Endothelial progenitor cells

A new key for endothelial dysfunction in migraine

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ABSTRACT

Objective: We aimed to study endothelial function with biochemical and ultrasonographic markers and its relation with endothelial progenitor cells (EPCs) in patients with migraine.

Methods: We performed a case-control study including 47 patients with episodic migraine (International Headache Society 2004 criteria) and 23 control subjects. We analyzed flow-mediated dilation (FMD) in the dominant brachial artery, calcitonin gene–related peptide (CGRP), and vascular endothelial growth factor (VEGF) levels by ELISA, nitric oxide stable metabolites (NOx) by high-performance liquid chromatography, and EPCs in peripheral blood samples, obtained during interictal periods (n = 47) and migraine attacks (n = 19). Frequency, severity, duration of attacks, and time of evolution of migraine were also recorded.

Results: Patients with migraine showed lower numbers of EPCs than control subjects (9.4 ± 5.0 vs 17.9 ± 6.0 colony forming unit–endothelial cells [CFU-ECs]; $p < 0.0001$) and higher levels of CGRP (164.2 ± 139.1 vs 37.1 ± 38.5 pg/mL), VEGF (473.4 ± 398.7 vs 72.6 ± 56.6 pg/mL), and NOx (1225.2 ± 466.1 vs 671.9 ± 358.6 μM) (all $p < 0.05$). During attacks, higher levels for CGRP (298.2 ± 100.3 pg/mL) and NOx (1,656.8 ± 259.5 μM) and lower numbers of EPC (7.2 ± 3.2 CFU-ECs) were observed (all $p < 0.05$). No changes were found for FMD in interictal periods or during headache. In relation to clinical parameters, EPCs decreased with the time of evolution of migraine ($r = -0.592$; $p < 0.0001$).

Conclusions: Patients with migraine show reduced numbers of EPCs and increased levels of CGRP, NOx, and VEGF than control subjects. Furthermore, EPC counts decrease as migraine progresses in time. These findings suggest altered endothelial function in patients with migraine. *Neurology*® 2012;79:474–479

GLOSSARY

CFU-EC = colony forming unit–endothelial cell; **CGRP** = calcitonin gene–related peptide; **EPC** = endothelial progenitor cell; **FMD** = flow-mediated dilation; **MA** = migraine with aura; **MWA** = migraine without aura; **NO** = nitric oxide; **NOx** = nitric oxide stable metabolites; **VEGF** = vascular endothelial growth factor.

Episodic migraine constitutes a neurologic disorder with vascular and neural mechanisms involved in its pathophysiology. Endothelial function, a predictor of vascular risk, has been studied in patients with migraine, becoming a challenge for investigators.[1] Most vascular risk factors have been associated with a decreased number of endothelial progenitor cells (EPCs) and endothelial dysfunction with cumulative risk.[2] Brachial artery flow-mediated dilation (FMD) has emerged as the most frequently used noninvasive tool for assessment of endothelial functioning. Previous FMD studies in migraineurs during interictal periods have shown contradictory results.[3–5]

Moreover, EPCs maintain the integrity of the endothelium when damage occurs, being considered as markers of endothelial function.[6] Furthermore, low EPC counts have also been found in patients with migraine.[7] Thus, a relation between migraine and vascular risk has been suggested.[8]

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Conversely, release of inflammatory vasoactive peptides during migraine attacks may induce endothelial damage.[9] Calcitonin gene–related peptide (CGRP) and nitric oxide (NO) cause arterial vasodilation and modulate endothelial function. NO pathway abnormalities and subsequent endothelial dysfunction have been reported in patients with migraine.[10] Furthermore, antagonists of CGRP and NO have been proved to abort migraine attacks.[11,12] Finally, vasodilation may also be mediated by vascular endothelial growth factor (VEGF), producing plasma protein extravasation that would increase the risk of migraine.[13]

Therefore, the objectives of this study were 1) to investigate the presence of endothelial dysfunction by ultrasonography and biomarkers in patients with migraine during interictal and ictal periods and 2) to analyze the relationship between endothelial function and clinical parameters.

METHODS

Study population. This was a case-control study including consecutive patients with episodic migraine with aura (MA) or without aura (MWA) attending a headache clinic at a tertiary hospital, in whom migraine was diagnosed by a neurologist according to the International Headache Society criteria[14] and healthy control subjects without migraine or other type of headache, matched by gender and age. All subjects were ≥18 years old. Control subjects were recruited from the hospital and university staff, students, and general population. Clinical variables were recorded, and FMD, CGRP, NOx, VEGF, and EPCs were determined. The period of recruitment was from April 2007 to December 2009.

Exclusion criteria were the following: 1) high blood pressure (known high blood pressure or ≥2 measurements greater than 140/90 mm Hg); 2) coronary disease (coronary lesions >50% determined by angiography, myocardial infarction, angina pectoris, or coronary recanalization); 3) diabetes mellitus (known diabetes mellitus or ≥2 fasting serum glucose determinations >126 mg/dL); 4) hypercholesterolemia (pharmacologically treated or fasting serum cholesterol >200 mg/dL); 5) infectious diseases; 6) chronic inflammatory conditions; 7) severe systemic diseases; 8) oligomenorrhea, polymenorrhea, or polycystic ovarian syndrome; 9) pregnancy or lactation; 10) obesity (body mass index >35 kg/m^2); 11) smoking habit (within the previous 12 months); and 12) recent consumption of vasoactive drugs (<4 times the medium half-life of the active substance). No patient was receiving preventive treatment for any of these conditions.

Standard protocol approvals, registrations, and patient consents. The study was approved by the Research Ethics Committee of Hospital Clínico Universitario of Santiago de Compostela. All patients and control subjects provided written informed consent.

Study protocol. After a screening visit, eligible subjects were invited to perform an ultrasonographic examination and blood sample extraction. Patients were headache-free from the previous 72 to the 24 hours after the visit. If a migraine occurred within the first 24 hours, measurements were repeated in another headache-free period. Subjects had not previously consumed anti-inflammatory or analgesic medication. In migraineurs, the complete protocol was repeated during a headache attack.

Measurements for control subjects and patients during the headache-free period were performed between 10:00 and 11:00 AM in a quiet, temperature-controlled room (22–24°C) by a single observer. Subjects were at rest in the supine position for the previous 10 minutes. A blood sample was extracted from the nondominant forearm. After blood collection, the ultrasonographic study was completed in the dominant forearm. Patients were admitted to the clinic during a migraine attack, and the duration of headache from onset was recorded. Symptomatic treatment was allowed after the study protocol was completed.

Clinical variables. Demographic and clinical data were recorded, including age, gender, type of migraine (MA or MWA), frequency of attacks, intensity of headaches (no effect on daily activities, pain limiting common tasks, or migraine preventing work), duration of attacks (4–8, 9–24, and 25–72 hours), and duration of the disease (years).[15] Clinical parameters of migraine were considered as an average of the patient's episodes.

FMD. FMD of the brachial artery was assessed by the same investigator blinded for biochemical and EPC determinations, after prior technical training and validation of data, compared with medical staff skilled in neurosonology. For that purpose, a high-resolution B-mode ultrasound device (Aplio 50 Toshiba SSA-700) with a 7.5-MHz linear array transducer was used. FMD evaluations were performed according to the International Brachial Artery Reactivity Task Force and the Working Group of the European Society of Hypertension guidelines.[16,17] The dominant brachial artery was imaged 3–5 cm proximal to the antecubital fossa in a longitudinal plane, perpendicular to the ultrasound beam. Baseline measurements were first performed (d1, as the mean of 5 artery diameter determinations during systole) and followed by a rapid inflation of a cuff placed around the proximal forearm to 300 mm Hg for 4 minutes. The location was marked, and a new determination was performed (d2, as the mean of new 5 determinations of the artery diameter during systole) 45–60 seconds after cuff release causing a reactive hyperemia. Brachial artery diameters were obtained from the near-to-far blood wall intima-media interfaces. FMD was expressed as the percentage of increase in the diameter from baseline (d2 − d1/d1 × 100).

EPCs. EPCs were measured according to the methods described elsewhere.[18] A 14-mL sample of venous blood was used for the isolation of EPC colonies. Samples were processed within 1 hour after collection by a unique investigator blinded for clinical, ultrasonographic, and biochemical data. Mononuclear cells were first isolated by Ficoll density gradient centrifugation. Five million peripheral blood mononuclear cells per well were then plated on fibronectin-coated 6-well dishes (BD BioSciences Discovery Labware) in EndoCult Liquid Medium (StemCell Technologies) containing penicillin (100 U/mL) and streptomycin (100 μg/mL) for 2 days to remove the adherent cell population including mature endothelial cells and monocytes. After 2 days, 1 million nonadherent cells were harvested and plated on fibronectin-coated 24-well plates (BD BioSciences Discovery Labware) in EndoCult Liquid Medium. Colonies formed 3 days later were counted and classified as early outgrowth colony forming unit–endothelial cells (CFU-ECs), consisting of a central cluster of rounded cells with elongated sprouting cells at the periphery, in a minimum of 3 wells per sample. Confirmation of

the endothelial cell lineage was performed as described previously.[2,19,20] Intraobserver correlation, assessed by the single investigator who analyzed 2 blood samples obtained from a single subject, was 0.91.

Laboratory tests. Blood samples, obtained simultaneously with the EPCs, were collected in chemistry test tubes, centrifuged at 3000 rpm for 15 minutes, and immediately frozen and stored at −80°C. Serum levels of CGRP (Peninsula Laboratories, LLC, Belmont, CA) and VEGF (R&D Systems Inc., Minneapolis, MN) were measured using commercial ELISA kits following the manufacturer's instructions.

NO is readily oxidized to nitrite (NO_2^-) and subsequently to nitrate (NO_3^-), which serve as index parameters of NO production. Serum levels of NOx (NO_2^- and NO_3^-) were determined by HPLC following a previously described method.[21] The intra-assay and interassay coefficients of variation were 5.9% and 7.4% for CGRP, 5.1% and 6.2% for VEGF, and 2.3% and 3.1% for NOx, respectively. Determinations were performed in a laboratory blinded to clinical and ultrasonographic data and EPC measurements.

Data and statistical analysis. Based on reproducibility data acquired in our laboratory before the study, a sample size of 46 patients with migraine in the headache-free period and 23 healthy control subjects was calculated to detect a between-group difference of 20% in FMD with a significance level of 0.05 and a power of 80%.

Based on previous studies, a sample size of 19 patients with an acute headache was calculated to detect a between-group difference of 20% in CGRP levels with a significance level of 0.05 and a power of 80%.[22] For other variables, the calculated sample size was smaller.

Results are expressed as percentages for categorical variables and mean (SD) or median (quartiles) for continuous variables, depending on the normal or not normal distribution of data. For all comparative analysis we have considered the following variables: FMD and levels of CGRP, VEGF, NOx, and CFU-ECs.

Differences between migraineurs and control subjects were analyzed using the Student *t* test. Differences among groups of the same population were determined by analysis of variance . Correlation between variables of the same group was calculated with regression lines with a 95% confidence interval. The Pearson coefficient for continuing variables (duration of disease) and the Spearman coefficient for categorical variables (intensity of pain and frequency of attacks) were used. A value of $p < 0.05$ was considered significant. Statistical analyses were performed with SPSS 16.0 for Mac software.

RESULTS

Clinical characteristics of study participants. Forty-seven patients with migraine were studied during the interictal period. Only 19 were also examined during an attack of headache. Twenty-three control subjects were included.

Mean age of patients with migraine was 37.8 ± 10.4 years; 46 patients were female (97.87%) and 1 was male (2.13%). Control subjects included 22 female (95.65%) and 1 male (4.35%). Mean age was 31.8 ± 11.0 years. Groups did not differ statistically in gender or age.

From the 47 patients with migraine, 33 presented MWA (70.21%) and 14 MA (29.79%). Time of evolution of migraines was 16.45 ± 13.40 years. Clinical parameters are shown in the table. For ictal studies, mean time from migraine onset was 7.57 ± 1.41 hours.

Table Clinical characteristics of patients with migraine

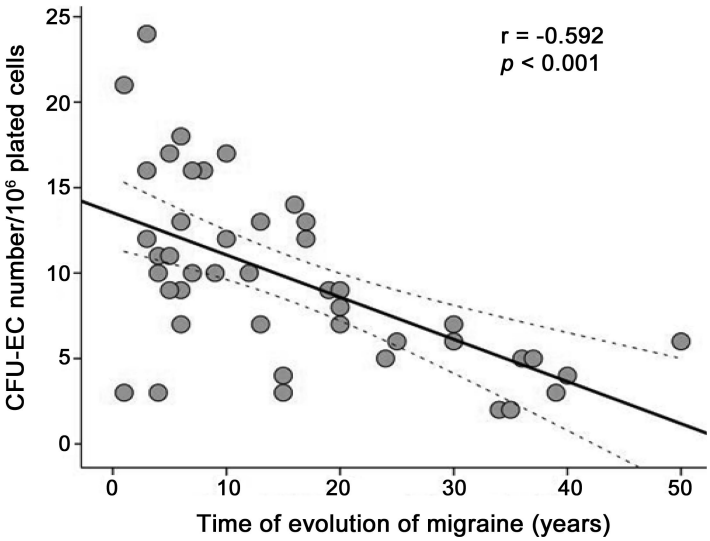
Characteristic	Value
Female gender, n (%)	46 (97.87)
Age, y, mean ± SD	37.8 ± 10.4
Type of migraine, n (%)	
Global	47 (100)
With aura	14 (29.79)
Without aura	33 (70.21)
Frequency of attacks, n (%)	
<4 d/mo	26 (55.23)
4–7 d/mo	18 (38.30)
8–15 d/mo	3 (6.38)
Intensity of pain, n (%)	
Mild	1 (2.13)
Moderate	16 (34.04)
Severe	30 (63.83)
Duration of pain, n (%)	
4–8 h	6 (12.77)
9–24 h	9 (19.15)
25–72 h	32 (68.08)
Time of evolution, y, mean ± SD	
Global	16.45 ± 13.4
With aura	17.7 ± 14.6
Without aura	16.3 ± 12.9

Comparative analysis between control subjects and patients with migraine during the interictal period. Patients with migraine showed lower CFU-EC counts (9.4 ± 5.0 vs 17.9 ± 6.0) and higher levels of CGRP (164.2 ± 139.1 vs 37.1 ± 38.5 pg/mL), VEGF (473.4 ± 398.7 vs 72.6 ± 56.6 pg/mL), and NOx (1225.2 ± 466.1 vs 671.9 ± 358.6 μM) than healthy subjects (all $p < 0.0001$), but no differences were found for FMD (15.3 ± 8.9 vs 17.5 ± 10.1%, $p = 0.308$).

Comparative analysis between MWA and MA during the interictal period. There were no differences between MWA and MA in relation to FMD (15.1 ± 9.1 vs 15.9 ± 9.0%, $p = 0.996$) and levels of CFU-ECs (9.1 ± 4.8 vs 10.2 ± 5.7, $p = 0.701$), CGRP (151.5 ± 140.2 vs 197.1 ± 136.6 pg/mL, $p = 0.295$), VEGF (533.2 ± 446.3 vs 318.8 ± 165.9 pg/mL, $p = 0.117$), and NOx (1188.4 ± 453.8 vs 1320.0 ± 504.2 μM, $p = 0.589$).

Comparative analysis between migraineurs in the interictal and ictal (migraine attack) periods. Nineteen patients (11 with MWA and 8 with MA) were stud-

Figure Correlation between colony forming unit-endothelial cell (CFU-EC) number and time of evolution of migraine



ied during a migraine attack. CFU-EC counts were lower (7.2 ± 3.2 vs 9.7 ± 5.3, $p = 0.022$) and serum levels of CGRP (298.2 ± 100.3 vs 160.7 ± 139.4 pg/mL, $p < 0.0001$) and NOx (1656.8 ± 259.5 vs 1252.9 ± 496.2 μM, $p < 0.0001$) were higher during headaches. No differences were found for FMD (18.9 ± 12.6 vs 15.1 ± 8.8%, $p = 0.381$) and VEGF serum levels (457.8 ± 271.5 vs 471.4 ± 384.3 pg/mL, $p = 0.359$).

Comparative analysis between ultrasonographic, biochemical, and EPC measurements and clinical parameters of migraine. ***Time of evolution of migraine.*** The numbers of CFU-ECs decreased progressively with increasing time of evolution of migraines ($r = -0.592$, $p < 0.0001$) (figure). In contrast, no correlations were found for FMD ($r = 0.046$, $p = 0.765$), CGRP (r = -0.226, $p = 0.140$), VEGF ($r = 0.047$, $p = 0.742$), or NOx ($r = 0.036$, $p = 0.815$).

Intensity of pain. No association was found between intensity of pain and FMD, CFU-ECs, CGRP, VEGF, or NOx. However, when only the moderate and severe pain groups were compared (considering that only one patient had mild headaches), serum levels of VEGF ($p = 0.040$) and NOx ($p = 0.003$) were higher for the severe pain group.

Frequency of migraines. We found no relationship between frequency of migraines and FMD or levels of CFU-ECs, CGRP, VEGF, or NOx.

Duration of headaches. No relationship was found between duration of migraine attacks and FMD or levels of CFU-ECs, CGRP, VEGF, or NOx.

DISCUSSION The purpose of this study was to investigate the relationship between migraine and endothelial function measured by FMD, levels of biochemical markers involved in vascular regulation, and EPC counts. In patients with migraine, we found lower EPC counts and higher levels of CGRP, VEGF, and NOx than in control subjects. During attacks, migraineurs showed lower EPC counts and higher levels of CGRP and NOx. No differences were found for FMD. Pain attacks did not modify the dilation capacity of the endothelium in our study. Interestingly, EPC counts decreased with the time of evolution of the disease.

Our study shows lower EPC counts in patients with migraine, supporting previous available data, which indicate a loss in number and function of EPCs in patients with migraine.[7] We included healthy subjects as controls, whereas Lee et al.[2] compared EPC numbers in subjects with migraine and tension-type headaches. Because we had previously excluded patients with the most prevalent vascular risk factors and other variables that may influence endothelium integrity, it is tempting to postulate that low EPC levels are more a result of pathophysiologic mechanisms related to migraine than a consequence of other conditions associated with endothelial dysfunction. EPCs are a reflection of the correct functioning of the endothelium, and their decrease could be in relation to lower endothelial repair ability. This fact would explain a long-term alteration in endothelial function in patients with migraine, according to results found in patients with cardiovascular and cerebrovascular diseases.[2,20] These values decrease even more during an attack of headache. However, the influence of the release of biochemical factors during migraines that may mediate the EPC mobilization should be taken into account and deserves further investigation. Previous studies showed alterations of indirect markers of endothelial dysfunction in migraines of recent onset.[23] The correlation found between EPCs and the time of evolution of migraine proposes worsening over years.

The role of CGRP in migraine is better known; it produces neurogenic inflammation, NO- and endothelium-independent relaxation, and transmission of nociceptive stimuli. NO regulates cerebral vessel tone and could constitute a signaling molecule leading to the propagation of inflammatory responses found in migraineurs.[10] Abnormalities in the NO pathway have been reported in patients with migraine.[24,25] Both have been extensively investigated in migraine, and NO donors can generate headache in patients with migraine.[26–28] Migraineurs showed higher levels of CGRP and NOx in our study. Induced inflammation by persistent stimulation of endothelium by CGRP and NOx could lead to a progressive decrease of EPC levels as occurs with other chronic diseases.[29] This effect might appear

with greater intensity during pain attacks according to the increased plasma levels of CGRP and NOx found during headache.

The potential role of VEGF in migraine is still not well established. VEGF constitutes a proangiogenic factor and may mediate vasodilation, leading to plasma protein extravasation and therefore to an increase in the risk of migraine. It can also promote EPC release.[30,31] We found decreased EPC counts and increased VEGF levels in migraineurs that could appear as a compensatory response. However, the exact mechanism by which VEGF is associated with migraine is barely known and needs further investigation. The dilation capacity of the endothelium did not differ in control subjects and patients with migraine in interictal or ictal periods. Several FMD determinations may have helped to define the involvement of the endothelium because FMD shows a high variability, and single determinations may reflect a punctual response of the endothelium more than a long-term process of endothelial dysfunction. Biochemical changes may also precede structural damage. Likewise, our study has a predominance of healthy women without previously known risk factors for endothelial dysfunction. Consequently, it is tempting to argue that results found for EPCs, VEGF, NOx, and CGRP suggest altered endothelial function in patients with migraine, although we found no differences in FMD. However, recent studies using an invasive ultrasonographic method with infusion of L-arginine, a NO precursor, have shown no correlation between cerebral and systemic vascular reactivity.[32]

No relation was observed between biochemical markers and EPCs in the interictal period and frequency of attacks or duration of pain. It might seem that patients with more frequent migraine attacks could trigger mechanisms resulting in endothelium dysfunction more often, but in our study only a small percentage of patients with migraine had more than 7 days of pain per month. The intensity of attacks was found to be related to higher levels of VEGF and NOx during the interictal period for moderate and severe pain groups. The more they increase, the easier it is for greater vasodilation with subsequently plasma protein extravasation that would increase the intensity of pain.

This study has several limitations such as the facts that FMD was only studied punctually and the FMD investigator was not blinded for clinical data. A bigger sample size, the use of vasoactive substances, or a simultaneous study of intracranial circulation could have powered our results. There are different methods for the study of EPCs, such as flow cytometry or cell culture for CFU-EC counts. At present, the most accepted method is flow cytometry, but several studies in stroke patients have obtained virtually the same results using both techniques.[18,33,34]

The high prevalence of migraine increases interest in its pathophysiology. Previous studies had separately shown the influence of different markers of endothelial function in migraines of recent onset and in patients from the general population.[4,7,23,24] Our results support these findings in patients free of other factors that could influence endothelium, suggesting their relation to patients with migraine independently of any other condition, but this may limit the generalizability of our results.

In our study, patients with migraine have lower EPC counts and higher levels of CGRP, NOx, and VEGF. Furthermore, EPC levels decrease with the longer duration of the disease. These findings suggest altered endothelial function in patients with migraine.

AUTHOR CONTRIBUTIONS

R. Leira and J. Castillo conceived and designed the research, analyzed and interpreted the data, and handled funding and supervision. X. Rodríguez-Osorio and R. Leira drafted the manuscript. J. Castillo performed statistical analysis. T. Sobrino and F. Martínez helped to analyze and interpret the data and made critical revision to the manuscript for important intellectual content. T. Sobrino and D. Brea acquired, analyzed, and interpreted the biochemical and cellular data and supervised the work. X. Rodríguez-Osorio, F. Martínez, and R. Leira helped to acquire, analyze, and interpret the clinical ultrasonographic data.

DISCLOSURE

The authors report no disclosures relevant to the manuscript. **Go to Neurology.org for full disclosures.**

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