Effect of Modification of the Carboxyl Groups of the Sialic Acid Binding Lectin from Bullfrog (Rana catesbeiana) Oocyte on Anti-tumor Activity

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The sialic acid binding lectin from bullfrog Rana catesbeiana oocyte (cSBL) is known to have anti-tumor activity. In order to investigate the relationship between the net charge of cSBL and its anti-tumor effect, cSBL was modified with a water-soluble carbodiimide (EDC) in the presence of three kinds of nucleophiles, taurine, glycine methyl ester and ethylenediamine. cSBL having four carboxyl groups was partially modified (ca. 2 residues). The anti-tumor activity of modified cSBLs was in the order of ethylenediamine-modified cSBL > glycine methyl ester-modified cSBL > taurine modified cSBL ≥ native cSBL. The results suggested that anti-tumor activity seems to increase with the increase in positive net charge, possibly enhancing the interaction of cSBL with sialoglycoprotein on the surface of tumor cells. The ribonuclease activity of ethylenediamine-modified cSBL decreased with the progress of the reaction, but the number of internalized molecules in the tumor cell increased. Thus, for anti-tumor activity, a higher incorporation of cSBL with reasonable RNase activity seems to be more important than total RNase activity.

Key words anti-tumor agent; ribonuclease; sialic acid binding protein; carbodiimide; chemical modification; carboxyl group

Fig. 1. Amino Acid Sequences of Four Frog RNases and Bovine Pancreatic RNase A

Rnase A: bovine pancreatic Rnase A; Rnase RC: Rnase from bullfrog liver; onconase, Rnase from leopard frog oocyte; jSBL, sialic acid binding lectin from Japanese frog oocyte; cSBL, sialic acid binding lectin from bullfrog oocyte. Amino acid residues essential for catalysis are denoted by white letters, and half-cystine residues are by white letters on a black background. Figures at the top and bottom of the matrix are Rnase A and cSBL numbering, respectively.

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cSBL is a basic protein with pI 9.2. It is not unreasonable to assume that the basic nature is one of the favorable factors of its binding with sialoglycoprotein on the cell surface. cSBL has four carboxyl groups, including a C-terminal carboxyl group of proline residue. The chemical modification of these carboxyl groups increases the pI of cSBL, and may enhance its anti-tumor activity. In order to test this hypothesis, we attempted the chemical modification of cSBL by a water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), in the presence of various nucleophiles, taunine, glycine methylester, and ethylenediamine. By the modification with taunine having a sulfonate group, the net charge of the modified cSBL did not change in comparison with native cSBL. By using glycine methylester as a nucleophile, the net charge increased, and by using ethylenediamine, the net charge increased further. In this report we tested the effect of modification with three nucleophiles on anti-tumor and RNase activity.

MATERIALS AND METHODS

Enzyme  cSBL was purified according to the methods of Nitta et al. 1, 2

Cells  Murine leukemia P388 cells, Ehrlich ascites cells, myeloma B16 cells and S180II sarcoma cells were obtained from Health Science Research Resources Bank (Osaka).

Reagents  RPMI1640 medium and bovine fetal serum were obtained from Life Technologies (Gaithersburg, MD, U.S.A.) and Intergen (Purchase, NY, U.S.A.), respectively. Yeast RNA was a product of Marine Bio (Tokyo). Mono S column was obtained from Amersham Pharmacia Biotech, AB (Uppsala, Sweden). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was a product of Sigma (St. Louis, MO, U.S.A.). Taurine, glycine methylester and ethylenediamine were obtained from Wako Pure Chem. (Osaka).

Chemical Modification of cSBL  The pH of the reaction mixture consisting of cSBL (0.25 mg/ml) and a nucleophile (0.6 μl) was adjusted to 5.0 by the addition of 1 M NaOH, then 40 mM EDC (final concentration) was added to the reaction mixture with stirring. The reaction was performed at room temperature for 30—120 min. The reaction was stopped by the addition of 1/5 volume of 1 M sodium acetate (pH 5.0), followed by dialysis against de-ionized water. In this report, we used abbreviations for cSBL modified by EDC in the presence of nucleophile, taurine (TA), glycine methylester (GM) and ethylenediamine (ED) as EDC-TA cSBL, EDC-GM cSBL and EDC-ED cSBL, respectively. The suffix means the reaction time of modification in minutes.

Estimation of Ethylenediamine Incorporated into cSBL  The amount of ethylenediamine bound to cSBL was determined by amino acid analysis after hydrolysis with 6 M HCl at 110 °C for 24 h. Ethylenediamine was eluted after phenylalanine in an amino acid analysis system, AccQ Tag System (Waters Milford, MA, U.S.A.) with increasing concentrations of acetonitrile.

Assay for RNase Activity  RNase activity was measured according to the previous method, 7 with RNA (0.25 mg/ml) as a substrate in 50 mM Tris–HCl buffer (pH 7.5) at 37°C. One unit of enzyme was defined as the enzyme amount which increased absorbancy 1.0 at 260 nm in 5 min under the experimental conditions.

Protein Concentration  cSBL concentration was measured spectrophotometrically assuming tentatively that the absorbancy at 280 nm of 0.1% solution is 1.0.

Measurement of CD Spectrum  The CD spectrum of the cSBL solution was measured by a J-600 spectropolarimeter (JASCO, Tokyo).

Anti-cell Proliferation Activity of cSBL  Anti-cell proliferation activity was measured with murine leukemia P388 cells or other tumor cells according to the method of Nitta et al. 3 P388 cells were cultured in RPMI1640 supplemented with 10% fetal calf serum. The cells were collected by centrifugation, then suspended in the medium and diluted to 2 × 10^5 cells/ml. In a 96-well plate, with each well containing 200 μl of the cell suspension, 10 μl of various concentrations of cSBL or modified cSBL was added. The viable cell number was counted after 48 h incubation at 37°C under 5% CO₂. Inhibition of cell proliferation was calculated as the percentage decrease of the increase in cell number against the increase in the control cell number. Anti-proliferation activity toward Ehrlich ascites cells, myeloma B16 cells, and S180II sarcoma cells was measured by a similar method.

Estimation of the Amount of cSBL or Modified cSBL  The amount of native and modified cSBL in P388 cells was estimated by RNase activity as a marker. One milliliter of P388 cells containing 2 × 10^5 cells was incubated with 2 μg cSBL or modified cSBL in the medium described above for 30 min at 37°C. The cells were collected by centrifugation at 200 × g and were then washed with PBS two times. The cell pellet was suspended in 0.5% Triton X-100 in 0.1 M Tris–HCl buffer (pH 7.5) and frozen and thawed twice, then centrifuged again. RNase activity of the supernatant was measured, and the amount of enzyme incorporated was calculated on the basis of the specific activity of the cSBL used.

RESULTS

Effect of EDC-Modification on RNase Activity of cSBL  The enzymatic activity of EDC-modified cSBLs is shown in Table 1. The RNase activity of EDC-modified cSBL decreased in the order of EDC-TA cSBL, EDC-GM cSBL and EDC-ED cSBL. This order coincided with the increase in net positive charge of the modified cSBL. The relationship between the nucleophile incorporated and enzyme activity was investigated in the case of EDC-ED modification. Incorporation of ethylenediamine increased time-dependently, and enzymatic activity decreased simultaneously. However, the incorporation of ethylenediamine was about 1.5, 2.2 and 2.4 residues/mol cSBL at the reaction time of 0.5, 1 and 2 h, respectively. The modification was performed for 120 min in the presence of 6 M guanidine–HCl to avoid the effect of a...
higher ordered structure that might prevent the modification. However, in the presence of such a denaturant, the incorporation of ethylenediamine was 2.1 residues/mol cSBL. The value did not exceed that in the absence of a denaturant. Thus, we thought that only two carboxyl groups among 4 (two Glu, one Asp and one C-terminal carboxyl group of Pro) are modified by EDC. The enzyme activity of EDC-ED cSBL120 was about 7% of the native cSBL. The CD spectrum among the 200 to 250 nm wavelength range of EDC-ED cSBL120 was almost the same as that of native cSBL (data not shown). We thus concluded that the decrease in ribonuclease activity didn’t derive from any significant conformational change by the incorporated ethylenediamine.

Effect of EDC-Modification of cSBL on Proliferation of P-388 Cell

The effect of EDC-modification of cSBL on the cell growth of mouse P388 cells was studied, and the results are shown in Fig. 2. The IC$_{50}$ for cell growth by the EDC-modified cBSLs in the presence of three nucleophiles are about 1.5, 1.5, 1.0 and 0.3 $\mu$M for native cSBL, EDC-TA cSBL$_{30}$, EDC-GM cSBL$_{30}$ and EDC-ED cSBL$_{30}$, respectively. EDC-TA cSBL had little effect on IC$_{50}$ value. The order of inhibitory effect coincided with the increase in net positive charge. However, to investigate further, we need to estimate the amount of incorporated nucleophile. Among the three nucleophiles, we were able to analyze easily only ethylenediamine incorporated into cSBL, and the inhibitory effect of EDC-ED cSBL was the largest of three. Further study was therefore mainly performed with ethylenediamine as a nucleophile (EDC-ED cSBL).

Relationship between the Incorporation of Ethylenediamine and Enzymatic Activity and Cell Growth Inhibitory Effect

In the modification of cSBL with EDC in the presence of ethylenediamine, enzymatic activity decreased time-dependently along with the incorporation of ethylenediamine. However, the incorporation of ethylenediamine seemed to reach 2 residues/mol cSBL. To avoid the interference of the modification by a higher ordered structure, we performed the modification reaction in 6M guanidine–HCl. Under this condition, the incorporation of ethylenediamine was roughly the same as that modified for 120 min (2.1 residues/mol cSBL). Thus, about half of EDC, which might modify four carboxyl groups, can be replaced with ethylenediamine. The cell growth inhibitory effect also increased with the incorporation of ethylenediamine. However, the dose–inhibitory effect curve of various degrees of modification were not so markedly different from each other in terms of the modified cSBL used (Fig. 3).

Quantitation of cSBL in P388

The amount of cSBL internalized into the P388 cells was estimated by measuring RNase activity in cell lysates. EDC-ED cSBL$_{120}$, which has shown the most potent effect of cell growth inhibition, was internalized by about 8 times that of the control cSBL, though the RNase activity in the cells was very low (50% of cSBL).

The Effect of EDC-ED cSBL on the Proliferation of Other Tumor Cells

The effect of EDC-ED cSBL$_{120}$ on the cell growth of the other tumor cells, Ehrlich ascites cells, myeloma B16, and S180II sarcoma cells, was tested (Fig. 4). The modified cSBL inhibited the cell growth of the three other tumor cells 10—30 times more effectively than that of native cSBL.
DISCUSSION

The mechanism of growth inhibition of cSBL toward tumor cells is not yet fully understood. However, the fact that sialidase-treated tumor cells (P388) are resistant to cSBL, suggests that the binding of cSBL with the sialoglycoprotein on the cell surface is an important process in the internalization of cSBL. The experiment of Nitta et al. demonstrated that cSBL treated with EDC-glycine methylester increased the cytotoxic effect of cSBL. The data reported here also suggests that the electrostatic interaction between cSBL and cell surface sialoglycoprotein might be considered an important factor affecting the cell toxicity. Rains et al. reported that the cell growth inhibitory effect by an RNase family enzyme can be attributed by the presence of free RNase (which did not combine with an internal RNase inhibitor). Bovine seminal RNase, which is known to exist as a dimeric form, also has an anti-tumor effect. It was thought that bovine seminal RNase bound with human placental RNase inhibitor very weakly, therefore, cellular RNase activity increased by the incorporation of seminal RNase. Although onconase hydrolyzes RNA at 1/100th the rate of hydrolysis by RNase A, it is an effective anti-tumor agent. Wu et al. proposed that onconase probably binds with some kind of receptor on the cell surface and is internalized, then hydrolyzes rRNA, causing the decrease in protein synthesis. The scheme is very similar to the hypothesis described in the case of cSBL, although there is some difference on the nature of the receptors. For both enzymes, the importance of RNase activity was stated. However, high RNase activity does not seem to be required for the anti-tumor effect of both enzymes. The experiment in this paper suggests that a higher affinity towards the cell surface receptor is more important than the higher RNase activity. However, it remains to be clarified why we have to assume the difference in receptors for both cases, onconase and cSBL, in spite of the similar primary structures of onconase and cSBL.

We were not able to identify the location of carboxyl groups modified by EDC in the presence of nucleophiles. However, Glu97 is one of the most probable candidates, because Glu97 corresponds to Glu111 of RNase A, which is known to be involved in the B2 base recognition site, and is important for RNA binding. Thus, the modification might be related to the inactivation. The assignment of these groups by means of site-directed mutagenesis will be reported in the following paper.