Arsenic trioxide promotes senescence and regulates the balance of adipogenic and osteogenic differentiation in human mesenchymal stem cells

Huanchen Cheng1,2, Lin Qiu2, Hao Zhang2, Mei Cheng2, Wei Li2, Xuefei Zhao2, Keyu Liu2, Lei Lei1*, and Jun Ma2*

1Department of Histology and Embryology, Harbin Medical University, Harbin 150081, China
2Institute of Harbin Hematology & Oncology, Harbin First Hospital, Harbin 150010, China
*Correspondence address. Tel/Fax: +86-451-86674518; E-mail: leil086@yahoo.com.cn (L.L); Tel: +86-451-84883432; Fax: +86-451-84883471; E-mail: haerbinxys@126.com (J.M.)

Arsenic trioxide (ATO) as an anti-tumor drug could induce differentiation and apoptosis in tumor cells. Mesenchymal stem cells (MSCs) play important roles in the hematogenesis of bone marrow. Many reports have shown that the disorder of MSC adipogenic and osteogenic differentiation occurs in some diseases. However, reports about the effects of ATO on MSCs are limited. In this study, we found that 1 μM ATO promoted MSC senescence mainly through p21, although it had no effect on apoptosis at this dose. Furthermore, ATO promoted adipogenic differentiation, but inhibited osteogenic differentiation in MSCs. Our study also showed that CCAAT/enhancer-binding protein alpha C/EBPα and peroxisome proliferator-activated receptor gamma PPARγ might be involved in the regulation of adipogenic and osteogenic differentiation induced by ATO. Our results indicated that ATO may exert an anti-tumor effect by influencing bone marrow micro-environment. Moreover, it may regulate the adipogenic and osteogenic differentiation of MSCs.

Keywords arsenic trioxide; mesenchymal stem cells; differentiation; C/EBPα; PPARγ

Received: September 28, 2010 Accepted: November 28, 2010

Introduction

Arsenic trioxide (ATO), a natural constituent of soil and water, is a well-known human carcinogen [1]. However, ATO has also been used to treat cancers and can induce complete remission of acute promyeloid leukemia (APL) [2]. ATO exerts an anti-tumor effect by exerting cytotoxicity on tumor cells, inducing differentiation at low doses and promoting apoptosis or autophagic cell death at high doses [3]. Kachinskas et al. [4] and Trouba et al. [5] both reported that ATO had a significant effect on the differentiation of cultured cells, such as keratinocytes and adipocytes. However, the reports on senescence, apoptosis, and differentiation of mesenchymal stem cells (MSCs) induced by ATO are limited.

MSCs constitute a population of adherent CD14−, CD34−, CD45−, CD73+, CD90+, and CD105+ cells. They could proliferate and differentiate into specific functional cell types, including osteocytes, adipocytes, and chondrocytes in vitro, which makes them promising tools for cell-based regenerative medicine and tissue engineering [6,7]. In recent years, MSCs have been extensively applied clinically, such as in the treatment of osteogenesis imperfecta, graft-versus-host disease, and acute myocardial infarction [8].

Generally, the adipogenic and osteogenic differentiation of MSCs maintains a dynamic balance in bone marrow. The imbalance of adipogenic and osteogenic differentiation of MSCs exists in some human diseases. For example, bone formation increased in patients with progressive osseous hyperplasia accompanied by heterotopic bone formation within their adipose tissue [9]. In contrast to patients with progressive osseous hyperplasia, bone formation decreased and bone marrow adipogenesis increased in patients with aging and immobility or after corticosteroid administration [10,11]. The regulation of adipogenic and osteogenic differentiation may provide a therapeutic target for diseases with inadequate bone formation and excessive marrow adipogenesis. Therefore, it is very interesting to study the interplay between adipogenic and osteogenic differentiation of MSCs. The regulation of MSC adipogenic and osteogenic differentiation may be useful in the treatment of some diseases in the future.

In this study, we determined the effects of ATO on cell viability, senescence, and apoptosis. Also, we detected the changes of adipogenic and osteogenic differentiation between control and ATO-treated MSCs. Furthermore, the expression of some important factors involved in senescence and differentiation regulation was detected by realtime RT–PCR and western blot.
Materials and Methods

MSC isolation and culture
Human bone marrow MSCs were collected from five healthy donors (median age 28) after written consent according to the guidelines approved by the local ethical committee and with the declarations of Helsinki in 1964. The MSCs were isolated by density gradient centrifugation, and then the monocyte and partial serum were transferred into DF-12 medium with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, USA). After culture for 2 weeks, >90% cells were CD90⁺, CD105⁺, and CD73⁺ as detected by flow cytometry. Then the cells were reseeded at a dilution of 1:3 when the cells reached 80% confluence, and the process was called one passage.

Cell viability and apoptosis detection
MSCs (2 × 10⁴ per well of 96-well plates) from the five donors were mixed and then treated with various concentrations of ATO for 48 h. Cell viability was determined by MTT assay as previously described [12]. The percentage of cell growth inhibition was calculated as (OD_{Vehicle} - OD_{Drug}) / OD_{Vehicle} × 100%. For apoptosis analysis, MSCs at passage 3 were treated according to the instructions of the Annexin V FITC Apoptosis Detection Kit (Beyotime, Shanghai, China), and were then detected with a flow cytometer.

SA-β-gal staining
The mixture of MSCs (1 × 10⁵) at passage 3 was plated in six-well chamber slides. SA-β-gal activity was detected the next day by the Senescence β-Galactosidase Staining Kit (Beyotime) according to the manufacturer’s instructions. Then the cells were observed under an inverted microscope. The percentage of senescent cells was calculated by the number of stained cells in 500 cells.

Real-time RT–PCR
Total RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The RNA samples were treated with Amplification Grade DNase I (TaKaRa, Tokyo, Japan) at room temperature for 60 min. First-strand cDNA was synthesized with the RevertAid™ First Strand cDNA Synthesis Kit (Promega, Madison, WI, USA). Real-time PCR was performed with SYBR® Premix Ex Taq™ (Takara) according to the manufacturer’s instructions. The PCR profile was as follows: 1 cycle at 94°C for 4 min, 40 cycles at 94°C for 25 s, 65°C (p21 and p53) or 62°C CCAAT/enhancer-binding protein alpha and peroxisome proliferator-activated receptor gamma (C/EBPα and PPARγ) for 36 s. GAPDH gene was used as an internal control to evaluate the relative expression. The signals were detected using an ABI 7500 system and quantitative results were obtained using the 2^(-ΔΔCt) method. All the primer sequences and annealing temperatures are listed in Table 1.

Western blot analysis
After exposure to ATO for 48 h, MSCs were collected and washed with cold phosphate-buffered saline (PBS) three times. Then lysis buffer (containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.1% sodium dodecyl sulfate supplemented with protease inhibitors) and phenylmethylsulfonyl fluoride were added. After lysing on ice for 30 min, the cells were centrifuged at 10,000 g for 15 min, and the supernatant (protein) was collected. The protein concentration was determined using Bradford assay. Fifty microgram protein of each sample was separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. Then the membrane was incubated with primary anti-p21, anti-p53, anti-C/EBPα, anti-PPARγ, or anti-β-actin antibodies (1:1000).

Table 1 Primer sequences used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Annealing temperature (°C)</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p21</td>
<td>5′-TGGCAGTAGGGCTATGGA-3′ (F)</td>
<td>65</td>
<td>178</td>
</tr>
<tr>
<td></td>
<td>5′-AACAGTCAGCCACGAGTAG-3′ (R)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P53</td>
<td>5′-ACCCAGGTTTCAAGTGAGGAG-3′ (F)</td>
<td>65</td>
<td>174</td>
</tr>
<tr>
<td></td>
<td>5′-GACCAACATTCAAAACACAGTAC-3′ (R)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/EBPα</td>
<td>5′-ACTTGGGTGGTCAAGTGGAGG-3′ (F)</td>
<td>62</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>5′-CATTGGAGGGTGTTGATTTGC-3′ (R)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPARγ</td>
<td>5′-AAGCCAAACTGAAACCAACGGA-3′ (F)</td>
<td>62</td>
<td>204</td>
</tr>
<tr>
<td></td>
<td>5′-GAAATGCTGGAGAATGCAAA-3′ (R)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′-GGATTGTTGCGTATGGAGG-3′ (F)</td>
<td>62/65</td>
<td>205</td>
</tr>
<tr>
<td></td>
<td>5′-GGAAGATGTTGATGAGGATT-3′ (R)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
F, forward primer; R, reverse primer.
(Cell Signaling Technology, Boston, USA) for 16 h at 4°C. Horseradish peroxidase conjugated anti-mouse IgG or anti-rabbit IgG (Cell Signaling Technology) antibodies were used as secondary antibodies. The signal was detected by the Lumi-ECL test system (Haiji, Harbin, China). β-actin was used as an internal control and the relative protein expression was analyzed by Quantity One 4.4.0 software (Bio-Rad, Hercules, USA).

**In vitro differentiation**
MSCs (5 × 10⁵) at passage 3 treated with 1 μM ATO for 48 h were plated in 6-well chamber slides after washing twice with PBS, and the inducing medium was added the next day. All reagents for inducing differentiation were purchased from Sigma (St Louis, USA). The medium for inducing adipogenic differentiation was Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS, 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine, 10 μg/ml insulin, and 200 μM indomethacin. The osteogenic inducing medium was DMEM with 10% FBS containing 10 mM β-glycerophosphate, 0.1 μM dexamethasone, and 50 μM ascorbic acid. The inducing medium was changed every 2–3 days. After 21 days of induction, cells were stained with Oil Red-O and Alizarin red for adipogenic and osteogenic differentiation, respectively. Then the Oil Red-O and Alizarin red were redissolved in isopropanol and deionized water, and the two solutions were semi-quantitatively analyzed at 490 and 595 nm by a plate reader (Bio-TEK-instruments Inc.), respectively. The OD values at 490 and 595 nm represent the abilities of adipogenic and osteogenic differentiation, respectively.

**Statistical analysis**
All the MSCs used for MTT, apoptosis, senescence, expression analysis, and differentiation were a mixture of five donors at passage 3. Each experiment was repeated three times and the average values were taken. Statistical analysis was done using an independent unpaired Student’s t-test and the statistical significance was accepted for a P value of <0.05.

**Results**

**Cell viability and apoptosis analysis**
MSCs at passage 3 were treated with ATO at concentrations of 1, 2, 4, 8, and 16 μM for 48 h, and cell viability was determined by MTT assay. The results showed that ATO at concentrations of 1 and 2 μM had almost no inhibition on MSC growth [Fig. 1(A)]. Similar to the effect of cell viability induced by ATO, 1 μM ATO did not induce the apoptosis of MSCs [Fig. 1(B)].

**ATO induces senescence of MSCs**
MSCs at passage 3 were treated with 1 μM ATO for 48 h and then plated in 6-well chamber slides. Cell senescence was detected the next day. The SA-β-gal staining results showed that 1 μM ATO induced senescence of MSCs. There were almost no senescent cells in control whereas the percentages of senescent cells were about 50%–70% in MSCs treated with 1 μM ATO [Fig. 1(C)].

To determine which molecule was involved in the senescence caused by ATO, the expressions of senescence-related p21 and p53 were detected by real-time RT–PCR and western blot in the ATO-treated MSCs and the control. The quantitative results showed that p21 was increased significantly (P < 0.01) after treatment with ATO, but p53 was not affected (P = 0.074). Similar to the expression at mRNA
level, western blot results showed that p21 was also increased significantly but p53 was not changed (Fig. 2).

**Figure 2** Analysis of expressions of p21 and p53 in MSCs treated with ATO  
(A) The expressions of p21 and p53 at mRNA level were analyzed by semi-quantitative RT–PCR and the corresponding quantitative data obtained using the 2^−ΔΔCt method were shown. GAPDH gene was used as an internal control. (B) The protein levels of p21 and p53 were detected by western blot and the corresponding quantitative data obtained using Quantity One 4.4.0 software were shown. β-actin was used as an internal control. P3 and P3-ATO indicated the control MSCs at passage 3 and the MSCs at passage 3 treated with 1 μM ATO for 48 h, respectively. All experiments were repeated three times and the average values were taken. The data were analyzed by Student’s t-test. Significant difference compared with P3. *P < 0.05, **P < 0.01 compared with P3.

**Figure 3** Adipogenic and osteogenic differentiation analysis of MSCs treated with ATO  
(A) Adipogenic differentiation stained with Oil Red O of MSCs at passage 3 treated with 1 μM ATO for 48 h. (B) Osteogenic differentiation stained with Alizarin red of MSCs at passage 3 treated with 1 μM ATO for 48 h. FAT, OST, and CON indicated adipogenic differentiation, osteogenic differentiation, and MSCs without induction, respectively. P3 and P3-ATO indicate the control MSCs at passage 3 and the MSCs at passage 3 treated with 1 μM ATO for 48 h, respectively. The adipogenic and osteogenic differentiation were both induced for 21 days and stained with Oil Red O and Alizarin red, respectively. Then the Oil Red-O and Alizarin red were re-dissolved in isopropanol and deionized water and the two solutions were semi-quantitatively analyzed at 490 and 595 nm by a plate reader. All experiments were repeated three times and the average values were taken. Magnification, 100 ×. The data were analyzed by Student’s t-test. *P < 0.05 and **P < 0.01 compared with P3-FAT and P3-OST, respectively.

ATO regulates the balance of adipogenic and osteogenic differentiation through C/EBPα and PPARγ  
MSCs at passage 3 were treated with 1 μM ATO for 48 h and plated in 6-well chamber slides with DF-12 medium, and the inducing medium was added the next day. Compared with the control, the lipid volumes were smaller and the lipid vesicle numbers were fewer in MSCs treated with ATO. Compared with adipogenic differentiation, calcium accumulation appeared earlier and the volumes were larger for osteogenic differentiation in MSCs treated with ATO than in the control. After being induced for 21 days, the cells were stained with Oil Red-O for adipogenic differentiation and Alizarin red for osteogenic differentiation. The staining results showed that ATO inhibited adipogenic differentiation but induced osteogenic differentiation [Fig. 3(A,B)]. To explore how ATO regulates adipogenic and osteogenic differentiation, we detected the expression of C/EBPα and PPARγ, which could play important roles in the balance of adipogenic and osteogenic differentiation. The real-time quantitative PCR results indicated that C/EBPα and PPARγ were both decreased significantly in MSCs treated with 1 μM ATO for 48 h [Fig. 4(A)]. Western blot results showed that C/EBPα and PPARγ were also decreased at protein level in MSCs treated with ATO [Fig. 4(B)].

**Discussion**

ATO can treat malignant blood diseases and tumors by inducing apoptosis, differentiation, and modulating DNA synthesis of tumor cells, except for its cytotoxicity [13,14].
In this study, 1 μM ATO showed minor cytotoxicity on MSCs, with the cell viability maintained at >95%. At this concentration, ATO almost had no effect on cell apoptosis, but promoted MSC senescence to percentages of about 50%–70%. These results showed that ATO could induce MSC senescence at low concentration (no cytotoxicity at this concentration), which indicated that ATO may firstly induce senescence and then exert an effect on cell apoptosis and death after its concentrations increased gradually. Senescence may be an important factor for the growth inhibition of tumor cells in ATO treatment. In ATO-treated MSCs, p21 was increased significantly whereas p53 was not changed, indicating that p21 may be important in the MSC senescence induced by ATO.

Wang et al. [15] reported that ATO inhibited 3T3-L1 preadipocyte differentiation through C/EBPα and PPARγ. Similarly, our study also showed that ATO inhibited MSC adipogenic differentiation but promoted its osteogenic differentiation. The quantitative results showed that the two key transcript factors C/EBPα and PPARγ, which were involved in adipogenic differentiation [16–19], were both decreased significantly in ATO-treated MSCs, which indicated that C/EBPα and PPARγ may regulate the adipogenic and osteogenic differentiation in ATO-treated MSCs. Fan et al. [20] reported that C/EBPα regulated adipogenic and osteogenic differentiation of mouse MSCs, and Takada et al. [21] showed that the decreased expression of PPARγ could impair adipogenesis and switch the MSC differentiation into the osteoblastic lineage, which confirmed the important roles of the two genes in MSC differentiation.

MSCs are an important component of the bone marrow micro-environment and play key roles in hematogenesis [22]. Moreover, ATO has clear clinical efficacy on some malignant tumors, especially for APL [2]. In this study, ATO induced MSC senescence and inhibited its adipogenic differentiation but promoted osteogenic differentiation, which implied that ATO may exert an anti-tumor effect by affecting bone marrow micro-environment. In addition, ATO could regulate MSC adipogenic and osteogenic differentiation, showing that ATO may be effective in the treatment of disease characterized by inadequate bone formation and excessive marrow adipogenesis. In addition, the study is useful for studying the regulation of MSC differentiation and providing theory for clinical application of ATO.

**Funding**

The work was supported by the grants from Harbin Technology and Innovation Foundation for Youth (2010RFQQS082 and 2009RFQQS027).

**References**

21 Takada I, Kouzmenko AP and Kato S. PPAR-gamma signaling crosstalk in mesenchymal stem cells. PPAR Res 2010, [Epub in pubmed].