Antineoplastic Efficacy of Doxorubicin Enzymatically Assembled on Galactose Residues of a Monoclonal Antibody Specific for the Carcinoembryonic Antigen

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ABSTRACT

We have developed a novel procedure to couple enzymatically the antineoplastic agent doxorubicin (Dox) on the galactose residues of a monoclonal antibody specific for the tumor-associated carcinoembryonic antigen. The synthesis of the immunoconjugate consists of covalent attachment of the NH₂ terminus of Dox to oxidized galactose residues of desialylated monoclonal antibody, followed by concurrent stabilization of Schiff bases by mild reduction with pyridine borane. The immunoconjugate preserved both antibody specificity and drug cytotoxicity. At equimolar concentrations, the immunoconjugate was 8 times more cytotoxic against two carcinoembryonic antigen-expressing carcinoma cell lines, LoVo and SW-480, than Dox alone. The intracellular drug accumulation was 8–8.5 times higher than that obtained with free Dox, and >50% of the drug delivered by the conjugate was retained for 24 h in the tumor cells. Only 4 days after treatment with a single dose of immunoconjugate carrying 2.5 ng of Dox, LoVo and SW-480 tumor transplants on the chorioallantoic membrane of embryonated hen eggs showed reduced tumor-induced angiogenesis and tumor progression by half, with no detectable damage to surrounding tissues. In contrast, the same amount of free drug induced insignificant changes in tumor progression and tumor-induced angiogenesis. Enzymatically mediated, glycosidic coupling of antineoplastic agents to antibodies specific for tumor-associated antigens may represent a novel platform for the development of more efficient anticancer agents with reduced side effects.

INTRODUCTION

Dox,³ an anthracycline derived from Streptomyces peucetius var. cœsius, is a powerful chemotherapeutic agent (1). The cytotoxic effects of Dox rely on its intercalation into double-stranded nucleic acids (2), with subsequent inhibition of DNA and RNA synthesis (3). Among other pleiotropic effects that depend upon the cell type, it is now generally accepted that the effects of Dox rely on its intercalation into double-stranded nucleic acids (2), with subsequent inhibition of DNA and RNA synthesis (3). Among other pleiotropic effects that depend upon the cell type, it is now generally accepted that the drug-induced insignificant changes in tumor progression and tumor-induced angiogenesis. Enzymatically mediated, glycosidic coupling of antineoplastic agents to antibodies specific for tumor-associated antigens may represent a novel platform for the development of more efficient anticancer agents with reduced side effects.

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MATERIALS AND METHODS

Antibodies. T84.66A3.1.1F2 hybridoma cell line secreting a mouse IgG1 antihuman CEA mAb was obtained from American Type Culture Collection (Rockville, MD). The antibody was purified from cell culture supernatants by affinity chromatography using a rat antimouse κ chain mAb-Sepharose column. Anti-Pgp mAb (Pgp, mouse IgG1, clone 4E3; Signet Laboratories, Dedham, MA) was used for FACS analysis. FITC-(Fab⁺)₂ goat antimouse IgG was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Rabbit anti-Dox serum (Western Chemical Research, Fort Collins, CO) was enriched for the IgG fraction by affinity chromatography on protein A-Sepharose column. Goat antirabbit IgG (Boehringer Mannheim) was radiola

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3 The abbreviations used are: Dox, doxorubicin; mAb, monoclonal antibody; CEA, carcinoembryonic antigen; Gal, galactose; CAM, chorioallantoic membrane; Pgp, P-glycoprotein; FACs, fluorescence-activated cell sorting; GAO, Gal oxidase; NANA, N-acetyl neuraminic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; PI, propidium iodide.

IgG1 at Dox, respectively; and 1.4 represents the extinction coefficient of mouse or rat the correction factors for absorbance of immunoglobulin-Dox conjugate and in PBS, washed with PBS, and incubated overnight at 4°C with 10
mL of 5% fat-free milk (Carnation, Nestle’s Food Company, Glendale, CA) for this, 1 mg of anti-CEA mAb was incubated for 4 h at room temperature, with continuous stirring, in 1 mL of conjugation buffer containing 0.1 mg of Dox and 0.1 mg of EDC. The conjugate was extensively dialyzed against PBS in SPECTRA/POR bags of 100,000 molecular weight cutoff (Sigma) and concentrated by speed vacuum centrifugation to 1 mg/mL with respect to immunoglobulin monomer. The chemical coupling of Dox to anti-CEA mAb was carried out using EDC (Imject Immunogen EDC Conjugation Kit, Pierce, Rockford, IL). For this, 1 mg of anti-CEA mAb was incubated for 4 h at room temperature, with continuous stirring, in 1 mL of conjugation buffer containing 0.1 mg of Dox and 0.1 mg of EDC. The conjugate was extensively dialyzed against PBS in SPECTRA/POR bags of 100,000 molecular weight cutoff, and the degree of coupling was calculated as follows:

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\text{Immunoglobulin:Dox ratio} = \frac{\{A_{280\text{ nm}} - (0.73 \times A_{495\text{ nm}})\} \times 1.4\} \times \{A_{495\text{ nm}} \times (8 \times 10^3)\}
\]

where A indicates the spectrophotometric absorbance; 0.73 and (8 \times 10^3) are the correction factors for absorbance of immunoglobulin-Dox conjugate and Dox, respectively; and 1.4 represents the extinction coefficient of mouse or rat IgG1 at \(\lambda_{280\text{ nm}}\). The absorption of Dox was measured at \(\lambda_{495\text{ nm}}\).

Electrophoretic Analyses. Isoelectric focusing of the unconjugated and desialylated anti-CEA mAb and anti-CEA-Gal-Dox conjugates was carried out on precast IEF 3-10 PhastGels using the PhastSystem apparatus (Pharmacia LKB). Gels were stained with Coomassie Blue R-250 according to the manufacturer’s instructions. The anti-CEA-Gal-Dox conjugate was also analyzed by SDS-PAGE using PhastGels 4–20% gradient polyacrylamide (Pharmacia LKB) under reducing conditions. Samples were left untreated or were treated with 0.01 units/\(\mu\)g PGN-ase F, and then 10 \(\mu\)g of the conjugate were electrophoresed for 1 h at 150 V. Gels were either stained with Coomassie Blue R-250 or electrotransferred under semidry conditions for 45 min at 450 mA onto Immobilon polyvinylidene fluoride membranes (Sigma) using a Multiple II apparatus (Pharmacia LKB). Membranes were blocked overnight at 4°C with 5% fat-free milk (Carnation, Nestlé Food Company, Glendale, CA) in PBS, washed with PBS, and incubated overnight at 4°C with 10 \(\mu\)g/mL affinity-purified IgG fraction from rabbit anti-Dox serum in PBS, 1% BSA, and 0.05% Tween 20. Membranes were washed with PBS-0.05% Tween 20 and bound rabbit anti-Dox IgG was detected after incubation for 2 h at room temperature with \(^{125}\text{I}-\text{goat antirabbit IgG (2 \times 10^5 \text{cpm/10-cm membrane) in PBS, 1% BSA, and 0.05% Tween 20, using Kodak X-OMAT films (Sigma).}

Thymidine Incorporation Assay. LoVo or SW-480 cells (5 \times 10^3) were cultured in 200 \(\mu\)L of complete medium and containing 1 \(\mu\)g/25 \(\mu\)L tritiated thymidine were incubated for 24 h with various doses of enzymatically or chemically engineered conjugates, Dox alone, or medium alone. In a parallel set of assays, graded amounts of unconjugated anti-CEA mAb (1–100 \(\mu\)g/mL) were added to the tumor cell cultures, 30 min before incubation with a constant amount of immunoconjugates and tritiated thymidine. After 24 h of culture, cells were harvested on filter paper, and the radioactivity was measured in a \(\beta\)-scintillation chamber (Pharmacia LKB).

Confocal Laser Scanning Microscopy. Tumor cells (1 \times 10^5 cells in 2 mL per well) were inoculated in the 12-well plates containing coverslips and cultured for 3–4 days with daily changes of medium until the cells were tightly adherent to the glass. Coverslips were rinsed with cold PBS-1% BSA, fixed for 10 min in methanol:acetone (1:1) at -20°C, and air-dried overnight. After rehydration in PBS-1% BSA, coverslips were incubated for 1 h at room temperature with 5 \(\mu\)g/mL anti-CEA or anti-Pgp mAbs, rinsed with cold PBS-1% BSA, and then incubated for 1 h at room temperature with 5 \(\mu\)g/mL FITC-(Fab’)-2 goat antimouse IgG. Coverslips were rinsed with cold PBS-1% BSA and incubated for 30 min at 37°C with 3 \(\mu\)g/600 \(\mu\)L/well RNase A in PBS-1% BSA to remove RNA. To visualize nuclei, coverslips were over-stained for 30 min at room temperature with 2 \(\mu\)g/600 \(\mu\)L PI in PBS-1% BSA per well; rinsed with cold PBS-1% BSA, followed by distilled water; and then mounted with Vectashield medium (Vector Laboratories, Burlingame, CA), sealed with Permount (Fisher Scientific), and analyzed in an inverted Leica confocal laser scanning microscope equipped with a fluorescence filter set for double excitation at 488/568 nm (Leica Lasertechnik, Heidelberg, Germany).

Control staining was performed using an isotype control IgG1 (Sigma) as primary antibody, and FITC-(Fab’)-2 goat antimouse IgG as secondary antibody.

FACS Analyses. To analyze the expression of CEA and Pgp, LoVo and SW-480 cells (2 \times 10^5) were incubated on ice for 30 min with anti-CEA mAb (10 \(\mu\)g), anti-Pgp mAb (2 \(\mu\)g), or the same amounts of mouse IgG1 isotype control. Cells were washed with cold PBS, incubated for 30 min on ice with FITC-(Fab’)-2 goat antimouse IgG, washed, and fixed with 1% paraformaldehyde in PBS, and the fluorescence intensity was measured by FACS, as described previously (35).

To determine the extent of intracellular drug accumulation, 1 \times 10^5 tumor cells in DMEM complete medium were exposed for 24 h to 0.2 \(\mu\)g of Dox or to a molar equivalent of Dox carried by the anti-CEA-Gal-Dox conjugate, washed, and fixed with 1% paraformaldehyde; and the fluorescence intensity was measured by FACS. In a parallel set of assays, 1 \times 10^5 cells were cultured for 24 h with 0.2 \(\mu\)g of Dox or with a molar equivalent of Dox carried by the anti-CEA-Gal-Dox conjugate, washed, and then recultured for another 24 h in DMEM alone. Cells were washed and fixed with 1% paraformaldehyde, and the fluorescence intensity was measured. The intrinsic fluorescence of Dox accumulated or retained intracellularly was acquired on an EPICS Profile II Analyzer (Coulter Corporation, Hialeah, FL) equipped with air-cooled argon ion laser emitting \(\lambda_{488\text{ nm}}\) at 15 mV in standard optical configuration. The mean of fluorescence intensity for the entire population of cells was measured among 5000 cells at \(\lambda_{488\text{ nm}}\) excitation versus \(\lambda_{580\text{ nm}}\) absorbance.

Tumor Growth in CAM System and Determination of Tumor Progression. LoVo and SW-480 cells were grown in DMEM complete medium and then detached with trypsin-EDTA. Tumor cells (2 \times 10^6 cells/2 mL) were inoculated for 12 h onto sterile blank concentration discs (1/4 inch = 6.4 mm; Difco Laboratories, Detroit, MI) in 12-well plates (Becton Dickinson), and discs were transferred to new 12-well plates containing fresh medium and cultured for another 7 days until cells formed a confluent layer. Fertilized 10-day-old white Leghorn hen eggs (Spafas Inc., Norwich, CT) were opened at the air sac, CAMs were transplanted aseptically with discs containing tumor cell inoculum, and the shells were sealed. Thirty eggs per cell line were prepared in this way. Eggs were incubated at 37°C and 98% relative humidity, and after 3 days, the 13-day-old eggs received a single-dose treatment of free Dox or conjugates applied in 25 \(\mu\)L of DMEM in the center of the disc. After 4 days, eggs were fixed in 10% buffered formalin solution and embedded in paraffin according to a standard procedure. Tumor progression was determined by morphometric measurements of tumor-CAM transplants using 7-\(\mu\)m cross-sections that were cut along the diameter of the supporting disc, then stained with H&E and scanned at \(\times 125\) magnification using a three-chip charged-coupled device color camera (DVC-960 MD; Sony Electronics, Inc.) adapted to a stereomicroscope (Carl Zeiss, Jena-Göttingen, Germany). Digitized images were measured planimetrically after previous calibration of the system with a standard \(\mu\)-slide (Carl Zeiss) using the software ImagePro Plus (Media Cybernetics, Silver Spring, MD). The cross-sectional areas were traced in mm². Statistical analysis of the measurements was carried out using the SPSS/PC+ software, version 7.0 (SPSS International BV, Gorinchem, the Netherlands). The defined variable was the cross-sectional area of CAM at the site of tumor transplantation. ANOVA (one-way ANOVA) was used to determine the difference in tumor progression between the groups. Measurements of tumor-CAM transplants were averaged for each group (5 CAMs/group) and calculated according to the t test for \(P < 0.05\).
RESULTS

Specificity of the Enzymatically Mediated Conjugation. Neuraminidases from *A. ureafaciens* and *C. perfringens* being able to cleave, respectively, Gal-(2-6)-NANA and Gal-(2-3)-NANA bonds (36), yielded fully desialylated mAb preparations, making accessible the Gal residues for oxidation by GAO. Mouse IgG1 anti-CEA mAb and rat IgG1 6.5.2 mAb showed four potential Gal acceptors for Dox. The coupling ratios for anti-CEA-Gal-Dox, 6.5.2-Gal-Dox, and anti-CEA-Dox conjugates were 3.7, 3.2, and 7.8 Dox molecules per molecule of immunoglobulin, respectively.

Isoelectric focusing analysis showed a slightly higher pl for the anti-CEA-Gal-Dox conjugate (6.2 and 6.5 pl), as compared to the desialylated-unconjugated mAb (6.1 and 6.3 pl), indicating a change in the net electrical charge of immunoglobulin after coupling the drug (Fig. 1a, Lanes 2 and 3). Also, the molecular mass of the heavy but not light chains of anti-CEA mAb was slightly increased (Fig. 1b, Lanes 2 and 3). Western blot analysis developed with rabbit anti-Dox IgG revealed the presence of Dox on the carbohydrate moieties of anti-CEA mAb but not on the conjugate treated with PNGase F, indicating that Dox was specifically coupled to the N-glycan moieties of mAbs (Fig. 1c, Lanes 1 and 2).

Cytotoxicity of anti-CEA-Gal-Dox Conjugates. Inhibition of DNA synthesis in LoVo and SW-480 cells, as determined by thymidine incorporation assay, was dose dependent for both anti-CEA-Gal-Dox and anti-CEA-Dox conjugates, as well as for free Dox. On a molar basis, at half-maximal inhibition of cell growth (IC₅₀), Dox delivered by the enzymatically engineered conjugate was 8.3 times more cytotoxic than Dox alone (Fig. 2). Both cell lines showed 15–20% growth inhibition upon incubation with 6.5.2-Gal-Dox conjugate (specificity control). Notable, although the coupling ratio for anti-CEA-Gal-Dox conjugate was 3.7, as compared to 7.8 for anti-CEA-Dox conjugate, the cytotoxicity of the enzymatically engineered conjugate was almost 4 times higher. Up to 80% inhibition of the cytotoxic effect exerted by anti-CEA-Gal-Dox conjugate was obtained on both tumor cell lines preincubated with 100 µg/ml of unconjugated anti-CEA mAb (data not shown), indicating that antibody specificity was preserved after enzymatic coupling of Dox.

Expression of CEA and Pgp on Colon Carcinoma Cell Lines. Using confocal laser scanning microscopy, we found that both LoVo and SW-480 cells express CEA and Pgp on the membrane (Fig. 3). The multidrug resistance associated Pgp is an ATP-driven transmembrane pump that facilitates active cellular efflux of toxic chemicals (37) and, thereby, lowers cytotoxicity of the drugs. FACS analysis showed that CEA was 3 times higher expressed on LoVo than on SW-480 cells (Fig. 4, top), whereas Pgp was 3 times higher expressed on SW-480 than on LoVo cells (Fig. 4, bottom).

Intracellular Retention of Dox Accumulation. Data illustrated in Fig. 6 indicate that both carcinoma cell lines accumulated 8–8.5 times more drug during 24-h exposure to equimolar concentrations of Dox.
delivered by the conjugate than by Dox alone (Fig. 5, top). After another 24 h of resting in medium alone, cells exposed to the enzymatically engineered conjugate retained 54–58.8% from the initial amount of drug accumulated (Fig. 5, bottom).

**Inhibition of Tumor Progression.** Untreated or low-dose Dox (2.5 ng)-, anti-CEA mAb (250 ng)-, or 6.5.2-Gal-Dox (250 ng)-treated LoVo-CAM transplants showed massive tumor growth with strong angiogenesis and profound invasion of CAMs (Fig. 6, a and b, e and f, g and h, and k and l, respectively). After 4 days from a single-dose application of 250 ng of Dox strongly inhibited both tumor progression and tumor-induced angiogenesis, leaving only small cell islets with pyknotic nuclei (Fig. 6, c and d), whereas 2.5 ng of Dox showed no detectable effect (Fig. 6, e and f, and Fig. 7). In contrast, 2.5 ng of Dox delivered by anti-CEA-Gal-Dox conjugate reduced tumor progression and tumor-induced angiogenesis of LoVo-CAM transplants (Fig. 6, i and j), as well as SW-480-CAM transplants (data not shown) by 42 and 50%, respectively (Fig. 7). At 250 ng of free Dox, tumor progression was completely inhibited, but severe fibrosis and sclerosis of the CAM parenchyma and stroma were observed. In contrast, although a slightly reduction in thickness of the CAM parenchyma was detected upon treatment with anti-CEA-Gal-Dox conjugate (Fig. 7, j versus d), inhibition of tumor progression and tumor-induced angiogenesis by the enzymatically engineered conjugate was not accompanied by visible alterations of media and intima of blood vessels or by damage of the CAM parenchyma and stroma, as analyzed microscopically.

**DISCUSSION**

Our study demonstrates that enzymatically mediated, glycosidic conjugation of Dox to a mAb specific for CEA tumor-associated antigen exerts efficient and specific cytotoxicity on two human colon carcinoma cell lines expressing CEA. Both enzymatically and chemically engineered immunoconjugates of Dox showed dose-dependent cytotoxicity on CEA-expressing cells. Although the chemical conjugation using EDC introduced twice as many Dox molecules per molecule of immunoglobulin than did the enzymatically mediated conjugation, the enzymatically engineered conjugate was 4 times more cytotoxic. This suggests that the enzymatically mediated coupling of Dox to the Gal residues of immunoglobulins provides a more efficient delivery of Dox to tumor cells than the chemical coupling does. Dox enzymatically assembled on the Gal residues of anti-CEA mAb exerted 8 times higher cytotoxicity against CEA-expressing carcinoma cells than the drug administered per se. The antiproliferative capacity of the enzymatic conjugate persisted for >72 h after a single exposure of $2 \times 10^5$ LoVo cells to 1 $\mu$g/ml conjugate (data not shown). A 15–20% nonspecific cytotoxicity was observed for both enzymatically and chemically engineered conjugates. This was presumably

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**Fig. 3. Identification of CEA and Pgp on colon carcinoma cells.** The expression of CEA and Pgp on the surface of LoVo and SW-480 colon carcinoma cells was analyzed by confocal laser scanning microscopy. Top, expression of CEA; bottom, expression of Pgp. Digitized microscopic images represent 1 of 8 horizontal cell sections of 0.5-μm thickness. The CEA and Pgp expressed by the tumor cells are indicated (green), as revealed by secondary antibody coupled to FITC. The nuclei are visualized (red), as stained with PI.
the result of endocytosis of the conjugates by fluid-phase pinocytosis.

At equimolar concentrations, Dox delivered by the enzymatically engineered conjugate showed a remarkable 8–8.5 times higher intracellular drug accumulation than the drug administered per se. This was presumably the result of targeting the drug to CEA-expressing tumor cells. It is noteworthy that higher level of CEA expression on LoVo than on SW-480 cells did not significantly influence the extent of intracellular accumulation of Dox. For as much as 0.2 µg/ml Dox per 2 × 10^5 cells, the 24-h intracellular retention upon delivery by the enzymatically engineered conjugate was >50%, whereas cells exposed to the same amount of free drug retained the entire amount of drug. However, cells exposed 24 h to >1 µg/ml free Dox per 2 × 10^5 cells retained only 20–25% of the drug (data not shown). This implies that at 0.2 µg/ml Dox per 2 × 10^5 cells, the entire amount of drug may have been tightly bound to DNA. It is also likely that cell saturation with the drug may correlate to maximal amount of drug bound to nucleus, and the unbound drug is exocytosed, especially when the Pgp multidrug resistance pump is present. Interestingly, Pgp expression on LoVo (4.8%) and SW-480 (15.8%) carcinoma cells did not make a significant difference between the two cell lines in terms of the extent to which Dox delivered by the immunoconjugate was retained intracellularly. One could consider that the pathway of cellular degradation and trafficking for Dox enzymatically assembled on the Gal residues of immunoglobulins may differ from that of Dox administered per se. Dox coupled to the sugar moieties of immunoglobulin may be incrementally released by endoglycosidases, thereby allowing a more efficient targeting to the nucleus, whereas the excess of free Dox entering the cell can be easier exocytosed by Pgp pump. This may explain why expression of Pgp on LoVo and SW-480 carcinoma cells significantly lowered the amount of drug when administered per se but did not lower the intracellular retention of Dox delivered by the immunoconjugate.

CAM experiments indicated that single-dose treatment of carcinoma transplants with anti-CEA-Gal-Dox conjugate carrying 2.5 ng of Dox reduced tumor progression and tumor-induced angiogenesis by half, whereas the same dose of free drug did not. Although the inhibition of tumor progression and tumor-induced angiogenesis was not complete at this dose, the drug delivered by the enzymatically engineered conjugate induced no detectable damage to surrounding CAM tissues. In contrast, severe sclerosis and fibrosis of media and intima of blood vessels occurred rapidly in the case of high dose of free Dox (250 ng).

Our results suggest that enzymatically mediated, glycosidic coupling of Dox to the Gal residues of antibodies specific for tumor-associated antigens may offer a more efficient anticancer therapy than chemically engineered conjugates and Dox administered per se. This is mainly because: (a) specific targeting of the drug to tumor cells that minimizes the side effects rather than being distributed randomly to surrounding tissues; (b) long-term cytotoxicity due to efficient intracellular drug accumulation and retention; and (c) bypass of Pgp multidrug resistance pump by means of different pathways of intracellular trafficking.
Fig. 5. Analysis of intracellular drug accumulation and retention by colon carcinoma cells. Colon carcinoma cells were treated with molar equivalents of Dox as carried by the anti-CEA-Gal-Dox conjugate or by free Dox, and the average of intrinsic fluorescence on 5000 cells was determined by FACS, as described previously (35). Top, percentage of drug accumulation upon 24 h of continuous incubation of LoVo and SW-480 cells with prodrugs; bottom, percentage of drug retention after resting the cells in medium alone for another 24 h.

Fig. 6. Inhibition of tumor progression upon treatment with various prodrugs. The macroscopic aspect, at ×125 magnification, of the tumor progression on CAMs after 4 days of culturing with or without treatment with prodrugs is illustrated. a, treatment of CAM with DMEM alone; c, free Dox (250 ng/25 μl); e, free Dox (2.5 ng/25 μl); g, anti-CEA mAb (250 ng/25 μl); i, anti-CEA-Gal-Dox conjugate (250 ng/25 μl) carrying 2.5 ng of Dox; and k, 6.5.2-Gal-Dox conjugate carrying 2.5 ng of Dox. The corresponding H&E stainings of CAM cross-sectional areas are illustrated in b, d, f, h, j, and l, respectively.
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