Adenovirus-Mediated Transfer of HPV 16 E6/E7 Antisense RNA to Human Cervical Cancer Cells\(^1\)

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INTRODUCTION

The most important risk factor for cervical cancer is human papillomavirus (HPV) infection, which has been shown to increase the relative risk of developing cervical intraepithelial neoplasia and cervical cancer in case control and cohort studies by 11- to 60-fold [1]. HPV has been identified in up to 90% of cervical cancers, and 60% of these are HPV 16 [2]. The viral DNA is usually integrated into host DNA, and HPV-specific RNA and proteins can be detected in the tumors and cell lines derived from them [3]. Inactivation of the \(p53\) gene by allelic loss or by point mutation is infrequent in primary cervical cancer, and the overall incidence of \(p53\) point mutations in these cancers is 1–6% [4].

The role of HPV in the pathogenesis of human cancers has been studied intensively in order to resolve whether HPV plays an etiological part in the development and progression of tumors. Two genes in HPV encode the oncoproteins E6 and E7. E6 can form complexes with p53 and promote p53 degradation [5]. HPV-positive cell lines express low levels of wild-type p53 [6]. The E7 protein binds to the Rb protein, preventing Rb from binding to its normal substrate, the E2F-1 protein [7]. The E6 and E7 proteins of HPV 16 and 18 have been demonstrated to be necessary to efficiently immortalize their natural host cells, primary human squamous epithelial cells [8]. Howley and colleagues have demonstrated that the inactivation of Rb and p53 are important steps in cervical carcinogenesis [6]. Furthermore, the continued expression of the E6 and E7 regions of the viral genome appears to be necessary for the maintenance of the malignant phenotype [9].

Gene expression can often be selectively inhibited by using complementary antisense messages [10]. von Knebel Doeberitz et al. [9] showed that expression of antisense HPV-18 E6 and E7 RNA could modify the growth of the cervical cancer cell line C4-I. This intriguing finding leads...
to the possibility of using antisense RNA to develop gene therapy techniques for human carcinomas that contain HPV DNA. Steele et al. [11] showed that a plasmid that expressed antisense RNA of HPV 18 decreased the growth rate of the human cervical cancer cell line HeLa, but the low transduction efficiency of the plasmid in the cell is problematic. Antisense E6 and E7 oligonucleotides have been shown to inhibit the growth of HPV-positive cancer cells but not HPV-negative cells [12]. However, when the oligonucleotides were withdrawn after 3 days of culture, the remaining cells recovered and grew as before. Any therapy with oligonucleotides might therefore require continuous administration.

Adenoviral vectors have many advantages over other viral vector systems and other in vitro techniques for introducing DNA into eukaryotic cells. However, there have been no reports of the efficacy of an adenoviral construct of antisense HPV E6 and E7. In the study presented here, the E6/E7 region of HPV 16 in the antisense orientation was inserted into an adenoviral vector. The effects of this antisense HPV 16 E6/E7 construct (Ad5CMV–HPV 16 AS) on production of E6/E7 mRNA and growth of human cervical cancer cells were evaluated.

**MATERIALS AND METHODS**

**Cell Lines and Culture Conditions**

The human cervical cancer cell line SiHa was obtained from the American Type Culture Collection (Rockville, MD). SiHa is an HPV 16-positive cell line and has a wild-type p53 [13]. Cells were grown in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum.

**Recombinant Adenovirus**

The HPV 16 genome, cloned into PBR322, was provided by the Japanese Cancer Research Resources Bank (JCRB, Tokyo). The entire E6/E7 region (nt 45 to 877) was amplified by polymerase chain reaction (PCR) using an upstream primer with a NotI site and a downstream primer with a HindIII site. This fragment was cloned in the antisense orientation into pAdE1CMV, a shuttle plasmid that contains the left end of Ad5 (0–16 map units, m.u.) with an E1 region (1.25–9.2 m.u.) substituted by an expression cassette containing the cytomegalovirus (CMV) promoter, a multicloning site, and a simian virus (SV40) polyadenylation signal. The recombinant plasmid with the E6/E7 region was cotransfected with pJM17 into 293 cells which are derived from primary embryonal kidney cells transformed by introducing sheared fragments of Ad5 DNA. This cell line contains an integrated E1 region and is thus highly permissive of the replication of the E1 replication-deficient adenovirus. A plasmid, pJM17 contains the entire Ad5 DNA molecule, with an insert in the E1 region that exceeds the packaging constraints of the adenovirus capsid. An adenoviral vector containing the same cassette but without the HPV 16 E6/E7 cDNA was used as the control (Ad5CMV–poly(A)). An adenoviral vector containing the same cassette but with p53 cDNA (Ad5CMV–p53) was also used to study the growth inhibitory effects.

**Adenoviral Infection**

Recombinant adenovirus was purified by double cesium gradient ultracentrifugation as described elsewhere [14]. Viral stocks were propagated in 293 cells. Cells were harvested 36–40 hr after infection and cell debris was removed by subjecting the lysed cells to CsCl gradient centrifugation. Concentrated virus was dialyzed and stored in aliquots at –80°C. Infection was carried out by adding the virus to high-glucose Dulbecco’s modified Eagle’s medium and to the cell monolayers. The viral titers were determined by plaque assays [14].

**Reverse Transcriptase–PCR Analysis**

Total RNA was prepared by lysing cell monolayers in guanidinium isothiocyanate and centrifuging over a 5.7 M CsCl solution. Reverse transcriptase (RT)–PCR was performed from 6 μg of total RNA to generate cDNA. These cDNA products were then amplified by PCR, using primers for the antisense E6/E7 of HPV 16 and the poly(A) signal of the adenovirus, yielding a 486-bp product. PCR products were analyzed on 2% agarose gels and stained with ethidium bromide.

**Cell Count Assay**

Cells were plated at a density of 5 × 10⁴/well in 12-well plates in triplicate. RPMI supplemented with 10% heat-inactivated fetal bovine serum was used as the growth medium. Cells were infected with either Ad5CMV–HPV 16 AS or the Ad5CMV–poly(A) control at a multiplicity of infection (m.o.i., viral particles per cell) of 100. Culture medium was used for mock infection. Triplicate cultures of SiHa cells with different treatments were analyzed. Cells were harvested by trypsinization and counted daily for 6 days. Cell viability was determined by trypan blue exclusion after harvest.

**[^1]H]Thymidine Incorporation Assay**

Cells were cultured at 2 × 10⁴ cells/well in 96-well flat-bottomed plates. Cells were infected with Ad5CMV–p53, Ad5CMV–HPV 16 AS, or Ad5CMV–poly(A) and cultured in medium for 5 days. Each well was pulse-treated with 1 μCi [¹H]thymidine (sp act, 6.7 Ci/mmol; Amersham Inc., Arlington Heights, IL) for an additional 24 hr, after which the cells were harvested with a Ph.D. cell harvester (Cam-
bridge Technology, Cambridge, MA). Individual filter discs were then processed for liquid scintillation counting. The data are presented as the means of triplicate samples.

**Tumorigenicity Assay**

SiHa cells were infected with Ad5CMV–HPV 16 AS or Ad5CMV–poly(A) at an m.o.i. of 30. An equal number of cells was treated with medium as a mock infection. Three hours after infection, the treated cells were harvested and rinsed twice with phosphate-buffered saline (PBS). For each treatment, 10 million (1 x 10^7) cells in a volume of 0.1 ml were injected subcutaneously into each nude (nu/nu) female mouse (aged 4–5 weeks; Harlan Co., Houston, TX). Ten mice were used for each treatment. The mice were examined every day and tumor formation and size were evaluated for 44 days. The tumors were measured every other day with calipers in two perpendicular diameters without knowledge of treatment groups. Tumor volume was calculated by assuming a spherical shape with the average tumor diameter calculated as the square root of the product of cross-sectional diameters.

**RESULTS**

The entire E6/E7 region was amplified and cloned in antisense orientation into the shuttle plasmid vector to form the plasmid pAdE1CMV–HPV 16 AS. The expected genomic structure of Ad5CMV–HPV 16 AS is depicted in Fig. 1B. Figure 1C shows that a 0.49-kb HPV 16 E6/E7 antisense-specific band and a 0.33-kb adenovirus-specific band amplified through PCR confirmed the identity of Ad5CMV–HPV 16 AS. This was further verified by sequencing analysis of the CsCl gradient-purified Ad5CMV–HPV 16 AS DNA. In RT-PCR analysis, the transcript of antisense E6/E7 RNA from Ad5CMV–HPV 16 AS was detected in Ad5CMV–HPV 16 AS-infected SiHa cells, while Ad5CMV–poly(A)-infected cells and mock-infected cells did not show any adenoviral antisense transcripts (Fig. 2).

The SiHa cells were treated with Ad5CMV–HPV 16 AS, Ad5CMV–poly(A), or medium only. In cell count assay, the growth of the Ad5CMV–HPV 16 AS-infected SiHa cells was greatly suppressed (Fig. 3). Taking the growth inhibition of the mock-infected SiHa cells as 0% on Day 6, that of Ad5CMV–HPV 16 AS-infected cells was 96%. The cells infected with Ad5CMV–poly(A) had a growth rate similar to that of the mock-infected cells. This growth assay was reproducible in five repeated experiments.

The growth inhibitory effect of Ad5CMV–HPV 16 AS was compared with that of an adenoviral p53 vector, Ad5CMV–p53. In [3H]thymidine incorporation assay, m.o.i. at the 50% growth inhibitory effect (IC50) of Ad5CMV–HPV 16 AS was similar to that of Ad5CMV–p53 (Fig. 5).

The growth inhibitory effect of Ad5CMV–HPV 16 AS was greatly enhanced by the combining that vector with Ad5CMV–p53 (Figs. 4 and 5).

To examine whether the Ad5CMV–HPV 16 AS virus can inhibit tumorigenicity of human cervical cancer cells, nude mice were injected with treated SiHa cells to initiate tumor formation. Each mouse received one injection of 1 x 10^7 cells that had been infected with Ad5CMV–HPV 16 AS or Ad5CMV–poly(A) at an m.o.i. of 30 for 3 hr. SiHa cells treated with medium alone were used as mock-infected controls. Tumors were formed only from the mock- or control virus-infected cells; mice that received Ad5CMV–HPV 16 AS-treated cells did not develop tumors (Table 1). This study was initiated with 10 mice per group and was completed in all.

**DISCUSSION**

Recombinant adenovirus is a gene delivery system that has great potential for cancer gene therapy. In this study, a recombinant adenovirus that expresses the antisense HPV E6/E7 gene was generated. The virus, Ad5CMV–HPV 16 AS, contains the CMV promoter, the total sequence of HPV 16 E6/E7 in the antisense orientation, and the SV40 polyadenylation signal. PCR assays and sequencing analysis of purified adenovirus DNA showed that Ad5CMV–HPV 16 AS was the newly generated recombinant adenovirus. RT-PCR analysis also showed that Ad5CMV–HPV 16 AS expressed the RNA of antisense HPV E6/E7 in the Ad5CMV–HPV 16 AS-infected SiHa cells. Expression of E6 and E7 genes has been linked directly to the proliferative capacity of cervical cancer cells and is also required for the maintenance of the transformed phenotype [15, 16]. Because most cervical carcinomas contain the transforming E6 and E7 genes, these genes make an attractive target for antisense inhibition. It was shown that HPV 16- or 18-positive cervical cancer cells were inhibited by oligonucleotides [12, 17] and plasmids [9, 11] that expressed RNA antisense to the transforming genes E6 and E7 of HPV. However, there have been no reports of the growth of cervical cancer cells being inhibited in vitro or in vivo by viral vectors expressing the antisense RNA of these genes. In the study presented here, we used an adenoviral vector because adenovirus has the feature of high infectivity in eukaryotic cells.

We examined the effects of Ad5CMV–HPV 16 AS in cervical cancer SiHa cells in culture and in tumors in nude mice. The results clearly demonstrate the effectiveness of the anti-HPV 16 E6/E7 adenoviral vector. The antisense HPV E6/E7 plasmid and the oligonucleotides anti-E6 plus anti-E7 lead to greater inhibition of growth of HPV-positive cells than the antisense HPV E7 plasmid or the anti-E7 oligonucleotides [11, 17]. The effect of antisense E6 and E7 is
FIG. 1. (A) Scheme for generation of recombinant HPV antisense adenovirus. The entire E6/E7 region of HPV 16 was amplified by PCR and cloned in antisense orientation into a shuttle plasmid, pAdE1CMV. The recombinant plasmid with the E6/E7 region was cotransfected with pJM17 into 293 cells. The transfected cells were maintained in medium until the onset of the cytopathic effect. (B) A map of Ad5CMV–HPV 16 AS genomic DNA, showing locations of the CMV promoter, HPV 16 E6/E7 antisense, the SV40 poly(A), and the PCR primers. Two pairs of primers that define 486-bp (HPV 16 E6/E7 antisense) and 335-bp (Ad5) DNA fragments were used in each reaction. (C) Newly generated HPV antisense recombinant adenovirus (Ad5CMV–HPV 16 AS) was identified by PCR analysis of the DNA samples. DNA templates used in each reaction were pAdE1CMV–HPV 16 AS (lane 2), Ad5CMV–poly(A) (lane 3), no DNA (lane 4), sample from the supernatant of 293 cells after cytopathic effect (lane 5), and the purified adenovirus (Ad5CMV–HPV 16 AS) (lane 6). Lane 1 shows size markers.
probably due to a reversal of the transformed phenotype brought about by the inhibition of E6 and E7.

The effects of antisense HPV E7 on cell growth are associated with the suppression of E7 expression and the antisense HPV E6 probably exerts its effect through the suppression of E6 as well as E7 [16]. In cervical cancers and cervical cancer cell lines containing HPV, mRNAs encoding the E6 and E7 proteins are transcribed from the same promoter in the form of a bicistronic transcript [18]. The bicistronic transcript is also spliced to produce two shorter transcripts E6 I-E7 and E6 II-E7 [19]. It has been proposed that it is a function of these spliced transcripts to stimulate E7 translation [18]. E7 protein is known to bind the product of the retinoblastoma susceptibility gene, Rb, thereby preventing pRb from regulating cell growth [20]. Inhibition of E7 protein expression allows pRb to perform its function of regulating cell growth [21]. E7 can directly interfere with cell cycle control by inducing S-phase entry by sequential activation of cyclin A gene and cyclin E gene expression [22]. Both E6 and E7 play important roles in affecting specific, though distinct check point controls in G1 [23, 24]. It has been suggested that the transforming function of E6 is independent of a direct association with p53 because mutants of E6 which fail to bind to p53 or promote p53 degradation are still able to cooperate with ras in an immortalization assay; yet, based on the use of chimeric E6 proteins, the transforming activity of E6 is localized to the carboxy terminal
region of the protein [23]. It has also been reported that anti-E7 oligonucleotides decrease E7 protein expression and increase Rb protein expression [17]. Because the changes in E6 and E7 mRNA and protein expression were not determined in this study, further experiments should address whether the mechanism of the growth inhibition of HPV 16-positive cells by Ad5CMV–HPV 16 AS is due to the p53- and Rb-dependent pathway or the p53- and Rb-independent pathway.

Some RNA molecules can act as enzymes to cleave other RNA molecules at specific sites [25]. One type of these RNA enzymes or ribozymes that has been studied in detail is the hammerhead ribozyme. Any RNA molecule can be targeted for cleavage by base pairing with a second RNA that is

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**FIG. 3.** Growth curves of the Ad5CMV–HPV 16 AS-infected human cervical cancer cells. The cells were inoculated at densities of $5 \times 10^4$well in 12-well plate 24 hr before infection. The cells were infected with Ad5CMV–HPV 16 AS or Ad5CMV–poly(A) at an m.o.i. of 100. Culture medium alone was used as mock infection. Triplicate wells for each treatment were counted daily from Postinfection Days 1 to 6. The means of cell counts for triplicate wells were plotted. Bars indicate standard deviation.

**FIG. 4.** Inhibition of the growth of SiHa cells as determined by $[^{3}H]$thymidine incorporation assay. The cells were inoculated at densities of $2 \times 10^3$well in a 96-well plate 24 hr before infection. The cells were infected with Ad5CMV–p53, Ad5CMV–HPV 16 AS, the combination, or Ad5CMV–poly(A). Culture medium alone was used as mock infection. The cells in triplicate wells for each treatment were trypsinized, harvested, and counted on Day 6 after infection. The means of cell counts for triplicate wells were plotted. Bars indicate standard deviation.
Ad5CMV–HPV 16 AS showed a potent growth inhibitory effect similar to that of Ad5CMV–p53. HPV 16 E6 is much more active in promoting p53 degradation than HPV 18 E6, and yet HPV 18 E6 is more efficient in the ras cooperation assay [23]. In addition, continuous E6 and E7 protein expression is required to maintain the malignant phenotype of these cells [23]. The adenoviral vector is capable of mediating high levels of antisense RNA expression that apparently are sufficient to overcome the capabilities of endogenous E6 and E7 protein expression. Restoration of p53 and Rb function, and inhibition of p53- and Rb-independent pathway by Ad5CMV–HPV 16 AS might be effective in reducing proliferation of these cells.

In this study, first-generation viruses from which E1A and E1B were deleted were used in vitro and ex vivo experiments. This adenovirus-mediated transgene expression in immunocompetent mice was transient and significantly reduced following a second administration of the virus 4 weeks after an initial injection [28]. Expression levels of the FIX gene with adenovirus vector declined to base level by 9 weeks and were not reestablished by a second injection 10 weeks after the initial injection [29]. To overcome these problems of immunogenicity of first-generation E1-deleted adenoviral vector, long-acting adenoviral vectors whose reexpression is designed to form a hammerhead structure. An inhibitory effect more potent than that produced by complementary antisense transcripts and oligonucleotides might be obtained by hammerhead ribozymes because such molecules not only bind to the target transcript but also can cleave it at a specific site [25]. Indeed, hammerhead ribozymes can bind and cleave RNA transcripts derived from the E6 and E7 genes of human papillomavirus type 16 and 18 [25, 26], can inhibit the replication of HPV virus, and can reverse the malignant phenotype of cells that express the ras oncogene [27]. However, studies comparing the two are necessary because ribozymes are not always more effective than antisense molecules. Therefore, tests to evaluate the antitumor potential of antipapillomavirus ribozymes by using adenoviral vectors should be addressed in cervical cancer.


FIG. 5. The concentration required for 50% growth inhibition (IC$_{50}$) of SiHa cells was determined by [3H]thymidine incorporation assay after infection with Ad5CMV–p53, Ad5CMV–HPV 16 AS, the combination, or Ad5CMV–poly(A) for 6 days. Each IC$_{50}$ is expressed in m.o.i. Bars indicate standard deviation.

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TABLE 1

Effect of Pretreatment with Ad5CMV–HPV 16 AS on Tumorigenicity of SiHa Cells in Nude Mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of tumors/ Mean volume (mm$^3$ ± SD) (%)</th>
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<tr>
<td>PBS</td>
<td>10/10 511 ± 164 (100)</td>
</tr>
<tr>
<td>Ad5CMV–poly(A)</td>
<td>9/10 370 ± 41 (72.4)</td>
</tr>
<tr>
<td>Ad5CMV–HPV 16 AS</td>
<td>0/10 0 ± 0 (0)</td>
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Note. SiHa cells were treated with Ad5CMV–HPV 16 AS and Ad5CMV–poly(A) at an m.o.i. of 30 and medium only for 3 hr. The treated cells were injected at 1 × 10$^7$ cells/mouse. Tumor sizes were determined 50 days after injection.
growth compared with invasive disease. Therefore, Ad5CMV–HPV 16 AS might be also potentially applicable to primary prevention, particularly in high-risk patients with preinvasive disease. The presence of HPV DNA indicates the presence of micrometastasis in lymph nodes and serum antibody levels against HPV correlate with a shortened survival [31, 32]. HPV-positive tumors tended to be more frequently of advanced stages than those that were negative for HPV [32]. These findings introduce the significance of systemic treatment of Ad5CMV–HPV 16 AS for invasive cervical cancer. To establish systemic treatment of Ad5CMV–HPV 16 AS, the important questions that remain to be answered are how to target and deliver Ad5CMV–HPV 16 AS to the site of tumors. Development of receptor-mediated system, tumor specific promoter system, and modification of structure proteins including fiber, penton, hexon etc. might yield tumor-specific gene expression by Ad5CMV–HPV 16 AS.

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REFERENCES


