

Original Article

Vitamin D analog EB1089 could repair the defective bone marrow-derived mesenchymal stromal cells in patients with systemic lupus erythematosus

Jing-Jing Xu¹, Yan-Bin Sun², Xiao-Li Zhang¹, Xiao-Fei Wang¹

¹Department of Rheumatology, Shengjing Hospital of China Medical University, Sanhao Street 36, Heping District, Shenyang City 110004, Liaoning Province, P. R. China; ²Department of Thoracic Surgery, First Hospital of China Medical University, Nanjing North Street 155, Heping District, Shenyang City 110001, Liaoning Province, P. R. China

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Abstract: Systemic lupus erythematosus (SLE) involves multiple factors, which result in the breakdown of self-tolerance and development of autoimmunity with organ damage. Bone marrow mesenchymal stem cells (BMMSCs) from the patients with SLE showed an impaired proliferative capacity compared with that from normal controls. In this study, we isolated BMMSCs from the patients with SLE and found that Vitamin D analog EB1089 could induce BMMSCs proliferation and mineralization deposition. Furthermore, we found that the expression of p-Smad 1/5/8 was promoted in BMMSCs with EB1089 treatment. In conclusion, our results support the notion that EB1089 promoted proliferation and osteogenic differentiation of BMMSCs by Smad 1/5/8 signaling pathway.

Keywords: Systemic lupus erythematosus, bone marrow mesenchymal stem cells, EB1089, proliferation, Smad signaling pathway

Introduction

Adult mesenchymal stem cells (MSCs) were first discovered by Friedenstein and associates, who identified an adherent, fibroblast-like population that could regenerate rudiments of normal bone *in vivo* [1]. The multilineage potential of MSCs plays an important role in wound healing and tissue regeneration through differentiation and the release of important growth factors and cytokines [2]. Bone marrow-derived mesenchymal stem cells (BM-derived MSCs) have therapeutic potential in a wide range of diseases [3, 4]. Previous studies suggest that autoimmune diseases may be described as a stem cell disorder. For example, Perez-Simon et al. [5] reported that the BMMSCs from chronic primary immune thrombocytopenia (ITP) patients showed an impaired proliferative capacity compared with that from normal controls. MSCs derived bone marrow in systemic lupus erythematosus (SLE) showed evidence of growth retardation *in vitro* [6].

1,25(OH)₂VD₃ has been found to induce a multiple-step differentiation of promyelocytes into

mature osteoclasts [7], to suppress parathyroid hormone expression and parathyroid cell growth [8], and to inhibit the growth and stimulate differentiation of keratinocytes [9]. EB1089 is an analogue of VD₃ where the side chain has been altered by the addition of two double bonds [10]. This analog has been shown to be 50-200 times more potent than VD₃ in anti-proliferative and differentiating activities on cancer cells [11, 12].

However, to our knowledge, no studies showed the effects of EB1089 on the defective bone marrow-derived mesenchymal stem cells. So in this paper, we studied the biological character changes of BM-derived MSCs from SLE patients after EB1089 treatment. The objective was to explore the role EB1089 in repairing defective BM-derived MSCs.

Materials and methods

Patients

Bone marrow samples were obtained from 19 patients with SLE. Written informed consent

The effects of EB1089 on the defective BMMSCs

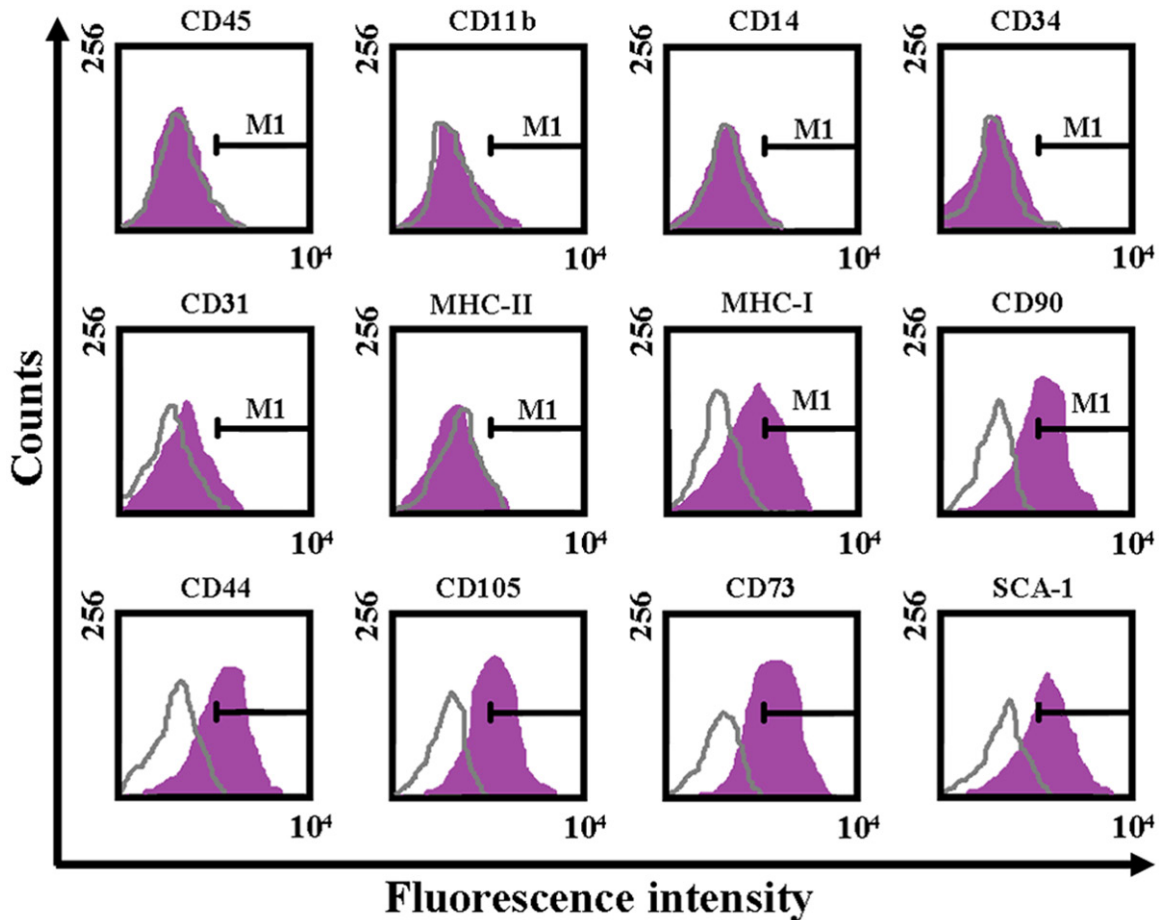


Figure 1. Immunophenotypic characterization of BMMSCs by flow cytometry (fluorescence-activated cell sorting).

was obtained from all the participants. This study was approved by the ethics committee of China Medical University.

Isolation of MSCs from bone marrow and cell culture

Bone marrow mononuclear cells from patients were isolated by Ficoll gradient and cultured at an initial density of 5×10^4 cells/cm² in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12; Gibco, Carlsbad, CA, USA), supplemented with 10% fetal calf serum (Gibco), 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, 10 ng/ml epidermal growth factor (EGF; PeproTech, Rocky Hill, NJ, USA), 2 ng/ml basic-fibroblast growth factor (bFGF; PeproTech), $1 \times$ insulin-transferrin-selenium (ITS; Gibco). The cultures were maintained at 37°C in a 5% CO₂ incubator, and the medium was changed after 48 hours and then every three days.

Phenotype assay

Cells from the patients with SLE were washed twice using PBS, stained for 30 min at 4°C using fluorochrome labeled antibodies against CD3, CD11b, CD14, CD19, CD31, CD34, CD105, CD106, CD133, CD25, CD44, CD45, CD73, CD80, CD86, CD90, Flk-1, c-Kit, Sca-1, MHC class I and MHC class II or with fluorochrome-matched control antibodies (Becton Dickinson, San Diego, CA, USA).

Chemicals

EB1089 (No. 3993/1) was purchased from R&D systems China (Shanghai, China) and supplied as a solution diluted in isopropanol at a concentration of 4×10^3 μM. As the methods of Wang et al. [13], dilutions were performed in absolute ethanol to obtain stock solutions of 100 μM. The aliquots of stock solutions were stored at -20°C and protected from light.

The effects of EB1089 on the defective BMSCs

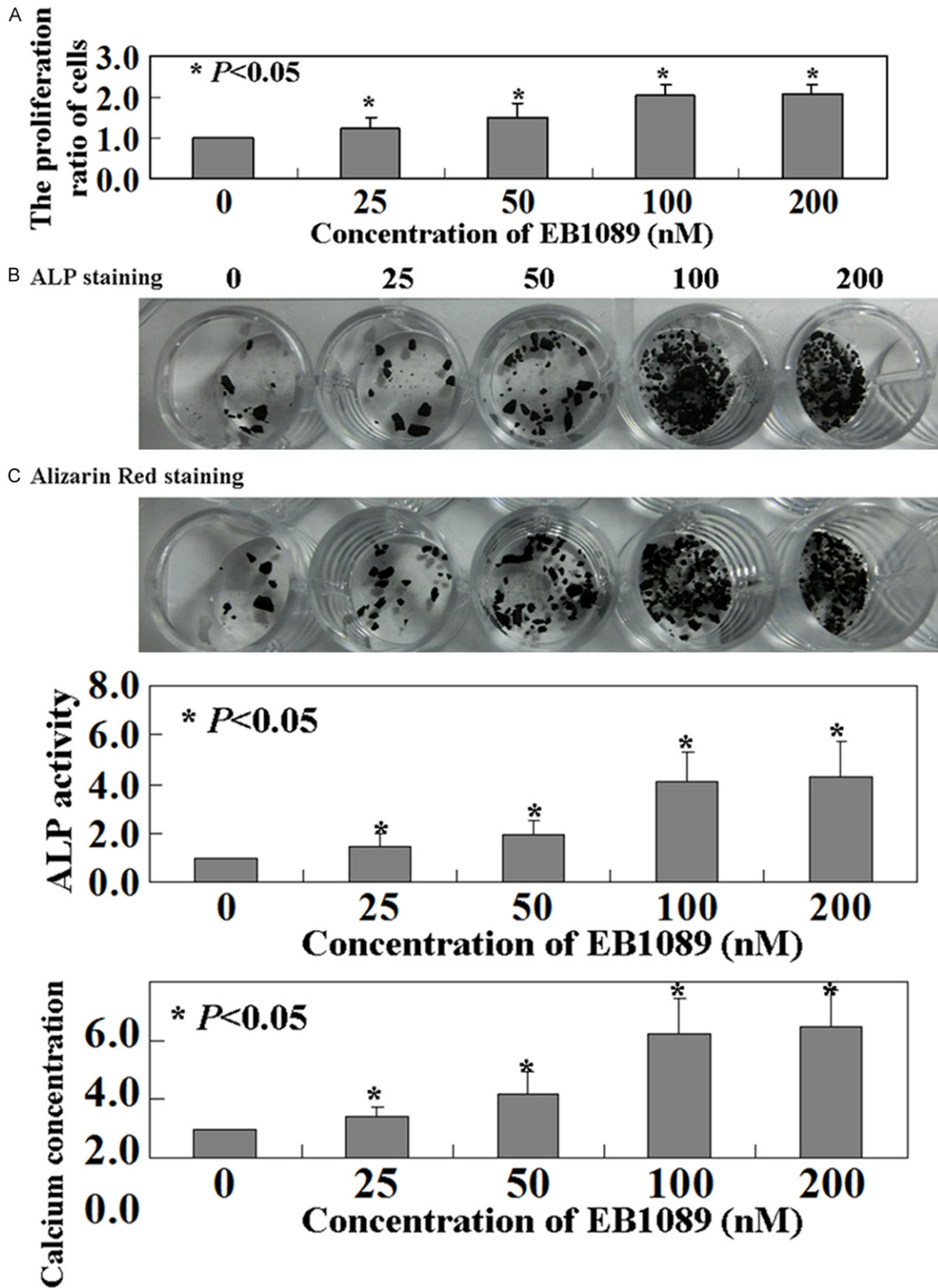


Figure 2. Proliferation of BMSCs was regulated by EB1089. A. Cell proliferation was assessed by MTT assay. B. ALP activity and C. mineralization were measured by ALP staining and Alizarin Red staining, respectively.

The effects of EB1089 on the defective BMMSCs

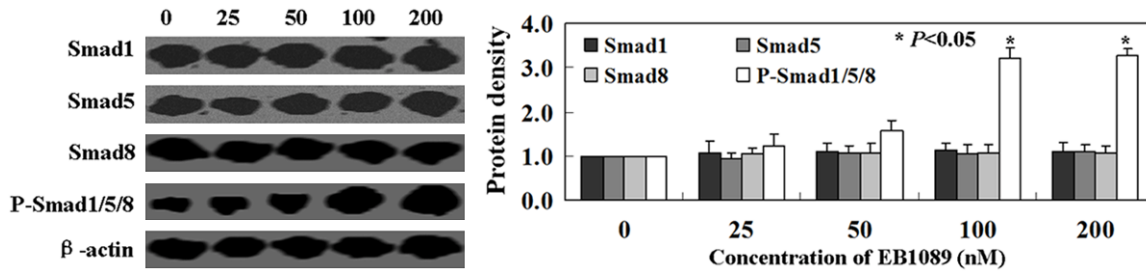


Figure 3. The mechanism of EB1089-induced proliferation in BMMSCs. Western blot analysis was performed using Smad 1, Smad 5, Smad 8, and p-Smad 1/5/8. β -actin was used as an internal control.

Cellular proliferation MTT assay

Cellular proliferation was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells (2×10^3 per well) were plated in 96-well microtiter plates and allowed to adhere. After 24 h, cells were treated with various concentrations of EB1089 (e.g. 0, 25, 50, 100, 200 nM for each). After 48 h, MTT was added to each well at a final concentration of 500 μ g/ml. The mixture was further incubated for 1 hour at 37°C, and the liquid in the wells was removed. Four hours later, cells were lysed with dimethyl sulfoxide (DMSO) and absorbance rates were measured at 550-560 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

In vitro mineralization assay

Cells were seeded in 6-well plates in triplicate at the density of 3×10^3 /cm². Alizarin Red S staining, which detects calcium deposition, was used as an indicator of mineralization. The cells were rinsed in PBS, and fixed in 70% ice-cold ethanol prior to staining with 40 mM Alizarin Red S (pH = 4.2, Sigma-Aldrich, Carlsbad, CA, USA) for 10 min at room temperature. Calcium content was quantified by measuring the amount of Alizarin Red S staining, which was bound to the mineralizing nodules. Alkaline phosphatase (ALP) staining was performed as previously described [14].

Western blot

Cells were washed once with phosphate-buffered saline, lysed for 30 min in lysis buffer (50 mM Tris-HCl, pH = 7.5, 150 mM NaCl, 1% Nonidet P-40) containing protease inhibitors (Cocktail; Roche, Basel, Switzerland) and phosphatase inhibitors (1 mM NaF and 1 mM

Na₃VO₄), and centrifuged at 15,000 \times g at 4°C for 15 min. Proteins were resolved by 10% SDS-PAGE, transferred to a nitrocellulose membrane, and detected using the following antibodies: anti-Smad 1 (Sigma), anti-Smad 5, anti-Smad 8, anti-phospho-Smad 1/5/8 (pSer⁴⁶³/Ser⁴⁶⁵). Immunostaining was detected using an enhanced chemiluminescence (ECL) system (Amersham Biosciences; Westborough, MA, USA).

Statistical analysis

Values are presented as mean and standard deviation (SD) for these experiments (mean \pm SD). Statistical significance was calculated with a Mann-Whitney-Wilcoxon statistical test (GraphPad 5.0). Statistical significance was set to a *P*-value less than 0.05. Each experiment was repeated three times.

Results

Bone marrow-derived MSC populations (BMMSCs)

Plastic adherent bone marrow stromal cells were isolated from pooled bone marrow from the patients with SLE, propagated for 1 week, hematopoietic cell depleted, and expanded further *in vitro* for 2 weeks. Flow cytometry analysis indicated that the majority of cells expressed the surface markers CD44, CD73, CD90, CD105, and MHC class I and didn't express CD45, CD11b, CD14, CD34, MHC class II and CD31 (Figure 1).

The proliferation of BMMSCs was regulated by EB1089

Compared to a control group with no EB1089, we found that increasing EB1089 in fixed incre-

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ments (25-50-100-200 nM) significantly increased the proliferation rate of BMMSCs ($P < 0.05$, **Figure 2A**). ALP activities rates increased from 1.5 ± 0.35 times to 4.4 ± 0.27 times in BMMSCs with EB1089 treatment respect to the control ones ($P < 0.05$, **Figure 2B**). After osteogenic induction of BMMSCs with EB1089 treatment for 3 weeks, mineralization nodules were observed with Alizarin Red S staining ($P < 0.05$, **Figure 2C**).

EB1089 improved BMMSCs viability via the Smad 1/5/8 pathway

To determine the effect of EB1089 on Smad 1/5/8 signaling, we performed Western blot analysis on BMMSCs. As shown by Western blot ($P < 0.05$, **Figure 3**), the phosphorylation of Smad1/5/8 was activated by EB1089 treatment in BMMSCs, while the total levels of Smad 1/5/8 showed no changes ($P > 0.05$, **Figure 3**).

Discussion

SLE involves multiple factors, which result in the breakdown of self-tolerance and development of autoimmunity with organ damage [15]. Sun et al. [6] found that bone marrow derived MSCs in patient with SLE are defective and may thus play an important role in the disease. More and more evidence showed the inhibitory effects of EB1089 on many cancer cells, such as gastric cancer [13], hepatocellular cancer [16], and prostate cancer [17]. van den Bemd et al. [18] found that both $1,25\text{-(OH)}_2\text{VD}_3$ and the analogs stimulated the growth of rat osteoblast-like cells (ROS 17/2.8). In this study, we isolated BMMSCs from the patients with SLE and found that Vitamin D analog EB1089 could induce BMMSCs proliferation and mineralization deposition.

The Smad signaling pathway is critical for mediating TGF- β superfamily signals from the cell surface to the nucleus [19]. P-Smad1/5/8 proteins translocate to the nucleus, bind to target genes, and regulate the transcription [20]. The level of Smad 1/5/8 activity was quantified in this study to explore the mechanism through which EB1089 induced-proliferation in BMMSCs. Our results showed that the expression of p-Smad 1/5/8 was promoted in BMMSCs with EB1089 treatment.

In conclusion, our results support the notion that EB1089 promoted proliferation and osteo-

genic differentiation of BMMSCs by Smad1/5/8 signaling pathway.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xiao-Fei Wang, Department of Rheumatology, Shengjing Hospital of China Medical University, 36 Sanhao Street, Heping District, Shenyang City 110004, Liaoning Province, P. R. China. E-mail: wangxf_sjhospital@126.com

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