Gene Therapy of Orthotopic Hepatocellular Carcinoma in Rats Using Adenovirus Coding for Interleukin 12

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The use of gene therapy to enhance antitumor immunity has emerged as a promising procedure to fight cancer. In this study we have tested the ability of an adenovirus carrying interleukin 12 (IL-12) gene (AdCMVIL-12) to eliminate tumoral lesions in 3 animal models of orthotopic hepatocellular carcinoma (HCC). Intratumoral injection of AdCMVIL-12 in animals with a single big tumor nodule implanted in the liver resulted in significant inhibition of tumor growth in a dose-dependent manner. Fifty percent of animals that received a dose of 5×10^9 plaque-forming units, showed complete regression of the tumor 2 weeks after treatment. In animals with 2 independent tumor nodules in the left liver lobe, injection in only one of them of 5×10^9 pfu AdCMVIL-12 induced, 15 days after therapy, complete regression of 50% of treated tumors and also of 50% of untreated lesions, with 60% long-term survival. Rats that were tumor free after therapy with AdCMVIL-12 showed protection against tumor rechallenge. A group of rats received the carcinogen diethylnitrosamine and developed multiple hepatic dysplasic nodules of 1 to 5 mm in diameter. These animals were treated by intrahepatic artery injection of either AdCMVIL-12 (5×10^9 pfu) or control vector. In this model AdCMVIL-12 induced complete tumor regression in 20% of treated rats and inhibited tumor growth in 60% of cases with an increase in rat survival. Activation of natural killer (NK) cells and inhibition of angiogenesis were found to be antitumor mechanisms set in motion by AdCMVIL-12. Our data indicate that experimental HCC can be efficiently treated by intratumoral or intravascular injection of adenovirus expressing IL-12. (HEPATOLOGY 2001;33:52-61.)

Hepatocellular carcinoma (HCC) is a common human malignancy that resists conventional chemotherapy and radiotherapy.[1-3] Current treatment of small, apparently solitary, nodules is based on partial hepatectomy or transplantation,[3,4] but there is no efficient treatment of patients with multiple tumor nodules.[1-3]

The use of gene therapy with immunostimulatory cytokines to enhance immunologic responses against tumor cells has emerged as a promising new approach to treat cancer.[5,6] This form of therapy can be accomplished either by *ex vivo* transduction of tumor cells or fibroblasts or by direct *in vivo* transduction of the tumor and adjacent tissue with cytokine genes such as interleukin 2 (IL-2), IL-4, IL-6, IL-7, IL-12, interferon gamma (INF-γ), tumor necrosis factor, and granulocyte macrophage–colony-stimulating factor.[5,6]

IL-12 (also named natural killer cell [NK] stimulatory factor) was originally identified as a factor secreted by Epstein-Barr virus–transformed human B cell lines that mediates several biological activities on T lymphocytes and natural killer (NK) cells.[7] IL-12 is produced by B cells, dendritic cells, and macrophages.[8,9] It acts on T cells and NK cells to induce proliferation and production of cytokines (especially IFN-γ) and to enhance generation and activity of cytotoxic lymphocytes. IL-12 is a key factor in the induction of macrophage activation leading to differentiation of uncommitted T lymphocytes to Th1 cells.[8,9] Systemic treatment with purified recombinant IL-12 has been shown to induce antitumor effects in different animal models.[10-14] The mechanisms of anti-tumoral activity of IL-12 include the activation of tumor-specific cytotoxic T lymphocytes (CTL) and NK cells and also the ability of this cytokine to inhibit angiogenesis *in vivo*.[10,14-17] The anti-angiogenic effect of IL-12 is mediated by IFN-γ, which in turn stimulates the production of the chemokine IFN-inducible protein-10[16,17] that, in addition to acting as a chemoattractant for lymphocytes, has a powerful inhibitory effect on proliferation and differentiation of endothelial cells.[18-20] Moreover, IL-12–stimulated NK cells are cytotoxic for activated endothelium thus contributing to block the formation of new tumoral vessels.[21]

Although systemic administration of IL-12 has been shown to be highly effective in inducing tumor regression and reducing metastases in diverse murine models of solid tumors,[10-14] important hematologic, hepatotoxic and muscular side effects limit its use as an anticancer agent.[22,23] A phase I clinical trial of systemic IL-12 treatment in subjects with renal cell carcinoma showed objective tumor responses in several patients, but a subsequent phase II trial of IL-12 in advanced renal cell cancer was discontinued because of severe toxicity, including death.[24,25] It seems therefore that the use of procedures allowing high local production of IL-12 at the site of interest (the tumor mass) with low systemic levels of the cytokine would achieve similar or better therapeutic effects with less toxicity. To this aim different strategies based on the transduction of

Abbreviations: HCC, hepatocellular carcinoma; IL, interleukin; IFN, interferon; NK, natural killer; CTL, cytotoxic T lymphocyte; DENA, diethylnitrosamine; MHC, major histocompatibility complex.

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tumoral cells with IL-12 genes have been attempted to treat various experimental neoplasms.[15,26-32]

Although some studies have already shown that intratumoral injection of vectors coding for IL-12 causes tumor regression in animals with subcutaneous tumors,[28-32] these results cannot be directly applied to orthotopic HCC. In contrast to orthotopic models, subcutaneous tumors display a high inherent immunogenicity caused by the presence in this localization of dendritic cells, which are very efficient at presenting tumoral antigens and eliciting immune responses.[15,33] Thus, the successful generation of antitumoral immunity against subcutaneous tumors does not necessarily indicate the same efficacy against tumors at an orthotopic site. Moreover, the therapeutic effects obtained in transplanted tumors may not apply to the case of tumors that arise in animals as a result of carcinogenic events. Since there are no data in the literature relating the effects of IL-12 gene transfer to orthotopic primary liver cancer, in the present study we have evaluated the antitumoral efficacy and effector mechanisms of adenovirus-mediated gene transfer of IL-12 in 3 models of this malignancy. Experimental models for HCC were generated either by inoculation of syngenic HCC cells into the liver of Buffalo rats to produce 1 or 2 tumor nodules or by chronic administration of the carcinogen diethylnitrosamine (DENA) to Wistar rats to obtain multifocal HCC. Our data show that treatment with adenovirus expressing IL-12 results in a marked antitumoral effect in all HCC models analyzed. This antitumor effect correlated with activation of NK cells and inhibition of angiogenesis *in vivo*.

MATERIALS AND METHODS

Animals and Cell Lines

Five- to 8-week-old male Buffalo rats and 6-week-old Wistar rats were obtained from Harlan (Barcelona, Spain). During the experimental period animals were housed in standard conditions and all animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences.

The 293 cell line (adenoviral E1 transformed human embryonic kidney cells), McA-RH7777 (HCC cell line from Buffalo rat), CC531 (colon cancer cell line from WAG rat), and YAC-1 (Moloney leukemia virus–transformed lymphoma cell line derived from A/Sn mice) were purchased from American Type Culture Collection (Rockville, MD). The 293 and CC531 cell lines were cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. McA-RH7777 cells were cultured in Dulbecco's modified Eagle medium supplemented with 20% heat-inactivated horse serum and 5% heat-inactivated fetal bovine serum. YAC-1 cells were cultured in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum.

Construction of Recombinant Adenoviral Vectors

Adenovirus carrying LacZ reporter gene (AdCMVlacZ) and IL-12 (AdCMVIL-12) under the control of CMV promoter were produced as reported previously.[34,35] Recombinant adenoviruses were isolated from a single plaque, expended in 293 cells, and purified by double cesium chloride ultracentrifugation. Purified virus was extensively dialyzed against 10 mmol/L Tris/1 mmol/L $MgCl_2$ and stored in aliquots at −80°C. Titration was made by plaque assay.

Induction of Orthotopic HCC and In Vivo Gene Therapy

Three experimental models have been developed for this study:

Model 1 (Single HCC Nodule). A total of 10^6 of McA-RH7777 cells were inoculated into the left liver lobe of Buffalo rats. A single tumor nodule (10-12 mm in diameter) was observed 2 weeks after inoculation of tumor cells. These animals were treated either with AdCMVIL-12 at different doses (5×10^9, 10^9, 10^8, 10^7 plaque-forming units/animal) or AdCMVlacZ (5×10^9 pfu/animal) as a control. Vectors were given by intratumoral injection. Two weeks after treatment, animals were sacrificed and liver samples were obtained for evaluation of treatment efficacy. Spleen was obtained for cytotoxicity assays.

Model 2 (Two Independent HCC Nodules). Two tumors were implanted in the liver by intrahepatic inoculation of 5×10^5 of McA-RH7777 cells into the left liver lobe of Buffalo rats. Two tumor nodules of 8 to 10 mm in diameter developed 10 days after inoculation of tumor cells. One tumor nodule was treated either with AdCMVIL-12 at 5×10^9 pfu/animal or control vector AdCMVlacZ at same dose. Vectors were given by intratumoral injection. Two weeks after treatment, animals were anesthetized and underwent laparotomy to observe the evolution of the tumor. Survival was checked daily in all of the animals.

Model 3 (Multiple HCC Nodules). Primary liver tumors were induced in Wistar rats (6 weeks old, 170 g) with DENA (Sigma, St. Louis, MO) as described previously.[36] Briefly, animals received 10 mg/kg/d of DENA for 12 weeks. The weight of the rats was recorded every week. Rats were given weekly doses of DENA in a volume corresponding to the estimated water consumption of 6 days of drinking water (0.01% vol/vol). Once the animals consumed the administered DENA solution, they were given DENA-free water the rest of the week. DENA solution was prepared each week. Tumor formation was monitored by weekly sacrifice of 1 rat during the period of DENA administration. At the end of the induction period, rats presented multiple tumor nodules in the liver ranging from microscopic neoplastic nodules to HCC nodules with diameter up to 5 mm. Twenty-four tumor-bearing rats were divided randomly into 3 groups: the treated group received 5×10^9 pfu of AdCMVIL-12 (n = 10) and control groups received either 5×10^9 pfu of control vector AdCMVlacZ (n = 6) or saline (n = 8). Vectors were given by infusion via hepatic artery. To this aim, rats were anesthetized with ketamine-atropine-diazepam and a butterfly with a 27-gauge needle was inserted into the gastroduodenal artery using an operating microscope (magnification ×16). After administration of AdCMVIL-12 or saline, the gastroduodenal artery was ligated and the presence of appropriate hepatic blood flow was confirmed. Two weeks after treatment, treated animals were anesthetized and laparotomized to observe the evolution of the tumor. Tumor burden was evaluated before and 2 weeks after treatment by 2 independent observers according to the score system described in Table 1. One month after treatment, 5 of 10 rats from the AdCMVIL-12–treated group and 2 of 6 rats from the AdCMVlacZ–treated group and 2 of 8 rats from the control group receiving saline were sacrificed. The evolution of the tumor was assessed as indicated above. Survival was checked daily in the remaining animals.

Cytokine Production

Tumor-bearing animals were treated by intratumor injection of 5×10^9 pfu/animal or control vector AdCMVlacZ at the same dose of

TABLE 1. Evaluation of Tumor Burden in Primary Liver Cancer Induced by DENA

Tumor Score	No. of Tumor Nodules	Size of Tumor Nodules	Metastasis
0	0	0	No
1	1-3	<2 mm	No
2	1-3	2-5 mm	No
3	3-5	2-5 mm	No
4	>5	2-5 mm	No
5	>5	>5 mm	No
6	>5	>5 mm	Yes

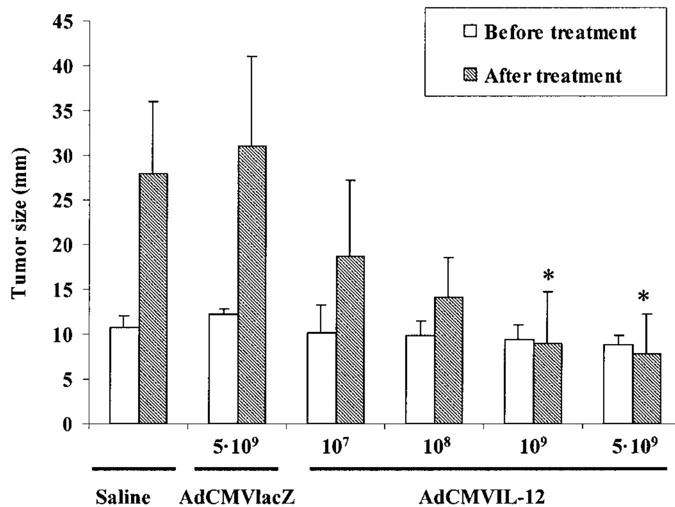

FIG. 1. *In vivo* gene therapy of established HCC cancer. Rats with orthotopic HCC tumor nodule (10-12 mm in diameter) were treated either with AdCMVIL-12 at different doses (5×10^9 pfu [n = 8], 10^9 pfu [n = 8], 10^8 pfu [n = 3], 10^7 pfu [n = 3]), or control adenovirus AdCMVlacZ at 5×10^9 pfu (n = 9), or saline (n = 6). Two weeks after treatment, the animals were sacrificed and liver samples were obtained for evaluation of tumors. Size of tumor was measured before and 2 weeks after treatment. $^*P < .01$ as compared with control animals.

saline. Blood samples were collected from the retro-orbital sinus at 1, 3, 5, and 7 days after treatment. Serum mouse IL-12 (the transgen contained in AdCMVIL-12) and rat IFN-γ (the main cytokine induced by IL-12) were determined by enzyme-linked immunosorbent assay with commercial kits (Biosource, Nivelles, Belgium) following the manufacturer's instructions.

Cytotoxicity Assay

Cytotoxicity assay was performed according to standard protocols as described before.[37,38] Viable splenocytes were tested for CTL activity after incubation for 5 days of 6×10^6 cells together with 2.5×10^5 McA-RH7777 cells (previously treated with 100 mg/mL of mitomycin C for 30 minutes at 37°C) in each well of a 24-well plate. On day 5, fresh tumor cells were harvested and 1×10^6 cells were labeled with 50 μCi ^{51}Cr for 1 hour. After labeling, the cells were washed 4 times, adjusted to 5×10^4/mL, and 100 μL of this cell suspension was added to a 96-well plate and incubated with effector cells for 5 hours at 37°C in different E/T cell ratios. To determine NK activity, splenocytes were obtained as effector cells and 3 types of target cells (McA-RH7777, YAC-1, and CC531) were used. In some experiments *in vitro* blockade of $CD4^+$, $CD8^+$, and major histocompatibility complex (MHC) class I^+ cells was performed by using purified anti-rat CD4 (clone OX-35, Pharmingen, San Diego, CA), anti-rat CD8a (clone OX-8 Pharmingen), and anti-rat RT1A (clone OX-18 Pharmigen) at final concentration of 100 μg/mL. Spontaneous and maximum release of ^{51}Cr from tumor cells were determined, the latter using 0.8% Triton X-100. After incubation, radioactivity was measured in 50-μL aliquots of the supernatants. Data represent the means of triplicate cultures. The percent of specific lysis was calculated using the formula (c.p.m. experimental sample − c.p.m. background)/(c.p.m. maximum lysis − c.p.m. background) × 100.

Assay for Antiangiogenic Activity of the Transgene

Fifty microliters of Matrigel (Becton-Dickinson, Bedford, MA) mixed with $VEGF_{165}$ (Peprotech, London, England) at final concentration of 20 ng/mL was injected into the middle lobe of livers of tumor-bearing animals. At same time, a single tumor nodule (8-10 mm in diameter) previously implanted in the left liver lobe was treated using 5×10^9 pfu of either AdCMVIL-12 or control vector AdCMVlacZ. After 10 days, rats were sacrificed and the middle lobe of livers that contained the Matrigel plug was removed, fixed in 10% buffered formalin and embedded in paraffin. The samples were sectioned and stained with hematoxylin-eosin for histologic analysis. The number of vascular cells was determined using a computerized digital analyzer Optimas 6.2 (Optimas Corporation, Bothell, Washington). The results are expressed as the mean number of cells in 10^5 μm^2.

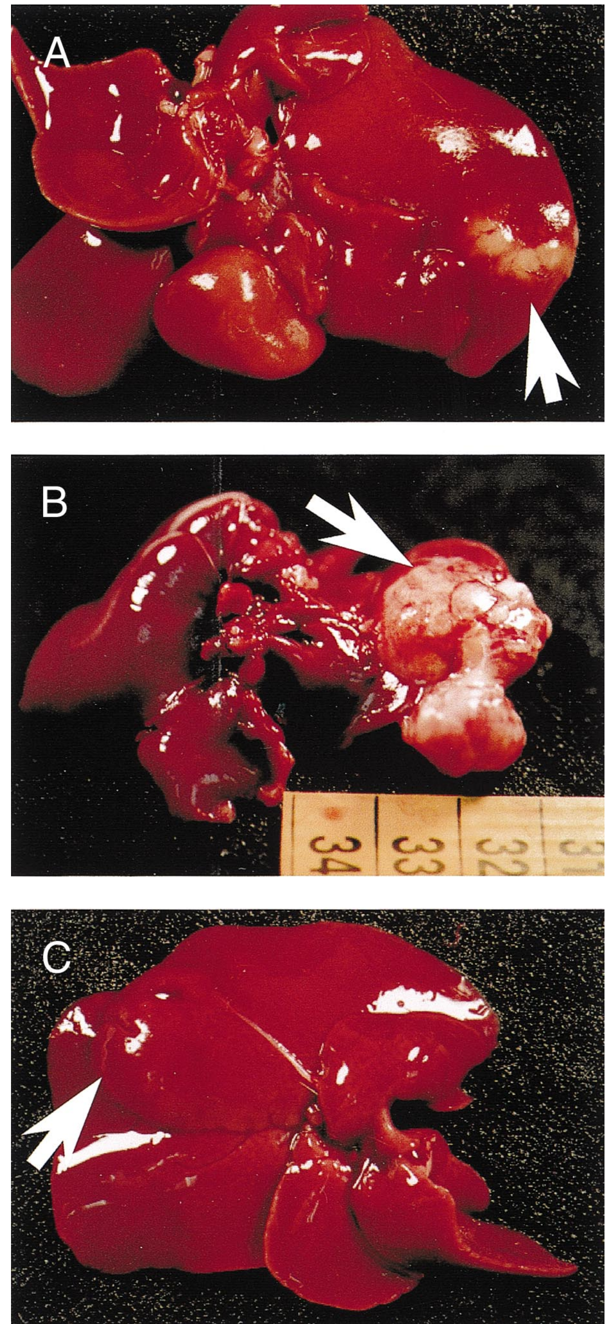

FIG. 2. Macroscopic aspect of the liver in rats before and 2 weeks after treatment with AdCMVIL-12 or AdCMVlacZ. (A) Liver from an animal before treatment showing a single tumor nodule of about 10 mm in diameter. (B) Liver from a control rat treated with AdCMVlacZ showing the presence of a large tumor of about 20 mm in diameter. (C) Liver from a rat treated with AdCMVIL-12 showing absence of tumor.

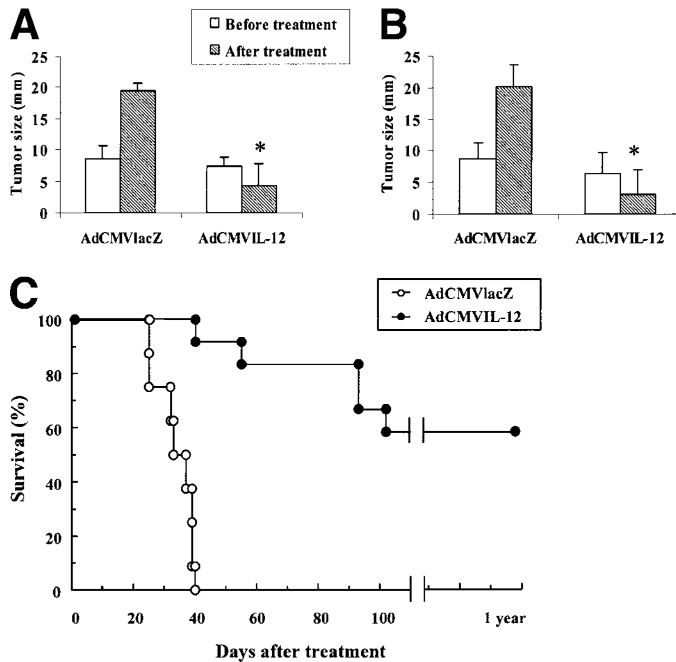

FIG. 3. Local injection of AdCMVIL-12 into one tumor induces distant antitumoral effect in animals with two orthotopic HCC tumor nodules. When tumors reached 8 to 10 mm in diameter 1 of the 2 tumor nodules was treated by intratumoral administration of 5×10^9 pfu of AdCMVIL-12 (n = 10) or control vector AdCMVlacZ (n = 10). The size of the tumors was measured before and 2 weeks after treatment and survival of animals was recorded. (A) Size of treated tumors in animals that received intratumoral injection of AdCMVIL-12 or control adenovirus AdCMVlacZ. (B) Size of untreated tumors in animals in which the distant tumor was treated with AdCMVIL-12 or control adenovirus AdCMVlacZ. (C) Long-term survival of animals after treatment with AdCMVIL-12 and AdCMVlacZ. $^*P < .01$ as compared with control animals.

RESULTS

In Vivo *Gene Therapy of Orthotopic HCC Tumor Nodule With AdCMVIL-12*

In a model of colon cancer we have previously shown that intratumoral injection of AdCMVIL-12 resulted in the production of high levels of IL-12 within the tumor mass and subsequent tumor regression.[35] To investigate whether AdCMVIL-12 could have therapeutic effects in immunocompetent animal models of HCC, we produced a tumor nodule in the liver by implantation of the syngeneic hepatocarcinoma cell line McA-RH7777 into the left liver lobe of Buffalo rats. When tumor size was 10 to 12 mm in diameter, animals were treated by intratumoral injection of AdCMVIL-12 at different doses (5×10^9, 10^9, 10^8, 10^7 pfu), control adenovirus AdCMVlacZ at 5×10^9 pfu or saline. Two weeks after treatment, animals were sacrificed and liver samples were obtained for evaluation of the therapy. Figure 1 shows that in all animals treated with saline or AdCMVlacZ the size of the tumors increased progressively. In contrast, inhibition of tumor growth was observed in animals treated with AdCMVIL-12 in a dose-dependent manner. Even the lowest dose of AdCMVIL-12 (10^7 pfu) reduced tumor growth. Four of 8 animals treated with high dose of AdCMVIL-12 (5×10^9 pfu) and 3 of 8 animals treated with intermediate dose of AdCMVIL-12 (10^9 pfu) exhibited a complete regression of the tumor (Fig. 1). Figure 2 shows representative photographs of livers with implanted HCC tumors before and 2 weeks after therapy with either AdCMVIL-12 or control vector. In rats in which the tumor was treated with control vector a lesion of more than 20 mm in diameter was present 15 days after therapy whereas no tumor or very small tumor nodules were found in AdCMVIL-12–treated rats. These data show a marked antitumoral effect of AdCMVIL-12 in this animal model of liver cancer.

Local AdCMVIL-12 Gene Therapy of HCC Induces Not Only Local But Also Distant Antitumoral Effects

Because in most cases of patients with HCC there are multiple tumor nodules in the liver, we evaluated whether injection of AdCMVIL-12 into a tumor nodule had any effect on distant lesions. To this aim we used the Buffalo rat HCC model with 2 tumor nodules in the liver. When tumors were 8 to 10 mm in diameter, one of the nodules was treated with 5×10^9 pfu of AdCMVIL-12 or AdCMVlacZ as control. We observed that all animals from the control group experienced a progressive tumor growth and died by day 40 (Fig. 3). In contrast, treatment with AdCMVIL-12 induced complete tumor disappearance of the treated lesion in 5 of 10 animals 2 weeks after therapy (Fig. 3A) and also a complete regression of the untreated tumor in 5 of 10 rats (Fig. 3B). The rest of animals that received AdCMVIL-12 had partial tumor regression. We also found that treatment with AdCMVIL-12 increased survival of tumor-bearing animals for more than 1 year as shown in Fig. 3C. These data indicate that, in our model, adenoviral gene transfer of IL-12 to a tumor nodule can exert a therapeutic effect not only on the treated lesion but also on distant nodules within the liver.

Long-Term Antitumor Immunity Induced by Intratumoral Injection of AdCMVIL-12

To know whether local treatment of HCC with AdCMVIL-12 could induce lasting immunologic memory, we rechallenged animals that were free of tumor after AdCMVIL-12

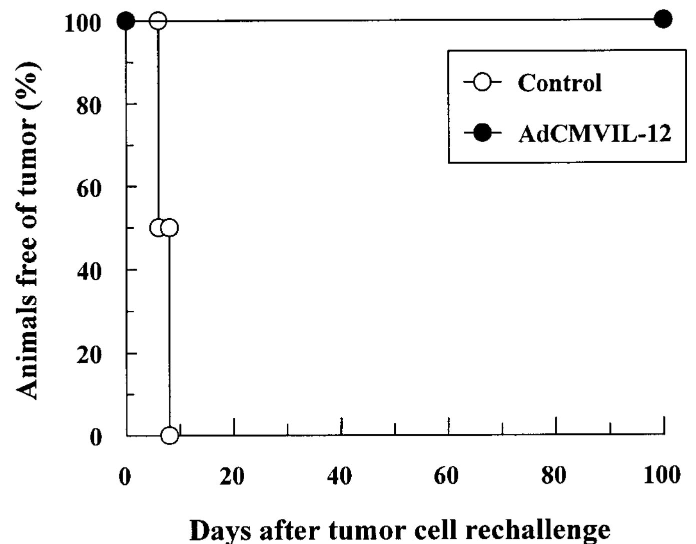

FIG. 4. Long-term protection against parental HCC cell rechallenge. McA-RH7777 cells (1×10^6 cells) were inoculated subcutaneously into the flank of animals that had completely eliminated the intrahepatic tumors after therapy with AdCMVIL-12 (n = 10). The same amount of McA-RH7777 cells were injected into the flank of age- and sex-matched naive animals (n = 6). The occurrence of subcutaneous tumor growth was documented and results were expressed as percentage of animals free of tumor.

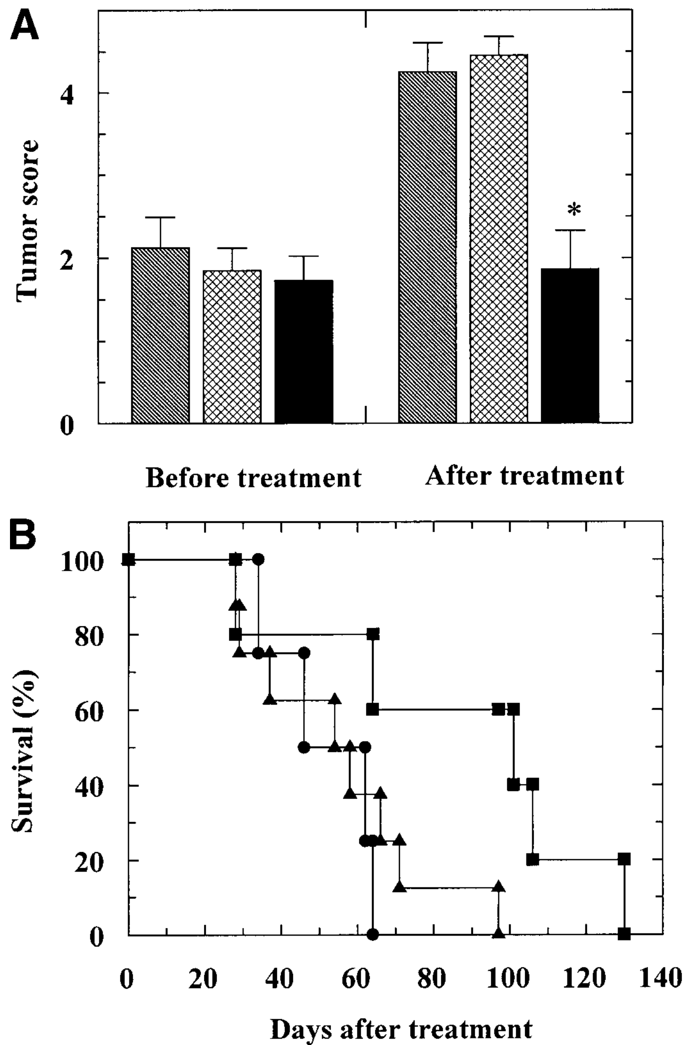

FIG. 5. *In vivo* gene therapy of established multifocal HCC induced by DENA. Rats with multiple dysplastic nodules of 1 to 5 mm in diameter in liver induced by the carcinogen diethylnitrosamine were treated by intrahepatic arterial infusion of either AdCMVIL-12 (5×10^9 pfu, n = 10), or control vector AdCMVlacZ (n = 6), or saline (n = 8). Tumor burden (score as described in the Materials and Methods and Table 1) was measured just before and 2 weeks after treatment. Four rats from the control group and 5 rats that received AdCMVIL-12 therapy were sacrificed 1 month after treatment. Liver samples were obtained for evaluation of tumors and blood samples were collected for biochemical analysis. The rest of the animals were left for assessment of survival. (A) Tumor score in animals with DENA-induced HCC before and after gene therapy with AdCMVIL-12 or control treatment. Tumor score was expressed as mean and standard error. *$P < .01$ as compared with control animals. (▧), Saline; (□), AdCMVlacZ; (■), AdCMVIL-12. (B) Survival of rats with DENA-induced HCC after gene therapy with AdCMVIL-12 or control treatment. (▲), Saline; (●), AdCMVlacZ; (■), AdCMVIL-12.

treatment with a new subcutaneous administration of McA-RH7777 cells 3 months after complete regression of primary tumors. Figure 4 shows that all animals (10 of 10) which had eliminated the tumor after AdCMVIL-12 therapy were resistant to new challenge with a subcutaneous injection of 10^6 parental McA-RH7777 cells, whereas all naive rats showed tumor growth at the site of injection 7 to 8 days after inoculation of cancer cells. These data indicate that *in vivo* gene therapy using AdCMVIL-12 vector may induce long-term protection against tumor recurrence.

In Vivo *Gene Therapy With AdCMVIL-12 of Multifocal HCC Induced by DENA in Rats*

The carcinogen DENA induces multiple tumor nodules in the liver through different steps of initiation, promotion, and tumor progression.[39-41] DENA-induced HCC is a very difficult

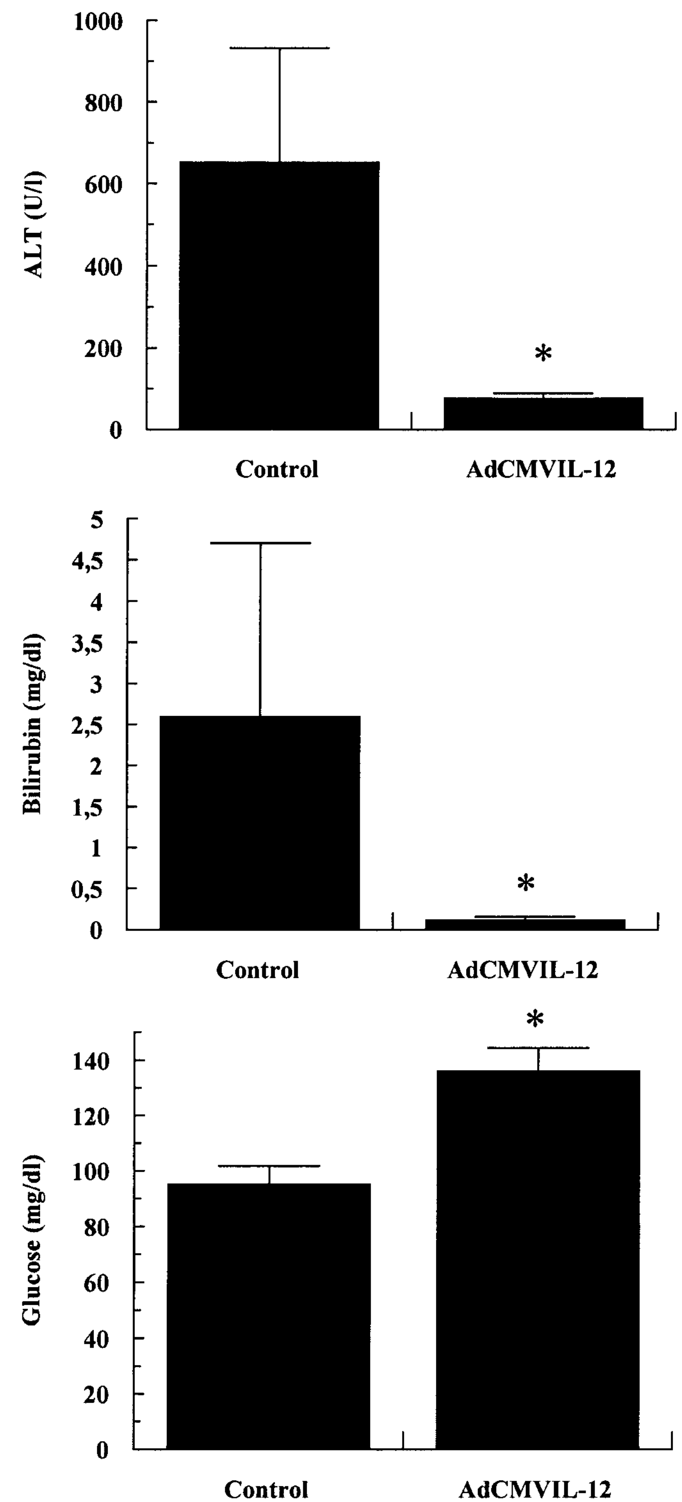

FIG. 6. Biochemical data in animals with DENA-induced HCC that received AdCMVIL-12 or control treatment (AdCMVlacZ or saline). Data are presented as mean and standard error. *$P < .01$ as compared with controls. ALT, alanine transaminase.

neoplasm to treat. This animal model of aggressive multifocal liver cancer is a hard test to evaluate the capabilities of gene therapy in liver cancer.[36,42,43] We therefore administered intra-arterially AdCMVIL-12 (5×10^9 pfu, n = 10), AdCMVlacZ (5×10^9 pfu, n = 6), or saline (n = 8) to rats with DENA-induced HCC. Tumor burden, expressed as tumor score (see Materials and Methods and Table 1), was assessed before treatment and 2 weeks later. Four rats from control groups and 5 rats that received AdCMVIL-12 therapy were sacrificed 1 month after treatment; livers were evaluated for tumor burden and blood samples were collected for biochemical analysis. The rest of the animals were followed up for survival. Figure 5A shows that tumor score increased in all animals from control groups during the 2 weeks that followed treatment. In contrast, among the 10 rats that received AdCMVIL-12 2 animals experienced complete tumor regression, in 6 rats tumor score remained stable, and in only 2 animals we observed an increase of tumor score. At the time of sacrifice of a representative sample of animals (4 weeks after treatment) 1 of 5 animals treated with AdCMVIL-12 was free of tumor, 1 rat showed tumor progression, and in 3 rats tumor score was stabilized. In contrast, tumor progression was observed in all control rats. According to these observations, treatment with AdCMVIL-12 resulted in a substantial increase of survival as shown in Fig. 5B. Although all control animals had died before day 96, survival of AdCMVIL-12–treated rats at this time point was 60%. All AdCMVIL-12–treated animals, however, died by day 130.

To evaluate biochemical changes 1 month after therapy we considered together the 2 control groups because they were comparable with respect to tumor burden and survival. As shown in Fig. 6 treatment with AdCMVIL-12 was associated with a decrease in serum level of alanine transaminase and bilirubin and an increase of glycemia as compared with controls, indicating AdCMVIL-12 therapy was not associated with hepatotoxicity but rather with improved liver biochemistry in animals with widespread liver cancer.

Production of Cytokines Following Intratumoral Injection of AdCMVIL-12

To examine if AdCMVIL-12 treatment induced production of cytokines in our model, a time course of expression of IL-12 and IFN-γ after intratumoral injection of 5×10^9 pfu of either AdCMVIL-12 or AdCMVlacZ or saline was performed along a period of 7 days. We found that tumor-bearing rats treated with AdCMVIL-12 produced IL-12 with maximal values at day 1 after the administration of AdCMVIL-12. Expression of IL-12 lasted for 3 days, because circulating levels of IL-12 could not be detected on day 5 and 7 (Table 2). In animals receiving control vector AdCMVlacZ or saline, serum IL-12 was undetectable (Table 2). Since IL-12 is a strong inducer of IFN-γ,[8,14] we also measured the production of IFN-γ in sera from treated animals. As shown in Table 2, animals receiving AdCMVIL-12 produced high serum level of IFN-γ at all time points. INF-γ was not detectable in sera from animals receiving control vector AdCMVlacZ or saline.

AdCMVIL-12 Elicits Antitumoral NK Cell Response

We have explored whether the antitumor effects induced by AdCMVIL-12 were mediated by activation of NK and/or cytotoxic T lymphocytes. To this aim mononuclear cells were isolated from spleens of tumor-bearing rats at days 1, 3, 5, and 8 after administration of either AdCMVIL-12 or control vector AdCMVlacZ. In assays to analyze specific antitumor CTL activity we could not detect significant cytotoxicity against McA-RH7777 cells in tumor-bearing animals treated with either AdCMVIL-12 or AdCMVlacZ. To ascertain that the lack of specific CTL activity was not caused by any possible immunosuppressive effect of high doses of AdCMVIL-12,[44] we performed the CTL assays in tumor-bearing animals receiving lower doses of AdCMVIL-12 (10^9, 10^8, and 10^7 pfu). Still, no CTL responses against McA-RH7777 cells were found (data not shown).

As mentioned in the Materials and Methods, NK cell activity was measured using YAC-1 (NK-sensitive cells), McA-RH7777 (parental tumor cells), and CC531 (unrelated colon cancer) as target cells. As shown in Fig. 7A and 7B significant cytotoxic NK activity against McA-RH7777 and YAC-1 cells was elicited by intratumoral administration of AdCMVIL-12. NK cytotoxicity was highest at day 3 after therapy and declined rapidly on subsequent days. Some cytotoxicity was also detected when using unrelated colon cancer cells (CC531) as targets although this effect was clearly less notorious than with the other 2 cell lines (Fig. 7C). However, in animals receiving control vector AdCMVlacZ only a modest elevation in NK activity was observed. Addition of antibodies against CD4 or CD8 did not inhibit the cytotoxic effect on McA-RH7777 cells and antibody against MHC class I only slightly decreased cytotoxicity against these target cells (Fig. 8). Our data indicate that in our animal model of HCC AdCMVIL-12 strongly stimulates NK activity against tumoral cells.

AdCMVIL-12 Elicits Antiangiogenic Effects

To know whether nonimmunologic mechanisms, particularly antiangiogenesis, were involved in the regression of HCC induced by AdCMVIL-12 we analyzed the vascularization that

TABLE 2. Production of IL-12 and IFN-γ in Sera Following Treatment With AdCMVIL-12

	Day 1	Day 3	Day 5	Day 7
IL-12				
AdCMVIL-12	2026.73 ± 933.86	123.48 ± 97.41	ud	ud
AdCMVlacZ	ud	ud	ud	ud
Saline	ud	ud	ud	ud
IFN-γ				
AdCMVIL-12	1234.19 ± 298.07	778.58 ± 163.45	413.29 ± 74.53	938.12 ± 364.31
AdCMVlacZ	ud	ud	ud	ud
Saline	ud	ud	ud	ud

NOTE. Tumor-bearing rats were treated by intratumoral injection of 5×10^9 pfu of either AdCMVIL-12 (n = 4) or AdCMVlacZ (n = 4) or saline (n = 2). Sera were collected on days 1, 3, 5, and 7 after treatment.

Abbreviation: ud, undetectable.

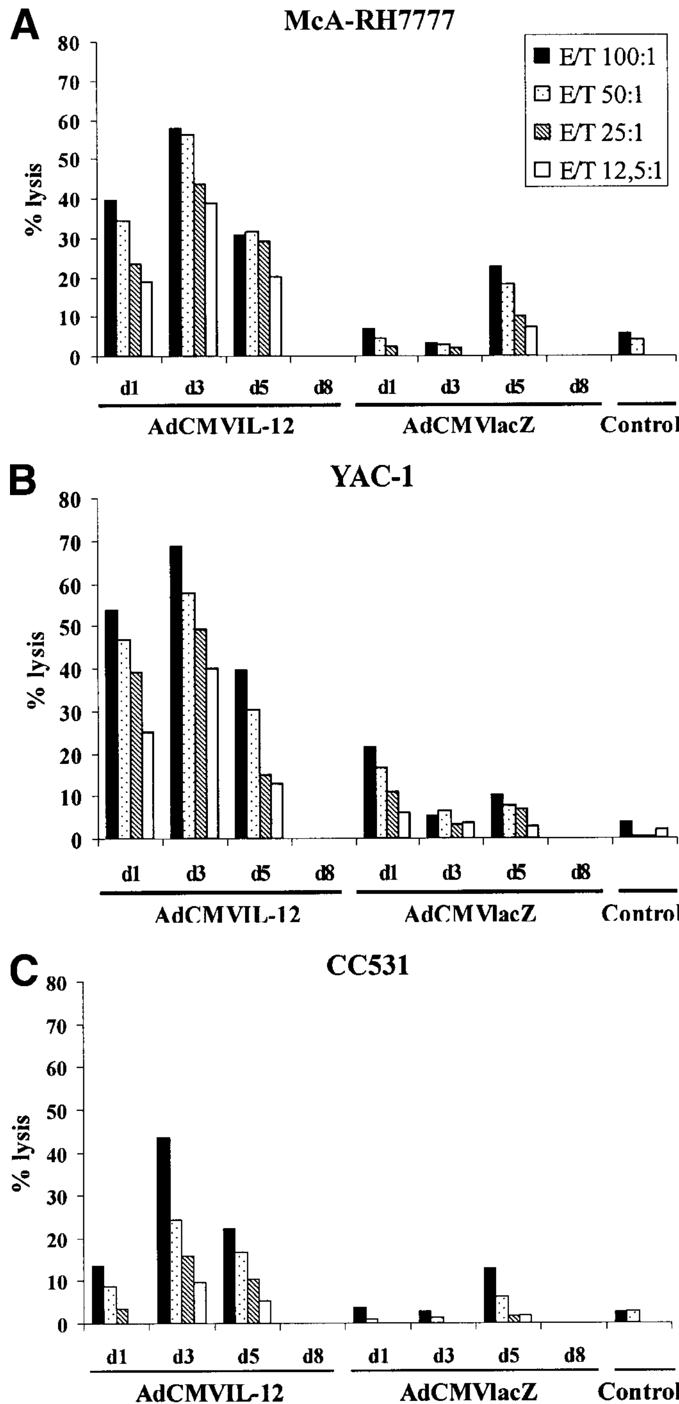

FIG. 7. NK activity in rats with HCC implanted in the liver that received intratumoral injections of either AdCMVIL-12, AdCMVlacZ, or saline. Splenocytes were isolated 1, 3, 5, and 8 days after therapy and assayed against ^{51}Cr-labeled parental tumor cells McA-RH7777 (A), NK-sensitive YAC-1 cells (B), and unrelated colon cancer CC 531 cells (C) at different effector to target cell ratios. Representative results of 2 independent experiments are shown.

develops in a Matrigel plug embedded in VEGF and placed into the liver. Animals with a tumor of 8 to 10 mm in diameter implanted in the liver were inoculated with VEGF-containing Matrigel into the same lobe and received at the same time an intratumor injection of 5×10^9 pfu of either AdCMVIL-12 or AdCMVlacZ. Ten days after treatment, the Matrigel plugs and surrounding liver tissue were obtained for histologic examination. As shown in Fig. 9A an intense invasion by endothelium-like cells was observed in Matrigel plugs from AdCMVlacZ-treated rats. In contrast, Matrigel plugs from rats that received AdCMVIL-12 were almost completely free of endothelium-like cells (Fig. 9B). Angiogenesis was quantitated in all plugs by measuring the number of endothelium-like cells on Matrigel sections using a computerized automatic analyzer. Figure 9C shows that AdCMVIL-12 treatment resulted in inhibition of VEGF-induced angiogenesis by 70%, as compared with cases treated with AdCMVlacZ.

DISCUSSION

IL-12 is a strong activator of cellular immunity, which has been shown to possess powerful antitumoral activities.[8-10] However, the use of recombinant IL-12 as an antitumoral agent is hampered by the toxicity of this cytokine when used at therapeutic doses.[22-25] This toxicity is mainly due to the ability of IL-12 to induce INF-γ production.[22,23] In contrast to the systemic administration of the recombinant protein, it seems possible that procedures allowing local synthesis of IL-12 may result in antitumoral effects without accompanying toxicity. This can be achieved by IL-12 gene delivery to the tumoral lesion or its surroundings. In this study we selected an adenoviral vector to transfer IL-12 to orthotopic HCC because of the ability of adenovirus to efficiently transduce hepatocytes and HCC cells.[34,36,45-48] Adenoviral vectors do not integrate into the host genome and the expression of the transgenes is transient lasting only 7 to 10 days.[49] This short period could nevertheless be sufficient for treatment of can-

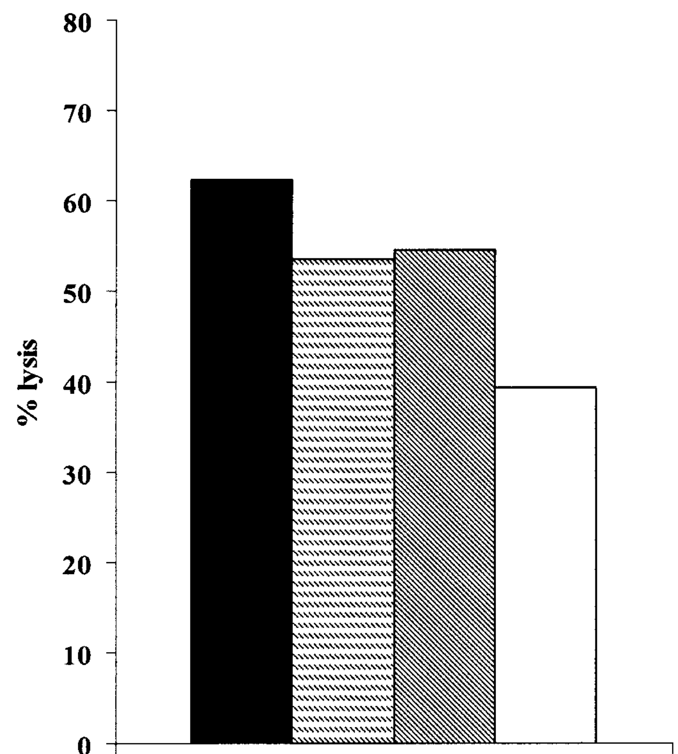

FIG. 8. Effect of anti-CD4, anti-CD8, and anti-MHC class I antibodies on NK activity. Splenocytes were isolated at days 3 from animals treated with AdCMVIL-12 (as in Fig. 7) and assayed against ^{51}Cr-labeled parental tumor McA-RH7777 cells in the presence of purified anti-rat CD4, anti-rat CD8a, and anti-rat RT1A at final concentration of 100 μg/mL, respectively. (■), Control; (▤), anti-CD8; (▧), anti-CD4; (□), anti-MHC I.

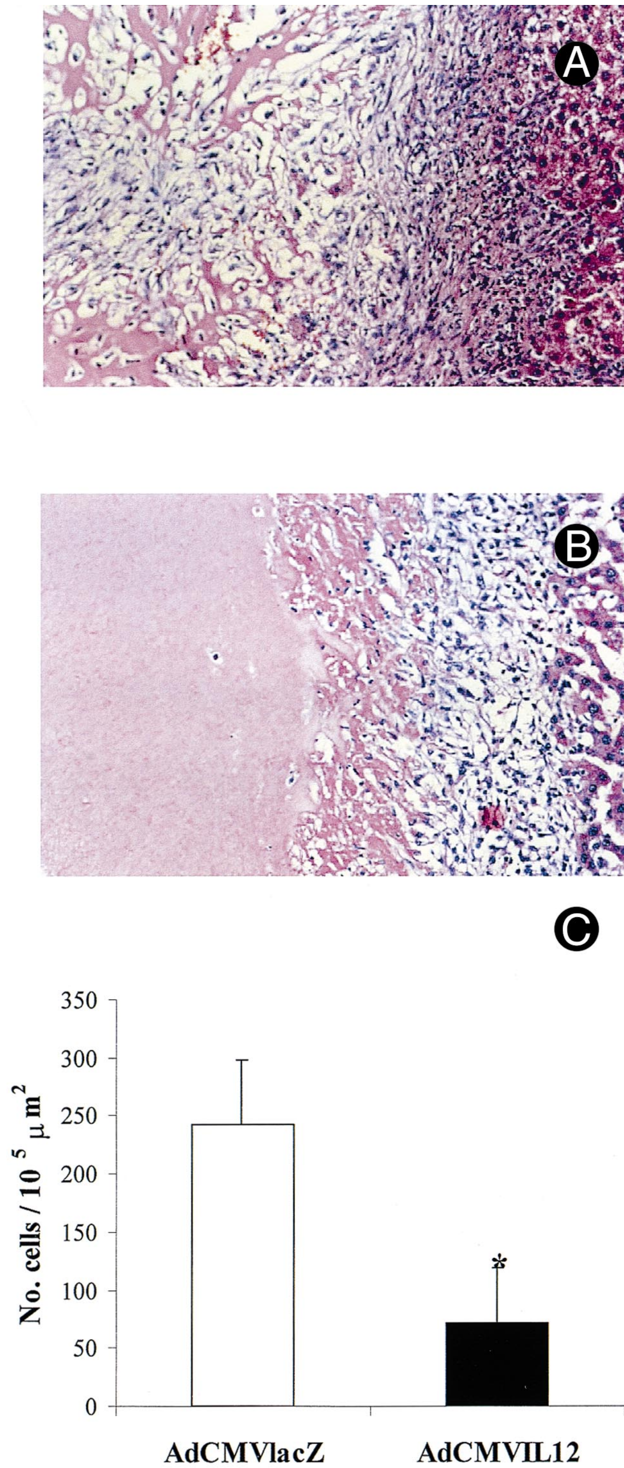

FIG. 9. Effect of AdCMVIL-12 therapy on VEGF-induced neovascularization of Matrigel plug implanted into the liver in animals with orthotopic HCC. A hepatic tumor (8-10 mm in diameter) was established in one lobe of liver. Animals were treated by intratumor injection of 5×10^9 pfu of either AdCMVIL-12 (n = 3) or AdCMVlacZ (n = 3) and Matrigel embedded with VEGF was injected into another lobe of liver at the same time. Ten days later, the Matrigel plugs and surrounding liver tissue were obtained for histologic examination. (A) Representative photomicrograph showing VEGF-induced neovascularization in Matrigel from animals that received control vector AdCMVlacZ. (B) Representative photomicrograph showing inhibition of VEGF-induced neovascularization in Matrigel from animals treated with AdCMVIL-12. (C) Quantitative analysis of angiogenesis was made by counting the number of endothelium-like cells in an area of 10^5 μm^2. Data are presented expressed as mean and standard deviation of 6 fields of each Matrigel plug. *$P < .01$ as compared with control animals.

cer, because after stimulation of an antitumor response with immunostimulatory cytokines the expression of the transgene may no longer be needed.

Our data show that intratumor injection of AdCMVIL-12 resulted in complete tumor elimination in 50% of rats with 1 big tumor (10-12 mm in diameter). In the model with 2 different HCCs implanted in the liver we found that AdCMVIL-12 therapy of only 1 tumor caused regression of the treated lesion but also of the untreated tumor nodule. Importantly this effect was accompanied by prolonged survival. Furthermore those rats that completely eliminated the tumor were resistant to new challenge with cancer cells given 3 months after complete regression of primary tumors, indicating the development of an efficient protective immunity against parental cell line. The antitumoral effect of AdCMVIL-12 is apparent not only on the treated neoplasm but also on other untreated tumors within the liver. These distant effects of locally administered AdCMVIL-12 may be a result of the ability of this vector to induce activation of immune responses and production of cytokines that can circulate from treated tumors to other intrahepatic neoplastic nodules. In our study the administration of AdCMVIL-12 resulted in a short-lived peak of serum IL-12 with highest values at day 1 and decline to undetectable level at day 5. However the production of IFN-γ was maintained at high levels at all the time points analyzed indicating a persisting activation of the immune system induced by IL-12. It is also worth noting that, due to the strong hepatotropism of adenoviruses, intratumoral injection of the vector results in transduction of the injected nodule but a proportion of the dose can escape to the general circulation infecting hepatocytes surrounding other liver tumors.[37] Thus IL-12 could be produced not only within the treated neoplasm but also around distant intrahepatic tumor nodules, making this therapeutic approach very attractive to treat patients with multinodular liver cancer.

Because transplanted tumors, even at an orthotopic site, might differ substantially from tumors that develop as a result of the occurrence of carcinogenic events, we have investigated the efficacy of AdCMVIL-12 therapy given via hepatic artery in DENA-induced liver cancer in rats. This is a model of multifocal HCC, which is extremely aggressive and difficult to treat. Recombinant IL-12 had already been shown to be able to display antitumoral effects in other models of carcinogen-induced tumors.[12,50] In previous studies we have used an adenovirus expressing the suicide gene thymidin-kinase (AdCMVtk) given by intraportal route to treat DENA-induced liver cancer.[36] Although this therapy had a potent antitumoral effect in this model it also caused a very high mortality among treated rats. In the present study AdCMVIL-12 induced 20% total tumor regression and significantly increased animal survival, although all animals died within 130 days. Thus, the results of this therapy are considerably better than those obtained with AdCMVtk and they open promising avenues to treat widespread HCC. The fact that despite early effects on tumor growth no long-term survival was achieved might be caused by the continuous generation of new neoplastic cells from preneoplastic lesions induced by DENA whereas the expression of IL-12 genes carried by first generation adenovirus was transient. Systemic administration of adenovirus can be successfully applied only once because of the development of neutralizing antibodies.[49] It seems possible that the development of transductionally efficient vectors, which could be administered repeatedly or the use of vectors allowing long-term (and drug-regulated) expression of the trans-

gene in the liver may improve the results of IL-12–based gene therapy of widespread liver cancer.

Activation of $CD8^+$ cells and stimulation of CTL activity have been shown to be involved in tumor regression induced by IL-12 in different animal tumor models.[10,35] In our system we could not detect specific antitumoral CTL activity after AdCMVIL-12 administration but we observed a strong stimulation of NK activity against parental tumor cells (McA-RH7777) and YAC-1 cells. It has been shown that stimulation of NK cells by IL-12 occurs via specific surface receptors[9] leading to enhanced production of IFN-γ and increased cytotoxic NK activity against tumor cells with low MHC class I expression,[9] a feature of McA-RH7777 cells used in this study (data not shown). Our data are in accordance with other published work in orthotopic prostate cancer model where treatment with IL-12 only induced activation of NK cells, but not tumor-specific CTL activity.[15] It is possible however that in our study AdCMVIL-12 might have produced low levels of specific CTL that would not be detectable in the CTL assay but that would be able to control *in vivo* tumor cell rechallenge after elimination of the primary neoplasm.

In this work we found that antiangiogenesis was one of the mechanisms contributing to tumor regression induced by gene transfer of IL-12 to the liver. The analysis of the vascularization of Matrigel plugs embedded with VEGF and implanted in the liver revealed that while Matrigel from animals treated with AdCMVLacZ exhibited abundant endothelium-like cells, there was 70% reduction in Matrigel capillaries and endothelium-like cells in rats that received AdCMVIL-12. These data are in agreement with recent reports showing that IL-12 inhibits new vessel formation in models of corneal vascularization in mice.[16,17] Neutralizing antibodies either to IFN-γ or IFN-inducible protein-10, a CXC chemokine induced by IFN-γ, substantially reduced the inhibition of angiogenesis induced by IL-12.[17] Thus, both IL-12 and the cytokine cascade activated by IL-12 may induce a diversity of biological changes including activation of NK cells and antiangiogenesis leading to tumor regression. These two effects, NK activation and antiangiogenesis, may in fact be closely related because a recent study shows that NK cells are required mediators of angiogenesis inhibition by IL-12 suggesting that cytotoxic damage of endothelial cells by NK cells is a potential mechanism by which IL-12 can suppress neovascularization.[21] However, the role of nonlymphocyte effector cells such as macrophages and neutrophils should also be considered because these cells may display antineoplastic effects and can be activated by the cytokine cascade set into motion by IL-12.[51]

In conclusion, AdCMVIL-12 is an efficient vector for treatment of HCC not only in animals with transplanted orthotopic tumor, but also in carcinogen-induced multifocal tumors at doses that do not induce obvious toxicity. Our data point to the convenience of considering IL-12–based gene therapy as a possible therapeutic option that should be evaluated in patients with widespread liver cancer.

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