Antagonism of p53-dependent Apoptosis by Mitogen Signals

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ABSTRACT

p53-mediated apoptosis is antagonized by growth factor stimulation. Here, we show that p53-dependent cell death induced by DNA damage was effectively prevented by mitogen activation. The levels of Bel-2, Bcl-xL, and Bax were not altered by cisplatin treatment and mitogen rescue. Instead, the protection against p53-regulated apoptosis was mediated by at least three distinct signaling pathways. Either phosphatidylinositol (PI) 3-kinase or mitogen-activated protein kinase (MEK) antagonized p53-induced apoptosis, and an additive preventive effect was observed when both kinases were activated. However, the combination of PI 3-kinase and MEK was not sufficient to completely prevent apoptosis induced by DNA damage. Mitogen activation further suppressed cisplatin-induced p53 expression, and the inhibition was mainly dependent on the Ca2+ pathway. Our results demonstrate that effective antagonism of p53-dependent apoptosis by mitogenic activation requires the presence of multiple signal pathways, including PI 3-kinase, MEK, and Ca2+.

INTRODUCTION

DNA damage induces accumulation of p53 followed by apoptosis in many different cells. The accumulation of p53 after DNA damage is mainly due to increased protein stability and enhanced translation (1). The increase of p53 is an essential step in DNA damage-induced apoptosis, as illustrated by the observation that ionizing irradiation does not induce apoptosis in p53−/− thymocytes (2, 3).

p53 stimulates the expression of a number of gene products that are known to participate in the apoptosis process. p53 is a direct activator of the Bax in a few types of cells (4). p53 also stimulates the expression of IGF1-binding protein-3 (5), which enhances apoptosis of the Bax in a few types of cells (4). p53 also stimulates the expression of a number of gene products that are involved in the apoptotic process (6).

p53-induced apoptosis is blocked by signals initiated from growth factors. p53-mediated apoptosis is suppressed by stimulation with IL-2, IL-3, IL-6, erythropoietin, granulocyte macrophage colony-stimulating factor, and IFN-γ (6, 8–14). Cytokines do not affect p53-mediated cell cycle arrest (15); instead, these cytokines prevent apoptosis through distinct mechanisms. For instance, the effect of IL-2 is attributed to the increased expression of Bcl-xL, and/or Bel-2 (13, 14), whereas the action of IL-3 is correlated with the altered expression of p21WAF1/CIP1 and GADD45 (6).

PI 3-kinase is well known for its major role in the apoptotic signal delivered by growth factors. The activation of PI 3-kinase confers protection from serum withdrawal-induced apoptosis by IGF-1 and nerve growth factor (16–18). PI 3-kinase also suppresses c-Myc-induced apoptosis (19). The antideath activity of PI 3-kinase is mediated through the activation of Akt/protein kinase B (16, 19, 20). A possible target of Akt is BAD, the phosphorylation of which led to cell survival (21). Despite of the dominant antiapoptotic activity of PI 3-kinase, the direct antagonism of p53-induced death by PI 3-kinase has yet to be demonstrated.

In this study, we explored the mechanism of how mitogenic signals antagonize p53-dependent apoptosis induced by cisplatin in transformed T cells. For proper mimicking of different mitogenic stimulations, combination of protein kinase C activator TPA and calcium ionophore A23187 were used. We demonstrated that the PI 3-kinase pathway directly antagonized p53-induced apoptosis. The effect of PI 3-kinase on the inhibition of p53-dependent apoptosis was further enhanced by coexpression of active MEK1. In addition, mitogen suppressed the p53 accumulation induced by DNA damage, independent of the PI 3-kinase and MEK pathways. Our results suggest that a combination of distinct mitogenic signals is required for effective antagonism of p53-mediated apoptosis.

MATERIALS AND METHODS

Reagents. Cisplatin, A23187, TPA, and staurosporin were obtained from Sigma Chemical Co. (St. Louis, MO). PD 98059, wortmannin, p38 MAP kinase inhibitor SB203580, and MG 101 (calpain inhibitor I) were purchased from Calbiochem (San Diego, CA). Anti-p53 antibodies (clones PAb421 and PAb1620) were obtained from Oncogene Science (Cambridge, MA). Anti-α-tubulin antibody was purchased from Amersham (Buckinghamshire, United Kingdom). Anti-Bcl-2 (N-19) and anti-Bax (P-19) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Bcl-xL antibody was a gift from Transduction Laboratories (Lexington, KY).

T-Cell Lines and Treatments. 101 is a T-cell hybridoma that is specific for a repressor cl 12-26 (22). DO11.10 is an ovalbumin 323–329-specific T-cell hybridoma. EL4 T lymphoma (ATCC TIB39; American Type Culture Collection, Manassas, VA) was a gift from Dr. Nan-Shih Liao (Academia Sinica, Taipei, Taiwan, Republic of China). H1299 cell line is p53-null large cell lung carcinoma. Cisplatin was dissolved in water. TPA and A23187 were dissolved in DMSO to make the stock concentration 500–1000 times the final concentration used. The final DMSO concentration in the cultured cells was <0.2% (v/v). The same amount of DMSO was added to the untreated control.

Plasmids and Transient Transfection. pCEP4-p53 containing the full-length wild-type p53 in the HindIII site of pSP72 was described previously (23). The constitutively active form of MEK1, pMCL-MEK1-N3/S218E/S222D (24), was a gift from Dr. Natalie G. Ahn (University of Colorado, Boulder, CO). Wild-type PI 3-kinase p110α was a gift from Dr. Michael D. Waterfield (Ludwig Institute for Cancer Research, London, United Kingdom). Active form of p110 was constructed by introducing a mutation of lysine to glutamic acid residue 227 according to Rodriguez-Viciana et al. (25). DNAs were transfected into H1299 cells using the calcium phosphate method.

Cell Death Measurement. The extent of apoptosis was determined by propidium iodide staining or by annexin V staining, as described previously (26, 27). For cell death involving transient transfection, cells were cotransfected with pCMV β-gal (Stratagene, La Jolla, CA) or with pGreen Lantern-1 (Life Technologies, Inc., Grand Island, NY). Cells were either stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside 72 h after transfection or directly observed under the fluorescence microscope. The numbers of blue, green, and unstained cells were determined by counting six different randomly

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1 The abbreviations used are: IGF, insulin-like growth factor; IL, interleukin; PI, phosphatidylinositol; MAP, mitogen-activated protein; MEK, MAP kinase kinase; TPA, 12-O-tetradecanoylphorbol-13-acetate.
RESULTS

Mitogen Activation Inhibited Cisplatin-induced Apoptosis in T Cells. We used cisplatin to trigger p53-dependent apoptosis in T cells. Three different transformed T cells were used, including T-cell hybridomas 10I and DO11.10 and T lymphoma EL4. These T cells have been previously used in apoptosis analysis (28), and all contain wild-type p53. Significant cell death was induced by cisplatin in all three T cells, as measured by DNA fragmentation (Fig. 1, A and B). Stimulation with TPA/A23187 weakly triggered apoptosis (5–15%) in these transformed T cells yet effectively prevented cisplatin-induced apoptosis (Fig. 1, A and B; data not shown for DO11.10). Because DNA fragmentation may not represent actual apoptosis well, annexin V was also used to quantitate cell death. Fig. 1C illustrates that an identical inhibitory effect of mitogen stimulation on cisplatin-induced cells was obtained with annexin V staining. Both TPA and A23187 were required for the optimum inhibition of cisplatin-induced cell death. TPA or A23187 alone was less effective in the prevention of cisplatin-triggered apoptosis (Fig. 1C). We further observed that mitogenic activation of T cells through T-cell receptors prevented cisplatin-mediated apoptosis. The extensive cell death (75%) induced by cisplatin was reduced to the level caused by anti-CD3 alone (Fig. 1D), which is known to induce apoptosis in transformed T cells (28, 29). It has been well documented that T-cell receptor-mediated death occurs independently of p53 (30). Therefore, similar to growth factors, mitogenic activation effectively antagonized cisplatin-induced apoptosis known to be mediated through p53. To simplify the study, we performed the following experiments with TPA/A23187 as a mitogen.

Cisplatin-induced p53 Accumulation Was Antagonized by Mitogen. The involvement of p53 was illustrated by a significant accumulation of p53 after cisplatin treatment (Fig. 2A). Little p53 was detected in untreated 10I T cells. Induction of p53 was eminent 8 h after cisplatin treatment (Fig. 2A). The protein levels of p53 peaked at 12 h and remained high 16 h after cisplatin treatment. The diminished p53 levels 24 h after cisplatin treatment were likely due to extensive cell death, as indicated by the same degree of reduction in α-tubulin level (Fig. 2B). Mitogen activation suppressed the accumulation of p53 in cisplatin-treated 10I cells. At least half of cisplatin-induced p53 expression was inhibited by mitogen (Fig. 2A).

Mitogenic Stimulation Did Not Significantly Alter the Expression of Bcl-2, Bcl-xL, and Bax. Bcl-2 is known to block p53-associated apoptosis in transformed cells (31, 32). Mitogenic activation also up-regulates the expression of antiapoptotic gene Bcl-xL, which is known to induce apoptosis in transformed T cells (28, 29). It has been well documented that T-cell receptor-mediated death occurs independently of p53 (30). Therefore, similar to growth factors, mitogenic activation effectively antagonized cisplatin-induced apoptosis known to be mediated through p53. To simplify the study, we performed the following experiments with TPA/A23187 as a mitogen.

Fig. 2. Mitogen activation inhibited cisplatin-induced p53 protein accumulation. A, T-cell hybridoma 10I was treated with cisplatin (cisplatin), TPA/A23187 (TPA), or cisplatin plus TPA/A23187 (cisplatin + T/A). The cells were harvested at the indicated times (in h), and the total cell extracts were prepared. Cell extracts (40 μg) were resolved by 12.5% SDS-PAGE and transferred to PVDF membrane (Millipore, Bedford, MA). The membrane was incubated with anti-p53 antibody PAb421 (Oncogene Science), followed by horseradish peroxidase-conjugated antimouse immunoglobulin antibody (Santa Cruz Biotechnology), and detected with ECL reagent (Amersham). B, expression of α-tubulin in the same extract, as detected by the specific antibody (Amersham).
inhibitory effect of mitogen on p53-associated apoptosis was apparently not mediated through an increase in Bcl-2 and Bcl-xL levels.

The other apoptosis-related gene product that may be induced by p53 is Bax (4). The levels of Bax in 10I were relatively stable after cisplatin stimulation or after mitogen activation (Fig. 3C). The activation of cisplatin-treated T cells did not change the levels of Bax expression. Despite the fact that Bax is regulated by p53 in some types of cells, no significant role for Bax was found in cisplatin/mitogen-stimulated T cells.

**PI 3-Kinase and MEK Synergistically Prevented p53-induced Apoptosis.** To identify mitogen signals that antagonize cisplatin-induced cell death, we used the specific inhibitors for each signaling pathway. The effectiveness of each inhibitor was confirmed independently (35). None of the inhibitors induced apoptosis at the concentrations used. Among the different inhibitors examined, the addition of wortmannin (100 nM), a specific PI 3-kinase inhibitor, moderately reversed the preventive effect of mitogen (Fig. 4A). The other inhibitor that weakly antagonized the preventive effect was PD 98059, a selective inhibitor of MEK. The inhibitions by wortmannin and PD 98059 were statistically significant (Fig. 4A). The effects of wortmannin and PD 98059 were additive. The presence of both inhibitors synergistically reversed mitogenic inhibition of cisplatin-induced apoptosis (Fig. 4A). No effect on mitogen-activated T lymphoma was observed with protein kinase C inhibitor staurosporin (10 nM) and p38 MAP kinase inhibitor SB203580 (10 μM). The effect of PI 3-kinase and MEK was further confirmed by direct transfection of the active PI 3-kinase and the active MEK1 into EL4 T cells (Fig. 4B). Green fluorescence protein was cotransfected as an indicator. The green cell ratio represented the fraction of transfected cells that were viable. Transfection with PI 3-kinase or MEK1 alone did not alter the green cell ratio (Fig. 4B). Cisplatin treatment led to a >50% decrease of viability in the vector control. Cisplatin-induced apoptosis was partially prevented by the expression of active PI 3-kinase or MEK1 (Fig. 4B). The antagonistic effect was specific for each kinase. PI 3-kinase-mediated protection was not inhibited by PD 98059, whereas MEK1-mediated protection was not eliminated by wortmannin. Greater antagonism was observed when both PI 3-kinase and MEK1 were transfected (Fig. 4B).

Cisplatin-induced cell death may be p53 independent, and because PI 3-kinase and MEK have not been shown to directly inhibit p53-induced apoptosis, the effectiveness of PI 3-kinase and MEK to suppress p53-initiated apoptosis was further investigated. Because all transformed T cells used in this study contained p53, we used a p53-null cell H1299 for such examination. With the cotransfection of pCMV-lacZ, the viability of the transfected H1299 cells was then assessed by determination of the blue cell ratio. Transient transfection of H1299 cells with wild-type p53 led to p53 expression (Fig. 5A) and significant cell death 72 h later (Fig. 5B). Coexpression of active PI 3-kinase or active MEK1 did not affect the expression of p53 (Fig. 5A). PI 3-kinase activation reduced p53-induced cell death by at least 50% (Fig. 5B). A weaker inhibition on p53-mediated apoptosis was also found with coexpression of active MEK1. The combination of active PI 3-kinase and active MEK1 further decreased p53-dependent cell death. Therefore, PI 3-kinase and MEK directly antagonized p53-induced apoptosis. Results from Figs. 4 and 5 support the hypothesis that PI 3-kinase and MEK contributed to the mitogenic suppression of cisplatin- and p53-induced cell death.

**Mitogenic Inhibition of p53 Induction by Ca**2+**-dependent Signals.** Cisplatin-induced apoptosis involves two distinct stages, the induction of p53 and the initiation of cell death by the accumulated p53. Our results illustrated that both stages were antagonized by mitogenic signals (Figs. 2A and 4A). Because the direct antagonism of p53-induced apoptosis by PI 3-kinase and MEK1 (Fig. 5) accounted for ~50% of mitogenic inhibition on cisplatin-induced cell death (Fig. 4), we next examined how mitogen signaling inhibited cisplatin-induced p53 accumulation (Fig. 2). The addition of either wortmannin or PD 98059 did not reverse the inhibition of p53 accumulation by mitogen (Fig. 6), supporting the hypothesis that PI 3-kinase and MEK contribute little to mitogenic inhibition of p53 accumulation. The mitogenic signals were further separated. For T cells treated with TPA alone, very minimal interference of p53 accumulation was observed (Fig. 6). In contrast, the presence of the Ca**2+ ionophore alone inhibited the accumulation of p53 almost as effectively as TPA/A23187 (Fig. 6). A potential candidate activated by Ca**2+ signaling is calpain (36, 37), which promotes the degradation of p53. This was supported by using inhibitor of calpain I, which reversed the antagonistic effect of A23187 (Fig. 6). Therefore, p53 induction was suppressed by mitogen activation mainly through a Ca**2+ -dependent signal that activates calpain-mediated degradation.

**DISCUSSION**

In this study, we examined the mechanism underlying the inhibition of p53-regulated apoptosis by mitogen activation of transformed T...
cells. DNA-damaging agents induced significant cell death in T-cell hybridomas and T lymphomas, which was effectively antagonized by TPA/A23187 and anti-CD3 (Fig. 1). No detectable changes in the levels of the antiapoptotic proteins, Bcl-2 and Bcl-xL, were seen in the cisplatin-treated or in the mitogen-rescued cells (Fig. 3, A and B). We also failed to observe any alteration in Bax level (Fig. 3C). These results indicate that mitogenic signals do not modulate the levels of Bcl-2, Bcl-xL, and Bax in transformed T cells, although the possibility that posttranslational modification of these proteins is involved cannot be ruled out.

Despite the fact that Bax is one of the most prominent mediators in p53-regulated apoptosis (38), not all p53-induced apoptosis requires activation of Bax (39). Our results are in accordance with the observations of Canman et al. (6), who showed that IL-3 antagonizes p53-mediated apoptosis but does not induce any change in the levels of Bcl-2, Bcl-xL, and Bax. There is also a discrepancy between our results and the observed induction of Bcl-xL in activated normal T cells (14, 33). We believe that such a discrepancy is due to the difference in the Bcl-xL level between the normal and the transformed T cells. The levels of both Bcl-2 and Bcl-xL are very low in normal T cells before activation (14), in contrast to the high endogenous level of Bcl-xL detected in transformed T cells (Fig. 3). Therefore, the contribution of Bcl-xL to the antiapoptotic action of mitogen in T lymphomas is suspected to be minimal.

The activation of PI 3-kinase has been shown to be the major survival signal from growth factors. Here, we observed an inhibition of p53-induced apoptosis by PI 3-kinase. The inhibition of PI 3-kinase partially reversed the preventive effect of mitogen on cisplatin-induced cell death (Fig. 4A). Moreover, transient expression of the active PI 3-kinase effectively antagonized cisplatin- and p53-initiated cell death in EL4 and H1299 cells (Figs. 4B and 5B). To our knowledge, this is the first demonstration of direct antagonism on p53-induced apoptosis by PI 3-kinase. On the contrary, the antiapoptotic role of MAP kinase was much less definite than that of PI 3-kinase. MAP kinase inhibits apoptosis induced by nerve growth factor deprivation in PC12 cells (40). MAP kinase is involved in the inhibition of ceramide-induced apoptosis by sphingosine 1-phosphate (41) and in the suppression of cardiac myocyte

![Graph A](image1)

**Fig. 4.** PI 3-kinase and MEK antagonized cisplatin-induced apoptosis. A, 10I T cells were treated with cisplatin (25 \( \mu \)M) in the presence of TPA/A23187 and inhibitors, and the extent of apoptosis was determined 22 h later as described in the legend to Fig. 1. The concentrations of inhibitors were as follows: wortmannin, 100 nM; PD 98059, 50 \( \mu \)M; staurosporin, 10 nM; and SB203580, 10 \( \mu \)M. None of the inhibitors alone induced apoptosis at the concentrations used. Statistical difference was determined with a t test. B, EL4 T cells were transfected with the vector alone (vector control), active PI 3-kinase (p110*), active MEK1 (MEK*), or a combination of the two kinases, together with pGreen Lantern-1 (Life Technologies, Inc.). Twenty-four h later, T cells were treated with cisplatin in the absence or presence of wortmannin (50 nM) or PD 98059 (25 \( \mu \)M), and the numbers of green and unstained cells were determined after another 48 h. The green cell ratio was calculated as the average green cell number in six randomly chosen fields divided by the average unstained live cell number of vector control in the same number of fields.

![Graph B](image2)

**Fig. 5.** PI 3-kinase and MEK suppressed apoptosis induced by ectopic expression of the wild-type p53. H1299 cells were transfected with pCMV-lacZ, pCEP4-p53, pCMV-p110*, or pCMV-MEK* using the calcium phosphate method. A, cells were harvested at the indicated times (in h), and p53 content was assessed with anti-p53 antibody by Western analysis. B, 72 h after transfection, cell viability was determined by staining with 5-bromo-4-chloro-3-indolyl-\( \beta \)-D-galactopyranoside. The blue cell ratio was calculated as the average blue cell number in six randomly chosen fields divided by the average unstained live cell number of the vector control in the same number of fields. The experiment was repeated twice with similar results.
apoptosis by cardiotrophin 1 (42). We detected a direct inhibition of cisplatin- and p53-induced cell death by active MEK1 (Figs. 4B and 5B). The antiapoptotic effect of MEK1 was independent of PI 3-kinase and vice versa. PI 3-kinase- and MEK1-mediated protections were not inhibited by PD 98059 and wortmannin, respectively (Fig. 4B). In addition, there was a synergistic protection against p53-induced apoptosis when both PI 3-kinase and MEK1 were activated. This is analogous to a recent report that both PI 3-kinase and MEK are required for the prevention of growth factor deprivation-induced apoptosis by IGF-1 in PC12 cells (18). On the basis of the established mechanism (19, 20), inhibition of p53-mediated cell death by PI 3-kinase is likely mediated through activation of Akt kinase. The exact mechanism of how MEK antagonizes p53-induced cell death would require further investigation.

Despite the prominent effect of PI 3-kinase and MEK on p53-induced cell death (Fig. 5B), the combination of both kinases suppressed only 50% of cisplatin-induced apoptosis (Fig. 4A). There was another level of regulation on p53-mediated apoptosis by mitogen. We further demonstrated that the stimulation with Ca\(^{2+}\) ionophore alone inhibited most of the cisplatin-triggered p53 increase, and such inhibition was likely mediated by calpain (Fig. 6). Therefore, our study suggests that the effective antagonism of p53-mediated cell death by mitogens requires at least three distinct signals: PI 3-kinase, MEK, and Ca\(^{2+}\). Genotoxic drugs are used to trigger apoptosis in cancer cells expressing wild-type p53. Our results reveal that cancer cells require multiple signals to escape p53-mediated cell death and suggest the possibility that blockage of one or more of these activation pathways in cancer cells would enhance the effectiveness of chemotherapy by genotoxic drugs.

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