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Early osteoinductive human bone marrow mesenchymal stromal/stem cells support an enhanced hematopoietic cell expansion with altered chemotaxis- and adhesion-related gene expression profiles

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ABSTRACT

Bone marrow (BM) microenvironment has a crucial role in supporting hematopoiesis. Here, by using a microarray analysis, we demonstrate that human BM mesenchymal stromal/stem cells (MSCs) in an early osteoinductive stage (e-MSCs) are characterized by unique hematopoiesis-associated gene expression with an enhanced hematopoiesis-supportive ability. In comparison to BM-MSCs without osteoinductive treatment, gene expression in e-MSCs was significantly altered in terms of their cell adhesion- and chemotaxis-related profiles, as identified with Gene Ontology and Gene Set Enrichment Analysis. Noteworthy, expression of the hematopoiesis-associated molecules CXCL12 and vascular cell adhesion molecule 1 was remarkably decreased in e-MSCs. e-MSCs supported an enhanced expansion of $CD34^+$ hematopoietic stem and progenitor cells, and generation of myeloid lineage cells in vitro. In addition, short-term osteoinductive treatment favored in vivo hematopoietic recovery in lethally irradiated mice that underwent BM transplantation. e-MSCs exhibited the absence of decreased stemness-associated gene expression, increased osteogenesis-associated gene expression, and apparent mineralization, thus maintaining the ability to differentiate into adipogenic cells. Our findings demonstrate the unique biological characteristics of e-MSCs as hematopoiesis-regulatory stromal cells at differentiation stage between MSCs and osteoprogenitor cells and have significant implications in developing new strategy for using pharmacological osteoinductive treatment to support hematopoiesis in hematopoietic stem and progenitor cell transplantation.

1. Introduction

Human bone marrow (BM) mesenchymal/stromal stem cells (MSCs) are multipotent stromal cells that can differentiate into osteoblasts (OBs) and adipocytes [1], and have the ability to expand hematopoietic stem and progenitor cells (HSPCs) when co-cultured in vitro [2,3]. The ability of human BM-derived MSCs to support hematopoiesis has been validated in clinical trials in the setting of HSPC transplantation (HSCT) [4,5]. Therefore, further research into the potential of BM-MSCs in hematopoiesis would lead to an improved outcome of HSCT.

By the use of genetic mouse models, stromal cells that show similar characteristics to MSCs were demonstrated to be crucial for

Abbreviations: BM, bone marrow; CC, C-C motif; CXC, C-X-C motif; e-MSC, early osteoinductive human bone marrow mesenchymal stromal/stem cell; Flt3-L, Flt3-ligand; G-CSF, granulocyte colony-stimulation factor; GO, gene ontology; GSEA, gene set enrichment analysis; Hb, hemoglobin; HSCT, hematopoietic stem and progenitor cell transplantation; HSPC, hematopoietic stem and progenitor cell; IL, interleukin; MNC, mononuclear cell; MSC, mesenchymal stromal/stem cell; OB, osteoblast; OICS, osteogenesis-inducing cocktails; PB, peripheral blood; qRT-PCR, quantitative reverse transcription PCR; SCF, stem cell factor; TPO, thrombopoietin; VCAM1, vascular cell adhesion molecule 1; VLA4, very late antigen 4; WBC, white blood cell.

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physiological hematopoiesis in BM microenvironments [6–10]. In addition, the pathological hematopoiesis of myelodysplasia results from a primary genetic abnormality in osteoprogenitor cells [11]. These findings imply that mouse osteoprogenitor cells are not simply in an intermediary differentiation stage between MSCs and OBs, but in a crucial functional stage for regulating hematopoiesis. However, the characteristics of such cells in humans are unknown.

The purpose of this study was to examine the hematopoiesis-supportive potential of early osteoinductive human BM-MSCs (e-MSCs) and to analyze the detailed gene expression profiles of these cells using microarray analysis.

2. Material and methods

2.1. Culture and osteogenic differentiation of human BM-MSCs

Normal human BM samples that were obtained from healthy adult volunteers with informed consent were purchased from All-Cells (Emeryville, CA). Human BM-MSCs were isolated and cultured based on a previously published method [3,12–15]. In brief, a single-cell suspension of 1×10^6 BM mononuclear cells (MNCs) was seeded into a 15 cm culture dish. The primary culture of adherent cells was passaged to disperse the colony-forming cells (passage 1), and cells at passage 1–3 were used as BM-MSCs. Prior to experiments, the surface antigen profile of CD11b, CD19, CD34, CD45, CD73, CD90, and CD105 was examined by flow cytometric analysis to confirm that these cells expressed MSC markers, but did not express hematopoietic cell markers [16]. To induce osteogenic differentiation of BM-MSCs, osteogenesis-inducing cocktails (OICS) of 100 μM ascorbic acid (Wako Chemicals Industries, Osaka, Japan), 1.8 mM potassium dihydrogen phosphate, and 100 nM dexamethasone (both from Sigma–Aldrich, St. Louis, MO) were added to the culture media (osteoinductive medium). Mineralization was evaluated by 1% Alizarin Red S staining. The study protocol was approved by the ethics committee of Kyoto University Hospital (#995).

2.2. Co-culture of human HSPCs and BM-MSCs

Human HSPCs were isolated from BM-MNCs using anti-CD34 immunomagnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and the purity was confirmed by flow cytometric analysis using an antibody against human CD34. BM-MSCs (2×10^4 cells/well) were seeded in a 24-well culture plate. In some experiments, BM-MSCs were treated with OICS for osteoinduction prior to co-culture. HSPCs (0.6×10^3 cells/well) were then applied and the cells were co-cultured in StemSpan Serum Free Expansion Medium (STEMCELL Technologies, Vancouver, Canada) supplemented with 100 ng/mL stem cell factor (SCF), 100 ng/mL Flt3-ligand (Flt3-L), 20 ng/mL interleukin (IL)-3 (all from Wako Chemicals Industries), and 50 ng/mL thrombopoietin (TPO) (Kyowa Hakko Kirin, Tokyo, Japan). After 10 days of co-culture, the number and surface marker expression of the expanded hematopoietic cells were examined by flow cytometric analysis. The antibodies used are listed in Supplementary Table 1.

2.3. Microarray analysis

Total RNA (1 μg/sample) from control unstimulated BM-MSCs (ctrl-MSCs) or BM-MSCs that were cultured in the osteoinductive medium for 2 days (e2-MSCs) or 5 days (e5-MSCs) was amplified using the Ambion Amino Allyl aRNA Kit (Ambion, Carlsbad, CA). Each sample of amplified RNA was labeled with Cy5, and labeled samples were co-hybridized with the 3D-Gene Human Oligo Chip 25k (Toray Industries, Tokyo, Japan) at 37 °C for 16 h. After washing the DNA chip, hybridization signals were scanned using a 3D-Gene Scanner 3000 (Toray Industries). All analyzed data were scaled by global normalization. Microarray data were analyzed using GeneSpring GX software (Tomy Digital Biology, Tokyo, Japan). All raw data were normalized and filtered based on the signal intensity values (20–100th percentile range). Hierarchical clustering and Gene ontology (GO) analysis was performed using GeneSpring GX software. Gene set enrichment analysis (GSEA) was performed using GSEA v2.0.14 software (http://www.broadinstitute.org/gsea/index.jsp). The gene set "Cell_Cell_Adhesion" was downloaded from the Molecular Signature Database (MSigDB; http://www.broadinstitute.org/gsea/msigdb/index.jsp). The genes included are listed in Supplementary Table 2. The analysis parameters were as follows: the number of permutations was 1,000, the permutation type was set to gene set, the gene set size filters were set to min = 15 and max = 500, and the metric for ranking genes was Diff_of_Classes. The complete microarray data are available in the NCBI Gene Expression Omnibus (GEO). The microarray accession number is 74837.

2.4. BM transplantation

Specific pathogen-free 6–8-week-old female C57BL/6 mice were purchased from CLEA Japan (Tokyo, Japan). BM nucleated cells (5×10^5/mouse) from C57BL/6 mice were transplanted into lethally irradiated (9 Gy) recipient C57BL/6 mice. OICS (dissolved in PBS) or PBS (control) was administered intraperitoneally to recipient mice on days 1–7 after BM transplantation. The survival of mice was observed each day until day 35 after BM transplantation. Peripheral blood (PB) was collected on days 0, 4, 7, 10, 13, 17, 21, 25, 28, and 35 after BM transplantation (n = 6) from the tail veins, and the number of white blood cells (WBCs) and the hemoglobin (Hb) levels were analyzed using an automated blood cell counter (Celltac α, Nihon Kohden, Tokyo, Japan). These studies were approved by the committee for animal research of the Kyoto University Graduate School of Medicine.

2.5. Statistical analysis

The unpaired Student's *t*-test was used for analysis, unless otherwise indicated. Data in bar graphs indicate the mean ± SD, and statistical significance is expressed as follows: *, $P < 0.05$; **, $P < 0.01$.

3. Results

3.1. Characteristics of early osteoinductive human BM-MSCs (e-MSCs)

First, we cultured human BM-MSCs in osteoinductive medium and examined their mineralization by Alizarin Red S staining. Mineralization was observed following 10 days of culture (Fig. 1A). Mineralization was not observed in BM-MSCs that were cultured in osteoinductive medium for just 2 or 5 days (Fig. 1A). When these early osteoinductive MSCs (e-MSCs) were cultured in adipogenesis-inducing medium for more than 2 weeks, significant fat deposition was observed (Fig. S1A–B), indicating their adipogenic differentiation ability. Thus, BM-MSCs cultured in osteoinductive medium for just 2 days (e2-MSCs) or 5 days (e5-MSCs) were considered to be immature cells that were in a differentiation stage between MSCs and osteoprogenitor cells. This was further supported by the observations that e2-MSCs and e5-MSCs did not show decreased expression of stemness-associated markers (Fig. S1C) or increased expression of osteogenesis-associated markers such as SP7 (also known as osterix), BGLAP (also known as osteocalcin), and SPP1

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