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Effectiveness of an Adriamycin Immunoconjugate that Recognizes the C-*erb*B-2 Product on Breast Cancer Cell Lines

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Abstract: Adriamycin (ADM) was chemically conjugated to a murine monoclonal antibody, A0011, which recognizes the c-*erb*B-2 product, via a disulfide bond using *N*-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) and 2-iminothiolane (2-IT). The molar ratio of ADM to the monoclonal antibody ranged from 15:1 to 25:1 and enzyme-linked immunosorbent assay (ELISA) showed that the binding activity of the conjugate was almost retained. We compared the efficacy of A0011 alone, ADM alone, the A0011-ADM conjugate, and a nonspecific murine IgM-ADM conjugate, against the human breast cancer cell lines SK-BR-3, MDA-MB-361, MCF-7, and BT-20. The A0011-ADM conjugate was observed to be ten times more cytotoxic to the cell lines overexpressing the c-*erb*B-2 product, namely, SK-BR-3 and MDA-MB-361, than free ADM, but it showed weak cytotoxicity against the cell lines with a low level of c-*erb*B-2 product expression, namely, MCF-7 and BT-20. However, free A0011 and nonspecific murine IgM-ADM conjugate showed no cytotoxicity toward any of the four cell lines, while the addition of a tenfold molar excess of A0011 inhibited conjugate cytotoxicity. These data suggest that conjugate cytotoxicity is antibody-mediated. Moreover, conjugate cytotoxicity at 10^{-6}M was correlated with antigen volume, and the data were fitted to the regression equation $y = -11.63 \log X + 116.38$ where the correlation coefficient = 0.950. Our results indicate that targeting therapy aiming at the c-*erb*B-2 product may be useful in the treatment of breast cancers overexpressing the c-*erb*B-2 product.

Key Words: Adriamycin, breast cancer, c-*erb*B-2, immunoconjugate, targeting therapy

Introduction

The c-*erb*B-2 protooncogene encodes a 185-kDa cell surface glycoprotein belonging to the tyrosine kinase receptor family,[1] and its product is known to be extensively homologous to the epidermal growth factor receptor (EGFR).[2] Amplification and/or overexpression of the c-*erb*B-2 protooncogene has been found in cancers of the breast,[3] ovaries,[4] stomach,[5] and lung,[6] and has been correlated with a poor prognosis. Moreover, artificial overexpression of c-*erb*B-2 in NIH 3T3 cells has been shown to result in malignant transformation.[7,8] These findings indicate that overexpression of c-*erb*B-2 may play a role in malignant tumor development. As breast cancer patients with overexpression of the c-*erb*B-2 product have a higher rate of recurrence and lower overall survival,[9] treatment involving not only surgery but also multidisciplinary therapy is required. As part of the multidisciplinary treatment, we developed targeting therapy aimed at the c-*erb*B-2 product. We selected the c-*erb*B-2 product as the target for two reasons: first, because it exists on the cell membrane; and second, because it is expressed on malignant cells and almost never found in normal tissues.

In our previous studies, we conjugated B4G7, a murine monoclonal antibody which recognizes human EGFR to gelonin, a 60S ribosome-inactivating protein, and examined its cytotoxic effect. The B4G7-gelonin conjugate killed EGFR-hyperproducing squamous carcinoma cells but not EGFR-deficient small cell lung cancer cells.[10] When this conjugate was injected intraperitoneally into nude mice bearing EGFR-hyperproducing cancer cells, it suppressed solid tumor growth.[11] However, since gelonin is not a human protein and cannot be used clinically, we developed targeting therapy using an antineoplastic agent, pepleomycin, and determined its in vitro cytotoxicity.[12] Although Adriamycin (ADM) is a clinically effective drug for treating breast cancer, its usage is limited by its dose-dependent side effects of bone marrow suppression and cardiac toxicity.[13,14] During the last decade, to minimize these side effects and increase its preferential concentration at the tumor site, ADM has been linked to a

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variety of antibodies which react with tumor-associated antigens.[15–22] We conjugated ADM to a murine monoclonal antibody, A0011, which reacts with the c-*erb*B-2 product via a disulfide bond. The cytotoxicity of this A0011-ADM conjugate was determined against four breast cancer cell lines that overexpress the c-*erb*B-2 product at various levels, and compared with free ADM and nonspecific murine IgM-ADM conjugate at matching doses. A0011 alone was also tested. The A0011-ADM conjugate was found specifically to kill breast cancer cell overexpressing c-*erb*B-2 and thus, we evaluated this new immunotherapy using an overexpressing oncogene as the target.

Materials and Methods

Chemical Reagents

A0011 is an IgM murine monoclonal antibody that immunoprecipitates 15 amino acids (HTANRPEDECVGEGL) in the extracellular domain of the c-*erb*B-2 product without any homology to other transmembrane receptors. The A0011 used in this study was purchased from Iwaki Glass, Tokyo, Japan, and Adriamycin was donated by Kyowahakko, Tokyo, Japan. *N*-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), 2-iminothiolane (2-IT), poly-L-lysine, glutaraldehyde, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma, St. Louis, MO, USA; murine polyclonal IgM was purchased from Cappel, West Chester, PA, USA; and alkaline phosphatase conjugated goat antimouse IgM $F(ab')_2$ was purchased from Zymed, San Francisco, CA, USA.

Cell Lines

The four breast cancer cell lines used for in vitro binding and cytotoxic studies, namely, SK-BR-3, MDA-MB-361, MCF-7, and BT-20, were obtained from the American Type Culture Collection, Rockville, MD, USA. All cell lines except MCF-7 were maintained in RPMI 1640 medium. MCF-7 was maintained in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were grown at 37°C in a humidified atmosphere with 5% CO_2 in air.

Preparation of the A0011-ADM Conjugate Antibody and ADM Modification

A volume of 1 mg/ml of antibody in 10 mM sodium phosphate buffer (PBS) containing 50 mM triethylamine, 50 mM NaCl, and 1 mM EDTA, at a pH of 8.0 was mixed with 2-IT at a final concentration of 5 mM. This mixture was incubated at 4°C for 90 min, and the excess reagent was removed by passage through a Sephadex G-50 column (Pharmacia, Uppsala, Sweden) equilibrated with 2 M NaCl/PBS.[22] ADM 1 mg/ml in distilled water was mixed with equimolar amounts of SPDP solution, being 50 mM in ethanol. The mixture was adjusted to pH 7.4 with triethylamine followed by incubation at room temperature for 6 h.

Conjugation of ADM to Antibody

The 2-iminothiolane (2-IT) thiolated antibody in 2 M NaCl/PBS was added to 10 equivalents of modified ADM. The reaction mixture was incubated at 4°C overnight, then centrifuged at 10000 × g, and the nonconjugated ADM was removed from the mixture by passage through a Sephadex G-50 column. The conjugate was dialyzed against 10 mM sodium phosphate buffer at a pH of 7.4. The molar ratio of antibody to ADM was determined by absorbance at 495 nm ($\varepsilon 495 = 8030$) for ADM, and at 280 nm (1 mg/ml = 1.4 absorbance units) for the antibody. To correct for the overlapping absorption of ADM at 280 nm, the following formula was used: antibody (mg/ml) = $[A_{280} - (0.72 \times A_{495})]/1.4$. The conjugate was finally sterilized by passage through a 0.22-μm filtration membrane (Millex-GV; Millipore, Bedford, MA, USA). To ensure that the ADM was conjugated to the antibody via a disulfide bond, the conjugate was treated with tenfold molar excess dithiothreitol (DTT), a reducing reagent, at room temperature for 30 min. Reduced conjugate was applied to a Sephadex G-50 column and the absorbance of each fraction at 280 and 495 nm was measured.

ELISA

The binding activity of A0011 and its conjugate was determined by ELISA using SK-BR-3 breast cancer cell lines. SK-BR-3 cells, 1×10^4 cells/well, were incubated at 37°C overnight in a 96-well flat-bottomed plate precoated with 50 mg/ml ploy-L-lysine. The wells were washed twice with PBS and fixed with 0.025% glutaraldehyde in PBS for 15 min at room temperature, followed by blocking with 0.2% gelatin, 0.1% fetal bovine serum, and 100 mM glycine/PBS for 1 h at room temperature. Serially diluted A0011 or its conjugate was added to the wells at 37°C. Each point was done in quadruplicate. After 1 h, the wells were washed twice with 0.05% Tween 20, 0.1% gelatin in PBS and incubated for an additional 1 h with alkaline phosphatase conjugated goat antimouse IgM (1:2500). The wells were then washed five times with Tween/PBS, and 1 mg/ml p-nitrophenol phosphate was added. The absorbance (405 nm) of the wells was measured in a

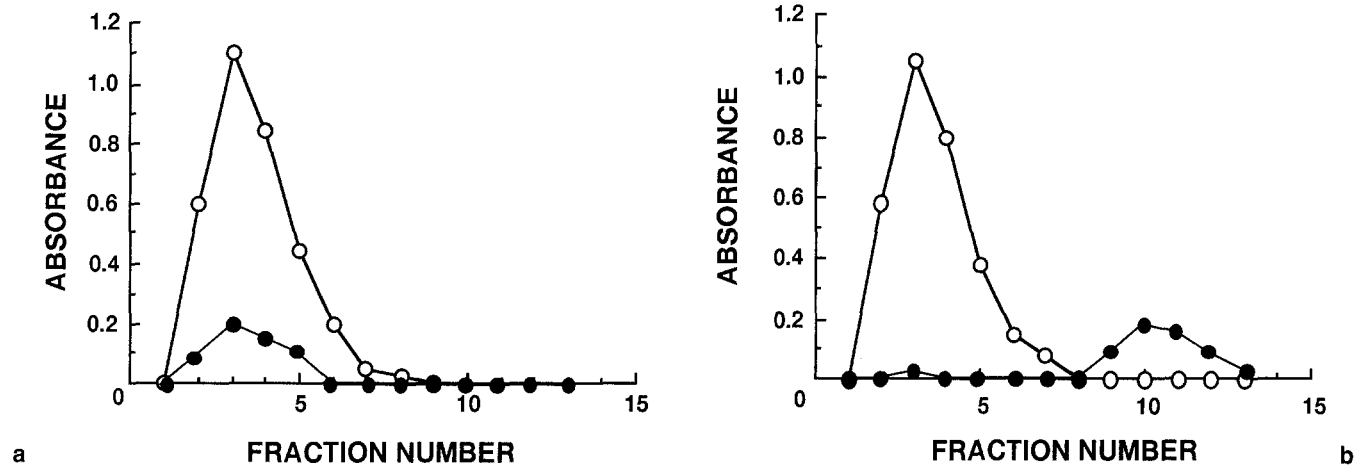

Fig. 1a,b. Gel filtration analysis of the A0011-Adriamycin (ADM) conjugate. **a** A0011 with absorbance at 280 nm and ADM with absorbance at 495 nm linked via a disulfide bond eluted from a Sephadex G-50 column as a single peak. **b** After reaction with a tenfold molar excess of dithiothreitol (DTT), the A0011-ADM conjugate was eluted from a Sephadex G-50 column as separated peaks. *Open circles*, 280 nm; *solid circles*, 495 nm

microplate reader 1 h later. The binding activity of A0011 to the other three breast cancer cell lines, MDA-MB-361, MCF-7, and BT-20, was investigated as described above.

Cytotoxicity In Vitro

The pharmacological activity of the conjugate was measured by the MTT assay. Briefly, exponentially growing cells, 1×10^4 cells/well, were transferred to a 96-well plate and incubated at 37°C overnight in complete medium. Subsequently, the cells were exposed for 15 min to the following serially diluted reagents: A0011-ADM conjugate, nonspecific murine IgM-ADM conjugate, A0011 alone, and ADM alone. Quadruplicate determinations were done for each dilution. The cells were then washed with PBS and resuspended with complete medium. After 3 days incubation, the cells were washed twice with 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 at 37°C for 3 h. At the end of this 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.

Competitive Inhibition of the Conjugate Effects by A0011

SK-BR-3 cells, 1×10^4/well, were exposed to a mixture of conjugate (10^{-6} M) and a zero- to tenfold molar excess of A0011. The cytotoxic effect was determined as described above.

Statistical Analysis

Statistical analysis was performed using Student's *t* test. All values are expressed as means ± SE.

Results

Preparation of the A0011-ADM Conjugate

ADM was conjugated to A0011 via a disulfide bond. First, ADM was coupled to SPDP, a heterobifunctional reagent, and the monoclonal antibodies (MAbs) were thiolated by 2-IT. Excess 2-IT was removed by passage through a Sephadex G-50 column. The thiolated MAbs were subsequently reacted with modified ADM, which led to the formation of a disulfide bond as a linker arm between ADM and the MAbs. Unconjugated ADM was removed by passage through a Sephadex G-50 column, but unconjugated MAbs could not be separated from the conjugates. Contamination may result in underestimating, but not overestimating the cytotoxic effect of the conjugates. Based on spectrophotometric analysis, the ADM:MAb molar ratio achieved ranged from 20 to 25, and the protein yields varied from 50% to 80%. Disulfide bond cleavage can be easily achieved by treatment with a reducing agent such as dithiothreitol (DTT). Regarding the untreated A0011-ADM conjugates, the 280 nm absorbance for antibody peaked simultaneously with the 495 nm absorbance for ADM (Fig. 1a). In the case of reduced conjugates with tenfold molar excess DTT, antibody absorbance showed the same curve as nonreduced conjugate, but the ADM absorbance peak shifted to the right (Fig. 1b).

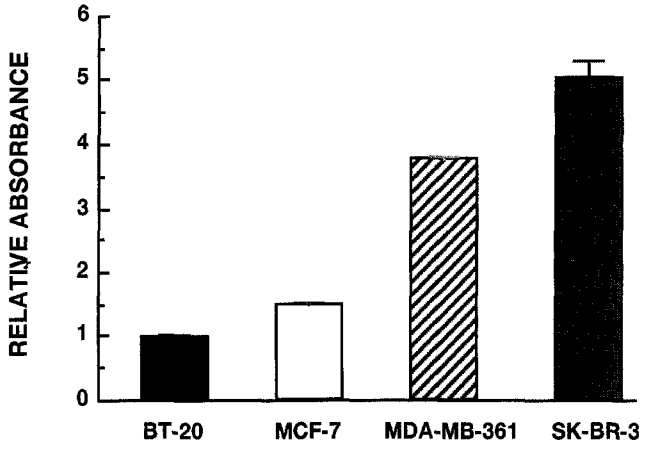

Fig. 2. Enzyme immunoassay analysis of A0011 binding to the various cells

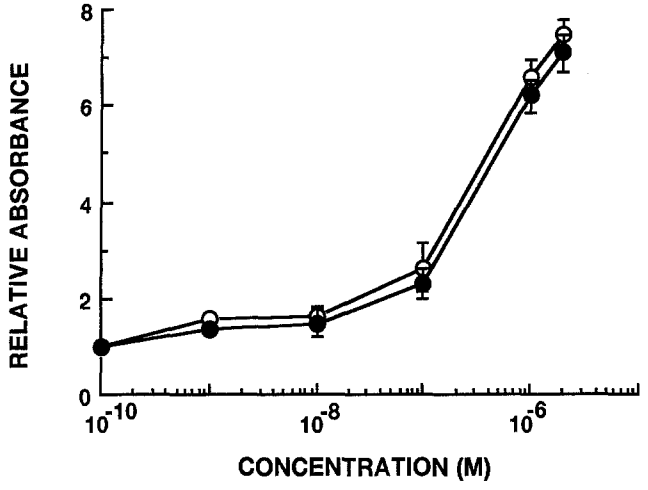

Fig. 3. Antigen binding activity of the A0011 and A0011-ADM conjugate. *Open circles*, A0011; *solid circles*, A0011-ADM

ELISA

The binding activity of A0011 to the four breast cancer cell lines was investigated by ELISA (Fig. 2). A0011 bound to the breast cancer cells in proportion to c-*erb*B-2 amplification. A0011-ADM conjugate retained most of its binding activity as compared with unconjugated A0011 (Fig. 3).

Cytotoxicity In Vitro

The cytotoxic activity of the A0011-ADM conjugate, A0011 alone, ADM alone, and murine IgM-ADM conjugate was assessed on SK-BR-3, c-*erb*B-2 overexpressing cells, using the MTT assay (Fig. 4). The A0011-ADM conjugate inhibited cell growth dose-dependently and was significantly more potent than equivalent amounts of ADM alone ($P < 0.01$). The cytotoxicity (IC_{50}) was 6×10^{-6}M for ADM and 7×10^{-7}M or A0011-ADM conjugate. Nonspecific murine IgM-ADM conjugate did not show any cytotoxicity at a high concentration of 10^{-6}M, and A0011 alone did not affect cell growth. The cytotoxicity of the conjugate was also evaluated against several breast cancer cell lines with various levels of c-*erb*B-2 expression (Fig. 5). The c-*erb*B-2 overexpressing cells, MDA-MB-361, were killed dose-dependently as in the case of SK-BR-3. Against MCF-7 and BT-20, which did not overexpress the c-*erb*B-2 product, the cytotoxicity of A0011-ADM conjugate was not recognized at the high concentration of 10^{-6}M. The cytotoxicity (IC_{50}) of ADM alone against MDA-MB-361, BT-20, and MCF-7 was 4×10^{-6}M, 7.5×10^{-7}M, and 9×10^{-7}M, respectively.

Competitive Inhibition of the Conjugate by Free A0011

A0011-ADM conjugate showed dose-dependent cytotoxicity against SK-BR-3 cells and the number of surviving cell at 10^{-6}M conjugate was almost 40%. To determine whether the effect of A0011-ADM conjugate was dependent on A0011, a zero- to tenfold molar excess of A0011 was mixed with 10^{-6}M conjugate before addition to the SK-BR-3 cells (Fig. 6). A0011 showed competitive inhibition of cytotoxicity dose-dependently and the number of surviving cells at 10^{-6}M conjugate with 5-fold molar excess A0011 rose to 80%. Conjugate mixed with more than a tenfold molar excess of A0011 did not kill the SK-BR-3 cells.

Correlation between Cytotoxicity and Binding Activity

The correlation between cytotoxicity and the binding activity (shown in Fig. 2) of conjugate at 10^{-6}M was

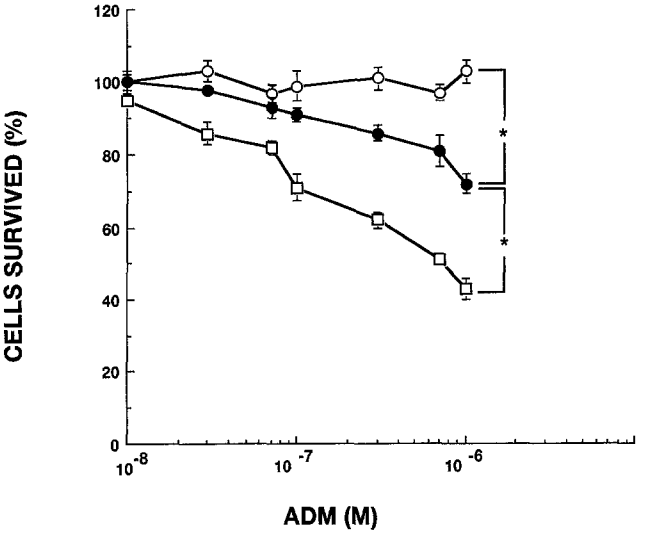

Fig. 4. Cytotoxicity of the A0011-ADM conjugate, nonspecific IgM-ADM conjugate, and ADM against SK-BR-3. Each data point represents an average of four determinations. *Open circles*, IgM-ADM; *solid circles*, ADM; *squares*, A0011-ADM; $*P < 0.01$

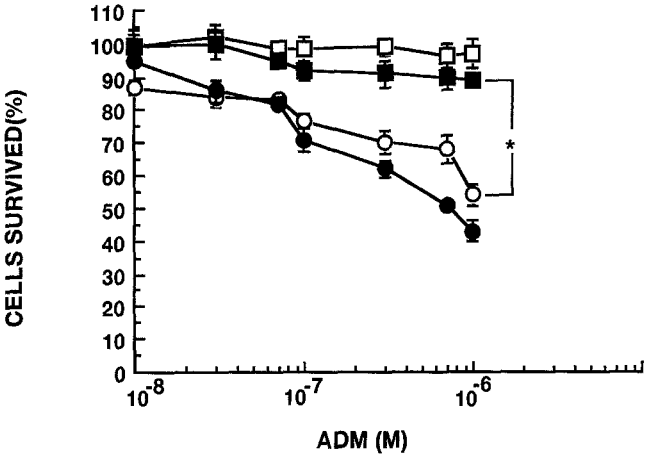

Fig. 5. Cytotoxicity of the A0011-ADM conjugate on the various cells. *Open squares*, BT-20; *solid squares*, MCF-7; *open circles*, MDA-MB-361; *solid circles*, SK-BR-3. $*P < 0.01$

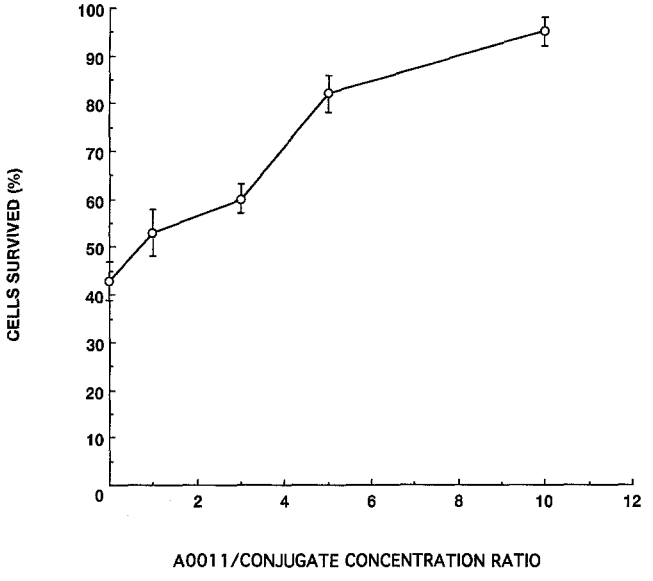

Fig. 6. Competitive inhibition of the A0011-ADM conjugate activity by free A0011. Each data point represents and average of four determinations

analyzed. Against breast cancer cells with high binding activity, A0011-ADM conjugate revealed more powerful cytotoxicity at the concentration of 10^{-6}M. The regression equation of the formula y = 116.38 − 11.63 logX where the correlation coefficient = 0.950 was fitted to the data (Fig. 7).

Discussion

In this study we described the preparation of anti-c-*erb*B-2 product-ADM immunoconjugate via a disulfide bond and evinced its specific and potent cytotoxicity to breast cancer cells overexpressing c-*erb*B-2. Despite the difficulty involved in directly demonstrating that ADM is covalently conjugated to antibody by a disulfide bond, our conclusion was supported by the following findings:

1. This bond is known to be easily cleaved under reduced conditions and, while ADM conjugated with the antibody via a disulfide bond eluted from gel filtration as a void volume, in the case of the reduced conjugate with excess DTT, ADM and the antibody eluted from gel filtration separately.
2. Nonspecific murine IgM-ADM conjugate showed no cytotoxicity against any of the four breast cancer cell lines, and the A0011-ADM conjugate was not as effective against cell lines which did not overexpress the c-*erb*B-2 product. Assuming that ADM was not linked to the antibody, these conjugates ought to have shown the same cytotoxicity as equimolar free ADM.

These findings confirm the conjugation between ADM and the antibody.

The c-*erb*B-2 product was selected as the target for the following reasons: First, it exists on the cell membrane. Second, it is strongly expressed in malignant cells, but poorly expressed in normal adult epithelial cells other than the renal tubuli.[23] Third, there have been reports concerning ligands for the c-*erb*B-2 product[24,25] which have been detected in the conditioned medium of c-*erb*B-2-positive cancer cells and found to induce tyrosine phosphorylation and stimulate cell growth. The c-*erb*B-2 product is a growth factor receptor that could play an important role in the autocrine cancer cell prolifieration. In other words, breast cancer

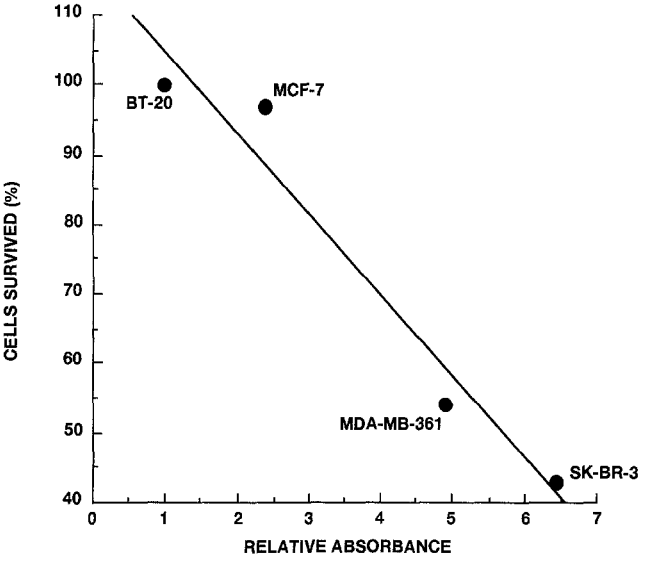

Fig. 7. Correlation between cytotoxicity and the binding activity of the conjugate (10^{-6}M). The regression equation of the formula y = −11.630x + 116.38, where the correlation coefficient = 0.950, was fitted to the data

cells with overexpression of the c-*erb*B-2 product may proliferate rapidly, which could lead to a high malignancy. Thus, using the c-*erb*B-2 product as the target, we were able to attack cancer cells with high malignant potential selectively.

It was recently reported that soluble c-*erb*B-2 protein was found in the circulating blood of breast cancer patients.[26] With in vivo application of the A0011-ADM conjugate, soluble receptors may cause the formation of immune complexes that inhibit the cytotoxic effect and lead to renal impairment; however, soluble c-*erb*B-2 protein was very small in quantity and probably different in conformation from the c-*erb*B-2 product on the cell membrane.

The effectiveness of targeting therapies using gelonin,[10,11,27,28] *Pseudomonas* exotoxin,[29] and ricin[27] which have been developed in the last decade is well documented; however, clinically these toxins are impractical because they are not human proteins. As a single agent, ADM is the most effective anticancer drug in the treatment of breast cancer, with an overall response rate of 43% in previously untreated breast cancer patients,[14] but it is associated with dose-dependent toxic effects, including bone marrow suppression, cardiac toxicity, and alopecia. In fact, it has been reported that leukopenia occurs in 68%–80% of treated patients, and the incidence of cardiomyopathy reached 20% at total doses above 550 mg/mm^2.[13] Thus, in order to utilize the anticancer activity of ADM, but minimize its harmful side effects, we employed ADM as the warhead of the immunoconjugate.

ELISA showed that the binding activity of the A0011-ADM conjugate was equal to that of A0011. A0011-ADM conjugate was more effective than an equivalent amount of free ADM to the c-*erb*B-2-overexpressing cells. In other words, the ADM activity of the conjugate was almost completely preserved. In conclusion, this conjugation procedure did not affect either the binding activity of the conjugate or the cytotoxic activity of ADM. Hence, as a link between ADM and the antibody, we selected a disulfide bond.

A0011-ADM conjugate exhibited specific cytotoxicity to SK-BR-3 and MDA-MB-361 breast cancer cell lines which overexpress the c-*erb*B-2 product, and its cytotoxicity was antibody-mediated, as shown by the following four findings:

1. Nonspecific murine IgM-ADM conjugate did not show any cytotoxicity to cells overexpressing the c-*erb*B-2 product.
2. A0011-ADM conjugate was not cytotoxic to MCF-7 or BT-20, which did not overexpress the c-*erb*B-2 product.
3. The effect of the A0011-ADM conjugate was competitively inhibited by free A0011.
4. The cytotoxicity of the conjugate was correlated with the binding activity of A0011.

These characteristics will cause the selective in vivo accumulation of conjugate in tumor tissue and decrease its side effects. Moreover, the cytotoxicity of the A0011-ADM conjugate was specific because the conjugate did not kill BT-20 cells overexpressing the EGF receptor, which is very similar to the c-*erb*B-2 product.[2]

A0011, being a murine IgM monoclonal antibody, is large in size, which causes high imunogenicity and difficulty in penetration into the core of a solid tumor mass. Thus, for clinical application, an IgG type antibody or smaller fragments including Fab should be developed.

In summary, A0011-ADM conjugate demonstrated specific cytotoxicity to breast cancer cells overexpressing the c-*erb*B-2 product. The results of this study therefore indicate that targeting therapy using the c-*erb*B-2 product as a target may be useful for treating potentially malignant breast cancers with c-*erb*B-2 overexpression.

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