Fujian Institute of Hematology, Fujian Key Laboratory of Hematology, Fujian Medical University Affiliated Union Hospital, Fuzhou, Fujian, P.R China

The effect to IL-3Rα, downstream PI3K/Akt signaling of all-trans retinoic acid and arsenic trioxide in NB4 cells

CHEN PING, WU JUAN-YING, HUANG HUI-FANG, CHEN YUAN-ZHONG

Received September 4, 2013, accepted October 4, 2013

Chen Yuan-zhong, Fujian Institute of Hematology, Fujian Medical University Affiliated Union Hospital, 29 Xinquan Road, Fuzhou, 350001, P.R.China
chenyz@mail.fjmu.edu.cn

Pharmazie 69: 297–300 (2014) *doi: 10.1691/ph.2014.3830*

All-trans retinoic acid (ATRA) and arsenic trioxide (As_2O_3) are the classic drugs used for induction therapy of acute promyelocytic leukemia (APL). IL-3Rα (CD123) is a specific marker of acute myeloid leukemia stem cells (AML-LSCs). The over-expression of IL-3Rα in patients with AML is related to high white blood cells counts, high percentages of blast cells, and poor prognosis. Moreover, in some studies, IL-3Rα has been considered a new detection marker of minimal residual disease in the bone marrow from patients with APL. In contrast to ATRA, As_2O_3 reduces both mRNA and protein expression of IL-3Rα and inhibits the activity of PI3K/Akt after 24 h, 48 h, and 72 h of exposure. Furthermore, NB4 cells adhered to the human stroma cell line HS-5 cells were used as an *in vitro* model of APL cells in the bone marrow microenvironment. Our results demonstrate that adhesion to HS-5 cells up-regulated IL-3Rα protein expression and activated the downstream PI3K/Akt signaling pathway in NB4 cells. Compared with ATRA, As_2O_3 more potently inhibits proliferation of NB4 cells adhered to stroma cells.

1. Introduction

Acute promyelocytic leukemia (APL) is a special subtype of acute myeloid leukemia (AML) and is characterized by the PML/RARα fusion protein. Clinically, all-trans retinoic acid (ATRA) and arsenic trioxide (As_2O_3), which are used for induction therapy of APL, can achieve similar hematologic remission rates in APL patients, but the molecular remission rate and disease-free survival rate are higher after As_2O_3 treatment than after ATRA treatment (Zheng et al. 2007). A major reason for this difference is that ATRA cannot kill PML/RARα+ leukemia-initiating cells (LICs), whereas As_2O_3 can specifically degrade the PML/RARα oncoprotein and destroy the self-renewal potential of PML/RARα+ LICs (Zhang et al. 2010). Whether ATRA and As_2O_3 differ in their ability to regulate other markers of myeloid leukemia stem cells remains unclear. IL-3Rα (CD123) refers to the α subunit of the IL-3 receptor and is used as a specific marker of acute myeloid leukemia stem cells (AML-LSCs) (Jordan et al. 2000). Clinical studies have revealed that high IL-3Rα expression is related to high white blood cells counts, high percentages of blast cells and poor prognosis in patients with AML (Testa et al. 2002). Moreover, IL-3Rα expression is associated with the proliferation ability of leukemia cells and can predict the relapse and survival of patients with AML after complete remission. In some studies, IL-3Rα was used as a marker of minimal residual disease (MRD) in the bone marrow of patients with APL (Wang et al. 2006). In our study, the influence of ATRA and As_2O_3 on IL-3Rα expression and the downstream PI3K/Akt signaling pathway in APL cell line NB4 cells was compared.

At present, a co-culture system with leukemia cells and bone marrow stroma cells is used as an *in vitro* model of MRD. The leukemia cells may derive some immature characteristics of stem cells from this co-culture system (Funayama K et al. 2010). However, whether the level of IL-3Rα changes in leukemia cells that are co-cultured with stroma cells has not been reported to date. We therefore used a model system in which the NB4 cells were attached to the human bone marrow stromal cell line HS-5, followed by an analysis of the expression of IL-3Rα and its downstream PI3K/Akt signaling pathway. In addition, the influence of ATRA and As_2O_3 on NB4 cells adhered to stroma was compared.

2. Investigations and results

2.1. Effects of ATRA or As_2O_3 on IL-3Rα expression in NB4 cells

IL-3Rα mRNA expression in NB4 cells treated with ATRA was dramatically reduced compared with the untreated control group (0.3001 ± 0.033 *VS* 1) after 24 h and returned to the untreated control group level after 48 h and 72 h. IL-3Rα protein expression remained unchanged at different time points after ATRA treatment. IL-3Rα mRNA expression was reduced to 0.253 ± 0.027, 0.236 ± 0.05, and 0.267 ± 0.038–fold compared with the untreated control group after As_2O_3 treatment for 24 h, 48 h, and 72 h, respectively (Fig. 1A). Changes in IL-3Rα protein expression were consistent with the changes in IL-3Rα mRNA expression, demonstrating that IL-3Rα protein expression was markedly reduced in response to As_2O_3 treatment (Fig. 1B).

2.2. Influence of ATRA and As_2O_3 on the PI3K/Akt signaling pathway in NB4 cells

Treating NB4 cells with ATRA for 24 h activated the PI3K/Akt signaling pathway, which is characterized by the up-regulated

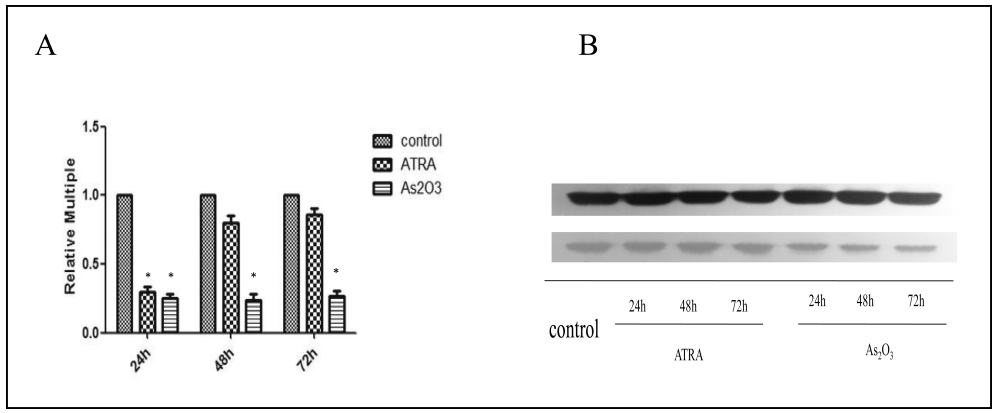

Fig. 1: Effects of ATRA or As2O3 on IL-3Rα expression in NB4 cells. Approximately 5×10^6 NB4 cells cultured alone were treated with 1 μM ATRA or 1 μM As_2O_3. A: The mRNA expression of IL-3Rα was determined by real-time quantitative RT-PCR; B: The protein expression of IL-3Rα was determined by Western blot. *: $p < 0.01$.

expression of p-Akt^{Ser473}, p- PDK1, and p-GSK3β. Expression of these proteins remained similar to the expression levels in the untreated control group after treatment for 48 h but was down-regulated after treatment for 72 h, suggesting that the PI3K/Akt signaling pathway was activated at an early stage during ATRA treatment. However, the expression levels of p-Akt^{Ser473}, p-PDK1, and p-GSK3β were down-regulated at all time points during As_2O_3 treatment, suggesting sustained suppression of the PI3K/Akt signaling pathway (Fig. 2).

2.3. Co-culture with HS-5 cells up-regulated IL-3Rα protein expression in NB4 cells

The expression of IL-3Rα mRNA in NB4 cells cultured alone or adhered to stroma was unchanged (1.0 *vs.* 0.781 ± 0.086, Fig. 3A). Western blotting assays indicated that IL-3Rα protein expression increased after co-culture (Fig. 3B).

2.4. Activation of the PI3K/Akt signaling pathway in NB4 cells after adhesion to HS-5 cells

After co-culture with HS-5 cells for 72 h, the expression levels of p-Akt^{Ser473}, p-GSK3β, and p-PDK1 were markedly up-regulated in NB4 cells (Fig. 4).

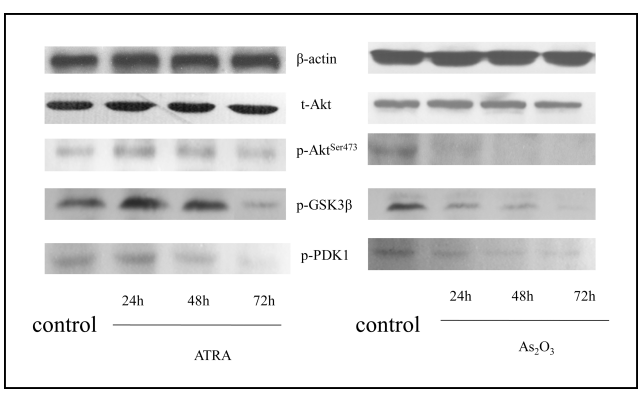

Fig. 2: Changes of the components in the PI3K/Akt signaling pathway in NB4 cells after treatment. Approximately 5×10^6 NB4 cells cultured alone were treated with 1 μM ATRA or 1 μM As_2O_3, and Western blot assays were performed to detect the protein expression of components of the PI3K/Akt signaling pathway.

2.5. Reduced sensitivity of NB4 cells to ATRA and As_2O_3 after adhesion to HS-5 cells.

The results of BrdU incorporation indicated that co-cultured NB4 cells (compared with NB4 cells cultured alone) exhibited reduced sensitivity to ATRA and As_2O_3. The inhibition rate of NB4 cells cultured alone or co-cultured treated with ATRA was $56.93 \pm 3.32\%$ *vs.* $10.81 \pm 1.59\%$, and treated with As_2O_3 was $59.89 \pm 4.76\%$ *vs* $33.81 \pm 5.59\%$, ($p < 0.01$)

3. Discussion

As a marker of myeloid leukemia stem cells, IL-3Rα is weakly expressed in normal HSCs. Because IL-3Rα is related to the proliferation of primary AML cells and is differentially expressed between LSCs and HSCs, IL-3Rα could represent a potential target in anti-leukemia therapy. In recent years, although drugs targeting IL-3Rα have been developed, whether this approach is appropriate has been debated (Frankel et al. 2008; Testa et al.2005; Jin et al. 2009). In a study examining the use of diphtheria toxin and rodent diphtheria toxin-murine interleukin-3 (DT-mIL3) as immunotoxic therapy for AML, DT-mIL3 specifically killed most primitive cells and leukemic long-term culture-initiating cells (LTC-ICs) with characteristics of early hematopoietic stem/progenitor cells. Although this therapy also killed normal hematopoietic progenitor cells, the therapy had no effect on normal LTC-ICs. Clinical studies on this therapy have not been conducted. Identifying available drugs that are IL-3Rα-targeting drugs remains a challenge. Our results demonstrated that ATRA had no influence on the protein expression of IL-3Rα and no early effect on the PI3K/Akt signaling pathway. In contrast, As_2O_3 significantly reduced the protein expression levels of IL-3Rα, thus suppressing the PI3K/Akt pathway.

In some *in vitro* studies, leukemia cells have been co-cultured with bone marrow stromal cells to mimic the residual leukemia cells in the bone marrow microenvironment. Adhering to stroma cells causes leukemia cells to develop some immature features (Funayama et al. 2010). Our results show for the first time that IL-3Rα mRNA expression is unchanged and that IL-3Rα protein expression is elevated in NB4 cells that are co-cultured with stroma cell line HS-5 for 72 h. This finding suggests that the bone marrow microenvironment might induce the expression of immaturity markers in leukemia cells, which could cause their proliferation under IL-3-insufficient or even IL-3-deficient conditions, increase their rate of proliferation, and thereby result in leukemia recurrence. In agreement with previous findings (Tabe et al. 2007), our results also revealed that co-culture could activate the PI3K/Akt signaling pathway, which is downstream of IL-3Rα, in NB4 cells. Thus, we speculate that IL-3Rα, a

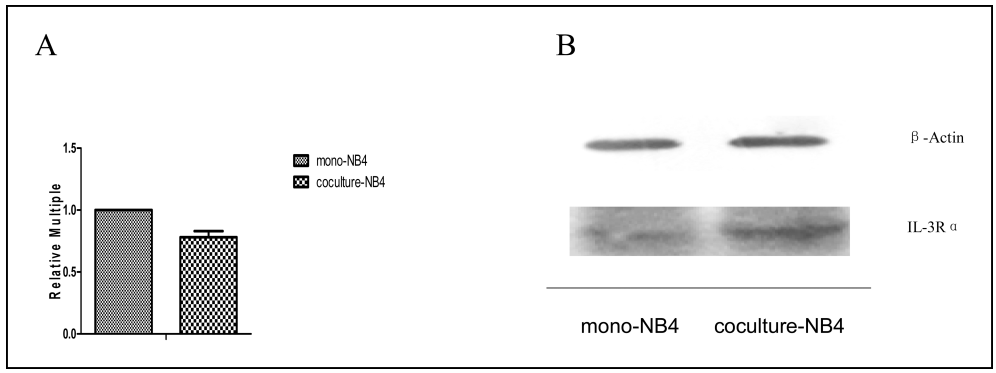

Fig. 3: IL-3Rα mRNA and protein expression in NB4 cells cultured with or without HS-5 for 72 h. Approximately 1×10^6 NB4 cells were cultured with or without HS-5 cells for 72 h. (**A**) Detection of IL-3Rα mRNA expression by real-time quantitative RT-PCR. (**B**) Detection of IL-3Rα protein expression by Western blot assays.

molecule upstream of the PI3K/Akt signaling pathway, may also be involved in the interaction between the bone marrow stroma and leukemia cells. Our results also confirmed that, when compared with As_2O_3, ATRA showed a reduced ability to inhibit the proliferation of co-cultured NB4 cells. In conclusion, As_2O_3 more potently inhibits proliferation of APL cells adhered to stroma cells.

4. Experimental

4.1. mRNA expression

Total RNA was extracted, followed by reverse transcription into cDNA according to the manufacturer's instructions (Promega, Madison, WI). Real-time quantitative PCR of IL-3Rα was performed following a reported procedure (Steelman et al. 1996). The primers were as follows: β-actin: forward, 5'-AGTGTGA CGTGGACATCCGC AA-3', reverse: 5'-ATCCACATCTGCTGGAA GGTGGAC-3'; and IL-3Rα: forward 5'-GACCTGTACTTGAACGTTGCC -3', reverse 5'- GAAACGACACCC-GATAC GTGT-3'. Data analysis was performed using the $2^{-\Delta\Delta CT}$ method. $2^{-\Delta\Delta CT} \geq 1.5$ was defined as up-regulation, and $2^{-\Delta\Delta CT} \leq 0.5$ was defined as down-regulation.

4.2. Protein expression

Approximately 5×10^6 cells were washed in PBS and suspended in five volumes of lysis buffer [20 mM HEPES (pH 7.9), 20% glycerol, 200 mM KCl, 0.5 mM EDTA, 0.5% NP-40, 0.5 mM DTT, 1% protease inhibitor cocktail (Sigma, USA)]. The lysates were then collected and stored at -70 °C. The protein concentrations in the supernatants were quantified with a kit (Beyotime Institute of Biotechnology, China). Supernatant samples containing 40 μg of total protein were resolved on 10–15% SDS-PAGE gels and transferred onto Immobilon-P PVDF membranes (Millipore, MA) by electroblotting; membranes were probed with primary antibodies and subsequently with horseradish peroxidase-conjugated secondary antibodies. The membrane blots were developed using an ECL kit (Amersham Biosciences, NJ).

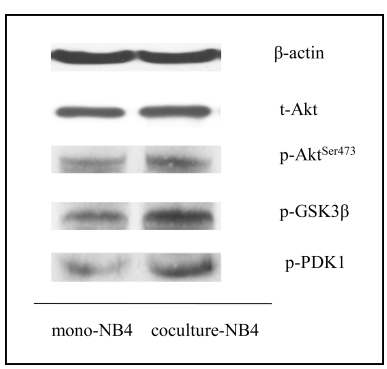

Fig. 4: Activation of the PI3K/Akt signaling pathway in NB4 cells cultured with or without HS-5 for 72 h. Approximately 5×10^6 NB4 cells were cultured with or without HS-5 cells for 72 h. p-Akt^{Ser473}, p-GSK3β, and p-PDK1 expression levels were detected by Western blot.

4.3. Co-culture system

HS-5 cells were transferred at a density of 5×10^4 cells per well to 12-well plates and incubated overnight to allow complete monolayer formation. NB4 cells were added to plates with a HS-5 monolayer at a density of 1×10^5 cells per well. Half of the medium in the co-culture system was replenished every two days. After co-culture for 72 h, NB4 cells adhered to HS-5 cells were separated from HS-5 cells using an anti-human CD45 magnetic bead method according to the manufacturer's instructions (Miltenyi, Germany). The purity of the NB4 cells obtained by this method was approximately 95%.

4.4. Cell proliferation measured

NB4 cells before and after co-culture were seeded into 96-well plates at a density of 1×10^4 cells/well and incubated with ATRA or As_2O_3 at a final concentration of 1 μM (total volume: 100 μl) at 37 °C for 72 h. Cell proliferation was measured with a BrdU Kit (Roche, Germany) according to the manufacturer's instructions.

4.5. Statistical analysis

SPSS version 11.5 for Windows was used for all analyses. Quantitative data are expressed as the mean ± standard deviation (X ± SD). Analysis of variance was used to compare group differences. Dunnett's *t*-test was used to compare the means of two specific groups. A value of $p < 0.05$ was considered significant.

Acknowledgements: This work was supported by grants from the National Natural Science Foundation of China (No. 81100382), the Fund of the Fujian Key Laboratory of Hematology (No. 2009J1004) and Fujian Provincial Education Funding (JB11059)

References

Frankel A, Liu JS, Rizzieri D, Hogge D (2008) Phase I clinical study of diphth-eria toxin-interleukin-3 fusion protein in patients with acute myeloid leukemia and myelodysplasia. Leuk Lymphoma 49: 543–553.

Funayama K, Shimane M, Nomura H, Asano S (2010) An evidence for adhesion- mediated acquisition of acute myeloid leukemic stem cell-like immaturities. Biochem and Biophys Res Commun 392: 271–276.

Jin L, Lee EM, Ramshaw HS, Busfield SJ, Peoppl AG, Wilkinson L, Guthridge MA, Thomas D, Barry EF, Boyd A, Gearing DP, Vairo G, Lopez AF, Dick JE, Lock RB (2009) Monoclonal antibody-mediated targeting of CD123, IL-3 receptor alpha chain, eliminates human acute myeloid leukemic stem cells. Cell Stem Cell 5: 31–42.

Jordan CT, Upchurch D, Szilvassy SJ, Guzman ML, Howard DS, Pettigrew AL, Meyerrose T, Rossi R, Grimes B, Rizzieri DA, Luger SM, Phillips GL (2000) The interleukin-3 receptor alpha chain is a unique marker for human acute myelogenous leukemia stem cells. Leukemia 14: 1774–1784.

Testa U, Riccioni R, Biffoni M, Diverio D, Lo-Coco F, Foà R, Peschle C, Frankel AE (2005) Diphteria toxin fused to variant human interleukin-3 induces cytotoxicity of blasts from patients with acute myeloid leukemia according to the level of interleukin-3 receptor expression. Blood 106: 2527–2529.

Wang YZ, Chang Y, Zhu HH, Qin YZ, Li JL, Fu JY, Li LD, Chen SS, Huang XJ, Lu DP, Liu YR (2006) Application of CD123 in detection of minimal residual disease in acute promyelocytic leukemia. Zhongguo Shi Yan Xue Ye Xue Za Zhi 14: 427–432.

Zhang XW, Yan XJ, Zhou ZR, Yang FF, Wu ZY, Sun HB, Liang WX, Song AX, Lallemand-Breitenbach V, Jeanne M, Zhang QY, Yang HY, Huang QH, Zhou GB, Tong JH, Zhang Y, Wu JH, Hu HY, de Thé H, Chen SJ, Chen Z (2010) Arsenic Trioxide controls the fate of the PML -RARa oncoprotein by directly binding PML. Science 328: 240–243.

Zheng X, Seshire A, Rüster B, Bug G, Beissert T, Puccetti E, Hoelzer D, Henschler R, Ruthardt M (2007) Arsenic but not all-trans retinoic acid overcomes the aberrant stem cell capacity of PML/RARalpha-positive leukemic stem cells. Haematologica 92: 323–331.