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Inactivation of Cdc2 increases the level of apoptosis induced by DNA damage

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SUMMARY

A number of lines of evidence have suggested a possible involvement of the mitosis-promoting protein kinase Cdc2 in the process of apoptotic cell death, and one recent study concluded that premature activation of Cdc2 is required for apoptosis. Here we have used a temperature-sensitive murine Cdc2 mutant cell line and Cdc2 inhibitor compounds to study the effect of inhibition of this protein kinase on apoptosis induced by DNA-damaging drugs. Inhibition of Cdc2 activity before or during exposure to DNA strand break-inducing drugs had the effect of increasing the level of subsequent apoptosis, as assessed by electron microscopy and flow cytometry. We conclude that, far from being required for cell death, a form of mammalian Cdc2 suppresses apoptosis induced by DNA damage. This form of Cdc2 appears to be active in G_2-arrested cells and is therefore presumably distinct from the mitosis-promoting Cdc2-cyclin B heterodimer.

Key words: apoptosis, Cdc2, cell cycle, checkpoint, mitoxantrone, olomoucine

INTRODUCTION

Cell death by apoptosis is a feature of a wide variety of normal physiological processes including, for example, neurogenesis and limb development in embryos, and the elimination of self-reactive T lymphocytes (Cowan et al., 1984; Glucksmann, 1951; Kerr, 1971; MacDonald and Lees, 1990; Wyllie et al., 1980). This mode of cell death is distinguished by specific ultrastructural changes, including a characteristic pattern of peripheral chromatin condensation within an intact nuclear envelope, and disappearance of the nuclear lamina (Kerr, 1971; Lazebnik et al., 1993; Ucker et al., 1992). Apoptotic death is also a common response to several classes of cytotoxic agents, including DNA-damaging drugs of importance in anticancer therapies (Hickman, 1992; Walker et al., 1990). Drug-induced DNA damage frequently results in cell cycle arrest in the pre-mitotic G_2 phase before the onset of apoptosis (Eastman, 1990; Lock and Ross, 1990). This observation, combined with broad similarities between mitosis and apoptosis, which both involve chromatin condensation and disassembly of the nuclear lamina, has led to the suggestion that these two processes might involve related molecular mechanisms (Eastman, 1990; Lock and Ross, 1990; Ucker, 1991). A recent report documenting work performed using a model of cytotoxic T-lymphocyte-mediated cell death, thought to represent a form of apoptosis, lent weight to this idea; the authors concluded that the Cdc2 protein kinase is required for cell death in this system (Shi et al., 1994). Furthermore, transient activation of Cdc2 has been reported as an early event in DNA damage-induced apoptosis (Shimizu et al., 1995).

In fission yeast the Cdc2 protein kinase determines the cell cycle timing of mitosis (Nurse and Thuriaux, 1980), and is also required in G_1 for commitment to the mitotic cell cycle, as opposed to the alternative developmental option of conjugation (Nurse and Bissett, 1981). Cdc2 has been conserved during eukaryotic evolution, and mammalian Cdc2 can perform both the G_1 and mitotic functions when substituted for its homologue in fission yeast (Lee and Nurse, 1987; MacNeill and Nurse, 1993; Nurse, 1990). Despite this apparent conservation of function, mammalian Cdc2 is thought to perform only the mitotic role in mammalian cells (Hamaguchi et al., 1992), with other cyclin-dependent kinases (CDKs) perhaps performing cell cycle commitment functions (Sherr, 1993; Vandenheuvel and Harlow, 1993).The mitosis-promoting kinase is a Cdc2-cyclin B heterodimer (Gautier et al., 1988; Labbe et al., 1989; Norbury and Nurse, 1992; Nurse, 1990), though Cdc2 also forms complexes with A-type cyclins in higher eukaryotes (Draetta et al., 1989; Minshull et al., 1990). Cyclin B is required for entry into mitosis in both simple and complex eukaryotes (Hagan et al., 1988; Minshull et al., 1989; Murray and Kirschner, 1989), while cyclin A appears to be restricted to multicellular organisms, and has been assigned roles in S phase entry or progression, entry into mitosis and suppression of cyclin B-associated activity (Girard et al., 1991; Knoblich and Lehner, 1993; Minshull et al., 1990; Pagano et al., 1992; Walker and Maller, 1991).

Activation of Cdc2 under different circumstances could conceivably lead either to mitosis or to apoptosis, but data from a variety of systems have cast doubt on the general applicability of this model. Apoptosis was found to occur in postmitotic neurons in the absence of detectable Cdc2 expression (Freeman et al., 1994), and in both immature thymocytes and serum-deprived fibroblasts, apoptosis proceeded without activation of the low levels of Cdc2 present in these cells (Norbury et al.,

1994; Oberhammer et al., 1994). Furthermore, disassembly of the nuclear lamina in apoptotic cells has been found to result from proteolytic degradation of the lamin proteins, which are by contrast solubilised as intact monomers in mitosis (Kaufmann, 1989; Oberhammer et al., 1994).

In this study we have investigated the involvement of Cdc2 in apoptosis induced by DNA-damaging drugs, using a murine temperature-sensitive mutant cell line with thermolabile Cdc2, or either of two Cdc2 inhibitor compounds to deplete Cdc2 activity. In line with our earlier findings in rat thymocytes (Norbury et al., 1994), we find that Cdc2 activity is not required for apoptosis in a murine mammary carcinoma cell line. Surprisingly, apoptosis proceeded more rapidly when Cdc2 was inhibited, revealing a hitherto unsuspected function for mammalian Cdc2 in the suppression of cell death.

MATERIALS AND METHODS

Cell culture and flow cytometry

HL-60, FM3a and *ts*FT210 cells (Mineo et al., 1986) were cultured in RPMI medium supplemented with 10% fetal calf serum in a 5% CO_2 atmosphere at 32°C (FM3a and *ts*FT210) or 37°C (FM3a and HL-60). Staining of methanol/acetone fixed cells with Hoescht 33258 was used to confirm that the cell lines were free from mycoplasma contamination. In some experiments olomoucine (OLO, kindly provided initially by L. Meijer, and subsequently obtained from Promega) was added from a 100 mM stock solution in dimethyl sulphoxide (DMSO); control cultures received an equivalent volume of DMSO. Where used, mitoxantrone (MTN) was added from a stock solution in RPMI medium. Cells were harvested by centrifugation at 3,000 ***g*** and prepared for flow cytometry analysis exactly as described by Hotz et al. (1994) by fixing in cold 50% ethanol for 1 hour at –20°C and then resuspending in phosphate-buffered saline containing 0.1% Triton X-100. This protocol allows low molecular mass products of DNA degradation to diffuse from the fixed cells before cytometry; the residual nuclear DNA content of apoptotic cells that have undergone internucleosomal DNA cleavage is consequently less than that of cells in which the DNA remains intact. The cell suspensions were then incubated for 30 minutes at room temperature after the addition of propidium iodide (40 μg/ml) and RNAse A (100 μg/ml) before analysis using a FACScan (Becton Dickinson). Each histogram represents 10,000 cells. A gate was set using forward light scatter to exclude small fragments, and the proportion of cells undergoing apoptosis in each population was estimated by quantification of cells with an apparent DNA content less than that of untreated cells in G_1. This method has been shown to be as sensitive in detection of apoptotic cells as methods based on in situ end labelling with terminal transferase (Hotz et al., 1994), and the validity of this assay for the cell lines used here was confirmed by comparison with results obtained using electron microscopy.

Electron microscopy

Mid-log phase cultures of FM3a and *ts*FT210 cells grown at 32°C were shifted to 40°C for 15 hours, and then held at 40°C for a further 24 hours in the presence or absence of 50 ng/ml MTN. Cells were harvested by centrifugation, and fixed by resuspension in 4% glutaraldehyde in 0.1 M phosphate buffer. Samples were post fixed in osmium tetroxide, dehydrated in ethanol, treated with propylene oxide and embedded in Spurr's epoxy resin. Thin sections were stained with uranyl acetate prior to examination with a Jeol 100CX electron microscope. For quantitation, cells within randomly-selected grid squares were counted and scored for the features of interphase, mitotic, apoptotic or necrotic cells. Cells scored as apoptotic exhibited intact nuclear membranes and nucleoli with peripheral condensation of the chromatin. Mitotic cells had clearly defined, individual condensed chromosomes and lacked both nuclear membranes and nucleoli, while necrotic cells exhibited cellular disintegration in the absence of the hallmarks of apoptosis.

RESULTS

In order to investigate the influence of Cdc2 activity on induction of apoptosis, we first compared the appearance of the temperature-sensitive murine Cdc2 mutant *ts*FT210 (Mineo et al., 1986; Th'ng et al., 1990) with that of the parental FM3a mammary carcinoma cell line, after exposure of the cells to DNA damage induced by the topoisomerase II inhibitor mitoxantrone (MTN; Fig. 1). The *CDC2* gene in *ts*FT210 cells carries a single point mutation that renders the Cdc2 protein kinase thermolabile in vitro after synthesis in mouse or fission yeast cells (Th'ng et al., 1990; C. Norbury, unpublished observations). Cdc2 protein is also degraded in *ts*FT210 cells at the restrictive temperature, and was undetectable by four hours after temperature-shift (Hamaguchi et al., 1992; Th'ng et al., 1990), resulting in accumulation of cells in G_2 (Figs 1d, 2c). The temperature-sensitive phenotype of *ts*FT210 can be transiently suppressed by introduction of a plasmid-borne copy of the wild-type *CDC2* cDNA driven by the SV40 promoter, indicating that no additional temperature-sensitive lesions exist in essential cell cycle functions in this line (Th'ng et al., 1990).

Electron microscopy of thin sections was used to detect the pattern of chromatin condensation at the nuclear periphery that is characteristic of apoptosis; this technique also allowed the identification of mitotic and necrotic cells. Exposure of the parental FM3a cells to 24 hours MTN treatment (50 ng/ml) at 40°C generated DNA strand breaks that induced a G_2 cell cycle 'checkpoint' arrest, revealed as a loss of mitotic cells from the population (Fig. 1d; see also Fig. 2b), with a small proportion becoming apoptotic or necrotic by the same time. After incubation at 40°C for 39 hours, the *ts*FT210 cells were arrested in G_2, lacking sufficient Cdc2 activity to enter mitosis (Fig. 1d; see also Fig. 2c). Strikingly, exposure of these cells to MTN resulted in approximately nine-fold more apoptosis than was seen in the parental cell line treated in the same way (Fig. 1d). This was an unexpected result, not least because we had imagined that complete inhibition of Cdc2 might have the effect of reinforcing the G_2 checkpoint arrest, and consequently increase cell survival after DNA damage. The level of necrotic cells was also elevated in the MTN-treated *ts*FT210 cells; these cells presumably include those that would have scored as apoptotic at an earlier time point, but which subsequently underwent secondary necrosis.

In order to characterize further the effect of Cdc2 inhibition on the onset of apoptosis, Cdc2 was inhibited for progressively increasing periods during a fixed period of exposure to DNA damage induced by the topoisomerase II inhibitors MTN (Fig. 2) and teniposide (VM26; not shown). At the doses used, these drugs induced a G_2 checkpoint arrest in FM3a after 48 hours of drug exposure at either 32 or 40°C (Fig. 2b; +MTN, 4n peak), or in *ts*FT210 cells exposed to the drugs for the same time at 32°C (Fig. 2c). In the absence of drugs, inactivation of Cdc2 in *ts*FT210 by shifting from 32° to 40°C also caused protracted G_2 arrest (Fig. 2c; –MTN);

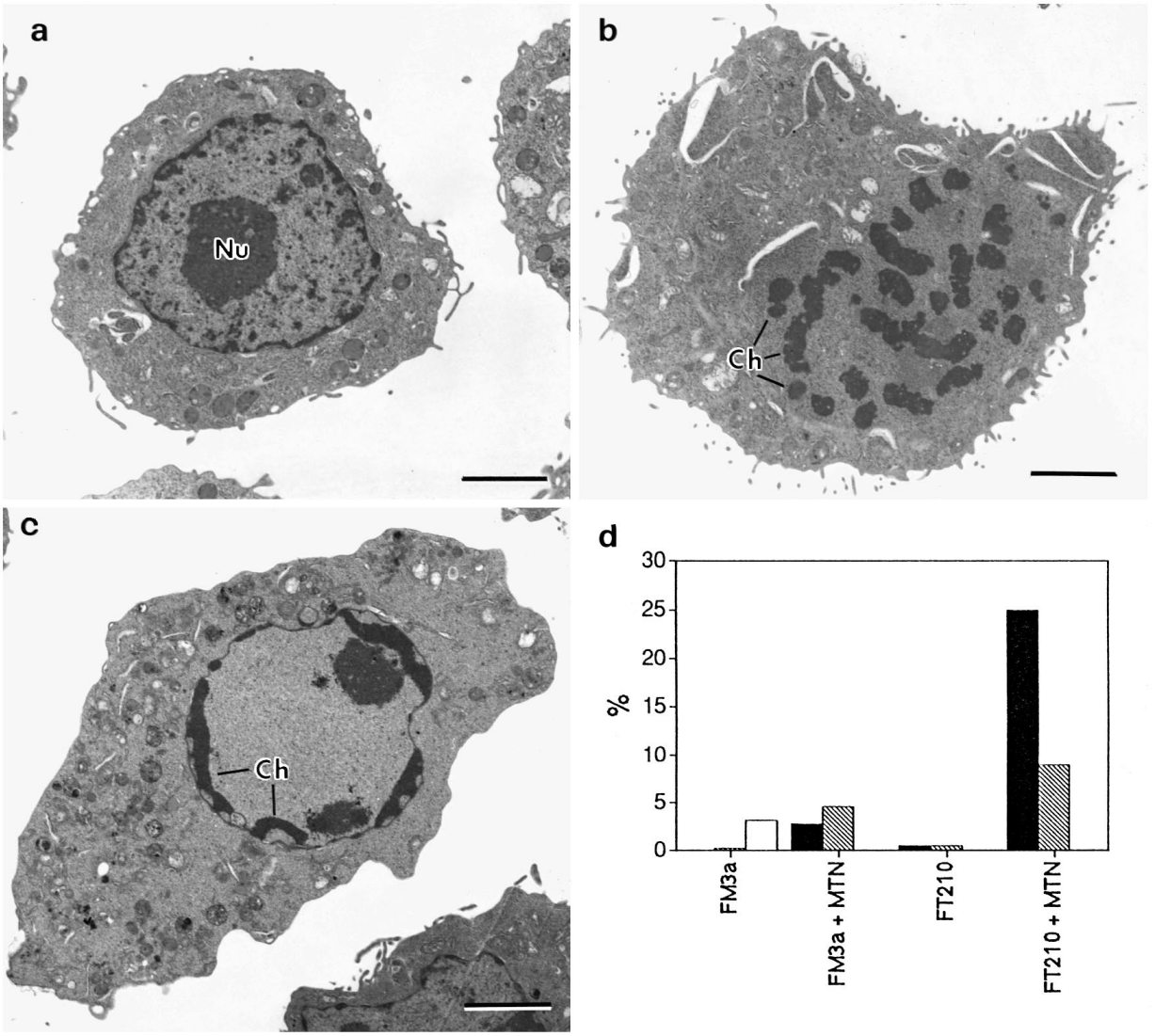

Fig. 1. Inhibition of Cdc2 before exposure to DNA damage causes acceleration of apoptosis. Mid-log phase cultures of FM3a and *ts*FT210 cells grown at 32°C were shifted to 40°C for 15 hours, and then held at 40°C for a further 24 hours in the presence or absence of 50 ng/ml MTN. Cells were harvested by centrifugation, then fixed; thin sections were examined by electron microscopy. (a) Example of an interphase FM3a cell showing the intact nuclear membrane, dispersed chromatin and large nucleolus (Nu). (b) Mitotic FM3a cell in early metaphase exhibiting condensed chromatin in individual chromosomes (Ch) and absence of both nuclear membrane and nucleolus. (c) Apoptotic *ts*FT210 cell showing the characteristic peripherally condensed chromatin (Ch) with an intact nuclear membrane and nucleolus. Bars, 3 μm. (d) Indices of apoptosis and mitosis in FM3a and *ts*FT210 cells in the presence (+ MTN) and absence of MTN. For each sample between 340 and 940 cells were scored; the proportions of mitotic (open bars), necrotic (hatched bars) and apoptotic cells (filled bars) are expressed as percentages of the total number of cells in each case.

after 63 hours at the restrictive temperature a small fraction of these G_2-arrested *ts*FT210 cells became apoptotic. By contrast, exposure of temperature-shifted *ts*FT210 cells to MTN led to very high levels of apoptosis, as measured by a flow cytometric method, in which accumulation of cells with a DNA content less than that of G_1 cells is used as a measure of apoptotic cell death (Fig. 2c; +MTN). The dead cells exhibited fragmented, condensed nuclei and internucleosomal DNA cleavage, features typical of apoptotic cell death (Fig. 1 and data not shown). After subtraction of background levels of apoptosis (those seen after temperature shift in the absence of drug), up to three-fold higher levels of MTN-induced apoptosis were seen in *ts*FT210 than in FM3a (Fig. 2d). This enhancement of apoptosis was particularly apparent when Cdc2 was inactivated before addition of the DNA-damaging drug, but *ts*FT210 cells shifted to 40°C either at the time of drug addition or shortly thereafter also displayed elevated levels of apoptosis by the end of the period of drug treatment. Similar results were obtained using VM26 or *cis*-diamminedichloroplatinum (II), which induces damage primarily by the generation of interstrand DNA crosslinks (not shown).

Could the observed enhancement of apoptosis upon inactivation of Cdc2 reflect an increased sensitivity in G_2 to the

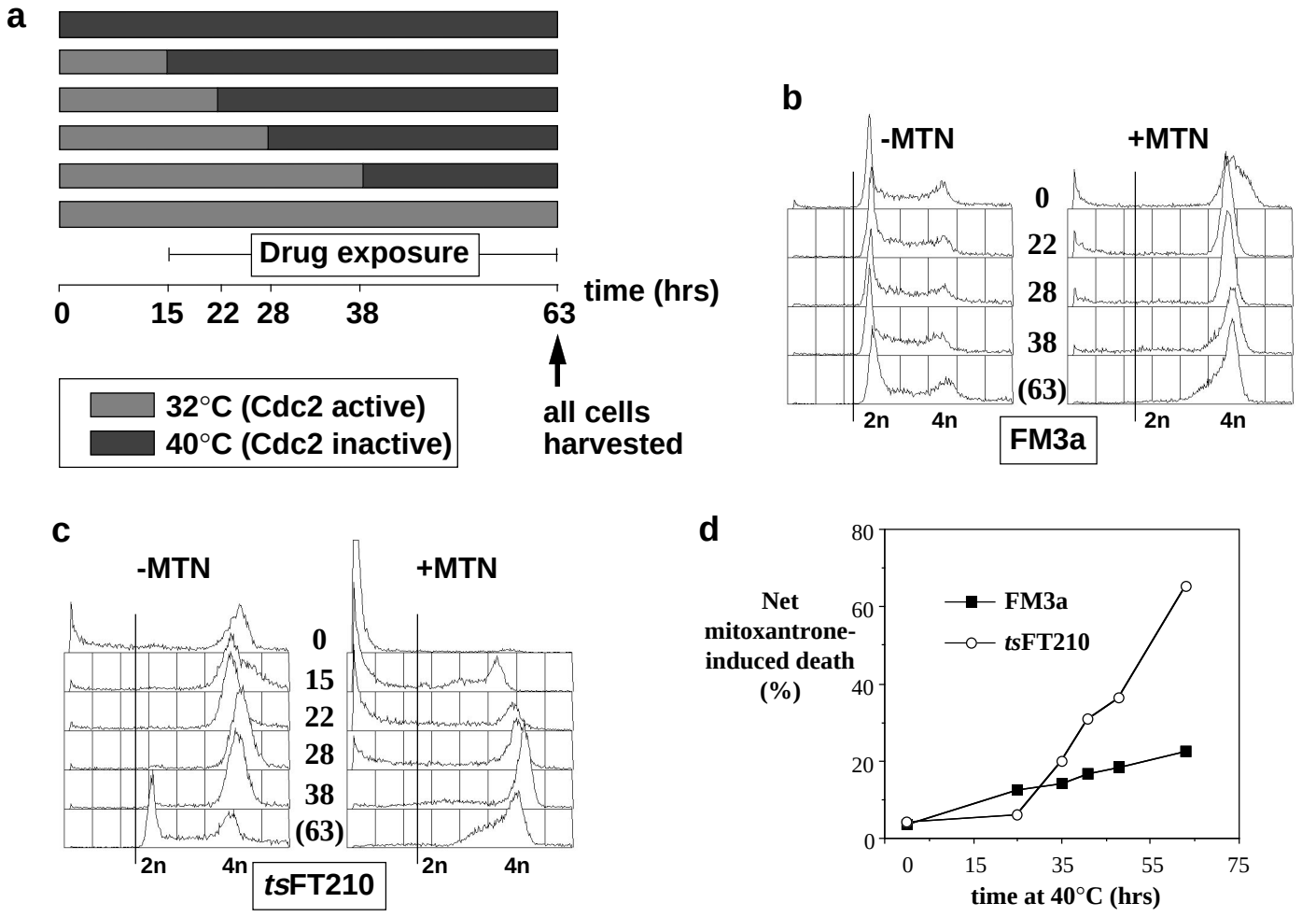

Fig. 2. The effect of progressively increasing the period of Cdc2 inhibition during a fixed period of exposure to DNA damage. (a) Experimental design. Parallel cultures of FM3a or *ts*FT210 cells were cultured at 32°C, or shifted to 40°C at 0, 15, 22, 28 or 38 hours, before being harvested at 63 hours and analysed by propidium iodide staining and flow cytometry. For each cell type, one set of parallel cultures was exposed to MTN (50 ng/ml) between 15 and 63 hours. Shifting to 40°C inactivates Cdc2 in *ts*FT210, but not in FM3a. (b) Effect of temperature shift during MTN treatment of FM3a cells. FM3a cells were cultured as described above in the presence (+) or absence (–) of MTN. Flow cytometry profiles are shown for pairs of cultures shifted to 40°C at each of the times shown (hours) either before (0) or after (22, 28, 38) addition of MTN; one pair (63) remained at 32°C for the full duration of the experiment. The positions of 2n (G_1) and 4n (G_2/M) peaks are indicated; the vertical line to the left of the 2n peak marks the boundary between cells with a normal DNA content and those (to the left of this line) in which apoptotic DNA degradation has begun. (c) Effect of temperature shift on MTN-induced death in mouse mammary carcinoma (*ts*FT210) cells with a temperature-sensitive Cdc2 protein. Cells were treated exactly as described in (b). Inactivation of Cdc2 before, or at early times during the period of exposure to MTN generates higher levels of apoptosis than seen in FM3a cells exposed to the same conditions (b). (d) Graphical representation of results presented in (b) and (c) (average of two separate experiments). Percentages of cells with less than normal G_1 DNA content were calculated from the flow cytometry data. Net MTN-induced death (ordinate) is calculated by subtracting death (percentage of all cells) resulting from temperature shift alone from that seen with the drug. MTN was added in all cases after 15 hours and was present for the subsequent 48 hours. Inhibition of Cdc2 before addition of MTN resulted in a three-fold increase in the level of MTN-induced death seen at 63 hours.

drugs used? This is unlikely as, in cells with normal Cdc2 function, MTN and VM26 appear to be maximally cytotoxic in cells traversing S phase (Chow and Ross, 1987; D'Arpa et al., 1990; Gorczyca et al., 1993). Furthermore, examination of the relative numbers of topo II-associated strand breaks showed no significant difference between FM3a and *ts*FT210 cells in the levels of DNA damage induced by VM26 at 40°C (not shown), suggesting that the observed enhancement of apoptosis represents an altered response to a fixed level of the primary apoptotic stimulus. Furthermore, MTN treatment of FM3a (or *ts*FT210 at 32°C) resulted in protracted G_2 arrest without induction of high levels of apoptosis comparable with those seen on MTN treatment of *ts*FT210 with inactive Cdc2 (Fig. 2b,c), demonstrating that G_2-arrested cells are not inherently sensitive to drug exposure. Thus the observed enhancement of apoptosis would appear to be a direct consequence of Cdc2 inactivation.

As *ts*FT210 may be unusual in its response to combined DNA damage and Cdc2 inhibition, we were interested to determine if a similar response would be seen in distinct cell lines, particularly those of human origin. Unfortunately, conditional human cell Cdc2 mutants are not yet available;

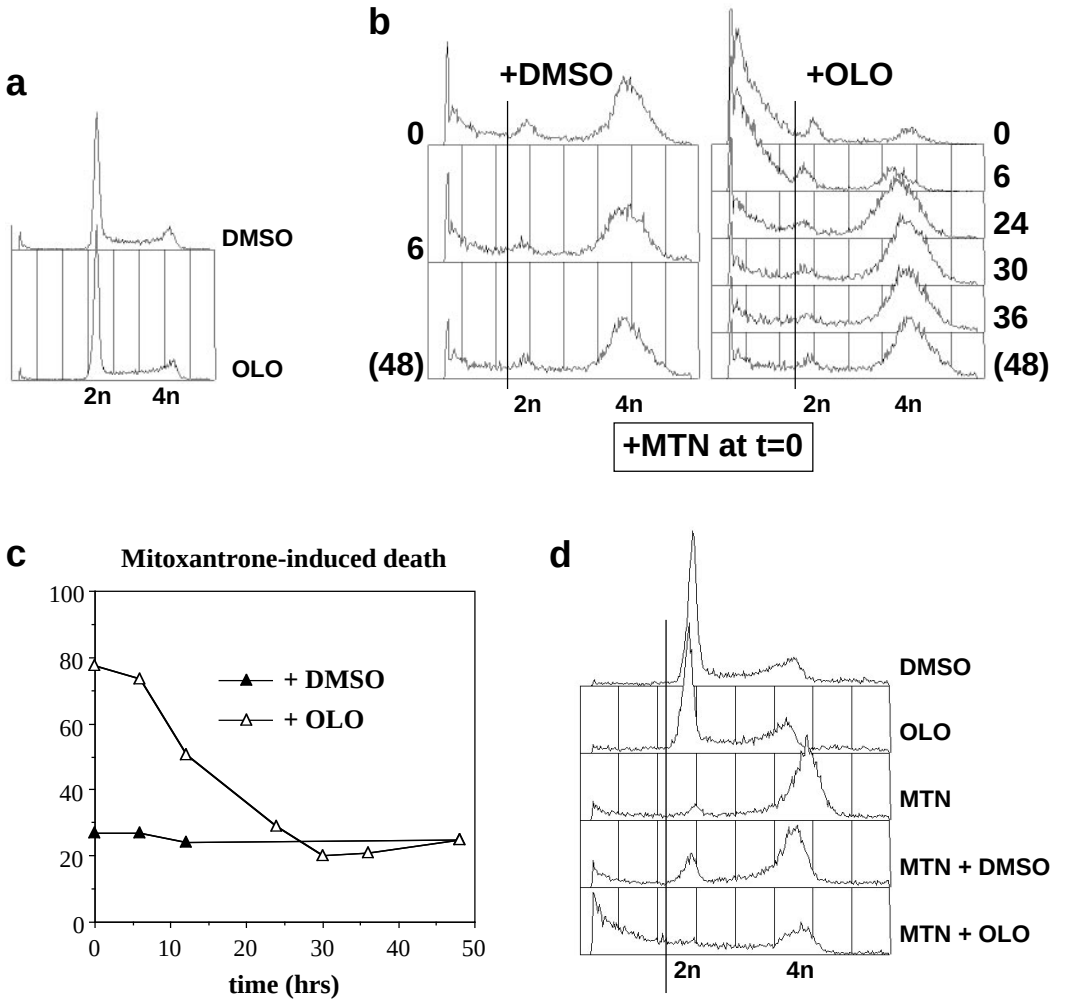

Fig. 3. Effects of the selective CDK inhibitor olomoucine (OLO) on human promyelocytic leukaemia (HL-60) cells and mouse FM3a cells. (a) Lack of significant cell cycle perturbation on addition of OLO to HL-60 cells. Cells were exposed to OLO (final concentration 50 μM), from a 100 mM stock dissolved in DMSO or an equivalent volume of DMSO for 24 hours before analysis by propidium iodide staining and flow cytometry. OLO treatment resulted in no significant alteration in cell cycle distribution. (b) Effect of OLO on MTN-induced death in HL-60 cells. All cells were exposed to MTN (25 ng/ml) for 48 hours, administered at time zero. OLO (final concentration 50 μM) or an equivalent volume of DMSO was added at the times indicated after MTN addition. Cells were harvested at 48 hours and processed for propidium iodide staining and flow cytometry. MTN treatment at this level generates a population of cells mainly arrested in G_2 at 48 hours (left hand panel). Simultaneous administration of OLO (in itself comparatively non-toxic; see a) resulted in a high level of apoptosis (right hand panel). (c) Graphical representation of the results presented in (b). Percentages of cells with less than the normal G_1 DNA content were calculated from the flow cytometry data, as in Fig. 2d. Addition of OLO at time 0 led to a three-fold increase in the level of death measured at 48 hours. (d) Effect of OLO on MTN-induced death in FM3a cells. Cells were exposed to DMSO (solvent control), OLO (50 μM), MTN (50 ng/ml) or the combinations indicated for 48 hours at 37°C, before being harvested and analysed by flow cytometry as above. As in HL-60, OLO alone had no significant effect on cell cycle distribution. Percentages of apoptotic cells were 12.5 (MTN), 14.0 (MTN + DMSO) and 47.8 (MTN + OLO).

instead, we made use of olomoucine (OLO), an inhibitory compound selective for cyclin-dependent kinases including Cdc2 in vitro (Glab et al., 1994). Biochemical demonstration of inhibition of Cdc2 by this drug in vivo presents technical difficulties, as the inhibition is reversible upon cell lysis; however, if Cdc2 is inhibited sufficiently in vivo, entry into mitosis should be blocked. When administered at 50 μM, OLO did not significantly affect the cell cycle distribution of FM3a or human promyelocytic leukemia (HL-60) cells (Fig. 3a,d). Nonetheless, simultaneous administration of OLO and MTN induced at least 3-fold more apoptosis at 48 hours than MTN alone, which again induced a G_2 arrest in most cells after the same time (Fig. 3b,d). The observed increase in apoptosis is necessarily p53-independent in the case of HL-60 cells, which lack functional p53 (Sen et al., 1993). Similar results (not shown) were obtained using the structurally distinct Cdc2 inhibitor butyrolactone I (Kitagawa et al., 1993).

DISCUSSION

We have shown that inhibition of Cdc2 during exposure of mammalian cells to DNA damage-inducing drugs accelerates the onset of apoptosis. One implication of this finding is that Cdc2 is not required for cell death. This is in line with recently reported negative correlations between apoptotic index and Cdc2 activity or expression (Freeman et al., 1994; Norbury et al., 1994; Oberhammer et al., 1994), but contrasts directly with the conclusions of Greenberg and colleagues (Shi et al., 1994), whose description of a requirement for Cdc2 in T-cell granule protease-induced death may reflect some feature of their specialized experimental system. A possible connection between the biochemistry of apoptosis and mitosis was also suggested by results obtained with a cell-free extract capable of inducing the morphological features of apoptosis in vitro (Lazebnik et al., 1993). Extraction of apoptosis-promoting activity required that cells were synchronized in mitosis but, significantly, Cdc2

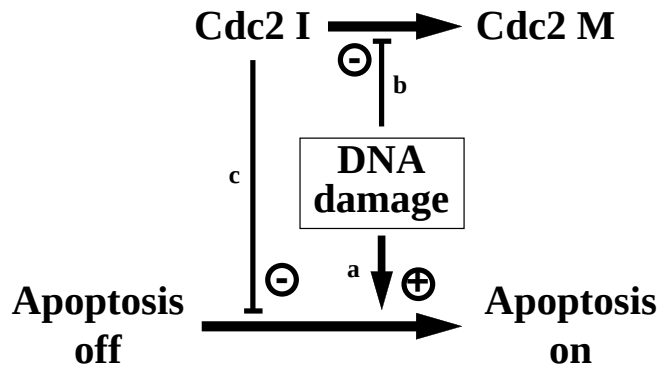

Fig. 4. A model for the interrelationship between DNA damage, Cdc2 activation and onset of apoptosis. DNA damage has the potential to induce apoptosis (a), which does not involve a general requirement for Cdc2 activation. In proliferating cells, DNA damage has the additional effect of blocking conversion of interphase (I) forms of Cdc2 to the mitotic (M) form (b). We propose that an interphase form of Cdc2 is capable of suppressing onset of apoptosis (c). For discussion see text.

did not appear to be a component of this activity. Thus it seems possible that in some experimental systems, though not in ours, passage through some aspect of mitosis (as distinct from Cdc2 activation) is required for the efficient expression of the apoptotic programme.

At the drug concentrations used in this study, the primary response of the cells to drug-induced DNA damage was to arrest in the G_2 phase of the cell cycle. This G_2 arrest appears to be a consequence of a checkpoint pathway leading to tyrosine phosphorylation and inactivation of the mitosis-promoting form of Cdc2 (our data not shown; see also Lock, 1992; Lock and Keeling, 1993; O'Connor et al., 1993; Thiebaut et al., 1994), though additional controls exist in some cell types that lead to reduced levels of cyclin B mRNA and protein in response to DNA damage (Muschel et al., 1993). During drug-induced inhibition of mitotic Cdc2, further depletion of Cdc2 by means of denaturation of a thermolabile Cdc2 protein had the effect of markedly elevating the level of apoptosis in response to a fixed level of DNA damage (Figs 1, 2). Thus an interphase form of Cdc2 distinct from the mitosis-promoting form appears to act to suppress apoptosis in cells arrested in G_2 in response to DNA damage (Fig. 4). Exposure of cells to moderate levels of DNA damage leads to activation of a checkpoint pathway that could block conversion of the putative apoptosis-suppressing form of Cdc2 to the mitosis-promoting form. In this way apoptosis and mitosis would be simultaneously suppressed, affording an opportunity for DNA repair and cell survival (Bernhard et al., 1994). At higher drug concentrations, apoptosis can be induced without significant cell cycle arrest (our data not shown; see also DelBino et al., 1991; Gorczyca et al., 1993). The activity of an interphase form of Cdc2 therefore appears to define a threshold level of DNA damage that determines the balance between the alternative responses of G_2 arrest and onset of apoptosis. Inhibition of this form of Cdc2 in cells exposed to DNA damage has the effect of reducing the threshold level of damage required to induce apoptosis. Interestingly, protracted inhibition of Cdc2 resulted in significant levels of apoptosis even in the absence of DNA-damaging drugs (Fig. 2c). Under these circumstances, the threshold level of DNA damage required to trigger apoptosis might be reduced to the background level found in G_2-arrested cells.

In yeasts, a non-mitotic function of Cdc2 is required for commitment to the cell cycle, with the alternative developmental fate being conjugation (Nurse and Bissett, 1981). We suggest that in mammals, too, Cdc2 has a non-mitotic function. In this case Cdc2 would act to promote continuation through the mitotic cycle, by suppressing pathway(s) leading to the alternative cellular fate of apoptotic cell death. A suppressive function for a CDK would not be without precedent; in fission yeast Cdc2/cyclin B suppresses S phase reinitiation (Broek et al., 1991; Hayles et al., 1994), while the distinct B-type cyclin Cyc17 suppresses conjugation (Obara-Ishihara and Okayama, 1994). Similarly, a role for cyclin A in the suppression of premature activation of Cdc2/cyclin B has been described (Walker and Maller, 1991), while in budding yeast Cdc28/Clb2 suppresses Swi4/Swi6-mediated transcription (Amon et al., 1993).

Administration of either of two Cdc2 inhibitor compounds, OLO or butyrolactone I, also had the effect of stimulating apoptosis in FM3a and human HL-60 cells exposed to MTN (Fig. 3). In comparison with distantly-related protein kinases, Cdc2 has been shown to be inhibited specifically by both of these inhibitors in vitro (Glab et al., 1994; Kitagawa et al., 1993). Other CDK family members are also likely to be inhibited by these compounds, however, so the enhancement of apoptosis that we have observed could in principle be due to inhibition of a CDK other than Cdc2. An alternative interpretation, supported by our results with the *ts*FT210 cell line described above, is that the levels of olomoucine and butyrolactone I used are sufficient to inhibit an apoptosis-suppressing form of Cdc2 effectively, but insufficient to inhibit the mitosis-promoting form of the protein kinase. Thus the effect of Cdk inhibition in enhancing apoptosis is not limited to one specific murine cell line, but may be of general significance in other mammalian systems. The enhancement of drug-induced apoptosis by simultaneous administration of Cdc2 inhibitors suggests a potential application for such compounds in increasing the cytotoxicity of anticancer drugs such as those used in this study. Further experiments will be required to establish the extent of this potential. It will be particularly interesting to determine the effects of such drug combinations on normal primary cells, in comparison with the transformed cell lines used here.

DNA damage-induced apoptosis depends on the function of the tumour suppressor gene product p53 in thymocytes (Clarke et al., 1993), but not in HL-60 human leukemic cells, in which p53 is inactive (Solary et al., 1994). As DNA-damaging drugs induce apoptosis in diverse tumour-derived cell lines, in which p53 function is frequently lost (Levine et al., 1991), it seems unlikely that there is a general requirement for p53 in DNA damage-induced cell death. We have not determined the p53 status of the FM3a and *ts*FT210 mammary carcinoma cells used in this study, though their failure to arrest in G_1 in response to DNA damage could indicate that they lack functional p53. The enhancement of apoptosis seen after administration of CDK inhibitors to MTN-treated HL-60 cells (Fig. 3) clearly does not require p53; it seems likely that the similar enhancement seen in *ts*FT210 after Cdc2 inhibition is also p53-independent.

It is not yet clear how the apoptosis-suppressing form of Cdc2 differs from the cyclin B-associated Cdc2 that drives cells into mitosis. In a number of systems, including mammalian cells, Cdc2 protein kinase activity has been found to increase to significant, though sub-mitotic, levels as cells proceed through interphase (see, for example, Marraccino et al., 1992). Thus there is at least the potential for non-mitotic funtions for Cdc2 in mammals, as in yeasts. One possibility would be that a different cyclin subunit, or some other as yet unidentified accessory protein, distinguishes the apoptosis-suppressing form. In this regard it is interesting that cyclin B- and cyclin A-associated kinase activities differ in their response to DNA damage (Muschel et al., 1993; O'Connor et al., 1993). Cyclin A-associated kinase activity appears to escape the checkpoint mechanism that mediates the inactivation of cyclin B/Cdc2, though the relative contributions of Cdc2 and the related Cdk2 to this checkpoint-resistant cyclin A-associated kinase may depend on the cell line used (Meikrantz et al., 1994; O'Connor et al., 1993). The results with olomoucine and butyrolactone I (Fig. 3), which inhibit Cdc2 by competition with ATP, suggest that Cdc2 protein kinase activity, rather than some hypothetical alternative function of the protein, is required to suppress apoptosis. If this is so, then identification of the relevant substrates for this form of Cdc2 should provide insight into the mechanism or mechanisms through which apoptosis is suppressed.

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REFERENCES

Amon, A., Tyers, M., Futcher, B. and Nasmyth, K. (1993). Mechanisms that help the yeast cell cycle clock tick: G2 cyclins transcriptionally activate G2 cyclins and repress G1 cyclins. *Cell* **74**, 993-1007.

Bernhard, E. J., McKenna, W. G. and Muschel, R. J. (1994). Cyclin expression and G2 phase delay after irradiation. *Radiat. Res.* **138**, S64-S67.

Broek, D., Bartlett, R., Crawford, K. and Nurse, P. (1991). Involvement of p34^{cdc2} in establishing the dependency of S phase on mitosis. *Nature* **349**, 388-394.

Chow, K. C. and Ross, W. E. (1987). Topoisomerase-specific drug sensitivity in relation to cell cycle progression. *Mol. Cell. Biol.* **7**, 3119-3123.

Clarke, A. R., Purdie, C. A., Harrison, D. J., Morris, R. G., Bird, C. C., Hooper, M. L. and Wyllie, A. H. (1993). Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature* **362**, 849-852.

Cowan, W. M., Fawcett, J. W., O'Leary, D. D. M. and Stanfield, B. B. (1984). Regressive events in neurogenesis. *Science* **225**, 1258-1265.

D'Arpa, P., Beardmore, C. and Liu, L. F. (1990). Involvement of nucleic acid synthesis in cell killing mechanisms of topoisomerase poisons. *Cancer Res.* **50**, 6919-6924.

DelBino, G., Skierski, J. S. and Darzynkiewicz, Z. (1991). The concentration-dependent diversity of effects of DNA topoisomerase I and II inhibitors on the cell cycle of HL-60 cells. *Exp. Cell Res.* **195**, 485-491.

Draetta, G., Luca, F., Westendorf, J., Brizuela, L., Ruderman, J. and Beach, D. (1989). Cdc2 protein kinase is complexed with both cyclin A and B: evidence for proteolytic inactivation of MPF. *Cell* **56**, 829-838.

Eastman, A. (1990). Activation of programmed cell death by anticancer agents: cisplatin as a model system. *Cancer Cells* **2**, 275-280.

Freeman, R. S., Estus, S. and Johnson, E. M. (1994). Analysis of cell cycle-related gene-expression in postmitotic neurons - selective induction of cyclin D1 during programmed cell-death. *Neuron* **12**, 343-355.

Gautier, J., Norbury, C., Lohka, M., Nurse, P. and Maller, J. (1988). Purified maturation-promoting factor contains the product of a Xenopus homolog of the fission yeast cell cycle control gene *cdc2*$^{+}$. *Cell* **54**, 433-9.

Girard, F., Strausfeld, U., Fernandez, A. and Lamb, N. J. C. (1991). Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. *Cell* **67**, 1169-1179.

Glab, N., Labidi, B., Qin, L. X., Trehin, C., Bergounioux, C. and Meijer, L. (1994). Olomoucine, an inhibitor of the cdc2/cdk2 kinases activity, blocks plant-cells at the G1-S and G2-M cell-cycle transitions. *FEBS Lett.* **353**, 207-211.

Glucksmann, A. (1951). Cell deaths in normal vertebrate ontogeny. *Biol. Rev.* **26**, 289-300.

Gorczyca, W., Gong, J. P., Ardelt, B., Traganos, F. and Darzynkiewicz, Z. (1993). The cell-cycle related differences in susceptibility of hl-60-cells to apoptosis induced by various antitumor agents. *Cancer Res.* **53**, 3186-3192.

Hagan, I., Hayles, J. and Nurse, P. (1988). Cloning and sequencing of the cyclin-related *cdc13*$^{+}$ gene and a cytological study of its role in fission yeast mitosis. *J. Cell Sci.* **91**, 587-595.

Hamaguchi, J. R., Tobey, R. A., Pines, J., Crissman, H. A., Hunter, T. and Bradbury, E. M. (1992). Requirement for p34^{cdc2} kinase is restricted to mitosis in the mammalian cdc2 mutant FT210. *J. Cell Biol.* **117**, 1041-1053.

Hayles, J., Fisher, D., Woollard, A. and Nurse, P. (1994). Temporal-order of S-phase and mitosis in fission yeast is determined by the state of the p34^{cdc2} mitotic B-cyclin complex. *Cell* **78**, 813-822.

Hickman, J. A. (1992). Apoptosis induced by anticancer drugs. *Cancer Metast. Rev.* **11**, 121-139.

Hotz, M. A., Gong, J. P., Traganos, F. and Darzynkiewicz, Z. (1994). Flow cytometric detection of apoptosis - comparison of the assays of in-situ DNA-degradation and chromatin changes. *Cytometry* **15**, 237-244.

Kaufmann, S. (1989). Induction of endonucleolytic DNA cleavage in human acute myelogenous leukaemia cells by etoposide, camptothecin and other cytotoxic anticancer drugs: a cautionary note. *Cancer Res.* **49**, 5870-5878.

Kerr, J. F. R. (1971). Shrinkage necrosis: a distinct mode of cell death. *J. Pathol.* **105**, 13-20.

Kitagawa, M., Okabe, T., Ogino, H., Matsumoto, H., Suzukitakahashi, I., Kokubo, T., Higashi, H., Saitoh, S., Taya, Y. and Yasuda, H. (1993). Butyrolactone-I, a selective inhibitor of cdk2 and cdc2 kinase. *Oncogene* **8**, 2425-2432.

Knoblich, J. A. and Lehner, C. F. (1993). Synergistic action of Drosophila cyclins A and B during the G2-M transition. *EMBO J.* **12**, 65-74.

Labbe, J. C., Capony, J. P., Caput, D., Cavadore, J. C., Derancourt, M. K., Lelias, J. M., Picard, A. and Dorée, M. (1989). MPF from starfish oocytes at first meiotic metaphase is a heterodimer containing one molecule of cdc2 and one molecule of cyclin B. *EMBO J.* **8**, 3053-3058.

Lazebnik, Y. A., Cole, S., Cooke, C. A., Nelson, W. G. and Earnshaw, W. C. (1993). Nuclear events of apoptosis in vitro in cell-free mitotic extracts - a model system for analysis of the active phase of apoptosis. *J. Cell Biol.* **123**, 7-22.

Lee, M. G. and Nurse, P. (1987). Complementation used to clone a human homologue of the fission yeast cell cycle control gene *cdc2*. *Nature* **327**, 31-35.

Levine, A. J., Momand, J. and Finlay, C. A. (1991). The p53 tumour suppressor gene. *Nature* **351**, 453-456.

Lock, R. B. and Ross, W. E. (1990). Possible role for p34^{cdc2} kinase in etoposide-induced cell death of Chinese hamster ovary cells. *Cancer Res.* **50**, 3767-3771.

Lock, R. B. (1992). Inhibition of p34^{cdc2} kinase activation, p34^{cdc2} tyrosine dephosphorylation, and mitotic progression in Chinese-hamster ovary cells exposed to etoposide. *Cancer Res* **52**, 1817-1822.

Lock, R. B. and Keeling, P. K. (1993). Responses of HeLa and Chinese hamster ovary p34^{cdc2}/cyclin-B kinase in relation to cell-cycle perturbations induced by etoposide. *Int. J. Oncol.* **3**, 33-42.

MacDonald, H. R. and Lees, R. K. (1990). Programmed death of autoreactive thymocytes. *Nature* **343**, 642-644.

Marraccino, R. L., Firpo, E. J. and Roberts, J. M. (1992). Activation of the p34^{cdc2} protein-kinase at the start of S-phase in the human cell-cycle. *Mol. Biol. Cell* **3**, 389-401.

MacNeill, S. A. and Nurse, P. (1993). Genetic-analysis of human p34^{cdc2} function in fission yeast. *Mol. Gen. Genet.* **240**, 315-322.

Meikrantz, W., Gisselbrecht, S., Tam, S. W. and Schlegel, R. (1994). Activation of cyclin A-dependent protein-kinases during apoptosis. *Proc. Nat. Acad. Sci. USA* **91**, 3754-3758.

Mineo, C., Murakami, Y., Ishimi, Y., Hanaoka, F. and Yamada, M. (1986).

Isolation and analysis of a mammalian temperature-sensitive mutant defective in G2 functions. *Exp. Cell Res.* **167**, 53-62.

Minshull, J., Blow, J. J. and Hunt, T. (1989). Translation of cyclin mRNA is necessary for extracts of activated *Xenopus* eggs to enter mitosis. *Cell* **56**, 947-956.

Minshull, J., Golsteyn, R., Hill, C. S. and Hunt, T. (1990). The A- and B-type cyclin associated cdc2 kinases in *Xenopus* turn on and off at different times in the cell cycle. *EMBO J.* **9**, 2865-75.

Murray, A. W. and Kirschner, M. W. (1989). Cyclin synthesis drives the early embryonic cell cycle. *Nature* **339**, 275-280.

Muschel, R. J., Zhang, H. B. and McKenna, W. G. (1993). Differential effect of ionizing-radiation on the expression of cyclin-A and cyclin-B in HeLa-cells. *Cancer Res.* **53**, 1128-1135.

Norbury, C. J. and Nurse, P. (1992). Animal cell cycles and their control. *Annu. Rev. Biochem.* **61**, 441-470.

Norbury, C., MacFarlane, M., Fearnhead, H. and Cohen, G. M. (1994). Cdc2 activation is not required for thymocyte apoptosis. *Biochem. Biophys. Res. Commun.* **202**, 1400-1406.

Nurse, P. and Bissett, Y. (1981). Gene required in Gl for commitment to cell cycle and in G2 for control of mitosis in fission yeast. *Nature* **292**, 558-560.

Nurse, P. and Thuriaux, P. (1980). Regulatory genes controlling mitosis in the fission yeast Schizosaccharomyces pombe. *Genetics* **96**, 627-637.

Nurse, P. (1990). Universal control mechanism regulating onset of M-phase. *Nature* **344**, 503-508.

Obara-Ishihara, T. and Okayama, H. (1994). A B-type cyclin negatively regulates conjugation via interacting with cell-cycle start genes in fission yeast. *EMBO J.* **13**, 1863-1872.

Oberhammer, F. A., Hochegger, K. and Froschl, G. (1994). Chromatin condensation during apoptosis is accompanied by degradation of lamin A and B, without enhanced activation of cdc2 kinase. *J. Cell Biol.* **126**, 827-837.

O'Connor, P. M., Ferris, D. K., Pagano, M., Draetta, G., Pines, J., Hunter, T., Longo, D. L. and Kohn, K. W. (1993). G2 delay induced by nitrogen-mustard in human-cells affects cyclin A/cdk2 and cyclin-B1/cdc2-kinase complexes differently. *J. Biol. Chem.* **268**, 8298-8308.

Pagano, M., Pepperkok, R., Verde, F., Ansorge, W. and Draetta, G. (1992). Cyclin A is required at two points in the human cell cycle. *EMBO J.* **11**, 961-971.

Sen, S., Takahashi, R., Rani, S., Freireich, E. J. and Stass, S. A. (1993). Expression of differentially phosphorylated Rb and mutant p53 proteins in myeloid leukemia cell lines. *Leukemia Res.* **17**, 639-647.

Sherr, C. J. (1993). Mammalian G1 cyclins. *Cell* **73**, 1059-1065.

Shi, L., Nishioka, W. K., Th'ng, J., Bradbury, E. M., Litchfield, D. W. and Greenberg, A. H. (1994). Premature p34^{cdc2} activation required for apoptosis. *Science* **263**, 1143-1145.

Shimizu, T., O'Connor, P. M., Kohn, K. W. and Pommier, Y. (1995). Unscheduled activation of cyclin B1/Cdc2 kinase in human promyelocytic leukemia cell line HL-60 cells undergoing apoptosis induced by DNA damage. *Cancer Res.* **55**, 228-231.

Solary, E., Bertrand, R. and Pommier, Y. (1994). Apoptosis induced by DNA topoisomerase-I and topoisomerase-II inhibitors in human leukemic HL-60 cells. *Leuk. Lymphoma* **15**, 21-32.

Th'ng, J. P., Wright, P. S., Hamaguchi, J., Lee, M. G., Norbury, C. J., Nurse, P. and Bradbury, E. M. (1990). The FT210 cell line is a mouse G2 phase mutant with a temperature-sensitive *CDC*2 gene product. *Cell* **63**, 313-24.

Thiebaut, F., Enns, R. and Howell, S. B. (1994). Cisplatin sensitivity correlates with its ability to cause cell-cycle arrest via a wee1 kinase-dependent pathway in Schizosaccharomyces pombe. *J. Cell Physiol.* **159**, 506-514.

Ucker, D. S. (1991). Death by suicide: one way to go in mammalian cellular development? *New Biol.* **3**, 103-109.

Ucker, D. S., Obermiller, P. S., Eckhart, W., Apgar, J. R., Berger, N. A. and Myers, J. (1992). Genome digestion is a dispensible consequence of physiological cell death mediated by cytotoxic T lymphocytes. *Mol. Cell. Biol.* **12**, 3060-3069.

Vandenheuvel, S. and Harlow, E. (1993). Distinct roles for cyclin-dependent kinases in cell-cycle control. *Science* **262**, 2050-2054.

Walker, P. R., Smith, C., Youdale, T., Leblanc, J., Whitfield, J. F. and Sikorska, M. (1990). Topoisomerase II-reactive chemotherapeutic drugs induce apoptosis in thymocytes. *Cancer Res.* **51**, 1078-1085.

Walker, D. H. and Maller, J. L. (1991). Role for cyclin A in the dependence of mitosis on completion of DNA replication. *Nature* **354**, 314-317.

Wyllie, A. H., Kerr, J. F. H. and Currie, A. R. (1980). Cell death: the significance of apoptosis. *Int. Rev. Cytol.* **68**, 251-306.

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