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Neurotrophic and neuroprotective potential of human limbus-derived mesenchymal stromal cells

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Abstract

Background aims. The purpose of this study was to examine neurotrophic and neuroprotective effects of limbus stroma-derived mesenchymal stromal cells (L-MSCs) on cortical neurons *in vitro* and *in vivo*. *Methods.* Cultured L-MSCs were characterized by flow cytometry and immunofluorescence through the use of specific MSC marker antibodies. Conditioned media were collected from normoxia- and hypoxia-treated L-MSCs to assess neurotrophic effects. Neuroprotective potentials were evaluated through the use of *in vitro* hypoxic cortical neuron culture and *in vivo* rat focal cerebral ischemia models. Neuronal morphology was confirmed by immunofluorescence with the use of anti-MAP2 antibody. Post-ischemic infarct volume and motor behavior were assayed by means of triphenyltetrazolium chloride staining and open-field testing, respectively. Human growth antibody arrays and enzyme-linked immunoassays were used to analyze trophic/growth factors contained in conditioned media. *Results.* Isolated human L-MSCs highly expressed CD29, CD90 and CD105 but not CD34 and CD45. Mesenchymal lineage cell surface expression pattern and differentiation capacity were identical to MSCs derived form human bone marrow and adipose tissue. The L-MSC normoxic and hypoxic conditioned media both promoted neurite outgrowth in cultured cortical neurons. Hypoxic conditioned medium showed superior neurotrophic function and neuroprotective potential with reduced ischemic brain injury and improved functional recovery in rat focal cerebral ischemia models. Human growth factor arrays and enzyme-linked immunoassays measurements showed neuroprotective and growth-associated cytokines (vascular endothelial growth factor [VEGF], VEGFR3, brain-derived neurotrophic factor, insulin-like growth factor -2 and hepatocyte growth factor) contained in conditioned media. Hypoxic exposure caused VEGF and brain-derived neurotrophic factor upregulation, possibly contributing to neurotrophic and neuroprotective effects. *Conclusions.* L-MSCs can secrete various neurotrophic factors stimulating neurite outgrowth and protecting neurons against brain ischemic injury through paracrine mechanism.

Key Words: *mesenchymal stromal cells, neuroprotection, paracrine*

Introduction

Mesenchymal stromal cells (MSCs) are well-characterized populations of fibroblast-like, plastic-adherent adult stem cells. They have the capacity to self-renew and differentiate into mature cells, including osteoblasts, chondrocytes, adipocytes, muscle cells, β-pancreatic islets cells and neurons (1). These cells can be isolated from various locations including bone marrow, adipose tissue, peripheral blood cells, fetal tissues, pancreas and the ocular limbus (2–4). More recently, MSCs have shown the potential to enhance neuronal growth and central nervous system (CNS) repair (5). Therefore, MSCs may be valuable for cell-based therapies in preclinical neurological disease CNS models (6,7). Intravenously administered or CNS-implanted MSCs promote functional recovery in animal stroke models, brain and spinal cord injury, multiple sclerosis and patients with stroke (8). Although some transdifferentiation of

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transplanted MSCs into CNS neurons and glia might replace damaged cells, exact mechanisms behind the MSC neuroprotective and growth-promoting effects remain poorly understood. However, newer evidence suggests that MSCs can secrete numerous neurotrophic factors to regulate neuron/neural stem cell survival and differentiation and stimulate endogenous protective paracrine responses (7,9–11).

The cornea is renewable tissue; as with other surface epithelium, it is regenerated throughout life. The cell source participating in its regeneration is the limbus, a transition zone between cornea, conjunctiva and sclera. The limbal epithelial basal layer may have corneal epithelial stem cells that are ultimate sources of corneal epithelial proliferation, differentiation and replacement during corneal epithelial wound-healing. Limbus-derived cell transplantation can effectively reconstruct corneal surfaces in patients with limbal stem cell deficiency. The adjacent limbus stroma contains mesenchymal cells with fibroblastic phenotypes, also serving as niche cells (2). Limbal stromal cells are required for maintaining corneal epithelial stem cell growth and differentiation by various secreted growth factors and inflammatory factors (12–14). In addition to this regenerative capacity, limbal stromal cells may have self-renewing and multipotent characteristics similar to those in bone marrow MSCs (2,3). Limbus stroma-derived MSCs (L-MSCs) have several advantages compared with bone marrow–derived MSC, including easy enrichment as the result of their plastic adherence and higher growth rates (2). The corneal limbus is one of the most richly innervated tissues in the body, and most nerve fibers enter the peripheral cornea at the limbus (15). Microarrays and reverse transcriptase–polymerase chain reaction analysis have shown that limbal stromal cells expressed neurotrophic factors such as neurotrophin-3, neurotrophin-5, nerve growth factor, basic fibroblast growth factor, brain-derived neurotrophic factor (BDNF) and glial cell line–derived neurotrophic factor (16). However, functional benefits of growth and neurotrophic factors by L-MSCs have not been elucidated regarding CNS neurons. In the present study, we determined neurotrophic and neuroprotective properties of stromal stem cells isolated from the human limbus. We present evidence that L-MSCs secrete soluble paracrine factors, promoting neurite outgrowth and protecting cortical neurons against hypoxic insults, which improve functional recovery after ischemic stroke.

Methods

Cells isolated from human limbus

Human limbal tissues were obtained from cadavers after cornea transplantation. Tissue collections for research were approved by the Tri-service General Hospital Institutional Review Board. The isolation of the limbal mesenchymal cells was performed according to previous guidelines, with some modifications (17). Limbal biopsy explants were minced into 1–2-mm^3 fragments, including epithelium and stromal tissue. To enrich stromal cell isolation, we first removed limbal epithelial sheets with the use of Dispase II (2.4 U/mL) at 37°C for 1.5 h and digested the remaining stroma with 0.25% trypsin–ethylenediaminetetraacetic acid at 37°C for 10 min. Limbal segments were grown in culture media composed of Dulbecco's modified Eagle's medium/F12 plus 10% fetal bovine serum (FBS). After 2 days of plating, we observed adherent spindle cells (limbal mesenchymal cells). On 80–90% confluence, they were serially passed at the density of 5×10^3 per cm^2. All cultures were used below passage 3 for experiments described later in the report. Bone marrow–derived and adipose-derived MSCs were generous gifts from Dr James (Graduate Institute of Medical Sciences, National Defense Medical Center, Taipei, Taiwan).

Flow cytometry analysis

Cultured cells were detached with trypsin/ethylenediaminetetraacetic acid and counted. Approximately 2×10^5 cells were divided into 5-mL aliquots in centrifuge tubes and then were washed with phosphate-buffered saline (PBS) containing 2% bovine serum albumin and 0.1% sodium azide (Sigma-Aldrich, St Louis, MO, USA). Subsequently, cells were stained with 2 μg/mL concentration of fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD29 (BD Biosciences, San Jose, CA, USA), mouse anti-human CD90 (BD Biosciences), mouse anti-human CD105, mouse anti-human CD34 (BD Biosciences) and mouse anti-human CD45 (BD Biosciences) at 4°C for 45 min. The stained cells were analyzed by means of fluorescence-activated cell sorting Calibur cytometry (BD Biosciences). CELLQuest software was used to create histograms. Each histogram represents 10,000 total events.

Transdifferentiation assays

To investigate the multidirectional differentiation of the fibroblast-like cells from human limbus, we also performed the inductions of osteogenic, adipogenic and chondrogenic differentiation.

Osteogenic differentiation

Cells were stimulated every 3–4 days in a growth medium supplemented with 0.1 μmol/L dexamethasone,