Immunoselective Cell Growth Inhibition by Antibody-Adriamycin Conjugates Targeting c-erbB-2 Product on Human Cancer Cells

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Monoclonal antibodies targeting c-erbB-2 protooncogene product p185 were conjugated with adriamycin via a pH-sensitive spacer. The resultant antibody-adriamycin conjugates showed immunoselective binding, internalization and cytotoxicity to p185-positive human breast cancer cell SKBr-3 and gastric cancer cell MKN-7, but not to normal human lymphocytes.

Key words adriamycin; monoclonal antibody; internalization

Adriamycin (ADM) is a popular anti-neoplastic drug because of its high toxicity to solid tumors. However, its therapeutic application has been limited by its severe side effects. Since such side effects are caused mainly by nonspecific adsorption by normal tissues,1) this cause could be reduced by conjugating ADM with a target selective vehicle such as an anti-cancer antibody.

c-erbB-2 is a protooncogene which encodes EGF receptor-like membrane protein p185.2) Amplification and overexpression of the c-erbB-2 gene has been shown in many human cancers, including 30% of lung, breast, ovary and stomach adenocarcinomas and where there is a 50- to 100-fold increase in p185 expression as compared with the normal cell level (reviewed in3). Thus, p185 is thought to be a suitable antigen for the immunotargeting therapy of human cancers. In this regard, we have here conjugated the monoclonal antibody (mAb) targeting p185 with ADM and demonstrated the cancer-specific cytotoxicity of the antibody-ADM conjugate.

MATERIALS AND METHODS

mAbs mAbs S2-61 and SER4 (both IgG1), which recognize c-erbB-2 product p185,3) and an isotype-matched control mAb B3 (IgG1), which recognizes rat gp125 antigen,4) were used in this report. mAbs were purified from the ascites fluid of mice by 50% (NH4)2SO4 precipitation followed by protein-G affinity chromatography.

cis-Aconityl-ADM-Coupled Antibody (cAA-Ab) cAA-Ab was prepared according to the method of Shen et al.5) as follows. Briefly, ADM hydrochloride (Kyowa Hakko Co., Tokyo) was mixed with cis-aconitic anhydride at a molar ratio of 1:0.75 in 0.1 M NaHCO3, and maintained at pH 9 at 0°C for 15 min. The resultant cis-aconityl ADM (cAA) was precipitated by the addition of 1 M HCl and collected by centrifugation (10000 x g, 10 min). cAA was then resolved in H2O, adjusted to pH 7 with 1 M NaOH, and then activated by mixing it with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide at a mol ratio of 1:1.5 following stirring at r.t. for 30 min. The activated cAA was mixed with purified mAb in 0.1 M NaH2PO4 (pH 9) at a mol ratio of 25:1 and incubated at r.t. keeping pH 9 with 1 M NaOH for 3 h. Finally, cAA-Ab was separated from low molecular materials by Sephadex G25 column chromatography equilibrated with phosphate-buffered saline (PBS), and was sterilized by filtration through a millipore membrane of 0.22 μm pore size. IgG and cAA content were determined on the basis of the absorbance at 280 and 476 nm for cAA-Ab. The mean substitution ratio (MSR) of ADM to Ab was calculated by the following formula:

\[ MSR \ (\text{mol ratio}) = \frac{0.036 \times OD_{476}^{\text{Ab}}}{OD_{280}^{\text{Ab}} - (OD_{476}^{\text{Ab}} \times 0.736)} \]

Binding Analysis of cAA-Ab The binding activity of cAA-Ab was determined by indirect immunofluorescence analysis using FITC-coupled rabbit anti-mouse immunoglobulin antibody (Dako, Copenhagen, Denmark), in which the cell fluorescence was quantified by a flow cytometer FACScan (Becton Dickinson, Mountain View, CA) with excitation at 488 nm and emission at 515—545 nm. The fluorescence intensity of 10000 viable cells was recorded. All determination was done at the same detection sensitivity, and the mean fluorescence intensity of each sample was computed as the relative amount of each binding.

Cell Growth Inhibition Analysis The cell growth inhibitory effect of cAA-Ab was analyzed on SKBr-3 human breast cancer cells and MKN-7 human gastric cancer cells as follows. Cells (4 x 104) suspended in 10 μl of a standard medium, Dulbecco’s modified Eagles’s minimal essential medium (Nissui Pharmaceutical Co., Tokyo) supplemented with 10% heat-inactivated fetal calf serum (FCS) (M.A. Bioproducts, Walkersville, MD), were mixed with a reciprocal dilution of cAA-Ab (100 μl) and incubated at 37°C for 1 h. After being washed with ice-cold PBS 3 times, the cells were resuspended in the standard medium and distributed in quadruplicate into a 48-well tissue culture plate (Costar) at 1 x 104 cells/500 μl standard medium/well. After being cultured for 4 d, the cells in each well were then harvested with actinase/EDTA treatment and were counted for viable cell number as determined by Trypan blue staining.

Analysis of the Response of Human Peripheral Blood Monocytes (PBMC) with Phytohemagglutinin (PHA) The cytotoxic activity of cAA-Ab for PBMC was determined by assaying the inhibition of PBMC proliferation with PHA (Sigma) as follows. Human PBMC were freshly prepared from blood obtained from a healthy volunteer

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by Ficol (Pharmacia) gradient centrifugation. The PBMC (2.0 × 10^6) were then mixed with various concentrations of cAA-Ab in 200 μl of standard medium at 37 °C for 1 h. After being washed twice with ice-cold standard medium, the cells were seeded at 2.5 × 10^5 cells/well in quadruplicate into a Falcon flat-bottomed 96-well tissue culture plate and cultured in 200 μl of standard medium containing PHA at 20 μg/ml. After 3 d of culture, the cells were pulsed with [3H]thymidine (Amersham Lab., Buckinghamshire, England) at 0.5 μCi/well for 4 h, and then harvested. The radioactivity of the cells was measured by standard liquid scintillation counting.

RESULTS AND DISCUSSION

We have successfully prepared three cAA-Abs by the method described in the above section. The resultant cAA-Abs from Sv2-61-IgG, SER4-IgG and B3-IgG (Sv2-61-cAA, SER4-cAA and B3-cAA) contained ADM at a MSR of 7.89, 6.85 and 9.18, respectively.

**Binding Analysis of cAA-Abs** We first examined the binding activity of cAA-Ab as compared with intact Ab. Representative binding curves of intact Ab and cAA-Ab in increasing concentrations were demonstrated on SKBr-3 in Fig. 1, and the same type of analysis was done on the other Abs and cAA-Abs, on MKN-7 and PBMC. From these analyses, maximal binding, which suggests the relative binding capacity on the cell, and the concentration resulting in 1/2 of maximal binding (titer), which suggests binding affinity, were read and then summarized in Table 1. As shown in Table 1, the relative binding capacities are 1.3–1.6 times higher in Sv2-61-related epitopes than in the SER4-epitope for both SKBr-3 and MKN-7 cells. Both anti-p185 Abs and cAA-Abs showed significant binding to SKBr-3 and MKN-7 with titers of 1.8–4.6 × 10^{-3} M, but showed no detectable binding to PBMC. If binding affinities (titers) were compared between intact IgG and corresponding cAA-Ab, no significant change (less than a 2 times increase in titer) was observed in either Sv2-61-cAA or SER4-cAA, suggesting that the modification of ADM had no inhibitory effect on antibody binding.

**Cell Growth Inhibition Analysis** We next examined the cell growth inhibitory effect of cAA-Abs targeting p185 (SER4-cAA and Sv2-61-cAA), the non-targeting control B3-cAA, and free cAA on both p185-positive target cancer cells and negative normal PBMC.

As shown in Fig. 2, SER4-cAA and Sv2-61-cAA sig-

![Graph showing binding curves of intact Ab and cAA-Ab](image)

**Table 1. Binding Characteristics of Intact Antibodies and cAA-Abs**

<table>
<thead>
<tr>
<th>Target cell</th>
<th>Titer (× 10^3 M)^a</th>
<th>Max binding (MFI)^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sv2-61-IgG</td>
<td>SKBr-3</td>
<td>2.6</td>
</tr>
<tr>
<td>Sv2-61-IgG</td>
<td>SKBr-3</td>
<td>2.8</td>
</tr>
<tr>
<td>Sv2-61-cAA</td>
<td>SKBr-3</td>
<td>1.8</td>
</tr>
<tr>
<td>Sv2-61-cAA</td>
<td>MKN7</td>
<td>2.7</td>
</tr>
<tr>
<td>Sv2-61-cAA</td>
<td>PBMC</td>
<td>ND</td>
</tr>
<tr>
<td>SER4-IgG</td>
<td>SKBr-3</td>
<td>2.8</td>
</tr>
<tr>
<td>SER4-IgG</td>
<td>MKN7</td>
<td>4.6</td>
</tr>
<tr>
<td>SER4-cAA</td>
<td>SKBr-3</td>
<td>4.5</td>
</tr>
<tr>
<td>SER4-cAA</td>
<td>MKN7</td>
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</tr>
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<tr>
<td>B3-cAA</td>
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<td>B3-cAA</td>
<td>PBMC</td>
<td>ND</td>
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\[a] Antibody concentration resulting in 1/2 of maximal binding in Fig. 1 and the same type of figures (data not shown).

\[b] Mean fluorescence intensity. ND: not detected; no detectable specific binding was observed.

![Graph showing cell growth inhibition](image)
Fig. 3. Competitive Inhibition of Cell Growth Inhibitory Effect of cAA-Ab by Intact Antibody
MKN-7 cells were treated with cAA-Ab at 0.2 μg cAA/ml (7.7–7.5 μg IgG/ml) in the absence or presence of intact antibodies (final concentration of 500 μg/ml), and were analyzed for growth activity as in Fig. 2. The columns and bars represent the mean value from four determinations and the S.D. of the mean, respectively.

Fig. 4. Effect of cAA-Ab on PHA-Response of PBMC
PBMC was treated with the indicated concentration of cAA-Ab or free cAA and was analyzed for proliferation with PHA as described in the Methods section. The percentage of [3H]thymidine incorporation as compared with that of the non-treated control cells is shown. The symbols represent average values from four determinations. All S.D. of the average values were less than 8.3%. (●), B3-cAA; (○), SER4-cAA; (□), SV2-61-cAA; (○) free cAA.

Fig. 5. Modulation of Cell Surface SER4-cAA on SKBr-3 and MKN-7
Intact cells (closed symbols) or formalin-fixed cells (open symbols) were mixed with SER4-cAA at 20 μg IgG/ml in a standard medium at 4°C for 2h. After being washed twice with ice-cold PBS, the cells were re-incubated in the standard medium at 37°C. At the indicated time after incubation, the cells were washed with ice-cold PBS once and processed for indirect immunofluorescence analysis as in Fig. 1. The percentage of the mean fluorescence intensity as compared with the 0 time value is shown as an index of cAA-Ab level on the cell surface. (●), SKBr-3; (△), MKN-7.

Ab is dependent on both antibody selectivity and the drug effect of cAA in the conjugate.

The effect of cAA-Ab on p185-negative normal human PBMC was also analyzed to confirm the specificity. As shown in Fig. 4, all cAA-Ab showed no significant inhibition of the PHA-response of PBMC. Only free cAA showed a somewhat inhibitory effect. These show that the nonspecific effect of anti-p185 cAA-Ab on normal human PBMC is negligible in the tested concentration.

The IC_{50} of free cAA in the PHA-response analysis (Fig. 4) was higher than that in the cell growth inhibition analysis (Fig. 2). This seems to be due to a difference in native sensitivity to cAA between the normal PBMC and cancer cells. In the same type of analysis, free intact ADM showed a 2.5–7 times greater effect than free cAA (data not shown).

Internalization of cAA-Ab by Target Cell Since the pH-sensitive cis-aconityl spacer in cAA-Ab is thought to be cleaved in an intracellular acidic compartment, such as the endosomes or lysosomes, resulting in the release of free ADM into cytosol, endocytosis (internalization) of cAA-Ab by target cells after its initial binding is an important factor in expressing its cytotoxic activity. Thus, we finally demonstrated the internalization of cAA-Ab by SKBr-3 and MKN-7 in Fig. 5. Cells were first coated with SER4-cAA at 4°C and then incubated at 37°C to allow the internalization of the cell surface-bound SER4-cAA. In the case of SKBr-3, the level of cell surface SER4-cAA decreased by 25% in 10 min and thereafter increased back to 90% of the initial level. By contrast, in the case of MKN-7, it continuously decreased to 54% of the initial level during 2 h of incubation (Fig. 5). In both cell types, the fixed cells showed only a slight decrease in cell surface SER4-cAA. In conjunction with the results that the total cAA-Ab content in the cells remained unchanged during the same type of analysis as in Fig. 5, as determined using 125I-labeled cAA-Ab, and the
immunofluorescence observation showed their intracellular localization (data not shown), the decrease in cell surface cAA-Ab substantially represents the internalization, but not the simple dissociation, of cAA-Ab.

The increase of SER4-cAA in SKBr-3 cell surface, following an initial decrease after 30 min incubation (as described above) may suggest that cAA-Ab are first internalized and then recycled to the cell surface. However, in MKN-7, no such apparent "recycle" was observed. Though we have no explanation for this different internalization pattern between SKBr-3 and MKN-7, it should concern the effect of cAA-Ab. The correlation between the cytotoxic activity of cAA-Ab and its intracellular fate has now been under investigation.

**Conclusion** It is noteworthy that anti-p185 cAA-Ab inhibit cancer cell growth at a concentration resulting in no inhibitory effect on normal cell activity. Taken together, anti-p185 cAA-Ab will be a good immunotargeting drug for p185-expressing human tumors.

**REFERENCES**