The Use of Emoxipin for Correction of Cyclophosphamide Cytotoxicity in Experimental Animals

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In experiments on animals with Lewis lung carcinoma, emoxipin decreased hematotoxicity of cyclophosphamide without reducing its antitumor efficiency (effect on primary tumor node). Combined administration of emoxipin and cyclophosphamide more effectively prevented the development of metastases compared to cytostatic monotherapy.

Key Words: *Emoxipin; cyclophosphamide; hematotoxicity; antitumor effect; metastasizing*

In modern therapy of oncological patients, the leading role is played by drug treatment. Optimization and improvement of the efficiency of known preparations is an important problem of oncopharmacology [1]. It can be solved via reduction of toxicity of antitumor drugs. Side effects of antitumor drugs can be determined by their toxic effects on cell membranes, disturbances in LPO regulation, and formation of free radicals [2,4,5]. The use of inhibitors of free-radical reactions as preparations of antioxidant therapy reducing side effects of cytostatics is pathogenetically substantiated.

Here we studied the efficiency of emoxipin (EM) as a hematoprotector and evaluated its influence on antitumor and antimetastatic effects of cyclophosphamide (CP).

MATERIALS AND METHODS

Experiments were carried out on 96 female C57Bl/6 mice weighing 18-20 g (Stolbovaya nursery, Russian Academy of Medical Sciences). Suspension of Lewis carcinoma cells (10^6 cells in Hanks solution) was injected intramuscularly (into hip muscles). The animals were divided into 5 groups: mice with Lewis lung carcinoma (LLC) without treatment (Group 1); animals with LLC receiving intraperitoneal injection of CP (Biokhimik company) in a dose of 100 mg/kg 2 times with 96-h interval starting from day 7 after cell transplantation (group 2, LLC+CP); mice with LLC receiving CP according to the same scheme as in group 2 in combination with EM (Moscow Endocrine Plant) administered daily intramuscularly in a dose of 12.5 mg/kg for 14 days starting from day 7 after tumor cell transplantation (group 3, LLC+CP+EM 12.5); mice with LLC receiving CP according to the same scheme as in group 2 in combination with daily intramuscular injections of 25 mg/kg EM for 14 days starting from day 7 after tumor cell transplantation (group 4, LLC+CP+EM 25); mice with LLC receiving CP according to the same scheme as in group 2 in combination with α-tocopherol (α-TP, Uralbiofarm) administered daily intramuscularly in a dose of 50 mg/kg for 14 days starting from day 7 after tumor cell transplantation (group 5, LLC+CP+α-TP). Each group consisted of 15-17 animals. Intact animals comprised a special group.

For evaluation of hematotoxicity, the blood was taken from 6 mice in each group under ether narcosis on days 14 and 22 of the experiment and the

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content of erythrocytes, hemoglobin, platelets, and leukocytes was determined. Bone marrow was isolated from the femur and a smear was prepared. Known antioxidant α-TP in a dose of 50 mg/kg was used as the reference preparation for EM. EM (3 hydroxy-6-methyl-2-ethylpyridine hydrochloride, an antioxidant exhibiting antihypoxic activity [3]) was used in two doses: 12.5 and 25 mg/kg. On day 22 after tumor transplantation, the animals were sacrificed under ether narcosis.

The efficiency of treatment was evaluated by the index of tumor growth inhibition calculated from the volume of primary tumor node and tumor weight at the end of the experiment. The antimetastatic effect was evaluated by the percent of animals with metastases, mean number of surface metastases per mouse, and degree of metastatic involvement of the lungs depending on the number and size of metastases. Index of metastasizing inhibition (IMI) was calculated [6].

The data were processed statistically using Student *t* test and χ^2 test.

RESULTS

In group 1 mice, anemia and leucopenia with drastic lymphopenia developed on day 14 of the experiment (Table 1). Administration of CP aggravated anemia and leukopenia in group 2 animals. The content of erythrocytes and hemoglobin decreased by 23.9 and 17.1% ($p<0.05$), respectively, while leukocyte count decreased by 65% (primarily at the expense of lymphocytes) compared to group 1 animals.

EM in both doses prevented the decrease in hemoglobin content after CP treatment, but did not prevent erythrocytopenia; in a dose of 12.5 mg/kg the preparation reduced the severity of leukopenia: leukocyte count increased by 1.98 times compared to group 2 (LLC+CP). Reference preparation α-TP had no effect on blood shifts resulting from tumor processes and CP treatment.

On day 22, thrombocytopenia developed in animals with LLC without treatment (platelet count decreased to 260.0±27.8 *vs*. 376.7±14.7 in intact animals, $p<0.05$) and in mice with LLC receiving CP (to 196.0±7.9), *i.e*. CP therapy aggravated thrombocytopenia by 24.6% compared to untreated animals ($p<0.05$).

Administration of EM in doses of 12.5 and 25 mg/kg increased platelet count by 67.3 and 75% compared to the corresponding parameter in group 2 (LLC+CP) and attained 328.0±38.1 and 343.3±33.3, respectively. More pronounced anemia in animals of groups 1 (LLC) and 2 (LLC+CP) was also noted on day 22: hemoglobin content and erythrocyte count decreased to 47.55±3.68 g/liter and 3.68±0.23×10^{12}/liter and to 43.55±2.9 g/liter and 2.3±0.18×10^{12}/liter, respectively. EM in a dose of 25 mg/kg and α-TP increased hemoglobin content by 24.4 and 27.3%, respectively ($p<0.05$), compared to group 2. Leukocyte count in untreated tumor-bearing mice by this term did not differ from that in intact mice (5.04±0.52×10^9/liter), with predominance of neutrophils over lymphocytes and remaining lymphopenia. In mice receiving CP monotherapy we observed leukocytosis (12.35±1.67×10^9/liter) at the expense of neutrophils, the number of lymphocytes did not differ from that in group 1 animals. In animals receiving EM in both doses and α-TP, blood parameters did not differ from thoes in animals receiving CP monotherapy.

On day 14, cell composition of the bone marrow in untreated mice with LLC did no differ from that in intact animals. In mice receiving CP monotherapy we observed activation of proliferative activity of immature granulocytes and inhibition of differentiation processes, which led to an increase in the content of blast forms (by 3 times) and maturating forms (myelocytes and metamyelocytes by 2.6 and 5.4 times, respectively) and a decrease in the count of segmented neutrophils by 5.2 times, compared to untreated animals (Table 2).

TABLE 1. Effect of EM on Hematological Parameters in Mice with LLC Treated with CP (Day 14 of Experiment)

Group	Hemoglobin, g/liter	Erythrocytes, ×10^{12}/liter	Leukocytes, ×10^9/liter	Neutrophils, ×10^9/liter	Lymphocytes, ×10^9/liter
Intact	143.4±3.1	8.23±0.18	5.84±0.48	1.7±0.21	4.10±0.32
1	61.17±1.90[a]	3.97±0.09[a]	3.52±0.30[a]	2.26±0.21[a]	1.28±0.15[a]
2	50.70±3.08[a,b]	3.02±0.20[a,b]	1.23±0.20[a,b]	0.96±0.18[a,b]	0.24±0.04[a,b]
3	66.0±2.9[a,c]	2.90±0.15[a,b]	2.44±0.20[a,b, c]	1.94±0.14[c]	0.47±0.13[a,b]
4	64.7±2.2[a,c]	2.7±0.1[a,b]	1.6±0.12[a,b,c]	1.18±0.09[b,d]	0.36±0.04[a,b]
5	54.8±4.2[a]	2.48±0.2[a,b]	1.22±0.12[a,b,c]	1.04±0.13[a,b,c]	0.16±0.01[a,b,e]

Note. $p<0.05$ compared to: [a]intact animals, [b]group 1, [c]group 2, [d]group 3, [e]group 4.

TABLE 2. Effect of Combined Treatment with CP and EM on Cell Composition of the Bone Marrow in Mice with LLC (Day 14 of Experiment)

Parameter	Group					
	Intact	LLC	LLC+CP	LLC+CP+ EM 12.5	LLC+CP+ EM 25	LLC+CP+ α-TP
Blasts	0.10±0.06	0.8±0.2	2.4±0.5[a,b]	0±0[b,c]	0.45±0.20[c]	2.9±0.9[a,d,e]
Myelocytes	6.0±2.3	7.0±1.4	18.4±0.6[a,b]	14.4±1.5[a,b]	13.9±1.2[a,b,c]	20.5±3.9[a,b]
Metamyelocytes	0.53±0.10	0.9±0.2	4.9±0.6[a,b]	6.2±0.7[a,b]	7.0±0.2[a,b,c]	8.1±1.2[a,b]
Stab neutrophils	30.5±3.5	36.5±4.8	46.0±1.2[a]	48.4±2.6[a]	48.8±3.3[a]	41.7±7.0
Segmented neutrophils	23.4±2.1	23.2±2.6	4.5±0.2[a,b]	13.25±2.90[a,b,c]	12.7±1.8[a,b,c]	5.6±1.0[a,b,e]
Basophilic normocytes	1.67±0.30	0.90±0.37	1.3±0.4	1.3±0.5	2.0±0.2[b]	1.7±0.7
Polychromatophilic normocytes	23.6±3.7	19.0±3.8	11.4±1.6[a]	11.9±2.1	12.1±1.8[a]	10.1±1.6[a]
Monocytes	4.9±0.5	8.2±2.2	7.0±0.6	6.5±1.8	12.9±2.3[a]	3.3±1.3[e]
Lymphocytes	6.5±1.9	2.2±0.3	4.8±0.6[b]	0.97±0.2[a,b,c]	1.7±0.3[a,c]	1.5±0.3[c]

Note. $p<0.05$ compared to: [a]intact group, [b]group 1 (LLC), [c]group 2 (LLC+CP), [d]group 3 (LLC+CP+EM 12.5), [e]group 4 (LLC+CP+EM 25).

The number of polychromatophilic normocytes decreased by 2 times compared to that in intact animals and did not differ from that in mice with LLC. In mice receiving CP and EM in a dose of 12.5 mg/kg, blasts disappeared and the count of segmented neutrophils increased by 2.9 times ($p<0.05$) compared to the corresponding values in intact mice. The number of polychromatophilic normocytes remained low. Similar changes were observed also in mice receiving CP and EM in a dose of 25 mg/kg: the number of blasts decreased by 5.3 times, the number of segmented neutrophils increased by 2.8 times ($p<0.05$) compared to animals receiving CP monotherapy (Table 2).

On day 22, the cell composition of the bone marrow in untreated animals with LLC differed from that in intact mice: lymphocyte count significantly decreased by 3.3 times. In group 2 receiving CP monotherapy, the number of segmented neutrophils increased 2-fold, while the number of basophilic and polychromatophilic normocytes decreased by 2.9 and 5.8 times, respectively, compared to group 1. EM in a dose of 25 mg/kg decreased the number of segmented neutrophils by 1.5 times, while in a dose of 12.5 mg/kg the preparation increased the number of polychromatophilic normocytes by 2.3 times compared to animals receiving CP monotherapy. Reference preparation α-TP did not correct the changes in the cell composition of the bone marrow. Hence, CP monotherapy damaged the myelokaryocyte and erythroid hemopoietic stems with the development of leuko- and erythrocytopenia on day 14 of the experiment.

Treatment with EM reduced the damaging effect of CP on granulocytopoiesis: the number of leukocytes in the peripheral blood increased and the number of polychromatophilic normocytes in the bone marrow significantly increased on day 22 of the experiment, which attests to attenuation of damage to the erythroid hemopoietic stem.

TABLE 3. Effect of Combined Treatment with CP and EM on LLC Metastasizing

Group	Animals with metastases, %	Mean number of metastases	Number of animals with lung metastases, %						
			0	I	II	III	IV	V	IMI, %
LLC	100	95.7±8.2	—	—	—	20.0	13.3	66.7	—
LLC+CP	100	5.0±1.2*	—	90.0*	10.0*	—	—	—	94.8
LLC+CP+EM 12.5	42.8*+	0.6±0.3*+	57.1*+	42.9*+	—	—	—	—	99.7+
LLC+CP+EM 25	50	1.0±0.5*+	50.0*+	50.0*+	—	—	—	—	99.5+
LLC+CP+α-TP	100x	5.2±1.2*xo	—	80.0*xo	20.0*xo	—	—	—	94.5xo

Note. $p<0.05$ compared to: *group 1 (LLC), +group 2 (LLC+CP), xgroup 3 (LLC+CP+EM 12.5), ogroup 4 (LLC+CP+EM 25)

Evaluation of the antitumor effect of CP+EM combination showed that inhibition of primary node growth in groups with combined treatment did not differ from that in the group receiving CP monotherapy.

In mice receiving CP alone, IMI was 94.8%, the incidence of metastasizing practically did not decrease compared to that in untreated animals. All animals had I (90%) or II (10%) degree of metastatic spreading, whereas in animals with LLC ($p<0.05$), III, IV, and V degree of metastatic spreading was observed in 20, 13.3, and 66.7% cases.

In mice receiving CP in combination with 12.5 or 25 mg/kg EM, the intensity of metastasizing decreased and IMI increased to 99.7 and 99.5%, respectively, compared to 94.8% in the CP group ($p<0.05$, Table 3).

The incidence of metastasizing significantly decreased to 42.8% only in the group receiving EM in a dose of 12.5 mg/kg; in all animals in this group, 0 or I degree of metastatic involvement of the lungs was noted (57.1 and 42.9%, $p<0.05$) compared to animals treated with CP alone. The degree of metastatic involvement of the lungs also decreased significantly in animals receiving CP+25 mg/kg EM: 0 (50%) or I (50%) degree was found.

Thus, EM reduced hematotoxicity of CP: it prevented the development of thrombocytopenia in the peripheral blood, attenuates the damaging effect of the cytostatic on granulocytopoiesis and erythroid hemopoietic stem. These effects increased the content of mature cell forms of the granulocytic stem, decreased the severity of leukopenia, and led to accumulation of polychromatophilic normocytes in the bone marrow. Antitumor efficiency of the cytostatics remained unchanged, while antimetastatic effect of the combination of CP with EM in both tested doses surpassed that of CP alone.

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