Ataxia telangiectasia and rad3-related kinase contributes to cell cycle arrest and survival after cisplatin but not oxaliplatin

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Abstract

The DNA cross-linking agents cisplatin and oxaliplatin are widely used in the treatment of human cancer. Lesions produced by these agents are widely known to activate the G_1 and G_2 cell cycle checkpoints. Less is known about the role of the intra–S-phase checkpoint in the response to these agents. In the present study, two different cell lines expressing a dominant-negative kinase dead (kd) version of the ataxia telangiectasia and rad3-related (ATR) kinase in an inducible fashion were examined for their responses to these two platinating agents and a variety of other DNA cross-linking drugs. The expression of the kdATR allele markedly sensitized the cells to cisplatin, but not to oxaliplatin, as assessed by inhibition of colony formation, induction of apoptosis, and cell cycle analysis. Similar differences in survival were noted for melphalan (ATR dependent) and 4-hydroperoxycyclophosphamide (ATR independent). Further experiments showed that ATR function is not necessary for removal of Pt-DNA adducts. The predominant difference between the responses to the two platinum drugs was the presence of a drug-specific ATR-dependent S-phase arrest after cisplatin but not oxaliplatin. These results indicate that involvement of ATR in the response to DNA cross-linking agents is lesion specific. This observation might need to be taken into account in the development and use of ATR or Chk1 inhibitors. [Mol Cancer Ther 2009;8(4):855–63]

Introduction

Platinum derivatives are among the most commonly used cross-linkers in clinical oncology. The most widely used of these is cisplatin, a coordination complex that consists of Pt (II) bound to two amine groups and two chloride ions. This agent, which is active in carcinomas of the ovary, lung, and testis as well as lymphoid malignancies, forms bivalent adducts with nucleophilic sites on purines in DNA (reviewed in refs. 1, 2). The predominant lesions produced are DNA intrastrand cross-links between adjacent purines, with smaller numbers of intrastrand cross-links between nonadjacent guanines in GpXpG sequences, interstrand cross-links between adjacent guanine residues on opposite strands, and DNA-protein cross-links (3, 4). Oxaliplatin, a platinum derivative bearing a 1,2-diaminocyclohexane ligand, was recently approved for colon cancer and is being investigated in other cisplatin-resistant cancers. The observation that oxaliplatin forms fewer DNA adducts than cisplatin at equimolar concentrations but causes equivalent or higher cytotoxicity at these concentrations (5–7) suggests that the lesions from each agent are handled differently by the cell.

The cellular response to these Pt-DNA adducts reflects at least four distinct processes: (*a*) damage recognition, (*b*) inhibition of replication/transcription, (*c*) replicative bypass of damaged DNA, and (*d*) cell cycle arrest. Differences in response to cisplatin versus oxaliplatin might reflect differences at any or all of these steps. For example, whereas the cross-links formed by these agents result in bending of the double-stranded DNA, the degree of bending (and hence the ability of the damage to be recognized) differs between cisplatin and oxaliplatin (8). Likewise, there seem to be differences in ability of DNA polymerases to bypass the lesions. Elevations of DNA polymerase β, the mammalian polymerase shown to catalyze significant replicative bypass of cisplatin-DNA adducts *in vitro* (9, 10), results in diminished killing but an increased rate of cisplatin-induced mutations (11). Importantly, DNA polymerase β has been shown to be more effective in translesion synthesis past oxaliplatin, which forms bulkier adducts, compared with cisplatin (12).

Ultimately, Pt-DNA adducts activate complex signaling pathways that cause cell cycle arrest and activate the DNA repair machinery. It was recently reported that oxaliplatin and cisplatin differ in the type of cell cycle arrests they induce, with cisplatin inducing a prominent activation of the replication checkpoint and oxaliplatin inducing prominent G_1-S and G_2-M arrests in p53 wild-type HCT116 colon cancer cells (13). The biochemical basis for this difference in cell cycle arrest and its potential effect on drug sensitivity remain unclear.

Experiments in *Schizosaccharomyces pombe* show a critical role for the genes *Rad1* and *Rad3* in cisplatin sensitivity

(14). Similar results have more recently been reported in *Saccharomyces cerevisiae* mutants (15). Consistent with these findings, several observations suggest that ataxia telangiectasia and rad3-related (ATR) kinase, a human PIKK that shares a conserved carboxyl-terminal kinase domain with human ATM, *S. pombe* rad3, and *S. cerevisiae* mec1, plays a major role in the response of mammalian cells to cisplatin. First, ATR kinase activity is increased in response to cisplatin (16, 17). Second, the ATR substrate BRCA1 (18) and the Chk1 substrate Rad51 (19) are localized to cisplatin-damaged DNA (20). In addition, inactivation of the ATR kinase sensitizes human cells to cisplatin in colony-forming assays (21, 22). Collectively, these observations suggest that ATR and the downstream kinase Chk1 play a role in response to cisplatin by phosphorylating BRCA and Rad51, respectively, which traffic to sites of DNA damage and activate homologous recombination repair (HRR; ref. 19). Although these results are consistent with recent reports that cells defective in HRR are hypersensitive to cisplatin (23, 24), additional roles for ATR in cisplatin sensitivity have not been ruled out. Most recently, Wilsker and Bunz reported that loss of ATR function strikingly enhanced the effects of several anticancer drugs but not others (25).

Based on the observation that inhibition of ATR or Chk1 signaling enhances drug sensitivity, there has been considerable interest in developing ATR and Chk1 inhibitors as a way to increase the therapeutic efficacy of specific cytotoxic drugs. It is, therefore, important to characterize the role of ATR kinase in response to these agents. In the present study, we examined the role of ATR in the response to cisplatin, oxaliplatin, and additional DNA cross-linking agents. We have observed that inhibition of ATR kinase activity sensitizes cells to cisplatin and melphalan but not oxaliplatin or 4-hydroperoxycyclophosphamide (4HC). Further experiments ruled out a role for ATR in removal of Pt-DNA adducts and instead suggested that different cross-linking agents differ in their ability to activate the ATR/Chk1 pathway.

Materials and Methods

Reagents

Cisplatin, doxycycline, Hoechst 33258, and melphalan were purchased from Sigma. 4HC and oxaliplatin were kindly provided by O. Michael Colvin and Susan Ludeman (Duke University, Durham, NC) and Sanofi Research, respectively.

Tissue Culture

GM847/kdATR and GK41 cells (kindly provided by Paul Nghiem, Harvard University, Cambridge, MA) were previously constructed from GM847 SV40-transformed fibroblasts and U2OS human osteosarcoma cells, respectively, by transfection with cDNA encoding kinase-inactivated ATR under the control of a doxycycline-responsive promoter (21, 26). GM847/kdATR cells were cultured in DMEM with 4.5 g/L glucose, 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin G, 100 μg/mL streptomycin, and 2 mmol/L glutamine (medium A) containing 400 μg/mL G418. GK41 cells were cultured in medium A containing 200 μg/mL G418 and 50 μg/mL hygromycin. At the start of each experiment, cells were grown in medium A for 48 h in the absence or presence of 1 μg/mL doxycycline.

Clonogenic Analysis

After pretreatment for 48 h in the absence or presence of doxycycline, aliquots containing 5,000 to 6,000 cells were plated in triplicate 35-mm dishes in the continued presence of doxycycline or diluent and allowed to adhere overnight. Varying concentrations of cisplatin, oxaliplatin, 4HC, or melphalan were added for the indicated times. Cells were then washed twice in serum- and drug-free medium and incubated for 12 to 14 d in medium A in the absence or presence of doxycycline to allow colony formation. After plates were stained with Coomassie blue, colonies containing ≥50 cells were manually counted. Control plates typically contained 140 to 180 colonies.

Quantitation of Apoptosis

After incubation in medium A lacking or containing 1 μg/mL doxycycline for 48 h, cells were exposed to 20 μmol/L cisplatin for 2 h, washed, and incubated for 0 to 72 h in drug-free medium A lacking or containing doxycycline. After incubation, adherent cells were released by trypsinization and pooled with nonadherent cells. To assess apoptosis, cells were washed twice with ice-cold PBS, fixed in 3:1 (v/v) methanol/acetic acid, deposited and dried on glass slides, and stained with 1 μg/mL Hoechst 33258. Samples were examined using fluorescence microscopy and a minimum of 300 cells per treatment were scored using criteria described previously (27, 28).

Flow Cytometry

A double thymidine block was used to synchronize GK41 cells at the G_1-S border. Cells were incubated in medium A lacking or containing doxycycline. Additionally, 2.5 mmol/L thymidine was added for two 17-h periods with an intervening 12-h thymidine-free incubation between treatments. After the removal of the second thymidine block, cells were incubated continuously with cisplatin or oxaliplatin and harvested at the designated time points. The control and treated cells were fixed in ethanol, stained with propidium iodide, and subjected to flow microfluorometry.

Measurement of Pt-DNA Adducts

Pt-DNA covalent adducts were quantitated by inductively coupled mass spectrometry as previously described (29). In brief, cells incubated in medium A lacking or containing 1 μg/mL doxycycline for 48 h were exposed to 40 μmol/L cisplatin for 2 h. After drug treatment, cells were washed twice with PBS and incubated in drug-free medium A containing or lacking 1 μg/mL doxycycline for 0 to 72 h. Following this incubation, cells were washed twice with ice-cold PBS and lysed in TEN buffer [10 mmol/L Tris-HCl (pH 7.4 at 21°C), 10 mmol/L EDTA, 150 mmol/L NaCl] supplemented with 0.4% SDS and 1 mg/mL proteinase K. As previously described (29), highly purified DNA was prepared by incubation of the lysates at 50°C for 16 h, followed

by DNA extraction with Tris-saturated phenol/chloroform (1:1), treatment with RNase A, reextraction with phenol/CHCl$_3$ and CHCl$_3$, and ethanol precipitation. The resulting DNA was resuspended in 750 μL 0.6 mol/L HCl and heated to 95°C for 30 min. The DNA concentration was estimated by measuring absorbance at 260 nm and elemental platinum was assayed by inductively coupled plasma mass spectrometry as described in detail (30).

Chk1 Phosphorylation

Protein expression was confirmed by immunoblotting using antibodies to phospho–Ser[317]-Chk1, phospho–Ser[345]-Chk1, and total Chk1 (Cell Signaling Technology). Cells were washed with PBS and solubilized for 30 min on ice in lysis buffer consisting of 20 mmol/L Tris-HCl (pH 8.0), 100 mmol/L NaCl, and 0.5% Triton X-100 containing protease and phosphatase inhibitors. Samples were sonicated and centrifuged at 12,000 × *g* for 30 min to pellet insolubles. Supernatant protein content was determined by the Bradford protein assay (31). After dilution with one third volume of 4× SDS-sample buffer, samples were heated at 65°C for 20 min, separated on a 12% SDS-PAGE gel, and electrophoretically transferred to nitrocellulose. Blotting was done as previously described (32) using peroxidase-coupled anti-rabbit secondary antibody and enhanced chemiluminescence detection.

Whole-Cell Lysis and Chromatin Fractionation

After incubation with cisplatin or oxaliplatin, each sample of cells was trypsinized, washed twice in PBS, and divided equally into two tubes. The first tube was used to prepare a whole-cell lysate as described above using 150 μL of lysis buffer and 50 μL of 4× SDS sample buffer. Chromatin fractionation was done on the second sample as described (33) with minor modification. Initially, ~3 × 10^6 GK41 cells were suspended in 150 μL of sucrose buffer [0.34 mol/L sucrose, 10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 3 mmol/L MgSO$_4$, 1 mmol/L DTT, 10% glycerol, and protease inhibitors] containing 0.15% (w/v) Triton X-100 and incubated on ice for 5 min. The sample was then centrifuged at 1,300 × *g* for 4 min at 4°C to pellet the nuclei. The nuclei were washed once with sucrose buffer, lysed in 200 μL nuclear extraction buffer [3 mmol/L EDTA, 0.2 mmol/L EGTA, 1 mmol/L DTT, 10 mmol/L HEPES (pH 7.5), and protease inhibitors], and incubated on ice for 10 min. Chromatin was then separated from soluble nuclear proteins by centrifugation at 1,700 × *g* for 4 min at 4°C. Chromatin was washed once with nuclear extraction buffer and centrifuged at 10,000 × *g* for 1 min. Chromatin was then resuspended in 200 μL of 1× SDS sample buffer and sheared by sonication. Samples were immunoblotted as described above using mouse monoclonal anti–replication protein A 32 (RPA32) antibody (AB-3, Calbiochem).

Results

DNA Cross-Linking Agents Differ in Sensitivity to ATR Inactivation

Previous studies have shown that doxycycline-induced expression of the kdATR allele exerts a dominant-negative effect on ATR signaling, with decreased DNA damage–induced ATR autophosphorylation and decreased total cellular ATR enzymatic activity when the mutant allele is overexpressed (21). To assess the effect of the kdATR allele on cisplatin action, GM847/kdATR cells and GK41 cells were incubated for 48 h in the absence or presence of doxycycline and then treated with cisplatin for 2 h. As indicated in Fig. 1A and B, exposure of GM847/kdATR and GK41 cells, respectively, to doxycycline enhanced cisplatin sensitivity, with the IC$_{50}$ or IC$_{90}$ values decreasing ~2-fold when the kdATR allele is overexpressed. These observations were consistent with previous studies examining longer cisplatin exposures (21, 26); however, the shorter exposure facilitated further biochemical analysis as described below. Control experiments showed that exposure of the parental GM847 cells to doxycycline alone had no effect on cisplatin sensitivity (data not shown), indicating that the doxycycline effect was due to overexpression of the mutant allele and not an independent drug effect.

Additional experiments examined the effect of the kdATR allele on the cytotoxicity of a variety of other agents known to induce DNA cross-links. We were particularly interested in the response to the related platinum drug oxaliplatin. Forced overexpression of kdATR sensitized GM847/kdATR cells to melphalan (Fig. 1C). In contrast, expression of the kdATR allele had little effect on the sensitivity of GM847/kdATR or GK41 cells to oxaliplatin (Fig. 1D and E, respectively). Similarly, no ATR-dependent difference was noted for survival after 4HC (Fig. 1F). These observations, which provide the first evidence that the effects of the kdATR allele vary depending on the nature of the DNA cross-links, suggest that ATR signaling may be an important mechanism affecting sensitivity to cisplatin but not oxaliplatin.

Colony-forming assays such as those used in Fig. 1 have been criticized because they do not distinguish between prolonged cell cycle arrest versus induction of cell death (34). To determine whether the enhanced antiproliferative effect of cisplatin observed in the presence of kdATR reflected increased cell death, cells were examined morphologically for evidence of apoptosis. Serial examination of cisplatin-treated cells revealed that 48 to 72 h after a 2-h treatment with 20 μmol/L cisplatin, a much higher percentage of cells expressing kdATR were apoptotic (Fig. 2). Thus, expression of the kdATR allele enhances cell death and does not merely cause prolonged arrest.

Removal of Pt-DNA Adducts Is Not Altered in Cells Expressing Kinase Dead ATR

Given the effect of ATR on survival after cisplatin damage, we next sought to assess the possibility that the enhanced killing in the kdATR-expressing cells arose from increased formation or diminished repair of Pt-DNA adducts. Examination of the formation and removal of Pt-DNA adducts in GK41 cells in the absence and presence of doxycycline showed that the initial adducts formed at the end of the 2-h cisplatin exposure and the adducts remaining 24 to 48 h after drug removal were indistinguishable in the absence and presence of doxycycline (Fig. 3). Similar results were observed in the GM847/kdATR cell line (data not shown).
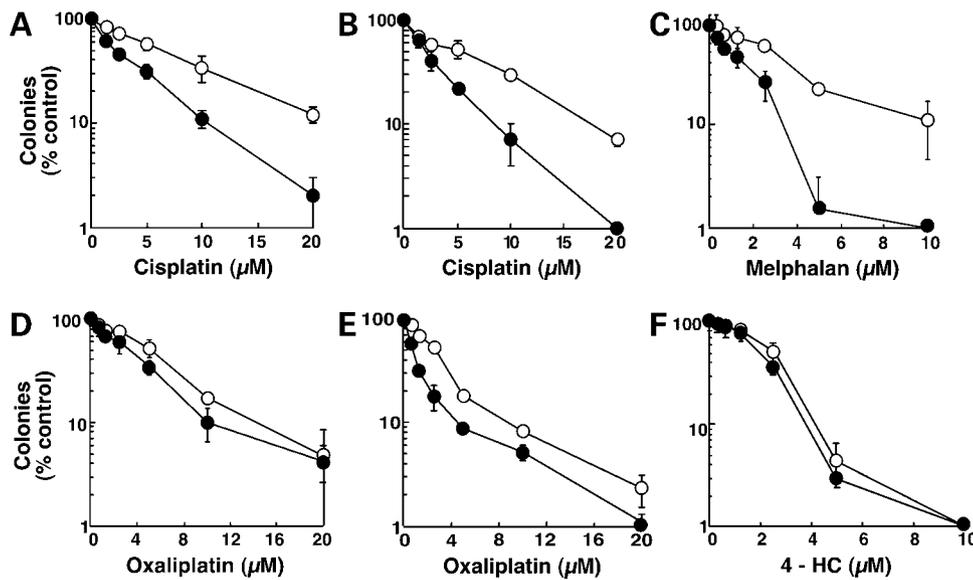
Figure 1. Effects of kdATR allele on sensitivity to various DNA cross-linking agents. GM847/kdATR cells (**A, C, D,** and **F**) and GK41 cells (**B** and **E**) grown in the absence (○) or presence (●) of doxycycline for 48 h were exposed to the indicated chemotherapeutic agent for 2 h (**A–C, F**) or 24 h (**D** and **E**), washed, and allowed to form colonies. *Error bars,* mean of triplicate plates. Results are representative of three independent experiments.

Cisplatin-Induced S-Phase Arrest is ATR Dependent

We previously showed that ATR kinase is necessary for S and G_2 arrests after ionizing radiation and topoisomerase poisons (21, 32). Although cisplatin-induced cross-links result in stalled replication forks as well as S- and G_2-phase arrests (34–36), the role of ATR in these cell cycle effects has been unclear. To assess this, we took advantage of the ability to synchronize GK41 cells just as they emerge from the G_1-S border by double thymidine block (26). After synchronization, cells were released into normal medium, exposed to 10 μmol/L cisplatin just as they emerged into the S phase, and harvested at the indicated time points for fluorescence-activated cell sorting analysis. In the absence of cisplatin, cells completed the S phase by 10 h after release from the thymidine block (Fig. 4A). After treatment with 10 μmol/L cisplatin, cells showed S-phase arrest at

10 h (59% of cells in the S phase versus 30% untreated; Fig. 4B). By 15 h, cells had progressed into late S-G_2. Importantly, this cisplatin-induced S-phase slowing was markedly diminished by expression of the *kdATR* allele (Fig. 4C), with only 38% of cells in the S-phase at 10 h (versus 30% in the control cells at 10 h). Control experiments showed that the addition of doxycycline had no independent effect upon S-phase progression in the absence of DNA damage. These results strongly suggest that arrest of DNA replication induced by cisplatin is ATR dependent.

ATR-Dependent Differences in Survival for Cisplatin and Oxaliplatin Reflect Differences in Cell Cycle Arrest

We were particularly interested in the differential survival between the two platinating agents (cisplatin and oxaliplatin) after inhibition of ATR signaling. To determine whether there was a difference in cell cycle response, we examined the effect
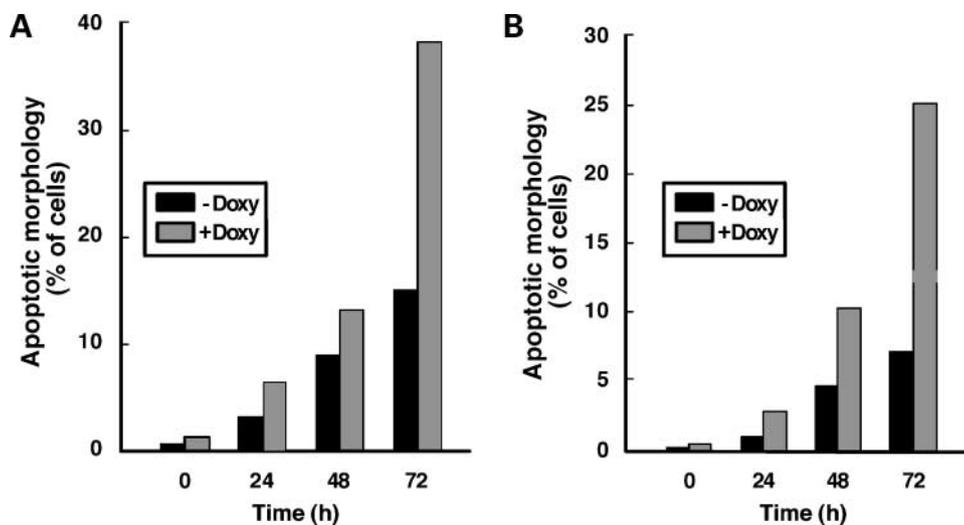

Figure 2. Effect of the kdATR allele on cisplatin-induced apoptosis. GM847/kdATR cells (**A**) or GK41 cells (**B**) were grown in the absence (−) or presence (+) of doxycycline for 48 h. Cells were then exposed to 20 μmol/L cisplatin for 2 h, washed, returned to complete medium with (+) or without (−) doxycycline, and harvested at the indicated time points for analysis. Apoptotic cells were quantitated as described in Materials and Methods.

of oxaliplatin on cell cycle progression of synchronized GK41 cells emerging from double thymidine block (Fig. 5). In contrast to 20 μmol/L cisplatin, which induced S-phase slowing just as 10 μmol/L did (data not shown; see Fig. 4B), 20 μmol/L oxaliplatin failed to result in a strong S-phase arrest (Fig. 5B). By 10 h, the peak of oxaliplatin-exposed cells reached the G_2 boundary; importantly, no difference was noted in the presence or absence of doxycycline (Fig. 5B versus C). Increasing oxaliplatin to 40 μmol/L also failed to produce an S-phase arrest (Fig. 5D and E). Similar differences between cisplatin and oxaliplatin were observed when bromodeoxyuridine-labeled GM847/kdATR were exposed to cisplatin versus oxaliplatin (data not shown). These results indicate that, in contrast to the S-phase arrest observed after treatment with cisplatin, the cell cycle effects of oxaliplatin are largely independent of ATR and occur at later points in the cell cycle.

ATR Dependence of Chk1 Phosphorylation After Cisplatin versus Oxaliplatin

The preceding differences in cell cycle response prompted us to compare Chk1 phosphorylation as a readout of ATR activation after cisplatin versus oxaliplatin exposure. As indicated in Fig. 6A, we observed an early (4 h) and sustained (8 h) phosphorylation of Chk1 Ser[345] after exposure to cisplatin (Fig. 6A, *lanes 5* and *7*). Much of this cisplatin-induced Chk1 phosphorylation was dependent on ATR kinase activity, as indicated by the fact that it was diminished in the presence of kdATR (Fig. 6A, *lanes 6* and *8*), preferably at 8 h. This is entirely consistent with the cell cycle responses noted in Fig. 4. In contrast, less Chk1 Ser[345] phosphorylation was induced by oxaliplatin (cf. *lanes 15* and *17* versus *5* and *7*) and the phosphorylation was less dependent on ATR activity, particularly at late time points (cf. *lanes 17* and *18* versus *7* and *8*). Blotting for Chk1 Ser[317] phosphorylation (Fig. 6A, *second panel*) revealed a similar pattern, with cisplatin-induced phosphorylation that occurred earlier (1 h, *lane 3*), was more intense and more sustained (cf. *lanes 5* and *7* versus *15* and *17*), and more clearly dependent on ATR activity, particularly at the late time points (cf. *lanes 7* and *8*). Collectively, these results indicate important differences in ATR activation after cisplatin versus oxaliplatin treatment that result in differences in Chk1 activation (Fig. 6A), cell cycle responses (Figs. 4 and 5), and survival (Fig. 1).

Binding of RPA, which has a high affinity for ssDNA after specific forms of DNA damage, to chromatin is required for the recruitment of several DNA damage checkpoint proteins, including ATR (35, 36). We examined RPA chromatin binding after exposure of GK41 cells to IC$_{90}$ concentrations of oxaliplatin and cisplatin and did not observe ATR kinase-dependent differences (Fig. 6B). Extensive phosphorylation of the chromatin-associated RPA was observed after cisplatin but not oxaliplatin by the 24-h time point. However, RPA phosphorylation, like chromatin loading, was independent of ATR kinase activity (Fig. 6B).

Discussion

Results of the present study show that ATR kinase activity is important in survival after exposure to cisplatin or mel-
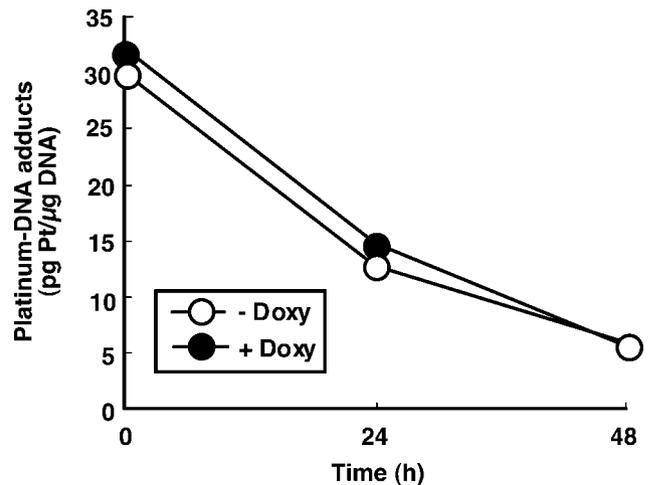

Figure 3. Removal of Pt-DNA adducts is not dependent on ATR kinase activity. GK41 cells grown in the absence (−) or presence (+) of doxycyline for 48 h were exposed to 40 μmol/L cisplatin for 2 h. After exposure, cells were either harvested immediately (time 0) or washed and returned to complete medium without (−) or with (+) doxycycline (*doxy*) for analysis at 24 and 48 h. At each time point, DNA was purified and analyzed for covalently bound platinum by inductively coupled mass spectrometry. Results shown are representative of three independent experiments.

phalan, but only minimally important after exposure to oxaliplatin or 4HC. Further analysis showed that ATR kinase function does not affect formation or removal of platinum-DNA adducts. Instead, differences in the ability of cisplatin versus oxaliplatin to activate ATR and its downstream kinase Chk1 were observed. These findings have potential implications not only for current understanding of the action of several widely used anticancer drugs, but also for further development and use of ATR and Chk1 inhibitors.

Most of the attention to cell cycle effects of cisplatin has focused on the G_1 and G_2 arrests induced by cisplatin (37–39). Although Lee et al. reported a prominent cisplatin-induced S-phase arrest in human breast cancer cell lines that could be abrogated by the kinase inhibitor UCN-01 (40), which inhibits Chk1, Tac1, and other kinases, it has been unclear which of the upstream DNA damage–activated kinases contributes to this arrest. The present study shows that ATR plays a critical role in this S-phase arrest (Fig. 4), a result that mirrors the recent observation that a *S. cerevisiae* mutant lacking the ATR-homologue mec1 exhibits diminished S-phase arrest after high-dose cisplatin (41). Our demonstration that ATR plays a critical role in cisplatin-induced S-phase slowing is consistent with the known checkpoint function of ATR (42) and adds to the growing evidence that ATR-dependent pathways contribute to survival after cells encounter various cytotoxic agents during the S phase.

As indicated in Figs. 1 and 2, ATR not only plays a role in cisplatin-induced S-phase slowing, but also in long-term survival of cisplatin-treated cells. One potential mechanism linking ATR and Chk1 activation with cisplatin sensitivity is HRR. Whereas most previous studies of cisplatin
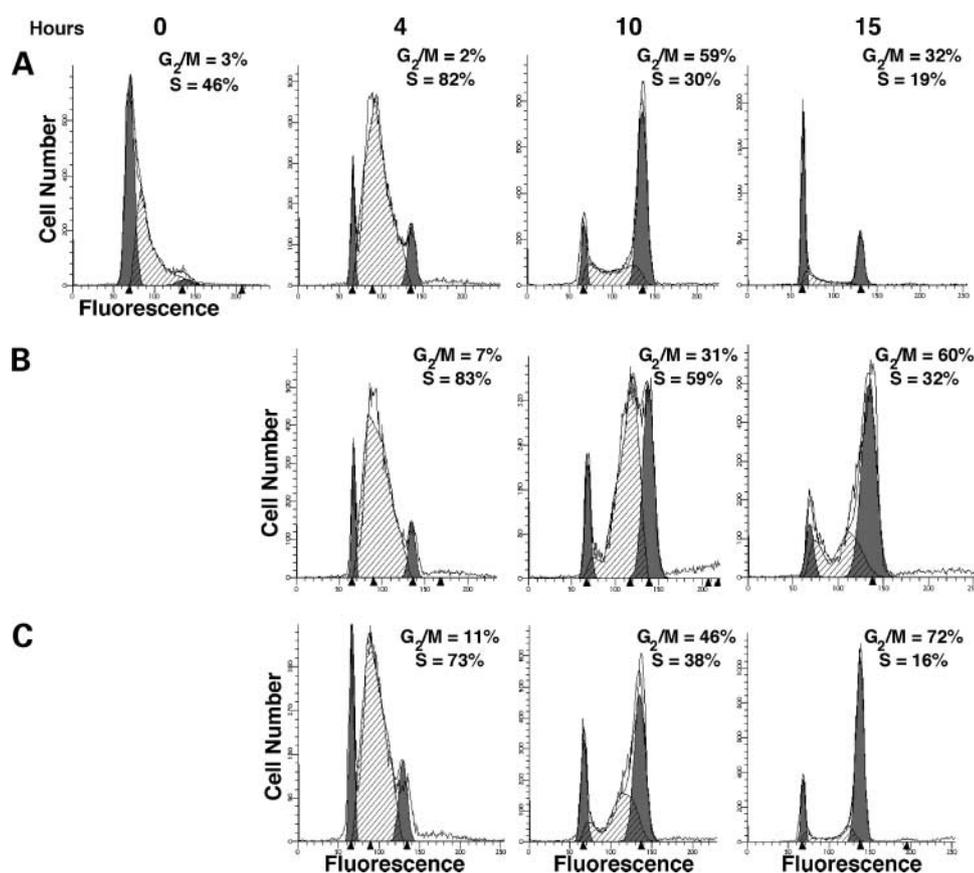
Figure 4. S-phase arrest after cisplatin is dependent on ATR kinase activity. GK41 cells were grown in the absence (**A** and **B**) or presence (**C**) of doxycycline for 48 h and synchronized at the G_1-S border by double thymidine block as described in Materials and Methods. At time 0 h, cells were harvested for flow cytometry or released from block into medium containing 10 μmol/L cisplatin (**B** and **C**) or diluent alone (**A**) for harvest at the indicated time points. Control experiments had determined that the addition of doxycycline had no independent effect on cell cycle progression after thymidine blockade (not shown). Results shown are representative of three different experiments.

sensitivity have focused on nucleotide excision repair, mismatch repair, and translesion DNA synthesis as potential mechanisms of circumventing cisplatin-induced cytotoxicity, a small number of recent studies have suggested that HRR also can affect cisplatin sensitivity (23, 24). Moreover, Wang et al. (43) have reported that HRR is dependent on ATR kinase activity and Sorensen et al. (19) observed that Chk1 plays a critical role in the phosphorylation and trafficking of the HRR protein RAD51 after hydroxyurea. Collectively, these observations suggest a model in which cisplatin induces sequential activation of ATR and Chk1, resulting in Rad51 phosphorylation and activation of HRR, which plays a critical role in cisplatin sensitivity. On the other hand, our results (Fig. 3) provide no evidence that ATR affects removal of Pt-DNA adducts. It is possible, of course, that selected subsets of adducts are preferentially affected by HRR and are sensitive to ATR activity. Alternatively, it is also possible that the effect of inhibiting ATR (Figs. 1, 2, and 4) or Chk1 (40) reflects the altered phosphorylation of another Chk1 substrate. Further investigation is required to distinguish between these possibilities.

Our findings of Chk1 activation after cisplatin differ from those reported by Wilsker and Bunz (25). They observed only modest phosphorylation of Chk1 Ser[317] with no significant phosphorylation on Ser[345] after cisplatin. However, there are important differences in experimental design. In that study, the concentration of cisplatin was lower and prolonged (0.6 μmol/L for 48 h versus 20 μmol/L for 2 h). Additionally, the authors used the colon cancer cell line DLD1, transfected to express a Seckel ATR mutant. The DLD1 cell line is DNA mismatch repair–deficient cell line. Given that both ATR and Chk1 are known to contain microsatellite repeats within their coding region that result in inactivating mutations, the functional integrity of the ATR and Chk1 proteins is unclear and mismatch repair seems important in the response to platinum damage (reviewed in ref. 44). In contrast, our results suggest a direct role for ATR kinase in phosphorylation of Chk1 Ser[345] after cisplatin damage (Fig. 6, *lanes 5–8*).

Expression of the kdATR allele sensitizes cells to cisplatin much more than oxaliplatin (Fig. 1), a closely related agent that also induces predominantly intrastrand cross-links. This difference in the ability of kdATR to affect sensitivity to these two agents seems to reflect the more robust and sustained activation of ATR by cisplatin versus oxaliplatin (Fig. 6A). A number of possible explanations might account for this difference. First, it has been reported that oxaliplatin forms about 2-fold fewer Pt-DNA adducts than cisplatin at equimolar concentrations (45). Because the differences in cell cycle response were also seen when the oxaliplatin concentration was 2- and 4-fold higher than cisplatin (cf. Figs. 4 and 5), it is unlikely that the observed differences in ATR/Chk1 pathway activation result solely from the number of

lesions formed. Instead, differences in the relative rates of translesion DNA synthesis past the oxaliplatin versus cisplatin adducts (12) might help determine whether ATR plays an important role. Whereas ATR is recruited to sites of ssDNA during the S phase in a RPA-dependent manner (36, 46), it is possible that recruitment of DNA polymerase β, which is particularly efficient in replicating through oxaliplatin adducts, is ATR independent and that translesion synthesis by this polymerase abrogates the need for ATR by allowing resolution of stalled replication forks and restoration of DNA integrity more effectively after oxaliplatin than after cisplatin. Although similar information is not available for melphalan versus 4HC adducts, a difference in the ability of translesion polymerases to replicate through these lesions might explain the varied effects of kdATR on survival after these agents (Fig. 1) as well.

During the course of the present studies, we also observed that cisplatin induced greater binding of RPA to chromatin and greater phosphorylation of the chromatin-bound RPA than oxaliplatin did (Fig. 6B). These differences, which were particularly pronounced at 24 h, might reflect the more persistent stalling of replication forks in
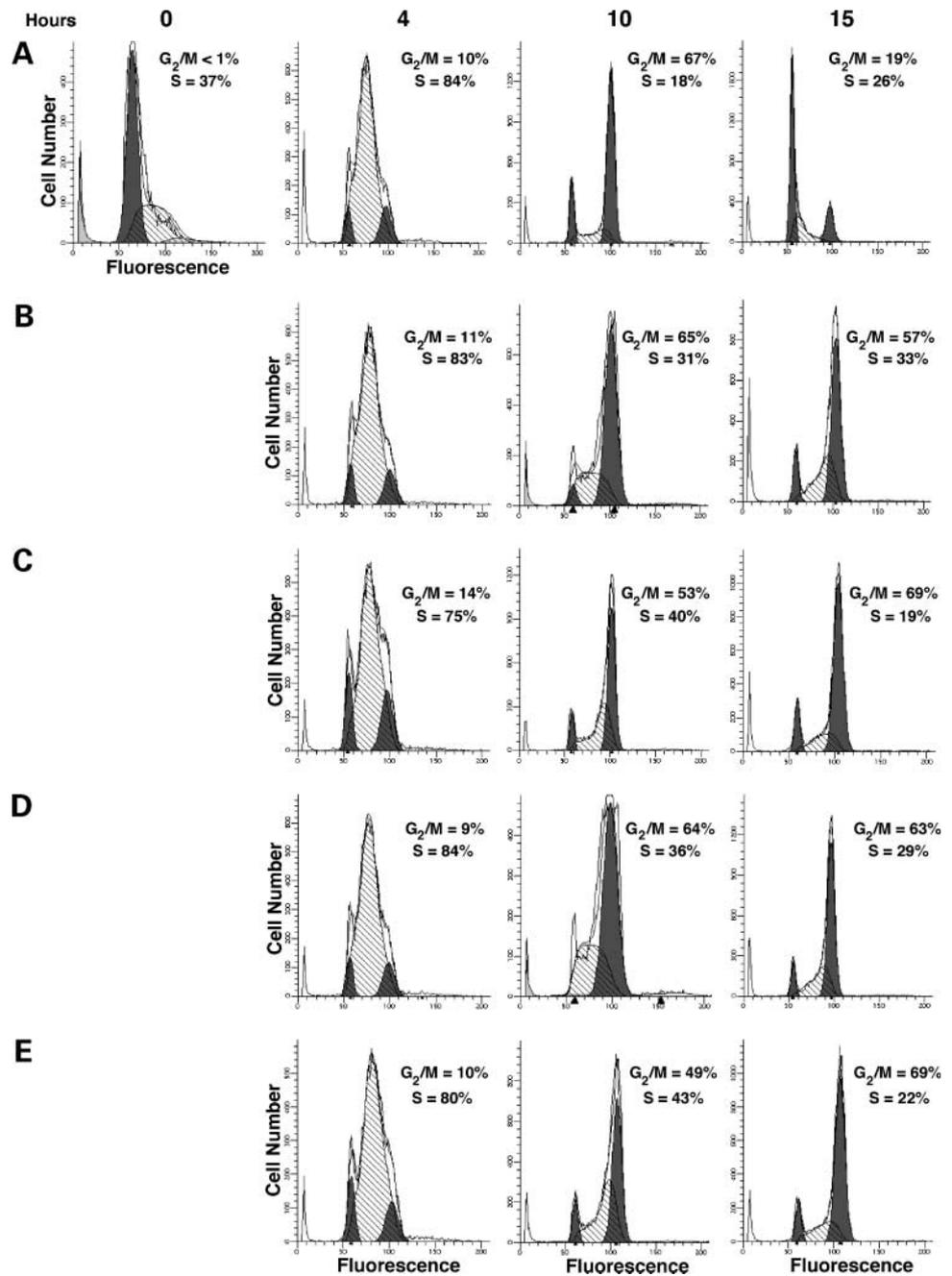

Figure 5. Cell cycle arrest after oxaliplatin occurs predominantly at the late S-G$_2$ boundary and is minimally dependent on ATR kinase activity. GK41 cells were grown in the absence (**A, B**, and **D**) or presence (**C** and **E**) of doxycycline for 48 h and synchronized at the G$_1$-S border by double thymidine block as described in Materials and Methods. At time 0 h, cells were harvested for flow cytometry or released from block into medium containing diluent alone (**A**), 20 μmol/L oxaliplatin (**B** and **C**), or 40 μmol/L oxaliplatin (**D** and **E**) for harvest at the indicated time points. Results shown are representative of three different experiments.
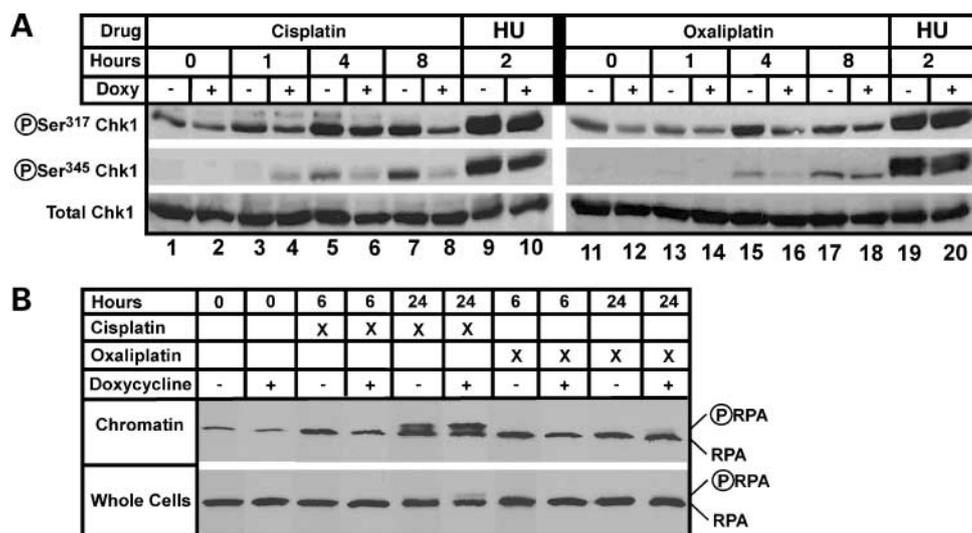
Figure 6. ATR-dependent Chk1 activation after platinum exposure is agent specific. **A,** GK41 cells were grown in the absence or presence of doxycycline for 48 h followed by continuous treatment with 20 μmol/L cisplatin, 20 μmol/L oxaliplatin, or 10 mmol/L hydroxyurea for the designated times. After cell lysates were prepared, phospho–Ser^{317}-Chk1 and phospho–Ser^{345}-Chk1 levels were compared using blots that were incubated with antibody and simultaneously exposed to the same piece of X-ray film. Total Chk1 served as a loading control. **B,** GK41 cells grown in the absence or presence of doxycycline for 48 h were treated with 20 μmol/L cisplatin or oxaliplatin for the indicated length of time. Whole-cell lysates were prepared from one aliquot of cells, and chromatin fractions were prepared from a second aliquot as described in Materials and methods. After the samples were subjected to SDS-PAGE and probed with anti-RPA32, nonadjacent lanes from a single X-ray exposure were rearranged to create this figure.

the face of cisplatin adducts compared with oxaliplatin. Moreover, the increased chromatin loading and phosphorylation of RPA observed 24 h after cisplatin were not affected by expression of kdATR (Fig. 6B, +*doxy*), suggesting that active signaling by ATR/Chk1 pathway does not contribute to the enhanced binding and phosphorylation of RPA. These results are consistent with current models suggesting that RPA recruitment to damaged chromatin occurs upstream of ATR (36, 47) rather than downstream. On the other hand, we cannot at present rule out the possibility that the difference in the amount of RPA loaded onto chromatin at 24 h reflects some other aspect of the difference in cell cycle response to these two agents.

Whatever the explanation for the varied ability of the kdATR allele to modulate the activity of different DNA cross-linking agents, the present observation that ATR plays an important role in cellular survival after treatment with cisplatin and melphalan but not oxaliplatin or 4HC has several potentially important implications. First, the present studies identify cisplatin and melphalan as two additional agents that can potentially be modulated by ATR and Chk1 inhibitors. Based on these results, preclinical study of cisplatin and melphalan with Chk1 or ATR inhibitors, which are currently in preclinical and early clinical development, seems warranted. Second, the present results suggest that the actions of other DNA cross-linking agents might not be universally modulated by ATR or Chk1 inhibitors. Accordingly, combinations of Chk1 or ATR inhibitors with other DNA cross-linking agents (typified by oxaliplatin or 4HC) will have to be examined on a case-by-case basis to determine whether the kinase inhibitor is able to modulate

the effects of the cross-linking agent. Third, the recent demonstration of ATR mutations that can potentially inhibit ATR action in colon, endometrial, and stomach cancers (48–50), coupled with the present results showing that cells defective for ATR-Chk1 signaling are more sensitive to the effects of cisplatin and melphalan, might make it reasonable to consider testing cisplatin or melphalan against cancers containing alterations in the ATR/Chk1 pathway.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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