angiography; MRI, magnetic resonance imaging; SCA, sickle cell anemia.
*MRI signs of cerebral small vessel disease as defined in methods.

ECFC Culture

Cultures of ECFCs were established from peripheral blood samples according to previously described methods.[18,20] Peripheral blood (45 mL) was collected into sodium heparin (9 mL) from 10 HbSS patients. Briefly, the anticoagulated blood was diluted 1:2 with PBS, which was underlaid with an equivalent volume of Ficoll-Paque PLUS (GE Healthcare) before centrifugation (317*g*, 30 minutes, room temperature). Only mononuclear cells were isolated and resuspended in EBM-2 medium containing the EGM-2 BulletKit (Lonza), 10% additional fetal bovine serum (Invitrogen), 1% penicillin/streptomycin (Invitrogen) and 1% L-glutamine (Gibco, Life Technologies). The cells (7×10^6 cells/well) were then seeded onto 12-well tissue culture plates precoated with type 1 rat-tail collagen (Sigma-Aldrich) and cultured in a humidified incubator

at 5% CO_2. Colonies of endothelial cells appeared between 10 and 21 days of culture. Cells from passages 3 to 5 were used for this study. ECFCs were identified by their typical cobblestone morphology and characterized positively for endothelial markers CD31, CD144, CD146, and VEGF (vascular endothelial growth factor)/KDR (kinase insert domain receptor) but negatively or low for endothelial activation antigens and CD34 and negatively for the myeloid cell marker CD45 and the endothelial progenitor marker CD133. Flow cytometric analysis was performed using a FACSCalibur flow cytometer and BD FACSDiva 7.0 software (BD Biosciences).

RNA Extraction

Total RNA was extracted from ECFCs using Trizol Reagent (Ambion Life Technologies) and a commercial RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. The quantification and purity of the RNA were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Only samples pure enough (A260/A230 ratio >1.8; A260/A280 ratio 1.8–2.0) and with concentration >100 ng/μL were used as templates for cDNA synthesis.

Synthesis of cDNA and Polymerase Chain Reaction Array for Human Endothelial Cell Biology

First-strand cDNA was synthesized from 1 μg of total RNA using the RT^2 First-Strand Kit (Qiagen). Subsequently, cDNA was combined with the RT^2 qPCR Master Mix, according to the manufacturer's instructions, and added to the pathway-specific RT^2 Profiler PCR Array (PAHS-015Z; Qiagen). This array includes 84 genes associated with endothelial cell biology and 12 controls (5 housekeeping genes, 1 assay for detecting genomic DNA contamination, 3 reverse transcription controls, and 3 positive polymerase chain reaction [PCR] controls). The levels of gene expression were quantified at the ABI StepOne Plus Real-Time PCR (Applied Biosystems). The PCR conditions were 1 cycle of 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 40 cycles of 60°C for 1 minutes. The threshold cycle values from samples of each gene and the internal controls were obtained and analyzed with the software RT^2 Profiler PCR Array Data Analysis v3.5 (Qiagen; http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). This web portal calculates the *P* values using a Student *t* test, on the replicate $2^{-\Delta C_t}$ values for each gene in the stroke group compared with the nonstroke group. The levels of gene expression were normalized in relation to the housekeeping genes Actin Beta (*ACTB*), Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*) and Ribosomal Protein Lateral Stalk Subunit P0 (*RPLP0*). For each sample, the assays were performed in duplicate and the mean expression value of each gene was used for the analysis. Genes were considered to be differentially expressed if they showed a fold-change of at least 2-fold up or down in comparison to the gene-expression levels of the control group (SCA patients without stroke) or $P<0.05$.

Validation of PCR Array by Quantitative Reverse Transcription PCR

The results from the PCR array were validated by quantitative reverse transcription PCR (qRT-PCR) performed for selected genes that showed differential expression between SCA patients with versus without stroke. To select the genes, 2 variables were considered: fold change >2 in gene expression or $P<0.05$ (considered significant). Ten genes were selected, 5 upregulated (Fms Related Receptor Tyrosine Kinase 1 [*FLT1*], Placental Growth Factor [*PGF*], Matrix Metalloproteinase 1 [*MMP1*], Protein C Receptor [*PROCR*] and Interleukin 6 [*IL6*]) and 5 downregulated (Intercellular Adhesion Molecule 1 [*ICAM1*], Selectin P Ligand [*SELPLG*], C-C Motif Chemokine Ligand 2 [*CCL2*], Kinase Insert Domain Receptor [*KDR*] and TNF Superfamily Member 10 [*TNFSF10*]) in patients with stroke. The same RNA samples from patients assessed through PCR array were used in validation experiments.

The RNA samples were treated with DNase I (Life Technologies), and cDNAs were synthesized from 1 μg of RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific), according to the manufacturer's protocol. The summary of the primer sequences, the size of the fragments obtained, and the primer concentrations are shown in Table 4. The qPCR reaction was performed in triplicate using ABI StepOnePlus Real Time PCR (Applied Biosystems). For PCR amplification, 6 μL of SYBR Green PCR Master Mix (Applied Biosystems), 3 μL (10 ng) of cDNA, and 3 μL of the specific primer were used. The cycling conditions were the same as those used in the PCR array. Amplification specificity was verified using a dissociation curve, and RNA expression was calculated relative to the expression of *ACTB* and *GAPDH* genes through the GeNorm program.

Confirmation of Differential Expression by Western Blot

To confirm the increased expression of the *MMP1* gene detected by the PCR array and qRT-PCR, we performed Western blot analysis. Pelleted cells were resuspended in RIPA buffer (50 mmol Tris-HCl, pH 7.4, 150 mmol NaCl, 0.1% SDS, 1% NP-40 and 0.5% sodium deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol sodium orthovanadate, 1 μg/mL leupeptin, and 1 μg/mL pepstatin A) incubated for 45 minutes at 4°C and centrifuged for 20 minutes at 15 000*g* at 4°C.

Protein concentrations were quantified by the Bio-Rad Protein Assay Kit (Sigma-Aldrich). Equal protein amounts were

Table 4. Sequencing Primers Used in the qRT-PCR Assays

Gene	Primer Sequences	Fragment Size (bp)	Primer (concentration in nmol/L)
CCL2	F: 5′-GCTCAGCCAGATGCAATCAAT-3′ R: 5′-CACTTGCTGCTGGTGATTCTTC-3′	110	300
FLT1	F: 5′-GAAAACGCATAATCTGGGACAG-3′ R: 5′-CTATGATTGTATTGGTTTGTCGATG-3′	150	300
ICAM1	F: 5′-GGAAATACTGAAACTTGCTGCCTA-3′ R: 5′-ACACATGTCTATGGAGGGCCAC-3′	81	150
IL6	F: 5′-CCAGGAGCCCAGCTATGAAC-3′ R: 5′-CCCAGGGAGAAGGCAACTG-3′	64	70
KDR	F: 5′-TGAAAGAAGGAGCAACACACAG-3′ R: 5′-AGAATTCCACAATCACCATGAGT-3′	147	150
MMP1	F: 5′-CAAGCCATATATGGACGTTCC-3′ R: 5′-CGCATGTAGAATCTGTCTTTAAAGA-3′	143	300
PGF	F: 5′-GAGGTGGAGCACATGTTCAGC-3′ R: 5′-GATCTTTAGGAGCTGCATGGTG-3′	126	70
PROCR	F: 5′-GAAAACACGAAAGGGAGCCA-3′ R: 5′-CTACAGCCACACCAGCAATGAT-3′	94	70
SELPLG	F: 5′-GCTGGTGCCATGCCTCTG-3′ R: 5′-GGCTTTCTCGGCTTCATCTG-3′	102	150
TNFSF10	F: 5′-TGAAGCAGATGCAGGACAAGTAC-3′ R: 5′-TCTTTCTAACGAGCTGACGGAG-3′	147	300
ACTB	F: 5′-TGACCCAGATCATGTTTGAGACC-3′ R: 5′-CAGAGGCGTACAGGGATAGCA-3′	81	150
GAPDH	F: 5′-AAGATCATCAGCAATGCCTCCT-3′ R: 5′-GGTCATGAGTCCTTCCACGATAC-3′	96	150

bp indicates base pair; F, forward; qRT-PCR, quantitative reverse transcription polymerase chain reaction; R, reverse. *CCL2* (C-C Motif Chemokine Ligand 2); *FLT1* (Fms-Related Receptor Tyrosine Kinase 1); *ICAM1* (Intercellular Adhesion Molecule 1); *IL6* (Interleukin 6); *KDR* (Kinase Insert Domain Receptor); *MMP1* (Matrix Metalloproteinase 1); *PGF* (Placental Growth Factor); *PROCR* (Protein C Receptor); *SELPLG* (Selectin P Ligand); *TNFSF10* (TNF Superfamily Member 10); *ACTB* (Actin Beta); *GAPDH* (Glyceraldehyde-3-Phosphate Dehydrogenase).

loaded onto 10% SDS polyacrylamide gels and electrophoretically transferred to nitrocellulose membrane. Nonspecific sites were blocked by incubation with a buffer containing Tris (10 mmol/L, pH7.4), NaCl (150 mmol/L), Tween 20 (0.1%), and fat-free dry milk (5%). Membranes were incubated overnight with anti-MMP1–specific primary antibody (Abcam) at 4°C, followed by horseradish peroxidase–conjugated secondary antibody (KPL conjugate peroxidase, goat anti-mouse IgG; Santa Cruz Biotechnology) at room temperature for 1 hour. GAPDH antibody (Santa Cruz Biotechnology) was used as a loading control. Immunoreactivities were visualized by the ECL Chemiluminescent Western Blotting Analysis System (Thermo Fisher).

Statistical analysis and graph construction for qRT-PCR and Western blot were performed with the GraphPad Prism 4 program (GraphPad Software). The Fisher-Pitman permutation test was used for the qRT-PCR analyses; $P<0.05$ was considered statistically significant after Benjamini-Hochberg adjustment for multiple tests.

Sequencing of the *MMP1* Gene Promoter Region

To identify variants that could affect expression levels of the *MMP1* gene in SCA patients, we analyzed the promoter region of the gene by direct Sanger sequencing. Total DNA was extracted from peripheral blood using the QIAamp DNA Blood Midi Kit, according to the manufacturer's instructions. The quantification and purity of the DNA were measured using a NanoDrop 2000 spectrophotometer (260/280 absorbance).

Primers and PCR Amplification of the Promoter Region of the *MMP1* Gene

The sequences covering the promoter region of the *MMP1* gene were obtained from the database of the National Center for Biotechnology Information and the Ensembl Genome Browser. For PCR amplification, 3 pairs of primers were used to cover the promoter region (Table 5). PCR was prepared with 40 to 100 ng of DNA, 25 pmol of forward and reverse primers, 75 pmol of dNTP, 25 pmol of $MgCl_2$, 250 pmol of enzyme buffer 1×, 0.1 U of Taq DNA polymerase (Invitrogen, Life Technologies), and ultrapure water to complete up to 25 μL. PCR was carried out in a Veriti 96-Well Thermal Cycler (Applied Biosystems–Applera Corp).

The amplification conditions were initial denaturation at 95°C for 5 minutes, 35 cycles at 95°C for 30 seconds, annealing for 30 seconds and extension at 72°C for 45 seconds, and finally extension at 72°C for 5 minutes. PCR fragments were analyzed by electrophoresis and compared with a 100-bp DNA ladder (Invitrogen) on an ethidium bromide–stained 1.5% agarose gel.

Sequencing Reaction

Sequencing reactions were performed with the same PCR primers. The reagents used for the sequencing reaction were the purified PCR product (40 ng); "Big Dye" 2× (ABI PRISM Big Dye Terminator v 3.1 Cycle Sequencing Kit; Applied Biosystems); 1× "Save Money" buffer (supplied by the same Big Dye

Table 5. Primers Used for *MMP1* Gene Promoter Sequencing

Fragment	Primer Sequences	Fragment Size (bp)
1	F: 5′-AGTCACGCTCAGTCTCTTTCCAG-3′ R: 5′-GAGACACACTCTGCCATGTAAACA-3′	856
2	F: 5′-TAGCACTTTATGACCATCAGAACC-3′ R: 5′-TTCACTCTCACCTCCAACTGGA-3′	711
3	F: 5′-CTCATGCCCCACTCTCCTTC-3′ R: 5′-TGGTCACATGCTGCTTTCCT-3′	611

bp indicates base pair; F, forward; R, reverse.

manufacturer), forward or reverse primer (5 pmol), and ultrapure water to complete the reaction to a final volume of 20 µL. The general cycle sequencing condition was 96°C for 1 minute, 96°C for 10 seconds, 57°C for 5 seconds, and 30 cycles at 60°C for 4 minutes. For purification, the samples were precipitated with 80 µL of 80% ethanol and centrifuged at 3297*g* for 45 minutes. The supernatant was discarded, followed by the addition of 150 µL of 70% ethanol and centrifugation for 10 minutes at 3297*g*. Finally, the supernatant was discarded and the samples were dried in a thermal cycler for 3 minutes at 64°C. The product of the purified sequencing reaction was then resuspended in 10 µL of Hi-Di Formamide (Applied Biosystems), vigorously shaken, and denatured at 94°C for 5 minutes. Nitrogen base sequence readings of the fragments were performed on an ABI PRISM 3700 automatic sequencer (Applied Biosystem).

The sequences obtained were analyzed and compared with the normal sequences of the gene with the aid of the programs Chromas Lite v2.5.1 (Technelysium) and CLC Sequence Viewer v6.5.1 (CLC Bio).

Results

In this study, we used the human endothelial cell biology RT^2 Profiler PCR Array to examine the differential expression of 84 genes in ECFCs from SCA patients with and without stroke. Twenty-nine differentially expressed genes were identified. Among these, 11 were upregulated and 18 were downregulated (Table 6) in patients with stroke compared with patients without stroke.

Validation Studies

Based on the PCR array results, comparing patients with and without stroke, 5 upregulated genes (*FLT1, PGF, MMP1, PROCR* and *IL6*) and 5 downregulated genes (*ICAM1, SELPLG, CCL2, KDR* and *TNFSF10*) were chosen for validation by qRT-PCR (considering fold change >2 or $P<0.05$). *ACTB* and *GAPDH* were used as housekeeping genes because the expression of these genes did not differ among patients. Individual RNA samples from each studied patient were assessed.

The qRT-PCR analysis demonstrated that all SCA patients with stroke (n=4) had threshold cycle values ranging between 18.0 and 19.3 cycles. Among the 6 SCA patients without stroke, 4 had threshold cycle values ranging between 27.0 and 30.6 cycles and 2 had threshold cycle values close to those of the group with stroke, at 19.8 and 21.7 cycles. Results obtained for the PCR array and for qRT-PCR are shown in Table 7. Graphs comparing both groups were generated using the program GraphPad Prism 4 (Figure 1).

Table 6. Genes That Showed Altered Expression Comparing SCA Patients With and Without Stroke by PCR Array

Gene	Fold Change	*P* Value	Pathway Involved
PF4	5.02	0.0680	Angiogenesis
KIT	3.72	0.8006	Angiogenesis
FLT1	3.49	0.0218*	Angiogenesis
PGF	2.93	0.0150*	Angiogenesis
PLAU	2.52	0.8081	Angiogenesis
THBD	2.08	0.1683	Platelet activation
MMP1	204.64	0.0004†	Angiogenesis/coagulation
PLAT	3.85	0.3670	Coagulation
PROCR	1.64	0.0479*	Coagulation
IL6	3.05	0.4405	Inflammatory response
IL1B	2.20	0.2231	Inflammatory response
SELE	−6.79	0.2093	Cell adhesion
ICAM1	−2.83	0.0531	Cell adhesion
SELPLG	−2.29	0.0090†	Cell adhesion
SELL	−2.27	0.2139	Cell adhesion
CCL2	−4.01	0.0799	Angiogenesis
MMP9	−2.67	0.1141	Angiogenesis
CCL5	−2.64	0.3832	Angiogenesis
TYMP	−2.50	0.0860	Angiogenesis
KDR	−1.62	0.0360*	Angiogenesis
IL7	−42.71	0.2101	Apoptosis
TNFSF10	−13.34	0.0253*	Apoptosis
TNF	−5.00	0.3930	Apoptosis
OCLN	−2.16	0.2451	Apoptosis
EDNRA	−2.12	0.2611	Apoptosis
TFPI	−2.33	0.1797	Coagulation
APOE	−3.06	0.2442	Inflammatory response
CX3CL1	−5.36	0.1323	Vasoconstriction and vasodilation
ACE	−3.41	0.1797	Vasoconstriction and vasodilation

PCR indicates polymerase chain reaction; SCA, sickle cell anemia.
*$P<0.05$.
†$P<0.01$.
PF4 (Platelet Fator 4); *KIT* (Kit Proto-Oncogene, Receptor Tyrosine Kinase); *FLT1* (Fms-Related Receptor Tyrosine Kinase 1); *PGF* (Placental Growth Factor); *PLAU* (Plasminogen Activator, Uroquinase); *THBD* (Thrombomodulin); *MMP1* (Matrix Metalloproteinase 1); *PLAT* (Plasminogen Activator, Tissue Type); *PROCR* (Protein C Receptor); *IL6* (Interleukin 6); *IL1B* (Interleukin 1 Beta); *SELE* (Selectin E); *ICAM1* (Intercellular Adhesion Molecule 1); *SELPLG* (Selectin P Ligand); *SELL* (Selectin L); *CCL2* (C-C Motif Chemokine Ligand 2); *MMP9* (Matrix metalloproteinase 9); *CCL5* (C-C Motif Chemokine Ligand 5); *TYMP* (Thymidine Phosphorylase); *KDR* (Kinase Insert Domain Receptor); *IL7* (Interleukin 7); *TNFSF10* (TNF Superfamily Member 10); *TNF* (Tumor Necrosis Factor); OCLN (Occludin); *EDNRA* (Endothelin Receptor Type A); *TFPI* (Tissue Factor Pathway Inhibitor); *APOE* (Apolipoprotein E); *CX3CL1* (C-X3- C Motif Chemokine Ligand 1); *ACE* (Angiotensin I Converting Enzyme).

We observed consistency for the endogenous *GAPDH* control between results obtained using the 2 different technologies for *FLT1*, *PGF*, *MMP1*, *PROCR*, *IL6*, *ICAM1*,

Table 7. Comparison of Results Obtained for the PCR Array and for qRT-PCR

	PCR Array		qRT-PCR	
Gene	Fold Change	*P* Value	Fold Change	Adjusted *P* Value
FLT1	3.49	0.0218*	2.17	0.2440
PGF	2.93	0.0150*	3.54	0.0238*
MMP1	204.64	0.0004†	25.21	0.0238*
PROCR	1.64	0.0479*	2.04	0.2440
IL6	3.05	0.4405	1.74	0.7354
ICAM1	−2.83	0.0531	−6.45	0.1666
SELPLG	−2.29	0.0090‡	1.28	0.7381
CCL2	−4.01	0.0799	−2.18	0.2440
KDR	−1.62	0.0360*	−5.02	0.2440
TNFSF10	−13.34	0.0253*	−4.64	0.1666

PCR indicates polymerase chain reaction; qRT-PCR, quantitative reverse transcription polymerase chain reaction.
*$P<0.05$.
†$P<0.001$.
‡$P<0.01$.
FLT1 (Fms-Related Receptor Tyrosine Kinase 1); *PGF* (Placental Growth Factor); *MMP1* (Matrix Metalloproteinase 1); *PROCR* (Protein C Receptor); IL6 (Interleukin 6); *ICAM1* (Intercellular Adhesion Molecule 1); *SELPLG* (Selectin P Ligand); *CCL2* (C-C Motif Chemokine Ligand 2); *KDR* (Kinase Insert Domain Receptor); *TNFSF10* (TNF Superfamily Member 10).

CCL2, KDR and *TNFSF10* genes. However, *SELPLG* showed a divergent result, presenting an opposing expression profile (Table 7). Statistically significant differences ($P<0.05$) with both technologies were observed for the *PGF* and *MMP1* genes (Figure 1).

MMP1 expression was significantly increased both in the PCR array and qRT-PCR. Levels of expressed MMP1 proteins were analyzed in 3 SCA patients with stroke and 6 SCA patients without stroke by Western blot (Figure 2A). We did not succeed in cultivating cryopreserved aliquots of ECFCs from 1 patient with stroke. As shown in Figure 2B, MMP1 was expressed in 3 patients with stroke and 2 patients without stroke. Figure 2C shows levels of MMP1 in patients with and without stroke. The MMP1 levels were undetectable in another 4 patients without stroke.

Western blot confirmed higher protein expression of MMP1 in ECFCs from SCA patients with stroke, in accordance with increased gene expression detected by qRT-PCR. The primary antibody used was ab38923, which binds to reduced and native MMP1 but does not cross-react with the other MMP family members (MMP2, MMP3, MMP9, etc.). This antibody reacts with the "pro" and active forms of MMP1, generating 2 bands of 62 and 54 kDa, respectively. The *P* value calculated by the Mann-Whitney *U* test was 0.2619. We emphasize that high *MMP1* expression levels were also detected in 2 SCA patients without stroke by RT-PCR, and the presence of the protein was confirmed by Western blot. Curiously, these 2 patients had other complications that are known to be associated with increased levels of MMP1. If these samples were excluded from the Western blot analysis, because they could interfere with the comparison between the groups with and without stroke, the calculated *P* value would be 0.0571. It is possible to observe a tendency for greater protein level in the stroke group. However, because of the great variability of band intensity in the stroke group and the small number of patients, the statistical significance was not observed.

Analysis of the Promoter Region of the *MMP1* Gene

To investigate a possible association between differential expression and single-nucleotide variants, direct DNA sequencing of the *MMP1* promoter region was carried out in DNA samples from the studied patients. Single-nucleotide variants at the *MMP1* promoter region associated with stroke or vascular disease, such as rs1799750, rs498186, rs475007, rs514921, and rs494379, have been described previously.[24–27] The results showed no statistically significant differences in the distributions of single-nucleotide variants between the studied groups ($P\geq0.05$).

Discussion

In this study, we correlate—for the first time—the differential expression of genes involved in endothelial cell biology with a specific clinical complication of SCA. Stroke is a common and potentially devastating manifestation of SCA that can affect children and adults. The analysis of gene expression profiles identified 29 differentially expressed genes; 11 were upregulated, with most being associated with angiogenesis (55%), inflammatory response (18%), and coagulation (18%) pathways. Downregulated expression was observed in 18 genes, most of which were associated with angiogenesis (28%), apoptosis (28%), and cell adhesion (22%) pathways. This finding is consistent with current hypotheses regarding the context of the pathophysiology of SCA. Angiogenesis contributes to neurorepair processes, including neurogenesis and synaptogenesis.[28–32] Angiogenesis requires proteolysis and remodeling of the extracellular matrix to allow endothelial cells to migrate and invade the surrounding tissue[33]; therefore, the action of MMPs in this process is crucial.

Interestingly, our results showed remarkable overexpression of the *MMP1* gene in the ECFCs of all SCA patients with stroke. In addition, Western blot analysis confirmed these data. MMPs are a large group of zinc-dependent proteases that degrade all extracellular matrix proteins and are involved in inflammatory processes, tissue reconstruction and repair,

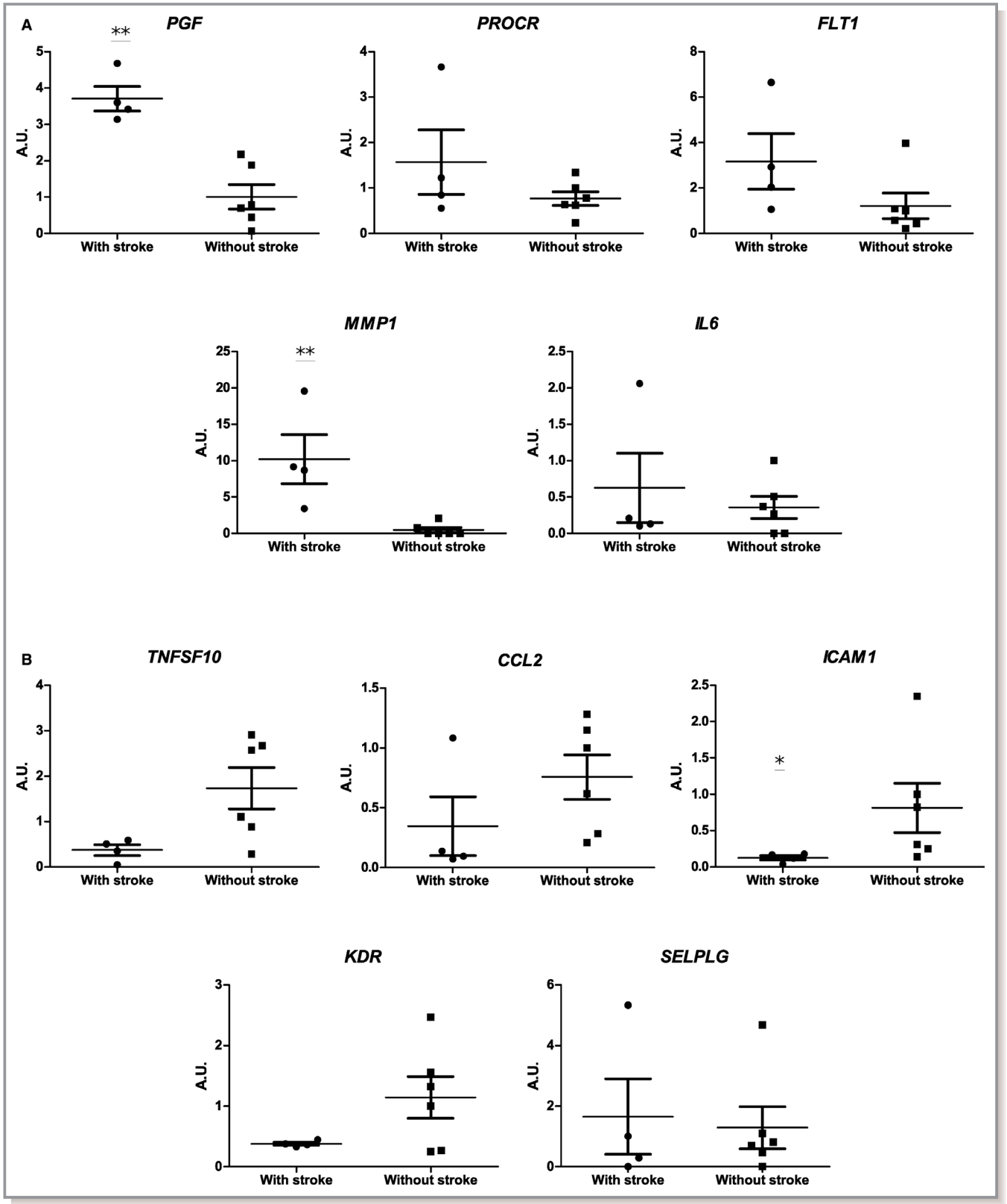

Figure 1. Relative mRNA levels in arbitrary units (AU) of hemoglobin S (HbSS) patients with stroke and without stroke by quantitative reverse transcription polymerase chain reaction. *P<0.05 and **P<0.01. **A,** Expression levels of upregulated genes Placental Growth Factor (*PGF*); Protein C Receptor (*PROCR*); Fms-Related Receptor Tyrosine Kinase 1 (*FLT1*); Matrix Metalloproteinase 1 (*MMP1*) and Interleukin 6 (*IL6*). **B,** Expression levels of downregulated genes TNF Superfamily Member 10 (*TNFSF10*); C-C Motif Chemokine Ligand 2 (*CCL2*); Intercellular Adhesion Molecule 1 (*ICAM1*); Kinase Insert Domain Receptor (*KDR*) and Selectin P Ligand (*SELPLG*).

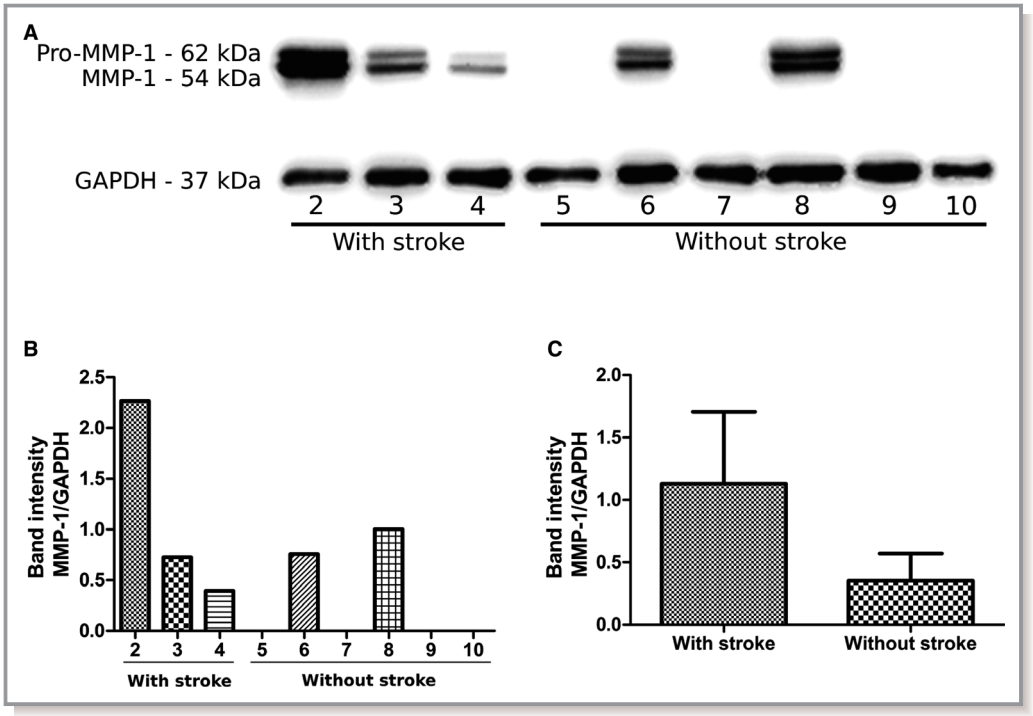

Figure 2. Validation of MMP1 (matrix metalloproteinase 1) expression by Western blot. **A**, Representative densitometric blotting of MMP1 and GAPDH in 3 patients with stroke and 6 without stroke. The upper band represents the "pro-" form, and the lower band represents the active form. **B**, Quantification of the intensity of the MMP1 band for each patient with and without stroke. **C**, Band intensity of MMP1 of patients, grouped according to the presence or absence of stroke. The results were normalized to the endogenous GAPDH protein levels. Mean±SEM: with stroke: 1.129±0.5765; without stroke: 0.3519±0.2189.

cell migration, and angiogenesis.[34,35] Their activities are regulated under physiologic conditions at the levels of transcription, zymogen activation, and inhibition by its interaction with the tissue inhibitor of MMPs.[36] MMPs play a role in the structure of blood vessels in the brain, and unregulated activity of these enzymes can contribute to neurologic disorders and stroke.[29] In acute stroke, the first MMP activated during hypoxia is MMP2, followed by activation of MMP9 by MMP3 and by free radicals generated soon after vessel occlusion. Active MMPs degrade the basal lamina and disrupt the tight junction proteins located in the clefts that join the endothelial cells, opening the blood–brain barrier and leading to vasogenic edema, hemorrhage, leukocyte infiltration, and progressive inflammatory reactions.[37,38]

MMP1 plays a role in the degradation of interstitial collagen types I, II, and III, major structural components of the fibrous plaque that are associated with the mechanism of atherosclerotic stroke. MMP1 protein has been shown to be expressed by macrophages, smooth muscle cells, and microvascular endothelial cells both at the boundary between the fibrous cap and the lipid core and in the shoulder region of the plaque. The expression of this protease by vascular cells seems to contribute to plaque instability.[39] Currently, no studies indicate the clinical significance of MMP1 in stroke in SCA patients. However, a single guanine (1G to 2G) variant located at the *MMP1* promoter region −1607 1G/2G (rs1799750) has been associated with IS risk. Experiments demonstrated that the promoter comprising the 2G alleles creates an E-26 virus transcription site and increases transcription capacity.[40] *MMP1* −1607 1G/2G seems to display association with IS risk in the Chinese population,[41] but no relation was identified in Tunisian patients.[27]

In the present study, the association of this variant with increased *MMP1* gene expression was not found. This observation raises the possibility that other factors may be regulating the expression of *MMP1* in the patients studied and/or that the effect of the variant can be associated with genetic ethnic background. The precise mechanism by which the overexpression of the *MMP1* gene may affect the risk of sickle cell stroke has yet to be elucidated. Curiously, high *MMP1* expression levels were also detected in 2 SCA patients without stroke who had liver disease. Hepatic dysfunction in patients with SCA is predominantly caused by vascular occlusion by sickled red blood cells with acute ischemia, cholestasis, hepatic sequestration crisis, transfusional iron overload, hepatitis C virus infection, and chronic hemolysis,

leading to the development of pigment stones.[42] MMPs play an important role in all stages of progressive liver injury. Hepatic ischemia and reperfusion injury results in MMP-dependent and independent release of reactive oxygen species, cytokines, and other proinflammatory mediators and consequently activates innate immune cells, leading to upregulation of the expression of liver vascular adhesion molecules. Proinflammatory factors, such as TNF-α (tumor necrosis factor α) and inducible NOS (nitric oxide synthase)–derived NO, can stimulate the expression and activation of MMPs, which modulate the activity of some of these factors through proteolytic cleavage, thus providing a possible feedback loop that would amplify and sustain the inflammatory environment.[43]

Interestingly, MMP1 seems to be a candidate for an antifibrotic role because it has been associated with the regression of liver fibrosis in rodents, through cleavage of the fibrillar extracellular matrix and promotion of apoptosis among the activated hepatic stellate cells.[44] In humans, MMP1 is expressed predominantly in monocytes and Kupffer cells in the early stage of nonalcoholic steatohepatitis and in hepatic progenitor cells and proliferating capillary endothelial cells during the advanced stage of the disease, contributing to the repair and regeneration of fibrotic liver.[45]

Distinct subphenotypes of clinical complications of SCD have been proposed: a vasculopathy subphenotype, comprising pulmonary hypertension, priapism, leg ulceration, and stroke, and a viscosity–vaso-occlusive subphenotype involving vaso-occlusive pain crisis, acute chest syndrome, and osteonecrosis.[13] Despite the small number of patients, it is interesting to note that 3 of 4 patients (75%) in the stroke group also had leg ulcer, which is well known to be secondary to vasculopathy.[46] In contrast, no one in the group without stroke has leg ulcer. We investigated other complications, such as pulmonary hypertension and albuminuria, and did not find any difference between groups.

Moreover, the analysis of the gene profiles of the ECFCs from SCA patients showed that the *PGF* gene was upregulated in patients with stroke compared with patients without stroke; a statistically significant difference was observed for both technologies used. PGF is a member of the VEGF family. In mammals, the VEGF family consists of 5 members: VEGFA, VEGFB, VEGFC, and VEGFD, and PGF. The biological effects of VEGFs are mediated by tyrosine kinase receptor VEGF receptors VEGFR1, VEGFR2, and VEGFR3, also known as FLT1 (Fms-related tyrosine kinase 1), KDR, and FLT4, respectively. PGF is a pleiotropic cytokine that stimulates the growth and migration of endothelial cells, angiogenesis, leukocyte infiltration, tumor growth, and revascularization of ischemic tissues.[41] In patients with SCD, increased serum levels of PGF have been described, leading to studies that suggest a possible association between inflammation and angiogenesis in SCD pathogenesis and with SCD complications.[42,43] Interestingly, Gaál and collaborators carried out a systematic evaluation of VEGFs regarding their angiogenic potential and their shortcomings in the murine central nervous system.[47] They showed that PGF was the most efficient and safe angiogenic factor, as the blood–brain barrier remained intact, and had minimal adverse effects, making it a potential candidate for therapeutic central nervous system revascularization.[44]

Among differentially expressed genes validated by qRT-PCR, we also highlight *ICAM1*. Endothelial *ICAM1* is essential for the activation and migration of leukocytes to sites of inflammation. SCD is an inflammatory state, and circulating endothelial cells from these patients display increased expression of adhesion molecules.[48] In contrast, downregulation of *ICAM1* expression has been observed in SCA patients with stroke, in comparison to patients without stroke. The analysis by PCR array showed a clear tendency toward a statistically significant difference (fold change: −2.83; P=0.053) that was not confirmed by qRT-PCR analysis (fold change: −6.45; P=0.1666). We performed *ICAM1* gene expression analysis of total RNA isolated from peripheral blood leukocytes. However, a statistically significant difference between the 2 groups was not observed (P>0.05).

We emphasize that a mainstay of our approach is the use of cultured ECFCs, which are not endothelial cells that reside naturally in the affected organs and thus are free of tissue specification phenotypes and acquired influences. However, studies have shown that after stroke, ischemic penumbral tissue releases angiogenic factors that induce proliferation of endothelial cells and that EPCs present in the systemic circulation are able to migrate and differentiate into mature endothelial cells in the ischemic area and promote neovessel formation.[49,50] In addition, emerging evidence suggests an important role of EPCs in liver angiogenesis and fibrosis. In a previous study, it was shown that circulating EPCs are significantly increased in patients with cirrhosis. EPCs stimulate angiogenesis by resident liver sinusoidal endothelial cells in cirrhosis through paracrine factors.[51] Recently, the contribution of EPCs to alcoholic liver injury via paracrine secretion of inflammatory and angiogenic mediators has been also reported.[52]

Furthermore, regarding treatment, all patients in the stroke group and only 1 patient in the control group (without stroke) were under regular blood transfusion. All stroke patients (n=4) had some vasculopathy including leg ulcers (n=3), retinopathy (n=2), pulmonary hypertension (n=1), and microalbuminuria (n=1) before their stroke events, indicating severe disease progression. Frequent red cell transfusions reduce vascular endothelial activation, inflammation, and the risk of recurrent or silent strokes.[53] Peripheral blood

samples for isolation of ECFCs were collected immediately before transfusion therapy. Moreover, in this study, we used ECFCs from passages 3 to 5. Chang Milbauer et al[19] stimulated Blood Outgrowth Endothelial Cells from 5 donors with IL-1β and TNF-α and monitored gene expression. In response, 122 genes significantly changed expression levels; however, all of them returned to baseline by 1 subsequent passage. Thus, we hypothesized that the effects of transfusion treatment may have been reduced after all these passages and no longer influenced the gene expression level and that the observed expression profile reflected only cell culture conditions and the genetics of the subjects.[19] We underscore that 2 patients from the control group and 1 from the stroke group were heterozygous for α-thalassemia (carriers of a 3.7 kb deletion in the α-globin gene). However, given the small number of the patients, it is not possible evaluate whether the presence of α-thalassemia could influence our results.

Our results suggest statistically significant differences in gene expression of ECFCs between SCA patients with and without stroke. The migratory features of these cells in SCA support the association of these cells with sickle cell stroke. The present study showed, for the first time, that phenotypic heterogeneity (in this case, SCA with versus without stroke) can have a genetic endothelial basis and most likely involves overexpression of the *MMP1* gene. Although our sample size was small, our analysis strongly suggest that angiogenesis is an important process in sickle cell stroke, worthy of further studies.

Although this study has some limitations, such as the small number of patients in each group and the lack of data before stroke, our preliminary results provide novel insights that can guide future interesting studies. Further research using transcriptomes in ECFCs shortly after stroke and in individuals at high risk of stroke and a study of the role of MMP1 in ECFC functions could contribute to better understanding of MMP1 involvement in the pathophysiology of vasculopathy and stroke in SCA.

Sources of Funding

This work was supported by the São Paulo Research Foundation (grant 2014/00984-3) and Coordination for the Improvement of Higher Education Personnel (grant 4570-2018).

Disclosures

None.

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