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Arsenic Trioxide- and Idarubicin-Induced Remissions in Relapsed Acute Promyelocytic Leukaemia: Clinicopathological and Molecular Features of a Pilot Study

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Arsenic trioxide (As_2O_3) effectively induces remissions in relapsed acute promyelocytic leukaemia (APL), but the safety of its long-term administration is unknown. The anthracycline idarubicin is highly active alone or in combination chemotherapy for the treatment of APL. To minimize arsenic exposure and based on the high sensitivity of APL cells to anthracyclines, we conducted a prospective study to evaluate induction with As_2O_3 followed by consolidation with idarubicin in the treatment of APL in relapse. Eight patients were treated with As_2O_3 at a daily dose of 10 mg until remission, followed by three monthly courses of idarubicin, at 6 mg/m^2/day for 5 days in the first course and 6 mg/m^2/day for 2 days in the subsequent two courses. All patients achieved morphological but not molecular remission after As_2O_3 treatment. During As_2O_3 therapy, an increase in white cell count peaking at a median of 17 days occurred in all the cases. Serial flow cytometric analysis of apoptosis, with mitochondrial APO2.7 antigen expression and the sub-G1 cell fraction on DNA histogram as markers, showed induction of apoptosis of APL cells in vivo. With both qualitative and real-time quantitative polymerase chain reaction, all patients were shown to attain molecular remission after subsequent idarubicin treatment. With a median follow up of 13 months, seven of eight patients have remained in complete clinical remission, with six patients in molecular remission as well. One patient who was in third remission became PCR-positive after being transiently negative. One patient died from an intracranial extramedullary relapse after achieving marrow molecular remission. We conclude that As_2O_3 induction followed by idarubicin consolidation is an effective therapy for APL in relapse. This regimen avoids the possible long-term toxicities of As_2O_3 and mutagenicity of combination chemotherapy, a strategy that might be suitable for this potentially curable leukaemia. Am. J. Hematol. 66:274–279, 2001.

Key words: acute promyelocytic leukaemia; arsenic trioxide; idarubicin

INTRODUCTION

Acute promyelocytic leukaemia (APL) is characterized by the translocation t(15;17)(q22;q21) and the reciprocal fusion of the *RARA* gene on chromosome 15 with the *PML* gene on chromosome 17. Front-line treatment of APL includes the use of *all-trans*-retinoic acid (ATRA) with or without concomitant chemotherapy for the induction of remission, followed by intensive post-remission consolidation chemotherapy [1,2]. Maintenance therapy may be beneficial in some patients [3]. Although this strategy results in a complete remission rate of 70%–95%, relapse still occurs in about 20%–30% of patients [4], who in most cases would be resistant to further ATRA treatment.

Arsenic trioxide (As_2O_3) has recently been reported to induce remissions in relapsed APL patients [5]. Studies in vitro showed that APL cells underwent differentiation

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with low concentrations of As_2O_3 but were induced into apoptosis at high concentrations [6]. Molecular mechanisms of apoptosis included down-regulation of *bcl*-2 [6] and degradation of the *PML/RARA* fusion protein [7]. In malignant lymphoid and myeloid cells, As_2O_3 also induced apoptosis via non-*PML/RARA* dependent pathways [8], including activation of *c-Jun* NH_2-terminal kinases [9] and disruption of microtubule formation [10]. Recently, a mitochondriotoxic effect has also been proposed [11], with As_2O_3 inducing a collapse in the mitochondrial transmembrane potential ($\Delta\Psi_m$) preceding the apoptosis [11].

Clinically, As_2O_3 was reported in three series to result in a complete remission in 29 (85%) of 34 patients with relapsed APL [7,8,12]. The treatment toxicities were mild and reversible. However, the post-remission treatment strategy is less well defined. Patients were in general treated with additional courses of As_2O_3 as consolidation therapy [5,12,13]. The cumulative use of As_2O_3 has raised a concern over toxicities [13].

To minimize arsenic exposure and considering the high sensitivity of APL cells to anthracyclines, we conducted a prospective study of eight patients with relapsed APL to evaluate the therapeutic efficacy of a single induction course with As_2O_3 followed by consolidation therapy with idarubicin.

MATERIALS AND METHODS

Patients and Treatment

Eight consecutive unselected patients with APL in relapse were included in the study. They had all relapsed from ATRA and chemotherapy-induced remissions. The diagnosis of APL was confirmed by the demonstration of t(15;17) or *PML/RARA*. For induction of remission, As_2O_3 was given as a 1-hr intravenous infusion at a daily dose of 10 mg until complete remission (CR) (blasts and abnormal promyelocytes <5% of cells in the marrow) was achieved. Post-remission therapy consisted of three monthly courses of intravenous idarubicin, given at 6 mg/m^2/day for 5 days in the first course and at 6 mg/m^2/day for 2 days in the subsequent two courses.

Flow Cytometric Analysis of Apoptosis In Vivo

The percentage of APL cells undergoing apoptosis in vivo during As_2O_3 treatment was monitored by flow cytometry (Coulter XL, Coulter Beckman, Fullerton, CA). Early apoptotic cells were enumerated by staining with the phycoerythrin conjugated monoclonal antibody 2.7A6A3 (Immunotech, Miami, FL), after prior permeabilization of the cell membrane by digitonin as previously described [14]. The antibody 2.7A6A3 detects a mitochondrial membrane antigen APO2.7 exposed early in apoptosis [15]. Late apoptotic cells were enumerated after staining with propidium iodide. The sub-G1 fraction of cells in the DNA histogram (analyzed by ModFit *LT*, Verity Software House, Topsham, Maine) represented late apoptotic cells with endonuclease-mediated DNA fragmentation. Data from over 10,000 cells were analyzed in each experiment.

Molecular Monitoring

Reverse transcription polymerase chain reaction (RT-PCR) for *PML/RARA* was performed with two pairs of nested *PML* and *RARA* primers, with a sensitivity of 10^{-5} to 10^{-6} [16]. RNA extraction and first-strand cDNA synthesis was performed as previously described [17]. To evaluate the size of the APL clone, quantification of *PML/RARA* was performed by a two-step real-time RT-PCR (Q-PCR) with the ABI Prism 7700 sequence detector (Perkin-Elmer, Foster City, CA), a technique that allows simple and rapid quantification of a target sequence during the exponential phase of the PCR. Briefly, PCR primers for the genes *PML* (5′ CCT GCA AGC TGC CGT GC 3′), *RARA* (5′ GAA CTG CTG CTC TGG GT 3′) and the dual-labeled TaqMan probe (5′ (FAM) ATG GCT TCG ACG AGT TC (TAMAR) 3′) were designed by the Primer Express software (Perkin-Elmer). The *PML* primer used in this assay was on exon 3. In APL cases where the *PML* breakpoint was in intron 3 (leading to a *PML* exon 3/*RARA* exon 3 fusion) [18], a 111-bp PCR product would be obtained, which was within the optimal detection range of Q-PCR. On the other hand, when the PML breakpoint was in intron 6 (leading to a *PML* exon 6/*RARA* exon 3 fusion), a 585-bp PCR product would be obtained, which was outside the optimal detection range of the assay. Therefore, Q-PCR was only evaluated serially in five patients that had the *PML* exon 3/*RARA* exon 3 fusion. In order to minimize variability in the results due to differences in patient samples, the *PML/RARA* fusion transcript copy number was normalized to an internal control by Q-PCR of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript (GAPDH forward primer, 5′ GAA GGT GAA GGT CGG AGT C 3′; GAPDH reverse primer, 5′ GAA GAT GGT GAT GGG ATT TC 3′; GAPDH TaqMan probe, 5′ (JOE) CAA GCT TCC CGT TCT CAG CC (TAMRA) 3′). First-strand cDNA synthesis was performed with 1.0 μg of total RNA, sequence specific primers, and MMLV reverse transcriptase according to the manufacturer's instructions. Subsequent Q-PCR amplification reactions were set up in a reaction volume of 50 μl with sequence-specific primers, TaqMan probes, and TaqMan Universal PCR Mix (Perkin-Elmer) according to the manufacturer's instructions. The threshold cycle (C_T) at which a significant increase in fluorescence signal was first detected was determined. In order to ensure that serial results were comparable, each patient would have his or her own standard curve, constructed by leukemic RNA extracted from the sample before treatment. For the post-

TABLE I. Clinicopathological and Molecular Features of Eight Patients Treated With As_2O_3 for Relapsed APL*

Patient no. (relapse)	Sex/age	Blood counts			Peak WBC		Time to remission	PCR results after				Survival (months)	Outcome
		Hb	WBC	Plat	Count	Time		As_2O_3	IDA(1)	IDA(2)	IDA(3)		
1 (R1)	F/24	12.3	4.5	62	70.2	14 d	40 d	+ve	ND	−ve	−ve	16+	CR
2 (R1)	F/45	11.9	1.3	88	2.0	30 d	48 d	+ve	−ve	−ve	ND	15+	CR
3 (R2)	F/44	12.5	2.6	32	25.0	18 d	40 d	+ve	−ve	−ve	+ve	15+	CR
4 (R1)	F/24	11.3	1.3	109	14.3	19 d	40 d	+ve	−ve	−ve	−ve	14+	CR
5 (R1)	M/25	7.7	15.1	14	57.1	16 d	37 d	+ve	−ve	−ve	ND[a]	12	EMD, dead
6 (R1)	M/22	8.7	4.6	39	26.7	20 d	51 d	+ve	−ve	−ve	−ve	12+	CR
7 (R1)	M/44	10.4	0.5	16	2.0	12 d	32 d	+ve	ND	ND	−ve	11+	CR
8 (R1)	M/35	12.1	48.2	73	152.5	7 d	28 d	+ve	−ve	−ve	−ve	6+	CR

*R1, first relapse; R2, second relapse; Hb, hemoglobin (g/dl); WBC, white blood cell count ($\times 10^9$/l); Plat, platelet ($\times 10^9$/l); d, days; IDA, idarubicin; CR, complete remission; ND, not done; EMD, extramedullary disease.
[a]Marrow turned PCR-positive on relapse as EMD.

As_2O_3 treatment samples, the *PML/RARA* mRNA copy numbers were read off from each patient's own standard curve and expressed as relative copy numbers of the pre-treatment specimen. The sensitivity of Q-PCR was tested by serial dilutions of leukemic RNA, obtained from a case of APL at diagnosis, in normal RNA.

RESULTS

Patients and Treatment

The clinicopathological features and treatment results of the eight patients are shown in Table I. Seven patients were in first relapse, and one was in second relapse. All patients achieved CR with As_2O_3 treatment, after a median time of 49 days. Mild reversible derangement of liver transaminases was the only side effect observed in two patients. There was an asymptomatic increase in white cell count in all the patients, which peaked at a median of 17 days. Consolidation with idarubicin caused significant neutropenia ($<0.5 \times 10^9$/l) and resulted in four episodes of sepsis requiring hospitalization.

Flow Cytometric Analysis of Apoptosis In Vivo

Cells undergoing apoptosis were serially assessed in three patients (Cases 5, 7, and 8). During As_2O_3 treatment, there was a progressive increase in white cell count (Fig. 1a,b). The proportion of cells undergoing apoptosis in vivo also increased during treatment, with APO2.7 expression detecting a higher percentage of apoptotic cells than the sub-G1 cell population. The apoptotic cells reached a peak at about the end of the second week after commencement of As_2O_3 treatment. The increases in white cells and apoptotic cells positive for APO2.7 showed an almost parallel trend (Fig. 1a,b). Cells in the sub-G1 fraction also showed a similar trend at a lower percentage. This pattern was observed in all the three cases investigated.

Molecular Monitoring

RT-PCR successfully amplified *PML/RARA* in all cases prior to treatment. After As_2O_3 induced remissions,

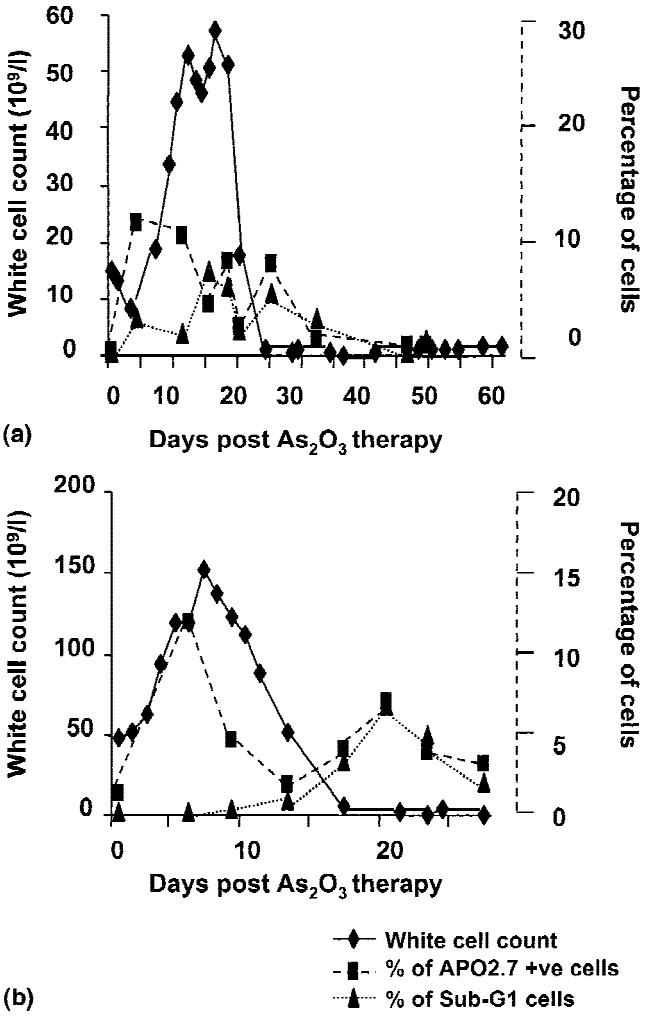

Fig. 1. (a) Changes of white cell count with apoptotic cells during As_2O_3 therapy in patient 5. A rise in cell count paralleled the increase in apoptotic cells, implying that the effect of As_2O_3 in vivo was a combination of differentiation and induction of apoptosis. (b) Patient 8, showing a similar pattern of change in white cell count and apoptotic cells.

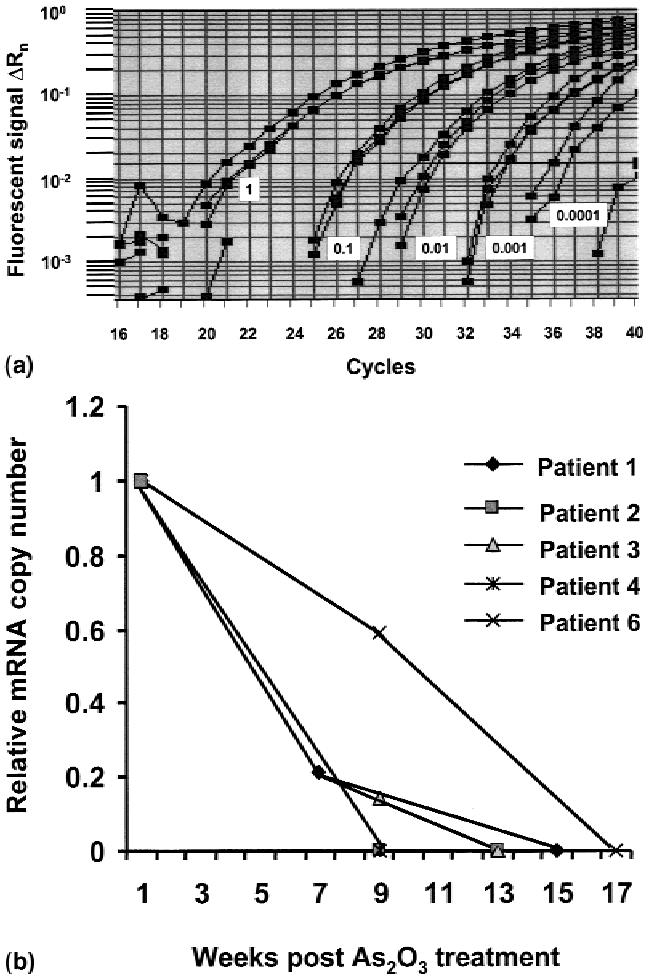

Fig. 2. (a) Sensitivity of real-time quantitative polymerase chain reaction (Q-PCR) of *PML/RARA*. At 10^{-4} dilution, only two of the triplicates showed concordant results, with the remaining one consistently showing a discordant result in repeated experiments. Therefore, the test was interpreted to show a sensitivity between 10^{-3} and 10^{-4}. (b) Decline of the *PML/RARA* fusion transcript with As_2O_3 treatment in five patients, as assessed by real-time Q-PCR.

all patients were still PCR positive, indicating the presence of minimal residual leukemia. However, all patients became PCR negative at various time points after idarubicin treatment. PCR remained to be negative on serial follow up in six of these patients (Table I). One patient (Case 3) who was in third remission reverted to PCR positivity 2 months after her last idarubicin treatment, although at the latest follow up she was still in complete clinical and haematological remission. Another patient (Case 5) remained PCR-negative until he suffered an extramedullary disease (EMD) relapse, when the marrow became PCR-positive again. The size of the APL clone was evaluated by Q-PCR. The sensitivity of Q-PCR was 10^{-3} to 10^{-4} (Fig. 2a), which was consistent with previous studies [19]. With Q-PCR, a decline of the *PML/RARA* fusion transcript was shown during As_2O_3 treatment, indicated a decrease in the size of the leukemic clone with treatment. The fusion transcript became undetectable after further consolidation with idarubicin (Fig. 2b).

Outcome

With a median follow up of 13 months, six of the eight patients have remained in morphological and molecular remission. One patient in third remission became PCR-positive after a transient period of negativity. She was the only patient in this series who had relapsed molecularly. Another patient (Case 5) had relapsed and died from an extramedullary disease (EMD) in the brain 12 months later.

DISCUSSION

As_2O_3 is highly efficacious in the treatment of APL in relapse, with a complete remission rate ranging from 57% to 100% [5,12,13]. However, the post As_2O_3 treatment strategy has not been well defined. Shen et al. [5] and Soignet et al. [12] reported that patients achieved clinical and in some cases molecular remission after prolonged and repeated courses of As_2O_3 treatment. However, acute and chronic arsenic toxicities were noted in patients given cumulative doses of over 1 g, including fluid accumulations, neuropathies, and muscle atrophy [13]. Furthermore, chronic arsenic administration might also lead to dose-related skin pathologies, gastrointestinal bleeding, and visceral malignancies [20]. Therefore, the long-term administration of As_2O_3 is potentially hazardous.

To reduce arsenic exposure, the use of consolidation chemotherapy after As_2O_3-induced remission is a logical approach. This may be similar to ATRA-induced remission in APL, where the administration of combination chemotherapy has been shown to result in significantly improved survival [2]. Of all the drugs used, anthracyclines appear to be the most important component. Idarubicin, either alone or in combination with non-cytarabine-containing chemotherapy [21], has been shown to be particularly active in the primary treatment of APL.

On the basis of these considerations, we have evaluated the therapeutic strategy of using idarubicin alone to consolidate As_2O_3-induced remission. During As_2O_3 treatment, there was an increase in white cell count in all of the patients. Using flow cytometry, we have also demonstrated the occurrence of apoptosis in vivo. Previous studies have used molecular markers, such as down-regulation of *bcl-2* [6] to indicate apoptosis. Here we have evaluated for the first time the actual percentage of cells undergoing apoptosis in vivo. Making use of the recent observations that As_2O_3 might also mediate its action through mitochondrial pathways [11], we have

used the mitochondrial antigen APO2.7 as a marker of early apoptosis. This antigen is not expressed in cells undergoing necrosis [15]. In fact, APO2.7 expression has been found to correlate well with other apoptotic markers such as annexin V and TUNEL staining [14]. Serial tests showed an increase in apoptotic cells with As_2O_3 treatment, which peaked at the about the same time as the white cell count (Fig. 1). Late apoptotic cells as represented in the sub-G1 fraction also correlated with APO2.7 expression. Interestingly, the increase in apoptosis and its peak almost paralleled those of the white cell count, implying that the apoptosis concomitant with the increase in white cell ultimately contributed to morphologic remission.

However, As_2O_3 induced only morphological remissions but not molecular remissions. The decrease in the size of the leukemic clone as shown by Q-PCR indicated that As_2O_3 ultimately induced apoptosis to result in morphologic differentiation. With idarubicin treatment, all patients achieved molecular remission. With a median follow up of 13 months, six of eight patients have remained in complete morphological and molecular remission. One patient in third remission achieved transient PCR negativity before molecular but not clinical relapse, after the completion of idarubicin treatment. She is currently being evaluated for further As_2O_3 therapy. The only patient who had relapsed died from EMD and not a marrow relapse. Therefore, the combination of As_2O_3 and idarubicin was effectively in eliminating marrow disease in seven of eight patients. This regimen may be less effective against more advanced relapse or extramedullary sites such as the brain, which are known to be important sanctuary areas for APL treated with ATRA and chemotherapy [22]. This implies that more effective treatment regimens have to be devised for these groups of patients.

We conclude that As_2O_3 induction followed by idarubicin consolidation was effective in achieving molecular remission in relapsed APL patients, although the follow-up period of this series is still short. However, this therapeutic strategy has important potential implications. Firstly, it minimizes the exposure of the patients to repeated courses of As_2O_3, as its long-term safety and side effects are unknown. Secondly, it forms the basis on which to formulate other chemotherapy consolidation regimens for As_2O_3-induced remission. Our strategy has the advantage of obviating the use of other chemotherapeutic agents, including topoisomerases that might be leukemogenic. Indeed, secondary leukemias have been reported in APL patients after intensive chemotherapy [23]. However, whether multi-agent combination consolidation chemotherapy for As_2O_3-induced remission is more effective and equally safe will have to evaluated by future studies.

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NOTE IN PROOF

The radiologic features of case 5 were reported in J Clin Oncol 2000;18:3435–3437.

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