ORIGINAL ARTICLE

A new recombinant vaccinia with targeted deletion of three viral genes: its safety and efficacy as an oncolytic virus

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To enhance further the safety and efficacy of oncolytic vaccinia virus, we have developed a new virus with targeted deletions of three viral genes encoding thymidine kinase and antiapoptotic/host range proteins SPI-1 and SPI-2 (vSPT). Infection of human and murine tumor cell lines yielded nearly equivalent or a log lower virus recovery in comparison to parental viruses. Viral infection activated multiple caspases in cancer cells but not in normal cells, suggesting infected cells may die via different pathways. In tumor-bearing mice, vSPT recovery from MC38 tumor was slightly reduced in comparison to two parental viruses. However, no virus was recovered from the brains and livers of mice injected with vSPT in contrast to control viruses. vSPT demonstrated

significantly lower pathogenicity in nude mice. Systemic delivery of vSPT showed significant tumor inhibition in subcutaneous MC38 tumor, human ovarian A2780 and murine ovarian MOSEC carcinomatosis models; however, the tumor inhibition by vSPT was reduced compared with parental viruses. These results demonstrated that although deletion of these three viral genes further enhanced tumor selectivity, it also weakened the oncolytic potency. This study illustrates the complexity of creating a tumor-selective oncolytic virus by deleting multiple viral genes involved in multiple cellular pathways.

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Introduction

Replicating viruses offer several advantages for tumor-directed gene therapy. Replicating viral vectors delivered initially to a small number of tumor cells will lead to an expanding infection within the tumor, significantly increasing the number of cells killed by the virus. Currently, a number of viruses, such as adenovirus, herpes simplex virus, measles virus and vaccinia virus, are being developed as replicating viruses for oncolytic virotherapy.^{1–7} Vaccinia virus has many attributes that provide advantages as a successful oncolytic virus. It has a large genome with the capacity to accommodate multiple foreign genes. It has a broad host range, replicates rapidly and can easily be recombined for making new recombinant viruses.^{7–10} The wild-type virus has only proved lethal in rare cases involving severely immunocompromized patients.¹¹ Vaccinia virus has been used as a live vaccine in the smallpox eradication program and recently as a vaccine against other infectious diseases and cancer. A good safety record has been established through the use of vaccinia virus as a smallpox vaccine. However, it has not been

widely accepted as a potential tumor-directed gene therapy vector because of concerns regarding the safety of a systemically administered replicating virus.^{7–10}

Vaccinia appears to target tumors selectively after systemic administration and thus displays a natural tumor tropism.^{12–16} Our previous studies have employed two different strategies to improve the tumor targeting of oncolytic vaccinia virus. One is to change the tumor microenvironment or the host to make the tumor more accessible to the virus. Recently, we have shown that hyperthermia augments vaccinia delivery to tumors after systemic injection, as hyperthermia increases the permeability of the endothelial vasculature to nanoparticles and viruses.¹⁷ The second strategy is to modify the virus genetically to improve its tumor targeting and oncolytic activity. We as well as other groups have demonstrated the positive effects of this strategy on recombinant vaccinia viruses in a number of studies. For example, we have explored a vaccinia mutant in which the genes encoding vaccinia growth factor (VGF) and thymidine kinase (TK) have been deleted.¹⁸ A TK- virus requires thymidine triphosphate for DNA synthesis from the nucleotide pool present in dividing cells. This leads to preferential viral replication in dividing cells and is the presumed explanation for the observed tumor specificity.^{13,19} When both viral genes were deleted, the resulting virus vvDD displayed remarkable tumor selectivity and sustained oncolytic ability in murine tumor models.¹⁸ This virus has been tested for toxicity

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and appeared to be safe in nonhuman primates.²⁰ We plan to initiate testing of this virus in human clinical trials in the near future.

In the last few years, viruses with deletions of antiapoptotic genes have been developed as potential oncolytic agents.²¹ It has been speculated that apoptosis may have been evolved as a defense against viral infection. Serpins have multiple roles including the regulation of immune and inflammatory responses and cell death.²² In vaccinia, *spi-1*, which encodes one of the viral serine protease inhibitors, is one of three related orthopoxvirus genes with sequence motifs that place them in the serpin superfamily. The *spi-1* gene was recently found to be necessary for replication of rabbit poxvirus in human lung A549 and pig kidney 15 cell lines. Subsequent studies suggested that its role in these cells was to prevent apoptosis.^{23,24} SPI-2, also known as CrmA, is the best-characterized orthopoxvirus serpin. It efficiently inhibits aspartic acid-specific cysteine proteases and less efficiently inhibits the aspartic acid-specific serine protease granzyme B and prevents interleukin-1- β formation and cytokine-induced apoptosis. Both proteins are expressed early in infection and reside intracellularly.²⁵ SPI-1 can act synergistically with SPI-2 to block granule-mediated cell killing pathways.²⁶ SPI-1, which has been shown to block apoptosis in RPV infections of cultured cells, and SPI-2, which can function to block apoptosis in several nonviral systems, may have partially overlapping functions *in vivo*. Our recent work deleting two viral genes (*spi-1*, *spi-2*) has shown that the mutant vaccinia (vSP) exhibits enhanced tumor selectivity.²⁷

On the basis of our previous studies, we hypothesized that combined deletions of the three viral genes encoding TK and two serpins might enhance the tumor selectivity of the mutant virus even further. Here, we describe this unique tumor-selective vaccinia with a triple targeted gene deletion of *tk*, *spi-1* and *spi-2*. We examined the replication of the virus, its cytotoxicity and the activation of caspases in infected cancer cells and normal cells *in vitro*. The biodistribution, viral pathogenicity and antitumoral effects were studied *in vivo* in murine cancer and human cancer xenograft models. Although some results of these studies are in agreement with our initial hypothesis, other results have revealed the complexity of functional interactions between viral gene products involved in multiple cellular pathways in the context of a replicating oncolytic vaccinia virus. It is hard to predict the behavior of a recombinant virus containing multiple viral gene deletions based solely on the behavior of each individual deletion within the virus. This concept may also apply to the design of oncolytic viruses in which multiple genes are inserted into the viral genome. Our present study should serve as a useful guide for future studies in designing new oncolytic viruses using a strategy exploring multiple viral and/or cellular genes whose products are involved in multiple cellular pathways.

Results

Construction of the vSPT

The parental virus, vSP, is a SPI-1/-2-dual gene deleted virus created previously by the insertion of the *lacZ* gene into the SPI-1 locus and the *GPT* gene into the SPI-2

locus.²⁷ A shuttle plasmid, pSEL-GFP, for insertional deletion at the *tk* locus was created (Figure 1a). The shuttle plasmid was used to insert *GFP* into the *TK* locus of the vSP by homologous recombination as described above, creating the triple gene deletion virus, vSPT. Multiple plaques of the virus with positive GFP expression were screened for deletions of the three genes. Polymerase chain reaction (PCR) reactions with gene-specific primers confirmed the deletion of these genes (Figure 1b). One of the positive plaques was amplified and purified for all further studies.

In vitro virus replication of vSPT

The ability of this new virus to replicate in cancer cells relative to the parental viruses (vJS6 and vSP) was examined. Three cancer cell lines, murine ovarian surface epithelial cells (MOSEC), murine colon adenocarcinoma (MC38) and human ovarian carcinoma (A2780) as well as normal human primary fibroblast (NHF) cells were infected with a series of vaccinia viruses vF13, vJS6, vSP or vSPT. The yield of infectious virus in cells at various time points was quantified by plaque assays in CV-1 cells. The results are shown in Figure 2. These results demonstrated that the yields of vSPT are comparable (in A2780) or a log lower (in MOSEC and MC38) than vSP or vJS6. The viral recovery of vSPT is also a log lower in normal cells (NHF) compared with wild-type virus. Strikingly, the replication of vSPT was up to 4 logs lower in normal cells compared with cancer cells. In this regard, it is noteworthy to point out that the wild-type virus vF13 also replicated poorly in NHF cells relative to cancer cells, suggesting the natural tumor tropism of the virus. These results demonstrated that although vSPT has gained specificity for tumor cells, the virus also has in general a reduced replication efficiency in tumor cells compared with the parental viruses from which vSPT was derived.

Cytotoxic effect of vSPT in vitro

We next tested the ability of vSPT to induce cytotoxic effects on cancer cells as well as human primary normal cells. Cells were cultured in 96-well plates and then infected with increasing doses of viruses. The viable cells were measured with a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay at 48 h after infection (Figure 3). The sensitivity to virally-induced cell killing or growth inhibition varied between the cell lines. In A2780 cells, a viral MOI of 1.0 resulted in an average of cancer cell growth inhibition of 50% by 48 h. At an MOI = 10, 70–85% of all cancer cells were inhibited or killed. Two viruses, vSP and vSPT, are relatively weaker than vJS6 and vF13 (Figure 3a). Similar results were obtained with additional cancer cell lines MC38 and HT-29 (data not shown). In MOSEC cells, for reasons that we do not fully understand, the two usually more potent viruses, vF13 and vJS6, appeared to be less effective than vSP and vSPT in killing cells of this particular line (Figure 3b). The human primary normal fibroblast MRC-5 cells were most resistant to vaccinia-mediated cell killing. Even infected with viruses at MOI = 10, about 60–75% of the MRC-5 cells survived (Figure 3c). These results suggested that the new virus vSPT is quite effective in killing infected cancer cells. In addition, cancer cells are usually more sensitive to vaccinia virus-induced cytotoxicity than normal cells.

Figure 1 (a) Schematic diagram of the parental viruses vJS6, vSP and new recombinant virus vSPT. Sites of left (vTK-L) and right (vTK-R) segments of vaccinia TK gene, ampicillin resistance gene, and xanthine-guanine phosphoribosyl transferase (*gpt*) gene are indicated. The *SPI-1* and *SPI-2* genes of vSP are interrupted by the insertion of the *lacZ* and *gpt* gene respectively. (b) PCR analysis of viral DNA from vSPT confirming the deletion of the genes in the recombinants. Lane M, 100-bp DNA ladder. The TK flanking sequence positive controls were shown in lanes 1 and 2. T1/T2 primers amplify a 150-bp fragment across the tk flanking region which demonstrate presence of flanking sequence of TK in wild-type vF13 (lane 1) and in vSPT (lane 2). Lanes 3 (vF13) and 4 (vSPT): T3/T4 primers amplify a 160-bp fragment across the region of recombination, the absence of which demonstrates the deletion of TK in vSPT. Lanes 5 (vF13) and 6 (vSPT): S1/S2 primers that amplify a 420-bp fragment across the region of SPI-1 recombination, which demonstrate absence of SPI-1 in vSPT; Lanes 7 (vF13) and 8 (vSPT): S3/S4 primers that amplify a 330-bp fragment across the region of SPI-2 recombination, which demonstrate the absence of SPI-2 in vSPT.

Figure 2 Viral recovery at various times after *in vitro* infection of normal and cancer cells. Cells in six-well plates were infected with vF13, vJS6, vSP or vSPT at MOI from 0.05 to 10, in triplicates. Data shown are from cells of MOSEC (a), MC38 (b), A2780 (c), and normal cells NHF (d). Viral recovery is presented as PFU/cells, as determined by plaque assays on CV-1 cells; average values are presented.

Activation of caspases in cancer cells, but not normal cells infected with vaccinia

Vaccinia virus-infected cells may die via multiple mechanisms. We have previously shown that a fraction

of vSP-infected cells were annexin-V positive, a sign of early apoptosis, whereas some cells died and released high-mobility group box-1 protein, a sign of necrosis.²⁷ To understand further the mechanisms of cell death in

Figure 3 Cytotoxicity assays *in vitro*. Cancer cells and normal human primary cells were infected with vF13, vJS6, vSP or vSPT at different viral dosage from MOI of 0.05–10, respectively. At 48 h post-infection, viable cells were determined with a MTT assay. The presented are data from A2780 (a), MOSEC (b) and normal cells MRC-5 (c). Representatives of at least three independent experiments are presented.

vSPT and other vaccinia viruses-infected cells, we have examined the activation of multiple cellular caspases in matched human cancer and normal tissue cells that were infected with vaccinia. Human lung cancer cells (H460) and human normal lung fibroblasts (MRC-5) were infected with vaccinia viruses at MOI of 5, and the activity of caspase-3, 8 and 9 were measured at 18 h postinfection, using Caspase Colorimetric Assay kits (R & D Systems). As shown in Figure 4, cancer cells and normal cells treated with the apoptosis-inducing agent etoposide (Etop) displayed significant amounts of activated caspases 3, 8 and 9 ($P < 0.0003$). In H460 cancer cells, vSP and vSPT-infected cancer cells displayed three activated caspases significantly higher than those in the control, mock-infected cells ($P < 0.005$). In vF13-infected H460 cancer cells, only caspase-9, but not casp-3 and -8, was significantly activated, mostly likely owing to the presence of caspase inhibitors SPI-1 and SPI-2 expressed from vF13. These results in cancer cells are in agreement with those in the literature and with our hypothesis. Surprisingly, none of the activated caspases was observed in the NHF (MRC-5) infected with the three viruses ($P > 0.05$).

In summary, these results showed clearly that caspases were activated in vaccinia-infected cancer cells whereas they were not activated in normal cells infected with vaccinia viruses. Interestingly, the data also suggest that cancer cells and normal cells may die via different pathways upon infection by viruses vSPT and vSP.

In vivo replication of vSPT

We then examined the ability of the virus to replicate in tumor and normal tissues after systemic administration in mice. Individual groups of tumor-bearing nude mice were injected intraperitoneally (i.p.) with 10^7 PFU of one of the four viruses, respectively. Eight days after viral injection, samples of brain, liver, spleen, lung, ovary and tumor tissues were harvested. Infectious viruses in those tissues were titrated on CV-1 cells, and viral yield was calculated as PFU per milligram protein (Table 1). For direct comparison, graphs of viral recovery for each of the four viruses in tumor (Figure 5a) and in normal tissues (Figure 5b) are presented.

As shown in Figure 5a, the viral yields of vSPT in tumor were similar to vSP ($P = 0.56$), but lower than vJS6 and vF13 ($P = 0.008$). Data in Figure 5b provide a direct comparison of viral recovery of four viruses from normal tissues. Viral yields of vSPT in normal tissues were much lower than for wild-type virus vF13 ($P = 0.008$ between vSPT and vF13, for all normal tissues tested). When compared to the two parental viruses, vSP and vJS6, the results with vSPT were very promising too. Viral yields of vSPT were lower than vSP in all normal tissues ($P = 0.008$). Compared with vJS6, yields were lower in the lung and liver ($P = 0.008$ and $P = 0.03$, respectively); similar trends existed in all other normal tissues examined although these differences did not attain statistical significance. In summary, the viral yields of vSPT were markedly reduced compared with vF13 and vSP in all normal tissues, and lower than vJS6 in at least two normal tissues, lung and liver. These results demonstrated that, even though vSPT was recovered at slightly lower amounts in tumor, its yield in most normal tissues was markedly lower than its two parental viruses vJS6 and vSP.

In vivo pathogenicity of vSPT

Nude mice were injected systemically with vF13, vJS6, vSP or vSPT at 1×10^8 PFU per mouse and followed for survival (Figure 6). As expected, the wild-type virus vF13 was extremely virulent, with nude mice surviving a median of 7 days. Consistent with our previous studies, both the vJS6 and vSP were attenuated compared with vF13 virus, as median survival of the nude mice increased to 15 and 32 days, respectively ($P < 0.0001$ compared with vF13). Significantly, vSPT was highly attenuated in nude mice, and all treated mice lived equal to or more than 50 days ($P < 1.0E-05$ with each of the other groups). These results are in excellent agreement with the data showing the reduced ability of the virus to replicate in normal tissues (Figure 5 and Table 1). As for the cause of animal death in vSP-treated group, it is at least partly owing to chronic infection in those nude mice, as we had observed some poxes in those mice quite late after viral administration. However, the exact causes of death have not been determined.

Figure 4 The activated caspases in vaccinia-infected human cancer cells and primary normal fibroblast cells. Human cancer cells (H460) and human normal lung fibroblasts (MRC-5) were mock-infected or infected with vaccinia vF13, vSP or vSPT at MOI of 5, respectively. At 18 h post-infection, cells were harvested and cell extracts were made and tested for activated caspases as described in Materials and methods. As positive controls, cells in the control dish were treated with an apoptosis-inducing agent etoposide (Etop) for 18 h. The experiments were performed twice and the data are presented as mean $\pm$ s.d. For both H460 and MRC-5 cells, Etop-treated cells showed significantly activated caspases as compared to mock-treated cells ($P < 0.001$). For MRC-5 cells, all three caspases in all three vaccinia viruses-infected cells showed no significant activation as compared with the control ($P > 0.05$). For H460 cells, all three activated caspases were upregulated in vSP- and vSPT-infected H460 cells ($P < 0.001$). However, only activated casp-9 was significantly increased in vF13-infected H460 cells ($P = 0.002$).

Table 1 Comparison of biodistributions of four vaccinia viruses in tumor and normal tissues^a

	vF13	vJS6	vSP	vSPT
Brain	5.2×10^2	2	17	0
Liver	5.1×10^3	0.32	2	0
Spleen	1.2×10^3	29	1.9×10^2	6
Lung	1.3×10^3	4.5×10^2	2.8×10^2	2
Ovary	3.1×10^7	9.5×10^5	2.5×10^7	3.1×10^4
Tumor	1.8×10^7	2.6×10^6	1.2×10^6	1.5×10^5

^aShown are the median viral yields, PFU/mg of total protein of tissues on day 8 after virus injection with vF13, vJS6, vSP and vSPT. Nude mice ($n = 5$ per group) were injected with virus at 1×10^7 PFU per mouse. On day 8 after virus administration, tumor and normal tissues were harvested and homogenized, and a standard plaque assay was performed on CV-1 cells. Data represent the median values. The P -values may be found in the Results section.

Antitumor effect in multiple tumor models

The ability of vSPT to function as an oncolytic virus was examined in three models of mouse and human cancers. In the first model, nude mice bearing subcutaneous (s.c.) MC38 tumors were injected systemically (intraperitoneally) with 1×10^8 PFU of vSPT, vJS6, vSP or a saline control (HBSS). As shown in the data in Figure 7a, significant antitumor effect was seen in the mice treated with vSPT, as well as in the groups treated with vJS6 or

vSP ($P < 0.001$ as compared with the saline-treated group). It is important to point out that mice started to die on day 13 after viral treatment with vJS6 owing to viral pathogenicity, whereas mice treated with vSP or vSPT remained healthy for the duration of the experiments, indicating reduced pathogenicity of the two viruses vSPT and vSP relative to vJS6. The antitumor effect of vSPT is attributable to the replication of virus alone because no therapeutic genes have been introduced in the virus, and the hosts were nude mice lacking functional T cells.

In the second model, we tested the oncolytic efficacy of vSPT in a human ovarian carcinomatosis xenograft in nude mice. A total of 5×10^6 cells of A2780 human ovarian carcinoma were injected i.p. into nude mice. Three weeks later, a total of 1×10^8 PFU/mouse of vSPT or saline was injected i.p. into the tumor-bearing mice. Mice were sacrificed 8 days later and tumors were examined (Figure 7b). As is evident, the average size of tumors in the control group was about 10 g, whereas the group treated with vaccinia vSPT was reduced to less than 4 g. Morphologically, the mice in the control group typically displayed distended abdomens owing to peritoneal growth of tumors, whereas the vSP-treated mice showed no similar abnormalities (data not shown). These results demonstrated that the oncolytic virus vSPT was efficacious in inhibiting growth in a human ovarian carcinomatosis model.

Figure 5 Recovery of vSPT from tissues of mice. A total of 2×10^5 MC38 cells were injected s.c. into athymic nude mice. When the tumors reached about 75–125 mm³, 1.0×10^7 PFU of specified vaccinia virus was injected i.p. into each mouse. Eight days after virus administration, tumor and normal tissues were collected. The virus titers (PFU/mg protein) were determined by titration of the virus in the tissue lysates on CV-1 cells. The median values of virus titers are presented in the figure. (a) Viral recovery from tumor tissues. $P=0.56$ when vSPT group compared to vSP group, and $P=0.008$ when vSPT group compared to vJS6 or vF13 group. (b) Viral recovery from normal tissues. The recovery of virus vSPT in normal tissues was much lower than vF13 ($P=0.008$), and than vSP ($P=0.008$). When compared to vJS6, the yields were lower in lung and liver ($P=0.008$ and $P=0.03$, respectively).

In the third tumor model, we have examined the oncolytic function of vSPT in a syngeneic murine MOSEC ovarian carcinomatosis in immunocompetant C57BL/6 mice. Mice were injected with 7×10^6 MOSEC cells. At 18 days after tumor cell inoculation, mice were divided into groups randomly, and 4×10^8 PFU of virus vJS6, vSP, vSPT or saline (HBSS) were injected. Tumor development and overall survival were monitored three times weekly (Figure 7c). All control animals developed tumor and died within 57 days. Tumor development was delayed significantly in mice treated with vSPT with survival extended up to 86 days ($P=0.0035$; versus HBSS-treated group). The animals treated with two parental viruses displayed prolonged survival; vSP treated animals surviving up to 113 days ($P=0.013$; vSP versus vSPT) and vJS6 treated animals surviving to a maximum of 123 days ($P=0.0018$; vJS6 versus vSPT). These results strongly suggest that in immunocompetent host, vSPT has lost some of its capacity to replicate and destroy the cancer cells, thus reduced its ability to prolong the survival of tumor-bearing hosts in comparison with its parental viruses vSP and vJS6.

Figure 6 vSPT results in decreased toxicity. Survival of nude mice after treatment with 1.0×10^8 PFU of vF13 (wild type; long dashed line), vJS6 (TK-; middle dashed line), vSP (SPI-1/-2-; dotted line), or vSPT (solid line) ($n=10$). Both vJS6 and vSP treated mice had significantly prolonged survival ($P<0.0001$) compared with vF13. However, mice treated with vSPT had the longest survival term ($P<0.00001$ compared with vF13).

Discussion

The previous studies have demonstrated that a TK-deleted vaccinia virus vJS6 exhibited reduced replication efficiency in nondividing cells both *in vitro* and *in vivo*.^{13,18} The viral pathogenicity was also decreased and significantly reduced recovery of the recombinant virus from normal tissues of nude mice was confirmed.¹⁸ However, the selectivity of this virus depends on the rapid proliferation of cancer cells to achieve its efficacy and it may not work as effectively in slowly growing human solid tumors. We have recently designed another recombinant vaccinia to achieve tumor selectivity based on a very different selective mechanism. One of the features of cancer is the ability to evade apoptosis.^{21,28} Therefore, we made another virus with deletion of two viral serpin genes, spi-1 and spi-2, the host range genes that provide antiapoptotic properties to infected cells. Our recent results showed that this virus (vSP) exhibited enhanced tumor-selective replication and reduced pathogenicity yet retained its strong oncolytic activity in a murine tumor model.²⁷

In this study, we addressed the hypothesis that a deletion of these three viral genes (*tk*, *spi-1*, *spi-2*) involved in two or three different cellular pathways would further enhance the selectivity and safety of vaccinia virus for both oncolytic virotherapy and as a vector for tumor-specific delivery of therapeutic genes. In comparison with the parental viruses vJS6 and vSP, the new virus vSPT displayed up to three logs of reduction in viral recovery from normal tissues. This reduced viral replication in normal tissues has resulted in greatly diminished viral pathogenicity. Therefore, vSPT exhibited an improved safety profile as it displayed the lowest recovery of infectious virus in a series of normal tissues examined. In addition, nude mice infected with vSPT had significantly increased survival compared with mice infected with the parental viruses. Importantly, we have been able to show that this virus was efficacious in inhibiting the growth of tumors of both mouse and human origins. The overall potency is best exemplified by the finding that a systemic injection of vSPT led to complete eradication of a subcutaneous

Figure 7 The therapeutic effect of vSPT in three tumor models. (a) Diminished MC38 tumor growth in nude mice treated with vSPT virus. When tumors reached about 75–125 mm³, mice were injected i.p. with 1.0×10^8 PFU of vSPT, vJS6, vSP or saline HBSS ($n=10$ each). The tumor sizes and health of mice were monitored regularly. The data are representatives of three independent experiments. $P < 0.05$ between the groups treated with HBSS and vSPT. (b) Treatment of A2780 human ovarian carcinomatosis in nude mice. The average tumor weights in treated and control animals. Eight days after inoculation with tumor cells, mice were injected with 1.0×10^8 PFU of the virus vSPT ($n=10$) or HBSS control ($n=10$). $P=0.001$, between the two groups by Student's t -test. (c) Treatment of MOSEC ovarian carcinomatosis in C57BL/6 mice. The tumor-bearing mice were treated with viruses 18 days after tumor cell inoculation. The formation of ascites and long-term survival of the animals were monitored. The statistical difference between vSPT-treated group versus other groups are all significant, as determined by log rank test, $P=0.0035$ (versus HBSS), $P=0.013$ (versus vSP group), and $P=0.0018$ (versus vJS6 group).

MC38 tumor in one out of five mice. In summary, these properties of the virus could have important implications for uses beyond oncolytic therapy, including its use as a vector for tumor-specific gene transfer and as a safer vaccine against cancer and infectious diseases including smallpox.

Although vSPT was safer, our results also indicated that vSPT lost one to two logs of activity for selective replication in tumor cells as compared with its parental viruses. On the basis of previous results on oncolytic viruses from clinical trials, it has been suggested that some oncolytic viruses have been so attenuated for safety reasons that they have lost their oncolytic potency or oncolytic 'punch'.^{6,21,29} Our study with vSPT in immunocompetent mice confirmed that this might have taken place with this recombinant vaccinia virus. Therefore, maximizing the safety and maintaining the oncolytic potency is a delicate balance. Further development of various viruses as oncolytic agents should be taken cautiously so as not to modify viruses to such extent that the oncolytic potential is either diminished or lost. Our next step to improve this vaccinia virus further would be to insert therapeutic genes to enhance its potency while maintaining its high tumor selectivity. Again, caution needs to be taken when multiple recombinant genes are inserted into the viral genome.

It is interesting to understand the mechanisms of cell death induced by oncolytic viruses. We have shown previously that wild-type and vSP-infected cancer and

normal cells may die via multiple mechanisms, including apoptosis and necrosis.²⁷ Recently, a new death pathway called autophagic cell death has been investigated in glioma cells induced by replicating adenovirus.³⁰ The inhibition of caspase 8 may initiate the autophagic signaling pathway.³¹ We have shown here that vaccinia viruses including vSPT induced the activation of multiple caspases 3, 8 and 9 in human cancer cells, but not in NHFs. These results suggest that normal cells and cancer cells infected by vaccinia may die via different pathways. Owing to the inhibition of caspase 8, at least some normal human cells infected by vaccinia virus may die via the autophagic cell death pathway. Our ongoing studies examining the mechanisms of death of cells infected with these vaccinia viruses will answer this question.

In summary, vaccinia has been actively studied as an oncolytic agent and as a vector for cancer gene therapy in addition to the traditional role as a vector for gene expression and vaccine development.^{7–10,32,33} In this study, our new virus has demonstrated enhanced tumor selectivity and is effective in inhibiting tumor growth in three tumor models in mice. Its oncolytic activity is somewhat diminished in comparison to two parental viruses. However, this new virus may serve as an excellent vector for cancer therapy by incorporating therapeutic genes such as suicide genes,^{19,34,35} or as a vector to express cytokines and chemokines, tumor-associated antigens and costimulatory molecules in the

setting of tumor immunotherapy.^{36–40} It can also be applied to combined immune cell-viral therapy.⁴¹ We plan to explore these types of studies with this and other recombinant vaccinia viruses in tumor models in mice.

Materials and methods

Cell lines and primary human cells

HeLa, HT-29, A2780, 143B (a TK- cell line) and NHF MRC-5 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). MC38 and CV-1 have been used frequently in this laboratory. Mouse ovarian surface epithelial cells (MOSEC) were a gift of Dr Katherine Roby.⁴² A2780 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). 143B cells were grown in minimum Eagles medium supplemented with 10% FBS. NHF were cultured in serum-free medium supplied with growth factors (Cambrex, East Rutherford, NJ, USA). MRC-5 cells were grown according to the suggestion of the provider. All other cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS, L-glutamine, penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA).

Vaccinia viruses

All recombinant vaccinia are derivatives of WR strain. The recombinant viruses vF13 (wild-type with *lacZ* gene inserted after the *F13L* locus), vJS6 (tk deletion), and vSP (deletion of *spi-1* and *spi-2*) have been used in the previous studies.^{13,27}

Creation of the vaccinia virus with triple deletion of tk and spi-1/-2 genes

vSP, a vaccinia virus that has the *lacZ* and *gpt* genes inserted into its SPI-1 and SPI-2 sites respectively, was used as the parental virus for recombination. A vaccinia shuttle plasmid was created that allowed for homologous recombination of pYSEL-GFP into the TK locus of vSP, creating the virus vSPT. Briefly, pSEL-GFP was digested with *HindIII* and *EcoRV*. The DNA segment containing GFP was self blunt-ligated into *HindIII* and *EcoRV* creating pYSEL-GFP. It is flanked by portions of the vaccinia TK gene, which allows for homologous recombination into this locus. To make the new virus, CV-1 cells were infected with vSP and then transfected with pYSEL-GFP. The mixture of viruses was then used to infect the TK-deficient 143B cells in selection medium containing 5-bromo-2'-deoxyuridine at final concentration of 25 μ g/ml (Sigma, St Louis, MO, USA). Six positive plaques were isolated and selected for 3–4 cycles in 143B cells.

DNA Extraction and PCR

Viral DNA was purified as described previously.¹⁸ The standard conditions for PCR were described previously.²⁷

PCR primers were designed to amplify the viral DNA fragments. For tk gene amplification, the positive control primers are T1/T2 set, T1 (tk227): GAACGGCGGACA TATTCAGT; T2 (tk379): GTCCATAGTCCCGTTCCGTA. For tk deletion analysis, the primers are T3/T4 set, T3 (tk360): TACGGAACGGGACTATGGAC; T4 (tk521):

GTTCGCCATACGCTCACAGA. For *Spi-1* gene, S1/S2 are used: S1 (SPI-1F1100), GACATGGACGTAGATATTC CGT; S2 (SPI-1R1520), GATAATAGAGAATCCTCC GTAT. For *Spi-2* gene, S3/S4 set was used: S3 (SPI-2F491): GAAGGAAATGAACCTCTGGAAT; S4 (SPI-2R822): GTGATTAAATGCCTTGCCGTAC.

Viral replication assays in vitro

Viral replication assays were performed as described.^{18,27} Briefly, 1×10^6 cells of MOSEC, MC38, A2780 and NHF in 6-well plates were infected with vF13, vJS6, vSP or vSPT at MOI 1.0 in 1 ml of MEM-2.5% FCS for 2 h at 37°C. Then cells were cultured in DMEM-10% FCS until harvesting at 6, 12, 24, 48 and 72 h after viral infection. After a three freeze-thaw cycles, the cell lysate was further homogenized using a FastPrep Cell Disrupter (Model FP120; Qbiogene, Inc., Carlsbad, CA, USA) to release virions, and then virus was titered on CV-1 cells.

Cytotoxicity assay in vitro

For viral cytotoxicity assay, cancer cells were plated at 1×10^4 cells per well in 96-well plates, and then infected with viruses at appropriate MOI as indicated. Forty-eight hours after infection, the cell viability was determined by a CellTiter 96 Aqueous Non-radioactive Cell Proliferation Assay (Promega Corp, Madison, MI, USA).

Measurement of the activities of caspases

The enzymatic activities of caspases (–3, 8 and 9) in the cell extracts were performed with colorimetric assay kits, according to the instruction of the manufacturer (R & D systems, Inc., Minneapolis, MN, USA).

Mice

Female nude mice and C57BL/6 were obtained from the NIH small animal facility (Frederick, MD, USA). They were housed in standard conditions and given food and water. Animal studies were approved by the Animal Care and Use Committee of the University of Pittsburgh.

Viral pathogenicity

Viral pathogenicity was assessed with tissue histology, *in vivo* viral replication, and mouse survival in nude mice. For assessment of virus replication, 8 days after i.p. injection of 1×10^7 PFU of vF13, vJS6, vSP or vSPT, whole sections of brain, liver, spleen, lung, ovary and tumor were homogenized in HBSS saline and stored at –70°C until use. Viral titers were determined and normalized to total protein (PFU/mg) as done previously.

Survival studies were performed on 6–7-weeks-old nude mice. Naïve mice were injected i.p. with 1×10^8 PFU of vF13, vJS6, vSP or vSPT in 200 μ l of HBSS and followed for survival.

Tumor models and antitumor Effects

In the first tumor model, 2×10^5 MC38 cells in 100 μ l of DMEM were injected s.c. into the right flanks of 7-week-old female nude mice and allowed to grow for 7–10 days. When the tumors reached 75–125 mm³ in volume, 1×10^8 PFU of vSPT or HBSS control were injected i.p. Tumor sizes were measured by an investigator blind to the treatment. Tumor volume was calculated as (width)² $\times$ length)0.52.

In the second tumor model, 5×10^6 A2780 cells in 500 μ l of HBSS were injected i.p. into nude mice. Three

weeks later, the animals were treated with 1×10^8 PFU of vSPT or HBSS as control. Animals were sacrificed after another 8 days when the control group exhibited distended abdomens due to tumor growth. The tumors in each mouse were dissected, collected and weighed as the end point.

In the third tumor model, C57BL/6 mice were injected with 7.5×10^6 MOSEC cells. At 18 days post-tumor cell inoculation, mice were divided into groups randomly, and 4×10^8 PFU of vSPT, vJS6, vSP or just saline buffer were injected i.p. The development of ascites and the survival of the mice were monitored three times a week.

Statistics

Statistical analyses were performed as described previously.²⁷ $P < 0.05$ was considered significant.

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