Cisplatin Treatment Induces a Transient Increase in Tumorigenic Potential Associated with High Interleukin-6 Expression in Head and Neck Squamous Cell Carcinoma

Kim J. Poth1, Alexander D. Guminski2, Gethin P. Thomas1, Paul J. Leo1, Ibtissam A. Jabbar1, and Nicholas A. Saunders1

Abstract

Head and neck squamous cell carcinoma (HNSCC) is characterized by the 5-year survival rate of ∼50%. Despite aggressive surgical, radiation, and chemotherapeutic interventions, 30% to 40% of patients die from the development of recurrent or disseminated disease that is resistant to chemotherapy. As a model of recurrence, we examined the effects of cisplatin on the ability of head and neck cancer cells to initiate tumors in a xenotransplant model. HNSCC cells were treated in vitro with cisplatin at a concentration that elicited >99% cytotoxicity and assessed for tumorigenic potential in nonobese diabetic/severe combined immunodeficient mice. HNSCC cells that survived cisplatin treatment formed tumors in nonobese diabetic/severe combined immunodeficient mice more efficiently than nontreated cells. Cisplatin-resistant cells were characterized using clonal analysis, in vivo imaging, and transcriptomic profiling. Preliminary functional assessment of a gene, interleukin-6 (IL-6), highly upregulated in cisplatin-treated cells was carried out using clonogenicity and tumorigenicity assays. We show that cisplatin-induced IL-6 expression can contribute to the increase in tumorigenic potential of head and neck cancer cells but does not contribute to cisplatin resistance. Finally, through clonal analysis, we show that cisplatin-induced IL-6 expression and cisplatin-induced tumorigenicity are stochastically derived. We report that cisplatin treatment of head and neck cancer cells results in a transient accumulation of cisplatin-resistant, small, and IL-6-positive cells that are highly tumorigenic. These data also suggest that therapies that reduce IL-6 action may reduce recurrence rates and/or increase disease-free survival times in head and neck cancer patients, and thus, IL-6 represents a promising new target in HNSCC treatment.

Introduction

The term head and neck cancer refers to a group of cancers arising from the epithelia of the upper aerodigestive tract (1). Approximately 90% of head and neck cancers are squamous cell carcinomas (HNSCC; ref. 2). Head and neck cancers are associated with a high rate of mortality and considerable morbidity, due to their anatomical location. Major risk factors for the development of HNSCC are tobacco use, alcohol consumption, and infection with human papillomavirus (1). Current treatment usually involves a combination of surgical resection with radiotherapy and chemotherapy (2). All of these treatment modalities have undergone improvements over the last several decades and have led to improvements in outcomes for some types of HNSCC (for example, advanced laryngeal and pharyngeal carcinoma; ref. 3). Despite these advances, the 5-year survival rate for HNSCC as a whole remains at ~50% (2). Surgery and radiation therapy have delivered reductions in patient mortality for localized disease. However, these interventions are of limited value against disseminated disease (4). Because of high recurrence rates and the propensity for locoregional spread, platinum-based regimens with or without taxanes are commonly used in HNSCC. However, drug resistance and recurrence still occur and remain barriers to curative treatments (see ref. 5 for review). Improved cure rates for HNSCC will require a greater understanding of the biology of tumor recurrence and drug resistance.

Cisplatin has been commonly used in the treatment of HNSCC as well as many other cancers (2, 6). Its modes of action are still incompletely understood, but seem to involve the induction of DNA damage due to the formation of platinum-DNA adducts that distort the structure of the DNA helix (6, 7). These adducts interfere with normal mitosis by disrupting appropriate
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chromosome segregation. Furthermore, cisplatin is thought to “hijack” transcription factors, which bind to the platinum-DNA adducts and are thus unavailable for their normal functions in gene regulation, as well as shielding the adducts from DNA repair proteins (7). Despite the advances made in understanding the mechanisms by which cisplatin induces cytotoxicity, few studies have focused on the biological properties of those tumor cells that survive cisplatin treatment. Because recurrent disease can only arise from those tumor cells that evade/survive cisplatin or radiation treatment, it remains important to understand the effect of cisplatin on the ability of surviving tumor cells to reestablish tumors in situ (i.e., initiate recurrent disease).

In the present study, we examined the ability of HNSCC cells to reestablish tumors in a xenotransplant model of HNSCC following a single cytotoxic dose of cisplatin. We report, for the first time, that a cytotoxic dose of cisplatin causes a transient increase in the ability of surviving cells to initiate tumors by approximately 100- to 600-fold in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. This transient increase in tumor-initiating (TI) activity returned to basal levels within several passages. Clonal analysis of cells before, or following cisplatin treatment, coupled with microarray analysis indicated that acute cisplatin treatment enriched for an interleukin-6 (IL-6)–positive and TI<sup>high</sup> fraction of small cells. Further examination indicated that the IL-6–positive, TI<sup>high</sup> small cells arose through a stochastic process and did not arise from a drug-resistant subpopulation of cancer stem cells. Most significantly, overexpression of IL-6 in HNSCC cells increased TI activity. Conversely, pharmacologic blockade of IL-6 signaling using an anti–IL-6 receptor monoclonal antibody (Actemra) reduced tumor initiation activity by cisplatin-treated HNSCC cells. Combined, these data suggest that cisplatin-induced IL-6 expression in HNSCC cells contributes to the transient increase in TI observed following cisplatin treatment.

Materials and Methods

Tissue culture and cisplatin treatment

HNSCC cell lines were obtained from the American Type Culture Collection and cultured as described (8). In the drug treatment experiments, cells were treated with cisplatin [cis-diaminedichloroplatinum(II) CDDP; InterPharma] for 48 hours.

Colony-forming assays

Colony-forming assays were done as described (9). Nontreated cells or cells treated with cisplatin for 48 hours were dissociated, counted, and plated at various densities (typically ranging from 1 x 10<sup>2</sup>-3 x 10<sup>4</sup> cells per well) into six-well tissue culture plates. Cells were incubated at 37°C for sufficient time to allow colony formation, and then colonies were stained with Coomassie blue and counted.

Cell viability assays

When required, cell viability was estimated using the CellTiter-Blue kit, according to the manufacturer's directions (Promega).

Animals and tumor initiation studies

Animal work was done in accordance with The University of Queensland ethics guidelines. Animals used for tumor initiation studies were NOD/SCID mice or nude mice (Animal Resources Centre). For tumor initiation studies, mice were injected s.c. on the neck scruff with 1 x 10<sup>4</sup> to 1 x 10<sup>6</sup> HNSCC cells in PBS. Mice were monitored regularly for tumor growth and sacrificed when tumors reached 1 cm x 1 cm.

Generation of green fluorescent protein–expressing cells and in vivo tumor imaging

Green fluorescent protein (GFP)–expressing FaDu cells (FaDu-GFP) were established following infection with the GFP-expressing pLL3.7 lentiviral construct as previously described (10, 11). Cells were checked at intervals through fluorescence microscopy or fluorescence-activated cell sorting to confirm continued stable expression of GFP. Before injection, NOD/SCID mice were shaved and treated with hair removal cream. NOD/SCID or nude mice were injected with FaDu-GFP cells s.c. in the neck scruff. Mice were anesthetized using isoflurane, and both X-ray and fluorescent images were acquired using a Kodak Image Station <i>in vivo</i> imaging system (Integrated Sciences). Images were captured and quantitated using the Kodak MI software.

Generation of single cell–derived clonal cell lines

Clonal cell lines derived from single nontreated FaDu or Detroit-562 cells and Detroit-562 cells treated with 5 μmol/L cisplatin for 48 hours were established by limiting dilution analysis. The plates were then monitored for colony formation. Under these conditions, a few wells produced a single colony. These colonies were expanded to generate clonal cell lines for further study.

RNA isolation

Total RNA was isolated from HNSCC cells using Trizol reagent (Invitrogen) as per the manufacturer's directions.

Reverse transcription and real-time PCR

Reverse transcription and real-time PCR conditions have been previously described (12). Primers for IL-6 were as follows: forward AGTGGAAACAAGCCAGGAC and reverse GTCAAGGG-TGTTATTGCAT. Cycling was done using a RotorGene 6000 thermal cycler (QIA-GEN), and data were analyzed using the RotorGene 1.7 software.

IL-6 protein quantification

IL-6 secreted into the culture medium by HNSCC cells was quantified using the BD CytoView Bead Array system according to the manufacturer's directions. Data
were acquired using a BD FACScarray flow cytometer, and analysis was done using FCAParray software (BD Biosciences).

**Generation of IL-6–overexpressing Detroit-562 cells**

293FT cells (Invitrogen) were reverse transected with packaging plasmids pVSV-G, pRSV-REV and pGP8.2 (13), and either pLV101-GFP control plasmid or pLV101-GFP-IL-6 using Lipofectamine 2000 (Invitrogen). Viral supernatant was harvested following incubation for 72 hours at 37°C. Cells were transduced with virus in the presence of 7.3 μg/mL polybrene. Transduced cells were selected using high-stringency sorting using a MoFlo flow cytometer, repeated three times. The stably expressing cells thus created were termed Detroit-pLV101 and Detroit-IL-6. The cell lines were assayed for IL-6 overexpression using qPCR and Cytometric Bead Array, as well as regular assessment of GFP expression using both fluorescence microscopy and fluorescence-activated cell sorting.

**Treatment of NOD/SCID mice with anti–IL-6R antibody (Actemra)**

Actemra (Roche) is a monoclonal antibody that inhibits IL-6 signaling by binding to the IL-6 receptor. Mice were inoculated s.c. with 1.3 × 10⁶ Detroit-562 cells that had been pretreated with 5 μmol/L cisplatin for 48 hours. Mice were then divided into two groups. The control group (n = 6) were injected i.p. with 100 μL PBS twice per week, whereas the Actemra group (n = 7) were injected i.p. with 2 mg (100 μL) Actemra twice per week. Mice were monitored for tumor incidence and growth, and sacrificed when tumors measured ~1 cm × 1 cm.

**Results**

**HNSCC cell lines exhibit a delayed cytotoxic response to cisplatin in vitro and in vivo**

Cisplatin concentration response curves were generated using resazurin-based CellTiter-Blue assays of mitochondrial integrity following a 48-hour exposure to cisplatin (Fig. 1A). Based on this analysis, cisplatin had an EC₅₀ value of ~6 μmol/L for FaDu and 11 μmol/L for Detroit-562 HNSCC cell lines. However, examination of the time-dependent effects of treatment with 2 and 5 μmol/L cisplatin on cell numbers indicated that cisplatin-induced cytotoxicity took several days to manifest, and hence, the EC₅₀ values determined after 48 hours of exposure to cisplatin were overestimated (Fig. 1B). Therefore, we assayed for long-term replicative viability using colony-forming assays (Supplementary Table S1). Using colony-forming assays, we found that treating cells with 2 μmol/L cisplatin could reduce colony-forming ability by >90% in several HNSCC cell lines, whereas 5 μmol/L cisplatin caused >99% loss of clonogenicity (Supplementary Table S1). The delayed cytotoxic response to cisplatin is important because to progress to in vivo tumor initiation studies, it is necessary to have a reliable and quantitative measure of the number of cells that survive cisplatin treatment and that can contribute to tumor initiation.

We next examined whether the delayed cytotoxic response to cisplatin also occurred in an in vivo setting.
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FaDu-GFP cells were treated with 5 μmol/L cisplatin for 48 hours, and 1 × 10⁶ treated and nontreated FaDu-GFP cells injected s.c. into NOD/SCID or nude mice. The fluorescent signal from the cisplatin-treated cells decreased below the limit of detection (i.e., to below 10% of the total injected cells) by day 16 postinjection, after which time it recovered to establish tumors (Fig. 1C). In contrast, the fluorescent signal from the nontreated cells decreased modestly to 75% of the total injected fluorescence by day 4 before recovering to establish tumors (Fig. 1C). These data indicate that a single, short exposure to cisplatin results in a profound, delayed cytotoxic response in vitro and in vivo.

**Transient increase in TI activity in HNSCC cells following a single cisplatin treatment**

The presence of surviving tumor cells with the ability to reestablish a tumor following treatment of HNSCC patients is an obligatory requirement for disease recurrence. We therefore tested the effects of a single cisplatin exposure on the ability of HNSCC cells to initiate and establish a tumor in vivo. We studied tumor initiation potential (i.e., the number of cells required to initiate a tumor) at both early and later time points following a single cisplatin exposure. We also examined the effect of the single cisplatin treatment on long-term in vitro survival. Similar numbers of cisplatin-treated (2 or 5 μmol/L) FaDu or Detroit-562 cells, compared with nontreated cells, were needed to initiate tumors when injected into NOD/SCID mice (Table 1). However, in vitro colony-forming assay data (Table 1) and the in vivo imaging data (Fig. 1C) indicate that the majority of the cisplatin-treated cells do not remain viable in the longer term, and hence, fewer viable cisplatin-treated HNSCC cells exist to initiate tumors following injection versus nontreated HNSCC cells. The CFE of the cells provided a surrogate marker for cell viability following injection and allowed comparisons of the proportion of surviving cisplatin-treated and nontreated cells available to participate in tumor initiation. Relative tumorigenicity was calculated by dividing colony forming efficiency (CFE) of nontreated cells by CFE of treated cells available to participate in tumor initiation.

### Table 1. Tumor initiation studies and concurrent clonogenicity assays show that cisplatin-treated HNSCC cells are more aggressive than their nontreated counterparts

<table>
<thead>
<tr>
<th>No. of tumors/number of mice injected (1 × 10⁵ cells injected)</th>
<th>CFE (%) Reduction in CFE compared with nontreated</th>
<th>Calculated number of colony-forming units for tumor initiation Increase in tumorigenicity (fold) compared with nontreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontreated Detroit-562</td>
<td>4/5 26 ± 1%</td>
<td>26,000 ± 260 1 ± 0.04</td>
</tr>
<tr>
<td>Detroit-562 2 μmol/L cisplatin</td>
<td>4/5 0.84 ± 0.27%</td>
<td>97% 840 ± 269 31 ± 9.9-fold increase*</td>
</tr>
<tr>
<td>Detroit-562 5 μmol/L cisplatin</td>
<td>5/5 0.048 ± 0.019%</td>
<td>99.8% 48 ± 19 650 ± 257-fold increase*</td>
</tr>
<tr>
<td>Nontreated FaDu</td>
<td>4/4 8 ± 1%</td>
<td>8,000 ± 1,000 1 ± 0.12</td>
</tr>
<tr>
<td>FaDu 2 μmol/L cisplatin</td>
<td>5/5 1.23 ± 0.39%</td>
<td>84.6% 1,230 ± 390 5.7 ± 1.8-fold increase†</td>
</tr>
<tr>
<td>FaDu 5 μmol/L cisplatin</td>
<td>4/5 0.05 ± 0.01%</td>
<td>99.4% 50 ± 10 112 ± 22-fold increase‡</td>
</tr>
</tbody>
</table>

NOTE: Cells were treated with 5 μmol/L cisplatin for 48 h in vitro. Following treatment, cells were replated to remove cells undergoing early cell death. Then after a further 16 h of incubation, cells were plated into six-well tissue culture plates at densities ranging from 1 × 10² to 3 × 10⁴ cells for colony-forming assays, and also injected s.c. into NOD/SCID mice (n = 5 mice per group). Mice were monitored regularly for tumor initiation and growth and were sacrificed when tumors measured 1 cm × 1 cm. CFE was used to infer the degree of postinjection loss of viability in the cisplatin-treated cells compared with nontreated cells. The estimated increase in tumor initiation is based on our estimate of the CFE multiplied by the number of cells that can initiate a tumor. In all cases, cisplatin-treated cells were significantly more aggressive than nontreated cells.

*P < 0.001, †t test.
†P < 0.01, ‡t test.
‡P < 0.05, ††t test.
Based on our measure of long-term viability (CFE), we estimate that the TI activity of the FaDu and Detroit cells was increased approximately 5- and 31-fold, respectively, in response to 2 μmol/L cisplatin and 112- and 650-fold in response to 5 μmol/L cisplatin (Table 1). These data indicate that cisplatin treatment induces a dose-dependent increase in TI activity in HNSCC cells.

Because tumor recurrence in patients can occur at very long time points posttreatment, we examined the late-phase response of cells to a single cisplatin treatment. Clonal cell lines were established following treatment of Detroit-562 cells with 5 μmol/L cisplatin for 48 hours. Clonogenicity, morphology, long-term viability, cisplatin sensitivity, and tumorigenicity were then examined. A total of 11 Detroit-562 clones were established following cisplatin treatment, but only 9 could be maintained through serial passaging. Following three to four passages in culture, many of these clones had regained CFE similar to the nontreated parental cell line (Table 2). The clones displayed morphologic differences in the size and appearance of the colonies as well as in the appearance of the individual cells (Supplementary Fig. S4).

Thus, a single treatment with cisplatin can induce dysmorphic changes in HNSCC cells that persist through many replication cycles. Cells from each of the nine clonal lines were injected into NOD/SCID mice and TI activity evaluated (Table 2). This showed that the TI activity of the FaDu and Detroit cells survived several passages in culture, many of these clones had regained CFE similar to the nontreated parental cell line (Table 2). The clones displayed morphologic differences in the size and appearance of the colonies as well as in the appearance of the individual cells (Supplementary Fig. S4).

Similar to the nontreated parental cell line, the clones still retained sensitivity to the cytotoxic effects of cisplatin such that re-treating long-term clones with 5 μmol/L cisplatin resulted in the majority of cells dying and detaching (data not shown). However, after several more passages in culture, three of the nine clones ceased growing to confluence, although the cells were still cycling and subsequently detaching from the culture vessel. This included clone 7, which had generated tumors with the highest efficiency out of the cisplatin-treated clones, suggesting that at least some of the derived clones were unstable in the long-term following a single cisplatin exposure. Combined, these data indicate that the effects of a single treatment with cisplatin are complex, with varying effects in the short and long term. In addition, these data indicate that the effects of a single exposure to cisplatin persist over a period of months through many replication cycles.

**Table 2.** Variable tumor initiation by cisplatin-treated single cell–derived clones injected into NOD/SCID mice

<table>
<thead>
<tr>
<th>% tumor formation</th>
<th>1 × 10⁴ cells injected</th>
<th>3 × 10⁴ cells injected</th>
<th>1 × 10⁵ cells injected</th>
<th>3 × 10⁵ cells injected</th>
<th>1 × 10⁶ cells injected</th>
<th>CFE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone number</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>25%</td>
<td>25%</td>
<td>100%</td>
<td>15%</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5%</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>75%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5%</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>100%</td>
<td>50%</td>
<td>100%</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>25%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>—</td>
<td>9.67%</td>
</tr>
<tr>
<td>6</td>
<td>75%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>1.14%</td>
</tr>
<tr>
<td>7</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>49.3%</td>
</tr>
<tr>
<td>10</td>
<td>75%</td>
<td>75%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>11.67%</td>
</tr>
<tr>
<td>11</td>
<td>100%</td>
<td>75%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>42%</td>
</tr>
</tbody>
</table>

NOTE: Detroit-562 cells were treated with 5 μmol/L cisplatin for 48 h, then cisplatin was removed and cells were incubated for a further 5 to 6 d to remove cells undergoing cell death. Cells were then plated at low numbers and monitored for colony growth. Wells in which a single colony grew were selected for expansion to form clonal cell lines, which were injected s.c. into NOD/SCID mice at varying numbers of cells per injection to study tumor initiation. Mice were monitored regularly for tumor initiation and growth and were sacrificed when tumors measured 1 cm × 1 cm. n = 4 for each group.

**Cisplatin-induced transient increase in tumorigenic potential is due to a stochastically derived population of cells that survive cisplatin and induce TI and IL-6**

The elevated TI activity observed in the immediate phase after a single cisplatin exposure could be due to (a) the enrichment for a rare, highly tumorigenic and cisplatin-resistant cancer stem cell population (see ref. 14 for a review) or (b) could be due to a stochastically derived population of cells that survive cisplatin treatment and induce TI activity in response to cisplatin. These two explanations reflect fundamentally different biological processes that have significant implications for the management of HNSCC. We therefore examined whether...
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Figure 2. Cisplatin treatment of both parental and clonal HNSCC cell lines induces IL-6 expression. A, comparison of increases in IL-6 gene expression in parental HNSCC cells following treatment with 5 μmol/L cisplatin for 48 h, assessed by cDNA microarray and real-time qPCR. Array values are mean fold change in IL-6 expression of cisplatin-treated cells compared with nontreated cells, n = 3. qPCR values are mean fold change in IL-6 expression in cisplatin-treated cells compared with baseline levels in nontreated cells. cDNA was generated from pooled RNA used for microarray experiments. n = 3 (FaDu); n = 4 (Detroit-562). B, IL-6 protein expression is increased in all cisplatin naïve HNSCC clones following treatment with 5 μmol/L cisplatin, assessed by the Cytometric Bead Array. Supernatant was collected from cultures of cells that were treated with 5 μmol/L cisplatin for 48 h, as well as nontreated cells. Results were normalized to cell number at the time the supernatant was collected, and ratios of secreted IL-6 in cisplatin-treated versus nontreated clones were determined. IL-6 was strongly upregulated in all clones following cisplatin exposure, by at least 30-fold compared with nontreated cells. Results are from two experiments each containing two replicate samples (except for FaDu clone 5, in which only one experiment was done). Columns, mean; bars, SEM.

For this reason, we sorted FaDu and Detroit-562 cells based on cell size 4 and 5 days postcisplatin treatment, respectively. This corresponds to the time taken for maximal cytotoxicity to occur in vitro following a 48-hour exposure to 5 μmol/L cisplatin (Fig. 1B). We found that the smallest 10% to 20% of cells yielded cells with higher CFE and higher tumorigenic potential compared with the largest cells (Supplementary Fig. S5). We therefore generated transcriptomic profiles from nontreated FaDu and Detroit-562 cells, as well as from the sorted small cell fraction of cisplatin-treated FaDu or Detroit-562 cells. Examination of the transcriptomic profiles showed no significant enrichment for the expression of genes encoding published cancer stem cell markers in the highly tumorigenic cisplatin-resistant cells from either cell line compared with nontreated cells (Supplementary Table S2). Notably, there was no significant enrichment for CD44 or BMI1, which have been reported to be enriched in putative HNSCC cancer stem cells (15). Hence, the lack of an association between putative cancer stem cell marker expression and the enrichment for enhanced TI activity would suggest that known cancer stem cell markers may not be associated with cisplatin-induced increases in TI activity.

These data suggest the transient enrichment for TI activity in cisplatin-resistant HNSCC cells is stochastic in nature, and that cisplatin can induce changes in the surviving cells that enhance TI activity. We now examined this concept more extensively. First, we determined whether the FaDu or Detroit-562 cell line contained a subpopulation of cells in which TI activity resided. We generated clones by randomly cloning individual nontreated cells and then expanding them and examining their ability to initiate tumors when injected into NOD/SCID mice. All clones (9/9) derived from FaDu and Detroit-562 cells were able to produce cell lines that could initiate tumors in mice (Supplementary Table S3). Given these cells generated colonies with high efficiency (approximately 20–50% efficiency), we can conclude that TI activity does not reside in a rare subpopulation of cancer cells.

Transcriptomic profiling of the cisplatin-resistant highly tumorigenic cells indicated that IL-6 expression was elevated 5- and 10-fold in the small tumorigenic cell fraction of cisplatin-treated FaDu and Detroit-562 cells, respectively, compared with nontreated parental cells. This finding has been confirmed by real-time PCR (Fig. 2A). Cisplatin-induced expression of IL-6 in cisplatin-resistant cells provided a further opportunity to determine whether cisplatin treatment enriched for a rare, highly tumorigenic IL-6–positive TI cell population or whether tumorigenicity and IL-6 expression were increased stochastically in response to cisplatin treatment. Therefore, we interrogated our panel of clonal cell lines derived from randomly plated, unselected, and nontreated FaDu and Detroit-562 cells. If a rare population of cancer stem cells exists that were IL-6 positive or induced IL-6 in response to cisplatin, then random isolation of individual...
cells would be unlikely to produce colonies that expressed IL-6 constitutively or induced IL-6 in response to cisplatin. Conversely, if cisplatin induced IL-6 expression through a stochastic process then, we would expect to see IL-6 expression induced in the surviving cells from all of the clones following cisplatin exposure. Figure 2B shows that expression of IL-6 was induced in all of the clonal cell lines following cisplatin exposure. These data indicate that cisplatin resistance is stochastic and that induction of IL-6 in response to cisplatin is also a stochastic process.

Elevated IL-6 expression following cisplatin treatment could contribute to cisplatin resistance and/or the transient increase in TI. To address this, we overexpressed IL-6 in Detroit-562 cells and examined its effect on both cisplatin resistance by CFE and TI activity in NOD/SCID mice. IL-6–overexpressing cells were able to initiate tumors when $3 \times 10^5$ cells were injected (i.e., with >3-fold higher efficiency) compared with both pLV101 control cells (Table 3) and the parental cell line (data not shown), both of which required at least $1 \times 10^5$ cells to initiate tumors. Furthermore, there was no difference in CFE between IL-6–overexpressing and control Detroit-562 cells both in the nontreated state and following exposure to $5 \mu$mol/L cisplatin for 48 hours, suggesting that IL-6 does not increase tumorigenic potential by increasing proliferation rate and does not contribute to cisplatin resistance in HNSCC cells. Finally, use of a therapeutic antibody that blocks IL-6 receptor signaling and reduces HNSCC growth in FIG. 3A) that have enhanced tumorigenic activity in vivo (Table 1). Significantly, these events are transient and stochastic in nature and are not easily explained by a cisplatin-resistant cancer stem cell model. To our knowledge, this is the first study to report that cisplatin can induce a transient increase in tumorigenic potential. This finding has implications for our understanding of tumor recurrence and suggests that therapies combining cisplatin with an IL-6 inhibitory antibody may have therapeutic value.

In the present study, we focused on the biological properties of cells that survived a cytotoxic dose of cisplatin because these are the cells that will contribute to tumor recurrence in patients. We found that following cisplatin treatment, surviving cells had increased TI activity, and clonal analysis showed that this was not a

### Table 3. IL-6 contributes to increased TI potential in HNSCC cells

<table>
<thead>
<tr>
<th></th>
<th>No. of tumors per $3 \times 10^3$ nontreated cells injected</th>
<th>No. of tumors per $1 \times 10^4$ nontreated cells injected</th>
<th>CFE of nontreated cells</th>
<th>CFE of cells treated with $5 \mu$mol/L cisplatin</th>
<th>IL-6 overexpression compared with pLV101 control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detroit-562-pLV101</td>
<td>0/4</td>
<td>2/4</td>
<td>44% (n = 2)</td>
<td>0.13% (n = 2)</td>
<td>240-fold overexpression</td>
</tr>
<tr>
<td>Detroit-562-IL-6</td>
<td>2/4</td>
<td>4/4</td>
<td>44.5% (n = 2)</td>
<td>0.12% (n = 2)</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Detroit-562 cells were transduced with either control lentivirus or virus encoding IL-6 and injected s.c. into NOD/SCID mice (n = 4). Mice were monitored regularly for tumor incidence and growth and were sacrificed when tumors reached 1 cm × 1 cm in size. Three times more control cells were required to initiate tumors compared with the IL-6–overexpressing cells. In addition, nontreated and cisplatin-treated ($5 \mu$mol/L for 48 h) cells were plated at densities ranging from $1 \times 10^2$ to $3 \times 10^4$ cells per well in six-well plates to determine CFE. Cells were incubated for sufficient time to allow colony formation, were stained with Coomassie blue, and colonies were counted. IL-6 expression in Detroit-562-IL-6 cells compared with control Detroit-562-pLV101 cells was determined by measuring the concentration of IL-6 secreted into the culture medium using the BD Cytometric Bead Array assay system.

Discussion

The major fatal complications of HNSCC are tumor recurrence and metastases (17). An obligatory step for tumor recurrence and/or the establishment of metastatic foci is the ability of the primary cancer cells to survive treatment and reestablish the tumor. We have explored the effects of cisplatin, the most commonly used drug in the treatment of HNSCC (5) on the ability of HNSCC cells to survive and initiate tumor growth in a xenotransplant model. Our findings support a model (Fig. 3C) in which cisplatin treatment results in a delayed, but profound, cytotoxic response accompanied by increased tumorigenic potential. Surviving cells then recover viability and proliferative capacity over time (Fig. 3C). The cytotoxicity induced by cisplatin is accompanied by the enrichment for cisplatin-induced IL-6–positive cells (Fig. 2A) that have enhanced tumorigenic activity in vivo (Table 1). Significantly, these events are transient and stochastic in nature and are not easily explained by a cisplatin-resistant cancer stem cell model. To our knowledge, this is the first study to report that cisplatin can induce a transient increase in tumorigenic potential. This finding has implications for our understanding of tumor recurrence and suggests that therapies combining cisplatin with an IL-6 inhibitory antibody may have therapeutic value.

In the present study, we focused on the biological properties of cells that survived a cytotoxic dose of cisplatin because these are the cells that will contribute to tumor recurrence in patients. We found that following cisplatin treatment, surviving cells had increased TI activity, and clonal analysis showed that this was not a

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Cisplatin Induces Increased Tumor Initiation by HNSCC

Property of a rare subpopulation of HNSCC cells (see below) but rather was a stochastic phenomenon. This finding has important clinical implications. For example, if cisplatin resistance and enhanced TI activity could be attributed to a biologically distinct subpopulation of drug-resistant, highly tumorigenic cells (e.g., cancer stem cells), then curative treatments of HNSCC patients would require targeted treatments against all of the constitutive subpopulations of cells within the tumor. If, on the other hand, drug resistance and tumorigenic potential (enhanced or otherwise) were stochastic, then curative treatment may be invoked by optimizing the dose and schedule of chemotherapeutics such that maximal cytotoxicity was achieved. However, it is noteworthy that our in vitro studies suggested that it may be difficult to achieve a dose of cisplatin that eradicates tumor cells completely and hence residual tumor cells will inevitably remain, following cisplatin treatment, that have the potential to prime recurrence. Thus, understanding the molecular basis for cisplatin-induced TI activity may be critical to the development of treatments that reduce tumor recurrence.

Our data indicate that the enhanced TI activity of cisplatin-resistant cells is transient and stochastic in nature. The evidence for enhanced TI activity in cisplatin-resistant cells is based on our observation that although similar numbers of cells must be injected to initiate tumors, the numbers of cells that survive to participate in tumor initiation, following cisplatin treatment, is extremely low relative to nontreated cells. Specifically, our concurrent colony-forming assays indicate that in the case of Detroit-562 cells treated with 5 μmol/L cisplatin, as few as 48 cells could be participating in tumor initiation (Table 1). Furthermore, we showed that cisplatin-surviving cells are characterized by an IL-6-positive TIlg phenotype associated with relatively small cell size. Significantly, these cells were not enriched for any known cancer stem cell marker (Supplementary Table S2) including markers previously reported to characterize HNSCC cancer stem cells (15). Moreover, colonies derived from randomly selected, nontreated individual cells were all tumorigenic in NOD/SCID mice, indicating that cells capable of forming colonies at high efficiency (approximately 20–50% CFE) were all capable of initiating tumors in vivo. Finally, we showed that all individual nontreated clones were sensitive to cisplatin-induced cytotoxicity, and that they all induced IL-6 in response to cisplatin exposure. Given that all clones behaved similarly, the simplest conclusion is that drug resistance, IL-6 induction, and the transient increase in TI activity are stochastically derived.

An important implication of the present study is that increased IL-6 secreted by cisplatin-surviving HNSCC cells contributes to the enhanced tumor initiation but not cisplatin resistance. This is supported by two observations in the current study. First, IL-6–overexpressing Detroit-562 cells were able to initiate tumors at lower numbers than controls (Table 3), or the parental cell line. However, IL-6–overexpressing cells retained sensitivity to cytotoxic doses of cisplatin. Similarly, when the monoclonal antibody Actemra was used to block IL-6 signaling in mice inoculated with cisplatin-treated HNSCC cells, it was found that the Actemra-treated mice had lower tumor incidence compared with controls. However, the tumors that did form in the Actemra group did not differ in growth rate to the tumors in control animals. In vivo tumor initiation studies are a surrogate assay for the clinical phenomenon of tumor recurrence. Thus, data presented
in this study suggest that the increased expression of IL-6 by HNSCC cells, in response to cisplatin, may increase the propensity for tumor recurrence in patients. In other words, those cells that survive cisplatin treatment have an enhanced capacity for tumor initiation due, in part, to cisplatin-induced IL-6 expression. This conclusion is supported independently by several clinical and laboratory observations. For example, high serum IL-6 levels are associated with worse prognosis for HNSCC patients, as well as with radioresistance (18, 19). In addition, IL-6 secreted by oral SCCs can downregulate the costimulatory molecule, CD80, leading to a tolerogenic immune environment (20). Furthermore, recent reports have shown that overexpression of IL-6 in HNSCC is sufficient to endow normally nontumorigenic MCF10A cells with tumorigenic capacity (21). Finally, mice inoculated with human HNSCC cell lines and treated with an anti–IL-6 receptor monoclonal antibody developed smaller tumors than mice given an antibody isotype control (16). Overall, these data indicate that cisplatin-induced IL-6 expression in HNSCC cells could compromise the effectiveness of standard treatment schedules. For example, cisplatin plus radiation is often given to HNSCC patients with curative intent, whereas cisplatin plus 5-fluorouracil is often given to HNSCC patients with metastatic disease (4, 17, 22–24). Although both these combinations often result in clinical improvement, the tumors frequently recur (25). Our data suggest that cisplatin-induced IL-6 expression and IL-6–enhanced TI activity may contribute to the poor outcomes seen in these patients. Hence, blocking IL-6 signaling with inhibitory antibodies (e.g., Actemra) in patients receiving cisplatin-based treatments may improve disease-free survival or overall survival.

Cisplatin treatment caused cytotoxicity, and the induction of IL-6 and high TI activity in the small surviving cell fraction in the acute period following a single cisplatin treatment. However, a single treatment with cisplatin also induced longer term effects on the HNSCC cells. For instance, studies of a second panel of clonal cell lines derived after 5 μmol/L cisplatin treatment showed that CFE increased markedly compared with CFE immediately after treatment (Tables 1 and 2), and that TI activity returned to close to parental levels within several passages in culture. In contrast, persisting effects resulting from the single cisplatin exposure included abnormal morphology and, in one third of the clones, altered in vitro growth characteristics such that they could not grow to confluence. This raises the issue of how cisplatin invokes long-term effects that manifest after the completion of many cell cycles. Cisplatin-induced cytotoxicity can be mediated by the induction of DNA damage resulting from the formation of platinum-DNA adducts, so-called “transcription factor hijacking” (7, 26) and “repair shielding” (27), enzyme inhibition (28), and generation of reactive oxygen species and subsequent endoplasmic reticulum stress (29). Although the mechanisms underlying the persisting effects observed in our clonal cell lines remain unclear, such prolonged effects on cell growth and morphology may be due to epigenetic changes induced by cisplatin or the induction of novel mutations as a result of errors occurring during DNA repair. In this regard, it has been noted that DNA Pol η has lower efficiency of error-free bypass of cisplatin-DNA adducts than adducts formed by other platinum compounds (6).

In conclusion, our study shows that cisplatin effects can be classified as acute or chronic. In the acute phase following a single cisplatin dose, surviving HNSCC cells have an enhanced ability to initiate tumors, which is attributable, in part, to cisplatin-mediated induction of IL-6. Subsequently, the chronic phase is characterized by a prolonged period in which cisplatin-induced cytotoxicity continues to manifest. Thus, therapies that reduce cisplatin-induced TI activity in the acute phase (i.e., IL-6 blocking antibodies such as Actemra) would be predicted to improve long-term survival or increase disease-free periods in HNSCC patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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