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Endothelial Progenitor Cells influence acute and subacute stroke hemodynamics

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ABSTRACT

Background: Endothelial Progenitor Cells (EPCs) are a circulating stem cell population with *in vivo* capacity of promoting angiogenesis after ischemic events. Despite the promising preclinical data, their potential integration with reperfusion therapies and hemodynamic evolution of stroke patients is still unknown. Our aim was to determine the association of EPCs with acute, subacute and chronic hemodynamic features.

Methods: In this prospective study, we included consecutive patients with ages between 18 and 80 years and non-lacunar ischemic stroke within the territory of a middle cerebral artery. All patients were subject to hemodynamic evaluation by ultrasound at baseline, seven days and three months. We quantified cerebral blood flow (CBF) and assessed early recanalization and collateral flow. Hemorrhagic transformation was graded in Magnetic Resonance imaging performed at seven days. EPCs were isolated from peripheral venous blood collected in the first 24 h and seven days, counted and submitted to functional *in vitro* tests.

Results: We included 45 patients with a median age of 70 ± 10 years. The angiogenic and migratory capacities of EPCs were associated with increased collateral flow in the acute stage and day seven CBF, without statistically significant associations with recanalization nor haemorrhagic transformation. The number of EPCs was not associated with any hemodynamic variable.

Conclusions: The functional properties of EPCs are associated with acute and subacute stroke hemodynamics, with no effect on haemorrhagic transformation.

1. Introduction

The treatment algorithm for acute ischemic stroke has had significant recent updates due to new effective strategies to promote recanalization. However, after the first few hours no therapy has demonstrated meaningful impact on clinical recovery. Endothelial Progenitor Cells (EPCs) are circulating cells that have emerged as a promising treatment strategy, with demonstrated *in vivo* capacity of promoting neovascularization and neurological improvement after stroke [1,2]. Nonetheless, their timing of action and integration within stroke hemodynamics are still unknown, which can be critical for optimization of their clinical effect.

Acute stroke hemodynamics have been recognized as one of the main determinants of clinical evolution [3,4]. Several pathophysiological mechanisms take place in the regulation of vascular responses with distinct implications and at different timepoints. In the hyperacute stage the main determinant of evolution is early recanalization, with a marked clinical impact [5,6]. Collateral pial circulation and cerebral blood flow (CBF) are also essential early hemodynamic mechanisms, installed in the attempt to extend the preservation of cerebral tissue after the vascular insult [7]. After the acute stage, and depending on timing of recanalization and resistance to ischemia, a loss of

Abbreviations: ACA, Anterior Cerebral Artery; CAC, Circulating Angiogenic Cells; CBF, Cerebral Blood Flow; CFU-EC, Collony Forminf Unit-Endothelial Cells; EGM-2, Endothelial Growth Medium-2; EGM-2 MV, Endothelial Growth Medium-2 Microvascular; EPC, Endothelial Progenitor Cell; FBS, Fetal Bovine Serum; HT, Hemorrhagic Transformation; ICA, Internal Carotid Artery; MCA, Middle Cerebral Artery; MNC, Mononuclear Cells; MRI, Magnetic Resonance Imaging; mRS, Modified Rankin Scale; NIHSS, National Institute of Health Stroke Scale; oEPC, Outgrowth Endothelial Progenitor Cell; PCA, Posterior Cerebral Artery; PH, Parenchymal Hemorrhage; TCCD, Transcranial Colour Coded Doppler; VA, Vertebral Artery

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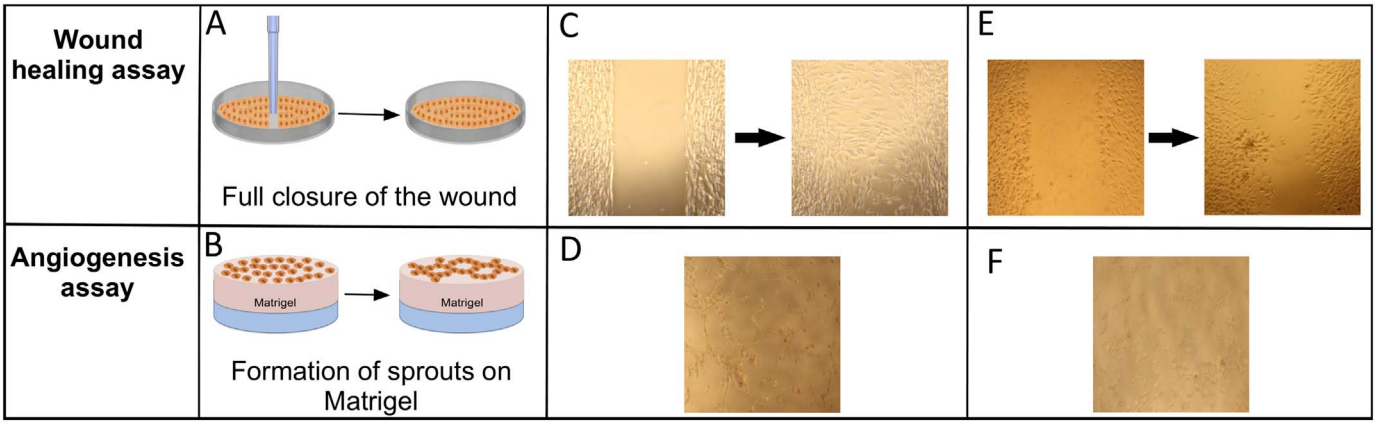

Fig. 1. Representation of *in vitro* wound healing and angiogenesis assays (A and B respectively). Examples with enhanced (C and D) and poor properties (E and F) in each test are shown.

autoregulation may precipitate hyperperfusion injury which may ultimately lead to hemorrhagic transformation [8]. Altogether, these are potentially modifiable hemodynamic responses, crucial to the clinical evolution after stroke.

The potential integration of EPC transplantation within the treating algorithm of stroke will have to take the hemodynamic response into account. As such, in this study we used a multidimensional approach to assess the interplay between EPCs and the several aspects of the patients' hemodynamic state.

2. Methods

2.1. Study population

We included consecutive acute ischemic stroke patients admitted in our department during a period of 27 months (June/2012 to August/2013 and June/2014 to July/2016) in a prospective observational cohort study. All patients with ages between 18 and 80 years and clinically defined non-lacunar strokes within the territory supplied by the Middle Cerebral Artery (MCA) that could have full clinical, neuroimaging and cellular evaluation within 24 h after the onset of symptoms were included (Supplementary Fig. 1 presents the flow chart for study participation, including exclusion criteria). All patients or legal representatives signed written informed consent for study participation. The study design was approved by the local ethics committee (Ref. 130-CE-2011).

At hospital presentation we collected demographic variables, vascular risk factors and quantified stroke severity using the National Institute of Health Stroke Scale (NIHSS) [9]. Functional outcome was graded in person at three months according to the modified Rankin scale (mRS) by vascular neurologist blinded to CBF and *in vitro* data.

2.2. Study design

At day zero (first 24 h after symptom onset) we collected patients' blood for cellular isolation and performed clinical and hemodynamic evaluation. At day 7 ± 2 days patients underwent MRI, repeated hemodynamic evaluation and blood collection for cellular studies. At 3 months ± one week participants had repeated clinical and hemodynamic evaluation. The primary objectives were to determine the associations between the number and functional properties of EPCs with CBF, hemorrhagic transformation, recanalization and flow diversion after ischemic stroke. We also aimed to understand the role of EPCs and CBF within clinical and demographic features as secondary objectives.

2.3. Isolation of EPC sub-populations

We isolated EPCs from 18 mL of peripheral venous blood collected in the first day and at day seven after stroke onset in accordance to previously validated protocols [1,2]. Mononuclear cells (MNC) were isolated from peripheral blood by density gradient centrifugation using Lymphoprep™ density gradient medium.

Three different types of EPCs were analyzed: circulating angiogenic cells (CACs), outgrowth Endothelial Progenitor Cells (oEPCs) and colony forming unit-endothelial cells (CFU-ECs). For CACs, MNCs were plated into 2 μg/cm^2 fibronectin-coated plates (24-well plates; 1.9 × 10^6 cells/well) and cultured in Endothelial Growth Medium-2 Microvascular (EGM-2 MV) containing 5% fetal bovine serum (FBS) during five days. Adherent cells were detached using trypsin, counted and used for functional assays.

For oEPCs and CFU-ECs, 10 × 10^6 MNCs in Endothelial Growth Medium-2 (EGM-2) with 10% FBS were seeded into one well of 2 μg/cm^2 fibronectin-coated 24-well plate. After 48 h, nonadherent cells were collected and 3 × 10^6 cells were replated into three fibronectin-coated 24-well plates. At day 5, colony-forming units were counted manually in four random fields (20 × magnification). The CFU-ECs were detached using trypsin and used for functional studies [10]. The adherent cells at 48 h continued cell culture for 14–21 days [11] to obtain oEPCs. Medium for oEPCs and CFU-ECs was changed every 48 h. All incubation periods were performed at 37 °C and 5%CO_2.

2.4. Functional tests of EPCs

The wound healing capacity of oEPCs and CFU-ECs was evaluated using the *in vitro* wound-healing (scratch) assay. In brief, cells were plated in 2 μg/cm^2 fibronectin-coated 96-well plates and cultured until they form a monolayer. Then, the wounds were created by scratching the cell layer with a 200 μL pipette tip. Migratory capacity was quantified as the percentage of wound closure after 24 h (Fig. 1).

The angiogenic capacity of oEPCs and CFU-ECs were determined by the sprout formation on Matrigel (using an IBIDI μ-slide angiogenesis kit). The total tube length and number of branching points (*i.e.* points featuring more than two connections) were manually measured in four random fields (20 × magnification) 24 h after plating (Fig. 1).

The migratory capacity of CACs was determined by transwell migration. In short, 2 × 10^4 CACs were placed in the upper chamber of a modified Boyden chamber (2 μg/cm^2 fibronectin-coated). The chamber was placed in a 24-well culture dish containing EBM-2 and human recombinant VEGF (50 ng/mL) and incubated for 24 h. The lower side of the filter was then washed with PBS and fixed with 4% paraformaldehyde. For quantification, cell nuclei were stained with 4′,6-diamidino-2-phenylindole. Cells migrating into the lower chamber were counted manually in 3 random microscopic fields (20 × magnification).