VARIATION OF SERUM G-CSF LEVEL IN APL TREATED WITH ALL-TRANS RETINOIC ACID

JANG Guosheng 姜国胜,* SUN Guanlin 孙关林, TANG Tianhua 唐天华, WU Wen 吴文
ZHOU Rongfu 周荣富, WU Weili 邹维礼, LI Xiusong 李秀松, SHEN Zhixiang 沈志祥
ZHANG Fenqin 张芬琴, CHEN Yu 陈钰, WANG Zhenyi 王振义

Rui-Jin Hospital, Shanghai Institute of Hematology, Shanghai 200025, China

ABSTRACT

Objective: To detect the level of serum G-CSF. from patients with acute promyelocytic leukemia pre- or post-treatment with ATRA and analyze the relationship between serum G-CSF and hyperleukocytosis. Methods: Enzyme-linked immunosorbent assay (ELISA) method was developed and used in detecting serum G-CSF. Linear correlation test and Spearman rank order correlation coefficient were used as the statistical analytical method. Results: The levels of serum G-CSF increased in 11.4% (4/35) of APL patients (equal of more than 0.095 ng/ml). It was also found that serum G-CSF level in 25 APL patients started to increase from the 6th day to 12th day and then gradually declined after treatment with ATRA. Both serum G-CSF and WBC numbers increase in 72% (18/25) patients; no obvious variation of WBC and increase of serum G-CSF and augmentation of WBC were seen in 12% (3/25) of the cases with APL. It was also demonstrated that serum G-CSF level was statistically related to the WBC number (r=0.275, $P<0.05$), promyelocytes (r=0.2015, $P<0.05$) or more matured granulocytes (r=0.2055 $P<0.05$) by Spearman rank correlation analysis. Conclusion: The results of this study strongly indicate that G-CSF variation in patients with APL after treatment with ATRA plays an important role in hyperleukocytosis of WBC increase.

Key words: APL, All-trans retinoic acid, Hyperleukocytosis, Serum G-CSF

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*Now working in Shandong Academy of Medical Sciences.
Correspondence to: Sun Guanlin, Rui-Jin Hospital, Shanghai Institute of Hematology; No.197, Rui-Jin Rood II, shanghai 200025, China; Fax:(0086-021)-64743206; Phone: (0086-021)-64370045.

It has been demonstrated that all-trans retinoic acid (ATRA) was effective for treating patients with acute promyelocytic leukemia APL cells.[1,2] High CR rate are obtained with (ATRA), in newly diagnosed APL and in APL relapsing after prior chemotherapy. A high response rate is also noted on patients resistant to chemotherapy. With more widespread use of ATRA, however, side effects are usually moderate, for example, dryness of skin, mucosa, bone pain and headache. Another more important side effect is moderate and transient hyperleukocytosis in APL patients after treatment with ATRA. In some patients, however, hyperleukocytosis may develop very rapidly, leading to clinical sighs of leukostasis and possible death. Although it is considered that hyperleukocytosis can induce serious complications in APL cases, its mechanism has not been clearly demonstrated.[3] The present study is with aim to detect the mechanism.

Because proliferation of normal hemapoitic cells and leukemia cells were regulated by some cytokines, for example, granulocyte colony-stimulating factor (G-CSF) is a glycoprotein that stimulate chiefly the proliferation and activation of neutrophilic granulocytes *in vivo* or *in vitro*,[4,5] therefore, it is of great value to exam serum G-CSF variation in patients with APL after ATRA treatment. In the present study, an enzyme linked immunosorbent assay (ELISA) was established and serum G-CSF of patients with APL pre- or post-treatment with ATRA, including period of hyperleucocytosis. Furthermore, we also analyzed some relationship between hyperleukocytosis and serum G-CSF. Result of the study was reported as follows.

MATERIALS AND METHODS

Cases of Patients with APL

35 cases of APL, 27 men, 8 women, and age median age 30 years (from 5 to 72 years). All of them

were treated by ATRA as same as reported earlier.[6] 18 healthy volunteers as controls.

Samples

Serum samples were obtained from the above patients and healthy volunteers. Serum was separated by centrifugation shortly after collection, and all samples were stored frozen (-80°C) until use. Blood samples for WBC counts and its multiplying was drawn on the same day as G-CSF assay.

G-CSF and Anti-G-CSF Serum

Rh G-CSF was supplied by professor San Bi He Mei (Sakitama Medical University, Japan), 0.3 μg/ml. For production of rabbit anti-rhG-CSF serum, 0.5 mg of the purified recombinant G-CSF was dissolved in 0.5ml of saline, emulsified with complete Freund's adjuvant, and injected subcutaneously three times at 2 week intervals. Serum collected from the immunized rabbit 5 days after the last injection was subjected to fractional precipitation with ammonium sulfate (separately 50%, 45% and 33% saturation). And then to SPA-sephorose 4B gel filtration for isolation of the IgG fraction. The purified IgG was conjugated to hoseradish peroxidase (HXP) by the method of being reported.[7] and then redissolved for use as a secondary antibody.

Elisa of G-CSF

Briefly, this assay is based on a double-antibody sandwich method. Plates pre-coated with 0.1 ml monoclonal mouse anti-rhG-CSF antibody (20μg/ml) are incubated + 4°C overnight with duplicates. After washing three times with 0.01M PBS containing 0.05% Tween 20, a bovine serum albumin solution and the plates covered and incubated at + 37°C for 2 hours. After a further three times washing, a standard or serum specimen was added in duplicates; RhG-CSF-standards ranging from 0.024~25 ng/ml. After covering, the plates were incubated 2 hours at + 37°C and then washed three times before adding the IgG-HRP. Conjugated anti-rhG-CSF (0.1ml/well, 1: 500). Then washed three times, adding 0.1ml/well color reaction mixture. After incubation for 20 minutes at room temperature in the dark, the reaction was stopped by adding 50μl 4N sulfuric acid, and the resulting optical density was measured at 492 nm using a MB 4000 enzume-linked detector. Analysis method of statistics: *t* test, linear correlation, Spearman rank order correlation coefficient (γs) are used as indicated in the test.

RESULTS

G-CSF Level in Samples

All 18 healthy volunteers had non-detectable serum levels of G-CSF. 4/35 APL patients were above the detection limit before treatment. 18 APL patients at WBC peak-starting period and WBC peak time separately were 15/18 and 15/18 above the assay sensitivity. 6/6 cases of patients with infectious disease were all above the detection limit. The being-detected percent and level median just as Table 1.

Detectable Percent of Serum G-CSF

In 25 APL patients, G-CSF level was measured at 3 days interval periods after ATRA treatment, results were indicated as detectable percent (equal or more than 0.095ng/ml), detailed results as Figure 1. G-CSF detectable percent of pretreatment. WBC peak and CR just as Figure 2.

Table 1. Serum G-CSF in healthy volunteers and in APL patients pre- or post- treatment with ATRA

	n	Serum G-CSF* detectable case	Serum G-CSF detectable percent(%)	Serum G-CSF median (ng/ml)
Normal controls	18	0	0	<0.095
Pretreatment APL	35	4	11.4	<0.13 (0.095~0.199)
Beginning of WBC increase	18	15	83.3#	0.100 (0.095~0.218)
APL at WBC peak	18	15	83.3#	0.108 (0.095~0.210)
Infectious cases**	6	6	100#	0.47 (0.15~3.20)

* Non-detectable indicate less than 0.095ng/ml.

** 3 cases with phenominia, 3 cases of APL patients accompanying infection.

\# $P<0.01$ as compared with controls or pre-treatment.

Correlation Analysis between Serum G-CSF and Total Number of WBC and Its Types or Body Temperature

Because of serum G-CSF in some samples were too low to be sensitive analysis was applied to the assay, Spearman rank correlation coefficient. The

results are detailed in Table 3.

On the other hand, concrete relationship between G-CSF level and WBC increase in 72% (18/25) patients, no obvious variation of WBC was observed in 16% (4/25) of the cases. Augmentation of WBC with no increase of serum G-CSF was seen in 12% (3/25) of the cases.

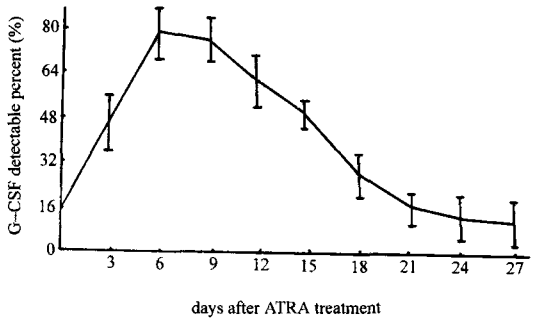

Fig.1. Variation of G-CSF detectable percent during ATRA treatment

Effect of Serum G-CSF with the Detectable or Not on WBC Number and Its Multiplying ($\bar{x} \pm s$)

By concret analysis, it was also found that WBC number or its multiplying in group with detectable G-CSF was obviously higher than that in group with nondetectable G-CSF, detailed results as Table 2.

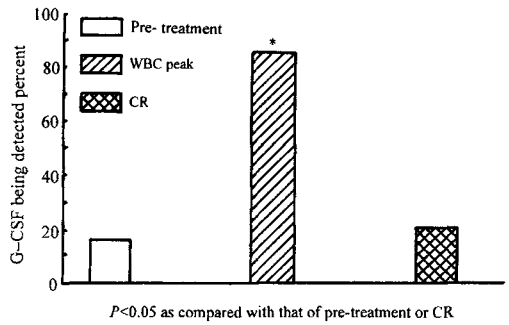

Fig. 2. WBC variation and its time related serum G-CSF detectable percent

Table 2. Effect of serum G-CSF with detectable or not on WBC number and its multiplying

	n	WBC $\bar{x} \pm s$ (x10'/l)	Promyelocytes $\bar{x} \pm s$ (x10'/l)	More matured granulocytes (x 10'/l)	Lymphocytes $\bar{x} \pm s$ (x 10'/l)
G-CSF detectable group	56	16.6 ± 16.3	9.35± 15.4	7.03 ± 6.71	4.9 ± 5.28
G-CSF non-detectable group	46	7.6 ± 9.1	1.53 ± 3.10	3.58 ± 3.09	2.17 ±2.0
		P<0.01	P<0.01	P<0.05	P<0.05

Table 3. Correlation between serum G-CSF and body temperature, WBC number or its types

N=100	G-CSF to WBC	G-CSF to promyelocytes	G-CSF to more matured granulocytes	G-CSF to lymphocytes	G-CSF to temperature
γs	0.275	0.247	0.2055	0.1869	0.0086
P	<0.05	<0.05	<0.05	>0.05	>0.05

DISCUSSION

Immunoassay studies have been reported for G-CSF with a sensitivity of 0.03 ng/ml,[5] 0.05 ng/ml[9] and 0.25 ng/ml[10] respectively. For the present study, it was 0.095 ng/ml. Early investigation indicated that serum G-CSF was non-detectable in all analyzed normal individuals analyzed. Our results of non-detectable levels of G-CSF in normal are in agreement with the other reports. For example, Watari, et al. using an immunoassay, found G-CSF >0.05 ng/ml in 4/56 normal individuals.[8] and Shirafuji, et al. did not, using a bioassay, find any detectable levels in 10 individuals.[11]

Although the newly developed ELISA was not so sensitive as to determine serum G-CSF levels in most normal persons, we did demonstrate with the use of this method that the G-CSF levels in most patients with acute myelocytic leukemia (AML) are often higher than that of the normal. In AML patients G-CSF could be detected in 54% and in ALL/AUL in 40% of analyzed samples.[9] Others, using an immunoassay found an increased G-CSF concentration in 5/12 patients with AML[6] Results of the present investigation showed that elevated G-CSF in APL percent was 11.4% (4/35), this differences may be due to different sensitivity. In addition, elevation of the G-CSF level was obviously

accompanied by an increased WBC number in some cases of bacterial infection in other reports[9] or the present study.

ATRA treatment could obtain high CR rates in newly diagnosed APL or in APL after prior chemotherapy.[12] It has been clearly demonstrated that ATRA was effective in inducing differentiation of APL cells.[13] With widespread use of ATRA, however, very common as well as more important side effects have been reported,[13] such as moderate and transient hyperleukocytosis, more than ten times as high as that of pre-treatment. The hyperleukocytosis often occurred after 15 to 20 days of treatment with ATRA. In some patients, however, hyperleukocytosis may develop very rapidly. In our experience with ATRA in newly diagnosed APL, approximately one-third of them showed rapidly increasing leukocyte counts in 7 day.

Through observing serum G-CSF and number of WBC in 25 cases of patients during the course of ATRA treatment, we found that all total number of WBC, promyelocytes, more matured granulocytes of lymphocytes increased obviously and reached the peak of their numbers 10 days after treatment with ATRA. Otherwise, serum G-CSF detectable level started to be increased on the 3rd day, and the peak of G-CSF level appeared on 6^{th} day, so the level of serum G-CSF and number of WBC or its types vary relatively with the same trend. Furthermore, it is interesting to note that level of serum G-CSF was significantly correlated with the total number of WBC, promyelocytes and the more matured granulocytes, but didn't correlate with lymphocytes. The possible mechanism was due to its main effect on the G-CSF of granulocytes.[4] By concrete analysis, both serum G-CSF and WBC increased in 72% (18/25) of the patients, with no obvious variation of WBC. But with increasing serum G-CSF in 16% (4/25) of the cases, no obvious increasing serum G-CSF was observed but WBC augmentation were seen in 12% (3/25) of the cases. The results indicated that relationship between serum G-CSF and number of WBC was sophisticated. Others also reported that level of serum G-CSF is not always parallel to variation of peripheral WBC.[14]

Because growth of normal hemapoitic cells or leukemia cells could be affected by many cytokines, detection of more cytokines at the same time will help to demonstrate the mechanism of hyperleukcytosis during that course of ATRA treatment.

Although not a common feature or not a concrete mechanism of serum G-CSF production, the leukemia cells have been showed to produce G-CSF[15] and it cannot be excluded that such a mechanism contributed to the increasing levels of serum G-CSF in APL patients after ATRA treatment.

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