ANTICANCER RESEARCH *25*: 765-778 (2005)

Activity and Therapeutic Potential of ORI-1001 Antisense Oligonucleotide on Human Papillomavirus Replication Utilizing a Model of Dysplastic Human Epithelium

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Abstract. *Human Papillomaviruses (HPVs) are small double-stranded DNA viruses that infect the cutaneous or mucosal epithelium. The high-risk genital HPVs are associated with squamous intraepithelial lesions of the anogenital region that can progress to cancer. Cervical cancer is the third leading cause of cancer death in women worldwide, yet there are no specific therapeutic treatments for HPV-associated malignancies. Development of specific antisense oligonucleotides as antiviral agents is an alternative therapeutic strategy. We utilized the organotypic raft culture system which recapitulates the entire HPV life cycle, including the production of infectious virions. We studied the effect of the ORI-1001 antisense phosphorothioate oligonucleotide designed against the E1 mRNA translation start site of low-risk HPV6 and HPV11, and tested it against high-risk HPV31b and HPV16 vegetative replication and oncogene promoter activity. ORI-1001 significantly inhibited HPV31b genome amplification. In contrast, HPV16 genome amplification was unaffected. In addition, ORI-1001 significantly downregulated transcriptional activity from a HPV31b p99 early promoter luciferase reporter construct, and inhibited E1 and E6E7 transcript expression from the wild-type genome. Our results support the idea that the antisense activity of ORI-1001 can target HPV31b functional activities in the differentiation dependent life cycle of this virus. Our results predict that binding stability between antisense oligonucleotides with partial homology to HPV genes may mediate targeting of multiple HPV types. Our studies also highlight the utility of the raft culture system in defining the parameters for testing antisense oligonucleotides against HPV.*

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Key Words: Human papillomavirus, antisense, raft cultures, replication, oncogenes.

Human papillomaviruses (HPVs) are small DNA viruses which commonly cause benign proliferations such as cutaneous and genital warts, as well as malignant epithelial lesions (1). Over 100 HPV types have been identified, out of which a subset of approximately 30 HPV types are associated with genital tract lesions (2, 3). Anogenital HPV types are classified as either "low-risk" or "high-risk" depending upon the potential of the lesions to progress to cancer (4). Low-risk types, such as HPV6 and HPV11, are associated with low-grade squamous epithelial lesions (condyloma accuminata), which rarely progress to cancer. On the other hand, high-risk types such as HPV16, HPV18, and HPV31 are associated with squamous intraepithelial lesions of the anogenital region, that can undergo malignant progression, most notably cervical cancer (5).

Despite its widespread occurrence, no specific antiviral agents for papillomavirus have been developed. Treatment of genital warts is nonspecific and consists predominantly of surgical, chemical or cryodestructive therapy (for review see reference 6). Recurring infections are common as HPVs infect the basal epithelium where the viral genome is maintained in an episomal form. Development of other control measures for HPV-associated cancer includes the development of prophylactic and therapeutic vaccines (for reviews see reference 7 and 8), as well as immunomodulatory agents (for review see reference 6).

Recent advances in the development of antisense oligonucleotides as novel therapeutic agents have generated an interest in applying this technology in the treatment of cutaneous viral diseases (for review see reference 9). Theoretically, the advantage of using antisense oligonucleotides as viral therapeutics is that such oligonucleotides have the potential ability to discriminate between healthy and infected cells by targeting specific viral gene products (10, 11). Antisense oligonucleotides have been shown to be active against Rous Sarcoma virus (12), vesicular stomatitis virus (13), herpes simplex virus type 1

(14) and type 2 (15), influenza virus (16), human T cell leukemia virus Type 1 (17) and human immunodeficiency virus (18). In addition, antisense oligonucleotides have been shown to inhibit cellular attachment of HIV (19) by nonspecific binding of the oligonucleotides to the viral receptor CD4 (20), as well as lentiviruses (21). A successful antisense-oligonucleotide based drug has already been approved for the human immunodeficiency syndrome (AIDS)-related cytomegalovirus (CMV) retinitis (22-26).

The possibilities of targeting both low-risk (27, 28) and high-risk (29, 30) HPVs by antisense strategies have been previously discussed. Antisense oligonucleotides targeted to the E2 gene of the low risk HPV6 and HPV11, as well as BPV-1, were found to inhibit E2-mediated transactivation *in vitro*, and suggested that the E2 protein was a potential target for antiviral therapy (27). A more recent study examined the effect of antisense oligonucleotides against the E1 start region of HPV11 and was shown to inhibit papillomavirus growth in a mouse xenograft model (28). However, the same oligonucleotides were shown to have "nonspecific" antiviral activity against HPV40, the target sequence of which differs by four bases when compared to HPV11 (28). In the same study, the non-specific antisense activity was also shown towards cytomegalovirus (CMV), although the anti-HPV oligonucleotides did not share any common sequences with the CMV genome. In these experiments, the oligonucleotides provided protection against CMV infection in a lethal mouse CMV model (28). Sequence-specific, non-antisense mechanisms could account for these antiviral activities (31). On the other hand, antisense oligonucleotides against the high-risk HPV18 E6 and E7 oncoproteins have been shown to specifically inhibit the growth of HPV-positive cells (29). Antisense experiments performed with an HPV16 containing cell line initially showed an inhibition of viral DNA synthesis (30), but genome replication resumed when the oligonucleotides were removed (29), thus demonstrating limitations in their efficacy and application. In another study, an HPV18 containing cell line was treated with an antisense oligonucleotide directed against the HPV 18 E6/E7 region (32). Although the transfected cells gained a more normal phenotype, the efficacy of the treatment was only manifested in cells with low copy numbers of the viral genome (29, 33, 34).

Ideally, antisense oligonucleotide therapeutics against HPV associated cutaneous as well as mucosal malignancies could be developed as creams or lotions which can be applied topically for localized treatment, thus reducing systemic side effects. As such, utilization of a HPV-infected human epithelial model would be an essential tool with which to test the activity of potential antisense oligonucleotides. In an effort to study the natural history and the HPV life cycle, and to evaluate potential anti-HPV agents, we have developed an *in vitro* epithelial model (35). The organotypic (raft) culture system supports the complete differentiation-dependent life cycle of HPV, concomitant with the production of infectious virions (36, 37). This type of a culture system is able to mimic the proliferation/differentiation cycles of the papillomavirus in the target tissue, the squamous epithelium. Such raft cultures are capable of reproducing the complete HPV18 (36), HPV16 (70), HPV45 (71) and HPV31b (37, 38, 39) life cycles *in vitro*, with the production of infectious virus. In addition, using the raft culture system we have recently generated infectious chimeric HPV18/16 virus (40).

We had two goals in the current study. First, we wished to determine whether the raft culture system could be utilized as a model system to test the efficacy and activity of antisense oligonucleotides against HPV, and if so what general parameters needed to be considered. Secondly, we utilized the raft culture system to study the activity of ORI-1001, an antisense phosphorothioate oligonucleotide directed against the E1 mRNA translation start site of HPV6 and HPV11 (31). The E1 gene codes for an early protein which is essential for the replication of the viral genome (41, 42). We have previously shown that E1 transcript levels are maximally upregulated during HPV virion biosynthesis (39). We tested and compared activity of ORI-1001 against HPV31b and HPV16 vegetative genome amplification. In addition, we also examined whether the antisense activity of ORI-1001 on HPV31b viral genome amplification correlated with E1 transcript expression. Transcript expression of the HPV31b E6 and E7 oncogenes was examined upon treatment with ORI-1001. Luciferase reporter assays were also used to determine the antisense activity of ORI-1001 on transcriptional activation from the HPV31 p99 early promoter.

Materials and Methods

Synthesis of ORI-1001. Sequence of ORI-1001 is depicted in Figure 1. Automated solid-phase synthesis of ORI-1001 was carried out as described previously (31). ORI-1001 was maintained as a 5 mM stock solution in sterile water at 4°C.

Organotypic epithelial raft cultures. The CIN-612 cell line was established from a cervical intraepithelial neoplasia (CIN) type I biopsy and contains HPV31b DNA (43). The CIN-612 9E clonal derivative maintains episomal copies of HPV31b genome at approximately 50 copies per cell (43). The W12 cell line (44) and the clone line W12 20863 (54) contain HPV16 DNA. CIN-612 9E, W12 and W12 (20863) cells were maintained in monolayer culture with E medium containing 5% fetal bovine serum in the presence of mitomycin C-treated J2 3T3 feeder cells (45, 46). Organotypic raft cultures were generated as has been previously described (36, 37, 40) and as depicted in Figure 2. These conditions have been shown to be optimal for HPV replication and production of infectious progeny (35, 36, 37, 38, 40, 47, 48). CIN-612 9E, W12 and

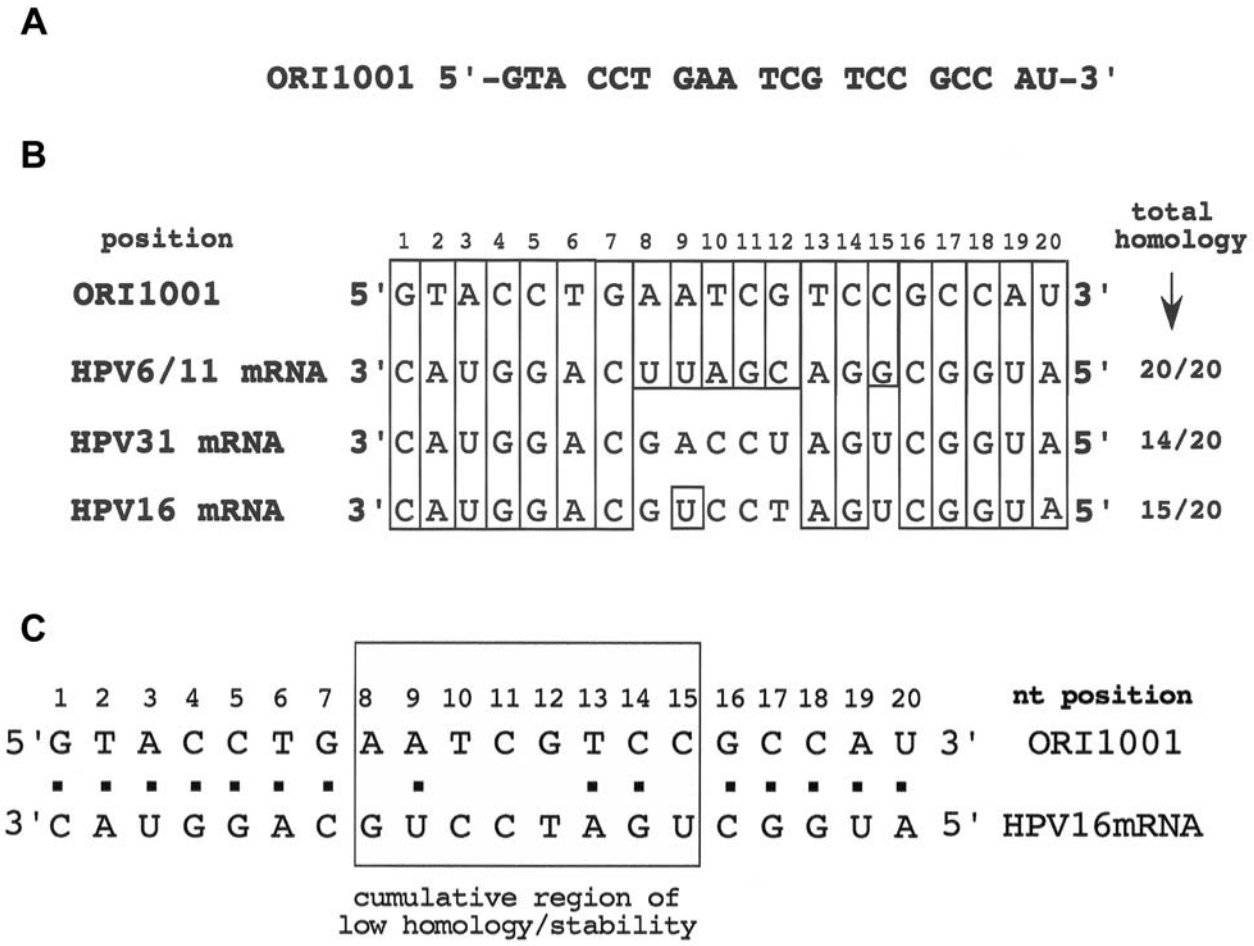

Figure 1. *A. Sequence of 20-mer ORI-1001 phosphorothioate antisense oligonucleotide designed against HPV6/11 E1 mRNA translation start site. The first fifteen ribonucleotides are 2'-deoxynucleotides. All internucleotidic linkages are phosphorothioates. The last five nucleotides are 2'-O-methoxy substituted ribonucleotides. B. Also shown, anti-sense sequence complementarity between ORI-1001 and analogous E1 sequences in HPV31 and HPV16. C. Diagram depicting region of low homology affecting interaction between ORI-1001oligonucleotide and HPV16 mRNA.*

W12 (20863) raft cultures were made by seeding $6x10^5$ cells on top of the collagen matrices. The next day, media was removed, and raft cultures were lifted to the air-liquid interface and fed every other day with E media supplemented with ORI-1001 concentrations as indicated, for a total of 10 days. For preparation of the treatment, the ORI-1001 stock was maintained on ice, and the stock solution was added to 12 ml of fresh E medium in a 15 ml Falcon tube, to achieve the desired final concentration. The treatment was then used to feed the raft cultures. Control treatments lacking ORI-1001 were used and are depicted as mock cultures. On day 10, the CIN-612 9E, W12 and W12 (20863) raft cultures were harvested by removing the epithelial layer and stored at -70˚C.

Nucleic acid extraction and Southern blotting. HPV31b and HPV16 viral genome replication in raft cultures were measured by Southern blot analysis. Total cellular DNA in raft tissue was extracted as previously described (39). For CIN-612 9E raft tissue, 2.5 μg of total cellular DNA were digested with *Hind*III which linearizes the HPV31b genome at nt 2455. For W12 and W12 (20863) raft tissue, 2.5 μg of total cellular DNA were digested with *Bam*HI which linearizes the HPV16 genome at nt 6152, separated by 0.8% agarose gel electrophoresis followed by transferring to GeneScreen Plus membranes (New England Nuclear Research Products, Boston, MA, USA), as previously reported (39). Plasmid pBS-HPV31 and pBS-HPV16 were linearized and hybridized with ^{32}P-labeled total HPV31b and HPV16 genomic DNA probes generated by random primer extension as described (39), followed by autoradiography. The intensity of the Form III linearized band was measured by scanning laser densitometry.

Ribonuclease protection assay. HPV31 E1 and E6E7 transcript analysis were performed as previously described (38, 47, 48). Briefly, radioactive antisense RNA probes were synthesized using the MAXI script/RPA II kit (Ambion Inc., Austin, Texas, USA). For detecting the E1 transcript, plasmid pCR31b-E1 was digested with *Sty*I, which yielded an antisense probe of 416 bp which was

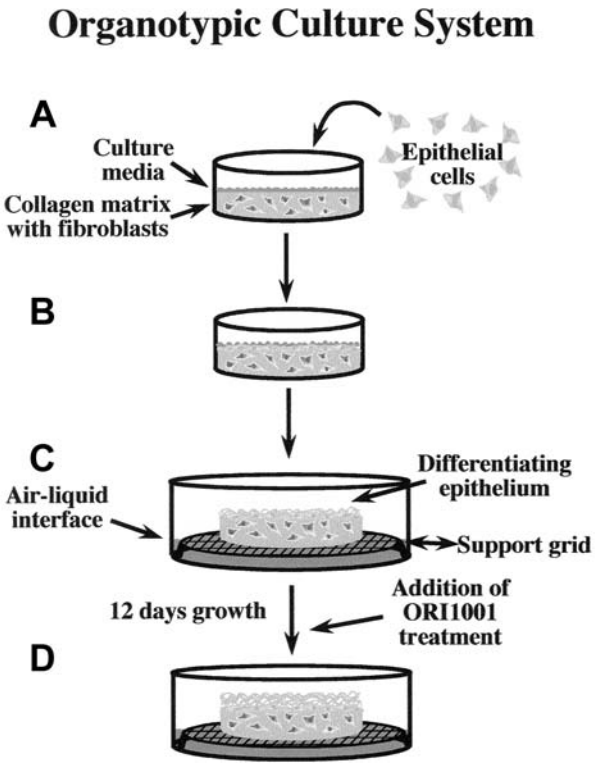

Figure 2. *Schematic of the preparation of the organotypic raft culture system. A. A dermal equivalent was prepared using rat tail collagen and fibroblasts and allowed to solidify. Epithelial cells were then added atop the dermal equivalent. B. Epithelial cells were allowed to settle in monolayer form overnight. C. Next day the dermal equivalents were lifted on wire support grids at the air-liquid interface. Media with the oligonucleotide treatment was added and the cells were fed by diffusion from beneath the wire support grid. Raft cultures were treated every other day for a total of 10 days and allowed to stratify. D. After 10 days the differentiated epithelium was harvested and used for nucleic acid manipulations and histological examination.*

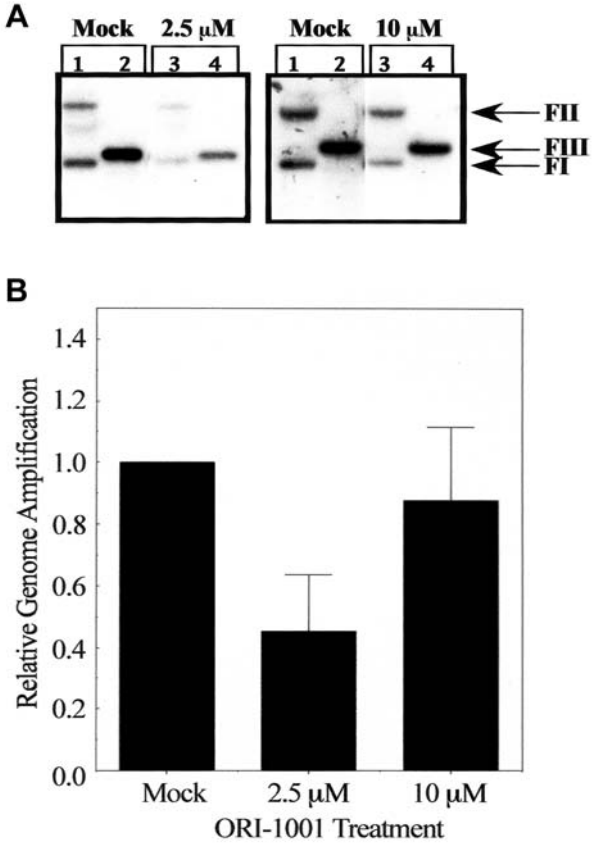

Figure 3. *A. ORI-1001 treatment decreases HPV31b genome amplification in a concentration dependent manner. Total DNA was isolated from CIN-612 9E raft cultures treated with 2.5 or 10 μM ORI-1001. 2.5 μg of the DNA was electrophoresed on a 0.8% agarose gel, transferred to nylon membrane, followed by hybridization with a HPV31b specific probe. Even numbered lanes were digested with HindIII which linearizes HPV31b DNA. Odd numbered lanes consist of undigested DNA. Lane 1-2, mock infected raft. Lane 3-4, rafts treated with 2.5 μmM and 10 μM ORI-1001 as indicated. Form I (FI) indicates supercoiled DNA, Form II (FII) indicates nicked circular DNA and Form III (FIII) indicates linearized DNA. B. Densitometric analysis of the results in A. For quantification, Form III bands at each of the treatments were scanned from individual experiments and compared with that of the mock sample which was set at 1. Treatment results with 2.5 μM ORI-1001 are cumulative of five individual experiments. Treatment results with 10 μM ORI-1001 are cumulative of three individual experiments. Error bars represent the standard error mean of the data.*

predicted to protect a 337 bp of the E1 transcript. For detecting the E6E7 transcript, plasmid pAccI was digested with, *Afl*III (49), which yielded an antisense probe of 365 bp predicted to protect a 325 bp fragment of the E6E7 transcript. Cyclophilin transcript expression was measured as an internal control (50, 51) using a cyclophilin antisense probe obtained from Ambion. The antisense probe generated was 138 bp and predicted to protect a 103 bp of the cyclophilin transcript. Probes were purified and hybridizations were performed as previously reported (38, 47, 48). Samples were analyzed by electrophoresis through 5% polyacrylamide-7 M urea gels followed by autoradiography. RNA Century standards were prepared as per manufacturer's recommendations (Ambion). The intensity of the protected fragments was measured by scanning laser densitometry. E1 and E6E7 transcript expression were normalized against the expression of the cyclophilin transcript.

Transfection and luciferase assay. The wild-type pGL2-31 URR construct was created as previously described (52). This construct contains the HPV31 upstream regulatory region cloned upstream of the firefly luciferase reporter gene. CIN-612 9E monolayer cells were transfected as previously described (53), with LipofectAMINE PLUS™ Reagent (Gibco-Life Technologies, Bethesda, MD, USA), using 1 μg of DNA construct, 6 μL of PLUS™ Reagent, and 4 μL of LipofectAMINE. 12 hours prior to transfection, cells were seeded at a density of $2x10^5$ cells per 35 mm dish in 5 mL of E medium. 12 hours post seeding, cells were washed with 1 mL PBS and transfected with serum-free media (KGM-2, Clonetics) containing 1 μg of empty vector

(pGL2-B) or the pGL2-31URR construct. After 4 hours incubation, the medium was replaced and cells were treated with the ORI-1001 at concentrations of 10 nM, 100 nM, 500 nM, 1 μM, and 2.5 μM. Mitomycin-C treated J2 fibroblasts were then seeded at a density of $5x10^4$ cells per dish. After 48 hours, cell lysates were prepared using Passive Lysis Buffer (Promega) and luciferase assays were performed with a Turner Designs TD 20/20 Luminometer using the Luciferase assay system (Promega) as recommended by the manufacturer. Luciferase activity values were normalized so that the relative luciferase activity is shown as a fold change over the luciferase activity of pGL2-31URR with no treatment, which was set to 1.

Histochemical analysis. Raft cultures were grown for 10 days as described, harvested, fixed in 10% buffered formalin, embedded in paraffin and 4-μm cross-sections were prepared. Sections were stained with hematoxylin and eosin as previously described (37).

Results

ORI-1001 downregulates HPV31b vegetative genome amplification. We investigated the effect of treating HPV31b infected raft culture tissue with increasing concentrations of ORI-1001 on HPV31b vegetative replication. We used the CIN-612 9E biopsy cell line derived from a low-grade cervical neoplastic lesion which maintains episomal copies of HPV31b (43). When grown in raft cultures the CIN-612 9E cell line reproduces neoplastic epithelial growth and differentiation, as well as the complete HPV31b life cycle including production of infectious viral particles (37). We treated CIN-612 9E raft cultures with ORI-1001 using concentrations of 2.5 μM and 10 μM. Following 10 days of growth and differentiation of the raft tissue, total DNA was extracted and Southern blots were performed with a HPV31 specific probe. As a measure of the fold-change in viral genome replication, the *Hind*III linearized HPV31 genomic Form III band was quantitated by densitometric analysis (Figure 3A). ORI-1001 affected the replication of HPV DNA in a dose-dependent manner (Figure 3B). ORI-1001 concentrations of 2.5 μM greatly decreased the HPV copy number when compared to mock infected raft tissue. In comparison, ORI-1001 concentrations of 10 μM moderately decreased the HPV copy number when compared to mock infected raft tissue. Short oligonucleotides which did not have homology to this region of HPV31 E1 mRNA do not affect HPV copy number (data not shown). The results shown in Figure 3 demonstrate a non-linear relationship between ORI-1001 treatment concentration and its effect on HPV31b genome amplification. We believe that the non-linear effects observed was due to a squelching effect caused by an overload of ORI-1001 during the 10 μM treatment. Thus, it is possible that oligonucleotide uptake and transport pathways may be affected at high concentrations, generating a less than optimal effect.

We wished to compare the antisense activity of ORI-1001 on HPV16 (another high risk virus) vegetative replication with that of HPV31b. Using the W12 parental cell line (44) we examined the effect of treating HPV16 containing raft cultures with 2.5 μM ORI-1001. ORI-1001 had negligible activity against HPV16 genome amplification (data not shown). In order to rule out the possibility that ORI-1001 was unable to affect HPV16 replication because the W12 line maintains low copy numbers of the episomal HPV16, we repeated the same experiment using the W12 (20863) clonal cell line (54), which maintains high copies of episomal HPV16 genome. ORI-1001 was also unable to affect HPV16 genome amplification in the W12 (20863) line also (data not shown), suggesting that the anti-HPV activity of ORI-1001 was more specific towards HPV31b (Figure 3 panel B).

Since ORI-1001 was designed as an antisense oligonucleotide against the identical E1 mRNA translation start sites of both HPV6 and HPV11 mRNA, we compared those sequences against the HPV31 and HPV16 analogous E1 translation start sites (Figure 1B). Sequence analysis revealed a significant degree of complimentarity between ORI-1001 and HPV31 as well as with HPV16 (70% *vs.* 75%). The ability of ORI-1001 to inhibit HPV31b genome amplification but not HPV16 was surprising given that there was an additional nucleotide at nt position 9 in the HPV16 mRNA sequence that was complementary to the antisense oligonucleotide than compared with HPV31. The ability of ORI-1001 to inhibit HPV31b genome amplification was likely due to the 5' and 3'- segments of ORI-1001 binding to the complimentary linear regions of E1 mRNA of HPV31 with greater affinity than it did with HPV16. There is complete homology of a heptanucleotide segment at the 5' end, and a pentanucleotide segment at the 3' end of ORI-1001, to complementary sites on the E1 mRNA of HPV31 (Figure 1B). Indeed, in another study, a heptanucleotide was shown to have potent antisense activity against the RNA coding for the SV40 large T antigen (55). Thus, binding of the antisense oligonucleotide with the E1 mRNA translation start site of HPV31 may interfere with efficient translation to the E1 protein, a downstream effect of the loss of E1 would result in the observed inhibition of HPV31b genome amplification (Figure 3). However, the inability of ORI-1001 to affect HPV16 genome amplification was intriguing, given a slightly greater degree of homology between the antisense oligonucleotide and HPV16 mRNA than with HPV31 mRNA. As was noted with the HPV31 mRNA sequence, the HPV16 mRNA sequence was predicted to have complete homology of a heptaneucleotide segment at the 5' end, as well as a pentanucleotide segment at the 3' end of ORI-1001 (Figure 1B). The only difference is in the presence of the uracil moiety at nt position 9 present in the HPV16 mRNA, which is able to base pair with the adenine moiety in the

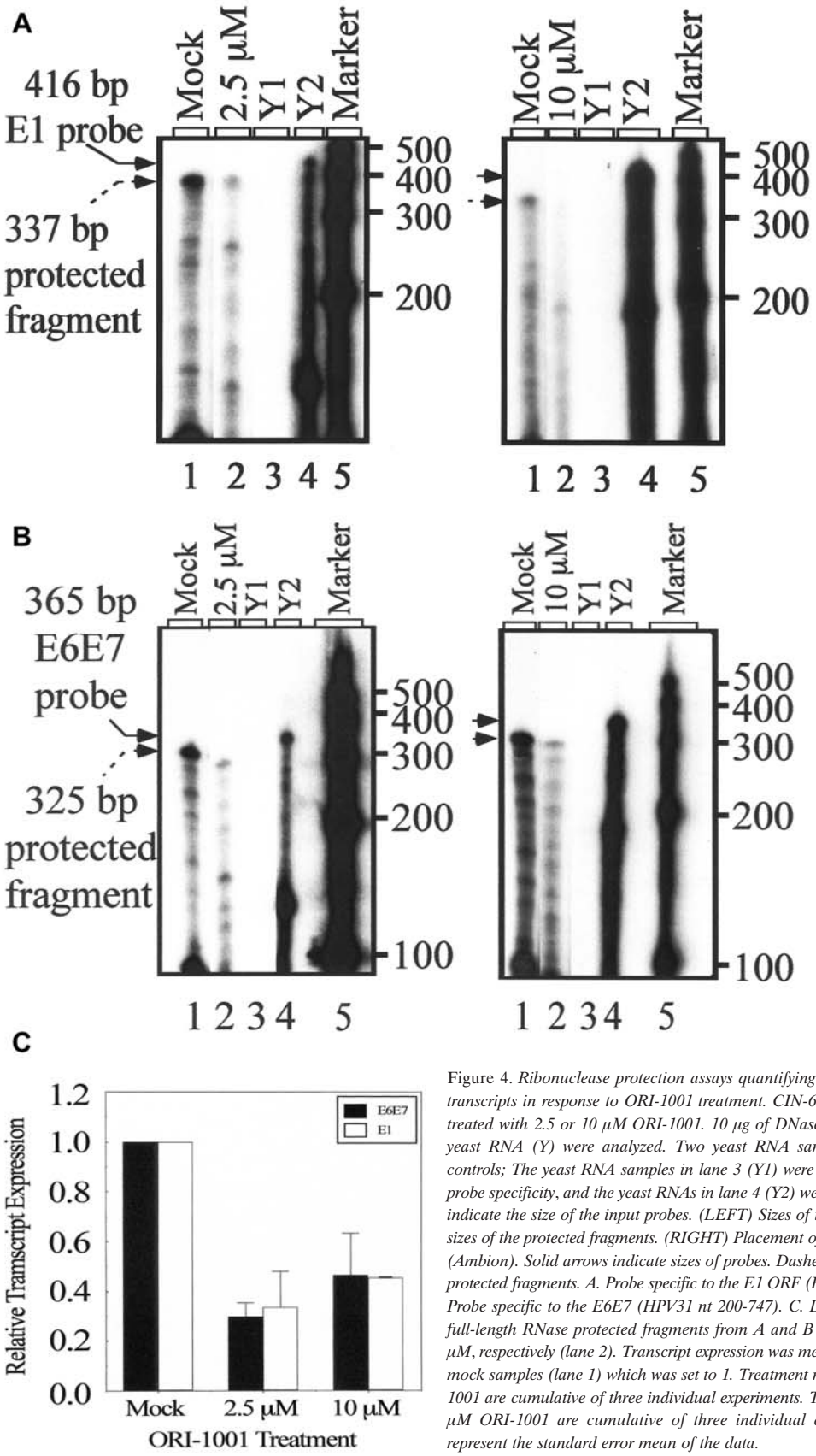

Figure 4. *Ribonuclease protection assays quantifying HPV31b E1 and E6E7 transcripts in response to ORI-1001 treatment. CIN-612 9E raft cultures were treated with 2.5 or 10 µM ORI-1001. 10 µg of DNase I-treated total RNA or yeast RNA (Y) were analyzed. Two yeast RNA samples were included as controls; The yeast RNA samples in lane 3 (Y1) were RNase digested to show probe specificity, and the yeast RNAs in lane 4 (Y2) were not RNase digested to indicate the size of the input probes. (LEFT) Sizes of the probes and predicted sizes of the protected fragments. (RIGHT) Placement of RNA Centrury Markers (Ambion). Solid arrows indicate sizes of probes. Dashed arrows indicate size of protected fragments. A. Probe specific to the E1 ORF (HPV31 nt 2064-2400). B. Probe specific to the E6E7 (HPV31 nt 200-747). C. Densitometric analysis of full-length RNase protected fragments from A and B treated with 2.5 and 10 µM, respectively (lane 2). Transcript expression was measured relative to that in mock samples (lane 1) which was set to 1. Treatment results with 2.5 µM ORI-1001 are cumulative of three individual experiments. Treatment results with 10 µM ORI-1001 are cumulative of three individual experiments. Error bars represent the standard error mean of the data.*

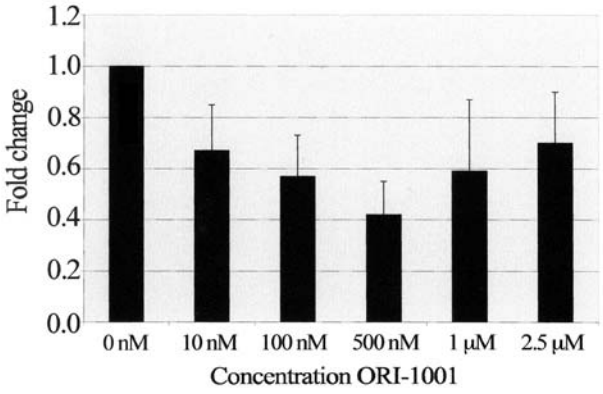

Figure 5. *ORI-1001 affects oncogene promoter activity from HPV31b p99 early promoter. The construct pGL2-31URR, consisting of nt 6921 to 121 of the upstream regualtory region (URR) of HPV31 in the pGL2-Basic vector (Promega) (see references 52 and 53), was transfected into CIN-612 9E cells using LipofectAMINE PLUS™ reagent (Gibco-Invitrogen). Cells were then treated with increasing concentration of ORI-1001 as indicated. Luciferase activity was measure after 48 hrs. Relative luciferase activity is shown as a fold change over the luciferase activity of pGL2-31URR with no treatment, which was set to 1. Treatment at each concentration was repeated three times. Error bars represent the standard error mean of the data.*

ORI-1001 sequence (with an adenine present at an identical position in the HPV31 mRNA). It is possible that an overall region of low stability may be created due to base pairing between the uracil moiety at nt position 9 on the HPV16 mRNA, equally with the adenine at either nt position 8 or nt position 9 of the ORI-1001 oligonucleotide, leading to mismatched base pairing and general destabilization of this interaction (Figure 1C). As a result, stable interaction between ORI-1001 and the HPV16 mRNA may be weak or impossible, which would agree with our observation that ORI-1001 affects genome amplification of HPV31 but not HPV16. Our results suggest that the degree of sequence homology between antisense oligonucleotides and their target mRNA may not be reliably used to predict an outcome. Overall, the results in Figure 3 strongly suggest that ORI-1001 can induce a negative effect at the level of HPV31b DNA vegetative replication in a concentration dependent manner.

Quantitation of HPV31b E1 and E6E7 transcript expression. We wanted to determine whether the decrease in HPV31b genome amplification (Figure 3) correlated with a similar decrease in early gene expression. We wanted to quantitate the expression of the E1 and the E6E7 transcripts, both of which are transcribed from the HPV31b p99 promoter of the upstream regulatory region (56). We reasoned that the HPV E1 gene product was necessary for the episomal replication of viral genomes. Thus, a loss in the E1 transcript would result in a corresponding loss of genome copy number and thus lead to a general inhibition of transcription. In addition, the E6 and E7 oncogenes transcribed from the p99 early promoter deregulate normal cell cycle processes and determine the carcinogenic potential of the HPV-infected tissue (57-65). CIN-612 9E raft cultures treated with 2.5 μM and 10 μM ORI-1001 were harvested and analyzed by ribonuclease protection assays (RPA), using antisense RNA probes specific to an internal region of E1 ORF, as well as the E6E7 spliced transcript. In untreated CIN612 9E raft cultures the E1-specific riboprobe was expected to protect a 337-nt fragment of RNA (Figure 4A). The E6E7-specific riboprobe was expected to protect a 325-nt fragment (Figure 4B). As an internal control, the expression of the cyclophilin transcript was determined. The protected fragments were quantitated using densitometry. ORI-1001 treatment significantly reduced the expression of both E1 and E6E7 transcripts (Figure 4C) at 2.5 μM as well as 10 μM concentrations compared to the mock sample. Expression of both transcripts was slightly higher in the 10 μM treated raft cultures when compared to the 2.5 μM treatments. This was not surprising since the 10 μM ORI-1001 treatment had less activity against HPV31b replication compared to 2.5 μM treated raft cultures (compare Figure 3B with Figure 4C). Control oligonucleotides which do not have homology to this region of the E1 mRNA did not affect transcript expression (data not shown). The data presented in Figure 3 and 4 cumulatively suggest that ORI-1001 significantly inhibited HPV31b genome amplification and the corresponding transcript expression.

ORI-1001 treatment results in downregulation of transcriptional activity from the HPV31 p99 early promoter. Thus far, we have demonstrated that ORI-1001 has an inhibitory effect on both HPV31b genome amplification as well as early gene expression. A consequence of the loss of E1 transcripts, and therefore E1 protein synthesis, would affect the HPV genome copy number, leading to a decrease in the number of templates available from which early transcription may occur. Since we observed suppression of both the E1 and E6E7 transcripts, we asked the question if ORI-1001 could directly affect the p99 promoter activity. In order to test this possibility, we carried out luciferase reporter assays. The HPV31 upstream regulatory region (URR) was cloned upstream of the luciferase reporter gene and transfected into CIN-612 9E monolayer cells. This cell line contains endogenously expressed HPV31b E1 protein. The transfected cells were treated with increasing concentrations of ORI-1001 (Figure 5). We observed a dose-dependent decrease in luciferase activity, with the greatest decrease at the 500 nM treatment level. At

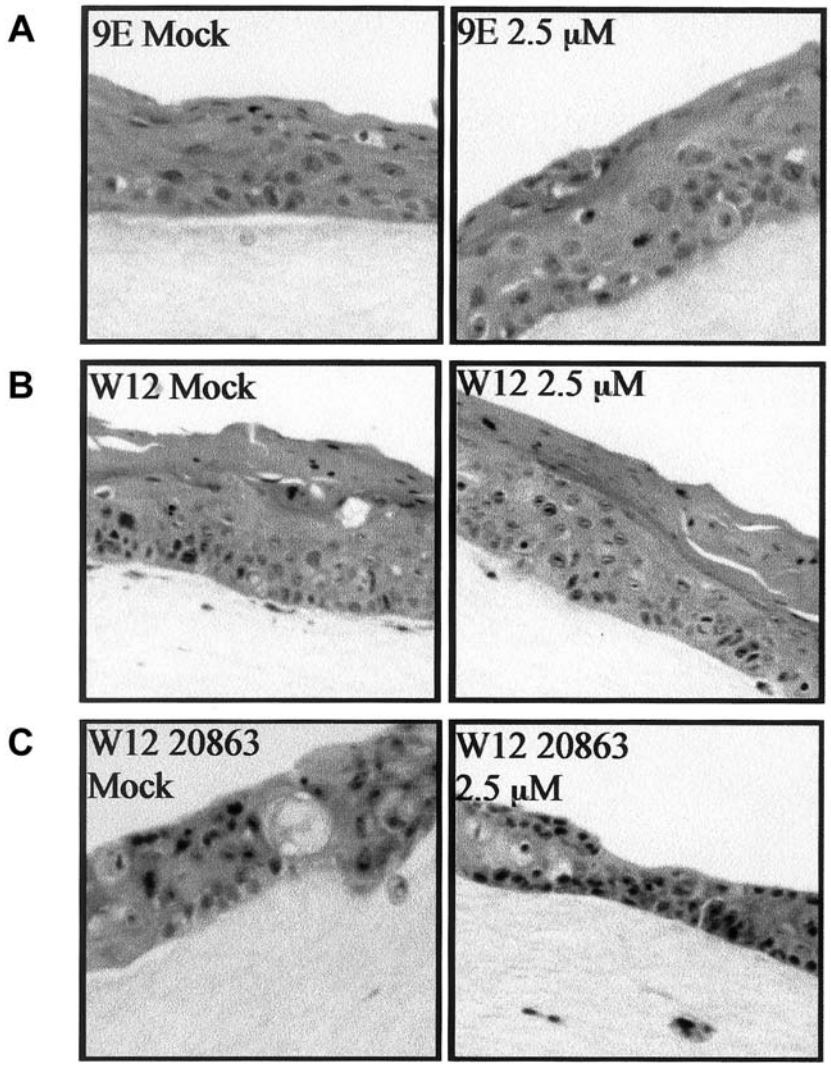

Figure 6. *Treatment with ORI-1001 has no effect on the morphology of the CIN I raft cultures. H&E staining and histological analysis of the impact of treating CIN-612 9E (Panel A) W12 (Panel B) and W12 (20863) (Panel C) raft cultures with 2.5 μM ORI-1001.*

concentrations higher than 500 nM the promoter activity was increased close to mock levels. The promoter activity can be correlated with titrating endogenous E1 transcripts in the CIN-612 9E cells by ORI-1001. Low concentrations of ORI-1001 (10 nM-500 nM) should titrate some E1 transcripts, but the cells should still have low levels to make functional E1 protein, which correlated with decreased promoter activity. Likewise, high concentrations of ORI-1001 (greater than 500 nM) should titrate more of the E1 transcripts than when treated with low levels of ORI-1001. We expected to observe a continuation of dose dependent decrease in promoter activity. However, at high concentrations, we observed promoter activity similar to that of mock treated controls. The inability to suppress promoter activity can be correlated with a possible squelching effect due to cellular overload of the oligonucleotide at these concentrations. Thus, the results in Figure 5 suggest the possibility that, in addition to its role in HPV genome replication, the HPV31b E1 protein may play a role in regulating transcriptional activity from the p99 early promoter. Interestingly, the BPV E1 protein has been shown to play a dual role in replication as well as transcriptional regulation from its own p89 promoter (66-68). The results we obtained (Figure 5) are in agreement with these studies. In addition, one might speculate that the papillomavirus E1 proteins may be endowed with common functions despite host specificity. Thus, inhibition of E1 synthesis can produce anti-HPV effects against a number of HPV types.

Treatment with ORI-1001 is not associated with cytopathic effects. Based on our results thus far, we have determined that ORI-1001 is a highly active antisense oligonucleotide against HPV31b genome amplification and transcriptional activity. Since the CIN-612 9E and the W12 cell lines used are derived from low grade CIN I cervical lesions, we could not rule out the possibility that the inhibitory activities of ORI-1001 on HPV31b was due to indirect cytotoxic effects on the tissue. Therefore, we further tested ORI-1001 for nonspecific, cytopathic properties. We perfomed H&E staining of ORI-1001 treated raft culture tissue sections and examined its effect on tissue morphology (Figure 6). Treatment with ORI-1001 was not associated with any generalized tissue cytopathic effects in both the CIN-612 9E, as well as W12 and W12 (20863) raft cultures. Since ORI-1001 was able to selectively downregulate HPV31b genome amplification and transcriptional activity (Figure 3 and Figure 4) without associated tissue cytopathic effects in CIN-612 9E raft cultures (Figure 6), we conclude that the anti-HPV activity of ORI-1001 on HPV31b was a direct result of targeting viral functions and not a by-product of indirect cytotoxicity.

Discussion

An ongoing effort has been directed towards the development of antisense oligonucleotide therapeutics as modulators of viral gene expression and replication. In general, these studies have generated valuable information about the ability of antisense oligonucleotides to target various stages of pathogenic viral life cycles. Until now, the development of antisense oligonucleotide therapies against HPV infections have not had the benefit of cell culture systems which support the differentiation-dependent life cycle of HPV. We have previously developed the organotypic raft culture system which is capable of reproducing the complete HPV life cycle, culminating in the production of infectious virions. Using the raft culture system we have also characterized the expression of early gene transcripts coding for E1, E2, E6 and E7 (69). Temporal expression patterns of HPV31b promoters over the vegetative life cycle of the virus was also described (38). In addition, HPV DNA can be transfected into primary human foreskin or ectocervical keratinocytes, with subsequent stratification and differentiation in raft culture (36). Such raft cultures are capable of producing HPV18 (36), HPV31b (37), HPV16 (70) HPV45 (71), as well as HPV31a, HPV33 and HPV39 virions (McLaughlin-Drubin and Meyers, unpublished), that were able to infect new cultures of primary human keratinocytes.

In the current study, we demonstrated the importance of the organotypic raft culture system for testing the anti-HPV activity of ORI-1001 antisense oligonucleotide, designed against the E1 mRNA start site of HPV6 and HPV11. Since the E1 gene product is required for the replication of the HPV genome, antisense targeting of this protein was a rational strategy. We demonstrated that ORI-1001 is an anti-HPV compound with activity against HPV31b. Treatment of CIN-612 9E raft cultures with ORI-1001 had a negative effect on HPV31b genome amplification as well as E1 transcript expression. The downregulation of E1 transcript expression could be correlated with HPV31b genome amplification. In contrast, genome amplification of HPV16 was unaffected by ORI-1001 treatment. We propose that the inhibitory activity of ORI-1001 towards HPV31b was due to a high degree of complimentarity between HPV31b E1 mRNA target sequence and that of ORI-1001. Stable binding of segments ORI-1001 with the complementary regions in the E1 mRNA translation start region may affect translation of the E1 protein thus lead to a loss of E1 from cells, and subsequently inhibit amplification of the HPV31b genome. In separate studies we have shown that indeed short oligonucleotides corresponding to the 5' and 3' regions of ORI-1001 inhibited HPV31b genome amplification (Alam *et al*., manuscript in preparation). Therefore, the mechanism of action of ORI-1001 is most probably targeted to an interference with translation initiation of the E1 protein. So far, arrest of protein translation as an antisense mechanism has been reported for only two studies. In the first study, the human ICAM-1 transcript was the target of antisense oligonucleotides in HUVEC cells (72). It was shown that the mechanism of inhibition of ICAM-1 protein expression was due to interference with the formation of the 80S translation initiation complex (72). In another study, antisense targeting of the human Hepatits C virus (HCV) resulted in reduced HCV core protein levels without affecting HCV RNA levels (73).

Interestingly, our results point out that the degree of complimentarity is perhaps not always a reliable prediction of stable interaction between the antisense oligonucleotide and the mRNA of interest, leading to an expected outcome. In our studies, ORI-1001 was unable to affect HPV16 genome amplification, despite having a slightly higher degree of homology with ORI-1001 as compared with HPV31. We believe that the overall regions of homology in question and their resultant binding energies may have significant effects upon stabilization of the interaction between the antisense oligonucleotide and target mRNAs being tested. Although we did not perform assays to measure delivery of ORI-1001 into the keratinocytes, it is also possible that ORI-1001 uptake was more efficient in the HPV31 positive cell line than in the HPV16 cell line, which would also help to explain why ORI-1001 was unable to affect HPV16 genome amplification. Another possibility is that E1 specific mRNAs are expressed at comparatively

higher levels in HPV16 positive cell lines than in the HPV31 cell lines, which would suggest that higher levels of antisense oligonucleotides may be required to observe a similar degree of inhibition in HPV16 genome amplification compared with HPV31.

A second possibility is that ORI-1001 may target E1 mRNA for degradation *via* activation of cellular Ribonuclease H (RNase H), since phosphorothioate oligonucleotides are excellent substrates for RNase H activity (74, 75, 76). In addition to the E1 transcript, the E6E7 transcript expression was also downregulated compared to that of untreated cells. However, upon treatment with ORI-1001, we were unable to detect any short E1 or E6E7 RNA species in the RPA-analysis performed in this study, thus indicating probable absence of cellular RNase H mediated cleavage activity. In studies targeting antisense mediated E7 expression, others have reported the appearance of shorter transcripts upon treatment, which was interpreted as RNase H-activated cleavage of the E7 RNA (77).

The modulation of the E1 and E6E7 transcript expression suggested the idea that part of the anti-HPV activities of ORI-1001 may be mediated via controlling transcriptional regulation from the p99 early promoter of HPV31b. Luciferase reporter assays demonstrated that the transcriptional activity from the p99 promoter was downregulated upon treating with increasing concentrations of ORI-1001. Since the luciferase reporter analysis was performed in the CIN-612 9E cell line, there are two possible explanations for the observed results. First, as discussed above, we believe that ORI-1001 likely binds to endogenous HPV31b E1 mRNA and inhibits translation of the protein. The second possibility is that ORI-1001 may bind to the E1 DNA coding region of the endogenous HPV31b genome, thus affecting the transcription of the E1 gene. Both instances would result in lowering of the endogenous E1 protein levels. As there are no antibodies available against the papillomavirus E1 proteins, we were unable to directly analyze expression of E1 protein levels to test our hypothesis.

There are numerous advantages to using the raft culture system for studying anti-HPV activity of antisense oligonucleotide. First, the development of anti-HPV oligonucleotide therapeutics as topical ointments or creams is highly desirable, in contrast to application via a systemic route. Toxicity studies in mice have shown that phosphorothioate oligonucleotides can produce immunologic stimulation (78), including marked splenomegaly (79, 80). Topical application of treatments would effect direct targeting of the infected epithelium. The raft culture system provides an excellent *in vitro* model system of both cutaneous and mucosal neoplastic epithelium on which to test the activity of such antisense oligonucleotides against HPV or other infectious agents. Second, treatment of HPV-associated raft culture tissue with anti-HPV therapeutics closely mimics the situation in a HPV-associated lesion *in vivo*. Third, we can generate HPV-containing cell lines with any oncogenic HPV type of choice for which the cloned genome is available, and potentially test the efficacy of antisense oligonucleotides against the virus of choice. Fourth, since raft cultures are capable of producing infectious HPV virions, we can directly correlate the efficacy of antisense oligonucleotide treatment with associated viral load.

The current study represents foundational work to test the efficacy of antisense oligonucleotides against HPVs in the raft culture system. We have demonstrated that the system can be used to separate antiviral effects from cytotoxic effects. We have generated some parameters for performing a more statistically rigorous study of anti-HPV oligonucleotides. Treatment with high doses of ORI-1001 may cause a cellular overload and hamper the ability of the cells to both absorb and transport the antisense oligonucleotides in a kinetically feasible form. These effects could be used to explain why the 10 μM treatments were less effective than 2.5 μM treatments in affecting HPV31b genome amplification and transcript expression. Based on our knowledge of the cellular and viral transcriptional systems we believe that molecular mechanisms are not always dose dependent. It would be imprudent to treat the cells with high dosages of the drug and modify their behavior in a pharmacologically irrelevant way.

We also observed that the effective oligonucleotide concentrations required for observing maximal effects on the HPV life cycle differed between monolayer and stratifying (thus multicellular) raft cultures. The monolayer cultures utilized in the luciferase assays demonstrated maximal effects at much lower concentration (500 nM) than that used in raft cultures (2.5 μM). Once again, the difference between the two systems underscores the importance of uptake and transport kinetics of the antisense oligonucleotides. Hence, multiple factors will need to be considered for effective testing of such anti-HPV compounds.

Our results also suggest that the classical field of antisense technology may be entering a new era in which complete homology between the antisense oligonucleotide and the mRNA region of interest is not necessary to mediate an effect. Our data does suggest that some of the homology plays a role, and is dependent on the stability of the interaction between the oligonucleotide and mRNA. Our studies have implications for targeting multiple HPVs with related homologies using a limited number of oligonucleotides.

In conclusion, development of anti-HPV therapeutics is very important for prevention and treatment of cervical cancer, where there exists a significant unmet medical need.

The results presented in the current study demonstrate that ORI-1001 is a potential candiate for further evaluation as a topical anti-HPV oligonucleotide agent in the treatment of HPV-associated lesions. In an effort to generate and identify oligonucleotides with specific activity against HPV, we are currently examining derivatives of ORI-1001.

Acknowledgements

We thank Danuta Huber, Lynn Budgeon and Neelam Desai for excellent technical assistance. This work was supported by an industry-university research contract from Origenix Technologies Inc., Quebec, Canada.

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Received January 13, 2005
Accepted February 18, 2005