Cystein Proteinase Inhibitor Stefin A as an Indicator of Efficiency of Tumor Treatment in Mice

T. A. Korolenko, O. N. Poteryaeva, O. V. Falameeva, and O. A. Levina

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The concentration of stefin A (cystatin A in mice) was measured in animals with experimental tumors (LS lymphosarcoma, HA-1-hepatoma, and Lewis lung carcinoma) during effective antitumor therapy. In mice with these tumors serum concentrations of stefin A increased, while the concentration of cystatin C (extracellular cystein proteinase inhibitor) decreased. The concentration of stefin A in tumor tissue in Lewis lung carcinoma was higher than in LS lymphosarcoma and HA-1-hepatoma ascitic cells, which can be explained by the degree of their malignancy. The content of stefin A in tumor tissue was similar to that in the liver and spleen of tumor-bearing animals, while its concentration in the liver and spleen of tumor-bearing animals was lower than in intact mice. The level of stefin A is an important marker of malignancy and an indicator of the efficiency of antitumor therapy.

Key Words: *human stefin A; mouse cystatin A; mouse tumors; antitumor therapy*

Cystatins (cystatins, stefins, and kininogenes) are natural reversible inhibitors of cystein proteinases involved in inflammatory and tumor processes [15]. Human stefins (stefin A, B, *etc.*) are a recently discovered group of endogenous intracellular inhibitors of cystein proteinases, named so in honor of J. Stefan Institute (Ljubljana) [6,8,15], where the primary structure of stefins A, B, and C was determined and stefin B gene was synthesized, cloned, and expressed in *E. coli* [14].

Stefin A (SA) is present in high concentrations in the skin epithelial cells, polymorphonuclear leukocytes, and lymphoid tissue [8,10]. SA was detected in low concentrations in extracellular fluids [3,4,8]. In humans cystatin C and SA are malignancy markers determining the outcome in some tumors [9,12,13]. SA belongs to the major tumor suppressors in humans [8-10]. SA in mice (cystatin A) is less studied [5,14]. We showed for the first time in mouse experiments that effective antitumor therapy increased the concentrations of both cystatin C and SA in tumor tissue [7].

Now we investigated the role of stefin A in the development of some mouse tumors and elucidated its value as a possible marker of tumor process and treatment efficiency.

Institute of Physiology, Siberian Division of Russian Academy of Medical Sciences, Novosibirsk. ***Address for correspondence:*** T.A.Korolenko @iph.ma.nsc.ru. Korolenko T. A.

MATERIALS AND METHODS

Experiments were carried out on male CBA, (CBA× C57Bl/6J)F_1, and A/Sn mice weighing 20-25 g (Institute of Physiology, Institute of Cytology and Genetics, Novosibirsk). Methods for reproduction of HA-1-hepatoma, LS lymphosarcoma, and Lewis lung carcinoma (LLC) were described previously [1,2,7]. Cyclophosphamide (CP) was injected intraperitoneally in a single dose of 50 mg/kg to mice with LS lymphosarcoma sensitive to CP therapy and in a dose of 150 mg/kg to mice with LLC resistant to CP (on day 10 after tumor transplantation). Mice with HA-1-hepatoma were intraperitoneally injected with macrophage stimulator carboxymethylated β-1,3-D-glucan (CMG, Institute of Chemistry, Slovak Academy of Sciences) in a single dose of 25 mg/kg.

Tissue homogenates were pretreated with 0.1% Triton X-100 [7]. The content of SA in tumor tissue, liver and spleen homogenates, and serum was measured using enzyme immunoassay kits (KRKA). Mouse SA cross-reacted with antibodies to human SA.

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Stained reaction products were measured on a multichannel spectrophotometer Star 30 Plate Reader (Kenstar) at λ=450 nm. The data were processed using Student's *t* test, the differences were significant at $p<0.05$.

RESULTS

The concentration of SA in the liver of intact mice was lower than in the spleen in all studied mouse strains (Tables 1 and 2). SA content in the liver of intact CBA mice was lower than in A/Sn and $(CBA\times C57Bl/6)F_1$ mice.

The concentration of SA in tumor tissue of mice with LS lymphosarcoma and HA-1-hepatoma approached SA levels in the liver and spleen of control mice (Table 1) and was 2-fold higher in tumor tissue of mice with LLC than in LS lymphosarcoma tissue and in HA-1-hepatoma ascitic cells (Tables 1 and 2). No differences between SA concentrations in the liver and spleen were detected in tumor-bearing mice of all three strains, while in intact mice the liver and spleen concentrations of SA differed (Tables 1 and 2).

In mice with LS lymphosarcoma the concentration of SA in tumor tissue and spleen were similar, but were lower than in the liver and spleen of intact mice (Table 1). In LLC tissue the concentration of SA was higher than in LS lymphosarcoma and HA-1-hepatoma (Tables 1 and 2). According to published data, in humans the concentrations of cystein proteinase inhibitors (cystatin C and SA) in tumor cells reflects the degree of their malignant degeneration [8,11].

The development of tumors in CBA and A/Sn mice caused a decrease of SA concentration in the liver and spleen in comparison with intact animals (Table 1), while in mice with LLC only the splenic level of SA was lowered (Table 2). These changes can be due to the toxic effect of the tumor on the body.

Effective treatment of mice with LS lymphosarcoma sensitive to CP (50 mg/kg, decrease of tumor weight to 16% of the control taken for 100%) was associated with an increase of SA level in tumor tissue (Table 1). Effective treatment of LLC (tumor resistant to CP alone) with a combination of high CP dose with CMG was associated with an increase in SA level in tumor tissue and liver (but not in the spleen; Table 2). CMG had a less pronounced effect on HA-1-hepatoma (reduced the number of ascitic cells to 80% compared to the control), which was not associated with significant changes in SA level in ascitic cells. The level of SA in the liver and spleen slightly increased (Table 1).

Serum level of cystatin C (the main extracellular inhibitor of cystein proteinases) in intact mice of all strains significantly (20-fold and more) surpassed SA concentrations in these animals, which confirmed pre-

TABLE 1. Effects of CP and CMG on Stefin A Content in Organs of CBA Mice with LS Lymphosarcoma and A/Sn Mice with HA-1-Hepatoma (*M*±*m*, nmol/g protein)

Group		Tumor tissue	Liver	Spleen
CBA	intact	—	0.291±0.005	0.492±0.005
	control (LS lymphosarcoma)	0.103±0.005	0.160±0.060**	0.117±0.030*
CP, 50 mg/kg		0.231±0.001^{+}	0.195±0.040	0.268±0.080^{++}
A/Sn	intact	—	0.478±0.050	0.569±0.090
	control (HA-1-hepatoma)	0.170±0.010	0.146±0.050**	0.188±0.020**
CMG, 25 mg/kg		0.544±0.220	0.408±0.130^{++}	0.332±0.030^{++}

Note. Ascitic cells are presented for HA-1-hepatoma. $p<0.01$ compared to *intact and $^{+}$control animals; $p<0.05$ compared to **intact and $^{++}$control animals. Here and in Table 2: 4-5 measurements per group.

TABLE 2. Effects of CP and CMG on Stefin A Content in Organs of $(CBA\times C57Bl/6)F_1$ Mice with Lewis Pulmonary Carcinoma (*M*±*m*, nmol/g protein)

Group	Tumor tissue	Liver	Spleen
Intact	—	0.391±0.010	0.631±0.010
Control (untreated)	0.264±0.020	0.264±0.060	0.304±0.020^{+}
CMG, 25 mg/kg	0.163±0.030	0.251±0.020^{+}	0.317±0.030^{+}
CP, 150 mg/kg	0.285±0.040	0.271±0.030^{+}	0.228±0.040^{+}
CP, 150 mg/kg+CMG, 25 mg/kg, simultaneously	0.454±0.030*	0.403±0.040*	0.219±0.030^{+}

Note. *$p<0.01$ compared to control (untreated) animals; $^{+}p<0.05$ compared to intact animals.

TABLE 3. Serum Concentrations of Stefin A and Cystatin C in Mice with Tumors ($M \pm m$, n=4-6)

Tumor type	Stefin A, nmol/liter		Cystatin C, nmol/liter	
	intact	untreated (tumor tissue)	intact	untreated (tumor tissue)
LS lymphosarcoma	0.510±0.005	0.660±0.031	25.00±0.22	10.4±1.2*
HA-1-hepatoma	0.790±0.008	1.120±0.035*	24.70±1.29	8.8±1.2*
Lewis lung carcinoma	0.470±0.001	0.760±0.019*	15.70±1.53	9.30±1.36*

Note. Intact mice: CBA for LS lymphosarcoma, (CBA×C57BL/6)F_1 for Lewis lung carcinoma, A/Sn for HA-1-hepatoma. *p<0.05 compared to intact mice.

dominantly intra- but not extracellular distribution of SA (Table 3). Serum SA level increased in A/Sn mice with HA-1-hepatoma and in (CBA×C57BL/6)F_1 mice with LLC (Table 3). The concentration of cystatin C was reduced in animals of all three strains with different tumors. This attest to opposite changes in serum concentrations of SA and cystatin C in tumor-bearing mice.

Thus, serum concentrations of SA increased in mice with HA-1-hepatoma and LLC, while the concentration of cystein proteinase inhibitor cystatin C decreased in all cases. SA level in tumor tissue was similar to its concentrations in the liver and spleen of the same mice, and was below the corresponding parameters in intact animals. Antitumor therapy was associated with an increase in SA concentration in tumor tissue, if the treatment was effective (LS lymphosarcoma and LLC), but not in HA-1-hepatoma. Hence, the level of SA can be considered as an important indicator of malignancy in mouse tumors. The increase in SA level in tumor tissue reflects the efficiency of antitumor therapy (except HA-1-hepatoma). Increased concentration of SA in the liver and spleen of the same animals with tumors necessitates further evaluation of the biological role of stefins in tumors.

Studies of the prognostic value of SA in various human tumors are in progress [8]. Distribution of cathepsin B and endogenous inhibitors of cystein proteinases (SA, stefin B, *etc.*) determines invasive activity of tumor cells in humans, but the mechanism of their adhesion to cell membrane remains unclear. SA are located in cells and on their surface, which determined their antiproteolytic function. The decrease in SA concentration is characteristic of many tumor cells, and therefore the use of drugs promoting its recovery is justified.

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