Cisplatin-Induced Growth Arrest of Head and Neck Cancer Cells Correlates With Increased Expression of p16 and p53

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Objectives: To determine expression of cell cycle and apoptotic genes, biochemical analysis of CCL23 and antisense cyclin D1-transfected CCL23 (CCL23AS) cells in the presence of cisplatin was performed. In addition, biochemical analysis of CAL27 cells before and after treatment with cisplatin was performed to determine expression of cell cycle genes.

Design: CCL23, CCL23AS, and CAL27 cell lines were treated with cisplatin. Western blot analysis, fluorescence-activated cell sorting, and apoptosis assays were performed.

Setting: In vitro study of head and neck cancer cell lines CCL23, CCL23AS, and CAL27.

Intervention: CCL23, CCL23AS, and CAL27 cells were treated with cisplatin.

Main Outcome Measures: Expression of p16, p21, p53, Bcl-xL, Bcl-xS, p27, DP1, MDM2, Bcl-2, c-Jun, and Jun-D were assessed using Western blot analysis.

Results: There was increased expression of p16, p21, p53, Bcl-xL, and Bcl-xS genes with cisplatin treatment in the CCL23 and CCL23AS cells. Expression of p27, DP1, MDM2, BCL2, c-jun, and jun-D remained unaltered after treatment. There was decreased phosphorylation of Rb protein with complete absence of hyperphosphorylated Rb in the maximally sensitized antisense cyclin D1-transfected (CCL23AS) cells. Fluorescence-activated cell sorter analysis revealed a decreased G2 phase of the cell cycle and an increased proportion of apoptotic cells in the CCL23AS cell line compared with parental CCL23 cells. Cell killing also occurred in the presence of caspase-3 inhibitor. While CCL23 cells contain wild-type p53, the CAL27 cells have a point mutation in codon 193 (A→T transversion) of exon 6. However, CAL27 cells still exhibited increased expression of p21 after treatment with cisplatin.

Conclusions: These results, in combination with increased expression of the p53 downstream effector p21, indicate that the cisplatin-induced cell cycle arrest operates through the p16/p53-dependent pathway, and a caspase-independent pathway may be involved. Combination treatment of head and neck squamous cell carcinoma via cell cycle inhibition and cisplatin holds promise as a potential therapy in the clinical setting.

Previous studies, including those from our laboratory, have shown that transfection of antisense cyclin D1 resulted in increased sensitivity of cancer cells to cisplatin. The cisplatin ID-50 (dose required to inhibit 50% of cell growth) value for antisense-transfected HNSCC cells was half that of the parental nontransfected cell line. The data support further investigation into the potential for combination therapy using cell cycle inhibitors and cisplatin for head and neck cancer. Cisplatin treatment results in adverse effects, including gastrointestinal distress, hearing loss, nerve damage, kidney damage, and bone marrow suppression. Because the toxic effects of cisplatin are significant, combining treatment modalities to allow a lower dose of cisplatin would be beneficial to patients with head and neck cancer.

The mechanism of action of cisplatin involves formation of adducts within the cell's DNA. Although blockage of mitogen-activated protein kinase and jun-K pathways observed in antisense cyclin D1–transfected cells may be involved in increased sensitivity to cisplatin, the precise molecular mechanism of growth arrest is not yet known. In the present investigation, we provide evidence that the chemosensitivity is associated with the activation of p16, p53, and BCL2–related BCLxS genes.

**METHODS**

**HNSCC CELL LINES**

The CCL23 cell line is a laryngeal squamous cell carcinoma (American Type Culture Collection, Gaithersburg, Md). CCL23AS is a stable transformant obtained with the transfection of antisense cyclin D1 plasmid into CCL23 cells. CAL27 is a tongue squamous cell carcinoma (American Type Culture Collection). Cell lines were maintained in Eagle's minimal essential medium (MEM; Gibco, Grand Island, NY) containing 1 mM glutamine, 100 IU/mL of penicillin G, 100 IU/mL of streptomycin sulfate, 0.5 µg/mL of fungizone, and 10% fetal calf serum (Omega Scientific, Tarzana, Calif).

**SEQUENCE ANALYSIS OF p53**

The genomic sequence for codons 5 through 8 of the p53 gene was amplified and sequenced using published polymerase chain reaction and sequencing primers. DNA (100 ng) was denatured at 95°C for 5 minutes and amplified using the following conditions: denaturation at 95°C for 30 seconds, annealing for 90 seconds, and extension at 72°C for 30 seconds. A 1°C step-down annealing temperature was used from 60°C to 57°C for 4 cycles each, followed by annealing at 56°C for 30 cycles. A final extension was carried out at 72°C for 7 minutes. Polymerase chain reaction products were purified with microcon filters (Millipore Inc, Bedford, Mass) and sequenced using an ABI 3730 analyzer (Applied Biosystems, Foster City, Calif).

**TREATMENT OF CELL CULTURES WITH CISPLATIN**

The parental CCL23, CCL23AS, and CAL27 cell lines were plated in 12-well plates at 37°C until cells reached 50% confluency. Cisplatin in phosphate-buffered saline (PBS) was added directly to the media at differing doses for each trial, ranging from 1 µg/mL to 10 µg/mL. The cells were incubated with cisplatin at 37°C for 6 hours for each trial. Media was aspirated out at the end of the treatment period and replaced with complete media. The cells were incubated at 37°C for another 16 hours for cells treated with 6 µg/mL of cisplatin and for 48 hours for cells treated with 3 µg/mL of cisplatin before harvest.

**WESTERN BLOT ANALYSIS**

The cell monolayer (106 cells in a 100-mm tissue culture dish) was rapidly rinsed twice with ice-cold PBS and lysed in 1 mL of ice-cold lysis buffer. The lysis buffer contained 0.1 mM phenylmethyl sulfonyl fluoride, 2 mM EDTA, 25 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 25 mM sodium fluoride, 5 µg of leupeptin, 0.2% Triton X-100 (Sigma-Aldrich Chemical Co, St Louis, Mo), and 0.3% Nonidet P-40 (Sigma-Aldrich Chemical Co) in 50 mM Tris-hydrochloride (Sigma-Aldrich Chemical Co)/150 mM sodium chloride (pH 7.5). The lysates were centrifuged at 12 000g at 4°C for 5 minutes, and the supernatants were collected. Aliquots of supernatants containing 20 µg of protein and prestained protein markers were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis in 10% gels under reducing conditions, and proteins were electrophoresed to polyvinylidene fluoride membranes (Millipore Corp, Billerica, Mass). After blocking nonspecific binding by incubation with 5% nonfat milk in PBS, the membranes were incubated with antibodies (Santa Cruz Biotechnology Inc, Santa Cruz, Calif) using the established protocol. Hybridization to specific proteins were detected with an alkaline phosphatase conjugated secondary antibody (Santa Cruz Biotechnology Inc).

**IMMUNOFLUORESCENCE STUDIES**

The cell lines were grown to 30% to 60% confluence on coverslips and then fixed after various treatments in 3.5% paraformaldehyde at 4°C for 5 hours and then washed (3 times for 5 minutes each) with PBS, also at 4°C. Immediately, cells were treated with freshly prepared 0.25% ammonium chloride for 5 minutes and washed 3 times with PBS for 5 minutes each. Cells were then permeabilized with 0.2% Triton X-100 for 10 minutes, followed by blocking with 1% bovine serum albumin in PBS for 15 minutes. Next, cells were treated with the monoclonal antibody for p53 (5 µg/mL; Calbiochen, San Diego, Calif) in 1% bovine serum albumin for 30 minutes, followed by treatment with Alexa-568 antimouse monoclonal antibody (1:400; Molecular Probes, Eugene, Ore) for 30 minutes. Cells were then washed and mounted on a microscope slide with Vectashield (Vector Laboratories, Burlingame, Calif). Fluorescence imaging was performed using a Leica DMIRB digital microscope (Leica Microsystems, Wetzlar, Germany) equipped with an ORCA CCD camera (HamamatsuCorp, Hamamatsu City, Japan). Images were recorded using Open Laboratory 3.1 (Improvision Inc, Boston, Mass) software and analyzed using the same software or Adobe Photoshop 7.1 (Adobe Systems Inc, San Jose, Calif).

**FLUORESCENCE-ACTIVATED CELL SORTER ANALYSIS**

The cisplatin-treated and untreated CCL23 and CCL23AS cell lines were analyzed using a Becton Dickinson FACScan analytic flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Briefly, 1 × 106 cells were suspended in ice-cold hypotonic DNA staining solution containing propidium iodide. Samples were protected from light and analyzed within an hour with the flow cytometer.
ANNEXIN V-FITC ASSAY FOR APOPTOSIS

The apoptosis assay was carried out with the Annexin V-FITC (fluorescein isothiocyanate) kit using the manufacturer’s protocol (Oncogene Research Products, Boston). Briefly, 5 × 10^5 cells in a 0.5-mL suspension of the culture medium are centrifuged at 1000g for 5 minutes, suspended in 0.5 mL of cold PBS, centrifuged again at the low speed for 5 minutes, and re-suspended in 0.5 mL of cold Annexin V binding buffer (10mM HEPES [N-2-hydroxyethylpiperazine-N’-ethanesulfonic acid] [pH 7.4], 150mM sodium chloride, 2.5mM calcium chloride, 1mM magnesium chloride, and 4% bovine serum albumin). Annexin V-FITC (1.25 µL) was added and incubated in the dark at room temperature for 15 minutes. The treated cells were centrifuged at the low speed for 5 minutes and suspended again in 0.5 mL of cold binding buffer. Propidium iodide (10 µL) was added, and the cells were analyzed on a FACScan analytic flow cytometer. Annexin V-FITC fluorescence was detected at 518 nm and the propidium iodide at 620 nm.

CASPASE-3 ASSAY

The release of caspase-3 was measured using the caspase-3 cellular activity assay kit (Calbiochem). Briefly, an aliquot (20 µL) of the CCL23 cell lysate was mixed with the caspase-3 substrate acetyl-DEVD-p-nitroanilide (Ac-DEVD-pNA) (200µM) in the assay buffer (100mM sodium chloride, 50mM HEPES, 10mM diithiorthreitol, 10% glycerol, and 0.1% CHAPS [3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate] [pH 7.4]) and incubated at 37°C for up to 180 minutes. Caspase-3 enzyme was measured as a release of pNA at 405 nm in an enzyme-linked immunosorbent assay plate reader. Control included purified caspase-3 enzyme (10 µU/µL) supplied by the manufacturer. Enzyme was added equivalent to that released by U937 cells treated with the apoptotic inducer etoposide. Caspase-3 activity was also measured in the lysate collected from CCL23 cells treated with cisplatin (6 µg/mL).

RESULTS

DETECTION OF A p53 MUTATION

There are 11 exons for the p53 gene, while tumor-associated mutations are mostly seen in the region encompassing exons 5 through 8. Comparison of the polymerase chain reaction sequences for exons 5 through 8 to the published p53 sequence showed the presence of wild-type p53 gene in CCL23 cells (data not shown). CAL27 cells contained a missense mutation in codon 193 of exon 6 (A→T transversion). This results in substitution of a neutral amino acid leucine for a positively charged histidine residue. This mutation could contribute to over-expression of the p53 protein in this cell line.

ELEVATED EXPRESSION OF p16 AND p53 WITH CISPLATIN TREATMENT

We had shown that there was reduced expression of cyclin D1 with the introduction of antisense cyclin D1, and these cells had an increased sensitivity to cisplatin. We can therefore expect perturbation of other cell cycle genes (Figure 1). Of the various proteins analyzed by Western blotting, expression of p16, p53, and p21 were elevated with the addition of cisplatin treatment in both the parental and antisense cyclin D1 cell lines (Figure 2A). The level of DP1 remained unaltered, and low-level expression of p27 and MDM2 was observed in both the cisplatin-treated and untreated cells. Data from our laboratory and others have shown that CCL23 cells express wild-type p53. Higher expression of p53 with cisplatin treatment may correlate with the activation of wild-type p53 in response to DNA damage by cisplatin. This is also reflected in the increased expression of the p21 protein, a downstream effector of p53. The higher-level expression of p16 with cisplatin treatment, particularly as seen in CCL23AS cells, again indicated a drug-induced response and possible presence of the functionally active p16 protein in the CCL23 and CCL23AS cells.

Western blot analysis of CAL27 after treatment with cisplatin is shown in Figure 2B. Because the high level of protein loading did not allow for assessment of increased p53 and p21 expression in Figure 2A, Western blot analysis for CCL23 cells was repeated and is shown for comparison. Although there was little change in the total expression of p53 in CAL27 following treatment with cisplatin, there was a marked increase in expression of p21. This suggests the
role of p53 mediation through p21 inhibition of the cyclin D1 or cyclin E pathway, as shown in Figure 1.

To confirm the expression level of p53, immunofluorescence studies were performed in CCL23 and CAL27 using a p53 antibody. In Figure 3A, p53 is seen in both the cytoplasm and nucleus of untreated CCL23 cells. After treatment with cisplatin, fewer live cells are seen, and increased expression of p53 is seen in the nucleus (Figure 3B). Untreated CAL27 cells show expression of p53 in the nucleus (Figure 3C), and treatment with cisplatin results in fewer live cells, with similar expression of p53 still in the nucleus (Figure 3D).

DEPHOSPHORYLATION OF Rb IN CISPLATIN-TREATED CELLS

The function of the Rb protein is dependent on the phosphorylation status of the protein, with the hyperphosphorylated form being functionally inactive and hypophosphorylated Rb being the active suppressor protein. With decreased expression of cyclin D1 and increased expression of p16, one would expect decreased CDK4 kinase activity in the nucleus resulting in decreased phosphorylation of the Rb protein. Figure 4 demonstrates dephosphorylation of Rb in cisplatin-treated cells, with complete absence of the hyperphosphorylated Rb in CCL23AS cells treated with 6 µg/mL of cisplatin (far right lane). In the parental CCL23 cells and CCL23AS cells treated with lower doses of cisplatin, there is higher expression of total Rb and hyperphosphorylated Rb, resulting in lack of separation of the 2 proteins (the 4 left lanes). Thus, in the CCL23AS cells, increased expression of p16 results in decreased CDK4 kinase activity, leading to a complete dephosphorylation of the Rb protein.

ENHANCED CELL DEATH IN CCL23AS CELLS FOLLOWING CISPLATIN TREATMENT

Fluorescence-activated cell sorter analysis was performed on the CCL23 and the CCL23AS cells following treatment with 3 and 6 µg/mL of cisplatin. Compared with the CCL23 cells, a decreased G2 peak was observed in the CCL23AS cells (28% vs 25%), indicating increased cell cycle arrest (Figure 5). There was an increase in apoptotic and necrotic cells in both the parental and antisense cyclin D1-containing cells treated with cisplatin. The level of apoptotic and necrotic cells in CCL23AS cells treated with 3 µg/mL of cisplatin (61%) equaled that of the parental CCL23 cells treated with 6 µg/mL cisplatin (58%). A very high level of apoptotic cells was seen in the CCL23AS cells (91%) treated with 6 µg/mL of cisplatin.

INDUCTION OF CASPASE-3 INDEPENDENT APOPTOSIS BY CISPLATIN

In normal cells, phosphotidyl serine is located on the cytoplasmic surface of the cell membrane. Induction of apoptosis results in the reorganization of phosphotidyl serine to the outside of the membrane and the molecule can be detected because of its interaction with the anticoagulant Annexin V. The translocation of phosphotidyl serine to cell surface seems to be an early event in apoptosis preceding nuclear breakdown and DNA fragmentation. Thus, Annexin V binding is a useful tool for the measurement of apoptosis. In the next set of experiments, FITC-conjugated Annexin V was used for the detection of phosphotidyl serine binding by flow cytometry. Propidium iodide was used to distinguish between early and late apoptosis.
Appropriate controls were performed with Annexin V-FITC alone and propidium iodide alone to minimize the overlap between these 2 signals. The analysis revealed induction of apoptosis with increasing concentration of cisplatin in both the CCL23 and CCL23AS cells (Figure 6). Again, half the cisplatin concentration (3 µg) was required in the CCL23AS cells, as opposed to 6 µg in the CCL23 cells for a similar level of apoptosis, confirming increased sensitivity of the CCL23AS cells to cisplatin.

A cell-permeable caspase-3 inhibitor (CPC3I; Oncogene Research Products) was used for the inhibition of apoptosis through the caspase-3 pathway. The inhibitor (50µM) was added to the cells at the time of cisplatin treatment. Although the inhibitor could reduce cell death in CCL23 and CCL23AS cells, there was cell killing (necrosis or senescence) in the presence of the caspase-3 inhibitor. Cell death in the presence of the inhibitor is noticeable in CCL23AS cells, indicating cell killing via a caspase-3 independent pathway in this cell system (Figure 6).

To confirm that a low level of caspase-3 is released in CCL23 cells with cisplatin treatment, a caspase-3 release assay was performed. The recombinant caspase-3 enzyme supplied was equivalent to that released by U937 cells treated with etoposide. Recombinant caspase-3 enzyme was added to lysate of CCL23 cells, and caspase-3 release was measured using the substrate. There was a release of 0.5 OD 405 units (optical density units measured at 405nM wave-

**Figure 3.** Immunofluorescence study of p53 in CCL23 and CAL27 cells following cisplatin treatment. A, Untreated CCL23 cells demonstrate expression of p53 in the cytoplasm and nucleus. B, Following treatment with cisplatin, there are fewer live cells, and p53 expression is increased in the nucleus. C, Untreated CAL27 cells show expression of p53 in the nucleus. D, Following treatment with cisplatin, there are fewer live cells, with similar expression of p53 in the nucleus.
untreated CCL23 cells did not release caspase-3. This supports our previous data showing minimal expression of caspase-3 in CCL23 cells compared with the control C6 cells and negligible caspase-3 expression in CCL23 cells after treatment with cisplatin.14

As an alternate apoptotic pathway, we examined the expression of Bcl-2 and found that the protein level was not altered in the different cell lines (data not shown). Therefore, Bcl-2-related proteins were studied. Bcl-x, a member of Bcl-2 family, has 2 alternatively spliced forms, Bcl-xL (241 amino acids) and Bcl-xS (178 amino acids).21 While Bcl-xL is anti-apoptotic, Bcl-xS is proapoptotic, and therefore the ratio of these 2 proteins could determine the extent of apoptosis. In the present investigation, although there was increased expression of both proteins, the expression of Bcl-xS was higher compared with Bcl-xL in the CCL23AS cells (Figure 8). Bcl-xL was completely absent in CCL23AS cells treated with 6 µg/mL of cisplatin. Thus, the Bcl-xS apoptotic pathway seems to be activated with cisplatin treatment.

COMMENT

In advanced head and neck cancers, cisplatin has been used both alone and in combination with other chemo-
Figure 6. Caspase-3 independent cell death in cisplatin-treated cells. A, CCL23 cells treated with 3 and 6 µg/mL of cisplatin (cisplatin-3 and cisplatin-6), followed by treatment with cisplatin + caspase-3 inhibitor (CPC3I). The graphs show increasing apoptosis with increasing doses of cisplatin. Although a decreased cell killing is seen in cisplatin-untreated cells with the addition of caspase inhibitor, there is cell death with cisplatin treatment even in the presence of caspase-3 inhibitor. B, CCL23AS (antisense cyclin D1–transfected cells) treated with 3 and 6 µg/mL of cisplatin, followed by treatment with cisplatin and CPC3I. Similar to the parental cells, antisense cyclin D1–containing cells show additional cell death in the presence of cisplatin that could be attributed to necrosis or senescence (lower right graph). FL1-H indicates annexin V–FITC fluorescence detected at 518nM; FL2-H, propidium iodide fluorescence detected at 620nM.
Several mechanisms of cisplatin resistance have been identified, including reduced drug accumulation, increased drug inactivation by sulfur-containing molecules, enhanced repair of cisplatin-DNA adducts, increased tolerance of cisplatin damage, and altered expression of regulatory proteins. While resistance to cisplatin in tumor cells is multifactorial, recent evidence indicates that important pathways involving altered expression of oncogenes and tumor suppressor genes also contribute to the phenomenon of resistance.

Oncogenes implicated in cisplatin resistance include the ras family, c-jun, c-fos, and c-myc. Cell cycle and apoptosis modulators have also been identified as mediators of cisplatin resistance. Warenius et al demonstrated that cisplatin resistance was correlated with c-myc–dependent expression of cyclin D1. In the 16 human carcinoma cell lines studied, those with higher levels of cyclin D1 expression exhibited higher resistance to cisplatin. A reduction in the expression of cyclin D1 could result in increased sensitivity of the tumors to cisplatin.

It has also been shown that cisplatin kills tumor cells by initiating apoptosis. While the specific mechanisms are not clear, investigators have focused on the key regulators of apoptosis. Tumor suppressor gene p53 is an essential regulator of apoptosis, and there is strong evidence that p53 plays a role in cisplatin sensitivity. Gallagher et al used p53 genetic suppressor elements to decrease p53 protein levels, resulting in an 8-fold increase in resistance to cisplatin in ovarian cancer cells. Several studies have confirmed the increased resistance to cisplatin in p53 mutant cancer cell lines. These studies support the theory that p53 mutations result in loss of G1/S checkpoint control and the inability of the mutated p53 to initiate apoptosis in response to DNA damage. Other investigators, however, have found that most HNSCC cell lines with a p53 mutation have increased sensitivity to cisplatin, whereas those with wild-type p53 tend to be more resistant. The latter data support the hypothesis that cells that lack functional p53 are unable to repair DNA damage and are thus more susceptible to DNA-damaging agents. In a recent report it was shown that the treatment of cells with interferon α/β increases the expression of p53 and that this expression can be augmented with a chemotherapeutic agent.

The efficacy of cisplatin in HNSCC is greatly enhanced when combined with another antitumor agent. Cell cycle inhibition using antisense cyclin D1 notably enhances the sensitivity of pancreatic head and neck cancer cells to cisplatin. The mechanism of action of this phenomenon of enhanced cisplatin sensitivity through cell cycle inhibition has not been well studied.

The mechanism of action of cisplatin involves formation of covalent adducts in which the chloride ligands of the drug are replaced by specific DNA bases. The adducts then inhibit DNA replication and transcription and activate the process of apoptosis. By inhibiting DNA synthesis in cells, cisplatin-DNA adducts slow cell division, with a selective effect on rapidly dividing tumor cells. In addition, the blockade of transcription and RNA synthesis by the cisplatin-DNA adducts also selectively inhibits rapidly dividing tumor cells.
Thus, enhanced expression of wild-type p53 seems to be an important pathway for apoptotic cell death in cisplatin-treated cells.

One study has shown that cisplatin-induced growth arrest in human cancer cells has characteristics of senescence rather than apoptosis. The senescence-associated β-galactosidase was expressed following cisplatin exposure, but there was no change in p53, p21, or p16 expression. This suggests that cancer cells lacking p53 function can also be killed by cisplatin via a p53-independent mechanism with similarities to replicative senescence.

To investigate these possibilities, we studied the expression of cell cycle and apoptotic genes in cisplatin-treated head and neck cancer cell lines: a parental and an antisense cyclin D1–transfected laryngeal carcinoma line (CCL23), as well as a tongue carcinoma line, CAL27. We demonstrate elevated expression of p16 and p33 in CCL23 and CCL23AS cells after treatment with cisplatin. This indicates that inhibition of HNSCC growth involves reduced growth through the Rb pathway and initiation of apoptosis by p53. Our results further suggest that the functional expression of p16 augments the effect of cisplatin in cell killing. It could therefore be speculated that the tumors expressing p16 may be good candidates for adjuvant therapy with cisplatin. Conversely, those tumors with absence of p16 expression might be treated with a combined p16 gene therapy and cisplatin protocol for an effective tumor treatment. We have previously shown that CCL27 cells transfected with antisense cyclin D1 demonstrated a slower rate of growth; however, the sensitivity to cisplatin was not increased. We believe this is because p16 is lacking in CCL27 cells (data not shown). Because p16 and p53 are functionally active in the nucleus, it is likely that cisplatin plays an essential role in the nuclear transport and stabilization of these proteins for cell cycle arrest and apoptosis.

Although the CCL23 cells contain human papillomavirus type 18, increased nuclear expression of p53 on cisplatin addition may be due to inactivation of the human papillomavirus E6 protein by cisplatin. CCL27 cells contain a missense mutation in codon 193 (A→T transversion) of exon 6 of the p53 gene. This mutation has also been observed previously in a brain tumor, premalignant head and neck cancer, and chondrosarcoma. Even in the presence of this mutation, there was increased expression of the p21 protein in CCL27 cells with cisplatin treatment. It is likely then that this mutational site is not involved in the activation of the p21 gene. Because in CCL27 cells there is increased expression of p21 and increased cell death after treatment with cisplatin, we hypothesize that there is p53-mediated cell-cycle arrest and apoptosis, since p21 is a downstream effector of p53. There is also the possibility of p21 activation via a p53-independent pathway.

In addition, our study suggests that apoptosis takes place via a caspase-3 independent pathway in cisplatin-treated cells. Bcl-XL prevents apoptosis by complexing with Bcl-2 (antiapoptotic) and Bax (proapoptotic) proteins. Bcl-xS is proapoptotic and, similar to Bcl-2-related proteins, is localized to mitochondria. Apoptotic activity of this protein is induced by its ability to form homodimers and heterodimers with Bcl-XL and Bcl-2. This could result in antagonizing the antiapoptotic activity of Bcl-2 and Bcl-XL. We have shown herein that in the presence of cisplatin, there is induction of Bcl-xS and a caspase-3 independent pathway of apoptosis in CCL23. Because CCL23 has relatively low expression of Bcl-XL, these cells are sensitive to cisplatin, since cisplatin-resistant cells tend to have high Bcl-XL expression. This leads to an interesting possibility that head and neck cancers can be treated with drugs that may induce Bcl-xS synthesis or inhibit Bcl-XL expression, in conjunction with cisplatin, for effective tumor control.

While it is true that many HNSCC cell lines have a p53 mutation and loss of p16, actual primary tumors are much more heterogeneous in nature. This phenomenon of tumor heterogeneity is likely a significant factor in tumor resistance to chemotherapy and radiation. Furthermore, while patients with advanced HNSCC are given the option of surgical resection vs chemoradiation, currently, it is not possible to predict which tumors will respond to chemoradiation and which will be resistant. A greater understanding of the mechanism of action of cisplatin will enhance our ability to predict a tumor’s response. The role of p16 and p53 in the cytotoxic action of cisplatin is important because these genes may have significance as prognostic markers to predict a tumor’s chemosensitivity. Inactivation of p16 and p53 seem to be early events in the development and progression of HNSCC. Therefore, the evaluation of the expression status of p16 and p53 may be a valuable tool for treatment planning. In the future, tumor biopsies may be routinely studied using real-time polymerase chain reaction to determine the expression of p16 and the status of p53 (mutant vs wild type). This will aid the clinician in selecting the type of treatment because tumors that lack p16 and p53 may not exhibit as great a sensitivity to cisplatin. The data also support the use of specific gene therapy with p16 and/or p53 that could enhance a tumor’s sensitivity to chemotherapy.

Combination treatment of HNSCC via cell cycle inhibition and cisplatin holds promise as a potential therapy in the clinical setting. The mechanism of action of antisense cyclin D1 and cisplatin appears to involve cell cycle arrest and initiation of apoptosis. Further understanding of the mechanisms involved in this phenomenon is needed. We demonstrate that the identification of genes involved in cisplatin-induced cell death may lead to more rational approaches for tumor treatment. Such a strategy holds potential for enhancing the efficacy and clinical usefulness of this valuable antineoplastic agent.

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