Secretable Chaperone Grp170 Enhances Therapeutic Activity of a Novel Tumor Suppressor, mda-7/IL-24

Ping Gao,¹,4 Xiaolei Sun,¹ Xing Chen,¹ Yanping Wang² Barbara A. Foster,³ John Subjeck,¹ Paul B. Fisher,⁵ and Xiang-Yang Wang¹,2,5

¹Departments of Cellular Stress Biology, ²Urologic Oncology, and ³Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, New York; College of Life Science, Beijing Normal University, Beijing, China; and ⁵Department of Human Genetics, Massey Cancer Center, Virginia Commonwealth University, School of Medicine, Richmond, Virginia

Abstract
Melanoma differentiation–associated gene-7 (mda-7)/interleukin-24 (IL-24) is a cancer-specific, apoptosis-inducing gene with broad-spectrum antitumor activity, making it an ideal candidate for a novel cancer gene therapy. A systemic and sustained antitumor immune response generated at the time of initial molecular-targeted therapy would provide additional clinical benefits in cancer patients, resulting in improved prevention of tumor recurrence. In this study, we explored the therapeutic efficacy of intratumoral delivery of a nonreplicating adenoviral vector encoding mda-7/IL-24 (Ad.mda-7) and a secretable form of endoplasmic reticulum resident chaperone grp170 (Ad.grp170), a potent immunostimulatory adjuvant and antigen carrier. Intratumoral administration of Ad.mda-7 in combination with Ad.grp170 was more effective in controlling growth of TRAMP-C2 prostate tumor compared with either Ad.mda-7 or Ad.grp170 treatment. Generation of systemic antitumor immunity was shown by enhanced protection against subsequent tumor challenge and improved control of distant tumors. The combined treatments enhanced antigen and tumor-specific T-cell response, as indicated by increased IFN-γ production and cytolytic activity. Antibody depletion suggests that CD8⁺ T cells may be involved in the antitumor effect of the dual molecule–targeted therapies. Therefore, introducing immunostimulatory chaperone grp170 in situ strongly promotes the “immunogenic” cell death when delivered to the mda-7/IL-24–induced apoptotic tumor cells, indicating that an improved anticancer efficacy may be achieved by concurrently targeting both tumor and immune compartments. Given multiple undefined antigens present endogenously within prostate cancer, these data provide a rationale for combining grp170-based vaccine strategy with mda-7/IL-24–targeted cancer therapy to induce durable systemic immunity. [Cancer Res 2008;68(10):3890–8]

Introduction
Melanoma differentiation associated gene-7 (mda-7), also known as interleukin-24 (IL-24), was initially cloned by subtraction hybridization applied to a differentiation therapy model of human melanoma cancer (1). A large body of evidence has shown that gene delivery of mda-7/IL-24 promotes growth inhibition and induces apoptosis in a broad array of human cancers. In contrast, mda-7/IL-24 does not induce growth suppressive or toxic effects in normal cells (2–4). In addition, mda-7/IL-24 is also capable of regulating cell cycle (5), inhibiting angiogenesis (6), and sensitizing cancer cells to radiation therapy (7, 8). Although the mechanism of cancer cell selectivity of mda-7/IL-24 requires further delineation (9–11), these pleiotropic properties places mda-7/IL-24 in a unique position for a novel cancer gene therapy. Indeed, a replication-incompetent adenovirus expressing mda-7/IL-24 (INGN-241) has now undergone evaluation in a phase I clinical trial for multiple solid tumors and has shown safety and significant clinical activity (4).

Currently, there is no curative therapy for prostate cancer (CaP) patients who develop recurrences or for those who have metastatic disease at the time of diagnosis. A systemic, specific, and sustained immune response against cancer at the time of initial therapy could address the most critical issue: prevention of tumor recurrence. However, CaP is considered poorly immunogenic despite the presence of antigens that may be tumor specific (12). In most cases, simply killing tumor cells by molecular or tumor-targeted treatments, including mda-7/IL-24 gene therapy, may not be sufficient to raise effective antitumor immunity. Without a proper “danger” signal, the apoptotic cells are largely ignored by the immune system (13), or may even induce tolerance (14, 15).

In recent years, some stress proteins have gained widespread attention due to their potential roles in cancer immunotherapy. The antitumor response has largely been attributed to the ability of stress proteins to form complexes with tumor-derived antigens and thereby facilitate the antigen cross-presentation and priming of T-effector cells. Different stress proteins are highly different in cellular functions and in their abilities to chaperone or bind antigens. Our previous studies have shown that grp170, the largest endoplasmic reticulum (ER)–resident chaperone (16), can interact with specific receptors on professional antigen-presenting cells (APC) and shuttle antigens into the endogenous processing pathway efficiently (17, 18). Tumor-derived grp170 or grp170 complexed with tumor-associated antigens are highly potent in eliciting antigen-specific or tumor-specific immune responses in various murine tumor models (17, 19). Grp170 also acts as a danger signal that stimulates phenotypic and functional maturation of dendritic cells (DC; ref. 20).

In view of multiple undefined antigens present endogenously within CaP cells, it is conceivable that the ability of grp170 to simultaneously deliver both adjuvanticity for the activation of innate immunity and antigenicity for CTL cross-priming could dramatically increase the intrinsic immunogenicity of the in vivo tumor killing by mda-7/IL-24. In this study, we sought to examine the potential of grp170-based immunotherapy to enhance mda-7/IL-24–mediated CaP therapy. We show that concurrent delivery

Requests for reprints: Xiang-Yang Wang, Department of Cellular Stress Biology, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263. Phone: 716-845-2375; Fax: 716-845-8899; E-mail: xiang-yang.wang@roswellpark.org.

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of secretable grp170 and mda-7/IL-24 effectively and markedly suppress established prostate tumors. Furthermore, the combined therapies augment a robust tumor-specific immune response, leading to a significantly improved control of distant and secondary tumors.

Materials and Methods

Mice and cell lines. Male C57BL/6 mice (ages 8–12 wk) purchased from NIH animal facilities were maintained in a pathogen-free facility at Roswell Park Cancer Institute. Animal care and experiments were approved by the Institutional Animal Care and Use Committee. TRAMP-C2 cell line was derived from a prostate tumor that arose in a transgenic adenocarcinoma of mouse prostate (TRAMP) mouse in the C57BL/6 background (21). The TRAMP-C2 cells, C2 cells transduced with OVA (C2-OVA), and B16 melanoma cells were maintained in DMEM containing 10% fetal bovine serum, 2 mmol/L L-glutamine, and 100 units/mL penicillin/streptomycin. Primary DCs were derived from primary culture of mouse bone marrow, as previously described (18). DC cell line DC1.2 was kindly provided by K. Rock (University of Massachusetts) and cultured in RPMI.

Adenovirus construction and characterization. The recombinant replication-defective adenoviral vectors encoding mda-7/IL-24 (Ad.mda-7) virus was created as described previously (2). The adenovirus carrying a secretable form of the grp170 gene (Ad.sgrp170) was constructed using BD Adeno-X Adenoviral Expression System (BD Bioscience). To distinguish the secretable grp170 from endogenous grp170, a His-tag was fused to the COOH terminus of mouse grp170, in which the KNDEL ER retention signal has been eliminated (22). This modified cDNA was inserted into the Nilh1/V Aval cloning sites of the pShuttle 2 plasmid and subsequently cloned into I-Ceu I/PcI-Sce I sites of adenoviral vector. All adenoviral vectors were produced in HEK293 cells, and infection titers were determined by plaque tittering on 293 cells. Viruses were concentrated and purified using AdenoPACK Maxi columns (Sartorius Stedim Biotech). Endotoxin levels were determined by using a chromogenic Limulus amebocyte lysate kinetic assay kit (Kinetic-QCL; Biowhittaker). Cells were infected with viruses with different multiplicity of infection (MOI) under standard culture conditions. Expression of the secreted grp170 in the supernatants of the infected cells was examined using antibodies against grp170 and His-tag as previously described (22). Expression of mda-7/IL-24 was examined by immunoblotting using murine anti–mda-7/IL-24 monoclonal antibodies (mAbs; Gene Hunter, Inc.).

Apoptosis assays. Annexin V binding assays were used to determine apoptosis induction as previously described (23). Briefly, 24 h after virus infection at an MOI of 300 plaque-forming units (pfu) per cell, cells were washed and stained with FITC-labeled Annexin V (BD Biosciences) for 15 min at room temperature and analyzed by flow cytometry. In addition, cells were subjected to immunoblotting analysis using antibodies for poly(ADP-ribose) polymerase (PARP; Santa Cruz Biotech).

Cell proliferation assay. Cells (2 × 10^4 per well) seeded in 96-well plates were treated with Ad.mda-7 at an MOI of 300. At the indicated times, media were replaced with 100 μL PBS containing 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT; Sigma). An equal volume of solubilization solution (0.01 N HCl in 10% SDS) was added 4 h later. The absorbance was read at 595 nm.

Tumor studies. C2 tumor cells (2 × 10^6) suspended in 100 μL sterile PBS were injected into the left dorsal flank of mice. When tumors reached 4 to 5 mm^3 (~1 wk after tumor inoculation), animals were randomly divided and received Ad.GFP, Ad.mda-7, Ad.sgrp170, or Ad.mda-7 plus Ad.sgrp170. Viruses were given i.t. in 30 μL PBS (5 × 10^6 pfu/mouse). For the group receiving the combined therapies, 2.5 × 10^6 pfu of each virus was given. All treatments were given every other day for a total of four doses. Tumor growth is monitored by measuring perpendicular tumor diameters using an electronic digital caliper. To determine the effect of the combined therapies on distant tumors, mice were established with tumors in both flanks. The viruses were delivered into tumors in the left flank only. Growth of contralateral tumors was followed to determine systemic antitumor immunity. Depletion of CD4+ and CD8+ T-cell subsets was accomplished by i.p. injection of GK1.5 and 2.43 mAbs, respectively, as previously described (22).

Enzyme-linked immunosorbent spot and CTL assays. Splenocytes were isolated from immunized mice 2 wk after immunization and stimulated with 1 μg/mL H-2Kβ restricted CTL epitope OVA257–264 (SIINFEKL) or mitomycin C–treated C2 cells to determine antigen-specific, IFN-γ–secreting T cells, as previously described (24). For CTL assay, splenocytes were stimulated with mitomycin C–treated tumor cells or 1 μmol/L OVA257–264 in the presence of IL-2 (20 units/mL) for 6 d. CD8+ T cells were used as effectors cells in a chromium release assay as described (24).

Statistical analysis. Statistical analysis of tumor growth inhibition, cytotoxicity assays, and enzyme-linked immunosorbent spot (ELISPOT) assay were done using paired or unpaired Student’s t test, as appropriate. P values of <0.05 were considered statistically significant.

Results

Construction and validation of adenovirus vector encoding secretable grp170. To enhance the immunogenicity of mda-7–mediated tumor cell–specific apoptosis and promote systemic antitumor immunity, we constructed a secreted form of mouse grp170 (sgrp170) by deleting the COOH terminal ER retention signal KNDEL (Fig. 1A). To distinguish the modified gene product

Figure 1. Adenovirus-mediated expression of secretable grp170 in TRAMP-C2 tumor cell. A, schematic representation of adenovirus vector encoding a secretable form of grp170 (Ad.sgrp170). The COOH terminal KNDEL signal was deleted from mouse grp170 cDNA to produce the secreted form of grp170. The His-tagged sgrp170 gene under the control of a constitutively active CMV promoter/enhancer was inserted into the replication incompetent adenoviral vector, in which the E1/E3 sequences have been deleted. Inverted terminal repeats (ITR), which flank the E1/E3 deleted genome, are necessary for the replication of adenoviral DNA. The C2 cells were infected with or without Ad.sgrp170 at different MOIs. Supernatants were collected at different time points and analyzed for the expression of sgrp170 using antibodies against grp170 (B) or His-tag (C).
from the endogenous grp170, the His-tag was fused to the COOH terminus of the grp170 gene. The fusion gene of grp170-His was inserted into E1/E3-deleted adenovirus-based vectors under the control of cytomegalovirus (CMV) promoter for constitutive and effective gene expression (i.e., Ad.grp170). The replication-defective virus vectors encoding this new fusion gene were successfully packaged and expanded in HEK293 cells. As a model for our studies, we selected the TRAMP-C2 cell line that was established from the spontaneous tumor of the autochthonous TRAMP model (21). Upon s.c. transplantation into syngeneic male C57BL/6 mice, TRAMP-C2 cells form slowly growing, vascularized, and poorly immunogenic tumors. After infection of C2 cells with Ad.grp170 at different MOIs, expression of the secretable grp170 gene was examined in the supernatants of the infected cells. Robust expression of the grp170 gene could be observed from day 1 at an MOI of 100 (Fig. 1B). The expression peaked at around 48 hours and remained stable for up to 4 days (data not shown). The secretable form of modified grp170 in the supernatants was further verified by immunoblotting using anti-His-tag antibodies (Fig. 1C). In addition, the infection of C2 cells with Ad.grp170 at an MOI of up to 500 had no observable effect on C2 cell viability in vitro (data not shown), indicating that grp170 overexpression per se does not exert a cytotoxic antitumor effect.

Adenovirus-mediated mda-7/IL-24 expression inhibits TRAMP-C2 tumor cell growth by inducing tumor apoptosis. In light of the limited information on the antitumor activity of mda-7/IL-24 in murine tumor cells, we therefore first determined whether adenovirus-mediated mda-7/IL-24 expression could induce growth suppression and apoptosis in TRAMP-C2 tumor cells (Fig. 2). Immunoblotting analysis confirmed the expression of mda-7/IL-24 gene in C2 cells infected with Ad.mda-7 at different MOIs (Fig. 2A, top). Ad.mda-7 generated multiple bands because of glycosylation, ranging in size from 20 to 30 kDa. A significant inhibition of proliferation (P = 0.001) was observed in C2 tumor cells treated with Ad.mda-7 at an MOI of 300 compared with that in cells treated with PBS or Ad.GFP (Fig. 2A, bottom). In addition, coinfection of C2 cells with Ad.grp170 had no effect on mda-7/IL-24–induced growth suppression in C2 tumor cells (data not shown).

Annexin V staining followed by fluorescence-activated cell sorting (FACS) was carried out to determine early apoptotic changes in C2 cells after virus infection. Whereas a significant increase in apoptotic cells was observed in C2 cells after Ad.mda-7 infection, no such change was evident in C2 cells treated with Ad.GFP or left untreated (Fig. 2B). In addition, cleavage of PARP was detected only in C2 cells infected with Ad.mda-7 (Fig. 2C), suggesting that overexpression of mda-7/IL-24 in mouse tumor cells induces apoptosis in a manner similar to that observed in human cancer cells (25). Furthermore, the Ad.mda-7 infection resulted in a profound increase in apoptosis of C2 tumor cells, as assayed by Annexin V staining and FACS. In contrast, no significant apoptosis was seen in normal DCs (Fig. 2D).

Intratumoral delivery of adenovirus encoding secretable grp170 enhances therapeutic efficacy of mda-7/IL-24–based gene therapy. We next assessed the therapeutic effects of i.t. injection of apoptosis-inducing mda-7/IL-24 in conjunction with immunostimulatory adjuvant grp170 on weakly immunogenic TRAMP-C2 tumor (Fig. 3). Immunization with irradiated C2 cells did not protect mice from subsequent tumor challenge (data not shown). C57BL/6 mice established with C2 tumors were treated with Ad.GFP, Ad.mda-7, Ad grp170, or Ad.mda-7 plus Ad grp170. It was observed that administration of Ad.GFP or PBS (data not shown) had little effect on C2 tumors, whereas treatment with either Ad.mda-7 or Ad.grp170 significantly delayed tumor growth. However, treatment with Ad.mda-7 combined with Ad grp170 exhibited much more potent tumor-suppressive activities (Fig. 3A). Additionally, tumors in 20% of mice treated with Ad.mda-7 plus Ad.grp170 showed complete and prolonged regression.

We additionally tested whether injection with Ad.mda-7 and Ad.grp170 could induce systemic immune responses that can control established tumors. The C2 tumors were inoculated into both the left and right flanks of mice. The tumor-bearing mice were treated in the right flank only. By comparison with the control group, mice receiving Ad.mda-7 plus Ad.grp170 showed a significant inhibition in tumor growth on the untreated, contralateral flank, whereas in mice injected with Ad.mda-7, growth of contralateral tumors was essentially unimpeded. Although injection of Ad.grp170 seemed to delay tumor growth to some extent, there was no statistical significance when compared with the control group (Fig. 3B).

Nearly half of CaP patients with clinically localized tumor undergo surgery to remove all or most of the cancer during the early phase of their disease. Therefore, we examined whether the combined in situ tumor therapies before surgery could prevent tumor growth after rechallenge. In this study, all treated tumors were surgically removed 1 wk after the last treatment. The mice were then challenged with C2 tumor cells 10 d later. As shown in Fig. 3C, mice receiving Ad.mda-7 plus Ad.grp170 were protected from rechallenge with C2 tumor, whereas those treated with Ad.GFP or Ad.mda-7 alone were still susceptible. It was seen that Ad.grp170 treatment failed to protect mice from rechallenge with the same tumor, suggesting that the mda-7/IL-24–mediated tumor apoptosis plays an important role in induction of antitumor responses. Moreover, treatment of local tumors with Ad.mda-7 plus Ad grp170 before surgery reduced lung metastases established by i.v. inoculation of C2 cells more effectively compared with the treatment with either Ad.mda-7 or Ad grp170 (Fig. 3D).

Coadministration of Ad.mda-7 and Ad grp170 induces an antigen and tumor-specific immunity. To facilitate immunomonitoring of antigen-specific immune response elicited by the combined therapies, we established TRAMP-C2 tumor cell line expressing a model antigen OVA. The expression of OVA gene in transduced C2 cells was confirmed by reverse transcription–PCR (RT-PCR; Fig. 4A). Mice established with C2-OVA tumors were treated with Ad.mda-7, Ad grp170, or Ad.mda-7 plus Ad grp170. Splenocytes were isolated from the treated mice 1 or 3 weeks after the last injection. The ELISPOT assay was used to examine the OVA-specific IFN-γ production by splenocytes upon stimulation with MHC I–restricted CTL epitope for OVA, i.e., OVA257-264 (SIINFEKL; Fig. 4B, top). Compared with those from Ad.mda-7–treated or Ad grp170-treated mice, a significant elevation in the level of IFN-γ was observed in cells from animals treated with Ad.mda-7 plus Ad grp170. It is also evident that splenocytes of Ad.mda-7–treated mice produced more IFN-γ compared with those from control mice, which agrees with the previous report showing the immunomodulatory effects of mda-7/IL-24 (26). However, levels of IFN-γ secreted by these cells were much lower than those derived from the group treated with combined therapies, even when examined 3 weeks after the treatment. Similar results were obtained when splenocytes from the treated mice were stimulated with mitomycin C–treated tumor cells (Fig. 4B,
suggesting that introduction of sgrp170 into Ad.mda-7-treated tumor promotes tumor-specific IFN-γ production. Furthermore, ELISPOT assay was performed to measure the tumor-specific secretion of IL-4 by splenocytes from the treated animals. It was observed that splenocytes of Ad.mda-7-treated mice produced higher levels of IL-4 compared with cells from animals treated with Ad.sgrp170 or Ad.mda-7 plus Ad.sgrp170 (Fig. 4C). Effector CD8+–T cell function, i.e., cytolytic activity, was assessed by chromium release assay using C2-OVA tumor cell as a target (Fig. 4D). At effector-target (E/T) ratios of 100:1 and 50:1, significantly increased cytolytic activities were observed in Ad.mda-7 plus Ad.sgrp170–treated group when compared with Ad.mda-7–treated or Ad.sgrp170–treated group. Similar results were obtained when OVA257–264-pulsed C2 cells were used as targets in the cytolytic assays (data not shown).

CD8+ cells are involved in the systemic antitumor effects provided by the combined gene therapies. We next examined the immune effector cells involved in the antitumor immunity generated by the combined in situ therapies. C2-OVA tumor-bearing mice were depleted of CD4+ or CD8+ cell subsets by antibody injections before the initiation of treatment (Fig. 5A). It was found that depletion of CD8+ cell abrogated the therapeutic efficacy of the combined treatments. However, antitumor immunity remained intact in mice depleted of CD4+ T cell or those treated with control IgG. To determine whether the antitumor immune response in C2-OVA tumor model was directed against only OVA antigen, we rechallenged C2-OVA tumor-free mice which had undergone the combined treatments with parental C2 tumor (Fig. 5B). Eighty percent of mice were resistant to the secondary tumor challenge, suggesting that the treatment of

Figure 2. Adenovirus-mediated mda-7 inhibits C2 tumor cell growth by inducing apoptosis. A, Ad.mda-7 infection suppresses proliferation of C2 cells in vitro. C2 cells were infected with Ad.sgrp170, Ad.mda-7 at different MOIs, or left untreated. Protein lysates (50 μg) were run on 12% SDS-PAGE and stained with anti–mda-7/IL-24 mAb (1:2,000; top). C2 cells were infected with Ad.GFP, Ad.mda-7, or left untreated. Cell proliferation was analyzed using MTT assay (bottom). B, Ad.mda-7 infection induces C2 tumor cell apoptosis. C2 tumor cells were collected 72 h after treatment and stained with FITC-labeled Annexin-V. The percentage of Annexin-V–positive cells was analyzed by flow cytometry (*, P < 0.01, Ad.mda-7 versus Ad.GFP or untreated control). C, Ad.mda-7 infection promotes PARP cleavage in C2 tumor cells. Cells were examined at different time points after treatment for cleavage of PARP by immunoblotting. β-Actin was used as the internal loading control. D, Ad.mda-7 induces apoptosis in tumor cells, but not in normal cells. TRAMP-C2 prostate tumor cells or normal DCs were infected with Ad.mda-7. Cells were stained with FITC-labeled Annexin-V 48 h later and examined using FACS; dotted line, control; solid line, Ad.mda-7–treated cells.
C2-OVA tumor with Ad.mda-7 and Ad.sgrp170 also induced immune responses against other endogenous antigens in addition to OVA. However, these mice developed aggressively growing tumors when rechallenged with B16 melanoma tumor (data not shown), indicating tumor specificity of the antitumor immune response.

Separate i.t. administration of Ad.mda-7 and Ad.sgrp170 is capable of inducing antitumor immunity. To determine whether injection of mda-7/IL-24 and sgrp170 at the same time is required for the generation of systemic immunity, a modified treatment protocol was used to deliver Ad.mda-7 and Ad.sgrp170 at different time points, as described in Fig. 6A. In contrast to Ad.mda-7-treated group, significant enhancement of mda-7/IL-24–targeted therapy by sgrp170 was observed in mice receiving the two therapeutic agents either together (T) or separately (S; Fig. 6B; \( P < 0.01 \) for Ad.mda-7 plus Ad.sgrp170 (T) or Ad.mda-7 plus Ad.sgrp170 (S) versus Ad.mda-7 group). Although administration of Ad.mda-7 and Ad.sgrp170 together seemed to provide an improved control of treated tumors compared with the delivery of these two molecules on different days, there was no statistical difference between these two treatment groups (\( P > 0.05 \)). Upon stimulation with the OVA257-264 peptide, splenocytes derived from C2-OVA-bearing mice treated with either Ad.mda-7 plus Ad.sgrp170 (T) or Ad.mda-7 plus Ad.sgrp170 (S) both displayed a robust, but comparable production of IFN-\( \gamma \) (Fig. 6C). However, cytolytic activity assays showed that concurrent delivery of Ad.mda-7 and Ad.sgrp170 at the same time promoted a more potent CTL response than separate administration of Ad.mda-7 and Ad.sgrp170 (Fig. 6D), suggesting that simultaneous introduction of the two molecules is preferred in the clinical setting.

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Figure 3. Intratumoral administration of adenovirus encoding mda-7/IL-24 and secretable grp170 induces a systemic antitumor response. A, Ad.sgrp170 promotes eradication of local C2 tumors by Ad.mda-7 in immunocompetent mice. Male C57BL/6 mice bearing C2 tumors (\( n = 10 \)) received Ad.mda-7, Ad.sgrp170, or Ad.mda-7 plus Ad.sgrp170 every 2 d for a total of four doses. Mice receiving Ad.GFP served as controls. Data are representative of three experiments (*, \( P < 0.02 \), Ad.mda-7 or Ad.sgrp170 versus Ad.GFP on day 42; **, \( P < 0.01 \), Ad.mda-7 plus Ad.sgrp170 versus Ad.mda-7 or Ad.sgrp170). B, inhibition of established distant tumors by the combined therapies. C2 tumor cells (1.5 \( \times \) \( 10^6 \)) were inoculated s.c. into the right and left flanks of mice at the same time. Only tumors in the left flank were treated. Growth of the tumors in the contralateral side was followed (*, \( P > 0.05 \), Ad.mda-7 or Ad.sgrp170 versus PBS; **, \( P < 0.01 \), Ad.mda-7 plus Ad.sgrp170 versus Ad.mda-7 or Ad.sgrp170). C, the combined therapies before surgery prevent the growth of secondary tumors. Tumors were removed by surgery 1 wk after the last treatment. Mice were then rechallenged with C2 cells in the opposite sides 10 d after surgical removal of the treated primary tumors (*, \( P > 0.05 \), Ad.mda-7 or Ad.sgrp170 versus control; **, \( P < 0.01 \), Ad.mda-7 plus Ad.sgrp170 versus Ad.mda-7 or Ad.sgrp170). D, reduction of experimental lung metastases after the combined therapies. Tumor-bearing mice were injected i.v. with 1 \( \times \) \( 10^6 \) C2 cells 3 d before initiation of the treatment. Treated tumors were removed by surgery 1 wk after the last treatment. Lungs were harvested 20 d after the surgery and examined for metastasis (*, \( P < 0.01 \), Ad.mda-7 plus Ad.sgrp170 versus Ad.mda-7 or Ad.sgrp170).
Discussion

To achieve ultimate tumor control, it would be ideal if integrating an immunotherapeutic protocol into molecular-targeted cancer therapies can generate systemic antitumor immunity. Based on our recent report demonstrating that extracellular targeting of grp170 dramatically improves the immunogenicity of poorly immunogenic tumors, including melanoma (22) and CaP, we postulate that tumor-specific killing by adenovirus-mediated mda-7/IL-24 expression, taken together with simultaneous release of tumor-derived grp170, will provide both danger signals and tumor-associated antigens to APCs (e.g., DCs), leading to strong tumor-specific immunity. In this study, using a mouse CaP model, we have shown that concurrent administration of secretable grp170 significantly enhanced therapeutic efficacy of the mda-7/IL-24–based gene therapy strategy via promoting antigen and/or tumor-specific immune responses. Furthermore, both tumor-specific apoptosis induction by mda-7/IL-24 and immunostimulatory adjuvant activities of sgrp170 contribute to the synergistic or additive antitumor effects provided by the combined strategies.

A significant increase in antigen-specific CD8+ T-cell frequency and tumor-specific cytolytic activity were displayed in mice treated with Ad.mda-7 plus Ad.sgrp170, compared with mice treated with either Ad.mda-7 or Ad.sgrp170, suggesting that targeting of ER

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chaperone grp170 to tumor milieu is a critical factor for initiation of tumor-specific systemic immune responses. Our earlier studies have shown that the largest ER chaperone grp170 is highly efficient in binding polypeptide chains (16, 22) and grp170 purified from tumor exhibits a more potent therapeutic efficacy than other stress proteins (17). The enhanced immunogenicity may be attributed to its highly efficient chaperoning capability, which is important for grp170-mediated interaction with APCs, antigen binding, and generation of antitumor immunity (19). Supporting evidence also came from other groups suggesting that the ability of chaperone-peptide complexes to generate an antigen-specific CTL response correlates with the affinity with which the chaperone binds substrates or peptides (27). Recent structure deletion studies revealed that grp170 contains two unique substrate-binding regions, i.e., the β-sheet domain and the COOH terminal helix domain (16). In light of the findings that stress proteins are capable of stimulating Th1-polarizing cytokine production (28–30) and preferentially activating antigen-specific CD8 T cells (31, 32), it is tempting to postulate that, in addition to facilitating antigen transport and subsequent uptake and presentation by APCs, the secreted grp170 acts as a Th1 polarizing adjuvant during mda-7/IL-24–induced tumor cell apoptosis, promoting IFN-γ–producing type 1 CD8+ T cells (Tc1). It has been shown that Tc1 cells are more effective in delaying tumor growth and progression than that of functionally distinct Tc2 cells (33).

In the present study, the significant increase in antigen-specific CD8+ T-cell frequency and cytolytic activity observed in mice treated with Ad.mda-7 and Ad grp170 strongly implicate CD8+ T-cell function in tumor growth suppression. In vivo antibody depletion study also provides supporting evidence. However, it should be noted that depletion of CD8+ DC or abrogation of CD8+ DC-dependent natural killer (NK) interaction/activation might also contribute to the diminished antitumor response in the treated mice. Future studies (e.g., adoptive T-cell transfer, NK cell depletion) should provide insights into the cellular mechanisms underlying the enhanced efficacy of the combined mda-7/secretable grp170 therapy. Given that CD8+ depletion does not completely abrogate the antitumor effects mediated by the combined therapies, it further indicates that other immune effector cells are likely to participate in the tumor control, such as NK cells (34). Our previous study showed that both NK cell and CD8+–T cells are required for tumor rejection elicited by vaccination with grp170-secreting tumor cells (22).

An early study by Miyahara et al. showed that adenoviral-mediated mda-7/IL-24 transfer induced anticancer immunity in UV-induced fibrosarcoma model (26). In agreement with the finding, we have shown that mda-7/IL-24 did exhibit immunostimulatory activities in our experiment, as indicated by enhanced IFN-γ production in OVA or tumor-specific T cells from Ad.mda-7–treated mice. However, the treatment failed to elicit effective systemic immunity against distant or secondary TRAMP-C2 tumors, which have been known to be poorly immunogenic. Interestingly, splenocytes from Ad.mda-7–treated mice consistently produced higher levels of IL-4 than those from animals treated with Ad.sgrp170 or Ad.mda-7 plus Ad.sgrp170, which lends support to an early study in which murine mda-7, also called IL-4–induced secreted protein (FISP), was postulated to be a type 2 cytokine (35). However, more studies are needed to determine the pleiotropic functions of this novel tumor suppressor.

This gene-based vaccine strategy tested here has several important advantages for clinical application. It has unique capacity to induce individual tumor-specific immune responses against a broad array of tumor antigens, obviating the need to prepare vaccines from surgically resected tumor specimens ex vivo. Furthermore, the in situ immunotherapy drastically reduces the possibility of tumor escape due to antigen loss or tumor heterogeneity, because the approach uses the tumor against itself and grp170 derived from tumor cells is directed against a diverse antigenic repertoire. In contrast to conventional stress protein–based vaccination approach that is limited by the availability of tumor specimens, the approach described here is universally applicable and more cost effective, because the vaccine is generated at the site of the patient’s own tumor using his own tumor antigens. Compared with other tumor-targeted gene therapies for inducing cell death in rapidly dividing

Figure 5. CD8+ cells contribute to the antitumor activities mediated by the combined therapies in vivo. A, depletion of CD8+ cell subset abolishes antitumor immunity. C57BL/6 mice (n = 6) with established C2-OVA tumors were depleted of CD4+, CD8+ cells by i.p. injection of GK1.5, 2.43 mAb, respectively. C2-OVA tumor–bearing mice were treated with Ad.mda-7 in combination with Ad.sgrp170 as described (CD8 depletion versus IgG control, P < 0.01). B, the combined in situ therapies results in a tumor-specific immune response. C2-OVA tumor-free mice after the combined therapies were rechallenged with parental TRAMP-C2 tumor (3 × 106 cells) in contralateral side.
cells, mda-7/IL-24–based approach should display enhanced safety in the clinic because of the cancer specificity of mda-7/IL-24 and its bystander activities (36).

Among the solid tumors, CaP is ideally suited for the first test of efficacy of this idea because this nonessential organ expresses a wide array of unique antigens and highly accessible to gene transfer by using digital or transrectal ultrasound guidance (37). Primary CaP is relatively slow growing and thus sequential gene therapy approaches can be incorporated safely into treatment strategies. Serum prostate-specific antigen can be easily used to monitor treatment response. In addition, the strategy may also prove effective against hormone-refractory CaP cells that are androgen-independent because mda-7/IL-24 causes release of tumor antigens from CaP regardless of their androgen sensitivity. Whereas surgery is necessary for cure, it is possible that early removal of localized CaP by radical prostatectomy may preclude an opportunity to generate effective long-term immune protection. We found that surgical removal of TRAMP-C2 tumor alone does not elicit tumor-protective immunity in mice (data not shown). In the present study, we show that antitumor immunity generated by the combined therapies still remains intact in animals after surgical removal of the treated local tumors. Therefore, one possibility would be to treat patients with clinically localized CaP but high risk for recurrence with the molecular-targeted therapies before surgical removal of the primary tumor.

Taken together, this study evaluated a dual molecular target-based therapy with significant promise for improving CaP therapy, which may also have potential applications for other neoplastic diseases. This novel approach exploits an immunostimulatory chaperone molecule, i.e., grp170, in combination with a nontoxic cancer-specific apoptosis-inducing gene, mda-7/IL-24. Given the encouraging phase I clinical studies with Ad.mda-7 (38) and minimal toxic side effects in mouse models and clinical trials with stress protein-based vaccines (39), this strategy merits further evaluation for potential clinical use.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Ping Gao, Xiaolei Sun, Xing Chen, et al.


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