HPV16 E6 RNA INTERFERENCE ENHANCES CISPLATIN AND DEATH RECEPTOR-MEDIATED APOPTOSIS IN HUMAN CERVICAL CARCINOMA CELLS

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ABSTRACT

In cervical cancer the p53 and pRb tumor suppressor pathways are disrupted by the human papilloma virus (HPV) E6 and E7 oncoproteins, since E6 targets p53 and E7 targets pRb for rapid proteosome-mediated degradation. We have investigated whether E6 suppression with small interfering (si)RNA restores p53 functionality and sensitizes the HPV16-positive cervical cancer cell line SiHa to apoptosis by cisplatin, irradiation, recombinant human tumor necrosis factor (TNF)-related apoptosis-inducing ligand (rhTRAIL) or agonistic anti-Fas antibody. E6 siRNA resulted in decreased E6 mRNA levels, enhanced p53 and p21 expression, demonstrating restoration of p53 functionality in SiHa, without inducing high levels of apoptosis (less than 10%). Cell surface expression of the proapoptotic death receptors 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 XIAP levels in these cells. We showed using siRNA that this enhanced apoptosis in E6-suppressed 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 development of cervical cancer (1). Worldwide, cervical cancer is the leading cause of cancer deaths among women (2). High-risk HPVs infect keratinocytes in the basal layer of 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 virus has evolved a mechanism to keep the host cell in a proliferative state (1). The HPV E6 and E7 proteins are the viral oncogenes which can immortalize primary human keratinocytes when cooperatively expressed (3). Following early HPV-induced steps of cellular immortalization, additional cellular events are necessary for complete transformation, reflecting the long-term and multi-step process of HPV-induced carcinogenesis (1). 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 (1, 3). E7 destabilizes pRb by targeting it for proteasome-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 (4).

Since 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 introduction of E2 protein into HPV transformed cells induces apoptosis or senescence, partly by inhibiting E6 and E7 transcription (1). 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 (5), thus potentiating the extrinsic apoptotic pathway (6). Binding of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (rhTRAIL) to DR4/DR5 as well as binding of Fas ligand (FasL) or agonistic anti-Fas antibody to Fas results in activation of the extrinsic apoptotic pathway via the formation of a death-inducing signaling complex (DISC). The DISC is composed of trimerized receptor molecules, Fas-associated protein with death domain (FADD) and procaspase-8 molecules. Following DISC assembly a cascade of caspases is activated, leading to cleavage of death substrates and eventually apoptosis (7). Moreover, p53 can also exert a potentiating effect on the intrinsic (mitochondria-mediated) apoptotic route by inducing expression of the mitochondria targeting proteins Noxa, Puma or Bax (6).
In the past, we showed that DR4, DR5 and Fas are expressed in human cervical cancers (8). Functionality of the extrinsic pathway was demonstrated in a panel of human HPV-positive cervical cancer cell lines. However, not all cell lines appeared to be sensitive to rhTRAIL- or anti-Fas antibody-induced apoptosis (5, 9). In the present study siRNA against HPV16 E6 was used to restore p53 functionality in rhTRAIL and anti-Fas antibody resistant human HPV16-positive 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 and cis-diamminedichloroplatinum(II) (cisplatin), combined with rhTRAIL or anti-Fas antibody.

**MATERIALS AND METHODS**

**Reagents and chemicals**

Dulbecco’s MEM, Nutrient Mixture F-12 (HAM) medium and trypsin stock (10x) solution were obtained from Invitrogen-Life Technologies (Merelbeke, Belgium). Fetal calf serum (FCS) was purchased from Bodinco (Alkmaar, the Netherlands), and 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) from Sigma-Aldrich (Zwijndrecht, the Netherlands) and cis-diamminedichloroplatinum(II) (cisplatin) from Bristol-Myers (Weesp, the Netherlands). RhTRAIL was home-made following a protocol as described previously (5), and anti-Fas monoclonal antibody (7C11) from Immunotech (Marseille, France). Dimethyl sulfoxide (DMSO) was purchased from Merck (Amsterdam, the Netherlands).

**Cell lines and cell culture**

The human cervical carcinoma cell lines HeLa S3 (HeLa) and SiHa were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were grown at 37°C in a humidified atmosphere with 5% CO₂ in 1:1 DMEM/HAM medium supplemented with 10% FCS. HeLa (HPV18 positive) and SiHa (HPV16 positive) 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 Na₂HPO₄·2 H₂O, 1.5 mM KH₂PO₄· pH 7.4).

**RNA interference**

SiRNA specific for HPV16 E6, previously described by Jiang and Milner (10), was synthesized by Eurogentec (Seraing, Belgium). The HPV16 E6 siRNA sequence was 5’-GAG-GUAUAUGACUUUGCUU-dTdT3’ (sense) and 5’AAGCAAAGUCAUAUACCUC-dTdT3’ (antisense). The p21 siRNA sequence was 5’CUUCGACUUUGUCACCGAG-dTdT3’ (sense) and 5’CUCCGUGACAAAGUGCGAA-dTdT3’ (antisense) (11). The XIAP siRNA sequence was 5’GUGGUAGUCCUGUUCAGC-dTdT3’ (sense) and 5’GCUGAAACAGGACUACCAC-dTdT3’ (antisense) (5). The negative control siRNA without any known homology with the human
genome was purchased from Eurogentec (Seraing, Belgium). HeLa (0.3x10⁶ cells/well) and SiHa (0.2x10⁶ cells/well) cells were transfected in 6-wells plates at 30-50% confluency with 8, 33 or 133 nM siRNA duplexes using Oligofectamine transfection reagent according to the manufacturer’s instructions (Invitrogen-Life Technologies, Breda, the Netherlands). After 24 h 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 FITC-labeled non-specific oligonucleotides (≥ 80%).

*mRNA quantitation*

Total cellular RNA was extracted and purified with the RNeasy kit from Qiagen (Venlo, the Netherlands). Real time RT-PCR was performed in 96-well plates using the SYBR Green method on a MyiQ real time detection system (Biorad) with GAPDH as internal control. A gradient RT-PCR was performed to assess primer specificity and to optimize annealing temperature (\(T_{\text{ann}}\)). 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 of 25 µl containing 12.5 µl IQ SYBR Green Supermix, 1 µl of each gene specific primer (5 µM) and 5 µl cDNA (1:50). The thermocycling program used for each run consisted of an initial 3 min denaturation at 95°C, followed by 40 cycles of 15 s denaturation at 95°C, 20 s primer annealing at the primer specific \(T_{\text{ann}}\) and 30 s fragment elongation at 72°C. Presence of unique reaction products was determined from the melting curves obtained at the end of 40 cycles of amplification.

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

*Cytotoxicity analysis*

The microculture tetrazolium (MTT) assay was used to assess cytotoxicity of E6 siRNA. 15,000 SiHa cells were incubated in a total volume of 200 µl. Treatment consisted of continuous incubation with negative siRNA or HPV16 E6 siRNA at 133 nM concentration. After 96 h, 20 µl of MTT (5 mg/ml phosphate buffered saline (PBS): 6.4 mM Na2HPO4; 1.5 mM KH2PO4; 0.14 mM NaCl; 2.7 mM KCl; pH=7.2) was added for 3.75 h. Next the plates were centrifuged and the supernatant aspirated. After dissolving the formazn crystals by adding dimethyl sulfoxide (Merck, Amsterdam, the Netherlands), the plates were read immediately at 520 nm using a microtiter well spectrometer (Bio-Rad microplate reader, Bio-Rad Laboratories BV, Veenendal, the Netherlands). Cell survival was defined as the growth of treated cells compared to untreated cells.
Irradiation

Exponentially growing cell cultures were irradiated using a $^{137}$Cs $\gamma$-ray machine (IBL 637, CIS biointernational, 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 trypan蓝izing, washed in cold PBS and diluted in cold PBS containing 2% FCS and 0.1% sodium azide. Cells were incubated on ice for 30 min with PE conjugated mouse anti-human DR4 or DR5 antibodies (Alexis Benelux, Breda, the Netherlands) at a final concentration of 20 µg/ml. PE conjugated mouse IgG1 (BD Pharmingen, Erembodegem, Belgium) served as isotype control. After washing, cells were resuspended in 200 µl PBS/2% FCS/0.1% sodium azide and analyzed (10,000 cells) by flow cytometry (Epics Elite, Coulter-Electronics, Hialeah, FL). The fluorescence intensity is a measure for DR expression on the cell surface. For every treatment condition three independent experiments were performed which were normalized to each other using the total fluorescence.

Detection of apoptosis

In a 96-well culture plate 5,000 SiHa cells were seeded in 100 µl culture medium. The next day cells were irradiated 24 h prior to 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 incubation with rhTRAIL or anti-Fas antibody at 37°C in a total volume of 200 µl 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 in three fields containing minimally 300 cells by fluorescence microscopy (5).

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 carbobenzyoxy-Asp-Glu-Val-Asp-AMC (7-amino-4-methylcoumarin) (DEVD-AMC) (Peptide Institute Inc., Osaka, Japan) in assay buffer (100 mM Hepes buffer, 10% sucrose, 0.1% 3[(3cholamidopropyl)dimethylammonio]-1 propanesulphonate (CHAPS), 5 mM dithiothreitol DTT and 0.0001% Igepal 630, pH 7.25) was added to cell lysates. Substrate cleavage leading to the release of free AMC was monitored at 37°C at 60 sec intervals for 25 cycles using a Varioskan multilabel counter (excitation 355 nm, emission 460 nm). Enzyme activity was expressed as nmol AMC released/min/mg protein.
Western blot analysis

Exponential growing cells were harvested, washed in cold PBS and lysed in SDS sample buffer (4% SDS, 20% glycerol, 0.5 M Tris-HCl, pH 6.8 and 0.002% bromophenol blue) containing 10% 2-β-mercaptoethanol, by boiling for 5 min in a waterbath. Proteins were separated on a SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore BV, Etten-Leur, the Netherlands) by semi-dry or wet blotting. Western blotting was performed as described by Santa Cruz Biotechnology (Heerhugowaard, the Netherlands) using skim milk as blocking agent. Equal loading of protein was checked by Coomassie Brilliant Blue (Bio-Rad Laboratories BV, Veenendaal, the Netherlands) 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) were purchased from Santa Cruz Biotechnology, mouse anti-caspase-8 (1C12), anti-caspase-3 (9662) and anti-cleaved caspase-3 (9661s) antibodies from Cell Signalling Technology (Leusden, the Netherlands), mouse anti-XIAP from Transduction Laboratories (Alphen aan de Rijn, the Netherlands). Secondary antibodies conjugated with horseradish peroxidase (HRP) were obtained from DAKO (Glostrup, Denmark). Chemiluminescence was detected using BM Chemiluminescence Blotting Substrate (POD) or Lumi-LightPLUS Western blotting substrate (Roche Diagnostics, Almere, the Netherlands). Protein expression levels were densitometrically analysed with ImageJ software.

Statistics

Statistical analysis was performed using the 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% ± 14% SD compared to negative control siRNA treated cells. The decrease was observed at 24 h post-transfection (Suppl. Fig. 1). We tested several HPV16 E6 antibodies as described recently (12) but were unable to detect HPV16 E6 in SiHa cells, known to have extremely low copies of HPV16 (13). Therefore, p53 protein levels were used as a functional read-out for efficacy of HPV16 E6 downregulation (12, 14, 15). Transfection of the cells with HPV16 E6 siRNA resulted in an upregulation of p53 protein levels compared to negative control siRNA and non-transfected matching control. Restoration of p53 was also associated with p21 upregulation in transfected SiHa, most clearly in E6-suppressed cells (Fig. 1A), reflecting transcriptionally active p53. No effect of HPV16 E6 siRNA on p53 and p21 was observed in HPV18-positive HeLa (data not shown), indicating selective HPV16 E6 silencing.
Figure 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. Non-transfected cells (control) are included. HPV16 E6 suppression induces cell growth inhibition but it is not significantly different from control or negative siRNA transfected cells. (B) Cytotoxicity assay of transfected SiHa cells. Cells were transfected with 133 nM E6 or negative control (neg) siRNA, 24 h later reseeded and 4 days later subjected to cytotoxicity analysis. Values are mean ± SD of 3 independent experiments.

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

**E6-suppressed SiHa cells are especially sensitized to the combination of death ligands and cisplatin**

We previously demonstrated that SiHa cells are resistant to rhTRAIL- and anti-Fas antibody-induced apoptosis (5, 9). Suppression of E6 in SiHa cells induced a small but significant increase in rhTRAIL or anti-Fas antibody-induced cell death (Fig. 2A). Current optimal therapy of 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 ligands-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 as 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 to negative control siRNA treated cells (Fig. 3A). Moreover, E6-suppressed cells became more sensitive to death ligand-induced apoptosis by cisplatin pre-treatment compared to 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 to the negative control siRNA transfected cells treated with the same concentration of cisplatin plus rhTRAIL \((P < 0.05)\) indicating a significant enhancement (Fig. 3A). An additional 20% apoptosis was observed in E6-suppressed cells treated with cisplatin plus anti-Fas antibody compared to matching negative control siRNA transfected cells \((P < 0.05)\) (Fig. 3B). Caspase-3 activity assay supported the apoptosis assay, since a substantial enhancement of caspase-3 activity was observed in E6-suppressed cells exposed to cisplatin, while the largest effect was seen when the E6-suppressed cells exposed to cisplatin and rhTRAIL (Fig. 3C). However, the 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 rapidly degraded (16). Therefore, caspase-3 activity assays performed on cells 24 h after apoptotic induction probably underestimates caspase activation due to loss of active caspase-3 in late apoptotic cells as measured with the acridine orange assay. The enhanced apoptosis of E6-suppressed cells in response to cisplatin is associated with p53 upregulation. Interestingly, cisplatin treatment resulted in a loss of p21 expression (Fig. 3D), suggesting the involvement of additional cellular factors in cisplatin-induced apoptosis in E6-suppressed cells.

**Figure 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. 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 means ± SD of 3 independent experiments (*\(P < 0.05\) - E6 vs negative siRNA transfected cells). (B) Cells were transfected with E6 or neg siRNA and 24 h later reseeded. The next day irradiated with 0 or 10 Gy and 24 h later 0.1 µg/ml rhTRAIL or 1.0 µg/ml anti-Fas antibody added for another 24 h. Data represent the means ± SD of 3 independent experiments (*\(P < 0.05\) – irradiated vs irradiated and rhTRAIL or anti-Fas antibody treated E6-suppressed cells).
Figure 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. 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. 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 were transfected and 24 h later reseeded. The next day cells were treated with 15 µM cisplatin for 2 h and then 0.1 µg/ml rhTRAIL for another 24 h, afterwards the cells were harvested and DEVDase activity was measured. Data represent the means ± SD of 3 independent experiments (*P < 0.05, **P < 0.01 – E6 vs. negative siRNA transfected cells) (A, B, C). (D) Western blot of p53 and p21 levels in SiHa transfected with E6 or negative siRNA and 24 h later reseeded. The next day, a 2 h pre-treatment with 15 µM cisplatin was followed by 0.1 µg/ml rhTRAIL for another 24 h. For each western blot one representative of 3 independent experiments is shown. Equal loading of protein was checked by Ponceau-S staining of the PVDF membrane.
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. E6 siRNA did not further enhance the effect of cisplatin on DR4 or DR5 expression levels (Fig. 4A, 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 the siRNA transfected cells only (Fig. 4C). These results demonstrated that cisplatin induced cell surface expression of DR4, DR5 and Fas, while E6 siRNA did not have any specific effect on receptor surface expression.

Figure 4. DR4, DR5 and Fas membrane expression in E6-suppressed SiHa cells. (A) DR4, (B) DR5 and (C) Fas membrane expression by flow cytometry. Cells were transfected, the next day 15 μM cisplatin added for 24 h. The mean fluorescence intensities were corrected for staining with a non-specific isotype control. Data represent the means ± SD of 3 independent experiments (*P < 0.05, **P < 0.01, ***P < 0.001, 1cisplatin treated cells vs matched control, 2transfected cells vs matched control).
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 to negative control 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 cleavage of caspase-3 into supposed active products (Fig. 5). However, this did not clearly correspond 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 (Fig. 5 and Fig. 3C). 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-suppressed cells treated with anti-Fas antibody, alone or in combination with cisplatin (data not shown).

Effect of p21 and XIAP downregulation 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 investigate the role of p21 and XIAP in apoptotic cell death of SiHa cells, we used an siRNA approach. Downregulation of p21 and XIAP at the protein level was confirmed by immunoblotting 48 h post-transfection (Fig. 6A). Transfected cells were pretreated with 15 µM cisplatin for 2 h followed by 0.1 µg/ml rhTRAIL for another 24 h then stained by acridine orange. Downregulation of nearly detectable p21 did not change apoptotic levels compared to negative siRNA transfected cells neither 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 the p21 downregulation could substitute cisplatin for its effect on rhTRAIL-induced apoptosis. However, cells transfected with combined p21/E6 siRNAs showed comparable low apoptotic levels as E6-suppressed cells after rhTRAIL treatment. This indicates that cisplatin has other non-p21 related effects that sensitizes E6-suppressed cells to rhTRAIL (Fig. 6A,B). XIAP suppression had no sensitizing effect on cisplatin- or rhTRAIL-induced apoptosis in SiHa cells, while XIAP suppression significantly enhanced apoptosis induction by cisplatin combined with rhTRAIL. This demonstrates that E6 suppression can be functionally replaced by 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 functionally have a similar effect on apoptosis when combined with cisplatin and rhTRAIL.

**Figure 5.** Caspases activation and XIAP downregulation in E6-suppressed SiHa cells treated with cisplatin and rhTRAIL. Cells were transfected and the next day pretreated for 2 h with 15 µM cisplatin followed by rhTRAIL for 24 h. For each western blot one representative of 3 independent experiments is shown. Equal loading of protein was checked by Ponceau-S staining of the PVDF membrane.

**DISCUSSION**

The present study shows that E6 suppression plus cisplatin strongly induced apoptosis in HPV16-positive human SiHa cervical cancer cells. This apoptotic effect was further enhanced by adding rhTRAIL or anti-Fas antibody. Enhanced caspase-8 and caspase-3 activation and the stronger XIAP cleavage suggest that both the intrinsic and extrinsic apoptotic pathway were activated. These results indicate that 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 HPV E6 open reading frame, three bicistronic variants of E6 and full length E7 have been described (cited in (17)), 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 as compared to siRNA that targets both E6 and E7 production (18, 19). HPV16 E6 silencing in SiHa cells by an siRNA sequence, targeting both full-length as well as the E6 splice variants, resulted in inhibition of cell growth and colony formation (20). An HPV16 E6 siRNA sequence against the full length transcript clearly induced apoptosis in SiHa but only
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at day 4 after siRNA treatment (21).

The siRNA in the present study targeted the full length HPV16 E6. This resulted in ~50% reduction in E6 mRNA, but did not induce much apoptosis when administered alone, comparable to the results of earlier studies using the same E6 siRNA sequence (10, 15). A range of siRNAs targeting different sequences of HPV16 E6 and/or E7 have been tested in cervical cell lines in vitro and in vivo studies. Depending on the E6 siRNA sequences used, the effect in SiHa cells can be either apoptosis induction (21) or senescence (14, 22). 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. demonstrated an even more effective HPV16 E6 knockdown in SiHa cells with other siRNAs (14). Despite the stronger reduction in E6 mRNA, a similar induction 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 (14). The lower concentration of more effective E6 siRNAs may, however, avoid off-target effects and therefore, especially reduce the small effect we observed with the negative control siRNA.

We showed that silencing of E6 following 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 (23-25) and is involved in Fas trafficking to the cell membrane (26). Additionally, we previously demonstrated that enhanced p53 expression following proteasome inhibition caused a strong DR5 membrane expression induction in SiHa cells (5). 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 further enhanced by E6 suppression, while the highest p53 levels were detected in E6-suppressed cells after cisplatin in SiHa cells. Two studies described that p53 activation following E6 RNAi was transient in HeLa and SiHa cells. Prolonged p53 stabilization can be achieved by cisplatin in E6-suppressed HeLa cells (19, 27). Moreover, the prolonged p53 activation resulted in an activation of the intrinsic pathway in E6-suppressed HeLa (28), which may occur in E6-suppressed SiHa cells as well. The decreased p21 levels following cisplatin treatment of E6-suppressed SiHa cells may affect apoptosis, as p21 not only mediates p53-induced cell cycle arrest but can also suppress apoptosis following exposure to DNA-damaging agents such as cisplatin, and death receptor-mediated apoptosis (29). However, downregulation of p21 had no effect on apoptosis in E6-suppressed SiHa cells following cisplatin and/or rhTRAIL treatment, which excludes a role for p21 in our setting.

Surprisingly, cisplatin-induced apoptotic levels in E6-suppressed cells could be further enhanced by adding rhTRAIL or anti-Fas antibody. We and others showed that SiHa cells were resistant to rhTRAIL or anti-Fas antibody-induced apoptosis (5, 22, 30). The resistance
was attributed to the inability to recruit FADD and caspase-8 to the DISC (5, 9, 30), which is probably caused by the binding of HPV16 E6 to FADD and the relatively low levels of caspase-8 in SiHa compared to other cervical cancer cell lines (9, 31). Although reduced caspase-8 levels have been related to E6-induced degradation in an osteosarcoma cells overexpressing exogenous HPV16 E6 (32), we found no caspase-8 upregulation following E6 suppression. RhTRAIL or anti-Fas 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 was observed (Fig. 5). It implies that caspase-3 functionality following rhTRAIL or anti-Fas treatment was inhibited by an anti-apoptotic protein, such as XIAP (5, 33). We here demonstrated that XIAP downregulation could only substitute E6 suppression without reducing apoptosis of the most effective combination treatment but not cisplatin or rhTRAIL. XIAP was very efficiently downregulated 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 further investigated.

The fact that viral E6 oncogene of high-risk HPV types are indispensable for cervical carcinogenesis and maintenance of the malignant phenotype (3), presents E6 as an ideal target for a specific cervical cancer therapy. RNAi offers the ability to selectively induce 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 monotherapy (34), and in combination with paclitaxel (15) or cisplatin (35). Other anticancer drugs aimed at the E6 and E7 activity may be more appropriate for the cocktail treatment (36). 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 MG132 sensitized cervical carcinoma cells to rhTRAIL-induced apoptosis (5). 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 cervixes compared to CIN3 lesions, indicating a therapeutic window (37). This option is realistic for further exploration in short-term, since the proteasome inhibitor bortezomib is already used in the clinic (38). In addition, both rhTRAIL and agonistic DR4 and DR5 antibodies are now being tested in phase I/II clinical trials (36, 39). Our results also present XIAP as putative target in combination with DR4 or DR5 targeting drugs. Interestingly, novel small molecule inhibitors that target XIAP are currently in early clinical development (40). Testing of combination treatments with E6/E7 targeting drugs on non-cancerous human cervical cells will be important. The cervical explants model is very laborious, while only E6/E7 immortalized cervical cell models have become available, which strongly limits options for drug toxicity testing in normal cervical cells.
In conclusion, our results demonstrate that E6 siRNA effectively suppresses E6 expression in the HPV16 SiHa cells. Cisplatin sensitizes E6 siRNA treated cells to apoptosis, which is strongly enhanced by adding rhTRAIL. These results may be translated into a clinic setting by XIAP or proteasome inhibitor utilization instead of an siRNA approach.

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