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Clumps of a mesenchymal stromal cell/extracellular matrix complex can be a novel tissue engineering therapy for bone regeneration

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Abstract

Background aims. The transplantation of mesenchymal stromal cells (MSCs) to damaged tissue has attracted attention in scientific and medical fields as an effective regenerative therapy. Nevertheless, additional studies are required to develop an MSC transplant method for bone regeneration because the use of an artificial scaffold restricts the number of transplanted cells and their function. Furthermore, regulating the degree of cell differentiation *in vitro* is desirable for a more effective regenerative therapy. To address these unresolved issues, with the use of a self-produced extracellular matrix (ECM), we developed clumps of an MSC/ECM complex (C-MSCs). *Methods.* MSCs isolated from rat femur were cultured in growth medium supplemented with 50 μg/mL of ascorbic acid for 7 days. To obtain C-MSCs, confluent cells were scratched with the use of a micropipette tip to roll up the cellular sheet, which consisted of ECM produced by the MSCs. The biological properties of C-MSCs were assessed *in vitro* and their bone regenerative activity was tested by use of a rat calvarial defect model. *Results.* Immunofluorescent confocal microscopic analysis revealed that type I collagen formed C-MSCs. *Osteopontin* messenger RNA expression and amount of calcium content were higher in C-MSCs cultured in osteo-inductive medium than those of untreated C-MSCs. The transplantation of osteogenic-differentiated C-MSCs led to rapid bone regeneration in the rat calvarial defect model. *Conclusions.* These results suggest that the use of C-MSCs refined by self-produced ECM, which contain no artificial scaffold and can be processed *in vitro*, may represent a novel tissue engineering therapy.

Key Words: *bone regeneration, complex mesenchymal stromal cells, extracellular matrix, mesenchymal stromal cells*

Introduction

Mesenchymal stromal cells (MSCs), which are self-renewing multipotent progenitor cells, have the potential to differentiate into osteoblasts, chondrocytes, adipocytes and other cell types. These cells have attracted attention as an effective bone regenerative therapy for many years [1,2]. Bone marrow–derived MSCs are the most commonly used stem cells for bone regeneration, both in experiments and in clinical practice [3]. The implantation of bone marrow–derived MSCs is known to promote bone formation at the sites of bone defects. Nevertheless, previously reported MSC transplantation procedures for bone defects are still unreliable, and obstacles have yet to be overcome.

One of the drawbacks of MSC transplantation therapy is the usage of an artificial scaffold. The ideal material for a scaffold generally exhibits good handleability and bio-compatibility and should also regulate cell proliferation and differentiation. Many studies have been conducted in attempts to develop such a scaffold [4–6], and have resulted in various scaffolds being approved for clinical use [7]. However, problems associated with biodegradability as well as inflammatory and immunological reactions are still being reported [8,9]. In addition, artificial scaffolds restrict the transplanted cell number or function, and their long-term side effects remain unclear. Therefore, a scaffold-free cell delivery system could be an excellent alternative that could avoid these problems.

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The second challenge in establishing an effective MSC transplantation therapy is adequately regulating the degree of cell differentiation *in vitro*. Difficulties are associated with regulating cell differentiation after implantation because the microenvironment surrounding the grafted tissue influences the function of the cells [10,11]. In large bone defects, periosteum and bone marrow, which provide signals to initiate the bone repair process [12,13], will be insufficient to stimulate the grafted cells to undergo osteogenic differentiation. The efficacy of exogenously applied osteo-inductive growth factors and their carrier biomaterials in resolving this problem was tested [14–16]; however, few definitive procedures for effective tissue engineering therapy have been described for bone regeneration. A suitable carrier for these growth factors has not yet been identified, which, in turn, results in a failure in delivering these cytokines or the maintenance of an effective dose and biological activity in the host tissue [9,17]. Subsequently, an ideal cell transplantation therapy should accomplish the cell processing *in vitro*.

To establish a promising regenerative therapy as described above, we generated clumps of an MSC/extracellular matrix (ECM) complex (C-MSCs). C-MSCs consist of cells and self-produced ECM, have good handleability, and can be transplanted into tissue lesions with no artificial scaffold. Moreover, they can be induced to undergo osteogenic differentiation *in vitro*. Accordingly, the aims of this study were to explore the biological function of C-MSCs *in vitro* and *in vivo* and to determine their bone regenerative efficacy in a rat calvarial defect model.

Methods

Rat MSCs

Male F344/DuCrlCrlj rats (Charles River Laboratories Japan) were used in this study after approval had been obtained from the Animal Care Committee of Hiroshima University. Rat MSCs were collected from the bone marrow of femurs taken from 3-week-old F344 rats. Cells from the bone marrow were suspended in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Biowest), 100 U/mL of penicillin (Sigma), 100 μg/mL of streptomycin (Sigma) and 500 ng/mL amphotericin B (Invitrogen) (growth medium [GM]) were then seeded on 100-mm plastic dishes (Corning). Non-adherent cells were removed after 24 h, and the adherent cells were subsequently expanded. Cells at the third passage were used in experiments as rat MSCs.

Flow cytometric analysis for MSC markers

Rat MSCs were incubated with a mouse monoclonal anti−Stro-1 immunoglobulin (Ig)M antibody (Millipore; #STRO-1), mouse monoclonal anti-CD73 IgG antibody (BD; #5F/B9), rat monoclonal anti-CD105 IgG antibody (Santa Cruz; #MJ7/18), mouse monoclonal anti-p75NGFR IgG antibody (Abcam; #MLR2), mouse monoclonal anti-CD34 IgG antibody (Santa Cruz; #ICO115) and mouse monoclonal anti-CD45 IgG antibody (BD; #OX-1) for 30 min at room temperature. The cells were then incubated with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM antibody (Vector Laboratory), FITC-conjugated goat anti-mouse IgG antibody (Vector Laboratory), and FITC-conjugated goat anti-rat IgG antibody (Vector Laboratory) for 30 min at room temperature. The expression profile of each molecule was determined by means of fluorescence-activated cell sorting (FACScan flow cytometer [BD]) with the use of Cell Quest software (BD).

Preparation of C-MSCs

MSCs were seeded at a density of 7.0×10^4 cells/well into 24-well plates (Corning) and cultured with growth medium supplemented with 50 μg/mL of L-ascorbic acid (Sigma) for 7 days. To obtain C-MSCs, confluent cells that had formed on the cellular sheet, consisting of the ECM produced by MSCs, were scratched with the use of a micropipette tip and then were torn off. The MSC/ECM complex was then detached from the bottom of the plate in a sheet shape and rolled up to make round clumps of cells. After 1-day incubation, 0.9∼1.2-mm-diameter C-MSCs were obtained. These were transferred into a 24-well ultra-low-binding plate (Corning) and maintained in GM or osteo-inductive medium (OIM; GM supplemented with 10 nmol/L dexamethasone, 50 μg/mL L-ascorbic acid (Sigma) and 10 mmol/L β-glycerophosphate (Sigma) [18]) until the end of the culture period. Some C-MSCs were digested with the use of 3 mg/mL collagenase (Sigma), and the cell number was counted.

Histological and immunohistochemical analyses of C-MSCs

Cultured C-MSCs were fixed with 1% paraformaldehyde and embedded in paraffin. Five-micrometer-thick serial sections were prepared. The samples were then stained with hematoxylin and eosin and observed under a light microscope. Regarding immunohistochemistry, the endogenous