Preparation of Potent Cytotoxic Ribonucleases by Cationization: Enhanced Cellular Uptake and Decreased Interaction with Ribonuclease Inhibitor by Chemical Modification of Carboxyl Groups†

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ABSTRACT: Carboxyl groups of bovine RNase A were amidated with ethylenediamine (to convert negative charges of carboxylate anions to positive ones), 2-aminoethanol (to eliminate negative charges), and taurine (to keep negative charges), respectively, by a carbodiimide reaction. Human RNase 1 was also modified with ethylenediamine. Surprisingly, the modified RNases were all cytotoxic toward 3T3-SV-40 cells despite their decreased ribonucleolytic activity. However, their enzymatic activity was not completely eliminated by the presence of excess cytosolic RNase inhibitor (RI). As for native RNase A and RNase 1 which were not cytotoxic, they were completely inactivated by RI. More interestingly, within the cytotoxic RNase derivatives, cytotoxicity correlated well with the net positive charge. RNase 1 and RNase A modified with ethylenediamine were more cytotoxic than naturally occurring cytotoxic bovine seminal RNase. An experiment using the fluorescence-labeled RNase derivatives indicated that the more cationic RNases were more efficiently adsorbed to the cells. Thus, it is suggested that the modification of carboxyl groups could change complementarity of RNase to RI and as a result endow RNase cytotoxicity and that cationization enhances the efficiency of cellular uptake of RNase so as to strengthen its cytotoxicity. The finding that an extracellular human enzyme such as RNase 1 could be effectively internalized into the cell by cationization suggests that cationization is a simple strategy for efficient delivery of a protein into cells and may open the way of the development of new therapeutics.

Ribonucleases (RNases)[1] constitute a large superfamily crossing over many species (*1*). Bovine pancreatic RNase A (EC 3.1.27.5), a prototype of mammalian extracellular RNases, is a digestive enzyme toward RNA and not cytotoxic like most of RNase A superfamily. However, some members of the RNase A superfamily have cytotoxic activity in addition to simple ribonucleolytic activity (reviewed in ref *2*). Remarkably, bovine seminal RNase (BS-RNase) and the amphibian RNase, Onconase isolated from *Rana pipiens*, are highly toxic to cancer cells (*3*). Onconase is currently undergoing phase III human clinical trials (*4*). The cytotoxic mechanisms for these two classes of RNases have been proposed as follows, the proteins are concentrated on extracellular matrix, internalized by nonreceptor-mediated endocytosis, and reach the cytosol, where they degrade cellular RNA, thus causing apoptotic and/or nonapoptotic cell death (*5*−*8*). Since, both Onconase and BS-RNase are relatively insensitive to a cytosolic RNase inhibitor (RI), and since RI gene is ubiquitously expressed in human tissues (*9*), the insensitivity of these RNases to RI could contribute to their cytotoxic activity (*5*, *6*).

In this regard, there may be several properties a cytotoxic RNase should have, first, the RNase should be an active catalyst; second, the RNase should be effectively internalized into the cytosol and translocated to the proper destination; and third, the RNase should be able to evade the endogenous RI. To enhance the cytotoxic activity by increasing the internalization efficiency, nontoxic RNases have been linked to cell-binding ligands, such as antibodies, transferrin, growth factors and cytokines (reviewed in ref *2*). These engineered RNases showed cell-type specific cytotoxicity, although the sensitivity of these RNases to RI would limit their cytotoxic potency if RI were present in internalization pathways in cells. To address the interaction with RI, mutants of RNase A in which the interaction with RI is disrupted have been designed (*10*, *11*). These engineered RNases are moderately cytotoxic.

Recently, Suzuki et al. (*12*) have made more cytotoxic RNases by combining two of these strategies. They linked RNase 1 (a human counterpart of RNase A) and eosinophil-derived neurotoxin (EDN or RNase 2, another human RNase) to transferrin. This allowed receptor-mediated endocytosis via the transferrin receptor and sterically hindered the interaction between RI and the RNase. Since BS-RNase and Onconase show comparable antitumor activity without the

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[1] Abbreviations: BS-RNase, bovine seminal RNase; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydorochloride; HEL, hen egg lysozyme; RI, RNase inhibitor, RITC, rhodamine B isothiocyanate.

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presence of cell targeting moiety (*2*), we hypothesize that the cytotoxicities of these naturally occurring RNases are derived not only from their low affinity for RI but also from their efficient internalization into the cytosol due to their effective adsorption to the cell surface.

A highly cationic protein would be efficiently adsorbed to the negatively charged cell surface by electrostatic interaction, resulting in the efficient internalization into the cell. Therefore, it is possible that highly cationic RNases which exhibit a low affinity for RI should show potent cytotoxicity. In fact, BS-RNase, a homodimeric protein with more than 80% sequence identity to RNase A, not only has low affinity for RI due to its unique dimeric structure but also is more cationic than RNase A (*3*). Amidation of carboxyl groups by a carbodiimide reaction (*13*) is a suitable way to change the net charge of the RNase systematically because various amines can be utilized for the modification. Furthermore, it is expected that the interaction of the RNase with RI would be sterically hindered by the modification of the carboxyl groups with amines having a side chain moiety. Therefore, we examined these possibilities using nontoxic bovine RNase A and human RNase 1. We prepared RNase derivatives with various net charges by amidation of carboxyl groups catalyzed by the carbodiimide reaction with ethylenediamine, 2-aminoethanol, and taurine, respectively. All of these chemically modified RNases acquired cytotoxic activity toward mouse malignant 3T3-SV-40 cells in various degrees and became relatively insensitive to RI. Cytotoxicity and cell binding ability of these RNases were correlated well with their net positive charges.

MATERIALS AND METHODS

Materials. Bovine RNase A was obtained from Sigma (Type II-A or XII-A). Human RNase 1 was a recombinant protein expressed and purified as described previously (*14*, *15*). BS-RNase was kindly provided by Prof. M. Irie (Hoshi College of Pharmacy, Japan). Hen-egg lysozyme (HEL) was donated by QP (Tokyo, Japan). 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydorochloride (EDC) was purchased from Dojin (Kumamoto, Japan). Ethylenediamine, 2-aminoethanol, taurine, and recombinant human placental RNase inhibitor (RI) were from Wako Chemical (Osaka, Japan).

Chemical Modifications. Coupling reactions of a protein with various amine nucleophiles by EDC were performed as described previously (*13*). Briefly, 150 mg of RNase A was dissolved in 5 mL of 2 M ethylenediamine, 2 M 2-aminoethanol, or 1 M taurine, respectively. The pH of each amine solution had been previously adjusted to 5.0 with HCl, before dissolving the protein. The reaction was initiated by adding 100 mg of EDC to each solution with stirring at room temperature. After 5 h of stirring, an additional 100 mg of EDC was added and stirring continued for an additional 12 h. The solutions were then exhaustively dialyzed against distilled water and lyophilized to give ethylenediamine-modified RNase A (RNaseA-NH_3^+), 2-aminoethanol-modified RNase A (RNaseA-OH), and taurine-modified RNase A (RNaseA-SO_3^-), respectively. HEL and RNase 1 were also modified with ethylenediamine similarly except that the reaction scale for RNase 1 was reduced to 1/10. Thus, ethylenediamine-modified HEL (HEL-NH_3^+) and ethylenediamine-modified RNase 1 (RNase1-NH_3^+) were obtained. To prepare the enzymatically inactive cationized RNase A maintaining the native conformation, one of the catalytic histidine residues (either His12 or His119) of RNaseA-NH_3^+ was alkylated by bromoacetate (*16*), yielding carboxymethylated RNaseA-NH_3^+ (CM-RNaseA-NH_3^+). To prepare the unfolded cationized RNase A, RNaseA-NH_3^+ was reduced with mercaptoethanol and *S*-alkylated with iodoacetamide (*17*), yielding *S*-carboxamidomethylated RNaseA-NH_3^+ (CAM-RNaseA-NH_3^+).

The number of modified carboxyl groups in the proteins was calculated from the number of amine moieties incorporated, determined by amino acid analysis using a Hitachi L-8800 amino acid analyzer for 2-aminoethanol and taurine and by colorimetric analysis of the number of free amino groups using 2,4,6-trinitrobenzenesulfonic acid (*18*) for ethylenediamine, respectively.

Protein concentrations were determined by UV spectroscopy assuming molar extinction coefficients of $\epsilon_{278} = 10\,105$ M^{-1} cm^{-1} for RNase A and its derivatives, $\epsilon_{278} = 7508$ M^{-1} cm^{-1} for RNase 1 and RNase1-NH_3^+, $\epsilon_{278} = 12\,450$ M^{-1} cm^{-1} for BS-RNase, and $\epsilon_{280} = 37\,717$ M^{-1} cm^{-1} for HEL-NH_3^+ (*17*, *19*).

RNase Activity and Interaction with RNase Inhibitor. Ribonucleolytic activities of the native and chemically modified RNases were determined in 0.1 M Tris-HCl buffer containing 0.1 M NaCl at pH 7.5 and 25 °C using yeast RNA as the substrate as described elsewhere (*14*).

Interaction of the RNases with RI was assessed by using agarose gel-based assay of the inhibition of ribonucleolytic activities against *Escherichia coli* ribosomal RNA (*10*, *12*). Briefly, 1 μL of RNase (10 ng) was added to 10 μL of 50 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 10 mM DTT, 4 μg of 16S- and 23S-ribosomal RNA form *E. coli* MRE600 (Boehringer Mannheim, Germany), and 40 units of RI (a 10-fold molar excess of RI; 1 unit of RI is defined as the amount required to inhibit the ribonucleolytic activity of 5 ng of RNase A by 50%), and incubated for 15 min at 37 °C. Samples were then subjected to 1.5% agarose gel electrophoresis using 20 mM Tris-0.5 mM EDTA-acetic acid buffer, pH 7.8. The gels were stained with ethidium bromide and visualized with UV. BS-RNase was used as a control.

Cytotoxicity Assays. Cytotoxicity assays were conducted with Swiss mouse albino 3T3 cells transformed with SV40 (3T3-SV-40; Dainippon Pharmaceutical Co., Japan). 3T3-SV-40 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 70 μg/mL of kanamycin. Cell viability was evaluated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma), as described previously (*20*, *21*). Briefly, aliquots (100 μL) of exponentially growing 3T3-SV-40 cells were seeded into 96-well plates (1500 cells/well) and incubated for 12 h at 37 °C, before 50 μL of the medium was replaced with the same volume of the serially diluted protein samples in culture medium. Incubation continued for 3 days before termination and cell viability was determined using the MTT assay according to the manufacture's instruction. The percent of cell growth was calculated in triplicate using cells grown without protein samples as the control.

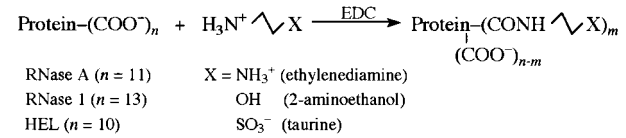

FIGURE 1: Chemical modification of RNases. Amidation of carboxyl groups of a protein with ethylenediamine, 2-aminoethanol, or taurine by water soluble carbodiimide EDC. The *n* and *m* indicate the numbers of original and modified carboxyl groups, respectively.

Rhodamine-Labeling of RNases and Fluorescent Microscopy. Native and chemically modified RNase As were labeled with Rhodamine B isothiocyanate (RITC, Sigma) (*22*). Briefly, 2 mg of protein were dissolved in 0.49 mL of 40 mM triethanolamine-HCl buffer, pH 8.4, and an appropriate amount of RITC in dimethylformamide (34 mg/mL) was added as described below. The mixture was stirred in the dark at room temperature for an appropriate period. The amount of the RITC solution added and the reaction period for the labeling of the respective proteins were as follows: RNase A (10 μL, for 3 days), RNaseA-SO_3^- (5 μL, for 4 h), RNaseA-OH (5 μL, for 3 h), and RNaseA-NH_3^+ (10 μL, for 1 h). RITC-labeled RNases thus obtained were desalted either by dialysis against distilled water or by Sephadex G-25 column chromatography using PBS as the elution buffer. The number of moles RITC incorporated per mole RNase A was determined using the extinction coefficient for rhodamine ($\epsilon_{558} = 1.29 \times 10^4$ M^{-1} cm^{-1}) (*22*) and amino acid analysis to determine protein concentration. The number of moles of RITC per mole of RNase was 1.0 for RNase A, 1.3 for RNaseA-SO_3^-, 1.8 for RNaseA-OH, and 1.3 for RNaseA-NH_3^+, suggesting that the electronic natures of these RNases were not seriously altered by such the light modification.

For fluorescent microscopy, approximately 2×10^4 cells of 3T3-SV-40 were plated in an eight-chamber tissue culture slide (NALGE NUNC International K. K., IL) and cultured in DMEM with 10% FBS for 24 h before the addition of RITC-labeled RNases. To have equal concentrations of the rhodamine moiety (2 μM), the final concentrations of RITC-labeled RNase A, RNaseA-SO_3^-, RNaseA-OH, and RNaseA-NH_3^+ in media were set to 2.0, 1.5, 1.1, and 1.6 μM, respectively. Thus, the fluoresence intensity of cells adsorbing (or incorporating) these RNases could be normalized by the affinity of cells for these RNases. After incubation for 120 min at 37 °C, the cells were directly examined using a laser scanning confocal microscopy (model MRC-1024; Bio-Rad).

RESULTS

Preparation of Chemically Modified RNases. As shown in Figure 1, the carboxyl groups of RNase A (the number of carboxyl groups, $n = 11$) were amidated with either ethylenediamine (converts the negative charge of the carboxylate anion to a positive charge), with 2-aminoethanol (eliminates negative charges), or with taurine (maintains negative charges) by the carbodiimide reaction, to give ethylenediamine-modified RNase A (RNaseA-NH_3^+), 2-aminoethanol-modified RNase A (RNaseA-OH), or taurine-modified RNase A (RNaseA-SO_3^-), respectively. Human RNase 1 ($n = 13$) and hen egg lysozyme (HEL, $n = 10$) were also modified with ethylenediamine to give ethylenediamine-modified RNase 1 (RNase1-NH_3^+) and HEL (HEL-NH_3^+). The number of modified carboxyl groups in RNaseA-OH and RNaseA-SO_3^- were directly determined by amino acid analysis which could determine the number of 2-aminoethanol or taurine moieties incorporated per RNase A molecule. For ethylenediamine-modified proteins (RNaseA-NH_3^+, RNase1-NH_3^+, and HEL-NH_3^+), the number of modified carboxyl groups (value of *m*, in Figure 1) was calculated from the increased number of free amino groups determined by a method using 2,4,6-trinitrobenzenesulfonate (*18*). As shown in Table 1, approximately 55−80% of carboxyl groups in each protein were amidated. From the values of *m*, the net charge of each modified protein at neutral pH was estimated (Table 1).

SDS−PAGE analysis under nonreducing conditions of the modified RNases (Figure 2) indicated that they migrated slightly slower than unmodified RNases as expected from the increase in molecular mass due to the modification. The bands of the modified RNases were much broader than those of the RNases indicating that the modified RNases were mixtures of heterogeneously modified proteins. Consistent with the estimated values of net positive charge which partly neutralize the negative charge of the SDS micell, the migration of the modified RNase As on SDS−PAGE showed the tendency to decrease in the order of RNaseA-SO_3^- (net charge to be +4), RNaseA-OH (+10), and RNaseA-NH_3^+ (+20) (Table 1 and Figure 2).

The cationic nature of the modified proteins was also examined by ion-exchange HPLC using a carboxylic cation-exchanger column, CM-Toyopearl 650S (4 × 150 mm, Tosoh, Tokyo), and eluting with a linear gradient of NaCl from 0 to 1 M in 0.05 M phosphate buffer, pH 7.0, over 100 min at a flow rate of 1 mL/min. Although both RNase A and RNase 1 were eluted as sharp peaks at concentrations of 0 M (pass through) and 0.14−0.17 M NaCl, respectively, the modified proteins gave very broad peaks with rather complex shapes (data not shown), indicating heterogeneity of the modified proteins. Concentration ranges of NaCl at which the respective proteins were eluted from the column are shown in Table 1. In general, as expected the concentration of NaCl needed for the elution of a protein increased with the increase in net positive charge of the protein. The exception was RNaseA-SO_3^- which showed considerably different behavior from RNase A, although both proteins possess the same net charge (+4). It is possible that there was some interaction between RNaseA-SO_3^- and the cation-exchange resin, such as a hydrophobic interaction, may be also changed by the modification of carboxyl groups with amines.

Thus, a series of RNase derivatives, in which the net positive charge was systematically altered, was obtained. In addition to HEL-NH_3^+ (net charge to be +23), histidine-carboxymethylated RNaseA-NH_3^+ (CM-RNaseA-NH_3^+, net charge to be +19) and reduced and *S*-carboxamidomethylated RNaseA-NH_3^+ (RCAM-RNaseA-NH_3^+, net charge to be +20) were prepared as cationized protein devoid of ribonucleolytic activity.

RNase Activity and Sensitivity to the RNase Inhibitor. Ribonucleolytic activities of RNases were assessed by using yeast RNA as a substrate in 0.1 M Tris-HCl buffer containing 0.1 M NaCl at pH 7.5 and 25 °C. The specific activity of the RNases were decreased by the modification of carboxyl groups (Table 1). RNaseA-NH_3^+ and RNase1-NH_3^+ retained

Table 1: Characteristic Properties of Native and Modified RNases

molecule	modified carboxyls (*m*/*n*)[a]	net charge[b]	concentration of NaCl for elution (M)[c]	relative RNase activity (%)[d]
RNase A	0/11	+4	0 (pass through)	100
RNaseA-SO_3^-	6/11	+4	0.06−0.40	63.2
RNaseA-OH	6/11	+10	0.22−0.48	17.7
RNaseA-NH_3^+	8/11	+20	0.33−0.58	1.6
RNase 1	0/13	+6	0.14−0.17	37.5
RNase1-NH_3^+	10.5/13	+27	0.40−0.77	0.38
CM-RNaseA-NH_3^+	8/11	+19[e]	ND[f]	<0.01
RCAM-RNaseA-NH_3^+	8/11	+20	ND[f]	<0.01
HEL-NH_3^+	7.5/10	+23	0.44−0.75	<0.01

[a] Number of modified carboxyls (*m*) versus number of available carboxyls (*n*). See Figure 1A. [b] The net charge of each protein at neutral pH was estimated form its *m* value. [c] Concentration of NaCl required to elute each protein from a CM-Toyopearl 650S (0.4 × 25 cm, a cation-exchange HPLC column; Tosoh, Japan) column, which was eluted with a linear gradient of NaCl from 0 to 1 M in 0.05 M phosphate buffer at pH 7.0 over 100 min at a flow rate of 1 mL/min. [d] Ribonucleolytic activity against 0.06% yeast RNA in 0.1 M Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl at 25 °C (*14*). [e] Due to the carboxymethylation of one histidine residue (either His12 or His119), the net positive charge was reduced by one. [f] Not determined.

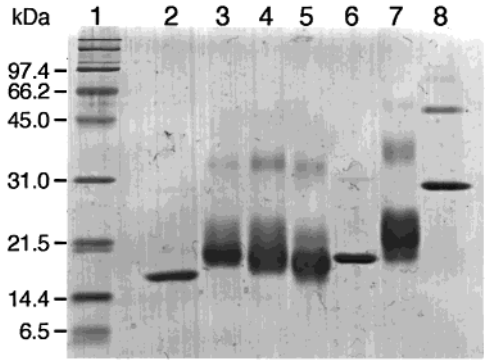

FIGURE 2: SDS−PAGE analysis of native and chemically modified RNases. Samples (1.5 μg/lane) were analyzed by SDS−PAGE under nonreducing conditions using a 15% polyacrylamide gel. Lane 1, Bio-Rad broad range molecular weight marker; lane 2, native RNase A; lane 3, RNaseA-NH_3^+; lane 4, RNaseA-OH; lane 5, RNaseA-SO_3^-; lane 6, native RNase 1; lane 7, RNase1-NH_3^+; lane 8, native BS-RNase. The gel was stained with Coomassie brilliant Blue R250.

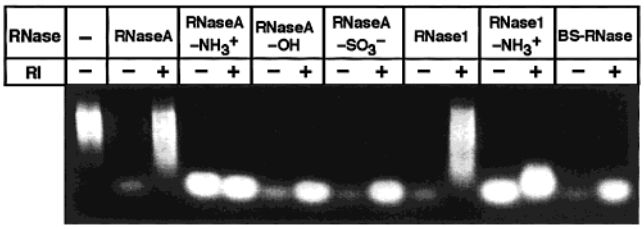

FIGURE 3: Agarose gel-based assay of enzymatic activity and RI resistance of RNases. Ribosomal RNA was treated with native or chemically modified RNases (10 ng) in the presence or absence of RI (40 units, a molar ratio of enzyme to inhibitor of 1:10) for 15 min at 37 °C. Samples were then subjected to 1.5% agarose gel electrophoresis.

only 1.6 and 0.38% activity of native RNase A, respectively whereas RNase-SO_3^- retained a higher activity (63.8%). As expected, the negative controls, HEL-NH_3^+, CM-RNaseA-NH_3^+, and RCAM-RNaseA-NH_3^+ were found to be completely inactive.

To assess the interaction of RNases with RI, the degradation of ribosomal RNA catalyzed by native and modified RNases as well as BS-RNase was examined in the absence and presence of human RI by means of an agarose gel-based assay (Figure 3). In the absence of RI, all RNases degraded ribosomal RNA. In the presence of 10-fold molar excess of RI, the catalytic activity of native RNase A and RNase 1

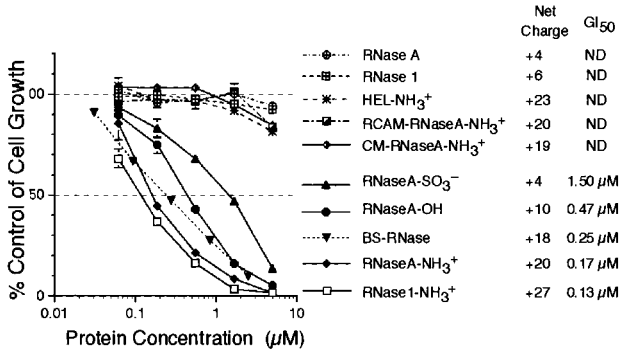

FIGURE 4: Cytotoxicity of ribonucleases. 3T3-SV-40 cells in DMEM supplemented with 10% FBS were seeded into 96-well plate (1500 cells/well) and left to adhere for 12 h and then treated with various concentration of each protein sample for 3 days. Cell growth of each well was monitored by MTT assay. Values are the mean from three cultures and are reported as a percentage of the buffer treated cells control, which was the mean value from medium without protein sample. On the right, the values of net charge and GI_{50} of each protein sample are shown. ND means not detected.

was almost completely inhibited by RI, whereas the catalytic activity of the modified RNases and BS-RNase was substantially reduced but not totally eliminated by RI. These results indicate that all modified RNases were relatively insensitive to RI like BS-RNase.

Cytotoxicity. The cytotoxicities of modified RNases (RNaseA-SO_3^-, RNaseA-OH, RNaseA-NH_3^+, and RNase1-NH_3^+), HEL-NH_3^+, CM-RNaseA-NH_3^+, and RCAM-RNaseA-NH_3^+ to 3T3-SV-40 cells were compared with those of native RNase A and RNase 1 as negative controls and with that of BS-RNase as a positive control. As shown in Figure 4, BS-RNase was cytotoxic to 3T3-SV-40 cells showing GI_{50} (the concentration required for inhibition of 50% of the cell growth) of 0.25 μM, while neither native RNase A nor RNase 1 were cytotoxic at the concentrations used in this assay. Surprisingly, all the modified RNases, which were not completely inactivated by RI, showed cytotoxicity (GI_{50} = 0.13−1.5 μM). Among the cytotoxic RNases, RNase1-NH_3^+ having the highest net positive charge (+27) was the most cytotoxic (GI_{50} = 0.13 μM), and RNaseA-SO_3^- having the lowest net positive charge (+4) was the least cytotoxic (GI_{50} = 1.5 μM). Despite the very low catalytic activity (Table 1), the highly cationic RNases, RNase1-NH_3^+ and RNaseA-NH_3^+ (net charges at neutral pH, +27 and +20, respectively), were much more cytotoxic than the naturally occurring

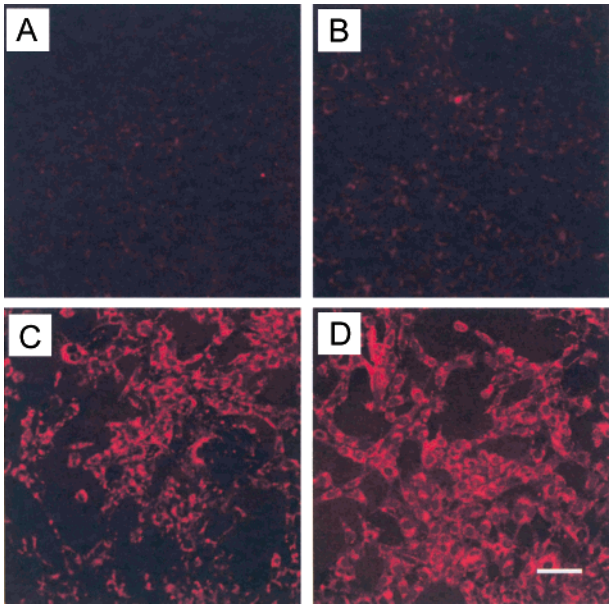

FIGURE 5: Fluorescent microscopic observation of 3T3-SV-40 cells treated with RITC-labeled RNase A derivatives. Confluent 3T3-SV-40 cells were incubated with each RITC-labeled RNase A derivative for 120 min at 37 °C, and the cells were examined under a laser scanning confocal microscopy. The concentration of Rhodamine moiety in each experiment was set to 2 μM, and thus the concentrations of RITC-labeled RNase A (A), RNaseA-SO_3^- (B), RNaseA-OH (C), and RNaseA-NH_3^+ (D) in media were 2.0, 1.5, 1.1, and 1.6 μM, respectively. The scale bar in panel D is equivalent to 50 μm.

cytotoxic BS-RNase. The control protein, HEL-NH_3^+, which is not an RNase but a small globular protein similar to RNaseA-NH_3^+ or RNase1-NH_3^+ with respect to molecular mass (14.3 kDa) and net positive charge (+23), was not cytotoxic. Furthermore, the enzymatically inactive cationic RNase A derivative having either denatured (RCAM-RNaseA-NH_3^+) or native (CM-RNaseA-NH_3^+) conformation was also not cytotoxic, indicating that ribonucleolytic activity of modified RNases are essential for their cytotoxicity.

Detection of Fluorescence-Labeled RNase in Cell Culture. To assess the interaction of native and modified RNase As with 3T3-SV-40 cells, RITC-labeled RNase at a concentration corresponding to 2 μM of the RITC moiety was incubated with 3T3-SV-40 cells for 120 min at 37 °C. The level of fluorescence was then examined using a fluorescent microscopy. As shown in Figure 5, the amount of RITC-labeled RNase associated with the cell increased with the increase in the net positive charge of the RNase A derivatives.

DISCUSSION

As suggested by Leland et al. (*10*), it is possible that RNases that retain catalytic activity in the presence of RI are cytotoxins by themselves. Furthermore, increasing the affinity of the RNases for cells could be more potent cytotoxins. We examined both these possibilities by modifying the carboxyl groups of the nontoxic bovine RNase A and human RNase 1 with amines having various side chains (Figure 1 and Table 1). The introduction of side chains caused by amidation of the carboxyl groups could lead to destabilization of the RNase−RI complex by steric hindrance while the concomitant cationization could induce efficient

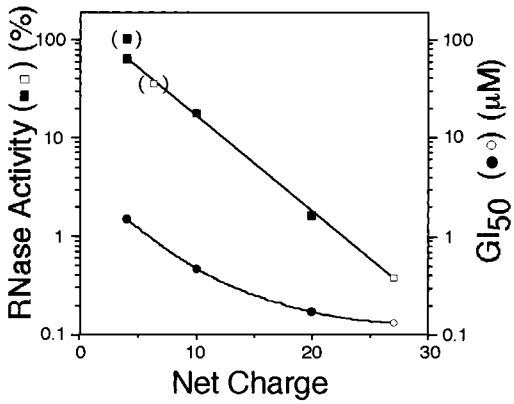

FIGURE 6: Correlation of RNase activity and of cytotoxicity with net charge of the chemically modified RNase A (filled circle and filled square) or RNase 1 (open circle and open square). The correlation between the RNase activity and net charge of native RNases is parenthesized.

binding of the RNase to an anionic cell surface and result in an enhanced cellular uptake. The following results were obtained.

First, all the chemically modified RNases showed decreased enzymatic activity (Table 1). Interestingly, as shown in Figure 6, the activities of all RNases including native ones correlated well with their net positive charges. These results suggest that the ribonucleolytic activity of RNases is mainly governed by the net positive charge. Since the substrate RNA is a polyanionic molecule, we speculate, from the analogy to the lytic activity of lysozyme (*23*, *24*), that the rate-determining step under the conditions employed is not a hydrolysis reaction of RNA substrate but the release of products from enzyme or turnover process. That is, when the net positive charge increases, the electrostatic interaction between the cationic enzyme and anionic products would be increased to reduce the product release or turnover rate. Of course the same interaction also facilitates the formation of an enzyme−substrate complex, and therefore, a well-known bell-shaped ionic strength-activity profile of RNases (*25*) may be explained.

Second, even in the presence of 10-fold molar excess of RI, all modified RNases were still catalytically as active as BS-RNase, but native RNase A and RNase 1 were completely inactivated by RI (Figure 3). The results suggest that some of the extended side chains introduced into the RNases via carboxyl groups in amide bonds interferes with the formation of RNase−RI complexes by steric hindrance.

Third, all modified RNases showed cytotoxic activity to 3T3-SV-40 cells (Figure 4), and the cytotoxic activity correlated well with the net positive charge of the modified RNase (Figure 6), indicating that within cytotoxic RNases, a more cationic RNase is more cytotoxic. Since highly cationic proteins completely devoid of ribonucleolytic activity (HEL-NH_3^+, CM-RNaseA-NH_3^+, and RCAM-RNaseA-NH_3^+) were not cytotoxic, it appears that positive charge alone is not sufficient for cytotoxicity. Despite the same net charge (+4), RNaseA-SO_3^- but not native RNase A was cytotoxic. These observations may suggest that the importance of the insensitivity of RNaseA-SO_3^- to RI for the cytotoxicity. However, the interaction with the cation-exchange resin, mimicking anionic cell surface in one sense, was different between RNaseA-SO_3^- and native RNase A

(Table 1). Therefore, there is another possibility such that the interaction with cells is different between RNaseA-SO_3^- and native RNase A and the difference in this interaction directs the former but not the latter to a pathway to reach the target RNA in the cytosol and cause damage.

Fourth, the amount of RITC-labeled RNase A derivative associated with cells increased with an increase in the net positive charge of the derivative (Figure 5), indicating that a more cationic protein binds more efficiently to cells by an electrostatic interaction between the cationic protein and anionic cell surface components such as sialic acids in carbohydrate.

From all of these results, the following conclusions can be drawn. Noncytotoxic RNase A and RNase 1 can be converted to potent cytotoxic RNases by cationization. These cationized RNases retained enzymatic activity, became relatively insensitive to RI, and underwent enhanced cellular uptake through the increased binding to the cell surface by electrostatic interaction. The importance of electrostatic interaction for cytotoxicity was confirmed by the observation that the cytotoxicity of RNaseA-NH_3^+ was completely abolished by addition of heparin, an anionic carbohydrate, into culture media (data not shown).

Factors which have been reported to affect the cytotoxicity of RNase include; efficiency of endocytosis into the cell (*2*), resistance to endogenous RI (*10*, *12*), intracellular routing or localization (*8*, *26*), and stability against proteolytic degradation (*27*). The cationized RNases reported here may satisfy two factors of them. Extremely low enzymatic activity of RNaseA-NH_3^+ or RNase1-NH_3^+ may be a disadvantage for their cytotoxicity as suggested by Bretscher et al. (*28*). Furthermore, the cationized RNases prepared here were heterogeneous with respect to both the modified site and the number of the modification per molecule of RNase. More sophisticated chemical modification or site-directed mutagenesis might yield a more cytotoxic cationized RNase derivative.

In the cytotoxic assay, we used mouse malignant cell line 3T3-SV-40. Preliminary experiments indicated that the RNaseA-NH_3^+ was also cytotoxic to other cell lines such as K562 (human erythroleukemia), A431 (human epidermal carcinoma), B16/BL6 (mouse melanoma), and Lewis lung carcinoma (mouse lung carcinoma) (data not shown). Cationization has been demonstrated to enhance the cellular uptake of immunoglobulin G (IgG) (*29*−*34*) and other proteins (*35*−*37*) as a result of adsorptive-mediated endocytosis. These results and our results suggest that cationization is generally applicable to protein internalization into the cell.

The development of methodology which enhances protein internalization into cells is of great interest for use in both basic research and clinical research. In addition of the cationization method proposed here, other methods such as glycosylation (*38*, *39*), hydrophobization (*40*), microinjection or cellular transfection (*41*), and receptor-mediated endocytosis (reviewed in refs *2* and *42*) have been investigated for delivery of proteins. Recently, several small regions of proteins called protein transduction domains (PTDs) have been identified that possess the ability to traverse biological membranes efficiently in a process termed protein transduction (*43*−*46*). Although the mechanism is unknown (*45*, *46*), cationization may be partly responsible for the protein transduction because PTDs contain a lot of cationic amino acids (Lys or Arg). For example, Tat PTD from human immunodeficiency virus possesses 8 cationic amino acids out of 11 residues.

The clinical use in humans may be limited by immunogenicity of cationized protein, because cationization increases immunogenicity of *heterologous* protein (*47*). Apple et al. (*36*) have shown that enhanced immunogenicity is due to the increased uptake of cationized protein by antigen-presenting cells. However, when cationized *homologous* proteins are administered in high doses, there is no measurable immune response or tissue toxicity in experimental animal (*31*, *48*). Therefore, recombinant human proteins may be preferred for cationization.

Adsorptive-mediated endocytosis following protein cationization is normally a nonspecific internalization pathway. However, when a specific cell surface ligand, such as antibody, growth factor, or cytokine, is fused to a protein for cell targeting, efficiency of the receptor-mediated endocytosis of the protein may be also increased by cationization, because the effective concentration of the protein fused to the ligand around the receptors on the cell surface would also increase with an increase in the electrostatic interaction between the cationized protein and anionic cell surface. Therefore, cationization of RNase may be a strong strategy for development of RNase-based chemotherapeutic agents.

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