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Original research

Secretomes of mesenchymal stem cells induce early bone regeneration by accelerating migration of stem cells

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ABSTRACT

Objective: We previously reported that secretomes from human bone marrow-derived mesenchymal stem cells (MSC-CM) have a strong potential to accelerate bone regeneration. The most important initial step for bone regeneration is osteoprogenitor cell migration to bone defects. We hypothesized that MSC: CM enhance the migration of endogenous stem cells earlier to the local lesioned part. In this study; we investigated the potential of MSCCM to induce in vivo early bone regeneration by accelerating cell migration in a rat calvarial bone defect model.

Materials and methods: Cytokine array analysis was performed to assess the types of cytokines included in MSC-CM. Bone defects (5 mm in diameter) were created in the calvarial bones of rats, and the damaged areas were implanted with atelocollagen suspended in MSC-CM or phosphate buffered saline. After 2 and 4 weeks, radiographic and histological analyses were performed. Furthermore, rat mesenchymal stem cells (rMSCs) were labeled with the lipophilic tracer 1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanine iodide (DiR), and the rats were photographed at various times after injection of the DiR-labeled rMSCs using in vivo imaging.

Results: MSC-CM contained many factors with respect to cell migration and tissue regeneration. Bone regeneration in rat calvaria was observed earliest in the MSC-CM implantation group. Migration of the labeled rMSCs from the tail toward the calvaria, where MSC-CM was implanted, was observed during the first 24 h after injection in the MSC-CM implantation group using in vivo imaging. Immunohistochemistry also indicated early cell migration.

Conclusion: MSC-CM enhanced the migration of endogenous stem cells facilitating earlier bone regeneration.

1. Introduction

Bone marrow-derived mesenchymal stem cells (MSCs) have undergone extensive clinical investigation as a bone regeneration tool [1,2]. However, several drawbacks of MSCs, including high capital investment, costly cell culture, complicated safety and quality management issues concerning cell handling, and patient discomfort with the invasive procedure called for cell collection [3]. Moreover, the survival duration of the implanted MSCs is short and post-transplantation disappearance is noted after several weeks [4].

Recent research revealed that various paracrine factors are secreted by implanted MSCs, including growth factors and chemokines, which lead to the induction of tissue regeneration by endogenous stem cells [4,5]. For example, hepatocyte growth factor (HGF), monocyte chemoattractant protein (MCP)-1, and stromal cell-derived factor-1 accelerate the migration of human bone marrow-derived mesenchymal stem cells (hMSCs) *in vitro* [6–8]. We previously reported that serum-free conditioned media from hMSCs contain numerous cytokines [9]. These secretomes regulate migration, angiogenesis, and osteogenesis in host MSCs; thus, they may accelerate bone and periodontal tissue regeneration [10–16]. Cell migration to local sites can trigger tissue regeneration processes such as angiogenesis. Indeed, cell migration is considered an important step in angiogenesis for bone regeneration [16]. However, there have been no studies investigating whether secretomes from hMSCs [MSC conditioned medium (MSC-CM)] can significantly influence cell migration and subsequently early bone

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regeneration. We have previously demonstrated that secretomes from hMSCs [MSC conditioned medium (MSC-CM)] recruit endogenous stem cells to the grafted site in rat calvarial bone defects using agarose [10]. However, there are no studies that demonstrate whether MSC-CM can significantly influence cell migration and subsequently early bone regeneration using atelocollagen.

Therefore, we hypothesized that cell migration comprises one of the crucial steps for early bone regeneration and that MSC-CM can induce earlier migration of endogenous stem cells to the lesioned area using atelocollagen instead of agarose. In this study, we evaluated the potential of MSC-CM in accelerating cell migration and facilitating early *in vivo* bone regeneration using a rat calvarial bone defect model.

2. Materials and methods

2.1. Cell preparation

All animal experiments performed in the present study were in strict accordance with the protocols approved in the Guidelines for Animal Experimentation of the Nagoya University School of Medicine (approval nos. 25,374 and 26,063).

hMSCs were acquired from Lonza, Inc. (Walkersville, MD, USA) and were cultured in MSC basal medium (Lonza, Inc.) that contained MSC-GM SingleQuots (Lonza, Inc.) at 37 °C in 5% CO_2/95% air. After primary culture, the cells were subcultured at approximately 1×10^4 cells/cm^2 density. hMSCs at the third to sixth passages were used.

We isolated Rat MSCs (rMSCs) from 7-week-old Wistar/ST male rats (Japan SLC, Shizuoka, Japan) as has been reported [17]. We sacrificed donor rats for the purpose of this experiment, and harvested the femora. We cut the edge of each bone under sterile conditions, and injected Dulbecco's modified Eagle medium (DMEM; Gibco, Rockville, MD, USA) into the bone marrow using an 18-gauge syringe, and flushed out the bone marrow cells to the opposite side; we repeated this procedure several times. After seeding the marrow into a tissue culture flask in DMEM containing an antibiotic–antimycotic solution (100 units/mL penicillin G, 100 mg/mL streptomycin, and 0.25 mg/mL amphotericin B; Gibco), we supplemented the medium with 10% fetal bovine serum. The floating cells were removed 3 days after seeding, and we replaced the medium with fresh medium. The adherent, spindle-shaped cells were passaged when the cells approached confluence. We collected the adherent cells using trypsin/ethylenediaminetetraacetic acid and resuspended in fresh medium, then they were transferred to new flasks at 1×10^4 cells/cm^2 density.

2.2. Preparation of conditioned media

A total of 80% confluent hMSCs were replenished with serum-free DMEM (Gibco, Rockville, MD, USA) containing antibiotic–antimycotic solution. The cell-cultured, conditioned media were collected after incubating for 48 h. The conditioned media were subsequently centrifuged at $440 \times g$ for 5 min at 4 °C. The supernatant was collected, centrifuged at $17{,}400 \times g$ for 3 min at 4 °C, and was then filtered at 0.22 μm (Millex®-GP; Merck Millipore Ltd., Billerica, MA, USA) to yield MSC-CM. The MSC-CM were stored at −80 °C prior to usage.

2.3. Cytokine antibody array

We performed cytokine array analysis for assessing the types of cytokines present in MSC-CM (RayBio® Human Cytokine Antibody Array G-Series 2000; RayBiotech, Inc., Norcross, GA, USA). We identified cytokines in MSC-CM and serum-free DMEM using 174 human cytokine array plates by laser scanning. Each scan was performed in duplicate, and data were calculated as the ratio of the cytokine level in MSC-CM to that in serum-free DMEM.

2.4. Rat calvarial bone defect model

Seven-week-old male Wistar/ST rats ($n = 24$) were anesthetized by intraperitoneally injecting with ketamine (60–90 mg/kg) and xylazine (100–150 mg/kg). For evaluating bone formation using micro-computed tomography (micro-CT; Rigaku, Tokyo, Japan) and histological and immunohistochemical assessments, two circular bone defects (5 mm in diameter) were created in the calvarial bone. A dental surgical drilling unit was used to create the bone defects, with a trephine constantly cooled with sterile saline. Next, the calvarial bone was carefully removed to avoid tearing of the dura. After thoroughly rinsing with saline to wash any bone fragments out, the experimental materials were implanted onto the defects.

Atelocollagen (Terudermis®; Olympus Terumo Biomaterials, Tokyo, Japan) was cut into the desired form and was suspended in 30 μL MSC-CM or phosphate buffered saline (PBS). The following groups were defined: (1) MSC-CM, MSC-CM/Terudermis®; (2) PBS, PBS/Terudermis®; and (3) Defect, unfilled defect. The periosteum and scalp were then closed with interrupted 4-0 nylon sutures.

2.5. Radiographic and histological analyses

Surgical sites were dissected and fixed in 4% paraformaldehyde (PFA). They were then subjected to micro-CT analysis using a laboratory X-ray CT device (micro-CT; Rigaku, Tokyo, Japan). We reconstituted three-dimensional images using the software supplied with the instrument. Furthermore, each calvarial bone sample was visualized using scanning electron microscopy (SEM; S-800, Hitachi, Ltd.). The explants were assessed radiographically and were then decalcified using K-CX solution (Falma Co., Tokyo, Japan). They were then dehydrated using graded ethanol, cleared with xylene, and embedded in paraffin. The specimens were sagittally cut to create 5-μm-thick histological sections in the buccal–palatal plane. We then performed hematoxylin and eosin staining and histologically analyzed the specimens using light microscopy (CK40; Olympus).

2.6. In vivo imaging analysis

rMSCs were harvested and cultured as previously described and labeled with the lipophilic tracer 1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanine iodide (DiR; Molecular Probes, Eugene, OR, USA) for the purpose of all imaging experiments [10,18]. The excitation of this fluorophore is achieved at 750 nm and it displays an emission peak at 782 nm. The cells were incubated in DiR (3×10^6 cells in 10 mL PBS containing 3.5 mg/mL dye and 0.5% ethanol) for 30 min at 37 °C. The cells were then washed twice with PBS and were intravenously injected into the caudal vein of Wistar/ST rats bearing the bone defects as described, which had been implanted with various materials immediately prior to rMSC injection. The rats were anesthetized by intraperitoneally injecting ketamine and xylazine prior to injection. DiR-labeled rMSC localization was monitored using Xenogen's IVIS® 200 Series Imaging System (Xenogen, Alameda, CA, USA) within living as well as sacrificed animals. Imaging was performed at 24 h, 72 h, and 1 week after injection of DiR-labeled cells.

2.7. Immunohistochemistry

We made 8-μm sections of the fresh-frozen samples in accordance with the Kawamoto method using a Multi-Purpose Cryosection Preparation Kit [19]. Each section was fixed with 4% paraformaldehyde (PFA)/PBS for 1 h at room temperature, permeabilized with 0.1% Triton-X-100 for 2 min on ice, washed twice with PBS (pH 7.4), and then stained with primary antibodies in blocking buffer overnight at 4 °C. The cells were then stained with secondary antibodies for 30 min, washed, stained with 4′,6-diamidino-2-phenylindole (Invitrogen, Tokyo, Japan) for 15 min. fluorescence microscopy (BZ-9000, Keyence,