Therapeutic Vaccination against Murine Lymphoma by Intratumoral Injection of Recombinant Fowlpox Virus Encoding CD40 Ligand

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Abstract

The interaction between CD40 ligand (CD40L, CD154) and its receptor CD40 on antigen-presenting cells is essential for the initiation of cell-mediated and humoral immune responses. Malignant B cells also express CD40 and respond to CD40L by enhancing expression of costimulatory molecules. In this study, we investigated the therapeutic antitumor effect of intratumoral administration of recombinant fowlpox virus encoding murine CD40L (rF-mCD40L) in a murine B-cell lymphoma model. BALB/c mice with established s.c. and widely metastatic A20 lymphoma tumors were treated with intratumoral injections of rF-mCD40L together with systemic chemotherapy. This combined chemoimmunotherapy resulted in complete tumor regression and long-term survival of the mice. Some tumor cells in the injected sites expressed the CD40L transgene and had increased expression of the CD80 and CD86 costimulatory molecules. The therapeutic effect was dependent on CD8 but not on CD4 T cells. Moreover, there was a requirement that the recombinant CD40L virus be injected directly into the tumor, as opposed to peritumoral or distant sites. Thus, rF-mCD40L injected directly into the tumor microenvironment enhances the immunogenicity of tumor B cells. The results support future plans for intratumoral injection of rF-mCD40L in patients with lymphoma. [Cancer Res 2007;67(14):7037–44]

Introduction

Patients with B-cell lymphoma initially respond to chemotherapy, radiotherapy, and monoclonal antibodies (mAb), but they are rarely cured by these treatments (1). Because malignant B cells retain many of the regulatory signals of normal B cells, it may be possible to bring them under the control of normal immunoregulatory circuits. Alternatively, they may become the target of active immune responses.

The induction of an immune response requires a specific antigen-presenting cell (APC) to process the antigen into peptides and to subsequently activate antigen-specific T cells via T-cell receptor and costimulatory molecule interactions (2, 3). CD40 on the APC and CD40 ligand (CD40L) on the T cell are two of the most important of these costimulatory interactions. CD40L is transiently expressed on the surface of activated CD4+ T lymphocytes (4, 5). CD40L induces APCs, such as B cells, dendritic cells, and macrophages, to express additional costimulatory molecules, such as CD80 and CD86, adhesion molecules, such as CD54 [intercellular adhesion molecule-1 (ICAM-1)], and also to up-regulate cytokine production (6–12). A number of attempts have been made to reconstitute this mutual activating system in tumor cells and their infiltrating immune cells. One approach has been to transduce tumor cells with genes coding for costimulatory molecules, cytokines, or chemokines (13–15). Malignancies derived from B lymphocytes provide a special opportunity because they can function as APCs and already express many costimulatory molecules, including CD40, CD80, and CD86. Cross-linking of CD40 on B lymphoma cells, either by coculture with CD40L-transfected cells or by transduction with recombinant virus vectors expressing the CD40L gene, can induce enhanced expression of costimulatory molecules. These phenotypic changes allow the malignant B cells to be recognized by T cells (16–19). Genes for immune regulatory molecules can be transduced into tumor cells very effectively by recombinant viral vectors. This can be accomplished either in vitro or by direct injection of the vectors in vivo into the tumor (18, 20–22).

A recombinant adenovirus vector encoding CD40L has been used to transduce this gene into human chronic lymphocytic leukemia cells ex vivo (18, 23). However, the use of recombinant adenovirus vectors in vivo can be impaired by preexisting antiviral antibodies (24). Fowlpox viruses are attractive vectors for gene delivery because they do not replicate or cause disease in mammals and are not impaired by preexisting antibodies. This vector can even be used repetitively without inhibition of transgene expression (25, 26). In the current study, we examined the antitumor effect of intratumoral administration of rF-mCD40L in the setting of preexisting and advanced lymphoma. Our results show that intratumoral injection of rF-mCD40L in combination with systemic chemotherapy can cure lymphoma by inducing a CD8 T-cell immune response.

Materials and Methods

Cell lines and mice. A20, a BALB/c B-cell lymphoma, and the rat hybridomas GK1.5 (anti-murine CD4) and 2.43 (anti-murine CD8) were obtained from American Type Culture Collection (ATCC). The isotype control rat hybridoma SFR8-B6 (rat IgG2b anti-human HLA Bw6) was provided by J. Parnes (Stanford University, Stanford, CA). CT26 is a murine colon carcinoma cell line. All cell lines were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin (Life Technologies, Inc.), and 50 μmol/L 2-mercaptoethanol and maintained at 37°C in a 5% CO2 atmosphere. During fowlpox virus infection, serum was omitted from the atmosphere. During fowlpox virus infection, serum was omitted from the atmosphere.

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©2007 American Association for Cancer Research. doi:10.1158/0008-5472.CAN-07-0224
Recombinant fowlpox virus vector. The murine CD40L gene was cloned from EL4 T cells (ATCC). Total RNA was isolated using RNeasy kit (Qiagen). First strand complementary DNA (cDNA) was synthesized using $A$ of total RNA. The cDNA was used for amplification of the CD40L gene by PCR using 30 cycles in 1× PCR buffer (Invitrogen), Taq polymerase, and 200 mmol/L deoxynucleotide triphosphate. Primers for the murine CD154 consisted of the forward primer 5'-GACTCTGACGACCTTTCAGTCAGCATGATAGAA-3' and reverse primer 5'-GTTTCTAGACGAGTTTGAGTAAAGCC-3'. These

Figure 1. Flow cytometric analysis of surface molecules on A20 cells infected with rF-mCD40L in vitro and in vivo. A, lymphoma cells were infected in vitro with WT-FP or rF-mCD40L at a multiplicity of infection of 200 for 12 h in serum-free medium and cultured for another 12 h in complete medium before staining with the indicated antibodies (solid histogram). Thin line, isotype staining control. Mean fluorescence intensity was calculated by subtracting the mean fluorescence values from the isotype control values and are indicated in each panel. B, tumor cells were prepared from tumor-bearing mice 24 h after intratumoral injection of rF-mCD40L or WT-FP and double stained with anti-CD154 and anti-CD80. Numbers in the figures, percentage of double-positive cells. Data reported here are one representative of three done with similar results.
amplified an 810-bp fragment encompassing the entire coding region of the murine CD40L gene. Recombinant fowlpox virus encoding the murine CD40L (rF-mCD40L) was constructed and manufactured by Therion Biologics Corporation. The rF-mCD40L recombinant was generated in chicken embryo dermal cells by in vivo recombination between a plasmid carrying the murine CD40L gene under control of the vaccinia 40K promoter and fowlpox virus (WT-FP) DNA. The plasmid directed the insertion of mCD40L and the GUS reporter gene in the FP14 site of the viral genome. Subsequently, the recombinant was plaque purified, amplified, and analyzed. CD40L expression was determined by in situ immunostain and Western blot. Nonrecombinant wild-type fowlpox virus (WT-FP) was used as a negative control. Purified viruses were stored at −80°C until use.

Female BALB/c mice (Harlan Sprague-Dawley; 6–8 weeks of age) were used as hosts for tumor challenge. CD8 knockout mice on the BALB/c background were a gift from Dr. G. Fathman (Stanford University). Mice were housed at the Laboratory Animal Facility at Stanford University Medical Center. All of the animal experiments were conducted according to the Stanford University Laboratory Animal Facility guidelines.

In vitro infection of tumor cells with rF-mCD40L and flow cytometry analysis. A20 cells (1 × 10⁶/mL) suspended in serum-free RPMI 1640 were infected with the rF-mCD40L or with WT-FP at a multiplicity of infection of 200 for 6 h at 37°C in a humidified atmosphere containing 5% CO₂. After the infection, cells were centrifuged and resuspended in complete medium and incubated for another 12 to 18 h. Transgene and costimulatory molecules on infected A20 cells were assessed by flow cytometry using a Becton Dickinson FACScan with CellQuest software. The following phycoerythrin- or FITC-conjugated murine mAbs were used: B7.1 (CD80), B7.2 (CD86), CD40L (CD154), and CD40 (all from PharMingen).

In vivo infection of tumor cells with rF-mCD40L. A20 tumor cells were implanted at a dose of 10⁶ cells s.c. into the flank of syngenic BALB/c mice and allowed to grow for 10 to 15 days. rF-mCD40L, at a dose of 1 × 10⁹ pfu in 0.1 mL, was injected directly into the s.c. tumor. Twenty-four hours later, the tumor was removed and made into a single-cell suspension. Cell preparation and staining for flow cytometry were done as previously described (16).

Tumor implantation and in vivo treatment protocol. A20 tumor cells were grown in vitro and frozen viably in aliquots as a master cell bank. For each experiment, cells from this bank were thawed and allowed to proliferate in vitro for 4 days before implantation. On the day of tumor inoculation, cells were washed thrice in PBS and diluted to the appropriate concentration. A20 cells were injected into BALB/c mice s.c. in the lower right flank with 10⁷ cells per mouse in 150 μL PBS (day 0). On day 15, mice bearing local tumors within a range 60 to 120 mm² were chosen and separated into groups for different treatments. rF-mCD40L or WT-FP [1 × 10⁹ plaque-forming units (pfu)] was injected into the tumor on days 15 and 22. Cyclophosphamide (Sigma Chemical Co.) was administrated i.p. on days 18 and 19 at a dose of 100 mg/kg body weight (Fig. 1). In tumor rechallenge experiments, 2 × 10⁵ CT26 or 1 × 10⁶ A20 were injected s.c. into alternate flanks on the same day. The local tumor size was measured twice a week using a caliper and expressed as the product of the two maximal perpendicular diameters (mm²). Animals were sacrificed when the tumor size reached or exceeded 500 mm², or when there were other signs of animal distress. Animal survival was followed for over 120 days.

T-cell depletion experiments. Mice were depleted of CD4⁺ or CD8⁺ T cells by injection i.p. of anti-CD4 (GK1.5 hybridoma) or anti-CD8 (2.43 hybridoma) mAbs. Ascitic fluid was harvested from mice implanted with individual hybridomas. These mAbs (250 μL of ascitic fluid per dose) were injected i.p. on days 12, 13, and 14, relative to the tumor inoculation.

![Figure 2](image-url)
Injections were then repeated weekly for the duration of the experiment. A group of mice received the irrelevant rat IgG2 antibody (SFR8-R6 hybridoma) as a control. These depletion conditions were validated by flow cytometry analysis of peripheral blood mononuclear cells using phycoerythrin-conjugated, nonblocking anti-CD4 (Caltag) and anti-CD8 (PharMingen) mAbs. Under these conditions, 99% of the relevant T-cell subsets were depleted whereas all other lymphocyte subsets remained within normal levels.

**Cytoxicity assays.** Splenocytes were isolated from both untreated, tumor-bearing mice as well as mice that had survived their tumors after being treated with the combination of rF-mCD40L and cyclophosphamide. Splenocytes were restimulated in vitro with irradiated (6,000 cGy) A20 cells at a tumor to splenocyte ratio of 5:1 for 5 days. Interleukin 2 (II-2; 10 units/mL; Chiron) was added to the cultures on day 3. Viable cells were harvested and incubated with 51Cr-labeled A20 at different effector to target cell ratios for 4 h in triplicate wells. 51Cr release was determined by analyzing the supernatants in a gamma counter (Wallac). The percentage of specific release was calculated according to the following formula: 100 × ([experimental release × spontaneous release] / [maximal release × spontaneous release]).

Spontaneous release and maximum release were determined in the presence of either medium alone or 1% SDS, respectively.

**Results**

**In vitro and in vivo infection of A20 tumor cells with rF-mCD40L.** As a first step, we determined the transgene and costimulatory molecule expression on A20 lymphoma cells infected with rF-mCD40L in vitro (Fig. 1A). Parent A20 cells express a low level of CD80 (B7-1), a high level of CD86 (B7-2), and a high level of CD40. As expected, they do not express CD40L. Following in vitro infection with rF-mCD40L, strong cell surface expression of the transgene product, CD40L, was detected on a majority of the A20 cells. No CD40L was detected on cells infected with the control WT-FP. Infection with rF-mCD40L and expression of the CD40L molecule was accompanied by up-regulation of CD86 expression, a doubling of the per cell expression of the CD86 molecule, as well as up-regulation of CD54 expression (data not shown). By contrast, expression of CD40L was accompanied by a decreased cell surface expression of CD40 (Fig. 2, P8S), presumably by internalization of the CD40 molecule (27, 28). CD40L expression and up-regulation of costimulatory molecules induced by rF-mCD40L were dose dependent. High doses of virus induced higher levels of CD40L expression as well as slight increases in virus-associated cell damage. Analysis of infected cells over time revealed that expression of the transgene product, CD40L, reached a maximum at 12 h, which was sustained for 18 h after infection. CD80 reached its highest expression at 24 to 48 h and maintained this level for 2 days (data not shown).

We next studied the delivery of the transgene into tumor cells in vivo. rF-mCD40L was injected directly into growing s.c. tumor nodules. After 24 h, the tumor was excised and prepared into a single-cell suspension. Compared with lymphoma B cells from tumors injected with WT-FP, we were able to detect the expression of the transgene product, CD40L, on 11.8% of excised tumor cells from the site that had been injected with rF-mCD40L. We did not detect the expression of CD40L on other cell populations such as dendritic cells or stromal cells but it is possible that nontumor cells were transduced with the transgene but were not included in the cell suspension that we were able to analyze by flow cytometry. Such local APCs, rather than the tumor B cells, could have been responsible for the sensitizing effect.

These cells also expressed increased levels of CD80 (Fig. 1B). However, the level of transduction and expression of the transgene and the resulting induction of expression of CD80 on the tumor cells was modest by comparison with levels achieved in vitro. With in vivo viral doses lower than 106 pfu, no transgene product could be detected on excised tumor cells.

***Therapeutic antitumor effect of rF-mCD40L.*** We tested the antitumor effect of rF-mCD40L in mice with established A20 tumors. Based on prior results with direct intratumoral injection of...
 naïve dendritic cells in this same tumor model (29), we designed the current experiment. In our experience with dendritic cell injection, we found it necessary to induce cell death in the tumor before initiating an immune stimulus. Therefore, we did the analogous experiment with rF-mCD40L. We initiated viral injections before the induction of cell death with chemotherapy because we assumed that tumor cells needed to be alive to express the transgene product as well as the other costimulatory molecules. As shown in Fig. 2 (rF-mCD40L), two intratumoral injections of 10^5 pfu of rF-mCD40L resulted in slight suppression of tumor growth compared with that in mice treated with intratumoral injection of WT-FP (Fig. 2, WT-FP) or PBS (Fig. 2, PBS). All mice treated with rF-mCD40L alone or WT-FP died of tumor progression within 5 to 7 weeks. Systemic administration of cyclophosphamide resulted in only temporary tumor shrinkage (Fig. 2, CTX). Most of these mice had tumor recurrence and eventually succumbed to systemic lymphoma. By contrast, the combination of intratumoral rF-mCD40L with systemic cyclophosphamide led to complete, long-term tumor regression in 100% of treated mice (Fig. 2, rF-mCD40L+CTX). These tumor-cured mice survived for over 120 days.

**Intratumoral injection is required for the antitumor effect of rF-mCD40L.** Injected virus could have traveled to draining lymph nodes and transduced APCs there. Alternatively, the effect may have been dependent on the transduction of tumor cells themselves rather than host APCs. We sought to distinguish between these possibilities by testing the requirement for direct intratumoral injection versus peritumoral injection. rF-mCD40L was administered by three different routes, intratumoral, peritumoral, and at a s.c. site distant from the tumor. As shown in Fig. 3D, rF-mCD40L + cyclophosphamide given at a distant site had no different effect on tumor growth than controls treated with cyclophosphamide alone (Fig. 3A). Similarly, in the group of mice treated with peritumoral injection of rF-mCD40L + cyclophosphamide, the majority of mice had tumor recurrence (Fig. 3C). However, mice that received the intratumoral rF-mCD40L + cyclophosphamide were cured of tumor and survived for over 120 days (Fig. 3B). This result implies that some cells within the tumor nodule need to be transduced with the CD40L gene; this target cell could be malignant B cells, other host APCs, or stromal cells within the tumor.

**CD8 but not CD4 T cells were involved in tumor regression.** We investigated the requirement for T cells in the antitumor effect induced by the combined chemoimmunotherapy treatment (Fig. 4). Animals were inoculated with tumor as before and treated either with chemotherapy alone (Fig. 4, CTX) or with chemotherapy plus intratumoral rF-mCD40L (Fig. 4). Mice were either depleted of CD4 (Fig. 4, CD4 depleted) or CD8 T cells (Fig. 4, CD8 depleted), or not depleted at all (Fig. 4, control antibody). T-cell depletions were begun on day 12 (just before treatment) and continued for the duration of the experiment. Flow cytometry analysis of peripheral blood validated that 99% of relevant T-cell subsets were depleted with anti-CD4 or anti-CD8 mAbs (data not shown).

Alternatively, the experiment was conducted in CD8 knockout mice (Fig. 4, CD8 knockout). The antitumor effect of the combined treatment in CD4-depleted mice was not impeded, with 9 of 10 surviving. However, the antitumor effect was completely abrogated in the CD8-depleted mice. Similar and confirmatory results were observed in CD8 knockout mice.

**Specific and long-term immunity against tumor.** To examine the generation of CTL responses against tumor cells, splenocytes from the mice that received the combination treatment and survived their tumors were examined for their killing activity against A20 tumor cells. As shown in Fig. 5, the effector cells from surviving mice, but not tumor-bearing mice, were able to lyse A20 tumor cells. To determine whether long-term immunity against tumor was generated, cured mice from the experiment of Fig. 4 (rF-mCD40L+CTX) were rechallenged with both A20 and CT 26, a syngenic colon tumor cell line. As shown in Fig. 6, surviving mice rejected the homologous A20 tumor but were not protected from the CT26 tumor. In normal BALB/c mice, both A20 and CT26 grew as expected and all mice died from tumor progression between 5 and 7 weeks.
Therefore, specific immunity against the A20 tumor was generated in these surviving mice.

Discussion

Many studies have shown that CD40L-modified dendritic cell or tumor cell vaccines can induce effective immunity against subsequent tumor challenge. However, it has been difficult to show effective immune therapy against established, growing tumors (30). In the present study, the combination treatment of intratumoral injection of rF-mCD40L plus systemic chemotherapy induced complete tumor regression and long-term immunity in mice with well-established lymphoma.

Induction of tumor-specific immunity using cell-based vaccines usually requires loading of antigen into an APC. B-cell lymphoma is a malignancy of cells that can, themselves, be APCs. This APC function can be augmented by manipulating and up-regulating costimulatory molecules. Malignant B cells express surface CD40, which can be activated by CD40L. In vitro, we confirmed that rF-mCD40L infection of tumor cells resulted in transgene (CD40L) expression as well as CD80 and CD86 up-regulation. In vivo, a small population of tumor cells expressed transgene upon the intratumoral injection of rF-mCD40L. Although the percentage of the transduced tumor population was low, it might have been sufficient to prime tumor-specific T cells. One might have expected the transduced tumor cells to stimulate other nontransduced tumor cells to up-regulate their costimulatory molecules, but we saw no evidence for this. Only the subpopulation of tumor cells expressing the transgene also up-regulated CD80 expression. Other cells, such as infiltrating dendritic cell or even stromal cells in the tumor microenvironment, may have been transduced with the transgene and could have been critical in the immunization process. Results of other investigators with CD40L gene transduction into nonhematologic tumors that do not express CD40 and thus cannot up-regulate costimulatory molecules must be explained by the transduction of tumor-infiltrating APCs (31, 32). Previous studies showed that tumor B cells activated with CD40L secrete several chemokines that directly enhance the migration of tumor-specific T cells to the tumor site (33, 34). The expression of costimulatory molecules on tumor cells may also enhance the retention of effector CD8 T cells at the tumor site (35).

Intratumoral administration of rF-mCD40L was necessary for the antitumor effect induced by chemoimmunotherapy. This result supports the hypothesis that either malignant B cells or infiltrating APCs are the critical target for gene transduction. The experiments of Kikuchi and Crystal provide strong evidence that the CD40-CD40L interaction is responsible for the priming effect, as coinjection of blocking anti-CD40 antibodies abrogated these priming effects (31, 36). Other investigators have shown that inoculation with CD40L engineered-tumor cells activates host dendritic cells and induces enhanced expression of B7-1 and ICAM-1 molecules (37). In these studies, interaction between CD40L on the tumor and CD40 expressed on dendritic cells enhanced expression of costimulatory molecules on the dendritic cell and stimulated the secretion of several cytokines by the dendritic cell. CD40L can prevent IL-10–mediated inhibition of dendritic cell function and dendritic cell apoptosis induced by tumor cells (38, 39). In the current experiment, we do not know which cell, tumor cell or local dendritic cell, is responsible for priming the antitumor effect.

Cured mice treated by rF-mCD40L + cyclophosphamide were protected from A20 tumor rechallenge, suggesting the induction of a long-lived antitumor immune response. Systemic antitumor immunity was also supported by the killing activity of splenocytes against A20 cells. Antibody-mediated T-cell subset depletion and
experiments in CD8 knockout mice showed that CD8 T cells are required for this antitumor effect. CD4 T cells were not required for the effector phase of the antitumor effect. CD4 T cells are usually involved in the induction and maintenance of a CD8 T-cell response. A number of studies have shown that CD4 or both CD4 and CD8 T cells are necessary for the immunity induced by the vaccination of viral CD40L-modified tumor cells (40, 41). However, recent studies showed that the function of T-helper cells can be circumvented by the engagement of CD40 on APCs (42–44). French et al. reported that CD40 antibody-mediated eradication of B-cell lymphoma is associated with the generation of CTL independent of T-helper cells (42, 45). Kikuchi et al. reported that tumor regression induced by the administration of CD40L and dendritic cell is dependent on CD8, but not CD4 T cells. In their study, as with ours, CD40L transgene was delivered to the tumor by a viral vector. This type of vector might help bypass the requirement for activated helper cells during CTL priming.

Recently, we and others have reported that dendritic cells directly injected into tumors can induce an antitumor, CD8 T-cell immune response (29). In these experiments, chemotherapy induced tumor necrosis and apoptosis, releasing tumor antigens and creating danger signals in the tumor environment. In the current study, we replaced dendritic cell with a CD40L virus. The results show that the antitumor effect of r-mCD40L + cyclophosphamide was equally powerful as dendritic cell + cyclophosphamide. This approach bypasses the requirement for the dendritic cell preparation, a technique that in humans would require custom-produced dendritic cells from each patient.

One concern in using viral vectors for immunotherapy is host immunity against the virus. Unlike adenovirus, which may be blocked by preexisting antibodies and can be used only once due to preexisting antiviral immunity, recombinant fowlpox vectors can be used multiple times without inhibition of transgene expression (25, 26, 46).

Taken together, the results of our experiments and those of other investigators establish that intratumoral injection of recombinant viral vectors expressing CD40L can cure established tumors by inducing a CD8 T-cell immune response against antigens on the tumor cells. Similar therapeutic results might be achievable in humans with lymphomas and other malignancies.

Acknowledgments

Received 1/17/2007; revised 4/13/2007; accepted 5/7/2007.

Grant support: NIH grants CA33899 and CA34233, and the Yu-Reichmann Foundation. R. Levy is an American Cancer Society Clinical Research Professor.

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We thank Dr. Shoshana Levy for helpful discussion throughout this work and Debbie Czerwinski for help with flow cytometry.


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