RELATIONSHIP BETWEEN G-CSF AND HYPERLEUKOCYTOSIS IN PATIENTS WITH APL AFTER TREATMENT WITH ALL-TRANS RETINOIC ACID

JIANG Guosheng 姜国胜, TANG Tianhua 唐天华, SUN Guanlin* 孙关林,
ZHANG Yukun 张玉昆 WU Wen* 吴文, WU Weili* 吴维礼,
REN Haiquan 任海泉, ZHAO Liangyu 赵良玉

Shangdong Academy of Medical Sciences, Jinan 250062, China
**Rui-Jin Hospital, Shanghai Institute of Hematology, Shanghai 200025, China*

ABSTRACT

Objective: To detect the G-CSF effect and its mechanism in hyperleucocytosis of patients with APL after treatment with all-trans retinoic acid (ATRA). Methods: MTT method was used to measure the proliferation of HL-60 cells or primary APL cells. cDNA-mRNA dot blot hybridization was used to detect the c-myc gene expression level of HL-60 cells after ATRA treatment. Cycle shift of HL-60 cells was measured by flow cytometric Analysis. Results: ATRA could inhibit obviously *in vitro* the proliferation of HL-60 cells of primary APL cells. The G_0/G_1 ratio of HL-60 at 24 hours or 96 hours post-treatment with ATRA was increased, proliferation of HL-60 cells at the same time was promoted obviously under the condition of rhG-CSF. C-myc gene expression was analyzed by dot blot hybridization; its expression levels of 8, 24 or 96 hours in culture with ATRA separately were higher, low, high or lower as compared with each other. Conclusion: G-CSF plays an important role in hyperleucocytosis of patients with APL after treatment with ATRA.

Key words: G-CSF, hyperleucocytosis, Acute promyelocytic leukemia

The patients with acute promyelocytic leukemia (APL) often died from early infection or coagulation disorders.[1] Complete remission (CR) rates of 50%~60% during the chemotherapy were reported in recent years.

Studies *in vitro* have shown that all-trans retinoic acid (ATRA) was capable of letting APL cells to differentiate at some low concentrations. This property has been applied *in vivo* in newly diagnosed and in relapsing APL. Otherwise, with less coagulation of disorders and bone marrow aplasia. Development of ATRA treatment has resulted in increasing CR rates to about 85%.[2]

With the widespread use of ATRA *in vivo*, side effects are usually moderate, for example, hyperleukocytosis in APL after treatment with ATRA.[3] It may develop very rapidly, leading to a clinical sign of leukostasis. We have investigated the mechanism of hyperleucocytosis by different ways, including detection of serum cytokines and others *in vitro* experiments. One of these important results showed that level of serum G-CSF was significantly correlated with the total number of WBC, including promyelocytes and more matured granulocytes.[4] With the aim to explore concrete mechanism of G-CSF which induced hyperleukocytosis further experiments were made in the present study and the results were reported as follows.

Accepted for publication: June 20, 1999
This work was supported by the National Natural Science Foundation of China (No. 39470318) and the Grant from the 9th Five-year Program for Scientific Research of China (96-906-0117).
Correspondence to: Jiang Guosheng, Shandong Academy Medical Sciences; No. 89, Jing-Shi Road, Jinan 250062, China; Phone (0086-531)-2956191ext.3288; Fax: (0086-531)-2954015; E-mail: jiyin@jn-public.sd.cninfo.net

MATIERIALS AND METHODS

The Effect of ATRA on HL-60 Cell or primary APL Cells

HL-60 cell line was kindly provided by Prof. Zhang Ling from Shangdong Academy of Medical Science, Jinan. Primary leukemia cells were obtained from patients with APL (92% promyelocytes in Bone Marrow Cells). RPMI-1640 medium, MTT, DMSO and other agents were from Sigma. 2×10^5 HL-60 cells or primary APL cells in a volume of 0.1 ml of 2×10^{-5} mol/L or 2×10^{-6} mol/L, ATRA were added and cultivated in 5% CO_2 at 37°C for 120 hours. The proliferation of HL-60 cell or primary APL cells before or after culture (8, 24, and 120 hours) was measured by MTT method. RPMI-1640 medium, MTT, DMSO and other agents were from Sigma.

The Effect of ATRA on the Cell Cycle Shift

Using the above mentioned culture method, the cell cycle of HL-60 cells before and 24 hours or 96 hours after culture was measured by flow cytometric analysis.

Response Variation of HL-60 Cells to rhG-CSF before or after Treatment with ATRA *in vitro*

0.1 ml HL-60 cells (2×10^5 cells in the volume) were cultured in RPMI-1640 medium supplemented with 0.1 ml ATRA (2×10^{-6} mol/L). The HL-60 cells 24 or 96 hours post-cultured with ATRA were washed three times (1000 r/min for 15 min), and 0.1 ml rhG-CSF (10 μg/ml) was added in each well of the flat-bottomed 96-well plate. At the same time, the HL-60 cells without treatment with ATRA were used as controls. MTT method was used to detect the proliferation.

Detection of c-myc Gene in HL-60 Cells

RNA extraction:

0.1 ml HL-60 cells (5×10^6/ml) were cultivated in a 96-well plate. 0.1 ml 2×10^{-6} mol/L ATRA was added with the cultivating condition of 5% CO_2 at 37°C for 0, 8, 24, and 96 hours. Then the HL-60 cells were centrifugated (2000 r/min for 5 min) for washing three times and the number of HL-60 were counted cells. Total RNA was prepared according to the conventional method of single-step method of RNA isolation by acid guanidine thiocyanate-phenol-chloroform extraction.[5]

Dot Blot Hybridization

The membranes were baked for 2 hours at 80°C. DNA fragments were prepared for producing radioactive DNA probe using a labeling kit (Pharmacia). The specific activity was around 1×10^8 cpm/μg DNA. Membrane was prehybridized in prehybridication solution (5 × Denhardts solution, 0.5% SDS, 20 mg/ml Salmon sperm DNA) for 1 hour at 65°C. Labeled probe was then added, hybridized 12 hours at 65°C. After hybridizing, the membrane was washed twice with 2 × SSPE, 0.1% SDS at room temperature for 10 min, followed by 1× SSPE, 0.1% SDS for 15 min at 65°C and 0.1 × SSPE, 0.1% SDS for 10 min at 65°C. The membrane was then air-dried and exposed to X-ray film for 36~48 hours at -20°C.

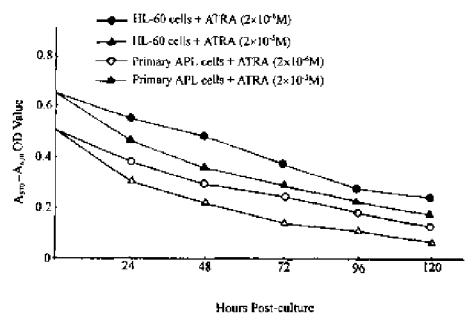
RESULTS

Effect of ATRA on the Proliferation of HL-60 Cells or Primary APL Cells

It was showed that ATRA could obviously inhibit *in vitro* the proliferation of HL-60 cells or primary APL cells.

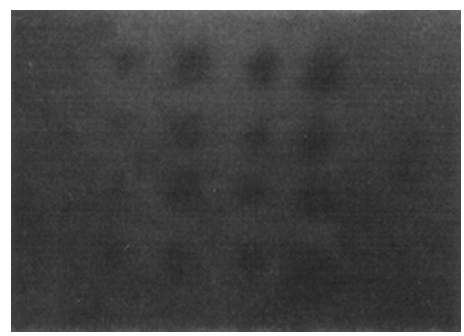
Variation of c-myc Gene in HL-60 Cells after Treatment with ATRA

Figure 2 shows that c-myc expression of HL-60 cells before ATRA treatment, 8 or 24 hours after ATRA treatment, was higher than that of 96 hours post treatment with ATRA. On the other hand, higher c-myc gene expression before or 24 hours post-treatment with ATRA was especially obvious.



*P<0.05~0.01 as compared with that of pre-treatment with ATRA.

Fig. 1. Effect of ATRA on proliferation of HL-60 cells and primary APL cells.



A B C D

A. Before ATRA treatment
B. 8 hours after ATRA treatment
C. 24 hours after ATRA treatment
D. 96 hours after ATRA treatment

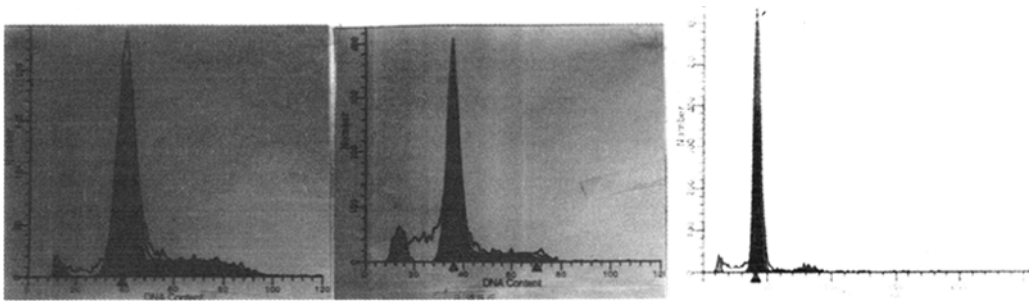
Fig 2. Variation of c-myc gene in HL-60 cells after treatment with ATRA

Cell Cycle Shift of HL-60 Cells before or after ATRA Treatment

The results in Table 1 and Figure 3 show that G_0/G_1 percent was increased 24 h or 96 h after ATRA treatment.

Table 1. Cell cycle shift of HL-60 cells after ATRA treatment

Term	Cell cycle percent(%)				
	G_0-G_1	G_2-M	S	G_2/G_1	% CV
Before treatment with ATRA	70. 6	0. 0	29. 4	2. 20	9. 14
24 h after treatment with ATRA	77. 9	2. 56	19. 4	1. 98	7. 07
96 h after treatment with ATRA	93. 7	1. 92	3. 38	1. 96	6. 03



A. Before ATRA treatment B. 24 h after ATRA treatment C. 96 h after ATRA treatment

Fig. 3. Cell cycle shift after ATRA treatment

Proliferation Sensitivity of rhG-CSF on HL-60 Cells after ATRA treatment

It was shown that HL-60 cells 24 hours or 96 hours after treatment with ATRA were more sensitive to rhG-CSF effect as compared with that before ATRA treatment. Exact results are detailed in Figure 4.

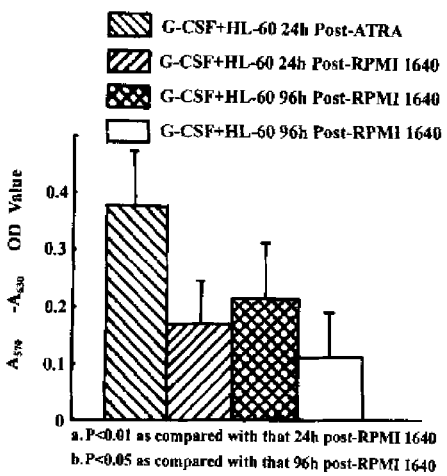


Fig. 4. Proliferation sensitivity of HL-60 cells after ATRA treatment.

DISCUSSION

It was well known that cytokines are correlated closely with the proliferation or differentiation of normal white cells or leukemia cells, especially with granulocytes or myelocytic leukemia cells. Hyperleukocytosis occurs in cases with APL after treatment with ATRA and often causes serious complications or even death, but the mechanism of hyperleukocytosis has not been clearly demonstrated. We first detedt4d serum level of G-CSF, IL-6 or TNF activity and found that only serum G-CSF was correlated with variation of WBC.[4,6-8] In the present study, we have done a lot of work to find out the exact concrete relationship between G-CSF and proliferation of leukemia cells, so we can evaluate the important role of G-CSF in hyperleukocytosis.

Our previous study showed that ATRA inhibited *in vitro* the proliferation of HL-60 or primary leukemia cells,[7] so it could not directly promote the proliferation of leukemia cells and cause the augmentation of the number of WBC in cases with APL after ATRA treatment. It was possible that promyelocytic leukemia cells were more sensitive to proliferation-related factors, for example G-CSF, after treatment with ATRA. In the present study, it is indicated that HL-60 cells 24 hours or 96 hours after-treatment with ATRA were indeed more sensitive to G-CSF *in vitro*. At the same time, cell cycle was measured by flow cytometric analysis. The results showed that G_0/G_1 of HL-60 cells was increased obviously after ATRA treatment, for example, 93% at 96 hours post-treatment with ATRA. By reviewing references,[9] it was easily found that the target term of G-CSF effect was G_0/G_1, so we can consider it possible that a higher serum level of G-CSF *in vivo* accompanied with G_0/G_1 increment must play an important role in hyperleukocytosis in cases with

APL after treatment with ATRA.

It was well demonstrated that c-myc gene plays an important role in proliferation and differentiation of leukemia cells for example, lower expression of that gene and without any cytokines came no proliferation; higher expression and less cytokines with cell apoptosis; higher expression and more cytokines with higher proliferation. In view of the above facts, dot blot hybridization was used to detect the c-myc gene expression in HL-60 cells before or after treatment with ATRA than at 96 hours after ATRA treatment. On the other hand, the expression at 24 hours with the low and it at 96 hours with the lower relatively. It is evident that the higher expression of c-myc accompanied with higher serum G-CSF could cause the higher proliferation of HL-60 cells and might be one of its roles in hyperleucocytosis.

The proliferation or differentiation of leukemia cells was affected by more cytokines and other factors *in vivo*. On the other hand, the results of the experiments *in vitro* were not always the same as that *in vivo*. Certainly, we still couldn't reach the conclusion that C-CSF was the chief or key factor to induce the hyperleucocytosis in APL after ATRA treatment. Further works will be done to find out the key mechanism of the hyperleucocytosis. But at least we can consider that G-CSF plays an important role in hyperleucocytosis and somehow relates with higher c-myc expression or G_0/G_1 ratio increment according to this present study.

REFERENCES

[1] Fenaux P. Management of acute promyelocytic Leukemia. Euro J Haematol 1993; 50: 65.

[2] Chen ZX, Xue YQ, Zhang R, et al. A Clinical and experimental study in alltrans retinoic acid treated acute promyelocytic leukemia. Blood 1991; 78: 1413.

[3] Huang M, Yu-chen Y, Shu-rong C, et al. Use of all trans retinoic acid in the treatment of acute promyelocytic Leukemia. Blood 1988; 72: 567.

[4] Jiang Guosheng, Sun Guanlin, Wu Wen, et al. Study on variation of serum G-CSF level in acute promyelocytic leukemia treated with all trans retinoic acid. Chin J Hematol 1994; 15:585.

[5] Groppe JC, Morse DE. Isolation of full length RNA templates for reverse transcription from tissues rich in RNase and proteoglycans. Anal Biochem 1993; 210:337.

[6] Jiang Guosheng, Sun Guanlin, Wu Wen, et al. Detection of interleukin 6 level in patients with acute promyelocytic leukemia. Tumor 1993; 13:247.

[7] Jiang Guosheng, Wu Wen, Zhou Rongfu, et al. Studies on relation between TNF and acute promyelocytic leukemia. Immunol J 1993; 9:350.

[8] Jiang Guosheng, Wu Wen, Zhou Rongfu, et al. Effection of ATRA and cytokines on proliferation of primary APL cells. Chin J Immunol 1994; 10:142.

[9] Doner P. Retinoic acid enhances colony stimulating factor-induced colonal growth of normal human myeloid progetitor. Exp Cell Res 1982; 138: 192.