The Enhanced Tumor Selectivity of an Oncolytic Vaccinia Lacking the Host Range and Antiapoptosis Genes SPI-1 and SPI-2

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Abstract

The ability of cancer cells to evade apoptosis may permit survival of a recombinant vaccinia lacking antiapoptotic genes in cancer cells compared with normal cells. We have explored the deletion of two vaccinia virus host range/antiapoptosis genes, SPI-1 and SPI-2, for their effects on the viral replication and their ability to induce cell death in infected normal and transformed cells in vitro. Indeed, in three paired normal and transformed cell types, the SPI-1 and SPI-2 gene-deleted virus (vSP) preferentially replicates in transformed cells or p53-null cells when compared with their normal counterparts. This selectivity may be derived from the fact that vSP-infected normal cells died faster than infected cancer cells. A fraction of infected cells died with evidence of necrosis as shown by both flow cytometry and detection of high-mobility group B1 protein released from necrotic cells into the culture supernatant. When administered to animals, vSP retains full ability to replicate in tumor tissues, whereas replication in normal tissues is greatly diminished. In a model of viral pathogenesis, mice treated with vSP survived substantially longer when compared with mice treated with the wild-type virus. The mutant virus vSP displayed significant antitumoral effects in an MC38 s.c. tumor model in both nude (P < 0.001) and immunocompetent mice (P < 0.05). We conclude that this recombinant vaccinia vSP shows promise for oncolytic virus therapy. Given its enhanced tumor selectivity, improved safety profile, and substantial oncolytic effects following systemic delivery in murine models, it should also serve as a useful vector for tumor-directed gene therapy.

(introduction)

Tumor-directed gene therapy has been limited by low transduction efficiency and relatively low levels of gene expression from current gene transfer vectors. This reduces its therapeutic potential despite modifications that allow tumor targeting and tumor-specific gene expression. A resulting trend in vector development for cancer therapy has been to explore replicating oncolytic viruses, such as adenovirus, herpes simplex virus, and vaccinia virus (1–5). With replicating viral vectors, levels of gene expression are higher and transduction efficiency is improved due to viral replication and subsequent spread to surrounding cells. Antitumor effects attributable to virus-mediated cell death are observed (1, 6–8). Virus-associated toxicity is a concern and various modifications have been explored in an effort to improve both tumor specificity and safety profiles (1).

Our laboratory has explored the application of tumor-selective replicating vaccinia virus (WR strain) for cancer therapy (4, 9). We have previously shown that a double deletion of the thymidine kinase (TK) and vaccinia growth factor (VGF) genes significantly decreases pathogenicity and increases tumor selectivity. A TK-virus requires TTP for DNA synthesis from the nucleotide pool present in dividing cells. The TK deletion leads to preferential viral replication in dividing cells. The VGF gene encodes the vaccinia growth factor, a secreted protein produced early in viral infection that acts as a mitogen to prime surrounding cells for subsequent viral infection. Deletion of this gene causes decreased viral replication in resting cells. Compared with the wild-type virus, the dual-deletion mutant displayed reduced viral recovery from resting NIH3T3 cells but equivalent viral recovery from dividing NIH3T3 cells in vitro. In tumor models in mice, the TK/VGF double-deletion mutant displayed higher tumor-targeting capacity and potent tumoricidal activity with reduced viral pathogenicity (10). However, most solid human tumors have a low percentage of cells in S-phase compared with rapidly growing murine tumors. Thus, this virus may not be as effective when applied in some types of human cancer. Therefore, other strategies for creating more efficient oncolytic vaccinia need to be explored.

Viruses have evolved a number of mechanisms, encoding a large number of specific proteins, designed to interfere with host antiviral defense to maximize viral replication (11, 12). Poxviruses encode a number of serine protease inhibitors (members of the serpin superfamily), which function to regulate key biological processes, including inflammation, fibrinolysis, and cell migration (13–15). Indeed, whereas some poxvirus serpins regulate inflammation or apoptosis, the function of other poxviruses remains unknown. Vaccinia encodes three serpins designated as SPI-1, SPI-2, and SPI-3 (16, 17). Of these serpins, SPI-1 (encoded by B22R) is implicated in the inhibition of apoptosis based on studies of rabbitpox SPI-1 (18, 19). SPI-1 binds calpentin G and functions to inhibit apoptosis through effects on mitochondria in some cell types (20). Wasilenko et al. (21) have shown that vaccinia virus infection directly affects the mitochondrial apoptosis cascade by influencing the permeability transition pore, as shown by using the Copenhagen strain that naturally lacks the SPI-2 gene. SPI-2 (encoded by B13R) inhibits the proteolytic activity of interleukin 1β.
replication before the cells die. In addition, the normal antiviral mechanisms, such as necrosis, senescence, autophagy, and mitotic catastrophe (29, 30). In normal cells, the default pathway in response to insults, such as viral infection, is to die via apoptosis. Based on these strikingly different properties of normal cells and cancer cells, we hypothesized that a vaccinia deleted of the SPI-1 and SPI-2 genes would selectively replicate in tumor cells and represent a safe and effective virus for oncolytic therapy. Normal cells infected with SPI-1- and SPI-2–deleted vaccinia virus (vSP) would undergo apoptosis early and thus reduce viral replication, whereas cancer cells infected with the same mutant virus in vitro would still be apoptosis resistant and thus allow for viral replication before the cells die. In addition, the normal antiviral cytokine milieu produced in vivo in response to virus exposure, including IFNα, may be more effective at controlling this serpin-deleted vaccinia replication in normal cells than in cancer cells, leading to selective survival in cancer cells in vivo. As products of the host range genes, other intrinsic properties of the two proteins may confer an advantage to the mutant virus, enabling better survival and proliferation in cancer cells. In either case, the SPI-1 and SPI-2 mutant virus could display significant selectivity in tumor cells.

In this study, we have shown that vSP is significantly attenuated in normal cells in vitro but retains or even enhances its replication competency in cancer cells. This selectivity may be derived partially from the fact that vSP-infected normal cells die faster than infected cancer cells, thus reducing the viral yield in normal cells. In s.c. tumor models in both immunodeficient and immunocompetent mice, systemically delivered vSP virus displayed significant tumor inhibitory activity as well as reduced toxicity. The mutant virus exhibits profound tumor selectivity and safety and should, therefore, have utility for cancer-directed oncolytic therapy and gene therapy.

Materials and Methods

Cell cultures. CV-1, HaCAT, HeLa, and H460 cell lines were obtained from the American Type Culture Collection (Manassas, VA). MC38, a nonmetastatic colon adenocarcinoma cell line derived from C57BL/6 mice, has been extensively used in our previous studies. Normal human epidermal keratinocytes and normal human bronchial epithelial cells were obtained from Cambrex Biosciences (East Rutherford, NJ). They were cultured under conditions provided by the supplier. Normal human primary fibroblast cells were obtained from Dr. Teresa Whiteside (University of Pittsburgh Cancer Institute, Pittsburgh, PA). The mouse embryonic fibroblasts (p53+/−; p53−/−) were gifts from Drs. Charles J. Sherr (St. Jude Children’s Hospital, Memphis, TN) and Tyler Jacks (Howard Hughes Medical Institute and Massachusetts Institute of Technology, Cambridge, MA). Most other cell lines were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L glutamine, and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin). Cells were maintained in an incubator at 37°C with 5% CO2.

Vaccinia viruses. All vaccinia viruses used in this study are derivatives of the WR strain. The pseudo-wild-type vF13L−, a WR strain virus with lacZ gene insertion and no viral deletions (31), as well as the single deletions of SPI-1 (ΔSPI-1) and SPI-2 (ΔSPI-2) have all been described (25). A double-deletion virus vDD-C, with deletion of TK and VGF and insertion of the suicide gene Escherichia coli cytosine deaminase (10, 32), has been previously described.

Creation of a vaccinia with dual mutation of SPI-1 and SPI-2 genes. The viruses deleting either SPI-1 gene (ΔSPI-1) or SPI-2 gene (ΔSPI-2) have been described previously (25). A shuttle vector for deleting SPI-2 was constructed using the plasmid pBR-SPI2 that contains a 1,020 bp SPI-2 DNA fragment cloned into the HindIII and BamHI sites of pBR322. The SPI-2 fragment was generated by PCR using vaccinia genomic DNA as a template and then cut with HindIII and BgII. The lacZ expression cassette was inserted into the EcoRV site in the SPI-2 gene, resulting in a vector for insertionional mutation of SPI-2 gene (pVΔSPI2). For constructing the recombinant virus with double deletions (vSP), the shuttle vector pVΔSPI2 was transfected into CV-1 cells. Cells were then infected with virus ΔSPI-1 at a multiplicity of infection (MOI) of 0.1. After three rounds of selection and amplification with confirmation of the deletion, one of the clones was selected for amplification and purification.

DNA extraction. Confluent CV-1 cells were infected with recombinant vaccinia virus. After 2 to 3 days when viral cytopathic effects were complete, supernatant was removed and cells were washed and harvested. The DNA purification was done as described previously (10).

PCR. Cloning of the SPI-2 gene used a forward primer of 5’-CTAGAAGGTGAACTCTGGAATTAGTGA-3’ (with HindIII site underlined); the reverse primer is, GTCAGAATCTGCTATAATCTCCAGTTGAAC (with BgII site underlined). Standard PCR used 50 μL of the PCR reaction containing purified vaccinia DNA, 1 μL of each primer (100 μmol/L), 1 μL of deoxynucleotide triphosphates (10 mmol/L), Invitrogen), 2.5 units of Taq polymerase (Promega Corp., Madison WI), and PCR buffer. PCR amplification parameters consisted of 15 seconds of denaturing at 94°C, 30 seconds of annealing at 55°C, and 2 minutes extension at 72°C for 35 cycles.

Virus replication in cultured cells in vitro. Confluent cells grown in six-well plates were infected at a MOI of 0.1 in 1 mL of medium supplemented with 2% FCS for 2 hours at 37°C. After washing with 1 × PBS, medium with 10% FCS was added and cells were incubated until harvesting at 24 hours postinfection. After three freeze-thaw cycles to lyse the cells and release virus, virus was quantified by plaque titration on CV-1 cells as described previously (10).

Apoptosis assays. For apoptosis assays, cells were infected with vaccinia viruses at a MOI of 1, 5, or 50 for over 1 hour in 1 mL of medium supplemented with 2% FBS. Following infection, the virus suspension was then aspirated and cells were washed once with 1 × PBS before addition of complete growth medium. The cells were harvested at 18 hours or at specified times postinfection. Cells were stained with Annexin V-phycocyanin and propidium iodide by using apoptosis kits under conditions provided by the manufacturer (BioVision, Inc., Mountain View, CA). The stained cells were further analyzed by flow cytometry using a Beckman Coulter XL four-color analyzer.

Western blot analysis for high-mobility group B1 protein. Human cancer cells and primary normal fibroblasts in six-well culture plates were infected with either no virus, vF13L−, or vSP at a MOI of 50 for over 1 hour in medium containing 2% FBS. The cells were washed once with 1 × PBS before addition of 1 mL of growth medium supplemented with 2% FBS. Small aliquots of medium were collected at specific times postinfection (6, 20, and 48 hours). The amount of human high-mobility group B1 protein (HMGB1) in the conditioned medium was analyzed by Western blot. Briefly, 12.5 μL of conditioned medium was used in each lane. Proteins were resolved in the 12% SDS-PAGE gel and then electroblotted onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The
membrane was first incubated with a rabbit anti-HMGBl polyclonal antibody at 1:2000 dilution (BD Biosciences, San Jose, CA), then incubated with secondary antirabbit whole antibody conjugated to horseradish peroxidase (Amersham Biosciences, Piscataway, NJ). Following application of West Pico chemiluminescent substrate (Pierce, Rockford, IL) to the membrane, signals were detected by exposure to X-ray films (Blue Basic Autorad film; ISC BioExpress, Kaysville, UT).

**Mice.** Female athymic nude mice and C57BL/6 immunocompetent mice, 6 weeks of age, were obtained from the NIH Small Animal Facility (Frederick, MD). They were housed in standard conditions and given food and water ad libitum. Animal studies were approved by the Animal Care and Use Committees of the host institutions.

**Biodistribution of the viruses.** For examination of viral replication and viral yields in tissues, nude mice were injected i.p. with 1 × 10⁷ plaque-forming unit (pfu) of vF13L+, vCD, or vSP. Eight days following viral treatment, mice were sacrificed and whole sections of normal tissues and tumor were homogenized in 1× HBSS and stored at −70°C until use. One milliliter of the appropriately diluted homogenate was incubated on CV-1 cells in six-well plates at 37°C in 5% CO₂ and titers were determined as described previously (10). Viral titers were normalized to total protein in the cell lysate and expressed as pfu/mg protein. For marker gene lacZ expression from the viruses, tumor and normal tissues were collected 5 days after virus administration and immediately frozen and stored at −70°C. The reporter gene assays were done essentially as described previously (33). Briefly, frozen tissue samples were thawed, homogenized, and lysed in 750 μl reporter gene lysis buffer with the β-galactosidase enzyme assay system (Promega). The relative enzyme activity is determined by light emission with a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). The concentration of total protein in each sample was determined using a bicinchoninic acid protein assay kit (Pierce) with bovine serum albumin as standard. The β-galactosidase activity is expressed in relative light units per milligram of protein (RLU/mg).

**Viral pathogenicity.** Viral pathogenicity was assessed with survival studies done on both nude mice and immunocompetent C57BL/6 mice. Naïve nude mice were injected i.p. with 1 × 10⁷ pfu of vF13L+, vDD-CD, or vSP in 200 μl of HBSS. C57BL/6 mice were injected i.p. with 1 × 10⁷ pfu of virus in 200 μl of HBSS per mouse. Mice were observed daily throughout the course of the experiment.

**Tumor models and antitumor effect.** MC-38 tumor cells (2.5 × 10⁵ cells in 100 μl of HBSS) were injected s.c. into the right flanks of 7- to 8-week-old female mice. When the tumors reached ~5 × 5 mm in diameter (750 μl in volume), 1 × 10⁶ pfu of vF13L+, vSP, vDD-CD, or HBSS saline were injected i.p. Using the health of mice was monitored daily and tumor sizes were measured thrice each week. Tumor volume was calculated as [(width)² × length] 0.52.

**Statistics.** Statistical analysis was done using the Mann-Whitney test for nonparametric data when appropriate. Tumor volumes between groups were assessed using ANOVA for repeated measures. Survival analysis was done using the method of Kaplan-Meier and differences between curves were assessed using the log-rank test. All statistics were generated using StatView Software (Abacus Concepts, Inc., Berkeley, CA) and P < 0.05 was considered significant.

**Results**

**Creation of vaccinia viruses with deletions in both SPI-1 and SPI-2 genes.** As described, a shuttle vector for the SPI-2 deletion vector was constructed and used to generate a deletion of SPI-2 in the parental virus ΔSPI-1. The resulting virus, vSP, contains a 585 bp deletion of the SPI-1 gene with insertion of a functional guanine phosphoribosyl transferase expression cassette. At the SPI-2 locus, the coding sequence was disrupted by an E. coli lacZ gene expression cassette. Recombinant virus plaques were selected and amplified as described. A number of assays were used to confirm the mutation of the target genes, including PCR assays using SPI-1- and SPI-2-specific primers.

*In vitro replication of vSP and vF13 viruses in normal and transformed cells.* Confluent normal cells and transformed cells were infected with vaccinia vF13 or vSP at a MOI of 0.1. The cells were harvested at various times following infection and the yields of infectious viruses from those infected cells were determined by lysing cells and titering on CV-1 cells. Three paired cell types were used: primary normal human epidermal keratinocyte and transformed keratinocyte cells (HaCAT); primary normal human bronchial epithelial cell and human lung cancer cells (H460); and mouse normal fibroblasts as well as p53−/− mouse fibroblasts derived from p53 knockout mice. The yields of virus in transformed cells were compared with their reciprocal counterparts. In transformed or p53−/− cells, vF13L+ and vSP had similar replication efficiency. However, vSP replicated less efficiently in normal cells when compared with the wild-type vF13L+. The ratio of infectious virus in each of three cell type–matched pairs was calculated (Fig. 1). In all cases, vF13L+ virus replicated with similar efficiency in both normal cells and transformed or mutated counterparts, with ratios between 0.8 and 2.7. The mutant virus vSP, however, replicated with greater efficiency in transformed cells and in p53−/− mouse fibroblasts when compared with normal counterparts (with a ratio between 11 and 122). Thus, a vaccinia virus deleted of SPI-1 and SPI-2 replicates preferentially in transformed or p53 null cells compared with normal cells. The replication of vaccinia, as well as many other viruses, is modulated by host production of cytokines and chemokines, which are themselves modulated by viral infection. Vaccinia virus expresses many soluble receptors and binding proteins for both cytokines and chemokines (34). In response to viral infection, cytokines, such as IFN and TNF, are produced by host cells *in vivo.* We examined the effects of some individual cytokines on the relative replication efficiency of the viruses under defined conditions *in vitro.* Confluent paired cells, normal human epidermal keratinocytes and HaCAT, were infected with vF13L+ or vSP at a MOI of 10 and then cultured alone, with IFN-α, with IFN-γ, or with both (IFN-α/IFN-γ). At 24 hours postinfection, cells were harvested and infectious virus was titered on CV-1 cells. In the normal human epidermal keratinocyte cells, the viral yields were assessed using the log-rank test for nonparametric data when appropriate. Tumor volumes between groups were assessed using ANOVA for repeated measures. Survival analysis was done using the method of Kaplan-Meier and differences between groups were assessed using the log-rank test. All statistics were generated using StatView Software (Abacus Concepts, Inc., Berkeley, CA) and P < 0.05 was considered significant.
remained the same under all conditions for both viruses. However, in HaCAT cells, cytokines moderately inhibited viral yields, with 2- to 3-fold reduction of both vF13L+ and vSP in the presence of either or both cytokines (data not shown). However, vSP still retained much of the preferential replication in transformed cells. Therefore, vSP displayed preferential activity of replication in transformed cells regardless of the absence or presence of IFNs.

**vSP induced both apoptosis and necrosis in infected normal and cancer cells.** Cells infected with vaccinia eventually die; this death does not occur via apoptosis in most cell lines studied (35–37). Vaccinia virus encodes a number of antiapoptotic genes, including SPI-1 and SPI-2. We hypothesized that deletion of these antiapoptotic genes may enable infected normal cells to die via apoptosis. In contrast, cancer cells are intrinsically resistant to death through apoptosis; thus, cancer cells infected with vaccinia lacking the viral antiapoptotic genes SPI-1 and SPI-2 may survive long enough for the virus to proliferate efficiently. We examined the mechanisms of cell death under similar conditions we previously used to analyze the replication efficiency of vSP compared with vF13L+ in three paired cell types. The key feature in this experiment was that there were no external stimuli for apoptosis, such as FasL, TNF, or IFN-γ. Under these conditions, we have analyzed cell death by Annexin V and propidium iodide staining at 6, 12, and 18 hours postinfection. Figure 2 shows the representative data from cells at 18 hours postinfection. In the control H460 cancer cells, 14% were dying/dead cells (cells that are Annexin V and/or propidium iodide positive). In the cells infected with either vF13L+ or vSP, 58% to 61% H460 cells are dying/dead cells, suggesting that vF13L+ and vSP infection induced cell death with similar kinetics in cancer cells. With normal human primary fibroblasts (Fig. 2B), 8% of control (mock-infected) cells are dying/dead. Normal human primary fibroblasts infected with vaccinia, either vF13L+ or vSP, are dying with a faster kinetics with 63% dying/dead in vF13L+-infected cells and 70% dying/dead in vSP-infected cells. More propidium iodide–positive cells were found in vSP-infected cells (37%) than in vF13L+-infected cells (14%). In summary, vaccinia infection caused normal human primary fibroblasts to die with faster kinetics compared with cancer cells. In addition, vSP seemed to induce more normal cells to die via necrosis (propidium iodide–positive cells) at this time point.

Necrotic cells release HMGB1, an abundant and conserved constituent of vertebrate nuclei (38, 39). We examined vaccinia-infected cell release of HMGB1 into culture medium. Cancer and normal cells were infected with vF13L+, vSP, or mock-infected, and then grown in complete medium containing 2% FBS. The conditioned medium was collected at various times following infection and was spun briefly to remove cell debris. Western blots confirmed the presence of HMGB1 in the culture medium (Fig. 3). Under normal growth conditions, there is no detectable HMGB1 in the medium from either cancer cell lines (H460 and HT-29); HMGB1 was also not released into the medium from mock-infected cells. At 6 hours after infection with either vF13L+ or vSP no visible HMGB1 was detected in the medium. However, 20 hours after infection, a faint band of HMGB1 was visible, indicating that some infected cells had begun to die via necrosis and had started to release HMGB1 into the medium. These results are consistent with those previously observed with only a small percentage of necrotic cells around this time point (Fig. 2A). By 48 hours postinfection,
both cancer cell lines infected with either vF13L+ or vSP released significant amounts of HMGB1 into the medium. When examined under microscopy, most of the cells are nonviable. As for normal human primary fibroblasts infected with either virus, smaller but visible bands of HMGB1 were detected in the medium at 20 or 48 hours following infection (data not shown). These results showed that vaccinia-infected cancer cells release significant amounts of HMGB1 into the culture medium in the late infection phase, reflecting a necrotic death. The deletion of SPI-1 and SPI-2 had little effect on the release of HMGB1 from infected cancer cell lines.

Reduced pathogenicity of vSP. Nude mice were injected with vaccinia vF13L+ or vSP at 1 × 107 pfu i.p. and then followed for survival (Fig. 4A). As expected, wild-type virus was extremely virulent, with vF13L+-treated mice all dying within 20 days. The median survival was 13 days. The exact mechanism of viral pathogenicity and death of mice is unclear. However, we know that WR vaccinia is a murine neurovirulent strain and that the virus replicates in brain, lung, spleen, ovary, and other organs (see below). Mice infected with vSP survived longer with a median survival time of 32 days. The pathogenicity of the viruses was also tested in immunocompetent mice (Fig. 4B). Both viruses were injected into C57BL/6 mice at a 10-fold higher dose, 1.0 × 108 pfu/mouse. All 10 mice injected with 1.0 × 108 pfu of vSP survived and remained healthy for at least 102 days, the date of sacrifice. Of mice injected with pseudo-wild-type vF13L+, 8 of 10 mice died within 1 week. The two mice that survived the initial phase of viral infection were treated with the mutant virus vSP. These results, obtained from both nude mice and immunocompetent mice, showed that vSP is significantly attenuated in mice and is much less pathogenic.

Enhanced tumor selectivity of vSP in vivo. The tissue distribution of the mutant vaccinia was examined using marker gene expression in tissues and by titering the infectious viruses recovered from tumor and normal tissues in tumor-bearing mice. S.c. MC38 tumor-bearing nude mice were injected with 107 pfu i.p. of vF13L+ or vSP. Five days following virus administration, tumor and normal tissues were harvested and lacZ expression was analyzed and expressed as RLU/mg protein (Fig. 5). In liver and spleen, there was 1 to 2 log of magnitude reduction of marker gene expression in vSP-infected animals relative to vF13L+. In brain, there was a trend toward decreased level of marker gene expression in vSP-treated mice. Increased β-galactosidase expression was seen in the tumor of vSP-infected animals relative to vF13L+; the difference is statistically significant (P < 0.05). These results together suggest that deletion of the two genes results in reduced viral gene expression in some normal tissues while causing enhanced viral gene expression in tumor tissue.

In a separate experiment, the infectious virus recovery from tissues was analyzed. Eight days following injection of virus, samples of tumor and normal tissues, including brain, liver, lung, spleen, and ovary, were harvested. The infectious viruses from these tissues were titered on CV-1 cells and viral yield was calculated per milligram protein (Fig. 6). Interestingly, the viral yield in tumors indicated that vSP generated amounts of infectious virus similar to wild-type virus. The second control mutant vDD-CD also generated similar amounts, consistent with a previous study done in this laboratory (10). In contrast, vSP was recovered at markedly lower titers compared with vF13L+ in all normal tissues examined except brain (P < 0.05). These results were consistent with marker gene expression studies as shown in Fig. 5. The data are also consistent with the in vitro observation using three paired types of normal/transformed or p53-null cells (Fig. 1). Thus, both marker gene expression and infectious virus

![Figure 3. Human HMGB-1 protein is released from vaccinia-infected human cancer cells. The cancer cells grown in six-well culture dishes were infected with vaccinia at a MOI of 50 for 2 hours. Following infection, the cells were washed with 1 × PBS and fed with 1 ml of medium supplemented with 2% FBS. At various time points, aliquots of medium were taken and stored at –20°C.](image1)

![Figure 4. Increased survival of mice treated with vSP. A, survival of nude mice treated with vaccinia. Mice were treated with 1.0 × 107 pfu of vF13L+ or vSP by i.p. injection (P < 0.0001). B, survival of C57BL/6 mice treated with vF13L+ or vSP at dose of 1.0 × 107 pfu of vF13L+ or vSP by i.p. injection. Eight of 10 mice treated with vF13L+ died within 7 days, whereas the remaining two survived for at least 102 days. No toxicity was seen in the group of mice treated with vSP. Kaplan-Meier survival statistics were done as described with the log-rank test (P = 0.0003).](image2)
injected i.p. with 1/C2 with those treated with saline alone (immunocompetent mice (Fig. 7). In nude mice, the growth of activity of the mutant virus in s.c. tumor models in both nude and Cancer Res 2005; 65: (21). November 1, 2005 9996 www.aacrjournals.org
tissues. It is important to point out that, when evaluated in the same experiment, vSP displayed even higher tumor selectivity in tissues of nude mice was confirmed. In addition, a significant significantly reduced recovery of the mutant virus from normal and tissues. MC38 tumors (75-125 mm³) were injected i.p. with 1 × 10⁷ pfu of vF13L+ or vSP. On day 5, mice were sacrificed and tissues were collected. The tissues were homogenized and β-galactosidase activity in the lysates was measured as described in Materials and Methods. The activities were normalized to protein concentration and then expressed as RLU/mg of protein. Columns, median values from 10 mice per group (*P = 0.058; **P < 0.05, between the groups of vF13L+ and vSP).

Potent antitumor effect of vSP. We examined the antitumor activity of the mutant virus in s.c. tumor models in both nude and immunocompetent mice (Fig. 7). In nude mice, the growth of tumors in those mice treated with vSP was inhibited compared with those treated with saline alone (P < 0.001). The virus vF13L+ displayed similar antitumor activity at an early stage after viral administration. All mice treated with vF13L+ died between days 8 and 16 due to adverse effects of the wild-type virus (Fig. 7A). In immunocompetent mice, significant antitumor activity was also observed for vSP when compared with the group treated with saline alone (P < 0.05; Fig. 7B). Thus, vSP displayed significant antitumor activity and less pathogenicity when compared with the wild-type vaccinia in both immunocompetent and immunodeficient mice.

Discussion
Our group previously showed that a TK- and VGF-deleted vaccinia virus reduced replication efficiency in nondividing cells both in vitro and in vivo (10). Viral pathogenicity was also decreased, and significantly reduced recovery of the mutant virus from normal tissues of nude mice was confirmed. In addition, a significant antitumor effect was observed following systemic injection in tumor models in nude mice. Therefore, a clinical trial using this virus to treat cutaneous malignancies has been proposed at our institution. The selectivity of this double-gene-deletion virus seems to depend on the rapid proliferation of cancer cells to achieve its efficacy. It may not work as effectively in slowly growing human tumors (4, 9).

Therefore, other modifications that enhance vaccinia tumor selectivity through different mechanisms are urgently needed. Tumor cells are generally more resistant to apoptosis, frequently sequestering p53 in the cytosol and overexpressing antiapoptotic proteins, including survivin, Bcl-2, and Bcl-X₇ (27, 28). However, tumor cells can still be induced to die by apoptotic or nonapoptotic mechanisms, such as necrosis, senescence, autophagy, and mitotic catastrophe (29, 30). Many viruses encode antiapoptotic proteins, and under some circumstances proapoptotic proteins, to enable expansion within their specialized niches of their host (11, 12). We designed a new mutant vaccinia to enable selective replication in cancer cells. We deleted two viral serpin genes, SPI-2 and SPI-1, that are host range genes providing antiapoptotic properties to infected cells. The site of disruption of apoptosis by these gene products (mitochondria and ICE) should be compensated for by mutations in cancer cells (e.g., p53). This virus termed vSP exhibited enhanced tumor-selective replication and reduced pathogenicity.

We have observed that normal cells infected with vaccinia died faster than those infected cancer cells (Fig. 2). In addition, more normal human primary fibroblast cells infected with vSP died via necrosis (propidium iodide positive) than apoptosis (Annexin V positive) compared with those infected with vF13L+. However, some caution should be exercised when we interpret these data with Annexin V staining. First, in the vaccinia life cycle, the exit and entry of various forms of vaccinia are complex processes (40). For example, the intracellular enveloped vaccinia exits the cells via fusion of outer membrane with the plasma membrane to produce a cell-associated enveloped virus particle by exocytosis. These processes might change the conformation and structure of the plasma membrane to create Annexin V–positive staining, which
Viruses can kill cells by either apoptosis and/or necrosis; frequently, the choice of pathway depends not only on the pathogen but also on the MOI as well as the cell type being studied. There are several potential mechanisms by which viruses activate apoptotic pathways. Mechanisms by which viruses cause infected cells to die via necrosis are not very clearly delineated. Cell death via necrosis is characterized by the release of cellular contents, including HMGB1, lactate dehydrogenase, S100 proteins, heat shock proteins, uric acid, and ATP (44). HMGB1 is a largely nuclear chromatin-binding protein that can be released from necrotic cells (38) and is secreted by activated macrophages and natural killer cells; it acts as a cytokine via binding to the receptors Toll-like receptor 2, Toll-like receptor 4, and receptor for advanced glycation end products, and triggers an inflammatory response associated with extensive tissue damage, thereby promoting tumor growth (38, 44–46). It is interesting to note that two animal RNA viruses, West Nile virus and infectious salmon anemia virus, induce host cell death via necrosis and release HMGB1 under certain conditions (47, 48). To our knowledge, our study is the first direct demonstration that infection with a DNA virus causes necrosis and release of HMGB1 from host cells. Many interesting questions remain to be addressed. How virus infection induces the release of HMGB1 from target cells and what role HMGB1 plays in the host immune response to viral infection are important subjects for further studies, especially in the context of using oncolytic viruses for cancer therapy. Another relevant question under intensive study has been the roles of cell necrosis and HMGB1 in tumor growth and the anticancer immune response (44–46).

The decreased pathogenicity of vSP is an important finding in the development of vaccinia for uses beyond oncolytic therapy, including its use as a safer vaccine against smallpox. Previous studies showed no viral attenuation on deletion of either serpin gene alone (49). These studies used an intranasal mode of infection that may have decreased sensitivity compared with systemic injections. Also, given the overlapping nature of many antihost response proteins, a single mutation may not have a significant effect on pathogenesis. We have clearly shown that the combined deletion of SPI-1 and SPI-2 markedly attenuates the virus, with no observed pathogenicity in immunocompetent mice following a systemic injection at a dose of $10^8$ pfu.

In this study, we have shown that a mutant vaccinia alone, in the absence of a therapeutic gene, is capable of causing antitumor effects from viral replication and subsequent cell death. This is not an immunologic response against the tumor as the effect is more profound in the immunodeficient mice. The most unique aspect is that this is achieved with a single systemic injection of the virus, thus having the advantage of no repeated injections. Also, given the overlapping nature of many antihost response proteins, a single mutation may not have a significant effect on pathogenesis. We have clearly shown that the combined deletion of SPI-1 and SPI-2 markedly attenuates the virus, with no observed pathogenicity in immunocompetent mice following a systemic injection at a dose of $10^8$ pfu.

In summary, vaccinia viruses are widely investigated as platforms for in vivo vaccine development and have gained recent interest as a vector for cancer gene delivery and oncolytic viral therapy (6–8, 50, 51). Here, we have created a tumor-selective replicating vaccinia virus by deleting both SPI-1 and SPI-2 genes.
This virus may be used as an oncolytic agent on its own or as a vector for cancer gene therapy by incorporating suicide or cytokine genes. An independent study deleting these two genes has shown the ability of this virus to function as an excellent vaccine (52). This virus may be used as an oncolytic agent on its own or as a vector for cancer gene therapy by incorporating suicide or cytokine genes. An independent study deleting these two genes has shown the ability of this virus to function as an excellent vaccine (52).

References
The Enhanced Tumor Selectivity of an Oncolytic Vaccinia Lacking the Host Range and Antiapoptosis Genes SPI-1 and SPI-2


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