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Research report

Bone marrow mononuclear cell transplantation promotes therapeutic angiogenesis via upregulation of the VEGF–VEGFR2 signaling pathway in a rat model of vascular dementia

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HIGHLIGHTS

- We treated 2VO rats with bone marrow mononuclear cells (BMMNCs).
- BMMNC transplantation promoted therapeutic angiogenesis in 2VO rats.
- The effects of BMMNCs in 2VO rats were abolished by pretreatment of SU5416.
- The angiogenic effect may result from upregulation of VEGF–VEGFR2 pathway.

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ABSTRACT

Bone marrow mononuclear cells (BMMNCs) are important for angiogenesis after stroke. We investigated the effects of BMMNCs on cognitive function, angiogenesis, and the vascular endothelial growth factor (VEGF)–VEGF receptor 2 (VEGFR2) signaling pathway in a rat model of vascular dementia. We transplanted BMMNCs into rats that had undergone permanent bilateral occlusion of the common carotid arteries (2VO) and observed their migration in vivo. On day 28, we assessed cognitive function with the Morris Water Maze test and examined vascular density and white matter damage within the corpus striatum by staining with fluorescein lycopersicon esculentum (tomato) lectin or Luxol fast blue. We evaluated expression of VEGF, rapidly accelerated fibrosarcoma 1 (Raf1), and extracellular-signal-regulated kinases 1 and 2 (ERK1/2) in the ischemic hemisphere by Western blot analysis on day 7 after cell transplantation. Contribution of the VEGF–VEGFR2 signaling pathway was confirmed by using VEGFR2 inhibitor SU5416. BMMNCs penetrated the blood–brain barrier and reached the ischemic cortex and white matter or incorporated into vascular walls of 2VO rats. BMMNC-treated 2VO rats had better learning and memory, higher vascular density, and less white matter damage than did vehicle-treated rats. The beneficial effects of BMMNCs were abolished by pretreatment of rats with SU5416. Protein expression of VEGF and phosphorylated Raf1 and ERK1/2 was also significantly increased by BMMNC treatment, but this upregulation was reversed by SU5416. BMMNCs can enhance angiogenesis, reduce white matter damage, and promote cognitive recovery in 2VO rats. The angiogenic effect may result from upregulation of the VEGF–VEGFR2 signaling pathway.

1. Introduction

Vascular dementia (VD) is the second most common cause of dementia after Alzheimer's disease and accounts for approximately 20% of dementia in China [1]. Chronic cerebral hypoperfusion is a major contributor to the memory dysfunction seen in patients with VD [2]. By increasing the number of functional blood vessels, therapeutic angiogenesis may reduce the extent of ischemia and improve cognition in these patients [3].

Stem-cell-based therapy has been proposed as a potential treatment for neurodegenerative diseases [4–6]. Bone marrow mononuclear cells (BMMNCs) are particularly attractive for such therapy because they are composed of different kinds of stem cells, can be rapidly isolated without cultivation, and can be used in autol-

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ogous applications [7]. BMMNCs comprise mesenchymal stem cells, hematopoietic progenitor cells, endothelial progenitor cells, and more committed cell lineages [8]. Several independent groups have demonstrated that BMMNC transplantation significantly reduces ischemic impairments and increases vascular density and blood flow in ischemic disorders such as cardiovascular disease [9,10], peripheral arterial disease [11], and diabetic foot [12].

The mechanism behind the angiogenic capacity of BMMNCs has not yet been defined. A recent study revealed that nitric oxide synthase, which is induced by vascular endothelial growth factor (VEGF), contributes to the angiogenesis that follows BMMNC transplantation in a rat model of VD [13]. VEGF plays an important role in vascular remodeling. Of its three main receptor subtypes, VEGF receptor-2 (VEGFR2) mediates most of the downstream angiogenic effects of VEGF, including microvascular permeability and endothelial cell proliferation, migration, and survival [14]. VEGFR2 triggers these events by activating intracellular tyrosine kinases of endothelial cells and multiple downstream signals, such as rapidly accelerated fibrosarcoma 1 (Raf1) [15] and extracellular-signal-regulated kinases 1 and 2 (ERK1/2) [16]. Whether BMMNCs can promote angiogenesis by upregulating the VEGF–VEGFR2 signaling pathway after VD is still unknown.

Several animal models of chronic cerebral hypoperfusion have been developed to mimic the pathological condition of clinical VD and explore the underlying mechanisms. Of these, the most-used model is bilateral carotid artery occlusion (2-vessel occlusion, 2VO) in rats [17]. Unlike other experimental animals (such as gerbil), rats have a complete circle of Willis that connects the carotid and vertebral systems. After the 2VO procedure, the circle of Willis in rats provides compensatory blood flow from the vertebral arteries to the regions that would normally be supplied by the ligated carotid arteries. Consequently, the 2VO procedure in rat causes global cerebral hypoperfusion rather than stroke [18]. In contrast to rats that undergo middle cerebral artery occlusion (MCAO), the most commonly used animal model of ischemic stroke [19], 2VO rats develop a diffuse brain lesion characterized by demyelination in the white matter [20] and cell loss in the hippocampal CA1 area. This injury impairs cognitive functions [21] without causing major motor deficits [17,22].

Although previous reports have indicated that BMMNC treatment is effective and safe for patients with acute ischemic stroke [23], no study has examined the angiogenic effects and possible therapeutic mechanism of BMMNCs in VD. We hypothesized that BMMNCs would improve functional outcome and promote therapeutic angiogenesis by upregulating the VEGF–VEGFR2 signaling pathway in a rat model of VD.

2. Materials and methods

2.1. Animals and ethics statement

Adult, male Sprague-Dawley rats (11–12 weeks old, 260–300 g) were purchased from the Animal Experimental Center of Zhengzhou University. They were housed in plastic cages (5 per cage) with free access to food and water and were maintained on a 12-h light/dark cycle at a constant temperature of $22 \pm 1\,^{\circ}C$. All protocols were approved by the Animal Care and Use Committee of Zhengzhou University. All efforts were made to minimize the number of animals used and their suffering.

2.2. Vascular dementia model

Rats were subjected to a previously reported 2VO model of VD in which the right and left common carotid arteries (CCAs) are permanently occluded [24,25]. Briefly, we anesthetized rats by intraperitoneal injection with 10% chloral hydrate (400 mg/kg) and made a midline incision in the ventral side of the neck to expose the CCAs. We gently separated the arteries from their sheaths and adjacent vagus nerves and then permanently occluded them with 5-0 silk suture under a surgical microscope. The neck wound was sutured closed and topical lidocaine applied. Successful 2VO was defined as an approximately 70% decrease in central blood flow, which was confirmed by laser-Doppler flowmetry [26]. Additionally, 55 sham-operated controls (sham) underwent the same surgical procedures but without carotid artery ligation.

2.3. Preparation of BMMNCs

Fresh BMMNCs were collected from femurs and tibias of male Sprague-Dawley rats (200–250 g; $n = 94$) and purified by Percoll gradient centrifugation as previously reported [27]. The rats were anesthetized by an overdose of chloral hydrate and then sacrificed. Right and left femurs and tibias were aseptically dissected and cut at both ends. Bone marrow was extruded with serum-free Dulbecco's modified Eagle's medium (DMEM/F12; HyClone, Logan, UT). The extracted bone marrow was subjected to density-gradient centrifugation (160 g, 25 min) in 1.083 g/mL Histopaque 1083 (Sigma–Aldrich, St. Louis, MO). The mononuclear cell layer was recovered from the gradient interface and washed three times by suspension in DMEM/F12 followed by 5-min centrifugation. The concentration of the cells was verified in a Neubauer counting chamber, and the number of viable cells was determined by trypan blue exclusion. To determine their migratory ability, we labeled a portion of the BMMNCs with bromodeoxyuridine (BrdU) by incubating them in culture medium containing 12 μg/mL BrdU (Sigma–Aldrich) for 24 h [28]. We assessed the purity of the isolated bone marrow cells as putative BMMNCs [29] by using fluorescence-activated cell sorting to determine the expression of various immunophenotypic markers. Ten million cells per rat ($n = 5$) were incubated at 4 °C for 30 min in phosphate-buffered saline (PBS) containing 2% fetal bovine serum and 1 μL of monoclonal antibody specific for CD34 (Santa Cruz Biotechnology, Santa Cruz, CA), CD45, CD90 (BD Biosciences, San Jose, CA), and CD117 (Abcam, Cambridge, MA, USA). To confirm the labeling rate, we incubated some BrdU-treated cells with 2 M HCl for 30 min to depurinate the DNA. Cells were then washed with PBS containing 0.1% fetal bovine serum albumin and 0.2% Tween-20 and incubated with 1 μL of anti-BrdU-fluorescein isothiocyanate (BD Biosciences) for 30 min.

2.4. Treatment and groups

The animals were randomly assigned to six groups: sham-operated rats injected with DMEM (sham + vehicle, $n = 29$), sham-operated rats injected with BMMNCs (sham + BMMNC, $n = 24$), 2VO rats injected with DMEM (2VO + vehicle, $n = 32$), 2VO rats injected with BMMNCs (2VO + BMMNC, $n = 32$), 2VO rats pretreated with VEGFR2 inhibitor SU5416 and injected with BMMNCs (2VO + SU5416 + BMMNC, $n = 32$), 2VO rats treated with SU5416 (2VO + SU5416, $n = 32$). In addition, we administered BrdU-labeled BMMNCs to a subgroup of 2VO rats (2VO + BrdU-labeled BMMNC, $n = 15$) to study the migration of BMMNCs.

Rats in the cell-treated groups were administered 3×10^6 viable BMMNCs diluted in 300 μL of DMEM via tail vein infusion on day 4 after CCA occlusion because cerebral blood flow most resembles that of humans with VD at this time point [18]. This dose of cells was chosen because we and others have shown that systemic treatment with 1×10^7 cells/kg (3×10^6 cells per rat in this study) effectively promotes recovery in a rat ischemic stroke model [28,30]. Vehicle-treated rats were infused with an equivalent volume of DMEM. SU5416-treated rats received 10 mg/kg VEGFR2 inhibitor SU5416 (Sigma–Aldrich) in DMEM by intraperitoneal injection immedi-