Large Nontransplanted Hepatocellular Carcinoma in Woodchucks: Treatment With Adenovirus-Mediated Delivery of Interleukin 12/B7.1 Genes

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Background: Cytokine-based gene therapy strategies efficiently stimulate immune responses against many established transplanted tumors, leading to rejection of the tumor. In this study, we investigated the therapeutic potential of cancer immunotherapy in a clinically more relevant model, woodchucks with primary hepatocellular carcinomas induced by woodchuck hepatitis virus. Methods: Large (2–5 cm), established intrahepatic tumors were given an injection once with 1 × 10⁹ plaque-forming units of AdIL-12/B7.1, an adenovirus vector carrying genes for murine interleukin 12 and B7.1, or of AdEGFP, the control virus, and regression of the tumors was then monitored. Five animals were used in total. Results: In four tumor-bearing animals, the antitumor response was assessed by autopsy and histologic analysis within 1–2 weeks after treatment. In all animals treated with AdIL-12/B7.1 therapy versus AdEGFP therapy, we observed substantial tumor regression (P = .006; two-sided unpaired Student’s t test) accompanied by a massive infiltration of T lymphocytes. These tumors also contained increased levels of CD4⁺ and CD8⁺ T cells and interferon gamma (IFN γ). In continuously growing tumor nodules given an injection of the control virus or in nontumoral liver, no such effects (i.e., tumor regression and increased levels of CD4⁺ and CD8⁺ T cells and IFN γ) were detected. In the fifth animal, monitored for long-term antitumor efficacy by magnetic resonance imaging (MRI) after intratumoral vector administration by MRI guidance, the tumor was almost completely eliminated (>95%) 7 weeks after treatment. Conclusion: Adenovirus vector-based immunotherapy appears to be an effective treatment of large nontransplanted (orthotopic) tumors that acquire malignant characteristics in a stepwise process, resulting in the real-world scenario of hepatocellular carcinoma in humans. [J Natl Cancer Inst 2001;93:472–9]

Among the various strategies used in cancer gene therapy, activation of the immune system by vector-mediated transfer of immunomodulatory genes is a most promising approach because tumor development in immunocompetent hosts suggests that the host’s immune system is failing to detect and eliminate malignant cells (1,2). This failure appears to result mainly from the evasive properties of the tumor (3), not from a lack of tumor antigens, because tumor cells transfected with immunomodulatory genes can induce specific antitumor immune responses resulting in tumor rejection (4,5). Adenoviral delivery of genes for cytokines and related immunomodulators, such as interleukin (IL) 2, IL-12, and IL-4, interferon gamma (IFN γ), and B7.1, can efficiently mediate tumor rejection and induce protective systemic immunity in several animal tumor models (6–12).

Of these cytokines, IL-12 appears to possess the strongest antitumor activity. The mechanism for IL-12-mediated tumor killing includes the activation of natural killer cells, T cells, lymphokine-activated killer cells, and macrophages (13). IL-12 stimulates IFN γ production from natural killer and T cells, promotes the cellular immune response by facilitating the proliferation and activation of Th1 T-helper cells (14), and inhibits angiogenesis in vivo (15). IL-12 interacts synergistically with the costimulatory molecule B7.1/CD80, normally expressed on professional antigen-presenting cells, to enhance protective antitumor immunity (9,16–19). B7.1-mediated antitumor activity in these studies was largely attributed to the stimulation of natural killer and CD8⁺ T cells, whereas the requirement for CD4⁺ cells in tumor rejection is highly dependent on the tumor model (20).

Previously, we constructed an adenovirus vector carrying genes for both IL-12 and B7.1, termed “AdIL-12/B7.1,” and demonstrated their synergistic effect on tumor growth by injecting the vector directly into breast adenocarcinomas transplanted from a polyoma middle-T-antigen (PyMT)-transgenic mouse model (9). In tumors derived from the transgenic model, one injection of the vector resulted in complete tumor regression in 70%–90% of the treated animals and induced long-term systemic immunity (9). However, the animal model in this study, as in the majority of cancer immunotherapy trials, is based on transplantation of established tumor cell lines into immunocompetent animals. In contrast, during carcinogenesis, a primary tumor develops from a single cell that becomes malignant in its natural environment (21), thus allowing the development of immune escape mechanisms that may negatively affect immunotherapeutic approaches in humans (21).

To address this problem, we tested the ability of the AdIL-12/B7.1 vector to reduce the volume of primary, nontransplanted woodchuck hepatitis virus (WHV)-induced hepatocellular carcinomas (HCCs) in woodchucks. WHV is closely related to the human hepatitis B virus in its structure, genomic organization, mechanism of replication, and the course of infection. Persistent WHV infection is associated frequently with the development of hepatic tumors (22). This experimental model has been applied extensively in the...
study of underlying mechanisms of human hepatitis B virus-induced hepatocarcinogenesis and in the development of prophylactic and therapeutic strategies of disease control (23,24).

**MATERIALS AND METHODS**

**Cell Culture**

The woodchuck liver cell line WH12/6 (25) was maintained in Ham’s F-12 medium supplemented with 10% fetal calf serum (FCS). Human MRC5 fibroblasts (cell line CCL 171; American Type Culture Collection, Manassas, VA) were grown in modified Eagle medium plus 10% FCS. All viruses were grown in 293 cells [adenovirus 5 E1-transformed human embryonic kidney cells (26)] maintained in modified Eagle medium/F-11 with 10% FCS. Media were supplemented with 2 mM L-glutamine, penicillin at 100 μg/mL, and streptomycin at 100 U/mL.

**Animals**

Adult American woodchucks (*Marmota monax*) were chronically infected with WHV, which is closely related to human hepatitis B virus. All chronic WHV carriers were provided by Marmorech, Inc., Ithaca, NY, and had one or more hepatic tumors that were 2 cm or larger in diameter, characterized by ultrasound and magnetic resonance imaging (MRI). In this study, a total of five animals have been used. Four animals were treated by intra-tumoral administration of Ad vectors after laparotomy, and one was given an injection under MRI guidance.

**Recombinant Adenovirus Vectors**

Construction of the recombinant adenovirus vector expressing both murine IL-12 subunits in early region 1 (E1) and B7.1 in early region 3 has been described previously (9). The control adenovirus AdEGFP encoding the enhanced green fluorescent protein was described (27). Adenovirus vectors were propagated, purified, and titrated as described previously (28,29).

**Enzyme-Linked Immunosorbent Assay and Flow Cytometry Analysis**

Hepatoma cells infected at a multiplicity of infection of 10 plaque-forming units (pfu) per cell were incubated in growth medium as indicated. Expression levels of secreted murine IL-12 were quantitated from infected cell supernatant by an enzyme-linked immunosorbent assay (ELISA) with the use of the mouse IL-12p70 DuoSet ELISA development kit (PharMingen, San Diego, CA) according to the instructions provided. The protected RNA bands were determined that murine IL-12 can be increased substantially by also infecting with IL-12 activity of IL-12 in transplanted tumors can inhibit tumor growth and induce tumor regression in various mouse tumor models (8,30–34). We have demonstrated previously (9) that the antitumor activity of IL-12 in transplanted tumors can be increased substantially by also inserting B7.1. When we injected this vector into the transplanted tumors at 2.5 × 10^7 pfu, a low virus dose, we observed complete tumor regression and induction of systemic immunity in the majority of animals. To determine whether AdIL-12/B7.1 could be used to treat established nontransplanted (orthotopic) tumors, we used the woodchuck animal model. Before the vector was given to woodchucks, however, we infected WH12/6 cells in vitro and determined that murine IL-12 and B7.1 were expressed 3 and 5 days later.
after infection (Fig. 1). Next, we selected large tumors in four woodchucks that were chronic WHV carriers and injected the tumors with AdIL-12/B7.1 or the control vector AdEGFP. The absolute tumor volumes of six tumor nodules, three injected with the adenovirus-based vector AdIL-12/B7.1 at a multiplicity of infection of 10 is shown. The murine IL-12 (mIL-12) concentration in supernatant was determined by enzyme-linked immunosorbent assay by use of the anti-mouse IL-12p70 antibody. All samples were assayed in triplicate. The 95% confidence intervals are indicated. For B7.1 detection, cells were stained with fluorescein isothiocyanate-conjugated anti-mouse B7–1 antibody and analyzed by flow cytometry; uninfected WH12/6 cells were used as controls (center peak). Compared with the negative control (human MRC-5 fibroblasts, left peak), uninfected WH12/6 cells (center peak) scored almost negative for B7.1 expression (4% after 72 hours). B7.1-positive cells (right peak), labeled M1, were 65% of the total cells on day 1, 92% on day 3, and 95% on day 5. FL1 = fluorescence channel 1 (green).

untreated and AdEGFP-injected tumors showed an approximately twofold increase in volume (Fig. 2, A). Fig. 2, B, presents representative MRI images from one woodchuck at three representative time points (before treatment and 4 and 7 weeks after treatment), showing the long-term antitumor response after direct intratumoral injection of 1 × 10^9 pfu of AdIL-12/B7.1 into a selected liver tumor nodule under MRI guidance. In this experiment, AdIL-12/B7.1 treatment had a pronounced effect on tumor growth over a 7-week period, leading to an approximately 95% reduction in tumor size from 8.6 cm^3 at the time of injection to 0.5 cm^3 by day 50 (Fig. 2, B-I to B-III). In contrast, no reduction in tumor size was noted in the surrounding untreated tumors. Thus, large established nontransplanted liver tumors can be treated efficiently by injection of an IL-12/B7.1-expressing adenovirus vector.

Association of Regression of Primary HCC and an Increase in Immune Effector Cells and IFN-γ Within the Tumor

Gene transfer studies (5,35,36) in several mouse models have indicated that rejection of transplanted tumors expressing
IL-12 and/or B7.1 is dependent on CD4+ and CD8+ T lymphocytes and natural killer cells. To analyze the immune response elicited by injection of AdIL-12/B7.1 into established nontransplanted liver tumors, we next examined liver and tumor biopsy samples from woodchucks 1–4 before and after treatment (Fig. 3, A).

Fig. 2. Short- and long-term antitumor efficacy of AdIL-12/B7.1 on woodchuck hepatitis virus-induced primary liver tumors. Panel A: selected tumor nodules 2–5 cm in diameter were intratumorally injected with $1 \times 10^9$ plaque-forming units (pfu) of AdIL-12/B7.1 or the control virus AdEGFP (three tumors for each vector). The animals were killed within 14 days after treatment and were monitored for tumor regression relative to untreated tumors. The average increase in tumor volume is shown (shaded bars). Tumor volume before treatment was set as 1 (solid bars). The 95% confidence intervals (CIs) are indicated by error bars. The difference in the relative increase of the size of AdEGFP- versus AdIL-12/B7.1-treated tumors was statistically significant (*) with $P = .006$: AdEGFP (2.1-fold [95% CI = 1.44-fold to 2.76-fold]; $P = .084$) versus AdIL-12/B7.1 (0.22-fold [95% CI = 0.03-fold to 0.41-fold]; $P = .015$). The absolute volumes from each injected tumor are shown in Table 1. Panel B: T1-weighted postcontrast magnetic resonance images of one representative woodchuck liver show neoplastic tumor nodules. One tumor indicated with white arrowheads was given a direct injection of $1 \times 10^9$ pfu of AdIL-12/B7.1 under magnetic resonance imaging guidance. The treated tumor is shown before (B-I) and 4 (B-II) and 7 (B-III) weeks after a single vector administration. Reductions in tumor size of 43% (4 weeks after injection) to 95% (7 weeks after injection) are shown in images B-II and B-III, respectively. Growth of the surrounding untreated nodules of equivalent size was unchanged.

Histologic sections from HCCs showed the typical features of neoplastic hepatic cells forming pseudotubulary structures with striking changes in the nuclear morphology, characterized by condensed chromatin and macronucleoli (Fig. 3, A–III). In contrast to nodules injected with the control vector AdEGFP (Fig. 3, A–IV), necrosis and massive inflammatory infiltration were detected only in AdIL-12/B7.1-treated tumors (Fig. 3, A–V). In the nontumoral liver parenchyma, we found typical morphologic patterns of chronic hepatitis after persistent WHV infection, characterized by moderate fibrosis and mild mononuclear infiltrate of portal or periporal tracts with occasional necrosis ([37] and Fig. 3, A–I).

Table 1. Tumor volume of established primary hepatocellular carcinomas after AdIL-12/B7.1 treatment

<table>
<thead>
<tr>
<th>Animal*</th>
<th>Treatment</th>
<th>Volume before treatment, cm³</th>
<th>Volume after treatment, cm³</th>
<th>Time, d†</th>
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<tbody>
<tr>
<td>3</td>
<td>AdEGFP</td>
<td>23.5</td>
<td>35.6</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>AdIL-12/B7.1</td>
<td>29.4</td>
<td>11.8</td>
<td>7</td>
</tr>
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<td>4</td>
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<td>7.45</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>AdIL-12/B7.1</td>
<td>37.8</td>
<td>6.8</td>
<td>10</td>
</tr>
<tr>
<td>1</td>
<td>AdEGFP</td>
<td>2.9</td>
<td>5.9</td>
<td>14</td>
</tr>
<tr>
<td>1</td>
<td>Untreated</td>
<td>5.9</td>
<td>10.6</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>AdIL-12/B7.1</td>
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<td>0.25</td>
<td>14</td>
</tr>
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<tr>
<td>1</td>
<td>Untreated</td>
<td>5.9</td>
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*In woodchucks 3 and 4, one selected tumor nodule was treated with AdEGFP and one with AdIL-12/B7.1.
†Tumor size was determined at 7, 10, and 14 days after treatment.
Fig. 3. Infiltration of immune effector cells in AdIL-12/B7.1-injected primary liver tumors. Panel A: representative liver and tumor sections from one woodchuck before (A-I and A-III) and after (A-II, A-IV, and A-V) treatment. One tumor nodule was given an injection of $1 \times 10^9$ plaque-forming units (pfu) of AdIL-12/B7.1, and one tumor nodule was given an injection of $1 \times 10^9$ pfu of the control vector AdEGFP. The animal was killed 10 days later and analyzed for treatment-associated morphologic changes. Liver parenchyma of chronic hepatitis after persistent woodchuck hepatitis virus infection before (A-I) and after (A-II) intratumoral injection are observed. No morphologic signs of adenovirus vector-related hepatic injury were detected. Pseudotubular structures of neoplastic hepatocytes from untreated hepatocellular carcinoma (HCC) (A-III) and after injection with AdEGFP (A-IV) or AdIL-12/B7.1 (A-V) are shown. Several areas of necrosis and massive inflammatory infiltration are shown in A-V. (Hematoxylin–eosin staining. Scale bars = 100 \mu m.) Panel B: analysis of T-lymphocyte and interferon gamma (IFN gamma) messenger RNA (mRNA) levels by ribonuclease protection assay. The RNA was obtained from liver tumor biopsy samples before and 10 days after intratumoral injection of the control virus AdEGFP (I) or AdIL-12/B7.1 (II). The average increases in CD4, CD8, and IFN gamma mRNA levels after each treatment are presented. The mRNA levels before treatment were set as 1 for each parameter. Results are reported as the mean ± 95% confidence interval.
However, no evidence of adenovirus-induced hepatic toxicity was observed in normal liver tissue 10 days after intratumoral vector administration (Fig. 3, A-II).

The histologic results were extended by RPA of liver and tumor biopsy samples obtained from woodchucks 1–4 (Fig. 3, B). Compared with the levels of T-lymphocyte-specific transcripts in hepatic tumors before treatment, RPA revealed an influx of CD4+ and CD8+ T cells into AdIL-12/B7.1-injected tumors (Fig. 3, B-II) that occurred at the peak of rejection. Compared with before-treatment samples, CD4+ lymphocytes showed an approximately sevenfold increase and CD8+ lymphocytes showed a moderate but important twofold increase. Moreover, AdIL-12/B7.1-injected HCC tissue showed an approximately twofold increase in the levels of IFN γ, the major determinant of the IL-12-mediated antitumor response. Thus, murine IL-12 expressed from an adenovirus vector is functional in woodchuck liver tumors. In contrast, no increase in CD4+ or CD8+ T cells or IFN γ occurred in tumors injected with control virus (Fig. 3, B-I) or in nontumoral liver samples (data not shown).

DISCUSSION

Previously, we showed that intratumoral injection of an adenovirus coexpressing IL-12 and B7.1 is an extremely efficient method of inducing the rejection of transplanted PyMT-induced tumors (9). To determine whether it is also active against primary tumors, we selected the well-characterized model of WHV-induced HCC in woodchucks. This model is clinically important because WHV is one of the most efficient hepatocarcinogens in woodchucks (38). WHV-induced hepatocarcinogenesis shows strong similarity to hepadnavirus-induced carcinogenesis in humans (23,39).

Because our previous data indicated that AdIL-12/B7.1-injected murine tumors were effectively rejected within approximately 2 weeks, the immune response against nontransplanted woodchuck liver tumors was first analyzed shortly after treatment. Consistent with the results obtained from the PyMT model (9), much of the tumor mass was rejected by day 14, suggesting that AdIL-12/B7.1 had a similar inhibitory effect on growth of transplanted and nontransplanted tumors at this early stage of treatment. The use of MRI guidance allowed us to locate and select large tumor nodules into which we could directly inject the vector, thereby avoiding the negative and stressful effects of surgery. MRI monitoring of a selected tumor revealed long-term tumor regression, resulting in nearly complete tumor elimination. However, there was a clear difference in the time course of tumor reduction between the tumor injected at one site under MRI guidance and the tumors injected at multiple sites during laparotomy.

In murine models, intratumoral injection of adenovirus vectors carrying IL-12 and/or B7.1 can stimulate systemic immunity that can act effectively on distal tumors (7–9). No clear systemic effect, however, has been observed in the woodchuck model, which may reflect a variation in the immunogenicity of different tumor nodules (Roggendorf M: unpublished data). Because these tumors are not of clonal origin and might express different tumor antigens, an effect on surrounding tumors might be obtained when vascular anastomoses allow the virus to spread between the tumors. Therefore, a lack of anastomoses between the tumor and the normal liver in this model might contribute to the lack of systemic response. In addition, higher doses of cytokine vectors were more efficient in inhibiting tumor growth than lower doses (9,30,40). If the body weight of a woodchuck and the tumor size of 2–5 cm in diameter are considered, the virus dose of 1 × 109 pfu, used for woodchucks, is low compared with doses used in mice. Therefore, we cannot exclude the possibility that the IL-12/B7.1 virus might induce systemic immunity in primary cancer at higher vector doses.

Our results suggest that regression of WHV-induced primary liver tumors is associated with massive intratumoral lymphocyte infiltration. We also show that intratumoral adenoviral IL-12/B7.1 expression in primary HCCs leads to increased intratumoral levels of IFN γ, consistent with results from a number of models indicating that the antitumor activity of IL-12 is IFN γ dependent (8,41,42). From recent observations, expression of major histocompatibility complex (MHC) class I molecules is altered in liver cells of animals chronically infected with WHV (43) and also commonly reduced on neoplastic cells of HCC nodules (Roggendorf M: unpublished data). Consequently, this reduction in MHC molecules may diminish the susceptibility of WHV-infected hepatocytes to virus-specific T cells, WHV-infected hepatocytes may evade the antiviral immunologic surveillance system, and thus liver damage may be perpetuated. The current model emphasizes the role of IFN γ for the modulation of peptide processing and stimulation of genes involved in antigen presentation by the MHC class I molecules (44). Thus, the activation of MHC class I-associated genes by local induction of IFN γ through tumor-infiltrating T lymphocytes may be a possible mechanism for efficient tumor rejection after IL-12/B7.1 immunotherapy in this model.

However, our observation varies from the observations made in established nontransplanted 3-MC-induced mouse tumors after IL-7/B7.1 gene delivery (45). In that model, failure to induce tumor regression depends on the inability of T cells to infiltrate nontransplanted tumors. The observed difference might result from a difference in T-cell infiltration of tumors induced by viruses or by carcinogens rather than a general difference between transplanted and primary, nontransplanted malignant tumors.

The animal model used in this study is expensive and difficult to use, and only a limited number of tumor-bearing WHV-infected woodchucks were available. Nevertheless, our results demonstrate that adenovirus vector-mediated immunotherapy is certainly not limited to artificial mouse tumor models and provide a powerful approach for the in vivo treatment of large, primary tumors in a model of hepatadnavirus-induced carcinogenesis, reflecting the real-world scenario of HCC in humans. Direct injection into selected HCC nodules under MRI guidance appears to be a relatively nontoxic and feasible method for administrating viral vectors in human clinical trials. However, our data emphasize the need to evaluate the mechanisms involved in effective intratumoral penetration by immune effector cells as a prerequisite for successful cytokine treatment of natural tumors.

REFERENCES


NOTES

Editor’s note: B. Tennant owns an equity interest in Marmotech, Inc., Ithaca, NY, the company that donated the woodchucks used in this study.

Supported in part by grant PU188/2–1 from the Deutsche Forschungsgemeinschaft and by the Internal Research Support Program from the University of Essen, Germany (to B. M. Putzer).

We thank Silke Bosk for excellent technical assistance in performing the magnetic resonance tomography, Li Jun for surgical assistance, and Michael Lowak for help in animal handling.

Manuscript received September 22, 2000; revised December 28, 2000; accepted January 9, 2001.