Chemoradiation of Cervical Cancer Cells: Targeting Human Papillomavirus E6 and p53 Leads to Either Augmented or Attenuated Apoptosis Depending on the Platinum Carrier Ligand

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ABSTRACT

Recent clinical trials comparing concurrent chemotherapy and radiation with radiation alone in cervical cancer have shown that chemoradiation reduces the risk of death by 30–50%. Despite the clinical success, treatment responses at the cellular level are still inadequately explored. A key event in cervical carcinogenesis is the disruption of p53 tumor suppressor pathway by human papillomavirus (HPV) E6 oncoprotein. We found that regardless of the HPV type in SiHa (HPV 16+), CaSkI (HPV 16+), HeLa (HPV 18+), and UT-DEC-1 (HPV 33+) cell lines, cisplatin, carboplatin, and a novel platinum compound, oxaliplatin, activated a p53 reporter and reduced the HPV E6 mRNA. Carboplatin and oxaliplatin treatment led also to stabilization of p53, whereas none of the platinums changed p73 levels. After irradiation (IR) alone, a decrease in HPV E6 mRNA levels and an activation of the p53-reporter were detected in SiHa, CaSkI, and HeLa cells, but not in UT-DEC-1 cells. Concomitant platinum treatment and IR led to poly(ADP-ribose) polymerase cleavage as a sign of caspase-3 activation and apoptosis. Clonogenic survival was enhanced by expressing a dominant negative p53 or ectopic HPV16 E6 in SiHa and HeLa cells treated with IR, carboplatin, or oxaliplatin or with a combination of IR + carboplatin or oxaliplatin. In contrast, dominant negative p53 or ectopic HPV 16 E6 sensitized the cells to cisplatin. Pt chemotherapeutics and radiation had a synergistic cytotoxic effect as determined by Bliss independence criterion. Taken together, p53 has a significant role in the cellular response to chemoradiation treatment in cervical cancer cell lines, but p53 activity may have a dramatically different effect on cell survival depending on the platinum carrier ligand.

INTRODUCTION

Over the past few years the concurrent chemotherapy and radiation (chemoradiotherapy) of locoregionally advanced cervical cancer has dramatically improved the local control and overall survival compared with the traditional therapy with radiation (1). The risk of death has been shown to be reduced by 30–50%, which led the National Cancer Institute to release a Clinical Announcement in February 1999 stating that, in high-risk early-stage and locally advanced cervical cancer, the new standard therapy should be concurrent cisplatin-based chemotherapy with radiation therapy. Cisplatin has become a standard part of the treatment, but we do not know whether the dose and schedule are optimal or whether it is possible to achieve even better efficacy with some other drugs or drug combinations. Clinical trials will ultimately resolve this issue, but before these can be based on a rational design, understanding of the molecular mechanisms of chemoradiation in this particular cancer is needed.

Cervical cancer develops through a multistep process through cervical cancer precursor lesions known to originate after HPV infection. Recent reports show that, with meticulous searching, HPV genes can be found in 99.7% of cervical cancer tissue (2). High risk HPV types, like types 16, 18, 33, 35, and 45, carry two major oncogenes E6 and E7, which bind and target p53 and pRb tumor suppressors for ubiquitin-mediated degradation (3). p53 plays a fundamental role in the cellular networking to genotoxic and cytotoxic stresses, which might affect genomic integrity and lead to abnormal cell division. p53 is normally tightly regulated by mdm2 protein and also by ubiquitin-mediated degradation (4). However, in cervical cancer cells, HPV E6 has displaced the mdm2 pathway (5, 6). If E6 expression is abolished, p53 can be restored to functional protein with transcriptional activity toward target effectors involved in cell cycle arrest and apoptosis (5, 7, 8). This makes cervical cancer unique among other cancers in which the predominant mechanism for inactivation of p53 is gene mutation. Theoretically, inhibiting E6-p53 binding or clearing E6 in tumor cells would render these cells more susceptible to cell cycle arrest or apoptosis by different stress-based treatments. In some other cancers, however, active p53 may either enhance or inhibit the response to therapeutic agents (9, 10).

Pt-DNA adducts are removed by NER, postreplication repair, and MMR (11). It has been shown that increased NER results in cisplatin resistance by increased removal of Pt-DNA adducts (12). However, intact MMR is required for cells sensitive to cisplatin but not for cells sensitive to oxaliplatin (13). MMR proteins hMSH2 and hMLH1 bind with high affinity to cisplatin adducts but not to oxaliplatin adducts (14). p53 is centrally involved in DNA damage recognition and repair (15, 16). In addition to the role of p53 in apoptosis and cell cycle arrest, the involvement of p53 in Pt-DNA adduct repair may have an impact in determining the intrinsic sensitivity of the cell to platinum drugs (17). p53 is a key protein also in the response to radiation genotoxicity (18). p53 may induce apoptosis in one cell type but is not required to engage the programmed death machinery in all cell types; thymocytes are killed by p53-dependent apoptosis after IR exposure (19), but p53 is not required for apoptosis caused by IR in endothelial cells (20). Cellular response to IR has been reported to be abrogated by HPV E6 through p53-dependent and p53-independent mechanisms (21). Reduced NER has also been observed when p53 is targeted with E6 or dominant negative p53 (22). However, when cervical cancer cells are exposed to IR, despite expressing E6, they are able to induce GADD45, one of many proteins involved in DNA damage repair, possibly through p53 activation (23).

Restoring the normal activity of p53 in cancer cells would reestablish the lost functions, and this intriguing possibility has motivated the researchers to search for molecules to reactivate p53 (24). Interestingly, many of the current chemotherapeutics as well as IR cause DNA damage or some other form of stress that activates p53 in...
wild-type cells. Earlier work has provided evidence for a link of functional status of p53 and both in vitro and clinical outcome. For example, p53 knockout mice carrying tumors have an increased resistance to treatment with etoposide, 5-fluorouracil, and doxorubicin (25). Similar results were obtained in studies with various cell systems and animal models (26, 27). Several reports have also shown that introduction of wild-type p53 or reactivating p53 confers sensitivity to radiation or chemotherapeutic drug treatment, but data from several other studies have confounded this straightforward association (9, 28, 29). Nevertheless, combination treatment with chemotherapeutics and p53-activating molecules has shown promising effects, not only in vitro but also in some clinical trials (30).

The present study was designed to evaluate different platinum compounds together with IR in cervical cancer cells. We found that all platinum compounds sensitized cervical cancer cells to ionizing radiation. p53 was involved in the apoptosis induced by all of the platinum compounds, but there was a striking difference depending on the drug used. The data show that p53 in HPV-positive cervical cancer cells is able to counteract the cytotoxic activity of cisplatin but can enhance the cytotoxicity of carboplatin, oxaliplatin, and IR.

MATERIALS AND METHODS

Cell Lines, Plasmids and Transfections. The human cervical cancer cell lines SiHa (HPV 16+), CaSki (HPV 16+), and HeLa (HPV 18+) were obtained from the American Type Culture Collection (Manassas, VA). The UT-DEC-1 cell line has been established from a HPV 33-positive mild vaginal intraepithelial neoplasia (31). Cells were grown as monolayers in DMEM supplemented with 10% fetal bovine serum, 200 mM L-glutamine, nonessential amino acids, and penicillin/streptomycin (Life Technologies, Inc., Paisley, Scotland).

The composition of the p53-reporter plasmid ptkGC(3/p53lac has been described earlier (32). In principle, a single p53 response element GC:p53 [GCCCGAGCTTGCTCTCG]3 from the human Rb gene promoter was inserted downstream of an HSV-tk basal promoter to activate luciferase reporter gene transcription. For creating stable lines, the p53 reporter expression cassette was cut out by SacI/SalI and inserted into the SacI/XhoI-activated expression vector pUB/Bsd (Invitrogen) providing an ampicillin resistance gene for plasmid amplification in Escherichia coli and a basicidinic resistance gene for eucaryotic selection.

ptkGC(p53lac-bsd was transfected to the cell lines with Fugene (Roche Diagnostics, Mannheim, Germany). Stable transfectants were selected with 5–10 μg/ml blasticidin (Invivogen, Carlsbad, CA). Resistant clones were analyzed with PCR to verify the presence of the intended plasmid sequences.

The SiHa DDp53 cell line carries a plasmid that expresses a truncated mouse p53 containing amino acid residues 1–14 and 302–390 under the control of the CMV promoter (DDp53). SiHa CMV is the control cell line with the parental vector pCMV-neo lacking the mutant p53 sequences. Both cell lines carry the neomycin resistance gene and were maintained in medium supplemented with Geneticin (800 μg/ml; Life Technologies, Inc.). Cisplatin (Bristol-Myers Squibb, Princeton, NJ), carboplatin (Bristol-Myers Squibb), and oxaliplatin (Sanofi-Synthelabo, Paris, France) were dissolved in a 0.9% NaCl solution (cisplatin and carboplatin) and water (oxaliplatin) to produce stock solutions of 0.5 μg/ml, 10 μg/ml, and 5 mg/ml, respectively. Stock solutions were diluted in the culture medium immediately before use. For p53 reporter assays and E6 mRNA experiments, drug concentrations were chosen based on IC50 of short term MTX cytotoxicity assays (data not shown). MTX assay was, however, not used to assess the treatment sensitivities because of apparent limitations of this test for this purpose (33).

The cells were irradiated using 4 MV photons. The dose was 5 Gy in one fraction. To get electronic equilibrium, the IR was given vertically from below the cell culture plates that were on top of a 10-min acrylic plate.

Cell Cytotoxicity Assays. The cytotoxic effect of the treatment was studied with the clonogenic assay. The clonogenic assays with DDp53 and HPV 16 E6 constructs were also chosen to better assess the long-term impact of p53 in the cells treated in low drug concentrations. Exponentially growing SiHa CMV and SiHa DDp53 cells (200 × 103 cells/well) were plated in 6-well plates 48 h before treatment. After 6 h drug treatment and/or IR, cells were washed with PBS preheated to 37°C, trypsinized, and suspended in medium without drugs. For each new well of the 6-well plate, 500 cells were plated for clonogenic growth. After 14 days, the plates were fixed with 1:1 acetic acid/methanol, stained with Giemsa, and counted. The experiments were repeated at least three times.

HeLa cells were selected to the transient transfections because their transfection efficiency (70–80%), as determined by green fluorescence protein expression, is superior to that of the other cell lines (below 40%). The cells were plated in 24-well plates (50 × 103 cells/well) 24 h before transfecting the cells with HPV16 E6 (kindly provided by K. Vousden, National Cancer Institute, Frederick, MD). pCMVneo or DDP53 and 0.4 μg DNA (in 1 μl Fugene) per well was used in all transfections. Twenty-four h after the transfection, the cells were treated with 2 h with 5 μM cisplatin, 10 μM oxaliplatin, or 140 μM carboplatin, washed with warm PBS, trypsinized and plated for clonogenic growth in 6-well plates (400 cells/well). After 7 days, the colonies were fixed, stained, and counted.

p53 Luciferase Assays. Stable ptkGC(p53lac-bsd SiHa, CaSki, HeLa, and UT-DEC-1 cells were seeded into 96-well plates (2 × 104 cells/100 μl/well). After allowing the cells to attach for 24 h, the platinum compounds were added to the wells, and some plates were additionally irradiated with 5 Gy. After treatment, the number of living cells was estimated by WST-1 assay (Roche, Mannheim, Germany). Thereafter, the cells were rinsed with PBS and overlaid with 100 μl of mixture containing 50% PBS and 50% Bright-Glo luciferase assay reagent (Promega, Madison, WI). Luciferase activity was quantitated with the aid of a Hybrid capture luminometer (Digene, Gaithersburg, MD). Luciferase readings were divided by WST-1 value to obtain normalized reporter activity.

Real-Time Quantitative Reverse Transcription-PCR. Total RNA was isolated with RNeasy kit (Qagen, Hilden, Germany). One μg of RNA was treated with DNAase I (Life Technologies, Inc.). The first-stranded cDNA was performed with the oligo(dT) primed first-strand synthesis in a total volume of 20 μl with a recombinant Moloney murine leukemia virus reverse transcriptase enzyme (PE Biosystems, Foster City, CA) or without enzyme to verify the absence of any DNA contamination. The primers for amplification were designed by Primer Express software (PE Biosystems), except for the housekeeping gene EF1α (34). The primers were: HPV 16 E6, forward, 5′-AAAGAGAACTGCAATTGTTCACAGGA-3′, and reverse, 5′-TGATATGTGGTTTGCGACCTGTGG-3′; HPV 18 E6, forward, 5′-TGCGGCGGTTTGGGA-3′, reverse, 5′-GTTCATGCGCGTGCGACACGT-3′; HPV 33 E6, forward, 5′-AAAACACGAAACATTGCATGATT-3′, reverse, 5′-GGCTACCCACAGGCTCTGACATGTA-3′, and reverse, 5′-GCGGTCGTTGAGAACTC-3′. Specificity of the PCR amplification was confirmed on an agarose gel and dissociation curve output in the amplification plot. The amplification was performed with SYBR green PCR master mix (PE Biosystems) in 25 μl of reaction volume consisting of 5 μl of the first-strand cDNA reaction and 0.4 μl primers. Reactions were run on GeneAmp 5700 sequence detection system (PE Biosystems) programmed to 5-min initial step at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. Each experiment was performed in triplicate. The Ct values in log linear range representing the detection threshold values were used for quantitation. Relative standard curves were generated by diluting the control samples to 1:1, 1:2, 1:4, 1:8, and 1:16. The SDS 5700 program in GeneAmp 5700 was used to calculate the relative expression levels of each sample. The amounts of HPV E6 transcripts were normalized against the readings for EF1α transcripts.

Western Blot. The total cellular protein extract (for PARP) and nuclear extracts (for p53 and p73) were prepared as described (5, 35). Higher concentrations of the drugs were used because of sampling the whole cell population. The Western blotting procedures were performed with Bio-Rad Bradford assay dye (Bio-Rad). Equivalent amounts (50 μg) of protein were separated on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride filters (Amersham). Equal loading was confirmed staining the filter with Perisoeau S. Proteins were detected with the following antibodies: DO-1 for p53, polyclonal rabbit anti-PARP H-250 (Santa Cruz). Anti-p73 rabbit sera raised against a peptide spanning amino acids 9–24 was kindly provided by B. Vojtesek Masaryk Memorial Cancer Institute, Brno, Czech Republic. Secondary horse-radish peroxidase-conjugated rabbit antimouse or swine antirabbit IgG antibodies were purchased from DAKO. The detection was carried out with ECL.

Statistical Methods. The interaction of drugs and X-ray was analyzed applying the Bliss independence criterion (36, 37). The dose effect of the drug...
alone was estimated by fitting a median-effect function into the data observed with different doses of the drug without IR treatment. Parameters of the median-effect function were estimated using the nonlinear regression model (procedure nlin in the SAS System for Windows, release 8.2/2001). The value of IC50 with 95% CI was calculated using the model. The same modeling was not possible for the effect of IR alone because of the one fixed dose. The IR effect was estimated from wells treated with 5 Gy IR without Pt. The IC50 was not estimated for the chemoradiation combination because the X-ray effect alone was >50%. The sensitivity of HeLa cells transiently transfected with DDp53 or HPV 16 E6 was compared with HeLa cells carrying the empty CMV vector, and the statistical analysis was done using Poisson regression analysis.

RESULTS


The possible synergism of radiation and chemotherapy was calculated based on the Bliss independence criteria. IR combined with all of the platinums resulted in a synergistic cytotoxic effect on SiHa cells (Fig. 1).

The clonogenic survival assays were performed on stably transfected SiHa cells carrying the DDp53 and HPV 16 E6 constructs or the empty vector to evaluate the role of p53 activity in the given treatments. The dominant negative effect of DDp53 construct involves the inhibition of transactivational tetramers by formation of inactive complex between the truncated protein and the wild-type p53 (38).

On the basis of the IC50 values, the SiHa CMV cell line was more sensitive to carboplatin and oxaliplatin than were the SiHa DDp53 of SiHa-ecE6 cell lines. In contrast, SiHa-ecE6 and SiHa DDp53 appeared to be even more sensitive than SiHa CMV to cisplatin, indicating that p53 activity in these cells sensitizes SiHa cells to carboplatin and oxaliplatin, but not for cisplatin cytotoxicity (Figs. 2 and 3; Table 1). In a separate experiment targeting the slope in the response curve for cisplatin at 5 Gy SiHa CMV and SiHa DDp53, the survival fractions were 55 and 41%, respectively, which was consistent with the observation that the silencing of p53 does not induce resistance to cisplatin in SiHa cells. Similarly, ectopic HPV16 E6 or DDp53 in HeLa cells induced resistance toward carboplatin and oxaliplatin but not toward carboplatin (Fig. 4).

Radiation alone was more effective in SiHa CMV than in cells with E6 or DDp53, indicating that the residual p53 activity in the parental cells have a role in their response to radiation (Figs. 2 and 3). After 5 Gy of radiation, cytotoxicity was observed in clonogenic assays but not in short-term MTT assays, indicating a delayed type of cytotoxicity (data not shown).

Concurrent Radiotherapy and Platinum Treatment Induce Apoptosis.

The platinum compounds and IR were tested for their ability to induce the cleavage of PARP, not only in SiHa CMV but also in SiHa DDp53 cell lines. In Western blot analysis, both of the cell lines were found to cleave PARP after concurrent chemoradiation (Fig. 5). PARP is cleaved by caspase 3, and this caspase activity reflects the ongoing apoptosis. The cells were also stained with Hoechst 33258 stain, and typical apoptotic fragmentation and shrinkage was detected (data not shown). Similar nuclear morphology was also seen in UT-DEC-1, HeLa, and CaSki cells after treatment, which indicated...
cisplatin, followed by carboplatin and oxaliplatin (Fig. 6). The regulation of E6 at 10 h and 24 h in SiHa cells was achieved with a drop in the relative E6 mRNA levels in all of the cell lines at 8 h. That these cells died predominantly by apoptosis after Pt + IR in these treatment conditions (data not shown). Taken together, the results from clonogenic assays and PARP analyses show that p53 was involved in, but not required for, the apoptotic effect of Pt + IR for these cervical cancer cell lines.

Both Radiation and Platinum Compounds Modulate HPV E6 mRNA Levels. The E6 mRNA levels were determined at several time points after platinum treatment alone or with 5Gy IR in SiHa cells (Fig. 6) and CaSki, HeLa, and UT-DEC-1 cells (Table 2). When cisplatin, carboplatin, and oxaliplatin were used alone, they caused a 90% decrease in E6 mRNA levels in SiHa cells at 24 h after the onset of treatment with high drug concentrations (Fig. 6). When the drugs were used together with IR, the E6 reduction was enhanced with low, but not with high Pt concentrations, when the E6 down-regulation was already strong. The rebound to normal E6 levels that was seen with IR alone was absent when the platinum were left in the cell culture medium. The E6 decrease was dose dependent with all of the platinums. At equimolar concentration 70 μM, the most effective down-regulation of E6 at 10 h and 24 h in SiHa cells was achieved with cisplatin, followed by carboplatin and oxaliplatin (Fig. 6).

IR alone (Fig. 7, Table 2) caused a rapid increase in the ratio of E6:EF1α at 2–6 h after treatment in SiHa (208 ± 8% at 2 h), HeLa (162 ± 5% at 6 h), and UT-DEC-1 (155 ± 14% at 6 h) cell lines (CaSki was not tested until 10 h). Shortly after the increase, there was a drop in the relative E6 mRNA levels in all of the cell lines at 8–10 h. After the initial decrease, the E6 levels returned to the levels measured in the control cells by 24 h (Fig. 7). In SiHa, CaSki, and HeLa cells, there was a second, more sustained, drop in the E6 levels at 36–48 h. At 72 h, the pretreatment levels were regained. In UT-DEC-1 cells, however, no decrease of E6 was seen after the transient drop at 8 h. Instead, an increase in the E6 levels was observed at 48–72 h. SiHa, CaSki, and HeLa cells were also able to slightly induce the p53 reporter at 36–48 h, whereas UT-DEC-1 cells showed a slight decrease in reporter activity.

p53-responsive Reporter Activation and Protein Stabilization Correlates with the Decrease of E6 mRNA. When 70 μM cisplatin, 1000 μM carboplatin, and 300 μM oxaliplatin were used alone in the medium of SiHa cells, a clear but slow p53-reporter activation was observed (Fig. 8). This activation was most pronounced with carboplatin. The CaSki, HeLa, and UT-DEC-1 cell lines showed a different magnitude of p53 activation, but they were all able to activate the reporter (Table 3). Different reporter sublines of a particular parental cell line showed reproducibly a similar activity, which indicated that the different reporter activities in the cell lines represented the intrinsic ability of the parental cell line to activate the p53 responsive promoter (data not shown). Combining IR with the platinums significantly increased the reporter activity (Fig. 8; Table 3). In kinetic comparison, the decrease of HPV E6 mRNA level correlated with the p53-reporter activation in all of the tested cell and drug combinations as well as after IR (Figs. 6–8; Table 2).

A similar level of E6 reduction led to p53 stabilization after 24-h treatment with 1000 μM carboplatin and 300 μM oxaliplatin in SiHa cells, but no clear stabilization with 70 μM or 100 μM cisplatin treatment was found in several repeated experiments (Fig. 9). This suggests that, besides E6-mediated degradation, an alternative pathway for decreasing the p53 levels in these cells has to be operative after cisplatin exposure. The Ddp53 construct stabilized also the wild-type p53 (but not p73) probably through binding of E6, but the huge overexpression leads to almost complete abrogation of p53 activity (5). At 12 h, 24 h, and 36 h, we also failed to see any significant p53 stabilization after 5 Gy of IR (data not shown). This prompted us to measure the amount of p73 in these cells because previous studies in other cell systems have found stabilization of p73 after cisplatin treatment (39). Neither Pt compounds nor IR altered the levels of p73 (Fig. 9). Because IR activation of p73 probably occurs through c-abl-mediated tyrosine phosphorylation and not by stabilization of the protein (40, 41), this was another reason to perform the kinetic comparison of the association of E6 levels and p53 activation.

The close correlation between E6 levels and IR induced p53-reporter activity suggests that p73 is not primarily responsible for the reporter activity after IR (Fig. 7).

DISCUSSION

The data presented here shows that p53 plays a significant role in concurrent radiation and chemotherapy with platinum compounds in cervical cancer cell lines. Depending on the p53 status, oxaliplatin and carboplatin had an opposite effect on the cells compared to cisplatin. IR alone transiently activated p53 in HeLa, SiHa, and CaSki cell lines, but not in UT-DEC-1 cell line, and, at 5 Gy, had a delayed type of cytotoxic effect. These findings have one important implication for the cervical cancer cells in general: depending on the drug, attempts to activate p53 in drug or treatment combinations may have either positive or negative effect to the desired cell cytotoxicity. Currently, several clinical trials have started on different chemotherapeutics with radiation. Although results from experiments in cell lines can never be directly transferred to clinical situations, the present data predicts that p53 status may have an important impact on the treatment response.

The finding that cisplatin did not increase p53 protein levels in Western analysis, despite p53-reporter activation, raised up the possibility that p73 might activate the reporter. The p53 luciferase construct used in the present study was designed to report p53 activity, but because of the consensus nature of its binding site, it is possible that it may also be activated by p73. p73 is inhibited by p53 mutants...
in mammalian cells, and, in addition, the DDp53 construct used in the present study binds p73 (42, 43). On the contrary, E6, like SV40 T antigen or adenovirus E1B, does not inactivate p73 (44, 45), although contradictory results have been reported (46). We also found that the DDp53 construct did not stabilize p73 in SiHa DDp53 cells in contrast to p53. In this stable cell line, DDp53 inactivates the transactiwalional properties of wild-type p53, but endogenous p53 degradation, mediated by E6, was probably inhibited by DDp53 binding to E6. This supports previous findings that p73 cannot be degraded by E6. In p53-null H1299 cells, mutant p53His175, which binds p73, causes increased resistance to cisplatin treatment (43). We observed that in SiHa and HeLa cells, which have wild-type p53, the DDp53 protein and ectopic HPV 16 E6 sensitizes these cells to cisplatin arguing in favor of p53’s predominance in response to the treatment. We also found that the amount of p73 protein levels did not change after platinum treatments. Therefore, we could not find evidence for the involvement of p73 in the response to the platinum drugs. Radiation, on the other hand, activates p73 through tyrosine phosphorylation and, therefore, is not necessarily detectable in protein levels (40, 41).

Table 1: Comparison of the IC50 values of cisplatin, oxaliplatin, and carboplatin in SiHa CMV, SiHa E6 and SiHa DD-p53 cell lines

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cisplatin</th>
<th>Oxaliplatin</th>
<th>Carboplatin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC50 (μM)</td>
<td>95% CI</td>
<td>IC50 (μM)</td>
</tr>
<tr>
<td>SiHa CMV and SiHa E6</td>
<td>3.6</td>
<td>3.2–4.1</td>
<td>7.5</td>
</tr>
<tr>
<td>SiHa CMV and SiHa E6</td>
<td>2.3</td>
<td>1.7–2.8</td>
<td>12.0</td>
</tr>
<tr>
<td>SiHa CMV and SiHa DD</td>
<td>3.2</td>
<td>2.4–3.9</td>
<td>7.3</td>
</tr>
<tr>
<td>SiHa CMV and SiHa DD</td>
<td>3.0</td>
<td>2.5–3.5</td>
<td>11.5</td>
</tr>
</tbody>
</table>

Fig. 4. Clonogenic survival of HeLa cells transiently transfected with HPV 16 E6, CMVneo, or DDp53 after treatment with 5 μM cisplatin, 10 μM oxaliplatin, or 50 μM carboplatin. After 2 h of drug exposure, the cells were washed with PBS, trypsinized, and replated for clonogenic growth. After 1 week, the cells were fixed, stained, and counted.

However, we found a close correlation between p53-reporter activity and HPV E6 mRNA levels in all of the tested cell lines. This would suggest that radiation-induced p53 reporter was indeed attributable to p53 activity, but some role for p73 cannot be completely ruled out.

Although we observed p53-reporter activation in all of the cell lines, it was by no means clear that this activation was associated with the biological response of the cells to the given treatment. Indirect evidence from experiments showing SiHa and HeLa cell sensitization to cisplatin by DDp53 or HPV 16 E6 suggests also that the p53 induction may confer resistance to cisplatin in these cells. One has to be cautious, in general, not to over-interpret any E6 transfection experiments, because E6 binds also to a number of cellular targets other than p53. Nevertheless, the clonogenic survival experiments with E6 and DDp53 produced similar results and, therefore, p53 is likely to be the main target of both constructs. These data support the hypothesis that p53 is a resistance factor for cisplatin in SiHa and HeLa cells induced by the drug itself. In p53-null SAOS-2 cells,
tetracycline-inducible expression of p53 has been reported to confer resistance to cisplatin in normal (10%) but not in low (<1%) serum conditions, and, interestingly, p53 activity was seen to significantly increase the DNA damage repair in normal-, but not in low-serum concentrations (47). In the present study, p53 is predominantly proapoptotic after carboplatin, oxaliplatin, and IR exposure in SiHa and HeLa cells. In contrast, p53 seems to protect the cells from cisplatin cytotoxicity. An intriguing possibility is that the p53 activity in cisplatin-treated cells was directed toward DNA repair.

Pt-DNA adducts are mainly believed to be removed by NER (48). Several proteins known to play central roles in NER have been reported to bind to p53, including XPD, XPB, and CSB (15). p53 is directly involved in both global and transcription-coupled excision repair and transcriptionally regulates a catalytic subunit of ribonucleotide reductase that provides deoxynucleotides for NER (49). p53 is able to decrease HPV E6 mRNA, possibly resulting in enhanced repair (53). In the present study, all of the platinum drugs and IR were able to decrease HPV E6 mRNA, possibly resulting in enhanced repair. Similarly, it has been previously reported that HTLV-1 Tax protein favors cisplatin-induced apoptosis through the inhibition of NER (54).

Table 2. Effect of different platinum compounds and γ-IR on the amount of HPV E6 mRNA in CaSki, HeLa, and UT-DEC-1 cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Time point</th>
<th>Irradiation</th>
<th>Cisplatin</th>
<th>Cisplatin + IR</th>
<th>Oxaliplatin</th>
<th>Oxaliplatin + IR</th>
<th>Carboplatin</th>
<th>Carboplatin + IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaSki</td>
<td>14 h</td>
<td>73 ± 5%&lt;sup&gt;a&lt;/sup&gt;</td>
<td>58 ± 6%</td>
<td>43 ± 2%</td>
<td>89 ± 3%</td>
<td>78 ± 2%</td>
<td>100 ± 4%</td>
<td>73 ± 6%</td>
</tr>
<tr>
<td>CaSki</td>
<td>30 h</td>
<td>90 ± 2%</td>
<td>n.t.&lt;sup&gt;a&lt;/sup&gt;</td>
<td>n.t.</td>
<td>61 ± 4%</td>
<td>59 ± 10%</td>
<td>105 ± 1%</td>
<td>53 ± 4%</td>
</tr>
<tr>
<td>CaSki&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30 h</td>
<td>120 ± 2%</td>
<td>n.t.</td>
<td>n.t.</td>
<td>31 ± 5%</td>
<td>37 ± 2%</td>
<td>16 ± 1%</td>
<td>25 ± 3%</td>
</tr>
<tr>
<td>HeLa</td>
<td>6 h</td>
<td>162 ± 5%</td>
<td>74 ± 4%</td>
<td>74 ± 14%</td>
<td>133 ± 9%</td>
<td>92 ± 23%</td>
<td>92 ± 7%</td>
<td>92 ± 8%</td>
</tr>
<tr>
<td>HeLa</td>
<td>12 h</td>
<td>59 ± 5%</td>
<td>24 ± 3%</td>
<td>23 ± 1%</td>
<td>67 ± 5%</td>
<td>74 ± 8%</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
<tr>
<td>HeLa</td>
<td>30 h</td>
<td>104 ± 5%</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
<tr>
<td>UT-DEC-1</td>
<td>6 h</td>
<td>155 ± 14%</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
<tr>
<td>UT-DEC-1</td>
<td>8 h</td>
<td>60 ± 4%</td>
<td>28 ± 1%</td>
<td>21 ± 0.2%</td>
<td>93 ± 0.4%</td>
<td>76 ± 2%</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
</tbody>
</table>

<sup>a</sup> Treatments: cisplatin 70 µM, oxaliplatin 100 µM, carboplatin 300 µM and 5 Gy γ-irradiation.

<sup>b</sup> The amount is expressed as percentage to the untreated control and the SDs were calculated from triplicate samples.

<sup>c</sup> n.t., not tested.

<sup>d</sup> Drug concentrations were 300 µM oxaliplatin and 1000 µM carboplatin.

![Fig. 7. Kinetic changes of p53 reporter activation and HPV E6 mRNA levels after exposure to 5 Gy γ-IR in SiHa (HPV 16), CaSki (HPV 16), HeLa (HPV 18), and UT-DEC-1 (HPV 33) cells. The measurements were performed in triplicate; error bars, ±SD. The drugs were left in the medium for the entire experiment.](image)

![Fig. 8. p53 activation induced by chemoradiotherapy in stably transfected SiHa reporter cells. In each measurement, the luciferase activities of the reporters cells were normalized with the cell number measured by WST-1 assay. Data points, readings from triplicate measurements ± SD. The drugs were left in the medium for the entire experiment.](image)
adducts, and this can explain why effects in MMR lead to cisplatin resistance but not to oxaliplatin resistance (11, 14). According to the present data, SiHa and HeLa cells were more sensitive to cisplatin when the p53 activity was abrogated by DDp53 or ectopic HPV16 E6. Therefore, if the activation of MMR discriminates between these cells with different sensitivities to cisplatin, p53 should decrease the MMR activity, rather than increase it, as one would expect.

According to the present thinking, the cytotoxic effect of Pt drugs is DNA damage related, like the cytotoxic effect of IR. However, it has been shown that oxaliplatin, in comparison with cisplatin, induces fewer DNA adducts despite equal or greater cytotoxicity in leukemia or colon carcinoma cell lines (55). We have shown here that the impact of Pt drugs and IR on HPV E6 levels may contribute to their cellular sensitivity. Likewise, the level of active p53 may either increase or decrease the sensitivity to Pt drugs by themselves and in combination with IR, depending on the platinum compound used. It seems that the association of cytotoxicity and DNA damage by Pt compounds and IR is not necessarily direct. However, it is to be emphasized that even cells with inactive p53 died as a result of these treatments, which shows that other mechanisms that are independent of p53 must play a role in the response to the treatment. Moreover, Pt compounds can indirectly induce apoptosis in cells that have no functional p53, which is in agreement with previous findings in other cell systems (56).

An earlier study has measured intratumoral cisplatin concentrations after 100 mg/m² iv. administration of the drug and found them to be in the range of 4.1–23.6 μg/g (57). We did not measure the Pt concentrations in the cell pellet, but 70 μM (21.2 μg/ml) cisplatin, although apparently a high concentration, may have some relevance to the reported in vivo situation. In the lack of definitive measurements, however, direct comparisons extrapolating the in vitro drug concentrations to clinical situations cannot be drawn. Moreover, it is likely that the tissue concentration of Pt compounds vary considerably in different depths of the tumor as predicted from large intervariability of Pt concentrations found in different tumors analyzed in studies evaluating intratumoral Pt. We observed that p53 activity and HPV E6 levels played a significant role in the cell survival in a certain therapeutic window. If the concentration of the Pt was very high, an immediate necrotic effect was observed that could not be modulated either by the inhibition of p53 by dominant negative p53 or by the overexpression of HPV 16 E6 (data not shown). Therefore, depending on the dose of the treatment, the cellular response may use distinct mechanisms manifesting through growth inhibition, apoptosis, or necrosis.

ACKNOWLEDGMENTS

We thank The Joint Clinical Biochemistry Laboratory of University of Turku, Turku University Central Hospital, and Wallac Oy for providing some of the facilities and equipment used in this study.

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