Anticancer Effect of a Lentiviral Vector Capable of Expressing HIV-1 Vpr

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ABSTRACT

A lentiviral vector capable of expressing the HIV-1 vpr gene (Vpr lentiviral vector) was constructed, and its in vivo anticancer effect was determined against cutaneous tumors derived from the AT-84 oral cancer cells in immunocompetent mice. A single intratumoral injection of the Vpr lentiviral vector not only significantly reduced the primary tumor volume but also completely regressed tumors in >40% of animals. More interestingly, the mice of which the primary tumors were completely regressed by the Vpr lentiviral vector were additionally protected from a secondary challenge of AT-84 cells. These data suggest that the Vpr lentiviral vector elicits its anticancer activity in part by the activation of the immune system. The above suggestion is additionally supported by the failure of the lentiviral vector to demonstrate anticancer activity in immunocompromised nude or SCID mice. The Vpr lentiviral vector offers a powerful new strategy for cancer gene therapy and may be useful for the control of solid tumors, such as human oral squamous cell carcinomas.

INTRODUCTION

Currently available methods of gene delivery suffer from several major limitations that hinder the high expectations of gene therapy. Nonviral gene therapy is inefficient and only accomplishes a transient expression of the transgene, whereas viral vectors do not offer a satisfactory combination of effectiveness of gene transfer, long-term gene expression, and bio-safety (1, 2). Therefore, the development of a new viral vector for gene therapy without the limitations of available methods has been pursued by a number of investigators. Despite safety concerns, a few laboratories have recently developed and refined lentiviral technology to mitigate the dearth of retroviral and adenoviral vectors (3). The lentiviral vector can induce long-term expression of the desired genes and may represent a significant step forward in the field of gene therapy.

Human oral cancer involves a variety of molecular genetic changes (4). Although changes in chromosomal translocation at the 11q13 locus, mutation of p53, and the genetic changes by the “high-risk” human papillomavirus infection are commonly found genetic disorders, other genetic alterations are also frequently noticed in human oral cancer (5–7). It is difficult to develop a unique gene therapy that is universally applicable for the treatment of all of the types of human oral cancer; therefore, a development of a vector capable of expressing cytotoxic gene(s) and/or activating immune system has been sought for the successful gene therapy of oral cancer.

It may be possible to achieve a combined approach using a gene vector to induce both cancer cell apoptosis and host immune response against cancer cells. Apoptosis, or programmed cell death, can be achieved by reinstallation of tumor suppressor genes in some cancer cells (8, 9). It has been demonstrated that apoptotic cancer cells can efficiently activate an immune response against them (10, 11). Although some tumor suppressor genes induce cancer cell apoptosis, both in vitro and in vivo tests indicate that tumor suppressor genes are not very efficient in inducing cancer cell apoptosis. Therefore, the use of more efficacious genes is necessary.

The HIV-1 vpr gene encodes a M, 14,000 nuclear protein, Vpr, which is expressed within infected cells and is packaged into virions (12, 13). The Vpr is required for importing the viral protein integration complex into the nucleus of nondividing cells (14, 15) and induces cell cycle arrest at the gap-2 checkpoint in a variety of mammalian cells including human squamous cancer cells (16–18). The cell cycle arrest is characterized by alterations in the activation and phosphorylation state of cell division cycle 2 (Cdc2) kinase (16, 19) and resembles the gap-2 checkpoint induced by genotoxic agents (20), which results in apoptosis (18).

We constructed a lentiviral vector capable of expressing HIV-1 Vpr (Vpr lentiviral vector), which induces cancer cell death in vitro more effectively than the doses of γ-irradiation that are normally used to treat cancer in the clinical arena (21). In this study, the in vivo therapeutic efficacy of the Vpr lentiviral vector was determined against cutaneous tumors derived from AT-84 murine oral cancer cells. A single intratumoral injection of the Vpr lentiviral vector not only rapidly reduced the primary tumor volume...
after the injection but also completely regressed tumors in >40% of animals. The C3H mice of which the primary tumors were completely regressed by the Vpr lentiviral vector were additionally protected from a secondary challenge of AT-84 cells. These data suggest that the Vpr lentiviral vector demonstrates its anticancer activity in part by the activation of the immune system. The above notion is additionally supported by the failure of the lentiviral vector to demonstrate anticancer activity in immunocompromised nude or SCID mice. We conclude that the Vpr lentiviral vector offers a powerful new strategy for cancer gene therapy and may be useful for the control of solid tumors such as human oral squamous cell carcinomas.

MATERIALS AND METHODS

Construction of Lentiviral Vector Capable of Expressing EGFP or Vpr. The lentiviral vector capable of expressing EGFP or Vpr was constructed using transducing, packaging, and envelope plasmids (21–23). The pHR’-CMV-EGFP and pHR’-CMV-Vpr-transducing plasmids were used to express EGFP and Vpr, respectively (21–23). Although Vpr protein carried by virions is able to cause cell apoptosis of some types of cells, the efficiency is significantly lower compared with a lentiviral vpr gene expression vector (24). The pCMVΔR8.2VprX containing mutant vpr gene was used as a packaging plasmid. The plasmid pSV-G encoding the amphoteric envelope G glycoprotein of the vesicular stomatitis virus was used as envelope plasmid. With this plasmid, the constructed lentiviral vectors can have a considerably wider range of host cells, e.g., human cells, including hepatocytes, and squamous cell carcinoma cells, as well as mouse cells (25–28). This envelope offers the additional advantage of high stability, which allows for particle concentration by ultracentrifugation (25). The EGFP or Vpr lentiviral vector was produced by cotransfecting 293T cells with the transducing plasmid pHR’-CMV-EGFP or pH’-CMV-Vpr, the packaging plasmid pCMVΔR8.2VprX, and the envelope plasmid pCMV-ΔSV-G using calcium-phosphate-mediated transfection (22, 23).

Concentration and the Determination of Viral Titers and Infectivity. Culture medium was collected from the 293T monolayer cultures at 48, 72, and 96 h after transfecting the above plasmids (18, 21). After ultracentrifugation at 25,000 rpm for 90 min, the pellets were resuspended in DMEM (Life Technologies, Inc., Grand Island, NY), pooled, and the viral titers were determined using the p24 assay (18, 21). The infectivity of the EGFP lentiviral vector was determined by measuring the corresponding TCID with a defined p24 count. Briefly, viral aliquots (with p24 count of 10 ng from five preparations) were diluted up to 1/10^4 in 200 μl of DMEM to infect the 293T monolayer cells. The number of EGFP-positive cells was then counted under a UV microscope 72 h after the infection. The TCID was determined by the Karber method: \( n = -\log_{(1-p)}(P) \), in which \( n \) is the average number of infectious virions per cell, \( P \) = proportion of EGFP-positive cells in the culture plates, and \( e = 2.71828 \) (2.71828). The number of infective virions per milliliter of viral stocks was calculated using p24 titers and the TCID. Using this method, the number of TCID per 1-pg p24 count was determined. Our results demonstrated that 1 pg of p24 count equals 1–10 infective units of EGFP lentiviral vector. In this paper, we set 1-pg p24 as 1 infectious virion. For Vpr viral titration, we also set 1-pg p24 as 1 infectious Vpr virion.

Western Blot Analysis and TUNEL Assay. The AT-84 cells infected by lentiviral Vpr expression vector and the control vector carrying EGFP gene at the MOI of 10 were lysed in 20 μl SDS-PAGE loading buffer [2% SDS, 10% glycerol, 50 mM Tris (pH 6.8), 100 mM dithiothreitol, 0.1% bromophenol blue]. After denatured in 100°C for 5 min, the samples were loaded on 15% gel. Then protein was blotted to nitrocellulose membrane and detected by anti-influenza HA antibody (12CA5; Boehringer-Mannheim), because the Vpr was fused at the NH2 terminus to the influenza HA nonapeptide (24).

The AT-84 cells were infected with the Vpr lentiviral vector, and viral apoptotic activity was determined using the TUNEL method. Reagents were purchased from Roche (Indianapolis, IN), and the TUNEL assay was performed as suggested by the manufacturer. Fluorescein-labeled apoptotic cells were detected using a UV microscopy. We also used annexin V protein binding method to detect apoptotic cells, and the results were similar to TUNEL assay.

Establishment of Tumor Model in Immunocompetent Mice Using a Murine Oral Cancer Cell Line AT-84. We obtained the AT-84 cancer cells from Dr. Stephen E. Karp (Sir Mortimer B. Davis Jewish General Hospital, Montreal, Canada). The AT-84 squamous cell carcinoma cells originated from a spontaneously occurring tumor in the oral mucosa of C3H mice (29). The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. Viable (as determined by trypan blue staining) AT-84 cells (10^5) were injected s.c. into the right flank of immunocompetent C3H mice (Charles River Laboratories, Wilmington, MA). Our pilot study showed that within 1 week, small palpable tumors were evident in 100% of animals; by 2 weeks, most tumors had reached 500-1000 mm^3 in volume. The size of the tumors was measured on three axes using calipers to calculate tumor volume. Selected tumors from different group were subjected for microscopic examination after H&E staining.

Intratumoral Injection of Culture Medium, EGFP Lentiviral Vector, or Vpr Lentiviral Vector and the Determination of Tumor Size. Either 0.1 ml of concentrated Vpr lentiviral vector (10^7 pfu/ml), EGFP vector (10^7 pfu/ml), or DMEM was injected intratumorally when the volume of primary tumors reached ~100–200 mm^3 (Experiments 1, 2, and 3) or 500-1000 mm^3 (Experiment 4). The growth of the tumors was monitored daily for 10–15 days, and tumor volume was measured daily as described elsewhere (30).

Determination of the Effect of Lentiviral Vpr Vector on the Protection of Mice from a Secondary Challenge of Tumor Cells. One major goal of gene therapy is to induce antitumor activity and to protect against recurrence and metastasis. To test whether C3H mice, of which the primary tumors were completely regressed by the Vpr lentiviral vector treatment, can be protected against a second inoculation with the same tumor cells, 14 Vpr-treated mice were rechallenged with the AT-84 cells 4 weeks after the treatment of tumors with DMEM. As controls, we included mice of which the primary tumors were treated with DMEM (n = 15) or EGFP lentiviral vectors (n = 14). These control groups still bore primary tumors at the time of injection of tumor cells into their left flanks. Moreover, a mouse of which the primary tumors spontaneously regressed after EGFP lentiviral vec-
tor treatment was also included in this experiment. The development of tumors was monitored for 4 weeks.

**Determination of the Effect of Vpr Lentiviral Vector on the Development of Primary Tumors in SCID and Nude Mice.** To determine whether Vpr-induced tumor regression is associated with immune activation, the therapeutic efficacy of DMEM, EGFP lentiviral vector, and Vpr lentiviral vector was tested against AT-84 tumors in nude mice (nu/nu, University of California Los Angeles, Medical Center Vivarium, Los Angeles, CA). Additionally, the therapeutic efficacy of the Vpr lentiviral vector was determined against AT-84 tumors in C.B.17 SCID (from Dr. Jerome Zack, University of California Los Angeles, School of Medicine, Los Angeles, CA) mice. The right flanks of mice were injected with AT-84 cells and treated with culture medium or lentiviral vectors in a manner similar to C3H mice as described earlier. The development of tumors was monitored and recorded for 2 weeks or for 35 days.

**RESULTS AND DISCUSSION**

**Lentiviral Vector Can Infect AT-84 Cells in Vitro.** To evaluate and document the therapeutic efficacy of the Vpr lentiviral vector, the infectivity of the lentiviral vector was desired in vitro. However, because the Vpr lentiviral vector can express the Vpr protein that induces cell cycle arrest and apoptosis, it is impossible to accurately assess the infectivity of this particular vector in vitro. Therefore, a similar lentiviral vector capable of expressing measurable reporter protein and not expressing or containing virion-associated Vpr is necessary for such evaluations.

Thus, the EGFP lentiviral vector was constructed, and AT-84 cells were infected with it. The infectivity of the vector was determined by counting the cells that showed fluorescence under UV microscopic examination. The monolayer culture of AT-84 cells was infected with the EGFP lentiviral vector at the MOI of 1, 3, 10, or 30. Approximately 2, 3, 10, or 20% of cells showed strong fluorescence when infected at the MOI of 1, 3, 10, or 30, respectively. These data indicate that the lentiviral vector can infect AT-84 cells and express a reporter gene in these cells (Fig. 1A).

The Vpr Lentiviral Vector Expresses Vpr and Induces Apoptosis of AT-84 Cells in Vitro. As shown in Fig. 1B, the Vpr lentiviral vector expressed Vpr in AT-84 cells, but EGFP vector did not. These data indicate that the Vpr lentiviral vector can infect and express Vpr in AT-84 cells, and the EGFP lentiviral vector does not carry virion-associated Vpr. The Vpr lentiviral vector induced apoptosis of AT-84 cells as shown in Fig. 1C. When the cells were infected with the Vpr vector at the MOI of 0.4 or 2, very few cells underwent apoptosis. Infection of cells at the MOI of 10 induced apoptosis in approximately 7–10% of cells when measured at 72 h after the infection. These data indicate that, like the EGFP vector, the Vpr vector can infect the AT-84 cells and induce apoptosis, presumably through the expression of Vpr protein in these cells.

The Vpr Lentiviral Vector not only Inhibits the Growth but also Reduces the Size of AT-84 Tumors or Completely Regresses Tumors in Immunocompetent C3H Mice. As shown in Fig. 2, when the Vpr vector was injected either at an early (Fig. 2, A, B, and C, tumors with volume of 100–200) or...
a later stage (Fig. 2D, tumors with volume of 600-1000 mm³) of tumor development, it rapidly inhibited the additional growth of tumors and also reduced the volume of the tumors. Interestingly, 40% of the tumors completely regressed within 2–3 weeks after the injection of the Vpr vector, regardless of the stage of tumor development at the time of injection (Table 1). Conversely, both medium and the EGFP lentiviral vector failed to inhibit the growth of the tumors as shown in Fig. 2. Intratumoral injection of DMEM or lentiviral vectors was conducted 7 days after the injection of tumor cells when the average tumor volume ~200 mm³. A, experiment 1. Nine mice were used, three mice for DMEM treatment and six mice for Vpr vector treatment. The vectors and DMEM were injected intratumorally 10 days after the injection of tumor cells when the average tumor volume ~200 mm³. B, experiment 2. Twelve mice were used in this experiment, 5 mice for EGFP vector treatment and 7 mice for Vpr vector treatment. Intratumoral injection of DMEM or lentiviral vectors was conducted 7 days after the injection of tumor cells when the average tumor volume ~200 mm³. C, experiment 3. Twenty-four mice were used in this experiment, 7 mice injected with DMEM, 8 mice injected with the EGFP vector, and 9 mice injected with the Vpr vector. The vectors and DMEM were injected intratumorally 5 days after the injection of tumor cells when the average tumor volume ~100 mm³. D, experiment 4. Twenty-four mice were used in this experiment, 5 mice for DMEM, 8 mice for EGFP, and 11 mice for Vpr vector treatment. The vectors and DMEM were injected intratumorally 5 days after the injection of tumor cells when the average tumor volume became ~1000 mm³.

Table 1: Effect of DMEM, EGFP, and Vpr lentiviral vector on the growth of AT-84 tumors in C3H mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice tested</th>
<th>No. of mice with inhibited tumor growth</th>
<th>No. of mice with complete tumor regression</th>
<th>No. of mice with continued growth of tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>DMEM 6</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Vpr vector</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>EGFP vector 5</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Vpr vector</td>
<td>7</td>
<td>1</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>DMEM 7</td>
<td>2</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>EGFP vector</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Vpr vector</td>
<td>9</td>
<td>7</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Experiment 4</td>
<td>DMEM 5</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>EGFP vector</td>
<td>8</td>
<td>0</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>Vpr vector</td>
<td>11</td>
<td>7</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>
gait, and so forth, were not observed in the Vpr-treated animals during the period of observation.

**Vpr Lentiviral Vector Does Not Demonstrate Anticancer Activity in Immunocompromised Nude and SCID Mice.** As indicated previously, the infectivity and apoptotic activity of the lentiviral vector is not very efficient. The infectivity of the EGFP vector at the MOI of 30 is only 20%, and only 10% of cells infected with the Vpr vector at the MOI of 10 underwent apoptosis. It is likely that intratumoral injection of the Vpr vector can infect only a fraction of the tumor cells in vivo. Therefore, the inhibition of tumor growth, the reduction of tumor size, or the complete regression of tumors may not be solely attributable to the cytotoxic (apoptotic) activity of Vpr. It is likely that other factors, such as activation of the host immune function, may be involved in the observed anticancer effect of the Vpr vector. To test this possibility, similar experiments were conducted using nude and SCID mice that have defective immune functions.

As shown in Fig. 4A, similar to DMEM or EGFP lentiviral vector, the Vpr lentiviral vector did not demonstrate anticancer activity in nude mice (Fig. 4A). Moreover, the Vpr lentiviral vector also failed to demonstrate anticancer activity in SCID mice, although it showed effective anticancer effect in C3H mice (Fig. 3B). These results strongly support the hypothesis that the Vpr lentiviral vector induces its anticancer activity, in part, by activating the immune system in laboratory animals. If so, then the laboratory animals of which the primary tumors completely regressed after Vpr vector treatment should be protected from a secondary challenge with the same type of cancer cells.

**Mice of which the Primary Tumors Treated with Vpr Lentiviral Vector Were Additionally Protected from a Secondary Challenge of Tumor Cells.** In mice of which the primary tumors completely regressed after Vpr lentiviral vector treatment, no tumors developed after a secondary challenge with AT-84 cells. However, 100% of control mice of which the primary tumors continuously grew after DMEM or EGFP vector treatment developed new tumors within 10 days after rechallenge with the same AT-84 tumor cells (Table 2). These data indicate that exposure of mice to the Vpr lentiviral vector may induce specific immune activation against the same type of cancer cells in these animals. However, the exact mechanism of immune activation by the Vpr vector remains to be studied.

One major problem in cancer therapies, including gene therapy, is metastasis of cancer cells to adjacent lymph nodes or other organs. Unless the metastatic cancer cells are completely eliminated, the cancer will recur. Therefore, many immunogene therapies, which can induce cytokine-activated immune effector cells, have been developed. The therapeutic efficacy of these immunogene therapies has been tested in cancer-bearing animals and humans to eliminate both primary and secondary (metastatic) cancer (30–32). However, the results of those immunogene therapies have not been very satisfactory, because most cancer cells poorly express tumor-
specific antigens at the cell surface. Cytokine-activated immune effector cells have been shown to respond to certain types of cancer in a very small percentage of patients (33–37). Thus, the development of an agent that can both eliminate primary tumors and activate the host immune system to prevent the development of metastatic tumors is preferable. The presented data strongly indicate that the Vpr lentiviral vector may be an excellent candidate for inhibiting the growth of both primary and metastatic tumors.

Biosafety is an important issue for using HIV-based lentiviral vector in clinic. Theoretically the probability of generating a replication-competent HIV by three-piece co-transfection is almost none (there is no HIV-1 viral envelope generating a replication-competent HIV by three-piece co-lentiviral vector in clinic. Theoretically the probability of growth of both primary and metastatic tumors.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice tested</th>
<th>Mice with secondary tumor formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>EGFP vector</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>EGFP vector*</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Vpr vector</td>
<td>14</td>
<td>14</td>
</tr>
</tbody>
</table>

* The mice of which the primary tumors completely regressed after EGFP vector treatment (spontaneous regression of tumors) were also completely protected from a secondary challenge with AT-84 cells as indicated in "Results."

REFERENCES


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