Effect of Cysteine Protease Inhibitor Ep-475 on TNF-α-Independent Cyclophosphamide-Induced Apoptosis in Mouse Lymphosarcoma LS Cells

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Cyclophosphamide 1.5-2.0-fold increased activity of cathepsins B and L in tumor tissue of mouse lymphosarcoma LS and caused tumor regression. The effect was most pronounced on day 5 after treatment. Twofold treatment with a selective cathepsin inhibitor Ep-475 slightly stimulated tumor growth in control mice and significantly reduced the antitumor effect of cyclophosphamide. Lysosomal cysteine proteases cathepsins B and L are involved, but do not play a key role in TNF-α-independent apoptosis in LS cells induced by cyclophosphamide.

Key Words: *mouse lymphosarcoma; apoptosis; cyclophosphamide; cathepsin B and L inhibitors; Ep-475*

Lysosomal proteases and their inhibitors play an important role in the regulation of tumor growth [8,11, 15]. Progression in human breast tumor and tumors of the large intestine, liver, and esophagus is accompanied by an increase in the concentration and activity of cysteine (cathepsins B and L), aspartyl (cathepsin D), and serine proteases and metalloproteinases. A correlation was found between increased secretion of proteases in tumor cells and their invasive or metastatic potential [5,10,12,14]. It was reported that lysosomal proteases are involved in apoptosis. The regulatory interaction were revealed between cathepsins B, L, and D [7] and caspases playing a central role in apoptosis [3]. Selective pharmacological and endogenous inhibitors of lysosomal cysteine proteases and specific antibodies against cathepsins B and L *in vitro* inhibit apoptosis in tumor cells induced by TNF-α [6]. Low content of cysteine proteases leads to activation of apoptosis, *e.g.* cathepsin L deficiency increases the percent of apoptotic cells in the brain [12].

Here we studied the role of cysteine proteases cathepsins B and L in TNF-α-independent apoptosis in transplantable mouse LS cells induced by alkylating antitumor agent cyclophosphamide (CP). Experiments were performed with a selective cysteine protease inhibitor Ep-475. Cysteine protease activity in mouse liver is completely inhibited 3 h after administration of Ep-465 (80 mg/kg) [13]. Ep-475 exhibits affinity for active sites in thiol proteases and irreversibly interacts with thiol groups in the active site of papain or cathepsin B [13].

MATERIALS AND METHODS

Experiments were performed on male CBA mice obtained from a vivarium of the Institute of Cytology and Genetics. The animals were kept in cages (6-7 mice per cage) under natural light/dark regimen and received granulated mixed fodder PK 120-1 (Laboratorsnab) and water *ad libitum*. Lymphosarcoma (10^6 cells/ml) was implanted into thigh muscles [1]. CP

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(Biokhimik) in a single dose of 25 or 30 mg/kg was injected into the caudal vein 11 days after tumor implantation. Recombinant TNF-α (Vektor Best, 1×10^{-4} U) was injected intravenously in 0.2 ml physiological saline.

Cysteine protease inhibitor Ep-475 (kindly provided by Prof. K. Hanada) in a dose of 80 mg/kg was injected intraperitoneally 3 and 48 h after CP administration. Control animals received only Ep-475 or solvent (10% dimethylsulfoxide) in the same period.

The size of tumor was measured with a trammel. Tumor volume was estimated by multiplication of 3 sizes. For biochemical study the animals were decapitated 3 days after administration of CP. Tumor weight was determined by the difference between the weights of treated (tumor implantation) and contralateral limbs. Activity of cathepsins B and L was measured in tumor tissue, liver, and other organs of experimental and control mice [10]. Fluorescence of the solution was estimated on a Perkin Elmer 650-10S spectrophotometer. Peptides Z-L-Phe-L-Arg-MCA and Z-L-Arg-L-Arg-MCA served as the substrates (Sigma). Cathepsin L activity was measured using a selective cathepsin B inhibitor CA-074 (Prof. K. Hanada). The results were expressed in nmol methyl coumarylamide (MCA) per 1 mg protein over 1 min. Caspase-3 activity in tumor tissue was measured colorimetrically using commercial reagents (Sigma).

The results were analyzed by Student's *t* test.

RESULTS

During the 1st day after administration of the preparation the intensity of tumor growth was similar in the control and experimental groups, but then tumor regression was revealed in CP-receiving animals. On day 5 tumor volume in these mice was 10% of the initial level. In mice receiving TNF-α the rate of tumor growth did not differ from the control (Fig. 1). Previous morphological and cytological studies and DNA electrophoresis showed that LS regression is realized via apoptosis of tumor cells [1]. Our experiments confirmed these data. Two days after administration of CP, caspase-3 activity in tumor tissue was 36.5±7.1 nmol/mg protein/min (*vs*. 5.5±1.3 nmol/mg protein/min in control animals, $p<0.001$). The fact that in mice receiving TNF-α the tumor progressively increases in size (similarly to control animals) suggests that TNF-α does not induce apoptosis in tumor cells, which requires no additional profs.

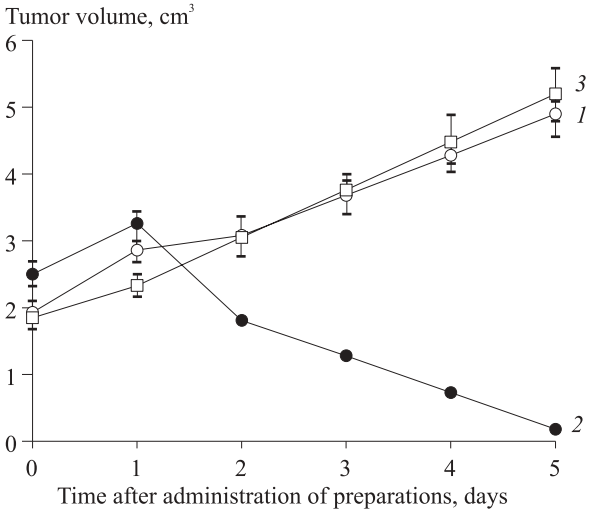

Fig. 1. Growth of intramuscular lymphosarcoma implants in the control conditions (*1*) and after intravenous injection of cyclophosphamide (*2*) or TNF-α (*3*).

Preliminary experiments showed that activity of cathepsins B and L in internal organs and, particularly, in the liver of intact mice decreases to zero 1 h after administration of Ep-475 in a dose of 80 mg/kg (Fig. 2). Cathepsin B and L activities increased to 50% of the baseline level 24 h after treatment and returned to

TABLE 1. Effect of Ep-475 on the Weight of Intramuscular LS Implants and Activity of Cathepsins B and L in Tumor Tissue, Liver, and Spleen of Tumor-Bearing Mice (*M*±*m*)

Parameter	Control	Ep-475	CP	Ep-475 and CP
Tumor weight on day 3 after therapy, g	3.10±0.09	3.50±0.22	1.40±0.07*	2.10±0.05^{+}
Cathepsin B activity, nmol MCA/mg protein/min				
tumor	0.870±0.026	0.620±0.171	1.260±0.097*	1.220±0.135*
liver	0.940±0.062	0.610±0.029*	1.000±0.047	0.670±0.013^{++}
spleen	1.210±0.135	1.030±0.048	1.340±0.127	1.070±0.029
Cathepsin L activity, nmol MCA/mg protein/min				
tumor	0.070±0.006	0.120±0.029	0.160±0.007*	0.150±0.012*
liver	0.420±0.015	0.330±0.017*	0.380±0.022	0.260±0.009^{+}
spleen	0.220±0.032	0.210±0.008	0.190±0.018	0.180±0.004

Note. *$p<0.05$ compared to the control; $^{+}p<0.01$ and $^{++}p<0.001$ compared to CP.

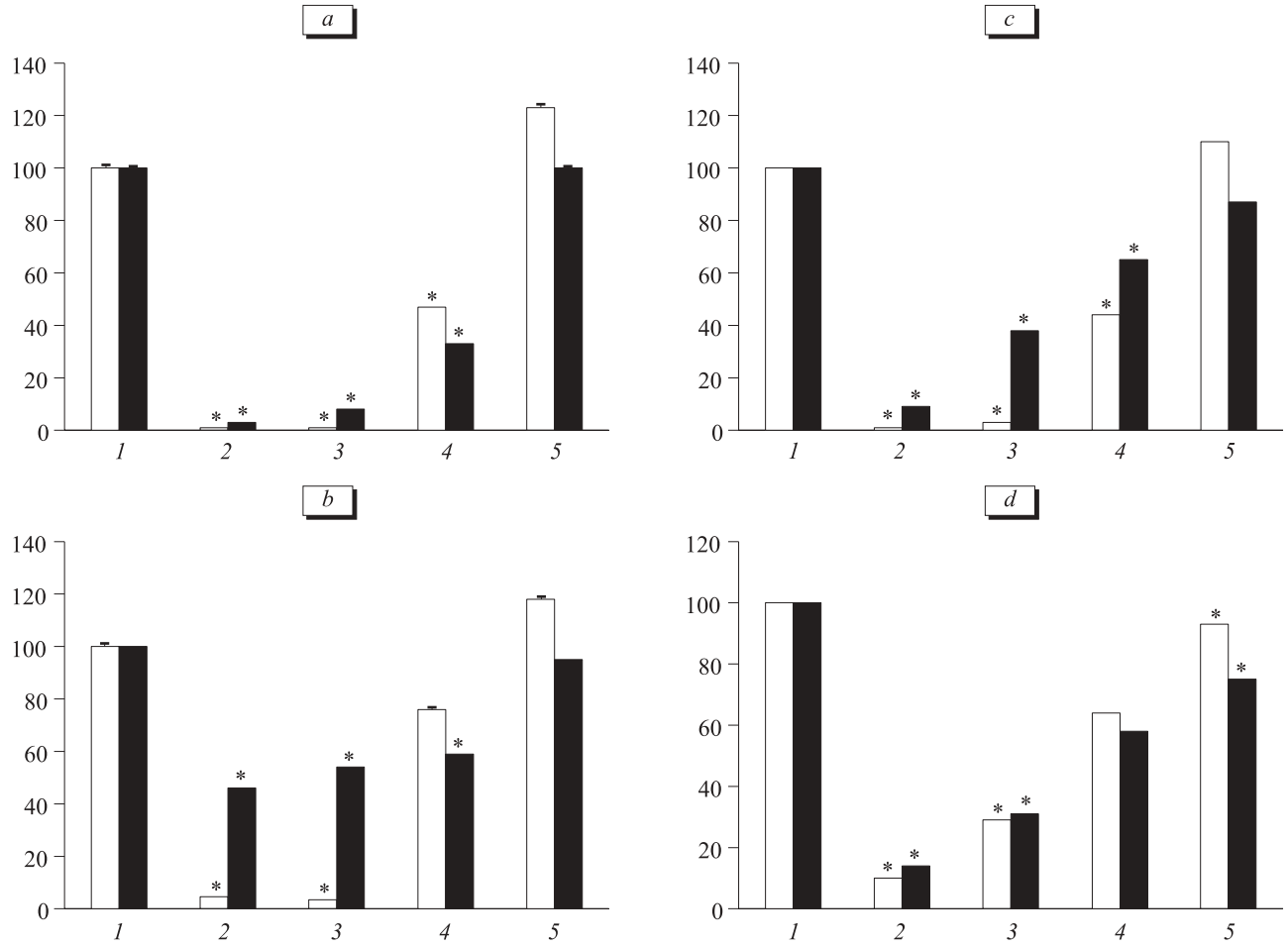

Fig. 2. Effect of Ep-475 injected intraperitoneally in a single dose of 80 mg/kg on activities of cathepsins B (light bars) and L (dark bars) in the liver (*a*), spleen (*b*), kidneys (*c*), and brain (*d*) of CBA mice. Control (*1*); Ep-475, 1 h (*2*); Ep-475, 3 h (*3*); Ep-475, 24 h (*4*); Ep-475, 48 h (*5*). Ordinate: specific activity of cathepsins (%). Each group comprised 6 animals. *$p<0.05$ compared to the control (100%).

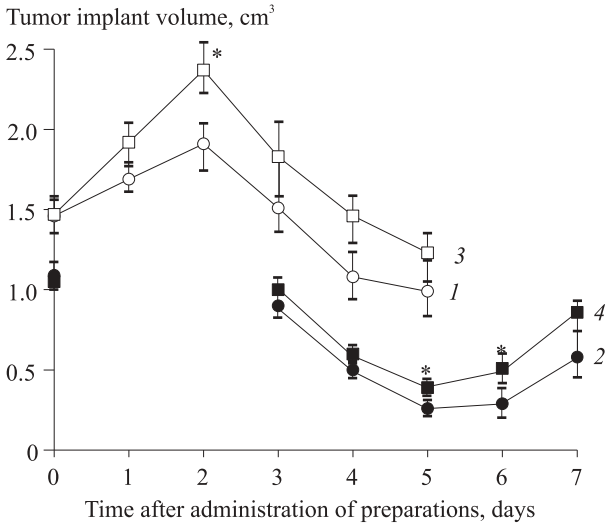

Fig. 3. Growth of intramuscular lymphosarcoma implants after administration of cyclophosphamide alone (*1*, *2*) or in combination with Ep-475 (*3*, *4*) in two experiments. Each group comprised 7-11 animals. *$p<0.05$ compared to cyclophosphamide-treated mice.

normal by the 2nd day. These data were taken into account in further studies. The inhibitor was administered 2 times (3 and 48 h after CP treatment) to exclude the effect of Ep-475 on CP metabolism.

On day 3 tumor weight in mice receiving Ep-475 surpassed the corresponding parameter in the control by 13% (statistically insignificant). During this period tumor weight in animals treated with the inhibitor after CP administration exceeded that in mice of the CP group by 50% ($p<0.05$, Table 1). Over the first 5-7 days after therapy tumor volume in mice receiving CP and Ep-475 was much higher than in animals of the CP group (Fig. 3).

After administration of CP activity of cathepsins B and L remained unchanged in the liver and spleen, but significantly increased in the tumor tissue. Ep-475 did not abolish the stimulatory effect of CP on enzyme activity in the tumor tissue (Table 1). Activities of cathepsins B and L most significantly decreased 2 days after Ep-475 administration. By contrast, enzyme activity increased in CP-treated animals during this

period. A relationship probably exists between cathepsin B and L activity and CP-induced changes in tumor cells. It can be hypothesized that this is related to the apoptogenic effect of CP. However, Ep-475 did not modulate the effect of CP and slightly stimulated tumor growth in control mice. The presented data argue against this hypothesis. Probably, cathepsins B and L in combination with or independently on caspases [4] are involved in the effector phase of apoptosis or aponecrosis [2] induced by CP and occurring spontaneously in several tumor cells.

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