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Hapten-directed targeting to single-chain antibody receptors

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Artificial recombinant receptors may be useful for selectively targeting imaging and therapeutic agents to sites of gene expression. To evaluate this approach, we developed transgenes to express highly on cells a single-chain antibody (scFv) against the hapten 4-ethoxymethylene-2-phenyl-2-oxazoline-5-one (phOx). A phOx enzyme conjugate was created by covalently attaching phOx molecules to polyethylene glycol (PEG)-modified β-glucuronidase. Cells expressing phOx scFv but not control scFv receptors were selectively killed after exposure to ß-glucuronidase derivatized with phOx and PEG (phOx-βG-PEG) and a glucuronide prodrug (*p*-hydroxy aniline mustard β-D-glucuronide, HAMG) of *p*-hydroxyaniline mustard. Targeted activation of HAMG produced bystander killing of receptor-negative cells in mixed populations containing as few as 10% phOx-receptor-positive cells. Functional phOx scFv receptors were stably expressed on B16-F1 melanoma tumors *in vivo*. Treatment of mice bearing established phOx-receptor-positive tumors with phOx-βG-PEG and HAMG significantly ($P \leqslant .0005$) suppressed tumor growth as compared with treatment with βG-PEG and HAMG or prodrug alone. phOx was unstable in the serum, suggesting alternative haptens may be more suitable for *in vivo* applications. Our results show that therapeutic agents can be targeted to artificial hapten receptors *in vitro* and *in vivo*. The expression of artificial receptors on target cells may allow preferential delivery of therapeutic or imaging molecules to sites of transgene expression.

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Monoclonal antibodies have been extensively investigated to target drugs, toxins, radionuclides and cytokines selectively to tumor cells to increase the therapeutic index of these agents.[1–3] Monoclonal antibody-based drugs, however, depend on the identification of specific disease-associated surface antigens, require the production and purification of large amounts of protein products and often induce deleterious humoral immune responses that can limit drug efficacy. Although gene-mediated therapeutics also require technical improvements, the advantages and limitations of antibody and genetic drugs largely differ. The development of transgenes that allow selective targeting of therapeutic agents to sites of selective gene expression may therefore complement antibody-based targeting strategies.

One approach to achieving selective targeting is to create artificial receptors with specificity for therapeutic or imaging agents. Artificial receptors can be created by expressing single-chain antibodies (scFv's) on the membrane of cells.[4–8] Thus, by choosing antibodies with the proper specificity, it may be possible to construct receptors that selectively bind and accumulate therapeutic or imaging agents to sites of gene expression. An even more generalized approach is to create receptors that bind a common feature of the agents to be targeted. This can be accomplished by linking a small recognition domain to the targets. 4-ethoxymethylene-2-phenyl-2-oxazoline-5-one (phOx) is a small molecule that has been extensively employed to derivatize proteins for the induction of experimental hypersensitivity. A high-affinity scFv against phOx[9,10] has been shown to localize FITC-labeled phOx molecules in cells.[11] Anti-phOx scFv has also been expressed on cells for magnetic bead selection of transfected cells[12] and for the *in vitro* decoration of lymphocyte stimulatory molecules.[5,13]

In the present study, the anti-phOx scFv was fused to the transmembrane (TM) domain and cytoplasmic tail of murine CD80, which we have previously shown to direct efficiently the expression of heterologous proteins to the cell surface.[14] The utility of surface receptor targeting was investigated by covalently attaching phOx moieties to β-glucuronidase (βG) to create a ligand for specific recognition and binding to cells that display phOx scFv receptors. The present study investigated whether the

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accumulation of phOx-modified βG at receptor-positive cells could allow preferential activation of a glucuronide prodrug (*p*-hydroxy aniline mustard β-D-glucuronide, HAMG) for selective cell killing (Fig 1). In addition, we tested the hypothesis that anti-phOx receptor targeting could provide therapeutic benefits *in vivo* in tumor-bearing mice. Finally, we examined whether phOx displayed suitable stability for *in vivo* applications.

Materials and methods

Reagents

The synthesis of *p*-hydroxyaniline mustard (pHAM) and HAMG have been described.[15] Recombinant *Escherichia coli*-derived βG was produced as described.[16] Succinimidyl succinate poly(ethylene glycol) (PEG) (5 kDa) was purchased from Sigma Chemical Company, St Louis, MO. phOx and dansyl were linked to fluorescein via a diaminopentane spacer to generate phOx-FITC and dansyl-FITC as described.[11] mAb 12CA5 against an epitope of the hemagglutinin (HA) protein of human influenza virus was from Roche, Mannheim, Germany. The construction of p2C11-γ1-B7 and phOx-γ1-B7 have been described.[6,8] A scFv was constructed as described[6] from plasmids encoding the V_k and V_H genes of the 27–44 mouse anti-dansyl hybridoma.[17] The anti-dansyl scFv was fused to the eB7 domain present in p2C11-e-B7,[8] and then inserted into the retroviral vector pLNCX (BD Biosciences, San Diego, CA) to generate pLNCX-dansyl-e-B7.

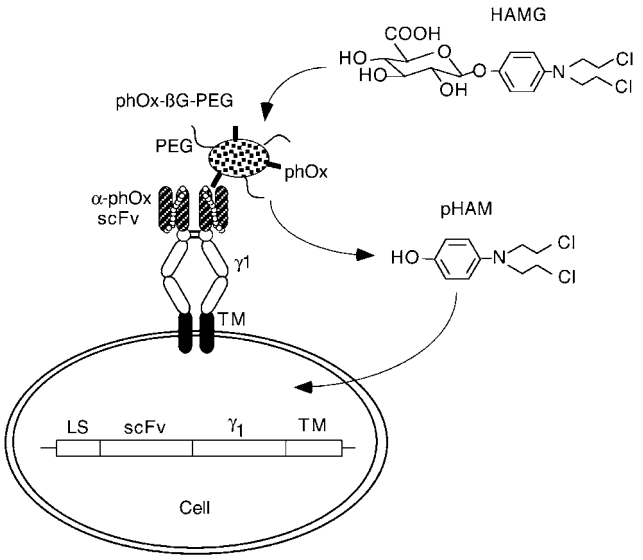

Figure 1 Hapten-directed enzyme prodrug therapy. phOx-βG-PEG can bind to phOx scFv receptors on cells. A nontoxic prodrug (HAMG) can then be enzymatically hydrolyzed to an alkylating agent (pHAM) at the cell surface, resulting in cell death. LS, leader sequence; scFv, single-chain antibody; γ1, hinge-CH_2-CH_3 region of human IgG_1; TM, transmembrane and cytosolic tail of murine CD80.

Cell lines and tissue culture

BALB/3T3 cells, B16-F1 melanoma cells and an anti-myc hybridoma (CRL-1729) were obtained from the American Type Culture Collection, Manassas, VA. mAb AGP3 against PEG was produced as described.[18] Cells were cultured as described.[8]

Transfection of receptor transgenes

BALB/3T3 fibroblasts were transiently transfected with phOx-γ1-B7 or p2C11-γ1-B7 using lipofectamine (Gibco Laboratories, Grand Island, NY). Stable 3T3/phOx and 3T3/2C11 cell lines were derived by the selection of cells in G418 and sorting for high expression. B16-F1 cells were also transfected with pcDNA3 or phOx-γ1-B7, selected in G418 and sorted for high surface expression to generate B16/pcDNA3 and B16/phOx cell lines. pLNCX-dansyl-e-B7 was packaged in Phoenix-Eco cells (generously provided by Dr Gary Nolan, Stanford University, CA). B16-F1 cells were infected with recombinant retroviral particles and selected in G418 to produce B16/dansyl cells.

Flow cytometer analysis

Cells were stained with FITC-conjugated goat anti-human Fc antibody or mouse anti-myc ascites followed by FITC-conjugated goat anti-mouse IgG $(Fab')_2$ fragments. The surface immunofluorescence of viable cells was measured with a FACScaliber flow cytometer (Becton Dickinson, Mountain View, CA) and fluorescence intensities were analyzed with Flowjo V3.2 (Tree Star, Inc., San Carlos, CA).

Immunobead adhesion assay

Transfected cells were incubated at 4°C for 30 minutes with Capture-Tec beads (BD Biosciences). The cells were washed five times with DMEM containing 0.5% bovine serum and stained with methylene blue in 50% methanol before examination under a light microscope.

Generation of ß-glucuronidase derivatized with phOx and PEG (phOx-βG-PEG) conjugates

Recombinant βG in 0.1 M borate buffer, pH 8.0 was reacted with various molar ratios of phOx (phOx/βG: 0, 5, 10, 20, 30, 50) for 2 hours at room temperature. After extensive dialysis against PBS, samples were analyzed for protein concentration by the bicinchoninic acid assay (Pierce, Rockford, IL) with BSA employed as the reference protein. βG activity was measured as described.[16] The number of phOx groups introduced into βG was estimated as described.[19] βG and phOx-βG were modified with succinimidyl succinate PEG at a weight ratio of 2.5:1 in 0.1 M borate buffer, pH 8.0 at room temperature for 2 hours as described.[18] Unreacted PEG was removed by gel filtration. The enzyme activity of phOx-βG-PEG conjugates was assayed by measuring hydrolysis of *p*-nitrophenol β-D-glucuronide as described.[18] The combined binding and enzymatic activities of phOx-βG-PEG conjugates were assayed as described.[16]

Briefly, phOx-βG-PEG conjugates were added to the wells of microtiter plates that were coated with 3T3/phOx cells. The wells were washed and the hydrolysis of *p*-nitrophenol β-D-glucuronide was measured.

Specific activation of HAMG by phOx-βG-PEG

phOx-βG-PEG (1.25 μg/ml) was added for 1 hour to 3T3/phOx and 3T3/2C11 cells in 96-well microtiter plates. The cells were washed twice with PBS and graded concentrations of pHAM or HAMG were added to the cells in triplicate for 24 hours at 37°C. The cells were subsequently washed once, incubated until hour 48 in fresh medium and then pulsed for 12 hours with [^{3}H]thymidine (1 μCi/well) in fresh medium. Results are expressed as the percent inhibition of [^{3}H]thymidine incorporation compared with untreated cells by the following formula:

$$\% \text{ inhibition} = 100\frac{\text{cpm sample} - \text{cpm background}}{\text{cpm control} - \text{cpm background}}$$

In vitro *bystander killing effect*

The βG activity of defined ratios of 3T3/phOx and 3T3/2C11 cells in triplicate wells of a microtiter plate was measured as described.[16] Triplicate wells were also washed with PBS and incubated with phOx-βG-PEG (5 μg/ml) for 1 hour. The cells were washed twice with PBS and then 50 μM HAMG or pHAM was added to the cells for 24 hours. Cells were incubated in fresh medium for an additional 24 hours before the incorporation of [^{3}H]thymidine into cellular DNA was measured as above.

In vivo *expression and activity of scFv chimeric protein*

C57BL/6 mice were s.c. injected in the right flank with B16/phOx or B16/pcDNA3 cells. Resected tumors (200–300 mm^3) were cut into small fragments and digested with 0.5 mg/ml collagenase in Hank's balanced saline solution containing Ca^{2+} and Mg^{2+} for 1 hour at room temperature. The cells were cultured in complete medium for 24 hours before the surface expression of scFv receptors was measured by flow cytometry as described above. Resected tumors were also embedded in OCT compound (Miles Inc., Elkhart, NJ) and snap frozen. Thin sections were fixed in acetone at −20°C for 10 minutes, blocked with 10% bovine serum at room temperature for 2 hours and incubated with Capture-Tec beads as described above.

Therapy of B16/phOx tumors

Groups of eight C57BL/6 mice were s.c. injected in the right flank with 2×10^6 B16/phOx cells. After 10 days, the mice were intravenously (i.v.) injected via the lateral tail vein with PBS, 150 μg phOx-βG-PEG or 150 μg βG-PEG. After 36 hours, mice were i.v. injected with a total of 500 μg AGP3 to accelerate the clearance of phOx-βG-PEG. Mice were i.v. injected 6 hours later with three fractionated doses of 10 mg/kg HAMG at 1 hour intervals. Control groups of tumor-bearing mice were treated with HAMG (10 mg/ml × 3) or PBS alone. All mice received a second round of therapy starting on day 14. Tumor volumes (length × width × height × 0.5) were estimated twice a week.

Stability of phOx-labeled conjugates

phOx-FITC or dansyl-FITC (0.041 mM) was incubated in mouse serum at 37°C. Samples were periodically analyzed by thin-layer chromatography in a solvent system of methanol/dichloromethane (1:1 vol/vol). Samples were also diluted in PBS and incubated with B16/phOx cells for 1 hour on ice. The cells were then washed and analyzed for immunofluorescence on a flow cytometer as described above. phOx-βG-PEG (100 μg/ml) was incubated in mouse serum at 37°C, diluted in PBS to 5 μg/ml and then added to 96-well microtiter plates coated with B16/phOx or B16-F1 cells for 1 hour. The wells were washed and the βG activity retained in the wells was determined as described.[18]

Statistical significance

Statistical significance of differences between mean values was estimated with Excel (Microsoft, Redmond, WA) using the independent *t*-test for unequal variances. *P*-values of less than .05 were considered to be statistically significant.

Results

Surface display of scFv-TM

The plasmid phOx-γ1-B7 encodes a chimeric protein consisting of the murine immunoglobulin κ chain signal peptide, a nine amino-acid HA epitope, an anti-phOx scFv, the hinge-CH_2-CH_3 region of human IgG_1, an 11 amino-acid myc epitope and the TM and complete cytosolic domains of murine CD80. p2C11-γ1-B7 encodes a similar chimeric protein with an anti-CD3 scFv in place of the phOx scFv and myc epitope. BALB/3T3 fibroblasts were transiently transfected with empty pcDNA3 vector, p2C11-γ1-B7 or phOx-γ1-B7, and then surface expression was analyzed by immunofluorescence staining. Figure 2a shows that both 2C11 scFv and phOx scFv were expressed on the surface of fibroblasts as detected by an antibody against the HA epitope present in the chimeric receptors. The functional antigen-binding activity of phOx receptors was examined by staining cells with phOx-FITC, a conjugate formed by linking FITC and phOx with a diaminopentane spacer. Figure 2a shows that cells expressing phOx but not 2C11 scFv receptors bound phOx-FITC, demonstrating that the phOx scFv retained antigen-binding activity. Fibroblasts transfectants were selected in G418 and sorted for high expression of the transgenes to obtain stable 3T3/phOx and 3T3/2C11 cells, respectively. The stable cells expressed similar levels of phOx and 2C11 scFv on their surface as determined by staining with antibody against the γ1 domain (Fig 2b). 3T3/phOx and 3T3/2C11 cells could be distinguished by the presence of an myc epitope in phOx-γ1-B7 (Fig 2b).

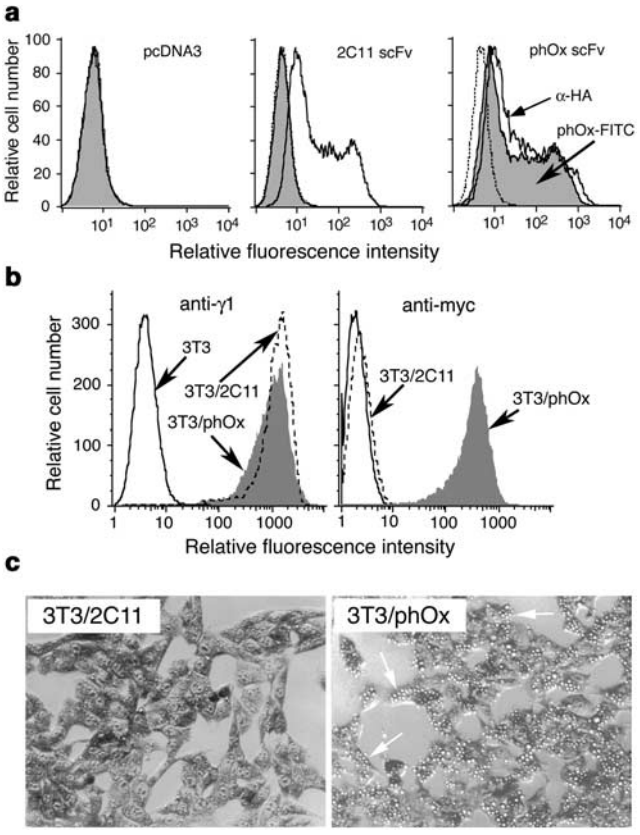

Figure 2 Expression and activity of phOx scFv receptors. (**a**) The immunofluorescence of fibroblasts transfected with pcDNA3, 2C11 scFv or phOx scFv plasmids after staining with control antibody (dotted line), anti-HA antibody (solid line) or phOx-FITC (shaded curve). (**b**) 3T3, 3T3/2C11 and 3T3/phOx cells were probed for scFv surface expression by staining with antibodies against the human γ_1 domain or an myc epitope. (**c**) 3T3/2C11 or 3T3/phOx cells were incubated with Capture-Tec beads. Arrows indicate examples of bound beads.

The antigen-binding activity of the phOx receptors was further confirmed by the ability of 3T3/phOx but not 3T3/2C11 cells to bind Capture-Tec (phOx-coated) beads (Fig 2).

Development of phOx-modified β-glucuronidase

We wished to test whether phOx receptors on cells could specifically bind phOx-modified proteins. Toward this goal, we introduced phOx moieties into β-glucuronidase. Reaction of βG with phOx resulted in the dose-dependent incorporation of phOx molecules with a corresponding decrease in enzyme activity (Table 1). The conjugate containing five phOx molecules per βG monomer (produced by reacting 10 mol phOx per mole βG) displayed the best compromise between phOx incorporation and retention of enzymatic activity as shown by assaying enzymatic activity of the conjugates after allowing them to bind to 3T3/phOx cells (Table 1). This conjugate was employed in subsequent experiments. To improve the pharmacokinetic properties of βG, PEG chains were introduced into βG and phOx-βG as described previously[18] to produce βG-PEG and phOx-βG-PEG.

Table 1 Characterization of phOx-modified βG

phOx/βG molar ratio	Measured phOx groups[a]	Enzyme activity[b] (% maximal activity)	Combined binding and enzyme activity[c] (% maximal activity)
0	0	100	7.8±0.2
5	2.4±0.6	86	78±0.3
10	5.1±0.4	72	100±9.4
20	7.7±0.1	42	55±1.6
30	8.8±0.7	23	25±1.6
50	13.7±1.6	9	9.4±0.3

Recombinant βG was modified with the indicated molar ratios of phOx.
[a]Mean number of phOx groups per βG monomer ± SE.
[b]Sample βG activity/unmodified βG activity × 100.
[c]Conjugates were incubated in wells coated with 3T3/phOx cells, washed and assayed for retained βG activity. Combined activity includes both conjugate binding and substrate hydrolysis.

The ability of phOx receptors to bind phOx-modified proteins was examined by incubating phOx-βG-PEG or βG-PEG with 3T3/phOx or 3T3/2C11 cells, washing the cells and assaying bound βG activity. Figure 3a shows that binding to phOx receptors required incorporation of phOx molecules in the conjugate because phOx-βG-PEG but not βG-PEG bound to 3T3/phOx cells. Neither phOx-βG-PEG nor βG-PEG bound to 3T3/2C11 cells, showing that phOx-βG-PEG binding to phOx receptors required the presence of the phOx scFv and was not the result of binding to other domains present in the chimeric receptors.

We examined whether specific targeting of phOx-βG-PEG to phOx receptors could allow selective hydrolysis of a glucuronide prodrug (HAMG). 3T3/phOx and 3T3/2C11 cells were incubated with phOx-βG-PEG, washed and then exposed to HAMG or pHAM. The rate of cellular DNA synthesis after drug treatment was measured as an index of cell viability. The IC_{50} value of HAMG to both 3T3/phOx and 3T3/2C11 cells was $>1000\,\mu$M, demonstrating that prodrug displayed low toxicity to these cells (Fig 3b). 3T3/phOx cells incubated with phOx-βG-PEG, however, were sensitive to HAMG with an IC_{50} value of $8.5\,\mu$M, similar to the IC_{50} value of pHAM ($3\,\mu$M). This result shows that phOx-βG-PEG on 3T3/phOx cells efficiently converted HAMG to pHAM. In contrast, phOx-βG-PEG did not appreciably convert HAMG to pHAM on 3T3/2C11 cells (Fig 3b), demonstrating that prodrug activation required specific capture of phOx-modified β-glucuronidase on cells.

An advantage of targeting a prodrug-activating enzyme to the surface of cells is the potential for substantial bystander killing of neighboring tumor cells. Bystander killing was examined in cultures containing different

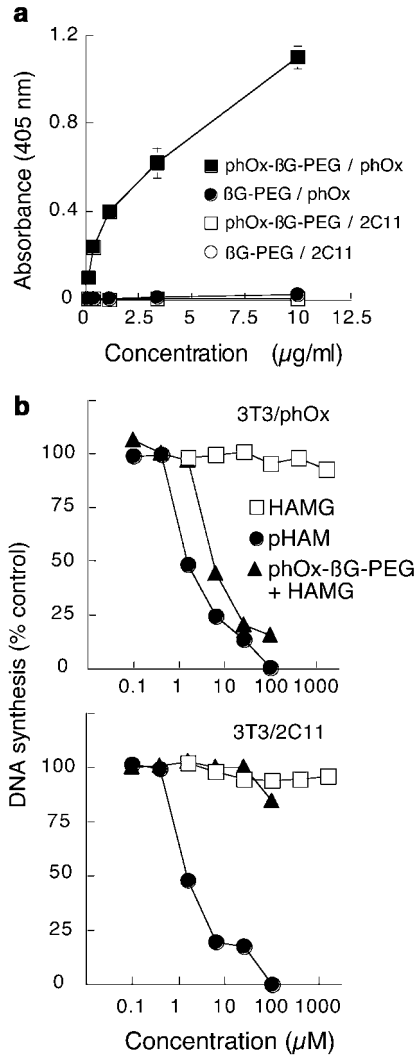

Figure 3 Specificity of phOx receptors. (**a**) Serial dilutions of phOx-βG-PEG (squares) or βG-PEG (circles) were incubated with 3T3/phOx (solid symbols) or 3T3/2C11 (open symbols) cells in triplicate and then assayed for hydrolysis of *p*-nitrophenol β-D-glucuronide. Bars, SE. (**b**) 3T3/phOx or 3T3/2C11 cells were incubated with medium or phOx-βG-PEG, washed and then incubated with graded concentrations of pHAM or HAMG in triplicate before the incorporation of [^{3}H]thymidine into cellular DNA was measured. Bars, SE.

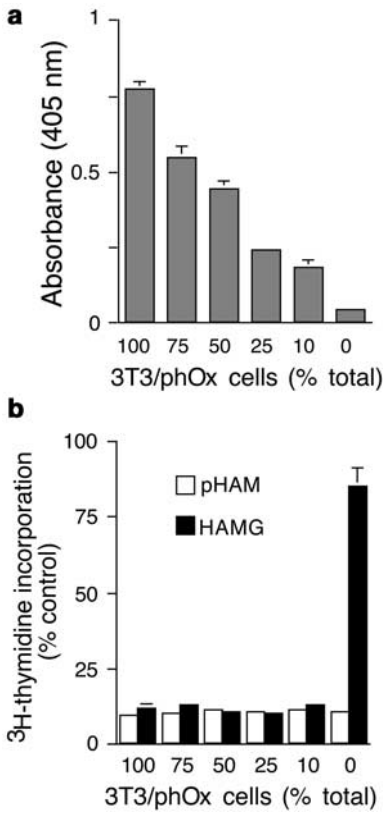

Figure 4 Bystander killing. (**a**) Mixed populations containing defined ratios of 3T3/phOx or 3T3/2C11 cells were incubated with phOx-βG-PEG or medium in triplicate before the hydrolysis of *p*-nitrophenol β-D-glucuronide was measured. Bars, SE. (**b**) Mixtures of 3T3/2C11 and 3T3/phOx cells exposed to HAMG or pHAM in triplicate were assayed for the incorporation of [^{3}H]thymidine into cellular DNA. Bars, SE.

ratios of 3T3/phOx and 3T3/2C11 cells. As expected, the global βG activity of mixed cultured treated with phOx-βG-PEG decreased as the percentage of 3T3/phOx cells was reduced (Fig 4a). Similar killing was observed after the addition of pHAM or sequential treatment of cells with phOx-βG-PEG and HAMG in cell populations containing from 100 to 10% 3T3/phOx cells (Fig 4b). The lack of a dose–response effect indicates that even less than 10% of 3T3/phOx cells may allow complete prodrug activation. This result shows that prodrug activated at cells expressing phOx receptors effectively killed neighboring cells, even when only 10% of the cells were receptor positive.

In vivo *antitumor activity*

To investigate the ability of phOx receptors to function *in vivo*, we generated B16-F1 melanoma cells that stably expressed phOx-γ1-B7 chimeric protein on their surface. B16-F1 cells that were transfected with empty vector were employed as a control because cells that express 2C11 receptors induce T-cell immunity and are rejected.[6,8] Cultured B16/phOx cells expressed high levels of phOx receptors on their surface as detected by indirect immunofluorescence (Fig 5a). phOx receptors were also stably expressed *in vivo* because tumor cells recovered from established B16/phOx tumors displayed high levels of phOx scFv (Fig 5a). phOx receptors on tumors were functional because sections produced from established B16/phOx tumors bound Capture-Tec beads (Fig 5b). Sections from control B16/pcDNA3 tumors, as expected, did not bind Capture-Tec beads.

The functional utility of phOx receptor targeting *in vivo* was examined in mice bearing B16/phOx tumors. Figure 6 shows that the mean tumor size in mice treated with phOx-βG-PEG and HAMG was significantly ($P \leqslant .0005$) smaller than in untreated mice, mice injected with HAMG

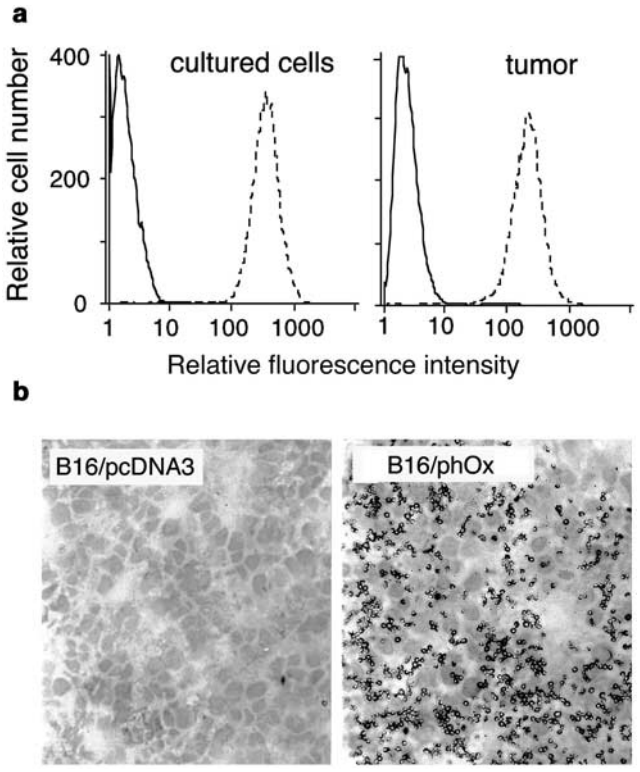

Figure 5 Cell surface display of functional phOx scFv receptors on melanoma tumor cells. (**a**) B16/pcDNA3 (solid line) or B16/phOx (dashed line) cells or cells recovered from tumors were stained with FITC-conjugated goat anti-human Fc antibody. (**b**) Thin sections of B16/pcDNA3 and B16/phOx tumors were incubated with Capture-Tec beads before the sections were counter stained with hematoxylin.

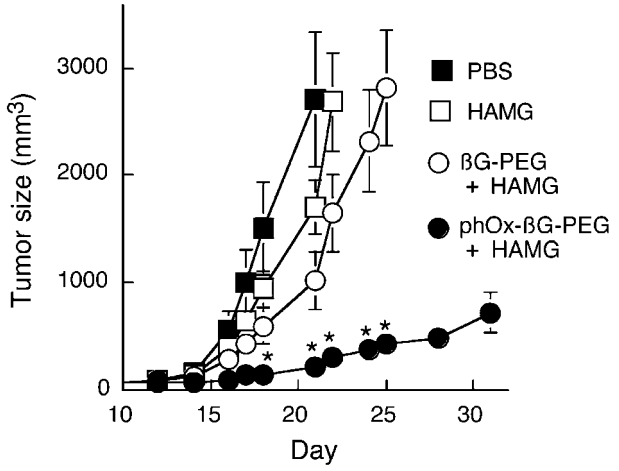

Figure 6 Therapy of B16/phOx tumors. Groups of eight C57BL/6 mice bearing B16/phOx tumor were i.v. injected with PBS (■), BHAMG (SYMBOL 63 \f "MathA" \s 11), βG-PEG and BHAMG (O) or phOx-βG-PEG and HAMG (●) as described in Materials and methods. Significant differences between phOx-βG-PEG and control groups are indicated; *$P \leqslant .0005$. Bars, SE.

alone as well as mice treated with βG-PEG and HAMG. Therapeutic efficacy required the presence of phOx moieties on βG, since tumors in mice treated with βG-PEG and HAMG grew at a similar rate as mice treated only with HAMG.

Stability of phOx

The stability of phOx was evaluated by incubating phOx-FITC and dansyl-FITC in mouse serum for various times and then probing for intact conjugate by staining B16/phOx or B16/dansyl cells for specific fluorescence. Figure 7a shows that incubation of phOx-FITC in the serum for 1 or 5 hours led to decreased fluorescence on

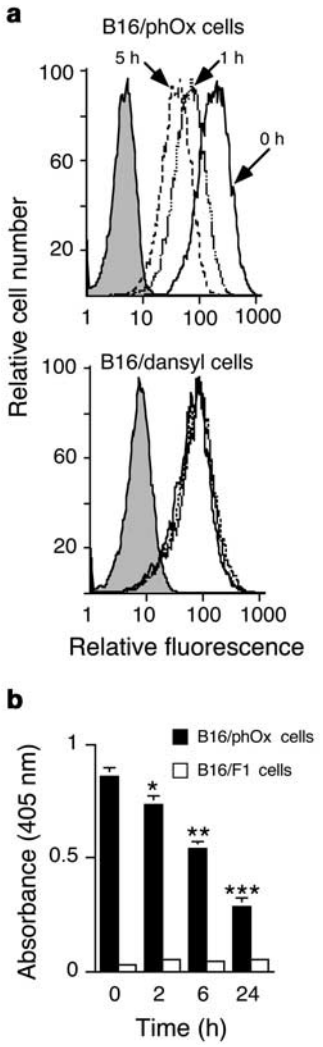

Figure 7 Stability of phOx conjugates. (**a**) phOx-FITC and dansyl-FITC were incubated in mouse serum for the indicated times at 37°C. B16/phOx (upper panel) and B16/dansyl (lower panel) cells were then stained with the conjugates and examined for surface immunofluorescence. B16/phOx cells stained with dansyl-FITC or B16/dansyl cells stained with phOx-FITC are indicated by the shaded curves. (**b**) phOx-βG-PEG was incubated in mouse serum for the indicated times before binding to B16/phOx or B16-F1 cells was assayed by measuring the hydrolysis of *p*-nitrophenol β-D-glucuronide. Significant differences between initial probe binding (0 hour) and later times are indicated: *$P \leqslant .05$; **$P \leqslant .005$; ***$P \leqslant .0005$. Bars, SE.

B16/phOx cells corresponding to 37 and 19% of the maximal fluorescence, respectively. In contrast, dansyl-FITC produced similar staining of B16/dansyl cells regardless of how long the probe was incubated in the serum (Fig 7a, bottom panel). phOx-FITC in PBS resolved as a single spot on thin-layer chromatography, whereas a slowly migrating spot was apparent after incubation of phOx-FITC in the serum (results not shown). In contrast, dansyl-FITC resolved as a unique spot on thin-layer chromatography before and after incubation in the serum (results not shown). The stability of phOx-βG-PEG was examined by incubating the conjugate in the serum and then assaying binding to B16/phOx cells. Figure 7b shows that compared to the original conjugate, binding deceased to 85% after 2 hours, 63% after 6 hours and 34% after 24 hours in the serum.

Discussion

This report evaluated the utility of phOx receptors for targeting hapten-derivatized ligands to cells. βG was converted to a ligand for phOx receptors by covalently attaching phOx moieties to this enzyme. Our results demonstrate that functionally active phOx-derivatized βG was bound by phOx receptors on cells, that localized enzyme was catalytically active, and that it could convert the glucuronide prodrug HAMG to pHAM, resulting in the death of tumor cells. In addition, the activated drug produced potent bystander killing of neighboring tumor cells that did not express phOx receptors. Treatment of established phOx-receptor-positive tumors by i.v. injection of phOx-derivatized βG and prodrug significantly ($P \leqslant .0005$) suppressed tumor growth. These results show for the first time that attachment of phOx moieties to a protein allows *in vivo* targeting to cells that express phOx receptors. This study also demonstrates that hapten-derivatized enzymes can activate prodrugs at tumor cells and provide therapeutic efficacy *in vivo*. phOx, however, appears to be susceptible to hydrolysis in the serum, indicating that more stable haptens should be more suitable for *in vivo* applications.

A major limitation of gene-mediated therapy of diseases is that only a fraction of target cells can be targeted with current delivery systems.[20] It is therefore important that therapeutic gene products are highly effective, even when expressed in a fraction of the target cells. For hapten receptor-based therapeutics, maximal effectiveness can be achieved by: (1) efficiently targeting the receptors to the cell surface, (2) stably retaining receptors on the cell membrane, (3) effectively capturing and retaining the hapten-modified ligand and (4) extending the therapeutic benefits to nontransduced target cells. The hapten receptor described in our study meets several of these criteria.

Efficient targeting of antihapten receptors to the cell surface is important to achieve high levels of receptors on cells. Previous studies have shown that fusion of the phOx scFv to the TM of the human platelet-derived growth factor receptor (PDGFR) or the human interleukin-6 receptor (IL-6R) allowed only low levels of scFv to reach the cell surface.[12,13] whereas fusion to the TM of the human Fc gamma II receptor (FcRII) allowed moderate levels of surface expression.[5] In contrast, we were able to direct high levels of phOx scFv to the plasma membrane of cells (Figs 2b and 5a). Two major differences in our chimeric phOx receptor may account for differences in surface expression levels. First, we included a γ1 spacer between the scFv and TM. We have previously demonstrated that inclusion of spacers between an scFv and TM can increase surface expression by reducing cleavage of the scFv from the cell surface.[6,8] Similarly, inclusion of 44 amino acids of the FcRII extracellular domain[5] probably explains the higher surface expression of that fusion protein as compared to the PDGFR and IL-6R fusion proteins.[12,13] Second, our phOx scFv receptor included the TM and intact cytoplasmic tail of murine CD80. In contrast, the PDGFR, IL-6R and FcRII fusion proteins retained only 5, 9 and 1 amino acids of the cytoplasmic tail of the receptors, respectively. Removal of all but five amino acids from our CD80 fusion protein resulted in the dramatic loss of surface expression (unpublished results), indicating that inclusion of an intact cytoplasmic tail is important for high surface expression of chimeric receptors.

The CD80 TM and cytosolic tail employed to anchor phOx scFv receptors allowed prolonged retention of chimeric proteins on the cell surface.[6] This is particularly important for localization of βG, since glucuronide prodrugs do not readily cross the plasma membrane of cells and require extracellular contact with enzyme for efficient activation.[21] Receptors that can undergo endocytosis, on the other hand, may be useful for targeting therapeutic agents with intracellular sites of action.

Effective localization of hapten-modified ligands requires prolonged retention of the ligands at cells. Construction of receptors with high-affinity antibodies can reduce the release of targeted ligands once they are bound. Prolonged retention also requires that the hapten be stably attached to the ligand. We found, however, that phOx-FITC was unstable in the serum. DNS-FITC, on the other hand, was stable in the serum, ruling out the possibility that alterations of FITC or the linker was responsible for the loss of phOx-FITC staining. phOx-FITC was stable in PBS, suggesting that some component of the serum was responsible for the loss of phOx antigenicity. Thin-layer chromatography analysis of phOx-FITC in the serum revealed the progressive appearance of a single new byproduct, indicating that the loss of antibody binding was due to enzymatic hydrolysis rather than to protein shielding of phOx. We suspect that the oxazoline-5-one ring of phOx ring is opening, and that this may be catalyzed by enzymes present in the serum.[22,23] phOx-βG-PEG also lost binding activity in the serum, but at a slower rate than phOx-FITC. The loss of phOx binding to cells likely reflected hydrolysis of phOx moieties because we have previously shown that βG is stable in the serum.[16] Although βG was derivatized with PEG primarily to enhance tumor

localization,[16,18,24] the PEG chains may also have shielded the phOx groups from enzymatic hydrolysis. In addition, multiple phOx groups were introduced into βG, allowing phOx-βG-PEG binding to phOx receptors as long as some phOx groups remained intact. The instability of phOx in blood, however, argues that alternative haptens may be more desirable for use *in vivo*, particularly for monovalent ligands.

Extension of the effects of therapeutic transgenes from transduced to nontransduced target cells can greatly increase the efficacy of gene-mediated therapies. A major advantage of gene-directed enzyme prodrug therapy with intracellular enzymes such as thymidine kinase or cytosine deaminase is that they do in fact provide bystander killing of nontransduced target cells.[25,26] Nevertheless, ganciclovir and 5-fluorocytosine must diffuse through gap junctions or be released from apoptotic cells to kill bystander cells.[25,27] These relative inefficient processes thus limit the degree of bystander killing that can be achieved. Extracellular activation of prodrugs, in contrast, produces very strong bystander killing,[28,29] since the activated drug can easily diffuse in the interstitial space to nontransduced target cells. In fact, we found that the expression of phOx receptors on as few as 10% of the cells produced similar killing as when all cell expressed phOx receptors, showing that hapten-directed enzymes do generate potent bystander effects.

A major strength of hapten-directed targeting is that multiple agents can be simultaneously targeted to cells expressing antihapten receptors by simply attaching hapten moieties to the ligands. For example, enzymes could be targeted for prodrug activation while cytokines could be employed to potentiate the immune response generated during prodrug therapy.[30,31] Similarly, radioisotopes could be targeted with hapten–chelate complexes to provide synergistic antitumor activity with appropriately selected prodrugs.[32,33] Imaging agents could also be targeted for noninvasive detection of sites of gene expression. These applications will benefit from advances in the fields of transcriptional targeting[34] and targeting vectors.[35,36]

In summary, hapten-directed targeting of agents to sites of receptor transgene expression takes advantage of the high selectivity of antibody–antigen interactions. scFv's can be efficiently expressed on cells by employing an extracellular domain to reduce cleavage and a TM domain with an intact cytoplasmic tail to anchor the receptors on the cell surface. Multiple ligands may be simultaneously targeted to cells with antihapten receptors.[5] It should also be feasible to image transgene expression *in vivo* by synthesizing phOx-chelating complexes containing radioisotopes or magnetic resonance contrast agents.

Abbreviations

HAMG, *p*-hydroxy aniline mustard β-D-glucuronide; pHAM, *p*-hydroxyaniline mustard; phOx, 4-ethoxymethylene-2-phenyl-2-oxazoline-5-one; phOx-ßG-PEG, ß-glucuronidase derivatized with phOx and polyethylene glycol; scFv, single-chain antibody; TM, transmembrane domain; γ1, H-CH_2-CH_3 region of human IgG_1.

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