Enhancement of Cancer Radiation Therapy by Use of Adenovirus-Mediated Secretable Glucose-Regulated Protein 94/gp96 Expression

Shanling Liu,¹,² He Wang,³ Zhonghui Yang,¹ Takashi Kon,¹ Jiangao Zhu,² Yiting Cao,¹ Fang Li,³ John Kirkpatrick,¹ Christopher V. Nicchitta,² and Chuan-Yuan Li¹

Departments of ¹Radiation Oncology, Immunology, and ²Cell Biology, Duke University Medical Center, Durham, North Carolina; ³West China 2nd University Hospital, Sichuan University, Chengdu, China

Abstract
Tumor-derived glucose-regulated protein 94 (GRP94/gp96) has shown great promise as a tumor vaccine. However, current protein-based approaches require the availability of large quantities of tumor tissue, which are often not possible. In addition, the efficacy of immunotherapy is often not ideal when used alone. In this study, we explored the therapeutic efficacy of a combined GRP94/gp96-based genetic immunotherapy and radiation therapy strategy in the weakly immunogenic and highly metastatic 4T1 murine mammary cancer model. An adenovirus encoding a modified, secretable form of GRP94 gene (AdsGRP94) was constructed and evaluated in various antitumor experiments. Lethally irradiated, virus-infected cells were used as vaccines. Adenoviral vectors were also injected directly into tumors in conjunction with tumor irradiation. Vaccination with lethally irradiated, AdsGRP94-infected 4T1 cells completely prevented subsequent tumor growth from challenge inoculations of as many as $10^7$ cells per mouse. In established tumor models, vaccinations alone had minimal effect on local and metastatic tumor growth. However, when vaccination was combined with radiation therapy and i.t. AdsGRP94 injections, local tumor growth and pulmonary metastasis were markedly inhibited. In some cases, complete tumor regression was observed. In these cases, the mice were resistant to subsequent tumor challenge and remain tumor free up to 10 months after initial therapy. Our results indicate that combined AdsGRP94-based immunotherapy and radiation therapy may be a potentially effective strategy for cancer treatment. (Cancer Res 2005; 65(20): 9126-31)

Introduction
In recent years, the stress protein glucose-regulated protein 94 (GRP94) gained widespread attention due to its potential in cancer immunotherapy. Tumor-derived GRP94/gp96 was shown to elicit potent antitumor responses in various murine tumor models (1, 2). Vaccines based on this protein are currently being evaluated in various clinical trials (3–5). The antitumor response has largely been attributed to the ability of GRP94 to form stable complexes with tumor-derived antigenic peptides and thereby facilitating the cross-presentation of MHC class I–restricted epitopes and priming of CD8+ effectors responses (6). In addition, a role for GRP94/gp96 in the activation of innate antitumor immune response has been established (7, 8). Indeed, it has recently been proposed that GRP94-elicited tumor immunity occurs primarily through the activation of innate immune responses (9). On the other hand, it was shown that GRP94 stimulated dendritic cell maturation and elicited cytokine and chemokine secretion from macrophages and dendritic cells in vitro (8, 10). It was also found that GRP94/gp96 could elicit a marked increase in tumor necrosis factor-$\alpha$ and IFN-$\gamma$ through activation of Th1 cytokine production in CD4+ cells (11). In this study, we sought to examine the potential of GRP94/gp96-based immunotherapy to enhance cancer radiation therapy. The main goal is to achieve both local tumor control and to prevent distant metastases. To achieve our goal, we engineered an adenovirus that can efficiently infect tumor cells and mediate the expression of a modified, secretable GRP94 gene. This approach was adopted to exploit the adjuvant-like properties of the protein and its ability to bind and present tumor-derived peptides to antigen-presenting cells (APC). In addition, it may obviate the need for large amount of tumor tissues and broaden the applicability of a stress protein–based immunotherapy strategy. We evaluated the effectiveness of this approach in combination with radiation therapy in a highly malignant, metastatic murine breast cancer model 4T1.

Materials and Methods

Cell culture. The highly metastatic, weakly immunogenic murine mammary cancer cell line 4T1 (H-2d) was obtained from Dr. Fred Miller (Michigan Cancer Foundation, Detroit, MI). Human embryonic kidney cell line Ad293 and murine colon cancer cell line CT26 were obtained from the American Type Culture Collection (Manassas, VA).

Mice. Female BALB/c mice (H-2d) were obtained from Charles River Laboratories (Raleigh, NC). Animals were maintained and cared for in accordance with the Duke University Institutional Animal Care and Use Committee guidelines.

Construction of recombinant adenovirus vectors. A recombinant adenovirus carrying a modified version of the GRP94 gene was constructed by use of the AdenoMax system from the Microbix Corp. (Toronto, Ontario, Canada) according to the procedure provided by the manufacturer. The amplification and purification was carried out by CsCl2 gradient centrifugation according to published procedures (12, 13). Virus titer was determined by plaque assay in Ad293 cells.

Vaccinations. To vaccinate mice, 4T1 cells were infected by AdsGRP94GFP or AdGFP (control) by adding virus to cells at a multiplicity of infection of 20 to 40 plaque-forming units (pfu) per cell in serum-free DMEM. Cells were incubated at 37°C for 1 hour before adding fresh DMEM.
to achieve fetal bovine serum concentration of 2%. Infected cells were irradiated with 100 Gy in a Mark 1 $^{137}$Cs irradiator (JS Shepard and Associates, San Fernando, CA), 24 hours after infection and injected into animals s.c. in the flank.

**Tumor growth delay.** For tumor growth delay assay, about $1 \times 10^6$ 4T1 cells were injected s.c. into BALB/c mice in 30 to 50 µL of PBS solution. Tumor growth was then followed evaluated by use of a caliper every 2 to 3 days. Tumor volume was calculated using the following formula: volume = length $\times$ width$^2$ $\times$ $\pi/6$. For histologic evaluation, tumors were removed at 2 weeks, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4-µm thickness, and stained with H&E.

**Cytotoxicity assay.** In vitro CTL activity assay was done following a published protocol (14).

**Enzyme-linked immunospot assay.** Enzyme-linked immunospot (ELISPOT) assays were done according to the manufacturer's manual (Mabtech, Mariemont, OH) to assess the IFN-γ production in splenocytes derived from vaccinated mice following in vitro stimulation (15).

**Treatment of established tumors.** BALB/c mice were inoculated s.c. with $1 \times 10^6$ of 4T1 cells in the right leg. Irradiation of tumor was given on days 8 and 10 at 10 Gy each. In some tumors, the mice were vaccinated. For vaccination, 4T1 cells were injected by AdsGRP94GFP or AdGFP and irradiated 24 hours later with 100 Gy of $\gamma$-rays. Irradiated cells were washed extensively with PBS and vaccination was done by i.p. injection of $5 \times 10^5$ cells, four times, on days 8, 10, 15, and 17. i.t. injection of virus ($1 \times 10^6$ pfu per mouse) was given on days 10, 15, and 17 in PBS. Tumor growth was measured every other day after tumor cell inoculation. Each experimental group contained 10 to 12 mice.

**Statistical analysis.** Statistical analysis of tumor growth inhibition, cytotoxicity assays, and ELISPOT assay were done using paired or unpaired Student's $t$ test, as appropriate. The survival analysis was done using Wilcoxon rank tests. $P_s < 0.05$ were considered statistically significant. All the tests are two sided.

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**Results and Discussion**

A modified GRP94 gene was cloned into an adenovirus shuttle vector. The modified gene was devoid of the COOH-terminal KDEL endoplasmic reticulum (ER) retention/retrieval sequence. Therefore, instead of being retained in the ER, the modified protein should be secreted. To identify the modified gene product more easily, the GFP was fused to the COOH terminus of the modified gene to form the fusion gene sGRP94-GFP (Fig. 1A). Virus vectors encoding this new fusion gene were successfully packaged and expanded in Ad293 cells. The newly prepared virus vectors were then used to infect 4T1 murine breast cancer cells. Expression of the fusion gene was examined in the supernatants and cellular lysates of the infected cells. Robust expression of the fusion gene could be observed from day 1 (Fig. 1B), consistent with a previous study (8). The expression peaked at around 48 hours and remained stable for up to 6 days. High-dose irradiation of the infected cultures did not seem to have any effects on the level of fusion protein expression. In addition, it was observed that infection of 4T1 cells by AdsGRP94GFP had no observable effect on 4T1 cell growth in vitro (data not shown).

To examine potential sGRP94-mediated antitumor immunity, 4T1 cells were infected with AdsGRP94GFP and then injected into the right flank of syngeneic BALB/c mice. Tumor formation was then followed in the injected mice. As shown in Fig. 2A, expression of sGRP94-GFP could delay tumor growth significantly ($P < 0.05$). The average times for tumors to reach 100 mm$^3$ were 45 days for AdsGRP94GFP group, 31 days for AdGFP group, and 18 days for the noninfected 4T1 group, respectively. In two of five mice injected with AdsGRP94GFP-infected 4T1 cells, tumors began to regress at 2 weeks and disappeared completely on days 24 and 32, respectively (Fig. 2B). When 4T1 cell were reinoculated ($1 \times 10^6$ cells per mouse) into these two mice, no tumor growth could be observed, indicating memory from the immunoeffector cells. In contrast, in control mice injected with noninfected control or AdGFP-infected 4T1 cells, tumors continued to grow aggressively.

Additional experiments were conducted to examine tumor histology. Two weeks after injection of 4T1 cells that have been infected with null vectors (control), AdsGRP94GFP, or AdGFP, tumors that emerged were excised and stained with H&E. Control 4T1 cells alone generated a few small areas of localized infiltrates of inflammatory lymphocytes (Fig. 2C, left). AdGFP-infected 4T1 cells produced more patches of moderately intense inflammatory infiltrates containing mainly of lymphocytes (Fig. 2C, middle). However, AdsGRP94GFP-infected 4T1 cells generated extensive and dense infiltrates of inflammatory cells (Fig. 2C, right). These findings suggest that in situ expression of GRP94 in AdsGRP94GFP-infected 4T1 tumors may promote an increase in the infiltration of immunoeffector cells, which may be responsible for the observed tumor rejection.

We further examined the prophylactic efficacy of sGRP94 expression on tumor growth inhibition. In these experiments, animals were immunized with lethally irradiated 4T1 cells infected with AdsGRP94GFP, AdGFP, or mock virus. After four weekly vaccinations, the host BALB/c mice (female) were challenged with s.c. injections of live 4T1 cells at 10$^7$ cells per mouse. In the null virus group, rapid tumor growth was observed. In the AdGFP group, delayed tumor growth was observed, indicating some degree of protective immunity. In contrast, the AdsGRP94GFP group showed complete resistance to 4T1 tumor cell challenge (Fig. 3A).

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**Figure 1.** Adenovirus-mediated secreted GRP94 gene expression. A, schematic representation of the adenovirus, AdsGRP94GFP, encoding the fusion protein between a modified, secretable GRP94 and GFP. The modification of GRP94 involves the deletion of the COOH-terminal KDEL sequence that anchored the whole protein to the ER. The fusion gene is placed under the control of a constitutively active cytomegalovirus promoter (CMV). B, AdsGRP94GFP-mediated expression of the fusion protein in murine breast tumor 4T1 cells. The cells were infected and irradiated 24 hours later on day 0. Supernatants were collected from the infected cells at different time points after irradiation. Negative control is supernatant collected from control vector AdGFP-infected 4T1 cells. Positive control, cellular lysates from AdsGRP94GFP-infected Ad293 cells. The antibody used was against GRP94; it was purchased from StressGen.
To quantify the ability of secreted GRP94 to stimulate antitumor immunoeffectors, splenocytes were obtained from mice that had undergone four weekly vaccinations (by use of 4T1 cells infected with null, AdGFP, or AdsGRP94GFP virus vectors) and assayed for their cytolytic activities against 3H-labeled 4T1 cells (Fig. 3B). When effector to target ratio is 100:1, significant increases in cytolytic activities were observed in AdsGRP94GFP group (with 79% cytotoxicity) when compared with AdGFP (with 81% cytotoxicity, \( P < 0.01 \)). In comparison, when a nonrelated colon cancer line CT26 was used as a target to evaluate splenocytes, no significant differences were observed among the three treatment groups despite a trend that suggested increased cytolytic activity in the AdsGRP94GFP group (Fig. 3C). At an effector/target ratio of 100:1, splenocytes from mice immunized with 4T1-AdsGRP94GFP showed 19.0% cytotoxicity against CT26 cells compared with splenocytes from mice immunized with 4T1 or 4T1-AdGFP, which showed 8.1% and 12.1% cytotoxicity, respectively.

Furthermore, the ELISPOT assay was used to examine the tumor cell–specific secretion of IFN-\( \gamma \), a recognized indicator for activated immune response from the splenocytes. As shown in Fig. 3D, a significant elevation in the level of IFN-\( \gamma \) was observed in cells obtained from animals immunized with AdsGRP94GFP-infected 4T1 cells compared with control AdGFP-infected 4T1 cells (\( P < 0.01 \) for 4T1-AdsGRP94GFP versus 4T1-AdGFP). It is of interest to note that splenocytes of 4T1-GFP-immunized mice secreted higher levels of IFN-\( \gamma \) compared with that from parental 4T1 cell–immunized mice (\( P < 0.01 \)). These results indicate that adenovirus and GFP proteins can also stimulate the host’s immune system.

To evaluate the efficacy of the secreted GRP94 immunotherapy approach in a more clinically relevant situation, experiments were conducted in mice with established 4T1 tumors. About \( 1 \times 10^5 \) 4T1 cells were injected s.c. into the hind limb of syngeneic BALB/c mice. A week later, a combination of radiation therapy, i.p. vaccinations, and i.t. virus injections was carried out as represented in the scheme shown in Fig. 4A. A variety of variables were then evaluated in the treated mice.

Tumor growth was measured every other day. The results were shown in Fig. 4B. In contrast to prophylactic immunization experiments as described in Fig. 3, vaccination alone had minimal effect on tumor growth in the established tumors. There was no significant difference between AdsGRP94GFP group and AdGFP control group, although there were differences between these groups and the nontreatment control group (\( P > 0.05 \) for AdsGRP94GFP versus AdGFP; \( P < 0.05 \) for AdsGRP94GFP or AdGFP versus nontreatment control group). However, in mice treated with both radiation and immunization (which included weekly i.p. vaccinations and i.t. viral delivery), significant enhancement of radiation therapy by immunotherapy was observed (\( P < 0.01 \) for AdsGRP94GFP + radiation therapy versus AdsGRP94GFP and AdsGRP94GFP + radiation therapy versus AdGFP + radiation therapy group).

Combined radiotherapy and vaccinations with 4T1-AdGFP could delay \textit{in situ} tumor growth significantly when compared with nonirradiated treatment (Fig. 4B). Interestingly, in the AdsGRP94GFP+ AdGFP vaccination–alone group, the survival rate was significantly different from the AdGFP group (\( P < 0.05 \) for AdGFP versus AdGFP) despite similar sizes of the local tumors, suggesting a possible effect of AdsGRP94GFP on metastatic tumor growth despite its lack of efficacy on primary tumor growth (Fig. 4C). In the group treated with radiation, i.t. AdsGRP94GFP gene delivery, and i.p. vaccinations of 4T1 cells infected with AdsGRP94GFP vectors,
tumor growth was significantly slower than all three control groups. Furthermore, two of the mice in this group were cured completely and survived tumor free for >300 days after tumor cell inoculation.

The fact that the radiation + AdGFP group showed a clear effect on tumor suppression but minimal effect on host survival indicates that metastasis play a significant roles in these mice. This is consistent with previous findings indicating that 4T1 is a highly metastatic tumor line. To quantitatively determine tumor metastases in various treatment groups, 4T1 cells stably transduced with the firefly luciferase gene (4T1-Luc) were injected into the tail vein of mice from various treatment groups. Tumor growth from the 4T1-Luc cells were then followed by use of a noninvasive Xenogen IVIS system (Cranbury, NJ) designed for bioluminescence detection and quantification. As shown in Fig. 4D, a clear difference was seen between the treatment and control groups with only the radiation + AdsGRP94GFP treatment group remaining completely metastasis-free during the whole course of the experiment. The bioluminescence observations were also confirmed by direct examination of dissected lungs from the 4T1-Luc-injected mice (Fig. 4D, bottom). These results indicate that robust systemic antitumor immunity was generated in the radiation + AdsGRP94GFP treatment group.

Further experiments were carried out to quantify growth delay with increasing doses of radiation therapy. Tumor-bearing mice were treated with different doses of irradiation along with a combination of i.t. virus injections and i.p. vaccinations of virus-infected 4T1 cells (Supplementary Fig. S1). When during the time to reach 5-fold the initial tumor volume was measured, the experiments clearly indicated robust dose response with increasing irradiation dosage. Secretable GRP94-based immunotherapy significantly enhanced the growth delay of radiation-treated tumors in a dose-dependent manner, causing increased delays of 5, 10, and 12 days when tumors were irradiated with 2 Gy, 2 Gy, or 2 Gy, respectively.

Figure 3. Secreted GRP94-induced protective immunity against tumor cell through prophylactic vaccination. A, protective immunity against 4T1 cells in mice that have been vaccinated by use of irradiated 4T1 cells that were infected with AdsGRP94GFP. The mice were vaccinated with four weekly injections of the tumor cell vaccines. To generate the vaccines, 4T1 cells were infected with AdsGRP94GFP, AdGFP, or null virus at a multiplicity of infection of 40 pfu per cell. Twenty-four hours after virus infection, the cells were irradiated with 100 Gy of γ-rays. About five million irradiated cells were then injected into the mice s.c. After four weekly vaccinations, live 4T1 cells (1 × 10⁶ cell per mouse) were injected s.c. and their growth was followed. Points, mean tumor volume of five mice; bars, SE. B, cytotoxic activities against 4T1 cells. Splenocytes from mice that have undergone the four weekly vaccinations were obtained, cultured, stimulated, and their activities against live 4T1 cells were measured using the JAM test. Statistically different activities were observed between the AdsGRP94GFP and AdGFP group (P < 0.03) or between the AdsGRP94GFP and null control group (P < 0.02) when the effector to target ratio is 100:1. C, cytotoxic activities against nonrelated CT26 cells. Splenocytes from mice that have undergone the four weekly vaccinations were evaluated for their activity against nonrelated CT26 cells syngeneic with the BALB/c mice. At effector to target ratio of 100:1, the difference between the experimental groups (AdsGRP94GFP versus AdGFP, AdsGRP94GFP versus null virus group) were not significant (P > 0.05 and 0.1, respectively). Points, mean percent cytotoxicity obtained from five mice (three replicates from each experiment); bars, SE. D, ELISPOT assay of 4T1-specific IFN-γ secretion from splenocytes. Splenocytes were derived from mice that have undergone four weekly vaccinations. Columns, mean from five mice; bars, SE. The difference between the AdsGRP94GFP and the control groups was significant (P < 0.01).
Gy, is ~ 2.0 as similar growth delays (~20 days) were achieved when 2 × 5 Gy radiotherapy + sGRP94 immunotherapy or 2 × 10 Gy radiotherapy alone was administered. These data suggest that sGRP94 immunotherapy can enhance radiotherapy across a wide dose range.

Additional experiments were also carried out to dissect the relative contributions of i.t. AdsGRP94GFP injection versus vaccination with AdsGRP94GFP-infected cells in enhancing radiation therapy. When during the time to reach 5-fold the initial tumor volume was measured in various treatment groups (Supplementary Fig. S2), i.p. vaccinations with 4T1 cells infected with AdsGRP94GFP virus had very similar and significant antitumor effects in both control (7 days) and radiation-treated groups (7.5 days), indicating that i.p. vaccinations had a significant antitumor effect independent of radiation therapy (Supplementary Fig. S2, left). On the other hand, i.t. injections of AdsGRP94GFP had almost no effect in nonirradiated tumors (~0.5 day) but a dramatic effect (11.25 days) in irradiated tumors, indicating a synergistic effect with ionizing radiation (Supplementary Fig. S2, right). These results support the notion that irradiation of tumor mass may release a significant amount of tumor-specific antigens that may be captured by sGRP94 and presented to APCs and other immunoeffector cells.

In summary, this study evaluated a novel adenovirus-mediated, secreted GRP94 gene/immunotherapy strategy. In established tumor models, a significant efficacy of the sGRP94-based gene/immunotherapy strategy was seen when it was combined with radiation therapy. These results provide the basis for further in-depth preclinical and clinical investigations of this highly promising treatment strategy for metastatic malignancies.

**Figure 4.** Efficacy of combined radiation and secreted GRP94-mediated immunotherapy in established murine 4T1 tumors. A, treatment scheme for the combined therapies. B, tumor growth delay as a result of the combined treatment. For this graph, day 0 is the day when radiation treatment started. Points, mean of 10 to 12 mice; bars, SE. GRP, vaccination with AdsGRP94GFP-infected 4T1 cells and i.t. injection of the AdsGRP94GFP (1 × 10⁸ pfu per injection). GFP, vaccination with AdGFP-infected 4T1 cells and i.t. injection of AdGFP (1 × 10⁸ pfu per injection). C, Kaplan-Meier plots of host BALB/c mouse survival as a result of the combined treatment. The group designations are the same as in (B). D, kinetics of de novo metastases in mice that have undergone combined radiation- and sGRP94-mediated immunotherapy. 4T1-Luc cells, which had been stably transduced with the firefly luciferase gene, were injected i.v. into mice bearing established tumors that had undergone various treatments. The growth of tumors from these cells was then followed with the aid of a very sensitive Xenogen IVIS bioluminescence detection system. Top, Kaplan-Meier plots of metastasis-free rate in the 4T1-Luc-injected mice. Points, value from five to seven mice. The treatment group designations are the same as those in (B). Bottom left, representative photographs of luciferase signal as a result of 4T1-Luc growth. Bottom right, confirmation from dissected lungs of tumor growth from the mice shown in left. Abbreviations: TF, tumor free; TG, tumor growth; RT, radiation therapy.
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