Cisplatin and Oxaliplatin Inhibit Gap Junctional Communication by Direct Action and by Reduction of Connexin Expression, Thereby Counteracting Cytotoxic Efficacy

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ABSTRACT

Cisplatin [cis-diamminedichloroplatinum(II)]/oxaliplatin [1,2-diaminocyclohexane(trans-1)oxolatoplatinum(II)] toxicity is enhanced by functional gap junctions between treated cells, implying that inhibition of gap junctions may decrease cytotoxic activity of these platinum-based agents. This study investigates the effect of gap junction modulation by cisplatin/oxaliplatin on cytotoxicity in a transformed cell line. The effects were explored using junctional channels expressed in transfected HeLa cells and purified hemichannels. Junctional channels showed a rapid, dose-dependent decrease in dye coupling with exposure to cisplatin/oxaliplatin. With longer exposure, both compounds also decreased connexin expression. Both compounds inhibit the activity of purified connexin hemichannels, over the same concentration range that they inhibit junctional dye permeability, demonstrating that inhibition occurs by direct interaction of the drugs with connexin protein. Cisplatin/oxaliplatin reduced the clonogenic survival of HeLa cells at low density and high density in a dose-dependent manner, but to a greater degree at high density, consistent with a positive effect of gap junctional intercellular communication (GJIC) on cytotoxicity. Reduction of GJIC by genetic or pharmacological means decreased cisplatin/oxaliplatin toxicity. At low cisplatin/oxaliplatin concentrations, where effects on connexin channels are minimal, the toxicity increased with increased cell density. However, higher concentrations strongly inhibited GJIC, and this counteracted the enhancing effect of greater cell density on toxicity. The present results indicate that inhibition of GJIC by cisplatin/oxaliplatin decreases their cytotoxicity. Direct inhibition of GJIC and reduction of connexin expression by cisplatin/oxaliplatin may thereby compromise the effectiveness of these compounds and be a factor in the development of resistance to this class of chemotherapeutic agents.

The cytotoxic activity of cisplatin [cis-diamminedichloroplatinum(II)] was recognized more than 37 years ago. Cisplatin enters cells passively in its uncharged form (Judson and Kelland, 2000) where the low chloride ion concentration in cytoplasm permits formation of mono- and diaquo forms, which are highly reactive (Jamieson and Lippard, 1999). Cisplatin, and oxaliplatin [1,2-diaminocyclohexane(trans-1)oxolatoplatinum(II)], a related platinum-based chemotherapeutic agent, reacts with GC-rich areas of DNA, forming both intrastrand and interstrand cross-links, leading to G2 arrest and to apoptosis or necrosis (Fuertesa et al., 2003). Oxaliplatin is effective against cisplatin-resistant cancers and thus is thought to have minimal cross-resistance with cisplatin (Wang and Lippard, 2005).

Cisplatin is the chemotherapeutic agent of choice for testicular cancer. It is also widely used in treatment of solid tumors originating in many other tissues, including lung, head and neck, and cervix and ovary (Fuertesa et al., 2003). Unfortunately, development of tumor resistance to cisplatin and related compounds is a major problem for effective ongoing chemotherapy (Wernyj and Morin, 2004). For this reason, it is important to explore mechanisms by which the efficacy of cisplatin cytotoxicity can be enhanced and resistance to it countered.

Connexin channels, which compose vertebrate gap junctions, mediate direct intercellular movement of cytoplasmic...
signaling molecules. There are approximately 20 isoforms of connexin protein, each of which forms channels with distinct regulation and permeability (Harris, 2001). The intercellular signaling mediated by connexin channels is important: every functional deletion of a connexin isoform produces a distinct pathology (Dobrowolski and Willecke, 2009; Zoidl and Dermietzel, 2010).

It has been reported that cisplatin toxicity is enhanced by the presence of functional gap junctions between the target cells (Jensen and Glazer, 2004; Peterson-Roth et al., 2009). Gap junctions enhance induction of apoptosis induced by cisplatin and by etoposide (an agent used with cisplatin in the standard combination bleomycin-etoposide-cisplatin chemotherapy for testicular cancer) (Kalvélyte et al., 2003). In bladder cancer lines, gap junction expression acts additively with cisplatin in promoting apoptosis, cell cycle arrest, and down-regulation of BCL-2 (Tanaka and Grossman, 2001).

A simple inference from these findings is that induction of apoptotic or necrotic cell death in a cell causes a molecular “death signal” to be transmitted to neighboring cells via gap junctions and kill them. Such an effect has also been observed with ionizing radiation, in which cells not irradiated but coupled to irradiated cells become damaged or die (Azzam et al., 1998, 2001). This is called a “bystander” effect. The gap junction dependent component of cisplatin toxicity suggests that reduction of intercellular spread of the death signal by inhibition of gap junctions would decrease cytotoxic activity of cisplatin, resulting in decreased therapeutic efficacy and resistance of cells to this compound.

Here, we report that both cisplatin and oxaliplatin inhibit activity of connexin channels by direct interaction with connexin protein and also reduce the level of expression. The inhibition of GJIC thus produced by cisplatin and oxaliplatin decrease the cytotoxicity, and by inference, the therapeutic efficacy of these compounds.

Materials and Methods

Cell Line and Cell Culture
The cell line used in these studies was a HeLa cell line stably transfected to express heteromeric connexin32/connexin26 (Cx32/Cx26) channels, described and characterized previously (Koreen et al., 2004). In this cell line, expression of both connexins is under the control of a single bidirectional tetracycline-inducible promoter. The Cx26 has a thrombin-cleavable C-terminal epitope tag (3.2 kDa) that includes a hemagglutinin (HA) epitope. Cells were grown at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 μg/ml G418 sulfate, and 200 μg/ml hygromycin B (all from Invitrogen, Carlsbad, CA). Connexin expression was induced with 1 μg/ml doxycycline (α-6-deoxyoctotetraacycline) for 48 h.

Cytotoxicity Assay
Toxicity was assessed by a colony-forming assay adapted from Jensen and Glazer (2004) for use at high and low cell density, corresponding to conditions in which junctional channel formation is permitted or not, respectively. For the high-density condition, cells were seeded at 30,000 cells/cm² so that cultures were 70 to 100% confluent at the time of drug exposure. Cells were treated with cisplatin/oxaliplatin for 1 h in the dark, washed with phosphate-buffered saline, harvested by trypsinization, counted, diluted, and seeded into six-well dishes. Colony formation was assessed 5 to 7 days later by fixation and staining with crystal violet. Colonies containing 50 or more cells were scored. For the low-density condition, cells were seeded at 100 cells/cm² directly into six-well plates and treated with cisplatin/oxaliplatin after attachment. They were rinsed and assessed for colony formation as described above. Colony formation was normalized to the colony-forming efficiency of non-drug-treated cells. There was no significant difference in plating efficiency between the low- and high-density cultures (data not shown).

Channel Purification and Reconstitution into Liposomes
These procedures were used according to previous protocols (Tao and Harris, 2004; He et al., 2009). After induction of connexin expression with doxycycline, cells were harvested, solubilized in n-octyl-β-D-glucoside (Calbiochem, San Diego, CA), and the connexin-purified by immunoaffinity chromatography using a monoclonal antibody directed against the HA epitope tag (clone HA-7, Sigma-Aldrich, St. Louis, MO). The connexin was released from the antibody by a pulse exposure to pH 4.0 buffer. Liposome formation and connexin incorporation into unilamellar liposomes followed previous protocols (Tao and Harris, 2007). Connexin was incorporated during liposome formation by gel filtration of phosphatidylcholine, phosphatidylserine, and rhodamine-labeled phosphatidylethanolamine (Avanti Polar Lipids, Alabaster, AL) at a molar ratio of 2:1:0.03 in urea buffer (see below) and affinity-purified connexin in 80 mM n-octyl-β-D-glucoside. Connexin-containing liposomes were collected in the void volume. The protein/lipid ratio corresponded to an amount of connexin equivalent to less than one hemichannel per liposome so that a significant fraction of the liposomes did not contain functional channels, for use as internal controls in the activity assay (see Transport-Specific Fractionation (TSF) Assay), and to allow sensitive detection of changes in channel activity.

“Parachute” Dye-Coupling Assay
This assay for gap junction function was performed as described in Goldberg et al. (1995) and Koreen et al. (2004). Donor and receiver cells were grown to confluence. Donor cells were double-labeled with 5 μM CM-DiI, a membrane dye that does not spread to coupled cells, and 5 μM calcine-acetoxyethyl ester, which is converted intracellularly into the gap junction-permeable dye calcine (both from Invitrogen). Donor cells were then trypsinized and seeded onto the receiver cells at 1:150 donor/receiver ratio. Donor cells were allowed to attach to the monolayer of receiver cells and form gap junctions for 4 h at 37°C and then examined with a fluorescence microscope. For each experimental condition, the average number of receiver cells containing dye per donor cell was visually determined and normalized to that of control cultures. For studies involving cisplatin and oxaliplatin, the donor and receiver cells were exposed to the agents only during the 4- to 5-h period in which the donor cells were plated onto the receiver cell monolayer.

Transport-Specific Fractionation Assay
TSF was used to assess activity of reconstituted connexin hemichannels (Harris et al., 1992; Bevans and Harris, 1999; Tao and Harris, 2004). TSF fractionates liposomes containing connexin channels into two populations within an iso-osmolar density gradient, based on channel permeability to urea and sucrose, uncharged solutes that permeate open connexin channels and have different densities at iso-osmolar concentrations. Liposomes are formed in, and entrapped, urea-containing solution, and are then centrifuged through iso-osmotic density gradients formed from urea and sucrose buffers. Equilibration of these solutes across the liposome through an open hemichannel occurs rapidly and increases the density of the liposome. Fractionation of liposomes into bands is monitored by the fluorescence of rhodamine-phosphothanolamine in the liposome membrane. The data are corrected for the presence of more than one channel per liposome.

TSF Dose-Response Relations. A change in the distribution of liposomes between the top and bottom bands, relative to controls, reflects a change in the fraction of channels that are permeable to the
gradient solutes (i.e., are functional). Because a channel that opens only infrequently for brief times will mediate sufficient exchange of solutes to cause liposome movement to the characteristic lower position, TSF is essentially an all-or-none assay for channel function per liposome. For this reason, the concentration of a compound that produces channel closure detectable in TSF is greater than that needed to see an effect in which reversible activity can be directly observed. Therefore, TSF does not give classic Michaelis-Menten binding parameters and does not allow determination of microscopic affinities. But, however, these latter measurements are very difficult to make and are not necessary for most applications. In summary, TSF permits measurement of the concentrations of a modulator that produce an effect in TSF. This concentration is an upper limit for the true half-maximal value.

Normalization of TSF Data. For each preparation of connexin, the fraction of liposomes in the lower TSF band in each experiment was normalized to the maximal value obtained for that preparation. This enabled comparison of modulatory effects across reconstitutions that produced different amounts of channel activity (fractions of liposomes with functional channels).

Curve Fitting of TSF Data. The activity-concentration data were fit with a four-parameter logistic function of the form

$$f(x) = \frac{a}{1 + \exp[b(x - c)]} + d.$$  

A Hill equation was not used because TSF does not produce a Michaelis-Menten dose-response curve (see above).

Western Blotting

Western blotting protocols were as in previous studies (Tao and Harris, 2004; He et al., 2009). In brief, cell lysates or purified connexin preparations were separated by SDS-polyacrylamide gel electrophoresis in 13% Tris-glycine gels and transferred to a polyvinylidene difluoride membrane. Primary antibody was mouse anti-HA clone HA-7 IgG (1:1000 dilution; Sigma-Aldrich), and secondary antibody was alkaline phosphatase-conjugated goat anti-mouse IgG (1:10,000 dilution). Immunopositive bands were visualized using Western Lightning chemiluminescence reagents (PerkinElmer Life and Analytical Sciences, Boston, MA). All Western blot exposures were in the linear range of detection, and the intensities of the resulting bands were quantified by Quantity One software on a GS-800 densitometer (Bio-Rad Laboratories, Hercules, CA).

Statistical Analysis

Data were statistically analyzed using unpaired Student’s $t$ test at a significance level of $P < 0.05$ and are presented as means $\pm$ S.E.

Results

Toxicity-Concentration Relation of Cisplatin and Oxaliplatin Depends on Cell Density. HeLa cells expressing Cx32/Cx26 were cultured under two conditions: a low-density condition in which the cells were well dispersed as single cells, and a high-density condition that allowed plentiful formation of gap junctions. Figure 1 shows the survival of cultures treated under low- and high-density conditions after 1-h exposure to cisplatin (Fig. 1, A and B) or oxaliplatin (Fig. 1, C and D). Cisplatin and oxaliplatin reduced clonogenic survival of cells at both low and high density in a concentration-dependent manner. However, at concentrations up to 5.0 $\mu$g/ml, survival was significantly greater at low density than at high density. Thus, the toxicity is greater when there is opportunity for gap junctional contacts between the cells.

Figure 1, B and D, shows quantitation of the differences in survival between the low-density and high-density conditions. When cisplatin/oxaliplatin levels were greater than 1.0 $\mu$g/ml, the difference in toxicity between low- and high-density cultures decreased; toxicity in high-density cultures decreased to that seen in the cultures in which the cells were unable to form gap junctions. This indicates that the positive influence of cell density on sensitivity to cisplatin/oxaliplatin decreases at higher drug concentrations; cells in the high-density cultures become less sensitive so that their survival...
rate decreases toward that of the low-density cells. One factor that may contribute to this is that with substantial cell death, the high-density cultures become lower density cultures and the enhanced toxicity due to high density is thus attenuated.

Density Dependence of Cisplatin and Oxaliplatin Toxicity Is Mediated by GJIC. The density dependence of cisplatin/oxaliplatin toxicity in connexin-expressing cells suggests a role for the intercellular communication mediated by gap junctions. To investigate whether the density-dependent effects of these agents involve GJIC, two methods were used to manipulate gap junction expression and function: lack of doxycycline induction of connexin expression and pharmacological inhibition of junctional channels. Western blot and parachute dye-coupling assay confirmed that doxycycline induced expression of connexin and the emergence of GJIC and that connexin expression and GJIC were not detected in cells not treated with doxycycline (Fig. 2).

Figure 3 shows survival in cisplatin or oxaliplatin of doxycycline-treated and -untreated cells. At low cell density, induction of connexin expression had no effect on cisplatin/oxaliplatin toxicity. In contrast, at high cell density, induction of connexin expression by doxycycline was much more sensitive to both agents, with survival approximately 2-fold less than that of doxycycline-untreated cells (Fig. 3, A and B). Pretreatment of high-density-induced cultures with 10 µg/ml 18α-glycyrrhetinic acid (18α-GA), a membrane-permeable reagent shown to substantially inhibit Cx32/Cx26 channels at this concentration (Fig. 2B), reduced the cytotoxicity of both cisplatin and oxaliplatin, resulting in substantially increased survival. In contrast, at low cell density, 18α-GA had little effect on survival after exposure to cisplatin or oxaliplatin (Fig. 3, C and D).

Specifically, at 1 µg/ml cisplatin, the low-density cell death was approximately 24%, whereas the high-density cell death was approximately 59%, so the high density increased toxicity by a factor of approximately 2.5. Block of the gap junctions by 18α-GA reduced the toxicity at high density to approximately 31%, eliminating approximately 80% of the increase in toxicity due to high-density conditions. The numbers for oxaliplatin are similar, with an approximately 2.2 increase in toxicity attributable to the high-density condition, of which approximately 52% was eliminated by 18α-GA.

Together, these results show that the enhanced toxicity at high density requires functional gap junctions, because the toxicity in high-density cultures is dramatically reduced when the connexins are not expressed or are blocked. Overall, the data show that the density dependences of cisplatin and oxaliplatin toxicity are mediated by GJIC.

Inhibition of GJIC by Cisplatin or Oxaliplatin Reduces the Effect of Density on Their Cytotoxicity. The experiments described above show that the enhancing effect of cell density on cisplatin/oxaliplatin cytotoxicity decreases at higher drug concentrations and that the density dependence of the cisplatin/oxaliplatin effects is mediated by GJIC. One question that arises is whether these agents have effects on GJIC itself, independently of their effects on cell survival. That is, cell death would obviously decrease the effective cell density and opportunity for GJIC, as mentioned previously, but is this the only way that cisplatin/oxaliplatin attenuate GJIC and thus their own toxicity? To address this issue, experiments were performed to assess the effects of cisplatin and oxaliplatin on dye-coupling between cultured cells under conditions that do not cause substantial cell death.

Junctional coupling was assessed by the parachute assay for dye coupling as described under Materials and Methods. Donor cells labeled with the membrane dye DiI and loaded with the junction-permeable dye calcein were seeded onto unlabeled receiver cells. Experiments were carried out in the presence of cisplatin or oxaliplatin, which are freely membrane-permeant. The seeded donor and receiver cells were incubated for 4 h, and the number of receiver cells per donor cell containing calcein was counted. GJIC was assessed as the number of receiver cells receiving calcein from a labeled cell, normalized to that for control conditions (without drug). There was some cell death during the experimental period (approximately 5 h), not exceeding 20%, which would not dramatically affect the results.

The data in Fig. 4 show that both cisplatin and oxaliplatin substantially reduce dye coupling in a concentration-dependent manner in the short term, the effect increasing over the range 1.0 to 7.5 µg/ml. Any cell death that occurred would tend to decrease the measured dye spread at the higher concentrations, so the real curves could be a bit less steep. For both compounds, minimal inhibition occurred below 1.0 µg/ml. The maximal inhibition, at 7.5 µg/ml was similar to that obtained at 10 µg/ml 18α-GA. The concentration range
over which cisplatin/oxaliplatin inhibit gap junction coupling was roughly the same range over which the effect of cell density on cisplatin cytotoxicity decreased (i.e., concentrations greater than 1.0 μg/ml). These findings suggest that inhibition of GJIC by these agents can attenuate their toxic effects.

Cisplatin and Oxaliplatin Directly Inhibit Connexin Channels. It is possible that the above-mentioned results could be accounted for by sublethal effects of cisplatin/oxaliplatin; if the physiology of the cells is compromised even in the short term by these agents, the cells could become uncoupled. Alternatively, the agents could alter GJIC by any of several mechanisms, including acting directly on connexin channels to close them and by changing the number of gap junction channels via effects on connexin expression or subcellular distribution. To identify the mechanism by which cisplatin/oxaliplatin affect GJIC, we explored their effects on purified and reconstituted connexin channels and on connexin expression in the cells.

Immunopurified Cx32/Cx26 hemichannels were reconstituted into unilamellar phospholipid liposomes, and the activities of the reconstituted channels explored by TSF, as described under Materials and Methods, which has been well characterized (Harris et al., 1992) and used effectively in studies of connexin channel modulation (Beverns and Harris, 1999; Locke et al., 2004a; Tao and Harris, 2004). TSF is essentially an all-or-none assay of per-liposome hemichannel activity as reflected by permeability to urea and sucrose. The effect of test compounds on channel activity was assessed by exposing connexin-containing liposomes to the compounds during a TSF centrifugation. The change in distribution of liposomes between the top and bottom positions, relative to a control gradient without the compound, is a quantitative measure of the fractional change in activity of the population of the channels.

Figure 5 illustrates the inhibition of the reconstituted connexin hemichannels by cisplatin or oxaliplatin in the TSF system. Figure 5, A and B, shows that the inhibition occurs in a concentration-dependent manner, developing with increasing cisplatin/oxaliplatin concentrations, with minimal inhibition at 0.5 μg/ml and maximal effect at approximately 5 μg/ml. These are consistent with the effects of these concentrations on cell coupling (Fig. 4), although the curves are a bit steeper. We attribute this difference to the fact that one experiment was carried in cells and that other experiment used purified protein in a noncellular environment. The effects seen on purified, reconstituted channels demonstrate that cisplatin/oxaliplatin can inhibit connexin channels by direct interaction with connexin protein.

Effects of Cisplatin and Oxaliplatin on Connexin Expression. Effects of cisplatin and oxaliplatin on connexin expression were determined by Western blotting. Relative to controls, expression of the connexin declines with treatment of the cells with cisplatin or oxaliplatin at 0.5 and 1 μg/ml for 48 h (Fig. 6); both compounds reduce connexin expression over this time period, with much greater inhibition by oxaliplatin than by cisplatin. Densitometric analysis of the immunoblots indicates that 0.5 μg/ml cisplatin suppresses the expression of connexins to approximately 50% and 0.5 μg/ml oxaliplatin does so to 90% of that in control cultures. From the above-mentioned studies showing that GJIC mediates the enhanced effect of cell density on toxicity, this difference...
ought to result in a lesser effect of cell density and less toxicity in oxaliplatin-treated cells. These effects are indeed seen in Fig. 1. These results suggest that cisplatin and oxaliplatin inhibit gap junction activity by direct interaction with connexin protein and that long-term treatment of cells with these agents result in inhibition of gap junction activity through reduction of connexin expression. The much greater effect on expression by oxaliplatin has implications for therapies that involve long-term exposure to these drugs.

Discussion

The present study investigates the effect of gap junctional communication on toxicity of cisplatin and oxaliplatin and its involvement in resistance of transformed cells to these antitumor drugs. The results show that both cisplatin and oxaliplatin inhibit activity of connexin channels by direct interaction with connexin protein and by reducing the level of connexin expression. The inhibition of GJIC by cisplatin and oxaliplatin decreases the cytotoxicity of these compounds, thereby generating a form of resistance to these antitumor reagents.

Fig. 4. Effects of cisplatin and oxaliplatin on dye coupling through gap junctions. The dye spread of cells treated with a range of cisplatin (A) and oxaliplatin (B) concentrations. Gap junctional communication was assessed as the average number of receiver cells containing calcein from each donor cell, normalized to controls without exposure to agents. Data points are mean ± S.E. for five-seven experiments. C, effects of 18-α-GA, cisplatin, and oxaliplatin on dye coupling. The bar graphs are means ± S.E. from five to seven dishes for each condition. *, P < 0.05, significantly different from control.

Fig. 5. Cisplatin and oxaliplatin directly inhibit heteromeric Cx32/Cx26 channels. Inhibition of connexin channels purified from cultured cells as a function of cisplatin (A) or oxaliplatin (B) concentration. The smooth curves are fits to the data of a four-parameter logistic function f(x) = a/(1 + exp(b(x - c))) + d, as described under Materials and Methods. Data points are means ± S.E. for five protein preparations.
This work confirms and builds on the work of others showing that a substantial component of cisplatin toxicity depends on gap junctional coupling among the target cells (Jensen and Glazer, 2004; Peterson-Roth et al., 2009). As in previous studies, we found that the toxicity of cisplatin/oxaliplatin was substantially greater when cells were at high density compared with low density. We demonstrate that this effect was absent in connexin-deficient cells and in coupled cells pretreated with a gap junction inhibitor. These genetic and pharmacological manipulations of connexins correlated with changes in gap junction function as determined by dye transfer.

This study uniquely shows that platinum-based agents exert a counter-therapeutic effect by inhibiting GJIC—by direct effects on the channel protein and by longer-term downstream effects on connexin expression. This inhibition of gap junctions by platinating reagents induces a mode of chemotherapeutic resistance; inhibition of gap junctions by platinating reagents must be considered a mechanism of resistance to their therapeutic effects.

Other mechanisms of cisplatin resistance include adaptive changes in its uptake/efflux, cytosolic redox state, and effectiveness of DNA repair or replication (Wang and Lippard, 2005). Decreased GJIC due to exposure to platinating reagents could also affect these cell autonomous mechanisms of resistance via downstream effects, but no information specifically addresses these possibilities.

The concentrations of cisplatin and oxaliplatin used in this study are consistent with those used in most in vitro studies (0.1–10 \( \mu \text{g/ml} \)). More importantly, the range over which we demonstrate cisplatin and oxaliplatin reduce gap junction function by direct interaction with connexin and reduction of connexin expression over time is 0.5 to 7.5 \( \mu \text{g/ml} \), which is approximately the plasma concentration range achieved during most cancer chemotherapies (including testicular cancer patients; Erdlenbruch et al., 2001). Thus, the fact that resistance develops with normal therapeutic levels indicates that it develops in the serum concentration range used in the present study.

This study provides proof-of-principle that platinum-based reagents directly inhibit connexin channels and in so doing compromise their toxic efficacy. It is likely that the effects on the connexins studied here occur for other connexins. Platinum is a highly nonselective, chemically reactive reagent, having widespread interactions with even weakly nucleophilic sites in proteins (e.g., thiol), RNA, and DNA (Fuertesa et al., 2003). It is unlikely that such a nonspecific reagent (unlike reagents that operate by ligand-receptor interactions) would distinguish among connexins, particularly given the sequence homology among them. Even if the effects are specific to Cx26 and Cx32, these connexins are known to persist in a variety of tumors, as noted below. To date, only one pharmacological agent has been shown to have differential effects among connexins (mel folquine; Cruikshank et al., 2004), and it acts in a classical ligand-receptor manner. This counter-therapeutic effect of platinating reagents can operate only in cells that are coupled by gap junctions. Our work and the work of others make clear that cells lacking gap junctions are already less sensitive to these agents than are coupled cells. Although it is generally held that tumor cells have reduced GJIC (see further comments below), changes in connexin expression with tumorigenesis are complex and heterogeneous. In some cases, GJIC is maintained or only modestly reduced. In these cases, one would expect the GJIC-mediated antitherapeutic effect of platinating reagents to play a role. For example, in testis, many connexins are expressed, including Cx26, Cx31, Cx32, Cx37, Cx40, and Cx43. In testicular cancer, Cx26 and Cx40 are up-regulated and Cx43 can be up-regulated or down-regulated, depending on the type of tumor (Brehm et al., 2002; Pointis and Segretain, 2005; Pointis et al., 2005, Ruttiger et al., 2008). In the lung, a similar situation pertains. Normally, many connexins are expressed, including Cx26, Cx32, Cx37, Cx40, Cx43, and Cx45. Small lung tumors have no change in expression of Cx37 and Cx45. In larger lung tumors, Cx32 and Cx40 are down-regulated, but Cx37, Cx43, and Cx45 are expressed (Isakson et al., 2001a,b; 2003; Udaka et al., 2006). In other cases, such as ovarian adenocarcinomas and cervical cancer, GJIC is dramatically reduced (Hanna et al., 1999; Umhauer et al., 2000; Aasen et al., 2005; Gershon et al., 2008). In this situation, because GJIC is already essentially absent, the effect of platinating reagents described in the present study would not occur, but in the former cases, the effect on the efficacy of the drugs due to effects on GJIC could occur.

Gap junction-mediated intercellular transfer of toxic effects has been demonstrated in many systems. Gap junctions have been shown to accelerate apoptotic cell death in normal and tumor cells exposed to toxic agents (Lin et al., 1998; Krutovskikh et al., 2002). Most relevant to our study, gap junctions have been shown to act synergistically with a variety of chemotherapeutic agents, including cisplatin, to cause cell death (Kalvelyte et al., 2003; Jensen and Glazer, 2004; Peterson-Roth et al., 2009). In addition, some toxic metabolites of prodrugs (e.g., 5-fluorouracil that has been converted from 5-fluorocytosine by cytosine deaminase) can pass between cells through gap junctions (Lawrence et al., 1998). It is also known that gap junctions are required for the bystander effect seen with low-dose ionizing radiation. In this phenomenon, a signal produced by ionizing radiation-damaged cells passes to unirradiated cells, triggering certain signaling pathways and producing cytotoxicity in the untreated cells (Mesnil et al., 1996; van Dillen et al., 2002).

Because the gap junction intercellular pathway can play a major role in the effects of cisplatin/oxaliplatin, the functionality of this pathway in human cancers probably an important determinant of the clinical response to platinum-based chemotherapy. This leads to several therapeutic considerations. One is that pharmacologic strategies designed to increase connexin expression or to enhance GJIC could sensitize malignant cells to platinum-based drugs and overcome the GJIC inhibitory effects of the drugs. Conversely, factors that reduce GJIC should decrease sensitivity to these agents, resulting in substantially reduced therapeutic efficacy. Because inhibition of GJIC by cisplatin/oxaliplatin may be a mechanism of development of resistance to these agents in tumors with gap junctions, the results reported here also suggest that clinical use of platinum-based drugs at lower doses or intermittent treatment may delay/reduce the development of resistance to these agents.

In addition to the GJIC-mediated toxic effects, gap junctions have been long implicated in the establishment of normal cellular growth control (Loewenstein and Rose, 1992; Yamasaki et al., 1995). More recently, they have been shown
to act as true tumor suppressors and to restore normal growth control to transformed and tumorigenic cells (Omori et al., 2001; Mesnil, 2002). For tumor cells with reduced GJIC, development of drugs and methods that can recover or increase GJIC provide a new and potent way to enhance treatment of these tumors. Relevant to the inhibitory effect of junctional communication by platinum reagents in the present study, several compounds, notably 4-phenylbutyrate, an inhibitor of histone deacetylases, have been shown to increase GJIC of otherwise GJIC-impaired tumor cells, which enhances toxic bystander effects as well as tending to restore growth control (Ammerpohl et al., 2007; Chen et al., 2008).

Although loss of GJIC is widely regarded to correlate with tumorigenic phenotypes, it is now clear that connexins can play distinct roles in specific stages of tumor progression. Specifically, increased levels of connexin expression and of GJIC are correlated with invasiveness, extravasation and metastasis in a variety of cancer cells. It has also been noted that primary tumors that are initially GJIC impaired become GJIC competent at the metastatic stage (Mesnil et al., 2005; Cronier et al., 2009). Thus, in this context, the down-regulation of intercellular communication by platinum compounds, although having an antitherapeutic effect on the cell survival, may have a positive therapeutic effect on metastasis. Thus, the results reported here are relevant to therapies directed at inhibition of several stages of tumor progression.

It was reported that platinum-based reagents, including cisplatin, affected GJIC by reducing the amount or inducing aberrant intracellular localization of connexin proteins, or by regulating the mitogen-activated protein kinase-dependent phosphorylation of specific sites on connexins (Fiorini et al., 2004; Zhao et al., 2004; Procházková et al., 2007). However, the effects reported here on purified, reconstituted connexin channels establish that inhibition can occur by direct interaction of cisplatin/oxaliplatin with connexin protein and that this has a detrimental effect on toxic efficacy. Therefore, although cisplatin/oxaliplatin can affect other aspects of cellular and junctional physiology, there is also an unambiguous direct effect at low micromolar concentrations on connexin channels themselves.

It is not surprising that pharmacological agents can have effects on cell physiology distinct from what is thought to be their primary action. The present study reveals that a previously unknown downstream effect of at least two platinum agents has important consequences for any process that involves toxic bystander killing—this by restoring growth control to transformed and tumorigenic cells (Ammerpohl et al., 2007; Chen et al., 2008).

References


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