Adenovirus-mediated CD40L gene therapy induced both humoral and cellular immunity against rat model of hepatocellular carcinoma

Tomonori Iida,[1,2,3] Hiroaki Shiba,[1] Takeyuki Misawa,[1] Toya Ohashi,[2] Yoshikatsu Eto[2] and Katsuhiko Yanaga[1]

[1]Department of Surgery, The Jikei University School of Medicine, Tokyo, Japan; [2]Department of Gene Therapy, Institute of DNA Medicine, The Jikei University School of Medicine, Tokyo, Japan

(Received May 2, 2008/Revised July 6, 2008/Accepted June 27, 2008/Online publication October 3, 2008)

Adenoviral-vector expressing CD40L (AxCAmCD40L)-mediated gene therapy was studied for treatment of hepatocellular carcinoma (HCC) using CD40 ligand (CD40L) complementary DNA in rats. The particular focus was whether humoral immunity took part in antitumor effect. When tumor cells transduced by AxCAmCD40L were implanted into the subcutaneous tissues of syngeneic rats, the tumor growth was suppressed. Intratumoral injection of AxCAmCD40L to pre-existing tumor in rats also led to significant reduction of tumor size. When tumor cells were re-implanted to prevention model rats and treatment model rats, no tumor growth was observed. Many studies to date have reported that cellular immunity induces antitumor immunity. However, the present study demonstrated that not only cellular immunity but also humoral immunity plays an essential role in a HCC model. These observations suggested that CD40L-mediated immune gene therapy for HCC was very effective treatment by activation of both cellular and humoral immune system. (*Cancer Sci* 2008; 99: 2097–2103)

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world,[1–4] and is resistant to chemotherapy and radiotherapy.[5] The only curative options are partial hepatectomy or total hepatectomy with liver transplantation for non-advanced HCC.[6,7] Intratumoral injection of ethanol or acetic acid can substitute resection for patients with decompensated liver disease who would not tolerate surgery or for those whose HCC is discovered when the process is not amenable to surgery.[4] Despite progress in early diagnosis, the prognosis of HCC remains poor. In addition, even after resection of HCC, the recurrence rate remains high, which leads to poor curability and long-term survival.[8] Thus, it is necessary to develop more effective therapy for HCC.

Immunogene therapy may offer a new therapeutic option for HCC. The immunogene therapy using immunostimulatory molecules aiming at enhancing antitumoral immunity is a good possibility for treating cancer. CD40 ligand (CD40L) is a 33-kDa type II membrane protein that belongs to the tumor necrosis factor (TNF) family and is predominantly expressed on $CD4^+$ T cells upon T-cell receptor (TCR) stimulation. Its receptor, CD40, belongs to the TNF receptor family and is expressed on antigen-presenting cells (APC)[9,10] including B cells, macrophages and dendritic cells. The interaction between CD40L and CD40 plays an important role in the activation of APC and the initiation of antigen specific T-cell responses, which lead to the initiation of both humoral and cellular immune responses.[11–13] Therefore, gene transfer of CD40L has been proposed as an efficient means to treat cancer.[14–19]

The immunity of a tumor-bearing patient frequently fails to eliminate cancers owing to either the lack of recognizable tumor antigens or the inability of tumor antigens to elicit an effective immune response.[20,21] The rationale for infected tumor cells with CD40L is to convert these cells into stimulators of APC, an effect leading to enhanced presentation of tumor antigens to T cells and activation of antitumor immune responses. *Ex vivo* infection of tumor cells with the CD40L gene or *in vivo* infection of the CD40L gene was able to induce antitumor immunity against various tumor cell lines in subcutaneous mouse models, and immunization with the *in vivo* modified tumor cells expressing CD40L had possibility to eliminate established tumors.[22–26] Actually, Schmitz *et al*.[14] reported that intratumoral injection of adenovirus vector expressing CD40L regressed the size of preexisting HCC cells (McA-RH7777) in rats.

In the present study, to analyze the potential of CD40L-based gene therapy for possible clinical application in the future, we have explored a gene-therapeutic approach to stimulate antitumor immunity by adenoviral-mediated transfer of CD40L into HCC tumor cells in a syngenic animal model. Therefore, we studied the antitumor mechanism with immunogene therapy using CD40L. Different from previous reports, our observation clearly indicated that not only cellular immunity but also humoral immunity played certain role in antitumor effect in CD40L mediated immune gene therapy for rat HCC.

Materials and Methods

Cell line. McA-RH7777, a rat hepatocellular carcinoma cell line, was obtained from the DAINIPPON SUMITOMO PHARMA laboratory products (Tokyo, Japan) and cultured in Alpha-Minimum Essential 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.

RCN9, a rat colon cancer cell line was obtained from the RIKEN Cell Bank (RCB0459, Tsukuba, Japan) and cultured in Roswell Park Memorial Institute medium (RPMI)-1640 medium (Sigma, St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (GIBCO BRL) and 1% penicillin-streptomycin (GIBCO BRL). Cells were incubated at 37°C with 5% CO_2.

Animals. Buffalo rats, 6-week-old males, purchased from CLEA Japan (Tokyo, Japan) were maintained in a specific pathogen-free room at 25 ± 3°C and treated according to National Institutes of Health guidelines. The ethical issues related to this study were discussed and performed in according with the local animal committee.

Construction of recombinant adenovirus vectors. AxCALacZ, a replication-defective recombinant adenoviral vector expressing the *Escherichia coli* β-galactosidase (β-gal) gene, was a gift from Dr I. Saito (Institute of Medical Science, University of Tokyo, Tokyo, Japan).[27] Mouse CD40L complementary DNA (cDNA) was a gift from Dr H. Yagita (Juntendo University School of Medicine). CD40L expressing adenoviral vector (AxCAmCD40L) was generated using adenovirus expression

[3]To whom correspondence should be addressed. E-mail: lgpsurgery@yahoo.co.jp

doi: 10.1111/j.1349-7006.2008.00953.x
© 2008 Japanese Cancer Association

vector kit (Takara Biomedicals, Tokyo, Japan) following the manufacturer's instructions. The β-gal and mouse CD40L gene were driven by the cytomegalovirus-enhancer chicken beta-actin hybrid promoter (CAG promoter)[28] and a rabbit beta-globin poly (A) signal located downstream from the gene. Both viral vectors were isolated from a single plaque, expanded in 293 cells, and purified by two rounds of cesium chloride (CsCl) centrifugation and titered with a bioassay using 293 cell line.[29] After purification, the adenovirus vectors were stored at –80°C.

***In vitro* CD40L expression on tumor cells by fluorescence-activated cell sorting (FACS) analysis.** McA-RH7777 cells were infected with AxCALacZ or AxCAmCD40L at different multiplicities of infection (MOI), 0, 0.01, 0.1, 1, 10 and 100 MOI, respectively. Two days after infection, the cells were examined for CD40L expression. The cells infected with adenoviral vectors were allowed to react with R-phycoerythrin (PE)-conjugated antimouse CD40L antibody (PharMingen, San Diego, CA, USA) for 30 min at 4°C. For detection of CD40 expression in parental McA-RH7777 cells, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated antimouse CD40 (PharMingen) for 30 min at 4°C. The cells were analyzed for fluorescence using FACS (Becton-Dickinson, San Jose, CA, USA).

Proliferation assay. McA-RH7777 cells were cultured in 6-well plate and infected with AxCALacZ or AxCAmCD40L at 1, 10 and 100 MOI, respectively. At 24 h and 48 h after infection, cells were harvested and seeded into 96-well plate (3.0×10^4 cells). The cell proliferation was measured with the Promega celltiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) following the manufacturer's instructions.

Animal models. To investigate the antitumor effect induced by the expression of mCD40L, McA-RH7777 cells (1×10^6 cells) transduced by AxCALacZ ($n = 15$) or AxCAmCD40L ($n = 20$), which were infected at 10 MOI, were implanted subcutaneously into the back of the animals as a prevention model. Tumor size was measured in two perpendicular tumor diameters using a caliper and was presented as a mean of two measurements, and the survival rate of animals were also checked. And then as a treatment model, McA-RH7777 cells (1×10^6 cells) were injected subcutaneously into the back of the animal. When tumor size reached about 6.0 mm in diameter, AxCALacZ ($n = 15$) or AxCAmCD40L ($n = 20$) (6×10^{10} PFU/animal) were injected into the tumor. Tumor diameter and the survival rate were checked by the same measurement methods. In each model, after 3 months from abrogation of tumorigenesis, the animals were re-injected McA-RH7777 cells (1×10^6 cells) at the opposite flank and observed at the same checked points.

Cytotoxic assay. The cytotoxic assay was performed according to standard ^{51}Cr releasing protocols as described elsewhere[30] to investigate CD40L expressing McA-RH7777 cells to induce tumor specific cytotoxic T lymphocyte (CTL) in prevention and treatment model. In 3, 7 and 14 days after subcutaneous implantation of McA-RH7777 cells infected AxCALacZ or AxCAmCD40L in prevention model and after intratumoral injection of either AxCALacZ or AxCAmCD40L in treatment model, animals were killed and their splenocytes were isolated. These viable splenocytes were incubated for 5 days at 6×10^6 cells together with 2.5×10^5 McA-RH7777 cells (previously treated with 100 µg/mL of mitomycin C for 30 min at 37°C) in each well of a 24-well plate. At 5 days after culture initiation, fresh tumor cells were harvested, and 1×10^6 cells were labeled with 50 mCi ^{51}Cr for 1 h at 37°C. After labeling, the cells were washed four times, adjusted to 5×10^4/mL and 100 µL of this cell suspension was added to 96-well plate and incubated with effector lymphocytes for 5 h at 37°C. The effector : target ratio ranged from 25:1 to 100:1.

To determine natural killer (NK) activity, fresh splenocytes were obtained as effector cells, and McA-RH7777 and YAC-1 were used as target cells.

In addition, we studied whether the cytotoxicity was induced by CTL or NK. Briefly, splenocytes from prevention and treatment model rats infected with either AxCAmCD40L or AxCALacZ were separated into CD8$^+$ cells and splenocytes by rat CD8a microbeads (Miltenvi Biotec, CA, USA). The CD8-positive cells were used as effector cells, and McA-RH7777 and YAC-1 were used as target cells. All determinations were made in triplicates, and the percentage of lysis was calculated using the formula (cpm experimental sample – cpm background)/(counts per minute [cpm] maximal lysis – cpm background) × 100.XXX

Detection of tumor cell-binding Ig in sera. Detection of tumor cell-binding immunoglobulin (Ig) in sera was described elsewhere.[31] Briefly, 25 µL of sera obtained from rats which are used AxCALacZ or AxCAmCD40L in prevention and treatment model or injected with phosphate-buffered saline (PBS) or parental McA-RH7777 cells as a control. The sera were added to 4×10^5 single-cell suspensions of McA-RH7777 cells. The cells were incubated for 30 min at 4°C, washed and re-suspended in PBS containing 1% bovine serum albumin (BSA) and 0.1% sodium azide. The cells were stained with PE-labeled anti rat immunoglobulin G (IgG; R&D Systems, MN, USA) for 30 min at 4°C. The stained cells were washed and analyzed using a FACS.

Cytotoxicity of sera obtained from prevention and treatment rats. Cytotoxicity of sera obtained from prevention and treatment model were detected by a modification of the method of Fujieda *et al.*[32] McA-RH7777 cells (1×10^5) in 96-well plate were incubated with 100 µL of culture medium as control or sera obtained from prevention or treatment model rats which are infected either AxCALacZ or AxCAmCD40L at 3, 7 and 14 days after injection or PBS (diluted 1:10 in culture medium) for 72 h at 37°C. Subsequently, 10 µL of 3-(4,5-dimethylthlthiazol-2-yl)-2,5-dipheny; tetrasodium bromide (MTT) (5 mg/mL) (Sigma) was added to each well. After 4 h of incubation, the medium was removed and the converted dye was solubilized with 0.1 N HCl in absolute isopropanol. Absorbance of converted dye was measured at a wavelength of 570 nm with background subtraction at 630 nm using microplate reader. RCN9 cells, rat colon cancer cell line were used as a control of target cells. All determinations were made in triplicate. The cytotoxicity against target cells was calculated as follow: cytotoxic activity (%) = 100(1 – S/M), where S is the absorbance of the sample and M is the mean absorbance of triplicate wells with target cells in culture medium without sera.

Histological studies. To analyze the antitumor effects after treatment with AxCAmCD40L, the animals were sacrificed at day 5 after intratumoral injection of 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).

Immunohistochemical studies. Paraffin sections of tumor tissue were cut and stained immunohistochemically using an affinity purified antimouse CD154 (eBioscience, San Diego, CA, USA) as a primary antibody and Biotin goat antiarmenian hamster IgG as a secondary antibody (eBioscience) for detecting CD40L expression. In addition, to detect whether CD4$^+$ or CD8$^+$ T cell, we used an affinity purified OX-38 and OX-8 (CHEMICON, USA) as a primary antibody and DAKO EnVision Kit/HRP as a secondary antibody (DAKO, Carpinteria, CA, USA). The assessments of these immunohistochemical approaches were performed according to the manufacturer's protocol.

Assessment of apoptosis in tumor tissue. Assessment of apoptosis was performed with DeadEnd Colorimetric TUNEL (terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate [dUTP] nick-end labeling) System (Promega, WI, USA) following the manufacturer's instructions. The paraffin-embedded sections were de-waxed and then digested with proteinase K for 10 min at room temperature. After washing with PBS, sections were labeled with TUNEL reaction mixture,

doi: 10.1111/j.1349-7006.2008.00953.x
© 2008 Japanese Cancer Association

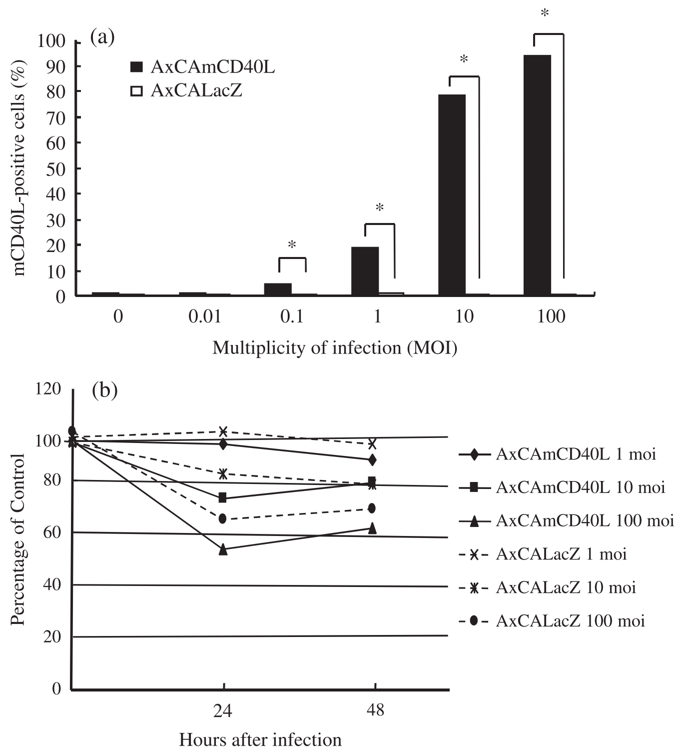

Fig. 1. *In vitro* study. (a) Positive cells infected with AxCAmCD40L demonstrated a dose-dependent expression of CD40L by fluorescence-activated cell sorting (FACS). However, CD40L positive cells were not detected in AxCALacZ infected cells at any dose. There were significant differences at 0.1, 1, 10 and 100 multiplicities of infection (MOI) in two groups. (b) Infection of McA-RH7777 cells with AxCAmCD40L temporarily changed growth rates of tumor cells at 24 h but recovered at 48 h. Data are given as mean percentage of the control (uninfected McA-RH7777 cells).

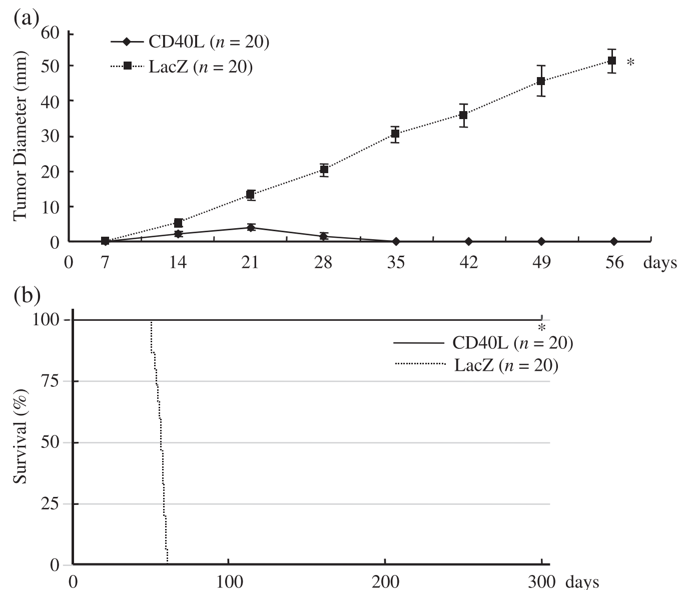

Fig. 2. *Ex vivo* gene transfer into McA-RH7777 cells with AxCAmCD40L abolished tumorigenicity *in vivo* as a prevention model. Cells were infected *in vitro* either with AxCAmCD40L or AxCALacZ at 10 MOI. Two days after infection, cells were injected subcutaneously at the back of the animal. (a) The tumor diameter of LacZ animals ($n = 20$) was developed progressively. On the other hands, CD40L treated tumor was disappeared ($n = 20$). There was a significant difference between these groups from the 21st day after injection. Data are presented as mean tumor diameter with standard error of the groups. *$P < 0.05$. (b) Survival of animals after injection of McA-Rh7777 infected with AxCAmCD40L or AxCALacZ. The animals which were injected with CD40L were all alive, but LacZ group was all dead. There was a significant difference at the survival course between two groups. *$P < 0.05$.

and incubated for 1 h at 37°C. Then, sections were reacted with 0.3% hydrogen peroxide in PBS for 30 min. The apoptosis was detected by diaminobenziol (DAB) stain.

Statistics. Repeated measure ANOVA, log rank and the non-parametric test were used for statistical studies. All *P*-values were considered statistically significant when the associated probability was less than 0.05.

Results

Expression of CD40L in infected cell line. McA-RH7777 cells infected with AxCAmCD40L expressed CD40L in a dose-dependent manner (Fig. 1a). At 0.01, 0.1 and 1 MOI, they showed low positive cells of mCD40L expression; with increasing MOI of 10 and 100 MOI, the expression level reached 78.5% and 93.4%, respectively. In contrast, no CD40L could be detected in tumor cells infected with AxCALacZ at any MOI (Fig. 1a).

McA-RH7777 cell proliferation after infection with AxCAmCD40L *in vitro*. The proliferation of cells that were infected by AxCAmCD40L was essentially same as that of AxCALacZ. This indicated that expression of CD40L was not a cause decreased cell proliferation. The proliferation was decreased at similar rate by infection of either AxCAmCD40L or AxCALacZ. There were not significant differences in each MOI of two groups. This may be due to toxicity of adenoviral infection (Fig. 1b).

Tumorigenesis of rat McA-RH7777 cells after ex vitro infection of adenovirus vectors, as a prevention model. The animals ($n = 20$) injected with McA-RH7777 cells infected AxCALacZ developed a tumor progressively. On the other hand, the animals ($n = 20$) that received tumor cells infected with AxCAmCD40L had temporary tumorigenesis but exhibited tumor regression 4 weeks after inoculation (Fig. 2a). There were remarkable differences in tumorigenesis between the two groups ($P < 0.001$). Every animal implanted tumor cells expressing LacZ were dead until 60 days, while all animals implanted with tumor cells expressing CD40L were alive up to 400 days with disease-free (Fig. 2b). There was significant difference in survival rate between the two groups ($P < 0.001$).

Efficacy of the treatment of pre-existing tumor in back by intratumoral injection of AxCAmCD40L. Tumor-bearing animals receiving AxCALacZ had progressive tumor growth, whereas the animals treated with AxCAmCD40L exhibited a significant inhibition of tumor growth ($P = 0.0016$, Fig. 3a). If we looked at independent animals, 12 of 20 AxCAmCD40L-treated animals showed complete disappearance of the tumor until 28 days, while the other eight animals exhibited a tumor growth rate similar to that of control animals, but AxCAmCD40L therapy caused a significant increase in survival of tumor-bearing animals, with 100% of treated animals surviving for more than 400 days ($P < 0.001$, Fig. 3b).

Persisted immune reaction against re-implanted tumor. To investigate persisted immune reaction against re-implanted tumor, after 3 months from abrogation of tumorigenesis, the surviving animals were re-implanted McA-RH7777 cells (1×10^6 cells) at the opposite flank and the same checked points were observed in each model. As a control, the rats injected subcutaneously with parental McA-RH7777 cells had tumors (29 ± 3.12 mm) on the 28th day. In the prevention model, the surviving animals (CD40L group, 20 of 20) had no tumor growth up to 28 days. The same results occurred in treatment model. To sum up, treated animals (CD40L group, 12 of 20) had long survival.

© 2008 Japanese Cancer Association

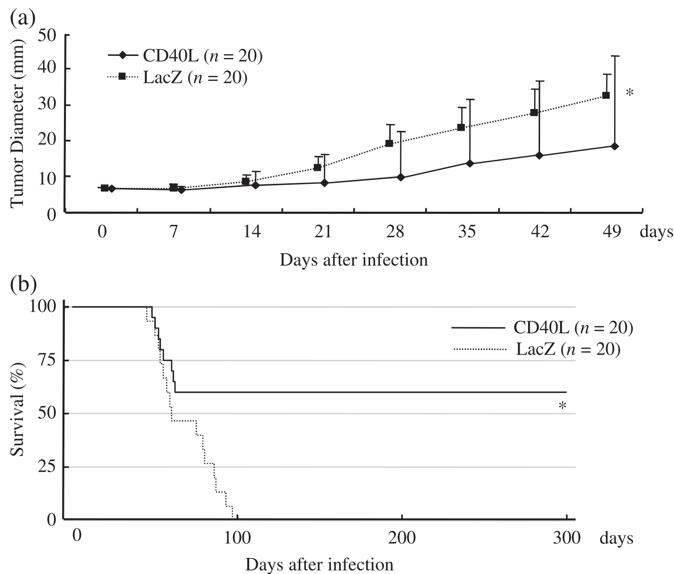

Fig. 3. *In vivo* gene therapy of established subcutaneous HCC tumor by intratumoral injection with AxCAmCD40L or AxCALacZ. Animals with a subcutaneously established tumor nodule (about 6.0 mm in diameter) were treated either with AxCAmCD40L at 6×10^{10} PFU ($n = 20$) or control adenovirus AxCALacZ at the same dose ($n = 20$). Animals were monitored to observe tumor development. (a) 12 of 20 AxCAmCD40L-treated animals showed complete disappearance of the tumor until 28 days, while the other 8 animals exhibited a tumor growth rate similar to that of control animals. There was a significant difference between CD40L group and LacZ in tumor diameter (*$P < 0.05$). (b) Survival of animals after intratumoral injection AxCAmCD40L or AxCALacZ showed a significant difference (*$P < 0.05$). 12 of 20 survival animals which treated with CD40L showed long survival time for more than 400 days. However, LacZ treated animals were all dead within 100 days.

CTL/NK assay. We explored whether prevention and treatment of HCC with AxCAmCD40L was associated with enhanced NK and CTL activity. As shown in Figure 4a,b, significant cytotoxic NK activity against YAC-1 and McA-RH7777 cells was elicited by intratumoral administration of AdCMVmCD40L. To analyze specific antitumor CTL activity, we selected $CD8^+$ cells using CD8a MicroBeads and explored ^{51}Cr releasing assays. The percentage of lysis for YAC-1 went down significantly, but the percentage of lysis for McA-RH7777 was similar to the case of using splenocytes. CTL and cytotoxicity was highest at day 3 after therapy and declined rapidly on subsequent days. In animals that received AxCALacZ, only a modest elevation in CTL and NK activity was detected. In the treatment model, a similar trend was demonstrated (Fig. 4c,d).

Antitumor effect induced by humoral immunity. To examine whether humoral immunity plays important role for antitumor effect in this rat models, we examined tumor cell-binding Ig in sera that were obtained from prevention and treatment rats by FACS in the first step. Tumor cell-binding Ig was detected in AxCAmCD40L prevention and treatment models. As shown in Figure 5a, there were significant differences between CD40L group and LacZ group in both models ($P < 0.05$). Next, direct cytotoxicity of sera obtained from prevention and treatment rats was examined *in vitro*. The cytotoxicity of McA-RH7777 expressing CD40L was significantly stronger than that of McA-RH7777 expressing LacZ, parental McA-RH77777 cells and PBS in both models ($P < 0.05$) and the antitumor effect was specific (Fig. 5b,c). In addition, cytotoxicity of sera did not induce in the case of different cell line, RCN9 cells.

Histological assay for antitumor effects by intratumoral injection of AxCAmCD40L. The histological sections from animals treated with AxCAmCD40L demonstrated significant lymphocyte infiltration in the tumor but the sections from control showed very little lymphocyte infiltration in HE staining (Fig. 6a,b). As for CD40L expression, the section of AxCAmCD40L-treated animals demonstrated strong CD40L expression in the tumor cells (Fig. 6c),

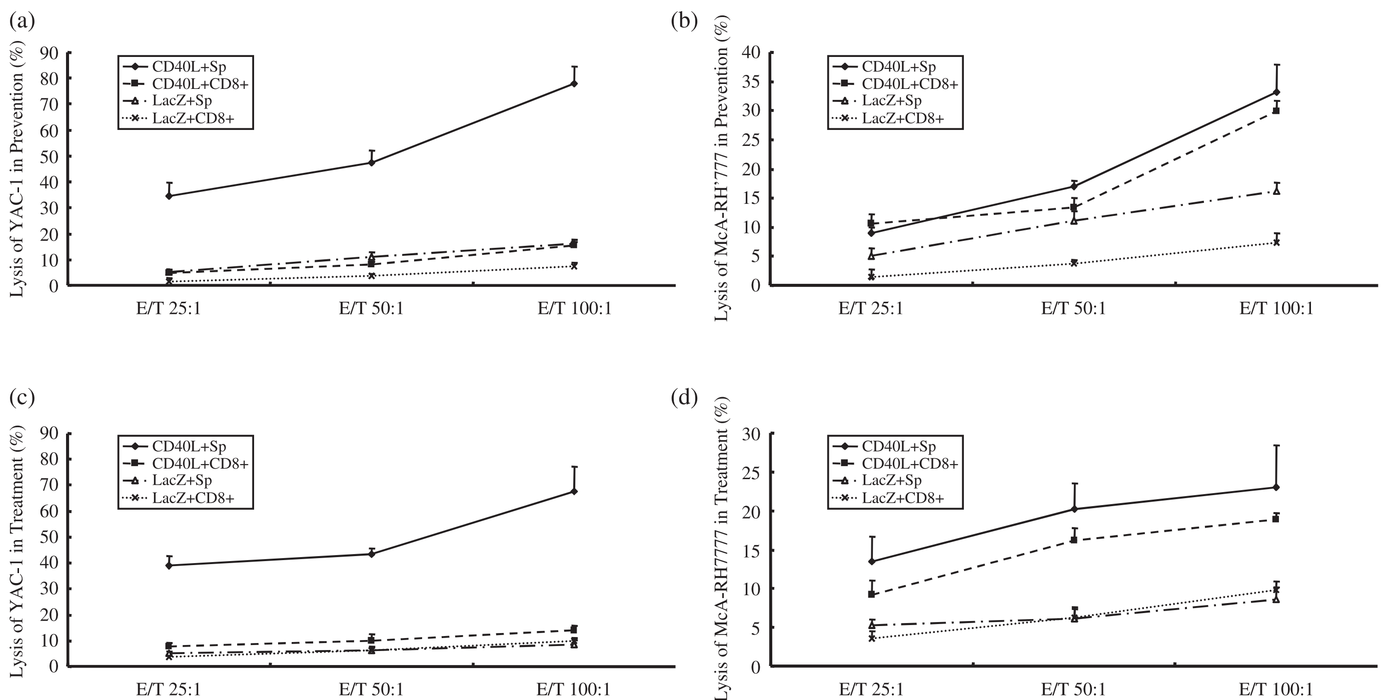

Fig. 4. Natural killer (NK) activity in animals with hepatocellular carcinoma (HCC) that received AxCAmCD40L, AxCALacZ or control phosphate buffered saline (PBS) in prevention model and treatment model. Splenocytes were isolated at three, 7 and 14 days after therapy and assayed against 51Cr-labeled parental McA-RH7777 cells in prevention model (a), parental McA-RH7777 cells in treatment model (b), NK-sensitive YAC-1 cells in prevention model (c) and YAC-1 cells in treatment model (d) at different effector to target cell ratios.

doi: 10.1111/j.1349-7006.2008.00953.x
© 2008 Japanese Cancer Association

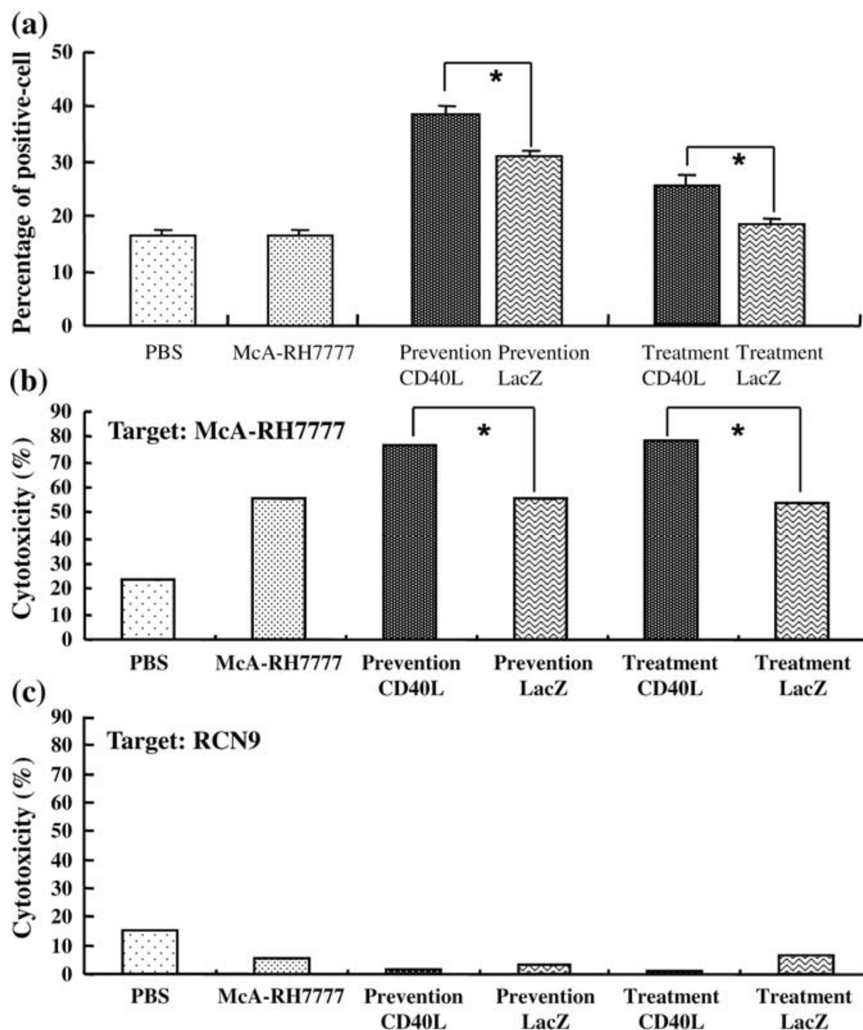

Fig. 5. The role of humoral immunity in antitumor effect. (a) Tumor cell-binding immunoglobulin in sera obtained from prevention, treatment, control phosphate-buffered saline (PBS) and tumor-bearing animals. The cells were stained with phycoerythrin (PE)-labeled anti rat immunoglobulin G (IgG) and analyzed using fluorescence-activated cell sorting (FACS). Tumor cell-binding Ig was detected in CD40L and LacZ expressing cells in both models. However, the cells expressing CD40L were enhanced and there were significant differences between AxCAmCD40L and AxCALacZ in both models ($P < 0.05$). *In vitro* direct cytotoxicity of sera obtained from prevention and treatment rats against McA-RH7777 (b) and RCN9 (c). Target cells were incubated with culture medium or sera which were obtained from animals implanted parental McA-RH7777, PBS or AxCAmCD40L or AxCALacZ in both models. All determinations were detected in triplicate. The cytotoxicity of sera obtained from AxCAmCD40L against McA-RH7777 was significantly stronger than the others in both models ($P < 0.05$). This effect was not detected against RCN9.

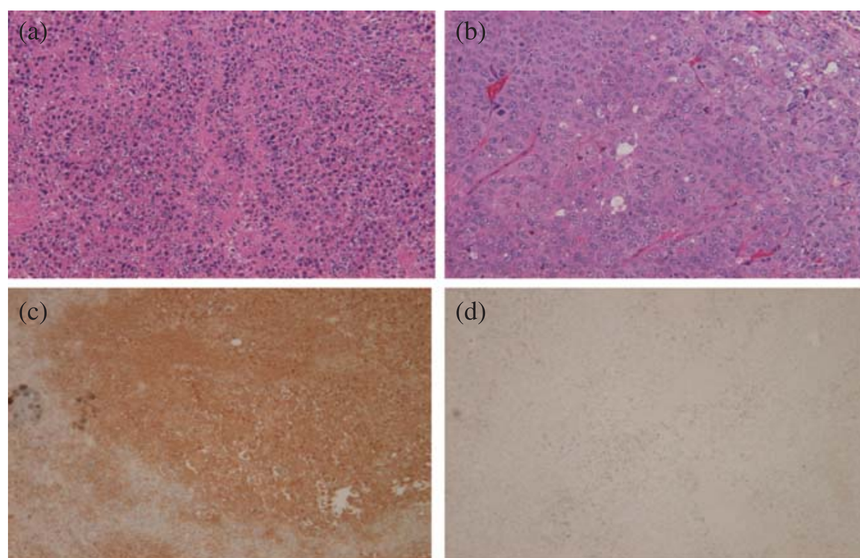

Fig. 6. Histological stain. To analyze the antitumor effects after treatment with AxCAmCD40L, the animals were sacrificed on day 5 after intratumoral injection of AxCAmCD40L or AxCALacZ. (a,b) Hematoxylin–eosin (HE) stain. Significant lymphocyte infiltration was observed in AxCAmCD40L-injected tumor (Fig. 6a) but not in the AxCALacZ-injected tumor (Fig. 6b). (c,d) Immunohistochemical study. Massive CD40L expression was observed in AxCAmCD40L injected tumor (Fig. 6c), but not in AxCALacZ (Fig. 6d) injected tumor. (Original magnification ×200).

but the section of control showed no expression in tumor cells (Fig. 6d). As for the results of the CD4 and CD8 stains, only CD8 was positive in CD40L group (Fig. 7c–f). In addition, TUNEL assay revealed strong stain in the tumor cells with AxCAmCD40L but very little stain in control (data not shown).

Discussion

The immunogene therapy strategy using genetically modified tumor cells expressing CD40L induced antitumor immunity has been demonstrated in several cancer models.[14–19] In this

© 2008 Japanese Cancer Association

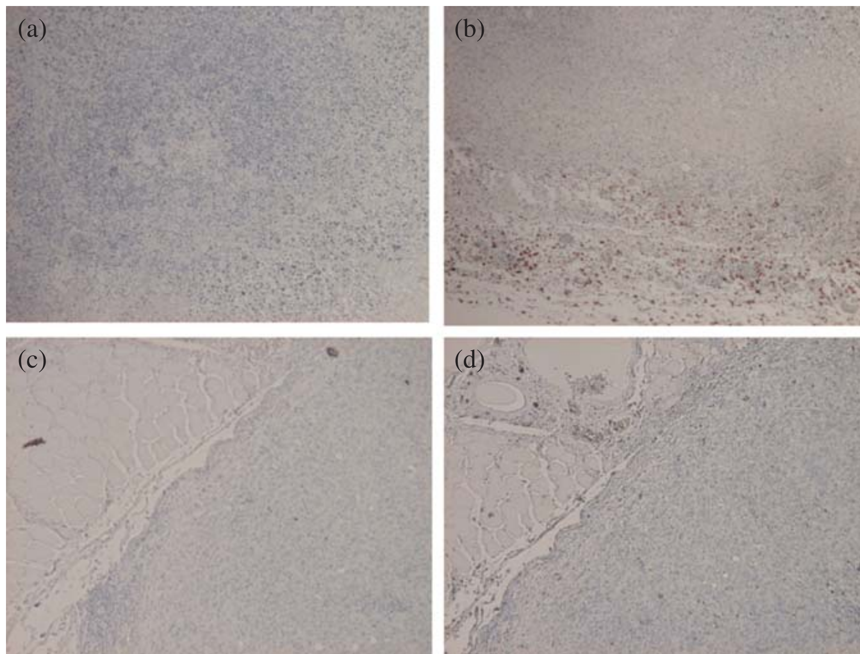

Fig. 7. CD4 and CD8 stain in treatment model. In CD40L treatment model, the positive findings were not CD4 (a) but CD8 (b). In LacZ treatment model, there were no positive findings (C and B).

study, we demonstrated that antitumor immunity induced by CD40L was significant in inhibiting tumor growth and long term survival in both prevention and treatment model and it got immunity memory. Tumor cells expressing CD40L eliminated tumorigenesis and acquired protective and systemic immunity against parental tumor cells that were re-implanted and showed long-term survival over 1 year. In the prevention model, we could induce sufficient antitumor immunity at low dose infection (at 10 MOI) in the absence of obvious side-effects such as weight loss, and bring out long-term survival. These suggest that this immunogene therapy is safe, with high transfer to the tumor and minimization of transfer to normal tissue. We also demonstrated expression of CD40L in rat HCC tissue, sequential infiltration of $CD8^+$ cells around the tumor by immunohistochemical stain and suggest that NK activity and tumor specific antibody were essential for the induction of antitumor effects in both models. Especially, the result that demonstrated humoral immunity plays an essential role is very important. In many studies using CD40L, antitumor effect caused by tumor-specific CTL has been emphasized. However, the present study demonstrated that tumor-specific antibody was superior to CTL. This result is very important finding to clarify antitumor mechanisms.

It is extremely import to confirm CD40L expression in tumor tissue because it means that transduction was successful. In our study, using immunohistochemical stain with anti CD40L, CD40L-treated animals showed strong expression of CD40L inside tumor tissue that was induced by intratumoral injection of AxCAmCD40L and continuously caused infiltration of lymphocytes at the marginal area. To our knowledge, this is the first report that CD40L expression transduced with adenovirus vector was detected in a rat HCC model visually by a immunohistochemical stain. In addition, anti $CD4^+$ and anti $CD8^+$ immunohistochemical stain revealed that the lymphocyte was not a $CD4^+$ T cell but $CD8^+$ T cell. CD40L is one of the strongest inducers of Th1 responses, by stimulating both innate and adaptive immunity, and recent findings imply that CD40L stimulation abrogates the suppressive effect of T regulatory cells. As concerns TUNEL assay, there were positive finding only in tumor tissue, not other tissue in this model. In contrast, control LacZ-treated animals did not show any positive stain. These results suggest that CD40L has been shown to induce apoptosis in tumor cells directly by mechanisms that are only beginning to be clarified while being an activator of immune cells. As we mentioned later, when it considers also enhanced Ig, it suggests not suppressor but NK and cytotoxic activity. In other words, these data suggest that the immunogene therapy with adenovirus vector expressing CD40L induced cell mediated immunity and caused antitumor effect. In this study, some of animals with CD40L injection developed a tumor progressively. There is a possibility that these animals could not express CD40L because of a technical or other unknown problem.

We studied CTL assay, NK assay and Ig to detect tumor specific anti tumor effect. Kikuchi *et al*. reported that intratumoral injection of adenovirus vector expressing CD40L suppressed the growth of established tumor cells in a CT26 colon cancer model. They demonstrated that that *in vivo* and *in vitro* modification of tumor cells to express CD40L elicited tumor specific CTL activity.[33] In contrast, our study did not demonstrate tumor specific CTL activity, in spite of the difference between AxCAmCD40L and AxCALacZ. However, tumor-cell-binding Ig was detected in sera obtained from the CD40L group. The antibody had specific cytotoxicity against parental McA-RH7777 cells *in vitro*. This demonstrated that tumor specific antibody was induced by AxCAmCD40L in both prevention and treatment HCC rat models. The amount of tumor-cell-binding Ig that was induced by AxCAmCD40L or AxCALacZ was higher than control parental cell and PBC. However, AxCAmCD40L produced more tumor-cell-binding Ig and more enhanced cytotoxicity than AxCALacZ. In addition, compared with RCN9 cells, a rat colon cancer cell line, there was significant difference. These results suggest the possibility that the humoral immunity participates in the antitumor effect in this model. In our model, at least NK cell and Ig were activated, then they induced antitumor effect.

Although antitumor immunity induced by CD40L was significant in inhibiting tumor growth in both the prevention and treatment model, synergistic antitumor effect of CD40L and IL-12 was not demonstrated. In addition, tumor-specific CTL was not cleared. However, we can suggest that NK cells and Ig played an important role in this model. The present study indicates that immunogene therapy using CD40L might be a new strategy of systematic therapy against HCC. Further studies, such as those enhancing the antitumor effect on metastatic cancers and specific antitumor CTL activity on the HCC model, are currently underway.

doi: 10.1111/j.1349-7006.2008.00953.x
© 2008 Japanese Cancer Association

Acknowledgments

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.

References

1 Lopez LJ, Marrero JA. Hepatocellular carcinoma. *Curr Opin Gastroenterol* 2004; **20**: 248–53.
2 Allen J, Venook A. Hepatocellular carcinoma: epidemic and treatment. *Curr Oncol Rep* 2004; **6**: 177–83.
3 Cormier JN, Thomas KT, Chari RS, Pinson CW. Management of hepatocellular carcinoma. *J Gastrointest Surg* 2006; **10**: 761–80.
4 Lodato F, Mazzella G, Festi D, Azzaroli F, Colecchia A, Roda E. Hepatocellular carcinoma prevention: a worldwide emergence between the opulence of developed countries and the economic constraints of developing nations. *World J Gastroenterol* 2006; **12**: 7239–49.
5 Kaido T. Characteristics and quality of randomized controlled trials in the treatment of hepatocellular carcinoma. *Dig Dis Sci* 2007; [Epub ahead of print].
6 Kassahun WT, Fangmann J, Harms J, Hauss J, Bartels M. Liver resection and transplantation in the management of hepatocellular carcinoma: a review. *Exp Clin Transplant* 2006; **4**: 549–58.
7 Yanaga K. Current status of hepatic resection for hepatocellular carcinoma. *J Gastroenterol* 2004; **39**: 919–26. Review.
8 Nuzzo G, Giuliante F, Gauzolino R, Vellone M, Ardito F, Giovannini I. Liver resections for hepatocellular carcinoma in chronic liver disease: experience in an Italian centre. *Eur J Surg Oncol* 2007; [Epub ahead of print].
9 Magott-Procelewska M. Costimulatory pathways as a basic mechanism of activating a tolerance signal in T cells. *Ann Transplant* 2004; **9**: 13–18.
10 Roy M, Waldschmidt T, Aruffo A, Ledbetter JA, Noelle RJ. The regulation of the expression of gp39, the CD40 ligand, on normal and cloned $CD4^+$ T cells. *J Immunol* 1993; **151**: 2497–510.
11 Bennett SR, Carbone FR, Karamalis F, Flavell RA, Miller JF, Heath WR. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* 1998; **393**: 478–80.
12 Schoenberger SP, Toes RE, van der Voort EI, Offringa R, Melief CJ. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* 1998; **393**: 480–3.
13 Grewal IS, Flavell RA. CD40 and CD154 in cell-mediated immunity. *Ann Rev Immunol* 1998; **16**: 111–35.
14 Schmitz V, Barajas M, Wang L *et al*. Adenovirus-mediated CD40 ligand gene therapy in a rat model of orthotopic hepatocellular carcinoma. *Hepatology* 2001; **34**: 72–81.
15 Loskog AS, Fransson ME, Totterman TT. AdCD40L gene therapy counteracts T regulatory cells and cures aggressive tumors in an orthotopic bladder cancer model. *Clin Cancer Res* 2005; **11**: 8816–21.
16 Liu Y, Xia D, Li F, Zheng C, Xiang J. Intratumoral administration of immature dendritic cells following the adenovirus vector encoding CD40 ligand elicits significant regression of established myeloma. *Cancer Gene Ther* 2005; **12**: 122–32.
17 Koppold B, Sauer G, Buening H *et al*. Chemotherapeutic agents enhance AAV2-mediated gene transfer into breast cancer cells promoting CD40 ligand-based immunotherapy. *J Cancer Res Clin Oncol* 2006; **132**: 787–94.
18 Loskog A, Totterman TH. CD40L: a multipotent molecule for tumor therapy. *Endocr Metab Immune Disord Drug Targets* 2007; **7**: 23–8.
19 Kimura T, Ohashi T, Kikuchi T, Kiyota H, Eto Y, Ohishi Y. Antitumor immunity against bladder cancer induced by ex vivo expression of CD40 ligand gene using retrovirus vector. *Cancer Gene Ther* 2003; **10**: 833–9.
20 Chouaib S, Asselin-Paturel C, Mami-Chouaib F, Caignard A, Blay JY. The host-tumor immune conflict. from immunosuppression to resistance and destruction. *Immunol Today* 1997; **18**: 493–7.
21 Hallermalm K, De Geer A, Kiessling R, Levitsky V, Levitskaya J. Autocrine secretion of Fas ligand shields tumor cells from Fas-mediated killing by cytotoxic lymphocytes. *Cancer Res* 2004; **64**: 6775–82.
22 Hernandez MG, Shen L, Rock KL. CD40-CD40 ligand interaction between dendritic cells and CD8+ T cells is needed to stimulate maximal T cell responses in the absence of CD4+ T cell help. *J Immunol* 2007; **178**: 2844–52.
23 Kikuchi T, Crystal RG. Anti-tumor immunity induced by in vivo adenovirus vector-mediated expression of CD40 ligand in tumor cells. *Hum Gene Ther* 1999; **10**: 1375–87.
24 Chiodoni C, Paglia P, Stoppacciaro A, Rodolfo M, Parenza M, Colombo MP. Dendritic cells infiltrating tumors cotransduced with granulocyte/macrophage colony-stimulating factor (GM-CSF) and CD40 ligand genes take up and present endogenous tumor-associated antigens, and prime naive mice for a cytotoxic T lymphocyte response. *J Exp Med* 1999; **190**: 125–33.
25 Couderc B, Zitvogel L, Douin-Echinard V *et al*. Enhancement of antitumor immunity by expression of CD70 (CD27 ligand) or CD154 (CD40 ligand) costimulatory molecules in tumor cells. *Cancer Gene Ther* 1998; **5**: 163–75.
26 Grossmann ME, Brown MP, Brenner MK. Antitumor responses induced by transgenic expression of CD40 ligand. *Hum Gene Ther* 1997; **8**: 1935–43.
27 Kanegae Y, Lee G, Sato Y, Tanaka M, Sakaki T, Sugano S. Efficient gene activation in mammalian cells by using recombinant adenovirus expressing site-specific cre recombinase. *Nucleic Acids Res* 1995; **23**: 3816.
28 Niwa H, Yamamura K, Miyazaki Y. Efficient selection for high expression transfectants with a novel eukaryotic vector. *Gene* 1991; **108**: 193.
29 Kanegae Y, Makimura M, Saito I. A simple and efficient method for purification of infectious recombinant adenovirus. *Jpn J Med Sci Biol* 1994; **47**: 157–66.
30 Kikuchi T, Akasaki Y, Homma S *et al*. Antitumor activity of interleukin-18 on mouse glioma cells. *J Immunother* 2000; **23**: 184–9.
31 Monach PA, Schreiber H, Rowley DA. $CD4^+$ and B lymphocytes in transplantation immunity. *Transplantation* 1993; **55**: 1356–61.
32 Fujieda M, Oishi N, Kurashige T. Antibody to endothelial cells in Kawasaki disease lyse endothelial cells without cytokine pretreatment. *Cli Exp Immunol* 1997; **107**: 120–6.
33 Kikuchi T, Miyazawa N, Moore MA, Crystal RG. Tumor regression induced by intratumor administration of adenovirus vector expressing CD40 ligand and naive dendritic cells. *Cancer Res* 2000; **60**: 6391–5.

© 2008 Japanese Cancer Association