Versatile Prostate Cancer Treatment with Inducible Caspase and Interleukin-12

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Abstract

To establish optimized conditions for immunity against prostate cancer, we compared the efficacy of multiple approaches in autochthonous and s.c. transgenic adenocarcinoma of the mouse prostate (TRAMP)-based models. Mice immunized with interleukin (IL)-12–containing apoptotic, but not necrotic TRAMP-C2 cell–based, vaccines were resistant to TRAMP-C2 tumor challenge and re-challenge, independently of the route of vaccination (s.c. or i.p.). Administration of γ-irradiated TRAMP-C2 cells preinfected with adenovirus containing both B7-1 and IL-12 genes, unlike adenovirus containing B7-1 alone, considerably prolonged C57BL/6 mice from TRAMP-C2 tumor growth and extended the life span of TRAMP mice. Vaccines that included dendritic cells, instead of IL-12, were equally efficient. Whereas injections of ligand-inducible caspase-1– and IL-12–containing adenoviruses cured small s.c. TRAMP-C2 tumors, nanopump-regulated delivery of viruses led to elimination of much larger tumors. The antitumor immune responses involved CD4+-, CD8+-, and natural killer cells and were strengthened by increasing the number of vaccinations. Intraprostatic administration of inducible caspase-1– and IL-12–containing adenoviruses resulted in local cell death and improved survival of adenocarcinoma-bearing TRAMP mice. Thus, tumor cell apoptosis induced by caspase in situ and accompanied by IL-12 is efficient against prostate cancer in a preclinical model. (Cancer Res 2005; 65(10): 4309-19)

Introduction

Due to advances in early detection and treatment of prostate cancer, the death rate from prostate cancer is at its lowest level since the National Cancer Institute began tracking cancer mortality in 1973 (1). Nevertheless, 20% to 25% of patients treated with surgery or radiation for localized prostate adenocarcinoma experience disease progression, presumably associated with occult, micrometastatic cancer. Evidently, a deeper understanding is needed of the immunologic mechanisms that result in prostate cancer prevention or regression in model systems.

An important question of immunotherapeutic design is whether apoptotic or necrotic tumor cells are better as a source of tumor antigens to stimulate specific antitumor immunity. Dendritic cells are 1,000 to 10,000 times more efficient at forming MHC-peptide complexes from phagocytosed cells than at forming them from preprocessed peptides (2). Potentially, both cell lysate-pulsed dendritic cells (3) and those exposed to apoptotic cells (4) induce T-helper 1 cell and CTL reactivity in vitro and mediate tumor regression in vivo. Because the optimum source of antigens remains a debatable issue (5–8), it may have to be readdressed for each tumor model.

 Suicide gene therapy represents an attractive approach to trigger tumor cell death in situ, providing a depot of potential tumor antigens while avoiding the side effects of radio- and chemotherapy. Herpes simplex virus type I thymidine kinase (HSV-tk) has been the most widely used suicide gene to date, partially because of its precedence and “bystander effect” (9, 10). However, alternatives are necessary for higher efficiency at killing slowly dividing tumor cells, such as prostate cancer cells. We have previously shown that lipid-permeable dimerizing ligand-inducible caspase–mediated apoptosis is cell cycle-independent, nontoxic to nontargeted cells and a minimally invasive way of creating a large source of dying tumor cells in vivo (11, 12). Moreover, inducible caspases can be restricted by tissue-specific promoters. Despite the clear theoretical advantages of inducible caspses, their application to transgenic cancer models and inducible caspase–driven immune responses have never been reported.

In addition to antigens, adjuvant(s), the number, type and activation state of antigen-presenting cells, tumor-specific helper/effector, and regulatory T cells influence the efficacy of immunotherapy (13). In general, inducers of an antitumor immune response must neither tolerate nor overstimulate (13, 14). Potential improvements on an antitumor immune response include the use of costimulatory molecules (e.g., B7-1) to drive T cells towards activation, cytokines [e.g., interleukin (IL)-12] to induce CTL-favoring T-helper 1 differentiation and expansion, and dendritic cells as antigen-presenting cells and an additional source of cytokines.

Intraprostatic administration of therapeutic molecules/cells into mice mimics vector delivery in prostate cancer clinical trials (15, 16). Direct injection into the prostate results in the wide dispersion of adenoviral vectors, especially within the urogenital tract (17, 18), likely providing extra protection against micro-metastases. To date, the majority of studies reporting intratumoral treatment of mice have been done in orthotopic preimplanted (19, 20) or s.c. (21) tumor models. The apparent recalcitrance to treat transgenic adenocarcinoma of the mouse prostate (TRAMP) mice intraprostatically (22) is likely related to the heterogeneity of pathology and variability of tumor progression rates in these animals (23, 24). Nevertheless, prostate cancer in TRAMP mice has proven to be sensitive to therapies, when the treatment is started at an appropriate time (23, 25). Depending on the protocol used, the life span of treated TRAMP animals can be extended by 6 to 8 weeks (26), and the incidence of metastasis can be reduced by 30% (27).
Although extensive apoptosis is capable of promoting dendritic cell maturation (28), modest apoptosis is unlikely to be immunogenic in the absence of exogenous “danger” signals. Therefore, we hypothesized that caspase-killed tumor cells supplemented with IL-12 might trigger robust immune responses against prostate cancer. Accordingly, by systematically testing several variables, including method of killing tumor cells, route of vaccine delivery, presence of a costimulatory molecule, cytokines, and/or dendritic cells, we developed an optimized prostate tumor vaccine protocol. Further, the same protocol was shown to be effective for the treatment of both ectopic and autochthonous prostate tumors in TRAMP-based animal models.

Materials and Methods

Animals. Six- to eight-week-old male C57BL/6 (BL/6) mice were purchased from the Donor Vendor Facility of Baylor College of Medicine (Houston, TX). B6.129S2-Cd4tm1Mak (Cd4 knock-out), B6.129S2-Cd8tm1Mak (Cd8 knockout), and nude (B6.Cg-Foxn1nU) mice were purchased from Jackson Laboratory (Bar Harbor, ME). TRAMP mice (BL/6 background) were generously provided by N.M. Greenberg (Baylor College of Medicine). All mice were housed in pathogen-free units in the Transgenic Mouse Facility at Baylor College of Medicine.

Cell lines. TRAMP-based TRAMP-C2 cells (referred to as TC2 in the illustrations), provided by N.M. Greenberg, no longer have detectable SV40 sequences (29). TRAMP-C2 cells, we developed an optimized prostate tumor vaccine protocol. Further, the same protocol was shown to be effective for the treatment of both ectopic and autochthonous prostate tumors in TRAMP-based animal models.

Generation of dendritic cells. Dendritic cells were generated from bone marrow of naïve BL/6 mice in complete culture medium supplemented with 20 ng/mL recombinant murine (rm) granulocyte-macrophage colony-stimulating factor, 10 ng/mL rmIL-4 and 50 μg/mL 2-mercaptopethanol. After 5 days in culture, cells were enriched by centrifugation over a 13.5% metrizamide gradient (Accurate Chemical & Scientific Corporation, Westbury, NY). The purity of dendritic cells was >90% as determined by flow cytometry based on CD11c, CD11b, B7-2, and I-A expression.

Transduction of cells with viral vectors. TRAMP-C2 cells were infected with several adenoviral vectors [multiplicity of infection (MOI) 40, if not indicated otherwise] and cultured for 5 days prior to their killing. Ad-B7-1, carrying murine B7-1, Ad-IL-12, expressing IL-12 polypeptide chains p35 and p40, AdBP1, containing B7-1 and IL-12, as well as control adenovirus (Ad-c), were gifts from F.L. Graham (McMaster University, Hamilton, Ontario, Canada; ref. 29). The purity of dendritic cells was >90% as determined by flow cytometry based on CD11c, CD11b, B7-2, and I-A expression.

Immunization of mice and their challenge with tumor. TRAMP-C2 cells [nontransduced or transduced with adenoviral constructs] were γ-irradiated with 60 Gy using a Gamacell 1000, model C irradiator (Atomic Energy of Canada Ltd., Kanata, Ontario, Canada), washed with Dulbecco’s PBS and injected i.p. or s.c. into the hind legs of BL/6, B6.129S2-Cd4tm1Mak, B6.129S2-Cd8tm1Mak nude, or 6- and 9-week-old TRAMP mice at 2 × 106 cells per mouse. In some experiments, purified dendritic cells were administered at 1.5 × 107 per mouse, alone or together with TRAMP-C2 cells. Control animals were injected with PBS.

Besides γ-irradiation, apoptosis-inducing methods of triggering cell death included infection of cells with Ad-HSV-tk (MOI 10) or Ad-iCasp1 followed by 2-day ganciclovir (100 mmol/L) or CID (200 mmol/L) AP20187; ARIAD Pharmaceuticals, Inc., Cambridge, MA) treatment, respectively. Necrosis was induced by three freeze-thaw cycles. In some cases, this was preceded by incubation of cells at 42°C for 30 minutes to induce production of heat-shock proteins followed by a 3-hour recovery period at 37°C.

Immunizations were repeated two to four times as described in Fig. 1. A. After the last immunization, mice (excluding TRAMP mice) were challenged with viable TRAMP-C2 cells (sufficient to induce tumor growth in 100% of BL/6 mice). Subcutaneous tumors were measured biweekly until animals required euthanasia. Tumor volumes were estimated using the formula: m1^2 × m2 / 2 × 0.5236, where m1 and m2 represented the maximum diameters of the tumor. Tumor-free mice were observed for at least 60 days following tumor cell rejection. Antitumor immune responses were evaluated 10 to 21 days after final immunization of mice bearing small tumors or reimmunization of tumor-free mice.

Treatment of mice with established tumors. One million viable TRAMP-C2 cells were inoculated s.c. into the hind legs of BL/6 mice. When minimum diameters of tumors reached at least 3.5, or 5.8 mm, Ad-B7-1 and Ad-iCasp1 were injected intratumorally as indicated in Fig. 1B. Ad-c was used alone or added to other viruses, so that each mouse received 3.5 × 1010 total viral particles. A nanopump (model KDS310; KD Scientific, Inc., Holliston, MA) was used to control the delivery of viruses by slow administration of viruses (5 μL/minute). Three days after each virus injection, CID (AP20187, 2 mg/kg body weight) or CID carrier (aqueous solution containing 27% polyethylene glycol 400 and 1.5% Tween 80) was given i.p. to mice that had been treated with Ad-iCasp1 or other viruses, respectively. Control animals were injected with PBS. Observation of mice, evaluation of antitumor responses and re-challenge of tumor-free mice were done as described above.

Twelve-week-old TRAMP mice were treated either by s.c. injections with γ-irradiated preinfected TRAMP-C2 cells as described above, or by two virus injections with a 3-week interval into a ventral prostate lobe, followed by CID or CID carrier administration. Doses of intraprostatically injected viruses were 5-fold lower than described in Fig. 1B. Long-term mouse survival was determined.

ELISPOT assay for IFN-γ. Ninety-six-well Multi-Screen-PA plates (Millipore, Bedford, MA) were coated with 10 μg/mL purified rat anti-mouse IFN-γ monoclonal antibody (clone R4-6A2; PharMingen) and incubated with 2 × 105 splenocytes per well in complete culture medium supplemented with 5 ng/mL rmIL-2 for 24 hours in the presence or absence of 2×103 TRAMP-C2 cells, pretreated with 10 ng/mL rmIFN-γ for 4 days and γ-irradiated with 100 Gy on the eve of the assay. Alternatively, splenocytes were stimulated with 4 × 103 or 2 × 103 dendritic cells preincubated with γ-irradiated (100 Gy) TRAMP-C2 cells at a ratio of 1:1 for 24 hours and centrifuged over Lympholyte-M gradient. Pokeweed mitogen (6 μg/mL; Sigma, St. Louis, MO) was used as a positive control. Plates were then incubated overnight at 4°C with 5 μg/mL biotinylated rat anti-mouse IFN-γ monoclonal antibody (clone XMGl2; PharMingen). Spots were visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium alkaline phosphatase substrate (Sigma) reaction, counted per triplicate well using a stereomicroscope and normalized for 105 cells.

CTL assay. Splenocytes were cultured for 7 days in Eagle’s MEM containing Earle’s salts, 1 mg/mL dextrose, 5 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 10 mmol/L HEPES, antibiotics, MEM essential amino acids, 0.15 mmol/L MEM nonessential amino acids, 50 μmol/L L-2-mercaptoethanol, 10% FBS, and 15% rat T-stim culture supplement without concanavalin A (Becton Dickinson) and every other day provided with 5 ng/mL rmIL-2 starting at day 2. Stimulation was done using 500 dendritic cells per well that had been preincubated with γ-irradiated (100 Gy) TRAMP-C2 cells at a ratio of 1:1 for 24 hours and centrifuged over a Lympholyte-M gradient. Effector cells were then collected and coincubated with 10 ng/mL rmIFN-γ-pretreated 3Cr-labeled TRAMP-C2 cells at ratios of 90:1, 60:1, 30:1, 15:1, and 7.5:1 in triplicate for 6 hours. At a 60:1 ratio, 50 YAC-1 cells were added per 3Cr-labeled target cell.
RM-1 and B16 cells were used as alternative targets. Radioactivity was measured by scintillation counter (Beckman Coulter, LS 6500; Beckman Instruments, Inc., Fullerton, CA). The percentage of cell lysis was calculated as follows: \[
\frac{\text{experimental release} - \text{spontaneous release}}{(\text{maximum release} - \text{spontaneous release})} \times 100%.
\]

**Histochemistry.** Subcutaneous tumor, prostate, spleen and liver tissues were fixed in 10% buffered formalin, embedded in paraffin, rehydrated and stained with H&E by routine procedure or for leukocyte acid phosphatase using Sigma kit reagents. Terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) assay was done according to the manufacturer’s instructions (Roche Diagnostics, Mannheim, Germany).

**Statistical analyses.** Data are presented as mean ± SD. The difference between experimental groups was considered to be significant at \(P < 0.05\) as was determined by Student’s t test or ANOVA when appropriate. Kaplan-Meier survival curves were analyzed using a log-rank test (GraphPad software).

## Results

### Comparison of Apoptotic Versus Necrotic Vaccines and Routes of Vaccine Delivery

Because immunization efficacy can depend on the accessibility of the vaccine to antigen-presenting cells, we immunized animals with apoptotic or necrotic tumor cells either i.p. or s.c. The costimulatory molecule, B7-1 (CD80), was tested because of multiple reports of improved immunogenicity of tumor cells expressing B7-1 (31–33), and IL-12 — as a cytokine that enhances a T-helper 1-biased immune response. Before injection, TRAMP-C2 cells were preinfected with AdB1, expressing B7-1 and IL-12, and killed by one of five methods (Fig. 1A) inducing primarily apoptosis, necrosis, or both (HSV-tk/ ganciclovir treatment; refs. 9, 34).

All three vaccines based on apoptotic tumor cells offered considerable protection against viable tumor cells, whereas neither the mock-vaccination nor the two necrosis-based vaccines showed any significant protection (Supplemental Fig. 1A). Moreover, there was no significant difference in results between vaccination via i.p. and s.c. routes (data not shown). Eighteen of 20 animals initially resistant to tumor challenge were also resistant to a re-challenge done 2 months later (Supplemental Fig. 1B), indicating long-term immunologic memory. Because secondary necrosis likely accompanied apoptosis, we cannot definitively conclude that apoptosis, in the absence of necrosis, was sufficient to prime the strong antitumor effects. Nevertheless, it is remarkable that even heat-shock did not improve necrosis-based vaccine efficacy. Having shown the equivalency of proapoptotic methods, in subsequent studies examining the immunogenicity of \(\text{ex vivo}\)–killed tumor cells, we used \(\gamma\)-irradiated TRAMP-C2 cells s.c.

### IL-12, but not B7-1, Is Necessary for Vaccine Efficacy

To determine the impact, if any, of IL-12 and B7-1 molecules on the observed immunogenicity, cells were either infected with AdB1P or adenoviruses expressing B7-1 or IL-12 alone, or neither (Ad-c) protein, or were uninfected. IL-12 secretion and B7-1 expression by Ad-IL-12- and Ad-B7-1-transduced cells were comparable to that of AdB1P-infected cells at MOI 10, 20 (data not shown), and 40. At optimum viral dose per cell (MOI 40), 90 ± 5% of TRAMP-C2 cells transduced with Ad-B7-1 and 85.0 ± 2.0% of those transduced with AdB1P expressed B7-1 ("background" due to nontransduced cells is subtracted) on day 4 after infection. The concentration of IL-12 in TRAMP-C2 cell-conditioned media on day 5 after infection with Ad-IL-12 or AdB1P fluctuated in the range of

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**Figure 1.** Vaccination and treatment schema. A, TRAMP-C2 cells were infected with adenoviral vectors (MOI 40), directing expression of IL-12, B7-1, both (AdB1P), or neither (Ad-c). After 5 days, \(2 \times 10^6\) transduced \(\gamma\)-irradiated (60 Gy) cells were injected s.c. or i.p. into BL/6 mice. Alternatively, cells were killed by the methods described, which included Ad-iCasp1 (to induce apoptosis), HSV-tk (apoptosis and necrosis), heat-shock in combination with freeze-thaw, or freeze-thaw alone (necrosis). Approximately 2 weeks later, vaccinations were repeated one to three times. Finally, mice were challenged s.c. with \(0.7 \times 10^6\) to \(1.5 \times 10^6\) viable TRAMP-C2 cells. B, C57BL/6 mice were injected with \(10^6\) viable TRAMP-C2 cells. Eighteen to 25 days later, established tumors were treated with Ad-IL-12, Ad-iCasp1, both, or Ad-c four times intratumorally followed by i.p. administration of CID or CID carrier and observed.

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Addition of Dendritic Cells Can Optimize TRAMP-C2 Cell–Based Vaccines

To further investigate potential improvements in our immunization design, we added dendritic cells to the vaccines, increased the number of immunizations from two to four and lowered the challenge dose of viable TRAMP-C2 cells from $2 \times 10^6$ to $7 \times 10^5$ cells per mouse. Controls with no dendritic cells were used for each distinct vaccine.

Vaccination with Ad-c-infected TRAMP-C2 cells had a minor inhibitory effect on tumor growth not previously seen, and notably better results were shown with Ad-IL-12 and AdBP1, likely due to the outlined changes in immunization protocol (Fig. 2B). As before, both Ad-IL-12 and AdBP1 had similar efficacy. The addition of immature dendritic cells to γ-irradiated TRAMP-C2 cells improved every vaccine, including nontransduced TRAMP-C2 cells, whereas dendritic cell injections without TRAMP-C2 cells had no significant effect. Remarkably, dendritic cell addition to noninfected TRAMP-C2 cells produced results comparable to those achieved when Ad-IL-12-transduced TRAMP-C2 cells were not supplemented with dendritic cells. The combination of dendritic cells and IL-12-containing TRAMP-C2 cells elicited the lowest mean tumor size, as well as a high proportion of tumor-free mice; however, the differences between outcomes of effective vaccinations were not statistically significant. Regardless of group, animals that rejected their tumors remained cancer-free after tumor re-challenge 2 months later (data not shown).

Apoptotic AdBP1-Transduced TRAMP-C2 Cells Can Prolong the Lives of Mice Bearing Autochthonous Tumors

As a vigorous test for vaccine efficacy, we immunized TRAMP mice with γ-irradiated AdBP1-infected TRAMP-C2 cells at 6, 10,
and 14 weeks of age. A significant reduction in the rate of tumor growth in the vaccinated animals was observed (data not shown). In a more stringent second experiment (Fig. 2C), mice were vaccinated in a similar fashion, but the initial vaccination was delayed until 9 weeks of age, at which point most animals display histologic evidence for prostatic intraepithelial neoplasia (35). The average TRAMP male on a BL/6 background requires euthanasia at about 32 weeks (36). However, by 33 weeks of age, only 10% (1 of 10) of vaccinated animals were considered moribund, whereas 72% (8 of 11) of nonvaccinated animals required euthanasia. In addition, the three surviving control animals had large prostate tumors, in contrast to the majority of vaccinated mice with barely palpable tumors. In a third experiment (Fig. 2D), mice were treated with γ-irradiated AdBP1- or Ad-IL-12-infected TRAMP-C2 cells four times, starting at 12 weeks of age, when 100% of TRAMP mice reportedly carry adenocarcinoma (23, 35). Initially, we observed a high death rate in all groups, likely due to an incurable stage adenocarcinoma in some mice at the beginning of treatment. Nevertheless, although 100% (10 of 10) of control mice were dead by 40 weeks, 50% of animals in the TRAMP-C2-Ad-IL-12- and TRAMP-C2-AdBP1-treated groups were still alive until 44 and 48 weeks of age, respectively. Again, therapeutic efficiencies of Ad-IL-12- and AdBP1-transduced TRAMP-C2 cells were not statistically different. Thus, IL-12-containing, apoptotic cell–based treatment can significantly prolong the life of mice prone to prostate cancer.

**Both CD4⁺ and CD8⁺ T Cells Are Required For Maximum Vaccine Efficacy**

The utility of IL-12 and resistance of mice to tumor re-challenge implicated T cell involvement. To gain direct evidence for this assumption, we did the immunization in T cell–deficient animals. As expected, vaccination with AdBP1-transduced γ-irradiated TRAMP-C2 cells failed to protect nude mice from TRAMP-C2 tumors (Fig. 3A). To investigate the role of T cell subsets in the antitumor response, we vaccinated animals deficient in CD4⁺ or CD8⁺ T cells twice. Unexpectedly, no difference was found between mean tumor sizes in vaccinated BL/6 or CD8 knockout mice (Fig. 3B). Moreover, 2 of 10 and 3 of 10 animals in these groups, respectively, were tumor-free. Two of 10 immunized CD4 knockout mice also remained tumor-free. However, the mean tumor volume in this group was close to that of nonimmunized mice. To better evaluate the CD8⁺ T cell requirement, we raised the number of vaccinations and reduced viable tumor cell number. In this case, CD8⁺ T cell deficiency mildly decreased the resistance of vaccinated mice to TRAMP-C2 cells: 44% of immunized CD8 knockout mice developed tumors, whereas none of the wild-type mice did (Fig. 3C). However, tumor growth in CD8 knockout mice was still dramatically slowed. Thus, although both T cell populations were required, CD4⁺ cells were more critical for efficient antitumor immunity than CD8⁺ cells.

**The Strength of the Antitumor Immune Response Depends on the Number of Immunizations and Type of TRAMP-C2 Cell–Based Vaccine**

To further investigate the antitumor effects of the vaccines used, we did ELISPOT and CTL assays with splenocytes isolated from immunized mice. TRAMP-specific CTL responses. TRAMP-C2 cells express a wide spectrum of mouse homologues of human prostate-specific gene products (37) and are susceptible to lysis by CTLs (38). Accordingly, we found CTL responses in all tumor-challenged (including nonvaccinated) versus naïve animals. However, no statistically significant differences were observed in cytotoxic responses of restimulated in vitro splenocytes from mice immunized with different apoptotic TRAMP-C2 cell–based vaccines at the highest effector/target ratios (Fig. 4A). Splenocytes from mice immunized with dendritic cell–containing vaccines caused an increased level of TRAMP-C2 cell lysis (up to 40%) even in the absence of restimulation (data not shown).
The best results were achieved in mice that were vaccinated with apoptotic TRAMP-C2 cells transduced with IL-12-containing adenoviruses and combined with dendritic cells (Fig. 4A). The presence of nonlabeled natural killer (NK)-sensitive YAC-1 cells showed that NK cells were responsible for about one-third of the detected TRAMP-C2 cell lysis, regardless of whether initial vaccines contained AdBP1 or Ad-IL-12 with or without dendritic cells (Fig. 4B). At the same time, the observed cytotoxicity was highly specific for TRAMP-C2 cells, because neither syngeneic B16 melanoma nor RM-1 prostate cancer cells were lysed efficiently (Fig. 4B). Thus, both CD8 and NK cells contributed to TRAMP-C2 cell killing, stimulated by the apoptosis-based vaccination.

**TRAMP-specific IFN-γ production.** Dendritic cells are potent inducers of IFN-γ production (39). Consequently, to monitor tumor-specific IFN-γ production in vaccinated mice, splenocytes were coincubated with either γ-irradiated TRAMP-C2 cells or dendritic cells preincubated with γ-irradiated TRAMP-C2 cells. The responses of splenocytes to 2 x 10^5 dendritic cells preincubated with γ-irradiated TRAMP-C2 cells, as well as to γ-irradiated TRAMP-C2 cells alone, were represented as mean ± SD spots per 10^6 splenocytes for each group of mice (n = 6). IFN-γ secretion was undetectable when TRAMP-C2 cells alone were added to wells; the background due to dendritic cells (no splenocytes) was 24.6 ± 5.2 (data not shown). Pokeweed mitogen (PWM) was used as a positive control in naïve and immunized (four times only) animals. *, statistically significant difference (P < 0.01) from control (unstimulated splenocytes).

Figure 4. Effect of immunization with apoptotic TRAMP-C2 cell–based vaccines on CTL responses and IFN-γ production by splenocytes of BL/6 mice. A–C, animals were immunized as described in the legend to Fig. 2A and B. A standard 6-hour ^51Cr-release assay was done after a 7-day stimulation of splenocytes in vitro with dendritic cells exposed to γ-irradiated TRAMP-C2 cells. Six independent experiments were done. Representative of three separate experiments with four mice per group. A, CTL responses in mice immunized with different vaccines with or without supplementary dendritic cells are compared. The results are shown as mean percent cytotoxicity against TRAMP-C2 cells at various effector/target ratios. *, statistically significant difference (P < 0.01) between groups in bracket and groups that were not vaccinated with TRAMP-C2 cells, calculated at the effector/target ratio 60:1. At this ratio, all groups were different (P < 0.01) from naïve animals. DCs, dendritic cells. B, YAC-1 cells were added at 50-fold excess to splenocytes at an effector/target ratio of 60:1. ^51Cr-labeled RM-1 or B16 cells were used as alternative targets for splenocytes from mice immunized with AdBP1-transduced TRAMP-C2 cells. Mean ± SD. *, statistically significant difference (P < 0.001) from RM-1 or B16 targets. C, cytotoxicity of splenocytes from mice immunized two or four times with TRAMP-C2 cells transduced with either Ad-IL-12 or AdBP1 is represented as mean ± SD. *, statistically significant difference (P < 0.001) between groups in brackets, calculated at the effector/target ratio 60:1. D, IFN-γ production by freshly isolated splenocytes from mice vaccinated two or four times with γ-irradiated TRAMP-C2 cells transduced with either Ad-IL-12 or AdBP1 was estimated by ELISPOT assay. The responses of splenocytes to 2 x 10^5 dendritic cells preincubated with γ-irradiated TRAMP-C2 cells, as well as to γ-irradiated TRAMP-C2 cells alone, are represented as mean ± SD spots per 10^6 splenocytes for each group of mice (n = 6). IFN-γ secretion was undetectable when TRAMP-C2 cells alone were added to wells; the background due to dendritic cells (no splenocytes) was 24.6 ± 5.2 (data not shown). Pokeweed mitogen (PWM) was used as a positive control in naïve and immunized (four times only) animals. *, statistically significant difference (P < 0.01) from control (unstimulated splenocytes).
Dendritic cells preincubated with apoptotic TRAMP-C2 cells had less ability to induce IFN-γ production than did tumor cells (Fig. 4D), regardless of the splenocyte/dendritic cell ratio (50:1 and 1:1) used (data not shown). Splenocytes from mice immunized with Ad-IL-12-infected TRAMP-C2 cells responded to TRAMP-C2 cell–exposed dendritic cells insignificantly better than did splenocytes from mice immunized with AdBP1-infected TRAMP-C2 cells (Fig. 4D). Furthermore, we compared the numbers of IFN-γ–producing cells among TRAMP-C2 cell–stimulated splenocytes after the various apoptotic cell–based vaccinations. A dramatic difference in the antitumor responses was found only between mice vaccinated with IL-12–expressing TRAMP-C2 cells and nonvaccinated animals (Supplemental Fig. 2). No significant differences were observed between mice immunized with either Ad-IL-12– or AdBP1-transduced TRAMP-C2 cells, or between mice that received the same vaccines with or without supplemental dendritic cells.

TRAMP-specific immune responses after four versus two immunizations. The more robust antitumor effects presented in Figs. 2B and 3C versus Figs. 2A and 3B might be explained by additional vaccinations and/or fewer TRAMP-C2 cells for the tumor challenge. To evaluate the role of number of immunizations in the strength of the immune response, we compared the responses of animals vaccinated two or four times with γ-irradiated Ad-IL-12– or AdBP1-transduced TRAMP-C2 cells. Four immunizations of mice with any of these vaccines resulted in greater CTL responses (Fig. 4C) and higher (P < 0.001) IFN-γ secretion (Fig. 4D) than two immunizations.

Intratumoral Administration of IL-12– and Inducible Caspase-1–Containing Adenoviruses Is an Efficient Treatment for Prostate Cancer

Having established the efficacy of the apoptosis- and IL-12-based approach to prostate tumor prophylaxis and eradication, we treated animals with preexisting s.c. or autochthonous tumors with Ad-Casp1/CID and Ad-IL-12.

Treatment of subcutaneous TRAMP-C2 tumors with inducible caspase-1– and IL-12-containing viruses. Intratumoral treatment of BL/6 mice began when their tumors reached either relatively small (4.5 × 5.2 mm or less in maximum diameter) or larger (4.5 × 5.5 mm and greater) sizes (Fig. 5A and B). All small tumors were rejected following treatment with both Ad-iCasp1 and Ad-IL-12, and one mouse treated with Ad-IL-12 alone also became tumor-free (Fig. 5A). Forty percent of mice bearing larger tumors also rejected them after Ad-iCasp1 and Ad-IL-12 treatment, and others showed reduced tumor growth rates (Fig. 5B). To test if the critical mass–related limitation of therapeutic efficiency could be partially overcome by improved inoculant delivery, we used a nanopump for slow [5 μL (7.10 × 10⁸ viral particles) / minute] virus administration into even larger tumors (5.8 × 7.3 mm and greater). Although these tumors could not be controlled by our standard virus delivery, 40% of mice treated by nanopump with both Ad-iCasp1 and Ad-IL-12 rejected their tumors (Fig. 5C). All cured animals remained tumor-free for at least 2 months after re-challenge with TRAMP-C2 tumors (data not shown).

Immune responses in TRAMP-C2 tumor-bearing mice treated with viruses. Mice treated with Ad-Casp1 in combination with Ad-IL-12 had significantly higher numbers of IFN-γ-producing splenocytes than mice in other groups (Fig. 5D). These animals, as well as mice, treated with Ad-iCasp1 alone, also showed increased tumor-specific CTL activity (Fig. 5E). In contrast to untreated tumors, tissues from animals treated with both Ad-iCasp1 and Ad-IL-12 were characterized by large areas of necrosis and peripheral lymphocyte infiltration intensively stained for acid phosphatase (Supplemental Fig. 3).

Intraprostatic treatment of TRAMP mice with inducible caspase-1– and IL-12-containing viruses. Intraprostatic treatment most accurately imitates clinical applications. Intraprostatically injected Ad-iCasp1 followed by i.p. CID administration, unlike Ad-iCasp1 alone, induces extensive prostate cell death in TRAMP mice that is detected by TUNEL assay 10 (data not shown) and 20 hours (Supplemental Fig. 4) after CID injection. Due to anesthesia-related mortality of animals, treatment with Ad-iCasp1 and Ad-IL-12 was limited to two prostate ventral lobe injections into TRAMP mice starting at 12 weeks of age. Nevertheless, this prolonged TRAMP mouse life span significantly beyond that of animals injected with Ad-c (Fig. 6A). H&E staining revealed larger areas of coagulative necrosis in treated than in untreated prostate tissues (Fig. 6C and B, respectively). Additionally, we observed lymphocyte infiltration, often surrounding these areas, and on the periphery of tumors. The infiltrated regions were saturated with acid phosphatase granules (Fig. 6G, H versus F), mainly characteristic of activated lymphocytes, including NK cells (40). Although frozen tumor sections have been analyzed previously for leukocyte acid phosphatase (41, 42), no reports on staining of formalin-fixed and paraffin-embedded organ tissues, as well as any tissues other than breast tumors for this enzyme have been found. To confirm the relevance and accuracy of our method, we examined spleen (Fig. 6D) and liver (Fig. 6E) sections. Whereas normal spleen tissues were heavily impregnated with acid phosphatase, representing both diffuse and granular patterns of its distribution, liver tissues were negative for acid phosphatase.

Thus, intratumoral administration of Ad-iCasp1 in combination with Ad-IL-12 treats both s.c. and orthotopic prostate adenocarcinomas, leading to considerably strengthened immune responses, dramatic suppression of tumor growth, and increased mouse survival.

Discussion

The use of dendritic cells in the induction of antitumor responses against poorly immunogenic tumors and in advanced disease is gaining support in clinical trials (43, 44). However, fiscal realities of our health care system dictate that any viable immunotherapeutic strategy should avoid the need to individualize therapy, relying instead on “off-the-shelf” reagents that reduce both time and cost. Because inducible caspases, which can create a source of tumor antigens in situ in tumor-bearing hosts, potentially satisfy this requirement, we compared an inducible caspase–based method with several well-described approaches to induce tumor cell death. High efficacy of apoptosis-inducing methods in a TRAMP-based model did not depend on the route of vaccine delivery and was consistent with Scheffer and colleagues’ study, showing immunity to apoptotic, but not necrotic, tumor cells (8). Seeking an optimum combination of inducible caspases with an adjuvant to trigger long-lasting immune responses, we tested B7-1 and IL-12. Conclusions about the comparative efficacy of distinct apoptotic TRAMP-C2 cell–based vaccines were founded on multiple criteria, including tumor growth, level of IFN-γ production and CTL activity. Despite previous observations of the positive therapeutic effects of B7-1 expression by tumor cells (31), especially when CD28+ NK and NKT cells had been activated by IL-12 (32, 33, 45), high levels of B7-1 (approximately 100-fold higher than on nontransduced cells)
on Ad-B7-1-infected TRAMP-C2 cells prior to irradiation had no detectable antitumor effects. Whereas low-level B7-1 expression could favor down-regulation of T cell responses (46), excessive costimulation might, in turn, bias T cell responses towards a T-helper 2 phenotype and even so-called "high zone tolerance" (i.e. clonal exhaustion; ref. 47). It is also possible that B7-1 is useful as an adjuvant only when targeted to viable tumor cells (31).

In contrast to B7-1, IL-12 had a dramatic positive effect in TRAMP-based models, and Ad-IL-12 was at least as effective as AdBP1 in both C57BL/6 and TRAMP animals. Although previous studies found that the combination of IL-12 and B7-1 was more efficient than B7-1 or IL-12 alone when respective adenoviral constructs were injected into mouse prostate or mammary adenocarcinomas (29, 48), our observation is in accordance with Aruga and colleagues’ report of tumor cells expressing IL-12 having higher immunogenicity than B7-1 (49). This may also support the ability of IL-12 to abrogate T cell tolerance induced by B7-1-CTLA-4 binding (50).

Reported effects of IL-12 include stimulation of CD4+-lymphocyte differentiation, NK activation, IFN-γ secretion by different cells, and CTL activation (51). Because these effects are dose- and model-dependent (52), the existing information regarding IL-12 applications to prostate cancer immunity (10, 48, 52–54) may not be generalizable or fully relevant to findings in TRAMP-based models, and immune responses in each particular study may require careful evaluation. We analyzed antitumor responses at least 2 weeks after the final vaccination to avoid transient T-, B-lymphocyte and antigen-presenting cell depletion following IL-12 administration (55, 56). In vivo protection from tumor rechallenge of up to 100% of vaccinated animals showed the efficiency of the apoptotic cell- and IL-12-based approach. Predictably, NK cells contributed significantly to protection against TRAMP-C2 tumors, especially in CD8-deficient mice (57). However, despite the expected role of CTLs (that accounted for approximately two-thirds of the total detected cytotoxicity), the
absence of CD8⁺ cells did not affect vaccine efficacy as dramatically as lack of CD4⁺ cells. Recently discovered insufficiency of CD8⁺-responses in CD4 knockout mice seemingly explains this observation (58). At the same time, prominent compensation of MHC class II-restricted immune responses in CD4 knockout mice (58) provided by CD8⁺ cells suggests the high probability of reciprocal compensatory MHC class I-restricted responses in CD8 knockout mice as well. In fact, although all vaccinated CD8 knockout mice eventually developed tumors by day 80 after tumor challenge (data not shown), tumor growth was retarded, confirming the presence of protective mechanisms (57).

To improve the vaccine microenvironment, we expanded the reservoir of dendritic cells. In addition to cytokine production and antigen presentation (2, 5), dendritic cells transfer antigens from live cells (39) and reduce tumor mass by direct cytotoxicity towards tumor cells (60), followed by phagocytosis of dead cells without consequent immunosuppression (61). Indeed, even without exogenous IL-12, dendritic cell addition dramatically slowed tumor growth in mice vaccinated with TRAMP-C2 cells. Apparently, additional adjuvants are no longer essential to trigger maturation of administered and preexisting dendritic cells when a large number of apoptotic cells is present at the site of injection (28). However, the total IL-12 amount may still be critical for involvement of distinct NK cell populations (45). Thus, although less economical than adenovirus injection alone, use of dendritic cells may have additive benefits for some prostate cancer patients.

We previously shown that inducible caspases could efficiently kill slowly dividing, prostate tumor–derived cells (12). We also reported that intratumoral injections of Ad-iCasp1 led to significant, but transient, reduction in TRAMP-C2 tumor growth and volume (30), and the survival of LNCaP tumor-bearing athymic mice was considerably prolonged by treatment with Ad-iCasp-9 (12). In the present study, we modified that approach for a standardized comparison with other vaccines. The results indicate that the efficiency of TRAMP-C2 cells pretreated with Ad-iCasp1 and CID in vitro was as high as the efficiency of HSV-tk-infected TRAMP-C2 cells. Furthermore, having suggested that IL-12 might improve outcome of cancer treatment with inducible caspases, we established the most rigorous test for therapeutic efficacy by evaluating the treatment of different-sized tumors. Apparently, 96 to 101 mm³ tumors were very close to or had reached a critical mass at which they became incurable with Ad-iCasp1 and Ad-IL-12. Coll et al. have shown that slow (20 μL/minute) delivery of a vector raised the level and duration of transgene expression (62), possibly due to better diffusion and weaker reflux of the inoculant along the needle track. In our experiments, the use of a nanopump expanded the range of treatable tumors and resulted in rejection of 40% of tumors with average volumes of 136 to 143 mm³. Markedly, although the IFN-γ release by splenocytes from mice treated with Ad-iCasp1 alone was significantly lower than in the case of virus combination, the level of TRAMP-C2-specific CTL activity was similar in both cases. Large central areas of coagulative necrosis and peripheral lymphocyte infiltration were present in tumors treated with both Ad-iCasp1 and Ad-IL-12. In situ induction of apoptosis by caspases could eliminate the need for costly and complicated preparation of patient’s cells and potentially sustain the high level of apoptosis in prostate tumor tissue between irradiations or when irradiation is unwarranted.

Serendipitously, we found that, in the absence of commercially available anti-CD8 and anti-CD3 antibodies efficiently binding to formalin-fixed tissues, staining for leukocyte acid phosphatase is useful for the retrospective screening of archived tissue samples. Leukocyte acid phosphatase content increases dramatically in formalin-fixed tissues, staining for leukocyte acid phosphatase is available anti-CD8 and anti-CD3 antibodies efficiently binding to formalin-fixed tissues, staining for leukocyte acid phosphatase is useful for the retrospective screening of archived tissue samples. Leukocyte acid phosphatase content increases dramatically in formalin-fixed tissues, staining for leukocyte acid phosphatase is available anti-CD8 and anti-CD3 antibodies efficiently binding to...
vacuoles inside cells in certain cases (67), in our experiments, untreated tumor tissues, surrounding necrotic areas and/or exhibiting signs of apoptosis, seemed to be negative for acidic phosphatase. Positive staining for acidic phosphatase was found in those areas of treated tumors that were infiltrated by lymphocytes, based on the results of parallel staining of adjacent sections with H&E.

Although autochthonous prostate tumors can potentially promote immune responses distinct from that induced by s.c. tumors, vaccination with apoptotic IL-12-containing TRAMP-C2 cells significantly increased survival of 6-, 9- and 12-week-old TRAMP mice. As expected based on Hurwitz et al.’s report (25), older mice seemed to be more resistant to the therapy applied. In part, because of the poor accessibility of the mouse prostate gland, intraprostatic immunotherapy in orthotopic prostate cancer models has been attempted in only a few studies. Those that involved IL-12 reported the result of a single virus injection into one of the dorsolateral prostate lobes of BL/6 mice with preestablished RM-9 tumors (48, 54). Although, TRAMP mice usually exhibit nonfocal patterns of adenocarcinoma development (23), notably, in our study, two injections of Ad-iCasp1 and Ad-IL-12 into a single ventral lobe were sufficient to initiate leukocyte infiltration of the prostate and to extend life span of >50% of animals by 12 weeks. Because the ventral lobes of the majority of TRAMP mice undergo malignant transformation at later stages than other prostate lobes, this indicated the induction of a generalized immune response.

For patients with clinically localized disease, intraprostatic injections of inducible caspases in combination with adjuvants may serve as a nonmutagenic presurgical treatment or an alternative to established forms of local therapy, such as radical prostatectomy and γ-irradiation. At the same time, an approach based on ex vivo γ-irradiation of excised tumor cells potentially could be used for postsurgical boosting of antitumor immunity against micrometastases in patients at high risk for or experiencing disease recurrence.

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