AdCD40L Immunogene Therapy for Bladder Carcinoma—
The First Phase I/IIa Trial

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Abstract

**Purpose:** Immunotherapy with Bacillus Calmette-Guerin (BCG) instillation is recommended for high-risk, non–muscle invasive bladder cancer. Bacillus Calmette-Guerin is not effective in advanced tumors, and better alternatives are warranted. Immunostimulating gene therapy with adenoviral vectors expressing CD40 ligand (AdCD40L) has shown efficacy in tumor models. CD40 ligand stimulates systemic immunity and may be effective in local and invasive human disease.

**Experimental Design:** Patients with invasive bladder cancer scheduled for cystectomy or patients with T4 tumors were enrolled in a phase I/IIa trial. Patients were treated with three cycles of intrabladder Clorpactin WCS-90 prewash, followed by AdCD40L instillation 1 week apart. Safety, gene transfer, immune effects, and antitumor responses were monitored.

**Results:** All eight recruited patients were treated as scheduled, and therapy was well tolerated. The main adverse effect was transient local pain during prewash. Postoperatively, urinary tract infections and one case of late septicemia with elevated potassium were reported. No adverse events were ascribed to vector therapy. Gene transfer was detected in biopsies, and bladders were heavily infiltrated with T cells. The effector marker IFN-γ increased in biopsies, whereas levels of circulating T regulatory cells were reduced. Histologic evaluation indicated that AdCD40L therapy reduced the load of malignant cells.

**Conclusions:** To our knowledge, this is the first report on immunogene therapy in bladder cancer and the first using AdCD40L in vivo. Local AdCD40L gene therapy was safe, boosted immune activation, and should be further evaluated as a single or an adjuvant therapy for urothelial malignancies. *Clin Cancer Res;* 16(12); 3279–87. ©2010 AACR.

Bladder cancer is the fifth most common cancer in the Western society. The common non–muscle invasive disease is treated with transurethral resection of the tumors, and in high-risk cases, adjuvant treatment is given with intravesical instillations of Bacillus Calmette-Guerin (BCG) or chemotherapy (1). More than half of the patients will relapse on this regimen, after which cystectomy is recommended (2). Cystectomy is also used initially for muscle invasive tumors. Because of the mortality and morbidity with major surgery, bladder-sparing effective therapies are warranted. CD40 ligand (CD40L; CD154) is a potent T helper 1 immune stimulator shown to elicit robust antitumor reactions (3–6). CD40L belongs to the tumor necrosis factor gene superfamily and is expressed under inflammatory conditions by immune cells and by stressed cells of nonhematopoietic origin (7). It preferentially ligates to CD40 on antigen-presenting cells. However, CD40 can also be detected on keratinocytes, fibroblasts, and carcinomas. CD40/CD40L ligation has a diverse outcome depending on the interacting cell types. CD40 stimulation of antigen-presenting cells such as dendritic cells leads to their maturation and increased capacity to present antigens to T cells, as well as secretion of cytokines, such as interleukin 12 (IL-12) and IFN-γ, leading to a T helper 1 response. Hence, upon CD40 triggering, dendritic cells orchestrate effector immune cells such as CTLs, natural killer (NK) cells, and M1 macrophages (8), all of which are important in antitumor immunity. Conversely, CD40 ligation on carcinoma cells impairs malignant cell growth and survival, and this can be enhanced by chemotherapy (9).

CD40 stimulation strategies with recombinant soluble CD40L, CD40L-expressing tumor vaccines, and anti-CD40 monoclonal antibodies have been evaluated for end-stage cancer in man with encouraging results (10–15). We have previously shown that AdCD40L gene therapy efficiently eradicates experimental bladder cancer (5, 6, 16), as well as spontaneous dog melanoma (17). Herein, we applied...
local transfer of the human CD40L gene through adenoviral vectors to patients with urinary bladder carcinoma in a one-armed, single-center phase I/IIa clinical trial.

Materials and Methods

Patients

Eligible were patients with urothelial cell carcinoma of the bladder who were ≥18 years old. Patients in phase I (n = 5) were scheduled for cystectomy because of high-risk tumor, whereas patients in phase IIa (n = 3) had stage T3 tumors. None of the patients had other malignancies 5 years before study nor had they recently received therapy for bladder cancer (within 3 mo for chemotherapy or 6 mo for BCG). Fertile women and patients with chronic urinary tract infections or systemic autoimmune disease were excluded. Written informed consent was obtained from all patients, and the study was completed according to Good Clinical Practice and the Declaration of Helsinki. The study protocol was approved by the Swedish Medical Products Agency (Eu-nr 2006-000985-34) and the regional ethical committee (DNr 2006:092). This study was registered at www.clinicaltrials.gov.

Adenoviral vector

An adenoviral vector serotype 5 carrying the human CD40L gene driven by a Rous sarcoma virus promoter (AdCD40L) was used for gene transfer (Vector Production Facility at the Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX). The vector had a concentration of $1 \times 10^{12}$ vector particles/mL and $1.44 \times 10^{10}$ infectious units/mL.

Treatment protocol

A biopsy confirmed tumor diagnosis at baseline. One week preceding treatment cycles, the first three patients underwent a run-in session in which Clorpactin WCS-90 alone was instilled to evaluate toxicity and immunologic effects. Patients in phase I received three preoperative cycles of AdCD40L at 1 week apart. Vector instillation was preceded by four brief washes with 0.1% Clorpactin WCS-90 (United-Guardian, Inc.) diluted in PBS, followed by one PBS rinse. After prewashing, AdCD40L diluted in 50 mL PBS (enough fluid to assure maximum bladder wall contact without creating too much urgency) was instilled and kept in the bladder for 30 to 60 minutes, depending on the patient. After treatment, the bladder was rested for 1 hour, followed by a final wash with PBS. The three first patients received $1 \times 10^{11}$ vector particles per treatment (low dose), whereas the remaining five patients received $1 \times 10^{12}$ vector particles (high dose). In phase I, patients underwent cystectomy 3 to 5 days after their last treatment. One of them refused cystectomy and only biopsies were done. In phase IIa, all tumors were removed at baseline, save for one marker tumor that was removed by transurethral resection 14 days after the last treatment (Supplementary Fig. S1). Prebiopsies and postbiopsies were evaluated by histology. Simultaneously, matched but untreated patients undergoing cystectomy (n = 7) were evaluated as controls to our patient cohort. We sampled blood for toxicity and immunologic analyses at baseline, before each treatment cycle, at day three post cycles, at cystectomy/resection, and at 30 days after final treatment.

Quantitative PCR

Vector DNA was analyzed from urine, serum, whole blood, and biopsies using vector-specific quantitative PCR. DNA was obtained by E.Z.N.A.Viral RNA Kit (Omega Biotek), which is suitable for DNA and RNA purification. Cytokine mRNA was evaluated in biopsies by cDNA synthesis using Superscript II (Invitrogen). Quantitative PCR was done using the Taqman system (iCycler, Bio-Rad Laboratories). Primer and probes were purchased from CyberGene AB and are shown in Supplementary Table S1. The vector copy number is shown per microgram viral DNA, whereas the differing amounts of cDNA content between the biopsy samples were corrected against β-actin. The mRNA copy numbers in 5 μL cDNA are shown in the figures.

Immunohistochemistry

Frozen sections of human biopsies were acetone fixed before staining with the following monoclonal antibodies: rabbit anti-CD40L (Santa Cruz Biotechnology), mouse anti-CD4 (Novo Castra Laboratories Ltd.), CD8 (Dako Corporation), CD40 and FoxP3 (Biologend), CD56 (MONOSAN), and CD68 (Serotec Ltd.). For detection, EnVision System Labeled Polymer–HRP and AEC Substrate Chromogen were used accordingly to manufacturer's instructions (Dako Corporation). All sections were counterstained with Mayer's hematoxylin (Histolab).

Translational Relevance

Cancer therapy using immune modulators is showing promise in early clinical trials for various tumors. For bladder cancer, live tuberculosis bacteria (Bacillus Calmette-Guerin) treatment is the gold-standard therapy for localized disease. Although bladder tumors are sensitive to immunotherapy, Bacillus Calmette-Guerin treatment does not induce systemic immunity and is therefore not an option for metastasized disease. CD40 ligand, on the other hand, is a potent initiator and accelerator of so-called T helper 1 immune responses characterized by the activation of CTLs and sustained immunity. Herein, we used an adenoviral vector to express CD40 ligand in the bladder of patients with urothelial cancer and observed immune activation and antitumor effects with low toxicity. Furthermore, we have successfully administrated AdCD40L to dog patients with malignant melanoma. Local AdCD40L therapy is potentially effective as a single or an adjuvant treatment for various solid tumors.
Cytometric bead array and enzyme-linked immunosorbent assay (ELISA)

Plasma cytokines were analyzed using the human Cytometric Bead Array Inflammation Kit (BD Biosciences) and ELISAs for soluble IL2 receptor (sIL2R), IFN-γ (DiaClone), and transforming growth factor β (TGF-β; Biosource). Antibodies to vector backbone were measured by Adenovirus IgG (Diagnostic Automation, Inc.).

Flow cytometry

Peripheral blood mononuclear cells from heparinized blood were analyzed by flow cytometry. T-cell activation was evaluated by stimulation with tumor-associated peptides. Briefly, $2 \times 10^5$ peripheral blood mononuclear cells were stimulated for 4 hours with a universal peptide mix containing 1 μg of each peptide derived from the tumor-associated antigens Her2 (HER262-76, 369-377, 654-662, 754-762, 776-788, 780-822, 818-836, and 883-899; ref. 18), Survivin (PepMixKit), NY-ESO-1 (PepMixKit), or negative control peptides (PepMixKit, JPT Peptide Technologies GmbH).

Peptide mixes were constructed to have binding affinity to multiple class I and II human leukocyte antigen (HLA) haplotypes. Cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences) using fluorescent-labeled monoclonal antibodies against CD3, CD4 (BD Biosciences), CD8, IFN-γ, CD107a, CD19, CD83, and FoxP3 from Biolegend.

Statistical evaluation

GraphPad Prism Software was used for statistical calculations.

Results

Safety and toxicity of AdCD40L gene therapy

Five patients with high-grade bladder carcinoma scheduled for cystectomy were enrolled in phase I, and three patients with T₄ tumors were enrolled in phase Ila. Seven patients were male, and one was female. The mean age was 70 years (range, 59-85 y). One of the patients in phase
I and two in phase IIa received previous BCG therapy without benefit (Supplementary Table S2). AdCD40L gene therapy was well tolerated. Adverse events, as evaluated by the common terminology criteria scale CTC, included CTC1 mild bladder pain or spasms in two and one patient, respectively, and were associated with Clorpactin WCS-90. There were no treatment-related changes in blood biochemical or hematologic parameters. Signs of cytokine storm, such as flu-like symptoms or fever, were absent, as were autoimmune manifestations. However, during treatment, some patients experienced adverse events such as postoperative pain following cystectomy (n = 1; CTC2-3), postoperative infection (n = 3; CTC2-3), spontaneously resolving asystole during anesthesia at cystectomy (n = 1; CTC4), insomnia (n = 1; CTC1), headache (n = 1; CTC1), constipation (n = 1; CTC2), common cold (n = 1; CTC1), and torticollis (n = 1; CTC2). One patient with urinary tract infection had concurrently increased neutrophils and monocytes (CTC2). Another patient had late septicemia (CTC4) combined with anorexia (CTC3) and nausea (CTC2), as well as elevated levels of calcium and potassium postoperatively probably because of surgery-related complications (CTC4).

**AdCD40L vector spreading and host antiviral immune responses**

The adenovector backbone was detected by quantitative PCR in the urine 1 to 4 hours postinstillation. Thereafter, the vector became undetectable independently of treatment dose (Fig. 1A). However, patients receiving high-dose instillation had higher levels of vector in urine than low-dose patients 1 hour posttreatment (Fig. 1B; P = 0.008). The vector can not be detected in serum at any time point save for one sample at the third treatment cycle of patient 10, in whom low levels were detected (data not shown). Biopsies from the tumor and seemingly normal epithelium were sampled pretherapy and posttherapy to evaluate the presence of the AdCD40L vector. Vector DNA can be detected at equal levels in biopsies from the tumor and normal tissue (Fig. 1C), and these levels were greater than baseline (P = 0.02 and P = 0.03, respectively). There was no difference (P = 0.3) in vector level in patients treated with low- or high-dose instillation, suggesting that the lower dose was sufficient for vector uptake (Fig. 1D). Antibody titers to adenovirus were modestly but significantly increased in most patients, with the exception of patient 03 who showed increasing levels (Fig. 1E; P = 0.02).

**CD40L protein expression in biopsies**

Biopsies from bladder epithelium were investigated for CD40L protein. CD40L is expressed at sites of inflammation by a variety of cells. In most patients, CD40L can be detected before treatment, but expression increased in the epithelial lining posttherapy (Fig. 2). Biopsies were also analyzed for CD40L mRNA, and the results were complementary to histochemistry (Fig. 3A; P = 0.03).

**CD40L-mediated cytokine burst**

The tumor biopsies were assessed for increased mRNA expression of other immune-related molecules. IFN-γ...
(\(P = 0.02\)) and IL-10 (\(P = 0.05\)) mRNA increased in all but one patient. There were no consistent alterations in IL-12, TGF-\(\beta\), or FoxP3 (Fig. 3B-F). Immediately following vector instillation, the proinflammatory cytokines IL-6 and IL-8, as well as sIL2R increased in the urine, but not plasma, but then rapidly disappeared (data not shown).

**Tumor-infiltrating inflammatory cells**

Biopsies were evaluated for the presence of immune cells before and after AdCD40L gene therapy (Fig. 4). The epithelium was heavily infiltrated with CD4+ T cells after treatment. CD8+ T cells were also enhanced, whereas NK cells (CD56+) somewhat increased in three patients. T regulatory (FoxP3+) and myeloid cell (CD68+) staining was inconclusive, with increases, decreases, and no change observed. However, T regulatory cells and myeloid cells increased in normal but not in tumor tissue post treatment. There was a modest to strong CD40 expression by the tumor cells and epithelial lining that was sustained for 30 days in most patients. Lymphocyte foci in treated bladders were strongly CD40 positive.

**Circulating immune cell populations**

There were no significant differences in the levels of circulating T cells, B cells, or NK cells pretherapy versus posttherapy. However, mature dendritic cells (CD83+) decreased in most patients, and T regulatory cells (CD3+CD4+FoxP3+) diminished in all patients after therapy (\(P = 0.008\)). T cells stimulated with tumor antigen peptide mixes responded with IFN-\(\gamma\) or CD107a. However, in most patients, these cells were undetectable in the circulation posttreatment (Fig. 5).

**Antitumor effects**

In phase I, biopsies were taken at baseline and immediately before cystectomy, and the removed whole bladder was also examined and multiple biopsies were taken for further investigation. In phase IIa, tumor biopsies were taken at baseline, and the remaining marker tumor was obtained for analysis at resection. In two of three patients in phase IIa, there was no change in tumor size (6 and 8 mm, respectively). In one patient, there was a 38% reduction in size of the marker tumor (40-25 mm). After
CD40L gene therapy, histopathology revealed that three of five phase I patients had no detectable tumor cells in the bladder (Fig. 6). However, residual dysplasia and metaplasia were present. One patient lacking tumor cells in the bladder still had remaining malignant cells in the resected distal ureter. All patients in phase IIa still had tumor cells in the resected marker lesion posttherapy. In seven of eight patients, there was a stronger inflammatory cell response after therapy. Moreover, in four of eight patients, the bladder was infiltrated with eosinophils posttreatment. Matched untreated patients (n = 7) undergoing cystectomy served as controls. All had remaining tumor in the removed bladder, and only two of seven patients had increased inflammation compared with pretreatment.

**Discussion**

To our knowledge, this is the first report on in vivo AdCD40L therapy. CD40 agonist-based tumor therapy has earlier been evaluated in a total of six published clinical trials. Tumor cell vaccines expressing CD40L alone or in combination with either IL-2 or GM-CSF or, alternatively, recombinant soluble CD40L trimers or anti-CD40 agonistic antibodies were used to treat patients with leukemia or solid tumors (10–15). Encouragingly, these studies did not reveal major severe side effects. Common adverse events were transient and included injection site reactions, flu-like symptoms, and in some cases, increased liver transaminases. Treatments reduced lymphocyte counts and increased lymph node size in patients with lymphoid tumors. Furthermore, several patients treated with tumor vaccines, recombinant CD40L, or anti-CD40 antibodies had stable disease at follow-up.

Our reason for assessing CD40 agonist treatment in patients with bladder cancer was 2-fold. Immunotherapy is a frequent form of local therapy in certain stages of the disease. Moreover, bladder carcinoma is an ideal candidate for local immunotherapy because of its localization, allowing nonsurgical administration and inspection. To date, two trials were reported on corrective p53 gene therapy for bladder cancer using adenovectors for gene transfer (19, 20). Both reports concluded that the procedure was safe. One trial used a transduction enhancer BIG-CHAP, which allowed for vector detection in most patients. However, corrective gene therapy has its limitations because of the ineffective vector transfer to tumor cells, and in the absence of a bystander activity, the corrective gene must be transferred to all malignant targets. In contrast, immunostimulatory gene therapy only requires that a fraction of cells at the tumor site be transduced to trigger antitumor T-cell immunity, which in turn can target transduced and nontransduced tumor cells.

In this study, AdCD40L gene transfer was applied after pretreatment with the enhancer Clorpactin. Both were well tolerated, and there were no adverse events directly ascribed to the vector. Local administration limited systemic CD40L exposure, which may explain the absence of flu-like symptoms or increased liver transaminases reported in earlier studies with CD40L-based strategies.

The vector was efficiently delivered to the bladder epithelium after treatment, and CD40L protein was increased in bladder tissue. Because CD40L can be prestored in a variety of cells and transferred to the plasma membrane.
within seconds to minutes after cell stress (7, 21), we cannot, however, definitively conclude that the detected CD40L only reflected transgene expression. Nonetheless, AdCD40L therapy stimulated infiltration of CD4+ and CD8+ T cells into bladder tissue, and the increase of IFN-γ mRNA indicated that these T cells were activated. The suppressor cytokine IL-10 was simultaneously increased, which can reflect natural or tumor-induced peripheral tolerance compensating for CD40L-induced immune activation. Correspondingly, increased numbers of FoxP3+ T regulatory cells were found after treatment in biopsies from seemingly normal epithelium but not in biopsies from tumor. Circulating T regulatory cells were decreased in all patients posttherapy, indicating that CD40L therapy in humans may counteract or redistribute T regulatory cells, as we (6) have shown in experimental models. Total circulating lymphocytes were not altered by treatment, although tumor-reactive T cells and mature dendritic cells were reduced in blood posttreatment in most patients. The latter may be a relocation of these cells into tumor tissue, but this could not be analyzed herein.

Malignant urothelial cell line cells expressing CD40 are growth inhibited or undergo apoptosis upon CD40L stimulation (9, 22). CD40 was expressed by tumor cells and bladder epithelium in our patient cohort, suggesting that the antitumor activity may have been the result of apoptosis. In immunodeficient mice, AdCD40L therapy does not cure but prolongs survival (9, 16), whereas in immunocompetent mice, complete tumor rejection is obtained (3–6, 16). We cannot distinguish between the effects of tumor-directed T cells and CD40-triggered tumor apoptosis histologically. Irrespective of mechanism, CD40-mediated apoptosis of urothelial cancer cell lines can be enhanced by chemotherapy (9), indicating the possibility of using AdCD40L gene therapy as adjuvant to conventional therapy for solid tumors.

The clinical consequences of our treatments were encouraging. In three of five phase I patients, there were no detectable malignant cells in the bladder after therapy, although one had scanty malignant cells remaining in the resected ureter. The two patients with refractory tumor and the patient with residual tumor in the ureter received low-dose vector treatment. Although some tumor cells may have been removed during transurethral resection before cystectomy, seven closely matched controls all had remaining high-grade tumor postcystectomy and only two had increased inflammation. The three patients in phase IIa had residual T4 tumors posttherapy but with a 38% reduction of tumor size in one. Future studies may include an extended duration and intensity of AdCD40L treatment.

In conclusion, local administration of AdCD40L vector therapy in patients with bladder cancer is safe and feasible; it stimulates T helper 1 immunity, suppresses regulatory cells, and seems to have antitumor activity. AdCD40L therapy is a promising type of biological therapy of bladder cancer.

**Disclosure of Potential Conflicts of Interest**

The authors did not report any potential conflicts of interest.
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Fig. 6. Hematoxylin-eosin-stained sections showing the histopathologic findings before and after AdCD40L therapy. Phase I shows high-grade tumors pretherapy. Patient 01 had few remaining malignant cells, whereas patients 02, 05, and 06 did not have high-grade tumors in the bladder posttreatment. However, patient 02 had remaining tumor in the ureter and metaplasia in the urothelial lining. Patients in phase Ila had all remaining superficial tumors, but patient 10 had reduction in tumor size. Patient 09 presample is a frozen section stained with hematoxylin. All postbiopsies showed increased inflammation except patient 03.

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