In Vitro and in Vivo Inhibition of Human Papillomavirus Type 16 E6 and E7 Genes

Theresa M. C. Tan1 and Robert C. Y. Ting
Institute of Molecular and Cell Biology, National University of Singapore, 10 Kent Ridge Crescent, Singapore 051 1, Republic of Singapore

ABSTRACT

Human cervical cancers are often associated with human papillomavirus (HPV). In HPV-positive cervical cancers, the oncoproteins E6 and E7 are consistently expressed. In this study, the effects of antisense inhibition of both proteins were examined. Phosphorothioate oligonucleotides (ODNs) AE6 and AE7 complementary to regions flanking the start codons of HPV16 E6 and E7 genes, respectively, were synthesized. These anti-HPV ODNs inhibited the growth of cervical cell lines CaSki and SiHa, which harbor HPV16 but had little effect on cells that do not. Both ODNs also affected the ability of CaSki cells to form colonies in soft agar. In nude mice, treatment with either AE6, AE7, or a mixture of both led to substantially smaller tumors. AE7 was observed to inhibit E7 synthesis. The AE6 ODN probably exerts its effect by suppressing the expression of E6 as well as E7. Cell cultures and tumors treated with AE6 showed a decrease in E7 expression. In addition, an antisense ODN targeted at the retinoblastoma gene was able to reverse some of the inhibitory effect of AE6 on CaSki cells, indicating that AE6 inhibited E7 synthesis. This study further demonstrates that anti-HPV ODNs may be useful therapeutically.

INTRODUCTION

The antisense concept involves the use of short complementary ODNs2 to interfere with the function of mRNAs. Such ODNs are usually between 12–20 bases in length. Hybridization of the antisense ODN with the target RNA by complementary base pairing provides high specificity and binding affinity resulting in the inhibition of expression of the protein product. The efficacy of antisense inhibition has been demonstrated for a variety of genes, particularly oncogenes (1–3).

HPVs are small DNA viruses with genomes of approximately 8 kb. Among the HPVs, a small subgroup including HPV16 and HPV18 are associated with cervical cancers (4). The transforming properties of these HPVs are encoded by the E6 and E7 genes (5–7). Both proteins may also play a role in the maintenance of the fully transformed phenotype.

Previous studies had shown that antisense ODNs to HPV16 and HPV18 E6 and E7 messages were useful in inhibiting cell growth and in affecting the transformed phenotype (8–10). However, the actual mechanism by which the anti-E6 and anti-E7 ODNs exert their effects is not well elucidated. We had shown previously that antisense ODNs complementary to E6 and E7 genes could specifically inhibit the proliferation of a cervical carcinoma cell line, CaSki (10). This study was carried out to further investigate the antisense inhibition of E6 and E7 in human carcinoma cells lines in culture, as well as to examine the effects of these ODNs on tumor growth in athymic nude mice.

MATERIALS AND METHODS

Synthesis of ODNs. Five different deoxyligonucleotides were used in this study (Fig. 1). Two of these, AE6 and AE7, hybridize with sequences flank

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m-E7 or AE7 formed fewer colonies on agar than did cells treated with MA or cells that were not treated (Table 4).

Tumor Growth in Nude Mice. At $5 \times 10^6$ cells, both CaSki and SiHa cells formed tumors in all mice. However, the latent period for CaSki cells was much longer than for SiHa and, hence, SiHa cells were used for all subsequent experiments. Once palpable tumors were established, the animals were divided into groups and treated with either PBS or with PS ODNs. In the initial experiment, the animals were treated for 10 days with 20 µg of MA, AE6, AE7, or with PBS.

RESULTS

Cellular Uptake of ODNs. Uptake of the biotinylated PS ODN AE7 was monitored over a period of 24 h. Fluorescence staining could be observed in SiHa cells 1 h after incubation with the ODN and was present throughout the 24-h period (Fig. 2). A similar pattern of ODN uptake was also observed for CaSki cells (data not shown).

Growth Inhibition of CaSki and SiHa Cells. In our earlier study, it was observed that 20 µM of AE6 or AE7 could selectively inhibit the proliferation of CaSki cells (10). In this study, the effects of various concentrations and combinations of ODNs on the proliferation of CaSki and SiHa cells were examined. Cells were treated with AE6, AE7, or a mixture of both in the ratio of 1:1. Concentrations ranging from 0.1 µM to 40 µM were used.

Treatment of CaSki and SiHa cells with AE6 resulted in inhibition of cell proliferation. This inhibition ranged from 6.5 to 67.2% for CaSki cells (Fig. 3A) and from 11.8 to 56.1% for SiHa cells (Fig. 3B) when the cells were treated with concentrations varying from 5 to 40 µM. Similarly, AE7 treatment also led to inhibition of cell growth ranging from 26.0 to 60.8% for CaSki cells (Fig. 3A) and from 21.1 to 50.2% for SiHa cells (Fig. 3B) at concentrations between 1 and 40 µM. However, a combination of AE6 and AE7 was more effective, and >50% inhibition was observed at 5 µM (Table 1).

Treatment of both CaSki and SiHa cells with the control ODNs MA, HPVC, or ARb led to inhibition of <20% (Fig. 3). In addition, AE6 and AE7 had little effect on the proliferation of C-33A, which does not harbor HPV DNA, and of HeLa, which contains HPV18 (Table 2). The presence of the cationic lipid DOTAP at 5 µg/ml did not affect the proliferation of all the cells.

In another series of experiments, CaSki cells were treated for 48 h with a mixture of AE6 and ARb (2:1 ratio). The presence of ARb diminished the inhibitory effect of AE6 on the proliferation of CaSki cells (Table 3).

E7 Expression in CaSki Cells. E7 expression could be detected in CaSki cells and this expression was induced by 100 ng phorbol 12-myristate-13-acetate (TPA)/ml medium. Expression of E7 was reduced after 5 h of incubation with 20 µM AE7 (Fig. 4). The decrease in E7 expression was still evident at 24 h. However, at 24 h, the reduction in E7 protein level was less than at 5 h. A similar change in E7 expression was also observed when the cells were treated with AE6. The expression of an unrelated protein, actin, was not affected at all.

Anchorage-independent Growth. Incubation of CaSki cells for 4 h with either AE6 or AE7 did not affect the viability of the cells (data not shown). However, cells that had been pretreated with either AE6 or AE7 formed fewer colonies on agar than did cells treated with MA or cells that were not treated (Table 4).

Fig. 1. Sequences of oligonucleotides used in this study and position of base pairing to the E6-E7 mRNA and Rb mRNA. HPVC correspond to the sense sequence of E6.

Fig. 2. Uptake of AE7 by SiHa cells at 1 (a), 4 (b), 8 (c), and 24 h (d).

Fig. 3. Expression of E7 in CaSki cells treated with AE6 and AE7 in combinations of 1:1 or 2:1 ratio at 5 h (a), 24 h (b), or 48 h (c and d).
Oligonucleotide concentration% of inhibition of CaSki cell proliferation ± SD% of inhibition of SiHa cell proliferation ± SD

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>CaSki (% inhibition)</th>
<th>SiHa (% inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 μM</td>
<td>32.9 ± 9.1</td>
<td>37.5 ± 5.5</td>
</tr>
<tr>
<td>0.5 μM</td>
<td>40.6 ± 8.3</td>
<td>37.3 ± 6.4</td>
</tr>
<tr>
<td>5.0 μM</td>
<td>58.7 ± 11.7</td>
<td>50.8 ± 14.0</td>
</tr>
</tbody>
</table>

Table 1: Effects of a mixture of AE6 and AE7 (1:1) on the proliferation of CaSki and SiHa cells

Measurement of E7, pRb, and Actin in Tumors. The expressions of E7 and pRb were determined in tumors excised from the nude mice. E7 expression was reduced in tumors treated with AE6 or AE7 (Fig. 6). This decrease in E7 was accompanied by the detection of pRb. pRb was not detectable in tumors treated with PBS or MA. The expression of an unrelated protein, actin, was not affected by any of the ODNs.

DISCUSSION

In this study, we report the results of PS ODN treatments of cervical cancer cells in culture and in tumors in nude mice. The results clearly demonstrate the effectiveness of anti-HPV16 ODNs, particularly for retarding tumor growth in nude mice. Before examining the effects of the anti-HPV ODNs in nude mice, we first examined the effects on cell cultures.

In cell cultures, the uptake of the PS ODN was rapid and efficient in the presence of the cationic lipid, DOTAP. The enhancement of ODN uptake by cationic lipids has been described for a variety of cell lines (11), including CaSki (12). In this study, DOTAP was used at 5 μg/ml, and this concentration was not toxic to all the cell lines used. The ODN remained associated with the cells for up to 24 h.

Both anti-HPV ODNs, AE6 and AE7, were able to inhibit the growth of CaSki cells (A) and SiHa cells (B) treated with various oligonucleotides. The cells were treated with AE6 (○), AE7 (●), MA (■), HPVC (□) and ARb (△).

At the end of the treatment, animals treated with AE6 or AE7 had smaller tumors. The animals were then left untreated for 12 days before being killed. Despite the 12-day period without treatment, animals that had been previously treated with AE6 or AE7 had smaller tumors with average weight one-half that of controls (Fig. 5A).

In another series of experiments, all animals were treated with 30 μg of ODN daily for 15 days while the control animals were similarly treated with PBS. At the end of the treatment, animals treated with AE6 or AE7 had substantially smaller tumors (Fig. 5B). The average weight of these tumors was 85% lighter than those treated with PBS.

To examine if a combination of AE6 and AE7 was more effective in retarding tumor growth, the animals were treated with 10 μg of either MA, AE6, AE7, or a mixture of AE6 and AE7 in a 1:1 ratio. After 10 days of treatment, animals treated with the mixture of AE6 and AE7 had smaller tumors than those animals treated with either AE6 or AE7 (Fig. 5C).

Measurement of E7, pRb, and Actin in Tumors. The expressions of E7 and pRb were determined in tumors excised from the nude mice. E7 expression was reduced in tumors treated with AE6 or AE7 (Fig. 6). This decrease in E7 was accompanied by the detection of pRb. pRb was not detectable in tumors treated with PBS or MA. The expression of an unrelated protein, actin, was not affected by any of the ODNs.

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<th>% of inhibition of SiHa cell proliferation ± SD</th>
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<tr>
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</tr>
<tr>
<td>5.0 μM</td>
<td>58.7 ± 11.7</td>
<td>50.8 ± 14.0</td>
</tr>
</tbody>
</table>

Table 2: Effects of antisense oligonucleotides on the proliferation of HeLa and C-33A cells

<table>
<thead>
<tr>
<th>Oligonucleotide concentration (μM)</th>
<th>% of inhibition of HeLa cell proliferation ± SD</th>
<th>% of inhibition of C-33A cell proliferation ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>AE6 (20 μM)</td>
<td>6.7 ± 7.1</td>
<td>6.6 ± 3.0</td>
</tr>
<tr>
<td>AE6 (40 μM)</td>
<td>13.8 ± 7.6</td>
<td>23.7 ± 13.3</td>
</tr>
<tr>
<td>AE7 (20 μM)</td>
<td>19.8 ± 8.4</td>
<td>0.7 ± 4.9</td>
</tr>
<tr>
<td>AE7 (40 μM)</td>
<td>18.8 ± 8.1</td>
<td>18.2 ± 3.5</td>
</tr>
<tr>
<td>MA (20 μM)</td>
<td>10.1 ± 8.4</td>
<td>ND*</td>
</tr>
<tr>
<td>MA (40 μM)</td>
<td>17.3 ± 8.1</td>
<td>ND</td>
</tr>
<tr>
<td>HPVC (20 μM)</td>
<td>16.4 ± 6.8</td>
<td>ND</td>
</tr>
<tr>
<td>HPVC (40 μM)</td>
<td>22.3 ± 7.0</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND, not determined.

Table 3: Effects of antisense oligonucleotides ARb:AE6 (1:2) on the proliferation of CaSki cells

<table>
<thead>
<tr>
<th>Oligonucleotide concentration (μM)</th>
<th>% of inhibition of cell proliferation ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 μM</td>
<td>19.6 ± 2.9</td>
</tr>
<tr>
<td>40 μM</td>
<td>20.2 ± 8.6</td>
</tr>
</tbody>
</table>
proliferation of CaSki and SiHa cells. Although Storey et al. (13) had reported nonspecific inhibition with similar ODNs, this study and that of Wong et al. (9) proved otherwise. Control ODNs did not significantly affect the growth of CaSki and SiHa cells, even at the highest concentration of 40 μM. In addition, AE6 and AE7 also decreased the ability of CaSki cells to grow on soft agar. The effects of AE6 and AE7 were specific to cells harboring HPV16, and there was little effect on the cervical line, C-33A, which has no HPV DNA, and HeLa, which contains HPV18.

In the nude mice, the anti-HPV ODNs specifically inhibited tumor growth and the control ODNs had little effect. Tumor growth was repressed by AE6 and AE7 throughout the entire period of treatment. At 12-days posttreatment, AE6-treated and AE7-treated animals still had substantially smaller tumors compared to the controls. The effect of AE6 and AE7 is probably due to a reversal of the transformed phenotype brought about by the inhibition of E6 and E7. Expression of E6 and E7 genes has been linked directly to the proliferative capacity of cervical cancer cells (14–16) and is also required for the maintenance of the transformed phenotype (6, 17).

The effects of AE7 on cell and tumor growth are associated with the suppression of E7 expression. In CaSki cells, the half-life of E7 is only 55 min (18). As such, it was possible to detect a decrease in E7 expression after only 5 h of incubation with AE7. However, the suppression of E7 expression was less after 24 h, and this led to only 50–60% inhibition of CaSki and SiHa cells after 48 h of treatment. E7 expression was also inhibited in tumors treated with AE7. This inhibition is specific to E7, and actin expression was not affected at all.

Table 4 Effect of antisense oligonucleotides on colony formation in soft agar

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>33.5</td>
</tr>
<tr>
<td>MA (20 μM)</td>
<td>35.5</td>
</tr>
<tr>
<td>AE6 (20 μM)</td>
<td>11.0</td>
</tr>
<tr>
<td>AE7 (20 μM)</td>
<td>9.5</td>
</tr>
</tbody>
</table>

Fig. 4. Western blot analysis of E7 and actin expression in CaSki cells. A, detection of E7 in the absence (Lane 1) and presence (Lane 2) of 100 ng/ml TPA. B, actin, and C, E7 expression after incubation with ODNs for 5 h. D, actin, and E, E7 expression after incubation with ODNs for 24 h. Lane 1, lysate of untreated CaSki cells. Lane 2, lysate of cells treated with a control ODN, MA. Lane 3, lysate of cells treated with AE7; and Lane 4, lysate of cells treated with AE6. The positions of Mr markers are indicated on the right and the position of actin (Ac) and E7 are indicated on the left.
Fig. 5. Mean weight of tumor (± SD) in nude mice after s.c. injection of SiHa cells. A, mean weight of tumors 12 days after treatment with PBS (n = 3 mice), 20 µg of MA (n = 3 mice), 20 µg AE6 (n = 5 mice), and 20 µg AE7 (n = 5 mice). B, mean weight of tumors at the end of 15 days of treatment with 30 µg of oligonucleotides. Animals were treated with PBS (n = 6), MA (n = 6), HPVC (n = 6), AE6 (n = 9), or AE7 (n = 9). C, mean weight of tumors at the end of 10 days of treatment with 10 µg of oligonucleotides. Animals were treated with PBS (n = 4), MA (n = 7), AE6 (n = 8), AE7 (n = 5), and AE6/AE7 (n = 8).
INHIBITION OF HPV16 E6 AND E7

Fig. 6. Western blot analysis for E7 (A), actin (B), and pRb (C) proteins in tumors treated with PBS (Lane 1), MA (Lane 2), AE6 (Lane 3), and AE7 (Lane 4). The positions of M, markers are indicated on the right and the position of E7, actin (Ac), and pRb are indicated on the left.

The AE6 ODN probably exerts its effect through the suppression of E6 as well as the expression of E7. In cervical cancers and cervical cell lines containing HPV, mRNAs encoding E6 and E7 proteins are transcribed from the same promoter in the form of a bicistronic transcript (19, 20). In vivo, the bicistronic transcript is also spliced to produce two shorter transcripts E6*I-E7 and E6*II-E7 (21). It has been proposed that it is a function of these spliced transcripts to stimulate E7 translation (19, 20). Because all three transcripts have the same 5' ODN sequence, one would assume that AE6 could affect all three because regions at the 5' end of the mRNA are most sensitive to antisense inhibition of translation (22—24).

Our data does indeed indicate that AE6 affects all three transcripts, thereby, inhibiting E7 translation. In vitro, inhibition of the full-length bicistronic E6-E7 transcript by AE6 results in the suppression of both E6 and E7 expression. This was achieved by the specific hybridization of AE6 with the E6 sequence and not through nonspecific interaction of AE6 with the E7 sequence (10). E7 is known to bind the product of the retinoblastoma susceptibility gene, pRb (25), thereby, preventing pRb from regulating cell growth. Inhibition of E7 will, thus, allow pRb to perform its function of regulating cell growth. For CaSki cells, the inhibition of cell proliferation by AE6 can be reversed by inhibiting pRb expression by using the ODN ARb, which has been shown to be effective in inhibiting the synthesis of pRb in human embryonic lung fibroblasts (26). This reversal suggests that part of the effects of AE6 is achieved via the inhibition of E7 expression. In addition, the expression of E7 in the tumors in nude mice probably caused the Rb protein to be unstable and, hence, Rb could only be detected in tumors treated with AE6 or AE7 because both ODNs suppress E7 expression. The reduction of E7 expression in tumors and cells treated with AE6 indicates that AE6 probably also inhibits E7 expression from the two shorter transcripts, E6*I-E7 and E6*II-E7, because in vivo, these two transcripts account for >80% of the major E6-E7 transcripts (21).

Antisense inhibition of E6 and E7 has been shown to be useful in inhibiting the growth of cervical cells harboring HPV16 or HPV18 DNA (8—10). This study also shows that anti-E6, as well as anti-E7 ODNs, are useful in impeding tumor growth in nude mice. A combination of AE6 and AE7 was more effective than each of the ODNs individually. These observations, together with the specificity of the antisense inhibition, augurs well for the therapeutic potential of antisense ODNs in HPV-related cervical cancers.

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