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ORIGINAL ARTICLE

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Epidermal growth factor receptor-dependent cytotoxic effect by an EGF–ribonuclease conjugate on human cancer cell lines

-A trial for less immunogenic chimeric toxin-

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Abstract Mammalian pancreatic ribonuclease (RNase) was conjugated chemically via a disulfide bond to human or murine epidermal growth factor (EGF). The conjugation between EGF and RNase was ascertained by SDS-PAGE using reduced and nonreduced conjugates. The EGF–RNase conjugate retained potent RNase activity and competed with ^{125}I-EGF for binding to EGFR to the same extent as unconjugated EGF. Both the human and murine EGF–RNase conjugates showed dose-dependent cytotoxicity against EGFR-overexpressing A431 human squamous carcinoma cells with IC_{50} values of $3 \times 10^{-7}\,M$ and $6 \times 10^{-7}\,M$, respectively, whereas free RNase had an IC_{50} of $10^{-4}\,M$. Against the EGFR-deficient small-cell lung cancer cell line H69, the EGF–RNase conjugate had no cytotoxic effect. The Human EGF–RNase conjugate showed dose-dependent cytotoxicity against other squamous carcinoma cell lines (TE-5, TE-1) and breast cancer cell lines (BT-20, SK-BR-3, MCF-7) and the cytotoxicity of the conjugate correlated positively with the level of expression of EGFR by each cell line. An unconjugated mixture of EGF and RNase had no greater effect than RNase alone on any cell line. Excess free EGF blocked EGF–RNase conjugate cytotoxicity against A431 cells. These results suggest that the EGF–RNase conjugate may be a more effective anticancer agent with less immunogenicity than coventional chimeric toxins.

Key words EGF · Ribonuclease · Conjugate · Breast cancer · Esophageal cancer

Introduction

Chimeric toxins are effective cytotoxic agents for cancer therapy. They commonly consist of plant or bacterial toxins conjugated to monoclonal antibodies [9] or ligands [7] that produce specific cytotoxicity against antigen-positive cell populations. There are some problems in the clinical application of chimeric toxins, one of which is the immunogenicity of the toxins which are usually composed of foreign proteins such as plant toxin, bacterial toxin and murine monoclonal antibody. We have developed a new class of agent composed of human EGF and mammalian RNase. Because both are endogenous proteins, this conjugate may elicit a less intense immune response in patients and thus may be safer.

Epidermal growth factor (EGF) [2] is a small endogenous protein with a molecular mass of 6045 Da. It is a mitogen for cells with specific receptors, and has been conjugated to the A chain of ricin and fragment A of diphtheria toxin and studied as a possible tumoricidal agent [3]. EGF has been also linked to *Pseudomonas exotoxin* and receptor-mediated endocytosis has been analyzed using this conjugate [6]. In addition, transforming growth factor alfa (TGFα)-*Pseudomonas* exotoxin conjugate has been created and its cytotoxicity via an EGF receptor (EGFR) assessed [7]. Breast and esophageal cancer patients with EGFR overexpression have a poor prognosis [21, 26]. These patients are not cured by surgery alone and need a multidisciplinary approach to treatment. As one element of a multidisciplinary treatment, we have developed a targeted therapy aimed at EGFR. Previously we conjugated B4G7 monoclonal antibody, which recognizes EGFR to gelonin [9] and pepleomycin [19]. Both immunoconjugates kill EGFR-hyperproducing squamous carcinoma cells dose dependently and their cytotoxic activity is dependent on the level of EGFR expression.

RNases are also endogenous proteins found in plants [14], bacteria [13], fungi [5], and mammals [12] including humans [31]. RNases play various roles: *S*-RNases degrade pollen RNA and take part in a self-incompatibility system in *Nicotina alata* [14]; the

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eosinophil cationic protein found in eosinophil granules kills parasites [15, 16]; and bovine seminal RNases inhibit tumor growth [10, 30]. Recently, RNases have been chemically conjugated to transferrin and monoclonal antibody [25] and their cytotoxic activity has been confirmed against antigen or receptor-positive cell populations.

We coupled human and murine EGF to mammalian RNase via a disulfide bond and determined the cytotoxicity of these conjugates against several cancer cell lines and investigated the possibility of utilizing the human endogenous conjugate for a new targeted therapy with less immunogenicity and more safety than conventional targeted therapies.

Materials and methods

Biochemical reagents

Bovine pancreatic RNase was purchased from Amresco (Solon, Ohio). Human recombinant EGF was purchased from Austral Biologicals (San Ramon, Calif). Murine EGF from mouse submaxillary glands was purchased from Toyobo Co. (Osaka, Japan). *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) and 2-iminothiolane (2-IT) were from Pierce Chemical Co. (Rockford, Ill). Sephadex G-25 and G-50 were from Pharmacia LKB Biotechnology (Uppsala, Sweden). ^{125}I-labeled human EGF was from Amersham (Amsterdam, UK), and finally 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was from Sigma Co. (St. Louis, Mo.).

Cell lines

Two breast cancer cell lines (MCF-7, BT-20) were obtained from the American Type Cell Collection. SK-BR-3 human breast cancer cell line, A431 human squamous carcinoma cell line and H69 small-cell lung cancer cell line were obtained from the Japanese Cancer Research Resources Bank. Two human squamous carcinoma cell lines (TE-1, TE-5) were kindly provided by Dr. T. Akaishi and Dr. T. Nishihira (Tohoku University, Miyagi, Japan). A431, TE-5, MCF-7 and BT-20 were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1 μg/ml amphotericin B and 100 μg/ml kanamycin. H69 and SK-BR-3 were grown in RPMI-1640 containing the same supplements as above. All cell lines were maintained in a humidified atmosphere of air containing 5% CO_2 in air at 37°C.

EGF binding assay

A confluent cell culture was placed on ice and washed twice with ice-cold Eagle's balanced salt solution (EBSS) buffer. Then, 0.5 ng/ml ^{125}I-EGF and a 1–5000-fold molar excess of EGF were added to the cells and incubated on ice for 2 h. After washing three times with ice-cold EBSS buffer, cells were solubilized with 1 ml 0.5 *N* NaOH and their radioactivity was counted in a Beckman gamma counter. The amount of EGFR of each cell line was evaluated using Scatchard plot analysis.

Conjugation procedure

Human or murine EGF was conjugated to RNase via a disulfide bond according to the method of Newton et al. [17]. Briefly, EGF (0.1 mg/ml) in 100 m*M* sodium phosphate buffer, pH 7.5, containing 100 m*M* NaCl, was incubated for 30 min with a five-fold molar excess of SPDP as freshly made solution (20 m*M*) in ethanol at room temperature. Excess SPDP was removed from SPDP-EGF by gel filtration on a Sephadex G-25 column equilibrated with 100 m*M* sodium phosphate buffer, pH 7.5, containing 100 m*M* NaCl. RNase (1 mg/ml) in the same sodium phosphate buffer as above was mixed with five-fold molar excess of 2-IT (30 m*M* in 0.8 *M* borate buffer, pH 8.5) for 90 min at room temperature. Unreacted 2-IT was removed via a Sephadex G-25 column equilibrated with the same phosphate buffer. Modified EGF was incubated overnight with an equimolar amount of modified RNase at 4°C. Then the reaction mixture was subjected to gel-filtration through a Sephadex G-50 column equilibrated with 100 m*M* sodium phosphate buffer, pH 7.5. The EGF–RNase conjugate was sterilized through a 0.22 μm membrane before addition to the cell lines.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Samples from the Sephadex G-50 column were subjected to SDS-PAGE according to the procedure of Laemmli [11]. The human EGF–RNase conjugate, human EGF and mammalian RNase were incubated for 15 min at 37°C in the presence of 1% SDS, 10% glycerol, 0.2 m*M* EDTA (SDS sample buffer) before application to the gel. The conjugate was reduced by heating for 10 min in boiling SDS sample buffer containing 20 mg 2-mercaptoethanol, and the reduced conjugate was also applied to the gel. The gels were stained with silver.

Competitive inhibition of ^{125}I-EGF binding to A431 cells by EGF and conjugate

Confluent A431 cells were transferred to a 96-well plate on ice and washed with ice-cold EBSS buffer. ^{125}I-EGF was added to the cells to a final concentration of 4×10^{-9} *M*. Simultaneously, a 1–100-fold molar excess of the human EGF–RNase conjugate or unlabeled human EGF was added to the cells and incubated on ice for 2 h. After three washes with ice-cold EBSS buffer, the cells were dissolved in 0.5 *N* NaOH at 37°C for 30 min and radioactivity was counted in a gamma counter. The amount of ^{125}I-EGF binding in the presence of a 100-fold molar excess of unlabeled EGF was defined as the background level and subtracted from the total binding to calculate the specific binding.

RNase assay

The activity of RNase was determined by measuring the release of acid-soluble nucleotides from yeast RNA [18]. Briefly, 100 μl sample was incubated with 100 μl yeast RNA (4 mg/ml) and 300 μl 0.1 *M* Tris/HCl buffer, pH 7.2, for 15 min at 37°C. The mixture was then incubated for 15 min on ice with 15% perchloric acid containing 20 m*M* lanthanum nitrate. After centrifugation at 10 000 g for 15 min, the absorbance at 260 nm of the supernatant was determined.

Cytotoxic effect of conjugate

The cytotoxic activity of the conjugate was assayed using an MTT assay. Briefly, exponentially growing cells were seeded into a 96-well plate (1×10^4 cells/well) and incubated overnight at 37°C , prior to addition of the reagents. The cells were then exposed overnight to serially diluted reagents (EGF–RNase conjugate, EGF alone, RNase

alone, mixture of EGF and RNase). Quadruplicate determinations were done for each dilution. The next day cells were washed twice with phosphate-buffered saline (PBS) and a fresh mixture of MTT (0.4% in PBS), and sodium succinate (0.1 *M* in PBS) was added to each well followed by incubation for 3 h at 37°C. At the end of incubation 150 µl dimethyl sulfoxide (DMSO) was added to each well to dissolve the MTT formazan. The Absorbance of each well was measured in a microplate reader using a test wavelength of 570 nm and a reference wavelength of 630 nm.

Results

Preparation of EGF–RNase conjugate

A single pyridine dithiopropionate group was introduced to EGF using a heterobifunctional reagent, SPDP. 2-IT-treated RNase was mixed with equimolar derivatized EGF leading to the formation of a disulfide bond between EGF and RNase. Unconjugated EGF was removed from the mixture by gel filtration on a Sephadex G-50 column. Reduced and nonreduced human EGF–RNase conjugate was subjected to SDS-PAGE with human EGF and mammalian RNase (Fig. 1). Under reducing conditions with 2-mercaptoethanol, the disulfide bond was cleaved and the conjugate showed two prominent bands corresponding to EGF and RNase (lane 4).

^{125}I-labeled human EGF was used to examine the binding activity of conjugate to EGF receptors. The human EGF–RNase conjugate showed a similar inhibition of ^{125}I-EGF binding to A431 cells compared to human EGF (Fig. 2). When EGF or conjugate was added to a tenfold molar excess, it reduced the ^{125}I-EGF binding to 30% and 40% of initial binding, respectively.

EGF-RNase conjugate showed potent RNase activity compared to free RNase. The conjugate hydrolyzed yeast RNA and produced acid-soluble nucleic acids as well as free RNase (data not shown).

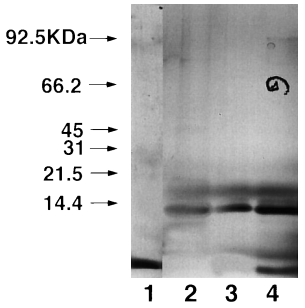

Fig. 1 SDS-PAGE of the EGF–RNase conjugate Human EGF, bovine pancreatic RNase and EGF–RNase conjugate were applied to a 15% polyacrylamide gel. EGF, RNase and conjugate were incubated for 15 min at 37°C in SDS sample buffer. The EGF–RNase conjugate was treated with 2-mercaptoethanol and the reduced conjugate was also applied to the gel. The protein bands were visualized by staining with silver. The arrows indicate the position of the molecular mass standards: phosphorylase B (92 500 Da), bovine serum albumin (66 200 Da), ovalbumin (45 000 Da), carbonic anhydrase (31 000 Da), soybean trypsin inhibitor (21 500 Da), lysozyme (14 400 Da). *Lane 1,* EGF (1.2 mg), *lane 2* RNase (0.7 µg), *lane 3* EGF–RNase conjugate (0.72 µg), *lane 4* reduced EGF–RNase conjugate (0.72 µg)

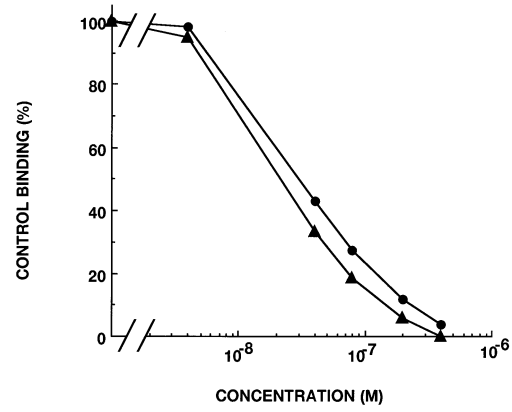

Fig. 2 Competitive inhibition of ^{125}I-EGF binding to A431 cells by EGF and the EGF–RNase conjugate. A431 cells (1×10^4 cells/well) were incubated in the presence of ^{125}I-EGF (4×10^{-9} *M*) with a 1 to 100-fold molar excess of EGF (●) or conjugate (▲). After 2 h incubation on ice, the cells were dissolved and their radioactivity was counted

Cytotoxic effect of the conjugate on the EGFR overexpressing cell line

The cytotoxicity of the conjugate was compared with RNase, EGF and a mixture of RNase and EGF against the EGFR-overexpressing A431 cell line. The human EGF-RNase conjugate killed A431 cells dose dependently with an IC_{50} of 3×10^{-7} *M*, while RNase, human EGF and a mixture of both showed little cytotoxic effect even at high concentrations (10^{-6} *M*; Fig. 3a). Free RNase exhibited its cytotoxicity at a concentration at of 10^{-5} *M* or more and its IC_{50} was 10^{-4} *M*. The murine EGF-RNase conjugate showed similar dose-dependent cytotoxicity against A431 cells with an IC_{50} of 6×10^{-7} *M*, but other reagents showed no marked cytotoxicity (Fig. 3b).

Effect of excess EGF on conjugate cytotoxicity

We examined whether the cytotoxicity of the EGF–RNase conjugate was ligand mediated. A431 cells (1×10^4 cells/well) were treated with 7×10^{-7} *M* human EGF–RNase conjugate and surviving cells were 30% of control. Conjugate 7×10^{-7} *M* and a 1–100-fold molar excess of human EGF were added to A431 cells simultaneously. After overnight incubation, surviving cells were determined using an MTT assay. Excess EGF inhibited the cytotoxic effect of 7×10^{-7} *M* EGF–RNase conjugate dose dependently. A tenfold excess of EGF reduced the cytotoxicity to 77% of

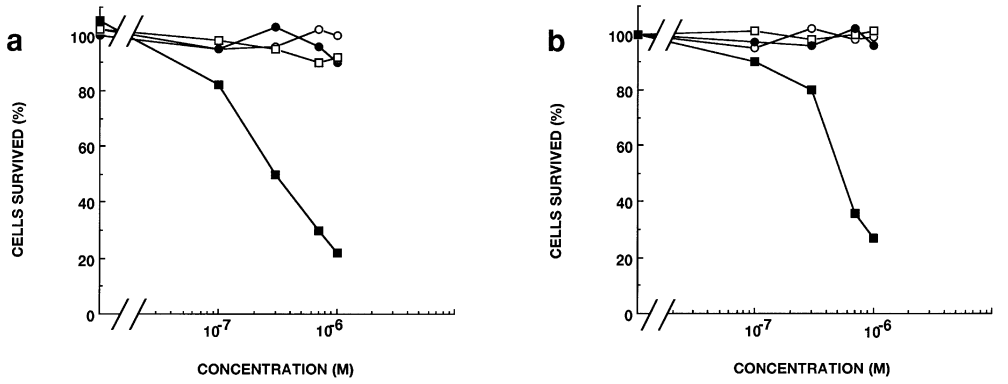

Fig. 3a, b Cytotoxicity of EGF, RNase and the EGF–RNase conjugate. **a** A431 cells in a 96-well plate (1×10^4 cells/well) were treated with various concentrations of human EGF, RNase, a mixture of EGF and RNase, and human EGF–RNase conjugate. After overnight incubation at 37°C, cytotoxicity was determined by an MTT assay. **b** the cytotoxicity of a murine EGF–RNase conjugate was determined by the same procedure (*EGF* ○, *RNase* □, *EGF–RNase conjugate* ■, *mixture of EGF and RNase* ●)

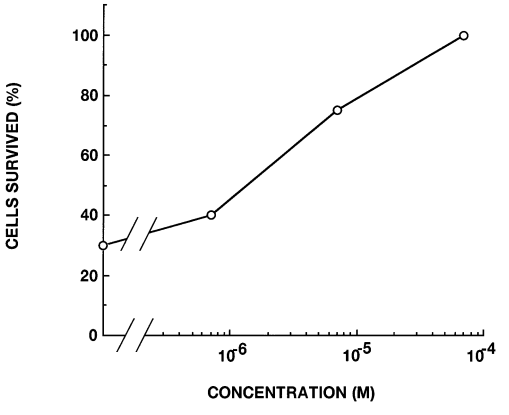

Fig. 4 Effect of excess EGF on conjugate cytotoxicity. EGF–RNase conjugate (7×10^{-7} *M*) was added to A431 cells with 0 to 100 times the amount of EGF in relation to the conjugate. The next day, cytotoxicity was determined by an MTT assay

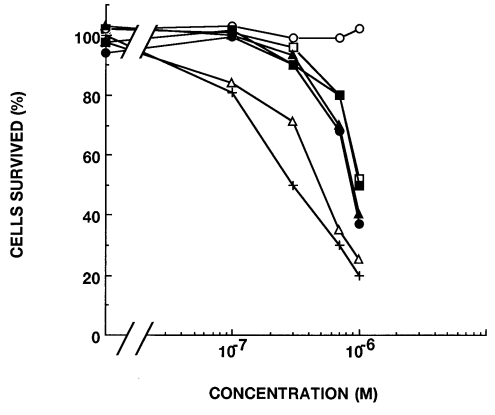

Fig. 5 Cytotoxicity of the EGF–RNase conjugate against various cell lines. The cell lines (A431, TE-1, TE-5, BT-20, SK-BR-3, MCF-7, H69) were transferred into 96-well plates (1×10^4 cells/well) and treated with serially diluted human EGF–RNase conjugate. The next day, cells were washed and an MTT assay was done. (*SK-BR-3* ●, *H69* ○, *TE-1* ■, MCF-7 □, *TE-5*▲, *BT-20* △, and *A431* +)

control and 100-fold excess of EGF inhibited the cytotoxicity completely (Fig. 4).

Cytotoxic effect on several cell lines with and without EGFR overexpression

The cytotoxicity of human EGF–RNase conjugate was assessed on several cancer cell lines (Fig. 5). Against the EGFR-overexpressing breast cancer cell line BT-20, the conjugate showed dose-dependent cytotoxicity with an IC_{50} of 5×10^{-7} *M*. Against TE-5 squamous carcinoma cells and SK-BR-3 breast cancer cells, the cytotoxicity of the conjugate was reduced compared with BT-20; the IC_{50} was 8.7×10^{-7} *M* and 8.5×10^{-7} *M*, respectively. Killing MCF-7 breast cancer cells and TE-1 squamous carcinoma cells needed a higher conjugate concentration. The conjugate showed no cytotoxic effect on EGFR-deficient H69 cells.

To determine whether the cytotoxicity of the human EGF–RNase conjugate depended upon EGFR, the IC_{50} of the conjugate against various cancer cell lines was compared with the number of EGFR of each cell line (Table 1). Against A431 with the highest EGFR numbers, the conjugate showed an IC_{50} of 3×10^{-7} *M*, whereas H69 without detectable EGFR had a higher IC_{50} of over 10^{-6} *M*. As the EGFR of the cancer cell lines increased, the IC_{50} value of the conjugate decreased.

Discussion

To develop a targeted therapy with less immunogenicity and toxicity, human or murine EGF was conjugated chemically to mammalian RNase via a

Table 1 Relationship between number of EGFR and cytotoxicity (*UD* undetectable)

Cell line	EGF receptor (sites/cell)	IC_{50} (M)
Squamous cell carcinoma		
A431	3×10^6	3×10^{-7}
TE5	1×10^5	8.7×10^{-7}
TE1	8.5×10^4	9.5×10^{-7}
Breast cancer		
BT-20	7.9×10^5	5×10^{-7}
SK-BR-3	2×10^5	8.5×10^{-7}
MCF-7	1×10^4	1×10^{-6}
Small cell lung cancer		
H69	UD	$> 1 \times 10^{-6}$

disulfide bond using SPDP and 2-IT. Both EGF–RNase conjugates showed dose-dependent cytotoxicity in vitro against cancer cells with EGFR overexpression.

A disulfide bond is easily cleaved under reducing conditions [8]. The EGF–RNase conjugate was reduced with 2-mercaptoethanol and applied to SDS-PAGE with nonreduced conjugate (Fig. 1). Under reducing coditions, the conjugate showed two prominent bands, corresponding to EGF and RNase. These findings suggest that EGF was coupled with RNase via a disulfide bond. A disulfide bond was also indicated as the linkage between EGF and RNase because this conjugation did not inhibit RNase activity or the EGFR binding activity of the conjugate. The EGF–RNase conjugate was an excellent competitor of ^{125}I-EGF binding to A431 cells when compared with EGF (Fig. 3) and an RNase assay showed that the EGF–RNase conjugate had almost the same activity as equimolar unconjugaed RNase (data not shown).

There are many reports of chimeric toxins which are effective in vitro and in vivo. Administration of these chimeric toxins to patients causes an immune response which produces antitoxin, antimonoclonal antibody or antiligand antibodies, because the components of these chimeric toxins are foreign proteins derived from plants, bacteria or mice. These antibodies can bind to the chimeric toxins and make immune complexes that inactivate the conjugate and cause renal damage. We developed a new class of agent composed of human EGF and mammalian RNase, both of which are endogenous proteins, with less immunogenicity and toxicity. Recently, an IL-2 fusion toxin has been developed and in an early clinical study only 1 of 15 patients developed low-titer anti-IL-2 antibodies during treatment [29], although all the patients receiving murine immunotoxin developed antibodies to murine immunoglobulin [22]. These findings suggest that chimeric toxin composed of only endogenous proteins could be less immunogenic than conventional targeted therapy.

EGF was selected as the ligand of the chimeric toxin for the following reasons: it is less immunogenic because it is an endogenous and small protein and it binds to cell surface receptors and is internalized [1, 23], so an EGF conjugate may also be internalized and carry out its action in the cytoplasm. Furthermore, EGFR overexpression is correlated with a poor prognosis in cancer patients [21, 26]. Thus since, as cancer cells with EGFR overexpression have a high malignant and a rapid proliferative potential, an EGF–RNase conjugate is an effective anticancer agent especially for cancers overexpressing EGFR which cannot be cured with conventional therapies.

RNase is an endogenous protein that is thought to play a variety of physiological roles. Furthermore, bovine seminal RNases and onconase extracted from *Rana pipiens* oocytes and early embryos have anticancer activity [32]. In our study, extracellular RNase killed cancer cells at extremely high concentrations (10^{-4} to 10^{-3} M). The cytotoxic mechanism of RNases is still not understood. Presumably, RNases somehow enter the cytoplasm, degrade intracellular RNA and kill cells by inhibiting protein synthesis. When extracelluar RNase is present at extremely high concentrations, it might be internalized through an endocytosis mechanism such as pinocytosis. Thus, RNases kill cells and are nontoxic enzymes unless they enter the cytoplasm. We therefore produced RNases with specificity for a certain cell population by coupling them to a ligand.

How the EGF–RNase conjugate kills cells is unknown. Probably, it is internalized after binding to cell surface receptors because it retains EGFR binding activity and the EGF–EGFR complex is internalized into the cell after EGF binds to EGFR on the cell surface [1, 23]. After internalization, the conjugate degrades intracellular RNA and inhibits protein synthesis which leads to cell death, because the conjugate retains potent RNase activity. The following experimental findings indicate that the cytotoxicity of the EGF–RNase conjugate is EGFR mediated: (1) the conjugate showed specific cytotoxicity against EGFR-overexpressing cells and had no cytotoxic effect on EGFR-deficient cells; (2) excess EGF inhibited the cytotoxicity of the conjugate; (3) free RNase and a simple mixture of EGF and RNase had no detectable cytotoxicity at concentrations (10^{-7} to 10^{-6} M) at which the conjugate showed effective cytotoxicity; and (4) the cytotoxicity of the conjugate was correlated positively with the number of EGFR. These findings are consistent with the above cytotoxic mechanism.

Because EGFR exist not only in cancer but also in normal cells, there is the potential problem of the EGF–RNase conjugate injuring normal organs when used in vivo. This may not be a problem for the following reasons: (1) the level of expression of EGFR by EGFR-overexpressing cancer cells is much higher than by normal cells [20] and thus the conjugate might react with cancer cells and have little effect on normal tissue; (2) cancer tissues have fragile blood vessels [24], so the conjugate might more easily penetrate these vessels; (3)

the adherens junction between cancer cells is not as tight as between normal cells [27, 28], thus the conjugate might bind easily to cancer cell surface receptors; and (4) TGFα–*Pseudomonas* exotoxin conjugate suppresses the growth of non-small-cell lung cancer xenograft formation in nude mice [4].

The EGF–RNase conjugate may be cytotoxic against EGFR-overexpressing cancer cells and yet have low immunogenicity and toxicity making it suitable for clinical application. We are purifying human RNases from liver obtained at autopsy. Conjugate consisting of only human proteins should be much less immunogenic and safer than mammalian conjugate and more suitable for patients. When RNases are conjugated to other ligands, for example IL-2 or Heregulin, this therapy may also be applicable to various other diseases.

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