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The Increase of Circulating Endothelial Progenitor Cells After Acute Ischemic Stroke Is Associated With Good Outcome

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Background and Purpose—Increased circulating endothelial progenitor cells (EPC) have been associated with a low cardiovascular risk and may be involved in endothelial cell regeneration. The present study was designed to evaluate the prognostic value of EPC in acute ischemic stroke.

Methods—Forty-eight patients with a first-ever nonlacunar ischemic stroke were prospectively included in the study within 12 hours of symptoms onset. Stroke severity was evaluated by the National Institutes of Health Stroke Scale, and functional outcome was assessed at 3 months by the modified Rankin Scale (mRS). Infarct volume growth between admission and days 4 to 7 was measured on multiparametric MRI. EPC colonies were defined as early outgrowth colony-forming unit-endothelial cell (CFU-EC). The increment of CFU-EC was quantified during the first week and defined as the absolute difference between the number of CFU-EC at day 7 and admission. The influence of CFU-EC increase on good functional outcome (mRS $\leq$2) and infarct growth was analyzed by logistic regression and linear models.

Results—Patients with good outcome (n=25) showed a higher CFU-EC increment during the first week (median [quartiles], 23 [11, 36] versus −3 [−7, 1], $P<0.0001$) compared with patients with poor outcome. CFU-EC increment $\geq$4 during the first week was associated with good functional outcome at 3 months (odds ratio, 30.7; 95% CI, 2.4 to 375.7; $P=0.004$) after adjustment for baseline stroke severity, ischemic volume and thrombolytic treatment. For each unit increase in the CFU-EC the mean reduction in the growth of infarct volume was 0.39 (0.03 to 0.76) mL ($P=0.033$).

Conclusions—The increase of circulating EPC after acute ischemic stroke is associated with good functional outcome and reduced infarct growth. These findings suggest that EPC might participate in neurorepair after ischemic stroke. **(*Stroke*. 2007;38:2759-2764.)**

Key Words: endothelial progenitor cells ■ ischemic stroke ■ neovascularization ■ neurorepair

Circulating endothelial progenitor cells (EPC), which derive from bone marrow and move to the peripheral blood, have been suggested to be a marker of cardiovascular risk and endothelial function.[1,2] The number of circulating EPC has been reported to be decreased in patients with cardiovascular risk factors such as smoking habit, hypercholesterolemia, diabetes and hypertension,[3–5] many of which have been identified as prognostic markers of poor outcome after ischemic stroke. Moreover, low EPC number has also been found in patients with unstable angina[6] and cerebrovascular disease.[7] EPC have been related to endothelial cell regeneration and neovascularization after tissue ischemia.[8,9]

The mobilization of circulating EPC has been shown to enhance the repair of damaged arteries.[10] The transfusion of EPC reduces neointima formation after vascular injury,[11] and the transplantation of progenitor cells after myocardial infarction improves ventricular function.[12,13] Because EPC have been involved in all these aforementioned repair processes, we tested the hypothesis that EPC might be associated with improved functional outcome and reduced brain injury in patients with acute ischemic stroke.

Methods

Study Population and Patients Characteristics

Between September 2005 and June 2006, one hundred patients with a first–ever nonlacunar hemispheric cerebral infarction in the territory of the middle cerebral artery of <12 hours duration and previously independent for their daily living activities were prospectively evaluated to be included in the study. Patients with chronic

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Partial results of this investigation were presented at the 35th Annual Meeting of the Society for Neuroscience in Washington DC, USA (November 2005) and the 15th European Stroke Conference in Brussels, Belgium (May 2006).

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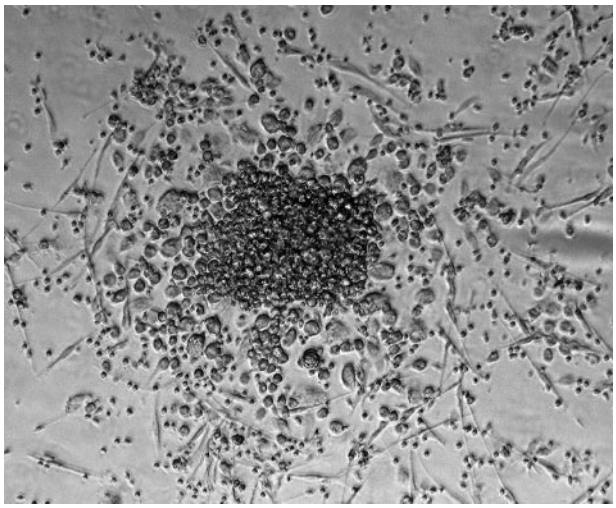

Figure 1. Micrographs of a CFU-EC in a phase-contrast microscope (x100).

inflammatory diseases (n=6), severe hepatic (n=2) or renal (n=1) diseases, hematological diseases (n=4), cancer (n=3) or infectious disease in the 15 days before inclusion (n=22) were excluded. Eight patients did not accept their participation in the study and 6 patients were lost during follow-up, so a total of 48 patients (male, 50%; mean age, 70.7 (10) years) were finally included in the study.

The protocol was approved by the ethics committee and informed consent was given by patients or their relatives.

Clinical Variables

All patients were admitted in the acute stroke unit and treated by the same stroke team according to the Guidelines of the Cerebrovascular Diseases Study Group of the Spanish Society of Neurology.[14] Medical history recording potential vascular risk factors, blood and coagulation tests, 12-lead ECG, chest radiography, and carotid ultrasonography were performed at admission. Stroke subtype was classified according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria[15] as atherothrombotic (n=8), cardioembolic (n=21), and undetermined (n=19). Fifteen patients received thrombolytic treatment with recombinant tissue plasminogen activator (rt-PA) following the Safe Implementation of Thrombolysis in Stroke-Monitoring Study (SITS-MOST) criteria.

Stroke severity was assessed by certified neurologists (M.B., M.R.-Y.) using the National Institutes of Health Stroke Scale (NIHSS) at admission, at 72 (±24) hours, and at 7 (±1) and 90 (±7) days.[16] Functional outcome was evaluated at 3 months using the modified Rankin Scale (mRS).

Neuroimaging Studies

All patients had a multiparametric MRI study at admission (median time from stroke onset, 5.6 [2.4, 8.5] hours) and on days 4 to 7. The increase in infarct volume was calculated as the absolute difference between the diffusion-weighted imaging lesion volume at baseline and the fluid-attenuated inversion recovery (FLAIR) lesion volume on days 4 to 7. Lesion volumes were calculated by using a manual

Table 1. Baseline Clinical Characteristics, Vascular Risk Factors, Stroke Subtype, Biochemical Parameters and Neuroimaging Findings in Patients With Good or Poor Outcome

	Good Outcome, n=25	Poor Outcome, n=23	*P*
Age, y	68.2 (12.6)	73.3 (4.1)	0.391
Male, %	56.0	43.5	0.282
Time from stroke onset to blood obtained, h	5.3 (2.1)	5.3 (2.3)	0.274
Vascular risk factors			
History of hypertension, %	36.0	69.7	0.020
History of diabetes, %	24.0	34.8	0.307
History of dyslipemia, %	24.0	30.4	0.430
Smoking habit, %	12.0	8.7	0.541
Alcohol consumption, %	16.0	13.0	0.352
History of ischemic heart disease, %	20.0	17.4	0.556
Biochemistry and vital signs on admission			
Body temperature (°C)	36.4 (0.3)	36.7 (0.4)	0.006
Systolic blood pressure (mm Hg)	144.7 (21.1)	160.0 (24.6)	0.039
Diastolic blood pressure (mm Hg)	81.2 (12.4)	84.7 (13.2)	0.268
Glucose levels (mg/dL)	119.1 (31.2)	148.9 (44.1)	0.015
Fibrinogen (mg/dL)	380.8 (103.6)	434.6 (125.9)	0.026
t-PA treatment, %	44.0	17.4	0.046
Neuroimaging findings			
Initial ischemic volume (mL)	12.1 (9.9)	38.3 (28.5)	<0.0001
Infarct volume day 4–7 (mL)	13.2 (10.1)	64.8 (29.6)	<0.0001
Clinical characteristics			
NIHSS on admission	6 (5, 9)	18 (14, 20)	<0.0001
Stroke subtype:			0.435
Atherothrombotic, %	16.0	17.4	
Cardioembolic, %	36.0	52.2	
Undetermined, %	48.0	30.4	

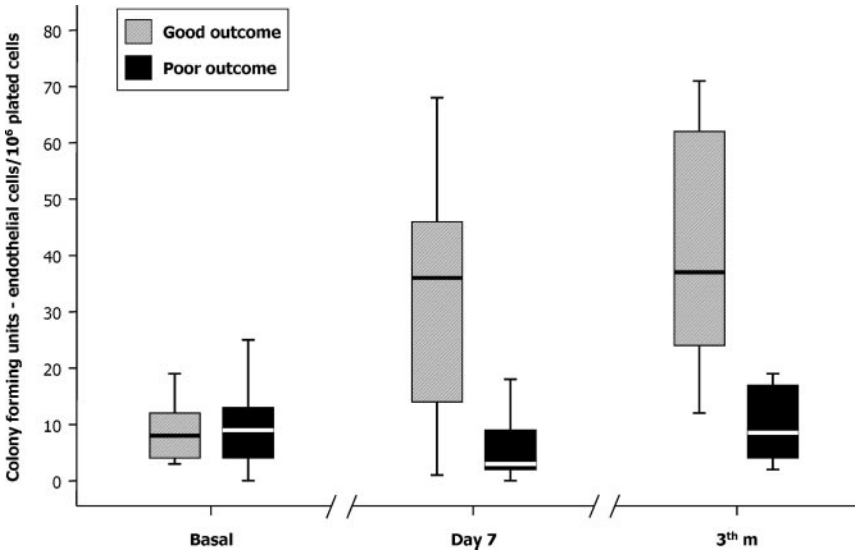

Figure 2. Temporal profile of median (quartiles) CFU-EC number in patients with good or poor outcome at 3 months.

segmentation method: the perimeter of the area of abnormal signal intensity was traced on each diffusion-weighted imaging or FLAIR map, and subsequently the volumetric software estimated the total volume using the thickness and the traced area on each slice; the window level and width were chosen to obtain the best contrast between the lesion and the normal surrounding tissue; each volume calculation was done 3 times, and the mean value was taken as definitive. All neuroimaging evaluations were made by the same neuroradiologist who had no knowledge of the patients' clinical and laboratory results.

Isolation, Cultivation and Characterization of EPC

A 14-mL sample of venous blood obtained at 5.4 (3.6, 7.4) hours from stroke onset, was used for the isolation of the EPC colonies. The samples were processed within 1 hour after collection by a unique investigator who had no knowledge of the patients' clinical and radiological results. Mononuclear cells were first isolated from peripheral blood 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, in order to remove the adherent cell population including mature endothelial cells and monocytes. After 2 days, one million of nonadherent cells (which contain the EPC) were harvested and plated on fibronectin-coated 24-well plates (BD BioSciences Discovery Labware) in EndoCult Liquid Medium. Colonies that form 3 days later were counted and classified as early outgrowth colony forming unit—endothelial cell or colony-forming unit-endothelial cell (CFU-EC). A CFU-EC consisted of a central cluster of rounded cells with elongated sprouting cells at the periphery (Figure 1). CFU-EC quantification, in a minimum of 3 wells per sample, was performed at admission, and at days 7 (1) and 90 (7) of evolution. We calculated the increment of EPC colonies during the first week as the absolute difference between the number of CFU-EC at day 7 and admission. Confirmation of the endothelial-cell lineage was performed as previously described.[1,7,17] The intraobserver correlation, which was assessed by the unique investigator who analyzed 2 blood samples obtained from a single subject, was 0.96.

Outcome Variables

The primary end point was good functional outcome (mRS $\leq$2) at 3 months. The absolute improvement in the NIHSS scores between baseline and days 7 and 90, and the increase in infarct volume were evaluated as secondary outcome variables.

Statistical Analysis

The results are expressed as percentages for categorical variables and as mean (SD) or median (quartiles) for the continuous variables depending on their normal distribution or not. Proportions were compared using the χ^2 test, and the Student t test or the Mann–Whitney test were used to compare continuous variables between groups. Spearman analysis was used for bivariate correlations.

The influence of the CFU-EC increase on functional outcome was assessed by logistic regression analysis after adjusting for the main baseline variables related to outcome in the univariate analyses (enter approach and probability of entry $P<0.05$). Because of a lack of linearity, CFU-EC increase was categorized by receiver operating characteristic analysis. Results were expressed as adjusted odds ratios with the corresponding 95%CI. The influence of the increment of CFU-EC on the growth of infarct volume, and on the change of NIHSS score, was evaluated by general linear models after adjusting for variables associated in the bivariate comparisons. Tables with bivariate analyses are not shown.

Results

Primary Outcome

Table 1 shows the main characteristics of patients by outcome groups. There was no difference in the number of CFU-EC at baseline between groups, but the number increased significantly at day 7 and 3 months in the good outcome group whereas remained stable during the 3 months follow-up in the poor outcome group ($P<0.0001$; Figure 2). The median CFU-EC increase during the first week was significantly

Table 2. Crude and Adjusted OR of Good Outcome at 3 Months for CFU-EC Increment ≥4 in the First Week

	OR (95% CI), *P*	OR (95% CI), Adjusted *P*
CFU-EC Increment ≥4 in the first week	42.0 (7.3 to 241.8), $P<0.0001$	30.7 (2.4 to 375.7), $P=0.004$
NIHSS on admission		1.3 (1.1 to 1.9), $P=0.002$
tPA treatment		0.5 (0.02 to 12.8), $P=0.701$
Initial ischemic volume		1.1 (1.0 to 1.2), $P=0.032$

Table 3. Unadjusted and Adjusted Increase in Mean (95% CI) NIHSS Score Improvement for CFU-EC Increment in the First Week

Change in the NIHSS Score Between Baseline and Day 7		
CFU-EC increment in the first week	−0.09 (−0.14 to −0.04), $P<0.0001$	−0.12 (−0.22 to −0.03), $P<0.0001$
NIHSS on admission		−0.19 (−0.44 to −0.05), $P=0.030$
tPA treatment		−0.18 (−2.7 to −0.04), $P=0.13$
Initial ischemic volume		0.03 (−0.02 to 0.08), $P=0.318$
Change in the NIHSS Score Between Baseline and Day 90		
CFU-EC increment ≥4 in the first week	−0.07 (−0.13 to −0.01), $P<0.0001$	−0.09 (−0.20 to −0.01), $P=0.012$
NIHSS on admission		−0.21 (−0.49 to −0.05), $P=0.003$
tPA treatment		−0.35 (−4.32 to 1.62), $P=0.860$
Initial ischemic volume		0.09 (−0.05 to 1.07), $P=0.763$

lower in patients with history of high blood pressure (−1 [−5, 9] versus 21 [3,37]; $P<0.0001$), whereas it was significantly higher in patients treated with tPA (36 [21,48] versus 0 [−4, 11]; $P<0.0001$). CFU-EC increase negatively correlated with stroke severity ($r=-0.465$, $P=0.001$), body temperature ($r=-0.469$, $P=0.001$), serum glucose ($r=-0.410$, $P=0.004$) and fibrinogen levels ($r=-0.418$, $P=0.003$), and with the diffusion-weighted imaging ischemic lesion ($r=-0.364$; $P=0.011$) at baseline. No relationship was found regarding age, sex, history of diabetes, smoking habit, cardiac diseases, time from symptoms onset to sampling, and blood pressure on admission.

CFU-EC increment of ≥4 during the first week was associated with good functional outcome at 3 months (odds ratios, 30.7; 95% CI, 2.4 to 375.7; $P=0.004$) after adjustment for stroke severity and diffusion-weighted imaging lesion volume at baseline, and for t-PA treatment (Table 2).

Secondary Outcomes

CFU-EC increase correlated with the improvement in the NIHSS score at day 7 ($r=-0.535$; $P<0.0001$) and day 90 ($r=-0.394$; $P=0.006$). Lineal regression models showed that by each unit increase in the CFU-EC during the first week, the NIHSS showed a mean improvement of −0.12 (95% CI, −0.22 to −0.03; $P<0.0001$) points at day 7 and of −0.09 (95% CI, −0.20 to −0.01; $P=0.012$) points at 3 months (Table 3).

Figure 3 shows the negative correlation between CFU-EC increase and infarct volume growth during the first week ($r=-0.656$, $P<0.0001$). For each unit increase in the CFU-EC the mean reduction in the growth of infarct volume was 0.39 (0.03 to 0.76) mL ($P=0.033$; Table 4).

Discussion

This is, to the best of our knowledge, the first prospective study which has evaluated the relationship between circulating EPC and brain injury in patients with acute ischemic stroke. Remarkably, the EPC increase during the first week was independently associated with good outcome at 3 months. This favorable effect on the primary variable was supported by positive effects on the reduction of infarct growth and neurological improvement at day 7 and 90.

We have demonstrated that circulating EPC increase in response to cerebral ischemia in patients after acute ischemic

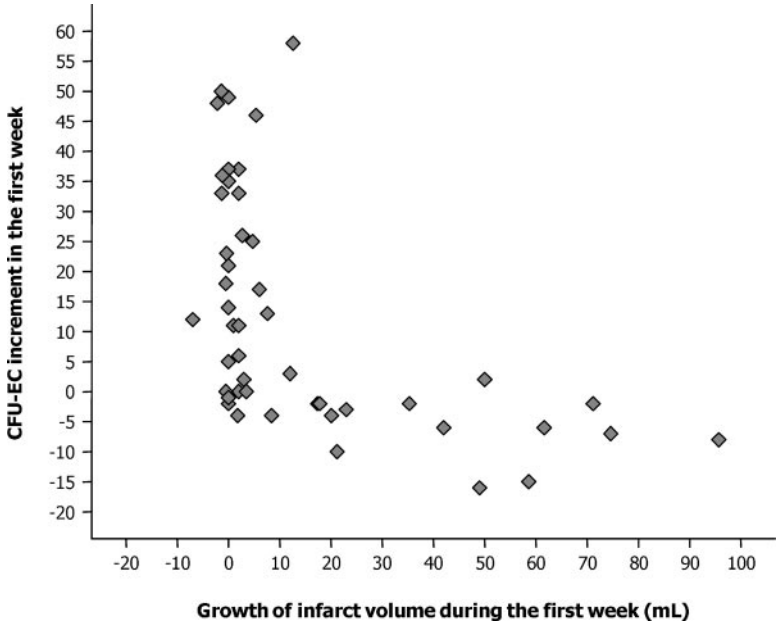

Figure 3. Scatterplot shows the correlation between the CFU-EC increment and the growth of infarct volume during the first week (Spearman correlation coefficient, $r=-0.656$, $P<0.0001$). Note the exponential relation between both variables.

Table 4. Unadjusted and Adjusted Increase in Mean (95% CI) Infarct Volume Growth for CFU-EC Increment in the First Week

CFU-EC increment in the first week	−0.64 (−0.95 to −0.32), $P<0.0001$	−0.39 (−0.76 to −0.03), $P=0.033$
Systolic blood pressure		0.37 (0.08 to 0.66), $P=0.013$
Body temperature		1.68 (−14.2 to 17.5), $P=0.832$
NIHSS on admission		0.31 (−1.08 to 1.7), $P=0.650$
DWI lesion volume at basaline		1.04 (0.73 to 1.34), $P<0.0001$

DWI indicates diffusion-weighted imaging.

stroke, and that the magnitude of this increase is directly related to a better functional outcome. These findings are in line with experimental and human studies which indicate that EPC might mediate endothelial cell regeneration and neovascularization,[8,10,11,17] and that EPC participate in the cerebral neovascularization processes present in adult brain after ischemia.[9,18,19] The fact that patients with good outcome showed higher CFU-EC number on day 7 and at 3 months, but not at admission, support the hypothesis that EPC can mediate processes of chronic vessel repair and neurorepair. Furthermore, the increment in the EPC during the first week was associated with an improvement in the NIHSS score at the same time. Likewise, a negative correlation exists between CFU-EC increase and infarct volume growth during the first week. However, the mechanisms by which EPC are associated with better neurological and functional outcome and with smaller growth of infarct volume deserve further studies.

Reduced circulating EPC have been associated with several cardiovascular risk factors such as hypercholesterolemia,[20] smoking habit,[4] diabetes[21] and Framingham score,[1] and with prognostic factors of acute stroke such as age, heart diseases, stroke severity, elevated blood pressure, serum glucose, fibrinogen and C-reactive protein.[4,5,22–24] Some of these factors were associated with a lower increase of EPC during the first week in this study, so we cannot completely rule out a confounding effect of these related variables. However, we did not find a prognostic effect of EPC at baseline, whereas the effect of EPC increment on outcome and infarct volume remained after adjustment for the main prognostic variables. Consequently, our results support the hypothesis that EPC may have an independent beneficial role in acute ischemic stroke.

The higher EPC increase in the small number of patients treated with rt-PA deserves further studies. rt-PA treatment is associated with overexpression of matrix metalloproteinase-9,[25] which is a potent stimulator of the increment of circulating EPC.[26] Because we did not perform systematic transcranial ultrasound, the potential effect of early recanalization on EPC increase is unknown.

In conclusion, a higher increase in circulating EPC during the first week is independently associated with a better clinical outcome in acute ischemic stroke patients. However, whether circulating EPC are able to incorporate into brain ischemic areas and to promote regenerative vasculogenesis in humans remains to be clarified. Finally, the role of EPC as a new therapeutic tool able to promote chronic neurorepair of brain tissue damaged by ischemia needs to be further explored.

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Disclosures

None.

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