Role of Cystatin C and Cysteine Proteinases in the Development of Mouse LS-Lymphosarcoma

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The growth of LS-lymphosarcoma in CBA mice was accompanied by a decrease in the content of the major extracellular inhibitor cystatin C in the tumor, plasma and, to a lesser extent, in tissued not involved in tumor process (liver and spleen). Cyclophosphamide in a dose of 50 mg/kg prolonged the life-span of animals and decreased tumor size by 80%. Cathepsin B and L activities in the tumor tissue increased by 3 and 7 times, respectively. Cystatin C content in the tumor tissue, spleen, and plasma also increased. Cystatin C assay in tumor tissue and plasma helps to predict the rate of tumor growth and to evaluate the efficiency of antitumor therapy.

Key Words: *cystatin C; cysteine proteinases; LS-lymphosarcoma; cyclophosphamide*

Increased activities of cysteine proteinases cathepsins B and L (CB and CL, respectively) were found in human breast carcinoma [12], lung [6], stomach [8], and colorectal [7] cancer, and mouse HA-1 hepatoma. CB and CL activities and expression of mRNA increase in metastasizing tumors [11]. Tumor cells synthesize proteolytic proenzymes. Procathepsin B activity in ascitic fluid in mice with HA-1 hepatoma 1000-fold surpasses that in the plasma.

Secretion of CB and CL by tumor cells leads to destruction of the extracellular matrix (collagen, elastin, and glycoproteins) and lysis of the basal membrane, which is important for tumor invasion [4].

Activity of cysteine proteinases is regulated by endogenous inhibitors. Stefins, cystatins, and kininogens were isolated and characterized [13]. It was hypothesized that pathological secretion of proteinases during neoplastic processes is accompanied by intensive utilization of endogenous inhibitors and an imbalance in the proteinases/proteinase inhibitors system; proteinase inhibitor deficiency leads to uncontrolled tumor growth [5,13].

The aim of the present study was to evaluate activity of cysteine proteinases CB and CL and the content of their major extracellular inhibitor cystatin C (CC) in mice with LS-lymphosarcoma receiving antitumor therapy with cyclophosphamide.

MATERIALS AND METHODS

Experiments were performed on 3-5-month-old male CBA mice (Institute of Cytology and Genetics). N-Nitroso-N-methylurea-induced LS-lymphosarcoma [1] was transplanted intramuscularly into the thigh of CBA mice (10^6 cells). Cyclophosphamide in a single dose of 50 mg/kg was injected into the lateral caudal vein, when the volume of lymphosarcoma reached 2-3 cm^3. The animals were decapitated 4 days after treatment. The blood, liver, and spleen were isolated. Hindlimbs were cut off and tumor weight was calculated as the difference between the weights of affected and intact hindlimbs.

The content of CC and CB and CL activities were measured in the tumor tissue and liver and spleen ho-

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mogenates. Tissue homogenates were prepared in 0.25 M sucrose with 0.001 M EDTA (pH 7.2-7.4). The samples were treated with Triton X-100 in a final concentration of 0.1%, because our previous experiments showed that this procedure 2.7-fold increases the rate of CC detection in tissue homogenates [14].

Activities of CB and CL were measured fluorometrically [3] using Z-Arg-Arg-MCA and Z-Phe-Arg-MCA (Vektor) as substrates, respectively. The selective CB inhibitor CA-074 was added, since these enzymes cross-react with the substrate in CL assay.

The content of CC was measured using enzyme-linked immunosorbent assay (ELISA) kits for human CC (KRKA). Our previous studies showed that mouse CC cross-reacts with anti-human CC antibodies [15]. ELISA was performed by the sandwich technique using horseradish peroxidase as the indicator enzyme. The reaction was visualized with 3,3',5,5'-tetramethylbenzidine. Colored products of the reaction were analyzed on a Star 30 Plate Reader multichannel spectrophotometer (Kenstar) at 450 nm.

The results were analyzed by Student's *t* test (Statistica software).

RESULTS

Cyclophosphamide decreased the weight of LS-lymphosarcoma by 4.8 times (Table 1). Previous studies showed that cyclophosphamide in a dose of 50 mg/kg caused tumor regression in 100% mice, but recurrence was observed in 50% (2-3 weeks) or 100% animals (35-40 days). No spontaneous regression was observed in untreated mice and the animals died 18-23 days after tumor transplantation [1].

Cyclophosphamide increased CB and CL activities in LS-lymphosarcoma cells by 4 and 7 times, respectively (Table 1). Increased CB and CL activities are probably responsible for destruction of tumor cells and tumor regression [11].

TABLE 1. Effects of Cyclophosphamide on the Weight of LS-Lymphosarcoma and Specific Activity of Cysteine Proteinases in Tumor Tissue (*M*±*m*)

Parameter	Without therapy	Cyclophosphamide
Tumor weight, g	2.90±0.34 (5)	0.60±0.14* (5)
Cathepsin B, nmol MCA/mg protein/min	0.217±0.029 (5)	0.866±0.160* (4)
Cathepsin L, nmol MCA/mg protein/min	0.202±0.015 (13)	1.383±0.337* (4)

Note. *$p<0.01$ compared to untreated animals. The number of measurements is shown in parenthesis.

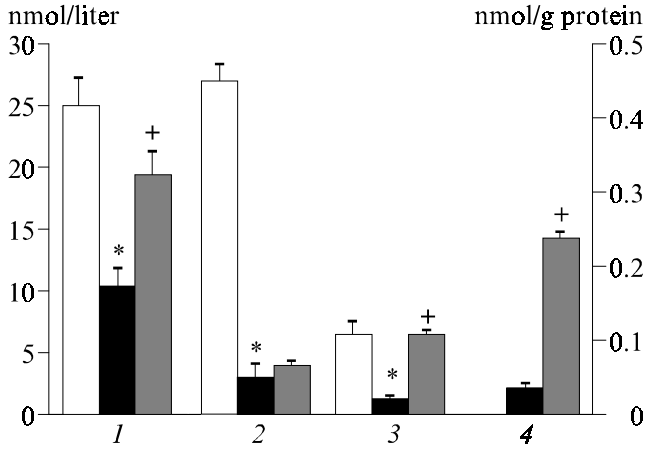

Fig. 1. Cystatin C content in the plasma (*1*, left ordinate), liver (*2*), spleen (*3*), and tumor tissue (*4*, right ordinate) in mice with LS-lymphosarcoma. Light bars: intact mice; dark bars: untreated mice with LS-lymphosarcoma; shaded bars: mice with LS-lymphosarcoma treated with cyclophosphamide. $p<0.01$: *compared to intact mice; +compared to untreated animals with LS-lymphosarcoma.

Immunohistochemical assays showed that CC is synthesized by practically all cells (especially, neuroendocrine cells, macrophages, *etc.*) [2]. In intact mice CC was found in the blood and urea. Tissue CC concentration decreased in the following order: brain—spleen—liver—thymus. Blood CC concentration in intact CBA mice was 25.00±0.22 nmol/liter, while in animals with LS-lymphosarcoma this parameter decreased by 3 times (Fig. 1). In untreated mice with LS-lymphosarcoma the concentration of CC in the tumor tissue was very low (0.040±0.003 nmol/g). The concentration of the inhibitor in the liver and spleen also decreased, which can be explained by toxic effects of the tumor (Fig. 1). Cyclophosphamide increased the content of CC in the tumor, spleen, and blood by 7, 4, and 2 times, respectively (Fig. 1). In humans, the decreased plasma concentration of CC can serve as a biological marker of tumor growth [9,10]. The increase in CC content in the plasma and other tissue can be used as a criterion of successful antitumor therapy.

Thus, the development of LS-lymphosarcoma is accompanied by imbalance between cysteine proteinases and their inhibitors; activity of cysteine proteinases increases, while the content of proteinase inhibitor decreases. CC content in the tumor tissue is very low. Antitumor therapy with cyclophosphamide increases CB and CL activities in the tumor tissue. CC concentration increases not only in the tumor tissue, but also in the spleen and plasma. We hypothesize that measurements of CC content in the tumor tissue, plasma, and spleen can be used for tumor prognosis and evaluation of the efficiency of antitumor therapy.

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