

# Synergistic tumoricidal effect of combined hMUC1 vaccination and hNIS radioiodine gene therapy

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## Abstract

We examined the merits of combinatorial hMUC1 vaccination and hNIS radioiodine gene therapy and evaluated its tumoricidal effects in an animal tumor model. CMNF (CT26 expressing hMUC1, hNIS, and firefly luciferase) cells were transplanted into 28 mice, and 4 and 11 days after tumor challenge, tumor-bearing mice were immunized i.m. with pcDNA3.1 or pcDNA-hMUC1 vaccine and subsequently administered PBS or <sup>131</sup>I i.p. [four groups (7 mice per group): pcDNA3.1 + PBS, pHMUC1 + PBS, pcDNA3.1 + <sup>131</sup>I, and pHMUC1 + <sup>131</sup>I groups]. Thirty-two days after tumor challenge, we rechallenged mice in the pcDNA3.1 + <sup>131</sup>I and pHMUC1 + <sup>131</sup>I groups with CMNF cells. Tumor progression and tumor-free mice (%) were monitored by bioluminescence. We investigated hMUC1-associated immune response generated by combination therapy. Marked tumor growth inhibition was observed in the pHMUC1 + <sup>131</sup>I group by bioluminescence at 32 days after tumor challenge. Mice in pHMUC1 + <sup>131</sup>I group showed complete hMUC1-expressing tumor suppression after tumor rechallenge, whereas mice in the pcDNA3.1 + <sup>131</sup>I group did not. The tumor-free mice (%) were much higher in the pHMUC1 + <sup>131</sup>I group than in the other three groups. Levels of hMUC1-associated CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells were higher in the pHMUC1 + <sup>131</sup>I group than in the other three groups. hMUC1-loaded

CD11<sup>+</sup> cells in the pHMUC1 + <sup>131</sup>I group were found to be most effective at generating hMUC1-associated CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells. The activities of hMUC1-associated cytotoxic T cells in the pHMUC1 + <sup>131</sup>I group were higher than in the other three groups. Our data suggest that pHMUC1 + <sup>131</sup>I combination therapy synergistically generates marked tumoricidal effects against established hMUC1-expressing cancers. [Mol Cancer Ther 2008; 7(7):2252–60]

## Introduction

Radiation destroys both cancer cells and other cells within tumor stroma, such as endothelial cells and intratumoral lymphocytes (1). Irradiated cancer cells undergo apoptosis or necrosis and secrete various proteins that stimulate immune cells, such as dendritic cells, macrophages, and T cells. In particular, antigen-presenting cells might recognize proteins generated by irradiating cancer cells and present tumor-specific antigenic peptides to T cells; thus, in turn, effector T cells might identify and kill antigen-expressing cancer cells (2–4). On the other hand, irradiated cancer or stroma cells show increased expressions of cell surface proteins, such as Fas, MHC class I molecules, and intracellular adhesion molecule-1 (5–7). These microenvironmental modifications could provide therapeutic advantages: (a) antigen-presenting cells might detect antigenic proteins derived from dying cancer cells and stimulate T cells in a MHC class-restricted manner and (b) activated T cells could migrate to cancer cells or immune-related organs through adhesion molecules in vascular lumen (8). Moreover, when other therapeutic modalities (e.g., immunotherapy and chemotherapy) are combined with radiotherapy, this radiation-induced immune response may be enhanced.

hMUC1 is highly overexpressed in various human cancers (9, 10). Moreover, because high expressions of hMUC1 are related with rapid tumor progression, many researchers have investigated the usefulness of hMUC1 DNA vaccines (11–13). However, although the therapeutic effect of hMUC1 immunization against cancer has been well investigated, hMUC1 vaccines alone are limited in terms of their abilities to inhibit cancer progression. Thus, new adjunctive modalities are required to enhance the therapeutic effects induced by hMUC1 immunization.

Sodium/iodide symporter (NIS) is a specialized active iodide transporter, and NIS-expressing cancer cells are known to accumulate several diagnostic or therapeutic radionuclides *in vitro* and *in vivo* (14, 15). More specifically, the transfer of the NIS gene and the functional expression of NIS protein enable cancer cells to accumulate therapeutic radionuclides, such as <sup>131</sup>I and <sup>188</sup>Re, from plasma and thus offer the possibility of radionuclide gene therapy. Some

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**Note:** Y.H. Jeon and Y. Choi contributed equally to this study.

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investigators have described the therapeutic effects of NIS-specific radionuclide gene therapy in animal models (16–19).

In the previous study, we established the CMNF cell line (a mouse colon cancer cell line; CT26/hMUC1-hNIS-*Fluc*) expressing the hMUC1, hNIS, and *Fluc* genes and examined the merits of combinatorial hMUC1 vaccine and radionuclide gene therapy (20). We hypothesized that hMUC1 antigenic peptides released by cancer cells killed by  $^{131}\text{I}$  would act as an antigen source and that these antigens would restimulate or maintain immune response against CMNF cells. We designed a combination therapy model that mimics the clinical situation and evaluated the synergistic tumoricidal effects of radiation-induced immune response induced by hNIS radioiodine gene therapy and hMUC1 immunotherapy in tumor-bearing mice.

## Materials and Methods

### Animals

Specific pathogen-free 6-week-old female BALB/c mice were obtained from SLC. All animal experiments were done after receiving approval from the Institutional Animal Care and Use Committee of the Clinical Research Institute at Seoul National University Hospital. In addition, the National Research Council guidelines for the care and use of laboratory animals (revised 1996) were observed throughout.

### Cell Line

Previously, we established the CMNF cell line, which is a stable clone of CT26 cells (a adenocarcinoma colon cancer cell line) that expresses hMUC1, hNIS, and firefly luciferase genes (CT26/hMUC1-hNIS-*Fluc*), and examined gene expression in this cell line by fluorescence-activated cell sorting,  $^{125}\text{I}$  uptake assays, and *in vitro* luciferase assays (data not shown; ref. 20).

### DNA Vaccine

The human pancreatic mucin1 gene, hMUC1 (accession no. J05582), was cloned into the *Bam*HI site of pcDNA3 vector (Invitrogen). Plasmid DNA was amplified in *Escherichia coli* DH5 $\alpha$  and purified by large-scale plasmid preparation using endotoxin-free Giga Prep columns (Qiagen). DNA was dissolved in endotoxin-free TE buffer for storage purposes.

### *In vitro* Clonogenic Assay

The procedure used has been described previously (21). Briefly, cells were grown in a 75 cm<sup>2</sup> flask and incubated for 7 h at 37°C in 5 mL HBSS containing 37 MBq/10 mL (1 mCi/10 mL) Na<sup>131</sup>I. The reaction was terminated by removing the radioisotope-containing medium and washing the cells twice with HBSS. Cells were then trypsinized, counted, and plated at densities of 250 or 1,000 per well in DMEM in six-well plates. They were then grown for 10 days, fixed with 3:1 methanol/acetic acid, and stained with crystal violet, and macroscopic colony numbers were counted. Survival rates are presented as colony numbers expressed as percentages of colony numbers in plates treated with HBSS only.

### Bioluminescence Imaging Acquisition

An IVIS100 imaging system (Xenogen), which includes an optical CCD camera mounted on a light-tight specimen chamber, was used for data acquisition and analysis. Firefly D-luciferin potassium salt (*Fluc* substrate) was diluted to 3 mg/100  $\mu\text{L}$  in PBS before use, and mice were injected i.p. with 100  $\mu\text{L}$  of this D-luciferin solution. Mice were placed individually in a specimen chamber containing the CCD camera, and light emitted by luciferase in mice was then measured. Grayscale photographic images and bioluminescent color images were superimposed using LIVING-IMAGE V. 2.12 (Xenogen) and IGOR image analysis software (WaveMetrics). To quantify emitted light, regions of interest were drawn over the tumor region and total photon effluxes over an exposure time of 3 s were determined. Bioluminescent signals were expressed in units of photons per cubic meter per second per steradian.

### *In vivo* Animal Experiments

The four experimental groups are the pcDNA + PBS, phMUC1 + PBS, pcDNA +  $^{131}\text{I}$ , and phMUC1 +  $^{131}\text{I}$  groups as described previously. The following experimental procedure was done for combined hMUC1 vaccination and hNIS radioiodine gene therapy (Fig. 2).

In 28 mice,  $1 \times 10^5$  CMNF cells were transplanted s.c. in right thighs. Four and 10 days later, mice were immunized with pcDNA3.1 (50  $\mu\text{g}/50 \mu\text{L}$ ) or phMUC1 (50  $\mu\text{g}/50 \mu\text{L}$ ) into a quadriceps muscle of the right hind leg and 14 days later were administered PBS or 2 mCi  $^{131}\text{I}$  i.p. [four groups (7 mice per group): pcDNA3.1 + PBS, phMUC1 + PBS, pcDNA3.1 +  $^{131}\text{I}$ , and phMUC1 +  $^{131}\text{I}$  groups]. Mice received a low-iodine diet with T4 supplementation in drinking water for 2 weeks post-tumor challenge to maximize radioiodine uptake in tumors and to reduce iodide uptake by thyroid glands. Tumor sizes were measured using a caliper at 7, 14, 17, 21, 24, 28, and 32 days post-tumor challenge and tumors were weighted in the pcDNA + PBS and phMUC1 + PBS groups at 32 days. For bioluminescence imaging acquisition, mice were repeatedly imaged at 3, 10, 14, 17, 21, 24, 28, and 32 days postchallenge using an optical CCD camera. For scintigraphic imaging acquisition, [ $^{99\text{m}}\text{Tc}$ ]pertechnetate (0.5 mCi) was injected i.p., and mice were imaged using a  $\gamma$ -ray camera (ON-410) at 14 days postchallenge.

### *In vivo* Tumor Rechallenge Experiment

Mice in the pcDNA +  $^{131}\text{I}$  and phMUC1 +  $^{131}\text{I}$  groups were rechallenged with  $5 \times 10^4$  CMNF cells s.c. in right thighs at 32 days postinitial challenge. Mice were repeatedly imaged at 3, 8, 12, and 16 days postrechallenge using an optical CCD camera. Postmortem tumor weights were measured at 16 days postrechallenge.

### Intracellular Cytokine Staining and Flow Cytometric Analysis

For the *in vivo* tumor protection experiments, mouse splenocytes (seven per group) were isolated from treated mice and stimulated *in vitro* for 72 h using 10  $\mu\text{g}/\text{mL}$  hMUC1 peptide (PDTRPAPGSTAPPAHGVTSAPDTRPAPGST) and interleukin-2 (50 units/mL). Stimulated splenocytes were treated with Golgistop (BD Pharmingen). Splenocytes were

then washed once in fluorescence-activated cell sorting buffer and stained with FITC-conjugated monoclonal rat anti-mouse CD8 (BD PharMingen). Cells were immunostained for cytokines using a Cytotfix/Cytoperm kit (BD PharMingen) and with PE-conjugated anti-IFN- $\gamma$  (BD PharMingen). Flow cytometric analysis was done using a Becton Dickinson FACScan using CellQuest software (Becton Dickinson Immunocytometry Systems).

#### **In vitro Splenocyte Cytotoxicity Assays**

For the *in vivo* tumor protection and long-term tumor growth inhibition experiment, the CytoTox 96 nonradioactive cytotoxicity assay (Promega) was used to measure the cytotoxic activities of splenocytes in treated mice (7 mice per group) according to the manufacturer's protocol with minor modification. Briefly, splenocytes of treated immunocompetent BALB/c mice were incubated in the presence of human interleukin-2 (50 units/mL) and 10  $\mu$ g/mL hMUC1 peptide (PDTRPAGSTAPPAHGVTSAPDTRPAGST). After 3 days, irradiated CT26 and CMNF target cells were plated at  $1 \times 10^4$  per well on 96-well U-bottomed plates (Costar), and splenocytes (effectors) were added to a final volume of 100  $\mu$ L in a 1:12 ratio. Plates were then incubated for 4 h in a humidified 5% CO<sub>2</sub> chamber at 37°C and centrifuged at  $500 \times g$  for 5 min. Aliquots (50  $\mu$ L) were transferred from all wells to fresh 96-well flat-bottomed plates, and an equal volume of reconstituted substrate mix was added to each well. The plates were then incubated in the dark at room temperature for 30 min. Stop solution (50  $\mu$ L) was then added, and absorbance values were measured at 492 nm. Cell death percentages at each effector-to-target cell ratio were calculated using  $[A \text{ (experimental)} - A \text{ (effector spontaneous)} - A \text{ (target spontaneous)}] \times 100 / [A \text{ (target maximum)} - A \text{ (target spontaneous)}]$ .

#### **Histopathology**

Tumor injection sites were removed from mice and preserved in 10% formalin solution (Sigma) until required. All tumor tissues were embedded in paraffin, sectioned at 4  $\mu$ m, and stained with H&E. Histopathologic reviews of tumor tissues were done independently by two pathologists.

#### **Restimulation of hMUC1-Associated CD8<sup>+</sup> T cells by Enriched CD11c<sup>+</sup> Cells from Immunized Mice**

Ten days after treating tumor-bearing mice (4 mice per group) with <sup>131</sup>I, splenocytes were harvested and CD8<sup>+</sup> T cells were separated using CD8 (Ly-2) microbeads (Miltenyi Biotec). The enriched CD8<sup>+</sup> T cells so obtained were analyzed by fluorescence-activated cell sorting and restimulated *in vitro* for 72 h with 10  $\mu$ g/mL hMUC1 peptide (PDTRPAGSTAPPAHGVTSAPDTRPAGST) and interleukin-2 (50 units/mL).

Thirteen days after treating tumor-bearing mice (4 mice per group) with <sup>131</sup>I, inguinal draining lymphoid cells were harvested and CD11<sup>+</sup> cells were enriched using CD11c (N418) Microbeads (Miltenyi Biotec). These enriched cells were then analyzed by fluorescence-activated cell sorting. Restimulated CD8<sup>+</sup> T cells [treated with Golgistop for 6 h (BD PharMingen)] were then harvested for coculture with the enriched CD11c<sup>+</sup> cells. The enriched CD11<sup>+</sup> cells

( $1 \times 10^5$ ) were then cocultured with restimulated  $1 \times 10^6$  hMUC1-associated CD8<sup>+</sup> T cells for 16 h. The restimulated CD8<sup>+</sup> T cells were then stained with CD8 and IFN- $\gamma$  antibody (BD PharMingen) and analyzed by flow cytometry.

#### **Statistical Analysis**

All data are expressed as mean  $\pm$  SD and are representative of at least two separate experiments. Intracellular cytokine staining, flow cytometry analysis, and tumor protection experiment findings were analyzed using the Kruskal-Wallis test and the Wilcoxon rank-sum test with Bonferroni's correction. Kaplan-Meier curves were used to estimate tumor-free mice. *P* values < 0.02 were considered significant.

## **Results**

#### **In vitro Clonogenic Assay**

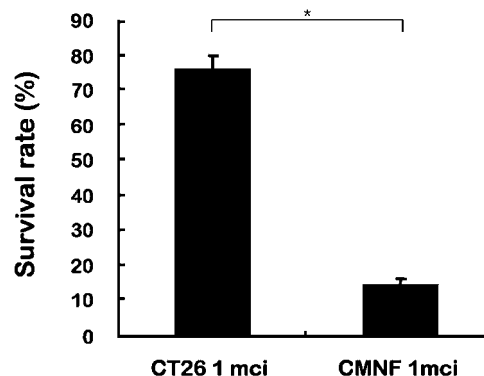
As shown in Fig. 1, the survival rates of CMNF (hNIS-expressing CT26) cells were markedly reduced to  $13.6 \pm 1.0\%$  in response to <sup>131</sup>I versus CT26 cells (*P* < 0.001).

#### **Therapy of Tumor Xenografts in Animals Treated with hMUC1 Vaccination and/or <sup>131</sup>I Therapy**

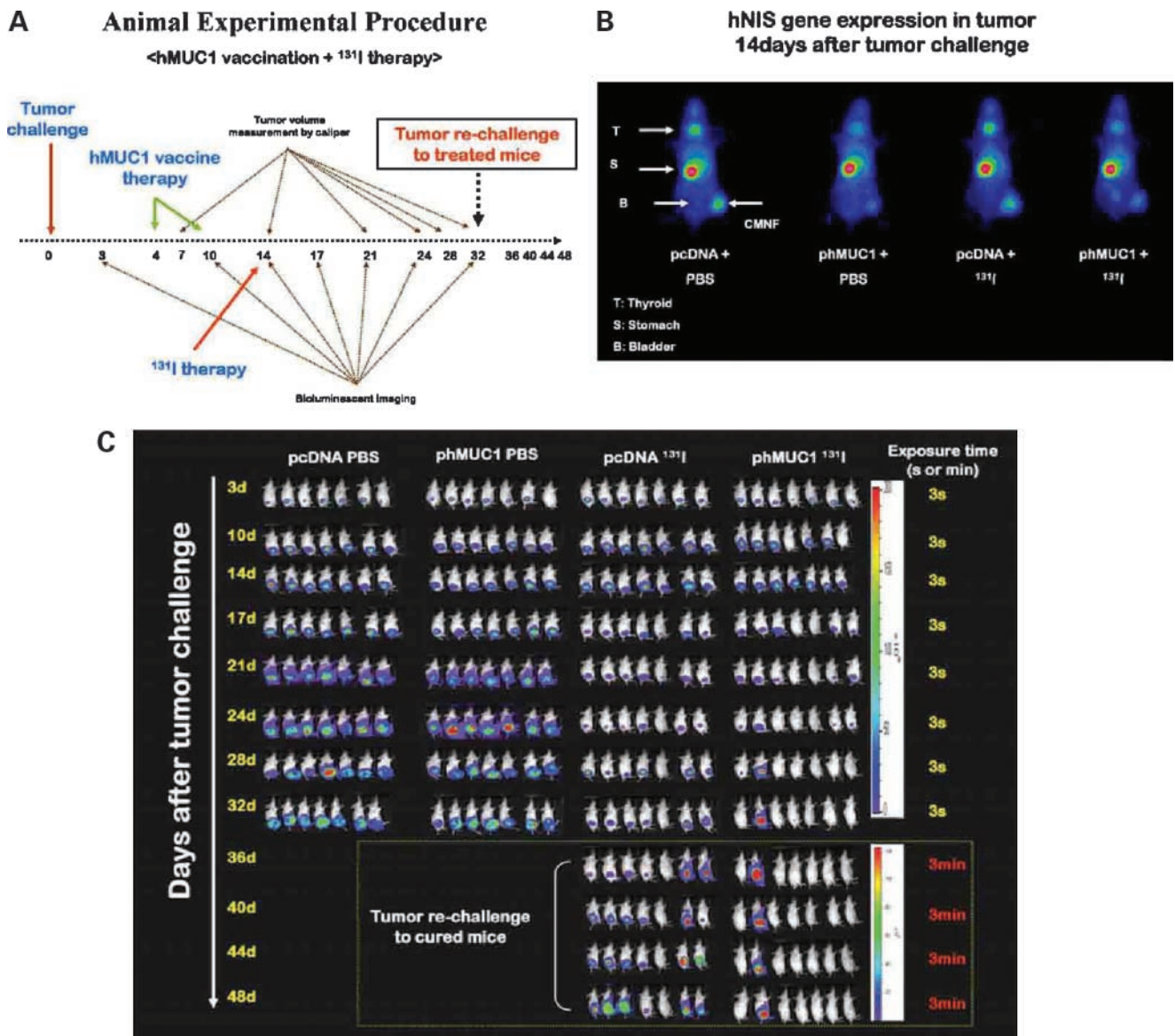
Minor tumor growth inhibition effects were observed in animals vaccinated with hMUC1 (phMUC1 + PBS and phMUC1 + <sup>131</sup>I groups) but not in the corresponding pcDNA vaccination groups by scintigraphic imaging (Fig. 2B). A slight tumor growth inhibiting effect was observed in the phMUC1 + PBS group but not in the pcDNA3.1 + PBS at 32 days postchallenge (Fig. 3A-C; *P* < 0.02, pcDNA + PBS versus phMUC1 + PBS). However, <sup>131</sup>I-treated animals (pcDNA + <sup>131</sup>I and phMUC1 + <sup>131</sup>I) showed rapid tumor growth inhibition (Figs. 2C and 3A-C) by bioluminescence imaging and according to caliper measurements.

H&E analysis showed different degrees of tumor necrosis (%) in the pcDNA + <sup>131</sup>I and phMUC1 + <sup>131</sup>I groups (70%

#### **In vitro cytotoxic activity: Clonogenic assay**



**Figure 1.** *In vitro* clonogenic assay after incubation with <sup>131</sup>I. CMNF cells were exposed to 37 MBq/10 mL (1 mCi/10 mL) <sup>131</sup>I for 7 h at 37°C. Cells were then grown for 10 d and stained with crystal violet, and colony numbers were counted. Survival rates (%) were colony numbers in plates treated with radionuclide expressed as percentages of colony numbers in plates containing buffer only. Columns, mean of triplicate experiments; bars, SD. \*, *P* < 0.001.



**Figure 2.** Animal experimental protocol and *in vivo* monitoring of antitumor effects of combined hMUC1 vaccination and  $^{131}\text{I}$  therapy. **A**,  $1 \times 10^5$  CMNF cells were transplanted s.c. into the right thighs of immunocompetent BALB/c mice. Tumor-bearing mice were immunized i.m. with each vector (pcDNA3-hMUC1 or pcDNA3.1) at 4 and 10 d post-tumor challenge, and scintigraphic images were acquired at 14 d postchallenge. At 14 d,  $^{131}\text{I}$  was injected i.p. Bioluminescent imaging and tumor volume measurements were done at the designated times. Thirty-two days postchallenge, cured mice in the pcDNA +  $^{131}\text{I}$  and pHMUC1 +  $^{131}\text{I}$  groups were rechallenged with  $5 \times 10^4$  CMNF cells. **B**, before administering a therapeutic dose of  $^{131}\text{I}$  to mice, scintigraphic images were acquired at 14 d postchallenge. T, thyroid; S, stomach; B, urinary bladder; CMNF, CT26/hMUC1-hNIS-FLuc. **C**, tumor growths were visualized by bioluminescent imaging at the designated times.

and 95%, respectively; Supplementary Fig. S1A)<sup>5</sup> at 32 days postchallenge. Bioluminescence imaging (Fig. 2C; 6 tumor-bearing mice in pcDNA +  $^{131}\text{I}$  group, 1 tumor-bearing mouse in pHMUC1 +  $^{131}\text{I}$  group, and 7 mice per group) and total photon efflux findings were significantly different in

the pcDNA +  $^{131}\text{I}$  and pHMUC1 +  $^{131}\text{I}$  groups at this time (Fig. 3A;  $P < 0.01$ , pcDNA +  $^{131}\text{I}$  versus pHMUC1 +  $^{131}\text{I}$  group). Seven  $^{131}\text{I}$ -treated mice were tumor free (one in the pcDNA +  $^{131}\text{I}$  group and six in the pHMUC1 +  $^{131}\text{I}$  group), but no mouse was tumor free in the non- $^{131}\text{I}$ -treated groups (Fig. 3D).

#### Tumor Rechallenge Experiment

Aggressive tumor growth was observed in the pcDNA +  $^{131}\text{I}$  group but not in the pHMUC1 +  $^{131}\text{I}$  group from 4 to

<sup>5</sup> Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

16 days after tumor rechallenge by bioluminescent imaging (Figs. 2C and 3A; pcDNA +  $^{131}\text{I}$  versus phMUC1 +  $^{131}\text{I}$ ,  $P < 0.01$ ). A significant difference in tumor weight was observed between pcDNA +  $^{131}\text{I}$  and phMUC1 +  $^{131}\text{I}$  groups (Fig. 3C;  $P < 0.001$ ) at 16 days post-tumor rechallenge. We also found a difference between these two groups in terms of degrees of necrosis (pcDNA +  $^{131}\text{I}$  and phMUC1 +  $^{131}\text{I}$ , 60% and 95% to 100%, respectively; Supplementary Fig. S1B).<sup>5</sup>

#### hMUC1-Associated CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells and CTL Activity

The number of T cells expressing CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> in the phMUC1 +  $^{131}\text{I}$  group was markedly higher than in the other three groups (pcDNA + PBS, phMUC1 + PBS, pcDNA +  $^{131}\text{I}$ , and phMUC1 +  $^{131}\text{I}$  groups,  $53.5 \pm 1.2$ ,  $91.7 \pm 1.7$ ,  $676.5 \pm 3.6$ , and  $1278.7 \pm 3.4$ , respectively, \*,  $P < 0.02$  and \*\*,  $P < 0.01$ ; Fig. 4A and B). As shown in Fig. 5A, the killing activities of CTL cells against hMUC1-expressing cancer was greatest in phMUC1 +  $^{131}\text{I}$  group among four therapy groups (pcDNA + PBS, phMUC1 + PBS, pcDNA +  $^{131}\text{I}$ , and phMUC1 +  $^{131}\text{I}$  groups,  $16 \pm 2\%$ ,  $20 \pm 1\%$ ,  $30 \pm 2\%$ , and  $60 \pm 2\%$ , respectively, \*,  $P < 0.02$  and \*\*,  $P < 0.01$ ).

#### Numbers of CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup>-Expressing T Cells Generated by hMUC1-Loaded CD11c<sup>+</sup> Cells

As shown in Fig. 6, enriched CD11c<sup>+</sup> cells from the phMUC1 +  $^{131}\text{I}$  group most effectively generated CD8<sup>+</sup>IFN- $\gamma$ -expressing T cells among four therapy groups (pcDNA +

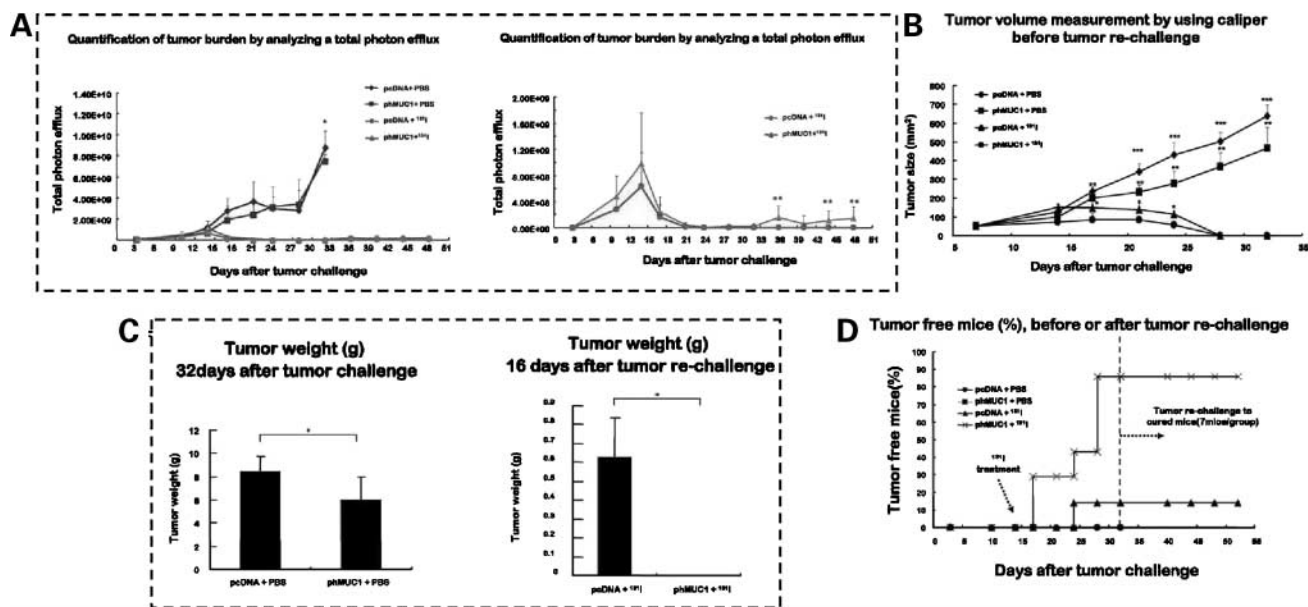
PBS, phMUC1 + PBS, pcDNA +  $^{131}\text{I}$ , and phMUC1 +  $^{131}\text{I}$  groups,  $7 \pm 3$ ,  $31 \pm 9$ ,  $86 \pm 19$ , and  $139 \pm 15$ , respectively, \*,  $P < 0.02$  and \*\*,  $P < 0.01$ ).

#### Long-term CTL Activity

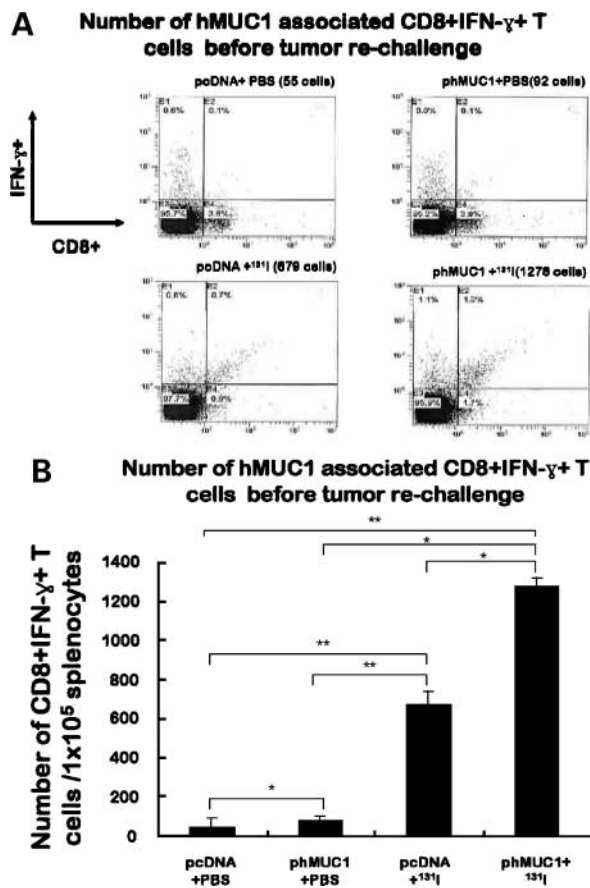
The killing activity of CTLs against hMUC1-expressing cancer in the phMUC1 +  $^{131}\text{I}$  group was 12 times that in the pcDNA +  $^{131}\text{I}$  group (Fig. 5B; pcDNA +  $^{131}\text{I}$  and phMUC1 +  $^{131}\text{I}$ ,  $8 \pm 2\%$  and  $96 \pm 5\%$ , respectively,  $P < 0.001$ ).

## Discussion

Cancer vaccinations using cancer DNA, tumor-derived cell, or dendritic cell vaccines have been applied to preclinical/clinical disease models (22–28). Of these, cancer DNA vaccines provide several advantages compared with the others: (a) DNA vaccines have the same effects as live attenuated vaccines in terms of their ability to induce CD8<sup>+</sup> T cell response but reduce safety concerns associated with live vaccines (29, 30). (b) DNA vaccines can be manipulated in a relatively cost-effective manner and are easily stored (31). Several researchers have reported that DNA vaccines can induce protection from tumors in immunocompetent mice and increase mouse survival (23, 24, 31–33). However, many researchers have not obtained satisfactory results in terms of long-term tumor protection and inhibition from recurrence in various cancer models due to the low immunogenicities of DNA vaccines or to the disruptions of regulator T cells or cytokine balances in the



**Figure 3.** Quantification or measurement of tumor growth. **A**, to quantify light intensities, regions of interest were drawn over tumor regions and total photon effluxes were measured.  $n = 7$  mice per group. \*,  $P < 0.02$  (pcDNA + PBS versus phMUC1 + PBS); \*\*,  $P < 0.01$  (pcDNA +  $^{131}\text{I}$  versus phMUC1 +  $^{131}\text{I}$ ). **B**, tumor volumes were measured using a caliper. Tumor volumes were defined as length (mm)  $\times$  width (mm). \*,  $P < 0.02$  (pcDNA +  $^{131}\text{I}$  versus phMUC1 +  $^{131}\text{I}$ ); \*\*,  $P < 0.02$  (phMUC1 +  $^{131}\text{I}$  versus phMUC1 + PBS); \*\*\*,  $P < 0.02$  (pcDNA + PBS versus phMUC1 + PBS). **C**, tumors were extracted from animals in the pcDNA + PBS and phMUC1 + PBS groups at 32 d postchallenge and weighted. \*,  $P < 0.02$ , at 16 d post-tumor rechallenge of cured mice in the pcDNA +  $^{131}\text{I}$  and phMUC1 +  $^{131}\text{I}$  groups, tumor masses were extracted and weighted; \*,  $P < 0.001$ . **D**, numbers of tumor-free mice before and after rechallenge are expressed as percentages. Experiments were done in triplicate. Columns, mean ( $n = 7$  mice per group); bars, SD.



**Figure 4.** Levels of hMUC1-associated CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T-cell immune response in immunocompetent mice treated with phMUC1 + <sup>131</sup>I. **A**, representative flow cytometry data. **B**, columns, numbers of IFN- $\gamma$  secreting hMUC1-associated CD8<sup>+</sup> T cells per  $1 \times 10^5$  cells. Splenocytes were stained with PE-labeled IFN- $\gamma$  and FITC-labeled CD8 antibody and analyzed by flow cytometry. \*,  $P < 0.02$ ; \*\*,  $P < 0.01$ . Experiments were done in triplicate. Columns, mean ( $n = 8$  mice per group); bars, SD.

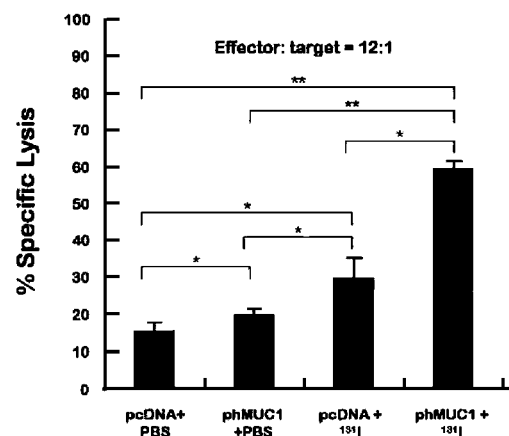
host (34, 35). Thus, we considered that cancer DNA vaccine immunotherapy may be augmented by other therapies.

Radionuclide therapy based on  $\beta$ -ray irradiation is a conventional cancer treatment. Current research is focusing on the use of radionuclide gene therapy to concentrate therapeutic radionuclides (<sup>131</sup>I, <sup>188</sup>Re) in specific cancer cells (14, 21, 36).  $\beta$ -rays emitted from <sup>131</sup>I travel 0.2 to 2.4 mm in tissue (37), which results in the death of cells near hNIS-expressing cancer cells by the crossfire effect.

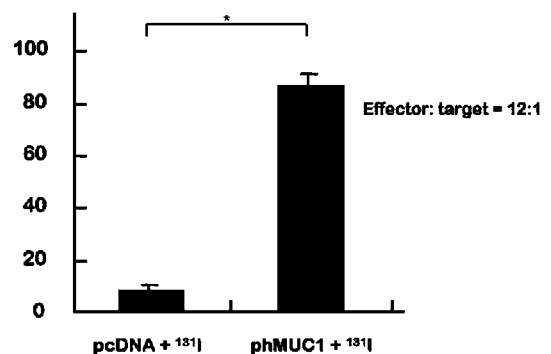
Cancer cells escape the attack of cancer-specific immune cells generated by cancer DNA vaccine because of no expression of tumor antigen or MHC class molecules and hindrance of regulatory T cells, etc. Therefore, cancer researchers have investigated new combination therapy to overcome weak therapeutics effects of cancer DNA vaccine. Recently, it was reported that radiation therapy plus cancer DNA vaccine is a promising strategy. Local radiation is the standard treatment for care of various type of cancer and has ability to directly not only kill cancer cells but also

induce radiation-induced immune response. Particularly, gene modification of cancer cell by radiation could provide cancer DNA vaccine the opportunity to overcome weak antitumor immunity. For example, Chakraborty et al. examined the phenotype marker and biological effects of irradiated cancer cell on killing activity of CD8<sup>+</sup> T cells (7). Irradiated cancer cell highly expresses Fas receptor and intracellular adhesion molecule-1 in a dose-dependent manner. Also, radiation provides increased the killing activity of tumor-associated CTL against cancer cell. Other group reported on similar finding with human cancer cell line (12 colon, 7 lungs, and 4 prostate). It was found that

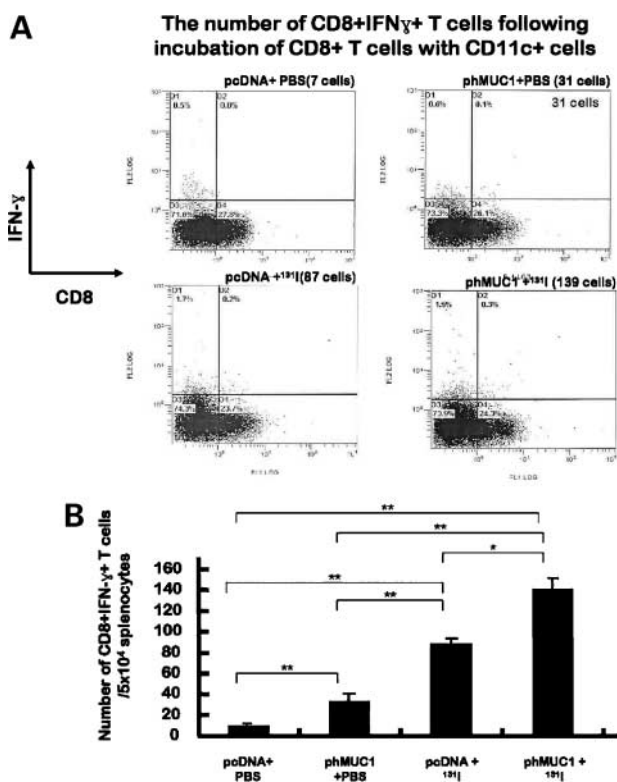
### A Cytotoxic T cell activity before tumor re-challenge



### B Cytotoxic T cell activity after tumor re-challenge



**Figure 5.** Cytotoxic effects of T cells against hMUC1-expressing cancer cells generated by combination therapy (phMUC1 + <sup>131</sup>I). **A**, splenocytes of treated mice were prepared and restimulated with interleukin-2 and hMUC1 peptides for 3 d. Irradiated CMNF cells (target cells) were then incubated with splenocytes (effectors) at an effector-to-target ratio of 12:1 for 4 h in 96-well plates. \*,  $P < 0.02$ ; \*\*,  $P < 0.01$ . **B**, to investigate long-term cytotoxic T-cell activity, splenocytes of mice were prepared at 16 d post-tumor rechallenge and restimulated with interleukin-2 and hMUC1 peptides for 3 d. Irradiated CMNF cells (target cells) were incubated with splenocytes (effectors) at an effector-to-target ratio of 12:1 for 4 h in 96-well plates. Supernatants were collected and amounts of LDH were determined using LDH assays. \*,  $P < 0.001$ . Experiments were done in triplicate. Columns, mean ( $n = 8$  mice per group); bars, SD.



**Figure 6.** Levels of hMUC1-associated CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells stimulated by hMUC1 antigen-loaded dendritic cells in the draining lymph nodes of mice treated with combination therapy. **A**, representative flow cytometry data. **B**, columns, numbers of IFN- $\gamma$ -secreting hMUC1-associated CD8<sup>+</sup> T cells per  $5 \times 10^4$  cells. Restimulated cells were stained with PE-labeled IFN- $\gamma$  and FITC-labeled CD8 antibody and analyzed by flow cytometry. \*,  $P < 0.02$ ; \*\*,  $P < 0.01$ . Experiments were done in triplicate. Columns, mean; bars, SD.

when those human cancer cell lines were irradiated with 10 Gy radiation, they observed the change of the surface gene expression (Fas, intracellular adhesion molecule-1, MUC1, CEA, and MHC class I) in human cancer (38).

Based on the recent fascinated reports, we have considered that hMUC1 vaccination plus hNIS radioiodine gene therapy may induce synergistic therapeutic effects similar with external local radiation in combination with cancer vaccine in established tumor model. Particularly, it was expected that hMUC1 vaccination plus hNIS radioiodine gene therapy could effectively inhibit tumor recurrence following complete tumor inhibition.

In this study, the therapeutic effects of a combined hMUC1 cancer DNA vaccine and hNIS radioiodine gene therapy were evaluated. Minor tumor growth inhibition was observed in the hMUC1 + PBS group, and slightly greater tumor growth inhibition was observed in the pcDNA3.1 + <sup>131</sup>I group by bioluminescence imaging and according to caliper measurements. The weak therapeutic effect of DNA vaccines has been substantially reviewed (31), and the inadequacy of radionuclide NIS gene single therapy has been well reported (17–19), which

suggests that monotherapies based on these modalities are probably insufficient to induce complete remission.

Theoretically, combinatorial cancer vaccine and radiotherapy may have a synergistic antitumor effect. Radiation would induce cell death through apoptosis or necrosis and these destroyed cells and their byproducts provide antigens that stimulate immune-related cells. Moreover, the radiation-induced tumor microenvironment could give various immune-related cells the opportunity to home in on affected tissues (8, 39). In the present study, marked tumor growth inhibition was observed in the phMUC1 + <sup>131</sup>I group but not in the monotherapy groups (Figs. 2C and 3A). Moreover, at 36 days post-challenge, 6 of 7 mice were tumor free in the phMUC1 + <sup>131</sup>I group.

This combination therapy generated the highest number of hMUC1-associated CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells (Fig. 4) and most enhanced the killing activity of cytotoxic T cells among four therapy groups (Fig. 5A). Moreover, this combination therapy was found to generate more hMUC1 antigen-loaded CD11c<sup>+</sup> cells than the other treatments (Fig. 6). Based on these findings, we suggest that the synergistic therapeutic effects of phMUC1 + <sup>131</sup>I therapy proceeds via the following mechanisms; there may have been many more hMUC1-loaded dendritic cells and hMUC1-associated effectors in the hMUC1 vaccination group than in the mock (pcDNA3.1) vaccination group. hMUC1-loaded dendritic cells could effectively recognize and cross-present hMUC1 antigenic peptides derived from apoptotic or necrotic cells induced by <sup>131</sup>I to effectors in the phMUC1 + <sup>131</sup>I group.

In our tumor rechallenge experiment, mice completely rejected tumor recurrence in combination therapy group (phMUC1 + <sup>131</sup>I) but not in monotherapy group (pcDNA + <sup>131</sup>I; Figs. 2C and 3A). hMUC1-associated CTL activity was also greater in the phMUC1 + <sup>131</sup>I group than in the pcDNA + <sup>131</sup>I group (Fig. 5B).

We already described the mechanism of the effects of therapy in tumor rechallenge study. Please refer to following description below.

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Our findings indicate that hNIS radioiodine gene therapy could be improved by several modifications. Using targeted gene therapy under the control of a tissue-specific promoter, the hNIS gene could be expressed in target tumor cells only, which would maximize therapeutic effect in cancerous tissues and minimizing damage to normal tissues (16, 18, 19). Moreover, more powerful therapeutic radionuclides like Re-188 could be used, and cotransfection with the thyroid peroxidase gene offers another means of enhancing radionuclide retention in cancer cells (14, 40, 41).

Further study is required before the efficacy of combined hMUC1 vaccination and  $^{131}\text{I}$  therapy can be examined in a patients. First, we should determine the proper dose (minimum dose) of  $^{131}\text{I}$  to maximize the immune response and cytotoxic activity against cancer cells and to minimize the damage of normal organs. Second, other immune modulators should be examined to improve on the weak immune response shown to hMUC1. For example, it has been reported that when adjuvants, such as CpG motif and a cytokine (granulocyte macrophage colony-stimulating factor, Flt3 ligand, and macrophage inflammatory protein-1 $\alpha$ ), were added to combination therapy to attract or cause the proliferation of antigen-presenting cells, antigen-specific immune responses against cancer were enhanced (31).

In summary, the present study shows that combined hMUC1 vaccination and hNIS radioiodine gene therapy can synergistically enhance hMUC1-associated immune response against hMUC1-expressing cancer cells and effectively inhibit tumor growth in an immunocompetent mouse model. In addition, this combination therapy seems to prevent tumor recurrence after complete remission. Further more experiments should be followed to be applied in cancer patients.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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