An adenoviral vector cancer vaccine that delivers a tumor-associated antigen/CD40-ligand fusion protein to dendritic cells

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To develop a method to overcome the anergy that exists in tumor hosts to cancer, we have designed an adenoviral vector for the *in vivo* activation and tumor antigen loading of dendritic cells. This adenoviral vector encodes a fusion protein composed of an amino-terminal tumor-associated antigen fragment fused to the CD40 ligand (CD40L). Subcutaneous injection of an adenoviral vector encoding a fusion protein of the human papillomavirus E7 foreign antigen linked to the CD40L generates $CD8^+$ T cell-dependent immunoresistance to the growth of the E7-positive syngeneic TC-1 cancer cells in C57BL/6 mice for up to 1 year. We also studied the s.c. injection of a vector carrying the gene for the human MUC-1 (hMUC-1) self-antigen fused to the CD40L. When this vector was injected into hMUC-1.Tg mice, which are transgenic for the hMUC-1 antigen, the growth of syngeneic hMUC-1-positive LL1/LL2hMUC-1 mouse cancer cells was suppressed in 100% of the injected animals. The hMUC-1.Tg mice are anergic to the hMUC-1 antigen before the injection of the vector. These experimental results show that it is possible to use vector injection to activate a long-lasting cellular immune response against self-antigens in anergic animals. The vector-mediated *in vivo* activation, and tumor-associated antigen loading of dendritic cells does not require additional cytokine boosting to induce the immune response against the tumor cells. This vector strategy may therefore be of use in the development of immunotherapy for the many carcinomas in which the hMUC-1 antigen is overexpressed.

E7 viral antigen | MUC-1 antigen | T cells | memory cells | immunotherapy

Xiang *et al*. (1) have used an oral plasmid DNA vaccine to induce immunological resistance to the engraftment of mouse colonic carcinoma cells that are positive for the human carcinoembryonic antigen (hCEA) gene in mice transgenic for hCEA. This plasmid encodes the extracellular domain (ecd) of the hCEA linked to the ecd of the CD40 ligand (CD40L). Oral administration of this plasmid DNA vaccine carried by an attenuated strain of *Salmonella typhimurium* resulted in effective tumor-protective immunity against hCEA-positive mouse colon cancer cells. The induction of immunity in these animals was shown to involve the activation of naïve T cells and dendritic cells (DCs). This vaccine was shown to be capable of activating an immune response against hCEA in animals that were anergic to this antigen and to be 100% effective in the prophylactic setting, but this response required the use of a second treatment, IL-2, which was antibody-targeted to T cells.

To administer the tumor-associated antigen (TAA)/CD40L vaccine in a way that could affect T cells in secondary lymphoid tissue in areas of the body other than the gastrointestinal tract, and to create a therapy that does not require the antibody-targeted IL-2, we constructed replication-incompetent adenoviral vectors encoding chimeric TAA/ecdCD40L transcription units. These transcription units encode either the human papillomavirus (HPV) E7 foreign tumor antigen or the human MUC-1 (hMUC-1) self-antigen fused to the 209-aa ecd of the CD40L. This region of CD40L contains all the sequences necessary for the formation of the CD40L trimer (2). These transcription units resembled the vaccine of Xiang in that they contained a leader sequence for secretion linked to the fusion protein composed of a TAA and the CD40L.

Our vaccine differed from that of Xiang in that it used an adenoviral vector rather than a plasmid in a *Salmonella* bacterial host strain for the delivery of the TAA/ecdCD40L transcription unit. Xiang used a leucine zipper domain in the region between the hCEA antigen and the full-length CD40L, whereas we used an 8-aa linker (NDAQAPKS) between the TAA (E7 or hMUC-1) gene and the ecdCD40L gene. The Xiang construct positions the hCEA antigen at the carboxyl terminus of the full-length CD40L, whereas our vector transcription unit attached the TAA to the amino-terminal end of the ecd of the CD40L. Our arrangement should provide better binding of the CD40L to the CD40 receptor on DCs and better secretion from the cells to the extracellular space. Xiang's method required the administration of a fusion molecule composed of IL-2 linked to a T cell-targeted antibody after the hCEA/CD40L DNA vaccine, whereas our TAA/ecdCD40L adenoviral vaccine was administered without an IL-2 boost. Xiang administered his plasmid DNA vaccine orally, whereas we administered our vector vaccine s.c.

The adenoviral system used in our work has several theoretical advantages over the *Salmonella* delivery system. The expression of the TAA/CD40L gene may be at higher levels and for longer periods of time with the adenoviral delivery than with the DNA vaccine. This adenoviral TAA/ecdCD40L vector injection may thereby induce a more vigorous immune response. Although immune-specific T cells that are elicited after immunization are thought to traffic throughout the body, a propensity still exists for tissue-specific homing by memory T cells to the lymphoid sites draining the natural area of infection (3). Thus, s.c. injection of the adenoviral vector carrying the highly immunogenic TAA/ecdCD40L in the region of the tumor cells may foster the optimal trafficking of sensitized cytotoxic T cells and the generation of memory cells.

For our experiments, we chose two TAAs. The first is the E7 protein of the most commonly encountered pathogenic (cancer-causing) genotype of the HPV. We selected this antigen because it is expressed in HPV-associated intraepithelial cervical dysplasia and in the primary cells of HPV-associated cervical cancer (4). In addition, several laboratories (5, 6), including the DiMaio laboratory (7) and our own laboratory (8), have shown that the E7 protein

Abbreviations: ecd, extracellular domain; DCs, dendritic cells; sig, a signal or leader sequence for secretion to the extracellular space; HPV, human papillomavirus; CMV, cytomegalovirus; TAA, tumor-associated antigen; hCEA, human carcinoembryonic antigen; pfu, plaque-forming units.

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is required for the maintenance of the malignant phenotype of cervical cancer cells.

The second TAA is the hMUC-1 epithelial antigen. This "self-antigen" is focally expressed from birth in normal epithelial cells, but is diffusely up-regulated on epithelial surfaces in 90% of cancers of the breast, ovary, colon, and lung (9). The overexpression of hMUC-1 has been shown to promote anchorage-independent growth of tumor cells (10). Mice that have been made transgenic for hMUC-1 have been shown to develop tolerance for hMUC-1 antigen-bearing syngeneic mouse cancer cells (11).

The experimental results described in this report show that the s.c. injection of the adenoviral vector carrying the E7/ecdCD40L fusion gene generates immunological resistance to E7-positive cancer cells for at least 1 year. In addition, the s.c. injection of the adenoviral vector carrying the hMUC-1/ecdCD40L fusion gene suppresses the growth of hMUC-1-positive mouse cancer cells in hMUC-1.Tg mice that are transgenic for the hMUC-1 gene. The induction of *in vivo* resistance to the growth of the hMUC-1-positive syngeneic mouse cancer cells was shown to involve a $CD8^+$ T cell immune response against the hMUC-1 self-antigen in hMUC1.Tg transgenic mice. These mice are initially immunologically unresponsive to the hMUC-1-positive mouse cancer cells. Thus, the hMUC-1/CD40L vector injections appeared to overcome anergy. This vaccine may be of use in preventing the recurrence of epithelial malignancies after surgery and for the immunotherapy of advanced epithelial cancers that recur after surgery.

Materials and Methods

Cell Culture and Mice. The hMUC-1.Tg mice were obtained from S. Gendler (Mayo Clinic, Scottsdale, AZ) (11).

Construction of the Adenoviral Vectors. The plasmid pDC406-mCD40L was purchased from ATCC. PCR was used to produce the carboxyl-terminal 209 aa of the ecd of the mouse CD40L (ecdCD40L), which contained neither the transmembrane domain nor the cytoplasmic domain. A spacer (NDAQAPKS) was placed at the 5′ (amino-terminal) end of the transcription unit for the ecdCD40L. This fragment was inserted into the plasmid pShuttle-CMV (ref. 12; CMV, cytomegalovirus) after restriction endonuclease digestion with *Hin*dIII and *Xho*I. This vector is designated pSCMVecdCD40L.

The E7 or ecdhMUC-1 TAA fragments were inserted into the pShuttle between the CMV promoter and the linker at the amino-terminal end of the ecdCD40L transcription unit after digestion of the pSCMVecdCD40L plasmid shuttle vector with *Not*I and *Xho*I. These plasmids were designated pSCMVE7/ecdCD40L and pSCMVecdhMUC-1/ecdCD40L. In a similar fashion, we fused the GFP gene with the ecdCD40L gene and inserted it downstream of the CMV promoter in the pShuttle-CMV vector (12). This plasmid was designated pSCMVGFP/ecdCD40L. We also constructed another set of pShuttle plasmids by inserting the human growth hormone signal sequence (sig) immediately downstream of the CMV promoter in the pSCMVE7/ecdCD40L, pSCMVecdhMUC-1/ecdCD40L, and pSCMVGFP/ecdCD40L plasmids to create the pSCMVsigE7/ecdCD40L, pSCMsigecdhMUC-1/ecdCD40L, and pSCMVsigGFP/ecdCD40L shuttle plasmids. The signal sequence was placed at the amino-terminal limit of the TAA/ecdCD40L protein to promote the release of the TAA/ecdCD40L protein from the vector-infected cells. The secretion is designed to amplify the effect of the vector beyond the infected cells. In addition, for the TAA/ecdCD40L protein to activate DCs and to be taken up by the DCs, the protein must be released from the vector-infected cells.

Ad-sig-E7/ecdCD40L, Ad-sig-ecdhMUC-1/ecdCD40L, and Ad-sig-GFP/ecdCD40L are replication-incompetent adenoviral vectors that are structurally similar, except for the TAA transcription unit between the sig, which is 3′ of the CMV promoter, and at the amino-terminal end of the secretable CD40L transcription unit. All these vectors lack the E1 and E3 adenoviral genes and were assembled by homologous recombination in bacterial host strains by using the AdEasy system (12) with the pSCMVsigE7/ecdCD40L, pSCMVsigecdhMUC-1/ecdCD40L, and pSCMVsigGFP/ecdCD40L shuttle plasmids.

Sig	HPV E7	NDAQAPKS	ecdCD40 ligand (Amino acid 52-261)
	HPV E7	NDAQAPKS	wtCD40 ligand (Amino acid 1-261)
Sig	GFP	NDAQAPKS	ecdCD40 ligand (Amino acid 52-261)

Fig. 1. Organization of the adenoviral transcription units. Elements of the adenoviral vector transcription units include Sig, human growth hormone signal sequence; HPV E7, the HPV E7 gene; NDAQAPKS, a peptide linker between E7 and the ecd of the CD40L gene; ecdCD40L, amino acids 52–261 of the CD40L gene, which contain the ecd of the CD40L without the transmembrane or cytoplasmic domains; wtCD40L, amino acids 1–261 of the full-length (wild-type) CD40L gene, which include the ecd, the transmembrane domain, and the cytoplasmic domain; and the GFP gene.

Tumor Model for Ad-sig-TAA/ecdCD40L Injections. We first conducted studies to evaluate the response of tumors to the Ad-sig-E7/ecdCD40L vector injections in C57BL/6 mice. All mice were treated by using the following protocol unless otherwise described. The Ad-sig-E7/ecdCD40L adenoviral vector [1×10^8 plaque-forming units (pfu)] or control adenoviral vectors (1×10^8 pfu), as shown in Fig. 1, were injected s.c. on days 0 and 7. One week after the last s.c. vector injection, 5×10^5 TC-1 tumor cells (13) were injected s.c. at a different site. For all injections, the vector or the cells were suspended in 100 μl of PBS. Tumor growth was monitored three times each week at the injection site of the tumor cells by caliper measurement and inspection. The mice were killed when their tumors became ulcerated or reached 1.5 cm in diameter. Tumor volumes are calculated by the product of length $\times$ width^2/2. Similar experiments were carried out with the Ad-sig-ecdhMUC-1/ecdCD40L vector, the LL1/LL2hMUC-1 cell line, and the hMUC-1.Tg transgenic mouse strain.

Adoptive Transfer of Lymphocytes from Tumor-Free Mice after Injection with TC-1 Cells, and s.c. Injection of the Ad-sig-E7/ecdCD40L Vector. ***Preparation of the C57BL/6 donor mice.*** C57BL/6 mice were injected s.c. with 5×10^5 TC-1 cells. When a small tumor nodule appeared at the injection site, measurements were conducted with calipers three times a week. Five days after the injection of TC-1 cells, 1×10^8 pfu of the Ad-sig-E7/ecdCD40L vector that expressed the secretable form of the E7/ecdCD40L fusion protein were injected s.c. at a site separate from the tumor nodule in the C57BL/6 mice. This vector injection was repeated in 7 days. Several weeks later, 1×10^7 TC-1 cells were then injected s.c. in the mice that were tumor-free. Of the 10 tumor-free mice that were injected with the vector and the second dose of tumor cells, 8 mice remained tumor-free. Four of the 8 mice that remained free of tumor for 1 year were killed. The splenic T cells were isolated by negative selection by using magnetic bead separation according to StemCell Technologies (Vancouver). The purified T cells were injected i.p. into C57BL/6 *nu/nu* athymic mice that carried s.c. nodules of TC-1 tumor cells (see Fig. 4*A*).

Preparation of the recipient C57BL/6 athymic* nu/nu *mice. C57BL/6 *nu/nu* immunocompromised mice (4- to 6-week-old females) were injected s.c. with 1×10^6 TC-1 cells. One week after the injection of the TC-1 cells, the mice were injected i.p. with spleen cells from the C57BL/6 mice that had been sensitized by s.c. injection of the Ad-sig-E7/ecdCD40L vector.

***In Vivo* Depletion of $CD4^+$ or $CD8^+$ T Lymphocytes.** In a separate experiment, *in vivo* mAb ablation of CD8 (clone 2.43; ATCC TIB 210) or CD4 (clone GK1.5; ATCC TIB 207) T cell subsets was performed by i.p. injection of 0.5 mg of antibody. This antibody was

purified from the culture supernatants of hybridomas. The CD8- or CD4-depleting antibodies were injected i.p. into the immunocompetent C57BL/6 donor mice on days −5, −3, and −1 before the first vector vaccination, and every 6 days thereafter (500 μg of purified antibody per mouse per injection) during vaccination, and also on days 6, 7, 8, 10, 12, and 14 after tumor challenge (14). On day 0 and day 7, 1×10^8 pfu of the Ad-sig-E7/ecdCD40L vector were injected s.c. Seven days later, 5×10^5 E7-positive TC-1 cells were injected s.c. into each mouse. Then, the mice were observed for 3 months.

Antibody suspensions were purified from hybridoma supernatants by passage through protein G columns according to the manufacturer's instructions (Pierce). $CD4^+$ and $CD8^+$ T cell depletion was monitored by flow cytometric analysis of splenocytes isolated from test animals. On the day of tumor challenge, $CD4^+$ and $CD8^+$ cell populations were reduced by 95% and 99%, respectively.

Mice were monitored for 3 months after tumor challenge and, at that time, the tumor-free mice were killed. The spleen T cells were then isolated as described above. Five million of the $CD4^+$ T cells from the CD8-depleted, sensitized animals were mixed with five million $CD8^+$ T cells from unsensitized animals. Similarly, five million of the $CD8^+$ T cells from the CD4-depleted, sensitized animals were mixed with five million $CD4^+$ T cells from the unsensitized mice. Ten million of these mixtures of sensitized $CD8^+$ T cells and unsensitized $CD4^+$ T cells, or sensitized $CD4^+$ T cells and unsensitized $CD8^+$ T cells, were injected into C57BL/6 nu/nu mice in which one million TC-1 cells had already been injected 5 days before. The animals were then monitored for survival.

Results

Construction and Analysis of the Ad-sig-E7/ecdCD40L Vector. The organization of the transcription unit of the Ad-sig-E7/ecdCD40L vector, and several control vectors generated for analytical purposes, is shown in Fig. 1. We exposed 293 cells to the following vectors: Ad-sig-E7/ecdCD40L, Ad-sig-GFP/ecdCD40L, Ad-E7, and Ad-wtCD40L (wtCD40L contains the transmembrane and cytoplasmic domains and the ecd of the CD40L). We then subjected the protein cell lysate of the infected cells to SDS/PAGE and Western blotting. The predicted molecular weights for each of the fusion proteins encoded by the CD40L transcription units were observed on the Western blot (data not shown).

Activation of DCs by the E7/ecdCD40L Fusion Protein. To test whether the E7/ecdCD40L fusion protein could bind to the CD40 receptor and activate cytokine release from DCs, we exposed bone marrow-derived DCs (15) to the following adenoviral vectors at a multiplicity of infection of 100: Ad-sig-E7/ecdCD40L, Ad-sig-GFP/ecdCD40L, and Ad-E7. The infected DCs were then inoculated in 24-well plates at 2×10^5 cells per ml. ELISA analyses confirmed that infection of DCs by the Ad-sig-E7/ecdCD40L vector stimulated the DCs to produce 18 pg of IL-12 per 2×10^5 cells per ml in 24 h, and 88 pg of IL-12 per 2×10^5 cells per ml in 48 h. This finding was statistically significantly higher than the level of IL-12 released after exposure of the DCs to the Ad-sig-GFP/ecdCD40L vector, the Ad-E7 vectors, or the PBS control ($P < 0.0001$ as shown in Fig. 2*A*). It is possible that the level of cytokine release in cells exposed to the Ad-sig-GFP/ecdCD40L vector is lower than that seen with the Ad-sig-E7/ecdCD40L vector because of a lower level of expression of the GFP/ecdCD40L, or because of the toxicity of the GFP to cells in which it is expressed at high levels.

Similarly, as shown in Fig. 2*B*, exposure of the DCs to the Ad-sig-E7/ecdCD40L vector also induced secretion of 335 pg of IFN-γ per 2×10^5 cells per ml in 24 h and 769 pg of IFN-γ per 2×10^5 cells per ml in 48 h. This level was statistically significantly higher than the levels induced by the PBS control or the Ad-E7 or Ad-sig-GFP/ecdCD40L vectors ($P < 0.0001$). We also exposed bone marrow-derived DCs to the Ad-sig-E7/ecdCD40L vector and showed that no IL-2 was secreted by the vector-exposed DCs (sensitivity of the assay was 0.3 pg/ml). These data suggest that the Ad-sig-E7/ecdCD40L vector can induce cytokine release from the DCs without the involvement of IL-2.

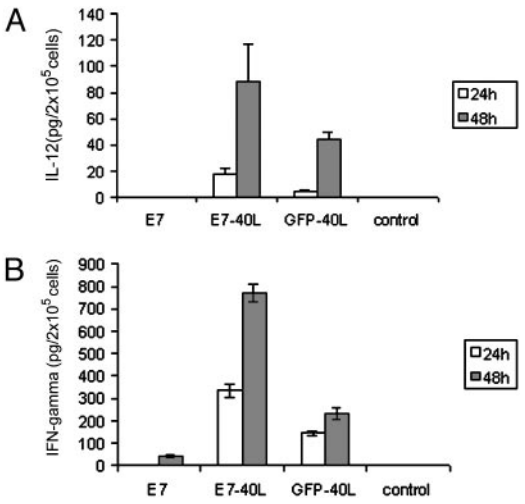

Fig. 2. Study of activation of cytokine release from DCs by exposure to the Ad-sig-E7/ecdCD40L vector. Bone marrow-derived DCs were exposed to the following vectors: E7, Ad-E7; E7–40L, Ad-sig-E7/ecdCD40L; and GFP-40L, Ad-sig-GFP/ecdCD40L. After addition of the vector at a multiplicity of infection of 100, the cells were placed in wells, and an ELISA was used to measure the production of IL-12 (*A*) and IFN-γ (*B*) by the vector-infected DCs during a 24- and a 48-h period.

To test whether the induction of IL-12 and IFN-γ release was caused by the binding of the E7/ecdCD40L protein to the CD40 receptor on the DCs, we added 10 μg of either nonimmune IgG or 10 μg of anti-CD40L antibody to DCs that had been exposed to the Ad-sig-E7/ecdCD40L vector. Addition of this antibody reduced the IL-12 released after exposure to the vector from 27 pg per 200,000 DCs in 24 h to 0 pg per 200,000 DCs in 24 h. The IFN-γ released in the presence of the nonimmune IgG was 366 pg per 200,000 DCs in 24 h, whereas, with the addition of anti-CD40L antibody, the level released was reduced to 71 pg per 200,000 DCs in 24 h. These experiments were carried out in triplicate. These results suggest that the TAA/ecdCD40L fusion protein that is released from Ad-sig-E7/ecdCD40L-exposed DCs can assemble itself into a functional trimer that binds to the CD40 receptor on the DCs, thereby activating the DCs to release IL-12 and IFN-γ.

We were also able to show that the exposure of bone marrow-derived DCs to the Ad-sig-E7/ecdCD40L vector induces an increase in the percentage of DCs that were positive for CD80 and CD86 from 8.4% to 27.7% (for CD80) and from 3.3% to 27.4% (for CD86). Thus, the Ad-sig-E7/ecdCD40L vector induced activation of the DCs.

Subcutaneous Injection of the Ad-sig-E7/ecdCD40L Vector Confers Protection Against Engraftment and Growth of the E7-Positive TC-1 Cancer Cell Line. Female C57BL/6 mice were injected s.c. with 1×10^8 pfu of the Ad-sig-E7/ecdCD40L or Ad-sig-GFP/ecdCD40L vectors once on each of 2 days, 7 days apart. The vector-treated mice were then injected s.c. with 5×10^5 cells from the E7-positive TC-1 cancer cell line (13) 10 days after the last vector injection. As shown in Fig. 3, 0% (0 of 5) of mice injected with the Ad-sig-E7/ecdCD40L vector formed palpable tumors during 2 months of observation after a single injection of TC-1 cells, whereas 100% (5 of 5) of the mice injected with the Ad-sig-GFP/ecdCD40L vector before the injection of the TC-1 cells formed s.c. tumors, which were >500 mm^3 by 15 days after TC-1 tumor-cell injection.

The differences between the sizes of the tumors in mice injected with the Ad-sig-E7/ecdCD40L vector and the Ad-sig-GFP/

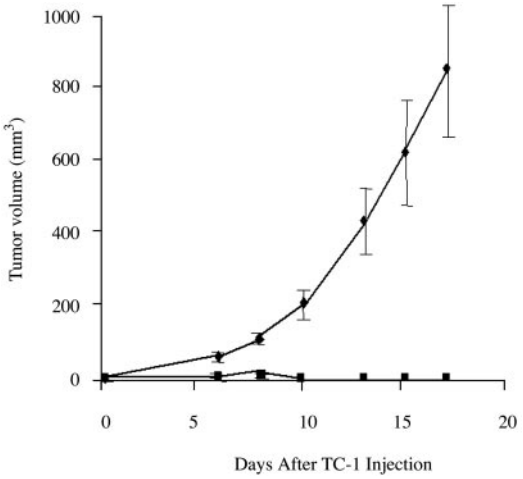

Fig. 3. Analysis of TC-1 E7-positive tumor cell line growth in mice after vector injections. Mice were injected s.c. with 1×10^8 pfu of vectors twice at 7-day intervals. Ten days later, 5×10^5 TC-1 cells were injected s.c. ($n = 5$). The following vectors were injected: ♦, Ad-sig-GFP/ecdCD40L (top line), and ■, Ad-sig-E7/ecdCD40L (bottom line). The growth of the s.c. nodule of TC-1 cells in the Ad-sig-E7/ecdCD40L-injected mice was statistically significantly different from the growth seen in the mice injected with the Ad-sig-GFP/ecdCD40L vector at the $P < 0.001$ level.

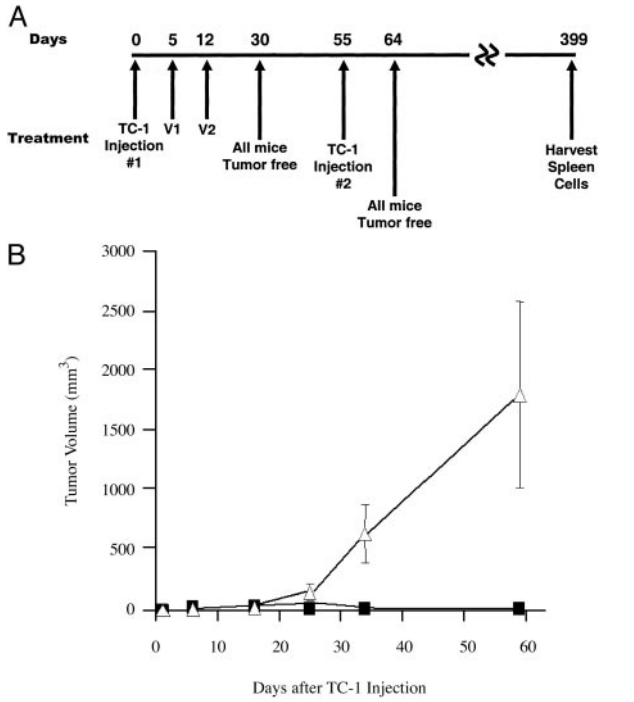

Fig. 4. (*A*) Time course of the events in the experiment presented in *B* and in Fig. 5*A*. TC-1, E7 tumor cell line; V1, first vector injection; V2, second vector injection. (*B*) Analysis of changes in the size of TC-1 E7-positive s.c. tumor nodules in C57BL/6 mice that had been growing for 5 days before injection with the Ad-sig-E7/ecdCD40L vector. As shown in *A*, 5×10^5 TC-1 cells were injected on day 0. Vectors (1×10^8 pfu) were injected on day 5 after the s.c. injection of TC-1 cells when the TC-1 tumor nodule is visible and injected again 7 days later ($n = 5$ per group). ■, Ad-sig-E7/ecdCD40L; △, PBS. The growth of the s.c. nodule of TC-1 cells in the mice injected with the Ad-sig-E7/ecdCD40L vector was statistically significantly different from that in the mice injected with PBS at the $P < 0.0001$ level.

ecdCD40L vector are statistically significantly different at the $P < 0.001$ level. The fact that the Ad-GFP/ecdCD40L vector injection does not protect the mice shows that the protective effect of the Ad-sig-E7/ecdCD40L vector, which prevents the growth of the TC-1 cell line, is not due to the adenoviral infection/transfection process.

When the tumor-free animals from the Ad-sig-E7/ecdCD40L-treated group were rechallenged with a larger dose of TC-1 cells (1×10^7 cells), a period of transient tumor growth was initially seen, which was followed by a decline in the size of the tumor nodule, ultimately leading to complete tumor regression in 100% of the five animals rechallenged with the higher dose of TC-1 cells.

Injection of the Ad-sig-E7/ecdCD40L Vector Induces Tumor Regression of Established TC-1 Tumors. Mice were first injected s.c. with 5×10^5 TC-1 cells on the hind flank. Five days later, the mice were injected s.c. at a different site with the Ad-sig-E7/ecdCD40L vector. This vector injection was repeated 7 days later. Control mice were injected s.c. with PBS 5 days after the injection of TC-1 cells (see Fig. 4*A* for time course of the experiment). As shown in Fig. 4*B*, two s.c. injections of the Ad-sig-E7/ecdCD40L vector resulted in transient growth followed by regression that was complete in 100% of the mice by 25 days after the second Ad-sig-E7/ecdCD40L vector injection. The second challenge of the immunized animals with a 20-fold increase in the number of the TC-1 tumor cells (see Fig. 4*A*) produced a brief transient growth and then complete regression of the tumor nodule in 100% of the mice injected with the Ad-sig-E7/ecdCD40L vector after the injection (data not shown). Although all the Ad-sig-E7/ecdCD40L vector-injected mice showed complete regression of tumors (see Fig. 4*B*), 100% of the animals injected with PBS developed progressive tumor growth at the TC-1 injection site within 14 days.

Splenic T Cells from Ad-sig-E7/ecdCD40L Vector-Injected C57BL/6 Mice Can Passively Transfer T Cell-Mediated Tumor Immunity to TC-1 Cells for up to 1 Year After Vector Injection. We monitored the mice from the experiment shown in Fig. 4*A* (time course of the experiment) for up to 1 year after TC-1 tumor injection, Ad-sig-E7/ecdCD40L vector injection (two injections separated by 7 days), and rechallenge with 1×10^7 TC-1 cells. Four of the eight animals that remained tumor-free for >1 year were killed and the T cells were isolated from the spleen by negative selection by using antibody and magnetic bead technology. Ten million of these splenic T cells were injected i.p. into C57BL/6 athymic nu/nu mice ($n = 7$), which had been injected s.c. 5 days previously with 5×10^5 TC-1 cells. As shown in Fig. 5*A*, the tumors in the nude mice given i.p. injections of the T cells from the Ad-sig-E7/ecdCD40L-sensitized donor mice grew into palpable s.c. nodules for 6 days and then regressed in all animals to very small tumors. The sizes of the s.c. tumor nodules were an average of 114 mm^3 on day 4, 234 mm^3 on day 6, 151 mm^3 on day 8, and 140 mm^3 on day 10 after the i.p. injection of the T cells from the sensitized immunocompetent animals shown in Fig. 4*B*. In three of the seven treated mice, the tumors regressed completely. In contrast, none of the s.c. tumors in the nude mice injected i.p. with T cells from unsensitized donor mice regressed. All the mice in the latter group died with progressive tumor growth within 3 weeks after TC-1 tumor cell injection. The mice treated with the i.p. injection of the splenic T cells from the sensitized donors were monitored for 3 months after the TC-1 challenge and remained tumor-free during that time.

A separate experiment was performed to determine the relative contribution of the $CD8^+$ vs. the $CD4^+$ T cells to the induction of immunoresistance to the TC-1 cells. The C57BL/6 mice were injected with the Ad-sig-E7/ecdCD40L vector twice, with each injection 7 days apart. Seven days after the last injection, 5×10^5 of the TC-1 cells were injected into a separate s.c. site. To deplete CD4 or CD8 T cells, the C57BL/6 donor mice were injected with antibodies against either $CD4^+$ T cells or $CD8^+$ T cells before and during the injection of the Ad-sig-E7/ecdCD40L vector (see *Materials and Methods* for the exact schedule of injections).

The efficiency of such antibody treatment for either the CD4 or

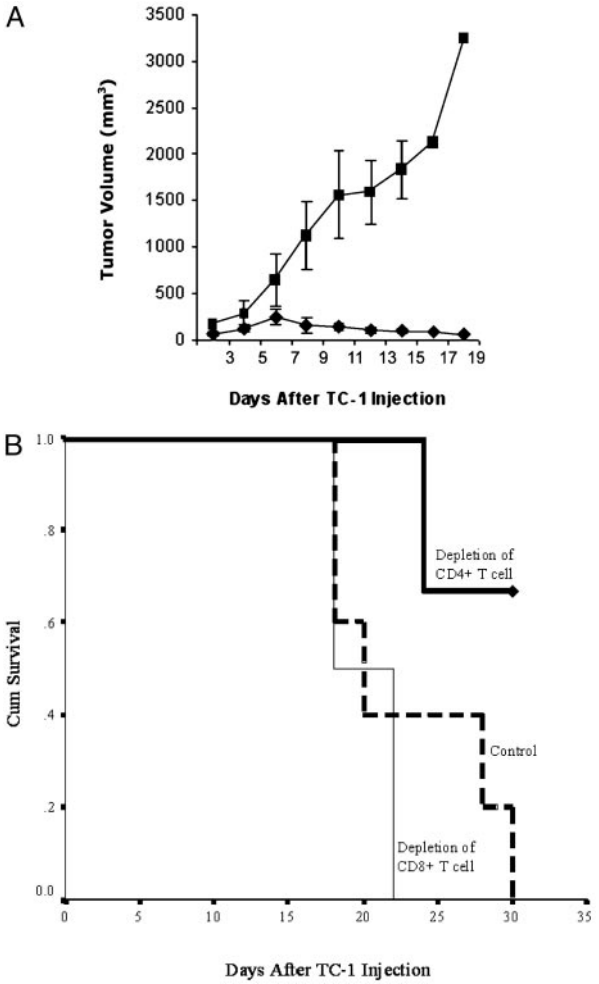

Fig. 5. (*A*) Passive transfer of immune resistance to TC-1 cell growth by using spleen cells collected from sensitized animals 1 year after vector injection. Splenic T lymphocytes were collected 1 year after Ad-sig-E7/ecdCD40L vector vaccination and challenge with TC-1 cells (see Fig. 4*A*). These sensitized spleen cells were then injected i.p. into C57BL/6 nude mice 5 days after injection of 500,000 TC-1 cells. ◆, C57BL/6 nude mice ($n = 7$) were injected s.c. with TC-1 cells and then injected i.p. 5 days later with 10×10^6 splenic lymphocytes from Ad-sig-E7/ecdCD40L-sensitized mice; ■, control animals were injected i.p. with 10×10^6 splenic T cells from unsensitized donor C57BL/6 mice 5 days after injection of TC-1 cells. (*B*) Survival of C57BL/6 nude mice after s.c. injection of TC-1 tumor cells and then i.p. injection of $CD4^+$ (thin unbroken line) or $CD8^+$ (thick unbroken line) T cell lymphocytes from Ad-sig-E7/ecdCD40L-sensitized C57BL/6 donors. Donor C57BL/6 mice were injected s.c. with the Ad-sig-E7/ecdCD40L vector at days 0 and 7. Seven days later, the mice were injected s.c. with 5×10^5 TC-1 cells. The mice were monitored for 3 months. At 5, 3, and 1 days before the vector injection, and every 6 days after the s.c. injection of the Ad-sig-E7/ecdCD40L vector, and also on days 6, 7, 8, 10, 12, and 14 after the injection of the TC-1 cells, the C57BL/6 donor mice were treated *in vivo* with antibodies specific for $CD4^+$ (thick unbroken line) or $CD8^+$ (thin unbroken line) T cell lymphocytes to deplete the respective T cell population. Then the sensitized $CD8^+$ or $CD4^+$ T cell lymphocytes from sensitized (Ad-sig-E7/ecdCD40L-injected) C57BL/6 donors were injected i.p. into C57BL/6 nude mice 7 days after s.c. injection of 5×10^5 TC-1 cells. A third group of C57BL/6 nude mice, which were control mice, did not receive passive transfer of T cells from sensitized mice (thick broken line) 7 days after s.c. injection of TC-1 cells. The mice were then monitored for cumulative survival.

CD8 depletion was determined to be 95% and 99%, respectively. Seven days after the last vector injection, the mice were injected with 5×10^5 TC-1 cells. Splenic $CD8^+$ or $CD4^+$ T cells were collected from the Ad-sig-E7/ecdCD40L vector-treated, $CD4^+$- or $CD8^+$-depleted donor mice, which had remained tumor-free for 3 months.

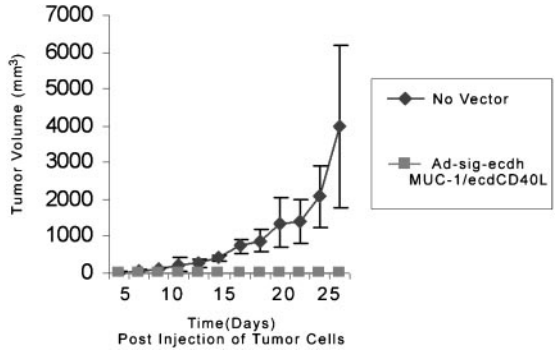

Fig. 6. The effect of the s.c. injection of the Ad-sig-ecdhMUC-1/ecdCD40L vector on the growth of the hMUC-1-positive LL1/LL2hMUC-1 cancer cell line in syngeneic hMUC-1.Tg mice. The Ad-sig-ecdhMUC-1/ecdCD40L vector was injected s.c. twice at 7-day intervals into hMUC-1.Tg mice, which were transgenic for the hMUC-1 gene. One week after the second vector injection, the mice were injected with 5×10^5 LL1/LL2hMUC-1 syngeneic mouse cancer cells that were positive for the hMUC-1 antigen. The growth of the LL1/LL2hMUC-1 cells in mice injected with the Ad-sig-ecdhMUC-1/ecdCD40L vector (■) was significantly different from the growth seen in mice not injected with vector (◆).

Five million of the $CD8^+$ T cells from CD4-depleted sensitized donors were mixed with 5 million of the splenic $CD4^+$ T cells from unsensitized donors. These cells are referred to as $CD8^+$ T cells from sensitized donors. Similarly, 5 million of the splenic $CD4^+$ T cells from CD8-depleted sensitized donors were mixed with 5 million of the splenic $CD8^+$ T cells from unsensitized donors. These cells are referred to as $CD4^+$ T cells from sensitized donors. Five million of each of these populations of cells were injected i.p. into C57BL/6 athymic nude mouse recipients in which TC-1 nodules had already been established.

As shown in Fig. 5*B*, the $CD4^+$ T cells (thin unbroken line) from the sensitized C57BL/6 mice did not protect the C57BL/6 nu/nu mice, whereas the $CD8^+$ cells from the sensitized C57BL/6 mice (thick unbroken line) prolonged the survival of the nude mice after injection of the TC-1 cells. Lymphocytes from unsensitized donor mice (broken thick line) did not protect the mice from TC-1 tumor growth. No statistically significant difference exists between the control- and CD8-depleted groups in Fig. 5 ($P = 0.21$).

Subcutaneous Injection of the Ad-sig-ecdhMUC-1/ecdCD40L Vector Overcomes Anergy for hMUC-1-Positive Cells in Mice That Are Transgenic for hMUC-1. The MUC-1 antigen is overexpressed in carcinomas of the breast, ovary, and pancreas and in other carcinomas (9). MUC-1 is also a self-antigen that is focally expressed on normal secretory epithelial cell apical surfaces. The overexpression of hMUC-1 in epithelial cancers is thought to disrupt E-cadherin function, leading to anchorage-independent growth and metastases (10). hMUC-1.Tg mice, which are transgenic for the hMUC-1 antigen, have been reported to be unresponsive immunologically to the hMUC-1 antigen (11).

We therefore s.c. injected the Ad-sig-ecdhMUC-1/ecdCD40L vector into hMUC-1.Tg mice. The hMUC-1.Tg mice had expressed the hMUC-1 antigen since birth (11). This experiment would therefore test whether the Ad-sig-ecdhMUC-1/ecdCD40L vector injection could produce resistance in anergic mice to the growth of syngeneic mouse cancer cells that were positive for the hMUC-1 antigen. As shown in Fig. 6, injection of the hMUC-1 mouse syngeneic tumor cell line, LL1/LL2hMUC-1, into the hMUC-1.Tg mice, which had not been injected with vector (◆), produced progressive growth of the LL1/LL2hMUC-1 s.c. tumor. These control animals had to be killed by 25 days after the s.c. injection of the tumor cells.

In contrast, in the hMUC-1.Tg transgenic mice that received s.c. injections of the Ad-sig-ecdhMUC-1/ecdCD40L vector, the growth

of the LL1/LL2hMUC-1 cell line was completely suppressed in all the animals tested (see ■, Fig. 6). Thus, the Ad-sig-ecdhMUC-1/ecdCD40L vector strategy can overcome anergy in 100% of the test mice without the need for additional cytokine booster treatments.

Discussion

The results of the experiments reported in this article show that the injection of the Ad-sig-E7/ecdCD40L vector into C57BL/6 mice induces T cell-mediated tumor immunity to the engraftment and growth of E7-positive tumor cells. The Ad-sig-E7/ecdCD40L also induces regression of established s.c. E7-positive tumor nodules in the C57BL/6 mice. Intraperitoneal injection of splenic T cells collected from Ad-sig-E7/ecdCD40L-vaccinated mice, which had remained tumor-free for >1 year after injection of the Ad-sig-E7/ecdCD40L vector and tumor challenge, induced regressions of TC-1 tumors already growing in immunocompromised athymic nude recipient mice (see Fig. 5*A*). This experiment and the experimental results summarized in Fig. 5*B* show that the effect of the Ad-sig-E7/ecdCD40L injections on E7-positive TC-1 cells is mediated by a $CD8^+$ T cell-dependent immune response that lasts for >1 year.

The study of the effect of s.c. injection of the Ad-sig-ecdhMUC-1/ecdCD40L vector into hMUC-1.Tg mice (11) allowed us to test whether the Ad-sig-ecdhMUC-1/ecdCD40L vector injection by itself could activate a $CD8^+$ T cell immune response against the hMUC-1-positive mouse cells in 100% of the animals otherwise anergic to the hMUC-1 antigen. This proved to be the case.

The Ad-sig-TAA/ecdCD40L vector strategy described in this article is unique in several ways. It has been shown to overcome anergy in a transgenic mouse model in 100% of the test mice without the use of cytokine boosting. In addition, it can generate cellular immunity for up to a year, which indicates that the vector strategy outlined in this article induces memory cells.

The Garen laboratory (16) has recently reported that the s.c. injection of 293 cells infected with an adenoviral vector carrying an E7/IgGFc transcription unit can suppress the growth of the TC-1 cell line in a syngeneic mouse model. An interesting parallel exists between the Ad-sig-E7/IgGFc vector of Garen and the Ad-sig-TAA/ecdCD40L vector described in this article: an *in vivo* continuous release strategy is used in both sets of experiments to generate an immune response against a foreign antigen.

The finding about the Ad-TAA/ecdCD40L vector that is different from the findings reported by Garen and his colleagues is the successful activation of an immune response against self-antigens without the need for cytokine booster treatments. The successful induction of T cell-mediated tumor immunity in 100% of the test anergic animals with the Ad-sig-ecdhMUC-1/ecdCD40L vector shows that the adenoviral vector delivery vehicle is superior to the oral DNA vaccine of Xiang (1) that was delivered in *Salmonella*. No need exists to use additional IL-2 treatments after the TAA-ecdCD40L vaccination in the case of the Ad-sig-ecdhMUC-1/ecdCD40L vector, whereas the IL-2 treatments are required to induce an antitumor immune response in 100% of the anergic animals with the *Salmonella* DNA vaccine approach. This experimental result confirms the superiority of the adenoviral vector approach.

The introduction of the secretory sequence at the amino-terminal end of the TAA/ecdCD40L transcription unit and the deletion of the transmembrane domain of the CD40L ensure that this protein will be secreted from the infected cells. Previous reports of the use (17) of Ad-CD40L vectors did not use a secretable CD40L transcription unit because the goal was to display the CD40L on the plasma membrane of the DCs. In this work (17), the effect of the vector was limited to the vector-infected cells and the cells that they directly stimulate. The use of the secretable vector in our work produces an amplification effect beyond the vector infected cells to uninfected DCs.

The statistically significant increase in secretion of IL-12 and IFN-γ at 48 h after exposure of the DCs to the Ad-sig-E7/ecdCD40L vector, as compared with the PBS control (see Fig. 2), shows that the E7/ecdCD40L fusion protein can bind to the CD40 receptor on DCs and stimulate the CD40 receptor sufficiently well to activate the DCs. The fact that the IL-12 and IFN-γ secretion after exposure to the Ad-sig-E7/ecdCD40L vector is statistically significantly greater than the secretion after exposure of the DCs to the Ad-E7 or Ad-sig-GFP/ecdCD40L vectors (see Fig. 2) shows that the increased secretion is not due to the effect of the adenoviral infection/transfection process on the DCs.

One possible complication of inducing an immune response against a self-antigen associated with cancer is that this could generate an autoimmune disease against the normal tissues that normally express that antigen. Several considerations suggest that this will not be a problem with the MUC-1 antigen. First, although the MUC-1 antigen is overexpressed diffusely at very high levels throughout neoplastic epithelial cells, MUC-1 is expressed only very focally and at very low levels in normal epithelial apical structures. Although experiments using a tandem-repeat protein of the MUC-1 antigen with a *Leishmania*-derived protein as adjuvant generated in chimpanzees $CD4^+$ helper and $CD8^+$ cytotoxic responses, no signs of autoimmune disease were detected for up to 1 year after the administration of the vaccine (18). Passive transfer of anti-MUC-12 antibodies does not cause autoimmune disease (19).

The results obtained with the Ad-sig-E7/ecdCD40L vector injections suggest that these vectors may be useful in the prevention of HPV-associated cervical cancer and for the treatment of metastatic cervical cancer. In addition, because hMUC-1 is a self-antigen that is overexpressed in 90% of carcinomas of the breast, ovary, colon, and lung, it is possible that the Ad-sig-ecdhMUC-1/ecdCD40L vector vaccine strategy described in this report could be of use in activating an immune response against a wide range of epithelial neoplasms in human patients.

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