Combining antiangiogenic therapy with immunotherapy exerts better therapeutic effects on large tumors in a woodchuck hepatoma model

Kai-Wen Huang\textsuperscript{a,b,c,1}, Hui-Lin Wu\textsuperscript{a,b,1}, Hsiu-Lin Lin\textsuperscript{d}, Po-Chin Liang\textsuperscript{d}, Pei-Jer Chen\textsuperscript{a,b,e}, Shih-Hui Chen\textsuperscript{f}, Hsin-I Lee\textsuperscript{g}, Pei-Yi Su\textsuperscript{h}, Wen-Hsuan Wu\textsuperscript{i}, Po-Huang Lee\textsuperscript{j}, Lih-Hwa Hwang\textsuperscript{a,f,2} and Ding-Shinn Chen\textsuperscript{a,2}

\textsuperscript{a}Hepatitis Research Center, Department of Surgery, and \textsuperscript{b}Department of Medical Imaging, National Taiwan University Hospital, Taipei 100, Taiwan; \textsuperscript{c}Graduate Institute of Clinical Medicine and \textsuperscript{d}Graduate Institute of Microbiology, National Taiwan University College of Medicine, Taipei 100, Taiwan; and \textsuperscript{e}Institute of Microbiology and Immunology, National Yang-Ming University, Taipei 112, Taiwan

Contributed by Ding-Shinn Chen, July 8, 2010 (sent for review April 30, 2010)

Cytokine and antiangiogenic gene therapies have proved effective in implanted hepatocellular carcinoma (HCC) models in which small tumor burdens were established in small rodents. These models, however, may not reflect large human HCCs, which are frequently detected at a stage when tumors are large and multifocal. In addition, HCC in patients is often associated with viral hepatitis. To investigate the effectiveness of a mixture type of gene therapy strategy on large tumors, we used the woodchuck model in which woodchuck hepatitis virus-induced HCCs are large and multifocal, simulating the conditions in humans. Adenoviruses encoding antiangiogenic factors (pigment epithelium-derived factor and endostatin) or cytokines (GM-CSF and IL-12) were delivered via the hepatic artery separately or in combination into woodchuck livers bearing HCCs. Our results showed that the mixture type of strategy, which contained two cytokines and two antiangiogenic factors, had better antitumor effects on large tumors as compared with monotherapy either with antiangiogenic or cytokine genes. The immunotherapy recruited significant levels of CD8\textsuperscript{T} cells that infiltrated the tumors, whereas the antiangiogenesis-based therapy significantly reduced tumor vasculature. The mixture type of gene therapy achieved both effects. In addition, it induced high levels of natural killer cells and apoptotic cells and reduced the levels of immunosuppressive effectors in the tumor regions. Hence, antiangiogenic therapy may provide the advantage of reducing immune tolerance in large tumors, making them more vulnerable to the immune reactions. Our study implies that in the future, the combination therapy may prove effective for the treatment of patients with advanced HCC.

antiangiogenic therapy \mid cytokine gene therapy \mid gene therapy \mid hepatocellular carcinoma \mid multifocal liver tumors

Hepatocellular carcinoma (HCC) is the third leading cause of cancer mortality worldwide, with a high incidence in Asia (1, 2). The most important risk factor for HCC is the persistent infection by hepatitis B virus (HBV) or hepatitis C virus (3, 4). For patients with HCC, surgical resection may provide a significant survival advantage. However, many patients are diagnosed in the late stage and cannot tolerate hepatectomy because of advanced cancer or poor liver function reserve (5). Hence, effective adjuvant or palliative therapies still remain to be developed.

To improve the treatment of HCC, preclinical animal studies are important because they allow us to evaluate the safety and effectiveness of unique therapeutic modalities. To date, animal models of HCC have been established mainly in rodents, either by implantation or by chemical induction. The primary liver tumor model, which develops spontaneously in its natural liver environment, is preferable to the implanted tumor models because it allows for the development of liver tumors that may exhibit immune escape properties, and multiple lesions are commonly seen in the primary liver tumor model. Nevertheless, none of the aforementioned rodent models can adequately represent the HCCs resulting from hepatitis viral infection.

The eastern woodchuck (Marmota monax) has been demonstrated to be a useful animal model for the study of hepatitis B and HBV-associated HCC (6). Chronic hepatitis can be induced in these animals by infection with the woodchuck hepatitis virus (WHV), a virus that shares a genomic structure and biological properties similar to human HBV (7). The woodchuck HCC, which usually develops within 1–4 y after WHV infection (8), is a naturally occurring tumor model that bears similarities to human HCC caused by HBV. The sizes of woodchucks and their tumors are more suitable for imaging diagnosis than those of smaller rodents, such as mice or rats, and thus enable an easier translation of interventional procedures to future human application. Hence, the woodchuck HCC model is more relevant than those in smaller rodents when searching for therapeutic strategies for human HCC.

We have demonstrated previously that the antitumor effect of combining GM-CSF and IL-12 gene therapies was superior to that of monotherapy with either cytokine (9). Significantly high levels of natural killer T (NKT) and natural killer (NK) cells and concurrently high levels of IFN-\(\gamma\) were induced by the combination therapy but not by either cytokine monotherapy, revealing synergy between the two cytokines with different mechanisms of action. Similarly, the combination of two antiangiogenic factors, each impairing angiogenesis in distinct pathways, can also be used to broaden therapeutic efficacy and prevent a potentially refractory state (10). Nevertheless, immunotherapy alone or antiangiogenic therapy alone may fail in the case of advanced tumors. Integrating tumor immunotherapy and antiangiogenic therapy has been applied in several studies for the treatment of advanced cancers (11–13). To augment the antitumor effects of gene therapy on larger HCCs associated with WHV infection, we assessed the antitumor effects of adenovirus-mediated concurrent delivery of IL-12 and GM-CSF genes, of endostatin (ED) and pigment epithelium-derived factor (PEDF) genes, or of the four genes together in woodchucks bearing large HCCs. Our data revealed that at the same total viral dose, the mixture type of strategy, which combined four genes together, exhibited synergistic and much better antitumor effects than either of the monotype gene therapy strategies.


The authors declare no conflict of interest.

1K.-W.H. and H.-L.W. contributed equally to this work.

2To whom correspondence may be addressed. E-mail: chends@ntu.edu.tw or lhhwang@ym.edu.tw.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1009534107/-/DCSupplemental.
Results

Woodchucks Chronically Infected with WHV Developed Multifocal Liver Tumors of Various Sizes. Sixteen woodchucks were enrolled in this study. All but 1 woodchuck showed progressive elevation of serum γ-glutamyl transpeptidase (γ-GT) levels, and ultrasound examination confirmed the presence of hepatic tumors in all 16 animals. The ultrasonic pattern of woodchuck hepatic tumors was characterized by a hypoechoic area. Abdominal computed tomography (CT) scanning demonstrated that hepatic tumors were mostly spheroid or ovoid in shape, strongly enhanced in arterial phase, and weakly enhanced in venous phase. No extrahepatic tumor metastases were detected. In total, 49 tumor nodules were found in these 16 woodchucks. Two animals had a solitary tumor, and the rest had multifocal tumors. Tumor diameters ranged from 3 to 60.6 mm. The gross findings of the tumors in one of the woodchucks are shown in Fig. S1A. Histological examination proved that all these tumors were HCC (Fig. S1B).

Adenovirus-Mediated Immunotherapy or Antiangiogenic Gene Therapy Significantly Regressed the WHV-Induced HCCs. Adenoviruses carrying a LacZ gene (Ad/LacZ), cytokine genes (Ad/GM-CSF and Ad/IL-12), antiangiogenic factor genes (Ad/PEDF and Ad/ED), or a combination of all four adenoviruses were infused into the hepatic artery of tumor-bearing animals. After adenovirus infusion, all animals had a poor appetite for 3–5 d and then recovered.

Tumor sizes were measured before and 14 d after adenovirus injection by laparotomy and intraoperative ultrasound examination. Some animals also received abdominal CT to monitor tumor progression. On euthanasia of the animals, tumor sizes were measured by calipers and the results were comparable to those obtained by ultrasound examination. As illustrated in Fig. 1A, the tumor volumes in the animals treated with Ad/LacZ increased by 74.0 ± 58.5% compared with the tumor volumes before treatment. In contrast, following treatment with Ad/(G + I), Ad/(P + E), or Ad/(4-in-1), tumor volumes were 24.2 ± 16.5% (P < 0.005 compared with the Ad/LacZ group), 44.0 ± 16.3% (P < 0.001), and 9.9 ± 7.7% (P < 0.005) of the pretreatment values, respectively. Because the total viral doses administered in these three treatment groups were the same, the tumor reduction rates indicate that the 4-in-1 gene therapy strategy induced better antitumor effects than any of the monotherapies, although the difference between the Ad/(G + I)-treated and the Ad/(4-in-1)-treated groups was not statistically significant.

Mixture Type of Gene Therapy Strategy Was More Effective Than Immunotherapy Alone for the Treatment of Large Tumors. When the tumors were arbitrarily stratified as small tumors (volume <8,000 mm³) or large tumors (volume >8,000 mm³), it was found that the Ad/(4-in-1)-treatment was much more effective on large tumors than the Ad/(G + I) treatment (13.08 ± 4.3 vs. 35.14 ± 3.5% of original tumor volume remaining; P < 0.005) but that the effects were comparable between the two treatment strategies on small tumors (7.57 ± 2.42 vs. 8.35 ± 2.31%; P = 0.677) (Fig. 1B). The results indicated that immunotherapy alone was sufficient to treat smaller tumors, whereas a combination of the two different strategies was needed to treat larger tumors. Representative photographs of tumor reduction detected by ultrasound examination or by abdominal CT scan are shown in Fig. 2.

Cytokine-Based Gene Therapy Increased the Amount of Lymphocytes Infiltrating the Tumors, and Antiangiogenesis-Based Gene Therapy Reduced Microvessel Density in the Tumors. Few immunological reagents are available for the study of woodchucks. After a series of pilot testing, we found that a rabbit anti-human CD3 antibody (DakoCytomation) was able to cross-react with woodchuck lymphocytes. Thus, we used it to detect the presence of CD3⁺ T cells in tumor tissues by immunohistochemical (IHC) staining. The data indicated that both Ad/(G + I) (Fig. 3B) and Ad/(4-in-1) (Fig. 3D) treatments significantly increased infiltration of CD3⁺ T cells into the tumors but that there was no significant difference between the two treatments. The Ad/(P + E) treatment (Fig. 3C) induced low but significant levels of T lymphocytes in the tumors as compared with the control group (Fig. 3E), which was likely attributable to the bystander effect of antiangiogenic gene therapy.

We examined whether the antiangiogenesis-based therapies reduced microvessel density (MVD) in the tumors. We used a mouse antibody against human α-smooth muscle actin (α-SMA) to detect the microvessels (14) because this antibody could cross-react with the α-SMA of various animal species. The data revealed that the MVD in the tumors was significantly reduced by the Ad/(P + E) (Fig. 4C) or the Ad/(4-in-1) (Fig. 4D) treatment but that it was not altered by the Ad/(G + I) treatment (Fig. 4B) as compared with the Ad/LacZ treatment (Fig. 4A). Interestingly, the MVD in the Ad/(4-in-1) group was slightly lower than that in the Ad/(P + E) group (Fig. 4E), suggesting that there were synergistic antiangiogenic effects in the 4-in-1 strategy.

Multiple Mechanisms Might Contribute to the Marked Antitumor Effects of the Mixture Type of Gene Therapy Strategy. To investigate whether there were other mechanisms involved in the antitumor effects of the 4-in-1 strategy, we analyzed the expression of some effector genes in the adenovirus-treated tumors by quantitative RT-PCR using the available woodchuck sequences. In the animals treated with Ad/(4-in-1), the expression of NKP46, a triggering molecule essential for the cytolytic antitumor activity of NK cells (15), was significantly higher (P < 0.01) (Fig. 5A), whereas the expression of cytotoxic T-lymphocyte antigen (CTLA)-4 (Fig. 5B) and programmed death (PD)-1 (Fig. 5C), both of which are inhibitory...
The tumor shrank after treatment.

Another large HCC nodule in the right liver lobe was observed by ultrasound and CT scan. Arrows depict the boundary of the tumor. The tumor observed by abdominal CT scan before (long and short axes). Another large HCC nodule in the right liver lobe was observed by abdominal CT scan before (long and short axes).

Levels decreased in all woodchucks with effective therapy. The WHV woodchucks had reduced, whereas the serum γ-GT levels continued to increase even though the tumor volumes in these animals increased after effective treatments; however, in some animals, the tumor volumes in these tumors were based on examination of 10 high-power fields (HPFs) by microscopic examination. Data represent mean value ± SEM. ***p < 0.001 compared with the Ad/LacZ control group (unpaired Student’s t test).

Finally, the results of TUNEL staining demonstrated that the Ad/(4-in-1) treatment induced much more apoptosis in the tumors than the Ad/(G + I) treatment, especially in large tumors. Taken together, the results indicated that the combination of immunotherapy with antiangiogenic gene therapy might involve multiple mechanisms to kill tumor cells. The synergism of combining two strategies was much more obvious in large tumors than in small tumors.

**Adenovirus-Mediated Gene Therapies Did Not Induce Significant Liver Damage.** Animals receiving cytokine or antiangiogenic gene therapy revealed no significant changes in the serum levels of aspartate aminotransferase (AST) or alanine aminotransferase (ALT) following adenovirus treatment as compared with the pretreatment data (Table S1). In most woodchucks, the serum γ-GT levels decreased after effective treatments; however, in some animals, the levels continued to increase even though the tumor volumes in these woodchucks had reduced, whereas the serum α-fetoprotein (AFP) levels decreased in all woodchucks with effective therapy. The WHV α-fetoprotein (AFP) and γ-GT (18, 19). Like most hepatitis B-induced HCCs in humans, they develop in their natural environment, with a background of active hepatitis, and immune escape mechanisms evolve that may negatively affect immunotherapeutical approaches (20). All these characteristics of woodchuck HCC and the outbred nature of the animals make the woodchuck a more clinically relevant animal model to study with the goal of developing prophylactic and therapeutic strategies for human HCC (7, 21, 22). Accordingly, our study used the woodchuck HCC model to investigate the feasibility and safety of adenovirus-mediated immunotherapy and antiangiogenic gene therapy for HCCs. The results are revealing and interesting. At the same viral dose, the combination of immunotherapy with antiangiogenic gene therapy was particularly effective for large tumors compared with either monotype of therapeutic strategy. The combination of antiangiogenic and immunotherapeutical regimens appeared to generate conditions that were beneficial to the regression of large tumor burdens but not observed in small tumors treated with the combined therapy or with immunotherapy alone (vide infra).

**Discussion**

Woodchuck HCCs induced by chronic WHV infection often present as multifocal nodules in the liver and are associated with elevated levels of serum AFP and γ-GT (18, 19). Like most hepatitis B-induced HCCs in humans, they develop in their natural environment, with a background of active hepatitis, and immune escape mechanisms evolve that may negatively affect immunotherapeutical approaches (20). All these characteristics of woodchuck HCC and the outbred nature of the animals make the woodchuck a more clinically relevant animal model to study with the goal of developing prophylactic and therapeutic strategies for human HCC (7, 21, 22). Accordingly, our study used the woodchuck HCC model to investigate the feasibility and safety of adenovirus-mediated immunotherapy and antiangiogenic gene therapy for HCCs. The results are revealing and interesting. At the same viral dose, the combination of immunotherapy with antiangiogenic gene therapy was particularly effective for large tumors compared with either monotype of therapeutic strategy. The combination of antiangiogenic and immunotherapeutical regimens appeared to generate conditions that were beneficial to the regression of large tumor burdens but not observed in small tumors treated with the combined therapy or with immunotherapy alone (vide infra).
The mechanisms underlying the profound antitumor effects of the mixture type of gene therapy strategy are believed to be multifocal. First, antangiogenic gene therapy could induce tumor regression by reducing tumor vasculature, which deprives the tumors of blood supply, leading to the induction of necrosis or apoptosis in tumor cells. Because the growth of large tumors requires more vasculature (23), antangiogenic therapy exhibits a greater antitumoral effect on large tumors. This may explain why the 4-in-1 strategy is superior to immunotherapy alone for the treatment of large HCCs in our study. Second, cytokine gene therapy can recruit macrophages or dendritic cells (DCs) to tumor sites, where they engulf apoptotic cells or tumor antigens released from the dead cells and stimulate antitumor immune responses. Third, for reasons that are currently unknown, the 4-in-1 strategy significantly stimulates NK cell activity in the tumors, which either exhibit immunosuppressive activities [i.e., Treg cells (16)] or exhibit an exhausted phenotype and impaired effector function [i.e., tolerant T cells (17)]. In the tumor environment, especially in large tumors, immunosuppressive factors, such as TGF-β or VEGF, are present in abundant levels (24, 25), which can impair the maturation of DCs from progenitors (26); the immature DCs then provide deficient costimulation and induce tolerance in CD8+ T cells (27). Previous research has shown that even when an appropriate priming event took place in situ and a potent antitumor response was generated, the effector cell population could revert to a tolerant state after entering the tumor (28). In our study, both Ad/(G + I) and Ad/(4-in-1) treatments elicited comparable levels of CD3+ T lymphocytes infiltrating the tumors (Fig. 3). However, the activated T cells in the Ad/(G + I)-treated animals might have become tolerant after entering the large tumors, as shown in Fig. 5 B and C [Ad/LacZ vs. Ad/(G + I)], which may account for the reduced efficacy of Ad/(G + I) treatment in large tumors. On the other hand, the antangiogenic therapy included in the 4-in-1 strategy could act as an auxiliary therapy that reduced the number of Treg or tolerant T cells in the tumors (Fig. 5 B and C); the mechanism, however, remains to be determined. As a result, functional effector T cells or NK cells induced by immunotherapy increased significantly, which could account for the markedly high levels of apoptosis in large tumors induced by the Ad/(4-in-1) treatment (Fig. 5D). In agreement with our observation, it was previously shown that combination of immunotherapy with an antangiogenic therapy using VEGF blockade could increase the ratio of effector T cells to Treg cells in the tumor microenvironment and result in enhancement of the efficacy of immunotherapy (29). Therefore, the presence of antangiogenic factors in the tumor environment may render tumors vulnerable to the immune system.

Compared with commonly used small animal models, the woodchuck is a complex and expensive animal model and is more difficult to handle. Nevertheless, it can provide more clinically relevant information to assess previously undescribed therapeutic approaches for hepatadivirus-induced HCC in humans. Thus far, only three studies have used woodchucks to evaluate the efficacy of viral vector-mediated gene therapies on the treatment of
the 4-in-1 therapeutical strategy the immune cells induced by the factors may eventually close the normalization window. Hence, in or excessive suppression of tumor vasculature by antiangiogenic therapy in the future.

Ad/(4-in-1) treatment are encouraging. The 90% tumor volume resembled the normal vasculature (32). The remaining vasculatures are remolded so that they more closely resemble the normal vasculature (32). The “normalized” blood vessels have increased transport capability, which more than compensates for the decrease in the total number of patent blood vessels and allows efficient entry of drugs or immune cells (32). However, the increase in vascular patency is transient. Sustained or excessive suppression of tumor vasculature by antiangiogenic factors may eventually close the normalization window. Hence, in the 4-in-1 therapeutic strategy the immune cells induced by the immunotherapy might infiltrate efficiently into tumors during this window period, which might explain why there was high T-cell infiltration in the face of poor vascularization.

Although adenoaviruses-mediated gene expression is transient and the vector cannot be injected repeatedly, our results from the Ad/(4-in-1) treatment are encouraging. The 90% tumor volume reduction by a single adenovirus injection may facilitate downstaging of the cancer status, thus increasing the manageability of advanced liver tumors.

In conclusion, using a clinically relevant animal model, we have demonstrated remarkable antitumor effects of a mixture of gene therapy strategies containing PEDF, ED, IL-12, and GM-CSF. The strategy is particularly meaningful for the treatment of HCCs with large tumor burdens and may encourage the clinical application of combined immunotherapy and antiangiogenic therapy in the future.

Materials and Methods

Animals and Screening of Tumors. Eastern woodchucks (M. monax) chronically infected with WHV were purchased from Northeastern Wildlife and housed in the animal facility at National Taiwan University College of Medicine with humane care. All experimental procedures were approved by and carried out in compliance with the guidelines set by the Animal Care and Use Committee of National Taiwan University College of Medicine. Serum γ GT activity was measured at 3-mo intervals to monitor development of HCC in woodchucks (33). Ultrasound examination of the liver was performed to confirm the presence of HCC. In total, 16 animals bearing one or more liver tumors were included in this study, with the smallest tumor being greater than 3 mm in diameter, as determined by ultrasound examination.

Construction of Adenoviral Vectors. Woodchuck GM-CSF cdNA was obtained by RT-PCR of RNA prepared from mitogen-stimulated woodchuck peripheral blood mononuclear cells, as previously described (34). Human ED and PEDF cdNA was obtained by RT-PCR of RNA from human liver. A DNA fragment encoding the 24 amino acids (MAAGPRTSVLLAFFALLPLWQTOEV) of the signal peptide of porcine growth hormone was cloned upstream of the ED or the PEDF fragment to produce an in-frame fusion protein, allowing for the secretion of ED or PEDF on expression. All the genes were under the control of a CMV promoter, and their sequences were verified by DNA sequencing. The adenoviral vector containing a woodchuck GM-CSF cdNA (Ad/GM-CSF), a human ED cdNA (Ad/ED), a human PEDF cdNA (Ad/PEDF), or a control LacZ gene (Ad/LacZ) was constructed using the AdEasy system (Stratagene), as previously described (35). Ad/IL-12, which contained a murine single-chain IL-12 gene encoding the two IL-12 subunits linked by a polypeptide linker (36), was kindly provided by B. L. Chiang at National Taiwan University Hospital. All the recombiant adenoviruses were amplified in 293 cells and purified using the AdEasy virus purification kit (Stratagene) following the manufacturer’s instructions.

Diagnostic Imaging to Determine Tumor Sizes. Liver tumors were detected, and the sizes were measured by intraoperative ultrasound (SSA-320A; Toshiba Co., Ltd.) before and 2 wk after adenovirus injection. The liver was exsanguinated and the longest and shortest axes of all the detected tumors were measured. Tumor volume was calculated using this formula: volume = shortest axis × longest axis × 0.52. During follow-up by ultrasonography, the same section plane was examined to ensure comparable measurements of the tumor size. In some animals, whole-body CT scanning was also performed to follow tumor progression. Biphasic whole-body CT was performed with a 64-slice CT scanner (Lightspeed VCT; General Electric) under a helical full scan (80 kV, 80 mA). Scans were ranged from the thoracic inlet to the pelvis and was conducted before and after i.v. administration of the contrast medium, Angiografin (1 mL/kg body weight; Schering). Postprocessing with reconstruction of the images at coronal, sagittal, and transverse planes was performed for 3D evaluation of the hepatic tumors.

In Vivo Animal Experiments. All the tumor-bearing woodchucks were randomly divided into four groups for treatment: (i) AdLacZ group (n = 4, tumor number = 9), which received 2 × 10^10 pfu AdLacZ per animal; (ii) Ad/(G + I) group (n = 7, tumor number = 24), which received immunotherapy with 1 × 10^10 pfu Ad/GM-CSF and 1 × 10^10 pfu Ad/IL-12 per animal; (iii) Ad/P + E group (n = 2, tumor number = 4), which received antiangiogenic therapy with 1 × 10^10 pfu Ad/PEDF and 1 × 10^10 pfu Ad/ED per animal; and (iv) Ad/(4- in-1) group (n = 3, tumor number = 12), which received 5 × 10^10 pfu of each of the four aforementioned adenoviruses per animal. After a 12-h fast, animals underwent midline laparotomy and were anesthetized with i.m. injection of 5 mg/kg of xylazine and 50 mg/kg of ketamine. The liver was exposed, liver tumors were located and measured by ultrasound, and the common hepatic artery was identified and looped by a fine tap. A 26-gauge injection needle was inserted into the hepatic artery under an operating microscope (20× magnification), and adenoviruses in 300 μL of PBS were infused into the artery. After injection, the laparotomy wound was sutured and the animals were returned to separate cages after i.m. administration of prophylactic antibiotics (0.2 MU/kg of penicillin).

Immunohistochemistry and Histological Staining. Tumor specimens were diagnosed as HCC based on the established classification of neoplastic liver lesions of animals. IHC staining was performed on 5-μm paraffin sections of posttreatment tumor tissues obtained 2 wk after adenovirus injection. After antigen retrieval by boiling the slides in Tris-EDTA buffer (pH 9.0) for 15 min, the sections were stained with rabbit anti-human α-SMA (1:100 dilution; DakoCytomation) to detect lymphocytes infiltrating into the tumors or with a mouse anti-human α-SMA monoclonal antibody (1:50 dilution; DAKO) to detect tumor vasculature. These two antibodies have been previously tested and proven to cross-react with corresponding antigens in woodchucks. For CD3 staining, the sections were washed and further incubated with a secondary antibody, rabbit anti-mouse IgG (1:200 dilution), and then developed similarly. Apoptotic cells were detected by TUNEL assay using an in situ detection kit (Roche) following the manufacturer’s instructions.

Huang et al.
sequences are listed in Table S2.

gene. The mRNA level of glyceraldehyde 3-phosphate dehydrogenase was dilutions with de ned copy numbers of a plasmid containing the target gene. The mRNA level of glyceraldehyde 3-phosphate dehydrogenase was also determined and used as an internal control for normalization. Primer sequences are listed in Table S2.

Acknowledgments.
This work was supported by Grant NSC98-3112-B-010-023 (to L.-H.H.) and Grant NSC93-3112-B-002-009 (to H.-L.W.) from the National Science Council of Taiwan and Grant 96-M-005 (to K.-W.H.) from the National Taiwan University Hospital.

Statistics. All the data are expressed as means with SD, and betweenthe study and control groups were determined by the Mann–Whitney U test or the unpaired Student’s t test where appropriate. All the grouped data were evaluated with computer software (SPSS/10, SPSS, Inc.). Two-tailed P values of less than 0.05 were considered statistically significant.

Serum Biochemical Parameters and AFP. Biochemical analysis for serum AST, ALT, and γ-GT activities was performed before and 2 wk after adenovirus treatment by means of an automatic biochemistry analyzer (8080R, Toshiba), whereas serum AFP levels were determined by an in-house established sandwich ELISA method (Materials and Methods).

Supporting Information

Huang et al. 10.1073/pnas.1009534107

SI Materials and Methods
The serum levels of woodchuck AFP were determined by sandwich ELISA. Each serum sample was diluted serially and incubated in a 96-well plate precoated with rabbit polyclonal anti-human AFP antibody (DAKO). A standard solution of woodchuck recombinant AFP purified from *Escherichia coli* was also diluted at concentrations of 3 to 50 ng/mL in diluent (1% BSA in PBS). Diluted serum samples and standards were incubated at room temperature for 4 h. After washing the plates five times with Tris-buffered saline containing 0.05% Tween 20, the HRP-conjugated rabbit polyclonal anti-human AFP antibody (DAKO) was added (1:1,250 dilution) and incubated at room temperature for 2 h. 3,3',5,5'-tetramethylbenzidine (TMB) substrate was then added for 10 min, and the reaction was stopped with 1 N H<sub>2</sub>SO<sub>4</sub>. Absorption of the samples and standards was measured at 450 nm. Diluted samples had values within the range of standards used to calculate the concentration of AFP.

Fig. S1. Representative photographs of woodchuck HCC. (A) Gross appearance of woodchuck HCC. The tumors were white to brown and paler than the surrounding parenchyma. The appearance of tumors resembled human HCC: soft, fragile, and sometimes well-encapsulated. tu, small tumor; Tu, large tumor. (B) Microscopic characteristics of woodchuck HCC. Histological analysis of the hepatic nodules showed the typical trabecular structures of HCC. Cancer cells were eosinophilic with H&E staining, and an increased nucleus-to-cytoplasm ratio was noted. (Magnification: 100×.)
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Animal</th>
<th>γ-GT before (U/L)</th>
<th>γ-GT after (U/L)</th>
<th>ALT before (U/L)</th>
<th>ALT after (U/L)</th>
<th>AST before (U/L)</th>
<th>AST after (U/L)</th>
<th>AFP before (μg/mL)</th>
<th>AFP after (μg/mL)</th>
<th>WHeAg before</th>
<th>WHeAg after</th>
<th>Virus titers before (genomes/mL)</th>
<th>Virus titers after (genomes/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad/LacZ</td>
<td>L1</td>
<td>151.0</td>
<td>78.0</td>
<td>17.0</td>
<td>21.0</td>
<td>109.0</td>
<td>142.0</td>
<td>8.2</td>
<td>22.5</td>
<td>212.8</td>
<td>220.63</td>
<td>3.2E + 10</td>
<td>3.1E + 10</td>
</tr>
<tr>
<td></td>
<td>L2</td>
<td>135.0</td>
<td>37.5</td>
<td>22.5</td>
<td>20.0</td>
<td>185.0</td>
<td>77.5</td>
<td>2,531.2</td>
<td>357*</td>
<td>155.6</td>
<td>162.34</td>
<td>2.2E + 08</td>
<td>5.2E + 08</td>
</tr>
<tr>
<td></td>
<td>L3</td>
<td>416.0</td>
<td>378.0</td>
<td>16.0</td>
<td>15.0</td>
<td>97.0</td>
<td>153.0</td>
<td>789.0</td>
<td>416.0</td>
<td>248.5</td>
<td>252.7</td>
<td>6.0E + 08</td>
<td>7.2E + 09</td>
</tr>
<tr>
<td></td>
<td>L4</td>
<td>51.0</td>
<td>32.0</td>
<td>11.0</td>
<td>15.0</td>
<td>69.0</td>
<td>185.0</td>
<td>270.7</td>
<td>153.4</td>
<td>283.9</td>
<td>270.7</td>
<td>1.6E + 09</td>
<td>2.0E + 10</td>
</tr>
<tr>
<td>Ad/(G + I)</td>
<td>GI1</td>
<td>5.0</td>
<td>12.5</td>
<td>20.0</td>
<td>20.0</td>
<td>37.5</td>
<td>77.5</td>
<td>59.1</td>
<td>11.9</td>
<td>178.85</td>
<td>178.85</td>
<td>9.9E + 10</td>
<td>2.1E + 11</td>
</tr>
<tr>
<td></td>
<td>GI2</td>
<td>38.0</td>
<td>29.0</td>
<td>26.0</td>
<td>15.0</td>
<td>49.0</td>
<td>162.0</td>
<td>68.2</td>
<td>23.7</td>
<td>17.71</td>
<td>1.83</td>
<td>5.8E + 06</td>
<td>7.6E + 06</td>
</tr>
<tr>
<td></td>
<td>GI3</td>
<td>30.0</td>
<td>19.0</td>
<td>16.0</td>
<td>27†</td>
<td>114.0</td>
<td>563†</td>
<td>386.2</td>
<td>185.8</td>
<td>168.24</td>
<td>ND</td>
<td>2.7E + 08</td>
<td>1.5E + 09</td>
</tr>
<tr>
<td></td>
<td>GI4</td>
<td>29.0</td>
<td>38.0</td>
<td>10.0</td>
<td>13.0</td>
<td>40.0</td>
<td>109.0</td>
<td>478.1</td>
<td>40.6</td>
<td>89.74</td>
<td>119.03</td>
<td>2.3E + 08</td>
<td>6.5E + 08</td>
</tr>
<tr>
<td></td>
<td>GI5</td>
<td>252.0</td>
<td>97.0</td>
<td>9.0</td>
<td>3.0</td>
<td>28.0</td>
<td>88.0</td>
<td>8,016.5</td>
<td>542.2</td>
<td>289.2</td>
<td>200.6</td>
<td>1.9E + 09</td>
<td>3.5E + 09</td>
</tr>
<tr>
<td></td>
<td>GI6</td>
<td>16.0</td>
<td>16.0</td>
<td>9.0</td>
<td>8.0</td>
<td>32.0</td>
<td>35.0</td>
<td>3,460.5</td>
<td>627.2</td>
<td>308.65</td>
<td>446.0</td>
<td>2.6E + 07</td>
<td>3.2E + 09</td>
</tr>
<tr>
<td></td>
<td>GI7</td>
<td>7.0</td>
<td>3.0</td>
<td>24.0</td>
<td>14.0</td>
<td>157.0</td>
<td>100.0</td>
<td>3,839.5</td>
<td>822.3</td>
<td>335.61</td>
<td>335.38</td>
<td>9.9E + 07</td>
<td>1.2E + 10</td>
</tr>
<tr>
<td>Ad/(P + E)</td>
<td>PE1</td>
<td>338.0</td>
<td>208.0</td>
<td>20.0</td>
<td>35.0</td>
<td>50.0</td>
<td>173.0</td>
<td>18.4</td>
<td>11.8</td>
<td>179.44</td>
<td>158.31</td>
<td>3.4E + 06</td>
<td>3.4E + 07</td>
</tr>
<tr>
<td></td>
<td>PE2</td>
<td>16.0</td>
<td>16.0</td>
<td>9.0</td>
<td>8.0</td>
<td>32.0</td>
<td>35.0</td>
<td>516.0</td>
<td>472.8</td>
<td>336.8</td>
<td>324.7</td>
<td>4.6E + 09</td>
<td>4.3E + 10</td>
</tr>
<tr>
<td>Ad/(4 in 1)</td>
<td>C1</td>
<td>643.0</td>
<td>141.0</td>
<td>21.0</td>
<td>14.0</td>
<td>55.0</td>
<td>71.0</td>
<td>197.0</td>
<td>170.5</td>
<td>195.82</td>
<td>218.23</td>
<td>1.6E + 10</td>
<td>2.6E + 10</td>
</tr>
<tr>
<td></td>
<td>C2</td>
<td>1,027.0</td>
<td>427.0</td>
<td>16.0</td>
<td>15.0</td>
<td>26.0</td>
<td>49.0</td>
<td>1,578.1</td>
<td>148.5</td>
<td>199.02</td>
<td>225.0</td>
<td>3.1E + 10</td>
<td>3.5E + 10</td>
</tr>
<tr>
<td></td>
<td>C3</td>
<td>64.0</td>
<td>71.0</td>
<td>20.0</td>
<td>14.0</td>
<td>216.0</td>
<td>240.0</td>
<td>70.3</td>
<td>22.8</td>
<td>283.9</td>
<td>268.8</td>
<td>5.1E + 08</td>
<td>9.1E + 09</td>
</tr>
</tbody>
</table>

ND, not determined; WHeAg, woodchuck hepatitis virus e antigen.
*L2 animal received surgical resection of one large tumor before gene therapy.
†GI3 animals had peritonitis.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WK-NKp46 Forward</td>
<td>CTCAAGAAGGGAGGAGTCAG</td>
</tr>
<tr>
<td>Reverse</td>
<td>CTGCACAGGAGCATCAC</td>
</tr>
<tr>
<td>WK-CTLA-4 Forward</td>
<td>GGAGCTCATGACCCACCAC</td>
</tr>
<tr>
<td>Reverse</td>
<td>TGGTTCTGGTTCAATGACAT</td>
</tr>
<tr>
<td>WK-PD-1 Forward</td>
<td>TGCCCGAGGAGGCTTGACTTCACAT</td>
</tr>
<tr>
<td>Reverse</td>
<td>TTCTCTGGTCACCTGAG</td>
</tr>
<tr>
<td>WK-GAPDH Forward</td>
<td>AACTCCCTCAAGATTGTCAG</td>
</tr>
<tr>
<td>Reverse</td>
<td>GCACACGGAAGCCAT</td>
</tr>
</tbody>
</table>

WK, woodchuck.