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DNA damage inducible-gene expression following platinum treatment in human ovarian carcinoma cell lines

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Abstract *Purpose*: DNA damage-inducible genes, such as gadd153, gadd45, p21 and c-jun, have previously been shown to be induced by the chemotherapeutic agent cisplatin. One of these genes, gadd153, has previously been reported to be differentially expressed in cisplatin-resistant cell lines and, therefore, to be a potential prognostic indicator for tumor response to cisplatin-based chemotherapy. It is not currently known whether such damage-inducible genes are turned on by the DNA damage itself (e.g. by the formation of Pt-DNA adducts) or by the downstream biological consequences of that damage. It is also not known whether the increased expression of these DNA-damage-inducible genes is related to immediate protective responses such as DNA repair or to more delayed responses such as cell cycle arrest or apoptosis. These experiments were initiated to characterize more fully the nature of the DNA damage-inducible response to cisplatin treatment and to determine whether any of these genes might be useful prognostic indicators of tumor response to cisplatin chemotherapy. *Methods*: The dose-response and time-course for the induction of the DNA damage-inducible genes gadd153, gadd45, p21 and c-jun were examined by Northern analysis in the human ovarian carcinoma cell line 2008 and its resistant subclone C13* following treatment with platinum anticancer agents. The extent of gene expression was correlated with cytotoxicity determined by growth inhibition assay, Pt-DNA adducts determined by atomic absorption spectrometry and inhibition of DNA synthesis determined by ^{3}H-thymidine incorporation. *Results*: All four genes were induced maximally in both sensitive and resistant cell lines at lethal cisplatin doses ($\geq ID_{90}$). Induction was maximal between 24 and 48 h following exposure to the drug for all genes except c-jun which was induced by 6 h. At 24 h following cisplatin treatment the overall levels of gadd153 were less in the resistant C13* cell line than in the parental 2008 cell line, while those of gadd45 were greater in C13* than in 2008. Maximal expression of p21 and c-jun was not significantly different in the two cell lines. The dose-response of these genes correlated with the cytotoxicity of cisplatin and the inhibition of DNA synthesis by cisplatin, rather than to the actual levels of Pt-DNA adducts. The more cytotoxic platinum analog, ormaplatin, also induced gadd153 and its induction was also based on cytotoxicity. *Conclusion*: These results suggest that the regulation of gadd153 and gadd45 expression occurs thorough separate pathways in the 2008 and C13* cell lines. The DNA damage-inducible gene response for all four damage-inducible genes tested appeared to be more directly correlated with downstream biologic effects of cisplatin damage than with actual Pt-DNA adduct levels. The time-course and dose-response for induction of these genes was more consistent with delayed responses such as apoptosis rather than more immediate responses such as DNA repair. Finally, these results strengthen previous suggestions that the expression of gadd153, and possibly other DNA damage-inducible genes, may be useful indicators of tumor response to cisplatin-based chemotherapy.

Key words Cisplatin · Gene expression · Drug resistance

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Introduction

Cisplatin (*cis*-diamminedichloroplatinum II) is a widely used anticancer agent with a broad range of antitumor

activity. It has significant activity in ovarian, testicular, bladder, head and neck, and lung cancer where it is most commonly used in combination with other drugs [20]. The activity of cisplatin is thought to be due to its ability to form inter- and intrastrand DNA crosslinks, predominantly at the N7 position of adjacent guanines [30]. The cytotoxic effect is likely due to inhibition of replication by cisplatin-DNA adducts. The events leading to cell death after cisplatin treatment are unknown. However, G2 arrest with subsequent triggering of the programmed cell death (or apoptosis) pathway has been proposed [32].

Resistance to the drug develops frequently and limits its efficacy. Possible mechanisms of acquired resistance may include: (1) decreased cellular accumulation [1, 3, 24], (2) inactivation of drug in the cell [18], (3) increased repair of Pt-DNA adducts [9], (4) enhanced replicative bypass, a component of postreplication repair [16, 23], and (5) inhibition or loss of the apoptotic response [11]. In addition, the development of resistance may be cell-type specific and related to the dosing schedule. These mechanisms appear to be multifactorial in many instances [2]. In analogy with the SOS response in bacteria, the DNA damage-inducible response in eukaryotic cells has been thought to be associated with increased repair or tolerance of the lesions. Thus, in theory, changes in gene expression could be associated with, or possibly even determine, the outcome after genotoxic damage.

Gadd153 and gadd45 are growth arrest DNA damage-inducible genes isolated by Fornace et al. [12] after UV treatment of Chinese hamster cells. They respond to a wide variety of DNA-damaging agents, including cisplatin, but are also induced in response to growth arrest from medium depletion or high confluency states in vitro [13]. The murine homolog of gadd153, CHOP-10, has been shown to be a member of the C/EBP (CCAAT/enhancer binding protein) family of transcription factors that are important in cell growth control processes such as differentiation [25]. Dimerization with other members of the C/EBP family occurs, but binding to a known classical C/EBP binding site in DNA does not. It is postulated that gadd153 may act as a negative regulator of other C/EBP family members. However, the exact biologic function of this gene remains unknown. Recently, the response of this gene has been correlated with chemosensitivity in an in vitro and in vivo model system [14]. The gadd45 gene, another growth arrest DNA damage-inducible genes, is frequently inducible in response to ionizing radiation, whereas gadd153 is only weakly induced. Gadd45 requires functional p53 for the response to ionizing radiation and has a p53 consensus binding site within its third intron [17]. Expression of gadd45 has been correlated with the presence of strong growth arrest and recently it has been shown to associate with PCNA where it may play a role in DNA repair [31, 37]. The response of the gadd genes appears to be regulated in part through common pathways, although the upstream and downstream elements of these pathways have yet to be determined [19, 37].

The cyclin kinase inhibitor, p21 (Waf1/Cip1/Sdi1), also lies downstream of p53 and forms the bridge between p53 and cell cycle control [35]. The expression of p21 leads to strong negative growth control signals and subsequent cell-cycle arrest. It is also implicated in control of DNA replication and repair through its interaction with PCNA [33]. The cellular response to DNA damage involves induction of p21 through p53-dependent pathways. However, other non-p53-dependent pathways also have been found in response to growth-arrest signals [29]. The immediate early gene, c-jun, has been found to be rapidly induced after UV treatment [7]. This is probably secondary to oxidative stress induced by reactive oxygen intermediates. The response to cisplatin is slower and appears to correlate with apoptosis [27]. At least part of the mechanism of induction likely includes involvement of the protein kinase C signal transduction pathway.

The human ovarian carcinoma cell lines, 2008 and the resistant subclone C13*, have been characterized by Howell and coworkers [4, 23]. The C13* cell line is 12-fold resistant to cisplatin at the ID_{50}. The factors responsible for this pattern of resistance include a 2-fold decrease in cisplatin accumulation [3] and a 4.5-fold enhancement of replicative bypass [23]. No difference in levels of total DNA repair activity has been seen [23, 38]. We assessed the response of the DNA damage-inducible genes, gadd153, gadd45, p21 and the immediate early gene, c-jun, in a well-characterized sensitive and resistant pair of cell lines. The induction of these genes in both a sensitive and resistant cell line appeared most correlated with the cytotoxic effect of cisplatin as opposed to actual levels of DNA damage. The overall induced levels of gadd153 were decreased in the resistant cell line while the levels of gadd45 were increased. The induced levels of p21 and c-jun were not significantly changed between cell lines.

Materials and methods

Cell lines and culture conditions.

The human ovarian carcinoma cell line 2008, and its cisplatin-resistant derivative, C13*, were generously provided by Dr. Paul Andrews (Georgetown University, Washington, DC). All cell lines were grown in RPMI-1640 supplemented with 10% fetal bovine serum and penicillin/streptomycin. Tissue culture reagents were obtained from the Lineberger Comprehensive Cancer Center Tissue Culture Facility (University of North Carolina, Chapel Hill, NC). Cells were grown in 100-mm culture dishes at 37 °C in an atmosphere containing 5% CO_2. Experiments were begun when cells were at 75% confluence.

Drug treatment.

Cisplatin, actinomycin D, and A23187 were purchased from Sigma Chemical Co. (St. Louis, Mo). Ormaplatin ((d, l)-*trans*-1,2-diaminocyclohexanetetrachloroplatinum(IV), also called tetraplatin) was synthesized by Dr. Steven Wyrick (School of Pharmacy, University of North Carolina) as described elsewhere [34]. Cisplatin and ormaplatin were prepared in 0.15 *M* NaCl immediately prior to use. A23187 and actinomycin D were dissolved in 95% ethanol. Logarithmically growing cells were treated with cisplatin or ormaplatin at the stated doses for 1 h in RPMI-1640 medium. The ID_{50} and ID_{90} concentrations for cisplatin are 3.1 μ*M* and 26 μ*M* for 2008 and 37 μ*M* and 212 μ*M* for C13*, respectively. The ID_{50} and ID_{90} concentrations for ormaplatin

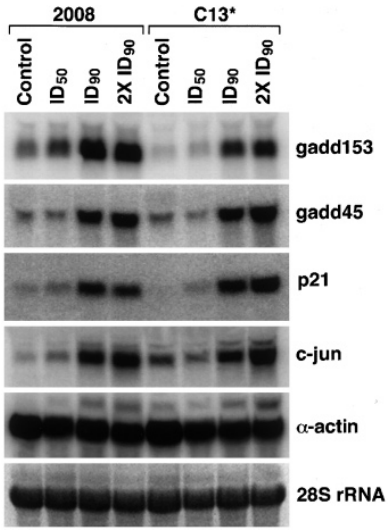

Fig. 1 Northern blot analysis of gene expression at various doses of cisplatin at 24 h following treatment. 2008 and C13* cell cultures were treated with various doses of cisplatin for 1 h and total cellular RNA was harvested at 24 h following the initiation of treatment. Control cells were untreated. mRNA levels of gadd153, gadd45, p21, c-jun and α-actin were analyzed by standard Northern hybridization (20 μg RNA per lane). A representative blot is shown. Both cell lines were run on the same blot and blots were reprobed several times. The last panel represents the negative of an EtBr stain of a gel (10 μg RNA per lane) to confirm RNA concentrations and loading

are 1.1 μ*M* and 15 μ*M* for 2008 and 3.4 μ*M* and 28 μ*M* for C13*, respectively, as determined by standard growth inhibition assays [28]. Subsequent incubation was done in the presence of fresh medium and all cells were refed every 24 h if the duration of the experiment was greater than 24 h.

Northern hybridization.

Cells were harvested at 1, 6, 24 or 48 h after the start of treatment. Sham-treated cells were harvested at 1 h. Total cellular RNA was isolated directly from 100-mm culture dishes with Tri-Reagent (MRC, Cincinnati, Ohio) according to the manufacturer's protocol, dissolved in formamide, and stored at -80 °C. RNA was isolated from single culture dishes for all time-points, except that two culture dishes were used for the 2× ID_{90} and 5× ID_{90} doses. RNA quantitation was performed spectrophotometrically by absorbance at 260 nm. The A_{260}/A_{280} ratios were consistently between 1.8 and 2.0 and EtBr staining after electrophoresis confirmed intact rRNA bands and equal loading. Total RNA (20 μg) was denatured and electrophoresed on a 1.4% agarose gel in the presence of 0.22-*M* formaldehyde unless otherwise noted. RNA was transferred in 10× SSC buffer (1.5 *M* NaCl, 0.15 *M* Na citrate, pH 7.0) to Zeta-Probe nylon membranes (Biorad, Richmond, Calif.) and UV fixed with a Stratagene UV crosslinker at 1200 J/m^2 (Stratagene, La Jolla, Calif.). Overnight hybridization with ^{32}P-labeled cDNA was carried out at 65 °C. Washes were done at 65 °C in 0.1× SSC/0.3% SDS. The blots were imaged with a Phosphorimager (Model No. 425F, Molecular Dynamics, Sunnyvale, Calif.). The ImageQuant software program from Molecular Dynamics was used to quantitate the bands.

The values for relative expression were calculated by normalizing to the 2008 ID_{90} sample for the dose-response studies at 24 h and the 2008 24 h sample for the time courses at 2× ID_{90}. The constitutive gene α-actin was decreased in all cisplatin-treated samples, even at the lowest cisplatin doses and shortest times utilized, in these studies. Because α-actin gene expression was clearly affected by cisplatin treatment, and because it was generally reduced to the same extent by cisplatin treatment in the 2008 and C13* cell lines, it was not used for normalization in these experiments. All quantitative comparisons between cell lines were made only with blots on which both cell lines were run simultaneously. Increases were normalized to each cell line's control (sham-treated) value on the same gel. Blots were stripped according to the manufacturer's protocol and reprobed several times.

c-DNA and labeling.

The probes used for hybridization were as follows. A 640-bp fragment of gadd153 cDNA was cut from the plasmid BH1 provided by Dr. N. Holbrook (NIA, NIH, Baltimore, MD.) by digestion with PstI and XhoI. A 1400-bp fragment of gadd45 c-DNA was cut from the plasmid pHu145B2 provided by Dr. A. Fornace (NCI, NIH, Bethesda, MD.) by digestion with KpnI and NotI. A 1070-bp fragment of c-jun cDNA was excised from the EcoRI site of a Bluescript II plasmid provided by Dr. J. Azizkahn (Lineberger Comprehensive Cancer Center, UNC, Chapel Hill). A 1350-bp α-actin cDNA was cut from the plasmid clone 91 provided by Dr. W. Marzluff (Department of Biochemistry and Biophysics, UNC, Chapel Hill) by digestion with PstI. A 380-bp fragment of p21 cDNA was cut from the plasmid pBS-p21B-clone #2 provided by Dr. Y. Xiong (Department of Biochemistry and Biophysics, UNC, Chapel Hill) by digestion with KpnI. The Prime-a-gene kit (Promega, Madison, Wis.) was used to label all cDNAs with [^{32}P]dCTP (specific activity, 6000 Ci/mmol; Amersham, Arlington Heights, Ill.) by the random priming method.

Inhibition of DNA synthesis assay.

DNA synthesis inhibition was measured by standard methods. Briefly, logarithmically growing cells were treated with cisplatin at various equitoxic doses for 1 h. For these experiments, the cells were grown in 60-mm culture dishes and cisplatin treatment was initiated when the cells were at 50% confluence. The cells were pulsed 24 h later with 6 μCi/ml [^{3}H]thymidine (specific activity, 85 Ci/mmol; NEN, Boston, Mass.) for 15 min. They were scraped into ice-cold harvest buffer (0.1 *M* NaCl, 0.01 *M* EDTA) and then lysed with an equal volume of lysis buffer (1 *M* NaOH, 0.02 *M* EDTA). The DNA was acid-precipitated with perchloric acid and incorporation of radiolabeled thymidine was measured by scintillation counting. The values presented are the mean of seven independent experiments.

Pt-DNA adduct quantitation.

Cells were plated in 100-mm culture dishes, grown, and treated with cisplatin for 1 h and the DNA purified from ten culture dishes immediately following treatment. DNA concentrations were determined by absorbance at 260 nm and Pt levels were determined on a Perkin Elmer Cetus Model 560 atomic absorption spectrophotometer with an HGA 500 graphite furnace and an AS-1 autosampler. The levels of Pt-DNA adducts from lower concentrations of cisplatin were estimated by extrapolation, since previous experiments in our laboratory have shown a linear correlation between Pt-DNA adduct levels and cisplatin concentrations in these cell lines.

Results

Gene induction in cultured human ovarian carcinoma cells

Standard northern hybridization was used to quantitate steady-state mRNA levels at various doses in the sensitive human ovarian carcinoma cell line, 2008, and the resistant subclone, C13*. The dose-response study was performed at 24 h. The sensitive and resistant cells were exposed to cisplatin at equitoxic doses for 1 h to achieve equivalent

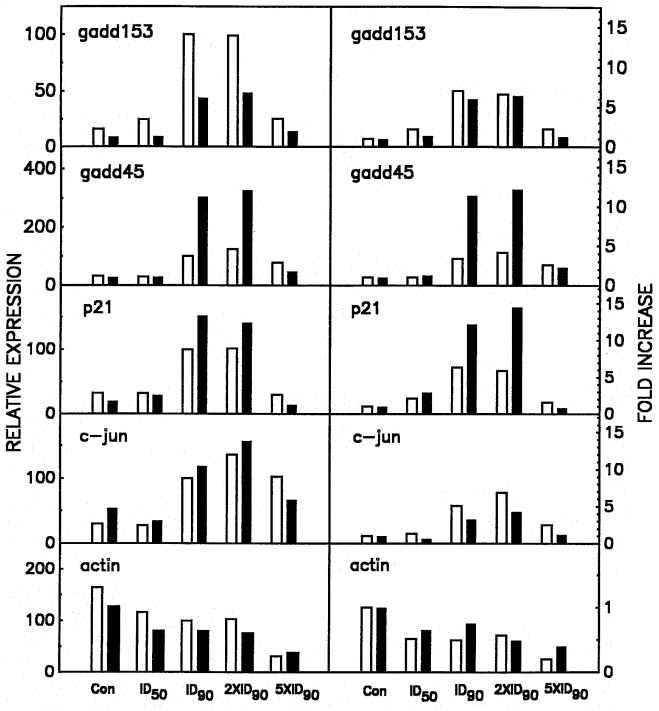

Fig. 2 Relative expression and fold increase of gadd153, gadd45, p21, c-jun and α-actin at 24 h after cisplatin treatment. Quantitation of gene expression was done with phosporimaging and the ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.) by volume integration of the amount of radioactivity associated with the appropriate bands. For relative expression values the 2008 ID_{90} sample was normalized to 100. Fold increases represent the treated sample values relative to each cell line's untreated control. All values are the mean of five or six independent experiments. Lanes represent the following doses: control (sham treated), ID_{50}, ID_{90}, 2× ID_{90}, 5× ID_{90} (*open bars* 2008, *filled bars* C13*)

biologic responses to the DNA-damaging agent. Gene expression was strongly dose-dependent for gadd153, gadd45, p21 and c-jun. Little or no increase above constitutive levels was seen at the ID_{50} dose. In contrast, maximal gene expression was seen at the ID_{90} and 2× ID_{90} dose (Fig. 1, 2). The constitutively expressed α-actin gene was reduced in both cell lines by approximately 30–50% in all cisplatin-treated samples, except at the 5× ID_{90} dose where it was decreased even further. At the supralethal dose of 5× ID_{90} there was a clear dose-related decrease in all DNA damage-inducible genes assessed, as well as in the constitutive α-actin gene. This generalized effect on transcription may have been due to specific inhibition of mRNA synthesis, since at 24 h overall total RNA synthesis as measured by [^{3}H]uridine incorporation was relatively unaffected at this dose (data not shown). Synthesis of rRNA is likely to account for the continued incorporation at the dose of 5× ID_{90}.

We used an equitoxic dose of 2× ID_{90} in both cell lines to define the time-course for induction. Cell lines were treated for 1 h with cisplatin, and RNA was harvested at various time-points. A typical Northern blot is shown in Fig. 3, and average data from two or three experiments are shown in Fig. 4. Transcripts of gadd153, gadd45 and p21

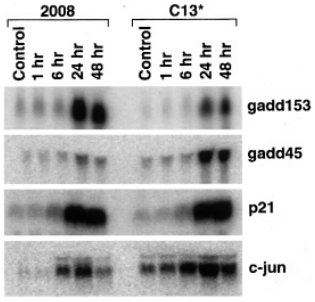

Fig. 3 Northern blot analysis of gene expression at various times following cisplatin treatment. 2008 and C13* cell cultures were treated with a 2× ID_{90} equitoxic dose of cisplatin for 1 h. Total cellular RNA was harvested at 1, 6, 24 and 48 h after the start of treatment. Control cells were untreated

were all induced to maximal levels between 24 and 48 h. In a single more detailed time-course study, maximal induction was observed at 40 h for both the 2008 and C13* cell lines, but the level of induction at 40 h was not much greater than that seen at 24 or 48 h for gadd153, gadd 45 or p21. The time-course for induction of c-jun was somewhat earlier, and significant induction was seen by 6 h in both 2008 and C13* cell lines. However, the induction at 24 h was still similar to that seen at 6 h. The induction of gadd153 at 12 h was approximately half the maximal expression (data not shown). Gadd153, gadd45 and p21 all still had increased expression at 48 h after drug treatment while c-jun expression had returned to near control levels 48 h after treatment.

Because the expression of all four genes was near maximal at 24 h, the relative expression in the sensitive 2008 and cisplatin-resistant C13* cell lines and the increase in the expression of each gene in C13* was measured at 24 h in five or six biological experiments. These results are summarized in Table 1. For gadd153, both basal and maximal gene expression following cisplatin treatment were significantly lower in the resistant C13* cell line. However, the fold increase in gadd153 expression following cisplatin treatment was about the same in the two cell lines. For gadd45 and p21, basal expression was somewhat lower in C13*. However, following cisplatin treatment maximal gene expression and the fold increase in gadd45 gene expression were significantly greater in C13*. For both p21 and c-jun, the trend was similar to that seen with gadd45 in that the induced level at the ID_{90} dose of cisplatin appeared to be greater in the resistant cell line, but the difference between the sensitive and resistant cell lines was not statistically significant.

Correlation of gadd153 with inhibition of DNA synthesis and Pt-DNA adduct levels

Maximal induction of gadd153, gadd45, p21 and c-jun occurred at doses in the ID_{90} to 2× ID_{90} range in both the sensitive and cisplatin-resistant cell lines (Fig. 2). The differences in gene expression at the ID_{90} and 2× ID_{90} doses were not significantly different for any of the genes studied. Since previous experiments in this laboratory [23] have shown that C13* cells tolerate more Pt-DNA adducts than 2008 cells at equal levels of toxicity, the fact that maximal gene expression occurred at equitoxic doses in

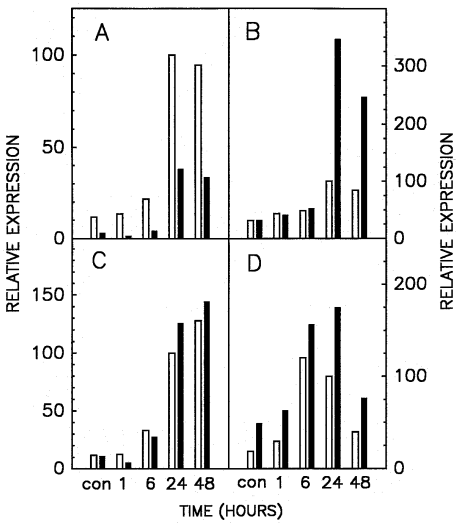

Fig. 4A–D Relative gene expression at various times following cisplatin treatment. Quantitation of the bands shown in Fig. 3 was done with phosporimaging as described for Fig. 2. The 2008 24-h sample was normalized to a value of 100 for all time-course experiments. The values are the mean relative expression of two or three independent experiments. **A** gadd153, **B** gadd45, **C** p21, **D** c-jun (*open bars* 2008, *filled bars* C13*)

Table 1 Induction of DNA damage-inducible genes after cisplatin treatment. ImageQuant software (Molecular Dynamics, Sunnyvale, Calif) was used to quantitate gene expression levels after phosporimaging and volume integration was used to determine the amount of radioactivity associated with the appropriate bands. Relative expression represents the mean value of samples in both cell lines relative to the 2008 ID_{90} sample which was normalized to 100. Both cell lines were run on an identical blot. Fold increases represent the mean of treated samples relative to each cell line's untreated control. All values are mean ± SEM. Numbers in parentheses are the number of independent experiments performed. Statistics were done with Student's *t*-test (two sample, unequal variance)

Genes	Cell line	Relative expression		Fold increase
		Untreated	ID_{90}	ID_{90}
gadd153	2008	16.1± 1.9 (6)	100.0± 0 (6)	7.0±1.0 (6)
	C13*	9.2± 1.7*(6)	44.2± 9.7**(6)	6.0±0.7 (6)
gadd45	2008	32.2± 5.3 (5)	100.0± 0 (5)	3.4±0.4 (5)
	C13*	28.0± 3.2 (5)	304.9±69.4* (5)	11.4±2.3*(5)
p21	2008	32.4±11.1 (5)	100.0± 0 (5)	6.4±3.0 (5)
	C13*	20.0± 7.9 (5)	152.1±39.4 (5)	12.2±4.7 (5)
c-jun	2008	30.3± 9.2 (5)	100.0± 0 (5)	5.4±1.6 (5)
	C13*	54.3±12.6 (5)	129.4±24.1 (5)	3.3±1.3 (5)

* $P \leq 0.05$. ** $P \leq 0.01$

both cell lines suggests that the induction of these genes was proportional to the cytotoxicity rather than the number of Pt-DNA adducts. To test this hypothesis directly we measured the number of Pt-DNA adducts by atomic absorption spectrometry and measured the inhibition of DNA synthesis as another measure of the cytotoxicity of those adducts. Figure 5 shows the correlation between gene expression and inhibition of DNA synthesis. Figure 6 shows the correlation between gene expression and Pt-DNA adduct number. For the purposes of these comparisons, it is important to focus on the portion of the curves leading up to maximal expression, since the decrease in

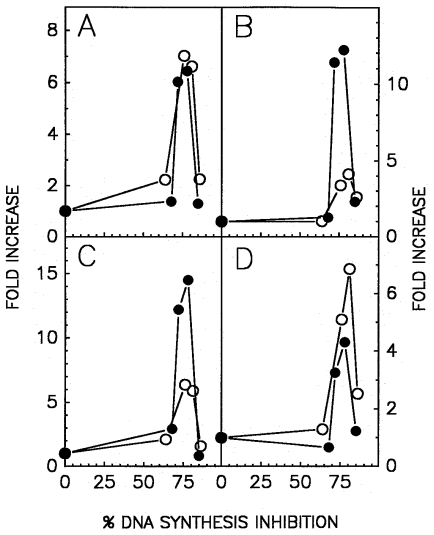

Fig. 5A–D Gene induction as a function of inhibition of DNA synthesis. The fold increase in gene expression over control (untreated) at 24 h following cisplatin for each cell line at doses ranging from ID_{50} to 5× ID_{90} was determined as described for Fig. 2 and is shown as a function of the corresponding level of inhibition of DNA synthesis. DNA synthesis inhibition was measured by standard [^{3}H]thymidine incorporation. **A** gadd153, **B** gadd45, **C** p21, **D** c-jun (*open circles* 2008, *filled circles* C13*)

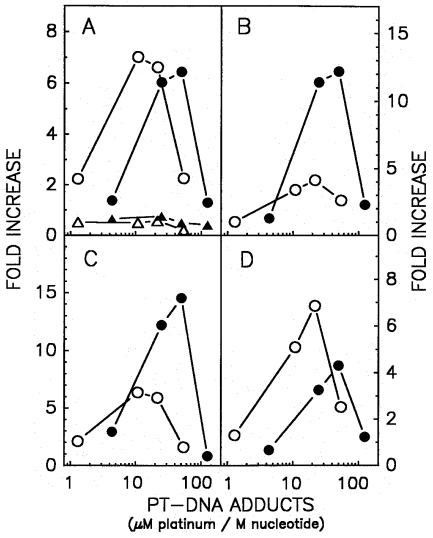

Fig. 6A–D Gene induction as a function of Pt adduct level. The fold increase in gene expression over control (untreated) following cisplatin at 24 h for each cell line at the doses ranging from ID_{50} to 5× ID_{90} was determined as described for Fig. 2 and is shown as a function of the corresponding level of Pt adducts as determined by atomic absorption spectrometry. **A** gadd153, **B** gadd45, **C** p21, **D** c-jun (*open circles* 2008, *filled circles* C13*, *open triangles* 2008, actin, *closed triangles* C13*, actin)

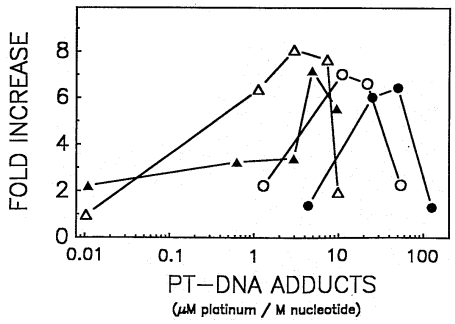

Fig. 7 Gadd153 induction by cisplatin and ormaplatin as a function of Pt adduct level. The fold increase in gene expression over control (untreated) for each cell line at doses ranging from ID_{50} to $5\times$ ID_{90} for cisplatin and ormaplatin was determined and is shown as a function of the corresponding level of Pt adducts as determined by atomic absorption spectrometry (*open symbols* 2008, *filled symbols* C13*, *circles* cisplatin-treated, *triangles* ormaplatin-treated)

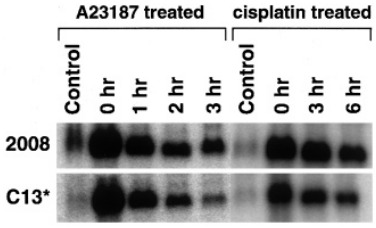

Fig. 8 Northern blot of gadd153 mRNA half-life. The half-life of gadd153 mRNA was determined by measuring the decay in levels after induction by the calcium ionophore A23187 for 4 h. At time 0 A23187 was removed from the incubation medium and 5 μg/ml actinomycin D was added. Total cellular RNA was harvested at 0, 1, 2 and 3 h following the addition of actinomycin D. The half-life of cisplatin-induced gadd153 mRNA was determined following treatment with a $2\times$ ID_{90} dose of cisplatin for 1 h. Actinomycin D (5 μg/ml) was added 24 h later to allow maximal induction of the gene to occur. Total cellular RNA was harvested at 0, 3 and 6 h following the addition of actinomycin D

gene expression at doses of $5\times$ ID_{90} and higher appeared to be due to a global inhibition of mRNA synthesis. As shown in Fig. 5, both sensitive and resistant cell lines had similar patterns of induction relative to the inhibition of DNA synthesis. This likely reflects the toxic manifestation of Pt-DNA adducts on the cell. As shown in Fig. 6, much higher levels of Pt-DNA adducts were required for maximal induction of all four genes in the resistant cell line. The number of Pt-DNA adducts required for maximal gadd153 and c-jun induction were approximately 2.8-fold higher in the C13* cell line. Gadd45 and p21 also appeared to reach maximal expression at higher adduct levels, but the data did not allow a quantitative comparison.

We also used the diaminocyclohexane (dach)-Pt derivative ormaplatin to assess the response of gadd153. Ormaplatin was used because dach-Pt adducts are significantly more cytotoxic than *cis*-diammine-Pt adducts in the 2008 cell line and the difference in cytotoxicity of the dach-Pt adducts in the C13* and 2008 cell lines is only 1.2-fold compared to the 4-fold difference for *cis*-diammine-Pt-DNA adducts [8]. The dose-response for induction of gadd153 by ormaplatin was very similar to that for cisplatin when compared at equitoxic doses (data not shown). As expected from the differences in cytotoxicity of the adducts, approximately threefold fewer dach-Pt adducts than *cis*-diammine-Pt adducts were required for maximal induction of gadd153 in the 2008 cell line, and the differences between the C13* and 2008 cell lines in the number of dach-Pt adducts required for induction were smaller than for *cis*-diammine-Pt adducts (Fig. 7).

mRNA half-life of gadd153

Earlier studies have shown that cisplatin and other DNA-damaging agents increase the activity of the gadd153 promoter and appeared to differentiate between a direct effect of DNA-damaging agents on gadd153 promoter activity, and a slower response to inhibition of DNA synthesis and cell cycle arrest which results from stabilization of gadd153 mRNA [22]. However, more recently Jackman et al. [19] have shown that stability of gadd153 and gadd45, but not c-jun, mRNA is increased in response to several DNA-damaging agents. Since the effect of cisplatin on mRNA stability has not been previously measured, we compared the stability of gadd153 mRNA following treatment of the 2008 and C13* cell lines with either cisplatin or the calcium ionophore A23187. While A23187 increases gadd153 mRNA levels at least in part by stabilizing the mRNA, the half-life of gadd153 mRNA decay following removal of A23187 is very similar to that found in untreated cells [5], but is much easier to measure because of the high initial gadd153 levels. As shown in Fig. 8, the half-life of gadd153 mRNA following cisplatin treatment was significantly prolonged compared to that following treatment with the ionophore (>4 h for cisplatin and ≈ 30 min for A23187). Thus, at least part of the increased expression of gadd153 in these cell lines following cisplatin treatment appears to be secondary to mRNA stabilization.

Discussion

Gadd153 has been shown to be induced by cisplatin treatment [14, 22] and has been proposed as a prognostic indicator of tumor response to cisplatin-based chemotherapy [14]. Therefore, we wished to better define the cellular response to cisplatin in a previously characterized cisplatin-sensitive and -resistant pair of human ovarian carcinoma cell lines. Others have shown that cisplatin also induces c-jun gene expression [27, 29]. The induction of the gadd genes is not a unique response to cisplatin, but is also seen following treatment with most genotoxic agents [13, 22] and in response to growth-arrest signals [37]. For the gadd genes both transcription rates and mRNA stability apparently determine the overall transcript levels following DNA damage [13, 19]. This is less clear when induction is related to growth-arrest signals alone [6], and, in fact, current data

suggest distinct and complex regulatory pathways for the control of gadd gene induction by DNA damage and growth arrest [37]. The induction of p21 by DNA damage and growth arrest may also occur through different signaling pathways and is influenced by, but not necessarily dependent on, p53 status [29]. The c-jun immediate early response gene has also been shown to be induced following cisplatin treatment in human myeloid leukemia cells and, at least in part, this induction is based on an increased transcription rate [27]. Thus, we determined the mRNA levels of the DNA damage-inducible genes gadd153, gadd45, p21 and c-jun, and the constitutive gene α-actin following cisplatin treatment.

We assessed the response of these genes in the 2008 cell line and its cisplatin-resistant subclone, C13*. Since previous work with these cell lines has revealed a decreased level of Pt accumulation [3] and enhanced replicative bypass [23] in the resistant C13* cell line, we were interested in whether alterations in inducible gene expression would correlate with the resistance phenotype and provide a more defined biochemical mechanism of drug resistance. Maximal expression of gadd153, gadd45, p21 and c-jun occurred around the ID_{90} to $2\times$ ID_{90} doses in each cell line following cisplatin treatment (Figs. 1, 2). The time-course for the response was similar for gadd153, gadd45 and p21, but somewhat earlier for c-jun (Figs. 3, 4). Maximal expression was seen as early as 6 h for c-jun, while for all the other genes it was not evident until 24 h following treatment. For gadd153 and c-jun these results are consistent with previous results on the kinetics of induction of these genes following cisplatin treatment [14, 27]. The time-course of the response for gadd45 and p21 induction following cisplatin has not been previously reported. It has previously been suggested that the gadd genes are coordinately regulated [13]. However, while induction of gadd153 and gadd45 were significantly induced in both the sensitive and resistant cell lines, it is interesting that both basal and maximal levels of gadd153 were decreased in the resistant C13* line in relation to the sensitive 2008 line, while the maximal level of gadd45 was increased in C13* in relation to 2008 when the cells were treated with equitoxic doses of cisplatin (ID_{90}) (Table 1). Thus, while coordinate regulation may occur under some circumstances, these differences would suggest that part of the control of expression of these genes may be through separate pathways. Defects in one of these pathways in the resistant cell line may be one possible mechanism whereby this coordinate control is abrogated. With respect to the other DNA damage-inducible genes, the expression levels of c-jun were not significantly different and those of p21, while showing a trend toward increased levels in the resistant line, also were not significantly different (Table 1). Thus, while there were significant similarities in the response of all four DNA damage-inducible genes to cisplatin treatment, the response cannot be called truly coordinate.

The mechanism for induction of gadd153 following treatment with cisplatin and other DNA-damaging agents has been shown to be related, in part, to an increase in the rate of transcription as determined by nuclear run-off [13] and gadd153/CAT expression vectors [30]. Recently, however, Jackman et al. [19] have also reported that greater mRNA stability of the gadd genes contributes to this response following methylmethanesulfonate (MMS) or UV damage. Since this effect in response to cisplatin has not been previously assessed, we determined the relative stability of gadd153 mRNA in our cell lines following cisplatin treatment. We found that the half-life of gadd153 mRNA was greater in the cisplatin-treated cells, compared with the non-DNA-damaged, A23187-treated cells, in both sensitive and resistant cell lines (Figure 8). However, since the half-life in both sensitive and resistant cells appears to be similar, differences in transcription rates must account for the lower basal and induced levels in the resistant C13* cell line.

The time-course for induction of all four genes following cisplatin treatment suggests that it is not the damage itself, but the biological consequences of the damage that are responsible for the induction of these genes. This becomes clearer when one compares the induction of these genes in the sensitive and platinum-resistant cells on the basis of the number of Pt-DNA adducts (Fig. 6) and on the basis of the cytotoxicity of these adducts (Fig. 2). In general, the induction of these genes appeared to be related to the cytotoxic effect of cisplatin rather than the actual levels of DNA damage as measured by the Pt-DNA adduct levels. Using DNA synthesis inhibition as an another marker of cytotoxicity, a consistent pattern of induction was again seen regardless of the resistance phenotype of the cell line (Fig. 5). For gadd153 this effect was also confirmed with the Pt-dach derivative, ormaplatin, where formation of the more cytotoxic dach-Pt-DNA adducts led to induction at lower adduct numbers compared with *cis*-diammine-Pt-DNA adducts (Fig. 7).

Previous studies have shown that the induction of these genes by DNA-damaging agents is strongly correlated with the onset of cell cycle arrest and/or apoptosis [8, 10, 27, 37]. Our results are consistent with either interpretation. Recent in vitro results have also indicated that gadd45 enhances nucleotide excision repair activity, leading to the suggestion that nucleotide excision repair might be a DNA damage-inducible response [31]. Our results are inconsistent with this hypothesis. In the first place, since the 2008 and C13* cell lines do not have significant differences in rates of DNA repair [23, 28], it is clear that the greater induction of gadd45 in the C13* line is not associated with increased repair. More importantly, repair of platinum and other bulky DNA lesions is a biphasic response, with the most rapid repair occurring in the first 6–8 h following DNA damage [9, 15]. Our results show that gadd45 induction following cisplatin treatment is insignificant during the first 6 h following damage. Thus, it is difficult to conceive that gadd45 contributes significantly to increased repair rates for Pt-DNA adducts.

The possible use of gadd153 expression as a predictor of clinical response in cancer patients has been evaluated in a preliminary study of head and neck carcinoma [21]. Our results suggest that, in theory, the expression of any of these genes could serve as a prognostic indicator of tumor

response. For example, maximal expression of gadd153, gadd45, p21 and c-jun occurred around the ID_{90} to $2\times$ ID_{90} doses in each cell line following cisplatin treatment. However, the ID_{90} dose is 26 μ*M* in the 2008 cell line and 212 μ*M* in the C13* cell line. Thus, at a dose that is achievable in vivo (≈50 μ*M*), one would only expect to see expression of those genes in the sensitive cells. Furthermore, all four genes appear to be induced by a wide variety of chemotherapeutic agents [10, 13, 22, 26, 27, 29]. However, of the four genes tested, gadd153 is likely to be the most useful prognostic indicator. c-Jun is induced by reactive oxygen species independently of damage to the DNA [7], making it inappropriate for a number of chemotherapeutic agents. The expression of gadd45 and p21 is at least partially dependent on the p53 status of the cell [36], and thus might differ in p53-negative tumors. Finally, the results with the 2008 and C13* cell lines suggest that even at equitoxic doses, the maximum expression of gadd153 may be less in platinum-resistant cells, although this needs to be confirmed by experiments with additional platinum-sensitive and resistant cell lines.

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