Original Article

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Effect of CXCR4 inhibitor AMD3100 on alkaline phosphatase activity and mineralization in osteoblastic MC3T3-E1 cells

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Summary **The aim of the study was to investigate the effect of C-X-C chemokine receptor type 4 (CXCR4) inhibitor AMD3100 on the osteogenic differentiation of pre-osteoblastic cell line MC3T3-E1. In this study we found that blocking SDF-1/CXCR4 signaling with AMD3100 strongly suppressed osteogenic differentiation in MC3T3-E1 cells, as evidenced by an early decrease in the activity of alkaline phosphatase (ALP), and down-regulation of mRNA expression of the osteogenic master regulator Runx2, ALP, osteocalcin, and progressive ankylosis genes. Moreover, we found that the regulatory effect of AMD3100 might be mediated *via* intracellular STAT3 activation. However, AMD3100 exerted no significant effect on generation of matrix mineralization at the terminal stage of osteogenic induction. In conclusion, our results demonstrated an inhibitory role of AMD3100 in osteogenic differentiation of MC3T3-E1 cells, especially in the early stage, which provides novel insights into the effect of CXCR4 antagonists on modulation of osteogenesis.**

Keywords: SDF-1/CXCR4 signaling, AMD3100, MC3T3-E1, osteogenic differentiation, signal transducer and activator of transcription 3

1. Introduction

Stromal-derived factor 1 (SDF-1, also known as CXCL12) was initially identified as a bone marrow stromal cell-derived factor, which specially binds to a G-protein-coupled receptor, C-X-C chemokine receptor type 4 (CXCR4) (*1,2*). The SDF-1/CXCR4 signaling pathway has been found to be important for the process of cellular inflammatory, immune response, blood homeostasis *et al.* (*3,4*). AMD3100 (plerixafor), a CXCR4 antagonist, which can inhibit binding of SDF-1 to CXCR4 and subsequent signal transduction, has been used as an effective hematopoietic stem cell mobilization agent in patients with non-Hodgkin's lymphoma (NHL) and multiple myeloma (MM) (*5-8*). It has been proposed that AMD3100 may also play an important role in treatment of many other SDF-1/CXCR4-regulated pathological processes such as cancer, human immunodeficiency virus infection, rheumatoid arthritis, atherosclerosis, and asthma (*9-13*).

Recent studies have suggested the SDF-1/CXCR4 pathway is involved in bone remodeling and osteoblast differentiation (*14-16*). Kitaori *et al.* (*17*) found that SDF-1/CXCR4 signaling has a critical role in recruitment of mesenchymal stem cells (MSC) to the fracture site during skeletal repair *in vivo*. Zhu *et al.* (*15*) and Hosogane *et al.* (*16*) showed that SDF-1/CXCR4 participated in the process of bone morphogenetic protein 2 (BMP-2)-induced osteogenic differentiation of primary MSCs or MSC cells lines *in vivo*. Therefore, special attention has been given to learn about the efficacy or toxicity of AMD3100 on osteogenesis.

Until now, only a few studies have demonstrated the effect of AMD3100 on osteogenesis of MSC at the early stage. The modulation of AMD3100 on differentiation of preosteoblasts, especially on mineralization has not been investigated. Therefore, in this study, we utilized the murine pre-osteoblastic cell line MC3T3-E1 to observe the effect of CXCR4 inhibitor AMD3100 on

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activity of alkaline phosphatase (ALP; an early marker of osteogenesis), expression of osteogenic factors, and mineralization (*18*). Furthermore, the possible mechanism of AMD3100 on ALP regulation by the transcription factor – signal transducer and activator of transcription 3 (STAT3) was also investigated.

2. Materials and Methods

2.1. *Chemicals and antibodies*

Dexamethasone, L-ascorbic acid, β-glycerophosphate, and the CXCR4 antagonist AMD3100 were all obtained from Sigma (St. Louis, MO, USA). All cell culture media and supplements were from Gibco (Carlsbad, CA, USA). Reagents for reverse transcription and those for real-time PCR reactions were from Toyobo (Shanghai, China). Anti-STAT3 and anti-phospho-STAT3 (Tyr705) rabbit monoclonal antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Secondary goat anti-rabbit IgG was obtained from Santa Cruz (Santa Cruz, CA, USA). Enhanced chemiluminescence (ECL) detection reagent was purchased from Millipore (Billerica, MA, USA).

2.2. *Cells and osteogenic induction*

MC3T3-E1 cells were acquired from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). For osteogenic induction, MC3T3-E1 cells were plated at a density of 1×10^4 cells/cm^2 in 24-well plates and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C under 5% (v/v) CO_2 in a humidified atmosphere. Osteoblast differentiation was induced by addition of 10 nM dexamethasone, 50 μg/mL L-ascorbic acid and 10 mM β-glycerophosphate as described previously (*19*). To block SDF-1/CXCR4 signaling in MC3T3-E1 cells, cells were incubated with the CXCR4 antagonist AMD3100 respectively at concentrations of 50, 100, 200, and 400 μM.

2.3. *ALP activity*

ALP activity was measured at 3, 6, and 9 days of osteogenic induction as described previously with minor modifications (*20*). Briefly, MC3T3-E1 cells were washed with one volume of phosphate-buffered saline (PBS) (pH 7.4) and lysed with addition of 100 μL/well of 25 mM Tris-HCl (pH 7.4) and 0.5% Triton X-100. Fifty microliter of cell lysate was incubated with 100 μL *p*-nitrophenyl phosphate at 37°C for 20 min. The reaction was stopped by addition of 50 μL NaOH (3 M) and absorbance was measured at 405 nm.

2.4. *Mineralization analysis*

Mineralization analysis was performed at 14 and 21 days of osteogenic induction as described previously (*21*). MC3T3-E1 cells were washed with PBS and fixed with 4% paraformaldehyde. Then the cells were washed with PBS and stained with 0.5% (w/v) alizarin red S solution for 1 h. After washing with PBS, the stained cultures were photographed. Then the cells were incubated with 10% (w/v) cetylpyridium chloride at 37°C for 1 h, optical density of the supernatant was measured at 562 nm.

2.5. *Quantitative real-time polymerase chain reaction (RT-qPCR)*

RT-qPCR was used to measure expression of osteogenic markers including ALP, progressive ankylosis (Ank), runt-related gene 2 (Runx2), and osteocalcin (OCN) genes at 6 and 9 days of osteogenic induction. Total RNA was extracted from cells using Trizol reagent. One microgram of total RNA from each sample was reverse-transcribed, and levels of target gene expression were quantified in real-time PCR detection system with a LightCycler 480 thermocycler (Roche Applied Science, Mannheim, Germany). Relative expression of target genes was calculated based on ΔCT values, which are differences in the number of threshold cycles between the target gene and the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences of target genes that were analyzed in this study are listed in Table 1.

2.6. *Western-blot*

For Western-blot analysis, MC3T3-E1 cells at 3, 6, 9, and 14 days of osteogenic induction were collected, and then whole cell lysate was obtained and the amount of total cellular protein was determined using the Bradford assay (*15,22*). Equal loading of 30 μg aliquots of total protein from each sample were fractionated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the following

Table 1. Quantitative real-time PCR primer sequences

Target genes	Primer sequences
ALP	Forward: 5'-TGGCTCTGCCTTTATTCCCTAGT-3' Reverse: 5'-AAATAAGGTGCTTTGGGAATCTGT-3'
Ank	Forward: 5'-ATGAGTCAGCCACCGAG-3' Reverse: 5'-GGAGGAAAGAGACGACAGTT-3'
Runx2	Forward: 5'-GCCGGGAATGATGAGAACTA-3' Reverse: 5'-GGTGAAACTCTTGCCTCGTC-3'
OCN	Forward: 5'-GCCATCACCCTGTCTCCTAA-3' Reverse: 5'-GCTGTGGAGAAGACACACGA-3'
GAPDH	Forward: 5'-CATCCCAGAGCTGAACG-3' Reverse: 5'-CTGGTCCTCAGTGTAGCC-3'

antibodies were incubated: anti-STAT3 (1:2,000), anti-phospho-STAT3 (Tyr705) (1:2,000), and anti-β-actin (1:1,000). Primary antibodies were detected with goat anti-rabbit IgG conjugated to horseradish peroxidase. Blots were developed with enhanced chemiluminescence (ECL) and exposed to X-ray film.

2.7. *Statistics*

Measurements in each experiment were run in triplicate. For quantitative data, results are reported as the mean ± S.D. To determine the differences between groups, one-way analysis of variance (ANOVA) was carried out using SPSS software (version 17.0), with significance accepted at $p < 0.05$.

3. Results

3.1. *CXCR4 inhibitor AMD3100 suppressed ALP activity during the early stage of osteogenic differentiation in MC3T3-E1 cells*

As seen in Figure 1, with osteoblast induction, ALP activity increased and achieved a peak at 6 days, and decreased slightly at 9 days, which is consistent with the previous studies (*16*). When treated with CXCR4 antagonist AMD3100, ALP activity significantly decreased at 3, 6, and 9 days (48%, 49%, and 53% decreases at 50 μM AMD3100, respectively, and 29%, 56%, and 73% decreases at 100 μM AMD3100, respectively) (Figure 1). Furthermore, detection of ALP activity in MC3T3-E1 cells at 6 days also showed that treatment with CXCR4 antagonist AMD3100 decreased osteogenic media-induced ALP activity in a concentration-dependent manner (49%, 56%, 67%, and 77% decreases with 50, 100, 200, and 400 μM of AMD3100, respectively) (data not shown). Taken together, these data suggest that CXCR4 inhibitor AMD3100 significantly inhibited ALP activity during the early stage of osteogenic differentiation in MC3T3-E1 cells.

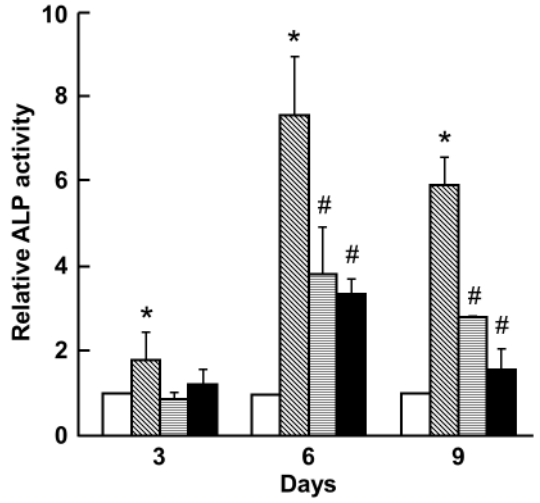

Figure 1. AMD3100 suppressed ALP activity during the early stage of osteogenic differentiation in MC3T3-E1 cells. MC3T3-E1 cells were treated with DMEM (open columns), osteogenic medium (shaded columns) and osteogenic medium supplemented with AMD3100 (horizontal and closed columns for 50 μM and 100 μM, respectively) and assessed for ALP activity at 3, 6, and 9 days (mean ± S.D., $n = 3$). * $p < 0.05$, *vs.* negative control group; # $p < 0.05$, *vs.* osteogenic induction group.

3.2. *CXCR4 inhibitor AMD3100 had no effect on matrix mineralization in MC3T3-E1 cells*

To evaluate calcium deposition in matrix, alizarin red staining was performed and quantified by a

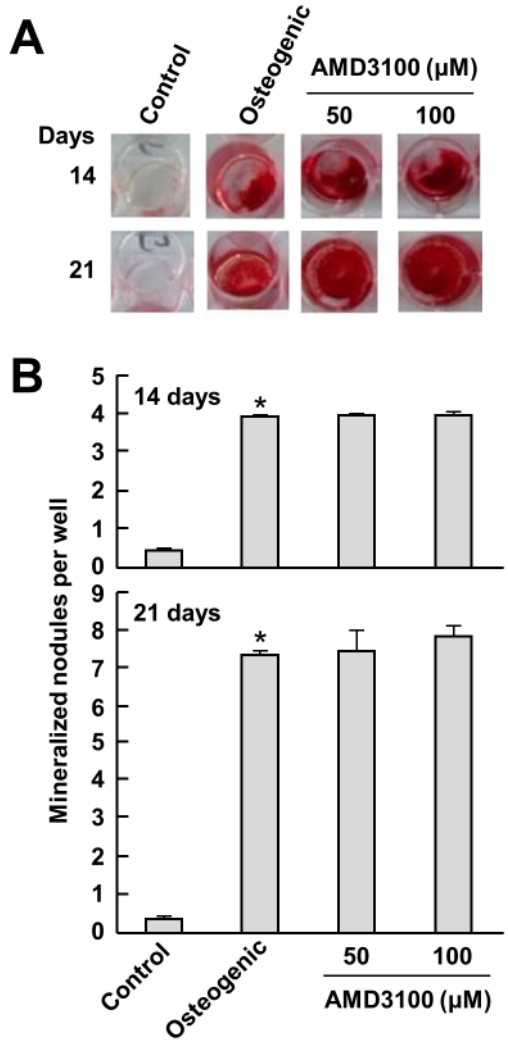

Figure 2. Effect of AMD3100 on matrix mineralization of MC3T3-E1 cells. (A) Typical observations for mineralization. Cells were cultured with DMEM, osteogenic medium and osteogenic medium supplemented with various concentrations of AMD3100 for 14 days (upper) and 21 days (lower). The resulting mineralization was assessed by Alzarin red S staining. **(B)** Quantitation of mineralization. Alzarin red S was solubilized in cell cultures of every group using cetylpyridinium chloride and quantified at 562 nm. Upper panel, 14 days; lower panel, 21 days. Data were mean ± S.D., $n = 3$. * $p < 0.05$, *vs.* negative control group.

colorimetric analysis based on solubilizing the red matrix precipitate with cetylpyridinium chloride at 14 and 21 days of osteoblast differentiation. Obvious matrix mineralization could be observed at 14 days of induction (upper panels in Figure 2A), and became extensive at 21 days as seen in Figure 2 (lower panels in Figure 2A). However, cetylpyridinium chloride analysis failed to detect minor increases or decreases in mineralization by treatment with AMD3100 at 50 and 100 μM (upper and lower panels in Figure 2B, respectively). These data suggest that CXCR4 inhibitor AMD3100 seemed to have no significant effect on generation of matrix mineralization at the terminal stage of osteogenic induction in MC3T3-E1 cells.

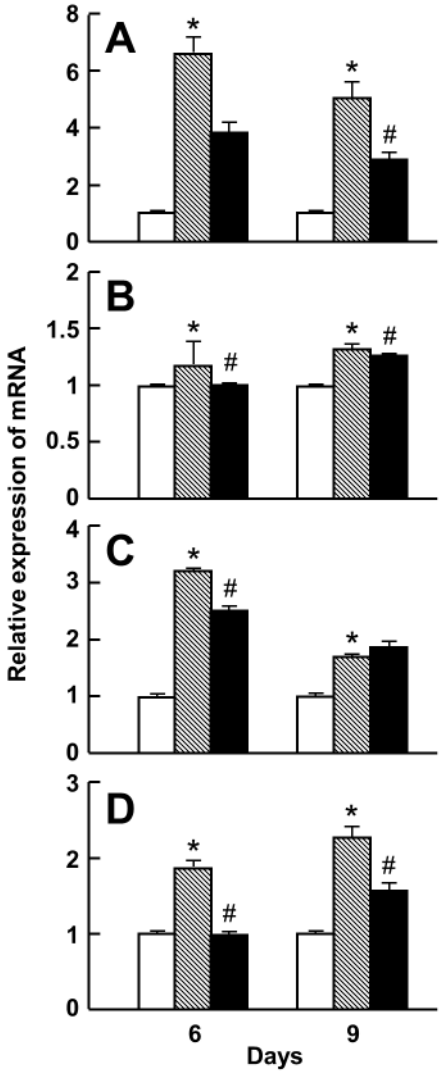

Figure 3. Effect of AMD3100 on osteoblast-specific marker genes expression in MC3T3-E1 cells. Cells were cultured with DMEM (open columns), osteogenic medium (shaded columns) and osteogenic medium supplemented with AMD3100 (400 μM) (closed columns) for 14 days, and then were harvested at 6 and 9 days during osteogenic differentiation for quantitative gene expression analysis of ALP **(A)**, Ank **(B)**, Runx2 **(C)**, and OCN **(D)**. Data were normalized to GAPDH expression and presented as fold difference relative to control cells cultured with DMEM (mean ± S.D., $n = 3$). * $p < 0.05$, *vs.* negative control group; # $p < 0.05$, *vs.* osteogenic induction group.

3.3. *Effect of CXCR4 inhibitor AMD3100 on osteoblast-specific marker genes expression in MC3T3-E1 cells*

The mRNA levels of four osteoblast-specific markers affected by CXCR4 inhibitor AMD3100 were determined by quantitative real time PCR analysis. As seen in Figure 3, compared with the osteogenic group, CXCR4 inhibitor AMD3100 (400 μM) suppressed the expression of both the early osteoblast differentiation markers (ALP and Runx2) and the late differentiation markers (Ank and OCN) at 6 and 9 days. Each difference reached a level of significance except for mRNA expression of Runx2 at 9 days.

3.4. *Effect of CXCR4 inhibitor AMD3100 on STAT3 in MC3T3-E1 cells*

To further understand mechanisms underlying the effect of AMD3100 on osteoblast differentiation, we examined the activation of STAT3 proteins in MC3T3-E1 cells treated with AMD3100 at 3 and 6 days of osteogenic induction. As seen in Figure 4, Western-blot results showed that osteogenic media stimulation increased the phosphorylation of STAT3 protein at 6 days compared with the control group, and treatment with CXCR4 antagonist AMD3100 almost abolished osteogenic media-induced STAT3 phosphorylation (Figure 4).

4. Discussion

In this study, we demonstrated that blocking the SDF-1/CXCR4 pathway could regulate osteogenic differentiation of pre-osteoblastic MC3T3-E1 cells. Our results demonstrate that CXCR4 inhibitor AMD3100 significantly inhibited the ALP activity during the early stage of osteogenic differentiation in MC3T3-E1 cells, but it seems to have no significant effect on generation of matrix mineralization at the terminal

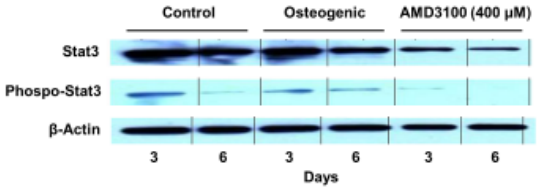

Figure 4. Blocking of SDF-1 signaling inhibited STAT3 expression during the early stage of osteogenic differentiation in MC3T3-E1 cells. Cells were treated with DMEM, osteogenic medium and osteogenic medium supplemented with AMD3100 (400 μM) for 6 days, and then levels of STAT3 and phospho-STAT3 were determined by Western-blot analysis.

stage of osteogenic induction in MC3T3-E1 cells. Meanwhile, ALP, Runx2, Ank, and OCN osteoblast-specific marker genes mRNA expression induced by the osteogenic media were decreased in MC3T3-E1 cells after treatment with AMD3100. Our results also show that the effect of AMD3100 on ALP might be mediated *via* inhibiting intracellular STAT3 activation at the early stage of osteogenic differentiation in MC3T3-E1 cells.

The high expression of SDF-1 by MSCs and osteoprogenitors indicates the intimacy between SDF-1 signaling and initiation of osteogenesis (*23*). High levels of SDF-1 have been detected in MSCs at the early stage of differentiation induced by dexamethasone or BMP2, while SDF-1's expression declines with cell maturation (*24*). Concomitantly, many studies revealed that abundant SDF-1 existing in osteoprecursors play an important role during postnatal bone development and bone regeneration (*25,26*). In this context, our detection that blocking of the SDF-1/CXCR4 signal axis inhibited osteogenic media-induced ALP activity and mRNA expression further suggested the involvement of SDF-1 signaling at the early stage of osteogenic fate determination of MC3T3-E1 cells, since ALP is expressed by MC3T3-E1 cells at the early phase of differentiation when committing to an osteogenic pathway. The result that CXCR4 inhibitor AMD3100 had no effect on matrix mineralization at the terminal stage of osteogenic differentiation in MC3T3-E1 cells may further prove the early role of SDF-1 signaling from another perspective.

The transcriptional regulation of osteoblast genes, such as ALP and OCN, takes place at their promoter regions *via* interaction with crucial transcription factors, such as Runx2, which is expressed at the earliest stage of osteogenic differentiation (*27*). In this context, our detection that blocking of SDF-1/CXCR4 signaling inhibited osteoinduced Runx2 expression further suggested the involvement of SDF-1/CXCR4 signaling in the osteogenic differentiation of MC3T3-E1 cells. Furthermore, our detection also showed that blocking SDF-1 signaling reduced expression of both the early osteoblast differentiation marker (ALP) and the late differentiation markers (Ank and OCN).

The finding of SDF-1 regulation of osteogenic differentiation led us to examine potential crosstalk between SDF-1 signaling and the osteogenic pathway in the context of promoting osteogenesis. The intracellular STAT3 pathway is the major sub pathway engaged in SDF-1 signaling, and studies have shown that STAT3 may play an important role in osteogenic differentiation (*28,29*). Moreover, Itoh *et al.* (*30*) detected that the interleukin-6 family-mediated STAT3 activation plays a critical role in osteoblast differentiation and bone formation. Our results that blocking of the SDF-1/CXCR4 axis inhibited the phosphorylation of STAT3 suggested involvement of STAT3 in mediating the SDF-1 effect on osteogenic differentiation in MC3T3-E1 cells. But, because of diverse species and cell types, the detailed mechanism involved in the STAT3 signaling-mediated osteogenesis still remains unknown (*31,32*). Mikami *et al*. (*31*) detected that a STAT3 siRNA suppressed the synergistic effect of BMP-2 and dexamethasone on ALP levels in mouse C3H10T1/2 pluripotent stem cells; but Levy *et al*. (*32*) indicated that eradication of STAT signaling considerably enhanced BMP-induced osteogenic differentiation of hMSCs, suggesting distinct roles of SDF-1 during different stages of differentiation in various cell types in response to different stimulants. Studies have shown that activation of the STAT3 pathway was rapid and transient (*32*). Similarly, our results show that blocking of SDF-1/CXCR4 signaling only had an effect on phosphorylation of STAT3 at the early stage of osteogenic differentiation in MC3T3-E1 cells. Furthermore, STAT3 and Runx2 are two of the major transcription factors that play essential roles in osteogenesis, they interact with each other in the nucleus, and Runx2 is a master regulator at the early phase of osteogenic differentiation; this interaction might affect osteogenic regulation (*33-35*). Further studies are needed to explain whether the interplay of STAT3 and Runx2 are involved in STAT3 signaling at the early stage of osteogenic differentiation.

In conclusion, our results indicate that blocking the SDF-1/CXCR4 pathway by AMD3100 exerts an inhibitory effect on the early osteogenic differentiation of MC3T3-E1 cells *via* the STAT3 pathway, but has no effect on generation of matrix mineralization at the terminal stage of osteogenic induction. These findings provide novel insights into the mechanisms underlying SDF-1/CXCR4 pathway inhibitors on osteogenic differentiation, which is helpful for re-evaluating their efficacy or toxicity on osteogenesis in the future.

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