Novel cancer treatments are needed to minimize deleterious effects and improve outcomes. Targeted lysis of cancerous cells by viruses without lysis of healthy cells (ie, oncolytic virotherapy) is a promising treatment option that may be more effective and have fewer adverse effects than traditional cancer treatments. A key factor in the success of oncolytic virus treatments is the susceptibility of neoplastic cells to viral infection because of mutations in genes required for function of antiviral defense mechanisms. Some of these mutations also alter cell signaling pathways, which confer selective growth advantages on cells. For example, cells of many tumors have upregulated expression of phosphorylated Akt, which contributes to altered gene

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**Objective**—To determine the oncolytic efficacy of an attenuated form of myxoma virus lacking the serp2 gene in canine tumor cells.

**Sample**—Primary cells were isolated from tumors that were surgically removed from dogs and from connective tissue obtained from the cadaver of a dog. Cells of various established cell lines from tumors and nontumorous tissues were obtained.

**Procedures**—Experiments were performed with cells in monolayer culture. Cell cultures were inoculated with wild-type myxoma viruses or myxoma viruses lacking the serp2 gene, and measures of cytopathic effects, viral growth kinetics, and cell death and apoptosis were determined.

**Results**—Myxoma viruses replicated in cells of many of the primary and established canine tumor cell lines. Canine tumor cells in which expression of activated protein kinase B was upregulated were more permissive to myxoma virus infection than were cells in which expression of activated protein kinase B was not upregulated. Myxoma viruses lacking the serp2 gene caused more cytopathic effects in canine tumor cells because of apoptosis than did wild-type myxoma viruses.

**Conclusions and Clinical Relevance**—Results of the present study indicated myxoma viruses lacking the serp2 gene may be useful for treatment of cancer in dogs.

**Impact for Human Medicine**—Results of the present study may be useful for development of novel oncolytic treatments for tumors in humans. (Am J Vet Res 2012;73:1252–1261)

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**Abbreviations**

- 7-AAD: 7-aminoactinomycin D
- Akt: Protein kinase B
- DEVD-AMC: Aspartic acid-glutamic acid-valine-aspartic acid–7-amino-4-methylcoumarin fluorogenic caspase 3 substrate
- CPE: Cytopathic effect
- FBS: Fetal bovine serum
- FITC: Fluorescein isothiocyanate
- MEM: Minimum essential medium
- MOI: Multiplicity of infection
- MYXV: Myxoma virus
- MYXVΔserp2: Attenuated myxoma virus lacking a viral serine proteinase inhibitor
- MYXV-gfp: Recombinant myxoma virus expressing green fluorescent protein
- MYXV-red: Recombinant myxoma virus expressing tandem dimer tomato red fluorescent protein
- serp2: Serine protease inhibitor 2 from myxoma virus
- VACV: Vaccinia virus
regulation, decreased apoptosis, and increased proliferation of tumor cells and enables productive infection of tumor cells by some oncolytic viruses.

Poxviruses have characteristics that make them excellent candidates for use in oncolytic virotherapy. These viruses preferentially infect cells in tissues with permeable vasculature, which is a feature of many aggressive tumors. Additionally, they can be genetically engineered to express antitumor agents without constraints on gene size, which is a limitation of many other viral vectors used for oncolysis. Poxvirus infection elicits strong cell-mediated immune responses, which cause lysis of infected cells, and effective humoral immune responses, which prevent establishment of latent or recurrent viral infections.

One poxvirus, MYXV, causes a lethal disease called myxomatosis in European rabbits. Myxoma virus is nonpathogenic in all other vertebrate species investigated, including humans and dogs. Myxoma virus productively infects cells of multiple human tumor cell lines in vitro. Its effectiveness as an oncolytic virus is supported by findings of other studies in which MYXV treatment was determined to be efficacious in mice with xenografts of human glioma and medulloblastoma. In immunocompetent animals, MYXV oncolysis is more efficacious when used in combination with the chemotherapeutic drug rapamycin.

Administration of chemotherapeutic drugs to animals with tumors might not be necessary if effective oncolytic viruses were available. As with all poxvirus-infected cells, MYXV-infected cells express several immunomodulatory proteins, some of which inhibit apoptosis. Although beneficial for viral pathogenesis during natural infections, expression of such genes is likely to hinder MYXV-based oncolysis. Thus, modification or deletion of antiapoptotic virulence factors in viruses may enhance apoptosis of tumor cells infected by those viruses. Indeed, VACV vectors that do not have the antiapoptosis genes SPI-1 and SPI-2 have enhanced oncolytic specificity and efficacy in mice with adenocarcinoma. However, because VACV is a pathogen of humans and mice, such mutant VACVs replicate in nontumorous cells in several organs, including the brain. Unlike VACV, MYXV does not cause disease in any species of animal except rabbits, which makes production and use of a recombinant MYXV with low replication efficiency unnecessary. Therefore, we wanted to determine the oncolytic properties of a recombinant MYXV that lacks expression of serp2, an antiapoptotic virulence factor. This virus causes milder disease in rabbits than wild-type MYXV but replicates at the same rate in rabbit cells in vitro and in vivo.

Testing of viruses for oncolytic efficacy is typically performed with mice that have induced or implanted neoplasms. This may not be the best method to evaluate oncolytic viruses for treatment of naturally developing tumors in humans. Investigation of oncolytic efficacy of viruses in dogs may be a superior method because dogs, like humans, naturally develop neoplasms. Furthermore, because dogs live in close contact with humans, neoplasms in dogs and humans that are caused by environmental factors may have similar biological behaviors. The purpose of the study reported here was to determine the oncolytic efficacy of MYXV in cells of established canine tumor cell lines and in primary canine tumor cells obtained from tumor explants and to compare results with those for MYXVserp2 to determine whether MYXV-mediated oncolysis is enhanced by removal of the serp2 gene.

Materials and Methods

Sample—Cells from the following established cell lines were obtained: rabbit kidney epithelial RK-13, human malignant glioma U-118 MG, canine osteosarcoma Abrams, canine transitional cell carcinoma Billey, canine osteosarcoma D-17, canine hemangiosarcoma Den, and canine hemangiosarcoma Fitz. In addition, primary tumor cells were isolated from naturally developing tumors surgically removed from dogs that were patients at the University of Illinois Veterinary Teaching Hospital. These tumors included 2 mast cell tumors and 1 each of hemangiosarcoma, soft tissue sarcoma, gastrointestinal stromal tumor, hemangioma, perianal adenocarcinoma, perianal adenoma, mixed mammary gland tumor, and renal carcinoma. Cells were also isolated from noncancerous fibrous connective tissue obtained from the cadaver of a dog undergoing necropsy for reasons unrelated to this study. Unfixed portions of each tumor surgically removed from dogs were dissected free of fat and necrotic tissue. These tumor sections were then minced into 1-mm cubes, placed in a 25-cm² cell culture flask, and incubated at room temperature (approx 22°C) for 10 minutes to allow tissue to adhere to bottoms of the flasks. Then, minimal essential cell culture media with Earle salts, 2mM glutamine, 50 U of penicillin G/mL, 50 µg of streptomycin/mL, 1mM sodium pyruvate, and 0.1mM nonessential amino acids (MEM) containing 10% FBS were added and flasks were incubated (37°C, 5% CO₂, and 100% humidity). Cells from noncancerous fibrous connective tissue (ie, fibroblasts) obtained from the cadaver of a dog were isolated in a similar manner. Trypsinization and passage of cultured cells was performed when cells were 95% to 100% confluent or sooner if tissue explants were degrading. Canine primary tumor and fibroblast cell cultures and cells from established cell lines were maintained in MEM with 10% FBS at 37°C, 5% CO₂, and 100% humidity.

Identification of tumor and cell types—Portions of each tumor surgically removed from dogs were fixed in neutral-buffered 10% formalin and submitted to the University of Illinois Veterinary Diagnostic Laboratory for histologic analysis. Tumors were paraffin embedded, sectioned, stained with H&E, and evaluated to identify tumor types.

Primary tumor cells isolated from tumors of dogs were cultured in 35-mm-diameter plates until they were 90% to 100% confluent. Cells were trypsinized, collected in MEM with 10% FBS, and pelleted by centrifugation at 400 × g for 5 minutes. Cell pellets were suspended in 1 mL of PBS solution, and 100-µL aliquots of cells were cytocentrifuged at 400 × g for 3 minutes onto charged glass slides. To ensure cultured cells were representative of the types of tumors from which
they were isolated (rather than adjacent nonneoplastic tissue), cells adhering to slides were evaluated after staining with Wright-Giemsa and cytochemical stains.

**Virus isolation**—Isolation and characterization of MYXV-serp2 (previously referred to as MYXVserp2::lacZ) was performed as described.29,30 Two recombinant MYXVs (MYXV-gfp and MYXV-red) (previously referred to as vMyxgfp31 and vMyx-tdTr,32 respectively) were also isolated; regulation of each fluorescent protein in these recombinant viruses was under the control of a synthetic vaccinia virus early-late promoter.

**Determination of CPEs of viruses**—Primary canine tumor cells and fibroblasts were inoculated with viruses. For comparison with results for canine primary tumor cells, RK-13 (fully permissive for virus replication)34 and U-118 MG (semipermissive for virus replication)34,b cells were inoculated with viruses. Cells were grown to confluence in 35-mm-diameter plates and inoculated with MYXV, MYXV-gfp, MYXV-red, or MYXV-serp2 at an MOI of 0, 0.1, 1, 5, or 10 infectious virus particles/cell. A low MOI of 0.1 infectious virus particles/cell was included to simulate likely in vivo virus particle-to-tumor cell ratios because sufficient numbers of infectious virus particles cannot be delivered homogenously in a solid tumor in vivo to ensure a high MOI. The MYXV-gfp and MYXV-red (recombinant viruses that express fluorescent proteins) throughout the life cycle were used to aid identification of virus-infected cells. Every 24 hours after inoculation, phase and fluorescent microscopic images of cells were acquired with a microscope (magnification, 100X) and software.1 Fluorescent images of MYXV-gfp–inoculated cells were acquired with a 480/40-nm bandpass excitation filter and an exposure time of 1,500 milliseconds. Fluorescent images of cells inoculated with MYXV-red were acquired with a 560/40-nm bandpass excitation filter and an exposure time of 400 milliseconds. Images were evaluated, and CPE scores of 1+ (≥ 50% of cells adherent to culture plate), 2+ (10% to 50% cells adherent to culture plate), or 3+ (< 10% of cells adherent to culture plate) were determined for each cell culture.

**Akt expression in cells**—Because increased expression of phosphorylated Akt is associated with greater susceptibility to MYXV-induced CPEs in human cancer cells,35 western immunoblot analysis of lysates of cells of some of the canine primary tumors (hemangiosarcoma and soft tissue sarcoma) and established canine tumor cell lines (hemangiosarcoma Den and transitional cell carcinoma Bliley) was performed to determine relative expression of Akt phosphorylated at serine 473; these cells were not inoculated with viruses. For comparison, protein extracted from human glioma U-118 MG cells, a cell line that moderately expresses phosphorylated Akt,34 was also assayed. Cells were grown in 35-mm-diameter plates to 95% confluency and then collected in cell lysis buffer with protease and phosphatase inhibitor.1 Total protein concentration was determined with a standard Bradford assay, and 50 µg of protein from each cell lysate was analyzed via SDS-PAGE (10%). The SDS-PAGE–separated proteins were transferred to a nitrocellulose membrane.4 Membranes were incubated in blocking buffer overnight (approx 16 hours) at 4°C, then incubated with rabbit antiphospho-Akt (serine 473) monoclonal antibody in accordance with the manufacturer’s protocol. Membranes were incubated for 1 hour at room temperature with a horseradish peroxidase–conjugated goat anti-rabbit IgG polyclonal antibody diluted 1:2,000 in blocking buffer. A chemiluminescent western blot detection kit was used to detect antigen-antibody complexes. Membranes were stripped in accordance with the protocol provided by the manufacturer of the detection kit, incubated with rabbit anti-Akt monoclonal antibody following the manufacturers’ instructions, and antigen-antibody complexes were detected. Experiments were repeated 3 times. Immunoblots were scanned and analyzed with photo editing software. Images were inverted (ie, grayscale values were reversed), and integrated density measurements were determined for standardized regions of interest including each protein band. Density values for phosphorylated Akt were normalized relative to density values for total Akt to determine mean percentage of phosphorylated Akt in each cell sample.

As a measure of virus maturation, expression of an MYXV protein (M130R) that is expressed during late stages of viral infection of cells was determined. Cells were collected 24 hours after inoculation with MYXV (MOI, 5 infectious virus particles/cell) and processed as described for determination of Akt expression, except that 25 µg of protein from each sample was separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with rabbit polyclonal anti-M130R primary antibodies.

**Virus growth curves**—Growth curve experiments were performed to determine whether MYXV could replicate in canine tumor cells and produce infectious viruses. To determine 1-step virus growth curves, cells of established canine tumor cell lines (hemangiosarcoma Den and transitional cell carcinoma Bliley) and primary canine tumor (hemangiosarcoma), primary canine fibroblast, and RK-13 cells were grown to confluence in 35-mm-diameter plates, inoculated with MYXV (MOI ≥ 5 infectious virus particles/cell), and incubated at 37°C in 5% CO2 for 1 hour. Virus inoculum was removed, and cells were washed with PBS solution 3 times. Then, 1.5 mL of MEM with 10% FBS was added to each plate. Cells were harvested with a sterile handheld flexible natural-rubber scraper attached to a glass rod (ie, rubber policeman) 4, 8, 12, 24, and 48 hours later and centrifuged (400 × g for 5 minutes). Cell pellets were resuspended in 1 mL of MEM without serum and stored at –80°C until determination of virus titers. Multistep growth curve experiments were performed to determine whether MYXV could spread from infected cells to adjacent noninfected cells in cultures. Multistep growth curve experiments were performed in a manner similar to that used for determination of 1-step growth curves, except growth curves were not determined for canine fibroblasts but were determined for canine primary soft tissue sarcoma cells, an MOI of 0.1 infectious virus particles/cell was used, and cells were harvested 0 (ie, 1 hour after virus inoculation), 12, 24, 48, 72, and 96 hours after MEM with 10% FBS was added to each plate.
To determine virus titers for 1-step and multistep MYXV growth curves, tumor cell culture samples were frozen and thawed 3 times, sonicated for 1 to 5 minutes in a water bath at room temperature, and a series of 10-fold dilutions were prepared. Four hundred microliters of each diluted sample was added to confluent RK-13 cells in 35-mm-diameter plates. Cells were incubated (37°C in 5% CO₂ for 1 hour) with agitation every 15 minutes, and a 3-mL overlay (1:1 mixture of 1% high-gelling temperature agarose solution and 2× MEM with 20% FBS; heated to 45°C) was added to the plates. Plates were incubated at 37°C in 5% CO₂ for 5 to 7 days. Plaques formed by MYXV infection of cells (small white foci) were counted. Mean ± SEM virus titers were calculated for results of ≥3 experiments and expressed as the number of plaque-forming units per milliliter.

Transmission electron microscopy—To determine whether MYXVs in all stages of morphogenesis were present in cells, MYXV-inoculated RK-13, hemangiosarcoma Den, transitional cell carcinoma Bliley, osteosarcoma Abrams, osteosarcoma D-17, and primary canine hemangiosarcoma and renal carcinoma cells were evaluated via transmission electron microscopy. Twenty-four hours after cultured cells were inoculated with MYXV (MOI, 5 infectious virus particles/cell), media and nonadherent cells were collected. Then, adherent cells were removed by trypsinization, pooled with nonadherent cells, and centrifuged (400 × g for 5 minutes). Supernatants were removed, cell pellets were suspended in Karnovsky fixative, and cell samples were stored at 4°C until imaging. Cell samples were shipped on ice packs (approx 4°C) to the University of Florida Interdisciplinary Center for Biotechnology Research, and cell samples were prepared and imaged with a transmission electron microscope and digital camera.

Cell death and apoptosis assays—Because CPEs in cells infected with viruses may be caused by several factors, including cell death, trypsin blue exclusion assays were performed to quantify cell death after inoculation with viruses. Confluent RK-13, hemangiosarcoma Den, transitional cell carcinoma Bliley, and primary canine hemangiosarcoma and soft tissue sarcoma tumor cells in 35-mm-diameter plates were mock inoculated (by replacing the media with 400 µL of MEM without viruses) or inoculated with the Lausanne strain of MYXV or MYXVΔserp2 (diluted in 400 µL of MEM at a high MOI [10 or 5 infectious virus particles/cell, respectively] or a low MOI [0.1 infectious virus particles/cell]). Cells were incubated for 1 hour, then the inoculum was removed and 1.5 mL of MEM with 10% FBS was added to each well. At 0, 16, 24, and 48 hours after adding MEM with 10% FBS to each well, medium containing nonadherent cells was collected. Adherent cells were washed in PBS solution, and wash solution from each culture was pooled with medium containing nonadherent cells. Then, adherent cells were collected by trypsinization and pooled with the medium containing nonadherent cells. Pooled cells were diluted 1:10 in trypsin blue dye solution. Blue (dead) and refractile, unstained (live) cells were manually counted by use of a hemocytometer. Proportion of dead cells was expressed as a percentage of total cells counted. Means of values for 3 to 12 replicates of each experiment were calculated.

Flow cytometry was performed to detect cells with annexin V binding to phosphatidylserine on cell membranes; this is a sensitive and specific method for identification of cells undergoing this early-stage event during apoptosis. Confluent RK-13, hemangiosarcoma Den, transitional cell carcinoma Bliley, and primary canine hemangiosarcoma and soft tissue sarcoma cells were mock inoculated or inoculated with MYXV or MYXVΔserp2 (MOI, 5 infectious virus particles/cell). Cells were collected 48 hours after inoculation. Aliquots of 10⁶ cells were incubated with FITC-conjugated annexin V and 7-AAD and assayed with a flow cytometer in accordance with the manufacturer’s instructions. Compensation control samples were evaluated for each cell line; these included unstained cells (negative control), cells treated with 1µM staurosporine overnight and then stained with FITC-conjugated annexin V (FITC positive control cells), MYXV-red–inoculated cells (phycoerythrin positive control cells), and cells fixed in buffered 2.5% formalin and then stained with 7-AAD (7-AAD positive control cells). A gate based on cell size (forward scatter-H) and complexity (side scatter-A) was applied to these data to exclude cell fragments from analysis. Data were analyzed with software. Percentage of cells with positive results for annexin V staining (ie, apoptotic cells) at 48 hours after inoculation was determined; cells with positive results for 7-AAD staining were eliminated from analysis because those cells may have undergone cell death by necrosis rather than by apoptosis.

Caspase-3 activation, a terminal event during apoptosis, was also quantified in those same cells via a caspase substrate–fluorochrome cleavage assay as previously described. Uninoculated cells incubated for at least 4 hours at 37°C in media with 1µM staurosporine were used as positive control cells for caspase-3 activation. The rate of DEVD-AMC cleavage was determined.

Statistical analysis—Data were analyzed via 1-way ANOVAs and pairwise multiple comparisons (Holm-Sidak method) with software. Values of P ≤ 0.05 were considered significant.

Results

Histologic and cytologic characterization of primary tumor cells—Histopathologic diagnoses determined for tumors surgically removed from dogs were summarized (Table 1). Cell morphology and cytochemical staining results were consistent with tumors from which cells had been isolated, except for cells of one of the mast cell tumors, which unexpectedly lacked staining for mast cell tryptase.

CPEs and early-stage virus-encoded protein expression in MYXV-inoculated tumor cells—The CPEs observed after MYXV infection of tumor cells included a change from a spindle-shaped polygonal morphology to a round and highly condensed appearance with detachment of cells from culture plates (Figure 1). The CPE scores for tumor cells 48 hours after inoculation with
MYXV (MOI, 5 infectious virus particles/cell) were summarized (Table 2). Most cells of established canine tumor cell lines and those obtained from primary tumors had CPEs by 48 hours after inoculation, but few CPEs were observed in primary canine fibroblasts obtained from noncancerous connective tissue.

Extensive CPEs were observed in hemangiosarcoma Den, transitional cell carcinoma Bliley, and primary canine soft tissue sarcoma cells 48 hours after virus inoculation, whereas few CPEs were observed in primary canine hemangiosarcoma cells at that time (Figure 1). Expression of recombinant virus-encoded green (MYXV-gfp) and red (MYXV-red) fluorescent proteins was observed in all canine tumor cell cultures 48 hours after virus inoculation. In contrast, faint red fluorescence was observed in extremely low numbers of primary canine fibroblasts at 48 hours after inoculation with MYXV-red (MOI, 0.1 infectious virus particles/cell), which indicated that viral infection was inhibited in these noncancerous cells. Growth curve kinetics of MYXV, MYXV-gfp, and MYXV-red in RK-

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Histopathologic diagnosis</th>
<th>Positive results for cytochemical staining</th>
<th>Negative results for cytochemical staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA-1</td>
<td>Hemangiosarcoma</td>
<td>Vimentin</td>
<td>Cytokeratin</td>
</tr>
<tr>
<td>STSA-1</td>
<td>Soft tissue sarcoma</td>
<td>Vimentin</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>GIST-1</td>
<td>Gastrointestinal stromal tumor</td>
<td>S100</td>
<td>Cytokeratin</td>
</tr>
<tr>
<td>HG-1</td>
<td>Hemangioma</td>
<td>Vimentin</td>
<td>CD18</td>
</tr>
<tr>
<td>MCT-1</td>
<td>Mast cell tumor</td>
<td>Giemsa</td>
<td>CD18</td>
</tr>
<tr>
<td>MCT-2</td>
<td>Mast cell tumor</td>
<td>Giemsa</td>
<td>Mast cell tryptase</td>
</tr>
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<td>Perianal adenocarcinoma</td>
<td>Mast cell tryptase</td>
<td>Vimentin</td>
</tr>
<tr>
<td>MM1-1</td>
<td>Mixed mammary gland tumor</td>
<td>Cytokeratin</td>
<td>CD18</td>
</tr>
<tr>
<td>RCA-1</td>
<td>Renal carcinoma</td>
<td>CD18</td>
<td>Vimentin</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>ND</td>
<td>ND</td>
<td>Alkaline phosphatase</td>
</tr>
</tbody>
</table>

Table 1—Histologic diagnoses and cytochemical staining results for tumors surgically removed from 10 dogs, connective tissue obtained from the cadaver of 1 dog, and canine primary cells obtained from explants of these tissues.

Figure 1—Representative photomicrographic images of monolayer cultures of various types of cells (cells of established cell lines rabbit kidney epithelial RK-13, human malignant glioma U-118 MG, canine osteosarcoma Den [HSA-Den], and canine transitional cell carcinoma Bliley [TCC-Bliley]; canine primary hemangiosarcoma [HSA-1] and soft tissue sarcoma [STSA-1] cells obtained from tumors surgically removed from dogs; and canine primary fibroblasts obtained from connective tissue of the cadaver of a dog) 48 hours after inoculation with viruses. A—Phase photomicrographic images of mock-inoculated cells. B—Phase photomicrographic images of MYXV-inoculated cells (MOI, 5 infectious virus particles/cell). Notice CPEs in cells. C—Fluorescent photomicrographic images of cells inoculated with MYXV-gfp (cells with green staining) or MYXV-red (cells with red staining; MOI, 0.1 infectious virus particles/cell). D—Representative phase photomicrographic images of cells inoculated with MYXV-gfp or MYXV-red (MOI, 0.1 infectious virus particles/cell). Bar = 0.1 µm.
13 cells were indistinguishable, which indicated the addition of fluorescent markers did not alter in vitro MYXV replication (data not shown).

Relationship between Akt phosphorylation and CPEs in MYXV-inoculated cells—Slightly greater expression of phosphorylated Akt was detected in hemangiosarcoma Den, transitional cell carcinoma Bliley, and primary soft tissue sarcoma cells than in human glioma U-118 MG cells (Figure 2), although results were not significantly different. These cells had moderate to severe CPEs after inoculation with MYXV (Table 2; Figure 1). In contrast, primary canine hemangiosarcoma cells had the lowest expression of phosphorylated Akt and had few CPEs after inoculation with MYXV. These findings indicated that the level of expression of phosphorylated Akt may be related to susceptibility of canine tumor cells to infection with MYXV.

Production of infectious virus particles in canine tumor cells versus that in RK-13 cells—One-step growth curves (MYXV MOI, ≥5 infectious virus particles/cell) indicated logarithmic increases in virus titers during 24 hours after inoculation of RK-13, hemangiosarcoma Den, and primary canine hemangiosarcoma cells (Figure 3). However, virus titers typically decreased after that time. High rates of MYXV replication were not detected in transitional cell carcinoma Bliley cells or primary canine fibroblasts. One-step growth curves were not determined for canine primary soft tissue carcinoma cells because of the large amount of virus particles needed to inoculate cells at an MOI ≥5 infectious virus particles/cell.

Table 2—Cytopathic effect scores for MYXV-inoculated cultures of cells of established cell lines, cells obtained from explants of tumors surgically removed from 10 dogs, and fibroblasts obtained from connective tissue collected from the cadaver of 1 dog.

<table>
<thead>
<tr>
<th>Cell name</th>
<th>Cell origin</th>
<th>CPE score</th>
</tr>
</thead>
<tbody>
<tr>
<td>RK-13</td>
<td>Rabbit kidney epithelium</td>
<td>2+</td>
</tr>
<tr>
<td>U-118 MG</td>
<td>Human glioma</td>
<td>2+</td>
</tr>
<tr>
<td>HSA-Den</td>
<td>Canine hemangiosarcoma</td>
<td>3+</td>
</tr>
<tr>
<td>TCC-Billey</td>
<td>Canine transitional cell carcinoma</td>
<td>3+</td>
</tr>
<tr>
<td>OSA-Abrams</td>
<td>Canine osteosarcoma</td>
<td>2+</td>
</tr>
<tr>
<td>OSA-D-17</td>
<td>Canine osteosarcoma</td>
<td>2+</td>
</tr>
<tr>
<td>HSA-Fitz</td>
<td>Canine hemangiosarcoma</td>
<td>3+</td>
</tr>
<tr>
<td>HSA-1</td>
<td>Canine hemangiosarcoma</td>
<td>1+</td>
</tr>
<tr>
<td>STSA-1</td>
<td>Canine soft tissue sarcoma</td>
<td>2+</td>
</tr>
<tr>
<td>MMT-1</td>
<td>Canine mixed mammary gland tumor</td>
<td>3+</td>
</tr>
<tr>
<td>GIST-1</td>
<td>Canine gastrointestinal stromal tumor</td>
<td>3+</td>
</tr>
<tr>
<td>PACA-1</td>
<td>Canine perianal adenocarcinoma</td>
<td>1+</td>
</tr>
<tr>
<td>PA-1</td>
<td>Canine perianal adenoma</td>
<td>3+</td>
</tr>
<tr>
<td>MCT-1</td>
<td>Canine mast cell tumor</td>
<td>ND</td>
</tr>
<tr>
<td>MCT-2</td>
<td>Canine mast cell tumor</td>
<td>2+</td>
</tr>
<tr>
<td>HG-1</td>
<td>Canine hemangioma</td>
<td>3+</td>
</tr>
<tr>
<td>RCA-1</td>
<td>Canine renal carcinoma</td>
<td>1+</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>Canine fibroblast</td>
<td>1+</td>
</tr>
</tbody>
</table>

Cytopathologic effects were determined on the basis of the severity of cellular damage scores (1+ [≥50% cells adherent to culture plate], 2+ [10% to 50% cells adherent to culture plate], or 3+ [≤10% cells adherent to culture plate]) observed 48 hours after inoculation of cell cultures with MYXV (MOI, 5 infectious virus particles/cell). ND = Not determined because results of cytochemical staining were inconsistent with type of tumor from which cells had been obtained.

Multistep growth curves (MYXV MOI, 0.1 infectious virus particles/cell) indicated moderate increases in virus titers after inoculation of cells (Figure 3). Moderate virus production was maintained in RK-13 and canine primary hemangiosarcoma cells through 96 hours after virus inoculation, indicating cell-to-cell spread of viruses in these cell cultures. Although an increase in virus yield was detected for hemangiosarcoma Den, transitional cell carcinoma Bliley, and canine primary soft tissue sarcoma cells, virus titers for these cells decreased after 24 hours. This finding was attributed to either rapid reduction in the number of viable cells or minimal cell-to-cell spread of viruses after initial virus replication.
Virion maturation in MYXV-infected cells—Because the lower yield of infectious viruses from canine tumor cells, compared with that from RK-13 cells, may have been attributable to arrest of the virus life cycle because of aberrant virus morphogenesis, virion maturation in cells was determined. Characteristics of cells of 3 MYXV-inoculated canine tumor cell cultures (hemangiosarcoma Den, transitional cell carcinoma Bliley, and primary hemangiosarcoma) were determined by evaluation of transmission electron micrographic images, and results were compared with those for RK-13 cells (Figure 4). Viral crescents, circular immature virions, immature virions with nucleoids, and intracellular mature virions were detected at 24 hours after virus inoculation in all cell cultures examined by transmission electron microscopy. This finding indicated MYXV replication completed a full cycle of maturation within canine tumor cells and the limited infectivity of lysates of MYXV-infected cells was not attributable to aberrant virus morphogenesis. In addition, the late-stage MYXV protein M130R was detected in inoculated RK-13 cells (positive control cells) and canine tumor cells, indicating virus maturation developed in canine tumor cells.

Cell death after MYXVserp2 inoculation versus that after MYXV inoculation—To determine wheth-

er deletion of the antiapoptotic viral serp2 protein from MYXV would result in a microscopically detectable increase in signs of cell damage, CPE results for MYXVserp2-inoculated cells were compared with those for cells inoculated with MYXV-gfp or MYXV-red. More severe CPEs were observed in RK-13, U-118...
MG, hemangiosarcoma Den, transitional cell carcinoma Bliley, and canine primary soft tissue sarcoma cells inoculated with MYXVAserp2, compared with CPEs in those cells inoculated with MYXV (Figure 5). In contrast, CPEs in canine primary hemangiosarcoma cells inoculated with those 2 viruses were similar. More severe CPEs were detected in osteosarcoma Abrams and canine primary perianal adenosarcoma and perianal adenoma cells inoculated with MYXVAserp2, compared with CPEs in those cells inoculated with MYXV (data not shown). Because 1-step growth curves for MYXV and MYXVAserp2 in RK-13 cells were indistinguishable, MYXVAserp2-enhanced CPEs in these cells were likely not attributable to differences in replication kinetics (data not shown).

Percentage of dead cells gradually increased from 0 to 48 hours after mock or virus inoculation in all cell cultures evaluated (data not shown). Significant differences in percentage of dead cells were not detected when results for mock-inoculated cells were compared with those for cells inoculated with a low virus MOI at any time point (data not shown). The percentage of trypan blue-stained (dead) cells in cultures of 5 types of cells (RK-13, hemangiosarcoma Den, transitional cell carcinoma Bliley, and canine primary hemangiosarcoma and soft tissue sarcoma) at 48 hours after mock, MYXV, or MYXVAserp2 inoculation (high MOI) were compared (Figure 6). Percentage of dead RK-13, hemangiosarcoma Den, and canine primary hemangiosarcoma cells in cultures inoculated with a high MOI of MYXV was slightly lower than that in mock-inoculated cultures, but differences were not significant. A significantly higher number of dead cells was detected in RK-13, hemangiosarcoma Den, transitional cell carcinoma Bliley, and canine primary hemangiosarcoma cell cultures 48 hours after inoculation with MYXVAserp2 (high MOI) than in cultures of those cells 48 hours after inoculation with MYXV (high MOI).

Hemangiosarcoma Den, transitional cell carcinoma Bliley, and canine primary hemangiosarcoma and soft tissue sarcoma cell cultures had higher percentages of cells in the early stages of apoptosis (ie, cells with positive results for annexin V staining and negative results for 7-AAD staining) than did mock-inoculated (negative control) cultures for TCC-Bliley and HAS-1 cells. Values are mean ± SEM. *Values that are significantly (P ≤ 0.05) different are indicated by brackets. †Positive control sample values are significantly (P ≤ 0.05) higher than negative control sample values. See Figure 1 for remainder of key.
A much higher percentage of mock-inoculated RK-13 cells had positive results for annexin V staining than was expected on the basis of the finding that CPEs had not been detected in those cells (Figure 5). Therefore, caspase-3 activation assays were performed. Although results were not significantly different, slightly higher rates of DEVD-AMC cleavage by caspase-3 were detected for tumor cells inoculated with MYXVAserp2, compared with that for mock- or MYXV-inoculated cells; this finding suggested that MYXVAserp2 increased apoptosis of canine tumor cells.

Discussion

The biological behavior of naturally developing tumors in dogs is more similar to the biological behavior of human neoplasms than is that of induced tumors in mice.37 Similar to human populations, dog populations are outbred and there are differences in genetics and immune responses among dogs that directly impact neoplastic transformation and immunoevasion by tumor cells. In addition, tumors of dogs can be caused by environmental factors to which humans can also be exposed. Results of the present study indicated canine tumor cells can be permissive to MYXV infection, as can human tumor cells.22 Interestingly, all of the canine tumor cell types evaluated in the present study were permissive to MYXV infection, as indicated by the finding that intracellular virus-controlled fluorescent protein expression was consistently observed after inoculation of cells with MYXV-gfp or MYXV-red (even at low MOIs). Nontumorous canine primary fibroblasts were resistant to MYXV-red infection. Importantly, there seemed to be a relationship between increased severity of CPEs and upregulated expression of phosphorylated Akt in canine tumor cells, which was similar to findings for human cancer cells reported by other authors.29 Thus, use of canine primary tumor cells for evaluation of the efficacy of MYXV as an oncolytic treatment seemed to be valid, and results of the present study may be relevant to oncolytic treatment of human cancers.

The primary objective of oncolytic virotherapy is to eliminate cancerous cells. For in vivo viral oncolysis, apoptotic cell death may be preferred to necrotic cell death because necrosis causes severe inflammation that can damage adjacent healthy tissues. Because MYXV encodes multiple proteins that can prevent cell death, we hypothesized that a mutant MYXV without these proteins would increase the rate of death of host cells. Although MYXV inoculation of cultures induced death in some cells, results were not significantly different from those for mock-inoculated cells for most canine tumor cell types evaluated. Percentage of dead cells was significantly increased in all but 1 (soft tissue sarcoma) of the canine tumor cell cultures inoculated with MYXVAserp2, compared with results for cell cultures inoculated with MYXV. Because the serp2 protein is antiapoptotic in RK-13 cells,29 it was anticipated that the lack of serp2 expression during MYXVAserp2 infection would increase the rate of apoptosis in canine tumor cells. As anticipated, results of annexin V staining and DEVD-AMC cleavage assays suggested MYXVAserp2 inoculation of canine tumor cell cultures increased the number of apoptotic cells, compared with results for MYXV-inoculated or mock-inoculated cell cultures.

Results of recent studies38,39 indicate the efficacy of an attenuated form of the zoonotic poxvirus VACV for oncolysis of canine mammary gland tumor xenografts in mice. In those studies,38,39 administration of 1 IV dose of an attenuated VACV (GLV-1h68) to mice resulted in significant reduction in the rate of tumor growth. In addition, the same canine primary soft tissue sarcoma cells used in the present study have been used by a collaborating investigator40 to evaluate oncolytic efficacy of another strain of VACV for treatment of canine soft tissue sarcoma xenografts in nude mice. Results of that study40 indicate systemic administration of poxviruses may be efficacious for treatment of dogs with tumors; systemic administration of oncolytic viruses may enable concurrent targeting of primary tumors and metastases. However, immunocompromised mice were used in those studies38–40; such mice do not develop the complex immune responses that develop during viral infection of immunocompetent hosts. Indeed, humoral immune responses to poxvirus infections are strong in susceptible hosts41; such humoral immune responses may limit the effectiveness of oncolytic viruses. Despite this possibility, results of clinical trials in which attenuated VACVs were administered to humans with cancer indicate that circulating antibodies to VACV (the smallpox vaccine virus) do not substantially affect treatment outcomes.52,63 Similarly, we anticipate that IV administration of modified poxviruses to dogs with cancer would be efficacious, despite clearance of the virus via cell and humoral immune responses.

Results of the present study indicated the poxvirus MYXV, with specific pathogenicity for rabbits, can cause damage and induce expression of viral-encoded proteins in canine tumor cells. In addition, increased expression of phosphorylated Akt seemed to correspond with increased CPEs in canine tumor cells inoculated with MYXV, as has been found for human tumor cells.35 Furthermore, inoculation of canine tumor cell cultures with MYXVAserp2 increased cell death by apoptosis, compared with results for cells inoculated with MYXV. Additional studies are warranted to determine effectiveness of MYXVAserp2 as an oncolytic agent in vivo. Results of the present study may be relevant to oncolytic virotherapy for humans with cancer.
References


