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

Alexandru C. Stan, Dorel L. Radu, Sofia Casares, Constantin A. Bona, and Teodor-D. Brumeanu

Department of Microbiology, Mount Sinai School of Medicine, New York, New York 10029

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, 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 primum movens of Dox activity is primarily exerted by its effect on stabilization of DNA-topoisomerase II complexes (4, 5).

We and others showed that the sine qua non condition for efficient therapy with Dox depends ultimately on the amount of intracellular drug accumulation (6). Several new strategies were aimed at increasing the intratumoral concentration of Dox: (a) encapsulation of Dox in polyisohexylcyanocrylate nanospheres (7, 8); (b) combined therapy with mannose, leukotriene, and bradykinins or their analogue, RMP-7, to increase selectively the transport of the drug into solid tumors (9–15); and (c) intratumoral or intraventricular administration of Dox, pre- or postoperation (16–19).

Immunotargeting tumors with Dox conjugated to antibodies has been considered an attractive anticancer strategy. Chemically engineered immunoconjugates of Dox showed up to 10 times increased toxicity than Dox alone. Tumor progression was inhibited by chemically coupled Dox to mAbs specific for C-erb-2 in breast carcinomas, Thy-1 in neuroblastomas, and CEA in colon carcinomas (20–22). To increase the number of Dox molecules per molecule of immunoglobulin, the drug was chemically coupled to aminodextran, i.e., Gentran 40, and the aminodextran-Dox intermediate was chemically attached to various antitumor antibodies (23–28). However, despite the large number of Dox molecules carried by such preparations, i.e., 30–40 Dox molecules per molecule of immunoglobulin, the therapeutic efficacy was below expectations. The major drawbacks were considered to be the loss of pharmacological activity of Dox due to drug oxidation during the coupling reaction, as well as a deficient catabolism of aminodextran-Dox intermediates, which, in turn, impairs delivery of active drug to the nucleus (29).

Herein, we describe a mild method to couple Dox enzymatically to the modified carbohydrate moieties of a mAb specific for CEA. The enzymatically engineered anti-CEA-Gal-Dox conjugate preserved both antibody specificity and drug cytotoxicity, and it was 8 times more cytotoxic against CEA-expressing carcinoma cells than Dox alone. Single application of anti-CEA-Gal-Dox conjugate on LoVo and SW-480 human colon carcinoma transplants on the CAM of embryonated hen eggs reduced tumor-induced angiogenesis and tumor progression by half, with no detectable damage to surrounding tissues.

MATERIALS AND METHODS

Antibodies. T84.66A3.1A.1F2 hybridoma cell line secreting a mouse IgGl 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 antimonuse κ chain mAb-Sepharose column. Anti-Pgp mAb (Pgp, mouse IgGl, 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 radiolabeled with ¹²⁵I(Amersham) according to a standard chloramine-T method. The 6.5.2 mAb, a rat IgGl anti-T-cell receptor clonotypic mAb (30), was purified from the cell culture supernatants by affinity chromatography on protein A-Sepharose column and was enzymatically coupled to Dox. The 6.5.2-Gal-Dox conjugate was used as specificity control for the anti-CEA-Gal-Dox conjugate.

Cell Lines. LoVo and SW-480 human colon carcinoma cell lines were obtained from American Type Culture Collection. LoVo is an aneuploid cell line with a modal number of 49 (31), and SW-480 is a hypotriploid cell line with 12% polyplody, established from a human colorectal adenocarcinoma Duke’s type B (32). Cells were grown at 37°C and 5% CO₂ in DMEM (Life Technology, Rockville, MD). The antibody was purified from cell culture supernatants by affinity chromatography on protein A-Sepharose, performed at the Mount Sinai School of Medicine—Confocal Laser Scan Microscopy core facility and was supported by NIH Shared Instrumentation Grant 1 S10 RR0 9145-01 and National Science Foundation Major Research Instrumentation Grant DBI-9724504.

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To whom requests for reprints should be addressed, at the Department of Microbiology, Mount Sinai School of Medicine, One Gustave L. Levy Place, Box 1124, New York, NY 10029-6574. Phone: (212) 241-7551; Fax: (212) 828-4151.

The abbreviations used are: Dox, doxorubicin; mAb, monoclonal antibody; CEA, carcinoembryonic antigen; Gal, galactose; CAM, chorioallantoic membrane; Pgp, P-glycoprotein; FACSc, 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 at 4°C with 5% fat-free milk (Carnation, Nestle Food Company, Glendale, CA) A graded amounts of unconjugated anti-CEA mAb (1–100 μg/ml) was removed by dialysis against PBS (pH 7.4). The reaction mixture was incubated for 48 h at 37°C under sterile conditions and stirred continuously with GAO (20 units; Sigma Chemical Co., St. Louis, MO), pyridine borane (80 mM), and 0.01 units/LKB) under reducing conditions. Samples were left untreated or were treated Electrophoretic Analyses. Isoelectric focusing of the unconjugated and containing 0.1 mg of Dox and 0.1 mg of EDC. The conjugate was extensively dialyzed overnight at 37°C with 50 milliunits each of the neuraminidase from Arthrobacter ureafaciens overnight at 37°C with 5% fat-free milk (Carnation Chemical Co., St. Louis, MO), pyridine borane (80 mM, Aldrich), and 0.1 mg of Dox (Sigma). Schiff bases formed between the aldehyde groups generated by oxidation with GAO at the sixth carbon of the tumor cell cultures, 30 min before incubation with a constant amount of IGU1 at the glass. Coverslips were rinsed with cold PBS-1% BSA, and then incubated for1 ha t room temperature with 5 μg/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 μg/600 μl/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 μg/600 μl 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 × 10⁵) were incubated on ice for 30 min with anti-CEA mAb (10 μg), anti-Pgp mAb (2 μg), 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 × 10⁵ tumor cells in DMEM complete medium were exposed for 24 h to 0.2 μg 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 × 10⁵ cells were cultured for 24 h with 0.2 μg 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 λ488 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 λ488 nm excitation versus λλ485 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 × 10⁵ cells/2 ml) were inoculated for 12 h on 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 (Sfapas 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 μl 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 immunohistochemical measurements of tumor-CAM transplants using 7-μm cross-sections that were cut along the diameter of the supporting disc, then stained with H&E and scanned at ×125 magnification using a three-chip charged-coupled device color camera (DXC-960 MD; Sony Electronics, Inc.) adapted to a stereomicroscope (Carl Zeiss, Jen-A-Göttingen, Germany). Digitized images were measured planimetrically after previous calibration of the system with a standard μ-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.

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RESULTS

Specificity of the Enzymatically Mediated Conjugation. Neuraminidases from A. ureafaciens and C. perfringens being able to cleave, respectively, Gal-α-(2-6)-NANA and Gal-α-(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 pI for the anti-CEA-Gal-Dox conjugate (6.2 and 6.5 pI), as compared to the desialylated unconjugated mAb (6.1 and 6.3 pI), 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 PGN-ase 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 i 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 conjugating 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 μg/ml conjugate (data not shown). A 15–20% nonspecific cytotoxicity was observed for both enzymatically and chemically engineered conjugates. This was presumably
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 mg/ml Dox per $2 \times 10^5$ cells, the 24-h intracellular retention upon delivery by the enzymatically engineered conjugate was $\geq 50\%$, whereas cells exposed to the same amount of free drug retained the entire amount of drug. However, cells exposed 24 h to $>1 \mu$g/ml free Dox per $2 \times 10^5$ cells retained only 20–25% of the drug (data not shown). This implies that at 0.2 $\mu$g/ml Dox per $2 \times 10^5$ 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. 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 immunon conjugate.

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.

![Graph showing drug accumulation and retention](image)

**FLUORESCENCE INTENSITY**

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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