

Anti-tumor Effect of an Immunotoxin Consisting of a Chimeric Anti-carcinoembryonic Antigen Antibody Conjugated to Ricin Toxin A

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Abstract: Immunotoxins, consisting of antibodies coupled to toxins, are extremely useful tools for the elimination of specific cell populations both *in vitro* and *in vivo* for research and therapeutic applications. The antibody is used to target the toxin to a specific cell population, which is distinguished by its cell-surface antigen. In this study, a mouse/human chimeric antibody to carcinoembryonic antigen (CEA) was chemically conjugated to deglycosylated ricin toxin A and the cytotoxic effect of the resulting immunotoxin was investigated *in vitro*. This immunotoxin showed a potent cytotoxicity against human CEA-expressing tumor cells, thus suggesting that this immunotoxin may therefore be potentially effective as a new anticancer agent.

Key words: Carcinoembryonic antigen (CEA), Chimeric antibody, Immunotoxin, Ricin toxin A (RTA)

Introduction

The eradication of tumor cells and their metastases is a major goal of tumor therapy. Targeting cytotoxicity to tumor cells is a potentially effective approach due to its simplicity, specificity, effectiveness and practicality in the therapy. Among the strategies in use, the tumor cell-specific antibody fused with a potent agent has proven to be effective.¹⁾ The agents used for such treatment include drugs, pro-drugs, toxins, cytokines or isotopes,¹⁾ and a potent protein inhibitor, the ricin toxin A (RTA), derived from the castor

bean, has recently been proven to be useful for this approach.^{2)–4)}

Carcinoembryonic antigen (CEA), a glycoprotein with a molecular mass of 180 kDa, is overexpressed in about 95% of all gastrointestinal and pancreatic cancers, as well as in most lung carcinomas. It is also expressed in breast carcinoma and squamous cell carcinoma of the head and neck.⁵⁾ Although the fusion of RTA to anti-CEA antibodies would be a promising strategy for the treatment of CEA-expressing tumors, there is still very limited information available on the antitumor activity of anti-CEA antibody/RTA fusion protein.⁶⁾

In a previous study, in order to reduce the immunogenicity of mouse monoclonal antibody in humans, we generated two mouse/human chimeric antibodies to CEA, designated Ch F11-35 and Ch F11-39, and thus showed that both chimeric antibodies exhibited the same high specificity and affinity for CEA as those of their parental mouse monoclonal antibodies.^{7,8)} In the present study, we prepared a new immunotoxin made with Ch F11-39 linked to RTA and evaluated its cytotoxic effect against human CEA-expressing tumor cells.

Materials and Methods

Materials. Deglycosylated ricin toxin A (dgRTA) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Human IgG1 (κ) myeloma protein was from The Binding Site (Birmingham, England) and was used as a negative control. Dithiothreitol (DTT) was from Katayama Chemical (Osaka); 4-succinimidylloxycarbonyl- α -methyl- α -(2-pyridyldithio)toluene (SMPT) from Pierce (Rockford, IL, USA); L-cysteine from Sigma (St. Louis, MO, USA); biotinylated goat anti-human IgG antibody from Vector (Burlingame, CA, USA); fluorescein-conjugated goat F(ab')₂ anti-human IgG from Cappel (Turnhout, Belgium); the dye 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) from Research Organics Inc. (Cleveland, OH, USA); and a human gastric cancer cell line, MKN-45 (CEA-expressing), from the Japanese Cancer Research Resources Bank (Tokyo).

Preparation and purification of immunotoxin.

The construction, expression, and immunochemical properties of the mouse/human chimeric antibody to CEA, designated Ch F11-39, have been described in detail elsewhere.⁷⁾ Ch F11-39 is of the human IgG1(κ) isotype and it was purified as previously described.⁸⁾ Ch F11-39 was conjugated with dgRTA by means of a coupling agent, SMPT.⁹⁾ Briefly, Ch F11-39 was treated for 1 h at room temperature with SMPT to introduce an average of between 1.5 and 1.8 activated disulfide groups per molecule of protein. The

derivative antibody was mixed with a 2.5-fold molar excess of dgRTA freshly reduced with DTT, concentrated to approximately 1.5 mg of total protein per ml, and then allowed to react for 3 days at room temperature. After stopping the reaction with L-cysteine for 6 h, the resulting immunotoxin, designated Ch F11-39-dgRTA, was purified by successive chromatography on columns of TSK-gel G3000SW (2.15 \times 60.0 cm, Tosoh, Tokyo) and HiTrap Blue HP (5 ml, Amersham Biosciences K. K., Tokyo) to remove free dgRTA and free Ch F11-39, respectively. The overall recovery of Ch F11-39-dgRTA after the final step was 25.6% of the starting material.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Ten micrograms each of purified protein was electrophoresed on 6% gel as described previously.⁷⁾ After electrophoresis, the proteins in the gel were visualized by silver staining.

Enzyme-linked immunosorbent assay (SPEIA).

The reactivity of Ch F11-39-dgRTA with purified CEA was estimated by SPEIA using CEA immobilized on 96-well plates as described elsewhere.⁷⁾ After incubation with Ch F11-39-dgRTA, Ch F11-39 or control human IgG, the plates were washed and then incubated with biotinylated goat anti-human IgG antibody.

Flow cytometry.

To confirm the antibody binding to CEA-expressing tumor cells, flow cytometry was also performed as described previously.¹⁰⁾ Briefly, after washing with ice-cold Dulbecco's PBS containing 5% normal goat serum, aliquots of 2×10^6 MKN-45 cells were mixed with 200 μ l of Ch F11-39-dgRTA, Ch F11-39, dgRTA, or control human IgG (1.0 μ g/ml each in Dulbecco's PBS containing 5% normal goat serum). The cells were incubated on ice for 1 h, washed three times, and then reacted with fluorescein-conjugated goat F(ab')₂ anti-human IgG on ice for 1 h. Next, the stained cells were washed, resuspended in 1.0 ml FACS Flow medium, filtered through a nylon mesh, and then analyzed with FACSCalibur.

Cytotoxicity assay. The efficacy of Ch F11-39-dgRTA in directing tumor cell killing was determined by a cytotoxicity assay using MKN-45 cells adhered to the well bottoms of 96-well plates.⁸⁾ Briefly, 1×10^5 MKN-45 cells were first seeded into 96-well plates (Costar, Cambridge, MA). After a 48-hour culture incubation, the plates were washed twice with Dulbecco's PBS to remove any non-adherent cells including dead cells. The plates were then incubated in the presence of Ch F11-39-dgRTA, Ch F11-39 alone, dgRTA alone or Ch F11-39 plus dgRTA for 24 hours at 37°C in 5% CO₂. The killed tumor cells were removed by washing for 3 minutes under vigorous shaking on a plate shaker. The remaining living cells were immediately quantified by a colorimetric assay using MTT as previously described.¹¹⁾ The % cell survival was calculated according to the formula: $100 - \% \text{ cell killing} = 100 - [(\text{OD of targeted cells alone} - \text{test OD}) / (\text{OD of targeted cells alone})] \times 100$.

Results

Purity and molecular weight. As shown in Fig. 1, the purified Ch F11-39, commercially available dgRTA and Ch F11-39-dgRTA all

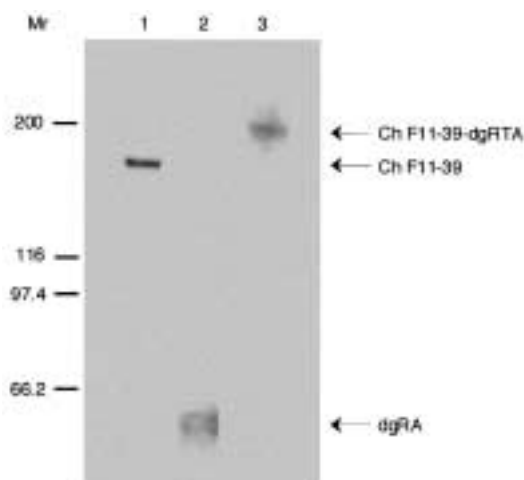


Fig. 1. SDS-PAGE analysis of Ch F11-39-dgRTA. Lane 1, Ch F11-39; lane 2, dgRTA; and lane 3, Ch F11-39-dgRTA. Vertical markers, molecular weight markers.

produced a single band in SDS-PAGE, thus indicating each preparation contained nearly pure protein. Since the bands of dgRTA and Ch F11-39-dgRTA were a little bit broad, their molecular weights were calculated for the center of the smear. Their estimated molecular weights were 150 kDa for Ch F11-39, 30 kDa for dgRTA and 180 kDa for Ch F11-39-dgRTA, respectively. As a result, the molecular weight of Ch F11-39-dgRTA corresponded to that calculated from the molecular weights of Ch F11-39 and dgRTA.

Reactivity with purified CEA. When tested by SPEIA using CEA immobilized on 96-well plates, Ch F11-39-dgRTA as well as Ch F11-39 reacted well with CEA, while control human IgG did not react with CEA (Fig. 2).

Binding to CEA-expressing tumor cells.

The binding of Ch F11-39-dgRTA to MKN-45 cells was investigated by flow cytometry. As expected from the reactivity in SPEIA, Ch F11-39-dgRTA significantly bound to MKN-45 cells, although its mean fluorescence intensity was slightly less than that of Ch F11-39 (Fig. 3). Neither the control human IgG nor dgRTA was observed to bind to MKN-45 cells (Fig. 3).

In vitro cytotoxic activity against CEA-expressing tumor cells.

The cytotoxic effect of Ch F11-39-dgRTA against MKN-45 cells is shown in Fig. 4. Ch F11-39-dgRTA reduced the % cell survival by 50% at a concentration (The IC₅₀) of 5×10^{-11} M. It was about 100-fold potent than dgRTA which had an IC₅₀ of about 5×10^{-9} M. The IC₅₀ of Ch F11-39 plus dgRTA was also approximately 5×10^{-9} M. On the other hand, Ch F11-39 alone did not show any cell killing even at the highest concentration tested (10^{-8} M).

Discussion

Although CEA is one of the most useful human tumor markers, several studies have shown that CEA is expressed on the epithelial cells of normal adult colon as well as on the surface of human colon cancer cells.¹²⁾¹³⁾

In normal colon tissues, however, CEA is

localized on the luminal surface of the single layer of columnar epithelial cells lining the upper parts of the crypts¹²⁾ and does not directly face either the blood flow or tissue fluid. On the other hand, in tumor tissues which no longer conform to the single layer organization by invading through the basement membrane in multicellular arrays, CEA

is usually localized on all sides of the cell in multicellular arrays and it directly faces the blood flow and/or tissue fluid. Hence, tumor CEA can be a useful target for immunotherapy or immunogene therapy using anti-CEA antibody.¹⁴⁾

Ricin, the highly toxic heterodimeric glycoprotein expressed in the endosperm of

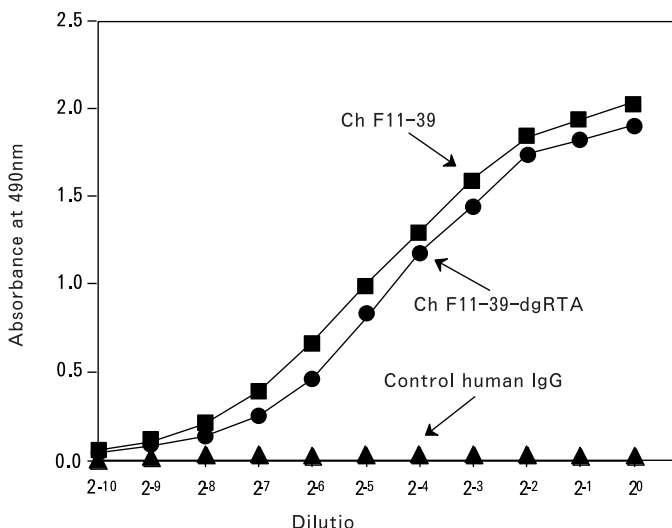


Fig. 2. Reactivity of Ch F11-39-dgRTA with purified CEA in ELISA. The starting concentration of each antibody for dilution was 10^{-8} M.

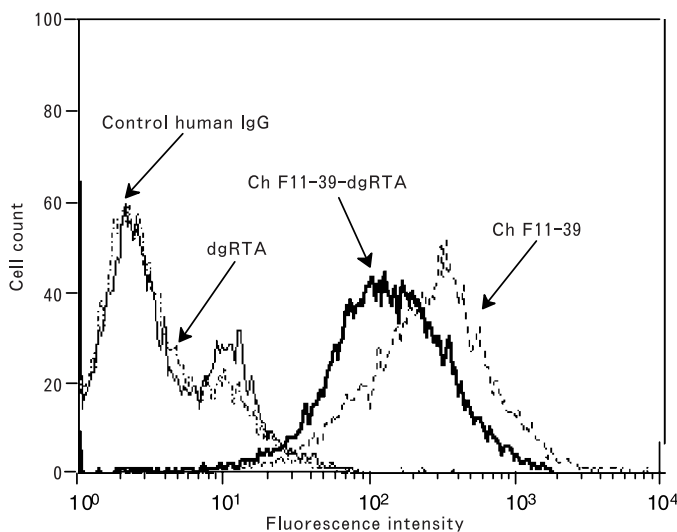


Fig. 3. Binding of Ch F11-39-dgRTA to CEA-expressing tumor cells in flow cytometry.

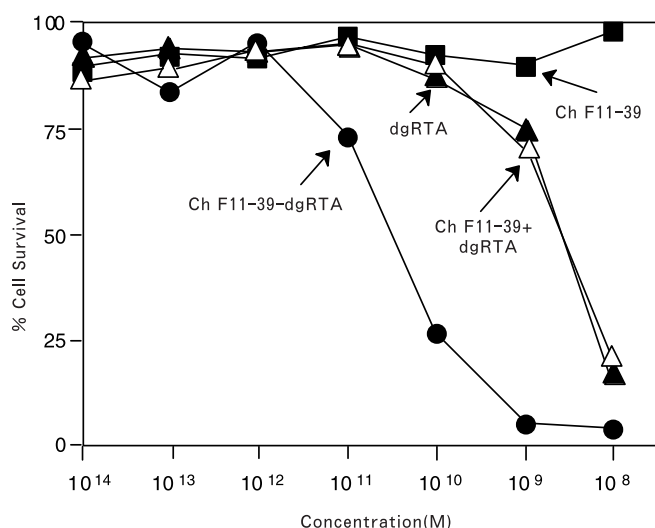


Fig. 4. Antitumor effect of Ch F11-39-dgRTA against CEA-expressing MKN-45 tumor cells. The starting concentration of each protein for dilution was 10^{-8} M.

castor seeds, is composed of a galactose-binding lectin B chain (the ricin toxin B or RTB) disulfide-linked to a RNA N-glycosidase A chain, RTA.¹⁵⁾ Chemically modified RTA has been conjugated to monoclonal antibodies to tumor-associated antigens and the resulting RTA-immunotoxins have been used for targeted therapy of cancer.¹⁵⁾ Several studies, however, have demonstrated that the therapeutic activity of the RTA-immunotoxins is diminished by its rapid elimination from the bloodstream due to a combination of two factors, namely entrapment by cells of the liver and breakdown of the RTA-immunotoxins.¹⁶⁾ Hepatic entrapment of the RTA-immunotoxins occurs because liver cells have receptors for the mannose- and fucose-terminating oligosaccharides that are present in the RTA moiety. In this context, dgRTA was used in this study to produce the RTA-immunotoxin, Ch F11-39-dgRTA.¹⁶⁾

When analyzed by SDS-PAGE, Ch F11-39-dgRTA showed a single band with a molecular weight of 180 kDa corresponding to that calculated from the molecular weights of Ch F11-39 and dgRTA. Ch F11-39-dgRTA also bound to purified CEA and CEA-expressing tumor cells, and exhibited a potent cyto-

toxicity against CEA-expressing tumor cells. From the IC_{50} values obtained by an *in vitro* cytotoxicity assay, Ch F11-39-dgRTA was about 100-fold potent than dgRTA or a combination of Ch F11-39 and dgRTA. As a result, our new immunotoxin, Ch F11-39-dgRTA, appears to be suitable candidates for the *in vivo* therapy of CEA-expressing cancers. The next step in assessing the practical utility of our immunotoxin is to determine whether or not it is able to selectively target CEA-expressing tumor cells in experimental *in vivo* situations.

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