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The effect of hypoxia on cytotoxicity, accumulation and DNA binding of cisplatin in Chinese hamster ovary cells

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The literature contains conflicting evidence regarding the hypoxic toxicity of cisplatin. We have found that in Chinese hamster ovary cells, cisplatin was up to 2.6 times more effective at reducing survival to 2% in hypoxic, compared with aerobic cells. Furthermore, using atomic absorption spectroscopy, it was determined that cells treated in hypoxia showed consistently higher accumulation (up to 1.5 times) and DNA binding (up to 1.7 times) than aerobic cells. Hypoxic cells also showed greater toxicity per platinum–DNA adduct than those treated in air, suggesting that increased binding of the drug, alone, cannot account for its increased hypoxic cytotoxicity. We suggest that the hypoxia selectivity of cisplatin involves several different mechanisms and possible explanations for these results are discussed.

Key words: CHO cells, cisplatin, DNA binding, drug accumulation, hypoxic.

Introduction

It is now well established that many solid tumors are heterogeneous with respect to oxygenation[1–3] and contain regions of hypoxic cells which, due to their inherent resistance to ionizing radiation, limit the success of radiotherapy.[4] It has been suggested that hypoxic regions of tumors may also be resistant to chemotherapeutic agents.[5] While there has been interest in cisplatin as a possible radiosensitizer of hypoxic cells, an examination of the literature reveals that there is some discrepancy with regard to its cytotoxic properties in hypoxic cells *in vitro* (for a summary, see Skov[6]). For example, while several groups have reported greater cytotoxicity of cisplatin in hypoxia,[7–9] others have reported no difference between air and hypoxia[10–12] and one has reported a greater aerobic toxicity.[13] Although some of these differences might be explained by experimental conditions, such as differences in cell type, drug concentrations used, the level and duration of hypoxia or the way in which hypoxia was achieved, the contributions of each of these factors have been difficult to determine. Indeed, measurements of cisplatin's aerobic toxicity alone have been shown to vary widely from laboratory to laboratory, even for the same cell lines and duration of drug exposure.[14] *In vivo* measurements of hypoxic versus aerobic toxicity are equally conflicting. One group reports no hypoxic toxicity *in vivo*,[15] another 2-fold greater aerobic toxicity[16] while yet another shows little effect of tumor oxygenation status.[17]

Of further interest, decreased accumulation of cisplatin has been consistently implicated as a possible mechanism for cisplatin resistance in a variety of human[18,19] and murine[20,21] cell lines and, thus, its modulation may play an important role in cisplatin toxicity. Decreased levels of platination of DNA have also been observed in cisplatin resistant cells.[22] The current study aims to clarify the relationship between the *in vitro* toxicity of cisplatin and the oxygenation status of a representative mammalian cell line, Chinese hamster ovary (CHO) cells. Drug accumulation was measured in the same cell population as toxicity and parallel experiments were performed to measure DNA binding of cisplatin in relation to toxicity in aerobic and hypoxic cells.

Methods and materials

Drug treatments

All experiments were performed with exponentially growing CHO cells grown in suspension cultures

More detail is found in Matthews.[23] This work was presented in part at the *40th Annual Meeting of the Radiation Research Society*, Salt Lake City, Utah, 1992. This research was supported in part by the National Cancer Institute of Canada with funds from the Canadian Cancer Society.

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in Eagle's α-minimum essential medium (MEM) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY). Cisplatin (obtained as a gift from Bull Laboratories, Australia) was stored at 4°C and fresh solutions were prepared immediately prior to treatments. Hypoxia was maintained through a continuous flow of humidified oxygen-free nitrogen for 1 h prior to addition of cells. Cells were then suspended in MEM with or without drug to a concentration of 2–4 × 10^5 cells/ml and treated at 37°C for up to 3 h. Aerobic vessels were similarly maintained. Toxicity measurements were performed as described elsewhere.[23]

Cellular accumulation

Cells were prepared for accumulation measurements using a similar method to Johnson *et al.*,[24] adapted for atomic absorption spectroscopy (AAS). At time intervals concurrent with those for toxicity measurements, 10 ml of cell suspension (~4 × 10^6 cells) was removed from the incubation vessels and the drug-containing medium removed by centrifugation at 93 *g* and 4°C for 7 min. The cell pellet was washed twice (subsequent washes were found to remove no further platinum from the samples) with 10 ml ice-cold phosphate buffered saline (PBS) and then cells were resuspended in 4.5 ml ice-cold PBS. The cell concentration was determined and 4 ml of cell suspension was centrifuged. The pellet was digested in 100 μl concentrated nitric acid at 37°C overnight. Homogenized samples were then diluted to 600 μl with distilled water and atomic absorption measurements were performed.

DNA binding

Excess cisplatin was removed using the washing procedure described above and cell lysis and DNA extraction was performed by a variation of existing methods in which platinum–DNA adducts have been shown to be stable.[24] Cells were lysed in 1 ml 10% sodium lauryl sulphate in 10 mM Tris (pH 8) containing 150 mM NaCl, 10 mM EDTA and 100 μg/ml proteinase K (Sigma, St Louis, MO), overnight at 37°C. DNA extraction was accomplished by two separate washes in equivalent volumes of neutralized phenol, followed by two washes in 24:1 chloroform:iso-amyl alcohol. DNA was precipitated by addition of twice the sample volume of 99.5% ethanol and stored overnight at −20°C. DNA was centrifuged at 9600 *g* for 3 h and DNA pellets air-dried before being rehydrolyzed in 200 μl 0.1M Tris (pH 8) with 50 mM EDTA. DNA concentration was determined by measurement of the optical density (OD) of the solutions at 260 and 280 nm, in an Aminco DW-2 UV/vis spectrophotometer. An OD_{260} of 1.0 corresponds to 50 μg/ml DNA. Sample DNA concentrations were in the range of 0.8–1.2 mg/ml. Ratios of OD_{260}/OD_{280} for the final DNA samples were in the range of 1.80–1.95. These samples were then analyzed by atomic absorption.

AAS

Platinum determinations were performed using a Varian SpectrAA 300 with graphite tube atomizer and Zeeman background correction, controlled by a PC running Varian AAS software. The instrument was operated in peak-height mode at 265.9 nm wavelength and 0.5 nm slit bandwidth using the following time–temperature program: 90°C for 30 s, ramp to 120°C in 5 s and hold for 10 s, ramp to 1100°C in 5 s and hold for 15 s, then 2800°C for 8 s, where absorption was measured. All measurements were performed on two 20 μl aliquot injections, in duplicate and were calibrated against absorbance measurements from commercially available standard solutions (Sigma). Absorbances were not affected by the changes in DNA or cell concentrations which occured between samples.

Results

Toxicity experiments

Cisplatin toxicity was measured at concentrations of 5, 10 and 15 μM for incubation times of 1, 1.5, 2 and 3 h. Toxicity curves for aerobic and hypoxic treatment with cisplatin are compared in Figure 1. Cisplatin was found to be more toxic in hypoxic cells than aerobic, particularly at longer treatment times. To quantitate the observed difference, each set of toxicity data was fitted by the method of least squares to either a simple exponential function or a linear-quadratic equation, depending on the visible shape of the curve. The concentration of cisplatin required to reduce the surviving fraction to 0.05 ($D_{0.05}$) was calculated for each data set (see Table 1), and the ratio of aerobic to hypoxic $D_{0.05}$ values, defined as the 'hypoxic cytotoxicity ratio' (HCR, after Brown[25]), was calculated. We have observed, as illustrated by HCR values shown in

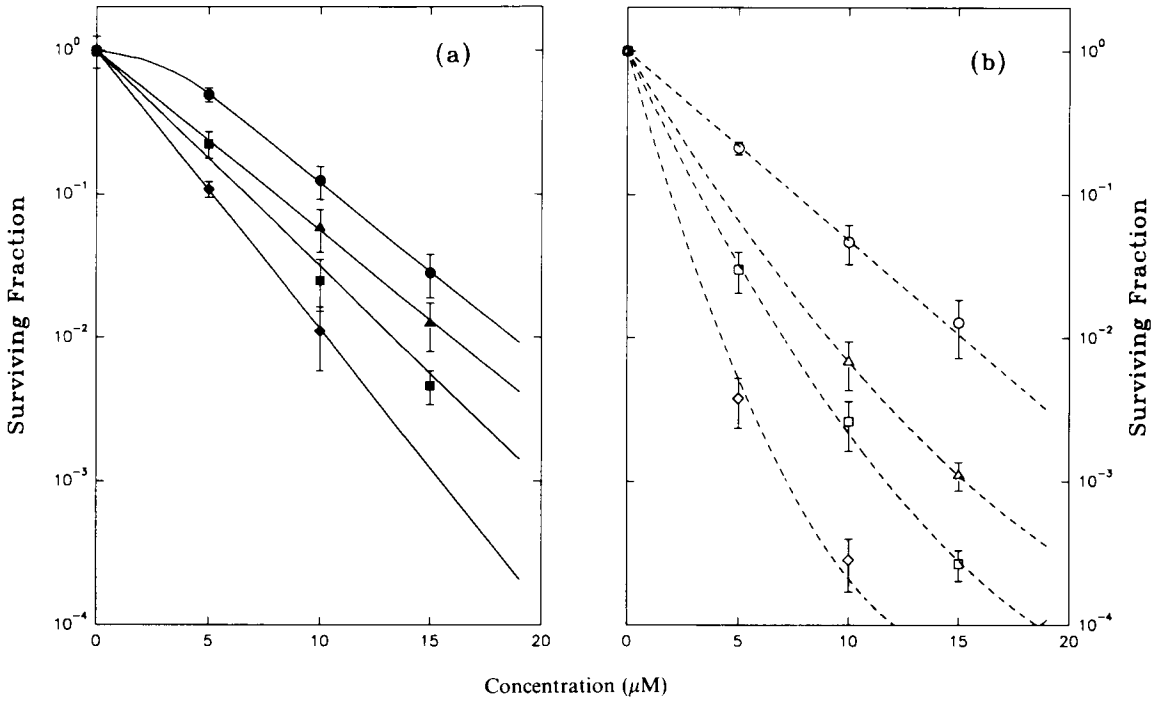

Figure 1. Survival of CHO cells as a function of the cisplatin concentration in aerobic (a) and hypoxic (b) environments. Error bars are SE of the mean of four independent experiments. Curves determined as described in text. ●, ○, 1 h exposure; ▲, △, 1.5 h; ■, □, 2 h; ◆, ◇, 3 h.

Table 1, that when treatment lasts longer than 1 h, cells are more sensitive to cisplatin under hypoxic conditions. There is also an apparent difference in the shapes of aerobic and hypoxic toxicity curves. Aerobic data followed a log–linear relationship (with the exception of the 1 h curve, which has a shoulder), while the hypoxic toxicity curves are steeper at lower concentrations (except incubation for 1 h which formed a straight line).

Cellular accumulation

Accumulation of platinum in cells was found to increase linearly with cisplatin concentration for all incubation times studied. Figure 2(a) illustrates this property for 1 and 3 h incubations (other data may be found in Matthews[26]). Furthermore, cells incubated in hypoxia were found to have consistently higher levels of platination than their aerobic counterparts. The slopes of accumulation versus cisplatin concentration lines were calculated by the method of least squares and the ratio of hypoxic to aerobic slopes was defined as the 'hypoxic accumulation ratio' (HAR) to give a measure of the level of enhanced accumulation due to hypoxic conditions. This value appears to increase with incubation period (as shown in Table 1), although not to the same extent as the HCR.

Table 1. Summary of toxicity, accumulation and DNA binding results

Incubation time (h)	$D_{0.05}$ (air) (μM)	$D_{0.05}$ (hyp) (μM)	HCR	HAR	HBR
1.0	13.1	9.9	1.3	1.2	1.4
1.5	10.4	5.6	1.9	1.2	1.4
2.0	8.7	4.3	2.0	1.3	1.5
3.0	6.7	2.6	2.6	1.5	1.7

DNA binding

Cells used in DNA binding experiments were incubated under identical conditions to those stated above, prior to phenol extraction of the DNA. The level of platination of the DNA was calculated in terms of the number of platinum atoms per 10^5 base pairs, with a detection limit for this assay of 0.75 atoms Pt 10^5 base pair. As illustrated in Figure 2(b), the amount of DNA-bound platinum increased linearly with cisplatin concentration and significantly more Pt was bound to DNA in cells treated

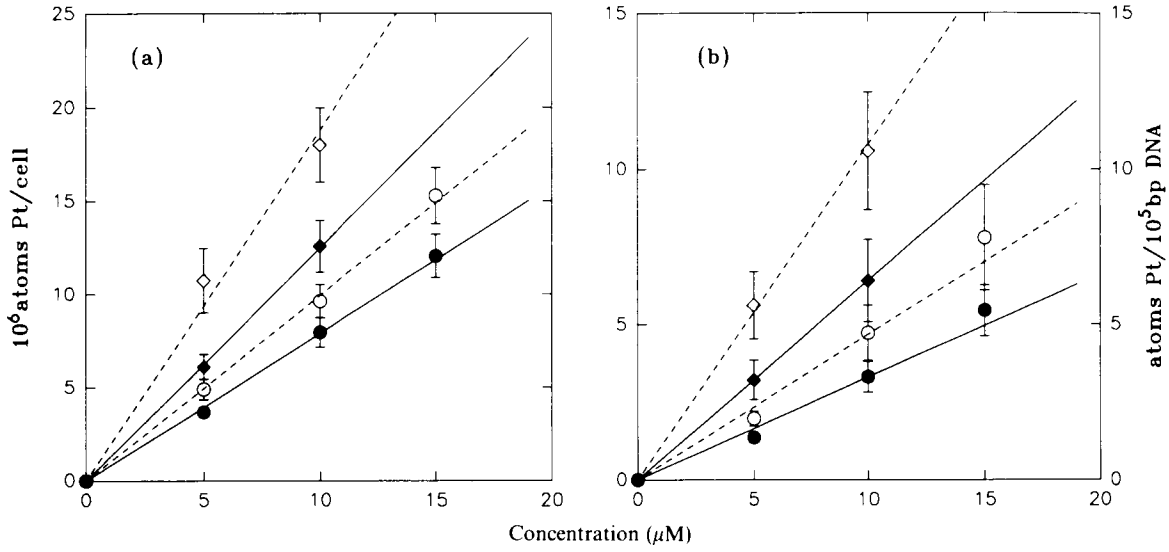

Figure 2. (a) Cellular accumulation and (b) DNA binding of cisplatin in aerobic (closed symbols, solid lines) and hypoxic (open symbols, dashed lines) CHO cells versus drug concentration, as determined by atomic absorption spectroscopy. Error bars are SE of the mean of four independent experiments. Lines calculated by method of least squares. ●, ○, 1 h exposure; ◆, ◇, 3 h (1.5 and 2 h data not shown, but is available in Matthews[26]).

in hypoxia. Thus, the HBR or hypoxic binding ratio was calculated using the same procedure as above and values are listed in Table 1. The HBR increases slightly with incubation time, in a similar fashion to the HAR.

Discussion

We have observed, as illustrated in Table 1, that cisplatin is more toxic to hypoxic than aerobic cells. Accompanied by this increase in cytotoxicity is an increase in intracellular accumulation and DNA-bound platinum levels in cells treated in an hypoxic environment.

Why the disagreement in published results?

As stated earlier, there are conflicting reports regarding cisplatin toxicity in hypoxia. It has been proposed[6] that these discrepancies might be related to differing experimental conditions, such as cell or drug concentrations or the method of producing hypoxia. In fact, the HCR of cisplatin increases with the length of exposure to hypoxia (Table 1), but for incubations of 1 h the HCR is 1.3, indicating that there is only a small increase in toxicity in hypoxia. Only for longer incubation times is the HCR significantly greater than 1. This, then, may explain some of the noted discrepancies. Several of the previous studies considered only 1 h incubations[10–13] in cisplatin, and were not necessarily intended to resolve any difference between aerobic and hypoxic toxicity. Indeed, Stratford *et al.*[7] have reported that enhancement of the cytotoxicity of cisplatin is seen only after a 2 h exposure to the drug. Thus, the mechanism responsible for the increased hypoxic toxicity may not respond to hypoxia immediately and may require at least an hour to manifest itself.

Alternatively, the reason for the longer incubation times required to observe an increased hypoxic toxicity may be related to the actual level of hypoxia. 'Hypoxia' can be defined as the oxygen concentration required to produce a 2- to 3-fold increase in surviving fraction of X-irradiated cells. However, radiobiological hypoxia, usually in the range of 0.1% O_2, may be insufficient to produce an increase in cisplatin toxicity. In fact, cisplatin is equitoxic to cells treated in 0.1% O_2 and those treated in air.[27] As the duration of hypoxia increases, the level of hypoxia is also increased. Therefore, the increased HCR at longer incubation periods may be a reflection of the decreased O_2 concentration and may help to explain some of the conflicting results in the literature.

The unlikely role of bioreductive processes in cisplatin toxicity

The fact that cisplatin toxicity is enhanced at very low oxygen tensions is reminiscent of other drugs, such as mitomycin C[28] (MMC) and misonidazole[29] (MISO), whose toxicity is known to be modulated by metabolic reduction. These compounds show HCRs of approximately 3 and 20, respectively, compared with the maximum value of approximately 2.6 obtained in this study for cisplatin. In fact, Stratford *et al.*[7] proposed that the biochemical reduction of *cis*-Pt(II) to a highly reactive Pt(I) intermediate might be responsible for the enhanced hypoxic cytotoxicity of cisplatin. The enhancement of MMC and MISO toxicity in hypoxia due to reductive processes is related to their reduction potentials. The $E^1_{1/2}$ for MMC is -270 mV and -389 mV for MISO. However, the one electron reduction potential for cisplatin is -1000 mV,[30] making it unlikely that reductive processes are responsible for the increased toxicity of cisplatin in hypoxia.

Intracellular accumulation of cisplatin in hypoxia

As illustrated in Figure 2(b), there is a marked increase in the level of platinum bound to DNA in cells exposed to cisplatin in hypoxia, compared with aerated cells. Also, the HBR and HCR increase in a similar fashion, although the binding ratios are considerably smaller than the cytotoxicity ratios. This suggests that increased binding of cisplatin to DNA is at least partly responsible for increased hypoxic cytotoxicity. However, Figure 2(a) shows that treatment of cells in hypoxia also results in an increased cisplatin accumulation over aerobically treated cells and that the HAR also increases with the duration of exposure. That some of the increased *toxicity* in hypoxia caused by the higher levels of DNA-bound platinum might be due to increased *accumulation* of the drug has been addressed by plotting the data as in Figure 3. This shows that the amount of platinum accumulated within the cell determines the amount which ultimately binds to the DNA and that the relationship between accumulation and DNA binding is not affected by the presence (or absence) of hypoxia. Thus, although cisplatin shows no greater affinity for DNA in hypoxia, an increased binding is observed due to increased cisplatin accumulation in hypoxic cells.

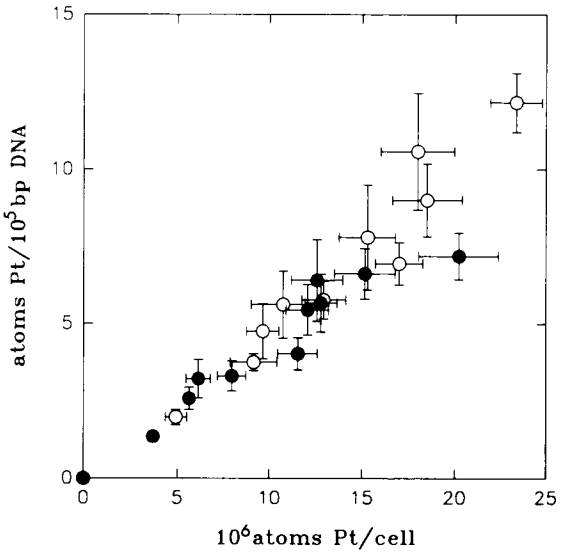

Figure 3. Total DNA-bound platinum versus cellular accumulation. CHO cells were treated in 5, 10 or 15 μM cisplatin for 1, 1.5, 2 or 3 h in aerobic (●) and hypoxic (○) vessels.

For many years, it was assumed that passive diffusion mediated cisplatin transport across the plasma membrane.[31] However, Andrews *et al.*[18] found that inhibition of oxidative phosphorylation *or* glycolysis separately produced no change in cisplatin accumulation, while inhibition of *both* of these processes decreased the intracellular accumulation, indicating that at least part of the mechanism of cisplatin transport is energy dependent. This is consistent with an increased cisplatin accumulation in hypoxia, where oxidative phosphorylation is prevented and anaerobic glycolysis is stimulated. An energy-dependent efflux mechanism, which would be inhibited by the lack of energy available in hypoxic cells, might account for our observations.

Possible mechanisms of hypoxic damage by cisplatin

Because accumulation and binding ratios for cisplatin are considerably smaller than the corresponding cytotoxicity ratios, we are led to believe that increased accumulation alone is not responsible for the increased hypoxic toxicity of cisplatin. Indeed, Figure 4(a) illustrates that cisplatin toxicity per atom of accumulation is higher in hypoxic than aerobic cells. Not only does more cisplatin accumulate in the cells, but it is more effective per atom at causing cell death. Since DNA is usually accepted to be the major target for cisplatin damage,

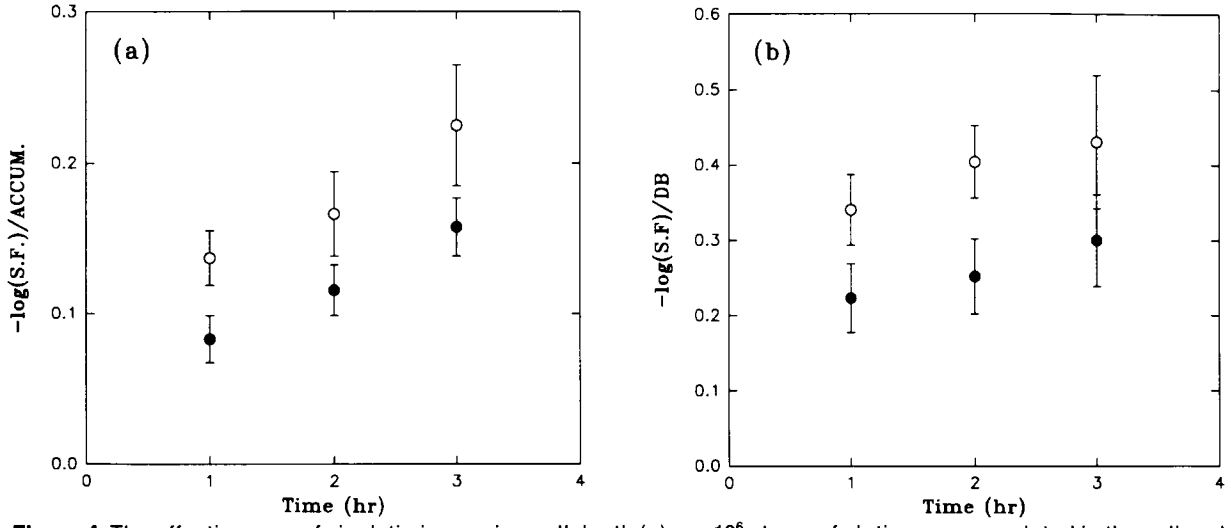

Figure 4. The effectiveness of cisplatin in causing cell death (a) per 10^6 atoms of platinum accumulated in the cell and (b) per platinum atom bound per 10^5 base pairs of DNA (5 μM data shown here). These data illustrate that each platinum adduct on DNA is more effective at causing cell death in hypoxic cells (○) than in aerobic cells (●).

we have also considered the relationship between toxicity and the level of platinum bound to DNA (Figure 4b). As in the case of accumulation, platinum–DNA adducts formed in hypoxia are more toxic per atom than those formed in air. Note that Figure 4 shows only data for 5 μM exposures to cisplatin. The 10 and 15 μM exposures showed higher toxicity per atom of accumulation, but less difference between aerobic and hypoxic cells with respect to toxicity per atom of platinum bound to DNA. These data suggest that there is a difference in the damage to hypoxic cells at low concentrations: either binding of cisplatin to DNA is not the only toxic feature of cisplatin or the DNA lesions produced in hypoxia are of a more damaging or less repairable nature.

Cisplatin is known to form several types of adducts with DNA: interstrand and intrastrand cross-links, DNA–protein cross-links and monofunctional adducts. Which of these lesion(s) are responsible for cisplatin toxicity is not known; however, it is generally accepted that monofunctional adducts are less damaging than cross-links. It has been suggested that quenching of monofunctional adducts by intracellular thiols, thus rendering them unable to produce more toxic inter- or intrastrand cross-links, may be a mechanism of resistance to cisplatin.[32] Thus, the decreased ability of hypoxic cells to produce reduced glutathione (GSH),[33] the principal non-protein intracellular thiol, may render hypoxic cells less able to quench monofunctional DNA adducts of cisplatin as existing pools are depleted. This would also explain the increased level of DNA-bound cisplatin, as GSH is known to bind free cisplatin, preventing it from binding to DNA.[34] However, we have found that cells depleted of GSH by buthionine sulphoximine (BSO) showed similar HCRs to those with normal GSH levels (unpublished results, 1992). Other intracellular protein thiols (such as metallothioneins, stress-induced proteins which have been implicated in cisplatin resistance[35]), are not depleted by BSO treatment and, thus, may play a role in the hypoxic toxicity of cisplatin.

DNA–protein cross-links as the mechanism for hypoxic toxicity

Although there is no evidence to cause us to expect that the relative levels of cisplatin interstrand or intrastrand cross-link formation would increase in hypoxia, there is evidence that DNA–protein cross-link formation is altered by hypoxic conditions. In the absence of any DNA-damaging agents, background levels of DNA–protein cross-links are higher in hypoxia.[36] Upon addition of cisplatin, these lesions could be rendered more difficult to repair by further cross-linking with cisplatin. This hypothesis is supported by the fact that the DNA–protein cross-links formed by cisplatin are known to be more persistent than interstrand cross-links or monofunctional adducts.[37] Furthermore, preliminary studies show that cells rendered hypoxic and treated with cisplatin immediately upon reoxygenation showed enhanced sensitivity to cisplatin than those not exposed to hypoxia (data not shown). The exact role of DNA–protein cross-links in the hypoxic toxicity of cisplatin, however, requires further investigation.

High mobility group (HMG) proteins 1 and 2, two non-histone chromosomal proteins, have recently been found to specifically recognize DNA damage by cisplatin[38] and have been found to be the major protein associated with the DNA–protein cross-links formed by cisplatin.[39] In addition, binding of HMG to cisplatin-modified DNA is quite strong and may involve thiol-containing residues.[40] It is possible then that HMG binding is altered under hypoxic conditions, due to thiol reactivity or protein conformational changes induced by hypoxia.

There is another class of proteins which may play a role in cisplatin toxicity in hypoxic cells, the oxygen regulated proteins (ORPs), which show increased expression due to hypoxic stress[41] and have been implicated in drug resistance. Sutherland[42] has demonstrated that the majority of ORPs produced in hypoxic CHO cells are not produced at higher than normal rates until after 2 h of hypoxia and are produced only at very low O_2 levels ($<0.03\%$). These properties are quite similar to the cytotoxic properties of cisplatin in hypoxia and, thus, the relationship between the two processes, ORP expression and cisplatin toxicity, warrants investigation.

Further studies

The observation that cisplatin is preferentially toxic to hypoxic cells requires further investigation. While the hypoxic selectivity is relatively small (HCR $\sim$ 2.6), it is on a comparable level to MMC (HCR $\sim$ 3), one of the earlier hypoxia-selective antitumor agents. Thus, the findings for cisplatin may serve as an example for the development of other hypoxia-selective analogs, based on a mechanisms other than or in addition to bioreduction. Indeed, we have found that some bis(platinum) complexes[43] with greater cross-linking potential than cisplatin[44] exhibit a similar hypoxia selectivity to cisplatin (in preparation). Investigation of the hypoxic toxicity of other platinum complexes, including some which are hindered from forming adducts with thiols, is in progress.

The clinical implications of the hypoxic selectivity of cisplatin are unclear. *In vivo*, cisplatin has been shown to produce an increase in tumor blood flow[45] and increase the oxygenation status of acutely hypoxic cells.[17] However, due to the very low level of oxygen required for enhancement of cisplatin toxicity, it is likely that only chronically hypoxic cells would be more susceptible. Furthermore, it is not known whether the *in vivo* hypoxic cytotoxicity of cisplatin is precluded by limited diffusion through tissue. This may explain the discrepancy between *in vivo* results[15–17] and the *in vitro* result presented here.

Conclusion

We have determined that cisplatin exhibits enhanced cytotoxicity in hypoxic cells and that this ratio increases with the length of the treatment time. Cisplatin's hypoxic selectivity appears multifaceted and one mechanism is an increased drug accumulation in hypoxic cells. Determination of other factors which contribute to this effect will produce new insights into the mechanisms by which cisplatin causes toxicity.

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