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ORIGINAL ARTICLE

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Regulation of p53 target gene expression by cisplatin-induced extracellular signal-regulated kinase

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Abstract The extracellular signal-regulated kinase (ERK) pathway is among several signal transduction pathways that are activated in response to exposure to the DNA damage-inducing chemotherapeutic agent cisplatin. We have previously reported that inhibition of cisplatin-induced ERK activity enhances sensitivity to cisplatin. Furthermore, we have demonstrated that cisplatin-induced ERK activation is required for optimal p53 protein accumulation following cisplatin-induced DNA damage. In the present study, we expanded our investigations to examine the effect of cisplatin-induced ERK activation on the expression of p53-targeted genes that have been shown to be important in the cellular response to DNA damage including Bax, Bcl-2, Bcl-$_{xl}$, Cyclin G, Gadd45, p21^{WAF1}, and Mdm2. In the ovarian carcinoma cell line A2780, cisplatin was shown to induce expression of p21^{WAF1}, Gadd45 and Mdm2, but cisplatin had no effect on expression of Bax, Bcl-2, Bcl-$_{xl}$, or Cyclin G. Inhibition of cisplatin-induced ERK activity by PD98059 resulted in decreased levels of p21^{WAF1}, Gadd45 and Mdm2. These results provide evidence that ERK activity during the cisplatin DNA damage response, regulates in part, these cell cycle control (p21^{WAF1}, Gadd45), DNA repair (Gadd45) and p53-regulatory (Mdm2) proteins.

Keywords ERK · Cisplatin · p53 · Apoptosis

Introduction

p53 has been labeled the "guardian of the genome" due to its involvement in the response to genotoxic stress and its role in preventing the unchecked accumulation of genetic changes [19]. p53 mediates the cellular response to DNA damage largely through the transcriptional activation of numerous p53 response genes that are involved with cell cycle control, DNA repair, and apoptosis [4]. Activation of cell cycle checkpoint genes by p53 can cause cell cycle arrest associated with DNA repair and can lead to enhanced cell survival [13]. In contrast, p53 can also mediate apoptosis by both transcription-dependent and transcription-independent pathways [5].

We have recently reported that cisplatin-induced extracellular signal-regulated kinase (ERK) activation acts as an upstream regulator of p53 protein accumulation following cisplatin DNA damage [25]. In addition, we have previously demonstrated that cisplatin-induced ERK activation provides a survival signal during cisplatin treatment [24]. Together, the results of these studies suggest that p53 may be a potential mediator of the ERK survival signal.

The focus of the current study was to examine whether cisplatin-induced ERK activity, in addition to contributing to p53 protein accumulation, also affects p53 function in terms of translational activation of select p53 target genes that may directly influence cell survival. We examined the effects of inhibition of cisplatin-induced ERK activity on the expression of proteins from p53 target genes [3, 9, 18, 20, 22, 35, 36] associated with cell cycle control (p21^{WAF1}, Gadd45, Cyclin G), apoptosis (Bax, Bcl-$_{xl}$, Bcl-2), DNA repair (Gadd45), and regulation of p53 stability (Mdm-2) in the ovarian carcinoma cell line A2780.

Materials and methods

Preparation of cellular extracts

The human epithelial adenocarcinoma ovarian cell line A2780 (gift from Dr. Thomas C. Hamilton, Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, Pa.) was maintained in RPMI-1640 medium containing penicillin/streptomycin/glutamine, 0.3 U/ml insulin (Life Technologies, Grand Island, N.Y.) and 10% fetal bovine serum (Intergen, Purchase, N.Y.) at 37°C in a

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humidified atmosphere containing 5% CO_2. Cells were grown in 100-mm Petri dishes for 72 h to 70–80% confluency. The cells were then treated with 10 μg/ml cis-platinum(II) diaminedichloride (cisplatin; Sigma, St. Louis, Mo.) dissolved in RPMI-1640 medium for 4, 8, 12, 18, 24, or 28 h. In studies using PD98059, cells were pretreated for 1 h with 100 μ*M* PD98059 (Calbiochem-Novabiochem, San Diego, Calif.) dissolved in dimethyl sulfoxide (DMSO), followed by continuous exposure to PD98059 during cisplatin treatment. Cells were washed with PBS and lysed in 500 μl ice-cold Triton lysis buffer (TLB) with protease inhibitors (TLB^+; 20 m*M* Tris, pH 7.4, with 137 m*M* NaCl, 25 m*M* glycerophosphate, 2 m*M* EDTA, 1 m*M* sodium vanadate, 2 m*M* sodium pyrophosphate, 1% Triton X-100, 10% glycerol, 1 m*M* PMSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 2 m*M* benzamidine and 0.5 m*M* DTT). Cellular lysates were clarified by centrifugation at 13,000 *g* for 15 min.

Western blot analysis

Cellular extracts (20 μg total protein) were resolved on 14% SDS-PAGE under denatured reducing conditions and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk, washed and incubated with rabbit polyclonal antibodies (Santa Cruz) against Bcl-2 (N19), Bcl-$_{xl}$ (L19), Gadd45 (C20), $p21^{WAF1}$ (C19) or Cyclin G (C18) at working dilutions of 1:500, 1:1000, 1:2000, 1:500 and 1:1000, respectively, or mouse monoclonal antibodies against Bax (B9), Mdm2 (SMP14), or p53 (D01) at working dilutions of 1:1000, 1:500, and 1:2000, respectively, in 0.5% nonfat milk. Membranes were washed, incubated with peroxidase-conjugated secondary antibodies (1:10,000; Sigma), washed again and visualized by enhanced chemiluminescence (Amersham Corporation, Arlington Heights, Ill.). The intensity of the bands was quantitated by densitometry (Personal Densitometer, Molecular Dynamics). Untreated specimens served as negative controls. Western blot analysis of β-actin was used as an internal loading control in all experiments. Since no significant difference in protein loading was observed, photographs of β-actin gels are not shown. Each experiment was repeated a minimum of three times.

Isolation of apoptotic DNA

For the isolation of apoptotic DNA, the method proposed by Herrmann et al. was used [15]. Briefly, A2780 cells grown in 100-mm Petri dishes at 80% confluency were pretreated for 1 h with DMSO (0.1%) or 100 μ*M* PD98059 in DMSO and then treated with or without 10 μg/ml cisplatin for 24 h. After treatment all cells were harvested, washed with PBS and pelleted by centrifugation. The pellets were treated for 10 s with lysis buffer (50 m*M* Tris-HCl, pH 7.5 with 1% NP-40 and 20 m*M* EDTA) and centrifuged for 5 min at 1600 *g*. The supernatants were collected and the extraction was repeated. Combined supernatants were brought to 1% SDS and treated for 2 h with 5 μg/ml RNase A at 56°C followed by digestion for 2 h with 2.5 μg/ml proteinase K at 37°C. After addition of two volumes of 10 *M* ammonium acetate DNA was precipitated with 2.5 volumes of ethanol, dissolved in TE buffer and quantitated using OD reading at 260 nm. DNA (equivalent amounts) from different treatments was separated by electrophoresis in 1.5% agarose gel with ethidium bromide. The gel was photographed under UV illumination.

Results

Accumulation of p53 protein during exposure to cisplatin

The cellular response to DNA damage includes both increased p53 protein accumulation and increased p53 activity [17]. In the wild-type p53-expressing ovarian carcinoma cell line A2780, p53 protein levels increased following exposure to cisplatin in a time-dependent manner. Treatment of A2780 cells with 10 μg/ml cisplatin resulted in a modest increase in p53 protein levels at 4 h followed by a gradual increase in levels (Fig. 1). A maximum increase of sevenfold over control p53 levels occurred at 18 h. The p53 protein levels decreased after 18 h and were near baseline by 28 h.

Effect of ERK inhibition on p53 protein levels

We have previously demonstrated that PD98059 inhibits cisplatin-induced ERK activation without affecting the activation of the MAP kinase JNK [8, 24]. A dose of 100 μ*M* PD98059 was chosen because this concentration has been shown to completely inhibit cisplatin-induced ERK activation without affecting JNK activity [8, 24]. PD98059 reduced the cisplatin-induced accumulation of p53 protein at each of the times examined in the time course (Fig. 2). The reduction was most dramatic at 18, 24 and 28 h. The p53 levels had reduced to at or below control levels by 24 and 28 h. PD98059 had no effect on baseline p53 protein levels. The fact that PD98059 did not completely inhibit the accumulation of p53 protein at time-points up to 18 h indicates that other upstream pathways, in addition to the ERK1/2 pathway, are involved in the regulation of p53 protein accumulation during cisplatin exposure.

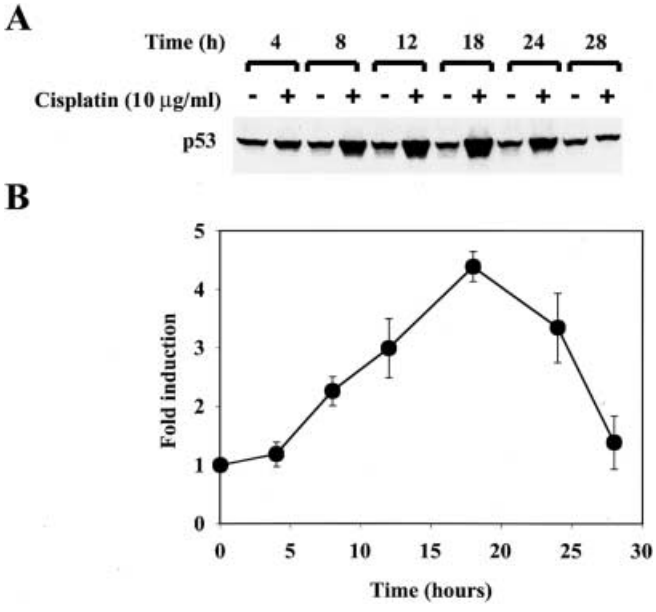

Fig. 1A, B Time-course of p53 accumulation during cisplatin exposure. A2780 cells were treated with or without 10 μg/ml cisplatin and harvested at the indicated times. The cell extracts were subjected to Western blot analysis for p53 protein. **A** Representative gel. **B** Fold increases in p53 protein determined by densitometry and calculated as the ratio of treated samples to untreated samples at each time-point. *Error bars* represent the standard error of the mean from three experiments

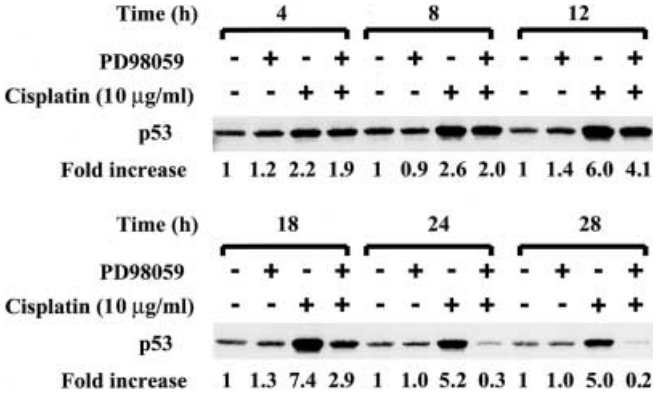

Fig. 2 Time-course of p53 accumulation during cisplatin and PD98059 treatment. A2780 cells were pretreated for 1 h with or without 100 μ*M* PD98059 and with or without 10 μg/ml cisplatin and harvested at the indicated times. The cell extracts were subjected to Western blot analysis for p53 protein. The fold increase of p53 protein was determined by densitometry and calculated as the ratio of treated samples to untreated samples at each time-point. The results shown are representative of at least three independent experiments

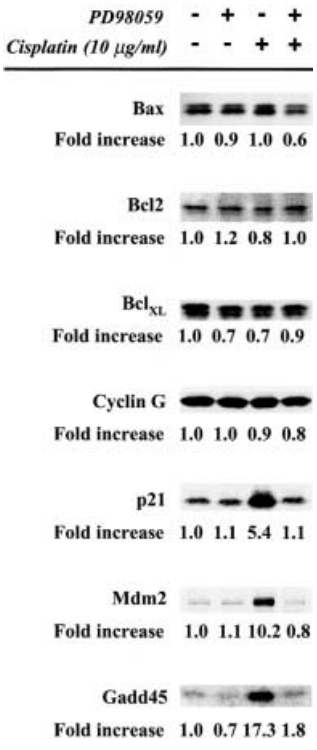

Fig. 3 Effect of PD98059 on cisplatin-induced protein accumulation of p53 target genes. A2780 cells were pretreated for 1 h with or without 100 μ*M* PD98059 and with or without 10 μg/ml cisplatin and harvested at 18 h. The cell extracts were subjected to Western blot analysis for Bax, Bcl-2, Bcl$_{xl}$, Cyclin G, p21^{WAF1}, Mdm2 or Gadd45 protein. The fold increase of each protein was determined by densitometry and calculated as the ratio of treated samples to untreated samples. The results shown are representative of at least three independent experiments

Effect of ERK inhibition on the expression of selected p53-regulated genes

The attenuation of p53 accumulation with ERK1/2 inhibition led us to explore the possibility that cisplatin-induced ERK1/2, in addition to regulating p53 accumulation, would also effect p53 function in terms of modulating expression of p53-regulated genes. In the cellular response to DNA damage, critical p53-targeted genes include those involved in cell cycle arrest, DNA repair and apoptosis. We examined the effect of PD98059 on the protein levels of p53-targeted genes known to affect the cell cycle, DNA repair and apoptosis. Western blot analysis was performed for p21^{WAF1}, Gadd45, Bax, Bcl-2, Bcl$_{xl}$, and Cyclin G. In addition, we examined the effect of PD98059 on Mdm2, the major protein involved in regulation of p53 stability following DNA damage [14]. Because 18 h of treatment with cisplatin resulted in a maximum increase in p53 protein levels in A2780 cells, we examined the effect of cisplatin-induced ERK inhibition on the expression of the p53-targeted proteins following 18 h of exposure to cisplatin (Fig. 3).

Three of the proteins examined, Bax, Bcl-2, and Bcl-$_{xl}$, are members of a family of proteins that control the disposition of a cell to undergo apoptosis [10, 20, 23, 36]. There was no change in the expression of any of these proteins with cisplatin treatment. The expression of Cyclin G was also not altered by cisplatin or PD98059. In contrast, expression of p21^{WAF1}, Mdm2 and Gadd45 proteins increased significantly following 18 h exposure to cisplatin. Increases of 5.4-, 10.2- and 17.3-fold were observed for p21^{WAF1}, Mdm2 and Gadd45 proteins, respectively. In all three cases, addition of PD98059 to cisplatin-treated cells resulted in a significant reduction in protein levels compared to cisplatin treatment alone. PD98059 had no significant effect on control baseline levels of any of the proteins.

We next examined the entire time course for p21^{WAF1}, Mdm2 and Gadd45 proteins and determined how PD98059 affected these proteins at each time-point (Fig. 4). Treatment of A2780 cells with 10 μg/ml cisplatin resulted in a time-dependent increase in all three proteins. Maximum increases in all three proteins in response to cisplatin occurred at 18 h. The level of p21^{WAF1} and Gadd45 proteins, in a similar manner to p53 protein levels, gradually decreased at 24 and 28 h. Levels of Mdm2 had decreased dramatically by 24 h of treatment with cisplatin. When cisplatin-induced ERK activation was inhibited by PD98059, the accumulation of all three proteins was significantly reduced. PD98059 had no effect on baseline levels of Mdm2 or Gadd45. However, following 24 and 28 h exposure to PD98059 alone, levels of p21^{WAF1} had increased slightly. This finding was consistently observed in repeated assays. The significance and mechanism involved in the change in p21^{WAF1} protein levels is unknown. Because increases in baseline p53 protein levels were not observed at 24 and 28 h, the increase in

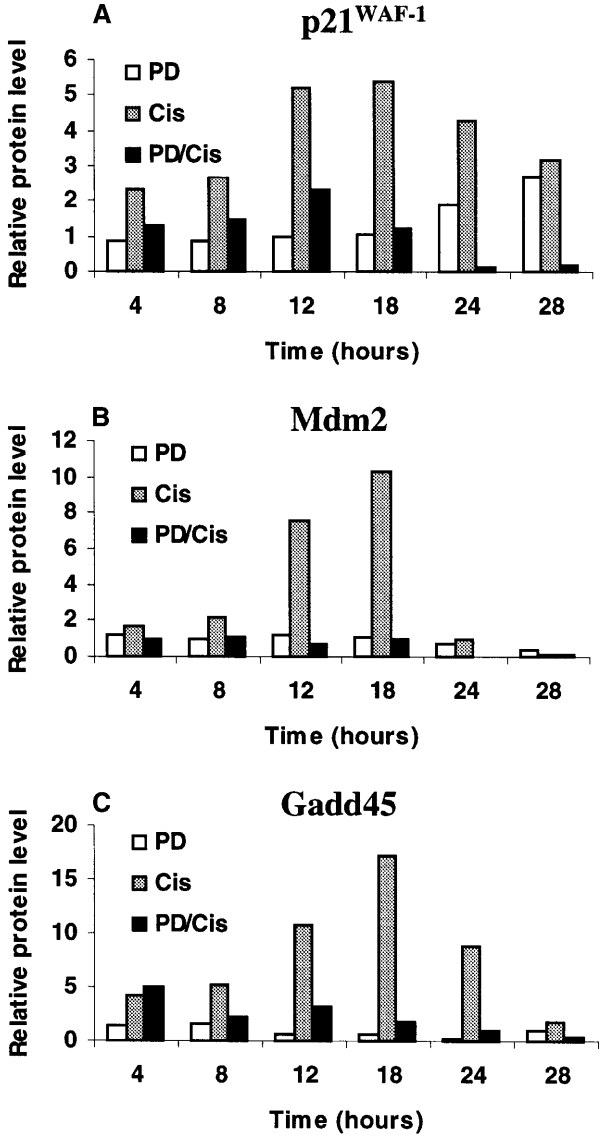

Fig. 4A–C Time-course of p53 accumulation during cisplatin and PD98059 treatment. A2780 cells were pretreated for 1 h with or without 100 μ*M* PD98059 and with or without 10 μg/ml cisplatin and harvested at the indicated times. The cell extracts were subjected to Western blot analysis for (**A**) $p21^{WAF1}$ protein, (**B**) MDM2 protein or (**C**) Gadd45 protein. Relative protein levels were determined by densitometry and calculated as the ratio of treated samples to untreated samples at each time-point (control untreated sample not shown) (*white bars* 100 μ*M* PD98059, *gray bars* 10 μg/ml cisplatin, *black bars* 100 μ*M* PD98059 and 10 μg/ml cisplatin). The results shown are representative of at least three independent experiments

$p21^{WAF1}$ protein baseline levels during exposure to PD98059 appears to be independent of changes in p53 protein accumulation.

Similar time courses were performed for Bax, Bcl-2, $Bcl\text{-}_{XL}$, and CyclinG to verify that changes were not occurring in these proteins at times other than 18 h after initiation of cisplatin treatment. There was no significant change in any of these proteins at any of the time-points (data not shown).

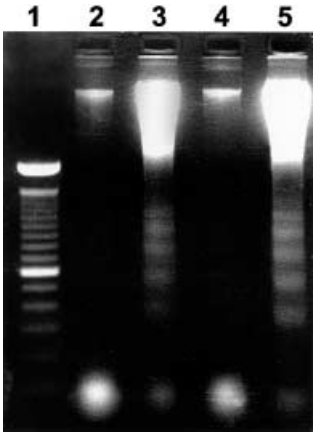

Fig. 5 Effect of PD98059 on cisplatin-dependent apoptosis. A2780 were pretreated for 1 h with DMSO or 100 μ*M* PD98059 and incubated for 24 h with or without 10 μg/ml cisplatin. After incubation DNA was isolated as described in Materials and methods. The DNA integrity of the samples is illustrated in the agarose gel (*lane 1* 100 bp DNA ladder, *lane 2* DNA from cells treated with DMSO only, *lane 3* DNA from cisplatin-treated cells following pretreatment with DMSO, *lane 4* DNA from cells treated with PD98059 only, *lane 5* DNA from cisplatin-treated cells following pretreatment with PD98059)

Effect of ERK inhibition on cisplatin-induced apoptosis

To determine whether the inhibition of ERK pathway has any effect on cisplatin-induced apoptosis in A2780 cells, we isolated DNA from the exposed for 24 h to 10 μg/ml cisplatin with or without PD98059 pretreatment (Fig. 5). Pretreatment of A2780 cells with DMSO or PD98059 in DMSO without cisplatin did not affect the DNA integrity of the cells (Fig. 5, lanes 2 and 4). The distinct apoptotic feature of a 180 bp DNA ladder was detected in cisplatin-treated A2780 cells (Fig. 5, lane 3). A more pronounced DNA ladder was observed in the presence of 100 μ*M* PD98059 (Fig. 5, lane 5), indicating enhanced apoptosis with ERK inhibition. We have previously demonstrated that cisplatin-induced cytotoxicity is also enhanced by PD98059 in this cell line, correlating with the increased apoptosis [8].

Discussion

Activation of p53 by cisplatin-induced DNA damage has been reported to have various effects on cellular sensitivity to cisplatin. In some studies, activation of p53 has been shown to provide cytoprotection against cisplatin [27, 32]. In contrast, increased resistance to cisplatin with disruption of normal wild-type p53 function has also been demonstrated [33]. Thus, the effect of activation of p53 on cisplatin sensitivity appears to be complex and variable depending upon the cellular context and can be associated with either survival or apoptosis [6, 11]. The expression of p53 target genes is also variable and is dependant on several factors such as the presence of mitogen signals [6, 7, 21] and the

presence of an apoptosis-susceptible phenotype [35]. In this study, we demonstrated that in A2780 cells, cisplatin induces select p53 target genes and that the induction of these genes is dependent, at least in part, on ERK activity.

In the cellular response to various types of genotoxic stress, activation of p53 has been shown to simultaneously downregulate Bcl-2 and upregulate Bax, and the ratio of these proteins is a critical determinant of apoptotic cell death [20, 35]. However, the role of these genes in response to cisplatin is unclear. Hong et al. have reported that levels of Bcl-2, Bcl-$_{Xl}$ and Bax are not altered by cisplatin treatment in T-cell hybridoma cell lines [16]. In the present study, we also demonstrated that cisplatin treatment had no effect on the protein content of Bcl-2, Bax, Bcl-$_{Xl}$, as well as CyclinG. These findings suggest that the expression of these proteins is likely not a critical component of the cellular response to cisplatin or the cytoprotective effect afforded by ERK activation.

Increases in protein levels of the p53 target genes Mdm2, p21^{WAF1} and Gadd45 were observed following cisplatin treatment and were also dependent upon ERK activity. Thus, optimal cisplatin-induced accumulation of Mdm2, p21^{WAF1} and Gadd45 proteins was dependent on ERK activity. Mdm2 is transcriptionally induced by p53 following DNA damage [3]. Mdm2 binds to p53 protein and inhibits the activation of p53 and targets p53 protein for degradation through the ubiquitin-dependent proteolytic pathway [14]. Thus, Mdm2 and p53 are predicted to form an autoregulatory feedback loop in which p53 limits its own activation through the production of Mdm2 [26]. Interference with the binding of Mdm2 protein to p53 protein can lead to increased accumulation of p53 protein. We have recently reported that ERK phosphorylates p53 protein at serine 15, the phosphorylation of which has been shown by other investigators to lead to decreased binding of Mdm2 to p53 [25, 28]. Cisplatin-induced ERK activity may therefore regulate both accumulation and function of p53 by this mechanism.

Accumulation of p21^{WAF1} following DNA damage has been classically associated with G_1 arrest [12]. However, p21^{WAF1} can be involved with both inducing and sustaining G_2/M arrest [2], the common cellular checkpoint affected during cisplatin treatment. By decreasing the level of p21^{WAF1} protein, inhibition of cisplatin-induced ERK activity may alter the G_2/M arrest resulting in decreased DNA repair or decreased recovery from the G_2/M arrest leading to enhanced apoptosis. This hypothesis is consistent with the findings of Abbott and Holt who demonstrated that activation of the upstream kinase of ERK, mitogen-activated protein kinase kinase 2, is essential for progression through the G_2/M checkpoint arrest caused by ionizing radiation [1].

The effect of ERK activity on Gadd45 expression could also have significant implications with regard to cell survival. Gadd45 has been shown to have a role in DNA excision repair [29]. A link between Gadd45-mediated DNA repair and cellular sensitivity to cisplatin has been demonstrated by Smith et al. who showed that blocking expression of Gadd45 in RKO human colon carcinoma cells with antisense vectors results in decreased DNA repair and sensitization of cells to killing by cisplatin [30]. It has also been shown that Gadd45 can suppress cell growth, presumably through the induction of a G_2/M cell cycle checkpoint via its association with Cdc2 and inhibition of Cdc2/Cyclin B1 kinase activity [34, 37]. Furthermore, overexpression of Gadd45 has been shown to protect cells from cisplatin killing in vitro [31]. Due to its role in both DNA repair and G_2/M arrest following DNA damage, Gadd45 is a potential mediator of cellular sensitivity to cisplatin.

In summary, cisplatin-induced ERK activation is required for not only optimal accumulation of p53 protein, but also optimal accumulation of Mdm2, p21^{WAF1} and Gadd45 protein during the cellular response to cisplatin in A2780 cells. Because the accumulation of any or all three of these proteins may influence the sensitivity of cells to cisplatin, Mdm2, p21^{WAF1} and Gadd45 are potential mediators of the ERK cytoprotective effect. Studies are underway in our laboratory to investigate how ERK-dependent accumulation of Mdm2, p21^{WAF1} and Gadd45 proteins may affect sensitivity to cisplatin in A2780 cells. The continued investigation of mediators of the ERK cytoprotective response, as well as other survival signals during the cisplatin response is critical in understanding the cellular response to cisplatin. Furthermore, identification of the molecular components involved in regulating the cellular sensitivity to cisplatin may provide potential targets for development of novel compounds that may be useful in enhancement of cisplatin cytotoxicity.

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