DRUGS EXPTL. CLIN. RES. **XXVII**(5/6) 279-283 (2000)

CHITOTRIOSIDASE AS A NEW MARKER OF MACROPHAGE STIMULATION IN A TUMOR MODEL TREATED WITH CYCLOPHOSPHAMIDE AND UKRAIN

KOROLENKO T.A.,[1] DJANAYEVA S.J.,[1] FALAMEYEVA O.V.,[1] WEVERS R.A.,[2] FILJUSHINA E.E.,[1] BUZUEVA I.I.,[1] KALEDIN V.I.,[3] SANDULA J.,[4] NOWICKY J.[5]

1) Institute of Physiology, Siberian Branch of the Russian Academy of Medical Sciences, Novosibirsk, Russia.
2) Institute of Neurology, University Hospital Nijmegen, Nijmegen, The Netherlands.
3) Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia.
4) Institute of Chemistry, Slovak Academy of Science, Bratislava, Slovakia.
5) Ukrainian Anticancer Institute, Vienna, Austria.

Summary: *Ukrain has previously been demonstrated to exert a malignotoxic effect* in vivo. *This antitumor drug has been effective in the treatment of some malignancies in experimental animals as a result of immunostimulation (macrophage stimulation). In the present study, serum chitotriosidase activity was measured as a biochemical marker of macrophage stimulation in several murine and rat models of macrophage stimulation. It was shown that zymosan, carboxymethylated glucan and Triton WR 1339 administration to CBA mice or Wistar rats was followed by a considerable increase in serum chitotriosidase activity. Murine LS lymphosarcoma development decreased serum chitotriosidase activity. Antitumor treatment by Ukrain or cyclophosphamide did not restore this index to the normal value.*

Introduction

The novel antitumor drug Ukrain, which has malignotoxic properties, has been effectively used in the treatment of murine HA-1 hepatoma, as recently shown (1). It was suggested that immunostimulation (macrophage stimulation) by Ukrain was responsible for this positive antitumor effect (1).

Chitotriosidase is a novel enzyme, which was identified and characterized several years ago, but its biological functions are still not well known (2-5). It has been suggested that this enzyme is related to macrophage stimulation, especially in lipid-laden tissue macrophages (6-7). Serum chitotriosidase activity was shown to be elevated more than 1,000-fold in the plasma of patients with Gaucher's disease. A small (2-5-fold) increase was observed in the serum of patients with other lysosomal storage diseases (7).

The aim of this study was to measure chitotriosidase activity as a new biochemical marker of macrophage stimulation in several experimental models, including macrophage stimulation (by zymosan, yeast polysaccharide carboxymethylated glucan and Triton WR 1339) and to measure tumor development treated by antitumor drugs (Ukrain and

0378-6501/2000/5/6 00279+5 $02.50/0
© 2000 Bioscience Ediprint Inc.

cyclophosphamide). In tumors, tumor cells coexist with macrophages and macrophage stimulation can be effective in host resistance during effective tumor treatment (8).

Materials and methods

Male CBA mice (RAMS Institute of Physiology and Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia) and Wistar rats were used. The experimental tumor used was transplantable LS lymphosarcoma, initially induced in CBA mice by methylnitrosurea. After a series of subcutaneous passages, it was transformed into ascitic form and crioconserved. The tumor cells were thawed when needed, adopted to the mouse milieu during the first passages and were subsequently used for transplantation to experimental animals. The tumor cells were transplanted into the thigh muscles (10^6 per mouse). When solid tumors developed (1.5-2.0 cm in diameter) mice were treated i.v. with cyclophosphamide (Sigma, USA, 25 mg/kg, single dose) or Ukrain (Nowicky Pharma, Vienna, Austria, 0.5 mg per mouse of 20 g, twice). The tumor tissue was excised 3-4 days later, weighed and analyzed for enzyme activity.

Three known macrophage stimulators were used. Zymosan (Sigma) was used at a dose of 100 mg/kg (single dose), Triton WR 1339 (Ruger Chemicals, Irvington, NJ, USA) was administered at 1,000 mg/kg i.p. and (Chemical Institute, Slovak Academy of Science, Slovakia) was injected to mice at a dose of 20 mg per mice of 20 g i.p. in a single administration.

Human serum and the serum of experimental animals was kept frozen (–20 °C) before enzyme activity determination.

Chitotriosidase activity was measured in the serum of Wistar rats and CBA mice, using a substrate 4-MUF-β-D-N,N',N"-triacetylchitotrioside (Sigma, a kind gift from Prof. R. Wevers, The Netherlands) according to the method described by Gue *et al.* (7) and Czartoryska *et al.* (5). Chitotriosidase activity was determined by incubating 10 µl serum with 200 µl of 22 µM/l substrate in McIlvain's phosphate-citrate buffer, pH 5.2, for 30 min at 37 °C. The reaction was stopped by adding 2.0 ml of 0.15 M glycine buffer, pH 10.6, and fluorescence of 4-methylumbelliferone was read in a Perkin-Elmer 650-10S spectrofluorometer (excitation: 360 nm; emission: 445 nm). The results were expressed as nmol MUF/ml released per hour. Statistical analysis of the data was performed using Student's *t*-test (differences were considered to be statistically significant at $p < 0.05$).

Results

Chitotriosidase activity was decreased in LS lymphoma mice compared with that in intact CBA mice (Tables I, II). Treatment by Ukrain tended to decrease

Table I *Influence of Ukrain (0.5 mg per 20 g mouse) and cyclophosphamide (25 mg/kg, single dose) administration to CBA mice with LS lymphosarcoma on serum chitotriosidase activity*

Group	Chitotriosidase activity
Control mice	387.0 + 30.4 (11)
LS lymphosarcoma	238.7 + 20.8** (13)
LS lymphosarcoma + Ukrain	167.6 + 48.2** (4)
LS + cyclophosphamide, 24 h	259.1 + 30.1** (6)
LS + cyclophosphamide, 48 h	281.0 + 66.5 (7)
LS + cyclophosphamide, 72 h	331.9 + 33.8 * (8)

Chitotriosidase activity expressed as nmol MUF/ml per hour. The number of mice is in paretheses. *$p < 0.05$ compared with untreated mice with LS lymphosarcoma. **$p < 0.05$ compared with control mice.

Table II *Serum chitotriosidase activity during stimulation of macrophages by zymosan (100 mg/kg) in Wistar rats*

Group	Chitotriosidase activity
Intact (control)	29.5 + 3.5 (9)
Zymosan, 48 h	43.2 + 4.1* (9)

Chitotriosidase activity expressed as nmol MUF/ml per hour. The number of animals is in parentheses. *p <0.05 compared with untreated rats.

enzyme activity (compared with untreated LS mice). Cyclophosphamide gradually increased serum chitotriosidase activity (reaching significant values 72 h after cyclophosphamide administration), although it was lower than the enzyme activity in control CBA mice (Table I).

Macrophage stimulation is closely related to nonspecific host defense mechanisms in tumors (8). We decided to compare the results obtained with other known models of macrophage stimulation and lysosome overloading. Morphometric study of liver revealed an increased number of macrophages during stimulation by carboxymethylglucan both on the second and seventh day after administration of this drug to mice (Table III). Simultaneously, an increased number of secondary lysosomes were found in all experimental groups as a morphological sign of macrophage stimulation (Table III). Similar data were obtained in the model of macrophage stimulation by zymosan in Wistar rats (1).

Increased serum chitotriosidase activity in CBA mice was found during macrophage stimulation by carboxymethylglucan (Table IV), indicating that this parameter reflected macrophage stimulation. Similar data were obtained in CBA mice and Wistar rats during macrophage stimulation by different agents (Tables II, IV).

Serum chitotriosidase activity was about five times higher in CBA mice than in Wistar rats. Both mice and rats revealed increased (1.5-2-fold) serum chitotriosidase activity during macrophage stimulation (maximum on the seventh day). No significant differences were found between the groups of macrophage stimulation induced by several of the agents used. Triton WR 1339-induced lysosomal overloading by lipids did not increase specifically serum chitotriosidase activity, unlike that in Gaucher's disease, which increased several hundred times.

Tumor development (murine LS lymphosarcoma) was followed by decreased serum chitotriosidase activity as a possible index of macrophage depression. Treatment of LS lymphosarcoma by CPA at a dose 25 mg/kg (significant reduction of tumor weight on the sixth day of treatment, with the possibility of subsequent metastases development) was followed by a tendency to normalization of serum chitotriosidase activity (especially at 72 h after single cyclophosphamide injection to mice with LS lymphosarcoma). It was shown that cyclophosphamide treatment (at a dose of 25 mg/kg) induced apoptosis

Table III *Morphometric study of CBA mouse liver during macrophage stimulation by carboxymethylglucan (CMG) (25mg/kg) (mean ± SE)*

Parameter	Control	CMG, 2 days	CMG, 7 days
Macrophage/mm^2	924.5 ± 38.0	1412.5 ± 92.0*	1782.8 ± 57.35*
Primary lysosome relative volume (%)	3.8 ± 0.50	1.05 ± 0.14*	1.2 ±0.08*
Secondary lysosome relative volume (%)	6.1 ±1.3	19.5 ± 2.72*	16.4 ±1.66*
Number of primary lysosomes 10 μM^2	18.8 ± 0.7	5.4 ± 0.60*	5.8 ± 0.48*
Number of secondary lysosomes 10 μM^2	1.7 ± 0.3	9.7 ± 1.13*	7.2 ± 0.74*

The number of animals is given in each group (50-75 micrographs per group). *p <0.05 compared with untreated mice.

Table IV *Influence of carboxymethylglucan (CMG) and Triton WR 1339 administration to CBA mice on serum chitotriosidase activity (mean ± SE).*

Group	Chitotriosidase activity
Control mice (CBA)	387.0 + 30.4 (11)
CMG, 48 h	738.7 + 67.7* (9)
CMG, 7 days	833.5 + 103.6* (6)
Triton WR 1339, 48 h	279.1 + 28.3 (4)
Triton WR 1339, 72 h	250.8 + 13.3* (4)
Triton WR 1339, 7 days	794.5 + 84.9* (4)
Triton WR 1339, 12 days	493.4 + 73.0 (5)

Chitotriosidase activity expressed as nmol MUF/ml per hour. The number of animals is in parentheses. *$p < 0.05$ compared with control mice.

(9). Thus, an increase in chitotriosidase activity can be related to apoptotic process.

Ukrain treatment of mice with LS lymphosarcoma did not significantly decrease tumor weight and chitotriosidase activity in this group was even decreased (compared with untreated LS lymphosarcoma mice), indicating that macrophage depression was not prevented. Possibly, the dose of Ukrain should be increased for treatment of murine LS lymphosarcoma and other routes of administration should be used to achieve more effective local activation of macrophages.

Discussion

The natural polymer of β-1,4-*N*-acetylglucosamine chitin, an essential structuralal component of the cell walls, is a chitotriosidase substrate (2-4). It has been suggested that chitotriosidase is intensively synthesized and excreted by activated macrophages. Serum chitotriosidase activity has been used for several years in the diagnosis of human lysosomal storage diseases, mainly Gaucher's disease (5). Marked increases of chitotriosidase activity in plasma is specific for Gaucher's disease, while elevated activity was also discovered in other lysosomal storage disorders, such as Niemann-Pick disease (7).

In all the models of macrophage stimulation in mice and rats used, elevated chitotriosidase activity was discovered, serving as a biochemical indicator of macrophage stimulation. Murine LS lymphosarcoma induced macrophage depression as evidenced by decreased serum chitotriosidase activity. Both drugs used (Ukrain and cyclophosphamide in the doses indicated) were not sufficiently effective against this type of tumor. The earlier protective effect of the same dose of Ukrain was shown against murine HA-1 hepatoma (1). During treatment by cyclophosphamide or Ukrain, serum chitotriosidase activity was lower than in the control CBA mice. Obviously, increased doses of these drugs could be more effective. An increase in the dose of cyclophosphamide up to 100 mg/kg resulted in more effective treatment of murine LS lymphosarcoma without formation of metastases (9). Unfortunately, with higher cyclophosphamide doses, toxicity was also increased.

In general, a new approach was introduced for monitoring the efficacy of antitumor therapy, using serum chitotriosidase activity, a new biochemical marker of macrophage stimulation, as a possible index of efficacy of antitumor therapy. This test may be useful in medical clinics for the testing of macrophage stimulation and depression of patients with tumor diseases.

Recently, increased chitotriosidase activity was discovered in the cerebrospinal fluid of patients with several inflammatory neurological diseases (5). The chitotriosidase assay is a simple and reliable test for

activated macrophages in cerebrospinal fluid and is a useful tool for the differential diagnosis of neurological diseases. In many cases of diseases of unknown origin, the increase in chitotriosidase activity in cerebrospinal fluid can indicate an inflammatory process in the central nervous system (5).

A new approach for the testing of activated macrophages has recently been introduced in atherosclerosis (9). Strong induction of members of the chitinase family was discovered locally during lipid loading and lesion of macrophages (9). Thus, it can be concluded that the chitotriosidase activity assay has several applications. It is a new biochemical test to study macrophage stimulation *in vivo* in tumor and inflammatory diseases.

Acknowledgement

We wish to thank Prof. R. Wevers (Nijmegen, The Netherlands) for the kind gift of the substrate for the determination of chitotriosidase activity and the Perkin-Elmer 650-10S spectrofluorometer to the Laboratory of Cellular Biochemistry at the Institute of Physiology. We also thank Prof. B. Chartoryska (Warsaw, Poland) for help and advice during determination of chitotriosidase activity. Many thanks to our colleague Dr. G.A. Paul, who provided human serum (Regional Diagnostic Center, Novosibirsk, Russia).

References

(1) Korolenko T.A., Svechnikova I.G., Filjushina E.E., et al. *Macrophage stimulation and antitumor effect of Ukrain*. Drugs Exptl. Clin. Res., **24**, 253, 1998.

(2) Rencema G.H., Boot R.G., Muijsers A.O., Donker-Koopman W.E., Aerts J.M. *Purification and characterization of human chitotriosidase, a novel member of the chitinase family of proteins*. J. Biol. Chem., **270**, 2198, 1995.

(3) Rencema G.H., Boot R.G., Strijland A., Donker-Koopman W.E., van den Berg M., Muijsers A.O.J.M. *Synthesis, sorting, and processing into distinct isoforms of human macrophage chitotriosidase*. Eur. J. Biochem., **244**, 279, 1997.

(4) Rencema G.H., Boot R.G., Donker-Koopman W.E., et al. *Chitotriosidase, a chitinase, and the 39-kDa human cartilage glycoprotein, a chitin-binding lectin, are homologues of family 18 glycosyl hydrolases secreted by human macrophages*. Eur. J. Biochem., **251** (1-2), 504, 1998.

(5) Czartoryska B., Tylki-Szymacska A., Gyrska D. *Serum chitotriosidase activity in Gaucher patients on enzyme replacement therapy (ERT)*. Clin. Biochem., **31**, 417, 1998.

(6) Hollak C.E.M., Van Weely S., Van Oerts M.H.J., Aerts J.M.F.G. *Marked elevation of plasma chitotriosidase activity. A novel hallmark of Gaucher disease*. J. Clin. Invest., **93**, 1288, 1994.

(7) GuoY., He W., Boer A.M., et al. *Elevated plasma chitotriosidase activity in various lysosomal storage discorder.* J. Inher. Metab. Dis., **18,** 717, 1995.

(8) Fachet J., Abel G., Jusupova S., et al. *Potentiation of non-specific host defence by polysaccharides like lentinan: Possible mechanisms*. Int. J. Immunotherapy, **5** (4), 167, 1989.

(9) Kaledin V.I., Nicolin V.P., Timofeera D.A., et al. *Cyclophosphane-induced apoptosis of murine lymphosarcoma cells: Experimental model in vivo.* Voprosy Oncol. (Rus)., **45**, 889, 2000.

(10) Boot R.G., Van Achterberg T.A., Van Aken B.E., et al. *Strong induction of members of the chitinase family of proteins in atherosclerosis: Chitotriosidase and human cartilage gp-39 expressed in lesion macrophages*. Arterioscler. Thromb. Vasc. Biol., **19**, 687, 1999.