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Delivery of fibroblast growth factor 2 enhances the viability of cord blood-derived mesenchymal stem cells transplanted to ischemic limbs

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Transplantation of cord blood-derived mesenchymal stem cells (CBMSCs) into ischemic regions could be a potential therapy for the treatment of ischemic disease, but its efficacy is limited by poor cell survival. We hypothesized that local delivery of fibroblast growth factor 2 (FGF2) to the site of CBMSC transplantation would enhance the viability of CBMSCs transplanted to ischemic tissues. Human CBMSCs were loaded onto fibrin gel with or without FGF2 and transplanted intramuscularly into either normal or ischemic hindlimbs of athymic mice. CBMSC transplantation combined with FGF2 delivery resulted in significantly lower apoptosis and higher survival of transplanted CBMSCs. The enhanced cell survival could be due to the local delivery of FGF2 and the enhanced secretion of anti-apoptotic factor. CBMSC transplantation and FGF2 delivery enhanced the expression of host-derived, platelet-derived growth factor-β and NG2, which induce endothelial cell homing and pericyte recruitment, respectively, and more effectively protected muscles from ischemic degeneration when compared to CBMSC transplantation alone. FGF2 delivery to the site of CBMSC transplantation can enhance the survival of CBMSCs transplanted into ischemic tissues. This approach could be used to improve the angiogenic efficacy of CBMSC transplantation therapy for ischemic disease.

[**Key words:** Angiogenesis; Cell survival; Cord blood-derived mesenchymal stem cells; Fibroblast growth factor 2; Hindlimb ischemia]

Stem cells, including bone marrow-derived mesenchymal stem cells, adipose-derived stem cells, and cord blood-derived mesenchymal stem cells (CBMSCs), hold great potential as cell-based therapies that promote vascularization for the treatment of ischemic disease (1–3). However, transplantation of stem cells alone generally results in limited angiogenic efficacy due to poor survival of the transplanted cells (4–6). Severe apoptosis and necrosis typically occur in ischemic regions due to the rapid metabolic changes caused by blood vessel occlusion (7,8). Stem cells transplanted into these regions are exposed to hypoxic conditions whereupon they immediately undergo ischemic apoptosis (7,8). As a result, significant death of engrafted cells occurs within days following transplantation (4–6). Therefore, the transplantation of stem cells into ischemic regions without additional supporting treatment may not result in significant therapeutic angiogenesis.

Many strategies that enhance the viability of stem cells transplanted into ischemic tissues have been developed. Recently, it was reported that the treatment of cells with heat shock protein or trimetazidine along with the preconditioning of cells to heat shock before transplantation into ischemic regions enhance cell survival (9–11). Cells treated with heat shock protein or trimetazidine are significantly protected against hypoxia-induced loss of cell viability, membrane damage and oxygen metabolism and experience significant increases in HIF-1α, survivin and Bcl-2 protein levels as well as Bcl-2 gene expression (9,10). Preconditioning to heat shock (42°C for 1 h) prior to cell implantation improves the survival of myoblasts implanted into the myocardium (11). Despite the potential advantages of protein treatment and heat shock preconditioning, low efficacy due to insufficient angiogenic cues in ischemic regions remains the largest obstacle to developing effective angiogenic cell therapies. Further, protein treatment or heat shock preconditioning before cell transplantation only guarantees enhanced cell viability. Angiogenesis in the pre-existing vasculature requires angiogenic factors such as fibroblast growth factor (FGF) or vascular endothelial growth factor (VEGF) at the initial stages. Therefore, enhancement of cell viability alone may not be sufficient enough to induce angiogenesis.

Fibroblast growth factor 2 (FGF2) induces angiogenesis and acts as an anti-apoptotic factor (12–14). In the present study, we tested the hypothesis that local delivery of FGF2 to the site of CBMSC transplantation in ischemic regions improves the viability of the transplanted CBMSCs. FGF2 protein injected into the body in soluble form undergoes rapid degradation due to its short half-life *in vivo* (15). Delivery systems that locally release FGF2 in a controlled manner, such as fibrin gel (16), may prolong the bioactivity of FGF2. CBMSCs in fibrin gel, with or without FGF2, were transplanted into normal or ischemic hindlimbs of athymic mice. CBMSCs transplanted into ischemic regions were evaluated for apoptotic activity, survival

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and angiogenic paracrine factor secretion along with the expression of factors responsible for endothelial cell (EC) homing and pericyte recruitment during the initial stages of ischemia (7 days).

MATERIALS AND METHODS

Cell culture Human umbilical cord blood (UCB) was obtained from the umbilical veins of normal full-term pregnant women following delivery with informed maternal consent. CBMSCs were then isolated and cultivated from human UCB as previously reported (17). Mononuclear cells were isolated from UCB by centrifugation through a Ficoll-Hypaque gradient (density 1.077 g/cm^3, Sigma, St. Louis, MO, USA). The separated mononuclear cells were washed, suspended in α-minimum essential medium (α-MEM, Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) and seeded at a concentration of 5×10^6 cells/cm^2. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2, and the culture medium was changed twice weekly. One to three weeks later, once the monolayer of fibroblast-like adherent cell colonies had reached 80% confluence, the cells were trypsinized (0.25% trypsin, HyClone), washed, resuspended in culture medium (α-MEM supplemented with 10% FBS) and sub-cultured (17). Cells were passaged every seven to eight days and then used after five passages. Prior to cell transplantation, CBMSCs were pre-labeled with enhanced green fluorescence (18).

Determination of the kinetics of FGF2 release from fibrin gel in vitro The kinetics of FGF2 release from fibrin gel were determined as previously described (16). FGF2 (1 mg/ml)-loaded fibrin gels were immerged in 2 ml micro-centrifuge tubes containing 1.5 ml of phosphate buffered saline (pH 7.4) and 0.02% (w/v) sodium azide. The samples (n = 4) were incubated at 37°C under continuous agitation. At various time points, the supernatant was withdrawn and fresh buffer was replenished. The concentrations of FGF2 in the supernatants were determined using an enzyme-linked immunosorbent assay (ELISA) kit (human FGF2 Duoset, R&D Systems, Minnepolis, MN). ELISA plates (Nunc, Polylabo, Strasbourg, France) were coated with the capture monoclonal antibodies and blocked with bovine serum albumin (1% w/v) containing sucrose (5% w/v) for 1 h. After adding the appropriately diluted samples to the ELISA plates, bound FGF2 was detected using antihuman FGF2 polyclonal antibodies. Streptavidine-conjugated horseradish peroxidase and the enzyme substrate (tetramethylbenzidine and peroxide) were then added to the plates, followed by incubation for 20 min. The enzyme reaction was stopped by the addition of acidic solution. The absorbances of the samples were read at 450 nm using a PowerWave X340 plate reader (Bio-TEK Instrument, Winooski, VT, USA). The amount of FGF2 in the supernatants was calculated from a calibration curve based on known concentrations of FGF2. The experiments were performed in quintet.

Model of mouse hindlimb ischemia Hindlimb ischemia was induced in mice as previously described (19). Four-week old female athymic mice (20–25 g body weight, Orient, Seoul, Korea) were anesthetized with xylazine (10 mg/kg) and ketamine (100 mg/kg). The femoral artery and its branches were ligated via skin incision using a 6-0 silk suture (Ethicon, Somerville, NJ, USA), along with the external iliac artery and all upstream arteries. The femoral artery was then excised from its proximal origin as a branch of the external iliac artery to the distal point whereupon it bifurcates into the saphenous and popliteal arteries. All animals received humane care in compliance with the Institute of Laboratory Animal Resources of Seoul National University (Institutional Animal Care and Use Committee No. SNU-100203-3).

Treatment of limb ischemia One day following arterial dissection, the mice were randomly divided into three groups (n = 5 for each group). Normal mice that did not undergo surgery served as a positive control. CBMSCs (2×10^6 cells/limb) were loaded in fibrin gel with or without FGF2 (25 μg FGF2/limb) and injected intramuscularly into the gracilis muscle of the medial thigh.

TUNEL assay Seven days after cell transplantation, TUNEL assays were performed to assess apoptotic activity in hindlimb ischemic tissues using an ApopTag Red *in situ* Apoptosis Detection Kit (Chemicon, Temecula, CA, USA) according to the manufacturer's instructions.

RT-PCR Seven days following cell transplantation, ischemic limb tissues were harvested, homogenized and lysed in TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted with chloroform (Sigma) and precipitated with 80% (v/v) isopropanol (Sigma). After the supernatant was removed, the RNA pellet was washed with 75% (v/v) ethanol, air-dried and dissolved in 0.1% (v/v) diethyl pyrocarbonate-treated water (Sigma). RNA concentration was determined by measuring the absorbance at 260 nm using a spectrophotometer. Reverse transcription was performed using 5 μg of pure total RNA and SuperScript™ II reverse transcriptase (Invitrogen), and the synthesized cDNA was amplified by PCR. PCR was carried out for 35 cycles of denaturing (94°C, 30 s), annealing (58°C, 45 s) and extension (72°C, 45 s), with a final extension at 72°C for 10 min. PCR products were visualized by electrophoresis on a 2% (w/v) agarose gel with ethidium bromide staining. The products were analyzed with a gel documentation system (Gel Doc 1000, Bio-Rad, Hercules, CA, USA). β-Actin served as an internal control.

Immunohistochemistry Ischemic limb muscles harvested seven days after treatment were embedded in Optimal Cutting Temperature compound (TISSUE-TEK® 4583, Sakura Finetek USA Inc., Torrance, CA, USA), and were then frozen and cut into 10 μm-thick sections at −22°C. Sections of ischemic tissues were immunofluorescently stained with antibodies against human nuclear antigen (HNA, Chemicon) and vascular endothelial growth factor (VEGF, Abcam, Cambridge, UK). The staining signals for HNA and VEGF were visualized using rhodamine- and FITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), respectively. The sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA) and examined using a fluorescence microscope (Nikon TE2000, Tokyo, Japan). All samples (n = 3 per group) were completely sectioned, and six slides were selected from the beginning, middle and end parts of each sample.

Histology Ischemic limb muscles retrieved seven days after treatment were fixed in formaldehyde, dehydrated with a graded ethanol series and embedded in paraffin. Specimens were sliced into 4-μm sections and stained with hematoxylin and eosin (H&E) to examine muscle degeneration and tissue inflammation. Masson's trichrome staining was also performed to assess tissue fibrosis in ischemic regions.

Western blot analysis The samples obtained from mouse hindlimb muscles were homogenized using a Dounce homogenizer (50 strokes, 4°C) in ice-cold lysis buffer (15 mM Tris HCl, pH 8.0, 0.25 M sucrose, 15 mM NaCl, 1.5 mM $MgCl_2$, 2.5 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 2 mM NaPPi, 1 μg/ml pepstatin A, 2.5 μg/ml aprotinin, 5 μg/ml leupeptin, 0.5 mM phenymethyl sulfonyl fluoride, 0.125 mM Na_3VO_4, 25 mM NaF and 10 μM lactacystin). Western blot analysis was carried out by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After transfer to an Immobilon-P membrane (Millipore Corp., Billerica, MA, USA), the samples were detected with antibodies against VEGF (Abcam), p53 (Abcam), PCNA (Abcam), PDGF-β (Abcam) and NG2 (Abcam), followed by subsequent incubation with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for one hour at room temperature. The blots were developed by enhanced chemiluminescence detection (Amersham Bioscience, Piscataway, NJ, USA). Band intensities on the Western blot were quantified by densitometric scanning using Image-Pro Plus software (Media Cybernetics, Inc., Silver Springs, MD, USA).

Statistical analysis Quantitative data are expressed as mean ± standard deviation. Statistical analysis was performed by ANOVA using a Bonferroni test. A *p*-value less than 0.05 was considered statistically significant.

RESULTS

Inhibited apoptosis and enhanced cell survival of transplanted CBSMCs FGF2 was released from fibrin gel *in vitro* for 10 days (Fig. 1). An initial burst of FGF2 was observed over the first 12 h, after which the FGF2 release rate remained almost constant (20,21). Localized and sustained delivery of FGF2 from fibrin gel over 10 days inhibited apoptosis and enhanced the survival of CBMSCs after transplantation into mouse ischemic hindlimbs. TUNEL assay revealed that apoptosis was significantly reduced in CBMSCs transplanted with FGF2 delivery, compared to CBMSCs transplanted alone (Figs. 2A and B). RT-PCR assay for human-specific BAX, a pro-apoptotic gene, revealed that CBMSC transplantation with FGF2 delivery experienced a 2.3-fold decrease in BAX expression compared to CBMSC transplantation alone (Figs. 2C and D). Western blot analysis revealed that expression of human-specific p53, a pro-apoptotic marker, was increased 1.8-fold in CBMSCs transplanted with FGF2 delivery compared to CBMSC transplanted alone (Figs. 2C and D). Immunofluorescent staining for HNA also showed that CBMSCs transplanted with FGF2 delivery showed significantly higher retention in ischemic regions compared to CBMSCs transplanted alone (Fig. 3).

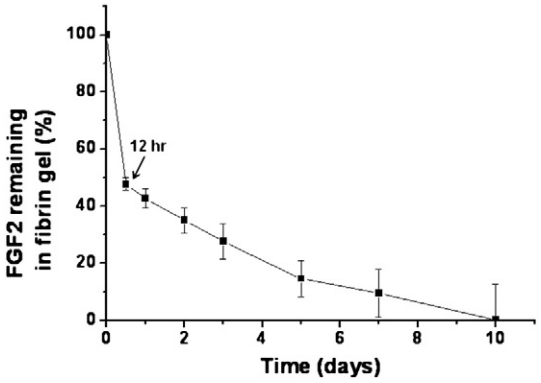

FIG. 1. Profiles of FGF2 release from fibrin gel. The values represent means ± standard deviation ($n = 4$).