Effect of an adenoviral vector that expresses the canine *p53* gene on cell growth of canine osteosarcoma and mammary adenocarcinoma cell lines

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Objective—To generate an adenoviral vector that expressed the canine *p53* gene and investigate its growth-inhibiting effect on canine osteosarcoma and mammary adenocarcinoma cell lines.

Sample Population—2 canine osteosarcoma cell lines (HOS, OOS) and 3 canine mammary adenocarcinoma cell lines (CHMp, CIPm, and CNMm).

Procedure—An adenoviral vector that expressed the canine *p53* gene (AxCA-cp53) was generated. *p53* gene expression was examined by use of reverse transcription (RT)-polymerase chain reaction (PCR) assay and immunohistochemistry. Susceptibility of cell lines to the adenoviral vector was determined by infection with an adenoviral vector that expresses β-galactosidase (AxCA-LacZ) and 3-indolyl-β-D-galactopyranoside staining. Growth inhibitory effects were examined by monitoring the numbers of cells after infection with mock (PBS) solution, AxCA-LacZ, or AxCA-cp53. The DNA contents per cell were measured by flow cytometry analysis. Apoptotic DNA fragmentation was detected by use of a terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay.

Results—AxCA-cp53-derived *p53* gene mRNA and P53 protein were detected by RT-PCR analysis and immunohistochemistry, respectively. Multiplicity of infection at which 50% of cells had positive 3-indolyl-β-D-galactopyranoside staining results ranged from 10 to 50. AxCA-cp53 induced growth inhibition in a dose-dependent manner. Arrest of the G_1-phase population and apoptotic DNA fragmentation were observed in cells infected with AxCA-cp53.

Conclusions and Clinical Relevance—AxCA-cp53 inhibits cell growth via induction of cell cycle arrest and apoptosis in canine osteosarcoma and mammary adenocarcinoma cell lines that lack a functional *p53* gene. AxCA-cp53 may be useful to target the *p53* gene in the treatment of dogs with tumors. (*Am J Vet Res* 2003;64:880–888)

The *p53* tumor suppressor gene and its aberration are considered to be a molecular basis for tumorigenesis. Approximately half of all human tumors, including breast cancer, osteosarcoma, colon cancer, and lung cancer, carry *p53* mutations.[1] In dogs, various types of tumors, including osteosarcoma,[2,3] mammary gland tumor,[4,5] lymphoma,[6] and colon cancer,[7] have *p53* mutations. *p53* gene-null mice are susceptible to tumors, even when they are extremely young, and loss of the *p53* gene strongly enhances tumor development driven by other oncogenic alterations.[8] Furthermore, many tumors that retain wild-type *p53* are defective in inducing or activating the P53 protein.[9] For example, the MDM2 protein, which inhibits P53 transcriptional activity and targets P53 for degradation, is associated with tumorigenesis through the inactivation of P53.[9]

The important activity of the P53 protein is to act as a sequence-specific transcriptional factor.[9] P53 protein induces other proteins that localize to the mitochondria, such as Bax[10] and NOXA,[11] and these proteins trigger cytochrome C release and activation of the Apaf-1/caspase-9 apoptosome.[9] P53 protein also induces the expression of death receptors, such as Fas[12] and Killer/DR5,[13] which mediate P53 protein-induced apoptosis. Furthermore, P53 protein enhances the transcription of $p21^{Waf1/Cip1}$ gene, the cyclin-dependent kinase inhibitor, and induces P53 protein-mediated G_1-phase population arrest.[14,15] On the other hand, P53 protein has activities that are independent of transcriptional factors, such as relocalization of death receptors to the cell surface[16] and regulation of translation by direct binding to the 5' untranslated region of certain mRNAs.[17] For these reasons, the *p53* gene may be a target for cancer gene therapy.

Various methods to introduce foreign genes have been investigated in vitro and in vivo, including liposome-mediated transfection,[18] DNA transfer by use of a gene gun,[18] and virus-mediated gene transfer.[19] The adenoviral vector is a virus-mediated gene transfer system.[19,20] Adenoviruses are double-stranded DNA viruses with a medium size genome (approx 36 kilobase pairs), which are suitable for carrying large or multiple foreign genes.[19,20] This vector has a broad host range in vitro and in vivo, with high infectivity.[19,20] Because ade-

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noviruses do not require integration into the host cell genome in their life cycle, they have low genotoxicity to host cells.[19,20] The adenoviral vector is easy to generate and manipulate and can be obtained in high titers, such as 10^{10} to 10^{12} plaque-forming units/mL.[19,20] Therefore, the adenoviral vector has been used to introduce foreign genes in various types of human gene therapies, including adenovirus-mediated *p53* treatment for cancer.[19,20] In humans, mild-to-moderate adverse effects following intratumoral injection of a adenoviral vector have included fever, arthralgia, dyspnea, hypertension, and tachycardia.[21] In an adenoviral gene treatment trial for ornithine transcarbamylase deficiency, a patient died after arterial adenovirus administration.[22,23] Therefore, the safety of adenoviral administration by intra-arterial and intratumoral injection should be investigated further.

In dogs, osteosarcoma is the most common primary bone tumor with frequent pulmonary metastasis at the time of admission.[24,25] The incidence of osteosarcoma in dogs is 50 times as common as the incidence in humans.[25] Most osteosarcomas in dogs are malignant, and osteosarcoma accounts for 80% of malignant bone tumors in dogs.[25] The prognosis is poor; approximately 90% of dogs with osteosarcoma die within 1 year if they are treated only with resection.[26,27] Chemotherapy for micrometastases of osteosarcoma is effective and improves survival, but it is not curative, and most dogs will die of osteosarcoma.[28-30] It has been reported that 40 to 50% of canine osteosarcomas have *p53* mutations.[2,3] Amplification of the *mdm2* gene and overexpression of the MDM2 protein have also been reported in canine osteosarcomas.[2,31]

Mammary gland tumor is the most common neoplasm in female dogs.[32,33] The incidence of mammary gland tumor in dogs is higher than in any other domestic animals and is 3 times as high as in humans.[32,33] Approximately 50% of the mammary gland tumors in dogs are benign, and the rest are malignant; half of the malignant tumors have undergone metastasis by the time they are initially diagnosed.[32,33] Resection is effective, if the tumors have no metastasis. However, if there is metastasis, the prognosis is poor. Although several chemotherapeutic drugs have been used for the treatment of metastatic mammary gland tumors in dogs, their efficacy is limited.[32,33] Chu et al[4] reported that 15% of primary carcinomas have *p53* mutations, whereas benign tumors do not have *p53* mutations. MDM2 protein overexperssion was also reported in some tumors that do not have *p53* mutations.[31] The purposes of the study reported here were to generate an adenoviral vector that expressed the canine *p53* gene and to investigate its growth inhibitory effect on canine osteosarcoma and mammary adenocarcinoma cell lines.

Materials and Methods

Cell culture conditions—Canine osteosarcoma cell lines (HOS and OOS)[34] and canine mammary adenocarcinoma cell lines (CIPm, CHMp, and CNMm) were established in our laboratory. HOS was established from a 7-year-old male mongrel dog with osteosarcoma of the scapula. OOS was established from a 10-year-old female Maltese with osteosarcoma of the mandible. CHMp was established from a 10-year-old female Shih Tzu with mammary adenocarcinoma (clinical stage IV of IV). CIPm was established from a 12-year-old female mongrel dog with mammary adenocarcinoma (clinical stage IV). CNMm was established from a 10-year-old female Maltese with mammary adenocarcinoma (clinical stage II). Passaged of all cells has been for > 3 years in our laboratory. During this period, the main morphologic characteristics of these cells did not change. All the canine cell lines were cultured in growth medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37°C in a humidified atmosphere of 5% CO_2. The 293 cells[a] were maintained in high-glucose Dulbecco modified Eagle's medium with 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 µg/mL). All cell lines were used within 15 passages from the beginning of this study, and when cells were passaged > 15 times, they were replaced with the new cell stock that was frozen at the beginning of this study.

Polymerase chain reaction analysis and single and strand conformation polymorphism analysis of the *p53* gene—To detect mutations of the *p53* gene in genomic DNA from cell lines, PCR-SSCP analysis was performed as described by Chu et al[4] with slight modifications. As controls, tissue specimens of spleen were obtained from 3 healthy dogs. Genomic DNA samples were extracted with silica-gel membrane.[b] Twelve primer pairs were used for PCR amplification **(Appendix)**, which cover the entire coding region except the first 19 nucleotides.[4] The genomic DNA (100 ng) were amplified by PCR in a volume of 50 µL by use of pairs of primers (15 pmol each) and 1.25 units of *Taq* DNA polymerase.[c] After denaturation at 94°C for 2 minutes, 35 cycles of the reaction (94°C for 1 minute [denaturation], 58°C for 1 minute [annealing], and 72°C for 1 minute [polymerization]) were performed, followed by a final extension procedure at 72°C for 7 minutes. The reaction products were mixed with the same volume of denaturing solution (95% [vol/vol] formamide, 0.05% xylene cyanol FF, 0.05% bromophenol blue), denatured at 95°C for 5 minutes, and directly thereafter placed on ice. The samples (5 µL/lane) were loaded onto 12.5% polyacrylamide gels.[d] Electrophoresis was performed at 15 W for 80 minutes, and the temperature was controlled by a Peltier-cooling system[e] at the optimally determined electrophoresis temperature for each primer pair. The gels were stained by use of a silver staining kit.[f] Products of PCR that had mobility shifts identified with PCR-SSCP analysis were cloned into a plasmid vector by use of the TA cloning method[g] and sequenced by the dideoxy chain termination method.[h] Seven clones of each PCR product were sequenced, and the nucleotide sequence of each clone was determined on both DNA strands in opposite directions.

Immunohistochemical analysis of endogenous P53 protein in canine cell lines—To detect the expression of the P53 protein in cell lines, immunohistochemical analysis was performed. Cells (HOS, OOS, CHMp, CIPm, and CNMm) were seeded in 8-chamber slides (1×10^4 cells/well) and allowed to adhere for 24 hours. Cells were washed 3 times in PBS solution with 0.9mM $CaCl_2$ and 0.5mM $MgCl_2$ and fixed in 4% (wt/vol) paraformaldehyde in PBS solution at 4°C for 30 minutes. After fixation, samples were washed twice with PBS solution and permeabilized with 0.2 (vol/vol) Triton X-100 in PBS solution. Samples were washed 3 times with PBS solution and incubated with a blocking solution (10% [vol/vol] horse serum in PBS solution) at room temperature (approx 25°C) for 1 hour, followed by incubation with a mouse antihuman P53 protein monoclonal antibody[35,i] at 4°C for 16 hours. Samples were washed 3 times with PBS solution, and immunodetection was performed by incubation with fluorescein isothiocyanate-conjugated horse antimouse IgG antibody[j] at room temperature for 1 hour. Samples were washed 3 times

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with PBS solution and were examined with a fluorescence microscope, using filters of 450 to 490 nm. Nonimmune mouse IgG[k] stained samples were used as a control.

Amplification of wild-type canine *p53* complementary DNA (cDNA)—Total RNA was extracted tissue specimens of spleen from Beagles by use of the acid guanidium-phenol-chlorofom method.[l] Single-strand **complementary DNA** (cDNA) was synthesized in a reaction primed with oligo(dT) by use of a commercially available kit.[m] For PCR amplification, primers were prepared on the basis of the canine *p53* gene sequence.[7] The sequences of the primers were 5′-ATGCAAGAGCCACAGACAGAG-3′ (forward) and 5′-AAGCAGGGGAAGTCAGTCTGAGTCGGG-3′ (reverse). The template cDNA was amplified by PCR in a volume of 50 µL, using the pair of primers (15 pmol each) and 2.5 units of *Pfu* DNA polymerase.[k] Amplification was performed by 40 cycles of denaturation (95°C for 30 seconds), annealing (60°C for 30 seconds) and polymerization (68°C for 3 minutes). To add 3' adenosines, 2.5 units of *Taq* DNA polymerase[n] were added to the reaction mixture of the PCR amplification and incubated at 72°C for 10 minutes. The product was cloned into a plasmid vector by use of the TA cloning method[e] (denoted as pCR2.1-cp53). The pCR2.1-cp53 was sequenced by the dideoxy chain termination method,[f] and the nucleotide sequence of each clone was determined on both DNA strands in opposite directions.

Construction and generation of the adenoviral vectors—An adenoviral vector that expressed wild-type canine *p53* was generated by use of a commercial adenovirus vector kit.[36,o] Briefly, the canine *p53* cDNA was extracted from pCR2.1-cp53 with *Eco*RI[p] restriction enzyme, blunt ends were formed with T4 DNA polymerase,[q] and it was inserted into an expression cassette cosmid vector, pAxCAwt, between the CAG promoter (ie, cytomegalovirus enhancer and chicken β-actin promoter) and the rabbit β-globin polyadenylation signal site.[36] The pAxCAwt is a cassette cosmid containing a nearly full-length adenovirus type 5 genome with E1 (nucleotides 454 to 3328) and E3 (nucleotides 28592 to 30470) deletions, a CAG promoter, and a rabbit β-globin polyadenylation signal site. The cosmid containing the canine *p53* cDNA inserted in sense orientation was selected by a restriction enzyme digestion pattern (double digestion with *Pac*I and *Sac*I[n]; denoted as pAxCA-cp53). The expression cosmid pAxCA-cp53 was cotransfected with the *Eco*T22I-digested DNA-terminal protein complex of Ad5-dlX[l] into the transformed human embryonic kidney cell line (293 cells), by use of the calcium phosphate method.[r] Ad5-dlX is a mutant of adenovirus type 5 that has an E3 deletion, and *Eco*T22I cuts the Ad5-dlX genome at 7 sites, all within the left third of the genome that contains E1. The recombinant adenovirus with E1 and E3 deletions and a canine *p53* gene expression unit were generated through homologous recombination occurring in 293 cells. After 24 hours of cultivation, the transfected cells were spread in three 96-well plates, using a 10-fold serial dilution mixed with untransfected 293 cells. After 10 to 15 days, viral clones were isolated and assessed by restriction analysis of the inserted genes. An adenoviral vector that expressed the canine *p53* gene was propagated in 293 cells, purified by sequential centrifugation in CsCl step gradients,[37] and dialyzed against PBS solution with 10% (vol/vol) glycerol. In this purification, > 99.9% of carryover proteins were removed.[37] Titers of virus stocks were determined by an end-point cytopathic effect assay.[37] AxCA-LacZ,[s] which expresses β-galactosidase of *Escherichia coli* under the same promoter, was used as a control vector. Titers of virus stocks used in this study were from 4 to 10×10^{10} plaque-forming units/mL.

Adenovirus infection—Cells were seeded in 8-chamber slides (1×10^4 cells/well), 6-well culture plates (HOS, 1×10^4 cells/well; OOS, 5×10^4 cells/well; CHMp, 1×10^4 cells/well; CIPm, 1×10^4 cells/well; CNMm, 5×10^4 cells/well), or 90-mm-diameter culture dishes (HOS, 1×10^5 cells/dish; OOS, 5×10^5 cells/dish; CHMp, 1×10^5 cells/dish; CIPm, 1×10^5 cells/dish; CNMm, 5×10^5 cells/dish) and allowed to adhere for 24 hours. To determine cell number in a well after 24 hours, cells in 3 spare wells of 6-well culture plates or in 3 spare 90-mm-diameter culture dishes were counted on a hemocytometer, and the mean number was calculated. Cell number in wells of 8-chamber slides was assumed to be 1×10^4 cells/well. Cells were infected by adding viral solutions, diluted in a culture medium to specific concentrations, to the cell monolayers (8-chamber slides, 50 µL/well; 6-well culture plates, 0.2 mL/well; 90-mm-diameter culture dishes, 1.0 mL). Cells were incubated at 37°C for 90 minutes, with a brief agitation every 15 minutes. After the infection, culture medium was added to wells (8-chamber slides, 0.5 mL/well; 6-well culture plates, 4 mL/well; 90-mm-diameter culture dishes, 10 mL), and the incubation continued at 37°C in a humidified atmosphere of 5% CO_2.

Reverse transcription and PCR analysis—Cells (HOS, OOS, CHMp, CIPm, and CNMm) were seeded in 90-mm-diameter culture dishes and infected with mock (PBS) solution, AxCA-LacZ, or AxCA-cp53 at the **multiplicity of infection (MOI)** of 100. After 24 hours of cultivation, total RNA was extracted[j] and treated with deoxyribonuclease I.[t] Single-strand cDNA was synthesized in a reaction primed with oligo(dT) by use of a commercially available kit[u] and amplified by PCR analysis. The primers used to amplify a cDNA fragment of the adenovirus-derived canine *p53* gene were **5′-AAAGAAGAAGCCACTAGATG-3′ (cp53-S)** and **5′-TGCACCTGAGGAGTGAATTG-3′ (Ad-R)**. The cp53-S primer can anneal endogenous canine *p53* mRNA, but the Ad-R primer is a specific primer for the expression cassette. Therefore, this pair of primers can detect transcription from the transfected adenovirus. As a control, the primers used to amplify the canine glyceraldehyde-3-phosphate dehydrogenase gene were 5′-TGCCGCCTGGAGAAAGCTGC-3′ (forward) and 5′-TCCCAGGAAATGAGCTTGAC-3′ (reverse). After denaturation at 95°C for 2 minutes, 30 cycles (95°C for 1 minute [denaturation] and 60°C for 1 minute [annealing and polymerization]) of the reaction were performed, followed by a final extension procedure at 72°C for 7 minutes. The PCR products were subjected to electrophoresis in a 4% agarose gel and observed by ethidium bromide staining.

Immunohistochemical analysis of P53 protein in adenovirus infected cell lines—Cells (HOS, OOS, CHMp, CIPm, and CNMm) were seeded in 8-chamber slides and infected with mock (PBS) solution, AxCA-LacZ, or AxCA-cp53 at the MOI of approximately 100. After 24 hours of cultivation, immunohistochemical analysis of P53 protein was performed, as described.

Canine cell line susceptibility to the adenoviral vector—The susceptibility of canine cell lines to the adenoviral vector was determined by use of the β-galactosidase assay. Cells (HOS, OOS, CHMp, CIPm, and CNMm) were seeded in 6-well culture plates and infected with mock (PBS) solution or AxCA-LacZ at the MOI of 1, 2, 5, 10, 20, 50, 100, 200, 500, and 1,000. Cells infected with AxCA-LacZ were assayed for β-galactosidase expression by use of the 3-indolyl-β-D-galactopyranoside staining method.[v] After 48 hours of infection, cells were washed once with PBS solution and fixed in 2% (vol/vol) formaldehyde and 0.2% (vol/vol) glutaraldehyde in PBS solution at room temperature for 15 minutes. Cells were washed 3 times with PBS solution and stained with PBS solution that contained 3-indolyl-β-D-galactopyranoside, potassium ferrocyanide, and potassium ferricyanide

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at 37°C for 3 hours. Blue-stained and nonstained cells in random viewing fields were counted, and percentages of stained cells in the total cell population (> 500 cells) were calculated. Three wells for each time point were examined.

Cell growth and viability assay—Cells (HOS, OOS, CHMp, CIPm, and CNMm) were seeded in 6-well culture plates and infected with mock (PBS) solution, AxCA-LacZ, or AxCA-cp53 at the MOI of 100 or 500. Both adherent and nonadherent cells were harvested each day (0 to 5 days after infection), stained with trypan blue, and numbers of live and dead cells were counted on a hemocytometer (total cell numbers counted were > 500). Three wells for each time point were counted.

Cell cycle analysis—To examine the cell cycle arrest imposed by AxCA-cp53 infection, DNA content per cell was measured by use of flow cytometry analysis.[38] Cells were seeded in 90-mm-diameter culture dishes and infected with mock (PBS) solution, AxCA-LacZ, or AxCA-cp53 at the MOI of 100 or 500. Both adherent and nonadherent cells were harvested using trypsin-EDTA each day, washed twice with PBS solution, and fixed in 70% (vol/vol) ethanol at 4°C for up to 5 days. Cells were washed 3 times with PBS solution, resuspended in PBS solution containing RNase A (250 U/mL), and incubated at room temperature for 20 minutes. Next, the same volume of PBS solution containing propidium iodide (100 µg/mL) was added. The DNA content was measured by flow cytometer,[w] and data from 20,000 cells were collected and analyzed by use of an analysis program.[u]

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay—To identify apoptotic cells, the **terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL)** assay was performed by use of a commercially available kit.[x] Cells were seeded in 90-mm-diameter culture dishes and infected with mock (PBS) solution, AxCA-LacZ, or AxCA-cp53 at the MOI of 100 or 500. Both adherent and nonadherent cells were harvested using trypsin-EDTA each day, washed twice with PBS solution, fixed in 2% (wt/vol) paraformaldehyde in PBS solution at room temperature for 30 minutes, and permeabilized with 0.2% (vol/vol) Triton-X 100 in 0.1% (wt/vol) sodium citrate at 4°C for 2 minutes. Cells were washed twice with PBS solution, and a labeling reaction was performed in the TUNEL reaction mixture (including terminal deoxynucleotidyl transferase and fluorescein-labeled dUTP) at 37°C for 1 hour in the dark. As negative controls, the labeling reaction was performed in the TUNEL reaction mixture without terminal deoxynucleotidyl transferase. Cells were washed twice in blocking buffer (0.1% [vol/vol] Triton-X 100 and 0.5% [wt/vol] bovine serum albumin in PBS solution), and flow cytometry analysis was performed.[u] Data from 10,000 cells were collected, and the percentages of labeled or unlabeled cells were calculated by use of an analysis program.[u]

Results

***p53* gene status in canine cell lines**—In HOS and CHMp cell lines, shifted bands were detected in exon 5 (primer pair D2; **Fig 1**). In the other cells, no shifted band was detected in all the primer pairs (data not shown). The PCR products that had mobility shifts identified by use of PCR-SSCP analysis were cloned and sequenced. The HOS cell line had 1 missense point mutation at codon 150 (TAT to AAT, Try^{150} to Asn). In the CHMp cell line, 1 allele had 1 missense point mutation at codon 149 (ATC to ATG, Ile^{149} to Met), and another allele had 1 missense point mutation at codon 167 (GAA to TAA, Glu^{167} to Stop). In the all cell lines, endogenous P53 protein was not detected by immunohistochemistry.

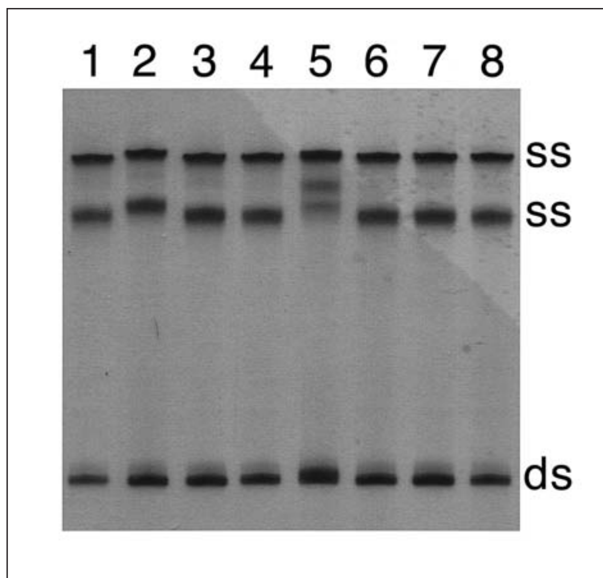

Figure 1—Results of gel electrophoresis following polymerase chain reaction (PCR) and single-strand conformation polymorphism (SSCP) analysis of the canine *p53* gene. Genomic DNA samples that were extracted from canine cell lines and spleen tissue specimens from clinically normal dogs (lane 1, spleen [dog 1]; lane 2, HOS; lane 3, OOS; lane 4, spleen [dog 2]; lane 5, CHMp; lane 6, CIPm; lane 7, spleen [dog 3]; lane 8, CNMm) were analyzed by PCR-SSCP analysis by use of primer pair D2. ss = Single strand DNA. ds = Double strand DNA.

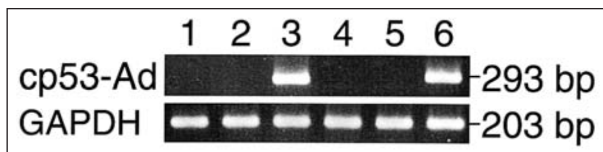

Figure 2—Ethidium bromide staining results of products obtained following reverse transcription and PCR analysis for adenovirus-mediated canine *p53* gene expression. The PCR analysis was performed by use of the primer pair of cp53-S and Ad-R. Lanes 1 to 3 contained CHMp cells; lanes 4 to 6 contained CNMm cells. Cells in lanes 1 and 4 were infected with mock (PBS) solution; cells in lanes 2 and 5 were infected with AxCA-LacZ (multiplicity of infection [MOI] of 100); cells in lanes 3 and 6 were infected with AxCA-cp53 (MOI of 100). cp53-Ad = AxCA-cp53 derived canine *p53* gene. GAPDH = Glyceraldehyde-3-phosphate dehydrogenase. bp = Base pairs.

Expression of the canine *p53* gene by an adenoviral vector—We constructed an adenoviral vector that contains wild-type canine *p53* under the control of a CAG promoter. Several groups[4,7,35] have reported nucleotide sequences that differ for the canine *p53* gene. The nucleotide sequence reported by Chu et al[4] had 99.8% similarity with pCR2.1-cp53 in the open reading frame, and 2 differences were found between their sequence and pCR2.1-cp53. Codon 6 of the canine *p53* gene in pCR2.1-cp53 was ACA (Thr^6), which differed from the corresponding codon TCA (Ser^6) reported by Chu et al,[4] but was the same sequence reported by Setoguchi et al.[7] Codon 143 of the canine *p53* gene in pCR2.1-cp53 was TGC, which differed from the corresponding codon TGT reported by Chu et al,[4] but the amino acid translated from this codon was identical (Cys^{143}). Because pCR2.1-cp53 originated from a clinically and genetically normal Beagle, we used this clone to represent the normal

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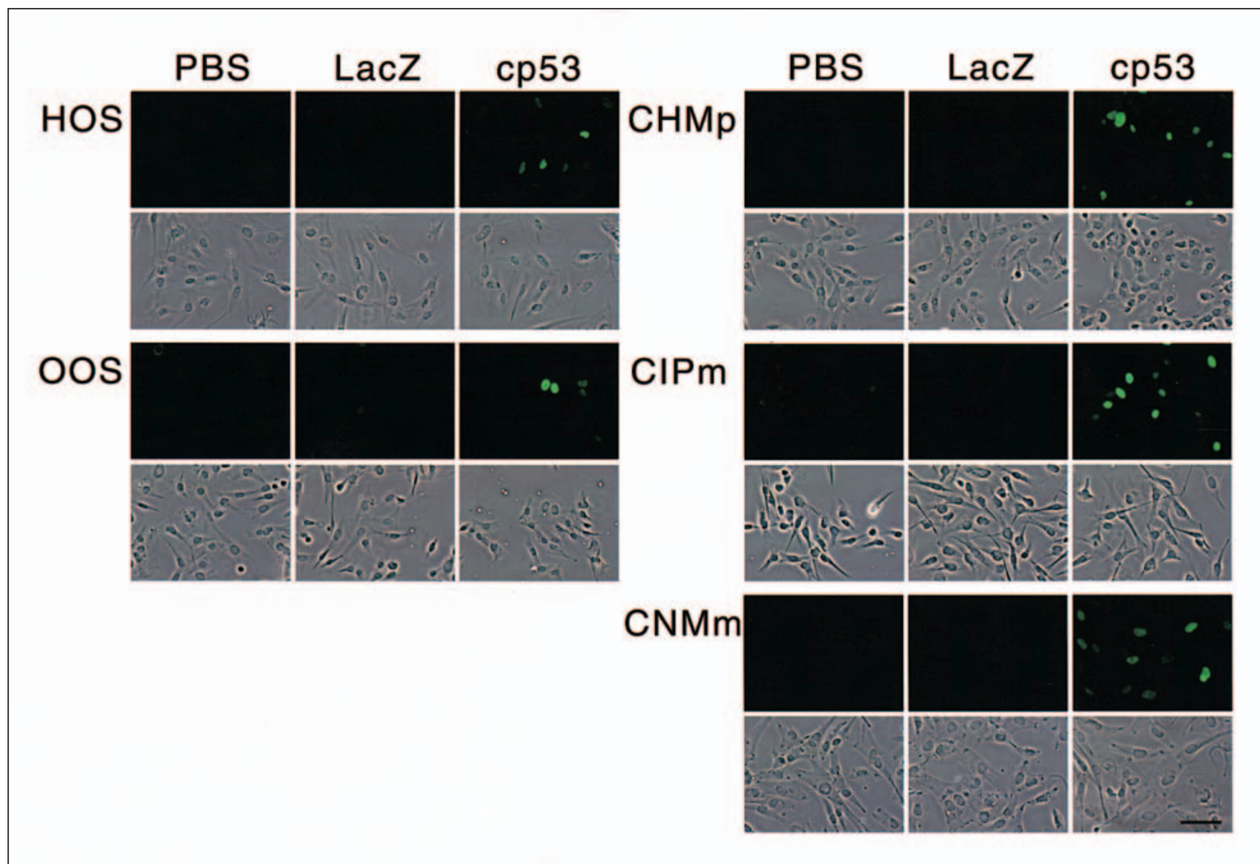

Figure 3—Immunohistochemical analysis results for P53 protein expression. Cells (HOS, OOS, CHMp, CIPm, and CNMm) were infected with mock (PBS) solution, AxCA-cp53 (cp53), or AxCA-LacZ (LacZ) at the MOI of 100. P53 protein expression was detected with fluorescein isothiocyanate-labeled antihuman P53 protein monoclonal antibody. Light field microscopy images of the same cells are shown in each lower panel. Bar = 100 µm.

canine *p53* gene. To examine the expression of the adenovirus-derived canine *p53* gene in canine cell lines, we performed RT-PCR analysis using cp53-S and Ad-R primers. Adenovirus-mediated canine *p53* gene mRNA was detected in cells infected with AxCA-cp53 but not in cells infected with mock (PBS) solution or AxCA-LacZ (**Fig 2**). Furthermore, P53 protein expression was also detected by immunohistochemistry. In cells infected with AxCA-cp53, the *p53* gene was overexpressed, compared with cells infected with mock (PBS) solution or AxCA-LacZ, and accumulation of P53 protein in the nuclei was observed (**Fig 3**).

Canine cell line susceptibility to the adenoviral vector—To estimate the susceptibility of canine cell lines to the adenoviral vector, cell lines were infected with AxCA-LacZ. Susceptibility was determined by measuring the percentage of blue-stained cells by use of 3-indolyl-β-D-galactopyranoside staining at 48 hours after infection. With an increase in the MOI, the percentage of blue cells increased (**Fig 4**). In HOS, OOS, CHMp, CIPm, and CNMm cell lines, the MOI at which 50% of cells had positive results was approximately 20, 20, 50, 10, and 10, respectively.

Effects of adenovirus-mediated canine *p53* gene on cell proliferation—Proliferation analysis was performed to examine the effect of the adenovirus-mediated canine *p53* gene on cell growth in vitro. The adenovirus-mediated canine *p53* gene had growth inhibitory effects on all cell lines (**Fig 5**).

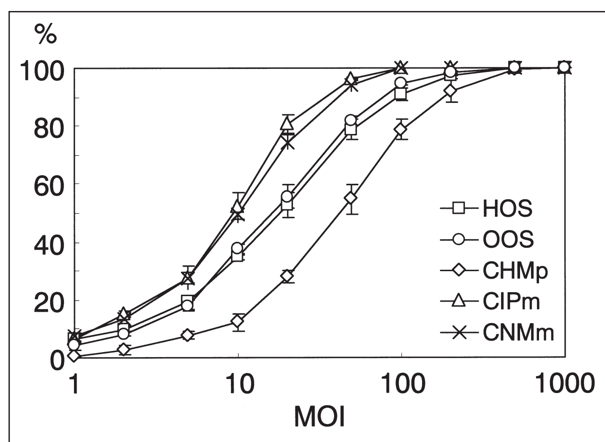

Figure 4—Mean (± SD, n = 3) percent susceptibility of canine cell lines to the adenoviral vector. Cells (HOS, OOS, CHMp, CIPm, and CNMm) were infected with AxCA-LacZ at the various MOI (1 to 1,000). After 2 days of incubation, cells were subjected to 3-indolyl-β-D-galactopyranoside staining. Mean percentages of stained cells in the total cell population were calculated.

In CIPm and CNMm cells infected with AxCA-cp53 at the MOI of 500, cell numbers decreased rapidly (Fig 5), and the viability of these cells was

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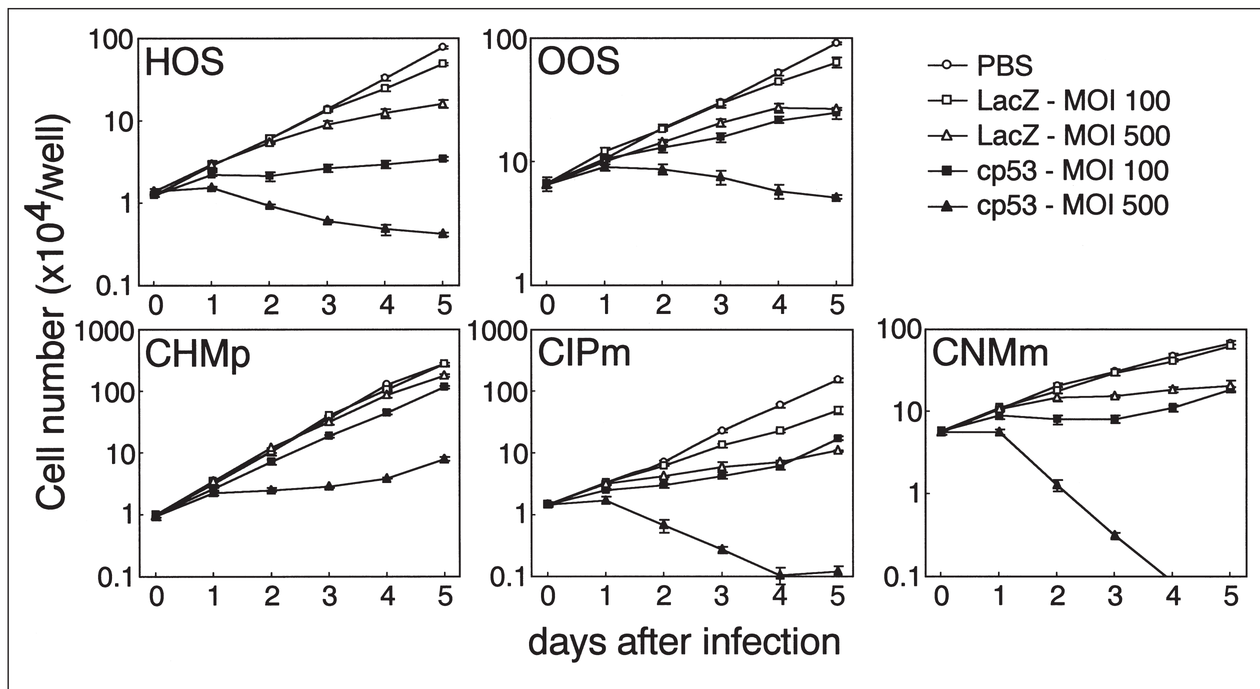

Figure 5—Mean (± SD, n = 3) proliferation assay results of the number of live cells following infection with adenoviral vectors. Cells (HOS, OOS, CHMp, CIPm, and CNMm) were infected with mock (PBS) solution, AxCA-LacZ (LacZ), or AxCA-cp53 (cp53) at the MOI of 100 or 500. Cells were harvested each day (0 to 5 days after infection), and the numbers of live cells were counted.

Table 1—Results of the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay

	Day 1 (%)			Day 2 (%)			Day 3 (%)		
Canine cell lines*	**PBS**	**LacZ**	**cp53**	**PBS**	**LacZ**	**cp53**	**PBS**	**LacZ**	**cp53**
HOS	–	–	–	–	–	–	–	–	–
Unlabeled (–)	99.5	99.7	98.6	99.6	99.4	61.7	99.6	97.1	33.9
Labeled (+)	0.50	0.37	1.43	0.37	0.66	38.5	0.41	2.99	66.2
OOS	–	–	–	–	–	–	–	–	–
Unlabeled (–)	99.9	99.8	99.4	99.7	99.7	98.2	99.8	99.6	88.1
Labeled (+)	0.15	0.16	0.62	0.29	0.27	1.87	0.17	0.45	12.0

*Cells were infected with mock (PBS) solution, AxCA-LacZ (LacZ), or AxCA-p53 (cp53) at the multiplicity of infection of 100.

decreased to < 5% (mean ± SEM, 0.7 ± 0.2 and 4.8 ± 0.4%, respectively) at 5 days after infection. In the HOS and OOS cells infected with AxCA-cp53 at the MOI of 500, cell numbers decreased slowly, and the viability of these cells was decreased to approximately 50% (mean ± SEM, 42.4 ± 0.6 and 48.2 ± 0.6%, respectively) at 5 days after infection. In the HOS, OOS, CIPm, and CNMm cells infected with AxCA-cp53 at the MOI of 100 and CHMp cells infected at the MOI of 500, cell numbers increased, but the growth rates were at least 75% slower than in cells infected with mock (PBS) solution. Adenovirus-mediated β-galactosidase also had a growth inhibitory effect, but its effect was weaker than that of AxCA-cp53 at the same MOI.

Cell cycle arrest by AxCA-cp53—Adenovirus-mediated P53 protein overexpression resulted in a decrease in the S-phase population, an increase in the G_1-phase population, and an increase in the **sub-diploid (subG_1)** phase population. For example, in the CIPm cells infected with mock (PBS) solution or AxCA-LacZ at 1 day after infection, S-phase, G_2/M-phase, and G_1-phase populations were approximately 20, 24, and 50%, respectively, whereas in cells infected with AxCA-cp53, S-phase, G_2/M-phase, and G_1-phase populations were approximately 3, 12, and 70%, respectively. Furthermore, subG_1-phase populations (dead cells) in cells infected with AxCA-cp53 increased from 15% at 1 day after infection to 93% at 3 days after infection, whereas the subG_1-phase populations in cells infected with mock (PBS) solution or AxCA-LacZ was < 10% on all 3 days. Similar results were also obtained at the MOI of 500 and in CNMm, HOS, OOS, and CHMp cells (data not shown).

Induction of apoptosis by AxCA-cp53—AxCA-cp53 induced DNA fragmentation, which is characteristic of apoptosis (**Table 1**). For example, positive TUNEL assay results for CIPm cells infected with mock (PBS) solution or AxCA-LacZ at the MOI of 100 were < 3%, whereas positive TUNEL assay results for CIPm cells infected with AxCA-cp53 at the MOI of 100 increased up to 66% at 3 days after infection. Induction of apoptosis was also detected in HOS, OOS, and CHMp cells (data not shown).

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Discussion

In our study, an adenoviral vector that expressed the canine-*p53* gene, AxCA-cp53, was constructed. AxCA-cp53-mediated P53 protein was accumulated in the nuclei and had a growth inhibitory effect on 2 canine osteosarcoma cell lines (HOS, OOS) and 3 canine mammary adenocarcinoma cell lines (CHMp, CIPm, CNMm) in a dose-dependent manner. These results indicate that AxCA-cp53 can induce the overexpression of P53 protein and inhibit the growth of canine tumor cell lines that lack a functional *p53* gene.

In CIPm and CNMm cells infected with AxCA-cp53 at the MOI of 500, almost all cells died, whereas in HOS and OOS cells infected with AxCA-cp53 at the same MOI, cell numbers decreased. Furthermore, in CHMp cells infected with AxCA-cp53, even at the MOI of 500, cell numbers increased. These variations in inhibitory effects of AxCA-cp53 may depend on the susceptibility of canine cell lines to the adenoviral vector. For example, when cells were infected with AxCA-LacZ at the MOI of 50, approximately 95% of the CIPm and CNMm cells had positive results for β-galactosidase activity; on the other hand, only half of the CHMp cells had positive results for β-galactosidase activity. These findings may be the result of the expression level of adenovirus receptors on the cell surface. The entry of human adenovirus type 5 used in our study into a cell is functionally divided into attachment and internalization. In the former, the fiber knob of the virus attaches to the coxsackievirus and adenovirus receptor.[39,40] Following attachment, the penton base of the virus interacts with integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$, and the virus enters a cell via a clathrin-coated pit.[41]

In our study, we could not find any difference in the effects of AxCA-cp53 between cells with *p53* mutation (HOS, CHMp) and cells without *p53* mutation (OOS, CIPm, CNMm) when compared at the same degree of transfection efficiency. For example, when the OOS and CHMp cells were infected with AxCA-cp53 at the MOI of 100 and 500, respectively, cell numbers slowly increased similarly. It is conceivable that wild-type *p53* was inactivated by degradation of translated P53 protein in these tumor cells without the *p53* mutation. Therefore, AxCA-cp53 could inhibit proliferation of these tumor cell lines. Many regulators of the P53 protein, which modify protein stability and activity, have been found; the MDM2 protein is a regulator of P53.[9] MDM2 binds to P53 protein and inhibits the transcriptional activity by concealing the activation domain.[42,43] The P53 protein is made ubiquitous by MDM2 and then targeted for degradation by the proteasome.[44,45] Furthermore, other protein kinases, phosphatases, acetyltransferases, and deacetylases affect the P53 protein and regulate its activity and stability.[46]

AxCA-cp53 had growth inhibitory effects on canine cell lines in our study. Adenovirus-mediated P53 protein overexpression resulted in a decrease in the S-phase population and increases in the G_1-phase and subG_1-phase populations. Apoptotic cells were detected by use of the TUNEL assay (Table 1). These findings indicate that the growth inhibitory effect of AxCA-cp53 is the result of induction of cell cycle arrest and apoptosis. In contrast in another study,[47] a bystander effect was thought to have an important role for in vivo application.

By use of immunohistochemistry, we could not detect endogenous P53 protein in cells with *p53* mutation (HOS, CHMp). Although overexpression of P53 protein has been positively associated with *p53* mutation in human tumors, not all tumors with *p53* mutation have P53 protein accumulation to a degree that is detectable by immunohistochemistry.[48,49] If a "Stop" mutation occurs and the product lacks the epitope recognized by anti-P53 protein antibody, there is no signal. Such a mutation was observed in CHMp cells at codon 167 (GAA to TAA, Glu^{167} to Stop), because the antibody (clone PAb 421[g]) used in our study recognizes amino acid residues 371 to 380 of the human P53 protein,[50] which corresponds to amino acid residues 359 to 378 of the canine P53 protein. Furthermore, there is a possibility that the point mutation detected in our study cannot stabilize the protein sufficiently to reach an amount that is detectable by use of immunohistochemistry.[48,49] Another possibility is that the threshold for detection by use of immunohistochemistry was too low to detect endogenous P53 protein in our study.

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[a]JCRB9068, Health Science Research Resources Bank, Osaka, Japan.
[b]DNeasy tissue kit, Qiagen, Hilden, Germany.
[c]AmpliTaq gold DNA polymerase, Applied Biosystems, Foster City, Calif.
[d]GeneGel excel 12.5/24 kit, Amersham Pharmacia, Buckinghamshire, England.
[e]GenePhor DNA separation system, Amersham Pharmacia, Buckinghamshire, England.
[f]PlusOne DNA silver staining kit, Amersham Pharmacia, Buckinghamshire, England.
[g]pCR2.1, Invitrogen, Carlsbad, Calif.
[h]BigDye primer cycle sequencing kit, Applied Biosystems, Foster City, Calif.
[i]Pab421, Calbiochem, Darmstadt, Germany.
[j]Vector Laboratories, Burlingame, Calif.
[k]Sigma Chemical Co, St Louis, Mo.
[l]RNA zol, Biotecx, Houston, Tex.
[m]ProSTAR ultra HF RT-PCR system and *PfuTurbo* DNA polymerase, Stratagene, La Jolla, Calif.
[n]AmpliTaq DNA polymerase, Applied Biosystems, Foster City, Calif.
[o]Adenovirus expression vector kit, Takara, Kyoto, Japan.
[p]New England BioLabs, Beverly, Mass.
[q]DNA blunting kit, Takara, Kyoto, Japan.
[r]CellPhect transfection kit, Amersham Pharmacia, Buckinghamshire, England.
[s]Riken gene bank, Tsukuba, Japan.
[t]Invitrogen, Carlsbad, Calif.
[u]GeneAmp RNA PCR kit, Applied Biosystems, Foster City, Calif.
[v]β-Gal staining set, Roche Diagnostics, Mannheim, Germany.
[w]FACScan flow cytometer and cell quest, Becton-Dickinson, Franklin Lakes, NJ.
[x]*in situ* Cell death detection kit, Roche Diagnostics, Mannheim, Germany.

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Appendix

Primers used for polymerase chain reaction and single-strand conformation polymorphism analysis of canine *p53* genomic DNA

Primer pair	Primer name	Primer sequence	Exon scanned	Electrophoresis temperature (°C)
A	2(S)	5′-ATGCAAGAGCCACAGTCAG-3′	Exon 2	10
	2(AS)	5′-GAGCCTGGCCTGCCCTC-3′	Exon 2	10
B	3(S)	5′-GCACTGACTTTCTGCTCTC-3′	Exon 3	10
	3(AS)	5′-GACCTCCCCACACCCAGT-3′	Exon 3	10
C1	4(S1)	5′-CTTGACTCTGGTCTCGCC-3′	Exon 4	10
	4(AS1)	5′-GGGTAGGTCTTCGGGGAA-3′	Exon 4	10
C2	4(S2)	5′-CCCTATCATCCTCTGTCC-3′	Exon 4	15
	4(AS2)	5′-GCCAGCCCCATGGAAACC-3′	Exon 4	15
D1	5(S1)	5′-GACCTGTCCATCTGTCCT-3′	Exon 5	15
	5(AS1)	5′-ATAGATGGCCATAGCGCGG-3′	Exon 5	15
D2	5(S2)	5′-ACCCCCACCCAATACCTG-3′	Exon 5	20
	5(AS2)	5′-GCCTTGTCCCATCTGTAG-3′	Exon 5	20
E	6(S)	5′-TGATTCCTCCCCGATGGC-3′	Exon 6	20
	6(AS)	5′-AGACCCCTCAGATGCCAA-3′	Exon 6	20
F	7(S)	5′-ACCCTGGGCCTACCTTCTA-3′	Exon 7	15
	7(AS)	5′-AGGGTGGCAGGCAGGTC-3′	Exon 7	15
G	8(S)	5′-GCTTCTCTCTTCTCACCTG-3′	Exon 8	15
	8(AS)	5′-CTCCTTCACCTCCTCTTGT-3′	Exon 8	15
H	9(S)	5′-GCTCAAAACATACTCTTCTCT-3′	Exon 9	10
	9(AS)	5′-TGCCTTATCTGTTCCTCCC-3′	Exon 9	10
I	10(S)	5′-AATGGTACTGTGGCTTCC-3′	Exon 10	10
	10(AS)	5′-CAAGCCGGCCCAGGTCA-3′	Exon 10	10
J	11(S)	5′-CTCCCACTTGCTAATATCGT-3′	Exon 11	10
	11(AS)	5′-TGAGGGTGTGCGTGTTGG-3′	Exon 11	10

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