Oncolytic Capacity of Attenuated Replicative Semliki Forest Virus in Human Melanoma Xenografts in Severe Combined Immunodeficient Mice


Abstract

Oncolytic viruses have gained attention as a novel form of cancer treatment. Many viral vectors in use today have been rendered safe by deletion of genes encoding viral structural proteins, thus making them unable to spread beyond the first infected cells. Hence, such replication-deficient constructs may lack efficacy. Here, we analyzed the oncolytic potential of the replication-competent vector VA7-EGFP, based on the avirulent Semliki Forest virus (SFV) strain A7(74), to kill cancer cells in culture as well as to target s.c. human melanoma xenografts in severe combined immunodeficient (SCID) mice. VA7-EGFP was able to infect most cancer cell lines studied, leading to complete lysis of the cells within 72 hours after infection. In SCID mice grafted with A2058 human melanoma, marked regression of the xenografts was observed following a single injection of 10^6 plaque-forming units of virus given either i.p., i.v., or intratumorally. Histologic analysis revealed the presence of virus not only in all treated tumors but also in the brains of the treated mice, causing progressing neuropathology beginning at day 16 after infection. Following initial oncolysis, clusters of viable tumor cells were observed embedded in connective tissue, and at later stages, encapsulated tumor nodules had formed. Infection of melanoma cells from explant cultures of these nodules revealed that a portion of the cells were resistant to virus. To be eligible for use in virotherapy, the ability of avirulent SFV to spread within tumor tissue may have to be improved and the biological safety of the virus may have to be addressed thoroughly in higher animals. (Cancer Res 2006; 66(14): 7185-94)

Introduction

Historically, viral oncolysis has probably been present throughout human evolution, and many observations of spontaneous tumor regression can probably be ascribed to preceding viral or bacterial infections (1–4). Viruses display many features that are advantageous in cancer killing, and with the advent of modern biotechnology and the capacity to manufacture virus stocks, the idea of exploiting viruses to kill tumors has become a reality.

Viral vectors based on adenovirus, herpes simplex type I, Newcastle disease virus, reovirus type 3, and vaccinia virus have already entered clinical trials (5–8), and many more are in the pipeline. However, most of the viruses used today are replication deficient, so-called replicons, and thus lack the ability to disseminate from cell to cell, which in turn limits their usefulness and may preclude their use in treatment of widespread, metastatic tumors. As the limitations of replicon-based virotherapy are becoming clearer, increasing attention has been directed toward replication-competent and conditionally replicating vectors. These viruses have retained the capacity to produce new virions and spread from cell to cell, but they are either intrinsically attenuated or have been rendered less pathogenic for safety reasons. Replication-competent viruses potentially allow for screening of the whole body as well as noninvasive administration at sites remote from the tumor.

Semliki Forest virus (SFV) is an enveloped, positive-stranded RNA virus of the family Togaviridae (genus Alphavirus), and it has a broad host range, including humans (9). Several strains of SFV exist, which differ markedly in virulence. Prompted by the key phenotypic features, we wanted to investigate the oncolytic potential of the avirulent SFV strain A7(74) in cultured cells and in experimental animals. First, in contrast to wild-type SFV, which is highly neurovirulent and lethal for mice of all ages, SFV A7(74) is relatively harmless to normal adult mice and forms only small perivascular foci of infection in the central nervous system (CNS) of these mice (10–12). However, in the brains of mice younger than 14 days, where neuronal processes are still maturing and active myelination is ongoing, SFV A7(74) is able to replicate and cause cytopathic death of neural cells similar to wild-type SFV (10, 13). Thus, avirulent SFV has the capacity to replicate in immature, actively growing neural cells while being severely restricted and controlled in adult, mature CNS tissue. Second, in pregnant mice, avirulent SFV can cross the blood-placental barrier and cause early abortion while leaving the mothers unaffected (14). This shows that avirulent SFV is able to transverse blood-endothelial barriers following peripheral inoculation and thus has the potential to reach poorly accessible or metastatic tumors. Finally, avirulent SFV induces rapid host cell protein synthesis shutoff and apoptosis in infected cells regardless of their p53 status (9, 15), and we previously showed significant cytotoxicity in several transformed cell lines of an expression vector based on SFV A7(74) carrying the gene for enhanced green fluorescent protein (EGFP; ref. 12). This vector, termed VA7-EGFP, replicated similarly to the parental SFV A7(74) and wild-type SFV in culture, indicating that, despite avirulence in mice, it had not lost its capacity to infect and kill transformed cells.
In this report, we have expanded the panel of susceptible cells by infecting and analyzing several human and murine cancer cell lines. Most cell lines tested were permissive to infection by avirulent SFV and also susceptible to SFV-mediated cell death. Infected cancer cells in culture died by both apoptotic and other mechanisms. We also show that a single injection into severe combined immunodeficient (SCID) mice of 10⁶ plaque-forming units (pfu) of VA7-EGFP, either i.v., i.p., or intratumorally, effectively reversed the growth of s.c. A2058 human melanoma xenografts. Despite extensive viral oncolysis, however, live tumor cells were found in resected xenograft nodules from treated mice together with infectious virus, indicating that equilibrium had formed between infected dying cancer cells and regrowing tumor tissue. We show the presence of host-derived connective tissue, which may have restricted virus spread and allowed for tumor regrowth. Moreover, a portion of the tumor cells in the xenograft nodules of the treated mice had become resistant to SFV, further limiting the success of the virotherapy.

Human melanoma is extremely prone to metastasize, often into the CNS, and current treatment modalities fail to provide long-term cures (16). There is therefore an urgent need to establish novel treatment forms that enable targeting of widespread tumors or tumors embedded in sensitive organs, such as the CNS. In this report, we have established the baseline oncolytic efficacy of replication-competent avirulent SFV using a xenograft model of human melanoma in SCID mice as a convenient platform to analyze tumor-virus interaction without interference from the immune system. Despite formidable oncolytic capacity of avirulent SFV, its ability to spread within tumor tissue may have to be enhanced and its biological safety profile may have to be addressed before it can become eligible for use in virotherapy in humans.

Materials and Methods

Recombinant virus and cell culture. The construction of replication-competent SFV vector VA7-EGFP has recently been described (12). Baby hamster kidney 21 (BHK-21) cell line was grown in Glasgow MEM (Sigma-Aldrich, St. Louis, MO) supplemented with 5% inactivated fetal bovine serum (FBS; Autogen Biosearch, Wilshire, United Kingdom). BMA-13 cell line was sustained in Eagle’s MEM (Sigma), 5% serum. Human colon adenocarcinoma LS174T [CCL-188; American Type Culture Collection (ATCC), Rockville, MD], human colon adenocarcinoma SW620 (CCL-227; ATCC), human lung carcinoma A549 (CCL-185; ATCC), human melanoma A2058 (CRL-11147; ATCC), human melanoma SK-MEL-5 (ICLC HTL01020; Common Access to Biological Resources and Information), and mouse neuroblastoma C-1300 were cultured in DMEM (Sigma), 10% FBS. All cultures were supplemented with 136 µg/mL streptomycin and 136 units/mL penicillin (Sigma) and maintained at 37°C in a humidified atmosphere containing 5% CO₂. Infection and analysis of cell lines. To characterize tumor cell line permissiveness and VA7-EGFP transduction capacity in vitro, cell lines listed in Table 1 were infected with virus at 1 multiplicity of infection (MOI) and analyzed at 6, 10 (or 12), 24, 48, and 72 hours after infection. For visualization of cytopathic effects under microscope and for immunostaining, cells were washed with PBS and fixed with 4% paraformaldehyde. To detect apoptosis, fixed cells were stained either in parallel using terminal deoxynucleotid transferase–mediated dUTP nick end labeling (TUNEL; in situ cell death detection kit, Roche, Basel, Switzerland) according to the manufacturer’s instructions or with diluted (1:250) mouse monoclonal antibody (mAb) against the 85-kDa cleaved form of poly(ADP-ribose) polymerase (PARP)-1 (BD Pharmingen, San Diego, CA) followed by biotinylated horse anti-mouse IgG antibody (Vectastain Elite Mouse kit, Vector Laboratories, Burlingame, CA) and Alexa Fluor 546–conjugated streptavidin (Molecular Probes, Invitrogen, Carlsbad, CA) at 2 µg/mL. In addition, to visualize general cell death and loss of cell integrity, cells in 24-well plates were incubated for 5 minutes in the presence of 50 µg/mL propidium iodide at 37°C before washing and fixation. Coverslips were mounted on microscope slides with Mowiol and observed under epifluorescence microscope (Leica DM IRB, Leica, Wetzlar, Germany). Images were processed using Adobe Photoshop 7.0 software.

For quantification of virus cytotoxicity, infected and uninfected cells in 96-well plates (4,000-15,000 per well in triplicate) in culture medium supplemented with 0.5% FBS were analyzed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer’s instructions (Cell Proliferation kit I, Roche). The degree of inhibition given in Table 1 was obtained by dividing the activity of infected cells with the activity of uninfected cells and multiplying by 100. Inhibition (0%) was assigned where the metabolic activity of infected cells exceeded that of the control cells. To compare viral replication kinetics between different cell lines, infected and uninfected cells (sustained in medium supplemented with 0.5% FBS) were processed and analyzed by fluorescein-activated cell sorting (FACS; FACSCalibur, BD Biosciences, San Jose, CA) as follows: at indicated time points, cell culture supernatant was harvested and the remaining cells were trypsinized. Trypsinized cells from each well were pooled with the supernatant from the same well. Cells were then either fixed with formalin (3.7%) and run separately or incubated with propidium iodide for 5 minutes before running. Fluorescence detection sensitivity (GFP and propidium iodide) was set using uninfected BHK-21 cells, and the same instrument settings were used for all runs. A minimum of 20,000 gated cells was counted.

Animal studies. Mice with SCID/SCID genotype were purchased from Harlan Laboratories (Horst, the Netherlands). A2058 human melanoma tumor cells were grown ex vivo under cell culture conditions described above, and tumors were established by injecting 2 × 10⁵ cells s.c. into the neck of mice ages 5 weeks as described previously (17). Fourteen days after tumor inoculation, when tumors had reached a size of ~500 mm³, 1 × 10⁶ pfu of VA7-EGFP virus was given either i.v., i.p., or intratumorally into groups of eight (i.v.) or four (intratumorally, i.p.) mice each. Three control mice received PBS only (i.v.). Injection regimen was 1 × 100 µL except for the intratumoral injections, which were 2 × 50 µL at different sites. Mice were monitored regularly for weight loss, symptoms of infection, and discomfort due to tumors. Tumor length and width were measured using a caliper and converted into volumes by the formula (length × width²) / 2. Sampled mice were sacrificed using CO₂. Animals were housed at the University of Turku Central Animal Laboratory (Turku, Finland) under specific pathogen-free conditions, and all experiments were conducted under protocol approved by the local ethics committee and in accordance with prevailing legislation and guidelines.

Histology. Mice were sampled at 5 and 13 days after infection or during the experiment as they developed discernible neuropathic symptoms. After blood was collected, mice were perfused with 30 mL 4% paraformaldehyde via the left ventricle. Tumors, spleen, muscle, pancreas, lung, liver, heart, brain, and spinal cord were taken and fixed in 10% neutral-buffered formalin. Tissue samples were mounted in paraffin blocks, and 4-µm sections were prepared. Parallel sections were stained with H&E, polyclonal SFV antibody (1:2,500), monoclonal anti-PARP antibody, TUNEL, or by the van Gieson technique. Fluorescent sections were counterstained with 0.1 mg/mL Hoechst 33342 (Sigma).

In situ hybridization. Presence of virus in tumor and brain sections was detected by in situ hybridization using a 5'-labeled probe specific for a region in the npg1 gene of SFV (sequence, 5'-GAAATCCGAGGAGTGCGTA-CAGGTAGGACTTGCGAGCGTG-3'). Similarly, expression of insert from the vector was detected using a probe specific for EGFP (sequence, 5'-TGCCCCGGTCGCGAGTGGGTCAGTGGTTGCGAGGCTAGCT-3'). Brieﬂy, after deparafﬁnization, sections were incubated in probe mixture, dried, and exposed to X-ray ﬁlm for 10 days. To determine probe speciﬁcity, parallel sections were incubated with 1,000-fold excess of nonlabeled probe before incubation with labeled probe. After exposure, composite images of H&E-stained sections and in situ results were created using Adobe Photoshop 7.0 software.

Virus titration. To determine virus concentration in blood and to conﬁrm that persisting virus was still able to kill melanoma cells, serum
stored at −70°C and tumor homogenate were used in plaque assay on both A2058 melanoma and MBA-13 cells as described (12).

**Explant cultures.** To confirm the presence of viable tumor cells after virus treatment, nine mice with established xenografts were given 1 × 10⁶ pfu virus i.p. and sampled for blood and tumor at various time points beginning at 19 days after infection. Tumors were processed as follows: using a razor blade, tumors or tumor nodules were divided, and one half was fixed in formalin and embedded in paraffin for histology, whereas the other half was minced in a culture well in a 24-well plate using a glass plunger in the presence of 300 μL Acutase (Sigma). The crude homogenate was incubated at 37°C for 15 minutes, after which 600 mL prewarmed DMEM supplemented with 20% FCS, 4 mmol/L L-glutamine, and 5 mg/L amphotericin B (Sigma) was added. The suspension was then split into a 24-well plate (300 μL/well), and growth medium was added to 1 mL. Plates were incubated under normal cell culture conditions at 37°C. Twenty-four hours later, samples of growth medium from each well were collected for virus titration, after which the medium was changed. Cells reaching optimal confluence were split into larger vessels and eventually cryogenically stored. During passage, explanted cells were accustomed to normal culture medium.

To confirm that the explanted cells were melanoma, they were compared under microscope to fresh A2058 cells. Moreover, cells were stained against both Melan-A antigen and the S100 protein using mouse monoclonal and rabbit polyclonal antibody, respectively. A2058 and SK-MEL-5 cells were stained the cells against the 85-kDa cleaved form of PARP-1, a marker for early apoptosis (18), and cleaved also in cells infected with Sindbis virus (19). Surprisingly, cleaved PARP was found only in others (A549 and LS174T) it seemed to be slightly delayed. The C-1300 neuroblastoma cell line was resistant to SFV as described previously (12).

Cultures of alphavirally infected cells typically contain increasing amounts of free-floating debris and detached, contracted cells, which by traditional methods, such as trypan blue exclusion or propidium iodide staining, appear dead. To quantify cell death in infected cultures, cells at 12 and 24 hours after infection were stained with propidium iodide and analyzed by FACS. The replication efficacy of VA7-EGFP in the cell lines was also measured by analyzing the number and fluorescence intensity of GFP-positive cells. The results presented in Table 2 and Supplementary Fig. S2 show that, in line with the increasing number of GFP-positive cells, also the number of propidium iodide–positive cells increased. Later during infection, the number of propidium iodide–positive cells started to decline, partly due to the appearance of propidium iodide–negative debris and also to the stringent fluorescence cutoff values in the data analysis. Comparing the number and fluorescence intensity of the GFP-positive cells revealed that, of the cell lines tested, BHK-21 cells provided the least permissive growth substrate for the virus, which is in keeping with our previous results (12). Although A2058, LS174T, SK-MEL-5, and SW620 were highly susceptible to infection, A549 was clearly less so and C-1300 was resistant.

To study the mode of cell death in the malignant cells, we first stained the cells against the 85-kDa cleaved form of PARP-1, a marker for early apoptosis (18), and cleaved also in cells infected with Sindbis virus (19). Surprisingly, cleaved PARP was found only in a small fraction of infected cells (Fig. 1A). As it has been reported that also cells devoid of PARP can undergo apoptosis during alphaviral infection and that normal cells may undergo atypical apoptosis without PARP cleavage (20, 21), we stained the cells by the TUNEL technique.

### Table 1. Cytotoxicity of VA7-EGFP in cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Type</th>
<th>10-12 h</th>
<th>Cell loss</th>
<th>MTT</th>
<th>24 h</th>
<th>Cell loss</th>
<th>MTT</th>
<th>48 h</th>
<th>Cell loss</th>
<th>MTT</th>
<th>72 h</th>
<th>Cell loss</th>
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</tr>
</thead>
<tbody>
<tr>
<td>BHK-21</td>
<td>Baby hamster kidney</td>
<td>30-40</td>
<td>0</td>
<td>80-90</td>
<td>19.7*</td>
<td>100</td>
<td>92.2*</td>
<td>100</td>
<td>99.1*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2058</td>
<td>Human melanoma</td>
<td>20-30</td>
<td>3.6</td>
<td>70-80</td>
<td>71.1*</td>
<td>100</td>
<td>96.7*</td>
<td>100</td>
<td>96.5*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A549</td>
<td>Human lung carcinoma</td>
<td>&lt;5</td>
<td>15.5</td>
<td>5-10</td>
<td>0</td>
<td>30-40</td>
<td>64.4*</td>
<td>70-80</td>
<td>84.6*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-1300</td>
<td>Mouse neuroblastoma</td>
<td>0</td>
<td>11.0</td>
<td>0</td>
<td>10.7</td>
<td>0</td>
<td>231*</td>
<td>&lt;5</td>
<td>14.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LS174T</td>
<td>Human colon adenocarcinoma</td>
<td>&lt;5</td>
<td>0</td>
<td>30-40</td>
<td>43.5</td>
<td>90-100</td>
<td>96.6*</td>
<td>100</td>
<td>96.6*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SK-MEL-5</td>
<td>Human melanoma</td>
<td>10-20</td>
<td>26.9</td>
<td>70-80</td>
<td>42.4*</td>
<td>100</td>
<td>86.6*</td>
<td>100</td>
<td>88.3*</td>
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</tr>
<tr>
<td>SW620</td>
<td>Human colon adenocarcinoma</td>
<td>&lt;5</td>
<td>14.6</td>
<td>30-40</td>
<td>23.3*</td>
<td>70-80</td>
<td>73.6*</td>
<td>100</td>
<td>96.5*</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**NOTE:** Cell lines were infected at 1 MOI and analyzed at indicated time points after infection. The shown data are estimations of cell detachment compared with uninfected control cells (cell loss) and the ratio of metabolic activity of infected cells to control cells (MTT). See Supplementary Fig. S1 for graphs.

*P < 0.05, between infected and uninfected cells.

### Results

**Infection of tumor cell lines in culture.** To expand the panel of susceptible cell lines from our previous study (12), several cancer cell lines were infected with VA7-EGFP and analyzed at various time points under microscope and by MTT assay. BHK-21 cells were included for comparison with our previous results. Results given in Table 1 and Supplementary Fig. S1 show that most cancer cell lines studied were susceptible to infection and virally mediated cell death. In cultures of susceptible cell lines (BHK-21, A2058, A549, LS174T, SK-MEL-5, and SW620), infection leads to marked morphologic changes in the cells and a progressive decline in cell numbers as determined under microscope. Cell detachment (cell loss) correlated with the loss of metabolic activity measured by the MTT assay, but in some cell lines (A2058, BHK-21, SK-MEL-5, and SW620) it preceded the decline in metabolic activity, whereas in others (A549 and LS174T) it seemed to be slightly delayed. The C-1300 neuroblastoma cell line was resistant to SFV as described previously (12).
independent of apoptosis, we incubated infected and control cells with propidium iodide before washing and fixing to reveal possible enhanced membrane permeability due to cellular stress. The results (Fig. 1C) showed that the virus-infected cells indeed took up propidium iodide more readily than the noninfected control cells, confirming that the total number of dying cells exceeded the number of apoptotic cells, suggesting that virus-infected cancer cells may also die by mechanisms other than apoptosis.

Table 2. Infection capacity of VA7-EGFP in cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>12 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% GFP-positive cells (± 1.1)</td>
<td>Intensity relative to infected BHK cells (%)</td>
</tr>
<tr>
<td>BHK-21</td>
<td>88.6</td>
<td>100</td>
</tr>
<tr>
<td>A2058</td>
<td>67.2</td>
<td>39.1</td>
</tr>
<tr>
<td>A549</td>
<td>10.3</td>
<td>51.4</td>
</tr>
<tr>
<td>C-1300</td>
<td>0.3</td>
<td>9.2</td>
</tr>
<tr>
<td>LS174T</td>
<td>10.5</td>
<td>45.2</td>
</tr>
<tr>
<td>SK-MEL-5</td>
<td>40.5</td>
<td>43.9</td>
</tr>
<tr>
<td>SW620</td>
<td>21.3</td>
<td>31.3</td>
</tr>
</tbody>
</table>

NOTE: A comparison of the infection efficacy of VA7-EGFP in different cell lines at 12 and 24 hours after infection (1 MOI) by FACS. Data shown are derived from defined populations of cells, which displayed similar light-scattering properties to uninfected control cells (Supplementary Fig. S2). Relative fluorescence intensity was obtained by comparing the geometric mean fluorescence value of GFP-positive cells normalized with background of BHK-21 cells. The proportion of propidium iodide-positive cells was derived from FACS data using live cells, whereas all other data were obtained from fixed cells.

Figure 1. Infection of A2058 human melanoma cells in culture. A, replication kinetics and cytotoxicity of VA7-EGFP over time. Phase-contrast images show cell morphology and extent of cell death at each time point, whereas fluorescence images show the presence of virus (GFP) and apoptotic cells (PARP). Only very few cells stained positive for PARP at the time points analyzed. B, TUNEL staining of infected cells. Apoptosis at 12 hours after infection compared with DNase-treated cells as positive controls. Only a fraction of infected cells stained positive. C, propidium iodide (PI) staining of cells. To confirm that cells were losing integrity during infection despite absence of apoptosis, cells at 18 hours after infection were stained with propidium iodide before washing and fixation. Images show increased uptake of propidium iodide by infected cells compared with uninfected control cells, indicating that cells suffered damage due to infection and may be dying by mechanism other than apoptosis.

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excised tumors were fixed in formalin, mounted in paraffin, and processed for histology. Figure 3 shows representative sections of whole tumors as well as close-ups of tumor borders to illustrate the difference between necrotic and live tumor tissue and to show connective tissue accentuated by van Gieson staining. The sections in Fig. 3 are from mice given virus i.v., but the histologic appearance of tumors was indistinguishable from those in the other treatment groups. Microscopic analysis of sections stained with H&E revealed widespread necrosis in tumors from treated mice, whereas control tumors contained less necrotic tissue mostly confined to the core of the tumor. The treated tumors also displayed extensive calcification not found in uninfected tumors. Despite extensive oncolysis of treated tumors, clusters of live melanoma cells could be found along the perimeter of the treated tumors at 5 days after infection, surrounded by or embedded within strands of connective tissue (Fig. 3C). Such clusters were not seen at 13 days after infection but were again detected from day 16 after infection onward. By 35 days after infection, separate encapsulated nodules were found, seemingly growing out from the original tumor (Fig. 3G). These nodules consistently contained a necrotic core and a mantle of live, proliferating cells in a pattern similar to control tumors (albeit smaller in scale). The capsules surrounding the nodules consisted of several layers of thin, elongated cells of murine origin, which were distinguished from melanoma cells by morphologic and histologic appearance as well as by staining of melanoma cells by Melan-A antibody (data not shown).

Apoptosis in tumors. Apoptosis was detected by TUNEL staining of serial sections of tumors from both infected and control mice (Fig. 4). In sections double stained with TUNEL and Hoechst, it was possible to distinguish between live (TUNEL-negative) cells and cells containing fragmented DNA, indicative of apoptosis (TUNEL-positive). In accordance with the histologic appearance of parallel sections stained with H&E, viable cancer cells could be found along the perimeter of both control tumors and tumor nodules from treated mice. Conversely, the TUNEL-positive cells were mostly confined to the central regions of both control tumors and nodule of treated tumors. In treated tumors 5 to 16 days after infection, apoptosis was extensive, covering most of the tumor tissue. However, it was not always possible to establish correlation between TUNEL-positive regions and areas of necrosis. This discrepancy may be typical for necrotizing tumor tissue and may reflect different stages of DNA fragmentation in tumors undergoing rapid destruction. It may also reflect different forms of cell death that occur simultaneously in dying tumors (23).

The results presented above are representative of several experiments and reflect intrinsic differences between each cell type, but depending on factors, such as state of passage, serum and metabolite concentration, and cell density, the kinetics of infection may vary. In the susceptible cell lines, however, all cells eventually died, and no regrowth could be observed even after prolonged incubation or after changing of culture medium or increasing serum or metabolite concentrations (data not shown).

**Treatment of SCID mice bearing s.c. A2058 melanoma xenografts.** To establish the capacity of avirulent, replication-competent SFV to reach and destroy a complex, three-dimensional tumor in a living host, VA7-EGFP vector was given either i.v., i.p., or intratumorally to SCID mice bearing s.c. human A2058 melanoma xenografts. Tumor sizes measured with a caliper corresponded well to the actual tumor weights determined after sampling (data not shown). As can be seen in Fig. 2, vector administration lead to a halt of tumor growth and a dramatic reduction in tumor volume irrespective of the route of administration (no statistical difference was seen in tumor volumes at any time between treatment groups by Student’s t test), in contrast to control tumors, which continued to increase in size until plateauing off 3 to 4 weeks after tumor inoculation.

**Clinical status of mice during treatment.** As has been described by Amor et al. (22), infection of SCID mice with avirulent SFV causes progressing neurologic symptoms, including ataxia and paralysis, and eventually death. In the present study, beginning at day 16 after infection, mice in the treatment groups started displaying varying degrees of tactile impairment and were subsequently sacrificed and sampled for virus titration and histology. Mouse developed symptoms in random order in all treatment groups, which did not provide clues about possible relationships between the vector administration route and the treatment groups, which did not provide clues about possible relationships between the vector administration route and the appearance of neurologic deficits. All infected mice showed fur ruffling, but some remained otherwise healthy throughout the study. Animals in all groups, including controls, lost 5% to 20% of weight presumably due to tumor burden and loss of appetite.

**Histologic analysis of tumor xenografts.** On sampling, treated xenografts appeared shrunken, compact, and yellowish. In some mice, the tumor had diminished into several separate, encapsulated nodules (Fig. 3G). In contrast, control tumors were large, peanut shaped, and extensively vascularized. On dissection, they felt rubbery and seemed hollow, with a central core full of blood and pus. To study the effect of infection on tumor morphology, excised tumors were fixed in formalin, mounted in paraffin, and stained with H&E revealed widespread necrosis in tumors from treated mice, whereas control tumors contained less necrotic tissue mostly confined to the core of the tumor. The treated tumors also displayed extensive calcification not found in uninfected tumors. Despite extensive oncolysis of treated tumors, clusters of live melanoma cells could be found along the perimeter of the treated tumors at 5 days after infection, surrounded by or embedded within strands of connective tissue (Fig. 3C). Such clusters were not seen at 13 days after infection but were again detected from day 16 after infection onward. By 35 days after infection, separate encapsulated nodules were found, seemingly growing out from the original tumor (Fig. 3G). These nodules consistently contained a necrotic core and a mantle of live, proliferating cells in a pattern similar to control tumors (albeit smaller in scale). The capsules surrounding the nodules consisted of several layers of thin, elongated cells of murine origin, which were distinguished from melanoma cells by morphologic and histologic appearance as well as by staining of melanoma cells by Melan-A antibody (data not shown).
PARP staining of tumors proved less informative, and we were unable to see any specific staining (data not shown). Similarly, mAb against activated PARP has been reported to function poorly when detecting apoptosis in paraffin sections of neoplastic colon epithelium (24). Taken together, a close analysis under microscope seems more reliable for study of tumor morphology and viability under these conditions.

Presence of virus in tumors. To confirm the presence of virus in treated tumors, sections were analyzed by immunohistochemistry and in situ hybridization using polyclonal rabbit antibody and RNA probe specific for SFV. Results from in situ hybridization of tumor sections shown in Fig. 5A-F revealed presence of virus in all treated tumors at all time points analyzed. The pattern of infection was patchy throughout the tumors, but virus RNA tended to localize to necrotic areas and the central regions of tumor nodules. Surprisingly, however, vector RNA was also detected in areas of the live zone along the perimeter of the nodules at later stages of infection. This pattern of infection was confirmed by immunohistochemistry, which also revealed that infected cells were concentrated at the interface between living and dying tumor cells and were never seen in connective tissue or cells of the tumor capsule (Fig. 5G-H). A few melanoma cells in SFV-positive areas appeared normal, suggesting that the infection in these areas had not yet progressed to a cytopathic stage at the time of sampling or, alternatively, that the tumor cells had become resistant to infection.

To address this issue, we analyzed the presence of virus in isolated tumors at different stages during the experiment by plaque titration. Virus isolated from tumors was able to infect cells with comparable efficacy with fresh stock virus, indicating that the virus itself had retained its oncolytic capacity while replicating in the tumor cells. Next, to test whether the cells in the regrowing tumors had become resistant to virus, we established explant cultures of cells from tumor nodules beginning at 19 days after infection and infected them with both fresh stock virus and circulating virus from treated mice. Out of more than 20 isolated nodules from a total of nine mice, stable cell lines could be obtained from only 3 nodules, as most of the isolated cells died due to presence of virus in the homogenate, confirmed by plaque assay (data not shown). Results revealed that, among these cells, a small population of cells existed that was completely resistant to virus (Supplementary Fig. S3). In fact, once virus has infected and destroyed the susceptible portion of the cells, the remaining resistant cells could be passaged further. We confirmed that the explanted cells were melanoma by immunostaining against Melan-A antigen and the S100 protein, both markers of melanoma, using fresh A2058 and SK-MEL-5 cells as positive and A549 cells as negative controls (Supplementary Fig. S3).

Expression of EGFP by the vector. Immunostaining of EGFP in tumor sections from day 5 after infection revealed expression of
the protein by the vector, but heavy necrosis of the tumor tissue made quantification difficult (data not shown). In situ hybridization of tumor sections using a probe specific for EGFP revealed presence of EGFP RNA up to 35 days after infection, but the protein was not expressed by circulating virus beyond day 5 after infection as assessed by infecting A2058 cells with serum from treated mice (data not shown).

Presence of virus in organs and blood. To determine the overall distribution and tropism of the vector in infected SCID mice, several organs and tissue samples (see Materials and Methods) were analyzed for the presence of virus by immunohistochemistry. Virus antigen could be detected in the brains and spinal cords of infected mice beginning at day 13 after infection (Fig. 5I) and in a few solitary muscle cells adjacent to tumors (Fig. 5I). Samples from all other organs were negative for virus, including the heart and muscle from the thigh of the mouse. In the brains of treated mice, virus antigen could be detected in the cytoplasm of single neurons and as patchy foci mostly in the cerebrum close to the cerebellum. In some sections of the spinal cord, virus was detected in a few large neurons of the gray matter. This infection pattern is typical for avirulent SFV A7(74) and consistent with previously published data about its replication in the CNS of adult mice (10–12).

Serum was used in a plaque assay to determine the titer of circulating virus in the treated mice. Virus was found in all infected mice at all time points analyzed with titers ranging from $5.5 \times 10^5$ to $3.4 \times 10^7$ pfu/mL. Together with the results presented above, these results suggest that the tumor xenografts constitute the major site of viral replication in treated mice and that the replication-competent virus reaches tumor tissue via the circulation. Although the actual source of virus dissemination during the persistent infection in this experiment remains unresolved, the CNS and the tumor itself are the most likely sites to serve as reservoirs for progeny virus. It is known that SFV can escape into the circulation when given intracranially and that replication-competent adenovirus can do the same from infected tumor xenografts in mice (25, 26).

Discussion

In the present study, we have studied the oncolytic capacity of replication-competent, avirulent SFV A7(74) expressing EGFP reporter gene in culture in a variety of tumor cell lines as well as in human melanoma xenografts in immunodeficient mice. The differences in viral replication kinetics between cell lines in vitro probably result from cellular factors that influence permissiveness to virus, including type and quantity of virus receptors, expression of antiviral proteins, and activity of components of the antiviral defense system. For example, increased number of viral receptors on cancer cells compared with normal cells has been shown to potentiate the oncotropism of coxsackievirus A21 (27). Correspondingly, small interfering RNA–mediated down-regulation of the high-affinity laminin receptor was shown to proportionally reduce the infectivity of Sindbis virus in ovarian cancer cells (28). On the other hand, alphaviruses can use several cell surface receptors for infection, including the high-affinity laminin receptor (29), heparan sulfate (30), and cell surface C-type lectins (31), which suggests that alphaviruses are not likely to be completely blocked at the level of entry and that the tropism of these viruses is largely determined by intracellular factors, which influence the viral replication machinery. Such factors include the double-stranded RNA–dependent protein kinase (PKR), RNase L, Mx class proteins, and components of the IFN-α/β-activated pathway (32). SFV is exquisitely sensitive to these, and replication of the virus is blocked in cells pretreated with recombinant IFN-α/β or overexpressing human MxA protein (33, 34).

Similarly, varying susceptibility of different cancer cell types to viral oncolysis may result from differences in expression of...
antiapoptotic proteins, although overexpression of such proteins can either limit SFV-mediated cell death or have virtually no effect at all (35, 36). Because cancer cells in general tend to be resistant to apoptosis, we wanted to analyze the mode of cell death in cells infected with avirulent SFV to see whether the virus would be able to overcome their resistance to programmed cell death. In summary, rather than undergoing cell death consistent with apoptosis, we observed that the majority of cells were dying by other mechanisms. Corroborating these findings, Smyth et al. (37) detected activated caspase in only a fraction of SFV-infected cancer cells throughout the course of infection. In addition, during infection with conditionally replicating adenovirus, cancer cells have been reported to die by mechanisms resembling necrosis, independent of major apoptotic pathways (38). Moreover, it has been shown that several cancer cell lines may undergo cell death by mechanisms that involve neither caspase activation nor DNA fragmentation, even in the presence of antiapoptotic Bcl-2 overexpression (39). Finally, Scallan et al. (35) found that the number of TUNEL-positive cells in cultures of SFV A7(74)-infected cells never exceeded 10% despite ongoing cell death in the majority of infected cells. The authors hypothesized that the observed discrepancy was due to asynchronous progression of infection in the cultures and the relatively brief interval during which apoptotic features are detectable. Backed by our own observations, we propose that, in addition to apoptosis, cells may die by other mechanisms induced by SFV.

To test whether avirulent SFV would display oncolytic efficacy also in vivo, we next established s.c. tumors of the aggressive human melanoma cell line A2058 in SCID mice. This model was chosen as melanoma is highly susceptible to virus and thereby provides the optimal setting for study of viral replication and oncolytic capacity within the acceptable time span in SCID mice (before the onset of clinical symptoms). In addition, as melanoma often metastasizes into the brain, it constitutes an attractive target for intervention with a neurotropic virus, such as SFV. We felt it was important to establish the oncolytic capacity of avirulent SFV in an immune-free environment to be able to understand basic interactions between virus and tumor. However, the SCID mouse does not represent the proper environment for further development of the virus into a tool for virotherapy. Lacking the adaptive immune system, SCID mice are unable to control the infection and develop slow-progressing neurologic symptoms due to persisting virus. Importantly, no symptoms appear in nude mice or immunocompetent mice, in which antibodies are critical in controlling and clearing the infection. Moreover, the cause of neuropathology in SCID mice remains obscure, as infection persists without

Figure 5. Virus tropism. SFV was detected by in situ hybridization (A-F) and immunohistochemistry (G-J). A to C, representative images of tumor sections from mice infected i.v. 5, 20, and 35 days after infection, respectively. D, representative images of tumor sections from mice infected i.p. 20 days after infection. E, representative images of tumor sections from mice infected intratumorally 20 days after infection. SFV is present in all tumors at all time points analyzed. F, hybridization control. G and H, serial sections from a treated mouse, i.v., day 20 after infection, stained with H&E and SFV antibody, respectively. SFV is localized at the interface between live and necrotic cells. I and J, presence of SFV in brain and muscle of treated mice, i.v., day 35 after infection and intratumorally, day 20 after infection, respectively. Bar, 2 mm.
apparent neuronal loss, which suggests that the animals die from other complications (22). However, because the neurologic symptoms are associated with viral infection, we acknowledge that the present vector may require modifications, achieved, for example, by abolishing its neuroinvasiveness or by further attenuating the virus (40, 41). Such modifications, however, are beyond the scope of this report.

In this study, the neurotropism of SFV did not restrict its ability to target s.c. melanoma, as within 3 weeks VA7-EGFP had caused regression of tumors to far below the starting volume. Despite initial tumor destruction, however, small isolated groups of dividing tumor cells were detected within strands of connective tissue in association with the tumor capsule. In this respect, it has been shown that, after an initial phase of cell death, mouse fibroblasts rapidly form strands of connective tissue into tumors, providing substrate for the formation of blood vessels, which in turn sustain tumor growth (42). As the connective tissue in the present study clearly was virus resistant, it most probably posed an anatomic barrier to virus spread, which in turn could have facilitated tumor escape and regrowth. Corroborating our findings, it has been shown that the thickness of the extracellular matrix surrounding tumor nodules as well as the progressive increase in components of the basement membrane substantially reduce tumor permissiveness to adenoviruses delivered via the circulation (43, 44). Furthermore, within lung carcinoma and melanoma xenografts, spread of replication-competent adenovirus is restricted, similar to what was observed here (26, 45). Incomplete transduction of tumors and tumor xenografts has been observed for a variety of replication-competent viruses, including attenuated measles virus (46, 47), herpes simplex virus (48, 49), and vesicular stomatitis virus (50), which suggests that physical barriers exist, which limit virus dissemination despite the ability to spread from cell to cell.

In the reports above, both live virus and tumor cells could be observed at late stages during treatment, suggesting equilibrium between infected dying cells and regrowing tumor cells. However, none of the studies addressed the issue of tumor resistance to virus experimentally. In the present study, not only did we isolate live virus from regrowing tumor nodules, but also cells that were completely resistant to virus could be extracted, which displayed similar morphology and immunochemical profile to parental A2058 cells. Although we cannot exclude the possibility that resistance was acquired by only growing the cells In vivo, it seems more likely that the cells in the xenografts were originally susceptible and only gained resistance as a result of the selective pressure exerted by the virus. In addition, as the tumorigenic potential of these resistant cells is unknown, we cannot be certain of their contribution to tumor regrowth. These aspects, however, are currently being investigated.

Nevertheless, the finding of virus resistance may have wider implications for the field of virotherapy and underscores the need to elucidate the basic biological interactions between virus and cancer cells during preclinical testing. Our findings, together with those from other studies, suggest that, on one hand, tumors In vivo become fenestrated and surrounded by physical barriers that hinder the spread of virus and that a dynamic equilibrium is established between viable dividing tumor cells and infected dying cells and on the other hand, that, in cases where tumor cells survive initial oncolysis, virus-resistant cells may form. Fortunately, as has been shown with several viral vectors, long-term survival may still be increased by repeated administrations of virus or by optimization of the vectors by including oncotoxic or immunostimulating inserts (8, 26, 28, 37). The results in this study warrant further analysis of virus-tumor interactions and testing of the oncolytic capacity of avirulent SFV in immunocompetent animals.

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