Immunogene Therapy by Adenovirus Vector Expressing CD40 Ligand for Metastatic Liver Cancer in Rats

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Abstract. *Background: We have explored a gene-therapeutic approach to stimulate antitumor immunity by adenoviral-mediated transfer of CD40 ligand (CD40L) to treat metastatic liver cancer in a rat model. Materials and Methods: Rat metastatic liver cancer cells were implanted into the back of rats bilaterally. When the larger tumor reached 8.0 mm in diameter, adenovirus vector-expressing mouse CD40L was injected intratumorally as treatment group (n=5), while LacZ was injected in the control group (n=5). Results: In the control group, the tumor gradually grew to be 20.7±1.6 (mean±SD) mm in intratumorally injected tumors and 21.8±3.7 mm in opposite tumors seven weeks after injection, respectively. In contrast, in the treatment group, the tumor was reduced to 3.6±8.2 mm and 3.7±8.2 mm. The tumor growth and survival rate were significantly different (p<0.001). Conclusion: Adenovirus vector-mediated CD40L gene therapy is an effective therapeutic method for metastatic liver cancer.*

Liver metastasis is one of the most important prognostic factors for digestive tract cancer. Hepatectomy is the most effective therapy for metastases of liver cancer and is potentially curative (1-4). However, the number of patients who benefit from hepatectomy is limited. Liver metastases from colorectal cancer are a good indication for hepatectomy, but such metastases are often widespread and are difficult to treat (1, 5, 6). Therefore, new systemic therapeutic approaches should be developed to further improve the outcome of metastases of liver cancer. Nevertheless, no effective systemic therapies exist for metastatic liver cancer which include gene therapy.

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Key Words: Gene therapy, adenovirus vector, CD40 ligand, rat, metastatic liver cancer.

Immunogene therapy using immunostimulatory molecules aiming at enhancing antitumoral immunity has a potential for the treatment of metastatic liver cancer. CD40 ligand (CD40L) is a type II membrane protein that belongs to the tumor necrosis factor family and is predominantly expressed on $CD4^+$ T-cells and binds to the CD40 receptor of the membrane of antigen-presenting cells (APCs) (7, 8). The interaction between CD40L and CD40 plays a crucial role in the activation of APCs and in the initiation of both humoral and cellular immune responses (7, 9, 10). Therefore, gene transfer of CD40L has been proposed as an efficient means of treating malignancies (11-16).

The immunity of a tumor-bearing patient frequently fails to eliminate malignant tumors due to either the lack of recognizable tumor antigens or the inability of tumor antigens to elicit an effective immune response (17, 18). The rationale for infecting tumor cells with CD40L is to convert these cells into stimulators of APCs, an effect leading to enhanced presentation of tumor antigens to T-cells and activation of antitumor immune responses.

To analyze the potential of CD40L-based gene therapy for possible clinical application to treat multiple metastases of liver cancer, we explored antitumor immunity by adenovirus-mediated transfer of CD40L in an animal model of metastatic liver cancer.

Materials and Methods

Cell line. RCN-9, a rat metastatic liver cancer derived from colon cancer, was obtained from the Riken Cell Bank (Tsukuba, Japan) and cultured in RPMI-1640 medium containing 10% fetal bovine serum (GIBCO BRL, Grand Island, NY, USA) and penicillin / streptomycin (GIBCO BRL). The cells were cultured at 37˚C with 5% CO_2.

Construction of recombinant adenovirus vector. The replication-defective recombinant adenovirus vector (AxCALacZ) expressing *Escherichia coli* β-galactosidase (β-gal), as control vector, was a kind gift from Dr. Saito (Institute of Medical Science, University of Tokyo, Tokyo, Japan) (19, 20). For treatment vector, an adenovirus-expressing mouse *CD40L* gene (AxCAmCD40L) was used. CD40L was a gift from Dr. H. Yagita (Juntendo University School of Medicine, Tokyo, Japan) and AxCAmCD40L was produced with the

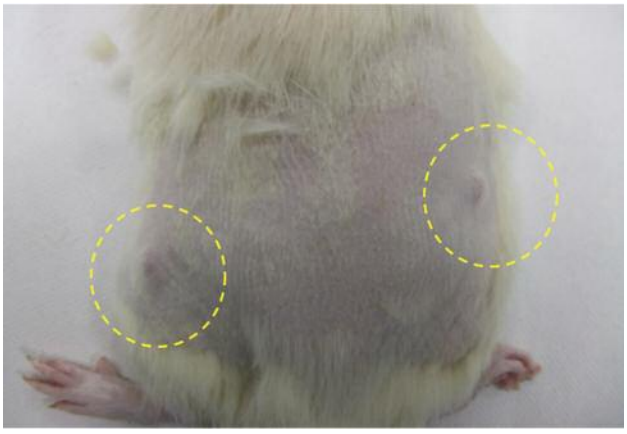

Figure 1. *A rat metastatic liver cancer model with two tumors in its back by bilateral subcutaneous injection of RCN-9.*

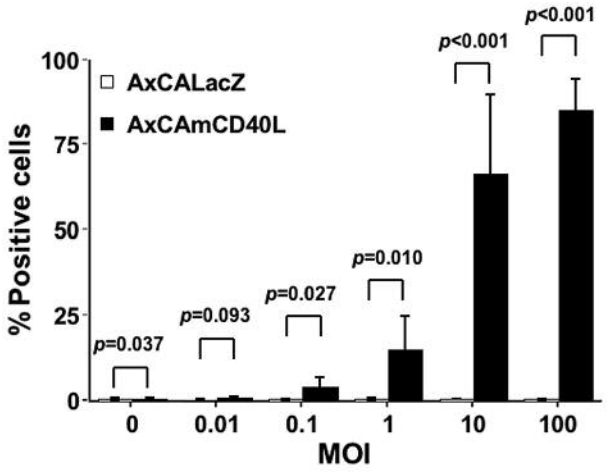

Figure 2. *Cells infected with AxCAmCD40L demonstrated a dose-dependent expression of CD40L. However, CD40L could not be detected in tumor cells infected with AxCALacZ at any dose. There is a significant difference of CD40L expression at 0.1, 1, 10 and 100 MOI between the two groups.*

adenovirus expression vector kit instrument from Takara Biomedicals (Tokyo, Japan) according to the manufacturer's instructions. These vectors were generated from adenovirus type 5, and the E1 and E3 regions were deleted to prevent viral replication. The *β-gal* gene and mouse *CD40L* were driven by the cytomegalovirus-enhancer-chicken β-actin hybrid promoter (CAG promoter) (21) and a rabbit beta-globin poly (A) signal located downstream from the gene. These vectors were purified by two rounds of CsCl centrifugation and then were stored at −80˚C until use. In this study, the vector was used at a concentration (22) of 6.0×10^{10} pfu/ml.

In vitro CD40L expression on tumor cells by FACS analysis. CD40L expression on tumor cells infected with AxCAmCD40L was measured by FACS analysis *in vitro*. RCN-9 was infected with AxCALacZ or AxCAmCD40L at 0, 0.01, 0.1, 1, 10 and 100 multiplicities of infection (MOI). At 48 hours after infection, the cells infected with vectors were allowed to react with R-phycoerythrin-conjugated anti-mouse CD40L antibody (Pharmingen, San Diego, CA, USA) for 30 minutes at 4˚C and CD40L expression was analyzed in both groups by fluorescence using FACS (Becton-Dickinson, San Jose, CA, USA). For detection of CD40 expression, RCN-9 was incubated with fluorescein isothiocyanate-conjugated anti-mouse CD40 antibody and analyzed by fluorescence using FACS. Irrelevant isotype–matched antibodies were used as controls.

Rat metastatic liver cancer model. Male Fisher344 rats (6 weeks old and 180 g), syngeneic with RCN-9, were housed in plastic cages with shredded paper bedding (Alpha-dri; Shepherd Specialty Papers Inc., MI, USA) in a biological cabinet at the Laboratory Animal Facility of Jikei University School of Medicine. The animals were maintained with a 12-hour light-dark cycle at a temperature of 22±2˚C and 55±5% humidity in a room with a filtered air supply.

A rat metastatic liver cancer model was established by injection of RCN-9 cells (1×10^6 cells) subcutaneously into the back of the animal bilaterally (Figure 1).

Immunogene therapy by AxCAmCD40L for metastasis liver cancer. When a tumor of the established bilateral back tumors became larger than 8.0 mm in diameter, the larger tumor in each animal was intratumorally injected with 6.0×10^{10} pfu/μml of AxCAmCD40L for the treatment group (n=5), or 6.0×10^{10} pfu/μml of AxCALacZ for the control group (n=5). The diameters of treated and untreated tumors in both groups were measured in the same way. The animals were followed-up without any treatment until death. The effect of immunogene therapy was assessed with regard to reduction of tumor diameters in the intratumorally injected and untreated tumors and by duration of survival.

Histological studies. For histological studies, tumor-bearing animals, from a separate group to those used for survival analysis, were killed on the 5th day after injection with either AxCAmCD40L or AxCALacZ. Tumor tissues were fixed in formalin and embedded in paraffin. Three mm-thick sections were made and stained with hematoxylin-eosin (HE).

Statistics. Non-paired Student's *t*-test, repeated measures ANOVA and Log rank test were used for statistical studies. All *p*-values were considered statistically significant when the associated probability was less than 0.05.

Results

In vitro CD40L expression on tumor cells by FACS analysis. RCN-9 cells infected with AxCAmCD40L demonstrated a dose-dependent expression of CD40L. At 0.01, 0.1 and 1 MOI, few cells exhibited positive CD40L expression, but the expression level reached 66.5±23.5% (mean±SD), and 85.3±9.2% at 10 and 100 MOI respectively. However, CD40L was not expressed in tumor cells infected with AxCALacZ at any dose. There was a significant difference in the proportion of cells expressing CD40L at 0.1 MOI or greater in the two groups (Figure 2). CD40 expression could not be detected in RCN-9 cells (data not shown).

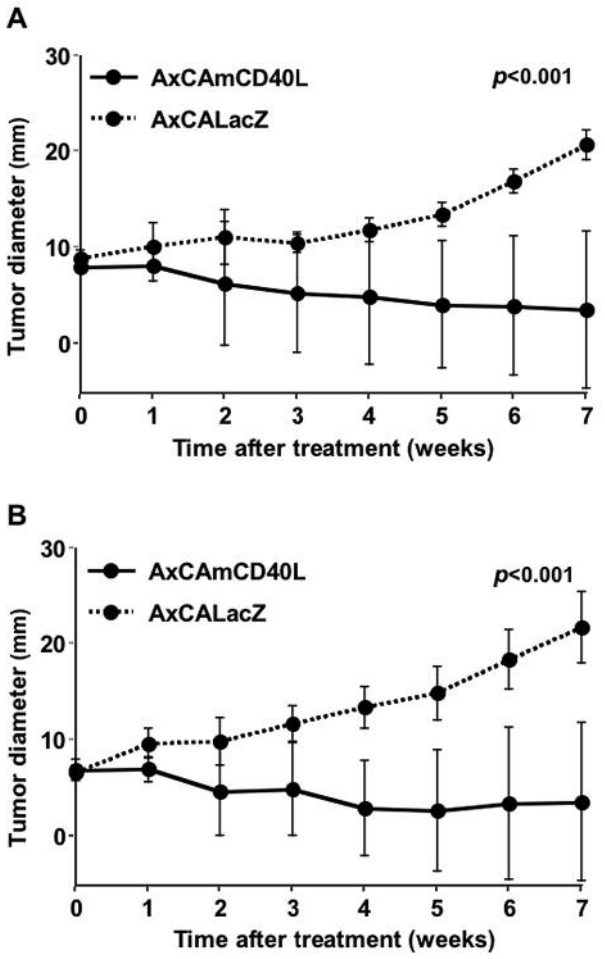

Figure 3. *The growth of intratumorally injected tumors was significantly lower in the AxCAmCD40L-treated group (A). Like intratumorally injected tumor growth, the tumor growth of corresponding untreated tumors was also significantly lower in the AxCAmCD40L-treated group (B).*

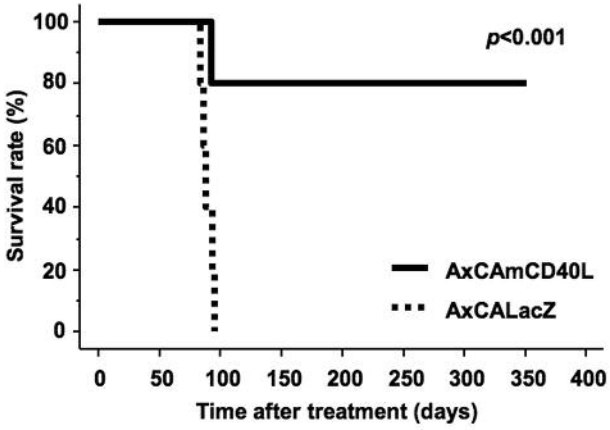

Figure 4. *In the control group, all animals died between 84 and 96 days after treatment, but as for the treatment group, only one animal died on the 92nd day. The treatment group had a significantly higher survival rate than that of the control group.*

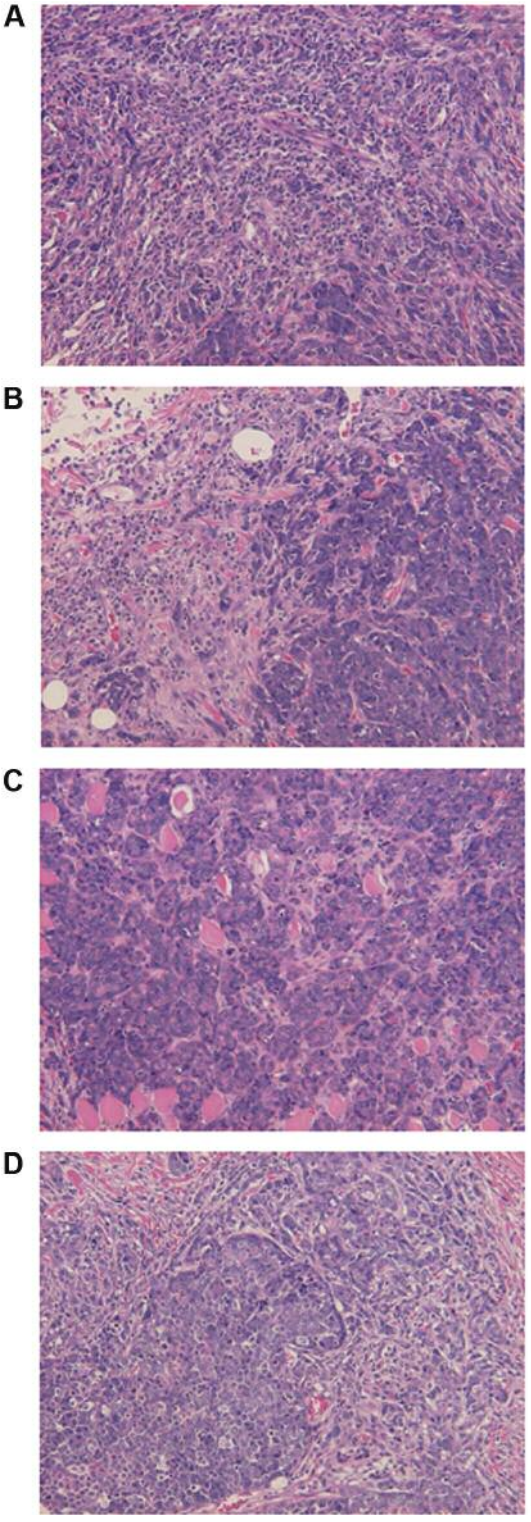

Figure 5. *The histological findings (×100) with HE staining demonstrated significant lymphocyte infiltration in both intatumorally injected tumor (A) and the corresponding untreated tumor (B) in the treatment group, but the control group showed very little lymphocyte infiltration in both intatumorally injected tumor (C) and the corresponding untreated tumor (D).*

Changes of intratumorally injected and untreated tumor diameters. As to changes in the tumor diameter, the initial diameter in the control group was 9.0±0.9 (mean±SD) mm, which gradually grew to 20.7±1.6 mm seven weeks after injection. In contrast, the tumor diameter in the treatment group was 8.1±0.4 mm, which gradually decreased to 3.7±8.2 mm (Figure 3A). The growth of intratumorally injected tumors was significantly lower in the treatment group ($p<0.001$). As to changes in the diameter of the corresponding untreated tumors, the initial diameter in the control group was 6.7±0.8 mm, which gradually grew to 21.8±3.7 mm at seven weeks after injection. In contrast, the tumor diameter in the treatment group was 6.9±1.1 mm, which gradually decreased to 3.7±8.2 mm at seven weeks after injection (Figure 3B). Like intratumorally injected tumor growth, the tumor growth of corresponding untreated tumors was also significantly lower in the treatment group ($p<0.001$). These data suggested that immunogene therapy by CD40L had high therapeutic efficacy for both intratumorally injected tumors and their corresponding untreated tumors.

Assessment of survival rate by immunogene therapy. In the control group, all five animals died between 84 and 96 days after treatment. In contrast, in the treatment group, only one out of five animals died by 3 months after treatment (Figure 4). The treatment group had a significantly higher survival rate than that of the control group ($p<0.001$).

Histological findings of intatumorally injected tumor and untreated tumor in treatment and control groups. The histological findings with HE staining from subcutaneous tumors of the treatment group demonstrated significant lymphocyte infiltration in both the intatumorally injected (Figure 5A) and the corresponding untreated tumors (Figure 5B), while the control group showed very little lymphocyte infiltration in both intatumorally injected (Figure 5C) and the untreated tumors (Figure 5D).

Discussion

Immunogene therapy using CD40L-induced antitumor immunity has been reported in several animal cancer models (11-16). CD40L is one of the strongest inducers of Th1 responses, by stimulating both innate and adaptive immunity. Recent findings indicate that CD40L stimulation abrogates the suppressive effect of T-regulatory cells. This major effecter mechanism synergizes to combat tumor growth. While being an activator of immune cells, CD40L has been shown to induce apoptosis in tumor cells directly by mechanisms that are only beginning to be clarified (15).

Metastasis to liver influences the prognosis of digestive tract cancer and hepatectomy is one of the most effective therapies (1-4). However, metastatic liver cancer of pancreatic origin (23), synchronous metastasis to liver from gastric cancer (24-26) and colorectal cancer with widespread liver metastasis are difficult to treat (1, 5, 6). Therefore, development of new therapeutic methods that have both local and systemic effects are necessary.

In this study, tumor-bearing animals receiving control vector AxCALacZ had progressive growth of both intratumorally injected tumor and untreated tumor, while the animals treated with AxCAmCD40L exhibited a significant reduction of both tumors. In other words, not only was a direct effect of treatment for a tumor by immunogene therapy using AxCAmCD40L found, but also an indirect effect was confirmed. Therefore, we believe that immunogene therapy using AxCAmCD40L could be expected to exert not only an antitumoral effect to limited parts of organs but also a systemic effect.

An important prerequisite to future clinical application of gene therapy for malignant tumors includes efficient gene transfer to the tumor and minimization of transfer to normal tissues. We have already reported the feasibility of iodized oil esters and degradable starch microspheres as embolic materials for satisfactory gene transfer to hepatocellular carcinoma (HCC) (27, 28). Unlike normal hepatocytes that receive dual blood supply from the hepatic artery and the portal vein, non-early HCC receives its blood supply exclusively from the hepatic artery (29, 30). It seems likely that the prolonged residence of the vector within the tumor vessels with the embolic agent increases contact between the vector and tumor cells, resulting in enhanced gene expression within the tumor and limiting gene transfer to the surrounding normal tissues. Immunogene therapy using CD40L for metastatic liver cancer may require modification of techniques such as injection of vector through the tumor-feeding artery.

In conclusion, adenovirus vector-mediated CD40L immunogene therapy is an effective therapeutic method for metastases of liver cancer in rats.

Acknowledgements

We thank Dr. I. Saito and Dr. Y. Kanegae, Institute of Medical Science, University of Tokyo, for providing the adenovirus vector; Dr. J. Miyazaki, Institute of Nutrition and Physiological Chemistry, Osaka University Graduate School of Medicine, for providing the CAG promoter; Dr. H. Yagita for providing mouse CD40L cDNA and the Laboratory Animal Facility of Jikei University School of Medicine for animal husbandry.

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Received March 14, 2008
Revised June 13, 2008
Accepted June 26, 2008