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Multipotent PDGFRβ-expressing cells in the circulation of stroke patients

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ABSTRACT

Tissue pericytes respond to injury, and support vascular and tissue regeneration. The presence of pericytes in the circulation may provide an attractive framework for tissue regeneration. Here, we detected multipotent pericyte-like cells in the circulating blood and determined its profiles during cerebral ischemia. Pericyte-like cells were isolated from the peripheral blood of acute stroke patients or asymptomatic individuals with vascular risk factors by fluorescence or magnetic activated cell sorting with anti-PDGF receptor-beta (PDGFRβ) antibody. The morphologic and molecular features of circulating PDGFRβ$^+$ cells were compared with tissue pericytes, and the associations with respect to quantity in the blood, culture outcome, and patient characteristics were analyzed. We found an increase in circulating PDGFRβ$^+$ cells in acute stroke patients compared to controls and a correlation with neurologic impairment. The isolated PDGFRβ$^+$ cells expressed mesenchymal stem cell markers, proliferated, and were multipotent under permissive culture conditions. The multipotent nature of these cells was comparable to fat-derived PDGFRβ$^+$ cells. These cells could be obtained by pharmacologic stimulation using bone marrow mobilizer. Circulating PDGFRβ$^+$ cells will be useful for future research involving endogenous recovery or autologous cell-based therapy.

Introduction

It has long been suggested that pericytes, which are originally defined by their morphology and close contact to endothelial cells, may have stem/progenitor cell potential and function as sources of cells for repair and tissue maintenance within various tissues (Dore-Duffy et al., 2006; Crisan et al., 2008). Pericytes have been described as tissue mesenchymal stem cells (MSCs) because they can differentiate into mature pericytes, osteoblasts, chondroblasts, fibroblasts, adipocytes, and smooth muscle cells (Pittenger et al., 1999; Farrington-Rock et al., 2004; Dellavalle et al., 2007; Tang et al., 2008). There is also evidence that pericyte-like cells circulate in the peripheral blood (Simper et al., 2002; Rajantie et al., 2004; Grunewald et al., 2006; Kokovay et al., 2006). Circulating pericytes may be related to other elusive, multilineage progenitor cells which have been previously described, such as MSCs, smooth muscle progenitor cells, recruited bone marrow-derived circulating cells, neuronal outgrowth cells, multipotent adult progenitor cells, CXCR4$^+$lin$^-$CD45$^-$ small cells, and mesoangioblasts (Simper et al., 2002; Zhao et al., 2003; Rajantie et al., 2004; Grunewald et al., 2006; Jung et al., 2008; Cesselli et al., 2009; Paczkowska et al., 2009; Koyanagi et al., 2010). However, until recently, the direct identification of circulating progenitor cells using a pericyte marker has not been shown.

Although no specific marker is available to define the pericyte phenotype clearly, PDGFRβ or NG2 has been used to identify pericytes and to distinguish immature pericytes from mature pericytes in preclinical and clinical studies (Shi and Gronthos, 2003; Dore-Duffy et al., 2006; Adams and Alitalo, 2007; Covas et al., 2008). Increased attention to circulating pericytes can yield a better understanding of the vasculogenic processes and their role underlying vascular diseases. Furthermore, circulating pericytes may be a source of multipotent stem cells with the potential of direct vasculo-neurogenesis and can be important for future therapeutic strategies. Whether this undifferentiated cell population is found in all individuals or is restricted to a subset of patients is as critical as the characterization of the phenotypic properties. Therefore, we set out to investigate the clinical relevance of the progenitor cell population in stroke patients and asymptomatic individuals with vascular risk factors.

Of particular interest from the perspective of the therapeutic utilization of these cells, we sought to purify pericytes using

Abbreviations: CE, cardioembolism; FACS, fluorescence activated cell sorting; G-CSF, granulocyte-colony stimulating factor; ICH, intracerebral hemorrhage; LAA, large artery atherosclerosis; MACS, magnetic activated cell sorting; MSCs, mesenchymal stem cells; NIHSS, National Institutes of Health Stroke Scale; PDGFRβ, platelet-derived growth factor receptor-beta; SVO, small vessel occlusion.

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fluorescence activated cell sorting (FACS) and magnetic activated cell sorting (MACS) with anti-PDGFRβ antibody from the adult peripheral blood, and then performed morphologic and molecular identification, multiplication, and mobilization into the peripheral blood.

Materials and methods

Study population

Two sets of subjects were enrolled: (1) those who had experienced an acute ischemic stroke and had been admitted to our tertiary care medical center (stroke group); and (2) those with more than one known vascular risk factors, but no stroke events (control group). Between June 2008 and January 2009, we prospectively studied 37 patients admitted to our hospital with first-ever strokes within 7 days from the onset of symptoms. The inclusion criteria were age between 30 and 80 years, signed written informed consent, and a radiologic-documented cerebral infarction. Exclusion criteria included an acute infarct of unusual causes, i.e., carotid dissection, hypercoagulable state, vasculitis, an incomplete workup, or an undetermined cause of stroke. Also excluded were subjects with the following factors that might have influenced blood cell profiles: treatment for thrombolysis; clinical or biochemical evidence of concomitant inflammatory disease; recent trauma; renal, hepatic, or hematological disorders; and autoimmune or malignant disease. The patient's neurologic status was assessed using the National Institute of Health Stroke Scale (NIHSS) scores on admission. Hemorrhage or infarct volumes were measured by computed tomography or diffusion-weighted images using a b value of 1000 s/mm^2, respectively. Subjects were allocated to four subgroups with two classification systems: one, the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification system (Adams et al., 1993), as follows: large artery atherosclerosis (LAA), cardioembolism (CE), and small vessel occlusion (SVO), and the other is Oxford Classification system (Bamford et al., 1991), as follows: lacunar (LAC), partial anterior circulation (PAC), total anterior circulation (TAC) or posterior circulation (POC). To eliminate confounding vascular risk factors, we also recruited 44 patients with vascular risk factors (control group) who had been referred to the outpatient clinic of the Department of Neurology. After completing a risk factor survey, we included patients who met the following characteristics: (1) no history of stroke; and (2) one or more vascular risk factors (hypertension, diabetes mellitus, dyslipidemia, a smoking history, or heart disease). We matched total risk burdens between cases and controls. Total risk burden was calculated using Framingham risk scores (Wilson et al., 1998). Patients in the control group underwent a similar laboratory and radiological workup for detection of cerebral vascular abnormalities. Excluded were patients with silent brain lesions associated with a significant stenosis in the neck or intracranial vessels.

Tissue sample acquisition

Human tissue samples were acquired with patient consent and approval of the Institutional Review Board of Seoul National University Hospital. Total human peripheral blood mononuclear cells were isolated from the venous blood (40 mL) of the 81 enrolled subjects by density gradient centrifugation using Histopaque-1077 (Sigma, St. Louis, MO, USA) as described previously (Chu et al., 2008; Jung et al., 2008). Human subcutaneous adipose tissue was acquired by elective surgery of three female subjects (ages: 35, 44, and 50 years). After removing mature adipocyte fractions and red blood cells, stroma-vascular fractions were utilized for our experiments (Lee et al., 2004; Kim et al., 2007). Tissue samples were subjected to FACS or long-term culture after MACS.

Fluorescence-activated cell sorter analysis

The fraction of pericytes in tissues was quantified by flow cytometry with a FACScan (Becton Dickinson, San Diego, CA, USA) using mouse monoclonal anti-human PDGFRβ-phycoerhtyrin (PE) (BD Biosciences; San Jose, CA), anti-rat PDGFRβ-PE antibody (Santa Cruz Biotechnology, Inc.), or NG2-FITC (Santa Cruz biotechnology, Inc., Santa Cruz, CA, USA). The cells were incubated with specific monoclonal antibodies (10 mg/mL) for 1 h at 4 °C. Irrelevant mouse IgG2a isotype was used as a control and values were subtracted to determine the percentage. In addition, tissue- or peripheral blood-derived pericytes were stained with PE-conjugated antibodies against CD31, CD34, CD45, CD73, CD90, and CD105 (Becton Dickinson; San Diego, CA, USA). Mouse isotypic antibodies served as the control. All experiments were performed by six times.

Magnetic-activated cell sorting

Magnetic-activated cell sorting was applied to freshly isolated peripheral blood mononuclear cells. Approximately 2×10^7 peripheral blood mononuclear cells were washed with PBS/5% fetal bovine serum (FBS) and suspended in a 1/100 dilution of anti-human PE-PDGFRβ antibody for 45 min on ice. After washing, the cells were incubated with anti-FITC or anti-PE microbeads for 15 min on ice and then separated on a Mini MACS magnetic column (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's recommendations.

Long-term culture and differentiation of PDGFRβ$^+$ cells

The PDGFRβ$^+$ cells sorted by MACS were plated at a density of $1–2\times10^5$ per well on 2% gelatin (Sigma)-coated 12-well plates. The cells were suspended using an EGM-2 BulletKit system (Clonetics) consisting of endothelial basal medium, 2% FBS, human vascular endothelial growth factor-1, human fibroblast growth factor-2, human epidermal growth factor, insulin-like growth factor-1, and ascorbic acid, and incubated at 37 °C in humidified 5% CO_2. With daily observation, first media changes were performed 5 days after plating. Thereafter, the media were changed every 2–3 days. Once the cell clusters or colonies had appeared, they were cultured until they formed larger colonies. These were then selected, trypsinized, and expanded over several passages using standard cell culture procedures. To compare the effect of various proliferation media on cell growth, we seeded 1×10^4 cells to gelatin-coated 100 mm plates and counted for 5 weeks. Various composites, such as EGM-2, DMEM, FBS, VEGF, IGF, EGF, FGF, recombinant human PDGF-BB, estradiol-17β, and other supplements under conditions of normoxia or hypoxia (5% O_2) were examined. At each passage, the number of cells was counted in triplicate on a hemocytometer. Six blood samples from different individuals were included in the growth curve assay. The sorted cells were subsequently cultured in DMEM/F12 supplemented with PDGF-BB (50 ng/mL) and 10% FBS to facilitate mature pericyte differentiation. For differentiation into neurons, the PDGFRβ$^+$ cells were cultured in the presence of B27, and 0.5% N1, 10 mol/L all-trans retinoic acid (Sigma) for 2 weeks.

Immunocytochemistry

The PDGFRβ$^+$ cells or differentiated cells were grown on poly-O-coated coverslips for 5 days and subjected to immunocytochemistry as previously described (Jung et al., 2008). The coverslips were stained with antibodies directed against indicated markers, or with control antibodies. Primary antibodies were as follows: anti-nestin (1:100; Chemicon, Temecula, CA); anti-TuJ1 (1:200; Chemicon); anti-neurofilament (anti-NF; 1:100; Chemicon); anti-mitogen-activated protein-2 (anti-MAP2; 1:1,000; Sigma, St. Louis, MO, USA); anti-CXCR4 (1:20; BD Biosciences); anti-PDGFRβ (1:200; Abcam,