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Cationic liposome-mediated transfection of CD40 ligand gene inhibits hepatic tumor growth of hepatocellular carcinoma in mice*

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Abstract: Objective: To evaluate the efficacy of cationic liposome-mediated CD40 ligand (CD40L) gene therapy for hepatocellular carcinoma. Methods: 1×10^6 of parental H22 cells or H22 cells transfected with the expression vector containing murine CD40L cDNA encoding the entire coding region (pcDNA3.1$^+$-mCD40L) were inoculated subcutaneously into the left flanks of syngenic BALB/C mice. The tumor-bearing mice (tumor nodules 10 mm in maximal diameter) received the treatment of the intratumoral injection of pcDNA3.1$^+$-mCD40L/Transfectam, pcDNA3.1$^+$, or phosphate-buffered saline (PBS), or no treatment. The mice were monitored for tumor growth weekly. We examined mCD40L messenger ribonucleic acid (mRNA) expression by reverse transcription polymerase chain reaction (RT-PCR) and the histologic changes in tumors at two weeks after intratumoral injection using immunohistochemical staining of tumor tissues. Results: All mice inoculated with parental H22 cells developed a tumor subcutaneously, and the tumor size increased progressively within three weeks. However, the mice receiving H22-CD40L cells exhibited complete regression of the tumor two weeks after tumor cell inoculation. The tumor-bearing animals with the treatment of pcDNA3.1$^+$ or PBS, or without treatment had progressive tumor growth, while those mice treated with pcDNA3.1$^+$-mCD40L exhibited a significant inhibition of tumor growth. RT-PCR analysis showed that 783-bp fragments corresponding to the mCD40L mRNA were amplified only from pcDNA3.1$^+$-mCD40L treated tumors. The tumor samples from pcDNA3.1$^+$-mCD40L-treated mice showed significant lymphocyte infiltration, apoptotic bodies, and confluent necrosis in the tumor tissues. Conclusion: The tumorigenicity of CD40L-expressing cells was abrogated when the cells were implanted subcutaneously. In vivo gene therapy of established liver tumor nodules in mice by the intratumoral injection of pcDNA3.1$^+$-mCD40L led to significant tumor inhibition. There was mCD40L mRNA expression in the tissues from pcDNA3.1$^+$-mCD40L-treated tumors. The intratumoral injection of pcDNA3.1$^+$-mCD40L induced a strong inflammatory, mainly lymphocytic infiltration of the tumor, and increased the necrotic rate of the neoplastic cells.

Key words: CD40 ligand gene, Hepatocellular carcinoma (HCC), Cationic liposome, Transfection
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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common neoplasms worldwide, and is highly prevalent in China (Tang, 2001). HCC is very resistant to chemotherapy, and the curative options are only partial hepatectomy and total hepatectomy with liver transplantation. However, many HCC tumors are not amenable to surgery. Thus, when trasnsplantation is not possible, no good therapeutic options are available for HCC. Therefore, it is urgent to develop new approaches to treat HCC.

Gene therapy may offer a new therapeutic option for HCC. The use of gene therapy with immunostimulatory molecules aiming at enhancing antitumoral immunity has emerged as a promising new approach to treat cancer. CD40 ligand (CD40L) is a member of the tumor necrosis factor family, which is expressed on activated T cells and binds to CD40 presented on the membrane of antigen-presenting cells (APC). CD40-CD40L interaction plays a crucial role in the activation of APC and in the initiation of both humoral and cellular immune responses (Mach

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et al., 1997; Grewal and Flavell, 1998; Roy *et al*., 1993). Thus, gene transfer of CD40L has been proposed as an efficient means to treat malignancies. In this study, we investigated the antitumor immunity in HCC using cationic liposome-mediated CD40L gene transfer.

MATERIALS AND METHODS

Animals and cell lines

Female BALB/c mice (6~8 weeks old) were purchased from the Second Xiangya Hospital (Changsha, China). H22 cell lines were provided by the Department of Biochemistry, the Fourth Military Medical University, China. Cells were maintained in RPMI 1640 medium, supplemented with 10 ml/L fetal calf serum (FCS), 1 mmol/L glutamine, and 100 kU/L penicillin.

cDNA and transfection

The expression vector containing murine CD40L cDNA encoding the entire coding region (pcDNA3.1$^+$-mCD40L) was produced and transfected into H22 cells as previously reported (Jiang *et al*., 2005a; 2005b). In brief, transfections were performed by the lipofection method with Lipofectace reagent. After two weeks of selection, G418-resistent clones were selected randomly from the surviving colonies. pcDNA3.1$^+$-mock was established by transfecting the pcDNA3.1$^+$ without a cDNA insert as control.

Tumorigenicity of H22 cells after infection with pcDNA3.1$^+$-mCD40L

To examine the tumorigenicity of H22 cells expressing CD40L in syngenic mice, 10 BALB/c mice were subcutaneously injected with 1×10^6 of parental H22 cells or H22 cells infected with pcDNA3.1$^+$-mCD40L. The mice were monitored for tumor growth weekly, and tumor size was determined by measuring two perpendicular diameters with a caliper.

Established tumors of HCC and in vivo gene therapy

For HCC establishment, 1×10^6 of H22 tumor cells were injected subcutaneously in the left flanks of mice. When the tumor size reached 10 mm in maximum diameter, they received the treatment with intratumoral injection of 100 μl of liposome-DNA complexes (10 μg pcDNA3.1$^+$-mCD40L and 0.2 μg liposome), 10 μg pcDNA3.1$^+$, or phosphate-buffered saline (PBS) alone, or no treatment. Tumor size was measured in two perpendicular tumor diameters using a caliper and was presented as the mean of two measurements.

Histologic sections

Tumor-bearing mice were killed on Day 14 after treatment of either liposome-pcDNA3.1$^+$-mCD40L complexes or control vector pcDNA3.1$^+$. Liver tumor tissues were fixed in formalin and embedded in paraffin. Three micrometers of sections were made and stained with hematoxylin-eosin (HE).

Assessment of apoptosis in tumoral tissue

Assessment of apoptosis was performed according to the manufacturer's protocol. The paraffin-embedded sections were dewaxed and then digested with proteinase K for 10 min at room temperature. After washing with PBS, sections were labeled with TdT-mediated dUTP-biotin nick end labeling (TUNEL) reaction mixture, and incubated for 1 h at 37 °C. Then, sections were reacted with Converter-AP for 30 min. The apoptosis rate was calculated as the percentage of apoptotic cells over total cells in six to nine high-power fields using an eyepiece graticule.

Detection of mCD40L expression by immunohistochemistry and reverse transcription polymerase chain reaction (RT-PCR)

Immunohistochemical staining for CD40L was performed on HCC tissues using a labeled streptavidin-biotin method. Deparaffinized sections were heated for 5 min at 100 °C in a pressure cooker to reactivate the antigen, and were treated with 0.3% (v/v) H_2O_2 in methanol for 30 min to abolish endogenous peroxidase activity. Sections were blocked with 1% (w/v) goat serum in PBS, then covered with rabbit anti-CD40L polyclonal antibody (1:100 diluted in PBS) overnight at 4 °C, and after washing, covered with second-step biotinylated antibody for 30 min, followed by being incubated with peroxidase-labeled streptavidin for 30 min. After washing, sections were then incubated with 0.05% (w/v) diaminobenzidene/ 0.15% (v/v) H_2O_2 and counterstained with 10% (w/v) hematoxylin.

The expression murine CD40L (mCD40L) messenger ribonucleic acid (mRNA) of tumor tissue was treated either with liposome-pcDNA3.1$^+$-mCD40L complexes or control vector pcDNA3.1$^+$ as previously reported (Jiang *et al*., 2005a; 2005b). In brief, 100 mg of tumor liver tissues were homogenized by using a Mini-Beadbeater, and total RNA was isolated. cDNAs were synthesized by extension of (dT)18 primers. A cDNA fragment coding for the full open reading frame of mCD40L gene was amplified by RT-PCR. Two primers specific for the mCD40L gene were used, namely, the sense primer (5′-GAC GCT AGC ATG ATA GAA ACA TAC AGC CAA CCT-3′) and the antisense primer (5′-GCC GAA TTC TCA GAG TTT GAG TAA GCC AAA AGA-3′). PCR conditions included 1 cycle at 94 °C for 5 min for pre-denaturation; 35 amplification cycles each consisting of denaturation at 94 °C for 60 s, annealing at 60 °C for 50 s, and extension at 72 °C for 90 s; followed by a further extension at 72 °C for 10 min. β-actin sense primer was 5′-ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG-3′, and antisense primer 5′-CGT CAT ACT CCT GCT TGC CTG ATC CAC ATC TGC-3′.

Statistical analysis

Kaplan-Meier curves were compared by the log rank test. Differences in tumor size between the different experimental groups were tested for statistical significance by a nonparametric test (Mann-Whitney test, 2-tailed) for unpaired samples. $P<0.05$ was considered significant.

RESULTS

Inhibition of tumor growth of mouse H22 cells in syngeneic animals by CD40L expression

We examined the tumor growth of H22 cells in syngeneic BALB/c mice to determine whether increased CD40L expression would have any effect on the growth of HCC cells in vivo. Ten syngeneic BALB/c mice were injected subcutaneously with 1×10^6 of parental H22 cells or H22 cells infected with pcDNA3.1$^+$-mCD40L. As shown in Fig.1, although all BALB/c mice inoculated with H22 cells transfected with pcDNA3.1$^+$-mCD40L developed subcutaneous tumors, the growth of tumor from H22 cells transfected with pcDNA3.1$^+$-mCD40L was significantly inhibited compared with that from parental H22 cells (at 4, 5, 6 weeks, $P<0.01$). These results indicate that the tumor growth of H22 cells infected with pcDNA3.1$^+$-mCD40L is suppressed in vivo, which might be mediated by host T lymphocytes.

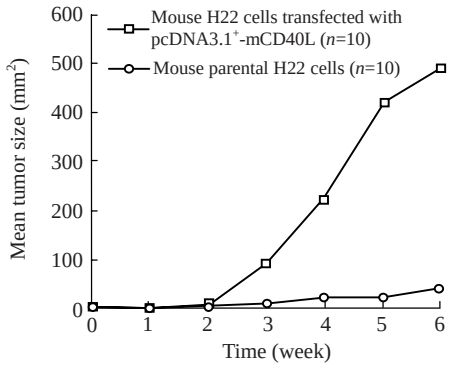

Fig.1 Growth rate of developing tumors after subcutaneous inoculation of mouse parental H22 cells or H22 cells transfected with pcDNA3.1$^+$-mCD40L in syngeneic BALB/c mouse

Efficacy of the treatment of established HCC tumors by intratumoral injection of pcDNA3.1$^+$-mCD40L

To investigate whether pcDNA3.1$^+$-mCD40L elicits a therapeutic effect in an HCC animal model, we generated a tumor nodule subcutaneously by implantation of the mouse HCC cell line, H22, into the left flanks of the syngeneic mice. The size of tumors was checked two and three weeks after treatment. As shown in Fig.2, the tumor-bearing animals receiving 10 μg pcDNA3.1$^+$ or PBS alone or no treatment had progressive tumor growth, while those mice treated with liposome-DNA complexes exhibited a significant inhibition of tumor growth.

Induced lymphocytic infiltration of the tumor and increased apoptosis rate of tumor cells by intratumoral injection of pcDNA3.1$^+$-mCD40L

Fig.3a shows the photomicrographs of tumors from pcDNA3.1$^+$-treated mice on Day 14, demonstrating the absence of necrosis and very little lymphocyte infiltration. In sharp contrast, the tumor samples from pcDNA3.1$^+$-mCD40L-treated mice showed significant necrosis and lymphocyte infiltration of the tumor (Fig.3b). In this group of mice, mononuclear cell infiltration was very intense and

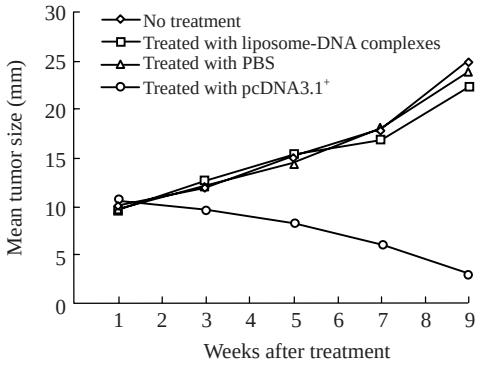

Fig.2 In vivo gene therapy of established HCC tumor
The tumor size was measured before initiation of treatment and every two weeks after therapy

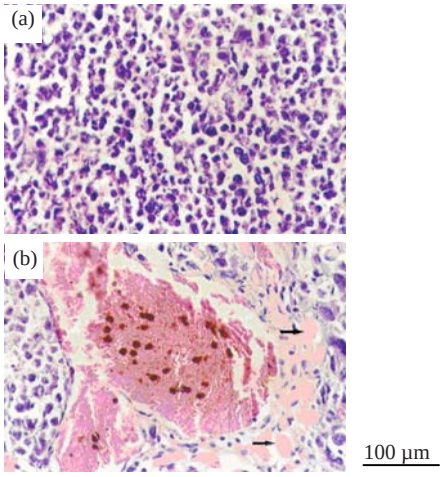

Fig.3 Histologic analysis of tumor in animals with HCC
(a) Sections of tumor after the treatment with control vector pcDNA3.1$^+$; (b) Sections of tumor after the treatment with pcDNA3.1$^+$-mCD40L. There are significantly lymphocytes infiltrating and necrotic area (→)

was accompanied by the presence of necrotic areas in the tumoral tissue. Apoptosis of tumor cells was analyzed by the TUNEL technique on Day 14 after therapy. Quantitative analysis showed that the apoptotic rate was 2.6% on Day 14 in the tumors treated with pcDNA3.1$^+$, and 5.6% in those treated with pcDNA3.1$^+$-mCD40L (Fig.4). These data indicate that the intratumoral injection of pcDNA3.1$^+$-mCD40L induced a strong inflammatory, mainly lymphocytic infiltration of the tumor, and increased the apoptotic rate of the neoplastic cells.

mCD40L expression in tumor tissue by immunohistochemistry and RT-PCR

Tumor tissues were examined for CD40L expression by immunohistochemistry. The positivity for CD40L was higher in tumor tissues after the treatment with liposome-pcDNA3.1$^+$-mCD40L complexes. In contrast, the tumor tissue with the treatment of control vector pcDNA3.1$^+$ or without treatment was rarely stained for CD40L. On a cellular level, CD40L expression mainly occurred in the form of diffuse cytoplasmic and membranous staining (Fig.5).

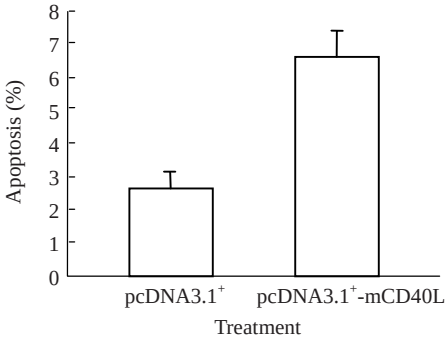

Fig.4 Analysis of the apoptotic rate of tumor cells treated with control vector pcDNA3.1$^+$ or pcDNA3.1$^+$-mCD40L on Day 14 after the treatment
Apoptosis was measured by the TUNEL assay. Data are given as mean percentage of apoptotic cells over total of cells in optical fields with standard error. $P<0.005$

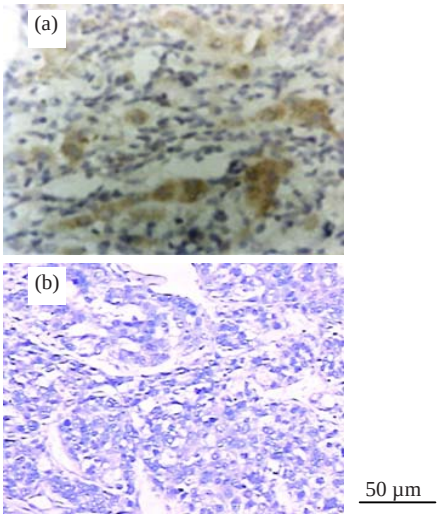

Fig.5 Immunohistochemical staining of tumor tissues with anti-CD40 antibody with the treatment of pcDNA3.1$^+$-mCD40L or without treatment
(a) Tumor tissues with the treatment of pcDNA3.1$^+$-mCD40L are strongly stained for CD40L. Many HCC cells showed diffuse cytoplasmic and membranous staining patterns; (b) In contrast, the tumor tissue without treatment was rarely stained for CD40L

RT-PCR analysis showed that 800-bp fragments corresponding to the mCD40L cDNA were amplified with the total cellular RNA only from tumor tissue

treated with liposome-pcDNA3.1$^+$-mCD40L complexes, but not from the tumor tissue treated with control vector pcDNA3.1$^+$ (Fig.6).

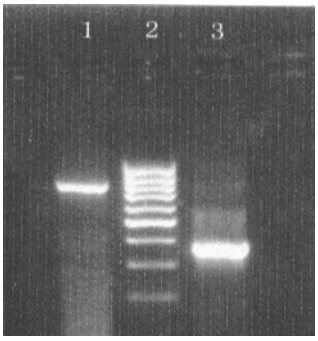

Fig.6 Detection of CD40L mRNA in tumor tissues by RT-PCR
Lane 1: 783-bp mCD40L-specific sequence; Lane 2: DNA markers; Lane 3: β-actin sequence

DISCUSSION

Gene therapy opens new possibilities for the treatment of incurable diseases, including HCC (Ruiz *et al*., 2001). However, the host's immunity frequently fails to eliminate malignant tumors caused either by the lack of recognizable tumor antigens or by the inability of tumor antigens to stimulate an effective immune response (Chouaib *et al*., 1997; Gabrilovich *et al*., 1996), so that most malignant tumors evade the host immune surveillance. The use of gene therapy with immunostimulatory molecules aiming at enhancing antitumoral immunity has emerged as a promising new approach to treat cancer (Ruiz *et al*., 1999; Melero *et al*., 2001).

CD40L-CD40 interactions in APC including dendritic cells (DCs)-induced activation of APC, promotion of effective antigen presentation, expression of costimulatory and adhesion molecules, and up-regulation of cytokine and chemokine production strongly promote Th1 differentiation, and provide a strong signal for interleukin (IL)-12 production, even in the presence of Th2 cytokines (Bullens *et al*., 2001; Li *et al*., 2006). The endogenous production of IL-12 resulting from the CD40L-CD40 interaction may play a role in the persistence of the antitumor effects (Harada *et al*., 1998; Toutirais *et al*., 2007). CD40 activation through CD40L-CD40 interaction may become a potential tool in malignant neoplasms (Ottaiano *et al*., 2002; Bereznaya and Chekhun, 2007). The ration for transducing tumors cell with CD40L is to convert these cells into stimulators of APC, an effect leading to enhance the presentation of tumors antigens to T cells and the activation of antitumor immune responses. In fact, CD40L-CD40 interactions have been demonstrated to overcome tumor-specific CD4$^+$ and CD8$^+$ tolerance and induce antitumor immunity (Sotomayor *et al*., 1999; Diehl *et al*., 1999). It remains possible that the gene therapy combining CD40L gene with another immunotherapeutic modality (e.g., IL-12, CD80) or with a tumor-associated antigen (e.g., alpha fetoprotein, AFP) would be still better. Using mCD40L gene or combining it with IL-12 gene, transduced leukemia cells therapy for chronic lymphocytic leukemia (Wierda *et al*., 2000), primary B-CLL (Wendtner *et al*., 2002; von Bergwelt-Baildon *et al*., 2004) and acute leukemia (Saudemont *et al*., 2002) has provided encouraging results. Treatment not only appears capable of inducing a cellular anti-leukemia immunity, but also may have a direct effect on leukemia cells by inducing latent sensitivity to Fas (CD95)-dependent leukemia cell apoptosis (Saudemont *et al*., 2002). Recently, many investigators have reported that ex vivo transduction of tumor cells with the mCD40L gene or in vivo transfection of the mCD40L gene was able to induce antitumor immunity against different tumor cell lines in subcutaneous tumor models, such as colorectal cancer (Georgopoulos *et al*., 2007), lung cancer (Noguchi *et al*., 2001; Tada *et al*., 2003), colon cancer (Sun *et al*., 2000; Buning *et al*., 2002), urologic malignancies (Loskog *et al*., 2001; 2002; Hussain *et al*., 2003), melanoma (Peter *et al*., 2002), ovarian carcinoma (Toutirais *et al*., 2007) and malignant mesothilima (Friedlander *et al*., 2003). Schmitz *et al*.(2001) used adenovirus-mediated CD40L gene therapy of orthotopic HCC, demonstrating that it abrogated HCC cells tumorigenicity when expressing mCD40L gene, and led to completely established tumor eradication and long-term survival in most of treated animals. Yanagi *et al*. (2003) demonstrated that Flt3L and CD40L significantly induced antitumor immunity against MH134 cells presumably through both innate and adaptive immunity. These data suggest that the administration of mCD40L gene might provide an efficient and safe treatment for HCC.

Our in vitro data show that the tumorigenicity of H22 cells was abolished by in vitro infection with pcDNA3.1$^+$-mCD40L. This effect appears to take place by a mechanism that does not involve a direct interaction of the transgene with CD40 on the tumor cell membrane. A main finding of our study was that in vivo gene therapy of established HCC by intratumoral injection could induce a potent antitumor immune response, leading to tumor eradication in pcDNA3.1$^+$-mCD40L-treated animals. Treatment with pcDNA3.1$^+$-mCD40L results in infiltration of immunocytes in the treated tumors and induction of apoptosis of tumor cells. Furthermore, we found that those animals with complete regression produced a protective antitumor immunity against tumor cell rechallenge.

In conclusion, our data show promising results of first-generation CD40L in the therapy of experimental liver cancer, suggesting that transduction of tumor cells with pcDNA3.1$^+$-mCD40L may provide an effective means to treat HCC. However, these data cannot be directly extrapolated to humans, for carefully planned phase I/II clinical trials are necessary to establish the efficacy and the toxicity of this therapeutic strategy in patients with liver cancer.

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