Effect of c-Abl tyrosine kinase on the cellular response to paclitaxel-induced microtubule damage

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Summary   DNA damage has been shown to activate c-Abl tyrosine kinase. We now report that, in addition to DNA damage, microtubule damage induced by paclitaxel results in activation of c-Abl kinase. In 3T3 cells, the presence of c-Abl kinase increased paclitaxel-induced cell death. In Abl-proficient cells, paclitaxel produced a marked and prolonged G2/M arrest which peaked at 24 h and a rapid and marked induction of p21[WAF1] which also peaked at 24 h. In Abl-deficient cells, the G2/M arrest induced by paclitaxel was less prominent and shorter in duration and the effect of paclitaxel on p21[WAF1] expression was reduced and delayed. Paclitaxel had no effect on p53 expression and MAPK phosphorylation. These findings indicate that, in 3T3 cells, c-Abl kinase facilitates cell death and regulates G2/M arrest in response to paclitaxel-induced microtubule damage in a pathway that is dependent on p21[WAF1] and independent of MAPK activity. © 2000 Cancer Research Campaign

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c-Abl is a non-receptor tyrosine kinase that is related to Src-homology domains 2 and 3 (SH2 and SH3) protein kinases (Wang, 1993). The finding that c-Abl associates with the retinoblastoma (Rb) protein has supported a role for c-Abl kinase in regulating the cell cycle (Kipreos and Wang, 1992). Other studies have demonstrated that c-Abl kinase phosphorylates the C-terminal domain of RNA polymerase II (Baskaran et al, 1996) and stimulates transcription (Kipreos and Wang, 1992).

Recent work has demonstrated that DNA damage induced by a variety of agents, including ionizing radiation, cisplatin, mitomycin C, methyl methane sulfonate, and cytarabine results in activation of c-Abl kinase (Kharbanda et al, 1995*a*; 1995b; Liu et al, 1996; Nehme et al, 1997). In response to DNA damage, c-Abl associates with DNA-dependent protein kinase (DNA-PK) and is activated by DNA-PK-dependent phosphorylation. Components of the DNA-PK complex are involved in the sensing and repair of DNA double-strand breaks.

The ATM protein has been shown to serve as an important link between DNA damage and activation of c-Abl kinase (Baskaran et al, 1997; Shafman et al, 1997). When cells are treated with ionizing radiation, c-Abl is activated, but only in the presence of functional ATM. The association between ATM and c-Abl seems to be constitutive; however, ATM activates c-Abl tyrosine kinase activity only following DNA damage.

Activation of c-Abl kinase has been shown to directly contribute to the regulation of growth arrest induced by ionizing radiation (Yuan et al, 1996). c-Abl has been found to interact with p53 and Rb (Goga et al, 1995; Welch and Wang, 1993). c-Abl activates the transactivation function of p53 and thus stimulates the

induction of p21[WAF1] (Goga et al, 1995). In response to DNA damage, c-Abl contributes to growth arrest by a p53-dependent and p21[WAF1]-independent mechanism (Kharbanda et al, 1998).

It was our hypothesis that DNA damage and microtubule damage result in activation of similar signaling pathways. Since DNA damage activates c-Abl kinase, we asked whether c-Abl kinase is involved in the signaling cascade activated by microtubule damage. We therefore determined the effects of the microtubule-damaging agent paclitaxel on c-Abl kinase and other signaling pathways. In addition, we investigated the effects of c-Abl kinase on paclitaxel toxicity and on paclitaxel-induced G2/M arrest.

MATERIALS AND METHODS

Cell culture

c-abl-null mouse fibroblasts (3T3-Abl$^{-/-}$ cells), polyclonal populations of reconstituted 3T3-Abl$^+$ cells, and 3T3 mock infected cells (3T3-mock cells) were obtained from Dr JYJ Wang (Wang, 1993). 3T3-Abl$^+$ and 3T3-mock cells were obtained by infection and selection of 3T3-Abl$^{-/-}$ cells with *c-abl* or empty recombinant retroviruses containing the hygromycin-resistance gene. 3T3-mock cells were used as a negative control in all experiments. All cell lines were maintained in Dulbecco's modified Eagle's medium (Irvine Scientific, Irvine CA, USA) supplemented with 100 mM L-glutamine and 10% heat-inactivated foetal bovine serum. The absence and presence of expression of c-Abl in 3T3-Abl$^{-/-}$, 3T3-mock, and 3T3-Abl$^+$ fibroblasts was verified by immunoblot analysis (data not shown).

Reagents

Paclitaxel, vinblastine, vincristine, etoposide, and doxorubicin were purchased from Sigma Chemical Co (St. Louis MO, USA) and dissolved in DMSO. Docetaxel was purchased from

Rhone-Poulenc Rorer Pharmaceuticals Inc (Collegeville PA, USA), and was prepared as a 50 mM stock solution in polysorbate 80. Vinorelbine was obtained from Glaxo Wellcome Inc (Research Triangle Park NC, USA), a stock solution of 9.26 mM was prepared in water. Cisplatin was obtained from Bristol-Myers Squibb Co (Princeton NJ, USA). Cisplatin was dissolved in 0.9% NaCl as 3.33 mM stock solution.

Growth rate assay

Since 3T3 cells do not form discrete colonies, sulforhodamine B growth rate assays were performed. Fibroblasts were seeded into 96-well plates at a density of 1500 cells well^{-1} in 100 µl medium. After 24 h, drugs were added in a final volume of 100 µl medium, and cells were exposed to microtubule-damaging agents for 24 h. Cells were allowed to grow for an additional 72 h after the beginning of drug exposure. Cell growth was stopped by adding 50 µl of 50% (w/v) trichloroacetic acid, and cellular proteins were stained with sulforhodamine B (Sigma Chemical Co) and measured by spectrophotometry (Skehan et al, 1990). Control plates were fixed to estimate the amount of cellular proteins at time 0. The relative growth rate, *r*, was calculated as reported previously (Monks et al, 1991). Each experiment was performed in triplicate, and IC$_{50}$ values were estimated by linear interpolation at $r = 0.5$. The growth rates of untreated Abl-proficient and -deficient cells were identical.

Quantitation of apoptotic cells

3T3 cells were treated with 160 nM paclitaxel for 24 h. 72 h after treatment, cells were collected by trypsinization and resuspended in PBS containing 4 µg ml^{-1} acridine orange and 4 µg ml^{-1} ethidium bromide. Subsequently, cells were assessed for apoptotic morphology by supravital fluorescence microscopy. Cells were scored as apoptotic according to established morphologic criteria (McGahon et al, 1995).

Cell cycle phase distribution

Approximately $1–2 \times 10^6$ cells were exposed to an equimolar concentration of 160 nM paclitaxel for 24 h. This corresponded to an IC$_{40}$ and an IC$_{70}$ concentration in 3T3-mock cells and 3T3-Abl$^+$ cells, respectively. At 0, 1, 2, 3, 4, 5 and 6 days after starting paclitaxel treatment, cells were harvested by trypsinization, washed twice with ice-cold PBS, and fixed in ice-cold 70% ethanol at a concentration of 10^6 cells ml^{-1}. Cells were counted and 10^6 cells per sample were centrifuged, resuspended in 300 µl of ice-cold PBS and treated with 0.1 mg ml^{-1} RNase A (Sigma Chemical Co) at 37°C for 30 min. Propidium iodide (Molecular Probes, Eugene OR, USA) at a final concentration of 50 µg ml^{-1} was then added to the cell suspensions. After a 30 min incubation on ice, cells were analysed on a FACScan flow cytometer (Becton-Dickinson, San Jose CA, USA). Multicycle AV Cell Cycle software (Phoenix Flow Systems, San Diego CA, USA) was used to calculate the fraction of cells in each phase of the cell cycle, as described previously by Dean and Jett (1974).

In vitro immune complex kinase assay for c-Abl kinase

Cells were grown to approximately 80% confluence and treated with an equimolar concentration of 160 nM paclitaxel for 24 h. At 12 and 24 h after the beginning of exposure to paclitaxel, cells were lysed in 1 ml lysis buffer (10 mM Tris-HCL (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 5 mM DTT, 1 mM sodium vanadate, 0.1 mM phenylmethylsulfonyl fluoride, and 5 mM aminocaproic acid) for 30 min on ice. The insoluble material was removed by centrifugation at 30 000 ***g*** for 20 min at 4°C. Equal amounts of protein were incubated with protein A-Sepharose and anti-Abl (2.5 µg) (K-12, Santa Cruz Biotechnology, Santa Cruz CA, USA) for 3 h at 4°C. Anti-Abl immune complexes were washed twice with lysis buffer (500 mM NaCl, lysis buffer/100 mM NaCl) and twice with kinase buffer (25 mM Tris-HCl (pH 7.4), 10 mM MgCl$_2$, and 1 mM DTT). The washed pellet was resuspended in 20 µl of kinase buffer with 50 µCi (γ-^{32}P) ATP (3000 Ci mmol^{-1}), 10 µM cold ATP and 1 µg of GST-CTD as substrate and incubated for 30 min at room temperature (Baskaran et al, 1996). Reactions were terminated by the addition of the SDS sample buffer and boiling for 5 min. The phosphorylated proteins were resolved by 5–15% SDS-PAGE and visualized by autoradiography. The amounts of c-Abl protein in the anti-Abl immunoprecipitates were analysed by immunoblotting with anti-Abl (8E9; Pharmingen, San Diego CA, USA). The antigen–antibody complexes were visualized by enhanced chemiluminescence.

Western blotting

Cells growing in log phase were treated with 160 nM paclitaxel for 24 h. At various points in time, cells were lysed in 100 µl lysis buffer as described above. 50 µg of total protein were subjected to electrophoresis on 8%, 15%, or 10% polyacrylamide gels, for analysis of phosphorylated MAPK, p21^{WAF1} and p53, respectively. Cellular proteins were transferred to a polyvinylidene difluoride membrane (Immobilon P; Millipore, Bedford MA, USA) by electroblotting. Membranes were exposed to polyclonal anti-phospho-MAPK antibody (New England Biolaboratories Inc, Beverly MA, USA), polyclonal anti-p21^{WAF1} antibody (Santa Cruz Biotechnology, Santa Cruz CA, USA), or monoclonal anti-p53 antibody (Santa Cruz Biotechnology), diluted 1: 1000 in 3–5% bovine serum albumin. After washing, blots were exposed to horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies diluted 1:3000 (Amersham Life Science Inc, Arlington Heights IL, USA) and the complexes were visualized by enhanced chemiluminescence.

RESULTS

Effect of paclitaxel on c-Abl kinase activity

DNA damage has been shown to activate c-Abl tyrosine kinase (Kharbanda et al, 1995a; 1995b; Liu et al, 1996; Nehme et al, 1997). We were interested in whether c-Abl tyrosine kinase is also involved in the cellular response to microtubule damage. 3T3-mock and 3T3-Abl$^+$ cells were treated with 160 nM paclitaxel for 24 h, and anti-Abl immunoprecipitates were prepared from cell lysates at 12, and 24 h. In vitro kinase assays were performed with the GST-CTD protein as substrate (Baskaran et al, 1996). GST-CTD phosphorylation was not detectable in untreated cells (Figure 1). In Abl-proficient cells, treatment with paclitaxel resulted in activation of c-Abl kinase at 12 and 24 h. As expected, paclitaxel did not activate c-Abl kinase in Abl-deficient cells. Paclitaxel had no
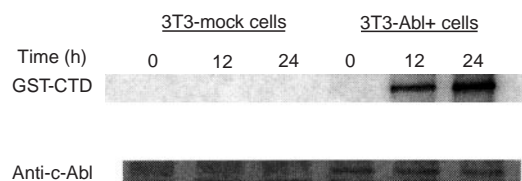
Figure 1 Effect of paclitaxel on c-Abl kinase activity. 3T3 cells were exposed to 160 nM paclitaxel and harvested at 12 and 24 h after the beginning of drug exposure. Time point 0 h represents untreated control cells. Upper panel, activation of c-Abl determined by an immune complex kinase assay with GST-CTD as the substrate. Lower panel, Western blot analysis using an anti-Abl antibody. The experiment was repeated three times with similar results

detectable effect on the levels of c-Abl protein in 3T3 cells (Figure 1, lower panel).

Effect of c-Abl kinase on paclitaxel sensitivity

In order to understand the role of c-Abl in the cellular response to microtubule damage, we determined the effect of c-Abl kinase on the sensitivity of 3T3 cells to microtubule-damaging agents (Table 1 and Figure 2). Abl-proficient cells were 3.7 ± 0.5-fold more sensitive to paclitaxel relative to Abl-deficient cells (IC_{50} 70 ± 20 vs 260 ± 50 nM, mean $\pm$ SD, $n = 4$; $P = 0.004$ by two-sided t-test). The effect of c-Abl on the sensitivity of 3T3 cells to docetaxel was much less marked and was not statistically significant. The presence of c-Abl kinase had no significant effect on the sensitivity of 3T3 cells to other microtubule-damaging agents, including vincristine, vinblastine, and vinorelbine. Similarly, c-Abl kinase had no effect on the sensitivity of 3T3 cells to cisplatin, etoposide and doxorubicin (data not shown).

Effect of c-Abl kinase on paclitaxel-induced apoptosis

Paclitaxel has been shown to induce apoptosis in fibroblasts and tumour cells (Fan, 1999). We have confirmed that paclitaxel triggers apoptosis in 3T3 cells by demonstrating paclitaxel-induced cleavage of poly (ADP-ribose) polymerase (PARP), a substrate for caspase 3 (Nicholson et al, 1995) (data not shown). In order to determine the effect of c-Abl kinase on paclitaxel-induced apoptosis, 3T3-mock and 3T3-Abl$^+$ cells were treated with 160 nM paclitaxel for 24 h and apoptotic cells were quantitated 72 h after the beginning of drug exposure. The presence of c-Abl resulted in a 1.9 ± 0.3 (mean $\pm$ SD)-fold increase in apoptotic cells in response to paclitaxel

treatment ($n = 3$, $P = 0.03$ by two-sided t-test). Interestingly, c-Abl had no effect on the number of apoptotic cells in response to treatment with 4 nM docetaxel for 24 h (data not shown).

Effect of c-Abl kinase and paclitaxel on cell cycle phase distribution

In order to understand the mechanism(s) by which c-Abl kinase regulates the sensitivity to paclitaxel, we determined the effect of paclitaxel on cell cycle phase distribution in Abl-proficient and -deficient cells (Figure 3). Cells were exposed to 160 nM paclitaxel for 24 h which corresponded to an IC_{40} and an IC_{70} concentration in 3T3-mock and 3T3-Abl$^+$ cells, respectively. Paclitaxel caused a marked G2/M arrest which peaked at 24 h after the beginning of drug exposure. At 24 h, the fraction of cells in G2/M phase was $82 \pm 9\%$ and $74 \pm 12\%$ in Abl-proficient and Abl-deficient cells, respectively (mean $\pm$ SD, $n = 3$, $P < 0.005$ by two-sided t-test). In Abl-proficient cells, the G2/M arrest persisted for a considerably longer period of time relative to Abl-deficient cells. The difference in the fraction of G2/M cells between Abl-proficient and Abl-deficient cells was most marked at 6 days after starting exposure to paclitaxel ($66 \pm 6\%$ vs $32 \pm 5\%$, mean $\pm$ SD, $n = 3$, $P = 0.002$ by two-sided t-test).

Effect of c-Abl kinase on paclitaxel-induced G2/M arrest

In order to better quantitate the effect of c-Abl kinase on paclitaxel-induced G2/M arrest, we calculated the area under the curve for fraction of cells in each phase of the cell cycle over time (percent $\times$ days). The AUC for the G2/M phase of paclitaxel treated cells was 260 ± 40 and 400 ± 30 (percent of cells in G2/M phase $\times$ days, mean $\pm$ SD) in Abl-deficient and Abl-proficient cells, respectively (Figure 4). This represents a 1.6 ± 0.2-fold difference in AUC which is statistically significant ($n = 3$, $P = 0.008$, by two-sided t-test).

Effect of c-Abl kinase and paclitaxel on p21WAF1 expression

Paclitaxel has been reported to induce p21^{WAF1} (Blagosklonny et al, 1995), a protein known to be a general inhibitor of cyclin-dependent kinases and a modulator of proliferating-cell-nuclear antigen activity (Harper et al, 1993; Hunter and Pines, 1994). Therefore, we investigated the effect of paclitaxel on the level of expression of p21^{WAF1} in 3T3-mock and 3T3-Abl$^+$ cells (Figure 5). p21^{WAF1} expression was determined by immunoblotting using an anti-p21^{WAF1} antibody. Cells were exposed to an equimolar concentration of paclitaxel (160 nM) for 24 h. In Abl-proficient cells,

Table 1 Effect of the c-Abl kinase on drug sensitivity. The sulforhodamine B growth-rate assay was used to generate dose–response curves. Results represent mean $\pm$ SD of at least three independent experiments performed with triplicate cultures. The dose-modifying factor represents the ratio of the IC_{50} values of 3T3-mock over 3T3-Abl$^+$ cells. IC_{50} values were compared by two-sided t-test

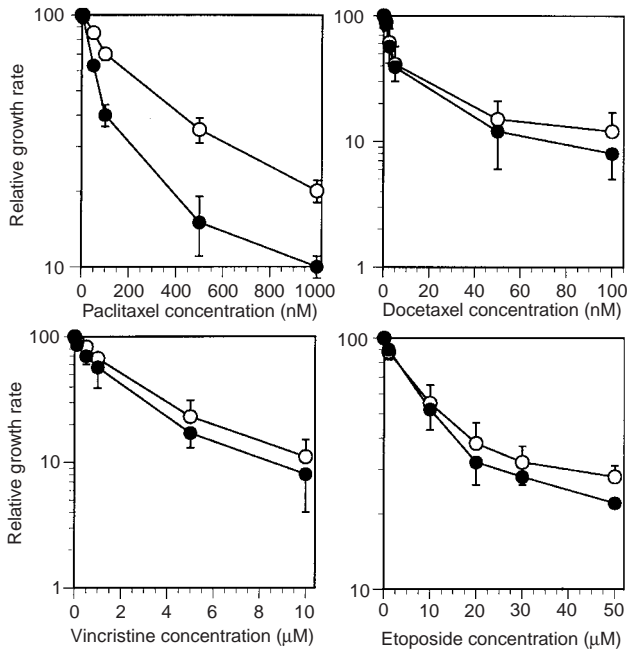| Drug	Exposure time (h)	3T3-mock cells (IC_{50})	3T3-Abl$^+$ cells (IC_{50})	Dose-modifying factor	*P*-value
Paclitaxel (nM)	24	260 ± 50	70 ± 20	3.7 ± 0.5	0.004
Docetaxel (nM)	24	4.1 ± 1.6	3.8 ± 1.2	1.1 ± 0.3	0.808
Vinblastine (nM)	24	43 ± 5	33 ± 8	1.3 ± 0.4	0.14
Vincristine (µM)	24	1.8 ± 0.4	1.5 ± 0.5	1.2 ± 0.3	0.46
Vinorelbine (nM)	24	85 ± 15	77 ± 21	1.1 ± 0.2	0.62

Figure 2 Effect of c-Abl kinase on drug sensitivity. The sulforhodamine B growth-rate assay was used to determine the effect of anticancer drugs in 3T3 cells. ● = 3T3-Abl⁺ cells; ○ = 3T3-mock cells. Data points represent mean ± SD of at least three independent experiments performed with triplicate cultures

paclitaxel caused a rapid and marked induction of p21^WAF1 which peaked at 24 h. In Abl-deficient cells, however, paclitaxel had no effect on the level of expression of p21^WAF1 at 24 h; at later points in time, overexpression of p21^WAF1 was observed.

Effect of c-Abl kinase and paclitaxel on p53 expression

p53 has been shown to regulate the expression of p21^WAF1 (El-Deiry et al, 1993). In paclitaxel-treated HCT-116 cells, p53 expression remained unchanged during the first 6 h, increased at 12 and 18 h, peaked at 24 h, and subsequently decreased rapidly (Stewart et al, 1999). To determine whether induction of p53 was required for paclitaxel-induced overexpression of p21^WAF1, we examined the effect of paclitaxel on p53 expression in 3T3-mock and 3T3-Abl⁺ cells (Figure 5). Cells were exposed to 160 nM paclitaxel for 24 h and cell lysates prepared at 24, 48, and 72 h were examined for p53 protein levels using an anti-p53 antibody. In three separate experiments paclitaxel had no significant effect on p53 expression in 3T3-mock and 3T3-Abl⁺ cells.

Effect of c-Abl kinase and paclitaxel on MAPK activity

Paclitaxel has been shown to activate MAPK without altering the level of MAPK expression (Ding et al, 1996; Wang et al, 1998; Shtil et al, 1999). MAPK has been shown to be an upstream regulator of p21^WAF1 (Blagosklonny et al, 1995). We therefore investigated the effect of c-Abl kinase and paclitaxel on MAPK activity in 3T3-mock and 3T3-Abl⁺ cells (Figure 5). MAPK activity was determined by immunoblotting using an antibody directed against
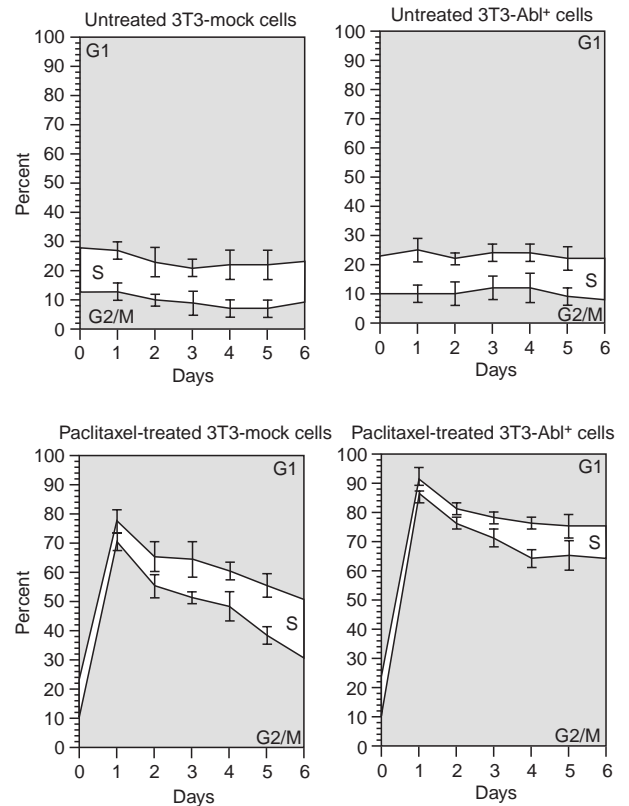

Figure 3 Effect of c-Abl kinase and paclitaxel on cell-cycle phase distribution. Cells were exposed to 160 nM paclitaxel for 24 h and harvested at the indicated times. The cell cycle phase distribution was determined by flow cytometry. Data points represent mean ± SD of three different experiments
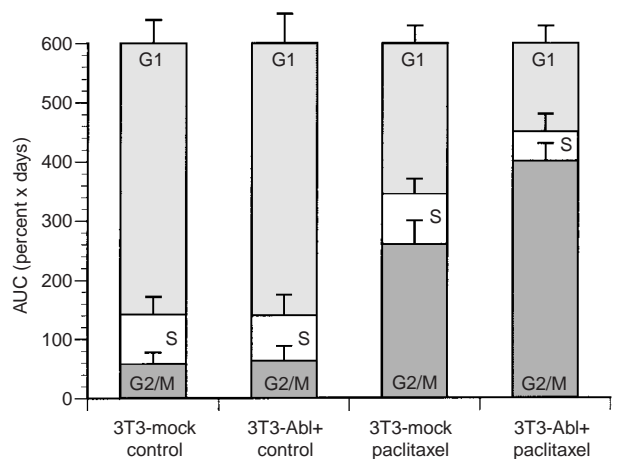

Figure 4 Effect of c-Abl kinase on paclitaxel-induced G2/M arrest. Each phase of the cell cycle is expressed as the area under the curve of fraction of cells over time (percent × days). The AUC for the G2/M phase was 1.6 ± 0.2-fold larger in paclitaxel-treated Abl-proficient cells relative to paclitaxel-treated Abl-deficient cells ($n = 3$, $P = 0.008$, by two-sided t-test). Columns represent mean ± SD of three different experiments

the phosphorylated form of MAPK. The fully phosphorylated MAPK protein served as a positive control. Cells were exposed to
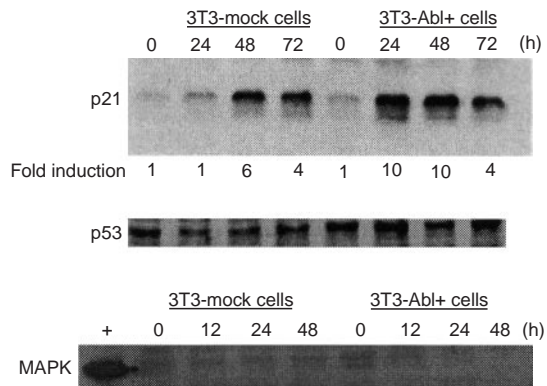
Figure 5 Effect of c-Abl kinase and paclitaxel on p21^{WAF1} and p53 expression and MAPK phosphorylation. Cells were exposed to 160 nM paclitaxel for 24 h and harvested at the indicated times after the beginning of drug exposure. Time-point 0 h represents untreated control cells. Cellular proteins were analysed by SDS PAGE and Western blotting using antibodies against p21^{WAF1}, p53 and the phosphorylated form of MAPK. The fully phosphorylated MAPK protein was used as a positive control (+). Experiments were repeated three times with similar results

160 nM paclitaxel for 24 h. Paclitaxel had no effect on MAPK phosphorylation in 3T3-mock and 3T3-Abl$^+$ cells. These findings indicate that, in 3T3 cells, upregulation of p21^{WAF1} by paclitaxel is mediated by a pathway that is independent of MAPK.

DISCUSSION

DNA damage caused by ionizing radiation and other DNA-damaging agents has been shown to result in activation of the c-Abl tyrosine kinase (Baskaran et al, 1997; Shafman et al, 1997). We now report that, in addition to DNA damage, microtubule damage induced by paclitaxel results in activation of c-Abl kinase. Even though both DNA damage and microtubule damage activate c-Abl kinase, the signaling pathways activated by the two types of cellular injury differ in several respects. For instance, in 3T3 cells, DNA-damaging agents activate the MAPK pathway (Nehme et al, 1997), whereas paclitaxel-induced microtubule damage did not.

Recent work has provided some insight related to the mechanism(s) by which DNA damage is detected. We have previously shown that activation of JNK and c-Abl by cisplatin is dependent on the integrity of DNA mismatch repair function, indicating that DNA mismatch repair proteins serve as a detector for DNA damage due to cisplatin (Nehme et al, 1997). The mechanism by which microtubule damage is detected, and the more upstream events that lead to activation of c-Abl in response to microtubule damage, are not understood.

In order to investigate the role of c-Abl in the cellular response to microtubule damage, we determined the effect of c-Abl kinase on the sensitivity of 3T3 cells to microtubule-damaging agents and found that loss of c-Abl kinase confers resistance to paclitaxel. Several mechanisms of acquired paclitaxel resistance have been described in cells made resistant by prolonged treatment at low drug concentrations. Paclitaxel-resistant CHO cells with structurally altered α and/or β tubulin and impaired ability to polymerize tubulin dimers into microtubules have been described

(Cabral and Barlow, 1991). A second mechanism of acquired paclitaxel resistance fits the general pattern of multiple drug resistance (MDR) (Horwitz et al, 1993). Our results indicate that loss of c-Abl kinase is another mechanism resulting in resistance to paclitaxel.

Interestingly, c-Abl had no effect on the sensitivity of 3T3 cells to docetaxel. This finding is intriguing, since the chemical structures of paclitaxel and docetaxel are very similar, and since both are anti-microtubule agents that promote accelerated assembly of excessively stable microtubules, thus affecting microtubule-dependent cellular functions like the control of mitosis and intracellular transport (Rowinsky and Donehower, 1996). However, the two taxanes differ in several respects. Docetaxel has a higher affinity for microtubules compared with paclitaxel (Lavelle et al, 1995). In preclinical models, paclitaxel and docetaxel differ with respect to in vitro cytotoxicity and tumour cell-kill in xenograft models (Schimming et al, 1999). Furthermore, in prostate cancer cells, docetaxel is capable of inducing bcl-2 phosphorylation and apoptotic cell death at 100-fold lower concentrations than paclitaxel (Haldar et al, 1997).

In addition to regulating the sensitivity of 3T3 cells to paclitaxel, c-Abl had marked effects on the cell cycle arrest induced by paclitaxel. In Abl-proficient cells, paclitaxel induced a marked and prolonged G2/M arrest. In Abl-deficient cells, paclitaxel induced a G2/M arrest that was much less pronounced and considerably shorter in duration. The presence of c-Abl kinase resulted in a 1.6-fold increase in paclitaxel-induced G2/M arrest, as quantitated by the ratio of the area under the curve of the fraction of cells in G2/M phase over time, indicating that the extent and duration of the paclitaxel-induced G2/M arrest are dependent on c-Abl tyrosine kinase activity.

In order to understand the mechanism by which c-Abl regulates the G2/M arrest caused by microtubule damage, we determined the effect of c-Abl and paclitaxel on p21^{WAF1} expression. In Abl-proficient cells, paclitaxel caused a rapid and marked induction of p21^{WAF1} which peaked at 24 h. Similar time courses of p21^{WAF1} induction by paclitaxel have been described in human breast and prostate cancer cells (Blagosklonny et al, 1995). In Abl-proficient cells, the time course of paclitaxel-induced overexpression of p21^{WAF1} corresponded well with the time course of paclitaxel-induced G2/M arrest which both peaked at 24 h. In Abl-deficient cells, paclitaxel caused no overexpression of p21^{WAF1} at 24 h; however, at later points in time, overexpression of p21^{WAF1} was observed. These findings indicate that c-Abl promotes the G2/M arrest in response to paclitaxel-induced microtubule damage by regulating the expression of p21^{WAF1}.

In Abl-deficient cells, paclitaxel caused a G2/M arrest at 24 h without inducing p21^{WAF1} expression. This observation can be explained by the activation of pathways that are independent of c-Abl and p21^{WAF1}. For instance, cellular injury has been shown to induce the ATM-mediated phosphorylation of chk2 as well as phosphorylation of chk1, both of which phosphorylate cdc25C, promoting its cytoplasmic sequestration (Shapirow and Harper, 1999). As a consequence, cdc25C cannot dephosphorylate cdc2, which remains in an inactive state, resulting in G2/M arrest.

The increase in p21 level at 48 and 72 h in paclitaxel-treated, Abl-deficient cells can be explained by the activation of Abl-independent pathways. For instance, increased p21^{WAF1} mRNA stability has been demonstrated in RKO cells following cellular injury (Wang et al, 2000). Investigation into the mechanisms

underlying this stabilization process revealed that proteins present in cytoplasmic lysates formed complexes with p21^WAF1 mRNA that were inducible in response to cellular injury. The RNA-binding protein HuR was identified within p21 mRNA–protein complexes (Wang et al, 2000).

In 3T3 cells, induction of p21^WAF1 by paclitaxel was not associated with overexpression of p53. This finding is in line with other reports, indicating that p21^WAF1 may be induced by p53-independent pathways. For instance, p53-independent induction of p21^WAF1 was observed in other cell lines, including breast and prostate carcinoma cells treated with paclitaxel (Blagosklonny et al, 1995), human breast carcinoma cells treated with etoposide (Sheikh et al, 1994), HaCat cells exposed to transforming growth factor β (Datto et al, 1995), and in KG-1 cells treated with γ-rays or tumour-necrosis factor α (Akashi et al, 1995).

One potential mechanism by which paclitaxel regulates the expression of p21^WAF1 is the MAPK signaling pathway. Two recent studies have reported that activation of the MAPK protein is involved in induction of p21^WAF1 in response to oxidative stress (Esposito et al, 1997), and to paclitaxel (Blagosklonny et al, 1995). Raf-1 kinase, a principal upstream regulator of MAPK activity, has been shown to be responsible for paclitaxel-induced overexpression of p21^WAF1 (Blagosklonny et al, 1995). However, our results in 3T3 cells show that induction of p21^WAF1 by paclitaxel was not mediated by the MAPK pathway, since paclitaxel had no significant effect on MAPK phosphorylation.

We conclude that, in addition to DNA damage, paclitaxel-induced microtubule damage results in activation of c-Abl kinase. c-Abl kinase plays an important role in the cellular response to microtubule damage; c-Abl kinase regulates the sensitivity of 3T3 cells to paclitaxel and regulates paclitaxel-induced G2/M arrest in a pathway that is dependent on p21^WAF1.

ACKNOWLEDGEMENTS

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