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Immunotherapy of Radioresistant Mammary Tumors with Early Metastasis Using Molecular Chaperone Vaccines Combined with Ionizing Radiation

Desheng Weng,*† Baizheng Song,* Shigeo Koido,‡ Stuart K. Calderwood,§ and Jianlin Gong*

In the current study, exposure of mammary tumor cells derived from mice transgenic for the polyomavirus middle T oncogene to ionizing radiation resulted in the generation of a tumor cell population that preferentially expressed cancer stem cell markers. In addition, these cells were more resistant to subsequent radiation treatments and appeared to acquire an enhanced capacity for dissemination to the lungs of mice. Therefore, we tested an immunotherapy approach to the treatment of local and disseminated mammary tumor cells in a murine model using a recently developed molecular chaperone-based vaccine that specifically targets the radioresistant subpopulation of tumor cells. Heat shock protein 70–peptide complexes (Hsp70.PC-F) were extracted from fusions of dendritic cells and radiation-enriched tumor cells, and the resulting chaperone vaccines were used to treat mice with pre-existing lung metastases. Immunization of mice with the Hsp70.PC-F vaccine resulted in a T cell–mediated immune response, including a significant increase in CD4 and CD8 T cell proliferation and the induction of effector T cells capable of targeting radioresistant tumor cells. Importantly, the growth of primary tumors was inhibited, and the number of tumor cells metastasizing to lung was reduced significantly by combining chaperone vaccine with radiotherapy. These results indicate that Hsp70.PC-F vaccine can induce specific immunity to radioresistant populations of mammary tumor cells and, thus, can complement radiotherapy, leading to synergistic killing. The Journal of Immunology, 2013, 191: 755–763.

Cancer stem cells (CSCs), whose existence has been advocated for decades by radiobiologists (1), are implicated as major factors in tumor recurrence after therapy (2). The identification of CSC markers, including CD44 and stem cell Ag 1 (Sca1), and the development of new animal models to measure self-renewal provide substantial evidence that links CSCs to radioresistance. The CD44 gene product is a cell surface glycoprotein that is involved with cell adhesion and migration. CD44 was the first CSC surface marker identified for CSCs in solid tumors (3). The breast CSCs were CD44+CD24low. Sca1 is a putative cell surface marker of hematopoietic stem cells that can be used to isolate multipotent progenitors from the mammary gland (4, 5). Sca1+ multipotent cells from an immortalized murine mammary cell line are more radioresistant than are their Sca1− counterparts (6). Culture of MCF-7 and MDA-MB-231 breast cancer cells in mammosphere media, in which stem/progenitor cells are preferentially selected to survive and form mammospheres (7), dramatically increased the subpopulation with CD44+CD24low surface markers (8). Importantly, tumor cells cultured under mammosphere conditions are more resistant to single-dose or fractionated radiation than are those cultured in the conventional monolayer culture, suggesting that cells bearing CD44+CD24low markers preferentially selected in mammosphere media are more radioresistant. Therefore, it is likely that elimination of CSCs could significantly improve the radiation response of tumors. CSCs could potentially be selectively targeted in therapy through inhibiting molecules in the signaling pathways that are exclusive to them and essential for CSC survival. Alternatively, CSC-targeted immunotherapy could be deployed against this population, because immunotherapy represents a non–cross-resistant strategy that can be complementary to conventional therapy. Recent findings indicate that CSC-targeted T cell–mediated immunotherapy can be developed (9–11). Pellegatta and associates (9, 12) reported that dendritic cells (DCs) loaded with lysates from GL261 neurospheres induced robust CD4 and CD8 T cells that infiltrated the tumor and led to the cure of 60–80% of animals with glioma. Xu et al. (10) found that CSCs from glioblastoma multiforme expressed increased levels of tumor-associated Ags, as well as MHC molecules; vaccination with DCs pulsed with CSC Ags induced a CTL response specific for CSCs and prolonged the survival of animals bearing 9L CSC brain tumors. These studies indicate that certain targets for immunotherapy against CSCs are already known, and others, although they remain unidentified, presumably exist.

Cancer cells can be immunogenic, and this property may be due to re-expressed embryonic Ags, as well as proteins bearing covalent alterations derived from mutated genes (13, 14). However, the
nature of most of these alterations is unknown and likely differ, even among individuals with tumors of similar histology. Optimal vaccines would be individualized and built around the antigenic repertoire of the individual patient. A number of approaches offers this potential, and heat shock protein (HSP) vaccines are notable members of this group (15–17). HSPs consist of a number of families of stress-inducible proteins whose main intracellular functions are as molecular chaperones (18–20). Thus, HSPs recognize unfolded sequences in target polypeptides and become bound to them. HSPs then aid in either the folding/refolding of such sequences or the targeting of unfolded polypeptides to the proteasome (20, 21). In this way, they maintain the functional quality of the proteome (19, 22, 23). However, as with other multidomain proteins, HSPs have multiple properties. For instance, they also can be released from cells and access the extracellular environment of tissues and associate with the surfaces of immune cells (24–26). These functions are partially dependent on the molecular chaperone functions of HSP, in that they can bind to intracellular antigenic peptides and transport the peptides through the extracellular milieu for later presentation to APCs (24–28). The immune roles of the HSPs also involve novel properties. These properties include the ability to bind to receptors on APCs, the capacity to chaperone bound peptides through the processes of endocytosis, and the promotion of tumor Ag cross-presentation (24, 29).

In the current study, we used a heat shock protein 70–peptide complex (Hsp70.PC) extracted from tumor cells that survived irradiation to target radioresistant tumor cells. Vaccination of heat shock protein 70–peptide complexes obtained from fusions of DC and irradiated tumor cells (Hsp70.PC-F) induced CTLs that preferentially killed the radioresistant tumor cells and improved the radiotherapeutic efficacy of tumor cells.

**Materials and Methods**

**Mice**

The mice (C57Bl/6 background) used in our experiments include female mice double transgenic for the human mucin 1 (MUC1) Ag and polyomavirus middle T (PyMT) gene, and 100 nM 5′-CTCTCCTCAGTTCCTCGCTCC-3′ reverse primer was used for the MT gene, and 100 nM 5′-CTCCACGGTCGTGGACATTGTAG-3′ reverse primer was used for the MUC1 gene. Primers for detection of the GFP gene included 5′-AAATGTCATCGACACCCAG-3′ (forward), 5′-TCTCTGAGAAGAATGTTGCGG-3′ (reverse), and internal positive control 5′-CTAGTCTCAATGGTCACGATCC-3′ (reverse). PCR was carried out with the primers and additional reagents: 10 μl 2X PCR mix, 4 μl tail DNA, and reagent-quality H2O. Size fractionation in a 1.5% agarose gel was used to analyze the PCR products (31).

**PCR**

PCR analysis was used to confirm the presence of the MUC1, PyMT, and GFP genes. Tail tissue DNA was extracted using the REDExtract-N-Amp Tissue PCR Kit (Sigma, Steinheim, Germany). One hundred nanomolar 5′-AGTCTAATCAATGACACCCAG-3′ forward primer and 5′-CTCTGAGAAGAATGTTGCGG-3′ reverse primer was used for the MT gene, and 100 nM 5′-CTGCCAGCAGCAGAACC-3′ forward primer and 5′-CTCTGGACATTGTAG-3′ reverse primer was used for the MUC1 gene. Primers for detection of the GFP gene included 5′-AAATGTCATCGACACCCAG-3′ (forward), 5′-TCTCTGAGAAGAATGTTGCGG-3′ (reverse), and internal positive control 5′-CTAGTCTCAATGGTCACGATCC-3′ (reverse). PCR was carried out with the primers and an additional reagents: 10 μl 2X PCR mix, 4 μl tail DNA, and reagent-quality H2O. Size fractionation in a 1.5% agarose gel was used to analyze the PCR products (31).

**MTT assay**

To determine the sensitivity of tumor cells to radiation, they were seeded in four replicates into 96-well tissue culture plates (1 × 104 cells/well) after receiving the indicated doses of radiation. Cells that did not receive radiation were incubated in culture medium and served as a control for cell viability (0 Gy). Cells were incubated at 37°C. Seventy-two hours after incubation, a 20-μl aliquot of MTT solution (5 mg/ml in PBS) was added to each well and incubated for another 4 h, followed by centrifugation at 800 rpm for 5 min. The supernatant in the culture was carefully aspirated, and 200 μl DMSO was added to each well and shaken for 10 min to dissolve the formazan crystals. The absorbance was measured at 590 nm by a microplate reader (33, 34). Cell viability was calculated as the percentage of MTT absorption as follows: percentage survival = (mean experimental absorbance/mean control absorbance) × 100%.

**FACS and immunocytochemical staining**

The mammary tumors were minced and incubated overnight in DMEM with 10% FCS, 2 mM l-glutamine, and 100 μg/ml both penicillin and streptomycin (Cellgro; Mediatech, Manassas, VA) in a Heracell CO2 incubator at 37°C and 5% CO2. The floating cells (most dead cells) were removed, and the adherent tumor cells were collected, cytocentrifuged, and stained with Abs against anti-CD44 (clone IMT), anti-Scal (clone D7; eBioscience, San Diego, CA), and Ki67 (clone MIB-1; Dako A/S, Hamburg, Germany) using a standard immunocytochemical (ICC) staining method. A similar method was used to detect the GFP+ metastatic colony cells from lungs of WT recipient mice that coexpressed CD44 and Scal. In addition, coexpression of CD44 and/or Scal, the epithelial-specific Ag (ESA; clone G8.8; eBioscience), and estrogen receptor (ER; clone MC-20; Santa Cruz Biotechnology, Santa Cruz, CA) was examined by FACS analysis.

**Clonogenic cell-survival assay**

Mammary tumor cells were trypsinized and diluted with growth medium to a single-cell suspension and plated into 10-cm tissue culture dishes (1 × 103 cells/dish). After the cells were attached to the dishes, they were irradiated (137Cs) source at a dose of 1.06 Gy/min at different dose levels and subsequently placed in an incubator until cells in the control group formed sufficient numbers of large clones (containing >50 cells). These clones were stained with 0.5% crystal violet and counted. The plating efficiency (PE) and the surviving fraction were calculated using the following formulas (35). PE = (number of colonies formed / number of cells seeded) × 100%. Surviving fraction = (number of colonies formed after treatment / number of cells seeded × PE) × 100%. The standard radiation survival curve was constructed, and the mean lethal dose, which represents the dose required to reduce the fraction of surviving cells to 37% of its previous value, was calculated by fitting the data with the multtarget–single hit model and linear-quadratic model (36, 37).

**Immunofluorescence staining**

Tumor cells plated into eight-well chambers were irradiated. After radiation, cells were fixed in 4% paraformaldehyde and treated with a 0.2% Nonidet P-40/PBS solution for 15 min at room temperature. Cells were washed with PBS and incubated for 2 h with anti-yH2AX (1:300 dilution; Upstate Biotechnology), followed by incubation with FITC-conjugated anti-mouse IgG (1:100 dilution) for 1 h. Slides were immersed in 0.05 mg/ml DAPI for 5 min and then mounted with coverslips using a Pro-Long Antifade Kit (Molecular Probes, Eugene, OR).

**Whole mount and H&E staining**

Mice were sacrificed at the indicated ages. For whole-mount preparation, mammary glands were harvested, and resected tissue was spread onto a slide and fixed in Carnoy’s fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid) for 2–4 h at 4°C. The tissue mount was washed in 70% ethanol for 15 min and 50% ethanol for 15 min, rinsed with distilled water for 5 min, and placed in a Carnime Alum staining solution overnight. Stained whole mammary glands were kept in 70% ethanol at 4°C for photographs. The solid masses, as indicated by the deep red staining with Carnime Alum, and areas > 1 mm2 were measured using Spot Advanced digital imaging software (Diagnostic Instruments, Sterling Heights, MI). Mammary glands also were embedded in paraffin, sectioned (5 μm), stained with H&E, and examined under a light microscope.
Colony-forming assay of lungs

To identify the disseminated cells in the lungs, sterile PBS, injected with a 24G needle via the right ventricle of the heart, was used to perfuse the lungs of blood before harvesting the lung tissue. The lungs were collected, minced, and digested in collagenase enzyme mixture solution, as previously described (38, 39). Single cells were cultured in 10% FCS DMEM for 2 wk and stained with anti-CD44 and anti-Scal Abs using standard ICC staining. Disseminated or metastatic tumor cells were determined by growth of tumor colonies in the plate with 0.5% crystal violet staining. Each GFP+ colony (>50 cells) was counted to quantify the disseminated cells for each mouse.

Injection and irradiation of mammary tumor cells in WT recipients

WT mice were anesthetized via i.p. injection of ketamine (100 mg/kg) plus xylazine (10 mg/kg). A total of 1 × 10^6 tumor cells from GFP+ MMT mice was injected into mammary fat pad (40). After 8–10 d, tumor growth reached around 1–5 mm size and the mice received 6- and 9-Gy ionizing radiation (IR) treatment using an X-RAD 320 (Precision X-Ray, N. Bradford, CT). The X-RAD 320 is a self-contained X-ray system for delivering a precise radiation dosage to the tumor in small animals. Some of the mice were also treated with Hsp70.PC vaccine obtained from DCs fused with irradiated MMT tumor cells. The mice were followed for up to 2 mo for the growth of tumors, which were measured twice weekly. At the end of the experiment, the mice were sacrificed, and the lungs, draining lymph nodes (LNs), and mammary tumors, if present, were harvested and examined.

T cell proliferation

Draining LNs were collected from immunized mice 7 d after the second vaccination or at the end of the experiment and LN cells (LNCs) were isolated. The LNCs were resuspended in RPMI 1640 medium containing 10% FCS, 2 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 5 × 10^{-5} M 2-ME in 96-well U-bottom plates in a volume of 200 µl/well for 5 d. T cell proliferation was assessed by [3H]thymidine incorporation after an additional 12-h incubation with 1 Ci/well [3H]thymidine. Radioactivity (mean ± SD of triplicates) was measured by liquid scintillation counting.

IFN-γ or tetramer staining

T cells from draining LNs were obtained after passing through a nylon wool column and then stained with anti-CD4/IFN-γ or anti-CD8/IFN-γ (BD Phar-mingen), according to the manufacturer’s instructions. For tetramer staining, T cells were incubated with PE-conjugated MUC1-8 iTAg (SAPDTRPA) or irrelevant iTAg (SIINFEKL) tetramer for 1 h at 4°C. After washing, the T cells were stained with FITC-conjugated anti-CD8a (clone 53-6.7) mAb for 40 min at 4°C. Cells were washed and fixed with 2% paraformaldehyde and analyzed by flow cytometry using CellQuest software (BD Biosciences).

CTL assay

Tumor cells from MMT mice, which were irradiated or not, monocytes (MCS), and unrelated tumor cells (2 × 10^5 cells/well) were prelabeled with 100 µCi Na_2[^3]CrO_4 for 60 min at 37°C, washed to remove unincorporated isotope, and used as targets. T cells isolated from draining LNs and/or spleens were purified through a nylon wool column and used as effector cells. The effector T cells or tumor target cells were resuspended in CTL assay medium at the indicated E:T ratios and placed in 96-well V-bottom plates for 5 h at 37°C. After incubation, the supernatants were collected, and radioactivity was quantitated in a gamma counter. Spontaneous release of [3H] was assessed by incubation of targets in the absence of effectors. Maximum and total release of [3H] were determined by incubation of targets in 0.5% Triton X-100. Percentage of specific [3H] release was calculated as ([experimental - spontaneous]/maximum - spontaneous) × 100.

Statistical analysis

Statistical significance was determined using the Student t test or χ² test. One-way ANOVA was used for analysis of data with more than two subgroups. The p values < 0.05 were considered statistically significant. SPSS Statistics v17.0 software (IBM, Somers, NY) was used for statistical analysis.

Results

Enrichment of tumor cells bearing stem cell markers by radiation

A number of studies showed that CSCs are more radioresistant than are other subpopulations of tumor cells (1, 2, 41). To identify the mammary tumor cells that are able to survive radiation, tumor cells were isolated from MMT mice and then sham irradiated or irradiated with escalating doses. The cells were cultured, and the surviving cells were counted. Tumor cell growth was inhibited by the parameter of MTT assay when the cells were irradiated by doses ≥3 Gy (Fig. 1A). Statistical significance was observed between nonirradiated tumor cells and cells irradiated with 6, 9, or 12 Gy (Fig. 1A). We next examined the populations of tumor cells surviving irradiation. In our previous studies, the subpopulations of mammary tumor cells expressing CD44 and Sca1 in MMT mice acted like CSCs, exhibiting increased tumorigenic and metastatic potential (40). Thus, we used CD44 and Sca1 as markers for CSCs to compare the subpopulations of tumor cells before and after radiation. Indeed, irradiation of tumor cells resulted in an enrichment in the population of cells expressing elevated levels of both CD44 and Sca1 (Fig. 1B). ESA expression was also increased in the irradiated cells, whereas the population of ER+ cells (marker for mammary-derived cells) remained constant (Fig. 1B). Increases in CSC surface markers were observed at 3 Gy, and they increased slightly as levels were increased to 12 Gy. The apoptosis of tumor cells after irradiation was also analyzed. The cells surviving after irradiation with the indicated doses of IR were triple stained with Sca1, CD44, and annexin V. Then, the gated Sca1 and CD44 double-positive cells were analyzed for apoptosis by annexin V staining. As shown in Fig. 1B, the numbers of apoptotic cells increased with an increasing dose of IR. The apoptotic cell fractions were 5.66 and 13%, respectively, for tumor cells irradiated with 6 and 12 Gy, suggesting that lethal damage also occurred in some of the double-positive cells (Fig. 1B, bottom panels). In addition, the population of tumor cells positive for CD44 or Sca1 increased with both fractionated 6-Gy and single 6-Gy IR (Fig. 1C, 1D). Approximately 18 and 25% of tumor cells subjected to either fractionated 6-Gy or single 6-Gy radiation were positive for CD44 or Sca1, respectively. These results show that tumor cells bearing CSC markers became preferentially enriched in cell populations exposed to IR.

Radioresistance of tumor cells bearing stem cell markers

The experiments in Fig. 1 indicate the enrichment of tumor cells bearing CSC markers, a finding that could suggest a potential survival advantage for these stem cells. To determine the radiosensitivity of this subset of tumor cells, we next treated mammary tumor cells, or not, with 6 Gy and allowed them to recover in culture. Two days later, the cultures were collected and irradiated again with the indicated doses of radiation and then subjected to a clonogenic cell-survival assay to determine their radiosensitivity. As shown in Fig. 2A, cells exposed to a priming dose of 6 Gy were more resistant to further radiation compared with control tumor cells. The mean lethal dose (D0) for initially nonirradiated tumor cells and 6-Gy–enriched surviving cells was 2.31 and 3.95 Gy, respectively, suggesting an increased radioresistance for IR-enriched cells with an increased CSC population. Consistent with this finding, the formation of phospho-γH2AX foci, an indication of triggering of the DNA damage response, was much less in the surviving tumor cells 3 h after radiation compared with nonpre-treated tumor cells (Fig. 2B, 2C). These experiments suggest a reduced acquisition of DNA damage in the IR-enriched tumor cells.

Increased metastatic potential of tumor cells selected by irradiation

We next examined the tumorigenic and metastatic potential of tumor cell populations enriched in CSCs by pre-exposure to 6 Gy. To facilitate identification of the disseminated tumor cells, we generated GFP+ MMT mice. The intracellular expression of GFP

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Statistics v17.0 software (IBM, Somers, NY) was used for statistical analysis.
did not alter mammary tumorigenesis (40). GFP-expressing tumor cells were isolated and irradiated with 6 Gy. Two days after radiation, the cells were transplanted into mammary fat pads of WT (non-MMT) mice. Tumors expressing GFP formed in WT mice injected with irradiated cells (Fig. 3A, upper panels). The tumors in the recipient mice resemble the mammary carcinoma under microscopy, with the formation of glandular structures (Fig. 3A, lower left panel), and they contain cells expressing stem cell markers (Fig. 3A, lower right panel). Although all mice injected with irradiated or nonirradiated cells eventually seeded primary tumors, those formed by nonirradiated cells progressed more vigorously than did those formed by irradiated cells (Fig. 3B). We also performed a cell number titration for tumorigenesis of the irradiated tumor cells. Tumors formed in mice inoculated with $50,000$ IR-selected tumor cells (Table I). Tumors did not form when 10,000 tumor cells were inoculated. These results suggest that the formation of primary tumor by IR-selected CSCs may not be as vigorous as those obtained by cell sorting (40). Contrary to the findings from the primary tumor, irradiated tumor cells appeared to possess increased potential for dissemination or metastasis. We used the ability to form cell colonies when minced lung tissue was incubated in tissue culture as one assay for

![FIGURE 1](image-url) Enrichment of tumor cells bearing CSC markers by irradiation. Mammary tumor cells were freshly isolated from MMT mice and irradiated. (A) Dose response of MMT tumor cells to IR treatment. MMT tumor cells received the indicated doses of IR, and cell viability was measured using an MTT assay. The statistical significance among groups that received 0 and 6, 9, or 12 Gy of IR was determined by one-way ANOVA. (B) Cells were double stained with anti-CD44 and Sca1, ESA, or ER Abs and analyzed by FACS. The percentage of double-positive cells is indicated. Bottom panels: Cells were triple stained with anti-CD44, Sca1, and annexin V Abs; Sca1/CD44+ cells were gated and analyzed for annexin V+ cells. (C and D) The cells were processed for ICC staining with anti-Sca1, anti-CD44, or Ki67 mAbs (original magnification ×40). Red color indicates cells positive for Sca1, CD44, or Ki67 that were counted and compared. *p < 0.01.

![FIGURE 2](image-url) Radioresistance of tumor cells enriched by irradiation. Tumor cells isolated from MMT mice were irradiated with 6 Gy or not and then cultured. (A) Two days after radiation, the surviving cells were irradiated further with the indicated doses of IR and assessed using a clonogenic cell-survival assay. (B and C) γH2AX foci with immunofluorescence staining (3 h). The nonirradiated tumor cells and IR-resistant cells were stained with anti-γH2AX mAb (original magnification ×40) (B) and the positive cells were counted and compared (C). *p < 0.01.
The validity of the assay as an index of dissemination was shown previously (40). As early as 4 d after tumor inoculation, colonies were observed in the lungs of recipient mice injected with 6 Gy–treated cells (Fig. 3C). Cells in the colonies expressed GFP, again indicating metastasis of mammary carcinoma cells from primary tumor. Colony number increased more rapidly in the preirradiated cells in the days postinoculation (Fig. 3C, 3D). In mice injected with control cells that were not preirradiated, substantial formation of lung colonies was not seen until day 26. The GFP+ lung colonies from both preirradiated cells and control cells contain populations of cells expressing CD44 or Sca1 (Fig. 3C, bottom panels). These results suggest that IR-enriched tumor cells possess enhanced dissemination ability.

FIGURE 3. Tumorigenic and metastatic potential of 6 Gy–enriched tumor cells. (A and B) A total of $1 \times 10^6$ 6 Gy–selected or unselected tumor cells isolated from GFP MMT mice were injected into the left and right mammary fat pads of syngeneic WT mice. The mice were sacrificed on the indicated day, and the mammary glands were harvested. The red arrow indicates the tumor in the mammary glands (A, upper panels). The mammary glands were further processed for sections that were stained with H&E or anti-CD44 mAb and examined under a microscope (original magnification $\times 60$). The red color indicates positive cells for CD44 (A, lower right panel), (B) The growth of tumor cells that received 0 or 6 Gy radiation. $p < 0.001$, t test. (C) Colony-forming assay of lung cells from recipient mice. Lungs were harvested and perfused to remove the circulating cells. The lung tissue was minced and digested in collagenase enzyme mixture solution, and cells were cultured in DMEM medium supplemented with 10% FCS for 2 wk and stained with 0.5% Crystal Violet. A colony that formed in the lung cell culture on day 30 from mice inoculated with tumor cells irradiated by IR was stained with PE-conjugated anti-CD44 (lower left panel) or Sca1 (lower right panel) mAbs and examined under a fluorescent microscope (original magnification $\times 10$). (D) The numbers of tumor colonies from the lungs of recipient mice were counted. *$p = 0.006$ on day 26, *$p = 0.013$ on day 30, t test.

### Table I. Tumorigenicity of MMT tumor cells selected by IR

<table>
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<tr>
<th>No. of Tumor Cells Injected</th>
<th>No. of Recipient Mice</th>
<th>Tumor Incidence</th>
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<td>$1 \times 10^6$</td>
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MMT tumor cells pre-exposed to 6 Gy radiation were injected into the mammary fat pad of recipient WT mice. The mice were followed for up to 60 d, and tumor growth was determined.

CTL-targeting radioresistant tumor cells induced by Hsp70. PCs extracted from DC-radioresistant tumor fusion cells

We next examined the feasibility of selectively eradicating radioresistant cells by immunotherapy. We previously described the preparation of a powerful vaccine from fusions of DC and tumor cells that can target subpopulations of tumor cells, such as CSCs (42). Therefore, DCs generated from WT mice were fused to radioresistant mammary tumor cells selected by 6-Gy IR, as described (43). The Hsp70.PC-F (from fusion of DC with radio-resistant tumor cells) were immunopurified with anti-Hsp70 Ab (43) and used to vaccinate mice. As a control, we used the Hsp70 vaccine from tumor cells that were not fused to DCs (Hsp70.PC-Tu). The Hsp70.PC-Tu vaccine is less active than the Hsp70.PC-F vaccine but is useful to control for the procedures used in vaccine preparation. After two immunizations with the Hsp70.PC-F or Hsp70.PC-Tu vaccine, the mice were sacrificed, and LNCs were isolated and assayed for T cell proliferation and CTL activity. Immunization with the Hsp70.PC-F vaccine (10 $\mu$g) resulted in more vigorous T cell proliferation in both WT and MUC1.Tg mice than in those immunized with the Hsp70.PC-Tu vaccine or treated with PBS alone (Fig. 4A). Similarly, immunization with the Hsp70.PC-F vaccine induced the highest levels of CD4 or CD8 T cells expressing IFN-γ, as well as CD8 T cells positive for MUC1 tetramer (Fig. 4B), suggesting the induction of effector T cells.

To determine the killing ability of CTLs induced by the Hsp70. PC vaccines, LNCs and splenocytes from mice immunized with
either of the Hsp70 vaccines or injected with PBS were incubated
with tumor cells, without or with 6-Gy irradiation, or MCs, and
cytotoxicity was measured using a standard method. Much higher
levels of CTL activity against radioresistant tumor cells were
observed in T cells from mice immunized with the Hsp70.PC-F
vaccine compared with those immunized with the Hsp70.PC-Tu
vaccine (Fig. 4C). In contrast, minimal killing was observed in
CTLs against autologous MCs, suggesting tumor specificity of the
CTLs (Fig. 4C). These results suggest that tumor antigenic pep-
tides derived from radioresistant tumor cells are effectively pre-
sented to the host T cells that possess the ability to kill the radio-
resistant breast cancer.

Combined radiotherapy and immunotherapy in the treatment of
mammary tumors with metastasis

In our previous investigation, immunization of mice with Hsp70.
PC-F vaccines prevented the growth of tumors (43). However,
immunotherapy alone is not sufficient to treat well-established
tumors (data not shown). Therefore, in the current study we eval-
uated the efficacy of combined HSP-based immunotherapy and
radiotherapy in the treatment of established mammary tumors. An
Hsp70.PC-F vaccine was extracted from hybrid cells after fusing
cells generated from bone marrow cells (44–46) and radioresistant
mammary tumor cells isolated from GFP+ MMT mice selected by
6 Gy. Mammary tumor cells from GFP+ MMT mice were isolated,
and $1 \times 10^6$ tumor cells were inoculated into the fat pad of
mammary glands of WT mice. The rationale for using WT mice
is that the MMT mouse is not a suitable model for radiotherapy
because of the growth of multiple spontaneous tumors. In addi-
tion, GFP expression in the tumor cells from MMT mice, when
transplanted in the WT mice, facilitates the detection of dissem-
inated tumor cells. The mice were treated with combined radio-
therapy and immunotherapy, as shown in Fig. 5A. All mice treated
with radiotherapy and control Hsp70.PC-Tu vaccine or PBS de-
tailed tumors in contrast, tumors were absent from six of nine
mice that were treated with combined radiotherapy and Hsp70.
PC-F vaccine (Fig. 5A). Consistent with these results, the highest
levels of T cell proliferation and CTL induction were observed in
mice treated with radiotherapy and the Hsp70.PC-F vaccine (Fig.
5B, 5C). Next, to investigate disseminated tumor cells, lung cells
from recipient mice were isolated, stained with anti-CD44 or anti-
Sca1 mAbs, and analyzed for GFP+ disseminated tumor cells. As
shown in Fig. 5D, a significant reduction in GFP+ tumor cells was
observed in the lungs of mice treated with radiotherapy and the
Hsp70.PC-F vaccine, suggesting the inhibition of metastasis. Simi-
lar results were observed in the colony-forming assay. The for-
mation of tumor cell colonies from mice immunized with Hsp70.
PC-F was significantly reduced compared with those from the
control groups (Fig. 5E). Together, these experiments indicate that
the antitumor immunity induced by Hsp70.PC-F combined with
radiotherapy promotes the inhibition of primary and disseminated
tumor cells.

Therapeutic value of T cells stimulated by Hsp70.PCs

To determine the therapeutic value of T cells stimulated by
Hsp70 vaccines, draining LNs were harvested from mice immu-
nized twice with either the Hsp70.PC-F vaccine or the control
Hsp70.PC-Tu vaccine or injected with PBS. T cells were isolated,
cultured for 2 d, and then used for treatment of mice with estab-
lished tumors in combination with radiotherapy. WT mice were
inoculated with $1 \times 10^6$ GFP+ tumor cells. Mice were treated with
radiation on days 8 and 11, followed by three injections of T cells
prepared from mice stimulated by the Hsp70.PC-F vaccine, tumor
vaccine, or PBS (Fig. 6A). Tumor development was observed in

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**FIGURE 4.** T cell response elicited by HSP70.PC-F vaccine prepared from 6 Gy–selected tumor cells. (A) T cell proliferation assay. Draining LNCs obtained from WT mice (left panel) or MUC1.Tg mice (right panel) immunized with HSP70.PC derived from fusion cells of DCs and 6 Gy–irradiated tumor cells (Hsp70.PC-F) or 6 Gy–irradiated tumor cells (Hsp70.PC-Tu). Control mice were injected with PBS. LNCs were cultured for 5 d, and [3H]thymidine was added during the last 12 h of culture. The incorporation of [3H]thymidine was measured. (B) LNCs obtained from immunized mice were analyzed by FACS for expression of IFN-γ in CD4 and CD8 T cells and/or MUC1 tetramer in CD8 T cells. The percentage of double-positive cells is indicated. (C) CTL assay. LNCs and splenocytes were isolated from mice immunized twice with Hsp70.PC-F, Hsp70.PC-Tu, or PBS and incubated with 51Cr-labeled IR–selected tumor cells, nonirradiated tumor cells, or MCs at E:T ratios of 60:1 (LNCs) or 100:1 (splenocytes). CTL activity was determined by [51Cr]-release assay.
all mice treated with radiation and T cells stimulated with the Hsp70.PC-Tu vaccine or PBS (Fig. 6B). In contrast, tumors were not found in six of seven mice treated with radiation and T cells stimulated with the Hsp70.PC-F vaccine (Fig. 6B). In addition, the T cells were able to kill the disseminated tumor cells. GFP+ colonies in the culture of lung cells from mice treated with radiation

FIGURE 5. Combined radiotherapy and immunotherapy in the treatment of mammary tumors with early metastasis. (A) Naive mice were inoculated with $1 \times 10^6$ of GFP+ MMT tumor cells in the mammary fat pad. The tumor was irradiated on days 8 and 10 using an X-RAD 320 X-Ray Biological Irradiator (Precision X-Ray). Subsequently, the mice were divided randomly into three groups and immunized with 10 µg Hsp70.PC-F or Hsp70.PC-Tu. Mice injected with PBS were used as control. The tumors were measured for up to 45 d, and tumor volume and tumor incidence are presented. (B) T cell proliferation. (C) CTL activity against IR-selected tumor, unselected tumor, or unrelated tumor cells (MC38) with E:T ratios of 100:1. CTL activity of LNCs (left panel) or splenocytes (right panel) against indicated targets was determined by [51Cr]-release assay. (D) The lungs collected from individual mice were carefully perfused to remove circulating cells before being processed to determine the GFP+ tumor cells by FACS. The percentage of GFP+ disseminated cells in total lung cells is indicated for each group. (E) Numbers of tumor cell colonies from the lungs of recipient mice. *p < 0.001, one-way ANOVA.

FIGURE 6. Combined radiotherapy and adoptive immunotherapy for the treatment of mammary tumors with early metastasis. (A) Diagram of experimental design, which includes priming T cells and their isolation, tumor inoculation, radiation treatment, and adoptive T cell transfer. (B) The recipient mice were sacrificed on day 60 after tumor inoculation. Primary tumor was determined, and tumor incidence is presented. (C) The GFP+ disseminated tumor cells in the lungs were assessed by colony-forming assay. *p < 0.001, one-way ANOVA.
and Hsp70.PC-F vaccine–induced T cells were significantly reduced compared with their counterparts treated with radiation and T cells stimulated by the Hsp70.PC-Tu vaccine or PBS (Fig. 6C). Together, these experiments show that T cells stimulated by the Hsp70.PC-F vaccine are able to enhance the curability of primary tumors by radiotherapy and eliminate secondary disseminated tumor cells, suggesting their therapeutic value in established cancer.

Discussion
Radiotherapy is one of the most widely used cancer treatments, ranking second only to surgery in its rate of use. Meta-analysis of tens of thousands of patients treated with radiation as a component of their breast cancer treatment showed that it improves overall survival for patients with early-stage and advanced disease (2, 47, 48). However, despite this progress in radiotherapy, many patients still die of distant metastasis and local recurrence. The failure of radiotherapy is, by definition, triggered by cells that survive radiation. The response of tumors, including breast cancers, to radiation is determined by intrinsic cellular radiosensitivity, micro-environmental factors (e.g., tumor hypoxia), and the means by which the radiation is delivered (49, 50). However, it has been well documented that the number of CSCs, or clonogens, in a given tumor is also an important determinant of radioresistance, or poor response to radiotherapy (1, 51–53). These results suggest a scenario in which a few CSCs can survive the radiation as a result of their intrinsic radioresistance, resulting in local recurrence and/or metastasis. Consistent with these findings, we show the preferential survival of tumor cells bearing stem cell markers where the surviving tumor cells are more resistant to irradiation and demonstrate increased ability to disseminate (Figs. 1–3). In the current study, the primary tumor grew much more slowly in mice inoculated with tumor cells irradiated with 6 Gy than in their counterparts injected with nonirradiated tumor cells. It is possible that the CSCs, although selected by irradiation, may also incur sublethal damage, leading to reduced proliferation during this treatment. The cells may need time to recuperate, reverse cell cycle arrest, andrepair radiation damage, resulting in slow growth of the primary tumors. Nevertheless, these tumor cells were more resistant to radiation by the criterion of clonogenic cell survival (Fig. 2). These experiments further suggest potential links between CSCs and radioresistance. Thus, therapy that targets the radioresistant clones and metastatic tumor cells bearing stem cell markers may be important for improving the curability of tumors.

CSCs could potentially be targeted for therapy through the molecules in the signaling pathways that regulate CSC renewal and pluripotency and are essential for their survival. However, such molecules may be difficult to identify because there are many similarities between normal stem cells and CSCs, including the molecules in the signaling pathways (54). Alternatively, CSC-targeted immunotherapy could be used, because it was recognized that immunotherapy can eliminate disseminated tumor cells, and it works most effectively in hosts with minimal tumor burden (55–57).

CSC-specific Ags would be ideal targets for immunotherapy. However, such Ags have not been identified. To circumvent this problem, we used molecular chaperone-based vaccines with enhanced immunogenicity through extraction of Hsp70.PCs from fusions of tumor and DCs (43, 58). In our previous studies, the formulation of Hsp70.PC-F vaccines was associated with elevated levels of Hsp90 and with abundant levels of the immunogenic tumor Ag peptides (43). The Hsp70.PC-F vaccine possesses powerful immune properties. These include stimulation of DC maturation, significant increases in CD8 T cells, and induction of effector and memory T cells able to break T cell unresponsiveness to nonmutated tumor Ags and provide protection to mice against challenge with tumor cells (43). In contrast, the immune response to vaccination with Hsp70.PC derived from tumor cells alone is muted against such nonmutated tumor Ag. Thus, the Hsp70.PC-F vaccines have enhanced immunogenicity and constitute an improved formulation of molecular chaperone-based therapy that can be complementary to radiotherapy. The rationale for extraction of Hsp70 from fusions of DCs and radioresistant tumor cells is the assumption that the chaperoned polypeptide cargoes of the HSP complexes will contain antigenic peptides specific for radioresistant tumor cells. Thus, CTLs specific for radioresistant tumor cells can be induced. Indeed, this molecular chaperone vaccine induced CTLs that preferentially killed radioresistant tumor cells bearing stem cell markers (Fig. 4). We used a tetramer assay to compare the induction of CTL against a tumorigenic peptide epitope. Although a tetramer specific for radioresistant tumor cells might be preferred, one is not available. Thus, we used a tetramer against the MUC1 peptide SAPDTRPA as a measurement of the induction of specific CTLs by the Hsp70.PC vaccine. In the current study, irradiation enriched the cell population expressing stem cell markers (Fig. 1). It was reported that tumor-associated Ags and MHC molecules are upregulated in CSCs (10). This may partially explain the enhanced lysis of irradiated tumor cells by CTLs induced by Hsp70.PC-F (Fig. 4C). Importantly, combined radioimmunotherapy improved the therapeutic effect of either modality alone by significantly inhibiting the primary tumor and eliminating disseminated tumor cells (Fig. 6). Six of nine mice treated with radiation and immunization with Hsp70.PC-F were free of tumors, and tumors grew very slowly in the remaining three mice. It is possible that the induction of CTLs against the tumor cells surviving the in vivo irradiation by Hsp70.PC-F may play an important role in the enhanced therapeutic effect. In addition to the specificity of T cell–mediated immune response by a chaperone vaccine, radiation has the potential to induce specific danger signals that are sensed by immune components, such as DCs, and lead to the activation of an adaptive immune response (59, 60). In this context, combined radiotherapy and immunotherapy has the potential for synergistic or additive effects against cancer.

The immunocompromised condition of many cancer patients may not be conducive to active immunotherapy because their immune system is likely to be suppressed by cancer and/or chemotherapy. To circumvent this problem, autologous T cells can be harvested and stimulated ex vivo and then infused back into patients. Our results show that such adoptive immunotherapy, using Hsp70.PC-F–stimulated T cells, is effective when combined with radiotherapy.

In summary, the current study explores the feasibility and efficiency of Hsp70.PC-F vaccine–based immunotherapy to target the populations of tumor cells that are likely to survive radiotherapy. In our model, immunotherapy, either by active immunization or adoptive transfer of T cells, can enhance the curability of established tumors by radiotherapy and eliminate disseminated tumor cells. Thus, radioimmunotherapy may hold promise for the treatment of breast cancer.

Disclosures
The authors have no financial conflicts of interest.

References