Macrophage Stimulator β-(1→3)-D-Carboxymethylglucan Improves the Efficiency of Chemotherapy of Lewis Lung Carcinoma

O. V. Falameeva, O. N. Poteryaeva, S. Ya. Zhanaeva, O. A. Levina, T. G. Filatova, T. A. Korolenko, V. I. Kaledin*, I. Sandula, and G. Kogan****

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We studied the effect of macrophage stimulator water-soluble β-(1→3)-D-carboxymethylglucan on the efficiency of cyclophosphamide chemotherapy in Lewis lung carcinoma. Cyclophosphamide inhibited the growth of primary tumor nodes by 57%. The preparation possessed pronounced antimetastatic activity: metastases were found in 40.9% animals. Combination therapy with cyclophosphamide and (1→3)β-D-glucan inhibited the growth of intramuscular tumors by 75-89% and reduced the incidence of metastases into the lungs by 92-94%. The therapeutic effect was most pronounced after simultaneous administration of these preparations: tumor growth was suppressed by 89.3% and metastases were found in only 7.5% animals (*vs.* 100% in the control). The potentiating effect of β-(1→3)-D-carboxymethylglucan is related to accumulation of cysteine proteinase inhibitors in the tumor tissue and plasma, but not to changes in blood cell composition.

Key Words: *Lewis lung carcinoma; cyclophosphamide; (1→3)β-D-glucan; cysteine proteinases; stefin A; cystatin C*

Despite considerable progress in chemotherapy of neoplasms, the search for new ways to increase its efficiency and reduce its general toxic effects is still an actual problem. There are clinical and experimental data on combined use of cytostatics and tumor necrosis factor-α (TNF-α) in the therapy of various tumors [1,3]. However, systemic administration of TNF-α is associated with various side effects [3]. The preparations stimulating TNF-α production in the tumor tissue are of considerable importance in this respect. Previous studies showed that macrophage stimulators potentiate the antitumor effect of cytostatics and decrease their leukopenic activity [1,3,4].

Water-soluble polysaccharides structurally similar to (1→3)β-D-glucan (*e.g.*, β-(1→3)-D-carboxymethylglucan, CMG) and stimulating secretion of cytolytic/cytostatic factors by macrophages and TNF-α synthesis hold much promise for clinical medicine [8,9]. Clinical observations and experiments with chemically induced mouse liver sarcoma and melanoma revealed antitumor and antimetastatic effects of water-soluble and -insoluble (1-3)-β-D-glucans [4,7-9]. As differentiated from systemic administration of recombinant cytokines (*e.g.*, interleukin-2), (1→3)β-D-glucans are low toxic [9,10,12].

Changes in the ratio between lysosomal cysteine proteinases (cathepsins B, L, *etc*.) and their endogenous inhibitors (cystatin C and stefin A) contribute to tumor growth and metastasizing [11, 13]. These para-

Institute of Physiology, Siberian Division of the Russian Academy of Medical Sciences, Novosibirsk; *Institute of Cytology and Genetics, Siberian Division of the Russian Academy of Sciences, Novosibirsk; **Institute of Chemistry, Slovakian Academy of Sciences, Bratislava. ***Address for correspondence:*** T. A. Korolenko@iph.ma.nsc.ru. Korolenko T. A.

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meters reflect the outcome of pathological processes and can be used as prognostic criteria of malignant transformation [11].

Here we evaluated whether CMG potentiates the antitumor and antimetastatic effects of cyclophosphamide (CP) during the therapy of Lewis lung carcinoma. We measured activity of cysteine proteinases (cathepsins B and L) and the contents of their endogenous inhibitors (extracellular cystatin C and intracellular stefin A), which probably serve as tumor markers and can be used as criteria for the efficiency of therapy.

MATERIALS AND METHODS

Experiments were carried out on 4-month-old male (CBA×C57Bl)F_1 mice weighing 26-30 g (Institute of Pharmacology). The animals were kept in plastic boxes under natural light/dark regimen and free access to food and water. Cryoconservated Lewis lung carcinoma cells were defrosted and inoculated intramuscularly to male C57Bl/6J mice. The developed tumor was transplanted to experimental animals. To this end, a suspension of tumor cells in physiological saline (5×10^6 cells/ml) was intramuscularly transplanted (0.1 ml) into the right thigh. Ten days after transplantation of the tumor the mice were divided into 8 groups. Control group received no antitumor therapy. Experimental mice received a single injection of CP (Biokhimik) and CMG (Institute of Chemistry) simultaneously or in various combinations (Table 1) [9]. In all series CP was injected in a single dose of 150 mg/kg 10 days after transplantation. The total dose of CMG was 25 mg/kg. The preparations were dissolved in physiological saline and injected intraperitoneally (1 ml/100 g). Twenty days after the start of the experiments, the mice were weighted and decapitated. The blood and internal organs (liver, spleen, and lungs) were obtained. Hindlimbs were cut off. Tumor weight was calculated as the difference between the weights of affected and intact hindlimbs. The lungs were fixed in 10% formalin. Hematogenic metastases into the lungs were counted using a binocular lens (×8).

The contents of cystatin C and stefin A and activities of cathepsins B and L were measured in tissue homogenates and plasma. Tissue homogenates were pretreated with 0.1% Triton X-100. Cathepsin B and L activities were estimated fluorometrically [13]. Cystatin C and stefin A contents were measured using enzyme immunoassay kits (antibodies against human cystatin C, KRKA). Mouse stefin A and cystatin C cross-reacted with antibodies against human cystatin C. Optical density was measured on a Star 30 Plate Reader multichannel spectrophotometer (Kenstar) at 450 nm. The results were analyzed by nonparametric Wilcoxon and Mann—Whitney tests.

RESULTS

Cyclophosphamide in a single dose of 150 mg/kg inhibited the growth of primary tumor nodes by 57% compared to the control. The preparation produced considerable antimetastatic effects: metastases were found in 40.9% animals, and their number per 1 mouse decreased to 7.1 (*vs.* 17.3 in the control, Table 1). Treatment with CMG had no effect on tumor growth, but 4-fold decreased the number of lung metastases (Table 1). Irrespective of the treatment schedule, CMG potentiated the antitumor and antimetastatic effects of CP. It should be emphasized that the therapeutic effect was most pronounced after simultaneous administration of these preparations: tumor growth was suppres-

TABLE 1. Effects of CP and/or CMG on the Growth of Lewis Lung Carcinoma and Number of Hematogenic Metastases in the Lungs in (CBA×C57Bl)F_1 Mice ($M\pm m$)

Group	Tumor weight, g	Number of lung metastases per mouse	Inhibition of tumor growth, %	Incidence of metastases, %
Control (n=20)	5.10±0.19	17.30±2.67	—	100
CP (n=20)	2.20±0.11*	7.10±1.19*	57.0	40.9*
CMG (n=20)	4.30±0.21	4.80±1.36*	15.6	27.8*
CP+CMG (25 mg/kg) at 3-day interval (n=20)	1.30±0.11$^+$	1.00±0.29$^+$	75.1	5.8$^+$
CP+CMG (25 mg/kg) simultaneously (n=10)	0.50±0.08$^+$	1.30±0.08$^+$	89.3	7.5$^+$
CMG (25 mg/kg)+CP at 3-day interval (n=10)	1.10±0.23$^+$	1.10±0.52$^+$	79.1	6.4$^+$
CMG (5 mg/kg, 5 injections)+CP at 3-day interval (n=10)	1.3±0.2$^+$	3.70±0.87$^+$	74.8	21.4$^+$
CP+CMG (5 mg/kg, 5 injections) at 3-day interval (n=10)	1.60±0.13$^+$	2.20±1.05$^+$	68.2	12.8$^+$

Note. *p<0.01 compared to untreated mice; ^+p<0.005 compared to control mice and animals receiving CP alone.

TABLE 2. Leukocyte Count, Activity of Cysteine Proteinases, and Content of Their Endogenous Inhibitors in Tumor Tissue from Mice with Lewis Lung Carcinoma (*M*±*m*, *n*=9-10)

Parameter		Control	CP, 150 mg/kg	CMG, 25 mg/kg	CP (150 mg/kg)+CMG (25 mg/kg) at 3-day interval
Leukocytes, 10^9/liter (3.90±0.28)		22.80±2.15*	8.8±1.2$^+$	16.60±3.66	9.80±1.45$^+$
PMNL,% (19.00±1.54)		57.70±2.08*	81.30±3.05*	60.00±3.57*	85.80±1.93*
lymphocytes, % (76.40±1.74)		37.70±1.86*	16.70±2.75$^+$	34.60±3.85	12.30±1.87$^+$
monocytes, % (4.60±0.49)		4.50±0.73	1.90±0.54$^+$	5.00±1.66	1.90±0.38$^+$
Cathepsin activities, U/mg protein	B	1.08±0.21	1.81±0.31	2.19±0.26^{++}	4.05±0.70^{++}
	L	1.26±0.17	5.35±1.13^{++}	2.17±0.48	3.22±0.68^{++}
Stefin A content, pmol/g protein		26.41±6.56	28.51±3.56	16.29±2.92	45.41±2.75^{++}
Cystatin C content, pmol/g protein		8.99±3.01	15.74±2.52^{++}	8.38±1.24	14.93±1.73^{++}

Note. Parameters in intact mice are shown in parentheses. *p<0.05 compared to intact mice; ^+p<0.01 and ^{++}p<0.05 compared to untreated animals.

sed by 89.3%, and metastases were found only in 7.5% animals (Table 1).

In control mice receiving no antitumor therapy we revealed leukocytosis, increased count of polymorphonuclear leukocytes (PMNL), and reduced content of peripheral blood lymphocytes compared to intact animals. CP 2.2-fold decreased leukocyte count, caused lympho- and monocytopenia, but increased the number of PMNL (Table 2). CMG had no effect on these parameters in untreated mice and did not modify the effect of CP. Hence, the stimulatory effect of CMG on antitumor activity of CP was not related to quantitative changes in blood cells (Table 2).

(1→3)-β-D-Glucans can bind surface receptors on macrophages and stimulate secretion of cytokines, including TNF-α and interleukin-1 [2,3]. Glucan receptors were also found on PMNL. Thus, the antitumor effect of these compounds can be related to an increase in the number of PMNL and glucan receptors [4-6].

Combined therapy with CP and CMG increased cathepsin B and L activities and the contents of cystatin C and stefin A in tumor tissue. CP alone increased cathepsin L, but not cathepsin B activity (Table 2). Activation of cathepsins is probably associated with intensification of apoptosis in tumor cells (if it occurs under these experimental conditions). On the other hand, the contents of cystatin C and stefin A increased in mice receiving CP and CMG (Table 2). It was reported that the content of cysteine proteinase inhibitors increases after successful antitumor therapy [11]. It remains unclear whether these changes are related to the increase in inhibitor content in tumor cells or accumulation of macrophages and other effector cells that infiltrate the tumor tissue. The increase in stefin A and cystatin C contents in the tumor tissue determines, or at least reflects its sensitivity to the therapy.

In experimental mice plasma cystatin C content was 1.7-fold lower than in intact animals (9.30±1.36 and 15.70±1.15 nmol/liter, respectively, p<0.05). After simultaneous administration of CP and CMG the concentration of cystatin C in experimental mice increased and did not differ from that in intact animals (16.10± 1.67 nmol/liter). Previous studies showed that the contents of cysteine proteinase inhibitors in tumor tissue and, particularly, in the blood can be used as prognostic criteria of tumor development and reflect the efficiency of antitumor therapy [11,12].

Our results indicate that macrophage stimulator CMG potentiates the antitumor effect of CP. Combination therapy with these preparations inhibits the growth of primary Lewis lung carcinoma nodes and decreases the number of metastases into the lungs. These changes correlate with the increase in cysteine proteinase activities (cathepsins B and L) and concentrations of their inhibitors (stefin A and cystatin C) in the tumor tissue. Therefore, these parameters can be used as criteria for the efficiency of antitumor therapy.

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