BIOPHYSICS AND BIOCHEMISTRY

Mouse Lymphosarcomas Sensitive and Resistant to Cyclophosphamide Therapy: Activity of Cathepsins B, L, and D during Various Schemes of Treatment with Cyclophosphamide and SE-Glycan

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We measured activities of cysteine (cathepsins B and L) and aspartyl proteinases (cathepsin D) in tumor tissue of mice with sensitive and resistant lymphosarcomas. In cyclophosphamide-resistant lymphosarcoma tissue activities of cathepsins B, L, and D were lower than in cyclophosphamide-sensitive lymphosarcoma. After treatment with cyclophosphamide in high doses enzyme activities in mice with cyclophosphamide-resistant lymphosarcoma increased more significantly than in animals with cyclophosphamide-sensitive lymphosarcoma. Sulfoethylated β-1,3-D-glycan potentiated the effect of cyclophosphamide in mice with both forms of lymphosarcoma. This drug in the lowest dose (10 mg/kg) was most effective.

Key Words: *cathepsins B, L, and D; resistant lymphosarcoma; glycans*

Cysteine (cathepsins B and L) and aspartyl proteinases (cathepsin D) are involved in the development of human malignant diseases [14]. Secretion of cathepsins B and L by tumor cells leads to destruction of the extracellular matrix and lysis of the basement membrane, which promotes invasion of tumor cells. The ability of tumor cells to produce proteinases correlates with their invasive and metastatic potential [6]. Intensive expression and increase in the contents and activities of cathepsins B, L, and D are observed in tissues of breast carcinoma, cancer of the lung, stomach, and rectum, and melanoma [11]. Previous experiments showed that activities of cysteine proteinases in tissues of mouse lymphosarcoma and Lewis lung adenocarcinoma increase after effective antitumor therapy [10]. Cysteine and aspartyl proteinases are closely related to the caspase system and interact with each other during the regulation of apoptosis. The inhibition of cathepsins B and D in human neuroblastoma cells with specific inhibitors induces apoptosis [7]. Cathepsin B plays an important role in TNF-α-induced apoptosis in hepatocytes [8]. Cathepsin D acts as a mediator of apoptosis in fibroblasts [13]. Cathepsin L is involved in apoptosis in spermatogonia induced by prolactin. The inhibition of cathepsin L in human glioma cells reduces their invasive potential and intensifies apoptosis induced by staurosporine [12].

Here we measured activities of cysteine (cathepsins B and L) and aspartyl proteinases (cathepsin D) in tumor tissue of mice with 2 forms of lymphosarco-

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ma (LS). Some tumor cells undergo apoptosis under the influence of cyclophosphamide (CP) [2], while others are resistant to apoptosis (RLS) and respond only to cytostatic and cytotoxic drugs in high doses [1]. We studied the effects of a new immunomodulator sulfoethylated β-1,3-D-glycan(SE-glycan) in various doses on the antitumor efficacy of CP.

MATERIALS AND METHODS

Experiments were performed on male CBA mice aging 4-6 months and obtained from vivarium of the Institute of Physiology. Washed from ascitic fluid LS and RLS cells were transplanted into thigh muscles (1.7×10^6 and 1.6×10^6 cells, respectively). SE-glycan (Institute of Chemistry, Slovak Academy of Sciences) was dissolved in NaCl and injected intraperitoneally in doses of 10, 25, and 50 mg/kg (various groups) on days 1, 4, and 7 after transplantation. On day 11 after transplantation the mice with LS and RLS intraperitoneally received CP (Konpo) in doses of 10 and 150 mg/kg, respectively. The preparation in these doses produces a similar therapeutic effect in animals with tumors. Tumor size was measured periodically with a caliper. The animals with RLS and LS were examined for 14 and 17 days, respectively. The mice were decapitated, and body weight and weight of tumors were measured. Activities of cathepsins B and L in tumor tissue were estimated using fluorescent substrates Z-Arg-Arg-NMCA and Z-Phe-Arg-NMCA, respectively (Research-and-Production Company Vektor) [6,9]. In experiments with Z-Phe-Arg-NMCA the selective cathepsin B inhibitor CA-074 was added to the incubation mixture. Fluorescence was measured on a Perkin Elmer 650-10S spectrofluorometer at excitation and emission wavelengths of 355 and 460 nm, respectively. The results were expressed in nmol methylcoumarylamide (MCA) per 1 mg protein over 1 min. Cathepsin D activity was measured spectrophotometrically on a Spekol 20 spectrophotometer (Karl Zeiss) using azocasein as the substrate [11]. The data were expressed in arbitrary laboratory units (A_{366}/g protein/min). The results were analyzed by Student's *t* test.

RESULTS

RLS ran a more malignant course than LS: RLS grew more rapidly and led to more early death of animals. On day 14 after transplantation tumor volumes were 5.0 ± 0.5 and 3.9 ± 0.3 cm^3, respectively. The growth of RLS was arrested over 3 days after CP administration, but continued on day 4. The mice were in an unsatisfactory state and displayed low locomotor activity. They were killed on day 15 after transplantation because of the risk of massive death. LS grew more slowly than RLS. Therefore, untreated mice with LS were observed 5 days longer than animals with RLS. It should be emphasized that in treated mice with LS the tumor did not grow and even decreased in volume by $^1/_3$ over 5 days. Then the size of LS progressively decreased. In this series the mice were killed 18 days after transplantation. Therefore, CP in doses of 10 and 150 mg/kg produced a similar therapeutic effect in animals with LS and RLS, respectively. This preparation inhibited the growth of RLS and LS by 24.4 and 17.1%, respectively (Table 1). SE-glycan is the water-soluble product formed after chemical modification (sulfoethylation) of high-molecular-weight glucose polymers [9] and used to potentiate the effect of CP. The culture of *Corinebacterium parvum*, antituberculous bacillus Calmette-Guerin (BCG) vaccine [4], and biologically active polysaccharide products carrageenan, prodigiozan, mannan [3], lentinan [9], and muramyl dipeptide [3] are immunomodulators and stimulators of nonspecific antitumor resistance most efficient during immunotherapy of tumors. Our previous studies showed that β-1,3-D-carboxymethylglucan potentiates the effect of CP on Lewis lung adenocarcinoma [9]. Further experiments on this model revealed some advantages of SE-glycan over carboxymethylglucan [5]. Threefold administration of SE-glycan in doses of 10, 25, and 50 mg/kg potentiated the therapeutic effect of CP. It should be emphasized that glycan in the lowest dose was most effective (10 mg/kg, Table 1). It is necessary to evaluate the efficacy of SE-glycan in low doses during combination therapy of tumors. Similar results were obtained in studies of another preparation with immunomodulatory activity. BCG vaccine capable of activating macrophages and administered in high doses was ineffective and even stimulated the growth of Krebs-2-carcinoma. SE-glycan not only potentiated the influence of cytostatics. SE-glycan administered before treatment with CP produced an antitumor effect in mice with LS. Threefold administration of SE-glycan in doses of 10 and 25 mg/kg inhibited LS growth by 33.3% on day 11 after transplantation.

Activities of cathepsins B, L, and D in tumor tissue of untreated mice with RLS were much lower than in animals with LS (Table 1). Various schemes of combination therapy increased proteinase activities in tumor tissues. However, activities of cathepsins B and D in RLS tissue were lower than in LS tissue (Table 1). It should be emphasized that RLS was characterized by a sharp increase in activity of proteinases (particularly cathepsin L) after treatment. Cathepsin activities in LS and RLS increased most significantly after combination therapy with 10 mg/kg SE-glycan and CP, which correlated with maximum growth arrest.

TABLE 1. Growth Arrest and Activities of Cathepsins B, L, and D in Tumor Tissue of Mice with LS and RLS during Therapy with CP and SE-Glycan in Various Doses (7-10 Animals per Group, $M \pm m$)

Group	Tumor and therapy	Tumor weight, g	Cathepsin B, nmol MCA/mg protein/min	Cathepsin L, nmol MCA/mg protein/min	Cathepsin D, A_{366}/mg protein/min
1	RLS, no therapy	4.10±0.26	0.490±0.113	0.040±0.007	4.40±0.94
2	RLS, CP (150 mg/kg)	3.10±0.18 $p_1<0.01$	1.210±0.159 $p_1<0.005$	0.160±0.031 $p_1<0.005$	7.1±1.5
3	RLS, CP (150 mg/kg) and SE-glycan (10 mg/kg, 3 times)	2.30±0.13 $p_1<0.001$ $p_2<0.01$	1.330±0.261 $p_1<0.05$	0.210±0.077	14.5±2.4 $p_1<0.01$ $p_2<0.05$
4	RLS, CP (150 mg/kg) and SE-glycan (25 mg/kg, 3 times)	2.40±0.19 $p_1<0.001$ $p_2<0.05$	1.050±0.139 $p_1<0.01$	0.150±0.021 $p_1<0.001$	8.70±1.47 $p_1<0.05$
5	RLS, CP (150 mg/kg) and SE-glycan (50 mg/kg, 3 times)	2.60±0.17 $p_1<0.001$	0.830±0.141	0.130±0.026 $p_1<0.01$	9.00±1.25 $p_1<0.05$
6	LS, no therapy	4.10±0.25	1.080±0.095 $p_1<0.01$	0.100±0.008 $p_1<0.01$	10.70±1.37 $p_1<0.01$
7	LS, CP (10 mg/kg)	3.40±0.47	1.950±0.364 $p_6<0.05$	0.160±0.029	17.00±2.46 $p_2<0.01$ $p_6<0.05$
8	LS, CP (10 mg/kg) and SE-glycan (10 mg/kg, 3 times)	2.10±0.52 $p_6<0.01$	2.420±0.368 $p_3<0.05$ $p_6<0.01$	0.150±0.026	196±3.2 $p_6<0.05$
9	LS, CP (10 mg/kg) and SE-glycan (25 mg/kg, 3 times)	2.90±0.48 $p_6<0.05$	1.460±0.146 $p_6<0.05$	0.110±0.011	16.40±1.66 $p_4<0.01$ $p_6<0.05$
10	LS, CP (10 mg/kg) and SE-glycan (50 mg/kg, 3 times)	3.2±0.4	1.680±0.154 $p_5<0.01$ $p_6<0.01$	0.090±0.011	17.60±1.75 $p_5<0.01$ $p_6<0.01$

Note. SE-glycan was injected intraperitoneally 1, 4, and 7 days after transplantation. Subscript at p: reference group.

The increase in cysteine protease activities during therapy probably reflects the organism's response to malignant disease, contributes to lysis of tumor cells, and prevents dissemination. It can be hypothesized that low activity of cysteine and aspartyl proteinases in RLS determines its malignancy and insensitivity to the cytostatic in a dose effective for LS.

Our results show that activities of cathepsins B, L, and D in tumor tissue of mice with RLS are lower than in animals with LS. After treatment with CP in high dose activities of cathepsins B, L, and D in RLS increased more significantly than in LS. However, enzyme activities in RLS tissue remained lower than in LS tissue. SE-glycan potentiated the effect of CP during therapy of mice with LS and RLS. This drug in the lowest dose (10 mg/kg) was most effective. The increase in proteinase activities in LS tissue during therapy can be related to apoptosis of tumor cells induced by CP. The degree of CP-induced apoptosis in RLS cells was much lower than in LS cells. RLS was characterized by the development of necrotic changes.

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