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Human Papilloma Virus 16 E6 RNA Interference Enhances Cisplatin and Death Receptor-Mediated Apoptosis in Human Cervical Carcinoma Cells[S]

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ABSTRACT

In cervical cancer, the p53 and retinoblastoma (pRb) tumor suppressor pathways are disrupted by the human papilloma virus (HPV) E6 and E7 oncoproteins, because E6 targets p53 and E7 targets pRb for rapid proteasome-mediated degradation. We have investigated whether E6 suppression with small interfering RNA (siRNA) restores p53 functionality and sensitizes the HPV16-positive cervical cancer cell line SiHa to apoptosis by cisplatin, irradiation, recombinant human tumor necrosis factor-related apoptosis-inducing ligand (rhTRAIL), or agonistic anti-Fas antibody. E6 siRNA resulted in decreased E6 mRNA levels and enhanced p53 and p21 expression, demonstrating the restoration of p53 functionality in SiHa cells, without inducing high levels of apoptosis (<10%). Cell surface expression of the proapoptotic death receptors (DRs) DR4, DR5, and Fas was not affected by E6 suppression. E6 suppression conferred susceptibility to cisplatin-induced apoptosis but not to irradiation-, rhTRAIL-, or anti-Fas antibody-induced apoptosis. Combining cisplatin with rhTRAIL or anti-Fas antibody induced even higher apoptosis levels in E6-suppressed cells. At the molecular level, cisplatin treatment resulted in elevated p53 levels, enhanced caspase-3 activation, and reduced p21 levels in E6-suppressed cells. Cisplatin in combination with death receptor ligands enhanced caspase-8 and caspase-3 activation and reduced X-linked inhibitor-of-apoptosis protein (XIAP) levels in these cells. We showed using siRNA that the enhanced apoptosis in E6-supressed cells was related to reduced XIAP levels and not due to reduced p21 levels. In conclusion, targeting E6 or XIAP in combination with cisplatin can efficiently potentiate rhTRAIL-induced apoptosis in HPV-positive cervical cancer cells.

Introduction

Infection with high-risk human papilloma virus (HPV), especially HPV16 or HPV18, is the major risk factor for the development of cervical cancer (Kadaja et al., 2009). Worldwide, cervical cancer is the leading cause of cancer deaths among women (Parkin et al., 2005). High-risk HPVs infect keratinocytes in the basal layer of the mucosal cervix epithelium, and the viral replicative cycle is tied to the keratinocyte differentiation program. Because they depend on the cellular DNA machinery to replicate their genomes, the viruses have evolved a mechanism to keep the host cell in a proliferative state (Kadaja et al., 2009). The HPV E6 and E7 proteins are the viral oncogenes that can immortalize primary human keratinocytes when cooperatively expressed (zur Hausen, 2000). After early HPV-induced steps of cellular immortalization, additional cellular events are necessary for complete transformation, reflecting the long-term and multistep process of HPV-induced carcinogenesis (Kadaja et al., 2009). The E6 and E7 proteins are involved in inducing and maintaining the malignant phenotype of cervical carcinoma by interference with the cell cycle regulatory proteins p53 and retinoblastoma (pRb), respectively (zur Hausen, 2000; Kadaja et al., 2009). E7 destabilizes pRb by targeting it for proteasome-

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ABBREVIATIONS: HPV, human papilloma virus; AMC, 7-amino-4-methylcoumarin; cisplatin, *cis*-diamminedichloroplatinum(II); DISC, death-inducing signaling complex; DR, death receptor; FADD, Fas-associated protein with death domain; FCS, fetal calf serum; MTT, 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; pRb, p53 retinoblastoma; PVDF, polyvinylidene difluoride; rhTRAIL, recombinant human tumor necrosis factor-related apoptosis-inducing ligand; RNAi, RNA interference; RT-PCR, reverse transcription-polymerase chain reaction; siRNA, small interfering RNA; XIAP, X-linked inhibitor-of-apoptosis protein; MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal; CIN, cervical intraepithelial neoplasia.

mediated degradation, resulting in cell cycle progression. In response to aberrant E7-driven proliferation, the host cell triggers apoptosis or senescence by p53 activation. E6 blocks this response by targeting p53 for degradation by the ubiquitin-proteasome system (Münger et al., 2004).

Because most HPV-induced malignancies still contain wild-type p53 and pRb, reducing E6 and E7 expression in HPV-transformed cells may restore the function of these tumor suppressor proteins, thereby preventing uncontrolled proliferation. Several studies have shown that the introduction of E2 protein into HPV-transformed cells induces apoptosis or senescence, partly by inhibiting E6 and E7 transcription (Kadaja et al., 2009). Another approach to selectively reduce E6 and E7 protein expression is by using RNA interference (RNAi). Selective silencing of viral E6 and E7 expression by short interfering RNA (siRNA) may functionally restore p53 and pRb. Elevated p53 protein levels can promote apoptosis in response to stress signals, such as irreparable DNA damage or other death stimuli, by transcriptional activation of target genes or through transcription-independent mechanisms. Elevated p53 protein levels can lead to increased cell membrane expression of the death receptors (DRs) DR4, DR5, and Fas (Hougardy et al., 2006), thus potentiating the extrinsic apoptotic pathway (Ashkenazi et al., 2008). Binding of recombinant human tumor necrosis factor-related apoptosis-inducing ligand (rhTRAIL) to DR4/DR5 as well as binding of Fas ligand or agonistic anti-Fas antibody to Fas results in the activation of the extrinsic apoptotic pathway via the formation of a death-inducing signaling complex (DISC). DISC is composed of trimerized receptor molecules, Fas-associated protein with death domain (FADD), and procaspase-8 molecules. After DISC assembly, a cascade of caspases is activated, leading to the cleavage of death substrates and eventually apoptosis (Pennarun et al., 2010). Moreover, p53 also can exert a potentiating effect on the intrinsic (mitochondria-mediated) apoptotic route by inducing the expression of the mitochondria-targeting proteins Noxa, p53 up-regulated modulator of apoptosis, or Bcl-2-associated X protein (Ashkenazi et al., 2008).

In the past, we showed that DR4, DR5, and Fas are expressed in human cervical cancers (Reesink-Peters et al., 2005). The functionality of the extrinsic pathway was demonstrated in a panel of HPV-positive human cervical cancer cell lines. However, not all of the cell lines appeared to be sensitive to rhTRAIL- or anti-Fas antibody-induced apoptosis (Hougardy et al., 2005, 2006). In the present study, siRNA against HPV16 E6 was used to restore p53 functionality in rhTRAIL- and anti-Fas antibody-resistant HPV16-positive human cervical carcinoma cells. We investigated whether selective silencing of E6 expression sensitized these cervical carcinoma cells to apoptotic induction by clinically relevant DNA-damaging agents (i.e., irradiation combined with rhTRAIL or anti-Fas antibody).

Materials and Methods

Reagents and Chemicals. Dulbecco's modified Eagle's medium, Nutrient Mixture F-12 HAM medium, and trypsin stock solution (10×) were obtained from Invitrogen (Carlsbad, CA). Fetal calf serum (FCS) was purchased from Bodinco (Alkmaar, The Netherlands), 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) from Sigma-Aldrich (St. Louis, MO), and *cis*-diamminedichloroplatinum(II) (cisplatin) from Bristol-Myers Squibb Co. (Stamford, CT). rhTRAIL was home-made following a protocol as described previously (Hougardy et al., 2006), and anti-Fas monoclonal antibody (7C11) was obtained from Immunotech (Marseille, France). Dimethyl sulfoxide was purchased from Merck (Darmstadt, Germany).

Cell Lines and Cell Culture. The human cervical carcinoma cell lines HeLa S3 (HeLa) and SiHa were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown at 37°C in a humidified atmosphere with 5% CO_2 in 1:1 Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 HAM medium supplemented with 10% FCS. HeLa (HPV18-positive) and SiHa (HPV16-positive) cells contain wild-type p53. Cells were detached with 0.05% trypsin/0.5 mM EDTA in phosphate-buffered saline (PBS) [0.14 mM NaCl, 2.7 mM KCl, 6.4 mM Na_2HPO_4, 2 H_2O, and 1.5 mM KH_2PO_4 (pH 7.4)].

RNA Interference. siRNA specific for HPV16 E6, previously described by Jiang and Milner (2002), was synthesized by Eurogentec (Seraing, Belgium). The HPV16 E6 siRNA sequences were 5′-GAGGUAUAUGACUUUGCUUdTdT-3′ (sense) and 5′-AAGCAAAGUCAUAUACCUCdTdT-3′ (antisense). The p21 siRNA sequences were 5′-CUUCGACUUUGUCACCGAGdTdT-3′ (sense) and 5′-CUCGGUGACAAAGUCGAAGdTdT-3′(antisense) (Spierings et al., 2004). The X-linked inhibitor-of-apoptosis protein (XIAP) siRNA sequences were 5′-GUGGUAGUCCUGUUUCAGCdTdT-3′ (sense) and 5′-GCUGAAACAGGACUACCACdTdT-3′ (antisense) (Hougardy et al., 2006). The negative control siRNA without any known homology with the human genome was purchased from Eurogentec. HeLa (0.3×10^6 cells/well) and SiHa (0.2×10^6 cells/well) cells were transfected in six-well plates at 30 to 50% confluence with 33 or 133 nM siRNA duplexes using Oligofectamine transfection reagent according to the manufacturer's instructions (Invitrogen). After 24 h, the cells were harvested and used for an apoptosis assay and, corresponding to treatment conditions, protein isolation, respectively. Transfection efficiency was determined by flow cytometry analysis of HeLa and SiHa cells transfected with fluorescein isothiocyanate-labeled nonspecific oligonucleotides (≥80%).

mRNA Quantification. Total cellular RNA was extracted and purified with the RNeasy kit from QIAGEN (Valencia, CA). Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed in 96-well plates using the SYBR Green method on a MyiQ real-time detection system (Bio-Rad Laboratories, Hercules, CA) with glyceraldehyde-3-phosphate dehydrogenase as an internal control. A gradient RT-PCR was performed to assess primer specificity and to optimize annealing temperature (T_{ann}). Primers used for E6 mRNA amplification were 5′-GGAATCCATATGCTGTATGT-3′ (forward) and 5′-CCCAAGCTTACAGCTGGGTTTCTCTACG-3′ (reverse). Amplification of the samples was carried out in triplicate in a final reaction volume of 25 μl, containing 12.5 μl of iQ SYBR Green Supermix, 1 μl of each gene-specific primer (5 μM), and 5 μl of cDNA (1:50). The thermocycling program used for each run consisted of an initial 3 min of denaturation at 95°C, followed by 40 cycles of 15 s of denaturation at 95°C, 20 s of primer annealing at the primer-specific T_{ann}, and 30 s of fragment elongation at 72°C. The presence of unique reaction products was determined from the melting curves obtained at the end of 40 cycles of amplification.

To determine RT-PCR efficiencies and initial starting quantities of the samples, a standard curve was generated using a 1:3 serial dilution from the total starting cDNA sample. Water controls were included to check for DNA contamination. Differences in the amounts of starting cDNA samples were corrected using glyceraldehyde-3-phosphate dehydrogenase as a housekeeping reference gene.

Cytotoxicity Analysis. The microculture tetrazolium MTT assay was used to assess the cytotoxicity of E6 siRNA. A total of 15,000 SiHa cells were incubated in a total volume of 200 μl. Treatment consisted of continuous incubation with negative control siRNA or HPV16 E6 siRNA at a concentration of 133 nM. After 96 h, 20 μl of MTT [5 mg/ml PBS (6.4 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 mM NaCl, and 2.7 mM KCl, pH 7.2)] was added for 3.75 h. Next, the

plates were centrifuged, and the supernatant was aspirated. After the formazan crystals were dissolved by the addition of dimethyl sulfoxide (Merck), the plates were read immediately at 520 nm using a microtiter well spectrometer and microplate reader (Bio-Rad Laboratories). Cell survival was defined as the growth of treated cells compared with that of untreated cells.

Irradiation. Exponentially growing cell cultures were irradiated using a ^{137}Cs gamma ray machine (IBL 637; Cis-Bio International, Gif-sur-Yvette, France) at a dose rate of 0.783 Gy/min.

Flow Cytometry. Cells were transfected with siRNA oligomers as described above, and 24 h after transfection, cells were subjected to flow cytometry. Cells were harvested by trypsinization, washed in ice-cold PBS, and diluted in ice-cold PBS containing 2% FCS and 0.1% sodium azide. Cells were incubated on ice for 30 min with phosphatidylethanolamine-conjugated mouse anti-human DR4 or DR5 antibodies (Alexis Benelux, Breda, The Netherlands) at final concentrations of 20 μg/ml. Phosphatidylethanolamine-conjugated mouse IgG1 (BD Pharmingen, San Diego, CA) served as an isotype control. After being washed, cells were resuspended in 200 μl of PBS/2% FCS/0.1% sodium azide and analyzed (10,000 cells) by flow cytometry (Epics Elite; Beckman Coulter, Inc., Fullerton, CA). The fluorescence intensity is a measure for DR expression on the cell surface. For every treatment condition, three independent experiments were performed that were normalized to each other using the total fluorescence.

Detection of Apoptosis. In a 96-well culture plate, 5000 SiHa cells were seeded in 100 μl of culture medium. On the next day, cells were irradiated 24 h before the addition of 0.1 μg/ml rhTRAIL or 1 μg/ml anti-Fas antibody for another 24 h. Otherwise, cells were pretreated for 2 h with 10 or 15 μM cisplatin, followed by 24 h of incubation with rhTRAIL or anti-Fas antibody at 37°C in a total volume of 200 μl of culture medium. Control cells were seeded with only medium or single-drug treatment. Apoptosis was defined by the appearance of apoptotic bodies and/or chromatin condensation and expressed as the percentage of apoptotic cells counted by fluorescence microscopy in three fields containing at least 300 cells (Hougardy et al., 2006).

Detection of Caspase Activity. Cell lysates (25 μg) were transferred to a microtiter plate and snap-frozen over liquid nitrogen. To initiate the reaction, 50 μM of the caspase substrate carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (AMC) (Peptide Institute Inc., Osaka, Japan) in assay buffer [100 mM HEPES, 10% sucrose, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, 5 mM dithiothreitol, and 0.0001% IGEPAL CA-630 (pH 7.25)] was added to cell lysates. Substrate cleavage leading to the release of free AMC was monitored at 37°C at intervals of 60 s for 25 cycles using a Varioskan multilabel reader (Thermo Fisher Scientific, Breda, The Netherlands) (excitation, 355 nm; emission, 460 nm). Enzyme activity was expressed as nmol AMC released $\cdot$ min^{-1} $\cdot$ mg protein^{-1}.

Western Blot Analysis. Exponentially growing cells were harvested, washed in ice-cold PBS, and lysed in SDS sample buffer [4% SDS, 20% glycerol, 0.5 M Tris-HCl (pH 6.8), and 0.002% bromphenol blue] containing 10% β-mercaptoethanol, by boiling for 5 min in a water bath. Proteins were separated on a SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Billerica, MA) by semidry or wet blotting. Western blot analyses were performed as described by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) using skim milk as a blocking agent. Equal loading of protein was checked by Coomassie Brilliant Blue (Bio-Rad Laboratories) staining of the SDS-polyacrylamide gel and Ponceau S (Sigma-Aldrich) staining of the PVDF membrane. The following antibodies were applied: mouse anti-p53 (DO-1) and mouse anti-p21 (F5) antibodies from Santa Cruz Biotechnology, mouse anti-caspase-8 (1C12), anti-caspase-3 (9662), and anti-cleaved caspase-3 (9661s) antibodies from Cell Signaling Technology (Danvers, MA), and mouse anti-XIAP antibody from Transduction Laboratories (Alphen aan de Rijn, The Netherlands). Secondary antibodies conjugated with horseradish peroxidase were obtained from Dako Denmark A/S (Glostrup, Denmark). Chemiluminescence was detected using BM Chemiluminescence Blotting Substrate (POD) or Lumi-Light^{PLUS} Western Blotting Substrate (Roche Diagnostics, Basel, Switzerland). Protein expression levels were densitometrically analyzed with ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical Analysis. Statistical analysis was performed using Student's t test. P values <0.05 were considered significant.

Results

HPV16 E6 siRNA Causes Functionally Enhanced p53 Levels in SiHa Cells. To determine the effect of selectively abrogating E6 mRNA expression on apoptotic induction, we used HPV16 E6 siRNA. Treatment with 133 nM E6 siRNA suppressed E6 mRNA levels to 53 $\pm$ 14% S.D. compared with those of negative control siRNA-treated cells. The decrease was observed at 24 h posttransfection (Supplemental Fig. 1). We tested several HPV16 E6 antibodies as described recently (Sima et al., 2008) but were unable to detect HPV16 E6 in SiHa cells, which are known to have extremely low copies of HPV16 (Herrington et al., 1992). Therefore, p53 protein levels were used as a functional readout for the efficacy of HPV16 E6 down-regulation (Sima et al., 2008; Yamato et al., 2008; Liu et al., 2009). Transfection of the cells with HPV16 E6 siRNA resulted in the up-regulation of p53 protein levels compared with those transfected with negative control siRNA and the nontransfected matching control. Restoration of p53 also was associated with p21 up-regulation in transfected SiHa cells, most clearly in E6-supressed cells (Fig. 1A), reflecting transcriptionally active p53. No effect of HPV16 E6 siRNA on p53 and p21 was observed in HPV18-positive HeLa cells (data not shown), indicating selective HPV16 E6 silencing.

The effect of E6 suppression on cell survival was studied. Cells transfected with negative control or E6 siRNA displayed only slightly less survival compared with that of nontransfected cells (Fig. 1B).

E6-Suppressed SiHa Cells Are Especially Sensitized to the Combination of Death Ligands and Cisplatin. We demonstrated previously that SiHa cells are resistant to rhTRAIL- and anti-Fas antibody-induced apoptosis (Hougardy et al., 2005, 2006). Suppression of E6 in SiHa cells

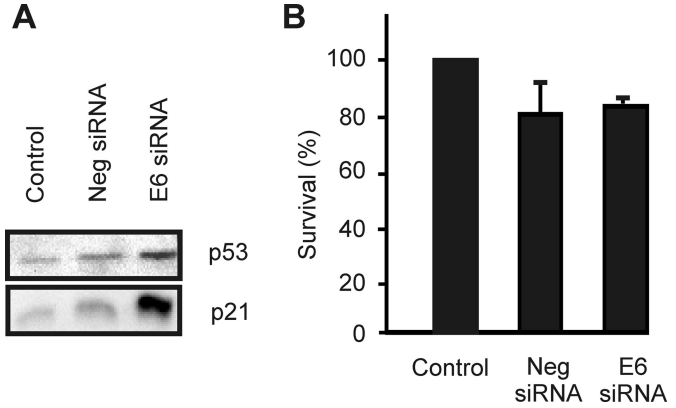

Fig. 1. Efficacy of HPV16 E6 siRNA sequence in SiHa cells. A, p53 and p21 expression in SiHa cells 24 h after transfection with E6 or negative control siRNA. Nontransfected cells (control) are included. HPV16 E6 suppression induces cell growth inhibition, but it is not significantly different from that of control or negative control siRNA-transfected cells. B, cytotoxicity assay of transfected SiHa cells. Cells were transfected with 133 nM E6 or negative control siRNA, 24 h later reseeded, and 4 days later subjected to cytotoxicity analysis. Values are mean $\pm$ S.D. of three independent experiments.

induced a small but significant increase in rhTRAIL- or anti-Fas antibody-induced cell death (Fig. 2A). Current optimal therapy for locally advanced cervical cancer consists of radiotherapy in combination with cisplatin-based chemotherapy. Therefore, irradiation was combined with rhTRAIL or anti-Fas antibody in E6-suppressed cells. Irradiated E6 siRNA-transfected SiHa cells were more sensitive to death ligand-induced apoptosis than irradiated negative control siRNA-transfected cells (Fig. 2b). This indicates that irradiation and E6 siRNA sensitize these cells in a selective and cooperative manner to death ligand-induced apoptosis, although to a relatively small extent because additional apoptosis did not exceed 10% ($P < 0.05$).

In contrast to irradiation, treatment with cisplatin alone induced more apoptosis in E6-suppressed cells compared with that in negative control siRNA-treated cells (Fig. 3A). Moreover, E6-suppressed cells became more sensitive to death ligand-induced apoptosis by cisplatin pretreatment compared with matching negative control siRNA-transfected cells. For example, up to 40% additional apoptosis was observed in E6-suppressed cells treated with 15 μM cisplatin plus rhTRAIL compared with negative control siRNA-transfected cells treated with the same concentration of cisplatin plus rhTRAIL ($P < 0.05$), thus indicating a significant enhancement (Fig. 3A). An additional 20% apoptosis was observed in E6-suppressed cells treated with cisplatin plus anti-Fas antibody compared with matching negative control siRNA-transfected cells ($P < 0.05$) (Fig. 3B). A caspase-3 activity assay supported the apoptosis assay, because substantial enhancement of caspase-3 activity was observed in E6-suppressed cells exposed to cisplatin, whereas the largest effect was seen in E6-suppressed cells exposed to cisplatin and rhTRAIL (Fig. 3C). However, caspase-3 activation did not exactly mirror the apoptotic levels. This might be due to the fact that caspase-3 activation is an early marker for apoptosis and is degraded rapidly (Albeck et al., 2008). Therefore, caspase-3 activity assays performed on cells 24 h after apoptotic induction probably underestimate caspase activation due to the loss of active caspase-3 in late apoptotic cells as measured by the acridine orange assay. The enhanced apoptosis of E6-suppressed cells in response to cisplatin is associated with p53 up-regulation. Cisplatin treatment resulted in the loss of p21 expression (Fig. 3D), suggesting the involvement of additional cellular factors in cisplatin-induced apoptosis in E6-suppressed cells.

Changes in DR4, DR5, and Fas Cell Surface Expression after E6 siRNA or Cisplatin Treatment. Cisplatin induced significant increases in DR4 and DR5 cell surface levels in SiHa cells. E6 siRNA did not further enhance the effect of cisplatin on DR4 or DR5 expression levels (Fig. 4, A and B). Negative control siRNA and E6 siRNA transfection resulted in strong induction of Fas cell surface levels. Cisplatin further enhanced Fas cell surface expression in siRNA-transfected cells only (Fig. 4C). These results demonstrated that cisplatin induced cell surface expression of DR4, DR5, and Fas, whereas E6 siRNA did not have any specific effect on receptor surface expression.

Cisplatin Increases rhTRAIL-Induced Activation of Caspase-8 and Caspase-3 in E6 siRNA-Transfected SiHa Cells. Next, we investigated the effect of cisplatin on intracellular caspase activation events in E6-suppressed cells in response to rhTRAIL treatment. Compared with negative control siRNA-transfected cells, E6 siRNA-transfected cells exposed to rhTRAIL or cisplatin plus rhTRAIL showed more caspase-8 cleavage, demonstrated by an increase in p43/p41 cleavage products and the appearance of the active caspase-8 fragment (p18). rhTRAIL induced the cleavage of caspase-3 into supposed active products (Fig. 5). However, this did not correspond clearly to the levels of apoptotic induction (Fig. 3A) nor to caspase-3 activity levels (Fig. 3C), suggesting that the functionality of caspase-3 was inhibited. In response to cisplatin, more cleavage of caspase-3 into active products and enhanced caspase-3 activity were detected in E6-suppressed cells. Treatment of the cells with a combination of cisplatin and rhTRAIL resulted in a further increase in caspase-3 activation, with the largest effect being visible in E6-suppressed SiHa cells (Figs. 3C and 5). Moreover, with this combination, reduced levels of full-length XIAP protein were observed, especially in E6-suppressed cells (55% reduction) (Fig. 5). Similar results were observed in E6-supressed cells treated with anti-Fas antibody, alone or in combination with cisplatin (data not shown).

Effect of p21 and XIAP Down-Regulation on Cisplatin and rhTRAIL Sensitivity. The apoptosis-inducing combination of E6 siRNA, cisplatin, and rhTRAIL resulted in a decrease in p21 and XIAP protein levels. To further investi-

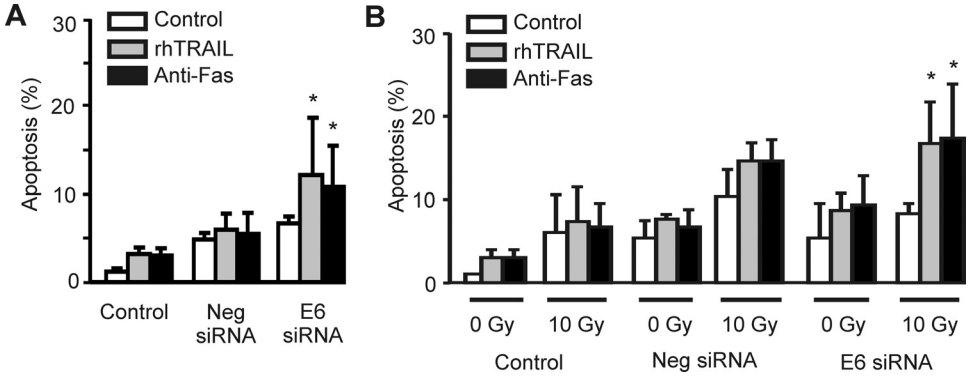

Fig. 2. Sensitivity of HPV16 E6-suppressed SiHa cells to irradiation-, rhTRAIL-, or anti-Fas antibody-induced apoptosis. A, cells were transfected and 24 h later reseeded. On the next day, cells were treated with 0.1 μg/ml rhTRAIL or 1.0 μg/ml anti-Fas antibody for another 24 h. Data represent the mean ± S.D. of three independent experiments (*, $P < 0.05$ for E6 versus negative control siRNA-transfected cells). B, cells were transfected with E6 or negative control siRNA and 24 h later reseeded. On the next day, the cells were irradiated with 0 or 10 Gy, and 24 h later 0.1 μg/ml rhTRAIL or 1.0 μg/ml anti-Fas antibody was added for another 24 h. Data represent the mean ± S.D. of three independent experiments (*, $P < 0.05$ for irradiated versus irradiated and rhTRAIL- or anti-Fas antibody-treated E6-suppressed cells).

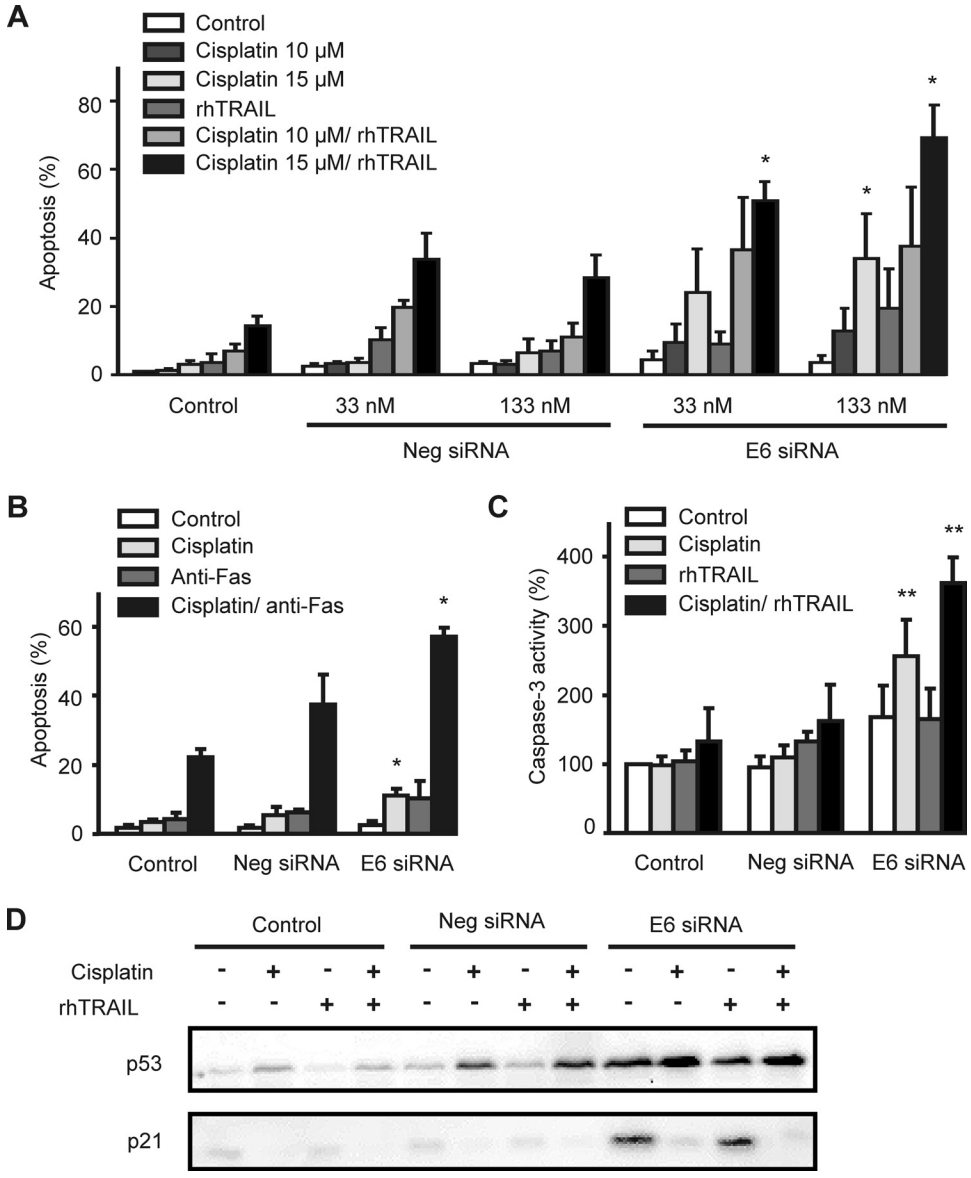

Fig. 3. Sensitivity of E6-suppressed SiHa cells to rhTRAIL or anti-Fas antibody-induced apoptosis is enhanced by cisplatin. A, cells were transfected and 24 h later reseeded. On the next day, cells were treated with cisplatin (10 or 15 μM) for 2 h and then 0.1 μg/ml rhTRAIL for another 24 h. Apoptosis was scored by acridine orange staining of cells. B, cells were transfected and 24 h later reseeded. On the next day, cells were treated with 15 μM cisplatin for 2 h and then 1 μg/ml anti-Fas for another 24 h. Apoptosis was scored by acridine orange staining. C, caspase-3 activity levels in transfected SiHa cells. Cells were transfected and 24 h later reseeded. On the next day, cells were treated with 15 μM cisplatin for 2 h and then 0.1 μg/ml rhTRAIL for another 24 h; afterward, the cells were harvested, and DEVDase activity was measured. Data represent the mean $\pm$ S.D. of three independent experiments (*, $P < 0.05$; **, $P < 0.01$ for E6 versus E6-negative control siRNA-transfected cells) (A–C). D, Western blot of p53 and p21 levels in SiHa cells transfected with E6 or negative control siRNA and 24 h later reseeded. On the next day, pretreatment for 2 h with 15 μM cisplatin was followed by 0.1 μg/ml rhTRAIL for another 24 h. For each Western blot, one representative of three independent experiments is shown. Equal loading of protein was checked by Ponceau S staining of the PVDF membrane.

gate the role of p21 and XIAP in apoptotic cell death of SiHa cells, we used a siRNA approach. Down-regulation of p21 and XIAP at the protein level was confirmed by immunoblotting 48 h posttransfection (Fig. 6A). Transfected cells were pretreated with 15 μM cisplatin for 2 h followed by 0.1 μg/ml rhTRAIL for another 24 h and then stained by acridine orange. Down-regulation of nearly detectable p21 did not change apoptotic levels compared with those of negative control siRNA-transfected cells, either with cisplatin, rhTRAIL, or a combination of both drugs. We already showed that E6 siRNA transfection resulted in strongly elevated p21 levels, whereas cisplatin abrogated this effect (Fig. 3D). Therefore, we used p21 siRNA in E6-suppressed cells to test whether p21 down-regulation could substitute for cisplatin for its effect on rhTRAIL-induced apoptosis. However, cells transfected with combined p21/E6 siRNAs showed low apoptotic levels comparable to those of E6-suppressed cells after rhTRAIL treatment. This indicates that cisplatin has other non-p21-related effects that sensitize E6-suppressed cells to rhTRAIL (Fig. 6, A and B). XIAP suppression had no sensitizing effect on cisplatin- or rhTRAIL-induced apoptosis in SiHa cells, whereas XIAP suppression significantly enhanced apoptosis induction by cisplatin combined with rhTRAIL. This demonstrates that E6 suppression can be replaced functionally with XIAP suppression (Fig. 6B). Next, SiHa cells were transfected with XIAP/E6 siRNAs and treated with cisplatin or rhTRAIL alone. Apoptosis levels remained low, and only in combination with cisplatin and rhTRAIL, a strong induction of apoptosis was observed, showing that combined XIAP/E6 suppression cannot replace either cisplatin or rhTRAIL. Taken together, our results indicate that XIAP reduction and E6 suppression have functionally similar effects on apoptosis when combined with cisplatin and rhTRAIL.

Discussion

The present study shows that E6 suppression plus cisplatin strongly induced apoptosis in HPV16-positive SiHa human cervical cancer cells. This apoptotic effect was enhanced further by adding rhTRAIL or anti-Fas antibody. Enhanced caspase-8 and caspase-3 activation and the stronger XIAP

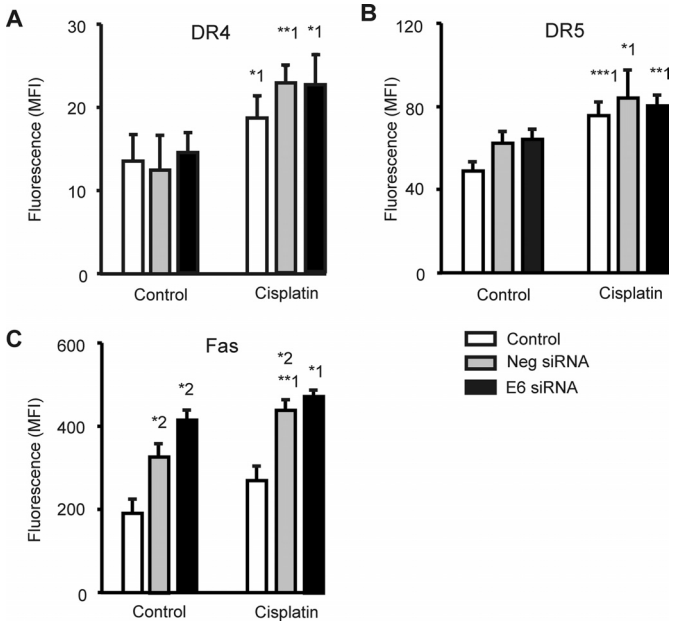

Fig. 4. DR4, DR5, and Fas membrane expression in E6-suppressed SiHa cells. A–C, DR4 (A), DR5 (B), and Fas (C) membrane expression by flow cytometry. Cells were transfected, and on the next day, 15 μM cisplatin was added for 24 h. The mean fluorescence intensities were corrected for staining with a nonspecific isotype control. Data represent the mean ± S.D. of three independent experiments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; 1, cisplatin-treated cells versus matched control; 2, transfected cells versus matched control).

cleavage suggest that both intrinsic and extrinsic apoptotic pathways were activated. These results indicate that the functional inhibition of E6 combined with cisplatin and death ligands may be an effective strategy to specifically enhance apoptosis and overcome resistance to rhTRAIL and anti-Fas antibody in HPV-positive cervical cancer.

Several studies have used an siRNA approach to suppress HPV E6 expression in cervical cancer cells. It is generally considered that E6 and E7 of malignant HPVs, including HPV16, are transcribed as a single bicistronic mRNA. However, as a result of alternative splicing of the HPV E6 open reading frame, three bicistronic variants of E6 and full-length E7 have been described previously (cited in Tang et al., 2006), allowing selective silencing of E6 without affecting E7. siRNA specific against full-length HPV18 E6 showed more growth suppression and cell death induction in HeLa cells compared with those of siRNA that targets both E6 and E7 production (Putral et al., 2005; Yamato et al., 2006). HPV16 E6 silencing in SiHa cells by an siRNA sequence, targeting both full-length and E6 splice variants, resulted in the inhibition of cell growth and colony formation (Yoshinouchi et al., 2003). A HPV16 E6 siRNA sequence against the full-length transcript clearly induced apoptosis in SiHa cells but only at day 4 after siRNA treatment (Butz et al., 2003).

The siRNA in the present study targeted full-length HPV16 E6. This resulted in ~50% reduction in E6 mRNA but did not induce much apoptosis when administered alone, which is comparable with the results of earlier studies using the same E6 siRNA sequence (Jiang and Milner, 2002; Liu et al., 2009). A range of siRNAs targeting different sequences of HPV16 E6 and/or E7 have been tested in cervical cancer cells in in vitro and in vivo studies. Depending on the E6 siRNA sequences used, the effect in SiHa cells can be either apoptosis induction (Butz et al., 2003) or senescence (Yamato et al., 2008; Eaton et al., 2011). These results imply that RNAi directed against different E6 splicing variants may exert different effects on apoptosis, cell growth, and colony formation. Yamato et al. (2008) demonstrated an even more effective HPV16 E6 knockdown in SiHa cells with other E6 siRNAs. Despite the stronger reduction in E6 mRNA, a similar induc-

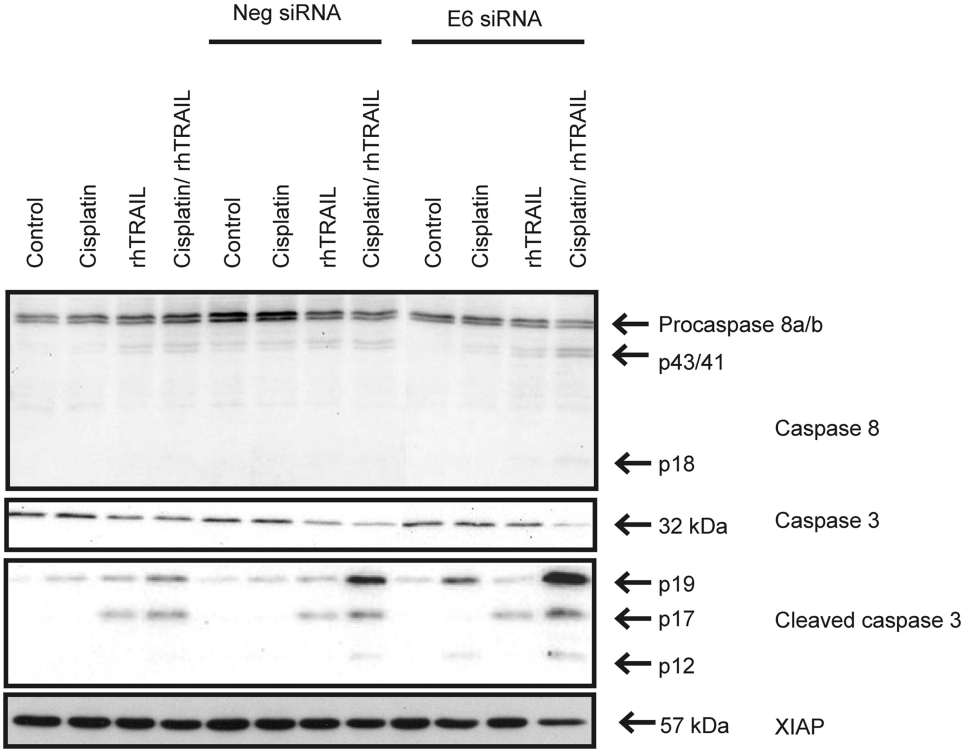

Fig. 5. Caspase activation and XIAP down-regulation in E6-suppressed SiHa cells treated with cisplatin and rhTRAIL. Cells were transfected and on the next day pre-treated for 2 h with 15 μM cisplatin followed by rhTRAIL for 24 h. For each Western blot, one representative of three independent experiments is shown. Equal loading of protein was checked by Ponceau S staining of the PVDF membrane.

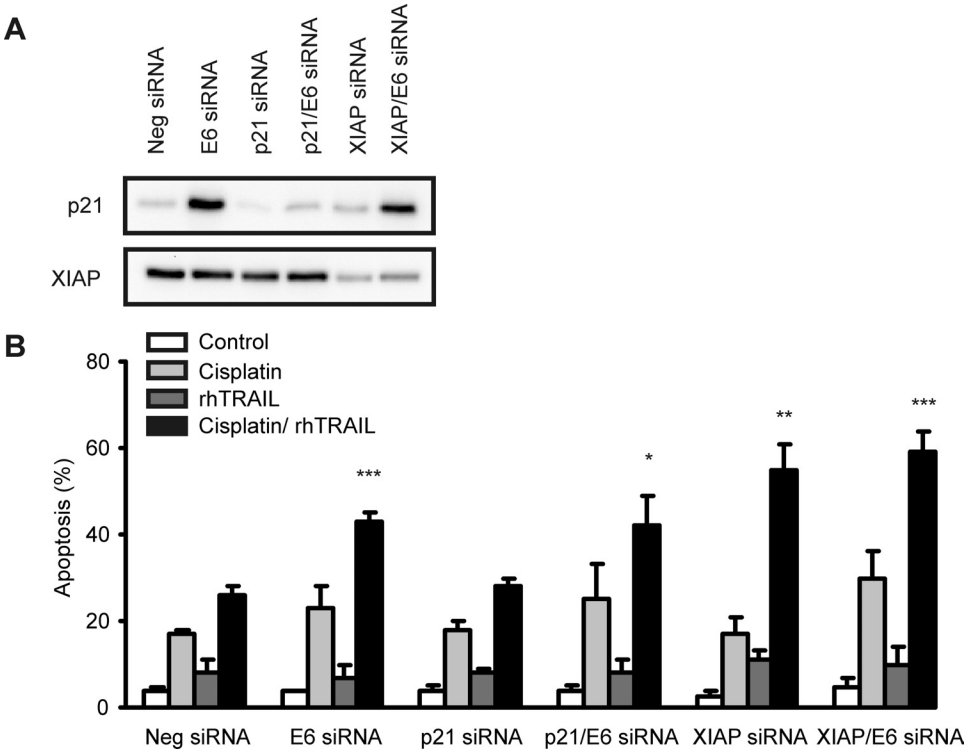

Fig. 6. XIAP down-regulation enhances sensitivity to a combination of cisplatin and rhTRAIL. A, p21 and XIAP expression 48 h posttransfection of E6, p21, p21/E6, XIAP, and XIAP/E6. Ponceau S staining served as a loading control. B, cells were transfected with 133 nM siRNA for single siRNA transfection and 66 nM of each siRNA in double transfection (total 133 nM) and 24 h later reseeded. On the next day, cells were pretreated with 15 μM cisplatin followed 2 h later by 0.1 μg/ml rhTRAIL. Apoptotic cells were counted 24 h later. Data represent the mean ± S.D. of three independent experiments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for E6, p21/E6, XIAP, or XIAP/E6 siRNA versus negative control siRNA-transfected cells).

tion of p53 was observed in SiHa cells when the more effective E6 siRNAs were compared with the E6 siRNA that was used in our study as well (Yamato et al., 2008). However, the lower concentrations of more effective E6 siRNAs may avoid off-target effects and, therefore, especially reduce the small effect that we observed with the negative control siRNA.

We showed that silencing of E6 after transfection with a single dose of E6 siRNA resulted in elevated p53 protein levels. It has been reported that p53 can transcriptionally activate DR4, DR5, and Fas (Guan et al., 2001; Liu et al., 2004; Zuckerman et al., 2009) and is involved in Fas trafficking to the cell membrane (Bennett et al., 1998). In addition, we demonstrated previously that enhanced p53 expression after proteasome inhibition caused strong DR5 membrane expression induction in SiHa cells (Hougardy et al., 2006). Upon E6 suppression, however, no major changes in DR4, DR5, or Fas membrane expression in SiHa cells were found. Notably, restoration of p53 expression and p53 transcriptional functionality by E6 siRNA was not sufficient to enhance apoptosis. Only in combination with cisplatin, an induction of apoptosis could be observed in E6-suppressed SiHa cells. At the molecular level, we found that cisplatin treatment caused elevated DR4 and DR5 membrane expression, which was not enhanced further by E6 suppression, whereas the highest p53 levels were detected in E6-suppressed cells after cisplatin treatment in SiHa cells. Two studies described that p53 activation after E6 RNAi was transient in HeLa and SiHa cells. Prolonged p53 stabilization was achieved by cisplatin in E6-suppressed HeLa cells (Koivusalo et al., 2005; Putral et al., 2005). Moreover, prolonged p53 activation resulted in the activation of the intrinsic pathway in E6-suppressed HeLa cells (Vogt et al., 2006), which may occur in E6-suppressed SiHa cells as well. The decreased p21 levels after cisplatin treatment of E6-suppressed SiHa cells may affect apoptosis, because p21 not only mediates p53-induced cell cycle arrest but also can suppress apoptosis after exposure to DNA-damaging agents, such as cisplatin, and death receptor-mediated apoptosis (Koster et al., 2010). However, down-regulation of p21 had no effect on apoptosis in E6-suppressed SiHa cells after cisplatin and/or rhTRAIL treatment, which excludes a role for p21 in our setting.

We were surprised to find that cisplatin-induced apoptotic levels in E6-suppressed cells could be enhanced further by adding rhTRAIL or anti-Fas antibody. We and others showed that SiHa cells were resistant to rhTRAIL- or anti-Fas antibody-induced apoptosis (Aguilar-Lemarroy et al., 2001; Hougardy et al., 2006; Eaton et al., 2011). The resistance was attributed to the inability to recruit FADD and caspase-8 to DISC (Aguilar-Lemarroy et al., 2001; Hougardy et al., 2005; Hougardy et al., 2006), which is probably caused by the binding of HPV16 E6 to FADD and the relatively low levels of caspase-8 in SiHa cells compared with those of other cervical cancer cell lines (Filippova et al., 2004; Hougardy et al., 2005). Although reduced caspase-8 levels have been related to E6-induced degradation in osteosarcoma cells overexpressing exogenous HPV16 E6 (Filippova et al., 2007), we found no caspase-8 up-regulation after E6 suppression. rhTRAIL or anti-Fas antibody treatment resulted in more cleavage of caspase-8 and a concomitant increase in caspase-3 cleavage in E6-suppressed cells. Only when E6-suppressed cells were exposed to cisplatin plus death ligands, a further enhancement of caspase-8 and caspase-3 activation as well as a moderate reduction (55%) in XIAP levels and apoptosis were observed (Fig. 5). It implies that caspase-3 functionality after rhTRAIL or anti-Fas antibody treatment was inhibited by an anti-apoptotic protein, such as XIAP (Salvesen and Duckett, 2002; Hougardy et al., 2006). We demonstrated here that XIAP down-regulation only could substitute for E6 suppression without reducing apoptosis of the most effective combination treatment but not cisplatin or rhTRAIL. XIAP was

down-regulated very efficiently and caused an almost similar effect as E6 suppression, suggesting that the partial reduction in E6 mRNA did not largely affect the soundness of our conclusions. The mechanism by which E6 suppression in combination with cisplatin and rhTRAIL induces caspase-8 and caspase-3 activation and reduces XIAP levels needs to be investigated further.

The fact that the viral E6 oncogenes of high-risk HPV types are indispensable for cervical carcinogenesis and maintenance of the malignant phenotype (zur Hausen, 2000) presents E6 as an ideal target for a specific cervical cancer therapy. RNAi offers the ability to selectively induce the degradation of a specific target mRNA, thereby preventing protein expression. Some studies illustrated that it is possible to inhibit cervical tumor growth in mice by E6 siRNA as a monotherapy (Chang et al., 2010) and in combination with paclitaxel (Liu et al., 2009) or cisplatin (Jung et al., 2011). Other anticancer drugs aimed at the E6 and E7 activities may be more appropriate for the cocktail treatment (Tan et al., 2012). The effect of E6, for example, can be diminished through proteasome inhibition, which results in higher p53 levels. Previously, we showed that proteasome inhibition by *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal (MG132) sensitized cervical carcinoma cells to rhTRAIL-induced apoptosis (Hougardy et al., 2006). Next, we tested MG132 in combination with TRAIL on normal cervical explants and cervical intraepithelial neoplasia (CIN3) explants and found much less apoptosis in normal cervices compared with that in CIN3 lesions, indicating a therapeutic window (Hougardy et al., 2008). This option is realistic for further exploration in the short term, because the proteasome inhibitor bortezomib is already used in the clinic (Jagannath et al., 2010). In addition, both rhTRAIL and agonistic DR4 and DR5 antibodies are now being tested in phase I/II clinical trials also in combination with radiotherapy and cisplatin in cervical cancer (Mahalingam et al., 2009; Tan et al., 2012). Our results also present XIAP as putative target in combination with DR4- or DR5-targeting drugs. It is interesting to note that novel small molecule inhibitors that target XIAP are currently in early clinical development (Amm et al., 2011). Testing of combination treatments with E6/E7-targeting drugs on noncancerous human cervical cells will be important. Unfortunately, the cervical explant model is very laborious, whereas only E6/E7-immortalized cervical cell models have become available, which strongly limits the options for drug toxicity testing in normal cervical cells.

In conclusion, our results demonstrate that E6 siRNA effectively suppresses E6 expression in HPV16-positive SiHa cells. Cisplatin sensitizes E6 siRNA-treated cells to apoptosis, which is strongly enhanced by adding rhTRAIL. These results may be translated into the clinical setting by XIAP or proteasome inhibitor utilization instead of an siRNA approach.

Authorship Contributions

Participated in research design: Tan, Hougardy, de Vries, van der Zee, and de Jong.

Conducted experiments: Tan, Hougardy, Meersma, and Schaap.

Performed data analysis: Tan, Hougardy, Meersma, Schaap, and de Jong.

Wrote or contributed to the writing of the manuscript: Tan, Hougardy, de Vries, van der Zee, and de Jong.

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