Review

Relationship between Cytokines and Leukocytosis in Patients with APL Induced by All-Trans Retinoic Acid or Arsenic Trioxide

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Leukocytosis or hyperleukocytosis occurs during ATRA or arsenic trioxide differentiation therapy, which is related to the RA syndrome. The number of WBC often increased by ten or more times as high as that of pretreatment, around 7 to 20 days after treatment with ATRA or arsenic trioxide. Usually, when number of WBC tended to peak, there was concomitance with down-regulation of promyelocytes, up-regulation of myelocytes and more mature neutrophils. The same trend of classification of BM was observed in most of the patients with APL to whom leukocytosis happened. Although the mechanism of leukocytosis has not been demonstrated clearly, so far the proliferation hypothesis by cytokines and rheological hypothesis by adhesion molecules were taken into consideration. Otherwise, hypothesis about more divisions of differentiated myelocytes induced by ATRA or arsenic trioxide remains unclear. Usually, this kind of leukocytosis or hyperleukocytosis itself requires no additional cytotoxic treatment. *Cellular & Molecular Immunology*. 2006;3(6):421-427.

Key Words: acute promyelocytic leukemia, leukocytosis, hyperleukocytosis, ATRA syndrome

Introduction

Acute promyelocytic leukemia (APL) is a kind disease with high mortality, often causing infection and haemorrhagic diarresis in early period. Although conventional chemotherapy is effective in making most of APL patients get complete remission (CR), it also results in some serious side effects, for example serious infection or haemorrhagic diarresis, which is contributable to the effect on both leukemia cells and normal hemopoietic cells. All trans-retinoic acid (ATRA) was produced in Shanghai and first used to treat patients with APL there (1), then it was kindly provided to treat APL patients abroad subsequently (2). The results showed that ATRA is able to induce CR in almost all patients with APL through *in vivo* differentiation of APL blasts. Overall, more than 90% of patients with newly diagnosed APL can achieve CR and about 75% can be cured by the combination of ATRA and chemotherapy (3). Now the addition of ATRA to standard treatment regimens for APL has more than doubled survival of patients compared with the use of cytotoxic chemotherapy alone (4-7). But with the widespread use of ATRA in treatment of APL, an activation of leukocytes inducing an increase in white blood cell count is observed during treatment with ATRA alone. The WBC count often increased by ten or more times as high as that of pre-treatment. Treatment with ATRA has also been associated with the development of leukocytosis in approximately one half of patients with APL (8-10). Recently, a discovery on the therapeutic effect of arsenic trioxide (As_2O_3) in APL has revived this ancient drug (11-15). It has been well demonstrated that As_2O_3 induced a high CR rate in both primary and relapsed APL patients (around 85-90%). Interestingly, during As_2O_3 treatment in APL patients, it also induced leukocytosis in about 50% of patients (16-18). As to the above-mentioned leukocytosis, the proliferation hypothesis and rheological hypothesis were taken into consideration (19, 20). Although some results so far have indicated that some cytokines and changes in cellular rheology were related to the variation of leukocytosis, no definite explanation for such a proliferative effect has been clearly established.

Leukocytosis induced by ATRA or arsenic trioxide

According to the experience of French and European, hyper-leukocytosis occurs during ATRA differentiation therapy in about 70% of *de novo* and 25% of relapsed APL cases. In

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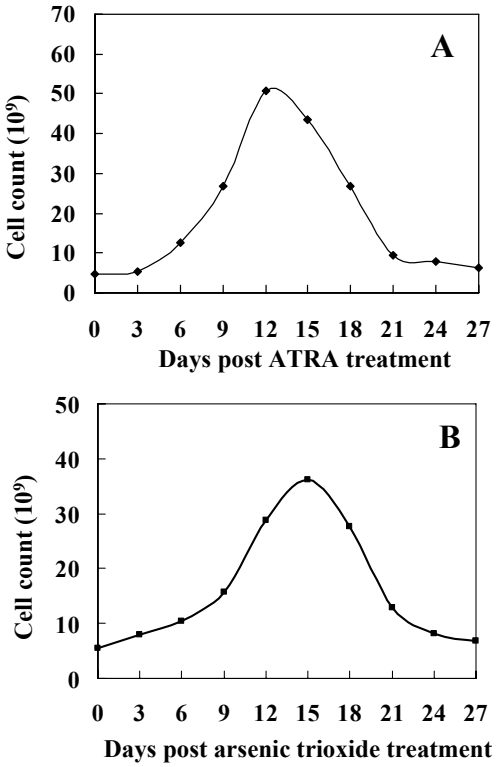

Figure 1. Characteristics of total WBC count in APL patients after treatment with ATRA or arsenic trioxide. Mean total leukocyte count of all patients with APL who underwent induction therapy with ATRA (A) or arsenic trioxide (B) at different time points. Cited from Jiang GS, et al., Chin J Cancer Res. 2003;15: 33-37 (10) and Jiang GS, et al., Shanghai Mian Yi Xue Za Zhi. 2003;23:190-193.

some cases, however, hyperleukocytosis may develop very rapidly, leading to serious side effects even death. The number of WBC often increased by ten or more times as high as that of pre-treatment, around 15 to 20 days abroad and 7 to 14 days in China respectively after treatment with ATRA (8-10). On avarage, the up-regulation of WBC sustains 12 to 15 days, and then down-regulated gradually (Figure 1A). The same trend of classification of bone marrow (BM) was observed in most of the patients with APL to whom leukocytosis happened.

Over the past several years, As_2O_3 as an effective salvage treatment for relapsed and/or refractory APL patients has been confirmed worldwide. Similarly, albeit less striking, changes in the morphology and immunophenotype of leukemic cells to ATRA have also been described (21-24). A group completed pilot clinical studies with this drug and has found that this new agent also induced both leukocytosis and related RA syndrome in substantial numbers of patients with APL (11). Fifteen of the 26 (58%) patients developed leukocytosis at some time points during induction therapy with arsenic trioxide. In these patients, the leukocyte count reverted to normal levels after a median of 29 days of treatment (Figure 1B). In some cases, the leukocyte count increased to high levels, the count exceeded 100,000 cells/ml. The median level of the leukocyte count at baseline (3,900 cells/ml) was somewhat higher in those patients who developed leukocytosis relative to those who did not (2,100 cells/ml; range 500 to 5,400 cells/ml). Eight of 26 (31%) patients developed the RA syndrome during induction.

On the other hand, some cell-surface antigens were also correlated to the leukocytosis. For example, APL blasts carry lymphocyte markers such as the B-cell marker CD19, which seems to be associated with high white blood cell counts (25). The T-cell marker CD2 is sometimes expressed in acute promyelocytic leukemia, and CD2 is reported to correlate with clinical characteristics. Among 29 APL, 6 were positive for CD2, APL patients with CD2 positive tended to have a higher leukocyte count than patients with CD2 negative, morphological characteristics as variant-APL (50% *vs* 0%) (26). Vahdat Linda et al. found no correlation between the types of PML/RAR-α transcript, morphology, or presence or absence of coagulopathy relative to development of the RA syndrome (27). They found a highly significant relation between expression of the surface marker CD13 relative to the peak leukocyte count and, to a lesser extent, relative to the development of the RA syndrome. No significant relations were observed with the other surface antigens (i.e., CD2, CD7, CD16, and CD33). Depending on the mentioned facts above, we concluded that either ATRA or arsenic trioxide could induce the occurrence of leukocytosis in APL patients, and some cell surface antigens were related to it.

Differentiation is closely related to the leukocytosis

Unlike conventional cytotoxic chemotherapy or radiotherapy, ATRA has initial biologic effects characterized by the differentiation of the malignant cells and phenotypically mature myeloid cells (1, 2, 28). A common feature of these drugs is the induction of cell differentiation, which has been associated with characteristic changes in morphology and immunophenotype (8, 9, 29, 30). The most remarkable feature was the progressive change of malignant cells in BM, with signs of their terminal differentiation and with Auer rods being sometimes observed in mature cells (2, 31). As to the relationship between differentiation and leukocytosis *in vivo*, the percent of promyelocytes was down-regulated 12 days after treatment with ATRA, but the percent of myelocytes, metamyelocytes and more mature myeloid cells was obviously up-regulated, especially for the percent of myelocytes or myelocyte-like cells (from median 0.006 to 0.216). The increment of myelocytes was in agreement with the peak of peripheral WBC count (29). The leukocytosis or hyperleukocytosis chiefly contributed to the up-regulation of myelocytes or myelocyte-like cells.

The clinical effectiveness of As_2O_3 in APL has also stimulated research activities aiming at an understanding of its mechanisms of action. *In vivo* and *in vitro* data suggested that induction of apoptosis and partial differentiation of APL cells are likely to constitute the cellular basis of the effects of

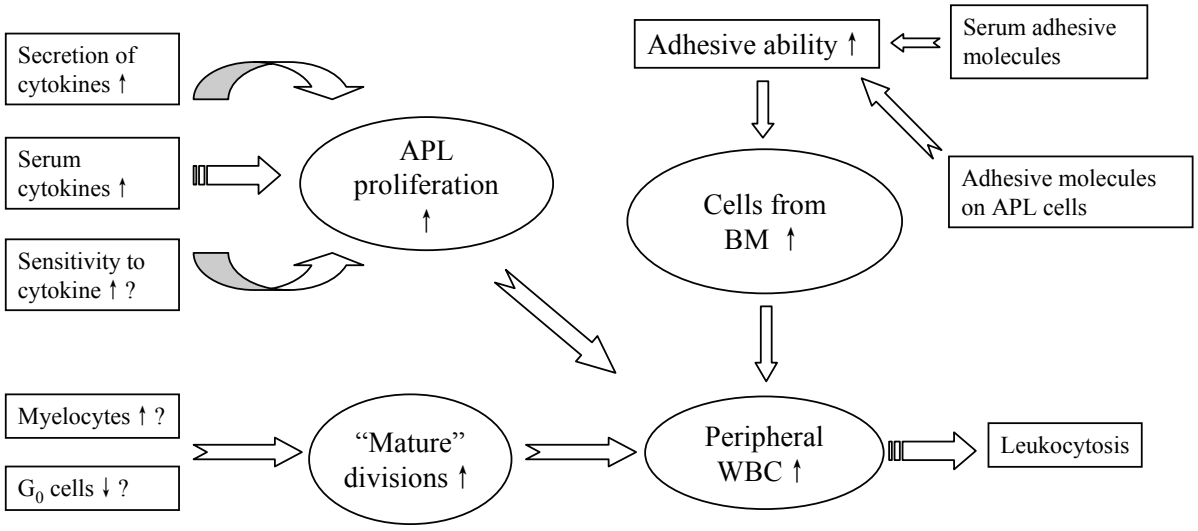

Figure 2. Mechanism model of leukocytosis in patients with APL after treatment with ATRA or arsenic trioxide. The proliferation hypothesis chiefly includes three aspects, for example, the proliferation of APL cells was up-regulated after *in vivo* exposure to ATRA or arsenic trioxide by the way of cytokine self-secretion and high level of serum cytokines, or possible up-regulation of sensitivity of APL cells to cytokine stimulation. The rheological hypothesis indicated by the up-regulation of adhesive molecules and adhesive ability by adhesive index, which results in the easy release of mature APL cells from BM into peripheral blood, whether there is a switch from G_0 into cell cycles and more post-differentiation divisions of myelocytes should be further elucidated by direct evidence, which is indicated as division hypothesis.

As_2O_3. Evidence from APL patients, initial clinical observations showed that during daily continuous intravenous infusion of As_2O_3 in APL, a large amount of myelocyte-like cells and degenerative, with the gradual reduction of leukemic promyelocytes. The percent of pro-myelocytes was down-regulated, while percents of myelocyte-like cells, metamyelocytes and more mature myeloid cells were up-regulated (30-32). Interestingly, the myelocyte-like cells were derived from abnormal promyelocytes because they still carried the PML/RARα gene as revealed by the fluorescence *in situ* hybridization (FISH) analysis. Soignet et al. performed a further *in vivo* analysis on the mechanisms of action of As_2O_3. They reported that As_2O_3 therapy induced a progressive decrease in the proportion of cells expressing CD33, an antigen associated with primitive myeloid cells, along with an increase in the proportion of cells expressing CD11b, an antigen restricted to mature myeloid elements (17). Lallemand-Breitenbach et al. demonstrated that As_2O_3 induced modest differentiation of APL cells *in vivo*, and significantly prolonged the survival of the diseased animals, one of the As_2O_3-treated mice showing mature granulocytes in the diminished tumor (33).

In order to further approach the complex mechanisms, *in vitro* effects of pharmacological concentrations of As_2O_3 on fresh APL cells and APL cell lines including ATRA-sensitive NB4 cells, and ATRA-resistant NB4-derived sublines MR2, R1 and R2, were studied. The initial study showed that As_2O_3 at 1.0-2.0 mM could significantly induce apoptosis in APL cells regardless of their sensitivity to ATRA (24, 34), which was widely confirmed by other groups (35). In order to establish an *in vitro* model of differentiation, NB4 cells were treated with 0.1-0.25 mM of As_2O_3 over a long time of culture. Indeed, after 6-9 days of treatment, cells started to present morphologic differentiation with CD11b expression and differentiation-associated cytochemical features. The differentiation seemed only a partial one since most cells were blocked at the myelocyte to metamyelocyte stage of maturation and no significant increase of nitroblue-tetrazolium (NBT) reduction, a classical marker of granulocytic maturation, was observed in NB4 cells (24).

The pharmacokinetic studies were performed among eight relapsed patients by measuring plasma drug levels with gas chromatography (16, 34). The results showed that after a peak level of 6.85 (5.54-7.30) mM, plasma arsenic was rapidly eliminated, with a t1/2a of 0.89 (SD: 0.29) h and a t1/2b of 12.13 (SD: 3.31) h. Hence, over most times during As_2O_3 treatment, plasma arsenic levels fluctuated between 0.1-0.5 mM. Such a phamacokinetic behavior did not alter after continuous use of arsenic trioxide. Among 20 cases studied, a similar CR rate (80%) was achieved as compared to that attained with a conventional dose. The mean Cpmax was 2.63 (range, 1.54-3.42) mM, and the ranges of t1/2a and t1/2b were 1.2-2.7 h and 6.23-14.9 h, respectively. Of note, the arsenic concentrations in the plasma of BM in five patients 24 h after administration of the drug at low dose were 0.061-0.49 mM (35). So the *in vivo* study showed that the differentiation of APL cells was all the same in agreement with the occurrence of leukocytosis, especially contributing to the up-regulation of myelocytes. The up-mentioned pharmacokinetic studies also indicated that the serum

concentration of ATRA or arsenic trioxide in APL could only induce the differentiation of APL cells.

Cytokines and proliferation hypothesis in leukocytosis

As to the leukocytosis or hyperleukocytosis induced by ATRA or arsenic trioxide, the easiest suggested cause for this side effect is a directly ATRA-induced proliferation of APL cells. However, no definite explanation for such a proliferative effect has been clearly established. Firstly, the *in vitro* inhibition of ATRA or arsenic trioxide on proliferation of HL-60, NB4 or primary APL cells, which is indicated by cell viability, up-regulation of G_0/G_1 and down-regulation of S (36), showed that the leukocytosis may be not contributed to the colony-stimulating activity of ATRA or arsenic trioxide directly. In further support of the proliferation hypothesis, Warrel et al. observed 11 cases with APL, 4 in 11 cases happened to hyperleukocytosis, 2 cases were changed to use chemotherapy, 2 cases continuously treated with ATRA, the S phase did not change obviously in one case, and the S phase was up-regulated from 2% to 10% in the other one (37). Otherwise, this kind of cell cycle profile was not observed in most of APL patients with leukocytosis. So it is suggested that leukocytosis induced by ATRA may not represent an increase in leukemia cell proliferation, but rather a transient increase in the lifespan of leukemia cells, which would otherwise die at the promyelocyte stage without relief from the differentiation blockade that is associated with this disease (11).

In view of the close relationship between cytokines and leukemia cell proliferation, which is modulated by the way of autocrine or paracrine secretion, the relationship between some cytokines and leukocytosis was detected in patients with APL after treatment with ATRA or arsenic trioixde. G-CSF is a polypeptide growth factor that regulates the production of neutrophilic granulocytes. Besides proliferative effects, G-CSF appears to modulate certain neutrophil functions as well as the distribution of neutrophils and progenitor cells within the body, whose abnormality will lead to the change of granulocytic hematopoiesis. Some data demonstrated the relationship between serum G-CSF level and hyperleukocytosis (38, 39). Serum G-CSF levels and circulating blood cells were detected in 47 patients of APL during the treatment with ATRA, both serum G-CSF level and WBC number increased in 68.1% of the cases, in 19.2% of the cases, serum G-CSF level was increased without obvious change in WBC number, and the reverse was true in 12.7% of the cases. The detectable percentage of serum G-CSF began to elevate on the 3rd day of treatment, and the peak of detectable percent was on the 9th day. Otherwise, WBC number began to elevate on the 6th day and peaked on the 11th day. The change of detectable percent of serum G-CSF was similiar to WBC or serum G-CSF elevated in advance slightly. Spearman's rank-order correlation coefficient showed there were a positive relationship between serum G-CSF level and WBC, myelocyte and its late stage. There was no relationship between serum G-CSF level and lymphocyte.

Because both serum G-CSF level and WBC number increased in 68.1% of the cases, it was suggested that besides G-CSF, other cytokines may be connected with the mechanism of WBC increasing. Therefore, further research of diffferent cytokine levels may help to study the mechanism of leukocytosis due to ATRA. The variations of serum IL-6 activity (40) and TNF activity (41) level were detected in cases with APL after treatment with ATRA, otherwise, the activity level of serum IL-6 or TNF was reduced with the treatment with ATRA, and had no significant correlation with the variation of WBC number. The results of augmentation of serum IL-1β or IL-6 were also observed by some other study group (42). On the other hand, most of studies showed that the serum IL-8 was reduced after treatment with ATRA (43). The level of serum cytokines was easily influenced by infection and other factors, so each was not always in line with the effect of ATRA alone.

Certainly, the detection of their secretion *in vitro* is a good way to elucidate the concrete relationship between them and proliferation of leukemia cells. A few data so far concerning cytokine secretion by APL cells after *in vitro* ATRA treatment have been up to now reported in the literature (19, 20, 44, 45), and their results demonstrated that the secretion of IL-1β and G-CSF, in some cases, often increased and correlated to the proliferation of leukemia cells, and the secretion of IL-8 decreased obviously. Otherwise there were no obvious variations of IL-6 and TNF-α secretion. On the other hand, they also found that primary APL cells could express mRNA for IL-1β, IL-6 and TNF-α and seem sensible to *in vivo* ATRA treatment, with achievement of CR, whereas the expression of mRNA for G-CSF, GM-CSF and IL-3 appears related to non-achievement of CR (45). RA induces a variety of effects in APL cells *in vitro*, including increased production of IL-1β (46), IL-8 (47), and receptors for G-CSF and GM-CSF (48, 49). Secretions of IL-1β, IL-8, G-CSF, TNF-α, IL-8, GM-CSF in primary leukemia cells of patients with APL were detected before or after treatment with ATRA, the results indicated that the secretion of IL-1β, IL-6 and TNF-α could be detected in each patient before ATRA treatment, but not G-CSF secretion. The secretion level of IL-1β, IL-6 and TNF-α did not change obviously in 7 days after exposure to ATRA *in vitro*, 4 in 12 patients with an up-regulation of IL-1β and G-CSF. IL-8 secretion level was down-regulated in 11/12 patients after exposure to ATRA as compared with that precious exposure. TNF-α, IL-6, GM-CSF was not modulated by ATRA. Visani et al. measured the IL-1α, IL-3, IL-4, IL-6, IL-10, G-CSF, TNF-α and GM-CSF, and only found the down-regulation of IL-6 and GM-CSF and up-regulation of IL-1α, the others did not change obviously (44). Matikainen et al. reported that ATRA could activate IL-1β enhancer and induce its expression, IL-1β could directly, or by inducing G-CSF synthesis, promote proliferation of leukemia cells. IL-1α transgenic mice also showed a hyperleukocytosis model (46). Although ATRA

could up-regulate the number of GM-CSF receptors and its affinity, the expression of GM-CSF and its secretion were not modulated by ATRA in primary APL cells, so it is not related to the hyperleukocytosis. Otherwise, Zeng et al. found that serum IL-6 increased obviously from 3-10 days, at peak in 10-12 days, then gradually decreased, and the variation of IL-6 was related to hyperleukocytosis (42). But Dubois et al. reported that IL-6 expression was not regulated by ATRA *in vitro* in primary APL cells (45). And there were still some cases with hyperleukocytosis without any obvious increase of IL-1β or G-CSF secretion after *in vivo* ATRA treatment (50), so further work should be done to detect other related factors.

In the patients with APL induced by arsenic trioxide, APL cells also showed a significant increase of IL-1β and G-CSF production, and a significant decrease of IL-6 and IL-8, however, there was no obvious variation of TNF-α, if compared with that of APL cells without exposure to arsenic trioxide. On the other hand, the proliferation ratio of APL cells *in vitro* was statistically correlated to the IL-1β secretion ratio or G-CSF secretion ratio. And it was also showed that the cell number ratio of patients with IL-1β or G-CSF augmentation 9 days after arsenic trioxide therapy *in vivo* was higher than that of patients without IL-1β or G-CSF augmentation respectively (51). So most importantly, hereby the up-regulation of serum level of IL-1β or G-CSF, which was resulted from the secretion of them by APL cells, was related to the leukocytosis in patients with APL, but not IL-6, TNF-α or IL-8.

Adhesion molecules and rheological hypothesis

Extravascular migration of myeloid cells may be partly related to up-regulated integrin expression, which could increase adhesion of these cells to vascular endothelium, thereby facilitating their extravasation (52-55). *In vitro*, this process has been associated with increased expression of integrins, cytokine release, and changes in cellular rheology (56-58). Wang et al. demonstrated that the MMP9 expression in HL60 cells was up-regulated by exposure to ATRA, and MMP9 could promote the migration of differentiating myeloid cells into extravascular tissues (59).

Another mechanism directly related to the differentiation of marrow leukemia cells could be a change in their microrheology, allowing their release from the BM and their transfer toward peripheral blood and tissues. Using a single cell aspiration assay into a glass restrictive channel, a group measured APL cell viscosity values in five *de novo* APL patients. A deformability index (DI) was defined as the ratio of mean normal neutrophil viscosity × 100/mean APL cell viscosity. Results were the following: 1) at diagnosis, two patients had high marrow DI (96% and 250%) and three patients had low marrow DI (16%, 17% and 40%); 2) when PB and marrow APL cells were simultaneously tested, PB APL cells displayed higher DI than marrow APL-cells; 3) the two patients with high initial marrow DI experienced an ATRA-induced hyperleukocytosis after only 1 day of treatment; 4) in the three patients with low initial marrow DI, the DI was increasing during ATRA therapy and hyperleukocytosis seemed to occur when a large amount of maturing APL cells reached a viscosity value similar to that of mature neutrophils. These results suggested that an asynchronism between rheological and morphological maturation in each APL cell might explain the occurrence of hyperleukocytosis in some patients during ATRA differentiation therapy (60).

On fresh leukemia cells taken from 30 patients with APL the membrane expression of a series of adhesion molecules including β2 integrins (CD11a/LFA-1, CD11b/Mac-1), selectin ligands (CD15/Le(x), CD15s/Le(x)) and tyrosine-phosphatase isoforms (CD45RA, CD45RO) were analyzed. The expressions of these molecules were also studied in nine of these patients following the APL cells' culture with and without ATRA. The fresh APL promyelocytes expressed CD45RA and CD15s on their surface, while CD11a, CD11b, CD15, and CD45RO were constantly absent. *In vitro* treatment with ATRA consistently increased the expression of CD15, CD11b, and CD45RO on leukemic promyelocytes; these changes were paralleled by a decrease of CD45RA display. The expression of sialylated antigen CD15s was fully independent from CD15 suggesting a differential enzymatic regulation within this selectin ligand system. ATRA was, however, incapable of promoting the up-regulation of CD11a in APL. As a result, asynchronous phenotype ($CD11a^-$, $CD11b^+$, $CD15^+$, $CD15s^{+/-}$, $CD45RA^-$, $CD45RO^+$) was generated that is normally undetectable on maturing myeloid cells. In order to provide a further control a case of acute agranulocytosis was also investigated, in which > 75% bone marrow cells were arrested at the promyelocyte stage; these bone marrow cells showed a surface phenotype identical to non-leukemic promyelocytes ($CD11a^+$, $CD11b^+$, $CD15^+$, $CD45RO^+$, $CD45RA^-$) with a spontaneous ability to differentiate *in vivo* towards the more mature stages of myeloid differentiation. We therefore suggest that in fresh and ATRA-induced APL cells distinct, regular phenotypic changes are identifiable that are probably associated with (15, 17) and not seen in normal and activated bone marrow. The up-regulation of adhesion molecules could result in the augmentation of adhesive ability of APL cells induced by ATRA, which makes them easily to release from bone marrow into peripheral blood and induce the leukocytosis (61).

Summary

The leukocytosis and the RA syndrome are commonly observed in some patients with APL after treatment with ATRA or arsenic trioxide. The *in vivo* study showed that the differentiation of APL cells was all the same in agreement with the occurrence of leukocytosis, especially contributing to the up-regulation of myelocytes. The pharmacokinetic studies also indicated that the serum concentration of ATRA or arsenic trioxide in APL only could induce the differentiation of APL cells. As to the mechanism of leukocytosis, there are some hypotheses (Figure 2). The proliferation hypothesis was demonstrated by the up-

regulation of serum level of IL-1β or G-CSF, which was resulted from the secretion of them by APL cells, but not IL-6, TNF-α or IL-8. The rheological hypothesis was taken into consideration, indicated by up-regulation of adhesive index and adhesion molecules of differentiationed leukemia cells, which could result in the release of APL cells from bone marrow to peripheral blood. Most importantly, the divisions hypothesis should be taken into consideration, because the leukocytosis chiefly contributed to the up-regulation of differentiated myelocytes and more matured neutrophils, and the normal myelocytes is usually the key proliferaton pool of granulocytes, with 3-4 times of divisions from promyelocytes to myelocytes, otherwise, the direct evidence remains unclear. In our opinion, another possible mechanism also should be further elucidated, or most of APL cells enter into cell cycles after exposure to ATRA or arsenic trioxide *in vivo*, usually about 60% of APL cells were in G_0 phase before exposure to them.

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