Treatment of Invasive Retinoblastoma in a Murine Model Using an Oncolytic Picornavirus

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Abstract
Retinoblastoma, the most common intraocular malignancy of childhood, metastasizes by initial invasion of the choroid and the optic nerve. There is no effective treatment for metastatic retinoblastoma, especially when the central nervous system (CNS) is involved, and prevention of this complication is a treatment priority. Seneca Valley Virus (SVV-001) is a conditionally replication-competent picornavirus that is not pathogenic to normal human cells but can kill human retinoblastoma cells in vitro with an IC50 of <1 viral particle (vp) per cell. A xenograft murine model of metastatic retinoblastoma was used to examine the therapeutic potential of SVV-001. Histopathologic analysis of ocular and brain tissues after a single tail vein injection of SVV-001 (1 × 10^13 vp/kg) showed effective treatment of choroid and ocular nerve tumor invasion (1 of 20 animals with invasive disease in the treated group versus 7 of 20 animals with invasive disease in the control group; P = 0.017) and prevention of CNS metastasis (0 of 20 animals with CNS metastatic disease in the treated group versus 4 of 20 animals with CNS disease in the control group; P = 0.036). There were no observed adverse events due to the virus in any of the treated animals. SVV-001 may be effective as a treatment of locally invasive and metastatic retinoblastoma. [Cancer Res 2007;67(22):10653–6]

Introduction
Retinoblastoma is the most common intraocular malignancy of childhood (1, 2). This disease can be inherited or can occur sporadically and results from mutations in the Rb gene either in the germline or in the tumor alone (3). Retinoblastoma can spread peripherally by invading the ocular coats or metastasize to the brain by invading the optic nerve (4, 5). No successful therapy is available for patients with central nervous system (CNS) metastasis, whereas only intensive chemotherapy followed by rescue bone marrow transplantation has been reported to successfully treat some patients with peripheral metastasis (6–9). Therefore, finding a successful, low-toxicity therapy to prevent and treat metastatic retinoblastoma is important for children with this complication.

Oncolytic virotherapy has emerged as a potential treatment for a variety of cancers (10). Seneca Valley Virus (SVV-001) is a naturally occurring RNA virus that belongs to the group of coronavirus in the family Picornaviridae. The virus has a short replication cycle and a simple genome and can be grown in high-titer preparations.

SVV is not a human pathogen and has no known deleterious effects on any animal, including mice or primates. Pediatric human tumor cell lines of neuroendocrine origin are permissive for SVV-001 infection and replication (11). Tumor cells that are capable of binding and internalizing the virus support replication of SVV-001 and are killed during the lytic phase of viral replication. The newly formed virions are then capable of infecting adjacent tumor cells, thus repeating the cycle. After tumor cell lines of neuroendocrine origin were implanted in the flank of nude mice, a single i.v. injection of SVV-001 in the tail vein resulted in infection of the tumor by the virus and a sustained reduction in the tumor volume (11). The use of SVV-001 for the treatment of invasive and metastatic disease using a murine xenograft model of aggressive retinoblastoma, a model that closely mimics this childhood cancer (12), is now reported.

Materials and Methods

Virus. Seneca Valley Virus (SVV-001; ref. 11) was obtained from Neotropix, Inc.

Cell lines. Y79 and Weri retinoblastoma cell lines, the U251 glioblastoma cell line, and the HEK 293 human embryonic kidney cell line were obtained from the American Type Culture Collection and maintained in high-glucose DMEM supplemented with 5% fetal bovine serum (FBS; Invitrogen-Life Technologies) at 37°C in 5% CO2 humidified air.

In vitro cytotoxicity assay. Cells were seeded at 2 × 10^5/ml in DMEM with 5% FBS and infected with SVV-001 from 0.1 to 5 viral particles (vp) per cell. The cultures were incubated for 48 h at 37°C. Cells were counted using a hemacytometer and viability was assessed by trypan blue exclusion.

Murine xenograft model of metastatic retinoblastoma. When Y79 cells are injected into the vitreous space of the eye of an immunodeficient Rag2 mouse, they form intraocular tumors that subsequently invade the choroid and optic nerve and metastasize to the brain, mimicking human retinoblastoma (12). Using a Hamilton needle, 2 × 10^4 Y79 cells were injected into the vitreous space of one eye of anesthetized Rag2 mice. Mice were observed weekly for tumor development. After 3 weeks, intraocular but not extraocular tumors formed. To study the treatment of only intraocular tumors, SVV-001 (1 × 10^13 vp/kg into the tail veins) was injected at 3 weeks in one group of 10 animals. The treated animals and an untreated group of 10 animals were sacrificed 2 weeks later and tissues were examined histopathologically. At that time, untreated animals had begun to develop extraocular extension of their tumors. Therefore, to study the effects of the virus on extraocular disease, SVV-001 (1 × 10^13 vp/kg into the tail veins) was injected into a group of 10 mice 5 weeks after Y79 cell injection and another group remained untreated. Two weeks later, the study was terminated because the untreated animals were showing evidence of discomfort from their large extraocular tumors. Because invasion into the choroid and optic nerve with metastases to the brain can occur as early as 5 weeks after injection of the retinoblastoma cells, tumor invasiveness and metastasis were analyzed statistically by combining the two treatment groups and comparing them to the two untreated groups.

Histology. The harvested eyes were fixed in formalin and sectioned. Slides were stained with H&E and examined microscopically.
Statistical analysis. Statistical analysis was performed using the Student t test and results were considered significant if P < 0.05.

Results and Discussion

Cytotoxicity of retinoblastoma cell lines incubated with SVV-001 in vitro. The cytotoxicity of retinoblastoma cell lines treated with SVV-001 was examined in vitro as described in Materials and Methods. SVV-001 is cytotoxic to Y79 and Weri retinoblastoma cells (Fig. 1). At the highest doses tested, the number of viable cells was reduced by >90% compared with control cells incubated without virus. There was >50% reduction in the number of viable cells at doses as low as 0.5 vp per cell. The virus had no discernible effect on U251 glioma or HEK 293 kidney cells at up to 5 vp per cell. SVV-001 also lacked effect on RTM1796 cells, a murine retinoblastoma cell line derived from a transgenic mouse with retinal tumors that express SV40 T antigen driven by the PAX6 promoter (data not shown). However, a murine neuroblastoma cell line is susceptible to the virus, indicating that some murine neuroendocrine tumor cells can be infected with the virus (11). The low dose of virus capable of killing the cells suggests that the virus proliferates in permissive cells, generating additional virus capable of infecting neighboring cells when it is released as the host cells lyse. This hypothesis has recently been confirmed (11). SVV-001 can infect and replicate in most human tumor cells that express neuroendocrine markers, such as gastrin-releasing peptide receptors, synaptophysin, neuron-specific enolase, and CD56 (11). Although the specific receptor for SVV-001 is currently not known, preliminary evidence indicates that the tropism of SVV-001 is primarily determined by either the binding or internalization receptors expressed on susceptible tumor cells because transfection of viral RNA into nonpermissive cells resulted in viral replication and cell lysis (11).

Response of a murine xenograft model of retinoblastoma to SVV-001 treatment in vivo. The next question addressed was whether SVV-001 could kill retinoblastoma tumor cells in vivo. Retinoblastoma is exclusively a human disease (13, 14). Rb1 mutations in animals do not result in retinal tumors. Transgenic models of the disease have been produced by knocking out multiple retinoblastoma family members and/or P53 (13, 15, 16). SVV-001 had no effect on these murine transgenic tumors and none of these animals showed any adverse events after SVV-001 injections (data not shown). An immunodeficient murine xenograft model was used to determine whether SVV-001 could kill retinoblastoma tumors in vivo. Y79 retinoblastoma cells (2 × 10⁴) were injected into the vitreous of 40 Rag2 immune-incompetent mice. The mice were monitored weekly for tumor growth. At 3 or 5 wk after injection of the retinoblastoma cells, SVV-001 (1 × 10¹⁵ vp/kg) was injected into the tail veins of 20 mice. Two weeks after SVV-001 injections, eyes and brain tissues were harvested and examined. The harvested tissues were fixed in formalin and sectioned. Slides were stained with H&E and examined microscopically. A, untreated animal showing large tumor protruding from the orbit and massive replacement of ocular structures by tumor when examined microscopically at low power. The optic nerve has been invaded with tumor. Magnification, ×4. B, animal treated with the oncolytic virus had intraocular tumor only when examined microscopically at low power. The optic nerve was free of tumor. Magnification, ×4. C, untreated animal showing diffuse choroid and optic nerve invasion and replacement of ocular structures by tumor (invasive tumor: 7 of 20 animals). Magnification, ×10. D, oncolytic virus–treated animal showing intraocular tumor only. A choroidal scar without tumor can be observed (invasive tumor: 1 of 20 animals; P = 0.017). Magnification, ×10.
model of retinoblastoma created by injection of human Y79 cells into the vitreous was tested. This model mimics intraocular vitreous seeds without primary retinal tumors and closely resembles the invasive patterns leading to CNS metastasis in human disease. Retinoblastoma cells massively invade the murine choroid and optic nerve 3 to 5 weeks after injection. By 5 weeks, the tumor spreads through the ocular coats and some animals will develop CNS metastases in the subarachnoid space. By 6 to 8 weeks, the animals show discomfort and must be sacrificed. If they are allowed to progress beyond 8 weeks after injection of the tumor cells, all animals ultimately develop CNS metastasis (12).

Y79 retinoblastoma cells ($2 \times 10^4$) were injected into the vitreous of 40 Rag2 immune-incompetent mice. The mice were monitored weekly for tumor growth. Three or 5 weeks after injection of the retinoblastoma cells, SVV-001 ($1 \times 10^{13}$ vp/kg) was injected into the tail veins of 20 mice; the remaining 20 mice served as untreated controls. Two weeks after SVV-001 injections, eyes and brain tissues were harvested and examined.

The gross physical appearance and the histopathologic analysis of eyes of untreated and SVV-001 treated mice revealed clear differences. All of the animals developed intraocular tumors by 3 weeks after intraocular injection of Y79 cells. By 5 weeks, the intraocular tumors of both treated and untreated animals had progressed. None of the treated animals had extracocular extension. By 7 weeks, tumors had completely replaced the eye structures. Of the treated animals, only one had a grossly visible tumor at the time of harvest. The remaining animals had small localized tumors that could only be observed histopathologically. There was preservation of the lens and globe. In marked contrast to the untreated control animals, all of the treated animals had normal activity and appetite and were not in any visible discomfort.

Histologic analysis of the enucleated eyes (Fig. 2A and C) of the untreated animals showed large globe-filling tumors (20 of 20) and diffuse tumor invasion of the choroid and the optic nerve (7 of 20). In contrast, the eyes of treated animals had a reduced intraocular tumor burden with some choroidal scarring suggesting tumor regression (Fig. 2B and D). Focal invasion of the choroid by viable tumor was present in only 1 of 20 eyes examined ($P = 0.017$). The presence of residual intraocular tumors may be attributed to the inability of SVV-001 to replicate in the vitreous. To test this possibility, SVV-001 was incubated with Y79 cells in the presence of vitreous in vitro. SVV-001 killed Y79 cells in the presence or absence of vitreous, suggesting that this hypothesis is unlikely. Because the vitreous seeds do not have a blood supply but grow as free-floating tumors, it is possible that the virus cannot gain access to the tumor. Children with retinoblastoma have primary retinal tumors that are vascular (17, 18); therefore, it is possible that peripheral injection of SVV-001 could be therapeutic for these primary tumors. Because of the lack of a suitable animal model to test this possibility, the answer to this question will have to wait for a clinical trial in children.

Histologic analysis of the brains of 4 of 20 xenograft mice that were not treated with SVV-001 showed tumor invading the subarachnoid space (Fig. 3A). No tumor (0 of 20) was detected in the brains of mice that received SVV-001 treatment (Fig. 3B). Although current imaging techniques do not allow us to say that tumor is present in the optic nerve or brain before beginning therapy, the absence of tumor in the optic nerve and brains of any of the SVV-001–treated animals suggests that systemically administered SVV-001 is able to infect tumors located in the CNS. No viral cytopathic changes or necrosis were observed in the brains, eyes, or any other organs.

The development of successful treatment and prevention of metastatic disease in children with retinoblastoma is of paramount importance. Because of its lack of apparent pathogenicity even in an immunodeficient host and its activity against retinoblastoma both in vitro and in vivo, SVV-001 is an attractive candidate for the treatment and/or prevention of metastatic retinoblastoma.

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