Cytokine Activation of Phosphoinositide 3-Kinase Sensitizes Hematopoietic Cells to Cisplatin-induced Death

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ABSTRACT

Cytokine growth factors regulate the normal proliferation of hematopoietic cells but can also override irradiation-induced growth arrest checkpoints through activation of a phosphoinositide 3-kinase (PI3K) signaling pathway. In the present study, we assessed the effect that erythropoietin and interleukin-3 have on cisplatin-treated hematopoietic cells.

When cultured in the presence of cytokine, cisplatin-treated 32D cells transiently accumulated in a G2-M phase arrest and ultimately died by a nonapoptotic mechanism. By comparison, reduction of cytokine-induced PI3K activity, either through cytokine receptor mutation or direct inhibition with LY294002, caused cisplatin-treated cells to enter a biphasic G1 and G2-M arrest. The arrest of these cells coincided with an absence of cyclin-dependent kinase (Cdk)1 and Cdk2 activity and significantly reduced cell death during cisplatin treatment. Indeed, LY294002 treatment during cisplatin exposure allowed the recovery of a viable, proliferating cell population after removal of cisplatin. In contrast, Cdns remained active in the G2-M-arrested population of cisplatin-treated cells with continuous cytokine activation of PI3K, and even transient exposure to cisplatin resulted in death of the entire population. These data suggest that cytokine activation of PI3K signaling pathways overrides cisplatin-induced growth arrest checkpoints, thereby sensitizing hematopoietic cells to DNA damage-induced death.

INTRODUCTION

Platinum-based drugs, such as cisplatin, are an important class of chemotherapeutic agents that are widely used to treat many types of human malignancies (1). Cisplatin cytotoxicity involves the formation of intra and interstrand DNA adducts, and the resulting DNA damage triggers cell cycle arrest, apoptosis, and/or necrotic cell death (2, 3).

Myelosuppression and anemia are common side effects of anticancer chemotherapy, including the use of cisplatin (4). Mammalian hematopoietic cells are absolutely dependent on cytokine growth factors for their survival and proliferation, and several of these growth factors have been successfully used in treatment of chemotherapy-induced hematopoietic anemias, e.g., Epo (5) is a principle regulator of erythroid cell development and can be used to reconstitute this compartment after chemotherapy-induced anemia (5). However, the effects that Epo or other cytokine growth factors have on hematopoietic cell responses to most chemotherapy agents are not well defined.

Many cytokine growth factors act through cell surface receptors of the Type I and II cytokine receptor family. This family of receptors generally functions through activation of Jak tyrosine kinases and subsequent activation of common signal transduction cascades, including Stat transcription factors, the Ras pathway, and PI3K pathways (6). Of these activities, only Jak kinase activation has been shown to be absolutely required for mitogenic signaling by cytokine receptors, e.g., the EpoR normally associates with and activates Jak2, and all biological activities of Epo are lost in the absence of this interaction (7–9). By contrast, EpoR mutants lacking activation domains for Stat5, Ras pathway, or PI3K pathways retain the ability to support survival and proliferation of erythroid cells in vitro and in vivo (10, 11). However, the PI3K signaling pathway is required to override DNA damage-induced growth arrest of hematopoietic cells (12).

Hematopoietic cells cultured in the absence of cytokine rapidly arrest in G1 phase of the cell cycle and die via apoptosis. By comparison, DNA damage induced by γ-IR results in a biphasic G1 and G2-M growth arrest and apoptotic cell death (13). The apoptotic response to γ-IR is p53 dependent but can be overridden by cytokine-induced expression of antiapoptotic proteins, such as Bcl-2 and Bcl-XL (13–15). By contrast, the γ-IR-induced growth arrest is p53 independent because this arrest occurs in hematopoietic cells that lack functional p53 protein (13, 16). Nonetheless, this growth arrest is efficiently overridden by cytokine treatment of γ-irradiated cells, and these populations continued to proliferate indefinitely without any apparent loss in viability. The G1 phase arrest of γ-irradiated hematopoietic cells was shown to result from an absence of active cyclin E/Cdk2 (17), a kinase complex that normally regulates progression through late G1 phase (18). The absence of cyclin E/Cdk2 activity correlated with a lack of phosphorylation at the activation site (threonine-160) of Cdk2 (17). Through activation of a PI3K/Akt-dependent signaling pathway, cytokines promote the phosphorylation of Cdk2 at threonine-160 and maintain cell cycle progression in the face of γ-IR-induced DNA damage.

In the present study, we assessed the effects of cytokine growth factors on the response to cisplatin treatment in murine, myeloid 32D cells. Treatment with Epo or IL-3 overrode cisplatin-induced growth arrests, but this effect resulted in increased necrotic death. Thus, by overriding DNA damage-induced cell cycle checkpoints, cytokines sensitize hematopoietic cells to cisplatin-induced death. Notably, inhibition of PI3K during cisplatin treatment limited cell death and enabled the recovery of viable proliferating populations on the removal of cisplatin.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. 32D cell clones stably expressing wild-type EpoR, truncated EpoR (EpoR[H3]), or Bcl-XL have been described (17, 19). All cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum plus either recombinant human Epo (5 units/ml) or recombinant murine IL-3 (1.4 ng/ml). In certain experiments, the PI3K inhibitor LY294002 (Calbiochem) was added to cell culture medium to a final concentration of 10 μM.

Analysis of Cisplatin and γ-IR Effects. Cells were collected, washed in PBS, and resuspended (5 × 10^6 cells/ml) in RPMI 1640 containing 10% fetal bovine serum and either IL-3 (1.4 ng/ml), Epo (5 units/ml), or no cytokine. All cultures were incubated at 37°C and 5% CO₂ until parallel cultures were treated with 5 μM cisplatin (Sigma), 4 Gy γ-IR from a 137Cs source, or left untreated. Treated cultures were returned to a 37°C, 5% CO₂ incubator for indicated times before analysis. Cell viability was assessed by exclusion of Trypan Blue (Life Technologies, Inc.).
For cell cycle analysis, cells were collected by centrifugation, resuspended at 1 x 10^6 cells/ml in PI staining buffer (0.1% sodium citrate, 0.1% Triton X-100, and 50 μg/ml P), and treated with 1 μg/ml RNase for 30 min at room temperature. Cell cycle histograms were generated from analysis of PI-stained cells by flow cytometry on a Becton Dickinson FACScan. For each culture, at least 1 x 10^6 events were recorded. The percentage of cells in each phase of the cell cycle was determined by analysis with ModFit Cell Cycle Analysis Software (Verity).

Analysis of apoptotic versus necrotic cell death was assayed by staining with PI and FITC-annexin V using the ApoAlert Annexin V kit (Clontech) according to the manufacturer’s instructions.

Cdk Kinase Assays. Cells were washed with PBS and lysed in Tween20 buffer [50 mM HEPES (pH 7.3), 150 mM NaCl, 1 mM EDTA, 0.1% Tween 20, 1 mM NaF, 10 mM β-glycerophosphate, and 0.1 mM Na3VO4] at 4°C. Insoluble material was removed by centrifugation, and specific proteins were precipitated with 2 μg of antiserum for Cdk1, Cdk2, or Cdk4 (Santa Cruz Biotechnology). Immune complexes were washed twice with Tween 20 buffer and twice with Cdk buffer [50 mM HEPES (pH 8.0) and 10 mM MgCl₂]. For each reaction, 10 μg of histone H1 (Roche) or recombinant Rb (Santa Cruz Biotechnology) were added. Reactions were initiated by the addition of 0.1 pmol ATP plus 10 μCi [γ-32P]ATP (ICN) and incubated at 30°C for 30 min. Reaction products were resolved by SDS-PAGE and visualized on a PhosphorImager (Molecular Dynamics).

Akt Kinase Assay. To assess cytokine-induced Akt activity in various culture conditions, cells were maintained in the absence of cytokine for 6 h and then stimulated for 10 min with the appropriate cytokine. Kinase assays were performed in vitro using an Akt Kinase Assay Kit (New England Biolabs), as described previously (12). To confirm specific effects on activation of the PI3K/Akt signaling pathway, total cell lysates were separated by SDS-PAGE and Western blotted with antiphospho-Akt antibodies (Santa Cruz Biotechnology).

RESULTS

To characterize the effects of cisplatin-induced DNA damage in hematopoietic cells, murine myeloid 32D cells expressing wild-type EpoR (EpoR[wt]) or truncated EpoR (EpoR[H]) were examined by flow cytometry. The truncated EpoR[H] lacks a PI3K recruitment site and is unable to override γ-IR-induced growth arrest, but EpoR[H] retains the ability to mediate Epo-induced proliferation in the absence of DNA damage (12, 13). As shown in Fig. 1, treatment of 32D-EpoR[H] cells with cisplatin plus Epo resulted in a biphasic G1 and G2-M arrest, whereas similar treatment of 32D-EpoR[wt] cells induced an arrest with significantly less G1 content. Thus, activation of EpoR[wt] reduced cisplatin-induced G1 phase arrest but did not prevent accumulation of cells in G2-M. By comparison, 32D-EpoR[wt] cells retained an asynchronous cell cycle profile when cultured in Epo after γ-IR, whereas 32D-EpoR[H] cells arrested in G1 and G2-M phases (Fig. 1A). The effect of IL-3 treatment was also assessed as a control for the activity of a wild-type cytokine receptor. All 32D cell lines cultured in IL-3 remained asynchronous after γ-IR but arrested in G2-M phases when treated with cisplatin. Notably, the viability of cisplatin-treated cultures arrested in G2-M phase (IL-3- or Epo-treated 32D-EpoR[wt] cells; Fig. 1B) was significantly less than those arrested at both G1 and G2-M phases (Epo-treated 32D-EpoR[H] cells; Fig. 1B). No viable cells were detected in cultures treated with cisplatin in the absence of cytokine (data not shown). All γ-irradiated cultures remained >95% viable when cultured in either Epo or IL-3.

To assess the kinetics of the response to cisplatin treatment, 32D-EpoR[H] cells were cultured in Epo or IL-3 and assayed by flow cytometry at various times after initiation of cisplatin treatment. As shown in Fig. 2A, IL-3-treated cultures progressed to a G1-M phase arrest by 48 h of cisplatin treatment, whereas Epo-treated cells retained a significant G1 population. However, Epo-treated cells eventually accumulated in G2-M phase at later time points. Epo-treated cells were reproducibly more viable than those treated with IL-3; however, both cultures were <20% viable after 120 h of continuous treatment (Fig. 2B). 32D-EpoR[wt] cells cultured in either IL-3 or Epo exhibited the same response to cisplatin treatment as shown for 32D-EpoR[H] cells cultured in IL-3 (data not shown).

DNA damage-induced apoptosis in hematopoietic cells is inhibited substantially by forcible expression of the antiapoptotic protein Bcl-XL (13). Indeed, 32D-EpoR[H] cells stably expressing Bcl-XL were somewhat more viable after cisplatin treatment than were the parental 32D-EpoR[H] cells. However, 32D-EpoR[H]/Bcl-XL cells also suffered significant losses in viability when treated with cisplatin and were reduced to <20% viability by 120 h of treatment when cultured in either Epo or IL-3 (Fig. 2B). As with parental cells, 32D-EpoR[H]/Bcl-XL cells cultured in Epo were more viable (Fig. 2B) and retained a larger G1 population (Fig. 2A) throughout cisplatin treatment than did cells cultured in IL-3, although cells in both cultures ultimately died.

Because Bcl-XL expression provided only modest protection from cisplatin-induced cell death, we tested whether death was caused by apoptosis or necrosis by staining cell preparations with PI and Annexin V. As shown in Fig. 3, 32D cells develop an apoptotic population when withdrawn from cytokine (no factor) as demon-

Fig. 1. Truncated EpoR less efficiently overrides cisplatin-induced G1 arrests. 32D cells stably expressing wild-type (EpoR[wt]) or truncated (EpoR[H]) EpoRs were cultured in Epo (5 units/ml) or IL-3 (1.4 ng/ml). Parallel cultures were supplemented with cisplatin (10 μM) exposed to 4 Gy γ-IR, or left untreated (no treatment). All cultures were maintained for 55 h, after which, cells were harvested, and DNA content was analyzed by flow cytometry (A). The percentage of viable cells in each phase of the cell cycle (G1-S-G2-M) is indicated. Before harvesting, cell viability was determined by trypan blue exclusion. In B, the percentage of viability and G1 phase distribution of cisplatin-treated cells were averaged from at least five separate experiments. Error bars represent the SE. The significance of differences between Epo and IL-3-treated 32D-EpoR-H cultures is indicated (P<0.018).

Both cultures were <20% viable after 120 h of continuous treatment (Fig. 2B). 32D-EpoR[wt] cells cultured in either IL-3 or Epo exhibited the same response to cisplatin treatment as shown for 32D-EpoR[H] cells cultured in IL-3 (data not shown).

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stratified by the presence of an Annexin-high, PI-low population (bottom right quadrant). No cell death was apparent in 32D-Bcl-XL cells withdrawn from cytokine. By contrast, continuous cisplatin treatment of either 32D or 32D-Bcl-XL cells cultured in IL-3 resulted in the development of PI- and Annexin-high populations (top right quadrant) consistent with necrotic breakdown of the plasma membrane. Results are shown for cells treated with IL-3 and cisplatin because these conditions induce the most rapid cell death. Similar results were obtained with Epo-treated 32D-EpoR[H]/Bcl-XL cells, although the onset of necrotic death was delayed compared with cultures in IL-3 (data not shown).

The difference in viability associated with the biphasic arrest versus G2-M arrest suggested that the ability of EpoR[wt], but not by EpoR[H], to override cell cycle checkpoints might contribute to cisplatin-induced cell death. In hematopoietic cells, the ability of cytokines to override a γIR-induced G1 cell cycle checkpoint is dependent on activation of a PI3K signaling pathway and subsequent regulation of Cdk2 catalytic activity (17). To assess whether a similar regulatory activity is exhibited by cytokines in cisplatin-treated 32D cells, Cdk1 and Cdk2 immune-complex kinase assays were performed on lysates of 32D-EpoR[H] cells cultured in Epo or IL-3 during 48 h of cisplatin treatment (Fig. 4, A and B). Both Cdk1 and Cdk2 catalytic activity were modestly reduced in cisplatin-treated cells cultured in Epo relative to cells not treated with cisplatin (50 and 80% reductions, respectively). By contrast, cisplatin treatment had little effect on Cdk1 or Cdk2 activity in cells cultured in IL-3. Thus, the delayed progression toward G2-M phase arrest and enhanced viability of Epo-treated 32D-EpoR[H] cells after cisplatin treatment correlated with reduced but not abrogated activities of both Cdk1 and Cdk2.

Previously, we had observed that inhibition of all cellular PI3K activity was more effective than receptor truncations at preventing cytokine-dependent suppression of cell cycle checkpoints (12, 17). Therefore, the ability of the PI3K inhibitor, LY294002, to alter the cell cycle profile and Cdk activity in 32D-EpoR[wt] cells after 50 h of cisplatin treatment was assessed. LY294002 has little effect on Cdk activity or cell cycle distribution in 32D cells in the absence of DNA-damaging agents (12, 17). However, LY294002-treated 32D-EpoR[wt] cells exhibited a biphasic G1 and G2-M arrest in cisplatin as compared with the largely G2-M arrest induced in cultures containing Epo or IL-3 only (Fig. 4C). The biphasic arrest of cells treated with cisplatin plus LY294002 was associated with a near absence of both Cdk1 and Cdk2 activity (Fig. 4D). These cells exhibited a reduced G2-M population compared with those treated with cisplatin alone, which may contribute to reductions in Cdk1 and Cdk2 activity within the total population. However, the near absence of Cdk1 and Cdk2 activity in cells treated with LY294002 plus cisplatin suggests that these Cdk1 are inactive in the G2-M-arrested population. By comparison, cells treated with cisplatin alone retained high levels of Cdk1 and Cdk2 activity that were comparable with cells actively growing in the absence of cisplatin. Cdk4 activity was not altered by the presence or absence of LY294002 and/or cisplatin. The specific inhibition of PI3K-dependent signaling pathways in LY294002-treated cells was confirmed by the significant reduction in cytokine-induced Akt activity, whereas cytokine-induced phosphorylation of Erk was not affected (Fig. 4E).

The more efficient G1 and G2-M phase arrest of LY294002-treated 32D cells during cisplatin treatment would be predicted to enhance viability if cisplatin-induced death of 32D cells is a product of inappropriate cell cycle progression in the face of unrepaired DNA damage. Consistent with this hypothesis, 32D-EpoR[wt] cells treated with Epo and LY294002 stabilized in a biphasic G1 and G2-M phase arrest by 48 h of cisplatin treatment (Fig. 5A) and maintained a high level of viability through 120 h of continuous cisplatin treatment (Fig. 5B). By comparison, parallel cultures lacking LY294002 progressed to a largely G2-M population by 48 h of cisplatin treatment and lacked significant viability after 72 h.

To determine whether the enhanced viability of LY294002-treated cells preserves their ability to proliferate after exposure to cisplatin, survival and cell cycle distribution were assayed after removal of cisplatin from the culture medium. 32D-EpoR[wt] cells that had begun to accumulate in G2-M phase after 24-h treatment with Epo and cisplatin (Fig. 5A) were washed out of cisplatin and returned to medium containing Epo alone (Fig. 5C). Cells in this culture continued to accumulate in G2-M phase after removal of cisplatin, similar to cells continuously treated with cisplatin. By 48-h viability was re-

Fig. 2. Truncated Epo enhances the G1 and G2-M arrest and viability of cisplatin-treated 32D cells. 32D-EpoR-H cells, or a derivative stably expressing Bcl-XL, were cultured in Epo (5 units/ml) or IL-3 (1.4 ng/ml). Cisplatin (5 μM) was added to each culture. In A, at various times after the addition of cisplatin, samples of each culture were assayed for DNA content by flow cytometry. The percentage of viable cells in each phase of the cell cycle (G1-S-G2-M) is indicated for each culture with asterisk (*). Error bars represent SE. Significant differences (P < 0.04) in viability between Epo- and IL-3-treated cultures are indicated by an asterisk (*).

The difference in viability associated with the biphasic arrest versus
Cytokine treatment of hematopoietic cells inherently provides significant protection against DNA damage-induced apoptosis because both bcl-2 and bcl-X{	extsubscript{L}} are immediate early gene targets induced by activated cytokine receptors (13, 22). As a consequence, additional antiapoptotic protection achieved by enforced expression of Bcl-X{	extsubscript{L}} provided only a modest increase in the viability of cisplatin-treated 32D cells. The lack of protection by Bcl-X{	extsubscript{L}} overexpression was also consistent with the observation that cisplatin primarily induced necrotic death of cytokine-treated 32D cells. Notably, this cisplatin-induced death was significantly enhanced by activation of PI3K and lack of a biphasic G{	extsubscript{1}} and G{	extsubscript{2}}-M phase growth arrest.

Sensitization to DNA damage-induced death has been observed in cells deficient in p53 pathways, e.g., ovarian carcinoma cells made deficient in p53 protein loose G{	extsubscript{1}} checkpoint control, accumulate in G{	extsubscript{2}}-M phase, and die via necrosis after cisplatin treatment (23). This outcome is essentially identical to the result of PI3K activation in cisplatin-treated 32D cells. Likewise, cells deficient in p53 or its transcriptional target, p21, can bypass arrest at G{	extsubscript{1}} phase after γ-IR and die through a failure of cytokinesis (24, 25). Thus, a mitotic catastrophe resulting from attempted cell division in the face of unrepaired DNA damage represents a common consequence of failed growth arrest checkpoints.

In cytokine-treated hematopoietic cells, failure to undergo growth arrest does not appear to be a consequence of altered p53 activity, because cells deficient in p53 continue to arrest after γ-IR in the absence of cytokine treatment (13, 16). Moreover, cytokine treatment overrides these checkpoints without altering expression of p53 target genes, such as p21 (17). This cytokine-mediated checkpoint failure was dependent on the activation of Cdk2 by a PI3K signaling pathway.

**DISCUSSION**

Through activation of a PI3K signaling pathway, cytokine receptors override γ-IR-induced G{	extsubscript{1}} and G{	extsubscript{2}}-M growth arrest checkpoints in hematopoietic cells (12, 13, 17). Likewise, the present study demonstrates that cytokine activation of PI3K overrides a G{	extsubscript{1}} phase growth arrest in cisplatin-treated 32D cells. However, these cells continued to accumulate in G{	extsubscript{2}}-M phase and eventually underwent nonapoptotic cell death. Thus, the PI3K-dependent suppression of growth arrest checkpoints sensitizes hematopoietic cells to cisplatin-induced death.

A similar sensitization has not been observed in γ-irradiated hematopoietic cells. Instead, cytokine treatment maintains hematopoietic cells in a viable, asynchronous state after γ-IR (12, 13, 17). These differing responses may reflect the greater difficulty in sensing and repairing cisplatin-induced versus γ-IR-induced DNA damage. Although γ-IR generates double-strand DNA breaks, cisplatin forms DNA adducts that become bound to high-mobility-group (HMG) proteins, preventing their recognition by DNA repair factors (2). Thus, growth arrest checkpoints also may be less efficiently established and/or more easily overridden in cisplatin-damaged as compared with γ-irradiated cells. This may explain why activation of truncated EpoR lacking PI3K recruitment sites (EpoR[H]) allows sustained growth arrest of γ-irradiated cells but only a weak arrest of cisplatin-treated 32D cells. Conversely, inhibition of all PI3K signaling within 32D cells by treatment with LY294002 resulted in more efficient G{	extsubscript{1}} and G{	extsubscript{2}}-M arrest during cisplatin treatment. This sustained arrest was associated with reduced death of cisplatin-treated 32D cells, consistent with the possibility that death results from attempted cell division in the face of unrepaired DNA damage.

Various studies have associated cisplatin-induced death with apoptotic and/or necrotic mechanisms (3). In several cell types, cisplatin-induced apoptosis could be inhibited by the expression of antiapoptotic proteins of the Bcl-2 family (20, 21). Similarly, enforced expression of Bcl-2 and related proteins in hematopoietic cells protects against γ-IR-induced apoptosis in the absence of cytokine (13). Cytokine treatment of hematopoietic cells inherently provides significant protection against DNA damage-induced apoptosis because both bcl-2 and bcl-X{	extsubscript{L}} are immediate early gene targets induced by activated cytokine receptors (13, 22). As a consequence, additional antiapoptotic protection achieved by enforced expression of Bcl-X{	extsubscript{L}} provided only a modest increase in the viability of cisplatin-treated 32D cells. The lack of protection by Bcl-X{	extsubscript{L}} overexpression was also consistent with the observation that cisplatin primarily induced necrotic death of cytokine-treated 32D cells. Notably, this cisplatin-induced death was significantly enhanced by activation of PI3K and lack of a biphasic G{	extsubscript{1}} and G{	extsubscript{2}}-M phase growth arrest.
Consistent with these findings, inhibition of PI3K in cisplatin-treated cells resulted in a loss of Cdk2-associated kinase activity. Inhibition of PI3K also reduced Cdk1-associated kinase activity in cisplatin-treated 32D cells. Normally, Cdk1 activity promotes G2-M phase progression (26). Thus, the lack of significant Cdk1 activity in 32D cells treated with cisplatin plus LY294002 was in keeping with the efficient G2-M arrest of these cells. By contrast, cisplatin treatment alone caused an accumulation of cells with 4N DNA content, but these cells retained high levels of Cdk1 activity that were comparable with those within an actively dividing culture. Thus, these G2-M arrests are mechanistically different depending on the presence (Cdk1

Fig. 4. PI3K enhances Cdk1 and Cdk2 activity in cisplatin-treated 32D cells. 32D-EpoR[H]/Bcl-XL cells were cultured in Epo (5 units/ml) or IL-3 (1.4 ng/ml). Parallel cultures were supplemented with cisplatin (5 μM) or left untreated (no treatment). After the addition of cisplatin (48 h), cultures were sampled and assayed for DNA content by flow cytometry (A). A sample of cells arrested in G1 phase was obtained after culture for 24 h in the absence of cytokine or cisplatin (no factor). The percentage of viable cells in each phase of the cell cycle (G1-S-G2-M) is indicated for each culture. Samples of cells assayed in A were lysed and assayed for Cdk1 or Cdk2 kinase activity (B). Alternatively, 32D-EpoR[wt]/Bcl-XL cells were cultured in various combinations of Epo, IL-3, cisplatin, or LY294002 (10 μM) for 48 h. Samples of each culture were assayed for DNA content (C) or were lysed and assayed for Cdk kinase activity (D). To confirm the specific inhibition of PI3K in LY294002-treated cultures, cytokine-induced Akt activity and Erk phosphorylation were assayed in cells treated with 10 μM LY294002 or in the absence of LY294002 (no treatment; E).

Fig. 5. Inhibition of PI3K enhances arrest and viability of cisplatin-treated 32D cells and allows recovery of proliferating cells after the removal of cisplatin. 32D-EpoR[wt] cells were cultured in Epo (5 units/ml) or Epo plus LY294002 (10 μM), and each culture was treated with cisplatin (5 μM). In A, samples of each culture were assayed for viability and DNA content at various times after the addition of cisplatin. The percentage of viable cells in each phase of the cell cycle (G1-S-G2-M) is indicated for each culture. In B, the average viability of cisplatin-treated cultures from four separate experiments is shown. Error bars represent SE. Significant differences (P < 0.01) between the viability of Epo versus Epo + LY294002 cultures are indicated by an asterisk (*). In C, a sample of the culture treated with Epo plus cisplatin for 24 h (A) was washed and returned to medium containing Epo (5 units/ml). DNA content and viability were assayed after 0, 24, or 48 h of continued culture in Epo. No viable cells were observed in samples obtained between 48 and 120 h of continued culture in Epo. Similar results have been obtained in three separate experiments. In D, a sample of the culture treated with Epo, LY294002, and cisplatin for 24 h (A) was washed and returned to medium containing Epo (5 units/ml) plus LY294002 (10 μM). After 48 h, the culture was washed again, and cells were returned to medium containing Epo (5 units/ml) alone. DNA content and viability were assayed after 0, 24, 48, or 120 h of continued culture in Epo. Similar results have been obtained in three separate experiments.

(17). Consistent with these findings, inhibition of PI3K in cisplatin-treated cells resulted in a loss of Cdk2-associated kinase activity.

Inhibition of PI3K also reduced Cdk1-associated kinase activity in cisplatin-treated 32D cells. Normally, Cdk1 activity promotes G2-M phase progression (26). Thus, the lack of significant Cdk1 activity in 32D cells treated with cisplatin plus LY294002 was in keeping with the efficient G2-M arrest of these cells. By contrast, cisplatin treatment alone caused an accumulation of cells with 4N DNA content, but these cells retained high levels of Cdk1 activity that were comparable with those within an actively dividing culture. Thus, these G2-M arrests are mechanistically different depending on the presence (Cdk1
active) or absence (Cdk1 inactive) of PI3K activity. Notably, the ability of PI3K activity to override a G2-M phase growth arrest in γ-irradiated hematopoietic cells (12) also appears to be mediated through regulation of Cdk1.4

Intriguingly, a more general role for PI3K signaling pathways in regulating sensitivity to cisplatin is suggested by the identification of a PI3K-kinase as a gene whose loss confers cisplatin resistance in Dictyostelium (27). Lack of PI3K-kinase activity would prevent synthesis of phosphotidylinositol 4,5-bisphosphate, the substrate for synthesis of phosphotidylinositol 3,4,5-trisphosphate by PI3K (28). Whether the loss of PI3K-kinase could reduce sensitivity to cisplatin in mammalian hematopoietic cells similar to that observed by directly inhibiting PI3K is presently unclear.

The significance of enhanced viability resulting from PI3K inhibition and growth arrest in cisplatin-treated 32D cells is demonstrated by the ability to recover a healthy, proliferating culture after the removal of cisplatin and reactivation of PI3K. The rescued cells exhibited typical growth patterns for 32D cells and appeared to have normal DNA content, although we have not yet addressed whether they have suffered mutations resulting from the cisplatin-induced DNA damage. By comparison, cultures even transiently exposed to cisplatin could not be rescued if treated in the presence of continuous cytokine-induced PI3K activity. Whether this cytokine-induced sensitization contributes significantly to anemias that are frequently associated with cisplatin chemotherapy remains to be determined. However, although Epo can be used to treat chemotherapy-induced anemias (5), some reports have indicated that the response to Epo is reduced in patients who are treated with cisplatin regimens as compared with noncisplatin-based chemotherapies (29, 30). Reduced cytokine responsiveness in cisplatin-treated patients would be consistent with the PI3K-dependent sensitization to cisplatin observed in this study. Inhibitors of PI3K have also been reported to sensitize cancer cells to chemotherapy, presumably by inhibiting the antiapoptotic effects of PI3K-dependent signaling pathways (31). Thus, by sensitizing cancer cells and protecting bone marrow hematopoietic lineage, PI3K inhibitors may have the potential to increase the therapeutic index of platinum-based chemotherapies.

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