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Transplanted Late Outgrowth Endothelial Progenitor Cells as Cell Therapy Product for Stroke

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Abstract Endothelial progenitor cells (EPCs) seem to be a promising option to treat patients with ischemic diseases. Here, we investigated the effects of late outgrowth EPCs, or endothelial colony-forming cells (ECFCs), a recently defined homogeneous subtype of EPCs, in a rat model of transient middle cerebral artery occlusion (MCAO). Either vehicle or 4.10^6 ECFCs, isolated from human cord blood, were intravenously injected 24 h after 1 h MCAO in rats assigned to control and transplanted groups respectively. ^{111}In-oxine-labeled ECFCs specifically homed to ischemic hemisphere and CM-Dil prelabeled ECFCs preferentially settled in the inner boundary of the core area of transplanted animals. Although incorporation of cells into neovessels was hardly detectable, ECFCs transplantation was associated with a reduction in apoptotic cell number, an increase in capillary density and a stimulation of neurogenesis at the site of injury. These effects were associated with an increase in growth factors expression in homogenates from ischemic area and may be related to the secretion by ECFCs of soluble factors that could affect apoptosis, vascular growth and neurogenesis. Microscopic examination of the ischemic hemisphere showed that ECFCs transplantation was also associated with a reduction in reactive astrogliosis. In conclusion, we demonstrated that ECFCs injected 24 h after MCAO settled in the injured area and improved functional recovery. The neurological benefits may be linked to a reduction in ischemia-induced apoptosis and a stimulation of ischemia-induced angiogenesis and neurogenesis. These findings raise perspectives for the use of ECFCs as a well-characterized cell therapy product for optimal therapeutic outcome after stroke.

Keywords Stroke · Late endothelial progenitors · Angiogenesis · Apoptosis

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Introduction

New approaches based on endothelial progenitor cell (EPCs) transplantation hold promises for the treatment of damaged ischemic brain [1]. EPCs are immature cells mobilized from bone marrow into the circulation that home into the site of injury and promote neovascularization, thereby improving blood flow and tissue recovery. Heterogeneous cell populations containing EPCs, such as bone marrow-derived progenitor cells, have recently provided

encouraging results in animal models of stroke [2–5] in that they improved neurological outcome and angiogenesis and increased protection against ischemia-induced apoptosis in the injured area. Bone marrow-derived progenitor cells also generated a trend towards functional improvement in a clinical phase I trial in ischemic stroke patients [6].

However, it should be underlined that these bone marrow-derived progenitor cells represent heterogeneous cell populations mainly containing haematopoietic and mesenchymal stem cells whereas endothelial progenitors are slightly represented. This heterogeneity is an important limitation to evidence the contribution of EPCs to therapeutic effects after stroke and encourages the evaluation of EPC-enriched cell preparations. Such preparations are obtained from peripheral blood or from cord blood. At least, two distinct types of EPCs are now recognized, referred to as early outgrowth EPCs and late outgrowth EPCs or endothelial colony-forming cells (ECFCs), according to their obtention from short term and long-term blood sample culture respectively. In vitro studies showed that these two cell populations had different proliferation rates, survival behaviours and lineages [7]. ECFCs represent a homogeneous and well-characterized cell population of endothelial origin with high proliferating capacity that is considered to be the relevant endothelial progenitors due to their specific vasculogenic activity [8]. ECFCs are considerably enriched in cord blood compared to adult peripheral blood and cord blood ECFCs display higher clonogenic potential related to cord blood cell immaturity [9]. In vivo, these potentialities have been explored using a mouse model of hindlimb ischemia, where ECFCs promote angiogenesis, and muscular regeneration [7]. However, the angiogenic, neurogenic and anti-apoptotic properties of ECFCs have never been explored in animal models of cerebral ischemia.

We therefore evaluate the effect of human cord blood-derived ECFCs intravenously transplanted into rats subjected to cerebral ischemia by transient middle cerebral artery occlusion (MCAO). In this purpose, we studied whether the transplanted ECFCs (1) enter the brain and home into the injured area, (2) reduce neurological deficit and neuron apoptosis associated with cerebral ischemia; (3) contribute to ischemia-induced angiogenesis and neurogenesis and (4) induce cell changes in the injured area.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (Centre d'Elevage Janvier, France) weighting 200 to 280 g were used for all experiments. This study, including care of the animals involved, was approved by the local ethic committee and was conducted according to the official edict by the French Ministry of Agriculture (Paris, France) and the recommendations of the Helsinki Declaration. These experiments were therefore conducted after approval of the protocol by the Institution's Animal Care and Use Committee (Université de la Méditerranée), in an authorized laboratory and under the supervision of an authorized researcher (PISANO 1359). Moreover, most of the experimental design requirements for preclinical studies of stem cell therapy proposed by the STEPS [10] group were fulfilled.

Middle Cerebral Artery Occlusion (MCAO)

MCAO was induced by a method of intraluminal vascular occlusion according to the technique of Longa et al. (1989) [11] with slight modifications [12]. Adequacy of MCAO and reperfusion was confirmed by laser-Doppler flowmetry (PF5010, Perimed, Stockholm, Sweden) using a flexible optical fiber probe (probe 418, Perimed) attached to the skull over the ipsilateral parietal cortex (6-mm lateral and 1-mm posterior of bregma. The surgical procedure was considered technically adequate if a more than 70% reduction in regional cerebral blood flow was observed after placement of intraluminal filament, followed by rapid restoration of the cerebral blood flow signal during reperfusion.

Physiological Parameters and Body Weight

Before MCAO, the tail artery was catheterized to monitor mean arterial pressure (M1166A, Helwett-Packard, Evry, France), plasma glucose concentration (Accu-chek Sensor, Roche Diagnostic, Meylan, France), hemoglobin, blood gases and acid-base status (ABL5, Radiometer Copenhagen, Neuilly-Plaisance, France) at the same 3 time points: before surgery, at the beginning of ischemia, and at the onset of reperfusion. Temperature sensors were inserted into the rectum and between the right temporal muscle and temporal bone (Mon-a-therm, subcutaneous sensor, Mallinckrodt, Tyco, Plaisir, France). Pericranial temperature was maintained constant at 37.5°C using servocontrolled heating blanket (Harvard Apparatus®, Les Ulis, France). Postoperative body weight was measured 7 and 14 days after MCAO.

Functional Tests

Functional tests were performed for all animals before MCAO, and 1, 4, 7, 10 and 14 days after MCAO. Neurological function was graded using the modified neurological severity score (mNSS) described by Chen et al. [2]. The mNSS is a composite score of motor, sensory, reflex, and balance tests ranging from 0 to 18 (normal

score, 0; maximal deficit score, 18). Somatosensory deficit was measured both before and after MCAO with adhesive-removal somatosensory test. A small adhesive-backed paper dot was used as tactile stimulus occupying the distal-radial region on the wrist of each forelimb. The time taken to remove the stimulus from the limb successively on right and left forelimb was recorded during 3 trials per day for 3 days before MCAO, individual trials being separated by at least 5 min. Once the rats were able to remove the dots within 10 s, they were subjected to MCAO. Somatosensory deficits were graded from 0 to 5 [0: animals do not feel attached paper dot and do not remove it; 1: animals feel attached paper dot but do not reach to remove it within 5 min; 2: animals remove attached paper dot within 3 to 5 min; 3: animals remove attached paper dot within 1 to 3 min; 4: animals remove attached paper dot within 10 to 60 s; 5: animals remove attached paper dot within 0 to 10 s].

Cell Preparation and Transplantation

Human umbilical cord blood samples (30–50 ml) from healthy donors were collected, in compliance with French legislation, in a sterile tube containing heparin and ECFCs were isolated as previously described [13]. Mononuclear cells (MNCs) were isolated by density gradient centrifugation. Briefly, blood was diluted 1:1 in phosphate-buffered saline (PBS) containing 2 mM ethylenediaminetetraacetic (EDTA) and layered over lymphocyte separation medium (Eurobio, Les Ulis, France). After a 30 min centrifugation at 400 g, MNCs were washed three times in PBS/EDTA. Cord blood MNCs were pre-plated in RPMI/10% fetal calf serum (FCS) for 24 h in plastic flasks. Non-adherent cells were then plated onto 0.2% gelatin-coated 24-well plates (10^5 cells per well) and maintained in endothelial basal medium-2 (EBM-2) supplemented with EGM-2 Single-Quots (EGM-2 medium, Lonza, Walkersville, MD, USA). The medium was changed every 4 days. The appearance of well-circumscribed colonies was monitored daily. For expansion, colonies were trypsinized and cells were replated on a 35 mm plate (passage 1). Subsequently, confluent cells were trypsinized and re-plated in T75 flasks for further passages. Cells at passages 2, 4 and 6 were used (P2, P4 and P6). All the cells were maintained under standard conditions (humidified atmosphere, 5% CO_2, 37°C). Under these conditions, culture of EPCs from cord blood-derived non-adherent MNCs resulted in the emergence of adherent colonies within 2 to 3 weeks. Cells rapidly expanded into a confluent monolayer displaying cobblestone morphology and homogeneous endothelial phenotype as shown by the uniform membrane expression of various endothelial antigens such as ICAM-1 (CD54), PECAM (CD31), S-Endo1 (CD146), CD34, VE-cadherin (CD144) and KDR (VEGFR-2), whereas cells expressed neither leukocyte markers (CD45, CD14) nor the immaturity marker CD133. They took up Dil-ac-LDL and showed lectin-binding affinity. Additional flow cytometry experiments (data not shown) showed that these cells also expressed CXC chemokine receptor (CXCR4) for the stromal-derived-factor-1 (SDF-1). E-selectin was weakly expressed whereas P-selectin expression was not detected in resting cells. Together, their morphology, time of appearance in culture, proliferation potential and phenotype are consistent with the features of late outgrowth endothelial cells (ECFCs) previously described [13]. Before cell transplantation, the ECFCs cell membrane was labeled using CM-Dil (Cell Tracker CM-Dil®, Molecular Probes). Briefly, cells were incubated successively 5 min at 37° and 15 min at 4°C in CM-Dil solution (2.5 μg/ml), washed three times in PBS and dissolved in PBS to a final concentration of 4.10^6 cells/ml. Human Umbilical Vein Endothelial Cells (HUVECs) and dermal microvascular endothelial cells (HDMECs) were used as cell control for transplantation. HUVECs were isolated according to the method of Jaffe et al. [14] and HDMECs were purchased from Lonza (Walkersville, MD, USA). HUVECs and HDMECs were seeded on gelatin-coated culture plates and grown in EGM-2 and EGM2-MV (Lonza) medium respectively. Cells were maintained under standard conditions (humidified atmosphere, 5% CO_2, 37°C) and used between 2 and 4 passages. Early outgrowth endothelial progenitor cells (early EPCs) obtained from human umbilical cord blood samples from healthy donors [15] were used as positive cell control for transplantation. Briefly, MNCs were isolated by density gradient centrifugation and were plated on fibronectin-coated 6-well plates. After 3 days, adherent cells that took up Dil-ac-LDL and showed lectin-binding affinity were defined as early EPCs. One day after MCAO, animals were randomly allocated to control (n=31) or ECFC-(n=37), early EPC-(n=4), HUVEC-(n=5) and HDMEC-(n=5) transplanted groups. Briefly, animals were anesthetized with 3.0 vol% sevoflurane in 30% O_2 with the use of a facemask. The femoral vein was isolated and then 4.10^6 ECFCs, HUVECs, HDMECs or 2. 10^6 early EPCs in PBS, or PBS alone, were delivered in the femoral vein of respectively early EPCs, ECFC-, HUVEC-, and HDMEC-transplanted and control animals.

Radioactive Labeling of ECFCs and Cell Tracking of Transplanted ECFCs

Cell radioactive labeling was performed as reported by Aicher et al. [16] with slight modifications. Briefly, ECFCs were trypsinized into single cell suspension, then 12.10^6 cells were labeled with 26 MBq ^{111}In-oxine (physical half-life: 2.8 days; γ energy: 171 keV, 245 keV; Mallinckrodt,

France) for 60 min at 37°C. Cells were washed twice in PBS to remove unbound ^{111}In-oxine. Labeling efficiency was 68% of total radioactivity. ECFCs were resuspended in 3 mL of PBS, and 4.10^{6} ^{111}In-oxine labeled ECFCs were injected into the femoral vein 24 h after MCAO. Seventy-two hours after ^{111}In-oxine labeled ECFCs injection, animals were sacrificed with a lethal dose of pentobarbital (Clin Midy, Gentilly, France), harvested organs were weighed and the specific radioactivity was counted (COBRA II auto-gamma, Camberra, Saint Quentin, France) in different tissues.

Cytokine Array Assays

Cytokine profiling in the supernatant of nearly confluent cultured ECFCs was tested using Raybio Human Angiogenesis Antibody Array C Series 1000 (RayBiotech, Inc, Tebu-bio) according to the manufacturer's instructions. A cytokine was considered to be secreted by ECFCs if it generated a spot intensity higher than 10% of this of the positive control provided by the manufacturer.

Morphological and Histological and Immunochemical Assessment

Three, 7 and 14 days after MCAO, animals were sacrificed with a lethal dose of pentobarbital (Clin Midy, Gentilly, France) and rat brains were fixed by trans-cardiac perfusion with 4% phosphate-buffered paraformaldehyde (Sigma-Aldrich). After decapitation, the brain was removed carefully from the skull and post-fixed 24 h in 4% phosphate-buffered paraformaldehyde at 4°C. Brains were successively cryopreserved in sucrose 10% and 30% for 6 and 72 h, snap-frozen and stocked at −80°C. Frozen sections were cut with a sliding microtome (CM1900, Leica, France SA) and stored at −20°C. All sections were examined by investigators blind to the experimental conditions using a light and fluorescent microscope (Eclipse TE 2000-U, Nikon France SA) equipped with a digital camera (DXM1200, Nikon France SA) and an image analyzer (Lucia 5.0 software, Nikon France SA).

Infarct volumes were measured from Nissl-stained sections (40 µm) collected at 0.4 mm intervals from olfactory bulb to cerebellum, 7 and 14 days after MCAO as described by Codaccioni et al. [12]. Infarct areas were first measured using SigmaScan Pro 5.0 software (SPSS Inc., Chicago, IL) and infarct volumes were calculated by multiplying the infarct area of each section by the distance (400 µm) between sections and adding together the volumes for each brain. To eliminate the contribution of postischemic oedema to the volume of injury, values were corrected for swelling according to the method of Lin et al. [17].

Apoptotic cell detection Apoptotic cells were detected on 6 µm-thick sections 3 days after injury by Terminal deoxynucleotidyl Transferase-mediated dUTP nick end labeling (TUNEL) as described in a previous work [12] showing that apoptosis peaked on day 3 after MCAO. Positive (DNaseI pre-treatment, Sigma-Aldrich®) and negative (reaction buffer incubation omitted) controls were performed to check efficiency of immunostaining performed under our conditions. Only cells containing apoptotic bodies are referred to as apoptotic cells. TUNEL positive cells were counted under high-power microscope and expressed as number of positive cells per square mm section. Apoptotic cells were measured in 5–10 fields of the ischemic inner boundary zone of the ipsilateral hemisphere and in the contralateral hemisphere.

Angiogenesis and neurogenesis assessment Existing capillaries were stained with alkaline phosphatase (ALP) as previously described by Dubowitz et al. [18] on 6 µm-thick sections. Quantitative analysis (n=4 per group) of capillary density was performed, 7 and 14 days after MCAO, by averaging the total number of ALP-stained blood vessels in 10 different microscopic fields around the infarct area in the ipsilateral hemisphere and expressed as a ratio with blood vessel count in the contralateral hemisphere. To explore neurogenesis 14 days after MCAO, 6 µm-thick brain sections were first treated with 10% H_2O_2 in PBS for 20 min, then pre-incubated in 6% normal bovin serum in PBS with 0.3% Tween 20 for 1 h at room temperature, and finally incubated overnight at 4°C with antibody against double cortin (DCX C-18, goat polyclonal IgG antibody, Santa Cruz Biotechnology; 1:100), a protein expressed in migrating neurons. Sections were further reacted with biotinylated rabbit anti-goat IgG (Sigma) at 1:200 for 1 h30. Immunoreaction product was visualized using hydrogen peroxide and diaminobenzidine (Sigma fast tablet sets®, Sigma-Aldrich). DCX positive cells were counted in 3 fields of the ipsilateral subventricular zone, and expressed as number of positive cells per square mm section (n=4 per group).

Quantitative evaluation of the macrophages/microglial cell and astrocyte number Fourteen days after MCAO, macrophages/microglial cells and astrocytes were examined at inner boundary of the core area on serial 6 µm-thick brain sections stained with anti-GFAP (Dako, 1:2000) and anti-CD 68 (Dako, 1:2500). Automated immunohistochemistry was performed with avidin-biotin-peroxidase complex on Ventana Benchmark XT. A quantitative evaluation of the cell number was performed on 2 consecutive sections from 6 animals in each group.

Western Blotting

On day 7, rats (n=4 per group) were decapitated, and ischemic forebrain area was immediately harvested and

homogenized by adding a 1:25 tissue to weight protein extraction buffer (50 mM TRIS, 1%Triton, 3.5 g/L NaCl, 15% protease inhibitor). Brain homogenates were then centrifuged and the supernatants stored at −80°C. For Western blotting assays, brain homogenates (60 µg) were mixed with an appropriate volume of 1× NuPAGE LDS Sample Buffer supplemented with 1× NuPAGE Reducing Agent (Invitrogen), heated at 90°C for 5 min, resolved by a 4–12% gradient Bis-Tris polyacrylamide gel electrophoresis (Invitrogen.) and transferred onto nitrocellulose membranes using NuPAGE iBlot Transfer Stacks. After blotting, membranes were blocked in phosphate buffered saline containing 0.05% Tween-20 (PBS-T) and 5% non-fat dry milk for 1 h. Then, membranes were probed overnight at 4°C with primary antibody for vascular endothelial growth factor (VEGF), brain derived neurotrophic factor (BDNF), neurotrophic growth factor (NGF) or insulin growth factor 1 (IGF-1) from goat or rabbit (Santa-Cruz), diluted (1:200) in antibody dilution buffer (PBS-T containing 1% non-fat dry milk). Membranes were washed three times for 10 min with antibody dilution buffer and incubated with peroxidase-conjugated secondary antibody (IgG peroxidase anti-goat or anti-rabbit, Sigma) for 1 h at room temperature. After extensive washing (6×5 min with PBS-T), antibody detection was accomplished by the ECL Plus system (Amersham Biosciences). Protein bands were quantified by a gel image analysis system.

Statistical Analysis

Values were reported as mean ± SD unless otherwise indicated. Physiological variables were analyzed by repeated-measure analysis of variance. Cerebral infarct volumes, TUNEL, cell densities and mNSS evolution were evaluated for normality and compared with unpaired student *t* tests. Adhesive-removal somatosensory scores and histological assessments were analyzed by the Kruskal-Wallis test followed by the Mann Whitney U test. Statistical analysis was performed with SigmaStat® 2.03 (SPSS Inc,). A value of $P<0.05$ was considered statistically significant.

Results

Physiological Parameters and Body Weight

There were no significant differences in physiological parameters between experimental groups (Table 1). Body weight of control and ECFC-transplanted rats were not significantly different before MCAO (control *vs.* ECFC-transplanted: 276±5 *vs.* 269±10 g; $P=0.201$, $n=5$) but became significantly higher in the ECFC-transplanted group 7 days after MCAO compared to control group (control *vs.* ECFC-transplanted: 230±36 *vs.* 282±20 g; $P=0.024$, $n=5$). The difference in body weight observed 14 days after MCAO

Table 1 Physiologic variables in experimental groups. Data are mean ± SD. MCAO = middle cerebral artery occlusion; MAP = mean arterial pressure; PaO2= arterial oxygen tension; PCO_2= arterial carbon dioxide. Before MCAO = During surgery; During MCAO = At the beginning of MCAO before awaking; At reperfusion = 3 min after reperfusion. No significant difference was observed between ECFC-transplanted and control group

		Control (n=20)	ECFCs (n=18)	*P*
Temperature (°C)	Before MCAO	37.3±0.6	37.2±0.1	0.86
	During MCAO	37.2±0.3	37.1±0.5	0.73
	Reperfusion	37.1±0.3	37.1±0.7	0.88
*p*H	Before MCAO	7.40±0.05	7.42±0.03	0.39
	During MCAO	7.42±0.04	7.43±0.04	0.54
	Reperfusion	7.40±0.07	7.41±0.02	0.48
PaCO2 (mmHg)	Before MCAO	43±3	43±4	0.76
	During MCAO	40±1	39±6	0.61
	Reperfusion	41±2	39±2	0.34
PaO2 (mmHg)	Before MCAO	133±15	133±10	0.65
	During MCAO	134±12	135±13	0.67
	Reperfusion	137±11	140±17	0.51
MAP (mmHg)	Before MCAO	86±18	84±11	0.54
	During MCAO	100±14	88±17	0.14
	Reperfusion	97±11	97±12	0.91
Glucose (mmol/l)	Before MCAO	7.9±2.0	8.0±1.7	0.69
	During MCAO	6.3±1.8	7.9±2.1	0.23
	Reperfusion	7.1±2.2	7.6±2.1	0.31
Hemoglobin (g/dl)	Before MCAO	14.2±0.1	14.3±0.2	0.73
	During MCAO	14.0±0.2	14.1±0.2	0.89
	Reperfusion	14.2±0.1	14.3±0.1	0.77

became non significant (control *vs.* ECFC-transplanted: 306±30 *vs.* 336±27 g; $P=0.14$, $n=5$).

Neurological Testing

Before transplantation, comparison of functional status between groups showed no significant difference in mNSS and adhesive-removal score. The mNSS score evolution in the ECFC-transplanted group showed a significant functional improvement at 7 ($P=0.034$), 10 ($P=0.020$) and 14 ($P=0.036$) days after MCAO compared to control animals; at 10 ($P=0.001$) and 14 ($P=0.001$) days after MCAO compared to HUVEC-transplanted animals and at 7 ($P=0.013$), 10 ($P=0.001$) and 14 ($P=0.007$) days after MCAO compared to HDMEC-transplanted animals. Early EPCs transplantation significantly improved mNSS and adhesive-removal scores at 7 ($P=0.008$), 10 ($P=0.048$) and 14 ($P=0.045$) days after MCAO compared to control animals (Fig. 1a). No difference was observed in mNSS score evolution either between control-, HUVEC- and HDMEC-transplanted groups or between early EPCs and ECFC-transplanted animals from 4 to 14 days after MCAO.

Adhesive-removal score evolution showed a selective left (contralateral) paw functional deficit in all groups 3 days after MCAO (Fig. 1b). Fourteen days after MCAO, compared to controls, ECFC- and early EPC-transplanted animals showed a left (contralateral) paw significant functional recovery ($P=0.033$ and 0.030 respectively). Similar results were obtained when ECFC- and early EPC-transplanted animals were compared to HUVEC- ($P=0.033$ and 0.032 respectively) and HDMEC-transplanted animals ($P=0.032$ and 0.031 respectively).

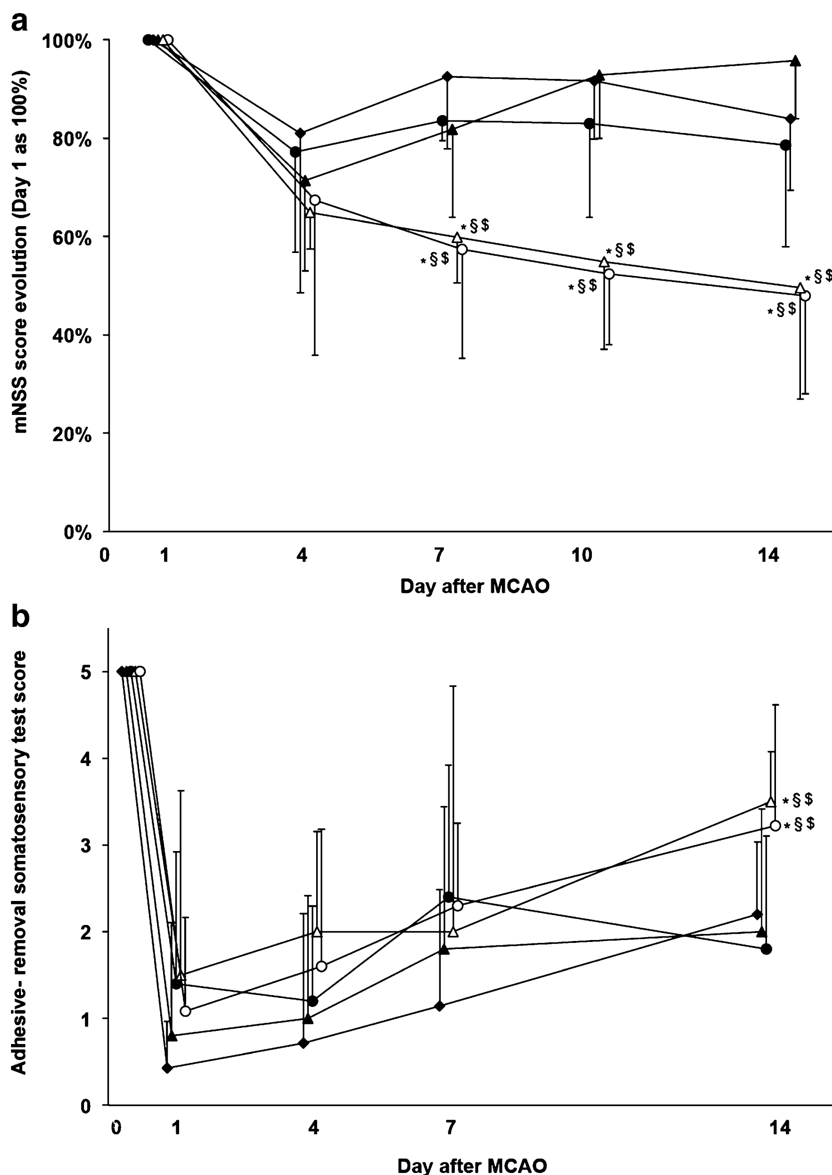

Fig. 1 **a** mNSS score evolution (mean ± sd, Day 1 as 100%) for control (●), HUVEC- (▲), HDMEC- (◆) CFU-EC- (Δ) and ECFC-(○) transplanted rats 4, 7, 10, and 14 days after MCAO. Significant functional improvement was observed 7, 10, 14 days after MCAO in ECFC and CFU-EC-transplanted groups compared to control (*), HUVEC- (§) and HDMEC- ($) transplanted groups. *, §, $, $P<0.05$. **b** Temporal profile of adhesive-removal scores of left paw with median ± sd values (*line*) in control (●), HUVEC- (▲), HDMEC- (◆),CFU-EC- (Δ) and ECFC-(○) transplanted rats 1, 4, 7 and 14 days after MCAO. We observed significant left paw adhesive-removal score improvement in ECFC- and CFU-EC- transplanted groups compared to control (*), HUVEC- (§) and HDMEC- ($) transplanted groups 14 days after MCAO. *, §, $, $P<0.05$

Distribution of Transplanted ECFCs in Rat After MCAO

Seventy-two hours after transplantation, ^{111}In-oxine-labeled ECFCs were detected mostly in liver, kidneys, and spleen (Fig. 2a). In the brain, the radioactive signal was significantly higher in the ipsilateral hemisphere than in the contralateral one (ipsilateral *vs*. contralateral hemisphere: $20\times10^3\pm3517$ *vs.* $11\times10^3\pm1257$ cpm; $n=3$, $P=0.014$) attesting to a ratio of 1.8±0.3 (Fig. 2b).

CM-Dil labeled cells (Fig. 2c) preferentially settled in the inner boundary of the core area in ECFC-transplanted rats 14 days after MCAO. Incorporation of CM-Dil labelled ECFCs into neovessels was hardly detectable on brain sections stained with DAPI (Fig. 2d). No labeled ECFCs were observed in control group brain sections.

Morphological, Histological and Immunochemical Analysis

Microscopic examination of sections of the two hemispheres with Nissl staining showed that infarct volumes were not significantly different between ECFC-transplanted and control rats 7 days (ECFC-transplanted *vs.* control: 103.8±48.7 *vs.* 95.2±62.2 mm^3; $P=0.85$, $n=5$) and 14 days (ECFC-transplanted *vs.* control: 189.4±111.3 *vs.* 188.0±30.3 mm^3; $P=0.98$, $n=5$) after MCAO. Three days after MCAO, apoptotic cell number was significantly lower in ECFC-transplanted rats compared to control rats (ECFC-transplanted *vs.* control: 48±18 *vs.* 130±50 /mm²; $P=0.018$, $n=6$) (Fig. 3). ALP staining in control (A) and ECFC-transplanted (B) animals 14 days after MCAO is represented in Fig. 4. Analysis of the ratio of capillary density between ipsilateral and contralateral hemisphere of each group (Fig. 4c) indicated a significant increase in the ECFC-transplanted group compared to the control group 14 days after MCAO (ECFC-transplanted *vs.* control: 2.80±0.34 *vs.* 1.73±0.17; $P=0.021$, $n=4$). Analysis of DCX staining in ipsilateral subventricular zone of control (Fig. 4d) and ECFC-transplanted (Fig. 4e) animals 14 days after MCAO indicated a significant increase (Fig. 4f) in neurogenesis in the ECFC-transplanted group compared to the control group (mean ± sd of DCX positive cell number in ECFC-transplanted *vs.* control: 35.75±6.34 *vs.* 18.75±4.57; $P=0.005$; $n=4$). Finally, in ECFC-

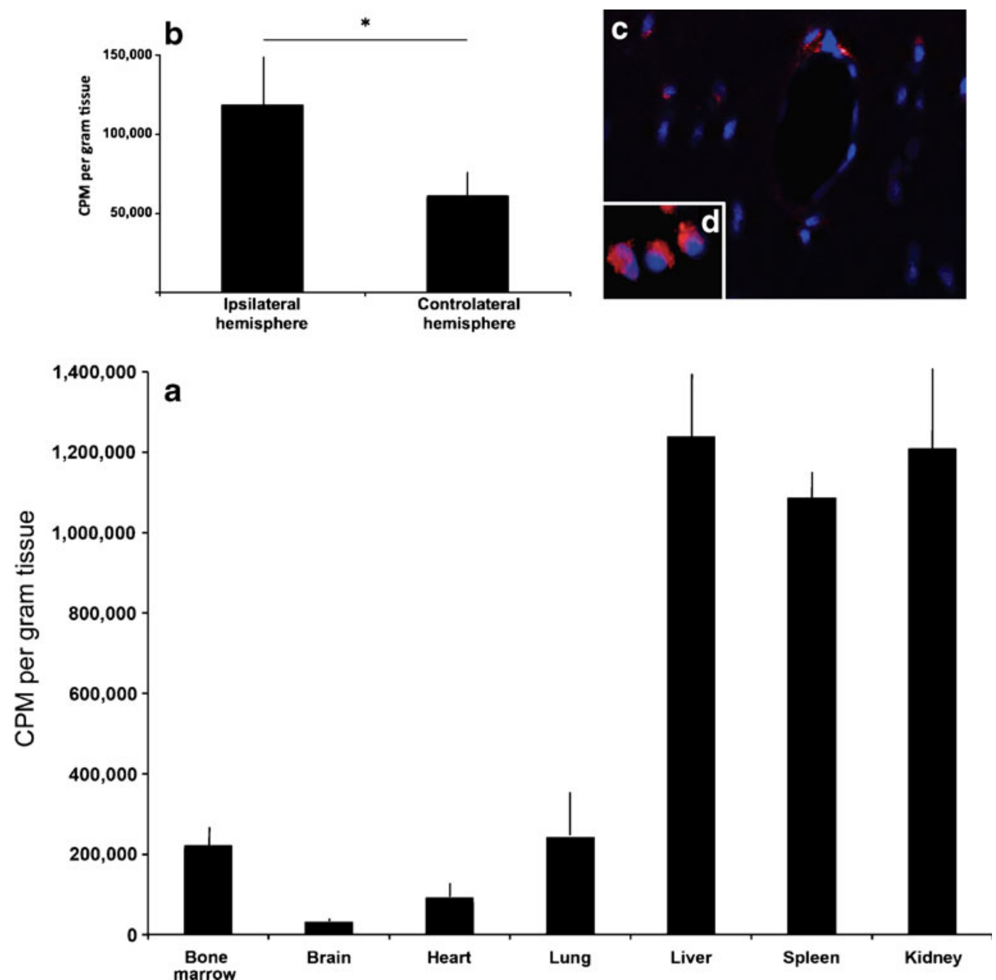

Fig. 2 **a** Distribution of radioactivity in different tissues 72 h after transplantation of ^{111}In-oxine-labeled ECFCs 1 day after MCAO ($n=3$). **b** Distribution of radioactivity in the ischemic (ipsilateral) and non-ischemic (controlateral) brain hemispheres ($n=3$, $P<0.05$). **c** ECFCs identification by CM-Dil cell labeling (magnification: ×40; DAPI counterstaining: blue) 14 days after MCAO on ipsilateral brain section of an ECFC-transplanted rat. **d** ECFCs localized near blood vessels (magnification: ×100; DAPI counterstaining: blue)

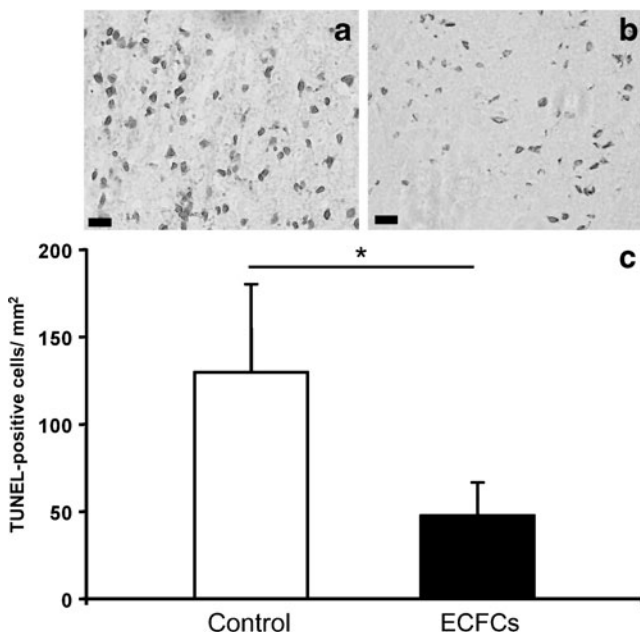

Fig. 3 Representative brain sections stained with TUNEL in control (**a**) and ECFC-transplanted (**b**) rats 3 days after MCAO. **c** Quantitative analysis (n=6 per group) shows that ECFCs significantly reduced ischemia-induced cerebral apoptosis. (* P<0.05; scale bar: 20 µm; magnification: ×40)

transplanted and control animals, we observed the joint presence of macrophages/microglial cells (Fig. 5a, b) and astrocytes forming a reactive astrogliosis (Fig. 5c, d). Quantitative analysis of microscopic examination (Fig. 5e) showed that, compared to controls, ECFC-transplanted animals exhibited a trend but not significant reduction in macrophages/microglial cell number (P=0.093, n=6) and a significant reduction in reactive astrogliosis (P=0.026, n=6).

ECFCs Secrete Factors That Could Affect Apoptosis, Angiogenesis and Neurogenesis and Induced Modifications of Growth Factor Expression in the Infarct Area

Cytokine profiling showed the presence of several secreted cytokines secreted into the supernatant of cultured ECFCs, particularly: growth-regulated oncogene-a (GRO-a), interleukin 8 (IL-8), tissue inhibitors of metalloproteinases (TIMP-1, TIMP-2) and metalloproteinases 2 and 9 (MMP-2 and MMP-9), epidermal growth factor (EGF), monocyte chemotactic protein-1 (MCP-1), platelet-derived growth factor homodimer BB (PDGF-BB), and angiopoietin 2 (ANG-2). The expression of VEGF and IGF-1 increased in brain homogenates from ipsilateral hemisphere of ECFC-transplanted rats 7 days after MCAO compared to the expression levels measured in control animals (Fig. 6c, d) whereas pro-BDNF expression decreased (Fig. 6b). No significant effect was observed on the expression of BDNF and NGF (Fig. 6a, e).

Discussion

This is the first report demonstrating the usefulness of ECFCs as a promising option for stroke therapy. We demonstrate that, after intravenous delivery, ECFCs enter the brain and home into the inner boundary of the ischemic area. ECFCs administered 24 h after 1 h of transient focal

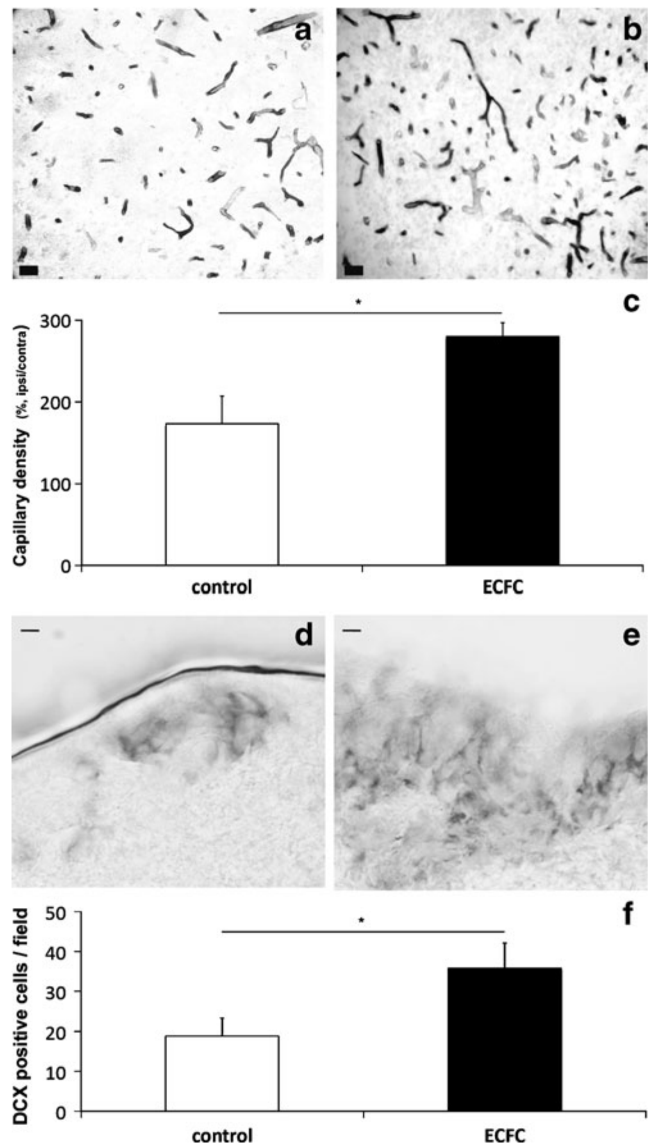

Fig. 4 Angiogenesis and neurogenesis assessment. Alkaline phosphatase (ALP) staining in control (**a**) and ECFC-transplanted (**b**) rats 14 days after MCAO. **c** Quantitative analysis (n=4 per group) shows that the ratio between ALP-stained blood vessels in the ipsilateral hemisphere and in the contralateral hemisphere was significantly increased in the ECFC-transplanted group compared to control group 14 days after MCAO. (*, P<0.05; n=4; scale bar: 20 µm; magnification: ×40). DCX staining in ipsilateral subventricular zone of control (**d**) and ECFC-transplanted (**e**) animals 14 days after MCAO indicated a significant increase (**f**) in neurogenesis in the ECFC-transplanted group compared to the control group (*, P=0,005; n=4; scale bar: 10 µm; magnification: ×100)

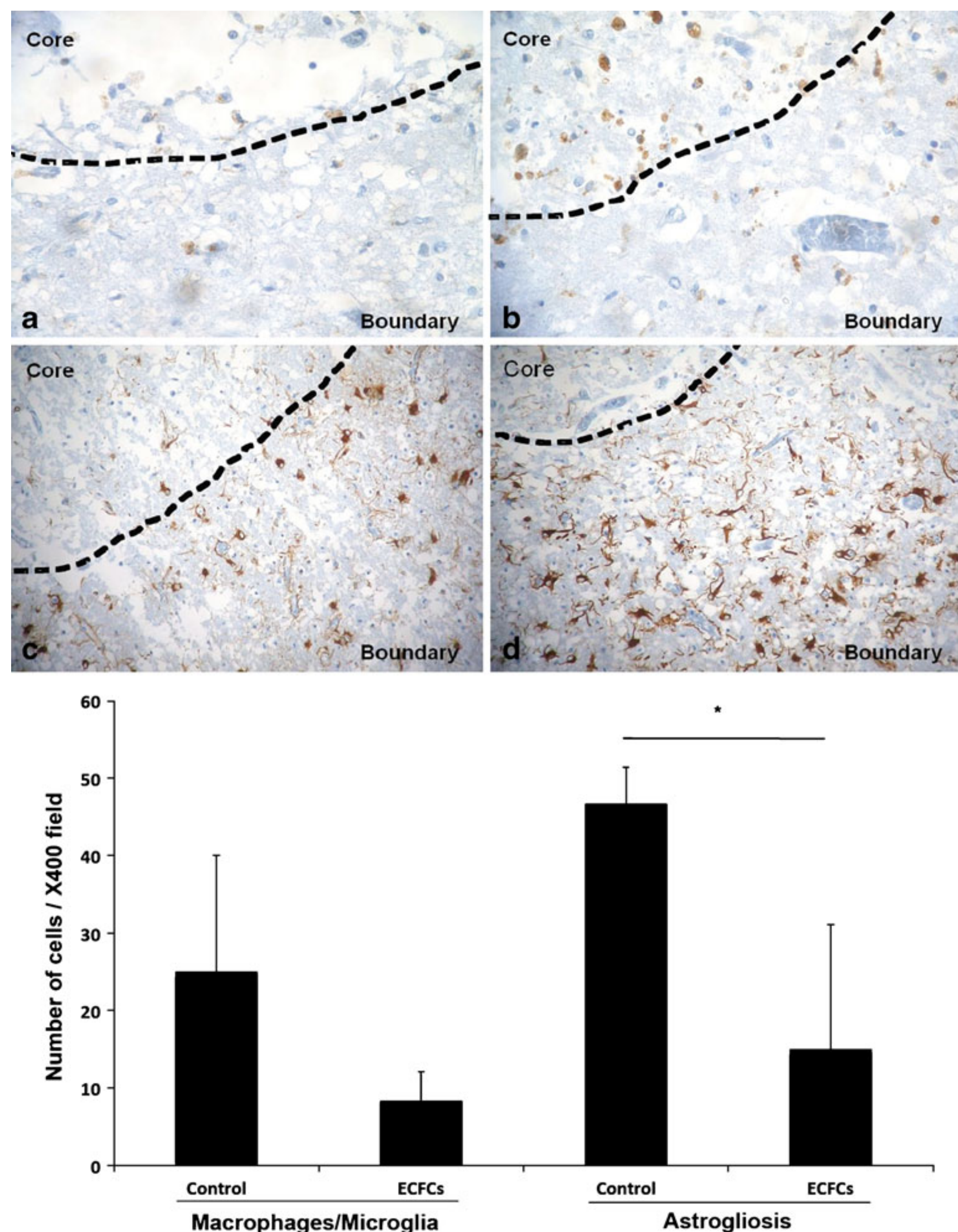

Fig. 5 Histological analysis 14 days after MCAO on ipsilateral brain sections. **a**, **b** Macrophages/microglial cells in ECFC-transplanted (**a**) and control (**b**) rat. **c**, **d** Reactive astrogliosis in ECFC-transplanted (**c**) and control (**c**) rat. **e** Quantitative analysis (n=6 per group, * P<0.05; magnification: ×40)

cerebral ischemia significantly improve functional recovery up to day 14 compared to vehicle. Reduction in ischemia-induced apoptosis, stimulation of ischemia-induced angiogenesis and neurogenesis and attenuation of reactive astrogliosis may contribute to the beneficial effect of ECFCs after MCAO.

The promises of using cord blood derived ECFCs for therapeutic purposes have emerged from the recent literature [19, 20]. This source of ECFCs allows circumventing critical limitations of using autologous ECFCs from peripheral blood. Indeed, adult ECFCs have limited proliferative potential and functional defects linked to cardiovascular risk factors.

In our study, cord blood ECFCs treatment reduced neurological deficits assessed by mNSS and the somatosensory tests performed up to 14 days after MCAO. The ECFC-induced improvement in neurological outcome occurred without significant reduction of infarct volume. This lack of correlation has frequently been observed at similar time points when marrow stromal cells (MSCs) [3, 21, 22] or human cord blood mononuclear cells (HUCBCs) [22] were used after MCAO. A reduction in infarct volume has been described with higher quantity of cells [23, 24] or when transplantation was performed at earlier time points [5]. Moreover, we observed that, in ECFC-transplanted rats, improvement of neurological deficits correlated, 7 days

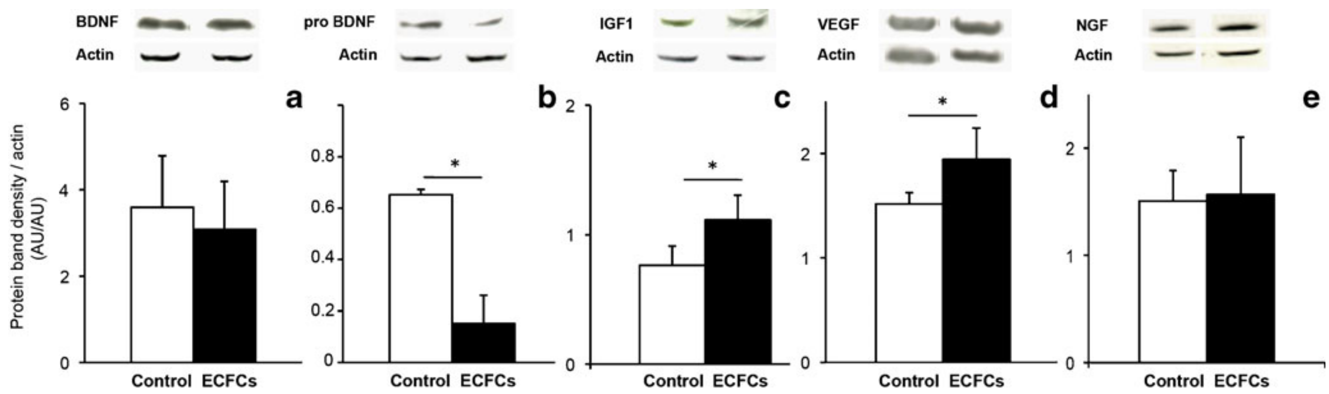

Fig. 6 Western blots showing BDNF (**a**), ProBDNF (**b**), IGF1 (**c**), VEGF (**d**) and NGF (**e**) levels in the ipsilateral brain hemisphere 7 days after MCAO in control and ECFC-transplanted animals. Upper panels show representative Western blots and lower panels show the quantitative analysis normalized to actin control (n=4 per group, * $P<0.05$)

after ischemia, with weight gain which is known to reflect the general well-being of the animals and the degree of brain injury [25, 26]. Duly, transplantation of early EPCs led to similar improvement of mNSS and somatosensory scores up to 14 days after MCAO. Mobilization of EPC of myeloid linage, detected using CFU-EC assays was shown to correlate with good outcome in patients with stroke suggesting that type of EPC may participle in repairing cerebral ischemia [27]. However transplantation of early EPC isolated from blood has never been tested in stroke models although they have been shown to improve blood flow recovery in mouse ischemic hindlimb [7]. In the perspective of cell-based therapy of acute ischemic diseases, early EPCs may have several limitations. In particular the collection of sufficient number of early EPCs may be greatly impaired due to the inability of such cells to proliferate in vitro by contrast to ECFC. In addition, consistent with their monocytic origin, early EPCs may display proinflammatory and procoagulant properties as recently suggested [28]. The beneficial effect of EPC seems to be progenitor cell specific since mature HUVECs and HDMECs provided no functional benefit compared to vehicle-treated rats.

Intravenous delivered ECFCs enter the brain, home in and were detectable for at least 14 days after MCAO in the inner boundary of the ischemic zone. Radioactive labeling experiments revealed accumulation of ECFCs into internal organs, as also reported after systemic injection of radio-labeled stem cells in ischemic myocardium [16]. Distribution of radiolabeled ECFCs was more pronounced in the ipsilateral hemisphere, suggesting an ischemia-induced homing of the transplanted cells. Immunochemical and CM-Dil labeling confirmed that ECFCs enter the ischemic brain, preferentially settle cerebral vessel periphery in the ischemic zone.

Homing of therapeutic progenitor cells to the ischemic zone after MCAO has been shown as early as 24 h after intravenous administration of MSCs [22, 24], and persisted at least 4 weeks after HUCBCs intravenous injection [3, 23]. Molecular mechanisms underlying the recruitment of EPCs to neovascularization sites after vessel injury have been extensively reviewed by Zampetaki et al. [29]. They involved upregulation of P- and E-selectins, VEGF, SDF-1 and ICAM-1 in the injured endothelium and expression of PSGL-1, β2 integrins and MMP-2 by EPCs. In a similar way, in our model, the homing of ECFCs to the ischemic zone may be related to the fact that ECFCs 1) may express E-selectin under specific conditions [13], expressed CXCR4 and secrete MMP/TIMP and 2) upregulated VEGF expression in the infarct area. ECFCs, which are not of haemotopoietic origin, do not express B2 integrins by contrast to early EPC.

The homing of ECFCs was associated with an increase in capillary density observed 14 days after MCAO and with an increase in neurogenesis observed 7 days after MCAO. To our knowledge, there is no study reporting such an increase in post-stroke angiogenesis and/or neurogenesis after EPCs transplantation. Increasing angiogenesis and neurogenesis by stem cell approaches in rodent models of stroke has only been described with MSCs (for review, see Zhang et al. [30]) and was related to their paracrine activity. Such paracrine mechanisms have been extensively associated to angiogenic capacity of early EPCs [7] and may contribute to the effect of such cells in our ischemia model. However, in mouse ischemic hindlimb, it has been established that, after local delivery [7], ECFCs promote angiogenesis rather through their high capacity to incorporate into growing vessels than through paracrine activity. In our experiments, we failed to detect any indubitable incorporation of ECFCs into rat neovasculature. Therefore, the hypothesis of a paracrine-dependent angiogenic and neurogenic effect of our injected ECFCs could not be fully excluded. Consistently, production of IL-8 by ECFCs was correlated to proangiogenic and mitogenic effect of these cells on mature endothelial cells [31]. In addition, with

showed that cultured ECFCs secreted an array of factors (GRO, IL-8, TIMP-1, TIMP-2 and MMP-2, EGF, MCP-1 and PDGF-BB), most of which involved in angiogenic and/or neurogenic processes [32–36]). A paracrine effect of ECFCs may also be hypothetized angiogenesis review on the basis of the upregulation of VEGF and IGF-1 expression observed 14 days after MCAO in the infarct area. Accordingly, increase in endogenous rat VEGF brain levels has been involved in MSC-induced angiogenesis after MCAO [37]. In a similar way, increase in endogenous rat IGF-1 has been involved in MSC-induced neurogenesis after MCAO [38]. No modification of BDNF expression after MCAO rescued by stem cell transplantation has been described, but, transplantation of MSCs after traumatic brain injury [39] increased BDNF brain levels. Finally, the hypothesis of a paracrine-dependent angiogenic effect of our injected ECFCs could be reinforced by an unpublished relevant observation from our lab showing that VEGF concentration, was strongly increased when ECFCs are submitted to hypoxic stimuli *in vitro* and by the work from Wang et al. [40] who showed that ECFCs modulate the differentiation of MSCs toward an endothelial lineage through a paracrine effect.

The improvement of stroke outcome after ECFCs transplantation was also accompanied by a reduction in ischemia-induced apoptosis 3 days after MCAO, as assessed by the lower number of TUNEL positive cells in the inner boundary of the core area of ECFC-transplanted rats. In the same way, Chen et al. [22] showed that MSCs delivered 1 day after MCAO reduced apoptosis 7 and 14 days after injury. Mechanisms underlying the antiapoptotic effect of stem cells have been poorly explored. In vitro, Hau et al. [41], using a cocultivation model, showed that HUCBCs reduced the hypoxia-induced apoptosis of neurons and hypothesized a direct contact between HUCBCs and injured neurons together with changes in neuroprotective cytokines. In our model, the ECFC-induced reduction in apoptosis may be related to the upregulation of IGF-I [42] which has been shown to prevent apoptotic neuronal cell death after experimental stroke. The decrease in proBDNF expression observed after ECFCs transplantation may also take part in the observed reduction in apoptosis since precursor neurotrophins such as proBDNF have been shown to have unique bioactivities which are opposite to those of their mature forms [43]. Finally, the ECFC-induced increase in VEGF tissue levels may also exert antiapototic effects since Deng et al. [44] showed that MSCs reduced neuronal apoptosis and promote neuronal proliferation through the release of VEGF after stroke in rats. Whatever the mechanism involved, as inhibition of apoptosis has been reported as an effective intervention to attenuate neurological injury after MCAO [45], it could be suggested that the anti-apoptotic effect of ECFCs injection might underlie, at least in part, their therapeutic effects after MCAO.

Histological analysis in ECFC-transplanted rat revealed a trend toward a reduced macrophages/microglial cell number compared to control rats. These cells exerted phagocytosis of neuronal debris and apoptotic neutrophils after stroke, suggesting a lessening need to remove cellular debris in less damaged regions. Whether macrophages/microglial cell infiltration is beneficial or deleterious to recovery after ischemic stroke is presently a matter of controversy. Attenuation of macrophages/microglial cell response to MCAO has been shown to induce an increase in infarct size [46]. Conversely, more recent studies pointed the fact that sustained microglial activation may contribute to tissue destruction after stroke by secreting a plethora of cytotoxic cytokines and free radicals [47]. Histological analysis also showed a reduction in ischemia-induced reactive astrogliosis, consistent with previously described effect of MSCs in a MCAO model [48] and on cultured astrocytes subjected to oxygen-glucose deprivation [40]. Astrocytes rapidly build a dense cell barrier surrounding the infarct, thus preventing the spread of damage, but, later, this barrier impedes in axonal outgrowth [48]. Therefore, in our model, one can speculate that reactive astrogliosis attenuation, observed at a remote time point, may play a role in ECFC-induced improvement of neurological outcome. This hypothesis could be supported by the recent focus of Chopp et al. [49] which suggests that one of the main mechanisms underlying improved outcome after stroke in rats treated with MSCs relies on the paracrine effect of MSCs on astrocytes and particularly on the reduction in astrocytic factors that inhibit axonal outgrowth after MCAO.

In conclusion, using a rat model of stroke, we have demonstrated that injection of the human progenitor cell subpopulation ECFCs improved functional recovery up to 14 days after the injury. This benefit may be mediated by either stimulation of ischemia-induced angiogenesis and reduction in ischemia-induced apoptosis and was accompanied by reduction of glial scar and macrophages/microglia cell infiltration. Although the molecular mechanisms of ECFC-mediated neurological improvement require further investigation, our findings raise perspectives for the use of ECFCs as a well-characterized cell therapy product for optimal therapeutic outcome after cerebral ischemia in stroke patients.

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