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The Use of Chelated Radionuclide (Samarium-153-Ethylenediaminetetramethylenephosphonate) to Modulate Phenotype of Tumor Cells and Enhance T Cell–Mediated Killing

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Abstract

Purpose—Exposing human tumor cells to sublethal doses of external beam radiation up-regulates expression of tumor antigen and accessory molecules, rendering tumor cells more susceptible to killing by antigen-specific CTLs. This study explored the possibility that exposure to palliative doses of a radiopharmaceutical agent could alter the phenotype of tumor cells to render them more susceptible to T cell – mediated killing.

Experimental Design—Here, 10 human tumor cell lines (4 prostate, 2 breast, and 4 lung) were exposed to increasing doses of the radiopharmaceutical samarium-153-ethylenediaminetetrame-thylenephosphonate (^{153}Sm-EDTMP) used in cancer patients to treat pain due to bone metastasis. Fluorescence-activated cell sorting analysis and quantitative real-time PCR analysis for expression of five surface molecules and several tumor-associated antigens involved in prostate cancer were done. LNCaP human prostate cancer cells were exposed to^{153}Sm-EDTMP and incubated with tumor-associated antigen-specific CTL in a CTL killing assay to determine whether exposure to ^{153}Sm-EDTMP rendered LNCaP cells more susceptible to T cell – mediated killing.

Results—Tumor cells up-regulated the surface molecules Fas (100% of cell lines up-regulated Fas), carcinoembryonic antigen (90%), mucin-1 (60%), MHC class I (50%), and intercellular adhesion molecule-1 (40%) in response to ^{153}Sm-EDTMP. Quantitative real-time PCR analysis revealed additional up-regulated tumor antigens. Exposure to ^{153}Sm-EDTMP rendered LNCaP cells more susceptible to killing by CTLs specific for prostate-specific antigen, carcinoembryonic antigen, and mucin-1.

Conclusions—Doses of ^{153}Sm-EDTMP equivalent to palliative doses delivered to bone alter the phenotype of tumor cells, suggesting that^{153}Sm-EDTMP may work synergistically with immuno-therapy to increase the susceptibility of tumor cells to CTL killing.

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Disclosure of Potential Conflicts of Interest
Conflict of interest with Cytogen Corp.

Metastasis to bone is a common and severe complication in advanced stages of numerous carcinomas. However, there is currently no standard of care for advanced-stage cancers postchemotherapy. Palliative radiotherapy or surgery is used to treat localized metastasis to bone, and bone-seeking radionuclides such as strontium-89 (^{89}Sr) and samarium-153 (^{153}Sm) provide some relief for patients with painful multifocal bone metastases. Recent preclinical and clinical studies (1–4) have shown that, in addition to its direct cytotoxic effects, external beam radiation (XRT) also modulates numerous classes of genes and up-regulates tumor-associated antigens (TAA), such as Fas, carcinoembryonic antigen (CEA), and mucin-1 (MUC-1), and cell surface molecules involved in antigen presentation and costimulation, such as MHC class I and intercellular adhesion molecule-1 (ICAM-1). Thus, exposing human tumor cell lines to sublethal doses of XRT enhances their susceptibility to killing by tumor antigen–specific CTLs (4, 5).

Recent clinical trials have studied the effectiveness of combining XRT and immunotherapy. A phase II clinical trial using XRT and a recombinant prostate-specific antigen (PSA)–expressing cancer vaccine showed at least a 3-fold increase in PSA-specific T cells over radiotherapy alone ($P < 0.0005$; ref. 6). The purpose of the present study was to determine whether a bone-seeking radionuclide could increase the expression of cell surface molecules and make human tumor cells more susceptible to T cell–mediated killing. One factor indicating that ^{153}Sm would be a better candidate than ^{89}Sr for use in combination with immunotherapy is that at 46 h, the half-life of ^{153}Sm is significantly shorter than the 50.6-day half-life of ^{89}Sr. The shorter half-life of ^{153}Sm would allow for repeated administration and faster recovery from pancytopenia. Safety of repeated ^{153}Sm-ethylenediaminetetramethylenephosphonate (EDTMP) administration has previously been shown in several trials, with the main toxicity being myelosuppression (also seen with ^{89}Sr; refs. 7–13).

^{153}Sm is chelated to EDTMP, forming a complex that binds avidly to hydroxyapatite in bone, especially in areas of high turnover such as metastatic lesions (14). Although palliation is the primary use of ^{153}Sm-EDTMP, two clinical trials suggest a possible survival benefit following ^{153}Sm-EDTMP treatment alone. In a phase I/II trial in hormone-refractory prostate cancer with symptomatic bone metastasis, patients receiving 2.5 mCi/kg ^{153}Sm-EDTMP had a median 9 months survival compared with a median 6 months survival for those receiving 1.0 mCi/kg ^{153}Sm-EDTMP ($P = 0.03$). A greater proportion of patients receiving the higher dose of ^{153}Sm-EDTMP also had decreases in serum PSA and prostatic acid phosphatase (PAP; ref. 10). A randomized dose-controlled trial showed increased survival among breast cancer patients administered with the approved palliative dose of ^{153}Sm-EDTMP (1.0 mCi/kg) compared with those receiving 0.5 mCi/kg (15). These studies on the therapeutic efficacy of ^{153}Sm-EDTMP alone and others exploring the effects in combination with various chemotherapies (16, 17) suggest that ^{153}Sm-EDTMP may be even more effective when used in combination with cancer vaccines.

In the study reported here, a variety of human tumor cells likely to metastasize to bone were exposed to palliative doses of ^{153}Sm-EDTMP to see if such exposure would modulate the phenotype of tumor cells and render them more susceptible to T cell–mediated killing. We treated 10 human tumor cell lines (4 prostate, 2 breast, and 4 lung) with increasing doses of ^{153}Sm-EDTMP and analyzed surface expression of five molecules known to be involved in T cell–mediated tumor cell lysis: Fas, CEA, MUC-1, MHC class I, and ICAM-1. Results showed that all 10 cell lines up-regulated at least two of these molecules. We chose to focus the remainder of our study on the human prostate cancer cell line LNCaP and analyzed changes in TAA and accessory molecule gene expression following treatment with ^{153}Sm-EDTMP. Cell killing assays were done to determine whether exposure to ^{153}Sm-EDTMP would render LNCaP cells more susceptible to antigen-specific CTL-mediated killing.

Overall, this study shows, for the first time, (*a*) the up-regulation of cell surface molecules in prostate, lung, and breast cancer cell lines after exposure to ^{153}Sm-EDTMP; (*b*) the increase in expression of tumor antigen genes and accessory genes after exposure to ^{153}Sm-EDTMP; and (*c*) the use of ^{153}Sm-EDTMP to functionally increase Ag-specific CTL-mediated killing in a prostate cancer cell line. Thus, these findings have important implications on the combined use of ^{153}Sm-EDTMP–mediated radiation and cancer immunotherapy that can benefit bone metastasis patients with metastases from a number of primary tumors with no identified standard treatment and with poor prognosis.

Materials and Methods

Tumor cell lines

Cells of human lung carcinoma (A549, SK-LU-1, NCI-H23, and Calu-1), breast carcinoma (T47D and MCF7), and prostate carcinoma (22Rv1, DU 145, PC-3, and LNCaP) were obtained from the American Type Culture Collection and cultured in medium designated by the American Type Culture Collection for propagation and maintenance. Cells were incubated at 37°C with 5% CO_2.

Samarium-153 dosimetry

^{153}Sm-EDTMP was provided by Cytogen Corporation. ^{153}Sm-EDTMP dosimetry estimates were derived by Dr. Jorge Carrasquillo (Memorial Sloan-Kettering Cancer Center, New York, NY). Doses of ^{153}Sm-EDTMP used in this study were chosen to model the calculated delivered dose to tumor in humans (18–80 Gy; refs. 14, 18). The dose administered to tumor cells (0–100 Gy) was calculated as the total given over the course of the experiment (i.e., 25 Gy × 4 d; Fig. 1).

Tumor irradiation

Human tumor cells were harvested while in log-growth phase and cultured for 24 h in alternate wells of a 24-well plate to prevent irradiation from adjacent wells. PBS was added to empty wells and interwell spaces. Predetermined concentrations of ^{153}Sm-EDTMP were added to the wells and harvested after 96 h for surface molecule analysis by flow cytometry. For XRT, tumor cells in suspension were placed on ice and irradiated (10 Gy) with a cesium-137 source (Gammacell-1000; AECL/Nordion) at a dose rate of 0.74 Gy/min. Nonirradiated control samples were also placed on ice. Both irradiated and nonirradiated cells were then washed in fresh medium and seeded in 75-cm^2 tissue culture flasks. After 96 h, the cells were harvested for surface molecule analysis by flow cytometry.

Flow cytometry analysis

Cell surface staining of tumor cells was done with primary-labeled monoclonal antibodies [CD95-FITC, CD54-PE, CD66-FITC, COL-1-FITC (19), CD227-FITC, and HLA-ABC-PE] and appropriate isotype-matched controls. All antibodies except COL-1 (20) were purchased from BD PharMingen. Stained cells were acquired on a FACScan flow cytometer using CellQuest software (BD PharMingen). Isotype control staining was <5% for all samples analyzed. Dead cells were excluded from the analysis based on scatter profile. Cell viability was tested by Annexin V–FITC staining.

Functional Fas assay

Human tumor cells were nonirradiated (0 Gy) or irradiated (10 and 20 Gy) and recultured, then harvested, and counted after 72 h. Tumor cells (2×10^6) were labeled with 75 μCi ^{111}indium oxine (Amersham Health) for 30 min at 37°C. The cells were washed and subsequently incubated for 18 h with varying concentrations of anti-Fas antibody, clone

CH11 (MBL). Control cells were incubated with IgM isotype control antibody (BD PharMingen), and Jurkat cells were used as a positive control for Fas-mediated cytotoxicity. Functional Fas was determined as previously described (1).

CTL cell lines

The A2-restricted, CEA-specific, $CD8^+$ CTL cell line (designated V8T) recognizes the CEA peptide epitope YLSGANLNL (CAP-1), as previously described (21, 22). A modification of the protocol described by Tsang et al. (23) was used to generate MUC-1-specific CTLs. This CTL line (designated T-VLG-P93L) was generated from a colon carcinoma patient; it recognizes the MUC-1 peptide epitope ALWGQDVTSV. The PSA-specific CTL line used in this study was derived from peripheral blood mononuclear cells of a healthy donor. Briefly, PSA-3A (VLSNDVCAQV; ref. 19) peptide-pulsed irradiated (30 Gy) dendritic cells were used as antigen-presenting cells for *in vitro* stimulation of autologous T cells at an effector to target ratio of 10:1. Cultures were maintained for 3 d in a medium containing 10% human AB serum, and 4 additional days in the same medium supplemented with 20 units/mL of recombinant human interleukin 2. After a 7-d *in vitro* stimulation cycle, cells were restimulated as described above. After three *in vitro* stimulation cycles, $CD8^+$ T cells were negatively isolated and used in the CTL assay, as described below.

Cytotoxicity assays

V8T (CEA CTL), T-VLG-P93L (MUC-1 CTL), and PSA-3A (PSA CTL) cells were used on day 4 of the restimulation cycle following Ficoll purification. Irradiated (25 and 50 Gy) and nonirradiated (0 Gy) LNCaP cells were cultured for 72 h, and then used as targets in a standard cytotoxicity assay. Initially, tumor cells were labeled using ^{111}indium oxine for 30 min at 37°C. A total of 2×10^3 radiolabeled tumor cells were then incubated with 6×10^4 CTLs (effector to target ration, 30:1) for 18 h at 37°C with 5% CO_2. Targets and CTLs were suspended in a complete medium supplemented with 10% human AB serum in 96-well U-bottomed plates (Costar). After incubation, supernatants were collected. The percentage of specific lysis was determined by the standard equation % specific lysis = [(experimental − spontaneous)/(maximum − spontaneous)] × 100.

RNA isolation

LNCaP cells were irradiated (25 and 50 Gy) or nonirradiated (0 Gy). After 96 h, cells were harvested from 24-well plates and washed twice, and total RNA was extracted and purified using the RNeasy midi kit (Qiagen, Inc.) according to the manufacturer's instructions. Real-time PCR was done as described previously (24).

Statistical analysis

Statistical differences were evaluated using ANOVA; repeat measurements were done using Statview 4.1 (Abacus Concepts, Inc.). Significant differences in flow cytometry data were determined using the Kolmogorov-Smirnov test.

Results

Phenotypic modulation of tumor cell membrane proteins following treatment with ^{153}Sm-EDTMP

It has been previously shown that sublethal irradiation of human tumor cell lines resulted in the up-regulation of a number of cell surface molecules, including Fas and MHC class I (1, 2, 4). To determine whether exposure to ^{153}Sm-EDTMP had this same effect, 10 human tumor cell lines (4 prostate, 2 breast, and 4 lung) were exposed to 0 or 25 Gy ^{153}Sm-EDTMP for 4 days and analyzed by flow cytometry for up-regulation of Fas, MHC class I,

ICAM-1, and the TAAs CEA and MUC-1. Up-regulation was defined as a ≥2-fold increase in cell surface expression. As shown in Fig. 1, 100% of the cell lines tested up-regulated Fas after exposure to ^{153}Sm-EDTMP, 50% up-regulated MHC class I, 40% up-regulated ICAM-1, 90% up-regulated CEA, and 60% up-regulated MUC-1. Fas functionality was tested in all cell lines by an antibody cross-linking assay (*hatched boxes,* functional Fas; *gray boxes,* nonfunctional Fas). These data show that exposure of tumor cells to ^{153}Sm-EDTMP results in the up-regulation of a variety of surface molecules that could potentially render these tumor cells more susceptible to CTL killing. Because ^{153}Sm-EDTMP is most commonly used to treat pain in prostate cancer metastatic to bone, further analysis focused on the prostate tumor cell lines LNCaP and PC-3.

Exposure of prostate cancer cell lines to ^{153}Sm-EDTMP leads to up-regulation of MHC class I and Fas

To evaluate the effects of ^{153}Sm-EDTMP exposure on prostate cancer cell lines, LNCaP and PC-3 cells were exposed to 0 or 25 Gy over the course of 96 h. Cells were then harvested and cell surface expression of MHC class I and Fas was measured by flow cytometry. As shown in Fig. 2, in LNCaP cells, the mean fluorescence intensity of MHC class I expression increased from 72 to 178 after exposure to 25 Gy ^{153}Sm-EDTMP, with no significant change in the percentage of cells positive for MHC class I. The mean fluorescence intensity of Fas expression increased from 37 to 84, with an increase in the percentage of positive cells from 9% with 0 Gy to 32% with 25 Gy ^{153}Sm-EDTMP. In PC-3 cells, the percentage of cells positive for MHC class I did not increase, but the mean fluorescence intensity increased from 180 to 447 after exposure to ^{153}Sm-EDTMP. The mean fluorescence intensity for Fas expression in PC-3 cells increased from 18 to 83 after exposure to ^{153}Sm-EDTMP, whereas the percentage of positive cells increased from 6.9% with 0 Gy to 12% with 25 Gy ^{153}Sm-EDTMP. Together, the data in Figs. 1 and 2 show that exposure of human prostate cancer cell lines to ^{153}Sm-EDTMP results in up-regulation of MHC-I and Fas.

Comparison of effects of exposure to XRT and ^{153}Sm-EDTMP on cell surface expression

Based on previous results showing an increase in cell surface molecule expression after exposure to XRT (1, 2, 4) and ^{153}Sm-EDTMP (Figs. 1 and 2), we compared the effects of ^{153}Sm-EDTMP exposure and XRT exposure on cell surface expression (Fig. 3). LNCaP cells were exposed to 0 or 10 Gy XRT (*left*) or increasing doses (0–100 Gy) of ^{153}Sm-EDTMP (*right*). As shown in Fig. 3A, after exposing LNCaP cells to 10 Gy XRT, the percentage of cells expressing Fas increased from 5% (0 Gy) to 21% (10 Gy). With ^{153}Sm-EDTMP, the percentage of cells expressing Fas increased after exposure to 12.5 Gy and peaked after exposure to 25 Gy—the only point at which the percentage of cells expressing Fas was higher with ^{153}Sm-EDTMP than with XRT. MHC class I expression was largely unchanged after ^{153}Sm-EDTMP exposure (Fig. 3B). Interestingly, although there was a minimal increase in ICAM-1 and CEA following XRT, there was a dose-dependent increase in the percentage of cells expressing both of these molecules after exposure to ^{153}Sm-EDTMP, with peak expression seen at ~25 Gy (Fig. 3C and D). The percentage of cells expressing MUC-I did not change after exposure to XRT (Fig. 3E) but decreased upon exposure to ^{153}Sm-EDTMP (except at 50 and 100 Gy). The percentage of cells expressing each of the surface molecules tested, except for MUC-1, peaked at 25 Gy ^{153}Sm-EDTMP. As a result, this dose was used in all subsequent experiments. The data in Fig. 3 show that the percentage of cells expressing Fas, CEA, and ICAM-1 increased after exposure to ^{153}Sm-EDTMP, and that for all molecules except MUC-1, peak expression (at 25 Gy) was greater than or equal to that seen with XRT. Treatment of prostate cancer cell line LNCaP with ^{153}Sm-EDTMP (25 and 50 Gy) was sublethal over 5 days as determined by

serial live cell counts (Fig. 4A). ^{153}Sm-EDTMP induces some degree of apoptosis; 24% of the cells undergo apoptosis by day 4 as determined by Annexin V staining (Fig. 4B).

Effect of ^{153}Sm-EDTMP exposure on gene expression in LNCaP cells

To confirm previous results and examine the effect of ^{153}Sm-EDTMP exposure on other potential accessory molecules and TAAs, we exposed LNCaP cells to 0, 25, or 50 Gy ^{153}Sm-EDTMP and analyzed them at the mRNA level 48 h postirradiation. After the RNA was extracted, quantitative real-time PCR was done on a variety of genes and normalized against the housekeeping gene *GAPDH*. As shown in Table 1, after exposure to 50 Gy ^{153}Sm-EDTMP, Fas, ICAM-1, and CEA were up-regulated 2-, 34-, and 13-fold respectively, which correlated with up-regulated surface expression at 96 h. We also examined other prostate TAAs. PSA levels increased 2.79-fold after exposure to 25 and 50 Gy ^{153}Sm-EDTMP. Similarly, prostate-specific membrane antigen (PSMA) gene expression increased 4.17-and 4.62-fold after 25 and 50 Gy ^{153}Sm-EDTMP exposure, respectively. PAP showed the highest up-regulation, with a 29-fold increase after 25 Gy, and a 36.2-fold increase after 50 Gy ^{153}Sm-EDTMP exposure. Expression of MUC-1 increased 3.67-fold after exposure to 25 Gy ^{153}Sm-EDTMP and 2.89-fold after exposure to 50 Gy ^{153}Sm-EDTMP. Taken together, these data show that exposure of human tumor cells to ^{153}Sm-EDTMP results in the up-regulation of a broad range of TAAs and other accessory genes. This experiment has been repeated thrice with similar results.

Exposure of LNCaP cells to palliative doses of ^{153}Sm-EDTMP renders them more sensitive to CTL killing

To determine whether exposure to ^{153}Sm-EDTMP would make tumor cells more susceptible to CTL killing, LNCaP cells were exposed to 0, 25, or 50 Gy ^{153}Sm-EDTMP. The cells were harvested 72 h later and made to undergo an antigen-specific CTL assay. CTLs specific for PSA, MUC-1, and CEA were incubated with ^{111}In-labeled LNCaP cells at an effector to target ratio of 30:1 (Fig. 5). After 18 h, the supernatant was harvested and specific lysis was calculated. Exposure of LNCaP cells to 25 Gy ^{153}Sm-EDTMP increased PSA-specific cell killing to 10%, a significant increase over that seen with 0 Gy (<5%; $P < 0.001$). A much greater increase in killing was seen after 50 Gy, where lysis increased to 80% ($P < 0.001$; Fig. 5A). There was a slight but statistically insignificant increase in MUC-1–specific killing after exposure to 25 Gy ^{153}Sm-EDTMP (Fig. 5B). However, exposure to 50 Gy ^{153}Sm-EDTMP increased cell killing to 28%, a statistically significant increase over 0 Gy ^{153}Sm-EDTMP (13%; $P < 0.001$). Interestingly, CEA-specific cell killing showed a significant increase after exposure to 25 Gy ^{153}Sm-EDTMP (38%) compared with 0 Gy ^{153}Sm-EDTMP (18%; $P < 0.001$). There was also a significant increase after exposure to 50 Gy ^{153}Sm-EDTMP ($P < 0.001$ versus mock-treated cells). There was no significant difference in killing between 25 and 50 Gy ^{153}Sm-EDTMP (Fig. 5C). These data show that exposure of cells to ^{153}Sm-EDTMP enhances antigen-specific CTL-mediated killing of LNCaP cells and that this effect can be extended to a variety of TAAs.

Discussion

Conventional cytotoxic therapies, such as radiation and chemotherapy, have historically been viewed as immunosuppressive. However, advances in the understanding of the development of antitumor immunity, as well as improved knowledge of the effects of radiation on tissues and the ability to deliver radiation selectively to areas of metastatic disease through the use of bone-targeted radionuclides, have revived interest in the possibility of combining radiation and immune-based therapies to achieve better local and systemic tumor control.

In this study, we have investigated the phenotypic and functional consequences of radiation mediated by ^{153}Sm-EDTMP on 10 human tumor cell lines (4 prostate, 2 breast, and 4 lung). The cell lines chosen were representative of classes of tumors commonly associated with metastasis to the bone: prostate, lung, and breast. In each class of tumors examined, we analyzed tumor cell lines that were derived from both primary lesions as well as from metastases. These tumor cell lines were heterogeneous in terms of their differentiation state and immunogenicity in xenograft models (25–28). Each of the five surface molecules monitored in this study (CEA, MUC-1, Fas, ICAM-1, and MHC class I) has been implicated in increased antitumor T-cell responses through diverse mechanisms. Fas is expressed on tumors, and it mediates direct killing of tumor cells by CTLs expressing Fas ligand (29). ICAM-1 is an adhesion molecule that is thought to enhance T cell–mediated killing as a result of increased T-cell binding (30, 31) and has also been found to directly costimulate activated T cells (32). CEA and MUC-1 are TAAs that are more highly expressed in tumor cells as opposed to normal cells (33). Cell surface expression of MHC class I is important for the presentation of tumor antigens such as these to CTL (34–36). It has been shown that tumors commonly down-regulate these molecules to escape immune recognition and elimination (37, 38). As a result, the up-regulation of these molecules by radiation could make tumor cells more susceptible to immune-mediated attack. This has been shown to be the case for external beam radiation (1, 2, 4), and in this study we hypothesized that a similar trend would be seen with the use of ^{153}Sm-EDTMP.

Data presented in this study clearly show that exposure to ^{153}Sm-EDTMP can induce phenotypic changes in tumor cells, and render these tumor cells more susceptible to T cell–mediated killing. In the first phase of our study, exposure to ^{153}Sm-EDTMP altered the phenotype of 10 human tumor cell lines, up-regulating at least two of the five surface molecules (Fas, CEA, MUC-1, MHC class I, and ICAM-1) on each cell line tested (Figs. 1 and 2). Among the 10 cell lines examined, the expression pattern of the tumor antigens CEA and MUC-1 seemed to be heterogeneous. After exposure to ^{153}Sm-EDTMP, however, 9 of the 10 cell lines up-regulated CEA, and 6 of the 10 cell lines up-regulated MUC-1.

We examined changes in the surface markers Fas, MHC-I, ICAM-1, CEA, and MUC-1 following exposure to ^{153}Sm-EDTMP because these proteins have been associated with increased sensitivity to T cell–mediated killing. It should be noted, however, that there was no consistent phenotypic pattern following ^{153}Sm-EDTMP exposure that correlated with increased sensitivity to CTL killing (Figs. 1 and 5). For example, the expression of the death receptor Fas increased in four of four prostate cancer cell lines following exposure to ^{153}Sm-EDTMP, although in three of those lines, the Fas signaling pathway was found to be defective (Fig. 1). Despite this, CTL killing of LNCaP tumor cells was significantly increased following ^{153}Sm-EDTMP treatments. In addition to phenotypic modulation, exposure of tumor cells to sublethal radiation has been reported to enhance degradation of existing proteins, which resulted in an increased intracellular peptide pool. Enhanced translation due to activation of the mammalian target of rapamycin pathway resulted in increased peptide production, antigen presentation, as well as CTL recognition of irradiated cells. In addition, novel proteins were made in response to irradiation, resulting in new peptides presented by MHC class I molecules, which were recognized by cytotoxic T cells (39). The mechanisms of radiation-mediated tumor cell sensitivity to CTL killing are complex, and perhaps different, among different tumor classes, and future studies will examine these mechanisms in more detail.

As previous studies had been conducted with XRT, it was important to compare changes in cell phenotype seen with XRT and ^{153}Sm-EDTMP. After exposure to 25 Gy ^{153}Sm-EDTMP, peak expression of all molecules except MUC-1 was equal to or greater than the expression induced by XRT (Fig. 3). Although XRT is effective in treating isolated bone

metastases because of its focused nature, it would not be an effective therapy for use in patients with widespread bone involvement. The bone-seeking nature of ^{153}Sm-EDTMP makes it a much more effective therapy for patients with widespread bone metastasis, and these data confirm the ability of ^{153}Sm-EDTMP to increase the expression of cell surface molecules at least as much as XRT, and support the hypothesis that ^{153}Sm-EDTMP would be effective in combination with immunotherapy. They also support the rationale for combining ^{153}Sm-EDTMP with other agents (e.g., chemotherapies and proteosome inhibitors), which are currently under active clinical investigation (16, 40).

It should be noted that treatment of prostate cancer cell line LNCaP with ^{153}Sm-EDTMP (25 and 50 Gy) was sublethal over 5 days as determined by serial live cell counts (Fig. 4A). ^{153}Sm-EDTMP induces some degree of apoptosis; 24% of the cells undergo apoptosis by day 4 as determined by Annexin V staining (Fig. 4B). These doses of ^{153}Sm-EDTMP are clinically relevant. During the clinical use of ^{153}Sm-EDTMP, the dose to normal bone is 0.25 Gy/mCi and the mean uptake in metastatic bone sites of prostate cancer is four times the normal bone; thus, to a 70-kg man receiving 70 mCi, an estimated dose of 70 Gy would be delivered to the involved bone; moreover, because the tumor is in close contact, the tumor would also receive large doses.

It is important to note that although Fas is up-regulated in LNCaP cells (Figs. 1–3), an antibody cross-linking assay determined that Fas is not functional in these cells. Previously, it has been reported that the up-regulation of Fas after irradiation sensitized tumor cells to killing by antigen-specific CTL (1). However, a subsequent study found that sensitivity to CTL killing was not always due to Fas expression, because human tumor cell lines that expressed nonfunctional Fas after irradiation exhibited enhanced killing by CTL (4). Our results agree with the latter finding because the LNCaP cells expressing nonfunctional Fas were killed efficiently (up to 90% lysis) by CTL specific for three different TAAs (PSA, CEA, and MUC-1) after exposure to ^{153}Sm-EDTMP (Fig. 5). Perhaps, in this case, enhanced killing by CTL was due to the up-regulation of the adhesion molecule ICAM-1 or of the TAAs.

This study also examined the effect of ^{153}Sm-EDTMP exposure on three proteins expressed in prostate cancer and recently identified as targets for immunotherapy (41)—PSA, PSMA, and PAP. As in our previous findings on the up-regulation of TAAs (Fig. 1), we showed an increase in gene expression for all three proteins after exposure to 25 and 50 Gy ^{153}Sm-EDTMP (Table 1). In the clinical trial reported by Gulley et al. (6) that examined radiation therapy plus a PSA-expressing vaccine, T-cell responses to PSMA, PAP, and MUC-1 were at least doubled after vaccination, although none of them was present in the vaccine, possibly as a result of antigen cascade (42–44). Gulley et al. hypothesized that PSA-specific T cells generated by the vaccine killed prostate cancer cells that then exposed other prostate-associated antigens during processing and presentation, eliciting CTLs specific for PSMA, PAP, and MUC-1. The up-regulation of PSMA, PAP, and MUC-1 by ^{153}Sm-EDTMP exposure suggests not only that an antigen cascade could produce T cells specifically targeted against these proteins, but that an immune response could be induced by combining ^{153}Sm-EDTMP irradiation with a vaccine expressing one or more of these molecules.

This is the first reported use of ^{153}Sm-EDTMP to functionally enhance the killing of human prostate cancer cells by antigen-specific CTL. We observed enhanced Ag-specific killing of the LNCaP cell line by three different HLA-A2–restricted CTL lines specific for PSA, CEA, and MUC-1 after ^{153}Sm-EDTMP exposure compared with nonirradiated cells (Fig. 5). In addition, we showed significantly enhanced CTL killing of particular surface antigens, whether or not their expression was enhanced by exposure to ^{153}Sm-EDTMP. For example,

CTL-mediated killing by MUC-1 – specific T cells increased, although surface expression of MUC-1 on LNCaP cells did not increase after ^{153}Sm-EDTMP exposure (Fig. 1). The mechanism of action is unclear but it may be a result of increased ICAM-1 expression, which enhances adhesion with the T cells. It may also be a result of enhanced MHC loading and peptide presentation because it has been shown that exposing cells to radiation increases production of peptides, as well as cell surface expression of MHC class I (39). Several studies have shown that both CEA and MUC-1 are expressed by prostate cancer cells (4, 6, 45–50), including the clinical trial described above that reported the generation of MUC-1–specific CTLs in prostate cancer patients after vaccination with a PSA-expressing vaccine (4, 6). Our data (Fig. 5) confirm and extend these findings.

It was observed that before treatment with ^{153}Sm-EDTMP, LNCaP cells expressed very low levels of CEA (2%; Fig. 3D); yet, these cells were lysed by CEA-specific CTLs (18%). This apparent difference can be explained by the differences in antibody detection of cell surface CEA by flow cytometry and T-cell recognition of a defined CEA epitope within the context of MHC-I HLA-A2. Cells, including tumor cells, present endogenously expressed proteins on their surface in the form of peptide MHC. In a previous study, Kass et al. (51) showed that many tumor cell lines or tumor samples express CEA intracellularly; yet, they can still be targets for CEA-specific CTL. Another example of this discordance is noted with the tumor antigen PSA. Because PSA is expressed essentially only in prostatic epithelial cells (normal and malignant) and the prostate gland is nonessential, this antigen is an enticing choice. Furthermore, more than 70% of tumor cells taken from bone metastasis in advanced prostate cancer patients express PSA with only 14% of patients having <50% of tumor cells from bone metastasis expressing PSA (52, 53). The fact that PSA is secreted and not membrane bound limits its use as a target for humoral immunity, but not its use as a target of specific cellular immune system attack. CTLs recognize and are activated by specific peptides in the context of the appropriate MHC class I molecule on antigen-presenting cells. This activation can, in turn, lead to killing of tumor targeted by the peptide-specific CTLs.

This study was the first to show that exposure to ^{153}Sm-EDTMP up-regulates cell surface molecules in prostate, lung, and breast cancer cell lines; increases expression of tumor antigen genes and accessory genes; and functionally increases antigen-specific CTL-mediated killing in a prostate cancer cell line. These findings have important implications for the combined use of ^{153}Sm-EDTMP and immunotherapy in patients with advanced cancer for whom there is no standard treatment.

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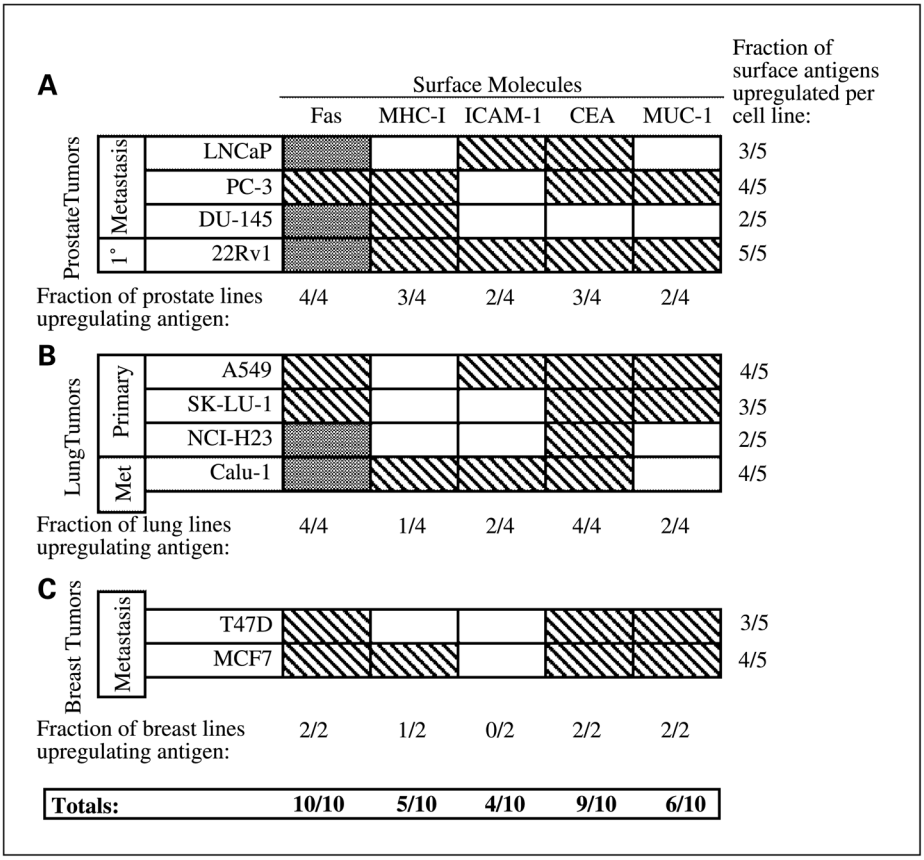

Fig. 1.

Effect of treatment with ^{153}Sm-EDTMP on surface molecule expression in 10 human tumor cell lines. Human prostate (*A*), lung (*B*), and breast (*C*) tumor cell lines were exposed to 0 or 25 Gy ^{153}Sm-EDTMP for 4 d. Cell surface expression of Fas, CEA, MUC-1, MHC class I, and ICAM-1was measured by flow cytometry. Hatch marks, increase in surface expression ≥2-fold; white, no change; gray, increased postirradiation surface expression of Fas, shown to be nonfunctional by antibody cross-linking assay. Met, metastasis; 1°, primary.

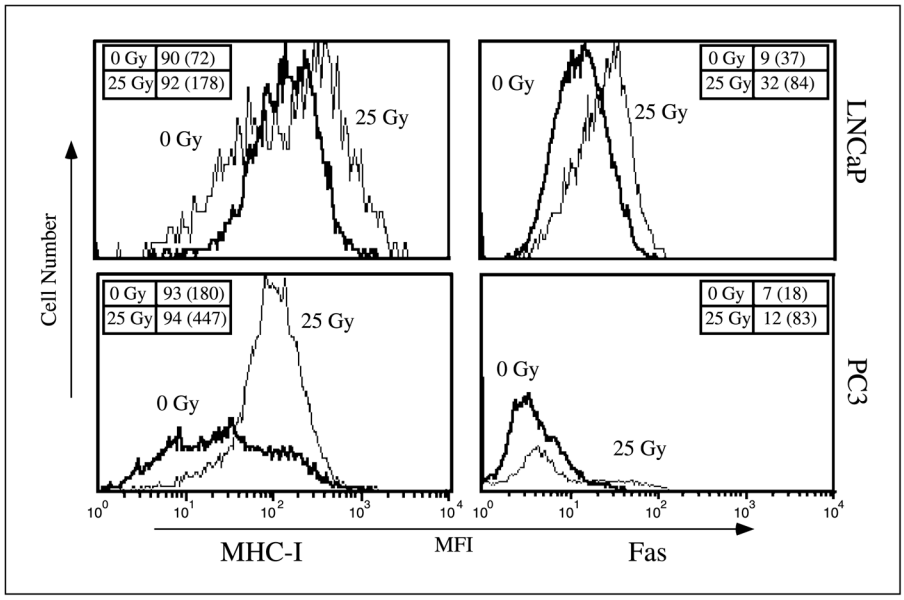

Fig. 2.

Up-regulation of MHC class I and Fas on two prostate cancer cell lines after exposure to ^{153}Sm-EDTMP. LNCaP (*top*) and PC-3 (*bottom*) cell lines were exposed to 0 Gy (*heavy line*) or 25 Gy ^{153}Sm-EDTMP (*thin line*) for 4 d. Cell surface expression of MHC class I (*left*) and Fas (*right*) was measured by flow cytometry. Inset numbers: % positive cells (mean fluorescence intensity (MFI)).

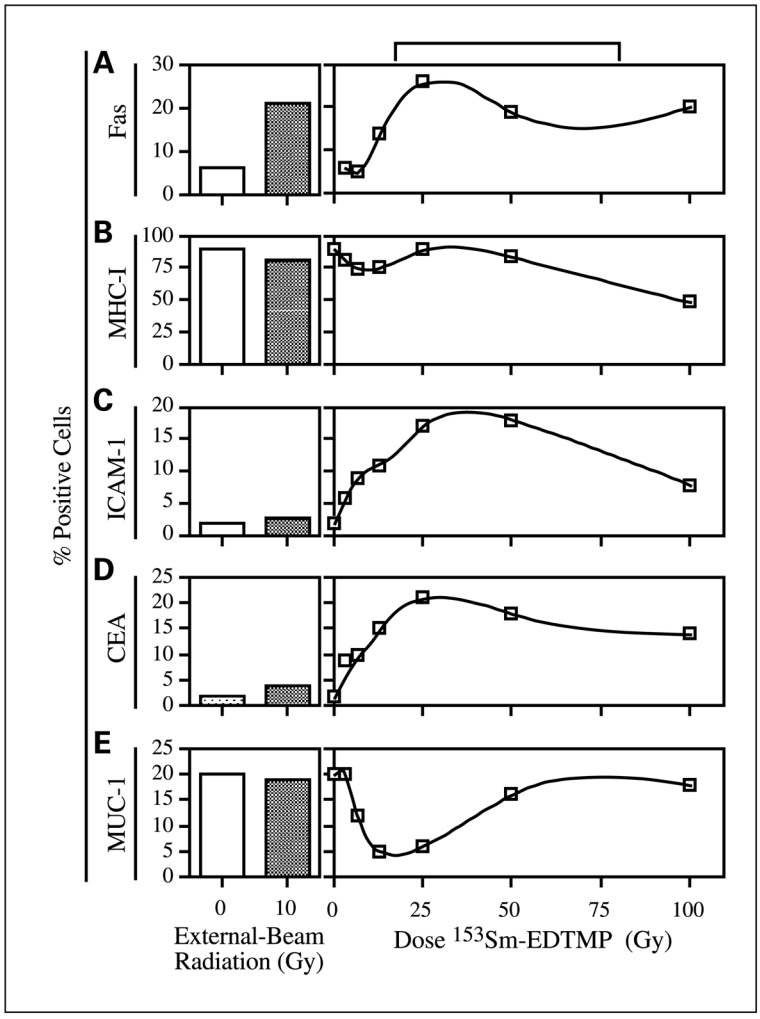

Fig. 3.

Comparison of effects of radiation with ^{153}Sm-EDTMP and XRTon cell surface molecule expression in LNCaP cells. LNCaP cells were exposed to 0 or 10 Gy XRT (*left*) or increasing doses (0–100 Gy) of ^{153}Sm-EDTMP (*right*). After 96 h, cells were harvested; stained for Fas (*A*), MHC-I (*B*), ICAM-1 (*C*), CEA (*D*), and MUC-1 (*E*); and analyzed by flow cytometry. Data are presented as percentage of positive cells. Bracket at top of figure, calculated delivered dose of^{153}Sm-EDTMP to tumor (18–80 Gy).

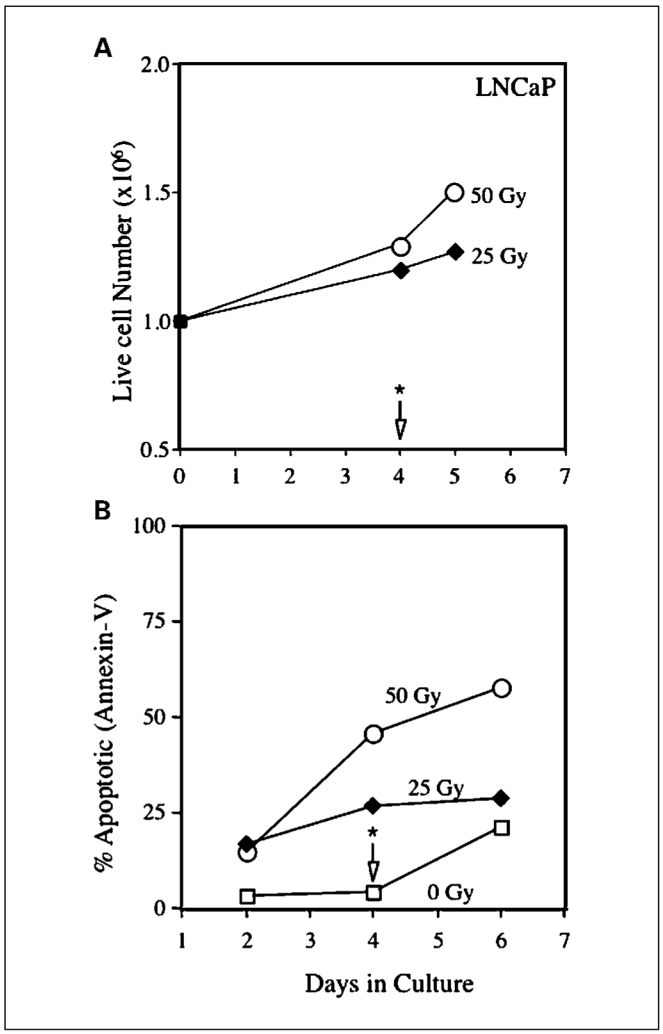

Fig. 4.

Treatment of prostate cancer cell line LNCaP with ^{153}Sm-EDTMP (25 and 50 Gy) was sublethal over 5 d. LNCaP cells were treated with ^{153}Sm-EDTMP (25 and 50 Gy); viability was determined by serial live cell counts (*A*); and apoptosis was determined by staining with Annexin V (*B*).

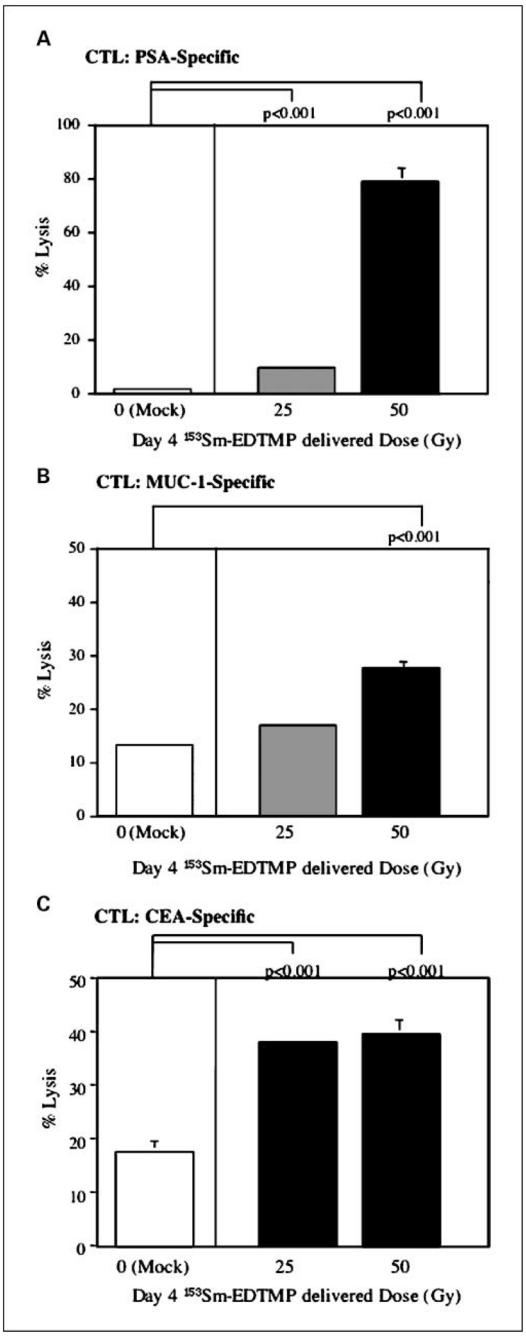

Fig. 5.

Effectof^{153}Sm-EDTMPon sensitivity of LNCaP cells to antigen-specific CTL killing. LNCaP cells were exposed to 0, 25, or 50 Gy^{153}Sm-EDTMP. Cells were harvested 72 h after exposure and labeled with ^{111}In. CTLs specific for PSA (*A*), MUC-1 (*B*), or CEA (*C*) were incubated with labeled LNCaP cells at an effector to target ratio of 30:1. After 18 h, supernatant was harvested and specific lysis was calculated.

Table 1

Up-regulation of accessory and tumor antigen genes following exposure to ^{153}Sm-EDTMP

	0 Gy	25 Gy	50 Gy
Accessory genes			
Fas	1	2	2
ICAM-1	1	29	34
Tumor antigen genes			
PSA	1	2.8	2.8
PSMA	1	4.2	4.6
PAP	1	29	36
CEA	1	10	13
MUC-1	1	3.7	2.9

NOTE: LNCaP cells were treated with 0, 25, or Gy ^{153}Sm-EDTMP. At 48 h, RNA was extracted from treated cells and quantitative real-time PCR was done on indicated genes and normalized against the housekeeping gene *GAPDH*. Numbers depict fold increase compared with the 0 Gy sample.