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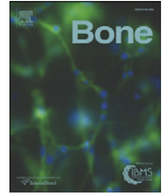

Original Full Length Article

Stem cell-conditioned medium accelerates distraction osteogenesis through multiple regenerative mechanisms

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ABSTRACT

Distraction osteogenesis (DO) successfully induces large-scale skeletal tissue regeneration, but it involves an undesirably long treatment period. A high-speed DO mouse model (H-DO) with a distraction speed twice that of a control DO model failed to generate new bone callus in the distraction gap. Here we demonstrate that the local administration of serum-free conditioned medium from human mesenchymal stem cells (MSC-CM) accelerated callus formation in the mouse H-DO model. Secretomic analysis identified factors contained in MSC-CM that recruit murine bone marrow stromal cells (mBMSCs) and endothelial cells/endothelial progenitor cells (EC/EPCs), inhibit inflammation and apoptosis, and promote osteoblast differentiation, angiogenesis, and cell proliferation. Functional assays identified MCP-1/-3 and IL-3/-6 as essential factors in recruiting mBMSCs and EC/EPCs. IL-3/-6 also enhanced the osteogenic differentiation of mBMSCs. MSC-CM that had been depleted of MCP-1/-3 failed to recruit mBMSCs, and consequently failed to promote callus formation. Taken together, our data suggest that MSCs produce a broad repertoire of trophic factors with tissue-regenerative activities that accelerate healing in the DO process.

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Introduction

Distraction osteogenesis (DO) is used in orthopedic and craniofacial surgeries to lengthen the skeleton and reconstruct skeletal deformities [1–4]. The DO procedure involves osteotomy followed by the gradual distraction of the two bone segments, which promotes neocallus formation in the gap between the segments. Neoangiogenesis and the recruitment of endogenous mesenchymal stem cells (MSCs) and osteogenic progenitors are required for large-scale DO-mediated tissue regeneration [4]. While DO provides significant clinical benefits, patients must endure a long treatment period (12 months on average) and use a large external fixator, which increases the risk of severe osteomyelitis by providing a possible entry route for pathogens. Therefore, it is desirable to reduce the duration of DO treatment.

Stem cell transplantation is a promising regenerative therapy. The transplantation of various adult MSCs and their derivatives into damaged areas promotes tissue repair in both humans and model animals [5,6]. Transplanted MSCs accelerate new bone formation in various preclinical animal models for bone defects, including DO models [7–11]. However, the engrafted stem cells have poor differentiation and survival rates, suggesting that they promote regeneration primarily through paracrine mechanisms [12,13]. Stem cells secrete a broad repertoire of trophic and immunomodulatory factors, known as the secretome [14]. Recent preclinical studies have reported that the secretomes from various stem cells have considerable potential for treating such intractable diseases as acute myocardial infarction [15], fulminant hepatic failure [16], renal failure [17,18], ischemic stroke [19], experimental autoimmune encephalomyelitis [20], hypoxic brain injury [21], and lung injury [22]. However, little is known about the secretomic signatures of the various types of stem cells. Identifying the key factors of various secretomes and their functions in secretome-mediated repair will contribute to the development of new regenerative therapies that do not require cell transplants.

In this study, serum-free conditioned medium (CM) derived from human MSCs (MSC-CM) was locally administered into the distraction gap in a high-speed DO (H-DO) mouse model. MSC-CM promoted the recruitment of murine bone marrow stromal cells (mBMSCs) and of endothelial cells/endothelial progenitor cells (EC/EPCs), and the establishment of a neoangiogenic network. These tissue-regenerative activities accelerated neocallus formation in the DO gap.

Materials and methods

Cell culture and CM preparation

Human bone marrow MSCs and human skin fibroblasts (FBs) were cultured as recommended by the suppliers, Lonza Walkersville and Health Science Research Resources Bank Japan, respectively. Briefly, cells were cultured in DMEM (Dulbecco's modified Eagle's medium,

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Sigma) containing 10% fetal bovine serum (FBS) (Lonza Walkersville) at 37 °C in a 5% CO_2 atmosphere and were subcultured every 6–7 days. When the cells reached 70–80% confluence, they were placed in serum-free DMEM and incubated for 48 h at 37 °C in 5% CO_2, after which the CM was collected and centrifuged for 4–5 min at 4 °C, 22,140 *g*. After brief re-centrifugation, the supernatant was collected and used as CM.

Mouse DO model

All animal experiments were performed in accordance with the Guidelines for Animal Experimentation of the Nagoya University School of Medicine. We used 8- to 10-week-old female ICR mice (Chubu Kagaku Shizai Corporation). The mouse DO model was generated and modified as previously described [23–25]. In this model, the external fixator consisted of two incomplete acrylic resin rings (outer diameter 20 mm, inner diameter 10 mm, and thickness 5 mm) and an expansion screw (600-301-30, Ortho Dentaurum). The total weight of the device, including the needles inserted into the tibia, was 2.7 g. The animals were anesthetized with an intraperitoneal injection of pentobarbital at 40 mg/kg body weight. The right limb was shaved and prepared with iodine solution. An anterior longitudinal incision was made on the right leg and the underlying muscles were bluntly separated, taking care not to remove all of the periosteum. A fibulotomy was performed with scissors. The tibia was attached to the fixator using one pair of 25-gauge needles proximally, one pair of 27-gauge needles distally, and acrylic resin. After the resin was completely polymerized (approximately 5 min), an osteotomy was performed at the middle of the diaphysis using very small cutting forceps, and the wound was closed with a 5–0 nylon suture. Distraction began after a 3 day latency period and continued for 8 days at a rate of 0.2 mm/12 h, increasing the tibia length by 3.2 mm. The model with this 8-day distraction time is referred to as the control (C-DO) model in the rest of the paper. The mice were sacrificed 15 days after surgery.

Mouse high-speed DO model (H-DO) with cell transplantation or CM treatment

To determine the effect of administering MSCs or MSC-CM during DO, we created a mouse H-DO model with a distraction rate of 0.4 mm/12 h, a length increase of 3.2 mm in 4 days. In the cell-transplanted group, 3×10^5 MSCs or FBs in 20 μl phosphate-buffered saline (PBS; Sigma) were transplanted into the distraction zone on day 5. The mice were sacrificed 5, 7, or 11 days after surgery. In the CM-treated groups, 20 μl collagen gel matrix (Cellmatrix Type I-A; Nitta Gelatin) containing 20 μl serum-free DMEM (control) or FB-CM or MSC-CM was injected transcutaneously into the center of the

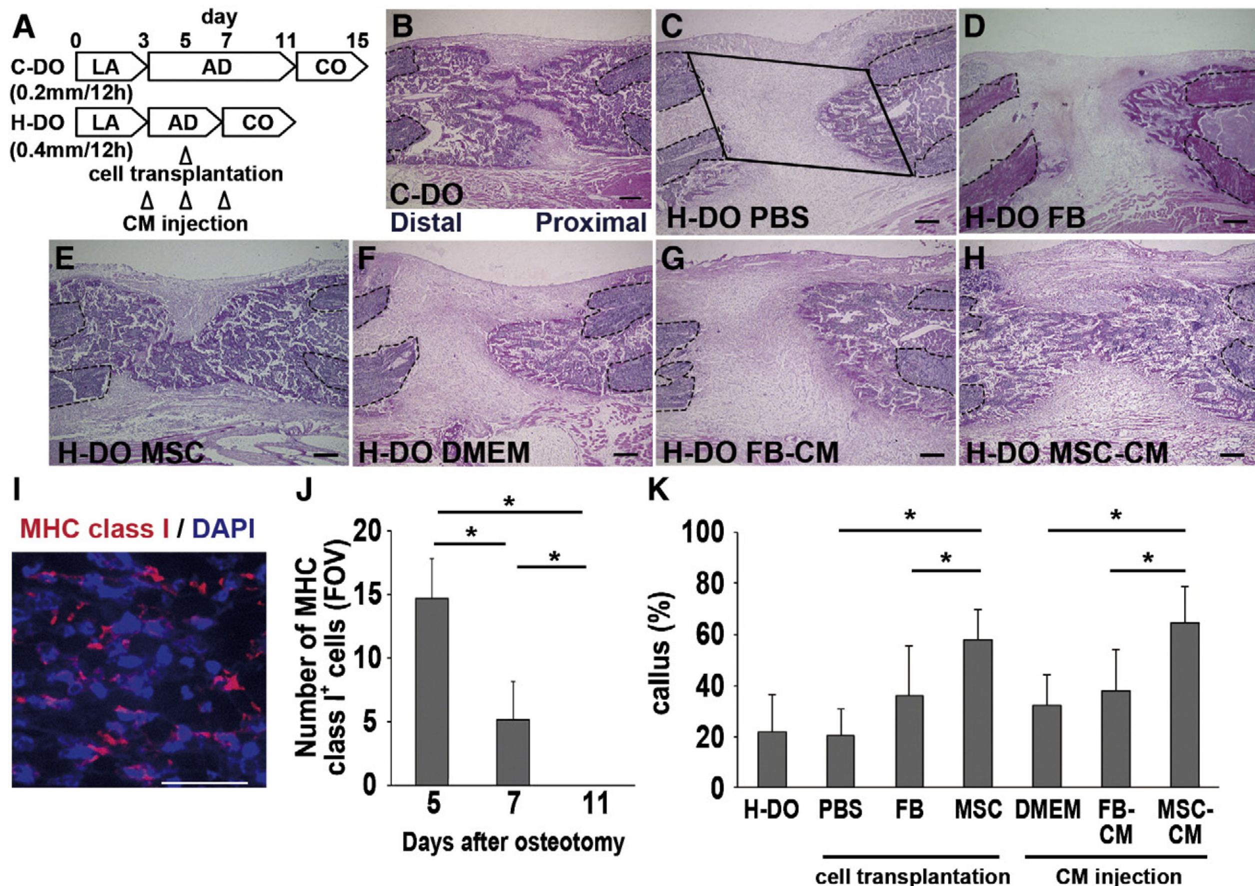

Fig. 1. MSCs accelerate callus formation through paracrine mechanisms. (A) Distraction protocols and experimental design. After a 3-day latency period, distraction was conducted over a period of 8 days (C-DO) at 0.2 mm/12 h, or a period of 4 (H-DO) days at a rate of 4 mm/12 h (H-DO), for a total length increase of 3.2 mm. Time points: white arrowheads, MSC transplantation or CM injections. LA: latency period; AD: active distraction period; CO: consolidation period. (B–H) Representative micrographs of hematoxylin–eosin (HE)-stained DO-gap sections, shown distal (left) to proximal (right); bar = 300 μm. (C) Schematic of the distraction zone. At the end of the consolidation period, neocallus had formed in the C-DO (B) but not in H-DO (C) gaps. Transplanted MSCs (E) but not FBs (D) promoted callus formation in the H-DO gap. Locally injected MSC-CM (H) but not DMEM (F) or FB-CM (G) promoted callus formation in the H-DO gap. (I) Immunohistochemical analysis of H-DO gaps: sections were prepared immediately after engrafting MSCs (post-surgery day 5) and stained with specific antibodies against human MHC class I (red) and DAPI (blue). Bar = 50 μm. (J) Quantification of transplanted human MHC class I^+ MSCs in the H-DO gap over time: MSCs had completely disappeared from the gap by the end of the consolidation period. FOV: field of view. Data represent mean ± SD; $^*p < 0.05$; n = 5 per group. (K) Histomorphometric analysis of callus formation in the distraction zone at the end of the consolidation period. Note that the callus area was significantly larger in the MSC-CM groups than in the DMEM or FB-CM groups. Data represent mean ± SD; $^*p < 0.05$; n = 10 per group.

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