ONCOLOGY

Delayed Effect of Antileukemic Polychemotherapy on Cell Death and Proliferation during Remission of Acute Lymphoblastic Leukemia in Children

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The effect of programmed antileukemic drug therapy on cells of the tumor clone is not selective, and the drugs affect also intact bone marrow and peripheral blood cells. The cytotoxic effect of antitumor therapy is attained through triggering apoptosis and does not depend on the type of drug therapy. The treatment induces long-lasting changes in spontaneous cell death processes. The disturbances in the kinetics of cell death caused by drug therapy are compensated by changes in proliferative activity of bone marrow cells.

Key Words: *acute lymphoblastic leukemia; apoptosis; granulocytes; lymphocytes; flow cytometry; short-term culturing*

Recent progress in the treatment of various forms of cancer is primarily related to the progress in drug development: correct choice of combinations of intensive drug therapy regimens, adequate doses, and concomitant therapy [6,7]. These achievements led to creation of protocols for the treatment of acute lymphoblastic leukemia (ALL) based on the use of intense high-dose drug therapy (BFM and COALL protocols, Germany, and many American protocols).

The effects of most modern drugs are dose-dependent: higher doses are associated with higher probability of adequate response to therapy and hence, higher incidence of remissions [10]. However, higher doses are fraught with toxic complications. Clinical significance of myelotoxic effect of antileukemic therapy is well established [2,7].

The main obstacle to increasing the dose is suppression of the bone marrow hemopoiesis as a direct cytotoxic effect of a certain drug or of long-term therapy with drug combination [4,5]. It was shown previously [3,4] that normal leukocytic composition of the blood in survivors with a history of ALL is associated with serious disorders in myelopoiesis, caused by suppressed activity of granulocytic/macrophagal precursors. The mechanism of these disorders remains little studied up to the present time.

Recent studies showed that the cytotoxic effect of the majority of antitumor drugs, including antileukemic drugs, is realized via induction of apoptosis [1,4, 9,11-13]. Similar processes are induced in other cells, including myeloid cells becoming the object of nonspecific effects of drug therapy [3,4,7]. Delayed effects of drug therapy on myelopoiesis in various periods after the treatment are unknown.

We investigated the effects of programmed polychemotherapy on normal hemopoiesis and cell death.

MATERIALS AND METHODS

Peripheral blood (PB) and bone marrow (BM) of 36 children aged 7-14 years during remission of ALL, who received treatment by protocols [7] ALL BFM-

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TABLE 1. Spontaneous Apoptosis of Peripheral Blood Leukocytes, Granulocytes, and Lymphocytes in Children with ALL Remissions at Different Terms after Discontinuation of Antileukemic Therapies (*M*±*m*)

Therapy		Leukocytes		Granulocytes		Lymphocytes	
		$\times10^9$/liter	spontaneous apoptosis, %	$\times10^3$/liter	spontaneous apoptosis, %	$\times10^3$/liter	spontaneous apoptosis, %
Control (*n*=20)		7.60±0.56	2.28±0.32	4.32±0.24	0.64±0.11	2.75±0.29	2.1±0.33
ALL MB-91, group	1 (*n*=4)	7.20±0.53	7.98±0.92	4.38±1.14	5.24±0.37	2.22±0.20	7.07±0.67
	2 (*n*=5)	7.30±0.89	6.62±0.61	3.97±0.45	4.78±0.56	1.83±0.66	6.37±0.75
	3 (*n*=5)	7.01±0.47	5.79±0.76	4.29±0.21	3.73±0.54	1.96±0.16	5.27±0.89
ALL BFM-90, group	1 (*n*=4)	7.10±0.78	7.28±0.26	3.66±0.07	5.2±0.6	2.29±0.29	7.01±0.32
	2 (*n*=6)	6.90±0.96	6.78±0.23	4.39±0.71	4.48±0.94	2.10±0.17	6.39±0.21
	3 (*n*=12)	7.50±0.78	5.06±0.64	4.24±0.27	3.04±0.53	2.04±0.16	4.73±0.67

90 (*n*=22) and ALL MB-91 (*n*=14) were studied. All children were divided into 3 groups, depending on the period elapsed after treatment: 1) 8 children during the first year after therapy was discontinued; 2) 11 children 1-2 years after therapy; and 3) 17 children more than 2 years after treatment. Control group (*n*=20) consisted of children without a history of cancer and polychemotherapy.

The morphology of PB was analyzed on an Argos Cobos 5 Diff automated analyzer. Spontaneous apoptosis of PB and BM cells after staining with propidium iodide was evaluated on a FacScan flow cytofluorimeter (Becton Dickinson). The kinetics of spontaneous PB apoptosis and morphological composition of BM were analyzed.

Colony-forming capacity of hemopoietic BM cells was evaluated by the Pike-Robinson method modified for the agar drop-liquid medium system. Mononuclear fraction was isolated from BM in Ficoll density gradient (1.077 g/ml). The test cells were explanted into agar drop. Feeder cells from 2 different donors suspended in a liquid medium served as the source of colony-stimulating activity. The number and proliferative potential of precursor cells were evaluated by the efficiency of cloning (EC) estimated per 1×10^5 explanted cells, ratio of colonies to clusters (PP — proliferative potential), and percentage of large and medium-sized colonies in cultures.

The rate of spontaneous apoptosis of BM and PB cells was evaluated using PermoCyte-FP™, WB 1010 (BioErgonomics) reagents [8]. PB leukocytes and BM mononuclear fraction isolated in Ficoll density gradient (1.077 g/ml) were fixed and permeabilized. After staining with propidium iodide fluorescence intensity was analyzed on a flow cytofluorimeter in range II per 10,000 cells, DNA distribution was evaluated, and the percentage of hypoploid (apoptotic) cells in the total population (for PB and BM) and in lymphocytic and granulocytic clusters (for PB) identified by photooptic characteristics were estimated.

RESULTS

The number of granulocytes and lymphocytes in PB of patients did not differ from normal; there were no appreciable differences between groups with different duration of the period after the treatment or between patients receiving different types of therapy. Study of spontaneous apoptosis showed a marked increase in the percentage of apoptotic granulocytes and lymphocytes in PB of children treated by chemotherapy (Table 1).

Analysis of spontaneous apoptosis of PB leukocytes in children with ALL remissions depending on the initial form of leukemia showed no significant differences in the levels of spontaneous apoptosis of granulocytes and lymphocytes in children with different immunocytological variants of ALL (Table 2).

The data indicate a remarkable intensification of spontaneous apoptosis in all PB cells in remote periods after chemotherapy.

In order to evaluate possible changes in the type of apoptosis in the whole cell population, the kinetics of apoptotic cells accumulation was studied during cell

TABLE 2. Relationship between Spontaneous Apoptosis of Peripheral Blood Cells in Children with ALL Remission and the Immunocytological Variant (*M*±*m*, %)

Leukemia variant	Leukocytes	Granulocytes	Lymphocytes
T-cell (*n*=3)	5.48±3.62	3.53±2.73	4.58±3.67
Pre-B (*n*=16)	6.63±0.78	4.26±0.58	6.12±0.76
Common (*n*=17)	6.42±0.39	4.43±0.62	6.58±0.83

TABLE 3. Time Course of Coefficient of Accumulation (CA) of Peripheral Blood Apoptotic Cells in Healthy Children and Children with ALL Remissions (*M*±*m*)

Cells	CA_{0-6}		CA_{6-12}		CA_{12-24}		$CA_{24\ h}$	
	control	ALL remission	control	ALL remission	control	ALL remission	control	ALL remission
Granulocytes	8.36±3.14	1.74±0.09*	2.80±0.15	1.87±0.17*	1.58±0.06	1.75±0.23*	23.1±4.7	5.8±1.10**
Lymphocytes	3.99±0.65	1.65±0.10*	2.49±0.15	1.56±0.09*	1.52±0.07	1.70±0.28*	14.65±2.00	4.38±0.82**

Note. *p<0.01, **p<0.05 *vs.* control.

incubation in a serum-free medium for 24 h. Two original parameters were analyzed: coefficient of accumulation (CA) of apoptotic cells during certain periods (after 6, 12, and 24 h of incubation) and the same parameter for 24 h. CA was estimated as the ratio of the percentage of apoptotic cells at the end of a certain period to the percentage of apoptotic cells at the beginning of this period.

Analysis of the kinetics of spontaneous apoptosis of control donor leukocytes showed the highest rate of accumulation during the first 6 h, after which it progressively decreased. The kinetics of apoptotic granulocytes and lymphocytes in children with a history of chemotherapy was different: the 24-h CA in them was significantly lower than in the reference group (Table 3).

The data indicate that antileukemic chemotherapy not only stimulate apoptosis of PB leukocytes, but also disorders the kinetics of spontaneous apoptosis.

The presence of normal count of PB leukocytes with increased capacity to apoptosis prompts us to elucidate some questions: how the compensatory processes run, what is the apoptosis/proliferation ratio, and which of hemopoiesis components is mainly responsible for compensatory functions. As the phenomenon of intensification of apoptosis was universal for all the studied groups, it was probable that a decrease in the number of precursor cells was a result of programmed chemotherapy. In order to verify this hypothesis, we investigated EC of bone marrow cells.

The efficiency of cloning increased with prolongation of the period elapsed after drug therapy, but did not reach the control level. The significant inverse correlation between EC intensification and the level of spontaneous apoptosis of BM cells in the first 2 groups confirms the role of apoptosis in the decrease in the number of clonogenic granulocyte precursors (the level of spontaneous apoptosis of BM cells/correlation coefficient was 7.55± 1.49/-0.78 in group 1 and 8.55±0.9/-0.74 in group 2, while in group 3 these values were 5.79±0.95/-0.20). The compensatory function in this pool seemed to belong to clonogenic precursors, which increased the proliferative potential. This was characteristic of all groups (Table 4).

Hence, the studies demonstrated a long-lasting delayed myelosuppressive effect of antileukemic polychemotherapy. More pronounced spontaneous apoptosis was observed during the first 2 years after therapy; later the differences leveled. No differences in the intensity of delayed myelosuppression after therapy according to protocols BFM-90 and MB-91 were detected.

The increased capacity to spontaneous apoptosis in all hemopoiesis components underlies the development of myelosuppression. The kinetics of spontaneous apoptosis of PB cells changed. The capacity to spontaneous apoptosis decreased with prolongation of the period elapsed after therapy. Despite enchanced spontaneous apoptosis, leukocyte count in PB of patients during all periods of observation was normal; this was a result of compensatory processes in hemopoiesis, which were characterized by increase of the proliferative potential of granulocytopoiesis precursor cells and the percentage of proliferating cells.

TABLE 4. Results of BM Cells Culturing after Discontinuation of Polychemotherapy (*M*±*m*)

Group	Cloning efficiency, arb. units	Proliferative potential, arb. units	Percentage of large and medium-sized colonies
Control (*n*=65)	87.7±8.3	0.77±0.20	16.5±1.6
1 (*n*=10)	27.50±3.31	1.26±0.21	22.84±1.76
2 (*n*=12)	27.56±4.23	1.34±0.18	16.36±1.78
3 (*n*=10)	29.89±3.88	1.385±0.230	14.25±1.32

Note. Control data were taken from report [4].

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