Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

Effect of combination of all-trans retinoic acid and arsenic trioxide on apoptosis of acute promyelocytic leukemia cells

Jiane Hu[1], Qiuyan Sun[2*], Wei Fang[2], Qinglin Wang[1]

[1] Department of Clinical Laboratory, Chongqing Three Gorges Central Hospital, Chongqing City, China
[2] Department of Pharmacy, Chongqing Three Gorges Central Hospital, Chongqing City, China

Correspondence to: b33pvp@163.com

Received February 22, 2019; **Accepted** April 28, 2019; **Published** April 30, 2019

Doi: http://dx.doi.org/10.14715/cmb/2019.65.4.16

Abstract: To study the effect of all-trans retinoic acid (ATRA) and arsenic trioxide (ATO) combination treatment on apoptosis of acute promyelocytic leukemia cells (NB4), inflammation and prognosis. The effect of ATRA - ATO combination on the proliferation of NB4 was determined using MTT assay. Apoptosis of NB4 cells was assessed with TUNEL assay. The effect of ATRA-As2O3 combination on the expressions of IL-6 and TNF-α in NB4 cells was determined using ELISA kits, while its effect on the quality of life of 25 acute promyelocytic leukemia patients admitted to our hospital was scored, as an index of prognosis. The combination treatment with ATRA and ATO significantly inhibited the proliferation of NB4 cells and promoted their apoptosis, relative to the model group. In addition, the combination treatment reduced serum IL-6 and TNF-α levels in patients with acute promyelocytic leukemia, and improve their quality of life and survival. Combination treatment with ATRA and ATO significantly inhibits the proliferation of NB4 cells and promotes their apoptosis, and reduces inflammatory responses in patients with acute promyelocytic leukemia, while improving their quality of life and prognosis.

Key words: Acute promyelocytic leukemia; All-trans retinoic acid; Arsenic trioxide; NB4 cells; Inflammation.

Introduction

Acute promyelocytic leukemia (APL) is a special type of acute myeloid leukemia which is characterized by rapid onset and progression, and it accounts for 10 – 15 % of leukemia cases, with majority of patients dying from complications before complete remission (CR) (1, 2) . It belongs to the most lethal M3 subtype of acute leukemia, and over 90 % of patients with APL have characteristic cytogenetic changes. Patients with chromosome dislocation have retinoic acid receptor (PML-RARα) fusion gene. The characteristic features of the disease are unlimited proliferation of promyelocytes and inhibition of apoptosis (3, 4). In the past 20 years, a lot of progress has been made on studies on APL. The discovery of the therapeutic effects of ATRA and ATO has become a milestone in the treatment of APL (5). All-trans retinoic acid (ATRA), also known as retinoic acid (chemical formula = $C_{20}H_{28}O_2$) is a metabolite of vitamin A in animals. In 1986, ATRA was used in the treatment of APL in China, with about 90 % complete remission. The complete remission of APL was increased to about 90 - 95 % with addition of anthracyclines, and the incidence of complications was significantly reduced (6). In the 1990s, ATO was used to treat APL, with significant therapeutic effects. Arsenic trioxide (ATO), one of the oldest poisons, is odorless and tasteless, and was named *pishuang* (arsenic) due to its white frost-like powder appearance (7, 8). With deepening of research, the treatment of APL has been continuously improved. The treatment has changed from the use of a single drug to the application of combination of two drugs, so as to promote the dual effect of apoptosis and differentiation of APL cells. The combination treatment plays an important role in maintaining complete remission and improved prognosis of APL (9). However, there are very limited studies on the effect of ATRA - ATO combination treatment on APL, and the effect of the combination treatment on the quality of life of the patients. The present study was carried out to investigate the effect of ATRA-ATO combination on proliferation and apoptosis of APL NB4 cells, and its effect on serum IL-6 and TNF-α levels in APL patients, as well as their quality of life and overall survival.

Materials and Methods

Reagents and Animals

Reagents

Fetal bovine serum (FBS), penicillin/streptomycin (P/S) and trypsin were purchased from American Invitrogen Company; PBS tablets and paraformaldehyde were products of Beijing Suo Laibao Technology Co., Ltd; MTT kits were purchased from Biyuntian Institute of Biotechnology, while IL-6 and TNF-α ELISA kits were products of Nanjing Institute of Bioengineering. Culture flasks and plates were obtained from Corning, USA, while TUNEL assay kits were purchased from R&D Systems, USA. Arsenic trioxide (ATO) and ATRA were purchased from American Sigma Corporation.

Instruments

Cell ultra-clean desk was purchased from Dongguan Zhuowei Purification Technology Co., Ltd. Ultra-low temperature refrigerator was product of Eppendorf, Germany. Cell culture incubator was purchased from Changzhou Jintan Youlian Instrument Research Institute, while 96-well microplate reader was obtained from China Mindray Company. Fluorescent inverted microscope was purchased from Nikon Corporation of Japan.

Cells

Human APL NB4 cells were purchased from Shanghai Gaining Biotechnology Co., Ltd (CM-H380). The culture medium was 1640 medium containing 10 % FBS+1% P/S. The NB4 cells were cultured in a 5 % CO_2 incubator at 37 ℃, and sub-cultured for 2 - 3 days (10).

Clinical data of APL patients

A total of 25 APL patients treated in our hospital from 2014 to 2017 were enrolled in the study. They were diagnosed using immunohistochemistry bone marrow biopsy in accordance with the diagnosis criteria of APL. Moreover, their promyelocytic leukemia/retinoic acid receptor fusion gene was positive. All patients signed written informed consent.

Methods

MTT assay for the effect of ATRA-ATO combination on the proliferation of NB4 cells

The NB4 cells in logarithmic growth phase were seeded at a concentration of 5×10^3 cells/well in 96-well plates, with 3 wells in each group. After exposure to combination of ATRA (1 μmol/L) and ATO (32 μmol/L) at 24 h, 48 h and 72 h, 10 μl of MTT solution (5 mg/ml) was added to each well and the wells were incubated for 4 h. Then, 100 μl of DMSO was solution was added to each well. After shaking, the crystals were completely dissolved. The absorbance of the solution was measured at a wavelength of 570 nm.

TUNEL assay for the effect of ATRA - ATO combination on apoptosis of NB4 cells

The cells were collected, rinsed with 1 × PBS and fixed with 4 % paraformaldehyde. Then, they were dehydrated in alcohol gradient (70, 95 and 100 % ethanol), and incubated with 50 μl of proteinase K working solution for 15 min, followed by washing twice with double distilled water. After incubating with 1 × TdT labeling solution for 5 min, 50 μl of labeled reaction mixture was added, followed by incubation for 1 h. Subsequently, 1 × TdT stopping solution was added to terminate the reaction at room temperature for 5 min, followed by washing with 1 × PBS for 5 min. Then, the nuclei were stained with DAPI staining solution in the dark for 15 min. The staining was observed under a microscope.

Determination of the effect of ATRA - ATO combination on serum levels of IL-6 and TNF-α in patients with APL using ELISA

Venous blood drawn from the patients was centrifuged to obtain serum, and standard curves were prepared. Blank wells and sample wells were set up, and to each well was added 50 μL of sample diluent, and the mixture was rinsed with washing solution after incubating for 30 min, and culture medium was discarded. To each sample well was added 50 μl of enzyme-labeled indicator. The samples were sealed with membrane, incubated for 30 min, rinsed with washing solution, and dried. To each well was added 50 μl of coloring solution A and 50 μl of coloring solution B (in the dark), and the wells were shaken and left for 15 min for color development. Thereafter, stopping solution was added to terminate the reaction, and absorbance was read at 450 nm (11).

Effect of ATRA combined with ATO on the quality of life of APL patients

The quality of life of APL patients was assessed after treating them with the combination of ATRA and ATO. The parameters assessed were physical function, role function (12), emotional function, cognitive function and social function. Each function was scored from 0 to 100, and the higher the score, the better the function.

Effect of ATRA - ATO combination on the prognosis of APL patients

Early death rate (EDR), complete remission rate (CR), recurrence rate, and time taken for complete remission were recorded in both groups. Early death rate (EDR) referred to death of the patient within 20 days of diagnosis and treatment; CR referred to granulocyte: promyelocyte ratio < 5 %, with white blood cells, platelets and hemoglobin within their normal ranges, and no leukemia cell population on the patient's peripheral blood leukocyte classification.

Statistical Analyses

Data are expressed as mean ± standard deviation (SD), and were analyzed using SPSS software version 17.0. Enumeration data was analyzed with χ^2 test. Measurement data were statistically analyzed using *t*-test. Values of $p < 0.05$ were assumed to indicate statistically significant differences.

Results

ATRA - ATO combination inhibited the proliferation of NB4 cells

The proliferation of NB4 cells was determined using MTT assay kit at 24, 48 and 72 h after treating with the combination of 1 μmol/L ATRA and 32 μmol/L ATO. The proliferation of NB4 cells was significantly inhibited at 48 h by the combination treatment, when compared with the control group ($p < 0.05$). The inhibition gradually and significantly increased with time ($p < 0.05$). These results are shown in Figure 1.

ATRA - ATO combination treatment enhanced apoptosis of NB4 cells

As shown in Figure 2, apoptosis in the combination treatment group was significantly increased, when compared with the control group ($p < 0.05$).

ATRA - ATO combination downregulated the expressions of IL-6 and TNF-α in APLpatients

The levels of IL-6 and TNF-α in APL patients were

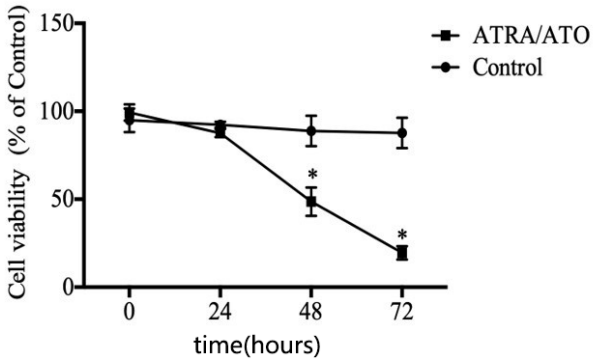

Figure 1. Effect of ATRA - ATO combination treatment on the viability of NB4 cells after 24, 48, 72 h. $^{*}p < 0.05$.

Table 1. Effect of ATRA - ATO combination on the levels of IL-6, TNF-α in acute promyelocytic leukemia patients.

List	Control	ATRA/ATO
IL-6 (pg/ml)	120.34 ± 10.46	75.84 ± 8.39*
TNF-α (pg/ml)	154.89 ± 11.23	93.27 ± 11.52*

$^{*}p < 0.05$ (ATRA - ATO treatment compared with control group).

Table 2. Effect of ATRA - ATO combination treatment on the quality in life of acute promyelocytic leukemia patients.

Group	Control	ATRA/ATO
Physical function	54.38 ± 8.89	62.34 ± 7.53
Role function	29.47 ± 5.23	49.47 ± 5.04*
Emotional function	67.36 ± 7.12	83.87 ± 8.39*
Cognitive function	67.39 ± 8.35	89.46 ± 9.18*
Social function	40.74 ± 5.84	58.62 ± 6.72*

*$p < 0.05$ (ATRA - ATO combination treatment group compared with control group).

determined with ELISA kit. The levels of IL-6 and TNF-α in APL were much lower in the patients treated with the combination of ATRA and ATO than in those in the control group ($p < 0.05$), as shown in Table 1.

ATRA - ATO combination treatment enhanced the quality of life of APL patients

The scores in role function, emotional function, cognitive function and social function were greatly improved in the APL patients after treatment with the combination of ATRA and ATO, relative to the control group ($p < 0.05$; Table 2).

ATRA - ATO combination improved the prognosis of APL patients

As shown in Table 3, the complete remission rate was much higher, and the recurrence rate was much lower in patients treated with ATRA and ATO combination than in the control group ($p < 0.05$). In addition, the time taken to achieve complete remission was significantly lower in the combination treatment group than that in the control group ($p < 0.05$. However, there was no significant difference in early mortality between the two groups.

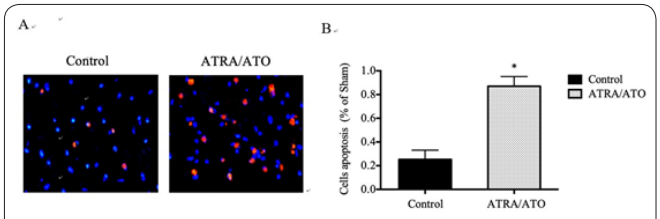

Figure 2. Effect of ATRA - ATO combination on apoptosis of NB4 cells. A: Nuclear staining as revealed in TUNEL assay (x20). B: Percentage of cell apoptosis. $^{*}p < 0.05$.

Discussion

Acute promyelocytic leukemia (APL) is an acute form of myeloid leukemia which is currently clinically treated using induction therapy, post-remission therapy, new therapy and bone marrow transplantation (13). It is pathologically characterized using immunology, cell morphology, molecular biology and chromosomology. In chromosomology, the pathological event is the mutual translocation of chromosomes 15 and 17. Indeed, 98 % of the patients have fusion genes. The PML-RARα proteins form dimeric complexes and cause disseminated intravascular coagulation. A large number of granular promyelocytes are produced in the bone marrow, leading to coagulopathy and bleeding tendency (14, 15). Prior to the use of targeted drugs in clinics, the mortality rate of APL was extremely high. However, with deepening of research on APL, many new treatment methods have emerged (16, 17). Acute promyelocytic leukemia (APL) was a type of acute myeloid leukemia with the worst prognosis until ATRA and ATO were applied in clinical practice (16, 17). The combination therapy of ATRA and ATO has become the first-line treatment for patients who are intolerant of anthracycline (18).

In a previous study, Liu *et al*. labeled a GFP model to study the effect of ATRA or ATO administration on the differentiation of APL cells. It was found that APL cells differentiated significantly and PML-RARα protein degradation was enhanced after administration of the drug (19). The complete remission of patients was significantly increased after treatment with ATRA or ATO (20). In another study (21), it was found that HOXA7 gene decreased after ATRA treatment, thereby inhibiting the proliferation of NB4 cells. Further studies found (21) that ATRA also changed the morphology of NB4 cells. Thus, HOXA7 may be a candidate gene for treating APL, which is an improvement on the molecular basis for the treatment of APL.

In the present study, APL NB4 cells were first used

Table 3. Effect of ATRA-ATO combination treatment on prognosis of acute promyelocytic leukemia patients.

Parameter	Control	ATRA/ATO
Early mortality (%)	3	2
Complete remission (%)	37	45*
Time taken to achieve complete remission (days)	38.5 ± 4.2	21.8 ± 4.6*
Recurrence (%)	17	6*

$^{*}p < 0.05$ (ATRA -ATO combination treatment group vs. control group).

to study the effects of ATRA- ATO combination on their proliferation and apoptosis. It was found that NB4 cell proliferation was inhibited after 48 h of ATRA and ATO treatment. Besides, the inhibition became more significant with time. The apoptosis of NB4 cells was detected with TUNEL assay. The apoptosis of NB4 cells significantly increased after ATRA and ATO combination treatment, suggesting that the combination treatment inhibited the proliferation of NB4 cells and promoted their apoptosis. This finding provides experimental basis for the use of these agents in clinical practice. In addition, it was found that the combination of ATRA and ATO significantly reduced the serum levels of IL-6 and TNF-α in patients, indicating that the combination treatment effectively controls patient's inflammatory response. After the combination treatment, the emotional, cognitive, and social functions of patients were significantly enhanced, indicating improved quality of life. The findings on the effect of the combined treatment on patients' prognosis suggest that it enhances complete remission, shortens the time taken to achieve complete remission, reduces recurrence, and effectively improves prognosis.

The results of this study suggest that ATRA combined with ATO can significantly inhibit the proliferation and promote apoptosis of NB4 cells. Furthermore, the combination of ATRA and ATO significantly enhances the quality of life of patients, shortens the time taken to achieve complete remission, and decreases recurrence of APL. Thus, the combination therapy merits clinical application.

Acknowledgements
None.

Interest conflict
There is no conflict of interest to be declared by the author.

Author's contribution
We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors, all authors read and approved the manuscript for publication.
Qiuyan Sun conceived and designed the study, Jiane Hu, Qiuyan Sun, Wei Fang, Qinglin Wang collected and analysed the data, Jiane Hu wrote the manuscript.

References

1. Testa U, Lo-Coco F. Prognostic factors in acute promyelocytic leukemia: strategies to define high-risk patients. Ann Hematol 2016; 95: 673-680.
2. Buckley SA, Kirtane K, Walter RB, Lee SJ, Lyman GH. Patient-reported outcomes in acute myeloid leukemia: Where are we now. Blood Rev 2018; 32: 81-87.
3. Pashaiefar H, Yaghmaie M, Tavakkolybazzaz J, Ghaffari SH, Alimoghaddam K, Pantea I, et al. The Association between PARP1 and LIG3 Expression Levels and Chromosomal Translocations in Acute Myeloid Leukemia Patients. Cell J 2018; 20: 204-210.
4. Watts JM, Tallman MS. Acute promyelocytic leukemia: what is the new standard of care? Blood Rev 2014; 28: 205-212.
5. Nguyen R, Houston J, Chan WK, Finkelstein D, Dyer MA. The role of interleukin-2, all-trans retinoic acid, and natural killer cells: surveillance mechanisms in anti-GD2 antibody therapy in neuroblastoma. Cancer Immunol Immunother 2018; 67: 615-626.
6. Derek MC, Christina B, Harry I. Retinoic acid and arsenic trioxide in the treatment of acute promyelocytic leukemia: current perspectives. Onco Targets Ther 2017; 10: 1585-1601.
7. Iland HJ, Wei A, Seymour JF. Have all-trans retinoic acid and arsenic trioxide replaced all-trans retinoic acid and anthracyclines in APL as standard of care. Best Pract Res Clin Haematol 2014; 27: 39-52.
8. Das M. Arsenic trioxide consolidation in APL. Lancet Oncol 2017; 18: 517.
9. Abaza Y, Kantarjian H, Garcia-Manero G, Estey E, Borthakur G, Jabbour E, et al. Long-term outcome of acute promyelocytic leukemia treated with all-trans-retinoic acid, arsenic trioxide, and gemtuzumab. Blood 2017; 129: 1275-1283.
10. Zhu B, Wang J, Zhou F, Liu Y, Lai Y, Wang J, et al. Zinc Depletion by TPEN Induces Apoptosis in Human Acute Promyelocytic NB4 Cells. Cell Physiol Biochem 2017; 42: 1822-1836.
11. Dunoyer-Geindre S, Rivier-Cordey AS, Tsopra O, Lecompte T, Kruithof EKO. Effect of ATRA and ATO on the expression of tissue factor in NB4 acute promyelocytic leukemia cells and regulatory function of the inflammatory cytokines TNF and IL-1β. Ann Hematol 2017; 96: 905-917.
12. Brown MB, Holcombe DC, Bolen LM, Thomson WS. Role function and job satisfaction of school psychologists practicing in an expanded role model. Psychol Rep 2006; 98: 486-496.
13. Sultan S, Irfan SM, Ashar S. Acute Promyelocytic Leukemia: a Single Center Study from Southern Pakistan. Asian Pac J Cancer Prev 2015; 16: 7893-7895.
14. Giri S, Pathak R, Aryal MR, Karmacharya P, Bhatt VR. Second primary malignancy in acute promyelocytic leukemia: a Surveillance, Epidemiology and End Results database study. Future Oncol 2017; 13: 1455-1457.
15. Wafa A, Moassass F, Liehr T, Al-Ablog A, Al-Achkar W. Acute promyelocytic leukemia with the translocation t(15;17) (q22; q21) associated with t(1;2) (q42~43; q11.2~12): a case report. J Med Case Rep 2016; 10: 203.
16. Tomita A, Kiyoi H, Naoe T. Mechanisms of action and resistance to all-trans retinoic acid (ATRA) and arsenic trioxide (As2O 3) in acute promyelocytic leukemia. Int J Hematol 2013; 97: 717-25.
17. Cao H, Xie J, Guo L, Han K, Pei Y, Li X, et al. All-trans retinoic acid induces autophagic degradation of ubiquitin-like modifier activating enzyme 3 in acute promyelocytic leukemia cells. Leuk Lymphoma 2018; 59: 1222-1230.
18. Zhu HH, Guo ZP, Jia JS, Jiang Q, Jiang H, Huang XJ. The impact of oral arsenic and all-trans-retinoic acid on coagulopathy in acute promyelocytic leukemia. Leuk Res 2018; 65: 14-19.
19. Liu X, Chen J, Yu S, Yan L, Guo H, Dai J, et al. All-trans retinoic acid and arsenic trioxide fail to derepress the monocytic differentiation driver Irf8 in acute promyelocytic leukemia cells. Cell Death Dis 2017; 8: 2782.
20. Abaza Y, Kantarjian H, Garcia-Manero G, Estey E, Borthakur G, Jabbour E, et al. Long-term outcome of acute promyelocytic leukemia treated with all trans retinoic acid , arsenic trioxide, and gemtuzumab. Blood 2017; 129: 1275-1283.
21. Guo Q, Jiang Q, Liu W, Bai Y. All-trans retinoic acid inhibits HOXA7 expression in leukemia cell NB4. Cell Mol Biol (Noisy-le-grand) 2016; 62: 63-66.