A Canine Conditionally Replicating Adenovirus for Evaluating Oncolytic Virotherapy in a Syngeneic Animal Model

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Oncolytic adenoviruses, which selectively replicate in and subsequently kill cancer cells, have emerged as a promising approach for treatment of tumors resistant to other modalities. Although preclinical results have been exciting, single-agent clinical efficacy has been less impressive heretofore. The immunogenicity of adenoviruses, and consequent premature abrogation of replication, may have been a partial reason. Improving the oncolytic potency of agents has been hampered by the inability to study host–vector interactions in immune-competent systems, since human serotype adenoviruses do not productively replicate in animal tissues. Therefore, approaches such as immunomodulation, which could result in sustained replication and subsequently increased oncolysis, have not been studied. Utilizing the osteocalcin promoter for restricting the replication of a canine adenovirus to dog osteosarcoma cells, we generated and tested the first nonhuman oncolytic adenovirus. This virus effectively killed canine osteosarcoma cells in vitro and yielded a therapeutic benefit in vivo. Canine osteosarcoma is the most frequent malignant disease in large dogs, with over 8000 cases in the United States annually, and there is no curative treatment. Therefore, immunomodulation for increased oncolytic potency could be studied with clinical trials in this population. This could eventually translate into human trials.

Key Words: adenovirus, virus replication, neoplasms, gene therapy, osteocalcin, osteosarcoma, immunosuppressive agents, biological therapy, dogs, humans

INTRODUCTION

Oncolytic virotherapy with adenoviruses replication competent in tumor but not in normal tissue is a promising approach for treatment of malignancies resistant to current treatments. Although there are convincing preclinical results, few Phase II and no Phase III clinical trials have been completed [1,2]. Further, due to the nature of translational research, agents utilized in the published trials may not be as effective as the most advanced agents in preclinical development. Therefore, it is currently unclear how well the approach will work in the clinic. In a Phase II trial with 40 head and neck cancer patients, an E1B55K-deleted oncolytic adenovirus injected intratumorally resulted in 3 complete, 2 partial, and 3 minor responses [3]. In another study the same virus resulted in 1 partial response when 5 patients with hepatocellular carcinoma were treated intravenously [4]. Higher response rates were seen when the agent was combined with chemotherapy [5]. Therefore, although there is clear evidence of efficacy, the available data suggest that even with multiple intratumoral injections, there is a need for improving the efficacy of treatments to achieve a therapeutic benefit for a larger proportion of patients.

Considering the strong immunogenicity of adenovirus [6], abrogation of replication due to immune activation may be a partial reason for the lower than expected clin-
cal efficacy. High neutralizing antibody titers are rapidly induced following treatment [7], which can affect the efficacy of adenoviral treatments in model systems [8,9]. A major problem in the field has been our inability to study host–vector interactions relevant to the oncolytic potential of adenoviruses [6,10]. Although it has been reported that murine epithelial cell lines may allow limited replication in vitro [11], and cotton rat or pig pulmonary tissues may be sensitive to human adenovirus-mediated toxicity [12,13], productive in vivo replication of human adenoviruses in animals has not yet been demonstrated. The opposite is probably also true, since nonhuman adenoviruses do not seem to replicate productively in human cells [14]. Further, xenograft studies are not helpful, as a syngeneic system is necessary for retaining immune competence. Importantly, studies with replicating herpesviruses have demonstrated the feasibility of immune suppression to achieve a more efficient antitumor effect, establishing the concept that immunomodulation can be synergistic with replicating viral systems [15,16]. Furthermore, anecdotal reports of increased adenovirus replication in immunocompromised individuals suggest that the synergy could be utilized in humans [17–20]. Moreover, it has been shown that transient immune suppression can prolong adenoviral transgene expression, reduce the neutralizing antibody and T-cell-mediated response, and increase anti-tumor efficacy in rodent and canine tissues [21–27]. Finally, there is a series of case reports suggesting increased tumor responses in humans, when injections of replication-competent adenoviruses were combined with immune suppression [17].

Osteosarcoma is the most common malignant tumor in dogs, with at least 8000 cases yearly in the United States [28]. Both metastatic and local diseases have been extensively studied and are strikingly similar to their human counterparts. Thus, canine osteosarcoma may be among the best characterized animal models for human malignant disease. If necessary, this tumor can also be induced with radiation [29]. Moreover, large dogs resemble humans in tumor to body mass ratios, site of origin, metastatic pattern, and osteosarcoma treatment [28]. Thus, canine osteosarcoma could be a useful animal model for testing immunomodulation approaches if a syngeneic conditionally replicating adenovirus (CRAD) could be developed. Canine adenoviruses have not been studied as extensively as human serotypes. However, the pathophysiology of canine adenovirus type 2 (CAV-2) resembles human serotypes 2 and 5 [30] and has been used for development of recombinant viruses [31–34].

An interesting and potentially useful parallel between dogs and humans is that there are patient populations with distinct levels of antibodies against CAV-2 or adenovirus 5, respectively. Dogs are often vaccinated with CAV-2 to protect from the more pathogenic CAV-1, the “canine hepatitis virus.” However, the proportion of dogs vaccinated depends on the country, region, and owner’s preference. Unvaccinated dogs with low CAV-2 antibody titers thus resemble patients with low anti-Ad5 titers, while vaccinated dogs could resemble patients with high titers or readministration of the agent.

Two types of CRADs have been reported. Type I CRADs exhibit genetic defects that are transcomplemented in tumor cells, such as deficiency of the p53-binding E1B55K protein or the pRb-binding constant region 2 of E1 [1,2]. Type II CRADs utilize tumor-specific promoters for controlling expression of crucial adenovirus genes such as the E1A, which acts as the central regulator of other early promoters and, consequently, of adenovirus replication. A number of promoters have been utilized for this approach and several human trials are in progress [1,2]. With regard to osteosarcoma, the osteocalcin promoter (OC) has been suggested as a useful candidate [35]. Activity of OC has been determined to be limited to osteoblasts in mature healthy humans, but high activity has been seen in prostate adenocarcinoma, bone metastasis, and osteosarcoma [35–37]. The biological reason for these tumor types expressing OC may be linked to their metastatic homing to bone, resulting in an osteoblastic or osteosclerotic radiographic appearance of the bone lesions [37,38]. As a large proportion of adenovirus administered intravenously localizes to the liver [39], it would be useful if the truncated promoter used here resembled the genomic promoter, which has been reported to have very low liver expression [40]. Although the OC promoter used here is a murine promoter, it has shown activity in both human and rat osteosarcoma cell lines [35,37,41,42]. Further, mammalian promoters typically readily cross species [35,37,41–43].

Here, we constructed a conditionally replicating canine adenovirus, OC-CAVE1, in which the OC promoter controls expression of the E1A of CAV-2. The novel virus was shown to replicate in, and kill, canine osteosarcoma cells in vitro and achieve a therapeutic benefit in an animal model of canine osteosarcoma. These results set the stage for testing of immunomodulation for increased replication and therapeutic benefit in clinical trials with dogs with osteosarcoma.

**RESULTS**

**The OC Promoter in a Plasmid Construct Is Induced in Canine Osteosarcoma Cell Lines**

We constructed pGL3-OC, in which the OC promoter controls the expression of luciferase, and compared it to pGLuc4 featuring the cytomegalovirus immediate early promoter (CMV). We transfected both plasmids into canine osteosarcoma lines D22, CF11, and D17 or ROS, the positive control for OC activity [35], and LoVo, a nonosteoblastic line expected to be OC-negative. After background readings obtained with the promoterless plasmid (pGL3-Basic) were subtracted, OC activity in the osteosarcoma lines was 2.1,
20.8, and 5.5% of CMV, respectively. The readings were 99.8 and 0.009% for ROS and LoVo (Fig. 1A).

The OC Promoter in an Adenovirus Construct Is Induced in Canine Osteosarcoma Cell Lines

Elements present in adenoviruses may interfere with the fidelity or activity of promoters. Therefore, we constructed human adenoviruses incorporating the OC promoter and used them to study the fidelity and activity of the promoter in the context of an adenovirus on canine osteosarcoma cells (Fig. 1B). OC promoter activity was 36.9, 18.9, and 81.4% of CMV for D22, CF11, and D17, while it was 34.0 and 4.9% for ROS and LoVo. Non-bone-marrow epithelioid cells. (C) A primary canine osteosarcoma sample was obtained fresh from the operation and homogenized. The osteosarcoma cells were then infected with two different doses of AdEasy-OC-Luc-CMV-GFP (OC) and AdEasy-CMV-Luc-CMV-GFP (CMV), followed by luciferase detection at 24 h. Mock-infected cells are also displayed. (D) To investigate if CAV-2 can replicate in and cause oncolysis of canine osteosarcoma cells, a TCID_{50} titration assay was performed. Cells were infected with serial dilutions of virus and the development of CPE was followed over 10 days. In the higher dilutions, CPE was observed at ca. 1 week, suggesting effective infection, productive replication, and oncolysis with CAV-2.
forming normal tissues do not express OC. To identify a line that would resemble normal tissues in this regard, we investigated further canine cell types, including mammary adenocarcinoma, melanoma, primary fibroblasts, and fetal kidney cells (data not shown). DK and DKCre [34] are dog kidney lines that have been used for propagation of CAV-2-based viruses and displayed OC activities of 2.2 and 2.6%, respectively, in comparison to CMV (Fig. 1B). Importantly, BTB mammary epithelioid cells were found to display low OC activity, 0.6% of CMV, as would be expected for non-bone-forming tissues, and were thus used in subsequent experiments as a surrogate for normal tissue.

The OC Promoter in an Adenovirus Construct Is Induced in Primary Canine Osteosarcoma

Data from human studies have suggested that there are frequently discrepancies between cell line and primary tumor data [1,2]. Therefore, we obtained a fresh primary canine osteosarcoma sample and infected it with adenoviruses incorporating the OC or CMV promoter. In accord with the cell line data, OC activity was 13.4 or 24.1% of CMV, depending on the dose of virus (Fig. 1C). Green fluorescent protein (GFP) expression was studied by fluorescence microscopy and was uniformly spread across the infected cells. The histology was that of a chondroblastic osteosarcoma (not shown).

CAV-2 Replicates in Canine Osteosarcoma Cells

Little is known about CAV-2 replication in canine tumor cells. Therefore, we performed a tissue culture infectious dose 50 (TCID₅₀) assay, and the virus was found to replicate in all canine osteosarcoma lines tested (Fig. 1D). As the end-point of the assay is emergence of cytopathic effect (CPE), as a function of time, in a dilution series, the result indicates the capacity of the virus to infect, replicate in, and cause cytotoxicity to canine osteosarcoma cells.

Construction of OC-CAVE1

We constructed a shuttle vector for introduction of OC for controlling expression of CAV-2 E1A (Fig. 2). A unique restriction site was created for introduction of the heterologous promoter into a site at which the distance of its TATA box from the transcription start site would closely resemble that of CAV-2. Homologous recombination in bacterial cells yielded a plasmid containing the genome of OC-CAVE1, which was released and propagated in DKCre cells, which transcomplement the E1 region of CAV-2. CMV-CAVE1 was created in an identical manner, except the ubiquitously expressed CMV promoter was utilized instead of OC. Titering of the viruses gave the following results: OC-CAVE1, 1.4 × 10¹² viral particles (VP)/ml, 6.3 × 10¹¹ TCID₅₀/ml; CMV-CAVE1, 3.8 × 10¹² VP/ml, 7.9 × 10¹¹ TCID₅₀/ml; CAVGFP, 2.0 × 10¹² VP/ml, 6.3 × 10¹⁰ TCID₅₀/ml; CAV-2, 2.0 × 10¹³ VP/ml, 6.3 × 10¹¹ TCID₅₀/ml.

OC-CAVE1 Replicates in and Kills Canine Osteosarcoma Cells

We used a crystal violet staining-based replication/killing assay to study the oncolytic potential of the novel viruses (Fig. 3). As crystal violet stains all cells, and dead cells typically detach, the staining indicates remaining live cells. In all cases, the E1-deleted control virus CAVGFP did not replicate in canine cells. The positive control, CAV-2, replicated in and killed all cell lines. In the OC-negative BTB cells (Fig. 3A), and the low-OC DK cells (Fig. 3B), OC-CAVE1 did not replicate, while the control viruses did. Importantly, in all osteosarcoma cell lines, OC-CAVE1 was able to achieve cell killing to a degree similar to those of CAV-2 and CMV-CAVE1 (Figs. 3C–3E).

An alternative approach to assessing cellular viability is studying their mitochondrial activity. This can be helpful with cells that do not easily detach even when visibly dead, such as D22 cells (unpublished observation by au-
FIG. 3. Crystal violet cell killing assay. Canine osteosarcoma cells (D22, CF11, D17) or nonmalignant cells (BTB, DK) were infected with an E1-deleted vector (CAVGF), the novel canine conditionally replicating adenovirus featuring the osteocalcin promoter for controlling E1A expression (OC-CAVE1), the isogenic control virus with a ubiquitously expressed promoter (CMV-CAVE1), or wild-type CAV-2. The growth medium was changed every 2 days until almost complete cell killing was visually evident at the lowest dose for any virus. Each cell line was evaluated individually. Crystal violet staining was then performed to detect attached cells. Killing of canine osteosarcoma cells with CAV-2-based agents did not always cause detachment although cells were dead upon visual inspection.
thors). This seemed to be true for many cell types used in this study, which suggests that the phenomenon may be related to canine osteosarcoma cells in general or CAV-2-based agents in particular. One possible explanation for the latter could be the lack of a known RGD motif in the CAV-2 penton base [33]. In accord with the crystal violet data, infection with OC-CAVE1 resulted in little or no reduction in mitochondrial activity of BTB or DK cells, while CAV-2 and CMV-CAVE1 achieved cell killing (Figs. 4A and 4B). In contrast, OC-CAVE1 killed osteosarcoma cells to a degree similar to those of CMV-CAVE1 and CAV-2 (Figs. 4C–4E).

**Replication of Human Adenovirus Serotype 5 on Canine Cells**

Although unlikely, it would be potentially useful if human adenoviruses would replicate in canine cells. This could facilitate utilization of the canine osteosarcoma model with candidate human clinical agents. Thus, we included a wild-type human serotype 5 virus in all MTS assays. However, no evidence of replication or cell killing was seen (data not shown).

**Therapeutic Efficacy of OC-CAVE1 in an Animal Model of Canine Osteosarcoma**

We tested the growth of D22, D17, and CF11 subcutaneously in nude and NIH-III mice. Following injection of 3 or 10 million D17 cells, tumors grew subcutaneously in all NIH-III mice but not in nude mice. CF11 did not grow in any mice. However, D22 grew steadily in nude mice and thus provided the most practical model. Five million cells were injected into both flanks followed by intratumoral injection of $1 \times 10^8$ VP of viruses on 3 consecutive days. OC-CAVE1 gave a significantly improved therapeutic effect over OptiMEM (the diluent) only ($P < 0.0001$). The efficacy of OC-CAVE1 was not different from that of CMV-CAVE1 ($P = 0.095$), but CAV-2 was more effective than OC-CAVE1 ($P = 0.0009$).

We conducted a further experiment, but this time a single intratumoral injection at a lower dose ($1 \times 10^8$ VP) was performed. Again, OC-CAVE1 delivered a significant therapeutic effect over OptiMEM only ($P < 0.0001$). Interestingly, with this dose, OC-CAVE1 was also improved over CAV-2 ($P < 0.0001$) and CMV-CAVE1 ($P = 0.0046$). As a further control group, we included an E1-deleted CAV-2-based vector CAVGFP. The therapeutic efficacy of OC-CAVE1 was improved also over this control ($P < 0.0001$).

**DISCUSSION**

Current clinical results with oncolytic adenoviruses suggest good safety, but single-agent treatment results have not been as impressive as the preclinical data. Considering the immunogenicity of adenovirus, immunomodulation may be a useful way to increase the oncolytic potency of CRADs [6,10]. The principle of temporary immunosuppression for increasing oncolytic potency has been validated with herpesviruses [15,16]. However, it has not been possible to test the approach with adenoviruses as no animal models have been reported to allow significant replication of human serotypes. Therefore, a syngeneic cancer model and a syngeneic CRAD are required.

We hypothesized that canine osteosarcoma would provide an excellent model for study of immunomodulation for increased CRAD efficacy, if a canine CRAD could be developed. Thus, we investigated the OC promoter in plasmid and adenovirus constructs and found high expression in all osteosarcoma cell lines (Figs. 1A and 1B). Normal non-bone-forming tissues do not express osteocalcin [36]. Hence, we sought to identify a canine cell type resembling normal tissues with regard to this aspect but also amenable to in vitro studies, which intact tissues are not. BTB mammary epithelioid cells were identified as such cells (Fig. 1B). Human studies have indicated that primary cancers may be different from cell lines in a variety of ways [1,2]. Thus, we obtained a clinical canine osteosarcoma specimen and found the cells to express high OC (Fig. 1C).

The biology of CAV-2 is not understood as well as that of human viruses. Thus, it was of interest to see if canine osteosarcoma cells would be infected with CAV-2 and if they could support productive replication. A TCID<sub>50</sub> assay was used to assess both attributes (Fig. 1D). We saw CPE develop gradually, with a maximum titer evident at day 10, suggesting subsequent rounds of replication, virus burst, and infection of further cells. If the CPE would have been due to the initial dose of virus, it would have been evident soon after infection.

These data established critical feasibility criteria and we therefore proceeded with construction of the canine CRAD, OC-CAVE1 (Fig. 2). The CRAD was modeled after human CRADs utilizing tumor-specific promoters for control of E1A [44–46]. Further, we retained the E3 region to maximize the oncolytic potential of the agent [6]. We also created an isogenic virus, CMV-CAVE1, with a high-activity CMV promoter, to control for the effect of introduction of a heterologous promoter in the CAV-2 E1 region. In cell killing experiments, no killing of OC-negative nonmalignant BTB cells by OC-CAVE1 could be detected (Figs. 3 and 4). Also, little or no killing of the nonmalignant DK cells was seen, while CAV-2 and CMV-CAVE1 killed both cell types effectively. DK cells express low OC activity, 2.2% of CMV (Fig. 1B). As all osteosarcoma lines expressed 18.9% of CMV or more, and were killed effectively, this established the therapeutic window for this construct. It was interesting to note that cell killing with OC-CAVE1 was similar to that of CAV-2 and CMV-CAVE1 for all osteosarcoma lines. CF11 cells were more sensitive to the recombinant viruses than to CAV-2. This may be due to the anti-tumor effects of high E1A expression, well documented in human cancers [47].
FIG. 4. MTS cell killing assay. Canine osteosarcoma cells (D22, CF11, D17) or nonmalignant cells (BTB, DK) were infected with an E1-deleted vector (CAVGFP), the novel canine conditionally replicating adenovirus featuring the osteocalcin promoter for controlling E1A expression (OC-CAVE1), the isogenic control virus with a ubiquitously expressed promoter (CMV-CAVE1), or wild-type CAV-2. The growth medium was changed every 2 days until almost complete cell killing was visually evident at the lowest dose for any virus. Each cell line was evaluated individually. Mitochondrial activity was then determined and compared to that of mock-infected cells to estimate the proportion of live cells. Bars indicate standard deviation.
Interestingly, CAV-2 and the CAV-2-based CRADs seemed to differ from human viruses in that cell killing did not always result in immediate cell detachment. Instead, upon visual inspection, the cell membrane skeletons sometimes remained attached, as displayed by the slight imbalance between crystal violet binding (Fig. 3C) and mitochondrial activity (Fig. 4C), for D22 in particular. This could be due to CAV-2 lacking an RGD motif in the penton base [32], but more studies are needed to clarify these findings. If confirmed, these findings could help shed light on penton base–integrin interactions in adenoviral entry.

We investigated the canine osteosarcoma lines for their capacity to form subcutaneous tumors in mice, and D22 cells in nude mice were identified as a useful model. With both high (Fig. 5A) and low (Fig. 5B) dose, OC-CAVE1 gave a significant anti-tumor effect. Interestingly, with the lower dose, the therapeutic effect of OC-CAVE1 was greater than that of CAV-2. Again, this could be due to high E1A expression contributing to the anti-tumor effect [47].

In conclusion, we have successfully created the first nonhuman CRAD, which effectively replicates in and causes oncolysis of canine osteosarcoma cells. For testing immunomodulation for improved oncolytic potency, the next step will be to test the agent with or without immunosuppression in clinical trials with dogs suffering from osteosarcoma. Notably, human prostate cancers frequently express OC [37]. It would be interesting if the same is true for dogs, as this disease could provide an additional syngeneic model. Candidate immune modulators could include cyclosporin and cyclophosphamide, both of which have been successfully used for transiently immunosuppressing dogs for increased adenoviral gene

![FIG. 5. Therapeutic efficacy of OC-CAVE1 in vivo. D22 canine osteosarcoma cells were injected into both flanks of nude mice (n = 5/group) followed by intratumoral injection of viruses. Tumor size was measured every 3 days. (A) 1 × 10^9 VP of the canine conditionally replicating adenovirus featuring the osteocalcin promoter for controlling E1A expression (OC-CAVE1), the isogenic control virus with a ubiquitously expressed promoter (CMV-CAVE1), wild-type CAV-2, or no virus (OptiMEM) was injected on 3 consecutive days. Tumors treated with OC-CAVE1 were smaller than tumors treated with OptiMEM (P < 0.0001). (B) A single 1 × 10^8 VP injection of OC-CAVE1, CMV-CAVE1, CAV-2, OptiMEM, or an E1-deleted vector (CAVGFP) was performed. OC-CAVE1 displayed therapeutic efficacy over OptiMEM (P < 0.0001), CAV-2 (P < 0.0001), CMV-CAVE1 (P = 0.0046), and CAVGFP (P < 0.0001). Bars indicate standard error.](image-url)
transfer and reduced T-cell-mediated and neutralizing antibody formation [21,22,48,49]. Promising results in dogs could then be translated into human Phase I trials.

**Materials and Methods**

**Cell lines.** Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) unless noted otherwise. All media were supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mM l-glutamine, 100 IU/ml penicillin, and 25 μg/ml streptomycin. All supplements were from Mediatech (Herndon, VA). D17, D22, and CFI1 canine osteosarcoma lines; DK dog kidney cells; and LoVo human colon cancer cells were from American Type Culture Collection (ATCC; Rockville, MD). D17 was grown in minimum essential medium with Earle’s salts and supplemented with 0.1 mM nonessential amino acids and 1 mM pyruvate. LoVo was grown in F12K medium. ROS17/2.8 cells (referred to as ROS) were courtesy of Dr. Thomas Gardner (Indiana University, Indianapolis, IN). 911 cells were from Dr. Alex van der Eb (University of Leiden, The Netherlands). 293 cells were purchased from Microbix (Toronto, Ontario, Canada) and cultured in Ham’s F12/DMEM. CMT12 and CMT27 canine mammary adenocarcinoma, CML10 canine melanoma (from Dr. Lauren Wolfe, Auburn University, Auburn, AL), FDK fetal dog kidney, NCF1 normal canine fibroblasts, and BTB canine mammary epithelioid cells (from Dr. R. Curtis Bird, Auburn University) were supplemented with 0.13% sodium bicarbonate and 0.02% amphotericin B. DKCre cells [31] were purchased from Microbix (Toronto, Ontario, Canada) and cultured in F12K medium. CMT12 and CMT27 canine mammary adenocarcinoma, CML10 canine melanoma (from Dr. Lauren Wolfe, Auburn University, Auburn, AL), FDK fetal dog kidney, NCF1 normal canine fibroblasts, and BTB canine mammary epithelioid cells (from Dr. R. Curtis Bird, Auburn University) were supplemented with 0.13% sodium bicarbonate and 0.02% amphotericin B. DKCre cells [31] were supplemented with 0.1 mM nonessential amino acids. Infections were performed in medium with 2% FBS.

**Luciferase expression with plasmids.** OC was isolated from pBlBS3.3 [35] (from Dr. Gerard Karsenty, MDACC, Houston, TX) and ligated into pG3L-Basic (Promega, Madison, WI) to create pG3L-OC. pG3L-Basic (no promoter), pCLuc4 (with CMV promoter, courtesy of Dr. Feng Johanning, University of Alabama at Birmingham), and pG3L-OC were transfected with Superfect (Qiagen, Valencia, CA) into D17, D22, CFI1, LoVo, ROS, and DK. Luciferase was measured 72 h later (Luciferase Assay System with Reporter Lysis Buffer; Promega). Readings obtained with pGL3-Basic were subtracted as background.

**Luciferase-expressing viruses.** For construction of AdEasy-OC-Luc-CMV-GFP, the OC-Luc fragment was cut from pG3L-OC and cloned into pAdTrack (Qbiogene). These shuttle plasmids were transfected with PmeI and cotransfected with pAdEasy-1 into B51s3.1 Escherichia coli cells. After confirmation of the structure, recombinants were linearized with PfaI and transfected into 911 cells for propagation, followed by two rounds of cesium chloride centrifugation. Functional titers (plaque-forming units, pfu) were determined by standard plaque assay on 293 cells. The resulting titers were 1.1 × 10^10^ pfu/ml and 3.0 × 10^9^ pfu/ml for AdEasy-OC-Luc-CMV-GFP and AdEasy-CMV-Luc-CMV-GFP, respectively. Depending on the cell line, infections were performed at 50–500 pfu/cell with both viruses for 1 h, followed by a medium change and detection of luciferase at 24 h as above, and results are presented as percentage of expression obtained with OC vs CMV.

**Replication of CAV-2 in canine osteosarcoma.** The Toronto strain of CAV-2 (Genbank U77082) was obtained from ATCC (Rockville, MD), titered, and propagated as below. TCID_{50} was performed with standard methodology (AdEasy Application Manual; Qbiogene). Briefly, serial 10-fold dilutions of CAV-2 were used to infect DK, D22, D17, or CFI1 cells, in replicates of 10 at each dilution, and the presence or absence of cytopathic effect was scored 9 days later. The titer was calculated as T = 10^{0.5 \cdot S}. For example, for 10/10 wells showing CPE, ratio = 1; for 2/10, ratio = 0.2. S is the sum of the ratios of all dilutions.

**Primary canine osteosarcoma.** Primary osteosarcoma material was obtained with owner permission from a dog undergoing surgery for osteosarcoma. Tumor viable by gross inspection was immersed in L-15 medium containing 20% horse serum (Hyclone) and 300 IU/ml penicillin, 0.3 mg/ml streptomycin, 0.75 mg/ml amphotericin B and transported on ice. Utilizing sterilize technique, we used two scalpels to mince the tissue and followed with 1 h of Disperse I (Roche Molecular Biochemicals, Indianapolis, IN) treatment to detach cells further. Dispersate was then removed, and cells were washed and allowed to attach overnight. The specimen was then infected with AdEasy-OC-Luc-CMV-GFP or AdEasy-CMV-Luc-CMV-GFP and 24 h later luciferase expression was determined as above. Representative tissue was fixed and subjected to histological analysis by one of us (G.P.S.) with expertise in osteosarcoma diagnosis.

**Construction of canine CRADs.** pTGS412 [31] was digested with EcoRI, and the 5612-bp fragment was gel purified and religated to make pCAVE1 (Fig. 2). A unique KpnI site was created into position 419 using standard PCR-directed mutagenesis (left flankning primer 5’-GTTCCTTCAGCGCTATGTTC-3’, antisense mutagenesis primer 5’-TCTTCTTCTGGTCTGAGCGCG-3’, sense mutagenesis primer 5’-CCGTTACCCAGGAAGGAGA-3’, right flankning primer 5’-CACGCCAAGCATCTGCTGAGA-3’). The mutagenesis primers overlap and incorporate the novel site. pCAVE1 was amplified with the former and latter two primers separately. The PCR products were mixed and subjected to a further round of PCR with the flanking primers. This PCR product and pCAVE1 were cut with Nhel and BsiXI and the fragment containing the new site was ligated into pCAVE1 to create pCAVE1Kpn. The KpnI site was then utilized for introduction of OC (from pBlBS3.3) or CMV (from pcDNA3; Invitrogen, San Diego, CA) to create pCAVE1KPNOC or pCAVE1KPCMV, respectively.

The shuttle plasmids were cotransfected with SwaI-linearized pTGS412 into B51s3.1 cells to create pOC-CAVE1 and pCMV-CAVE1, respectively, using methodology described in detail previously [31]. The recombinants were digested with NolI and transfected into DKCre cells for propagation, followed by purification with double cesium chloride gradients [31]. Each stage of the cloning was confirmed with multiple restriction enzyme digests and sequencing as necessary. For pOC-CAVE1 and pCMV-CAVE1, NotI, EcoRI, EcoRV, and HindIII digestions were performed and the E1 region, including the promoters, was completely sequenced.

CAV-2 and CAVGFP were also propagated on DKCre cells and purified as above. CAVGFP is an E1-deleted CAV-2-based vector [31]. To determine the VP concentration, the viruses were diluted in 10 mM Tris (pH 8.0), 1 mM EDTA, 0.1% SDS and incubated at 56°C for 10 min, and the absorbance at 260 nm was measured. One absorbance unit corresponds to 1.1 × 10^{12} VP/ml. TCID_{50} on DKCre cells was utilized for functional titering (see above).

**Crystal violet cell killing assay.** Cells in 12-well plates were infected with CAVGFP, OC-CAVE1, CMV-CAVE1, or CAV-2 for 1 h at 37°C in 250 μl. Cells were then washed once and incubated at 37°C in 2% medium. On day 2.5 (BTB), day 5 (DK), day 7 (D17), day 8 (CF11), or day 12 (D22), crystal violet staining was performed as described [50].

**In vitro cytotoxicity assay (MTS).** Cells in 96-well plates were infected in quadruplicate in 50 μl. On day 4 (BTB), day 7 (DK), day 8 (CF11, D17), or day 12 (D22), cell viability was measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega). A wild-type human adenovirus 5 strain (Ad3000wt) was obtained from ATCC, propagated on 293 cells, titered as above (1.2 × 10^{12} VP/ml, 1.3 × 10^{11} pfu/ml), and included in all MTS experiments.

**Animal experiments.** CD1/mu or NIH-III mice (Charles River Laboratories, Wilmington, MA) were injected subcutaneously with 5 million (D22) or 3 and 10 million (D17, CF11) cells into both flanks. When tumors were ca. 5 mm in diameter, intratumoral injections were performed in a volume of 50 μl utilizing three needle tracts per injection. Tumor size was measured with a caliper every 3 days and volume was calculated as V = 0.5 × d_{2} × (d_{1})^{2}, where d_{1} is the longest diameter and d_{2} the shortest. Mice were inspected daily and when pain or discomfort were evident, the experiment was terminated.

**Statistics.** The analysis of the tumor size data was performed using a repeated-measures growth model with PROC MIXED (SAS v8.02; SAS Institute, Inc). doi:10.1016/S1525-0016(02)00049-7


