In Vivo Antitumor Effect of CD40L-transduced Tumor Cells as a Vaccine for B-Cell Lymphoma

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ABSTRACT

CD40–CD40 ligand (CD40L) interactions play a critical role in the activation of cellular immunity. CD40L enhances the antigen presentation function of CD40-expressing B cells. We have used a murine B-cell lymphoma model (A20) to study the in vivo antitumor effect of the administration of tumor cells transduced with a recombinant adenovirus encoding CD40L (AdvCD40L). After infection with AdvCD40L, A20 tumor cells up-regulate several T-cell costimulatory molecules (CD80, CD86, ICAM-1, and LFA-3) and Fas expression. Animals vaccinated with irradiated tumor cells transduced with AdvCD40L are protected against a lethal dose of parental A20 tumor cells. Animals with pre-existing tumors treated with AdvCD40L-transduced tumor cells display inhibition of the tumor growth, and this treatment confers a survival advantage. In vivo depletion studies demonstrate that both CD4\(^+\) and CD8\(^+\) T cells mediate the antitumor immunity provided by AdvCD40L-transduced tumor cells. These results show that genetic modification of tumor B cells with CD40L can be a useful strategy to promote systemic immunity against B-cell malignancies and provide an in vivo system to allow for additional evaluation and refinement of this approach.

INTRODUCTION

One of the major goals of tumor immunotherapy is the generation of tumor-specific T-cell responses that eventually will contribute to eradication of tumor. The initiation of a T-cell antigen-specific immune response requires multiple signals provided by recognition of tumor-specific T cells (2, 3). Recent efforts have focused on changing the accessory molecules necessary for an efficient activation of antigen-specific T cells (2, 3). Effective activation of autologous CTLs that are able to lyse unmodified tumor B cells (11). Hence, genetic modification of tumor B cells by herpes simplex virus encoding CD40L stimulates the in vitro generation of autologous CTLs that are able to lyse unmodified tumor B cells (11). Moreover, genetic modification of tumor B cells by herpes simplex virus encoding CD40L appears as an attractive approach to stimulate autologous T-cell responses directed against parental tumor cells. Recently, leukemia B cells infected with an adenovirus encoding CD40L have been used in patients as a way to activate systemic antitumor immunity (12). As a result of this maneuver, patients experienced reductions in leukemia cell counts and lymph node size, and developed leukemia-reactive T cells. However, these clinical responses were not complete. To date there has been no in vivo animal lymphoma model in which to study additional refinements of this general approach.

In this study, we have used a murine B-cell lymphoma model to analyze the in vivo antitumor effect of tumor B cells transduced with CD40L as a vaccine. We show that after transduction with an adenovirus encoding CD40L (AdvCD40L), tumor cells abundantly express costimulatory molecules, and when used as a vaccine, they induce a protective systemic immune response in vivo against unmodified tumor cells. Our results additionally support the use of tumor cells engineered to express CD40L as a therapeutic vaccine for patients with B-cell lymphoma.

MATERIALS AND METHODS

Animals. Female BALB/c mice (Harlan-Sprague Dawley, San Diego, CA; 6–8 weeks of age) were used for in vivo experiments. Animals were housed at the Laboratory Animal Facility at Stanford University Medical Center (Stanford, CA). All of the experiments were conducted according to the Stanford University Laboratory Animal Facility care guidelines.

Cell Lines. A20 is a BALB/c B-cell lymphoma (13) expressing MHC class I and II H-2\(^d\) molecules, and was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Tumor cells were cultured in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 10% heat-inactivated FCS (Hyclone, Logan, UT), 100 units/ml penicillin, 100 \(\mu\)g/ml streptomycin (both from Life Technologies, Inc.), and 50 \(\mu\)M 2-mercaptoethanol (Sigma, St. Louis, MO; complete medium). Cells were grown in suspension culture at 37°C in 5% CO\(_2\). The adenovirus-transformed human embryonic kidney cell line 293 was grown in DMEM supplemented with 10% FCS, 100 units/ml penicillin, 100 \(\mu\)g/ml streptomycin, and E3 deletions, and prepared as described elsewhere (14). Briefly, for the production of the adenovirus encoding murine CD40L (Adv CD40L), the

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3 The abbreviations used are: APC, antigen-presenting cell; CD40L, CD40 ligand; Adv, adenovirus; MAb, monoclonal antibody; PE, phycocerythrin; CTL, cytotoxic T lymphocyte; AdvY-gal, β-galactosidase gene.
murine CD40L cDNA was obtained by PCR as described (15) and cloned into the pXCI plasmid. Linearized pXCI containing the murine CD40L cDNA was cotransfected with linearized pGT3652 (in which the E3 gene is deleted) into BJ5183 bacteria cells by heat shock. Plasmids resulting from the homologous recombination between the fragment containing the murine CD40L and the E1 region of pGT3652 were selected by restriction digest mapping, amplified, and transfected into 293 cells using LipofectAMINE Plus (Life Technologies, Inc. Gaithersburg, MD). Viral plaques were isolated and used to generate primary and amplified viral stocks. Viral clones containing the murine CD40L cDNA were confirmed by PCR and sequencing analysis. Expression of the CD40L protein was confirmed by flow cytometry analysis of infected HeLa cells using a MAAb against murine CD40L (PharMingen, San Diego, CA). The virus was expanded in 293 cells and purified by cesium chloride gradient ultracentrifugation. Puriﬁed virus was dialyzed extensively against PBS containing 10% glycerol, and aliquots were stored at −70 °C. Viral titters were determined by plaque assay on 293 cells. The adenovirus encoding Adβ gal was generated as described (16).

In Vitro Transduction of Tumor Cells with Recombinant Adenovirus

Encoding CD40L. A20 cells cultured in complete medium but containing only 2% FCS were infected with the AdvCD40L or Advβ-gal at a multiplicity of infection of 1000 for 24 h in a volume of 1–2 ml in a humidified atmosphere containing 5% CO2 at 37°C. Expression of CD40 and CD40L was monitored by flow cytometry using PE-conjugated MAbs (PharMingen). After the infection, cells were resuspended in complete medium and incubated for another 24 h, and the expression of costimulatory molecules was assessed by flow cytometry. For this purpose, an aliquot of cells was washed twice in PBS-1% BSA-0.05% sodium azide, and stained for 30 min on ice with a panel of PE-conjugated MAbs speciﬁc for murine B7.1 (CD80), B7.2 (CD86), ICAM-1 (CD54), CD48 (the mouse homologue of LFA-3), and Fas (CD95; all from PharMingen). Appropriate isotype controls were used in all of the experiments. After incubation, the cells were washed and then ﬁxed with 2% paraformaldehyde, and the cells were analyzed using a Becton Dickinson FACScan (Mountain View, CA) with the CellQuest software.

Tumor Challenge and in Vivo Experiments. A20 tumor cells were thawed from a common frozen stock and passaged in vitro in complete medium for 4 days before use. On the day of tumor challenge, cells were washed three times in RPMI 1640 (no supplements) and diluted to the appropriate concentration. Groups of mice (10/group) received 2 × 106 cells in a volume of 0.2 ml s.c. As s.c. tumors grow, palpable regional lymphadenopathy and splenomegaly could be observed, indicating systemic spread of tumor.

For prophylactic experiments, mice were vaccinated twice s.c. at 2-week intervals with 1 × 106 A20 cells infected previously in vitro with AdvCD40L or with control Advβ-gal. After the infection, the cells were washed extensively with RPMI 1640 and irradiated (5000 cGy) immediately before injection into the mice. One week after the last vaccine, animals were challenged with live tumor cells as described above. Animals were followed daily for survival. Survival analysis was performed using Prism software (GraphPad, San Diego, CA), and statistical diﬀerences in survival were calculated using the long-rank test.

For therapeutic experiments, mice were ﬁrst injected s.c. with 2 × 105 A20 cells in one ﬂank. On days 5 and 11 after tumor challenge mice received two s.c. injections on the opposite flank of 1 × 106 irradiated tumor cells transduced with AdvCD40L or Advβ-gal virus. Tumors were measured three times weekly in two dimensions (length and width) with a caliper, and tumor volumes were calculated according to the formula: (width)2 × length × 0.52. Tumor volumes are reported as mean mm3 ± SE. Ps were determined by using a two-tailed t test. Animals were followed for survival as described above.

T-Cell Depletion Experiments. Mice (10/group) were vaccinated twice with irradiated AdvCD40L-transduced tumor cells and challenged 1 week after the last vaccine with live tumor cells. Animals were depleted of CD4+ and CD8+ cells by i.p. injection of anti-CD4 (GK1.5 hybridoma) or anti-CD8 (53–6,72 hybridoma) ascitic ﬂuid. Antibodies (200 μl of ascitic ﬂuid/dose) were injected on days −3, −2, −1, and 0, relative to the tumor challenge, and then every other day for a week followed by four weekly injections. A group of mice received rat IgG2 antibody (H22–15-5 hybridoma) as a control. These depletion conditions were validated by flow cytometry analysis of splenocytes using PE-conjugated MAbs anti-CD4 (Caltag, Burlingame, CA) and anti-CD8 (clone 53–5.8; PharMingen); 99% of the relevant cell subset was depleted, whereas all of the other subsets remained within normal levels.

Cytotoxicity Assays. Ten days after the last vaccine spleenocytes and lymph nodes were isolated from 2 representative mice of each group, pooled, and restimulated at 5 × 106 cells/ml with irradiated (5000 cGy) A20 cells (1 × 107/ml) for 6 days. IL-2 (10 units/ml; Chiron, Emeryville, CA) was added to the cultures on day 3. Viable cells were harvested and tested in a 4-h standard 51Cr release assay for the ability to lyse A20 cells. Briefly, 51Cr-labeled A20 or P815 cells were incubated with effector cells at different E:T ratios in triplicate wells, and 51Cr release was determined by analyzing the supernatants in a gamma counter (Wallac, Turku, Finland). The percentage of specific release was calculated according to the formula: 100 × ([experimental release − spontaneous release]/(maximal release − spontaneous release)).

ELISA for Detection of Anti-Idiotype Antibodies. Serum from vaccinated mice was added to 96-well MaxiSorp plates (Nunc, Naperville, IL) coated with puriﬁed recombinant single chain Fv A20 protein (10 μg/ml; kindly provided by H. Veeken, Freeburg University, Freeburg, Germany). Anti-idiotype antibody was detected by using horseradish peroxidase-conjugated goat antimouse IgG (Caltag). A serum known to contain anti-A20 idiotype antibodies was used as positive control. Absorbance was determined at 405 nm using a Vmax microplate reader (Molecular Devices, Menlo Park, CA).

RESULTS

Induction of Costimulatory Molecules on A20 Tumor Cells after Transduction with AdvCD40L. The expression of CD40 on A20 lymphoma cells was ﬁrst conﬁrmed by ﬂow cytometry. As shown in Fig. 1, A20 cells express high levels of CD40 (Fig. 1A) but do not express CD40L (Fig. 1B). A20 cells infected with AdvCD40L (24 h), cell surface CD40L expression could be detected on a substantial proportion (24%) of cells (Fig. 1D) but not on cells infected with the control virus Advβ-gal (Fig. 1C). After infection with the recombinant adenoviruses (48 h), A20 tumor cells were evaluated for the expression of costimulatory molecules. As shown in Fig. 2, Advβ-gal-infected A20 cells express no CD80 (B7-1), low levels of CD86 (B7-2; only 30% of the cells), and moderate levels of ICAM-1 and LFA-3. However, after the infection with AdvCD40L, 75%, and 72% of the cells expressed the CD80 and CD86 molecules, respectively, and cells displayed a signiﬁcant increase in the expression of other T-cell costimulatory molecules (mean ﬂuorescence intensity; Advβ-
compared with animals treated with Adv on days 5 and 11 after tumor challenge. Tumor growth was slower in tumor cells transduced with Adv_H9252 than in uninfected cells (Δ). One week after the last vaccine mice were challenged with 2 × 10^5 parental A20 tumor cells and followed for survival. A group of naive mice received tumor challenge only (●). B, mice vaccinated with AdvCD40L-transduced tumor cells (□) that rejected the tumor were rechallenged with the same dose of parental A20 tumor cells as above. A group of naive mice received tumor challenge only (●).
molecules or cytokines into the tumor cells (18, 19). In the last several years, efforts in tumor immunotherapy have been directed at modifying the tumor cells to promote antigen-presentation by the malignant cells themselves. One of the molecules used to accomplish that goal is CD40L.

Through its interaction with CD40 on B cells, CD40L is able to increase the expression of a number of adhesion and costimulatory molecules. This phenotypic change also has functional consequences, because this interaction makes B cells better APCs and increases their capacity to stimulate T cells (7). This effect has been demonstrated not only in normal B cells but also in lymphoma B cells as well (9, 20, 21). In our study, we have shown that A20 lymphoma cells, transduced with a recombinant virus encoding CD40L, can significantly up-regulate several adhesion and costimulatory molecules that potentially may convert those cells into better APCs and stimulate tumor antigen-specific T cells. Vaccination with these CD40L-modified tumor cells induced a protective systemic antitumor immunity against a B-cell lymphoma. Moreover, the treatment of mice with pre-existing tumors with CD40L-modified tumor cells also had a substantial beneficial effect. This result is in agreement with that reported previously by Dilloo et al. (22). However, in that study the tumor cells were not directly modified with CD40L but mixed with syngeneic fibroblasts that were transduced with CD40L-encoding retrovirus. Recent studies have suggested that the efficacy of a cellular vaccine may be greater when the transgene encoding a costimulatory molecule is directly expressed by the tumor cell (23).

The mechanism by which tumor cells modified with CD40L induce antitumor immunity may be related to direct priming of T cells. Very recently, it has been shown that tumor B cells activated with CD40L secrete several chemokines that directly enhances the migration of tumor-specific T cells to the tumor site (24). A20 tumor cells transduced with CD40L express high levels of costimulatory molecules (CD80, CD86, ICAM-1, and LFA-3). This ensures a proper stimulation of tumor-specific T cells and may contribute to the immunogenicity of the tumor cells (25). Furthermore, expression of costimulatory molecules may also enhance the recruitment of effector CD8+ T cells at the tumor site (26).

Direct stimulation of dendritic cells may be another mechanism that explains the antitumor effect of the tumor cells transduced with CD40L. Interaction of CD40L with CD40 expressed on dendritic cells enhances the expression of costimulatory molecules and stimulates the secretion of several cytokines that additionally activate a number of effector cells (T cells and natural killer cells; Ref. 27). After infection of A20 cells with AdvCD40L we were able to detect CD40L expression in 24% of the tumor cells (Fig. 1). This may be enough to promote an antitumor effect, because only a minor fraction of cells need to express CD40L to activate the immune system and induce tumor inhibition (28).

![Graph A](image1.png)

**Fig. 4.** Survival of mice with pre-existing tumors treated with irradiated AdvCD40L-transduced tumor cells. Groups of 10 mice were injected with 2 × 10^5 A20 tumor cells s.c. on day 0. On days 5 and 11, mice were treated with 10^6 irradiated A20 cells transduced in vitro with AdvCD40L (□), Advβ-gal (○), or uninfected cells (△), and the size of the tumors (A) and survival (B) were monitored; bars, ±SD.

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)

**Fig. 5.** CTL activity induced by irradiated tumor cells transduced with AdvCD40L. Lymph node and spleen cells from mice vaccinated with irradiated tumor cells transduced with AdvCD40L or Advβ-gal viruses were restimulated in vitro for 6 days with irradiated (5000 cGy) parental A20 tumor cells. Cytotoxic activity against A20 or P815 cells was measured in a standard 4-h 51 Cr release assay. Data are represented as mean specific lysis of triplicate values (%) at different E:T ratios. AdvCD40L-vaccinated versus A20 (□) or P815 (■) target cells. Advβ-gal-vaccinated versus A20 (○) or P815 (□) target cells; bars, ±SD.

**Fig. 6.** Effect of in vivo depletion of T-cell subsets on survival after vaccination with AdvCD40L-transduced tumor cells. Groups of 10 mice were vaccinated twice, 2 weeks apart s.c., with 10^6 irradiated A20 cells transduced in vitro with AdvCD40L and challenged with 2 × 10^6 parental A20 tumor cells 1 week after the last vaccine. Mice were depleted of T cells by i.p. administration of GK 1.5 (anti-CD4; □) or 53–6.72 (anti-CD8; △) antibodies. Control groups of mice received rat IgG (H22–15–5; ○) or tumor challenge only (●).
In line with other studies (22), our depletion studies suggest that T cells (both CD4+ and CD8+), are the effector cells of the antitumor effect observed after vaccination with tumor cells transduced with CD40L. This may be related to the fact that tumor A20 cells express both MHC class I and II molecules (13), thus making them potential targets for both CD4+ and CD8+ T cells. CD4+ T cells may also be critically involved in the induction and maintenance of the CD8+ CTL response. Moreover, our in vitro studies show that mice vaccinated with tumor cells transduced with AdvCD40L but not with a control virus had generated effector T cells able to specifically recognize parental A20 tumor cells. However, the role of a humoral response against the tumor cells is less clear. Our data suggest that antibodies directed against the idiotype protein (a known A20 tumor antigen) do not play a role in the antitumor effect observed.

One of the effects of CD40L interaction on A20 cells is the up-regulation of the Fas molecule. This may enhance the sensitivity of the tumor cells to apoptosis by interaction with Fas ligand. Interestingly, administration of soluble CD40L in the A20 tumor model stimulates the infiltration of T cells expressing Fas ligand into the tumor (29). This may contribute to enhanced apoptosis of tumor cells as has been shown for human B-cell lymphomas (30). Those apoptotic cells may then be uptaken by dendritic cells that in turn activate tumor-specific T cells (31), in a process facilitated by activation of dendritic cells via CD40 ligation (32).

In summary, we have shown that tumor cells transduced with a recombinant virus encoding CD40L induce systemic immunity against a B-cell lymphoma. These data additionally support the use of CD40L gene transfer into B-cell tumors as an active immunotherapeutic strategy for patients with B-cell malignancies. The current studies offer results in an in vivo model system that can be used to study additional refinements in this approach.

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