Effects of Human Papillomavirus Type 18-specific Antisense Oligonucleotides on the Transformed Phenotype of Human Carcinoma Cell Lines

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ABSTRACT

DNA of human papillomavirus type 18 is present in several human cancer cell lines that were derived from oral or cervical tumors, and it is known that several features of the transformed phenotype can be inhibited by expression of antisense RNA to human papillomavirus (HPV). The present study was performed to find out whether antisense oligonucleotides were also inhibitory. Synthetic oligonucleotides were made that were complementary to regions of the start codons of the E6 and E7 genes of HPV-18. These were added to cultures of the oral cancer cell line 1483 and the cervical cancer cell line C4-1, each of which contain DNA of HPV-18. As controls we used the oral cancer cell line 183 and the monkey kidney cell line Vero, which do not contain HPV. Anti-E6 and anti-E7 oligonucleotides, in concentrations between 1 and 5 μM, significantly inhibited the growth of the 1483 and C4-1 cells, but not the 183 or Vero cell lines. Treatment of the 1483 cells with a combination of 2.5 μM of each of the antisense oligonucleotides was a more effective inhibitor than 5 μM of either one used alone. Antisense oligonucleotides had no effect on the ability of 1483 cells to form foci in soft agar, nor on their plating efficiency or serum requirements. Microscopic examination of 1483 cells showed that the antisense E7 oligonucleotide produced cell-rounding, detachment from the surface of the culture flask, and cell death, while the antisense E6 oligonucleotide had none of these effects. Random-sequence oligonucleotides had no effects of any type on any cells that were growing in culture. However, if random-sequence oligonucleotides were added to cells at the time they were passed to a new culture vessel, they produced severe nonspecific toxic effects. These results show that the use of synthetic oligonucleotides is an effective way of producing antisense-mediated changes in the behavior of human cancer cells that contain DNA of HPV-18.

INTRODUCTION

Papillomaviruses are small DNA viruses that induce the hyperproliferation of epithelial cells. Although nearly 70 different genotypes have been isolated from various human lesions, only a small subset of these viruses is commonly associated with neoplastic and preneoplastic lesions. However, various epidemiological studies indicate that up to 90% of all human oral and genital tumors and cell lines derived from these tumors harbor specific types of HPV (1-3). The types of HPV that are associated with these cancers are able to immortalize primary human keratinocytes and to transform rodent cells (4-6). These observations suggest that HPV may play an important role in the development of some human carcinomas.

Two viral genes, E6 and E7, are required for the acquisition and maintenance of a fully transformed phenotype (6-8). Previously it has been shown that endogenously expressed E6 and E7 antisense RNA will inhibit the growth of cervical and oral cancer cells that contain DNA of HPV-18 (9, 10). In those experiments, the oral cancer cells, line 1483, did not survive transfection by antisense-expressing plasmids, implying that they may be highly sensitive to anti-HPV mole-

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2 The abbreviations used are: HPV, human papillomavirus; FBS, fetal bovine serum.

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**RESULTS**

**Oligonucleotide Uptake.** Fluorescence microscopy revealed that each of the cell lines was capable of taking up the oligonucleotides (Fig. 2). Within 30 min after exposure, faint fluorescence was seen on the outer membrane, and by 1 h each of the cell lines showed a diffuse cytoplasmic staining. Within 2 h, up to 75% of cells showed a very intense nuclear staining. By 4 h after exposure, the nuclear fluorescence was no longer seen, and had been replaced by a coarse granular staining of the cytoplasm. After 4 h, no fluorescence was detectable. Each of the different cell types showed the same time course, pattern of staining, and proportion of cells that took up the oligonucleotides.

**Growth Rate.** When plated at an initial density of $1 \times 10^4$ cells/ml, each cell line grew to a maximum density within 10 days. The growth of the 1483 and C4–1 cells was inhibited by the presence of the antisense oligonucleotides, but not the random sequence oligonucleotides, at each of the time points examined. Representative growth curves are shown in Fig. 3.

The effect of different concentrations of oligonucleotides on 1483 cells was examined by counting cells after exposure for 8 days. On a molar basis, the antisense E7 oligonucleotide was a more effective growth inhibitor than the antisense E6 oligonucleotide. Combined treatment with 2.5 $\mu$M of each of the antisense oligonucleotides was the most inhibitory, with around 90% inhibition of growth (Fig. 4). When the composition of the antisense oligonucleotides was altered to make them complementary to a slightly 3' region that still spanned the start codon, no difference was observed in the inhibitory effect (Table 1). The Vero cells and the 183 cells were not affected significantly by any dose or combination of any of the oligonucleotides. When treatment of the HPV-containing cells with antisense oligonucleotides was stopped after 3 days, cultures resumed a growth rate similar to that of the untreated controls (Fig. 5).

**Serum Requirements.** Neither antisense nor random sequence oligonucleotides had any effect on the serum requirements of any cell line that was tested when the initial cell suspensions were permitted to recover for 5 h at 37°C in media without any oligonucleotides. How-

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**Fig. 1.** Genomic location and DNA sequence of oligonucleotides that were used. Numbers, number of the nucleotide in the genome of HPV-18 (19). The start codons of the E6 and E7 genes are underlined.

**Fig. 2.** Uptake of random sequence oligonucleotides in 1483 cells, as seen by UV fluorescence microscopy. Fluoresceinated oligonucleotides were added to the culture, which was then photographed at x 400 after 30 min (A), 1 h (B), 3 h (C), and 4 h (D).
Antisense Inhibition of Cancer Cells

Fig. 3. Effect of antisense oligonucleotides on growth of carcinoma cells. Cells (1 × 10⁴) were plated in duplicate into 24-well dishes and allowed to recover for 2 days. Media were changed and cells were cultured either in the absence of oligonucleotides (○), in the presence of 5 μM antisense oligonucleotides (▲), or in the presence of random sequence oligonucleotides (●). Cell types and specificity of the antisense nucleotides are indicated. The media were changed daily and fresh oligonucleotides were added. Cells were observed for morphological changes and were trypsinized and counted in a hemocytometer. Numbers are averaged from all experiments.

However, when cell suspensions were plated in media that contained 5 μM of either the antisense or random sequence oligonucleotide, there was a significant interference in ability of all cells to attach and form monolayers (Fig. 6).

Plating Efficiency. In the 1483 cells, when a combination of 5 μM oligonucleotides was added after 5 h of recovery and incubation, the plating efficiency was decreased approximately 40%. This occurred with both the antisense and the random sequence oligonucleotides. Neither the antisense nor the random sequence oligonucleotides affected the plating efficiency of the HPV-negative cells when the cell suspensions were incubated for 5 h prior to addition of oligonucleotides. However, when either the random sequence or antisense oligonucleotides were added to the culture media immediately, plating efficiencies of each cell type dropped significantly (Fig. 7).

Anchorage Independence. No significant changes were observed in the ability of the 1483 cells or the 183 cells to form foci in soft agar, in the presence of 5 μM either the antisense or random sequence oligonucleotides, regardless of whether the oligonucleotides were added to the cultures immediately or after a short incubation (Table 2).

Morphology. Although the antisense E6 oligonucleotide reduced the growth rate of the HPV-positive cells, it did not cause any morphological alterations. Treatment with the antisense E7 oligonucleotide, however, caused the cells to round up and detach from the surface of the culture vessel within 2 days after the initial exposure.

Table 1. Effects on cells of antisense oligonucleotides that exactly span the start codons of E6 and E7 genes of HPV-18 with oligonucleotides that span a slightly 3' sequence

<table>
<thead>
<tr>
<th>First base of oligonucleotidea</th>
<th>% Inhibition of 1483 cells Day 4d</th>
<th>Day 6e</th>
<th>% Inhibition of C4-1 cells Day 4f</th>
<th>Day 6f</th>
</tr>
</thead>
<tbody>
<tr>
<td>127</td>
<td>51</td>
<td>77</td>
<td>56</td>
<td>87</td>
</tr>
<tr>
<td>124</td>
<td>47</td>
<td>71</td>
<td>78</td>
<td>91</td>
</tr>
<tr>
<td>612</td>
<td>54</td>
<td>79</td>
<td>73</td>
<td>89</td>
</tr>
<tr>
<td>608</td>
<td>47</td>
<td>71</td>
<td>78</td>
<td>91</td>
</tr>
</tbody>
</table>

* Numbering is from the sequence of Seedorf et al. (19). The oligonucleotide starting at base 127 exactly spanned the start codon of the E6 gene, and the oligonucleotide starting at base 612 exactly spanned the start codon of the E7 gene.
* Control cultures with no oligonucleotides had 87 cells/ml.
* Control cultures with no oligonucleotides had 280 cells/ml.
* Control cultures with no oligonucleotides had 160 cells/ml.
* Control cultures with no oligonucleotides had 235 cells/ml.
Fig. 4. Effect of different doses of oligonucleotides on 183 cells (solid bars) and 1483 cells (open bars). Cells (1 x 10⁴) were plated in duplicate into 24-well dishes in oligonucleotide-free media. After 2 days, oligonucleotides were added to some of the cultures. Media were changed daily and fresh oligonucleotides were added. Each experiment was repeated at least 3 times. The results are expressed as mean ± SE percent inhibition of growth in comparison to nontreated control cultures. Oligonucleotides were either antisense E6 (ASE6), antisense E7 (ASE7), a combination of antisense E6 and antisense E7, or a combination of random sequence oligonucleotides, as indicated.

Table 2 Effects of oligonucleotides on focus formation by oral cancer cells in soft agar

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Foci</th>
<th>183 cells</th>
<th>1483 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>-</td>
<td>10.5</td>
<td>130.0</td>
</tr>
<tr>
<td>Antisense E7 (5 µM)</td>
<td>-</td>
<td>11.0</td>
<td>112.0</td>
</tr>
<tr>
<td>Antisense E6 (5 µM)</td>
<td>-</td>
<td>11.0</td>
<td>121.5</td>
</tr>
<tr>
<td>Antisense E6 + antisense E7 (5 µM)</td>
<td>-</td>
<td>9.0</td>
<td>126.5</td>
</tr>
<tr>
<td>Random E6 + random E7 (5 µM)</td>
<td>-</td>
<td>9.5</td>
<td>112.5</td>
</tr>
</tbody>
</table>

(Fig. 8). The detached cells were permeable to 0.5% trypan blue. Neither the 183 cells nor Vero cells were morphologically altered in the presence of any of the oligonucleotides.

**Interferon.** Each cell type produced interferon in a dose-dependent manner when cultured in the presence of 0.1 to 100 µg/ml of Poly(I):Poly(C). In the presence of 10 µg/ml of Poly(I):Poly(C), the levels of interferon ranged from 50 laboratory units in the case of C4–1 cells to 2,000 units in the case of Vero cells. In the presence of 100 µg of Poly(I):Poly(C), the 1483 cells produced 16,000 units of interferon. However, none of the cells produced any detectable levels of interferon when exposed to antisense or random sequence oligonucleotides.

**DISCUSSION**

Antisense technology provides a valuable tool that can be used to interfere with the expression of specific genes. First described over 10 years ago (14, 15), these modulators have sequences that are complementary to sequences of the mRNA of the gene of interest and frequently led to modifications of the phenotype of the cell.

In this study, we report the results of the phosphorothioate-modified antisense oligodeoxynucleotide treatment of human oral tumor cells. It has been previously shown that most oral and genital tumors harbor specific types of HPV, namely, types 16 and 18 (3), and that the E6 and E7 proteins of these viruses are instrumental in the immortalization and transformation of cultured human and rodent cells (6, 16–18). In cells that are transformed by HPV, the E6 and E7 genes are continually expressed (19–21), and inhibition of these genes in cervical tumor cells by expression of antisense RNA may lead to a reversion to a more normal phenotype (9, 10). Thus, it is of considerable interest to determine whether inhibition of viral gene expression in a HPV-18-containing oral cancer cell line will result in any changes in the cells.

We selected both HPV-positive and HPV-negative human cancer cell lines for the analysis of the effects of antisense E6 and E7 oligonucleotides on the transformed phenotypes of these cells as measured by standard in vitro assays. Both of the oral cancer cell lines chosen in these experiments were recently derived from human oral cancers and have been carefully characterized (11). The major difference between these 2 cell lines is that the 1483 cells contain approximately one copy of HPV-18 per cell, while the 183 cells do not carry any viral DNA (10, 22). The cervical cancer cell line C4–1 has also been shown to carry DNA of HPV-18 (9, 23). We synthesized oligonucleotides to sequences that overlap the initiation sites of the E6 and E7 genes. Previous experiments, in various systems, document initiation sites to be generally an effective target (14, 24–26). Uniform phosphorothioate modification was made to increase the stability of these molecules in the presence of cellular factors (27, 28).

Before examining the effect of the oligonucleotides on the phenotype of the cells, it was important to show that none of the cell lines was resistant to uptake of oligonucleotides. For this purpose, the oligonucleotides were labeled with fluorescein, which has does not interfere with the expression of specific genes. First described over 10 years ago (14, 15), these modulators have sequences that are complementary to sequences of the mRNA of the gene of interest and frequently led to modifications of the phenotype of the cell.

In this study, we report the results of the phosphorothioate-modified antisense oligodeoxynucleotide treatment of human oral tumor cells.
Fig. 5. Comparison of effects of continuous exposure of carcinoma cells to oligonucleotides with exposure for 4 days followed by recovery for 4 days. Cells were either untreated (●), exposed to 5 μM antisense oligonucleotides continuously (▲), or exposed to 5 μM antisense oligonucleotides for 3 days only (●). Cell types and specificity of oligonucleotides are indicated. Experimental conditions were as in Fig. 3, except that after the fourth day the media did not contain any oligonucleotides.

Fig. 6. Effect of oligonucleotides on serum requirement of cells. Cells (1 × 10⁴) were resuspended in media supplemented with 0, 0.5, 1, 2, 5, or 10% fetal bovine serum. Oligonucleotides were added to a final concentration of 5 μM either immediately, or after incubation for 5 h. Cultures were incubated and fed with fresh media and oligonucleotides daily for 10–14 days. Percent confluency of the monolayer was estimated after staining. Open boxes, untreated control cultures; stippled boxes, cells treated with antisense E6 + antisense E7 oligonucleotides; filled boxes, cells exposed to E6 + E7 random sequence oligonucleotides.

used, and were comparable with those reported in other types of cell (29, 30). Thus, the differences in the effects of antisense oligonucleotides on different cells may not be attributed to the manner of their processing, but must be due to more specific effects.

The growth rate of the HPV-18-containing 1483 cells and C4-1 cells was inhibited by the antisense but not by the random sequence oligonucleotides, at all time points and concentrations tested. Furthermore, this effect was specific for HPV-containing cells since the 183 and the Vero cells, which do not harbor HPV, were not affected by either the antisense or the random sequence oligonucleotides. The antisense E7 oligonucleotide was more effective than the E6 oligonucleotide at all concentrations tested. However, a combination of the E6 and E7 antisense oligonucleotides resulted in the greatest level of inhibition. This observation may support the fact that the E7 gene...
product plays a larger role in transformation and its maintenance in human cancer cells. The HPV-18 E7 protein alone is sufficient for the immortalization of rodent fibroblasts (6, 17) and, in cooperation with an activated ras or fos oncogene (17, 18, 31), can fully transform primary cells. The E6 protein, however, has a weaker transforming activity, perhaps acting mainly as a transcriptional activator (32) to increase the transformation potential of the E7 gene (33). Both genes are required for high frequency immortalization of human epithelial cells (7). The mechanism of action of these virus proteins is probably through their ability to bind to the products of cellular suppressor proteins, p53 and Rb (34-36).

The effect of the E6 antisense oligonucleotides appeared to be restricted to inhibition of growth of susceptible cells. However, the antisense E7 oligonucleotide also caused cellular detachment and permeability to trypan blue. This suggests that inhibition of E7 gene expression, in some HPV-positive cell lines, interferes not only with their growth but also with their survival.

The serum requirements of cells were not affected by antisense oligonucleotides, and the plating efficiency of the 1483 cells was reduced only slightly and inconsistently. However, in both assays, a significant inhibition was observed when any oligonucleotide, antisense or random sequence, was added immediately to the cultures of any cells, instead of after an interval of 4-5 h after passage. Since this effect apparently influences the early stages of cell growth, it seems likely that it is caused by a nonspecific interference with the initial attachment of the cells to the substrate. Therefore, all assays that measure some property such as the growth rate or plating efficiency should be performed on cells that have been allowed to settle and attach prior to treatment with these molecules. Anchorage independence was not affected by oligonucleotides, which is consistent with results using oligonucleotides and cells transformed by bovine papillomavirus (data not shown). At this time, it is unclear whether negative result indicates that antisense oligonucleotides cannot affect the ability of cells to form foci in soft agar, or whether it simply shows that agar prevents oligonucleotides from reaching and being taken up by target cells.

A possible mechanism of action of antisense molecules has been suggested to involve the induction of interferon by the presence of double stranded RNA (25, 37). However, the lack of interferon in the cells that were treated with antisense molecules argues against interferon as being an important mediator, particularly as the cells were capable of producing high levels of interferon when induced by Poly-(1):Poly(C). In HeLa cells that were inhibited by endogenously produced antisense RNA, no reduction was seen in levels of HPV RNA (10). Similarly, no differences were seen in levels of RNA or E6 or E7 proteins of HPV-18 in C4-1 cells by von Knebel Doeberitz et al. (9), and Storey et al. (24) found no reduction in levels of E7 protein of HPV-16 in cells that were inhibited by oligonucleotides. Thus, the mechanism by which these antisense molecules mediate their inhibition is unknown.

The present results contrast somewhat with those in a similar system by Storey et al. (24). They were able to inhibit the synthesis of DNA in cervical cancer cells that carry DNA of HPV-16 by use of oligonucleotides that were very similar to the ones used in the present study, and inhibited the growth of the cells. However, their effects on growth appeared to be nonspecific, being seen also with the C33-1 line of cervical cancer cells that reportedly does not contain DNA of HPV-16. In the present study, we used 2 cell lines as controls that do not contain DNA of HPV, and neither showed any susceptibility to antisense oligonucleotides, and previously did not show any susceptibility to expressed antisense RNA (10). Also, von Knebel Doeberitz et al. (9) did not find any susceptibility to antisense RNA of HPV-18 in another line of cells, C127, that do not have DNA of HPV. The explanation for the nonspecific effects seen by Storey et al. (24) remains unclear, but might depend on the choice of cells that were used as a negative control.

As noted previously by several groups, the inhibition of cancer cells by antisense nucleic acids has implications for the development of
Fig. 8. Cellular morphology. Cells were grown, in the presence or absence of antisense or random sequence oligonucleotides, at the concentrations indicated. Cells were either the HPV-positive 1483 cells (A) or the HPV-negative 183 cells (B). Media and oligonucleotides were changed daily, and photographs were taken after 8 days. Data are representative of 4 separate cultures.
new methods of cancer therapy (38). Antisense oligonucleotides have been infused into animals to biologically effective levels without any marked nonspecific toxicity (27), and future research should focus on the potential for in vivo effectiveness as well as clarifying the mechanisms of action.

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REFERENCES

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