Combination Cancer Therapy by Hapten-Targeted Prodrug-Activating Enzymes and Cytokines

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Combination therapy can help overcome limitations in the treatment of heterogeneous tumors. In the current study, we examined whether multiple therapeutic agents could be targeted to anti-dansyl single-chain antibodies (DNS scFv) that were anchored on the plasma membrane of cancer cells. Functional DNS scFv could be stably expressed on CT-26 colon cancer cells both in vitro and in vivo. Dansyl moieties were covalently attached to recombinant β-glucuronidase (βG) and interleukin 2 (IL-2) via a flexible poly(ethylene glycol) linker to form DNS–PEG–βG and DNS–PEG–IL-2 conjugates. The conjugates displayed enzymatic and splenocyte-stimulatory activities, respectively, that were similar to those of the unmodified proteins. The conjugates selectively bound CT-26 cells that expressed anti-DNS scFv (CT-26/DNS cells) but not CT-26 cells that expressed control scFv (CT-26/phOx cells). DNS–PEG–βG preferentially activated a glucuronide prodrug (BHAMG) of p-hydroxy aniline mustard at CT-26/DNS cells in culture and accumulated in subcutaneous CT-26/DNS tumors after intravenous administration. Systemic administration of DNS–PEG–IL-2 or DNS–PEG–βG and BHAMG significantly delayed the growth of CT-26/DNS but not control CT-26/phOx tumors. Combination treatment with DNS–PEG–βG and BHAMG followed by DNS–PEG–IL-2 therapy significantly suppressed the growth of CT-26/DNS tumors as compared to either single-agent regimen. These results show that at least two DNS-modified therapeutic agents can be selectively delivered to DNS scFv receptors in vitro and in vivo, allowing combination therapy of DNS scFv-modified tumors.

INTRODUCTION

Conventional cancer treatment is often limited by unacceptable toxicity to normal tissues and associated side effects (1, 2). Methods to selectively target therapeutic agents to tumor cells can improve treatment efficacy and reduce the exposure of normal tissues to cytotoxic agents. Antibody-directed enzyme prodrug therapy (ADEPT), in which an immunoenzyme is employed to preferentially activate prodrugs at cancer cells, has demonstrated advantages for tumor therapy in animal models including high accumulation of drug in tumors (3), bystander killing of antigen-negative tumor cells (4), and improved efficacy compared with conventional chemotherapy with reduced side effects (5). Our previous results also demonstrated that an immunoenzyme composed of monoclonal antibody RH1 conjugated to β-glucuronidase could target rat AS30D hepato-cellular carcinoma tumors and specifically convert a glucuronide prodrug (BHAMG5) of p-hydroxy aniline mustard to active p-hydroxy aniline mustard (phAM) (6, 7). Rats cured of malignant hepatocellular carcinoma ascites by ADEPT with BHAMG possessed long-lasting protective immunity to subsequent tumor challenges, suggesting that ADEPT can synergize with the immune system to provide increased therapeutic efficacy (6, 7). The immunogenicity of synergistic malignancies can also be increased by targeting cytokines to the tumor microenvironment (8). For example, antibody–IL-2 fusion proteins can effectively suppress tumor growth (9) and amplify the T cell mediated immune responses induced by cancer vaccines (10, 11). Based on these findings, an attractive therapeutic strategy can be envisioned in which the major bulk of tumors is reduced by ADEPT and targeted immunocytokine therapy is then employed to potentiate the immune response generated during prodrug therapy, thereby eliminating residual and disseminated disease.

One approach to target therapeutic agents to tumors is to create artificial anti-hapten receptors on cancer cells that can bind to hapten-derivatized molecules. We previously demonstrated that expression on tumor cells of a membrane-anchored

1 Abbreviations: DNS scFv, anti-dansyl single chain antibody; phOx scFv, anti-4-ethoxymethylene-2-phenyl-2-oxazoline-5-one single chain antibody; CT-26/DNS, CT-26 cells that express membrane DNS scFv; CT-26/phOx, CT-26 cells that express membrane phOx scFv; DNS, dansyl (5-dimethylamino-1-naphthalenesulfonyl); phOx, 4-ethoxymethylene-2-phenyl-2-oxazoline-5-one; PEG, poly(ethylene glycol); DNS–PEG, PEG modified with a dansyl moiety; DNS–PEG–βG, Escherichia coli β-glucuronidase modified with DNS–PEG; DNS–PEG–IL-2, interleukin 2 modified with DNS–PEG; pHAM, p-hydroxyaniline mustard; BHAMG, tetra-n-butylammonium salt of glucuronide p-hydroxyaniline mustard.
scFv with specificity for the hapten 4-ethoxymethylene-2-phenyl-2-oxazoline-5-one (phOx) allowed selective trapping of phOx-derivated βG at the cells for activation of glucuronide prodrugs (12). phOx, however, is unstable in serum, resulting in the progressive loss of phOx antigenicity. In contrast, a bivalent dansyl probe [(DNS)₂-DTPA-111 In] was stable in serum and could be specifically bound by tumor cells that expressed DNS scFv receptors in vitro and in vivo (13). The DNS moiety is therefore more suitable for in vivo hapten-directed targeting.

In the present study, we wished to test the hypothesis that multiple therapeutic agents can be simultaneously targeted to cells expressing anti-hapten receptors. This was examined by expressing DNS scFv receptors on CT-26 colorectal cancer cells and attaching DNS molecules to recombinant βG and IL-2 via flexible poly(ethylene glycol) spacers (Figure 1a). We then tested the hypotheses that (1) DNS-modified molecules could be selectively bound by anti-DNS scFv on cells, (2) accumulation of DNS-modified βG at DNS scFv-positive cells would allow activation of glucuronide prodrugs, (3) DNS-modified IL-2 could stimulate splenocyte proliferation at receptor-positive cells, and (4) treatment of established tumors with hapten-modified βG for prodrug activation in combination with hapten-modified IL-2 could provide improved therapeutic efficacy as compared with either individual treatment regimen. The results of our study show that hapten-directed therapy does allow more than one therapeutic agent to be targeted to tumors for combination cancer therapy.

The syntheses of pHAM and BHAMG have been described (14). Recombinant βG containing a his-tag was purified by metal chelate chromatography on His-Bind resin (Novagen) as described (15). Recombinant mouse IL-2 was produced in Pichia pastoris (kindly provided by Dr. Mi-Hua Tao, Institute of Biomedical Sciences, Academia Sinica, Taipei) and purified by metal chelate chromatography on His-Bind resin (Novagen, San Diego, CA). Dansyl chloride, dansyl cadaverine, fluorescamine, and p-nitrophenyl-β-d-glucuronide were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). PEG₃₄₀₀-disuccinimidyl succinamide (3400 Da) was purchased from Nektar Therapeutics (Birmingham, AL). Dansyl chloride and 4-ethoxymethylene-2-phenyl-2-oxazoline-5-one (phOx) were from Sigma-Aldrich Chemical Co. (St. Louis, MO). Dansyl chloride and 4-ethoxymethylene-2-phenyl-2-oxazoline-5-one (phOx) were linked to fluorescein via a diaminopentane spacer to generate pHAM-FITC and dansyl-FITC as described (12). The anti-poly(ethylene glycol) antibody, E11 (IgG 1 ), which binds to the repeating (O-CH₂-CH₂) subunits of the PEG backbone and can detect and clear PEG-modified proteins, has been described (16).

Cells and Animals. CT-26 colon carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM supplemented with 10% heat-inactivated bovine calf serum, 100 units/mL penicillin and 100 μg/mL streptomycin. BALB/c mice were obtained from the National Laboratory Animal Center, Taipei, Taiwan. Animal experiments were performed in accordance with institute guidelines.

Transfection of Receptor Transgenes. pLNCX-DNS-B7 and pLNCX-phOx-B7 plasmids encode membrane-bound scFv with specificity for DNS and phOx, respectively (12). To produce pseudotyped retrovirus, pLNCX-DNS-B7 and pLNCX-phOx-B7 were cotransfected in GP2-293 cells (Clontech, Mountainview, CA) with pVSVG (Clontech), which expresses the G protein of vesicular stomatitis virus (VSV). RT under the transcriptional control of the cytomegalovirus immediate early gene promoter. Two days after transfection, the culture medium was filtered, mixed with 8 μg/mL Polybrene and added to CT-26 colon carcinoma cells. The cells were selected in G418 and sorted on a flow cytometer to generate CT-26/DNS and CT-26/phOx cells that expressed similar levels of DNS or phOx scFv receptors, respectively.

Figure 1. Combination cancer therapy by hapten-targeted prodrug-activating enzymes and cytokines. (a) Anti-DNS scFv expressed on the surface of tumor cells can bind and trap DNS–PEG–βG on the cells. The glucuronide prodrug BHAMG can then be selectively hydrolyzed to the active alkylating agent pHAM at the tumor cells. Likewise, binding of DNS–PEG–IL-2 to anti-DNS scFv on the cancer cells can also allow accumulation of the cytokine at the tumor site to boost tumor immunity. (b) The immunofluorescence of CT-26 (shaded curve), CT-26/DNS cells (solid line), and CT-26/phOx (dashed line) cells is shown after staining with 1 μM DNS–FITC (left panel) or phOx–FITC (right panel). (c) Established CT-26 (shaded curve), CT-26/DNS (solid line), and CT-26/phOx (dashed line) tumors were excised from BALB/c mice and disaggregated by collagenase treatment. The cells were cultured for 24 h and stained with DNS–FITC (left panel) or phOx–FITC (right panel), and the immunofluorescence of the cells was measured on a flow cytometer.
Flowjo V3.2 (Tree Star, Inc., San Carlos, CA). In vivo expression of scFv receptor was examined by injecting BALB/c mice sc with $2 \times 10^6$ CT-26/DNS or CT-26/phOx cells. After 14 days, tumors (200–300 mm$^3$) were resected, cut into small fragments, and digested with 0.5 mg/mL collagenase in Hanks balanced saline solution containing Ca$^{2+}$ and Mg$^{2+}$ for 1 h at room temperature. The cells were cultured in complete medium for 24 h before the surface expression of scFv receptors was measured in a FACS caliber flow cytometer.

**Generation and Characterization of DNS–PEG–βG and DNS–PEG–IL-2.** PEG$_3400$–disuccinimidyl succinamide (SSA$_2$–PEG) was reacted with dansyl cadaverine at a molar ratio of 2:1 in CH$_2$Cl$_2$ containing 0.1% triethylamine at room temperature for 1 h. The resulting DNS–PEG$_{3400}$–succinimidyl succinamide (DNS–PEG$_{3400}$–SSA) was detected under UV illumination, eluted from silica gel with dichloromethane–methanol (9:1 v/v), and then dried (16). βG and IL-2 were passed through a Sephadex G-25 column equilibrated with 0.1 M NaHCO$_3$, pH 7.5 and then concentrated by ultrafiltration to 1 mg/mL. DNS–PEG$_{3400}$–SSA (2.5 w/w βG, 2 w/w IL-2) was added for 2 h at room temperature. One-tenth volume of a saturated solution of glycine (pH 8.0) was added to stop the reaction. Unreacted PEG was removed by gel filtration on a Sephacryl S-200 HR column. Protein concentrations were determined by the bicinchoninic acid assay (Pierce, Rockford, IL) with bovine S-200 HR column. Protein concentrations were determined by the bicinchoninic acid assay (Pierce, Rockford, IL) with bovine S-200 HR column.

**In Vivo Clearance of DNS–PEG–βG.** Groups of 3 BALB/c mice bearing established CT-26/DNS (right flank) or CT-26/phOx (left flank) tumors were iv injected with 150 μg of βG or DNS–PEG–βG at time zero. Blood samples (30 μL) were removed at 0.17, 1.5, 4, 7, 11, 23 h before iv injection at hour 24 of 150 μg of mAb E11 to accelerate the clearance of DNS–PEG–βG from the circulation. Additional blood samples were taken at subsequent times (3.5, 6, 24 h), and the serum concentration of DNS–PEG–βG was measured by a βG enzyme microassay as described (18). Sample concentrations were calculated by comparison of absorbance values with a standard curve constructed from known concentrations of DNS–PEG–βG or βG.

**Tumor Localization of DNS–PEG–βG.** BALB/c mice bearing established CT-26/DNS (right flank) or CT-26/phOx (left flank) tumors were iv injected with 150 μg of DNS–PEG–βG. After 24 h, mice were iv injected with 150 μg of mAb E11 to accelerate the clearance of DNS–PEG–βG. After 3.5 h, the tumors were excised, embedded in Tissue-Tek OCT in liquid nitrogen, and cut into 10 μm sections. Tumor sections were stained for βG activity with the β-glucuronidase reporter gene staining kit (Sigma). Briefly, the sections were placed in fixation solution (10 mM MES, pH 5.6, with 300 mM mannitol and 0.3% formaldehyde) at room temperature for 45 min, washed three times with wash solution (10 mM sodium phosphate, pH 7.0, with 0.2 mM EDTA), and stained with 5-bromo–4-chloro–3-indolyl–β-d-glucuronicide (X-Gluc) at 37 °C for 3 h. The sections were then stained with nuclear fast red (Sigma) as a counterstain. All sections were examined on an upright microscope (Olympus BX41, Japan).

**Cocktail Therapy.** Groups of 5 BALB/c mice were sc injected in the right flank with 10$^6$ CT-26/DNS or CT-26/phOx cells. After 7 days, the mice were iv injected with PBS or 150 μg of DNS–PEG–βG. Twenty-four hours later, the mice were injected with 150 μg of E11 to accelerate the clearance of DNS–PEG–βG. Mice were iv injected 4 h later with three fractionated doses of 15 mg/kg BHAMG at 1 h intervals. Control groups of tumor-bearing mice were treated with BHAMG (15 mg/kg × 3), or PBS alone. All groups of BHAMG-treated mice were iv injected 24 h later with three fractionated doses of 15 mg/kg BHAMG at 1 h intervals. On day 10, specific groups of mice were ip injected with 20,000 units of DNS–PEG–IL-2. These mice received a total of 5 rounds of DNS–PEG–IL-2 over 10 days. Tumor volumes (length × width × height × 0.5) and body weight were measured twice a week.

**Data Analysis.** Statistical significance of differences between mean values was estimated with Excel (Microsoft, Redmond, WA) using the independent t-test for unequal variances.

**RESULTS**

**Surface Display of Functional Anti-Dansyl scFv Receptors.** The retroviral vectors pLNXX-DNS-eB7 and pLNXX-phOx-
eB7 encode membrane-anchored forms of DNS scFv and phOx scFv receptors in which anti-DNS or anti-phOx scFv is fused to the Ig-like C type domain, the transmembrane, and the cytoplasmic tail of murine CD80 (12). CT-26 colon cancer cells were infected with recombinant retroviral particles, selected in G418, and sorted for high expression of DNS or phOx scFv receptors to obtain stable CT-26/DNS and CT-26/phOx cell lines. The surface expression and antigen-binding activity of the receptors were examined by immunofluorescence staining using DNS–FITC or phOx–FITC probes. Similar levels of DNS and phOx receptors were detected on the cells as compared with cells maintained in culture, demonstrating that functional receptors were stably expressed in vivo (Figure 1c).

**Characterization of DNS and PEG Modified Proteins.** DNS moieties were covalently attached to βG and IL-2 via a flexible poly(ethylene glycol) linker to minimize steric barriers between DNS and the scFv receptors on cells. Addition of PEG to proteins can also prolong their circulation half-life and improve in vivo stability. An average of 3.5 and 2.5 molecules of DNS–PEG3400 were attached to each molecule of βG or IL-2 to form DNS–PEG–βG and DNS–PEG–IL-2, respectively. The electrophoretic mobilities of DNS–PEG–βG and DNS–PEG–IL-2 were slower than those of unmodified βG and IL-2 as determined by SDS–PAGE, demonstrating successful conjugation of DNS–PEG3400 to βG and IL-2 (Figure 2a). The bioactivity of the conjugates was assayed by measuring the hydrolysis of p-nitrophenol-β-d-glucuronide by DNS–PEG–βG or the stimulation of splenocyte proliferation by DNS–PEG–IL-2. Both conjugates displayed activities similar to those of the unmodified proteins (Figure 2b,c). The ability of DNS–PEG–βG and DNS–PEG–IL-2 to bind to DNS scFv receptors was examined by incubating the conjugates with CT-26/DNS and CT-26/phOx cells and then staining the cells with rabbit anti-βG or anti-IL-2 antibodies followed with FITC-conjugated second antibodies. Both DNS–PEG–βG and DNS–PEG–IL-2 bound to CT-26/DNS cells (Figure 2, upper panel) but not to control CT-26/phOx cells (Figure 3, lower panel), demonstrating that DNS–PEG-modified proteins could specifically bind DNS scFv receptors.

**Specific Bioactivity of DNS–PEG–βG and DNS–PEG–IL-2.** To examine whether targeting DNS–PEG–βG to DNS scFv receptors could allow selective activation of the glucuronide prodrug BHAMG to pHAM at cancer cells, CT-26/DNS and CT-26/phOx cells were incubated with DNS–PEG–βG, washed, and then exposed to BHAMG or pHAM. The rate of cellular ATP synthesis after drug treatment was measured as an index of cell viability. The IC50 value of BHAMG to both CT-26/DNS and CT-26/phOx cells was >2000 μM, demonstrating that produg displayed low toxicity to these cells (Figure 4a). CT-26/DNS cells that were preincubated with DNS–PEG–βG were sensitive to BHAMG with an IC50 value of 6.25 μM, similar to the IC50 value observed when the cells were treated with pHAM (IC50 = 5.72 μM), showing that DNS–PEG–βG efficiently converted BHAMG to pHAM at CT-26/DNS cells (Figure 4a, upper panel). By contrast, incubation of CT-26/phOx cells with DNS–PEG–βG before addition of BHAMG did not result in additional cell killing as compared to cells treated with BHAMG alone (Figure 4a, lower panel), demonstrating that produg activation required specific capture of DNS–PEG–βG by DNS scFv receptor-positive cells.

We also examined whether DNS–PEG–IL-2 that was captured on cells by DNS scFv receptors could stimulate splenocyte proliferation. CT-26/DNS and CT-26/phOx cells were incubated with DNS–PEG–IL-2, washed twice with PBS, and then incubated with murine splenocytes. Figure 4b shows that incubation of DNS–PEG–IL-2 with CT-26/DNS cells but not CT-26/phOx cells, induced splenocyte proliferation, demonstrating that DNS–PEG–IL-2 targeted to DNS scFv receptors retained T cell stimulatory activity.

**In Vivo DNS–PEG–βG Clearances and Localization.** We previously showed that the PEG modification of βG prolonged serum half-life, reduced spleen uptake, and enhanced tumor localization of antibody–βG conjugates (15). The long serum half-life of PEG-modified βG, however, prevents administration of produg when maximal tumor accumulation of the immunoconjugate is achieved. Intravenous anti-PEG antibodies can accelerate the clearance of PEG-modified proteins from the circulation and allow earlier administration of produg (15). DNS–PEG–βG displayed an initial serum half-life of 4.4 ± 0.2 h as compared to 0.84 ± 0.03 h for unmodified βG. Intravenous administration of the anti-PEG mAb E11 rapidly reduced the concentration of DNS–PEG–βG in blood by 37-fold (2.6 to 0.07 μg/mL) within 3.5 h after administration (Figure
5a). The localization of DNS–PEG–βG in CT-26/DNS and CT-26/phOx tumors after E11-accelerated clearance was examined by staining tissue sections for βG activity. CT-26/DNS tumor sections clearly displayed βG activity (Figure 5b, right panel) whereas only background activity was observed in CT-26/phOx tumors (Figure 5b, left panel), demonstrating that βG was selectively retained in CT-26/DNS tumors after E11-mediated clearance of DNS–PEG–βG.

In Vivo Antitumor Activity. The antitumor activity of prodruk treatment in combination with DNS–PEG–IL-2 was examined in BALB/c mice bearing 50–100 mm³ CT-26/DNS or CT-26/phOx tumors. Prodrug therapy consisted of an intravenous bolus of DNS–PEG–βG, clearance with mAb E11, and fractionated intravenous administration of BHAMG on days 8 and 9. Starting on day 10, some mice received 20 000 units of DNS–PEG–IL-2 every 2 days over 10 days. Groups of tumor-bearing mice were also treated with DNS–PEG–IL-2 alone, BHAMG, or PBS. Figure 6 shows that targeted prodruk therapy (p ≤ 0.005) or DNS–PEG–IL-2 therapy (p ≤ 0.005) significantly delayed the growth of CT-26/DNS tumors but not control CT-26/phOx tumors as compared to therapy with BHAMG or PBS. Combined treatment with prodruk and DNS–PEG–IL-2 further significantly (p < 0.001) delayed CT-26/DNS tumor growth as compared to each individual treatment and cured one of 5 mice. No significant weight loss was observed in any of the treatment groups (data not shown).

DISCUSSION

This report evaluated simultaneous targeting of hapten-derivatized therapeutic molecules to anti-hapten scFv receptor-positive cells for combination cancer therapy. Our results demonstrate that functionally active DNS–PEG-derivatized βG and IL-2 could specifically bind to DNS scFv receptors on cancer cells to selectively convert the glucuronide prodruk BHAMG to pHAM and stimulate the proliferation of splenocytes, respectively. Combination treatment of established CT-26/DNS scFv receptor-positive tumors by prodruk therapy and DNS–PEG–IL-2 significantly suppressed tumor growth as compared to treatment with prodruk therapy or DNS–PEG–IL-2 alone. Our results therefore show that two therapeutic molecules can be targeted to tumor cells to increase therapeutic efficacy.

Successful targeting of hapten-derivatized molecules requires efficient expression of functional DNS scFv receptors on cells as well as prolonged retention of the hapten-derivatized molecules at the cells. The B7-1 TM and cytosolic tail employed to anchor the DNS scFv receptors on cells allow enhanced surface expression and reduced shedding of chimeric scFv receptors in vitro and in vivo (19, 20). The anti-DNS scFv possesses high affinity (K_D = 1.4 × 10⁻⁸ M⁻¹) for DNS (21), allowing prolonged retention of DNS-modified molecules on
The specificity and stability of the hapten is also critical for the successful localization of hapten-modified molecules to anti-hapten scFv receptors on cells. We previously employed phOx for hapten-directed enzyme−prodrug therapy (HDEPT) but found that phOx is susceptible to hydrolysis in serum, resulting in the progressive loss of phOx antigenicity (12). DNS is a small molecule which has been extensively employed to derivatize proteins. (DNS)2−DTPA−111In was stable in serum at 37 °C for 24 h and specifically bound to B16-F1 cells that expressed DNS scFv receptors in vitro and in vivo (13), indicating DNS-derivatized molecules possess sufficient stability and specificity to allow effective targeting to DNS receptors in vivo. The results of the current study support the notion that DNS is suited for in vitro and in vivo targeting applications.

Effective localization of hapten-modified ligands requires ensuring adequate half-lives of the therapeutic agents in vivo. PEG modification of βG can extend its serum half-life, decrease normal tissue uptake, and increase tumor uptake of antibody−βG−PEG conjugates for targeted activation of glucuronide prodrugs (24). Our present results showed that DNS−PEG-modified βG exhibited more than 5-fold increase in serum half-life as compared with unmodified βG. PEG modification was also critical for retention of enzymatic activity and for attachment of multiple DNS moieties on the proteins. Direct linkage of 3.7 DNS molecules to βG resulted in the loss of 80% of enzyme activity (unpublished results) whereas attachment of 3.5 DNS−PEG molecules to βG allowed retention of over 90% of enzyme activity. The large size of the hydrophilic DNS−PEG3400 molecules may retard interactions with lysines in the active site of βG, resulting in less damage to enzyme activity. Attachment of DNS through a PEG spacer may also allow more conformational flexibility in binding of DNS to the anti-DNS scFv receptors on cells, thereby promoting multivalent interactions between the DNS-modified proteins and the scFv receptors. Multivalent binding is highly desirable. For example, Cortens and colleagues reported that in a two-step approach for radioimmunotargeting of cancers, the tumor uptake of bivalent DTPA−111In was better than that of monovalent DTPA−111In due to increased avidity (25). Similarly, we found increased uptake of a divalent (DNS)2−DTPA−111In probe by anti-DNS scFv receptors on B16/DNS tumors (13). The ability to attach multiple DNS groups on βG and IL-2 with retention of nearly full biological activity is therefore an important advantage of using PEG as a spacer.

The slow clearance of PEG-modified conjugate from the circulation can cause low tumor-to-blood ratios that delay prodrug injection. Antibody−βG−PEG conjugates can be rapidly cleared from the circulation and uptake of conjugate in the liver can be induced by systemic administration of an IgM anti-PEG antibody without affecting the accumulation of the conjugate in tumors (24). In the present study, we found that the clearance of DNS−PEG−βG from the blood could be accelerated by systemic administration of an IgM anti-PEG antibody. High concentrations of DNS−PEG−βG can therefore be maintained in the blood to maximize tumor accumulation until anti-PEG antibody is given shortly before prodrug administration.

PEG-modified IL-2 has been demonstrated to possess a markedly prolonged circulating half-life and increased antitumor potency as compared to unmodified IL-2 (26, 27). For example, Morrison et al. showed that specifically targeting antibody−IL-2 to cancer cells can provide a specific and effective T cell response capable of eliminating tumors (28), and Zimmerman et al. showed that a lower dose and less frequent administration of PEG−IL-2 could provide the same tumor-growth-inhibiting effect compared with IL-2 (29). In our study, DNS−PEG−IL-2 required tumor-targeting to exhibit antitumor activity since CT-
26phOx tumors were completely refractory to DNS–PEG–IL-2 (Figure 6b).

Combination cancer treatment by chemotherapy and immunotherapy is attractive because the treatments have different mechanisms of action and toxicities, possess potential therapeutic synergies, and have general applicability to many cancer types (30). It was not evident a priori, however, that combination therapy with IL-2 and BHAMG would produce additional therapeutic benefits over each individual agent since alkylating agents such as pHAM, the hydrolys product of BHAMG, can induce dose-limiting leukopenia (31), which could in turn antagonize the effects of IL-2 therapy. We previously showed that treatment of rats bearing established malignant hepatocellular carcinoma ascites by ADEPT with BHAMG produced only a limited reduction in white blood cell numbers as compared to treatment with pHAM (6), suggesting that local generation of alkylating agents at the tumor site may spare bone-marrow progenitors and allow initiation of an antitumor immune response against antigens released from dying tumor cells. An important finding of this study was that ADEPT did not cure tumor-bearing rats depleted of CD4+ and CD8+ T cells, demonstrating that T cells provide substantial antitumor activity in ADEPT with BHAMG therapy (7). In addition, Lode, H.N et al. also showed that tumor-targeted IL-2 amplifies T cell mediated immune responses (10). Based on these results, T cells likely play an important role in the combination treatment with DNS–PEG–βG and BHAMG followed by DNS–PEG–IL-2 therapy.

In summary, our results demonstrate that selectively targeted DNS–PEG–βG/BHAMG to reduce the bulk of tumors and subsequent DNS–PEG–IL-2 therapy to potentiate the immune response resulted in improved therapeutic efficacy. A major strength of hapten-directed targeting is the ability to employ multiple agents that possess different mechanisms of actions. Importantly, a single small anti-DNS scFv receptor gene can be employed for gene-expression imaging (13) as well as for delivering multiple therapeutic agents for the combination therapy of tumors.

ACKNOWLEDGMENT

This study was supported by grants from the National Research Program for Genomic Medicine, National Science Council, Taipei, Taiwan (NSC92-2112-B-037-001 and NSC91-2320-B-037-046), and the Genomic and Proteomic Program, Academia Sinica, Taipei, Taiwan (94M007-2).

LITERATURE CITED


BC0600160