

Original Research Report

## The nude rat as an orthotopic model for cervical cancer

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### Abstract

**Objective.** The purposes of this study were to establish intracervical tumors of the nude rat as an orthotopic experimental model for human cervical cancer and to preliminary evaluate the effects of the adenoviral vector, Ad5CMV-*p53*, on orthotopic cervical tumor size.

**Methods.** Human cervical cancer SiHa and ME-180 cells were injected into the cervix of the nude rat. Four days later,  $1 \times 10^9$  plaque forming units (PFU) of Ad5CMV-*p53* were injected into the cervix. The rats were later sacrificed to determine cervical tumor size.

**Results.** Eight of ten nude rats developed SiHa cell tumors; all ten nude rats developed ME-180 cell tumors. Four of ten SiHa cell tumors metastasized to the pelvic cavity; no ME-180 cell tumors did. The growth of Ad5CMV-*p53*-infected cells was greatly suppressed. The Ad5CMV-*p53* treatment significantly reduced both cell tumor volumes in nude rat cervixes.

**Conclusion.** The nude rat cervix grows tumors similar to human cervical cancer tumors and makes an excellent experimental model. Transfection of cervical cancer cells with the wild-type *p53* gene via Ad5CMV-*p53* is a potential therapeutic approach to cervical cancer. © 2005 Elsevier Inc. All rights reserved.

**Keywords:** Gene therapy; Cervical cancer; Orthotopic model; Nude rat; Animal models of cancer; ME-180

### Introduction

Cervical cancer is the third most common malignancy in women worldwide and a major cause of mortality and morbidity in women in the developing world [1]. Despite progress in early detection and treatment, survival rates have not markedly improved in the last 10–20 years. Neoadjuvant chemotherapy following surgery or radiation therapy has been developed for locally advanced cervical cancer; however, further progress in controlling local and distant metastatic disease is needed. To gain further insight

into and to develop new therapeutics for human cervical cancer and lymph-node metastasis in particular, an appropriate *in vivo* model is essential.

Early experimental models involving implanting human cervical cancer cells subcutaneously in athymic nude mice failed to yield metastatic disease. Orthotopic transplant models of human prostate cancer [2], pancreatic cancer [3], bladder cancer [4], lung cancer [5], colon cancer [6], breast cancer [7], and renal cell cancer [8] have demonstrated local tumor progression and distant metastases. These results provided some useful information to recognize the biological behavior of each cancer. Among gynecologic malignancies, orthotopic animal models of endometrial cancer [9] and ovarian cancer [10] have been reported, but that of cervical cancer remained to be established.

Clinical trials of cancer gene therapy, primarily using adenoviral vectors, have been developed for non-small cell lung cancer, head, and neck cancer, hepatocellular carcinoma, prostate cancer, ovarian cancer, and malignant

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glioma [11]. In most clinical trials, vectors are locally injected to achieve the high level of gene expression in targeted cancer tissues. Subcutaneous tumor models in nude mice are commonly used to evaluate the *in vivo* antiproliferative effect of each vector. Our group has reported the use of the adenoviral vector in nude mice and in the rhesus monkey [12].

The modality of tumor progression in the subcutaneous tumor model is different from that in the orthotopic tumor model. The subcutaneous tumor model causes central tumor necrosis because of its low blood supply in the subcutaneous tissue, large tumor size because of its low potency of metastasis, and solitary tumor growth without invasion because of the lack of surrounding visceral organs. Cervical cancer invades the parametrial tissue first, regional lymph nodes second, and finally, distant organ sites. Invasion of cervical cancer to the surrounding visceral organs induces lethal complications, namely, renal failure by hydronephrosis, local bleeding, bladder metastasis, and rectal metastasis. The orthotopic cervical cancer tumor model follows the same progression; therefore, it might be considered adequate to evaluate new treatments.

We previously reported that adenovirus-*p53* inhibits the growth of human cervical cancer subcutaneous tumors in nude mice by the induction of apoptosis [13]. For simulation of clinical trials of gene therapy for cervical cancer, we established for the first the orthotopic cervical cancer model in the athymic nude rat cervix. Human cervical cancer cells ME-180 and SiHa showed different tumor growth modalities. Here, we report the development of the orthotopic model of human cervical cancer and the effect of adenovirus-*p53* on the growth of orthotopic cervical cancer cell tumors in the nude rat cervix.

## Materials and methods

### Experimental design

The overall design for these experiments is shown in Fig. 1.

### Cell line and recombinant adenovirus

Human cervical cancer lines SiHa and ME-180 were obtained from the American Type Culture Collection (Rockville, MD). SiHa and ME-180 were infected with HPV 16 and HPV 68, respectively, and have wild-type *p53*. Cells were grown in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum. The recombinant *p53* adenoviral vector ad5CMV-*p53* contains the cytomegalovirus promoter and wild-type *p53* cDNA inserted into the *E1*-deleted region of modified adenovirus Ad5 [14]. This study used an adenoviral vector without the *p53* cDNA (ad5CMV-*poly A*) as a control. The replication defective *E1*-deleted Ad5CMV-*LacZ* virus

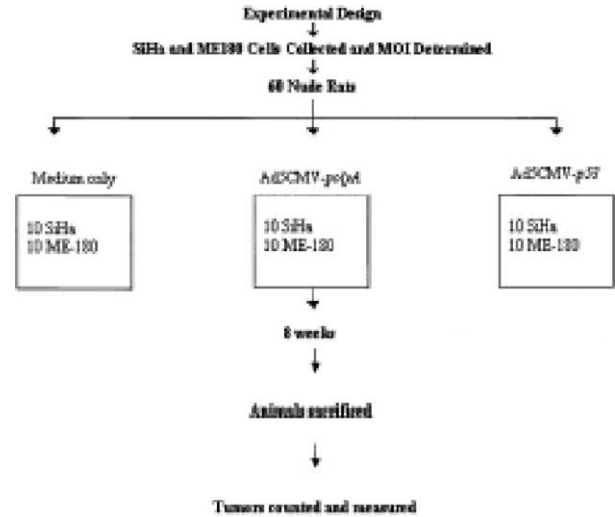


Fig. 1. Overall experimental design. Twenty nude rats were treated with RPMI medium only, 20 with Ad5CMV-*poly A*, and 20 with Ad5CMV-*p53*. Within each group, ten rats were infected with SiHa cells, and ten with ME-180 cells. After 8 weeks, all the rats were sacrificed and their tumors were counted and measured.

was used to determine transduction efficiencies. The 50% transduction efficiency was assessed by scoring 500 X-gal-positive cells in each of three replicate dishes and then determining the 50% of  $\beta$ -gal-positive blue cells. Viral stocks were propagated in 293 cells. All viruses were purified with double cesium chloride gradients using standard methods and titered with plaque assay [15].

### Cell count assay

Cells were plated at a density of  $5 \times 10^4$  cells/well in 12-well plates. Cells were infected with either Ad5CMV-*p53* or the Ad5CMV-*poly A* viral control. Culture medium alone was used as the mock infection control. Each sample was analyzed in triplicate. After the sixth day of culture, cells were harvested and counted to determine the 50% growth inhibitory concentration (IC50). Cell viability was determined by trypan blue exclusion. The cells were inoculated at densities of  $5 \times 10^4$  cells/well in each 12-well plate 24 h before infection.

### Western blot analysis

Total cell lysates were prepared by lysing cell monolayers in plates with sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer after rinsing the cells with phosphate buffered saline. Each lane was loaded with 5  $\mu$ g of cell lysate protein as determined by BCA protein assay (Pierce, Rockford, IL). After electrophoresis at 20 mA for 2 h, the proteins in the gels were transferred to Hybond-ECL membrane (Amersham Corp., Arlington Heights, IL). The membranes were then blocked with 1% dry milk and 0.1% Tween 20 (Sigma Chemical Co., St. Louis, MO) in phosphate buffered saline and

probed with two primary antibodies, mouse anti-human *p53* monoclonal antibody D07 (DAKO) and mouse anti-human actin monoclonal antibody (Amersham). The membranes were developed according to the Amersham ECL protocol.

#### Immunohistochemical analysis

The Ad5CMV-*p53*-injected SiHa cell tumors were analyzed to detect *p53* protein expression. Tissue samples were fixed with 10% formalin for 24 h, and tissue sections were de-paraffinized. Tissue sections of SiHa cell tumors were treated with 3% H<sub>2</sub>O<sub>2</sub> in methanol for 5 min. Immunohistochemical staining was performed with the Vectastain Elite kit (Vector Laboratories Inc., Burlingame, CA). The primary antibody was a mouse anti-human *p53* monoclonal antibody DO7 (DAKO Co., Carpinteria, CA), and the secondary antibody was a biotinylated goat anti-mouse immunoglobulin G (IgG) (Vector Labs). Avidin and biotinylated horseradish peroxidase macromolecular complex reagent (Vector Labs) was used to detect the antigen–antibody complex. The cells were stained with diaminobenzidine and then counterstained with Harris hematoxylin (Sigma Chemical Co., St. Louis, MO).

#### Inhibition of orthotopic cervical tumor growth in nude rats

To simulate the clinical trial of gene therapy for cervical cancer, an orthotopic cervical cancer model was established in the nude rats (Harlan, Houston, TX). The University of Texas M. D. Anderson Cancer Center guidelines for the care and use of the animals were followed. Ten million SiHa and ME-180 cells in 50  $\mu$ l of RPMI medium were injected through an insulin syringe with a 27 1/2 gauge needle through the anterior vaginal fornix into the space among the anterior vaginal fornix, the lower limit of the bladder wall, and the anterior wall of the cervix. The vaginal portion of cervix is too hard and small for proper cell injection. Injection of cells through the posterior fornix into the cervix is often used to induce intraperitoneal injection through the pouch of Douglas. For these reasons, we injected cells through the anterior vaginal fornix into the space among the anterior vaginal fornix, the lower limit of the bladder wall, and the anterior wall of the cervix. This resulted in good tumor growth. The 50  $\mu$ l of Ad5CMV-*p53* ( $1 \times 10^9$  plaque forming unit, PFU), Ad5CMV-*poly A* ( $1 \times 10^9$  PFU), or culture medium only was injected into the anterior cervix, which is the same portion that was previously injected with cervical cancer cells on days four, five, and six. Ten rats were used in each group. Eight weeks after the injection of cells, all rats were sacrificed and the tumor size was measured. Tumor volume was calculated assuming a spherical shape, with the average tumor diameter being the square root of the product of cross-sectional diameters.

## Results

#### Adenoviral infection of cervical cancer cells

To determine the adenoviral transduction efficiency of SiHa and ME-180 cell line, cells were infected for 2 days with Ad5CMV-*LacZ*, an adenovirus that expresses the  $\beta$ -gal gene. There appeared to be a linear relationship between the number of infected cells and the number of adenovirus particles used in the infection. Both cell lines inoculated with a single dose of Ad5CMV-*LacZ* at 10 multiplicity of infection (MOI) or greater exhibited 100% blue cells (Fig. 2). The 50% transduction efficiencies of this vector were 0.3 and 1.1 MOI in SiHa and ME-180 cells, respectively, and both cells showed high transduction efficiencies.

#### Expression of exogenous *p53* in cervical cancer cells

To determine the expression of the *p53* protein in the Ad5CMV-*p53*-infected cervical cancer cells, Western blotting and immunohistochemical analysis were done using the mouse anti-human *p53* monoclonal antibody DO7. Western blot analysis was performed to compare the amount of *p53* protein produced following the infection with Ad5CMV-*p53* (Fig. 3A). Multiple dishes of SiHa cells were infected with Ad5CMV-*p53* at 2 MOI. Following 12 h of incubation, the medium which contained virus was replaced with fresh medium, and each dish was cultured. The *p53* protein expression peaked 3 days after infection and began to decline thereafter. However, the intensity of the *p53* band at day 15 remained significantly higher than that in non-infected cells. Immunohistochemical analysis of SiHa cell tumors injected with Ad5CMV-*p53* revealed characteristic staining of *p53* protein in the nucleus 6 h after injection,

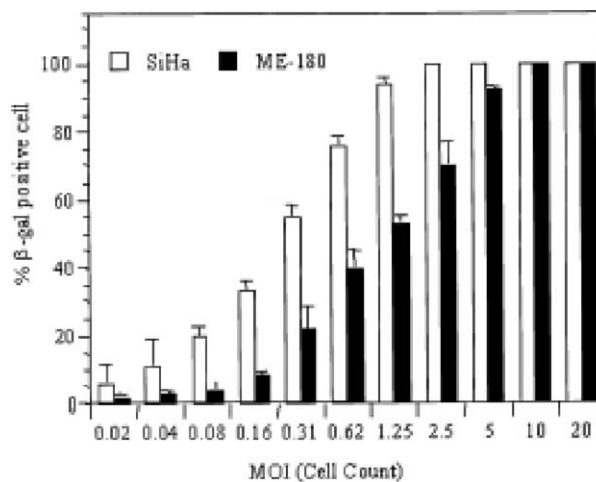


Fig. 2. Transduction efficiencies of human cervical cancer SiHa and ME-180 cells. A recombinant  $\beta$ -gal adenovirus, Ad5CMV-*LacZ*, was used to infect the cells at different MOIs ranging from 0.02 to 20. The percentages of  $\beta$ -gal positive cells were determined after scoring of 500 cells each on triplicate dishes.

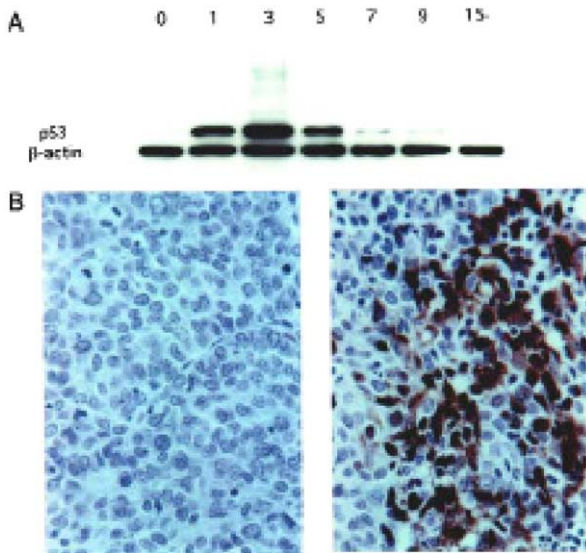


Fig. 3. (A) Time course of *p53* expression in SiHa cells. Multiple dishes of SiHa cells were infected with Ad5CMV-*p53* at 2 MOI. Cell lysates were prepared at indicated time points after infection and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blots were probed with anti-*p53* and anti-actin antibodies. (B) Immunohistochemical staining of Ad5CMV-*poly A*-injected SiHa cell tumor (left) and Ad5CMV-*p53*-injected SiHa tumor (right) 6 h after injection. Magnification,  $\times 100$ .

whereas AdSCMV-*poly A*-injected tumor cells failed to show *p53* staining (Fig. 3B).

#### Effect of exogenous *p53* on cervical cancer cell growth

The growth of the Ad5CMV-*p53*-infected SiHa and ME-180 cells was greatly suppressed when estimated by cell count assay 6 days after infection (Fig. 4). The cells were treated with Ad5CMV-*p53*, Ad5CMV-*poly A*, or medium only. The IC<sub>50</sub> for SiHa and ME-180 is 4 and 15 MOI, respectively. Western blot and immunohistochemical analyses demonstrated that production of the *p53* protein was significantly increased in Ad5CMV-*p53*-infected cells when compared with control cells, suggesting that the exogenous *p53* mRNA may be efficiently translated. Human cervical cancer cells have a functional genotype equivalent to that of cells in which the *p53* gene product is inactivated by the complex of the E6 protein with the *p53* protein. The adenoviral vector is capable of mediating high levels of *p53* expression, which are apparently sufficient to overcome the capabilities of the endogenous E6 to inactivate the *p53* protein.

#### Inhibition of orthotopic tumor growth in nude rat

To address the feasibility of *p53* gene therapy for orthotopic tumors, the efficacy of Ad5CMV-*p53* in inhibiting tumor growth was evaluated in an orthotopic cervical cancer nude rat model using human cervical cancer SiHa and ME-180 cells. Eight of the ten rats treated with medium only developed SiHa cell tumors in

the cervix, and four of the same ten rats developed metastatic lesions in the pelvic cavity as shown in Figs. 5A and E. All ten rats injected with ME-180 cells and treated with medium only developed ME-180 cell tumors in their cervixes; however, none developed metastatic lesions of the pelvic cavity as shown in Fig. 5B. Central necrosis, which frequently occurs in subcutaneous tumor models, is not generated in any of SiHa and ME-180 tumors as shown in Fig. 5C. Treatment with Ad5CMV-*p53* significantly reduced sizes of SiHa cell tumors compared with those treated with medium only and Ad5CMV-*poly A* ( $P < 0.001$ , unpaired *t* test) (Figs. 5F and 6A). SiHa cell tumors appeared in five of ten animals after treatment with Ad5CMV-*p53*. Ad5CMV-*p53* decreased metastases of SiHa cell tumors from four out of ten rats to one out of ten rats. The SiHa tumor sizes treated with medium only were not significantly different from those with Ad5CMV-*poly A*. On the other

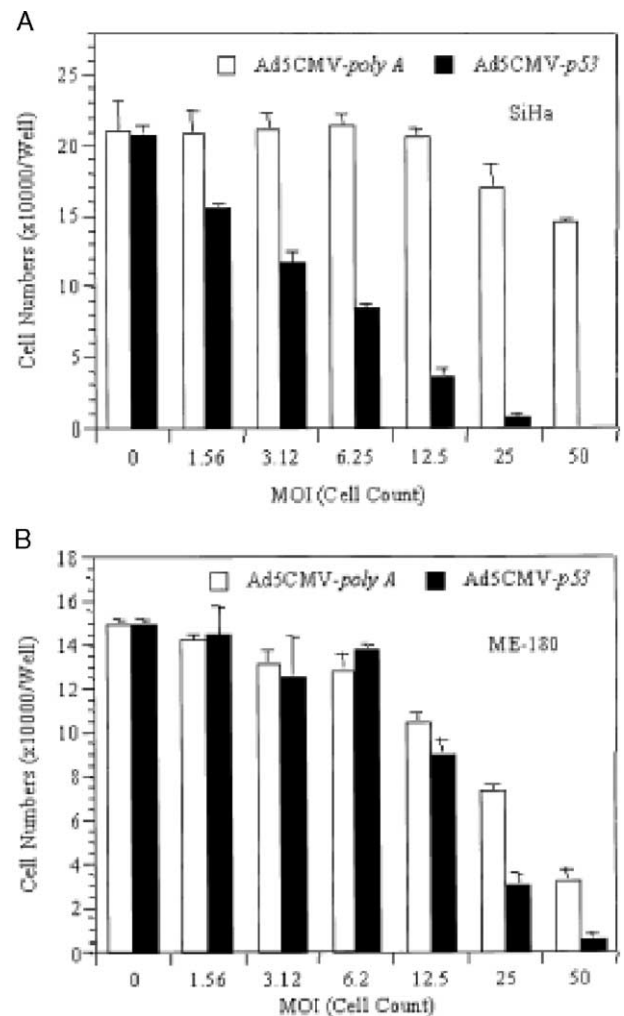


Fig. 4. (A) Inhibition of the growth of SiHa cells as determined by a cell count assay. The mean cell counts for triplicate wells were plotted. Bars indicate standard deviation. (B) Inhibition of the growth of ME-180 cells as determined by a cell count assay. The mean cell counts for triplicate wells were plotted. Bars indicate standard deviation.



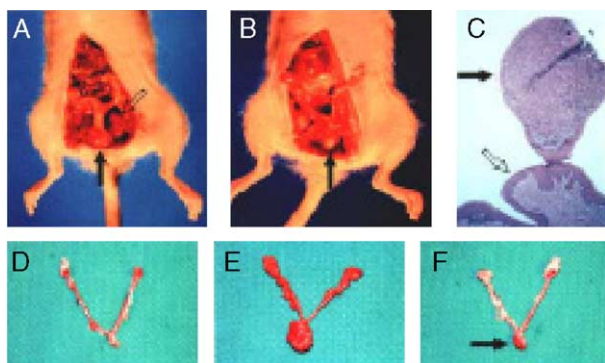


Fig. 5. Orthotopic cervical cancer model in the nude rat. (A) SiHa cell tumor in the rat cervix. The rat was sacrificed eight weeks after the injection into the anterior cervix with  $1 \times 10^7$  SiHa cells. Closed ( $\blacktriuparrow$ ) and open ( $\hat{\triuparrow}$ ) arrowheads indicate intracervical tumor and pelvic metastasis, respectively. (B) ME-180 cell tumor in the rat cervix. The rat was sacrificed 8 weeks after the injection into the anterior cervix with  $1 \times 10^7$  ME-180 cells. Closed arrowhead indicates intracervical tumor. (C) Hematoxylin–eosin staining section of SiHa cell tumor in the rat cervix shows no central necrosis. Closed and open arrowheads indicate SiHa cell tumor and normal rat cervix, respectively. Magnification,  $\times 40$ . (D) Normal rat cervix and uterine body. (E) Medium-injected SiHa cell tumor in the rat cervix. (F) Ad5CMV-*p53*-injected SiHa cell tumor. The rat was injected into the anterior cervix with 50  $\mu$ l of Ad5CMV-*p53* ( $1 \times 10^9$  PFU) 4, 5, and 6 days after the injection of SiHa cells. Eight weeks after the injection of cells, the rat was sacrificed. Ad5CMV-*p53* reduced tumor growth in the rat cervix (closed arrowhead) compared with those injected with medium or Ad5CMV-*poly A*.

hand, ME-180 tumors appeared in eight of ten animals after treatment with Ad5CMV-*p53* (Fig. 6B). Ad5CMV-*p53* significantly reduced ME-180 tumor sizes compared with medium only and AdCMV-*poly A* ( $P < 0.001$ , unpaired *t* test). Metastasis of ME-180 cell tumor did not appear in any treated animals. The ME-180 tumor sizes treated with medium only were not significantly different from those treated with Ad5CMV-*poly A*.

## Comment

While an animal model of cervical cancer, such as the subcutaneous implantation of cervical cancer cells in a xenograft model [13], has been used in experimental oncology, there has been no report of an orthotopic cervical cancer model. In this article, we report an orthotopic cervical cancer model with metastases in the nude rat using two distinct human cervical cancer cell lines. The interpretation of experiments using subcutaneously implanted cervical tumors is limited because of different tissue-specific factors. The orthotopic bladder tumor model has been successfully used for evaluation of the efficacy of certain cancer treatments [16–20]. However, the tumor cell lines used to develop the orthotopic bladder tumor models, the number of instilled tumor cells, and the response variables were different. Currently, the SiHa and ME-180 cervical tumors implanted in nude rats are used for orthotopic tumor implantation. The amount of

instilled cells was  $10^7$  cells in both tumors. Shapiro et al. [21] investigated the dose response of tumor implantation in the bladder tumor model and found a maximum tumor growth rate of  $>90\%$  after instillation of  $10^6$  tumor cells, whereas instillation with  $2.5 \times 10^5$  cells led to a tumor growth rate of 30%. The rat cervix is distinctly difficult to inject tumor cells into because of its smaller size and harder tissue on the injection site than the bladder. Tumor instillation of  $<5 \times 10^6$  cells led to a decrease in the tumor growth rates in SiHa and ME-180 cell tumors. Therefore, we used  $10^7$  cells in both cell tumors. Low tumor volumes in SiHa cell tumors can compromise the evaluation of

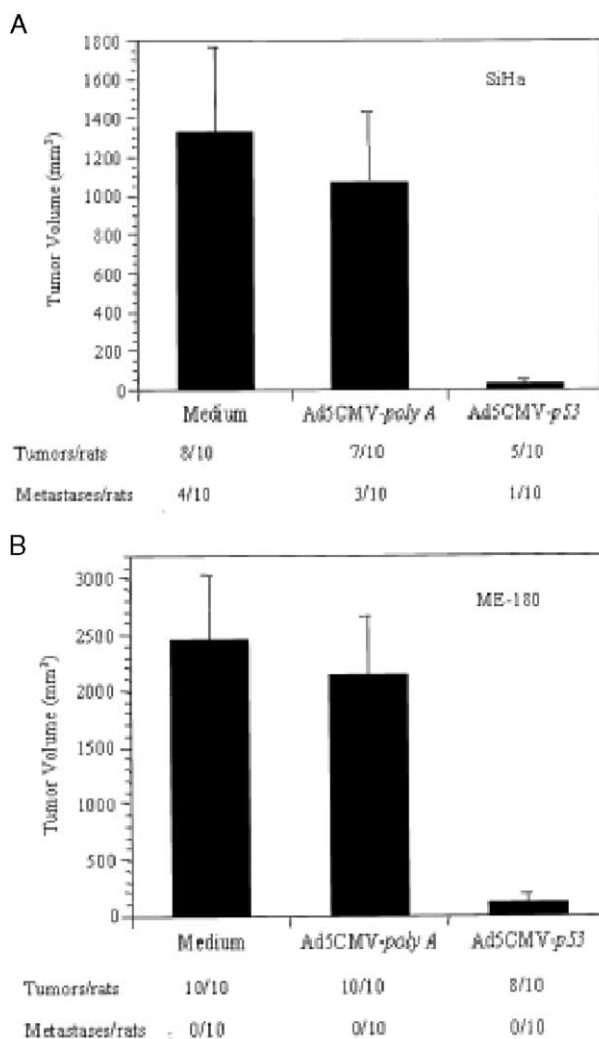


Fig. 6. Anti-tumor effects of intracervically administered Ad5CMV-*p53* in nude rats. Rats were injected into the anterior cervix with  $1 \times 10^7$  cervical cancer cells/rat. Four, five, and six days later, 50  $\mu$ l of Ad5CMV-*p53* ( $1 \times 10^9$  PFU), Ad5CMV-*poly A* ( $1 \times 10^9$  PFU), or medium alone was injected into the anterior cervix which is the same portion to that was previously injected with cervical cancer cells. Ten rats were used for each treatment group. (A) Effect of three injections of Ad5CMV-*p53* on orthotopic cervical tumor growth of SiHa cells in rats. Points indicate mean. Bars indicate standard deviation. (B) Effect of three injections of Ad5CMV-*p53* on orthotopic cervical tumor growth of ME-180 cells in rats. Points indicate mean. Bars indicate standard deviation.

experimental results and may increase the number of animals needed for investigation. However, SiHa cell tumors induced high rates of metastasis in the pelvic cavity. Therefore, SiHa cell tumors are adequate to evaluate the metastatic potential of cervical cancer and the effectiveness of pharmaceuticals to treat metastatic lesions. On the other hand, ME-180 had high tumor volumes, so this cell line is adequate to evaluate the treatment of the primary tumor in the cervix without increasing the number of animals needed for investigation. Furthermore, none of the tumors showed any central necrosis, which often appears in subcutaneous tumor models. It is well known that subcutaneous tissue lacks blood supply to grow large subcutaneous tumors without central necrosis and that the cervix has an abundant blood supply, which led to the growth of the orthotopic cervical tumors without central necrosis. Collectively, these SiHa and ME-180 cell tumors orthotopically established in the nude rat cervix might be good candidates to simulate clinical progression and metastasis of cervical cancer and clinical treatment of cervical cancer by pharmaceuticals.

Successful tumor implantation is dependent on adequate injection into the anterior cervical surface through the anterior vaginal fornix. We tried traumatizing the cervical surface with a knife or a pair of scissors and the clumping the vagina by agarose gel and clips. However, when using this method, the rats had a lower occurrence (20–30%) of intracervical tumors. Finally, we successfully employed an anterior intracervical injection to create the orthotopic cervical tumor.

Tumor outgrowth detected by abdominal palpation and vaginal inspection is thought to be the most probable response variable in this tumor model. However, these methods did not provide reliable parameters for assessing treatment effects on tumor growth. Using these parameters as response variables may give inadequate results because a reduced tumor growth in one group may be the result of successful treatment or may simply reflect failure of tumor implantation. To overcome these difficulties, other authors tried to monitor intravesical tumor growth and treatment effects by imaging methods like transrectal ultrasound [22] or resonance imaging [23,24]. These methods proved to be a suitable means for monitoring intravesical tumor growth, but each imaging procedure required anesthesia and catheterization. Apart from this, imaging methods, especially magnetic resonance imaging, are expensive. Consequently, the ME-180 tumor model is adequate to monitor treatment effects because 100% of animals established intracervical tumors. In addition to SiHa and ME-180 cells, HeLa, C4I, C4II, MW751, HT-3, and C33A are commonly used in the study of cervical oncology. In this study, we did not try these cell lines, but further investigation of them might provide promising results for orthotopic cervical cancer models. From these results, we conclude that our model is a simple, cheap, and useful method to evaluate the orthotopic rat cervical cancer model.

Most clinical trials of gene therapy for cancer deliver vectors by intratumoral injections. The innate host responses following administration of adenoviral vectors to humans result from the administration route [25]. Therefore, host responses after intracervical tumor injection might differ from those after subcutaneous tumor injection. These differences between injection sites could be further elucidated by using immunocompetent syngenic animal models. Human cervical cancer cells do not metastasize in immunocompromised subcutaneous animal models [12]. However, SiHa cell intracervical tumors metastasized to pelvic organs – probably to regional lymph nodes – and Ad5CMV-*p53* suppressed SiHa cell tumor metastasis. Thus, the orthotopic SiHa cell tumor model could evaluate the efficacy of treatment methods for regional lymph-node metastasis. The uterine cervix encompasses many organs including blood vessels, lymph nodes, and the ureter, bladder, and rectum. These organs may develop a serious adverse reaction to the intracervical injection of adenovirus vectors. Therefore, it is important to investigate orthotopic cervical cancer models – and determine maximum tolerant dose of adenovirus – before using them to simulate clinical trials of gene therapy for cervical cancer in patients.

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