Suppression of cervical carcinoma cell growth by intracytoplasmic codelivery of anti-oncoprotein E6 antibody and small interfering RNA

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Abstract

Cervical cancer is caused by high-risk types of human papillomaviruses (HPV) that encode the E6 and E7 oncoproteins. Silencing of E6 gene expression in HPV-positive cell lines by transfection of small interfering RNA (siRNA) with cationic lipids restores the dormant p53 tumor suppressor pathway. Because cationic lipids can also be used for intracytoplasmic delivery of proteins, we tested whether the delivery of monoclonal antibodies that bind to HPV16 E6 and neutralize its biological activity in vitro could restore p53 function in tumor cells. Here, we show that the 4C6 antibody is efficiently delivered into the cell cytoplasm using a lipidic reagent used for siRNA transfection. The delivery of 4C6 resulted in the nuclear accumulation of p53 protein in CaSki and SiHa cells but not in HeLa cells. Furthermore, the antibody-mediated p53 response was dramatically increased when a peptide corresponding to the 4C6 epitope and bearing a COOH-terminal cysteine residue was added to the transduction mixture. We found that a fraction of the added peptides were dimers that allowed the formation of antibody polymers adsorbed onto the lipidic matrix. With this system, the proliferation of CaSki and SiHa cells was strongly diminished, but no apoptosis was detectable. Remarkably, cell growth was almost totally suppressed by the addition of E6-specific siRNA to the transduction complex. The results indicate that the activity of E6 oncoprotein can be down-regulated in vivo by lipid-mediated antibody delivery and that antibodies and siRNA act synergistically when codelivered. This novel targeting strategy is simple to implement and may find therapeutic applications. [Mol Cancer Ther 2007;6(6):1728–35]

Introduction

A clear association between certain human papillomavirus (HPV) types and genital cancer has been well established and high-risk types of HPV have been identified in nearly all cervical cancer patients worldwide (1). HPVs encode the E6 and E7 proteins, which, in high-risk types such as 16 and 18, are essential for malignant transformation and the maintenance of the malignant phenotype of cervical cancer (2, 3). The transforming properties of the E6 and E7 oncoproteins are attributed to their ability to bind and inactivate the p53 tumor suppressor protein and members of the retinoblastoma protein family, respectively. Because these viral oncoproteins play pivotal roles in disrupting the cell cycle (4), they are considered potential targets for the therapeutic treatment of HPV-positive cervical cancers.

Evidence from in vitro studies shows that the transformed phenotype of carcinoma cells is lost when the expression of the E6 and E7 oncoproteins is inhibited by gene therapy approaches (5). Although antisense RNA and ribozymes were found to be effective, there is growing evidence that the use of small interfering RNAs (siRNA) that target E6 mRNA transcripts is the method of choice for inducing growth suppression, senescence, and apoptosis of HPV18-positive HeLa and HPV16-positive SiHa or CaSkıı cells (6–11). However, due to the bicistronic nature of the E6-E7 mRNA in these cell lines, it is not clear if anti-E6 siRNA on its own suppresses the expression of E6 or if targeting only E6 is sufficient for achieving growth inhibition and cell death. Recently, it has been shown that the inhibition of E6 activity in SiHa cells, using an intracellularly expressed recombinant antibody fragment that binds specifically to E6, led to changes in nuclear structure and the appearance of markers of apoptosis (12).

We previously reported the isolation of anti-HPV16 E6 monoclonal antibodies that specifically recognize E6 expressed in CaSkıı and SiHa cells. Certain antibodies that bind to the NH2-terminal region of E6 were found to totally block the E6-mediated degradation of p53 in vitro (13, 14). These antibodies were cloned as single-chain Fv fragments and accumulated as toxic aggregates when expressed in the cytoplasm (15). Recently, it has been shown that certain cationic lipids can be used for the intracytoplasmic delivery of proteins, including antibodies (16–18). Because cervical cell lines can be transfected readily with siRNA, we...
investigated whether lipid-based reagents used for siRNA transfection would also be effective for the intracellular delivery of anti-E6 antibodies. We found that the intracytoplasmic delivery of an anti-E6 neutralizing antibody by means of a commercially available cationic lipid is able to restore p53 levels in CaSkii cells. In addition, antibody-lipid mixtures generated in the presence of a peptide bearing a COOH-terminal cysteine and harboring the relevant epitope led to the formation of complexes that were readily internalized and possessed anti-E6 activity. Enhanced cell growth suppression occurred when both antibody and siRNA were included in the cationic lipid-based complexes. This new delivery system, which is likely to be applicable to other monoclonal antibodies, represents a powerful tool for analyzing the oncogenic activity of HPV16 E6 in cervical cell lines and may have potential for cancer cell therapy.

**Materials and Methods**

**Cell Culture and Peptides**

Cell lines were grown according to the American Type Culture Collection guidelines and maintained in DMEM (Invitrogen) supplemented with 1-glutamine (2 mmol/L), penicillin (100 IU/mL), streptomycin (25 μg/mL), and 10% heat-inactivated FCS at 37°C in a humidified 5% CO2 atmosphere. Fresh cells were thawed from frozen stocks after 10 passages. The synthetic peptides KRTAMFQDDPFQRPRC (K15C), KRTAMFQDPFQRPR (K14R), and KQRFHNIQRGRTGC (K14C) were purchased from NeoMPS.

**siRNA and Transfection**

Synthetic siRNAs against mRNAs encoding HPV16 E6 and HPV18 E6 as well as control siRNA were obtained from Ambion. The target sequence for HPV16 E6 was 5'-CCGCUUGUGUGAUCGUAATTC-3' (nucleotides 386–404), and for HPV18 E6 5'-CUAACUACACUGGGUAUAAT-3' (nucleotides 381–399). At 16 to 24 h before transfection, the cells were seeded at 10^6 per well in 12-well plates. For the transfection, 6 μL siRNA (2 μmol/L stock solution) was mixed with 94 μL of medium without serum. After addition of 6 μL HiPerFect (Qiagen), the mixture was incubated at room temperature for 10 min and subsequently added to cells fed with fresh complete medium. The final concentration of siRNA was 10 nmol/L. The incubated cells were harvested 48 h after transfection.

**Antibody Assays**

Monoclonal antibodies 4C6 (IgG1κ), 6F4 (IgG1κ), and 3F8 (IgG2aκ) were purified either by affinity chromatography with immobilized antigen as described (13) or by protein A chromatography (Amersham Biosciences) according to the manufacturer’s instructions. Mouse polyclonal IgGs were obtained from Sigma-Aldrich. All antibody preparations were dialyzed against 20 mmol/L HEPES (pH 7.5) and 150 mmol/L NaCl before use and kept at 4°C at a concentration of ~3 mg/mL. The ability of antibodies to bind to E6 expressed in reticulocyte extracts was tested as described (13). Transduction experiments were carried out in 24-well plates or in four-well LabTek II chambered cover glasses (Nalge Nunc) with 1.5 x 10^5 cells/mL seeded the day before. Typically, 5 μg of pure antibody were mixed with 9 μg of peptide, diluted in 20 mmol/L HEPES buffer (pH 7.5), in a total volume of 20 μL. After addition of 10 μL HiPerFect, the ingredients were thoroughly mixed by pipetting and incubated at room temperature for 20 min. At the end of the incubation period, the mixture was added dropwise onto the cells that had been washed twice and covered with 0.5 mL of serum-free medium. After 4 h of incubation at 37°C, the serum-free medium was replaced with fresh medium containing 10% serum and the cells were further incubated at 37°C for 48 h. In the case of the 3F8 antibody, 10 μg of antibody were used per assay and the cells were incubated for 24 h before adding the complete medium. For the cotreatment with siRNA, 3 μL of 2 μmol/L stock solution of siRNA were added to the antibody-peptide-HiPerFect mixture before incubation for 20 min at room temperature. The particle sizes of the transduction mixtures were determined on incubation at room temperature by dynamic light scattering using a Zetamaster 3000 instrument (Malvern Instruments) following the manufacturer’s instructions.

**Analysis by Immunofluorescence Microscopy and Western Blotting**

The cells were washed with PBS and either fixed with 4% paraformaldehyde for microscopy or harvested by trypsinization for preparing total cell extracts as described previously (19). The transduced antibodies were visualized with Alexa Fluor 488–labeled goat anti-mouse immunoglobulins (Molecular Probes), and p53 was detected with anti-p53 rabbit polyclonal serum FL-393 (Santa Cruz Biotechnology) followed by Alexa Fluor 568–labeled goat anti-rabbit immunoglobulins (Molecular Probes). Approximatively 100 μg of soluble cell proteins were loaded on gels for Western blotting. The p53, p21, poly(ADP-ribose) polymerase (PARP), and actin polypeptides were revealed with enhanced chemiluminescence (Amersham Biosciences) using anti-p53 (Ambion), anti-p21 (C-19, Santa Cruz Biotechnology), anti-GADD45α (H-165, Santa Cruz Biotechnology), anti-PARP-1 (20), and anti-actin (Sigma-Aldrich) antibodies for detection. Hoechst 33258 was used for nuclear counterstaining.

**Quantitative Reverse Transcription-PCR**

Total RNA was extracted from transfected cells (10^6) using the RNeasy Plus Mini kit (Qiagen) according to the manufacturer’s instructions. Extracted RNA (1 μg) was reverse transcribed with random hexamers using the Transcriptor First Strand cDNA Synthesis kit (Roche). Amplification of HPV16 E6 and β-actin (internal control) sequences was done with a LightCycler using the FastStart DNA Master SYBR Green I kit (Roche). The primers used for HPV16 E6 were 5'CCCAGAAGTGACCAGTACC-3' and 5'AAGCAAGTGCTATACCTCACC-3'. These primers do not allow amplification of the E6 splice variant E6*. The β-actin primers were obtained from Ambion. The PCR efficiencies of these primer pairs were calculated by doing
dilution experiments with sample cDNA. The data were analyzed using the comparative ΔΔCt method (21).

**Biacore Measurements**

The binding affinities of the monoclonal antibodies were measured at 25°C with a Biacore 2000 instrument (Biacore AB). Purified antibodies (40 μg/mL) were immobilized on the CM4 sensor chip by amine activation chemistry as described (Biacore applications handbook). The binding efficiency was evaluated by adding the corresponding epitope peptide at various concentrations (0.4–50 nmol/L) in 20 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 3.4 mmol/L EDTA, and 0.005% surfactant P20. An antibody-free surface was used as control for refractive index change and nonspecific binding. The association and dissociation rate constants were evaluated using Biaevaluation 4.1 software (Biacore AB), and data were fitted to a 1:1 Langmuir binding model. For determining the presence of dimers in the peptide solution, approximatively 3,000 relative units of antibody were immobilized on the CM4 chip. Following injection of saturating amounts of the peptide (5 μmol/L), the monoclonal antibody used for coating was added at a concentration of 200 nmol/L. The control surface was generated by injection of buffer instead of peptide.

**Clonogenic Survival Assay**

Clonogenic survival assays were done essentially as described (22). Briefly, CaSki or HeLa cells were plated in triplicate in 12-well plates (10⁵ per well) and, after 24 h, treated with siRNA or antibody as described above. After 48 h of incubation at 37°C, the cells from the triplicate wells were harvested by trypsinization and 1 of 50 of the pooled cell suspension was replated in triplicate in 60-mm-diameter tissue culture dishes. After 6 to 7 days of incubation in complete medium, the growing cells were either counted manually or stained with crystal violet after fixation with formaldehyde.

**Results**

**Intracellular Delivery of Anti-E6 Antibodies**

Previous experiments in our laboratory have shown that all commercially available cationic lipids are not equally well suited for the transfection of siRNA. Of five formulations (jet-SI, X-tremeGENE, Gene Silencer, SiPort, and HiPerFect) tested in parallel in CaSki cells for their ability to deliver anti-HPV16 E6 siRNA targeting a sequence of E6 mRNA between the E6* splice sites, HiPerFect was the most effective in terms of the number of positive cells when probed with an anti-p53 fluorescent antibody 48 h after transfection (Fig. 1A). This increase in the level of p53 protein in the nuclei of treated cells reflects the specific down-regulation of E6 activity (reviewed in ref. 10 and references therein). Because HiPerFect is chemically similar to the lipospermine DOGS that has been successfully used for the delivery of anionic proteins (17), we tested the ability of HiPerFect to transduce three purified anti-HPV16 E6 antibodies, 4C6, 6F4, and 3F8 (Fig. 1B). Whereas 4C6 and 6F4 are both capable of neutralizing HPV16 E6-mediated p53 degradation in vitro, the 3F8 antibody does not affect p53 degradation (Fig. 1C; ref. 14). The 4C6 and 3F8 antibodies could be readily detected in the cytoplasm of CaSki cells 48 h after treatment (Fig. 2A). This

**Figure 1.** Inhibition of the E6-mediated p53 degradation by siRNA or antibodies. **A**, nuclear accumulation of p53 in CaSki cells after treatment with siE6. The cells were transfected with siE6 or control siRNA (siC) and analyzed by immunofluorescence microscopy 48 h after transfection. **B**, analysis by SDS-PAGE and Coomassie blue staining of the purified antibodies used in this study. **C**, effect of the anti-E6 antibodies on in vitro E6-mediated p53 degradation. In vitro – translated p53 protein and in vitro – translated E6 protein were mixed and either no antibody or 3F8 (top) or 4C6 (bottom) antibodies (1 μg) were added to the mixture. After incubation, aliquots were taken at the time indicated and analyzed by SDS-PAGE and autoradiography. Electrophoretic migration of p53, E6, and molecular weight standards (Amersham Biosciences).
homogeneous distribution of 4C6 and 3F8 in about 5% and 30% of the treated cells, respectively, was not observed with 6F4. Analysis of the transduced cells by immunofluorescence revealed that p53 levels were increased significantly in cells bearing the 4C6 antibody, whereas no such effect was observed with the 6F4-treated or the 3F8-treated cells. To determine whether the increase in p53 levels with 4C6, which could be clearly detected 24 h after transduction, was due to specific inhibition of HPV16 E6, we did the same experiments in HeLa cells that are HPV18 E6 positive and HPV16 E6 negative. Although both 4C6 and 3F8 antibodies could be clearly identified within these cells, no p53 signal was detected even after prolonged incubation (Fig. 2B). These observations suggest that the 4C6 antibody delivered by means of HiPerFect is capable of specifically inhibiting E6-mediated p53 degradation in HPV16-positive carcinoma cells.

The 4C6 and 6F4 antibodies bind to a region of E6 close to its NH2 terminus (23), whereas 3F8 recognizes the COOH-terminal domain of E6 (14). The precise location of the epitopes recognized by 4C6 and 6F4 antibodies has been previously mapped by competition ELISA experiments using peptides: the strongest competition for both was obtained with peptide K14R that corresponds to residues 4 to 17 of HPV16 E6 (24). By using the same peptide containing an additional COOH-terminal cysteine (K15C), we showed by Biacore that the equilibrium affinity constant for 4C6 was in the nanomolar range (Supplementary Table). To test whether this peptide could affect the binding capacity of the delivered 4C6 antibody, we added it in excess to the 4C6-cationic lipid complexes in the transduction experiments. In this case, we observed a significant increase in antibody uptake with >50% of the cells being positive when probed by immunofluorescence microscopy (Fig. 2C). In addition, the level of p53 in the transduced cells was also notably increased compared with

**Figure 2.** Nuclear accumulation of p53 in CaSki and HeLa cells after antibody delivery. The CaSki (A and C) and HeLa (B) cells were transduced with the indicated relevant antibodies in the presence (C) or absence (A and B) of K15C peptide. The treated cells were processed as indicated in the legend of Fig. 1. The antibodies in the cytoplasm (Ab) were visualized by costaining with Alexa Fluor 488–labeled anti-mouse goat immunoglobulins. The exposure time of the pictures was 3 s, except that of 4C6 + K15C, which was 1.8 s. Right, merged images, including blue nuclear counterstaining. Magnification, ×400.

**Figure 3.** E6 down-regulation by concurrent treatment with siRNA and 4C6 antibody. Intensity of p53 restoration and E6 mRNA level in carcinoma cells treated with siRNA and/or 4C6 antibody. A, CaSki (top) and SiHa (bottom) cells were treated with either siE6, 4C6 and K15C, or siE6, 4C6, and K15C. After 48 h of incubation, the p53 protein level was analyzed by immunofluorescence microscopy as indicated in the legend of Fig. 1. B, E6 mRNA level in CaSki cells treated as in A. The E6-specific mRNA in the cell extracts was quantified by real-time reverse transcription-PCR and normalized to that obtained with cells treated with HiPerFect alone (100%). Columns, mean value of four independent experiments.
that obtained with 4C6 alone (Fig. 2A). Because addition of K15C peptide was not beneficial for the delivery of the 3F8 antibody, we concluded that K15C selectively favors complexion of the 4C6 antibody with HiPerFect.

**Restoration of p53 following siRNA Treatment and/or 4C6 Antibody Delivery**

To compare the efficiencies of siRNA and antibody treatments on p53 protein restoration in greater detail, we did comparative immunofluorescence assays in CaSki cells treated with either HPV16 E6-specific siRNA (siE6) or 4C6 and K15C or with all three molecules together. Compared with single treatment (siE6 or 4C6 and K15C), we observed a clear reproducible increase in the p53 level in CaSki cells after concurrent treatment (Fig. 3A). This was also the case with SiHa cells, although the total number of positive cells after mixed treatment was lower than that after siE6 treatment. As we suspected that the antibody-peptide complex and siE6 might act synergistically in down-regulating E6, we measured the relative levels of endogenous E6 mRNA by quantitative reverse transcription-PCR with E6-specific primers. Compared with untreated cells, the reduction in E6 message in siE6- and siE6/4C6/K15C-treated CaSki cells was 60% and 72%, respectively (P < 0.05; Fig. 3B). A reduction of <5% was observed in cells treated with 4C6 and K15C alone. Because the total numbers of p53-positive cells were comparable, it seems that the cationic lipid-siRNA complexes formed in the presence of antibody and peptide are taken up efficiently by the cells and that these additives do not affect and may slightly potentiate the efficiency of siRNA delivery.

p53 restoration was confirmed by Western blotting experiments with whole-cell extracts of CaSki and SiHa cells treated with different combinations of 4C6, 3F8, K15C, and siE6. As observed by immunofluorescence at 48 h after treatment, the p53 level was reproducibly maximal when 4C6, K15C, and siE6 were all present in the mixture (Fig. 4). However, the signal obtained when siE6 was omitted from this mixture suggests that the association of 4C6 with K15C is sufficient for p53 restoration. Interestingly, the light chains of the transduced antibodies could be detected readily in the extracts and this confirmed the lack of p53 stimulation by the 3F8 antibody in CaSki cells (Fig. 4, lanes 2 and 7). The results illustrate the clear advantage of adding K15C to the 4C6 antibody for inhibiting E6 in CaSki cells and show that siRNA addition to 4C6-containing protein complexes does not affect the delivery of functional antibodies.

**Analysis of the Antibody/Peptide/Lipid Complexes**

To gain further insight into the beneficial effect of K15C on antibody delivery, we investigated whether the peptide influences the formation of the cationic lipid-antibody complexes that are added to the cells by observing subconfluent CaSki cells treated for ~4 h with siE6, 4C6, or 4C6 and K15C. Whereas siRNA-HiPerFect or antibody-HiPerFect complexes were almost undetectable at the magnification used, we could clearly observe the appearance of aggregates in the presence of K15C (Fig. 5A). These aggregates were relatively homogeneous in size (~500 nm in diameter; Supplementary Table) even when siE6 was present in the mixture. Interestingly, the aggregates were almost undetectable when K15C was replaced by K14R, an identical peptide lacking the COOH-terminal cysteine residue (Supplementary Fig. S1A). Thus, it seems that K15C allows the formation of antibody-lipid complexes of higher order, possibly via disulfide-bridged dimers, which are more amenable to internalization by the cells (25). The presence of peptide dimers in the K15C preparation was confirmed by Biacore measurements (Supplementary Fig. S1B). Depending on the preparation, up to 25% of the peptide molecules were found to be dimerized (data not shown). The addition of chemically dimerized K15C to the transduction mixture also gave rise to the formation of similar aggregates (Supplementary Fig. S1A). Thus, it seems that the presence of K15C dimers in the transduction mixture allows the formation of antibody polymers connected to the lipidic matrix (Supplementary Fig. S1C) and that these complexes of defined size are well suited for efficient transduction.

![Figure 4](https://example.com/f4.png)

**Figure 4.** Analysis of the p53 restoration in CaSki and SiHa cells after treatment with siRNA and/or anti-E6 antibodies by Western blotting. The antibodies (4C6 and 3F8), peptide (K15C), and siRNAs (control siRNA and siE6) used in parallel for delivery are indicated. Forty-eight hours after treatment, the cells were lysed and crude extracts containing a similar amount of protein were analyzed by SDS-PAGE and Western blotting. The presence of the p53, actin, and antibody light chain (L) polypeptides on the blot was revealed with specific anti-p53, anti-actin, and antiserum antibodies and a peroxidase-labeled anti-immunoglobulin reagent (Materials and Methods).
Induction of Cell Growth Arrest and Synergistic E6 Down-regulation

The accumulation of p53 is generally associated with the transcription of a series of p53-responsive cellular genes (including p21 and GADD45α) that mediate the induction of growth arrest and eventually cell death (26). To determine whether the abundance of p53 observed after delivery of 4C6 and/or siRNA influences cell survival, we did Western blot analyses of the p53 target genes p21WAF1/CIP1 (27) and GADD45α (28) and of PARP, an enzyme that is cleaved by caspases soon after induction of apoptosis (29). As shown in Fig. 5A, induction of p21 could only be detected in extracts bearing a significant amount of p53 and the integrity of the 113-kDa PARP polypeptide was not affected even under the conditions of maximal restoration of p53. Cleavage of PARP could be clearly observed in CaSki cells treated in parallel with staurosporine (Fig. 5A), a drug that triggers apoptosis in cervical carcinoma cells (30). We could not detect the GADD45α polypeptides in the extracts of antibody- or siRNA-treated cells, in contrast to what is seen after single treatment with doxorubicin (results not shown). To further analyze possible apoptotic changes in the cells treated with the combined mixture of siE6, 4C6, and K15C that reproducibly led to highest level of p53 restoration (Figs. 3A and 5A), we checked the shape of the nuclei of the treated CaSki cells and of staurosporine-treated cells under the microscope. We did not observe shrinkage or fragmentation of the nuclei of cells bearing the antibodies, even after 96 h of treatment, in contrast to the effects seen with staurosporine (Fig. 5B). Although p53 remained clearly detectable after prolonged incubation (Fig. 5B), this suggests that the siRNA-antibody treatment was not sufficient to stimulate an apoptotic response in CaSki cells.

To assess whether the cell cycle (and hence proliferation) was affected, we did a series of clonogenic assays with CaSki and HeLa cells treated with either siE6, 4C6, and K15C or all three molecules together. At day 7, the cells treated with HiPerFect alone almost reached confluency, whereas those treated with siRNA and/or antibody formed colonies (Supplementary Fig. S2). The delivery of 4C6 in conjunction with K15C provoked an ~50% reduction in the number of growing cells as observed after siE6 transfection (Fig. 5C). However, a reduction of 80% was attained when siE6, 4C6, and K15C were applied

Figure 5. Biological effect of the codelivery of siRNA and anti-E6 antibodies. A, analysis of the p21 (left) and PARP (right) levels after treatment. The cells were treated as indicated and the corresponding extracts were processed as indicated in the legend of Fig. 4. The p21 and PARP polypeptides were revealed with relevant polyclonal antibodies. The slow migrating band detected with the anti-p21 antibodies corresponds to a cellular protein and served as loading control. PARPc, cleaved PARP protein; ST, staurosporine. B, nuclear shape of CaSki cells after treatment with 4C6 and K15C for 2 d (picture 1) or 4 d (pictures 3 and 4) compared with cells incubated with staurosporine for 12 h (picture 2). Green staining, expression of p53; blue staining, cell nuclei. Typical shrinkage of nuclei (arrows) observed after staurosporine treatment. Magnification, ×630. C, clonogenic survival of carcinoma cells treated with siRNA and/or 4C6 antibody. CaSki and HeLa cells seeded in triplicate were either transfected with HPV16 or HPV18 siE6, transduced with 4C6 and K15C, or treated with concurrent addition of HPV16 siE6, 4C6, and K15C. Forty-eight hours after treatment, the cells were replated and the rescued cells were counted after 1 wk of growth. Relative survival rates (in %) were obtained by dividing the cell counts in each case by that of cells treated with HiPerFect alone (Ø). Gray columns, CaSki cells; white columns, HeLa cells. Columns, mean values of five independent experiments.
together. A comparable cumulative effect, albeit to a lesser extent, was observed with similarly treated SiHa cells (Supplementary Fig. S2). Because HeLa cells were almost unaffected by such treatments, we conclude that 4C6 potentiates the siRNA-mediated suppression of cervical carcinoma cell growth.

Discussion

Since the discovery by Goodwin and DiMaio (31) that the growth-regulatory machinery, active in normal cells, is intact in HPV-positive carcinoma cells but masked by the expression of E6 and E7 proteins, there has been considerable interest in targeting these genes for reverting the masked cell cycle machinery. Among the promising gene approaches developed thus far to specifically target E6 and/or E7 (5), it was found that siRNA led to ablation of E6 in HPV18-positive HeLa cells leading to apoptosis, albeit under harsh conditions of cell transfection (7, 10). Studies done with Si6 or short hairpin RNA showed that treated HeLa or SiHa cells did not die but stopped proliferating and became more sensitive to chemotherapy (9, 22, 32). This suggests that additional cell type–specific pathways probably need to be involved if therapeutic applications are to be achieved. Although RNA interference seems to be a reliable method for down-regulating oncoproteins in diseased cells, protein therapy would circumvent the need for transfer of nucleic acid–based material into cells (33). A gene therapy approach is also problematic when the aim is intracytoplasmic expression of gene-encoded neutralizing polypeptides, such as antibody fragments, which do not fold under reducing conditions. In contrast to a recent report (12), we were unable to express soluble recombinant anti-HPV16 antibodies in carcinoma cells and we therefore investigated the possibility of transducing complete anti-E6 antibodies into CaSki and SiHa cells using cationic lipids (17). Our results show that, when antibody 4C6 directed to the NH2 terminus of E6 was delivered in this manner, it becomes more sensitive to chemotherapy (9, 22, 32). This suggests that additional cell type–specific pathways probably need to be involved if therapeutic applications are to be achieved. Although RNA interference seems to be a reliable method for down-regulating oncoproteins in diseased cells, protein therapy would circumvent the need for transfer of nucleic acid–based material into cells (33). A gene therapy approach is also problematic when the aim is intracytoplasmic expression of gene-encoded neutralizing polypeptides, such as antibody fragments, which do not fold under reducing conditions. In contrast to a recent report (12), we were unable to express soluble recombinant anti-HPV16 antibodies in carcinoma cells and we therefore investigated the possibility of transducing complete anti-E6 antibodies into CaSki and SiHa cells using cationic lipids (17). Our results show that, when antibody 4C6 directed to the NH2 terminus of E6 was delivered in this manner, it was possible to neutralize E6-mediated p53 degradation in vivo. Such a neutralization was not observed with an antibody directed to the COOH-terminal region of E6. Because antibodies cannot diffuse into the nucleus, it seems that the inhibition of E6 activity leading to p53 degradation takes place in the cytoplasm of carcinoma cells (34, 35). However, we recently found that an anti-RNA polymerase II antibody transduced under identical conditions accumulated in the nucleus (data not shown). This suggests that nuclear E6 may also be affected by the 4C6 treatment. Because E6 is a multifunctional protein (36, 37) interfering with the transcriptional program of numerous genes in HPV-positive cell lines (38), it will be of interest to determine how the 4C6 antibody affects HPV16-positive carcinoma cells, especially because it has been shown that this antibody, even when used at a high concentration, does not cross-react with cellular proteins. 

We found that a commercially available cationic lipid, recommended for siRNA transfection, is particularly well suited for the delivery of purified monoclonal antibodies into CaSki, SiHa, and HeLa cells. Because a different anti-E6 neutralizing antibody was not equally well transduced, it is possible that unique peptidic sequences displayed on the surface of immunoglobulins favor interaction with the lipid in the multilamellar complexes taken up by the cells (39). This approach has thus far been used only for fluorochrome-labeled polyclonal antibodies and enzymes, probably because of the ease with which these can easily be detected (18, 40–42). Our observation that addition of dimerized peptides increased antibody uptake probably through the formation, during the sequential mixing of the transduction products, of an “antibody network” connected to the cationic lipid skeletons (Supplementary Fig. S1C) was of considerable importance. Furthermore, the long-term inhibitory effect on E6 observed by means of these higher-order complexes was enhanced by the addition of low concentrations of siRNA, indicating that both the antigen present in the cells and that synthesized de novo could be concurrently targeted, leading to a synergistic effect. It was also found possible to code deliver to CaSki cells the 4C6 antibody together with a siRNA targeting the mitotic kinesin Eg5 (43). In this case, numerous cells showed an aberrant nuclear organization and a high content of p53 (Supplementary Fig. S3), indicating that both E6 and Eg5 functions were inhibited. It may thus be possible to use other siRNAs specifically ablat ing cellular proteins up-regulated in cervical carcinoma cells (44) for code delivery with the 4C6 antibody in an attempt to induce detectable phenotypic changes of therapeutic value.

Our results show the feasibility of a new method for the efficient delivery of neutralizing antibodies and siRNA to cells. The presumed formation of an antibody network linked together by peptide dimers was probably critical for observing the biological effect, although it was not essential for the delivery of a control antibody. Because it may be possible to deliver with this system an additional protein cargo into cells by tagging it to the peptide epitope, it will be interesting to determine the fate of bound internalized peptides in an endolysosomal cell system where proteolysis and acidification take place (45, 46). We are currently testing whether other monoclonal antibodies specific for discrete linear epitopes of intracellular targets identified by proteomic studies or profiling technologies (reviewed in ref. 47 and references therein) could be used for similar purposes.

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