Effect of Adenovirus-Mediated Heat Shock Protein Expression and Oncolyis in Combination with Low-Dose Cyclophosphamide Treatment on Antitumor Immune Responses

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Abstract

Heat shock proteins such as gp96 have the ability to chaperone peptides and activate antigen-presenting cells. In this study, we tested whether adenovirus-mediated overexpression of secreted or membrane-associated forms of gp96 in tumor cells would stimulate an antitumor immune response. Studies were carried out in C57Bl/6 mice bearing aggressively growing s.c. tumors derived from syngeneic TC-1 cells, a cell line that expresses HPV16 E6 and E7 proteins. We found that secreted gp96 can induce protective and therapeutic antitumor immune responses. Our data also indicate that the antitumor effect of secreted gp96 expression seems to be limited by the induction of suppressive regulatory T cells (Tregs). TC-1 tumor transplantation increased the number of splenic and tumor-infiltrating Tregs. Importantly, treatment of mice with low-dose cyclophosphamide decreased the number of Tregs and enhanced the immunostimulatory effect of secreted gp96 expression. We also tested whether an oncolytic vector (Ad.IR-E1A/TRAIL), that is able to induce tumor cell apoptosis and, potentially, release cryptic tumor epitopes in immunogenic form, could stimulate antitumor immune responses. Although tumor cells infected ex vivo with Ad.IR-E1A/TRAIL had no antitumor effect when used as a vaccine alone, the additional treatment with low-dose cyclophosphamide resulted in the elimination of pre-established tumors. This study gives a rationale for testing approaches that suppress Tregs in combination with oncolytic or immunostimulatory vectors. (Cancer Res 2006; 66(2): 960-9)

Introduction

Heat shock proteins (HSP) have a unique ability to activate both innate and adaptive immunity, which is attributed to their ability to interact with a number of receptors on dendritic cells and macrophages that serve as “professional” antigen-presenting cells (APC; ref. 1). Proteins or peptides bound to HSPs are redisplayed in complexes with MHC class I molecules upon internalization by APCs, triggering the production of proinflammatory cytokines and subsequent activation of T cell–mediated immune responses. These properties make HSPs attractive vehicles for the delivery of antigens into antigen-presenting pathways. HSPs capable of providing these functions include hsp70, hsp90, gp96, and calreticulin [for a review, see ref. (2)]. There are numerous approaches by which HSPs can enhance specific immunity against infectious agents or cancers. Originally, HSPs directly isolated from tumor tissue were used to elicit antitumor immunity. As a variation to this scheme, it has been shown that peptides or proteins complexed or fused with syngeneic or allogeneic (mycobacterial) HSPs can elicit potent antibody and cytotoxic responses. The HSP gp96, genetically modified to be displayed on the surface of or secreted from tumor cells, activated potent antitumor responses and tumor regression (3, 4).

Since 1997, genetically engineered adenoviruses that selectively replicate in and kill tumor cells have been used for the treatment of cancer [for a review, see ref. (5)]. Our lab has previously developed oncolytic vectors based on adenovirus genomes deleted for E1A and E1B genes. HPV E6 or E7 expressing tumor cells support DNA replication of E1-deleted adenosiviruses, with 6, 7). This tumor-specific replication of viral genomes can be converted into tumor-specific transgene expression using homologous recombination in adenovirus genomes (Ad.IR system; ref. 8). We have used the Ad.IR system to express adenovirus E1A (to allow for production of progeny virus) and tumor necrosis factor–related apoptosis inducing ligand (TRAIL; to induce apoptosis and efficient release of progeny virus and viral spread; ref. 9). The resulting oncolytic adenovirus vectors (Ad.IR-E1A/TRAIL) was able to eliminate pre-established metastases in xenograft models after a single systemic application (9) and did not cause unspecific toxicity in mice or baboons (10).

Adenovirus vectors and, particularly, oncolytic adenoviruses, could potentially increase the potency of HSP-based immunotherapy strategies. Interaction of adenovirus capsids with cellular receptors induces the expression of pro-inflammatory cytokines/chemokines, such as tumor necrosis factor–α (TNF-α), IFN-γ, interleukin (IL)-1, IL-6, IL-12, and monocyte chemoattractant protein-1 and 2, which results in the recruitment of effector cells of the innate and adaptive immune system to the site of infection (11). These cytokines also activate the functions of APCs. Furthermore, presentation of adenovirus proteins of the incoming adenovirus particle and/or de novo expression of adenovirus proteins in tumor cells could provide an adjuvant effect on the activation of tumor-specific T cells. With regard to oncolytic adenoviruses, it is thought that tumor cell lysis has the potential to release tumor antigens as apoptotic bodies or in complex with tumor-derived or expressed HSPs functioning as chaperones for antigen presentation to dendritic cells and (in the context of adenovirus infection) to subsequent activation of antitumor T cell responses. This hypothesis is supported by several studies. An oncolytic Ad5-based vector showed a strong antitumor efficacy towards rectal carcinomas in immunocompetent mice, which was accompanied by an acute inflammatory reaction (e.g., CD8+ T cell infiltration, increased TNF-α and IFN-γ levels), whereas the
Adenovirus-Mediated Oncolytic and gp96 Expression

Antitumor efficacy of this vector against the same cancer cell line was significantly lower in athymic mice (12, 13). We have recently shown that transplantation of mouse breast cancer cells (C3L5), that underwent viral oncolysis upon infection with Ad.IR-E1A/TRAIL, into C3H mice induced a systemic antitumor immune response that resulted in tumor rejection. This response was significantly greater than with mock-infected or first-generation control vector-infected cell vaccines (14). A recent study showed that HPV E6/E7 expressing TC-1 mouse tumor cells that underwent apoptosis after herpes simplex virus infection increased the efficacy of dendritic cell vaccines more than TC-1 cells that died upon UV-B radiation (15). The latter study underscores the adjutant effect of viral infection. Conversely, a series of studies argue that tumor cell death via apoptosis and uptake of apoptotic bodies by APCs can cause immunologic ignorance to tumor antigens (16). It is thought that phagocytic uptake of apoptotic cells by macrophages/APCs and subsequent signaling results in a decreased ability to efficiently stimulate T effector cell responses (17), increased anti-inflammatory cytokine production (18, 19), decreased proinflammatory cytokine production (20), and/or possibly aid the generation of regulatory T cells (21, 22). Notably, the studies supporting an immunosuppressive role of tumor cell apoptosis were not done in the context of adenvirus infection.

Tumors employ several mechanisms to evade an immune response, including the down-regulation of tumor-selective antigens, MHC, and costimulatory molecules. Among these mechanisms, the escape of tumors from immunologic control via T suppressor cells (Tregs) is attracting increased attention. Human and murine Tregs are CD4+CD25+ and express a number of other markers including Forkhead P3 (FoxP3), CTLA4, glucocorticoid-induced TNFR-related protein (GITR), L-selectin (CD62L), neuropilin-1, and OX40 antigen (CD134). As early as the late 1970s, studies showed that administration of cyclophosphamide could improve antitumor responses. Cyclophosphamide is a chemotherapeutic agent used to treat various types of cancer. The high doses (in humans >120 mg/kg, in mice >400 mg/kg) of drug required for effective chemotherapy cause immunosuppression. However, at low doses (in mice, <100 mg/kg), cyclophosphamide treatment results in enhanced immune responses against a variety of antigens (23–27), a property that was attributed to the ability to selectively kill Tregs (28–31). In mouse experiments, it was shown that upon cyclophosphamide injection, the number of Tregs decreased by day 4. By day 10, the absolute number of Tregs returned to normal, indicating that the effects of cyclophosphamide are transient without prolonged reduction of tolerance in the body (31). Administration of immunostimulating modalities during this period of Treg inhibition would theoretically allow for enhanced antitumor immune responses with a decreased likelihood of autoimmunity.

The objectives of this study are to analyze, in a model with HPV E6/E7 expressing TC-1 tumor cells, the effect of (a) adenovirus-mediated overexpression of membrane-bound or soluble forms of gp96, (b) Ad.IR-E1A/TRAIL-induced tumor cell death and a combination of both on the induction of an antitumor immune response, and (c) of low-dose cyclophosphamide treatment on the antitumor efficacy of our adenovirus-based approaches.

Materials and Methods

Cell lines and antibodies. TC-1 cells (syngeneic to C57Bl/6 mice; ATCC CRL-2785) were used in these studies. Cells were maintained in RPMI 1640 + 10% fetal bovine serum and supplemented with t-glutamine, penicillin, and streptomycin. Antibodies used were a rabbit polyclonal anti-gp96 (Stressgen, Victoria, British Columbia, Canada), a rat monoclonal anti-gp96 antibody (Stressgen), a mouse anti-rat FITC-conjugated antibody, and donkey anti-rat hors eradish peroxidase–conjugated antibody (The Jackson Laboratory, Bar Harbor, MN).

Generation of recombinant adenovirus and Ad.IR vectors expressing a soluble or membrane-anchored form of gp96. The human gp96 cDNA cloned in the plasmid pGEMEX-gp96 (kindly provided by Dr. P.K. Srivastava, University of Connecticut Cancer Center, Farmington, CT) was amplified by PCR whereby the KDEL endoplasmic reticulum retention signal was eliminated. This modified cDNA was cloned into pAd.RSV to generate pAd.sgp96. To generate pAd.IRE1A/gp96, the lacz gene in pAd.IRE1A/Bgal (32) was replaced with the sgp96 gene. To generate the membrane gp96-expressing vectors, the PCR-amplified, KDEL-deleted cDNA was cloned into the pDisplay plasmid (Invitrogen, Carlsbad, CA) in frame with the membrane anchor. The resulting mg96 fragment was released and cloned into pAd.RSV to generate pAd.mgp96, or in replacement of the lacZ cassette, into pAd.IRE1A/Bgal, in order to generate pAd.IR-E1a/mgp96. Adenoviral vectors were produced in 293 cells by recombination with pBHG10 and titrated by OD 260 spectrometry as well as by quantitative Southern blotting for viral genomes using an 8 kb probe corresponding to a region in the backbone of all adenoviral vectors as described elsewhere (33). The infectious titers were determined by plaque titrating on 293 cells. All vectors had a genome to plaque-forming unit (pfu) ratio of ~20:1.

Western blotting. For the detection of mg96, proteins were extracted from transduced cells, and for the detection of the soluble form of gp96, a rabbit polyclonal anti-gp96 antibody (Stressgen), and protein G Sepharose beads (Sigma, St. Louis, MO) were used to pull down the complexes. After polyacrylamide electrophoresis, proteins were blotted onto nitrocellulose membranes and probed with a rat monoclonal anti-gp96 antibody followed by anti-rat immunoglobulin-HRP conjugated antibody.

Immunofluorescence analysis of gp96. Cells were plated in eight-well chamber slides, transduced with adenovirus vectors expressing the soluble or membrane forms of gp96, or control vectors, and fixed after 48 to 72 hours. Cells were first incubated with a rat monoclonal anti-gp96 antibody (Stressgen), followed by a mouse anti-rat FITC-conjugated antibody (The Jackson Laboratory). Cells were visualized under UV light and photographed.

Crystal violet assay. Cells were plated in 24-well plates and transduced at different multiplicities of infection (MOI) with adenoviral vectors in triplicate. Viable cells in each well were stained at the indicated time points with crystal violet as described elsewhere (8). Results were expressed as the percentage of viable cells compared with mock-treated controls.

Animal experiments. C57Bl/6 mice (The Jackson Laboratory) were used. Animal experiments were done in a number of different settings to evaluate the antitumor efficacy of gp96 expression as a soluble or membrane form from TC-1 cells.

“Vaccination” setting I. Cells were transduced with adenovirus vectors at an MOI of 100 pfu/cell. Sixteen hours later, cells were collected, washed, and irradiated with 6,000 rad. Mice were injected with 1 × 10^6 irradiated cells at days 0 and 14, and challenged with 2 × 10^3 untreated tumor cells on day 24. Tumor growth was monitored after that.

Vaccination setting II. Mice were injected s.c. into the right inguinal flank with 5 × 10^3 untreated TC-1 cells. When tumors reached a diameter of 2 mm, one group of mice was i.p. injected with 500 μL of 4 mg/mL of cyclophosphamide in PBS; the other group received 500 μL of PBS. Four days later, mice were s.c. injected (into the left inguinal flank) with 1 × 10^6 TC-1 cells transduced ex vivo with the induced adenovirus vectors. Following vaccination, the volume of the primary tumor was measured twice a week. The tumor volume was calculated using the formula [largest diameter × (smallest diameter)^2]. Mice were sacrificed when the tumor volume reached 1,000 mm^3.

“Therapeutic” setting. Two strategies were used. In the first setting, cells were transduced with 100 pfu/cell of the indicated adenovirus vectors. Sixteen hours later, (for Ad.sgp96 and Ad.mgp96 vectors) or 72 hours later
(for AdIR vectors), mock- or adenovirus-transduced cells were collected, washed, and mice were s.c. injected into the right inguinal region with $1 \times 10^6$ cells. In another setting, transduced cells were mixed with 20% of nontransduced cells.

**Analysis of Tregs.** Splenocytes were analyzed by flow cytometry using the following antibodies: rat monoclonal antibody (mAb) anti-FoxP3-PE (clone FIH16s, ebioscience, San Diego, CA), rat mAb anti-CD4-PE, and rat mAb anti-CD25-FITC (all from ebioscience). All samples were treated with Fe-block (CD16/CD32). Corresponding isotope controls yielded no significant staining. Cryosections of TC-1 tumors and spleens were analyzed by immunofluorescence using rabbit anti-FoxP3 antibodies (provided by Dr. Koji Matsushita, University of Tokyo; ref. 34) and rat mAb anti-CD25 antibodies (clone 16.15, ebioscience). Binding of primary antibodies was visualized with goat anti-rabbit Alexa fluor 568 (red) and goat anti-rat Alexa fluor 488 (green) antibodies (Molecular Probes, Eugene, OR). Nuclei were stained with 4',6-diamidino-2-phenylindole (Sigma).

**ELISpot.** Splenocytes of naïve C57Bl/6 mice were pulsed with 10 μg of the HPV16 E7 49-57 carrying the H-2D b-restricted peptide (RAHYNIVTF; ref. 35) or an unrelated control peptide (C57Bl/6 mice are H-2D b and the E7 49-57 peptide contains a CTL epitope). On day 14 after vaccination with mock-infected TC-1 cells or cells transduced with AdCo, Ad.sgp96, or AdIRE1A/TRAIL, vaccinated animals (with and without cyclophosphamide) were sacrificed, splenocytes were collected and 1 $\times 10^6$ cells were mixed with 1 $\times 10^5$ ex vivo pulsed splenocytes for in vitro sensitization. After 24 hours of incubation in 96-well plates, cells were plated in anti–IFN-γ-coated wells of ELISpot plates (Millipore, Bedford, MA). Twenty-four hours later, plates were washed and the spots of IFN-γ-producing T cells were counted.

**Results**

**Vector construction.** gp96 is an endoplasmic reticulum–resident protein that is released upon necrotic cell death or virus infection and subsequently confers antigen-transport to and activation of dendritic cells. To increase the immunostimulatory potency of gp96, we constructed a secreted form of gp96 (sgp96) by deleting the COOH-terminal KDEL endoplasmic reticulum–retention signal. Notably, gp96 has an NH2-terminal leader peptide (L) allowing for secretion. For comparison, we also generated a membrane-localized form of gp96 (mgp96) that was recently shown to induce potent antitumor responses (4). To create mgp96, a COOH-terminal PDGFR transmembrane domain was linked to the gp96 OBF deleted for the KDEL signal (Fig. L4). We constructed two sets of vectors for constitutive and replication-activated (tumor-specific) gp96 expression. We hypothesized that restricting gp96 expression to tumor cells would increase the safety profile of our vectors, considering that unrestricted gp96 expression (in nontumor cells) without cell death can cause autoimmune reactions (36). For constitutive gene expression, E1/E3-deleted Ad5-based vectors expressing mgp96 and sgp96 under the control of the ubiquitously active RSV promoter were generated (Ad.mgp96 and Ad.sgp96). For tumor-specific gp96 expression, we constructed AdIR vectors (AdIR-E1A/mgp96 and AdIR-E1A/sgp96). In addition to gp96, these vectors express E1A in a tumor-specific manner, which will confer high-level transgene expression in turn replication of vector genomes only in tumor cells. E1A also has the potential for up-regulating HSPs in tumor cells, which, in turn, would improve antigen presentation to dendritic cells (37). Other control adenovirus vectors (AdCo) included AdGFP (14) and Ad.bGal (8). Control AdIR vectors included AdIR-GFP (14) and AdIR-E1A/AP (32).

**Transgene expression.** As a model for our studies, we selected TC-1 cells. TC-1 cells are immortalized murine epithelial cells that stably express HPV-16 E6 and E7 proteins, which can support DNA replication of E1-deleted adenovirus vectors and serve as easily detectable tumor antigens. Upon s.c. transplantation into syngeneic C57Bl/6 mice, TC-1 cells form aggressively growing, vascularized tumors. Infection of TC-1 cells with Ad.GFP at a MOI of 30 pfu/cell conferred transgene expression in nearly 100% of cells (Fig. 1B). The level of viral DNA replication of AdIR-GFP in TC-1 cells was ~10-fold lower than in human tumor cells (data not shown). Consequently, transgene (GFP) expression levels in TC-1 cells from replication-activated AdIR vectors were significantly lower than from adenovirus vectors that contained the transgene under the direct control of the RSV promoter (Fig. 1B). This is also reflected in Western blot analyses for gp96 expression in TC-1 cells upon infection with Ad.mgp96, Ad.sgp96, Ad.IRE1A/mgp96, or Ad.IRE1A/sgp96 (Fig. 1C). This analysis also confirmed that mgp96 is exclusively found in cell lysates, whereas the vast majority of sgp96 is secreted and detectable in the supernatant. Furthermore, Fig. 1C shows that TC-1 cells express endogenous gp96 at high levels, and that this is not triggered by adenovirus infection. Membrane localization of mgp96 expression was shown by immunofluorescence of adenovirus-transduced TC-1 cells using gp96-specific antibodies (Fig. 1D).

**Effect of gp96 expression on TC-1 cells in vitro.** We first tested in vitro whether expression of gp96 affects TC-1 viability (Fig. 1E). In CPE assays, there was no difference in cytotoxicity between control adenovirus-infected and Ad or AdIR vectors expressing either gp96. This indicates that gp96 overexpression per se does not exert an antitumor effect. Notably, compared with Ad.sgp96 and Ad.mgp96, all vectors containing adenovirus E1A displayed slightly more cytotoxicity when infected onto TC-1 cells at a MOI of 100 pfu/cell.

**Gp96-mediated stimulation of antitumor immune responses.** Next we analyzed the effect of gp96 expression in TC-1 cells on growth of s.c. tumors. In an initial study, TC-1 cells were infected ex vivo at a MOI of 100 pfu/cell. A total of 2 $\times 10^5$ infected cells were then transplanted s.c. into C57Bl/6 mice and tumor volume was measured over a period of 25 days (Fig. 2A). Although tumor growth of Ad.mgp96 infected TC-1 cells was comparable to mock- and Ad.bGal-infected cells, tumors derived from Ad.sgp96-infected cells grew significantly slower ($P < 0.01$). In a first attempt to assess whether gp96 expression could induce an antitumor response to TC-1 cells that are not transduced with adenovirus, we transplanted adenovirus-infected cells together with a mixture (20%) of nontransduced cells (Fig. 2B). The outcome was comparable to that seen with 100% transduced cells. To corroborate these results, we conducted the following vaccination experiment. We injected TC-1 cells transduced ex vivo with adenovirus vectors, repeated the vaccination 2 weeks later, and challenged the mice with 2 $\times 10^5$ untransduced TC-1 cells 14 days after the second vaccination [in this experiment, vaccination cells were sterilized by γ-irradiation (4,000 rad) to avoid outgrowth of tumors and provide gp96 expression]. The tumor volume was measured and survival was recorded (Fig. 2C). Mice vaccinated with Ad.sgp96 had a slightly better survival but the differences between groups vaccinated with Ad.bGal-, Ad.mgp96-, and Ad.sgp96-infected cells were not significant ($P = 0.42$ for Ad.bGal versus Ad.sgp96).

We hypothesized that the failure of vaccination to generate effective protective immune responses could have been due to the suppressive effect of Treg cells that are induced by TC-1 tumors. We therefore studied the presence of Tregs in our model and evaluated whether Tregs can be killed by low-dose cyclophosphamide treatment.
Splenocytes of TC-1 tumor-bearing or naïve mice with and without cyclophosphamide treatment (2 mg/mouse) were analyzed by flow cytometry for the presence of CD4+, CD25+, and FoxP3+ cells (Fig. 3). In control mice (without tumors and without cyclophosphamide treatment), the percentage of CD4+, CD4+CD25+, and FoxP3+ cells in the spleen was 18%, 2%, and 3.2%, respectively. The presence of s.c. TC-1 tumors significantly increased the percentage of splenic FoxP3+ and CD25+ cells within the CD4+ cell population in mice that were not treated with cyclophosphamide (P = 0.018 for FoxP3+, P = 0.05 for CD25+/CD4+, and P = 0.004 for CD25+).

Figure 1. Characterization of gp96-expressing adenovirus vectors. A, schematic representation of adenovirus vectors. The COOH-terminal KDEL signals were deleted from the gp96 cDNA to produce the secreted form of gp96 (sgp96) or replaced with the PDGF transmembrane domain (TM) to produce mgp96. Gp96 contains an NH2-terminal leader (L) peptide. Ad.sgp96 and Ad.mgp96 express the soluble form of gp96 and the membrane-anchored form of gp96, respectively, under the control of the RSV promoter. Ad.IR-E1A/sgp96, Ad.IR-E1A/mgp96, Ad.IR-E1A/TRAIL, and Ad.IR-E1A/AP contain the Ad5 E1A gene linked via an IRES to sgp96, mgp96, TRAIL, and alkaline phosphatase (AP) genes, respectively. The bicistronic transgene cassettes are flanked by inverted homology regions (IR). The orientation of the bicistronic transgene cassettes towards the RSV promoters is in the 3’→5’ direction. If Ad.IR vectors infect cells that support Ad DNA replication (e.g., TC-1 cells), (replication-dependent) homologous recombination between the IRs links the RSV promoter to the 5’-end of the transgene cassette, which results in gene expression [for a more detailed explanation of Ad.IR vectors, see ref. (47)]. PA, SV40 polyadenylation signal; ITR, inverted terminal repeats. B, transduction of TC-1 cells by adenovirus vectors. TC-1 cells were infected with Ad.GFP and the replication-activated Ad.IR-GFP vector (14) at the indicated MOIs. The percentage of GFP-expressing cells was analyzed 24 hours after infection by flow cytometry. Columns, mean from three experiments; bars, SD <10% for all groups. C, analysis of gp96 expression by Western blotting with gp96-specific antibodies. Cells were infected with the indicated adenovirus vectors at an MOI of 100 pfu/cell and gp96 expression was analyzed 48 hours later in cell lysates and supernatants. For analysis of cell lysates, a total of 10 μg of protein was loaded on 10% polyacrylamide/SDS gels and separated (left). For analysis of secreted sgp96, 500 μL of culture supernatant was immunoprecipitated using anti-gp96 antibodies and protein A Sepharose. The precipitated proteins were separated by polyacrylamide gel electrophoresis (right). Arrow, endogenous gp96 (endoplasmic reticulum-localized); stars, recombinant sgp96 and mgp96. D, analysis of mgp96 expression by immunofluorescence. Cells were infected with Ad.bGal (a control vector), Ad.mgp96, and Ad.IR-E1A/mgp96 as described in (B) and analyzed by staining with a rat anti-gp96 antibody and an anti-rat-FITC conjugated antibody. Note the membrane localization of gp96. E, in vitro toxicity of gp96 vectors (crystal violet). TC-1 cells were infected with adenovirus vectors at the indicated MOIs. Cell viability was assessed by crystal violet staining 9 days after infection.
for FoxP3/CD4+; Fig. 3A, right). Four days after cyclophosphamide injection, the percentage of CD4+ in the spleen was higher compared with control mice without cyclophosphamide treatment (Fig. 3A, left). However, the amount of Tregs, as the percentage of CD25+ cells within the CD4+ population, decreased after cyclophosphamide treatment (Fig. 3A, middle; P = 0.0006). Furthermore, the percentage of FoxP3+ and FoxP3+/CD4+ cells in the spleen was significantly decreased in cyclophosphamide-treated mice (P < 0.000001; Fig. 3A, right). Sections of tumors were analyzed by immunofluorescence for FoxP3 and CD25 (Fig. 3B). These analyses showed markedly less FoxP3+ and CD25+ cells in TC-1 tumors of cyclophosphamide-treated mice. Taken together, our data indicate that TC-1 tumors attract Tregs and increase the number of Tregs in spleen and that low-dose cyclophosphamide treatment specifically kills Tregs.

Having established that low-dose cyclophosphamide treatment can eliminate Tregs in spleen and tumors, we did the following vaccination study: mice were injected with TC-1 cells. When s.c. tumors reached measurable size, half of the mice were injected with low-dose cyclophosphamide or PBS, followed by cell vaccinations 4 days later. Cell-based vaccines were TC-1 cells mock-treated, infected ex vivo with a control adenovirus vector expressing GFP (Ad.Co) and with Ad.mgp96 and Ad.sgp96 at a MOI of 500 pfu/cell. No significant difference was seen between the groups of mice that were not treated with low-dose cyclophosphamide (Fig. 4A). Cyclophosphamide injection alone slightly accelerated tumor growth (Fig. 4B, "Mock" + CY). Importantly, tumor growth was greatly delayed in mice vaccinated with adenovirus infected TC-1 cells (Fig. 4B), whereby there was a significantly stronger delay in tumor growth (and prolonged survival) in mice vaccinated with sgp96-expressing TC-1 cells (P < 0.01 for Ad.Co + CY versus Ad.sgp96 + CY; Fig. 4C and D). A similar outcome was seen in studies done with Ad.IR vectors expressing sgp96 (data not shown).

In conclusion, the secreted form of sgp96 is able to induce an antitumor immune response that slows the outgrowth of wild-type s.c. tumors. This effect can be greatly enhanced by preinjection of low-dose cyclophosphamide which has the potential to selectively kill regulatory T cells.

**Induction of an immune response of TC-1 cells infected with Ad.IR-E1A/TRAIL.** One of the goals of our study was to test whether Ad.IR-E1A/TRAIL-mediated oncolysis, in combination with sgp96 expression, triggered synergistic antitumor immune responses. Towards this goal, we evaluated first whether vaccination with TC-1 cells infected with Ad.IR-E1A/TRAIL could protect against tumor cell challenge. Upon infection of cultured TC-1 cells at an MOI of 100 pfu/cell, the oncolytic vector, Ad.IR.E1A/TRAIL, efficiently killed TC-1 cells by apoptosis as verified in caspase-3 activation assays (data not shown). Vaccination studies were conducted as described for Fig. 4. Cell-based vaccines were TC-1 cells infected ex vivo with a control adenovirus vector or Ad.IR-E1A/TRAIL at a MOI of 500 pfu/cell (Fig. 5; for mock controls, see Fig. 4). No significant difference was seen between the two groups of mice that were not treated with cyclophosphamide. Importantly, in three out of five cyclophosphamide-treated mice vaccinated with Ad.IR-E1A/TRAIL-infected TC-1 cells, tumor growth was completely suppressed for 30 days. Suppression of tumor growth after...
Ad.IR-E1A/TRAIL infection was significantly greater than that of Ad.Co infection, indicating that TC-1 cells which die by Ad.IR-E1A/TRAIL mediated apoptosis could act as strong vaccines. Interestingly, TC-1 cells infected with Ad.Co at a MOI of 500 pfu/cell also showed a vaccination effect in cyclophosphamide-treated mice, which could be due to the expression of viral proteins and/or virus-mediated cell death.

To further characterize antitumor immune responses seen in Figs. 4 and 5A, splenocytes of vaccinated mice were analyzed by ELispot assay for the frequency of IFN-γ-producing T cells specific to the HPV16 E7_{49-57} peptide (RAHYNIVTF) carrying an H-2D^b-restricted epitope (ref. 35; Fig. 5B). Overall, this study indicated that the number of E7-specific T cells correlated with the tumor-destructive immune responses described above. The fraction of E7-specific IFN-γ-producing cells is larger in cyclophosphamide-treated mice with Ad.IR-E1A/TRAIL or Ad.sgp96 than with mock- or Ad.Co-infected TC-1 cells.

In summary, the oncolytic vector, Ad.IR-E1A/TRAIL, that induces TRAIL-mediated apoptosis in the context of adenovirus infection, can trigger therapeutic T cell responses if mice are pretreated with low-dose cyclophosphamide.

**Analysis of in vitro synergy of gp96 expression and viral oncolysis.** A recent report suggested that overexpression of HSP70 supports the oncolytic effect of a replication-competent human adenovirus vector in vitro (38). To evaluate whether we would see a similar effect in our system, we infected TC-1 cells with Ad.IR vectors expressing E1A + gp96, E1A + TRAIL, and E1A + alkaline phosphatase (AP; as a control). The oncolytic vector Ad.IR.E1A/TRAIL efficiently killed TC-1 cells (Fig. 6A). When combined with Ad.IR-E1A/sgp96, however, we could not detect a stronger cytolytic effect of Ad.IR-E1A/TRAIL or Ad.IR-E1A/AP in this cell line (Fig. 6A), as well as in several other cell lines tested (data not shown). The discrepancy with the study by Haviv et al. might be due to the use of different HSP's (hsp70 versus gp96) or due to different HSP expression levels.

**Analysis of antitumor synergy between Ad.IR-E1A/sgp96 and Ad.IR-E1A/TRAIL.** To study a potential synergy between gp96 expression and viral oncolysis in inducing antitumor immune responses, we performed two studies. First, we injected mice with a mixture of TC-1 cells infected ex vivo with adenovirus vectors and noninfected cells (80:20) and measured tumor outgrowth (Fig. 6B). Cells were infected at a total MOI of 500 pfu/cell with Ad.IR-E1A/AP, Ad.IR-E1A/sgp96 + Ad.IR-E1A/AP, Ad.IR-E1A/TRAIL + Ad.IR-E1A/AP, and Ad.IR-E1A/sgp96 + Ad.IR-E1A/TRAIL (Fig. 6B). Although the combination of the oncolytic vector and the gp96-expressing vector slightly slowed tumor growth, this effect was not significant compared with the other groups (Ad.IR-E1A/sgp96 + Ad.IR-E1A/TRAIL versus Ad.IR-E1A/sgp96 + Ad.IR-E1A/AP; P = 0.021; Ad.IR-E1A/sgp96 + Ad.IR-E1A/TRAIL versus Ad.IR-E1A/TRAIL + Ad.IR-E1A/AP; P = 0.052).

Secondly, in a vaccination scheme, TC-1 cells were infected ex vivo at a MOI of 500 pfu/cell with Ad.IR-E1A/sgp96 + Ad.IR-E1A/AP, Ad.IR-E1A/TRAIL + Ad.IR-E1A/AP, and Ad.IR-E1A/sgp96 + Ad.IR-E1A/TRAIL injected into mice with pre-established TC-1 tumors 4 days after treatment with low-dose cyclophosphamide (Fig. 6C). As in Fig. 5, Ad.IR.E1A/TRAIL-mediated oncolysis had a strong vaccination effect. Surprisingly, gp96 expression reduced the vaccination effect of Ad.IR-E1A/TRAIL-infected TC-1 cells.

In conclusion, TC-1 cells infected with Ad.IR-E1A/sgp96 induce a tumor-destructive immune response in cyclophosphamide-treated mice. This immune response was not increased by additional infection with Ad.IR-E1A/TRAIL.
Discussion

The goal of this study was to overexpress an HSP from adenovirus vectors in tumor cells and to test whether this, alone or in combination with TRAIL-mediated tumor cell apoptosis (from Ad.IR-E1A/TRAIL), would stimulate an antitumor immune response. As outlined earlier, we hypothesized that HSP would efficiently chaperone cryptic tumor cell antigens (released upon oncolysis) to APCs and that this, in the context of adenovirus-induced release of proinflammatory cytokines, would induce a strong T cell response.

Based on two earlier reports demonstrating the successful application of a gp96 genetically modified to be displayed on the surface of expressing cells for cancer immunotherapy (4), we focused on gp96 as a test HSP. In addition to a membrane-anchored gp96, we also expressed a secreted form of gp96 to enable presentation of tumor antigens to APCs that are not directly in contact with infected tumor cells. It is known that endogenous gp96 is abundant in tumor cells and our data shown in Fig. 1C confirm this for TC-1 cells. Furthermore, endogenous gp96 expression could theoretically be further up-regulated by adenovirus infection and/or E1A expression from Ad.IR-E1A/TRAIL (37). In spite of this, we found that TC-1 cells mock-infected or infected with control vectors (Ad.bGal or Ad.IR-E1A/AP) could not induce antitumor immune responses in therapy or vaccination schemes (Figs. 4 and 6B). Furthermore, we were unable to detect a significant antitumor effect if a membrane-anchored form of gp96 was expressed. However, the secreted gp96 form when overexpressed from an adenovirus vector could induce an in vivo response capable of significantly delaying tumor growth. Several reasons could account for the lack of a response after Ad.mgp96 treatment. The secreted gp96 form might facilitate the transport of tumor antigens to regional lymph nodes and its uptake by APCs.

![Figure 4](cancerres.aacrjournals.org/content/canres/66/2/966.full.html#fig4)

**Figure 4.** Effect of cyclophosphamide after vaccination with TC-1 cells that overexpress gp96. C57Bl/6 mice were s.c. injected with $2 \times 10^5$ TC-1 cells. When tumors reached a diameter of 2 mm, one group of mice (B) received an i.p. injection of 2 mg cyclophosphamide (CY). The other group (A) received PBS injections. Four days later, mice were vaccinated with $1 \times 10^6$ TC-cells, mock-infected or infected ex vivo with Ad.Co (a bGal-expressing first-generation vector), Ad.mgp96, or Ad.sgp96 at an MOI of 500 pfu/cell. Growth of primary tumors was recorded over a period of 35 days. Mice were sacrificed when tumors reached a volume of 1,000 mm$^3$ (for PBS-injected groups; C) or 200 mm$^3$ (for cyclophosphamide-treated groups) and survival was recorded (D).

The relatively high MOI of 500 pfu/cell was chosen for TC-1 cell infection to inhibit outgrowth of vaccination tumors. Notably, Ad.Co infection at this MOI caused cell death within 5 days in cultured TC-1 cells ($n = 5$). Each line represents one mouse/tumor.
whereas membrane gp96 can only interact with APCs residing in the tumor. Furthermore, as outlined in more detail below, TC-1 cells attract Tregs (through expression of specific cytokines as suggested for human tumors; ref. 39) and these tumor-associated Tregs could interfere with T cell activation at the tumor site.

We also reasoned that it would be beneficial if a secreted form of gp96 is exclusively expressed by tumor cells that undergo apoptosis and release antigen, which, with gp96 acting as a chaperone, is taken up by APCs. We therefore employed the replication-activated Ad.IR system that selectively activates transgene expression in tumor cells. Although TC-1 cells support replication of E1-deleted adenovirus vectors and allow for transgene expression from Ad.IR vectors, the levels of both replication and transgene expression are lower than in human cells. This is not surprising as mouse cells are less permissive for adenovirus replication than human cells [whereas 7 days after infection with first-generation adenovirus at an MOI of 500 pfu/cell, ~5,000 pfu/cell of de novo produced adenovirus particles were found in HeLa cell lysates, <5 pfu were found in TC-1 cells (data not shown)]. Therefore, experiments done with human replicating adenovirus vectors, including Ad.IR vectors, in mouse tumor cells underestimate the potential that the same vectors might have in human tumor cells. Overall, however, the ability of sgp96 expressed from Ad.gp96 or Ad.IR-E1A/gp96 to stimulate an effective prophylactic vaccination in the TC-1 cell model was disappointing compared to studies with HSPs in other tumor models (4). One explanation for this outcome is that a T cell response against TC-1 cells is suppressed in vivo. Several mechanisms could account for this, including the activation of Tregs (21, 22). A role of Tregs is indicated by our studies showing that (a) TC-1 tumor transplantation increases the number of splenic Tregs, (b) infiltrating Tregs are present in TC-1 tumors, (c) low-dose cyclophosphamide treatment decreases the number of tumor-infiltrating and splenic Tregs, and (d) cyclophosphamide treatment greatly enhances the effect of sgp96 to trigger a protective immune response. Although it is thought that low-dose cyclophosphamide acts through a mechanism of selective toxicity against Tregs (40), other effects of low-dose cyclophosphamide on T cell homeostasis are likely. Notably, in the period of observation (5 weeks), we did not observe signs of autoimmunity such as hypopigmentation of the coat.

Our hypothesis, that the immunogenicity of tumor cells could be increased by viral oncolysis goes back to studies by Lindenmann and Klein in 1967 (41). The underlying mechanisms include the release of proinflammatory cytokines after adenovirus uptake into tumor cells or cells of the immune system (including APCs and macrophages), the ability to release cryptic antigens as apoptotic bodies, and the coexpression of viral proteins in tumor cells which are potent immunogens (CTL specific to adenovirus proteins might help liberate tumor antigens for cross-priming of APCs). A number of studies have supported this hypothesis (12–14). We showed here that a vaccination effect of Ad.IR-E1A/TRAIL can be achieved by treatment of mice with low-dose cyclophosphamide and that cells
that die from Ad.IR-E1A/TRAIL infection stimulate a stronger immune response than cells that die from adenovirus-mediated toxicity (after Ad.Co infection). The latter indicates that TRAIL-mediated apoptosis (in the context of adenovirus infection) is able to release antigens in a more immunogenic form.

Earlier studies reported the synergistic effect of hsp70 and viral oncolysis (42, 43). In our experiment, we did not see the additive effect of sgp96 expression and Ad.IR-E1A/TRAIL-mediated oncolysis. The reason for the finding that tumor cells, which overexpress sgp96 and die by apoptosis, are less immunogenic, is unclear. We speculate that the combination of antigen released by apoptosis, and antigen chaperoned to APC by sgp96, might overload the ability of the APC to process and present antigen in regional lymph nodes. There may be a relative lack of maturation signals when more antigen is present in immature APCs and this might lead to tolerance/anergy [for a review see ref. (44)]. The "overflow" hypothesis is further supported by studies with recombinant gp96 and tumor-derived peptides showing that the immunostimulatory effect of gp96 is dose-dependent; low doses of gp96 generate immunity, whereas doses 10 times the immunizing dose do not (45). Clearly, more experiments with vectors that express different sgp96 levels and studies in other tumor models are required to support our preliminary observation of a lack of synergy between Ad.IR-E1A/sgp96 and Ad.IR.E1A/TRAIL. The conflict between our data and data by Chen's group (42, 43) might also be related to the use of different HSPs (hsp70 versus gp96). These HSP's can use different receptors to mediate their effects. Hsp70 binds to CD14 or Toll-like receptors, which may function to enhance innate immunity via APC activation, whereas gp96 binds to CD91, which may interfere with phagocytosis of apoptotic cells (46).

In conclusion, this study shows that a secreted form of gp96 has immunostimulatory activity. Our data indicate that the effect of viral oncolysis and adenovirus-mediated gp96 expression depends on the tumor cell type and the mechanism by which tumor cells die. The finding that an oncolytic or immunostimulatory vector, in combination with cyclophosphamide treatment, induces an immune response that is able to eliminate pre-established tumors has important implications for tumor gene therapy trials.

Figure 6. In vivo analysis of a potential synergy between Ad.IR-E1A/sgp96 and Ad.IR-E1A/TRAIL. A. Cytolytic effect of the oncolytic adenovirus vector Ad.IR-E1A/TRAIL after infection of TC-1 cells alone or in combination with gp96-expressing adenovirus vectors. Confluent TC-1 cells (in 24-well plates) were infected at the indicated MOIs and cytotoxicity was assessed by crystal violet staining 3 days after infection. The remaining (stained) cells were destained with 20% acetic acid, lysed, and the absorbance of the lysate was measured at OD570. Results were expressed as the percentage of cells remaining compared with untreated controls (if two vectors were used for infection, the MOI of each vector was 50 or 150 pfu/cell, respectively; n = 3).
B. TC-1 cells were transduced at a total MOI of 500 pfu/cell. Sixteen hours later, adenovirus-transduced cells were harvested, washed, and 8 × 10^6 transduced cells were mixed with 2 × 10^6 nontransduced cells and s.c. injected into mice. As controls, two groups of mice received 8 × 10^6 or 2 × 10^6 nontransduced cells. Tumor growth was monitored at the indicated time points. Each line represents one tumor. If two vectors were used, the MOI of each vector was 50 or 150 pfu/cell.
C. Mice were s.c. injected with 2 × 10^6 TC-1 cells. When tumors reached a diameter of 2 mm, all mice received an i.p. injection of 2 mg cyclophosphamide (CY). Four days later, mice were vaccinated with 1 × 10^6 TC-1 cells, mock-infected or infected ex vivo with the indicated adenovirus vectors at a total MOI of 500 pfu/cell. Growth of primary tumors was recorded over a period of 35 days (n = 5) except for the Ad.IR-E1A/TRAIL + Ad.IR-E1A/sgp96 group, where 10 mice were analyzed. Each line represents one tumor.
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References


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