Cross-talk between DNA damage and cell survival checkpoints during G_2 and mitosis: pharmacologic implications

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Abstract

In this study, we wanted to clarify the role of survivin-mediated survival signaling during G_2 and M in tumor cells treated with DNA-damaging agents. As a cellular model, we selected MOLT-4 human T-cell lymphoblastic leukemia cells that overexpress survivin and nonfunctional p53. Treatment with melphalan, a classic DNA-damaging agent, led to the induction of the DNA damage checkpoint and growth arrest in the G_2 phase of the cell cycle. Checkpoint abrogation by caffeine was accompanied by mitotic entry and rapid apoptotic cell death, whereas cells remaining in G_2 remained viable during the same time interval. Unexpectedly, when the spindle checkpoint was activated following G_2 abrogation, two different effects could be observed. If the microtubules of the melphalan-treated cells were destabilized by nocodazole, cells became arrested in prometaphase with low survivin levels and entered apoptosis. In contrast, if the microtubules of the melphalan-treated cells were stabilized by taxol, cells were still arrested in prometaphase, but apoptotic execution was inhibited. This effect is, most likely, directly mediated by survivin itself given its well-established antiapoptotic functions. In conclusion, depending on the way the spindle checkpoint was activated in cells with damaged DNA, cells could be either protected by survivin or die during mitosis. We suggest that the efficacy of DNA damage checkpoint abrogators used in combination with DNA-damaging agents may critically depend on whether DNA damage is able to invoke spindle checkpoint response and to activate survivin-associated survival signaling during mitosis. [Mol Cancer Ther 2005;4(12):2016–25]

Introduction

DNA damage activates different cell cycle checkpoints in G_1, intra-S, G_2, and early mitosis that are part of cellular surveillance mechanisms and protect cells from deleterious effects induced by genotoxic agents (for review, see ref. 1). Other cell cycle checkpoints monitor proper assembly of the mitotic spindle and prevent missegregation of chromosomes during the metaphase to anaphase transition (for recent review, see ref. 2). It is currently not clear if the DNA damage checkpoint is composed of only one pathway that responds to different types and levels of DNA damage and operates from late S until early prophase or if there are several partially independent pathways that are differently activated by under-replicated DNA in late S, DNA strand breaks in G_2, and chromosome damage in early prophase (3). The different components of the DNA damage checkpoint may be either p53 dependent or p53 independent (reviewed in ref. 4). In tumor cells that frequently have nonfunctional p53, DNA damage principally leads to cell cycle arrest in the G_2 phase due to inhibitory phosphorylation of the master mitotic kinase Cdk1/Cdc2 on Thr^{14} and Tyr^{15} (5) and/or by exclusion of Cdk1 from the nucleus by 14-3-3σ-mediated nuclear export (6). Other studies showed the existence of a mitotic checkpoint that is activated by chromosomal damage in early prophase (7). It has also been shown that DNA damage can lead to defects in kinetochore attachment, which might result in activation of the spindle assembly checkpoint (8). However, few studies have been carried out to clarify the possible interaction(s) between the DNA damage and the spindle checkpoint(s), especially in situations where cells enter mitosis with a damaged genome and might possibly activate both checkpoints. This possibility is particularly interesting given the recent advent of very effective DNA damage checkpoint abrogators, such as UCN-01 and flavopiridol, which have entered clinical trials in combination with DNA-damaging agents (9). Furthermore, it is well established that many tumor cells have only partially functional cell cycle checkpoints, which may influence the cytotoxic effect of antitumor drugs, such as DNA-damaging agents. Aberrant

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cell cycle checkpoint function is a double-edged sword that might not necessarily be only beneficial. We would expect an improved therapeutic activity of antitumor drugs if cells with drug-induced damage continued cell proliferation with unrepaired lesions, which would result in cell death. Alternatively, inactive cell cycle checkpoints might allow some of the tumor cells to survive drug treatment with damaged genomes favoring the selection of a subpopulation with increased genomic instability and drug resistance or with a more aggressive tumor phenotype.

In addition to cell cycle checkpoints, the cellular response to DNA damage also involves survival signaling that may decide if cells will have the opportunity to repair potentially lethal damage and survive or if they die before they have had time to repair the damage. Progression through G_2 and mitosis is guarded by antiapoptotic signaling mediated by survivin, a member of the inhibitor of apoptosis protein family (10). Survivin is highly expressed in G_2-M cells and associates with the mitotic spindle in early mitosis (11). Survivin is one of the most common proteins to be overexpressed in human tumors (12) and has been proposed to protect cells from a default induction of cell death elicited by antitumor drugs in G_2 phase (10) and during the G_2-M progression (13). In this way, high survivin levels may favor aberrant progression of transformed cells going through mitosis with damaged DNA. Survivin expression is negatively regulated by p53 (14, 15), for which reason survivin-associated antiapoptotic signaling is particularly important in tumor cells with nonfunctional p53. Because survivin is overexpressed in tumor cells and is low or absent in normal cells, this protein seems to present an ideal target for cancer-targeted therapy. Recently, search for safe and effective survivin antagonists for clinical use has been launched that is aimed at selective and effective destruction of tumor cells (11).

In this study, we wanted to clarify the potential role of survival signaling during mitosis in cells with damaged DNA. We were specifically interested in survivin-associated survival signaling during G_2 and M. As a model, MOLT-4 cells were treated with a classic DNA-damaging drug, melphalan. These cells are typical of tumor cells that overexpress survivin on both the protein and the mRNA levels (13, 16) and have nonfunctional p53. The G_2 damage checkpoint was induced by melphalan treatment, and the G_2-arrested cells forced into mitosis by the cell cycle abrogator caffeine. In contrast to previous studies (17), we activated the DNA damage checkpoint first and then activated the spindle checkpoint by either nocodazole or taxol. This situation, in which tumor cells with genotoxic lesions progress into mitosis with unrepaired DNA lesions, is of particular interest because it mimics the clinical use of DNA-damaging agents in combination with checkpoint abrogators, such as UCN-01 and flavopiridol (9). Our results suggest that a crucial factor in the efficacy of DNA damage checkpoint abrogators used in combination with DNA-damaging agents is whether the DNA damage is able to invoke a spindle checkpoint response associated with active survival signaling during mitosis.

Materials and Methods

Drugs, Chemicals, and Antibodies

Melphalan (L-PAM), caffeine, taxol, nocodazole, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, propidium iodide (PI), carbamoyl cyanide *m*-chlorophenylhydrazone, and RNase A were purchased from Sigma (St. Louis, MO), 3,3′-dihexyloxacarbocyanine iodide was from Molecular Probes (Eugene, OR), whereas the caspase-3 activity kit and purified caspase-3 were from BioMol (Hamburg, Germany). Media, antibiotics, and serum were from Life Technologies (Paisley, United Kingdom). Monoclonal anti-MPM-2 antibodies were from DAKO (Carpinteria, CA), and monoclonal anti-Cdk1, monoclonal anti–cyclin B1, polyclonal rabbit anti-survivin, and polyclonal goat anti-actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit anti–poly(ADP-ribose) polymerase and polyclonal rabbit anti-cleaved caspase-3 were from Cell Signaling (Beverly, MA). Horseradish peroxidase–conjugated anti-mouse, anti-rabbit, and anti-goat IgG antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). FITC-conjugated anti-mouse and anti-rabbit IgG antibodies were from Amersham Pharmacia Biotech (Uppsala, Sweden).

Cells

MOLT-4 cells were maintained in RPMI supplemented with 10% fetal bovine serum and antibiotics (100 μg/dm^3 streptomycin and 100,000 units/dm^3 penicillin). The cells were grown at 37°C in a humidified 5% CO_2-95% air atmosphere. In these conditions, the doubling time was ~12 hours.

Cytotoxic Activity

The cytotoxic activity of melphalan was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Briefly, cells were seeded in 24-well plates (1×10^4 per well) and exposed to melphalan for 18 hours. Following two washes with warm growth medium, cells were incubated for an additional 48 hours. The IC_{95} is defined as the inhibitory drug concentration causing 95% reduction of A_{540} versus that of control. Alternatively, the cytotoxic activity of melphalan was determined by counting of cells with Coulter counter (Hialeah, FL) with similar results.

Flow Cytometry

Distribution of cells through the cell cycle was determined by flow cytometry as described (18) using an EPICS Profile II Flow Cytometer (Coulter) equipped with an argon laser to give 488 nm light. The cells were fixed in 70% ethanol at −20°C, rehydrated in PBS, and stained with PBS containing PI (20 μg/mL) and RNase A (100 μg/mL) for 30 minutes at room temperature. The percentage of cells in each phase of the cell cycle was calculated by MultiPlus software (Phoenix Flow Systems, San Diego, CA).

Caspase-3 Activity Assay

Drug-treated or control cells (1×10^6) were lysed in 50 μL lysing buffer [50 mmol/L HEPES (pH 7.4), 0.1% CHAPS, 5 mmol/L DTT, 0.1 mmol/L EDTA] for 5 minutes on ice and

centrifuged at 20,000 × *g* for 10 minutes at 4°C, and supernatants were collected. Protein concentration was determined by the bicinchoninic acid assay and supernatants were examined for caspase-3 activity using the caspase-3 kit according to the manufacturer's instructions. Fold increase in protease activity was determined by comparing drug-induced values and nontreated controls. Calibration curves were generated using purified caspase-3 and activity units were calculated (units/μg protein/h).

Western Blotting

Cells were lysed in radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/L Tris-HCl (pH 8)] containing protease inhibitor cocktail (Roche, Meylan, France) and phosphatase inhibitors (50 mmol/L NaF, 50 mmol/L β-glycerophosphate, 1 mmol/L sodium orthovanadate) for 15 minutes on ice. Cell lysates were centrifuged at 20,000 × *g* for 10 minutes at 4°C and supernatants were collected. Protein concentrations in cell lysates were determined by the bicinchoninic acid assay. Equal amounts (usually 50 μg/lane) were loaded in Laemmli buffer and separated by SDS-PAGE electrophoresis in either 12% or 5% to 20% linear gradient acrylamide gels and transferred onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech). After transfer, membranes were blocked in 5% nonfat milk in TBS buffer [10 mmol/L Tris-HCl, 150 mmol/L NaCl (pH 8)] and washed in TBST buffer (TBS buffer containing 0.05% Tween 20). Membranes were incubated with primary antibodies diluted in TBST containing 0.5% bovine serum albumin at 1:100 [anti-MPM-2, anti–poly(ADP-ribose) polymerase, anti-caspase-3, anti-survivin, and anti-Cdk1] or 1:1,000 (anti-actin) for 1 to 3 hours at room temperature. After three washes in TBST, membranes were incubated with secondary antibodies diluted at 1:40,000 in TBST for 1 hour at room temperature. Results were revealed by the enhanced chemiluminescence kit (Amersham Pharmacia Biotech). Equal protein loading was verified by rehybridization of membranes and reprobing with anti-actin antibodies.

DNA Fragmentation

Internucleosomal DNA fragmentation in cells undergoing apoptosis was assayed essentially as described (19). High molecular weight DNA fragmentation was determined by inverted pulse-field gel electrophoresis. Cells were washed in PBS and embedded in 0.75% low melting agarose (FMC Bioproducts, Rockland, ME). Plugs were then incubated in lysis buffer [10 mmol/L Tris-HCl (pH 8), 10 mmol/L NaCl, 25 mmol/L EDTA, 0.9% sarcosyl, 0.1% SDS, 1 mg/mL proteinase K] for 24 hours at 50°C, washed thrice in washing buffer [10 mmol/L Tris-HCl (pH 8), 50 mmol/L EDTA], and stored in 50 mmol/L EDTA (pH 8) at 4°C until analysis. Samples, including 50 to 1,000-kbp λ DNA marker (Bio-Rad, Hercules, CA), were analyzed in a 1% FastLane agarose gel (FMC Bioproducts) in 0.5× Tris-borate EDTA buffer [45 mmol/L Tris-borate, 1 mmol/L EDTA (pH 8.3)] with temperature maintained at 6°C using field inversion gel electrophoresis Mapper system (Bio-Rad). The pulse-wave switcher was programmed to provide initial 9-second forward and 3-second reverse pulses, with linear ramp 3:1 and a constant voltage of 180 V throughout 12 hours. Gels were stained and photographed under UV illumination.

Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End Labeling Assay

Following drug treatment, cells were collected by centrifugation and fixed in 1% formaldehyde in PBS for 15 minutes on ice. After centrifugation, the pellet was washed in PBS and resuspended in 70% ethanol, and samples were stored at −20°C. After rehydration in PBS, cells were resuspended in 50 μL reaction buffer containing 5 units terminal transferase, 2.5 mmol/L cobalt chloride, 0.2 mol/L sodium cacodylate, 25 mmol/L Tris-HCl (pH 6.6), 0.25 mg/mL bovine serum albumin, and 1 μg biotin-dUTP (Roche Molecular Biochemicals, Mannheim, Germany) and incubated at 37°C for 30 minutes. The pellet was washed with rinsing buffer (PBS containing 1% Triton X-100, 0.5% bovine serum albumin); resuspended in 100 μL staining buffer, which contained 2.5 μg/mL FITC-avidin, 4× SSC buffer [1× SSC buffer: 0.15 mol/L NaCl, 0.015 mol/L sodium citrate (pH 7)], 0.1% Triton X-100, and 5% (w/v) nonfat dry milk; and incubated for 30 minutes at room temperature in the dark. The pellets were then washed twice with rinsing buffer, resuspended in PBS containing 5 μg/mL PI and 100 μg/mL RNase A, and incubated at room temperature for 30 minutes. The red (PI) and green (fluorescein) fluorescence was measured with Becton Dickinson (Le Pont de Claix, France) FACScan flow cytometer; the data from 10^4 cells were collected and analyzed by the MultiPlus software.

Mitochondrial Transmembrane Potential Measurements

Control or drug-treated cells were incubated with 3,3′-dihexyloxacarbocyanine iodide at the final concentration of 20 nmol/L at 37°C for 15 minutes and PI was added to each sample (5 μg/mL final concentration) followed by immediate analysis of fluorochrome incorporation in a Ventage cytometer (Becton Dickinson). Fluorescence of 3,3′-dihexyloxacarbocyanine iodide and PI was recorded. The position of cells that have lost their mitochondrial potential was determined by exposure to the classic mitochondrial poison carbamoyl cyanide *m*-chlorophenylhydrazone, an uncoupler of oxidative phosphorylation (20), followed by staining as described above.

Results

Treatment of MOLT-4 Cells with Melphalan Leads to Prolonged G_2 Arrest

Cells were treated with 2 μg/mL melphalan, which corresponds to the IC_{95} dose, for 18 hours and post-incubated in drug-free medium, and the cell cycle distribution was determined by flow cytometry. MOLT-4 cells that have nonfunctional p53 due to a mutation in Trp^{248} (21) progressively accumulated in the G_2-M region of the cell cycle with a 4N DNA content (Fig. 1A). At this dose, the majority of growth-arrested cells remained viable

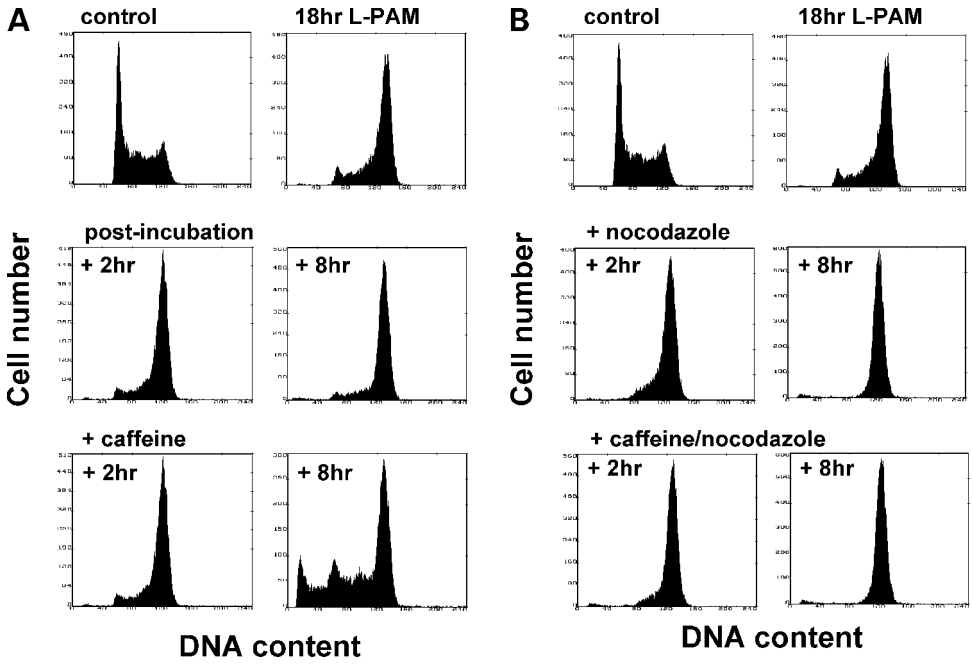

Figure 1. DNA histograms of MOLT-4 cells treated with 2 μg/mL melphalan for 18 h and postincubated in the absence or presence of 1 mmol/L caffeine (**A**) or with nocodazole in the absence or presence of 1 mmol/L caffeine (**B**).

for at least 24 hours with an apoptotic fraction of ~6% to 8% as determined by PI exclusion. It should be added that cells treated with 2 μg/mL melphalan did not resume proliferation even after prolonged incubation in drug-free medium (up to 2 weeks) and ultimately all died. To confirm the activation of the DNA damage checkpoint in the melphalan-treated cells, we analyzed the expression of mitotic markers, including the cyclin B1 levels, the phosphorylation status of the major mitotic kinase Cdk1, and the presence of the mitotic MPM-2 epitope by Western blot analysis. We observed a clear increase in cyclin B1 levels after 18 hours of melphalan exposure in comparison with untreated control cells (Fig. 2A). In parallel, cell cycle arrest in melphalan-treated MOLT-4 cells was associated with the appearance of the phosphorylated, inactive forms of Cdk1 associated with phosphorylation of Tyr^{15} and Thr^{14} (Fig. 2A). The absence of a detectable MPM-2 signal further confirmed that the melphalan-treated cells had not entered mitosis but were arrested in G_2.

The DNA Damage Checkpoint Abrogator Caffeine Can Induce G_2-M Transit of Melphalan-Treated Cells

Although most melphalan-treated MOLT-4 cells arrested in G_2, microscopic analysis of Hoechst-stained cells showed low levels of mitotic cells (~4% of the total cell population) at times longer than 18 hours of drug exposure (data not shown). This suggested that MOLT-4 cells have only a partially functional DNA damage checkpoint and are unable to maintain G_2 arrest following prolonged melphalan exposure. We therefore wanted to establish if the melphalan-induced G_2 arrest could be modulated by caffeine, a well-known abrogator of the DNA damage checkpoint. To this end, cells were treated with melphalan for 18 hours as described above followed by postincubation with a nontoxic dose (1 mmol/L) of caffeine. At this dose, caffeine did not influence the cell cycle distribution or the doubling time when given as a single agent (data not shown). In contrast, addition of caffeine to G_2-arrested cells induced G_2 exit and subsequent accumulation in the sub-G_1 region (Fig. 1A). Analysis of biochemical markers by Western blot analysis showed that postincubation of

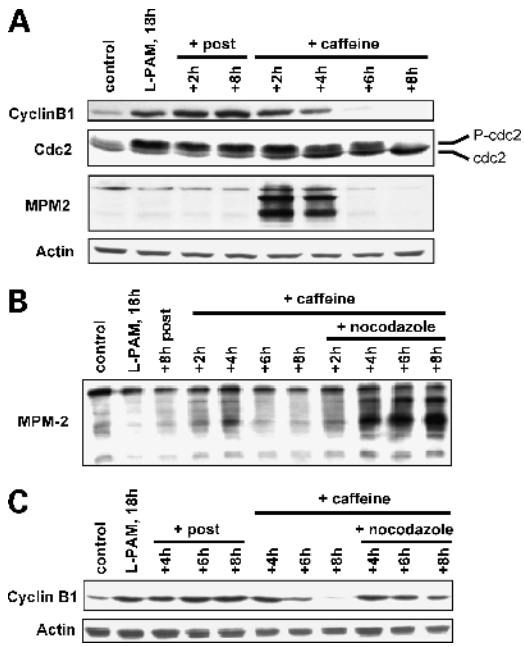

Figure 2. Western blot analysis of Cdk1 phosphorylation, cyclin B1 expression, and presence of the mitotic MPM-2 phosphoepitope in MOLT-4 cells treated with 2 μg/mL melphalan for 18 h and postincubated in the absence or presence of 1 mmol/L caffeine (**A**) or with a combination of 1 mmol/L caffeine and 50 ng/mL nocodazole (**B** and **C**).

melphalan-treated cells with caffeine was accompanied by activation of Cdk1 as determined by the conversion of the slow-migrating inactive phosphorylated form into the faster-migrating dephosphorylated active form. In contrast, no apparent activation of Cdk1 kinase was observed throughout an 8-hour postincubation period in the absence of caffeine (Fig. 2A). Activation of Cdk1 kinase was followed by a rapid decrease of cyclin B1 protein levels and the appearance of the MPM-2 epitope (Fig. 2A). These results suggest that caffeine induced not only mitotic entry but also mitotic transit of the melphalan-treated cells at least to anaphase when the MPM-2 signal disappears (22).

Cells Treated with Both Melphalan and the Microtubule Inhibitors Nocodazole and Taxol Arrest at the Spindle Checkpoint

We next wanted to determine if melphalan-treated MOLT-4 cells induced to enter mitosis become arrested at the spindle checkpoint. To this end, combined treatments of melphalan followed by caffeine and nocodazole as well as caffeine and taxol were employed. Flow cytometry analysis showed that postincubation of melphalan-treated cells with 50 ng/mL nocodazole led to accumulation of cells in the G_2-M region similar to cells postincubated in drug-free medium (compare Fig. 1A and B). Addition of both caffeine and nocodazole to melphalan-treated cells also resulted in accumulation of cells in G_2-M (Fig. 1B) without any cells in the sub-G_1 region. Further characterization by Western blot analysis showed that cells postincubated with both caffeine and nocodazole accumulated with high cyclin B1 levels and a strong MPM-2 signal, whereas caffeine alone induced G_2 exit and mitotic progression with transient high MPM-2 signal and decreasing cyclin B1 levels (Fig. 2B and C). Microscopic examination of Hoechst 33342–stained melphalan-treated cells that were postincubated with both caffeine and nocodazole or with both caffeine and taxol revealed that the cells were arrested in mitosis with condensed chromosomes before chromatid separation, which corresponds to prometaphase (Fig. 3A). Quantitation of mitotic fractions in all combinations confirmed that in the presence of caffeine melphalan-treated cells rapidly progressed to mitosis, but cells postincubated with both nocodazole and taxol arrested in prometaphase for at least 6 hours (Fig. 3B). These results clearly show that the mitotic spindle checkpoint can be activated in cells treated with genotoxic agents by both nocodazole and taxol.

Caffeine-Induced G_2-M Transit Is Accompanied by Apoptosis

We next wanted to establish how the sequential activation of the DNA damage and spindle checkpoints in melphalan-treated MOLT-4 cells influenced the cell death kinetics. The appearance of G_1 and sub-G_1 cells (Fig. 1A) following the combined treatment with melphalan and caffeine suggested that G_2 exit and mitotic transit may lead to rapid onset of apoptotic cell death possibly due to the presence of high levels of genotoxic lesions in the mitotic cells.

First, we determined the number of apoptotic cells by PI exclusion assay. As shown in Fig. 3C, within an 8-hour postincubation in the absence of modulators, the fraction of dead cells increased only from 6% to ~8%, whereas addition of caffeine almost doubled the number of dead

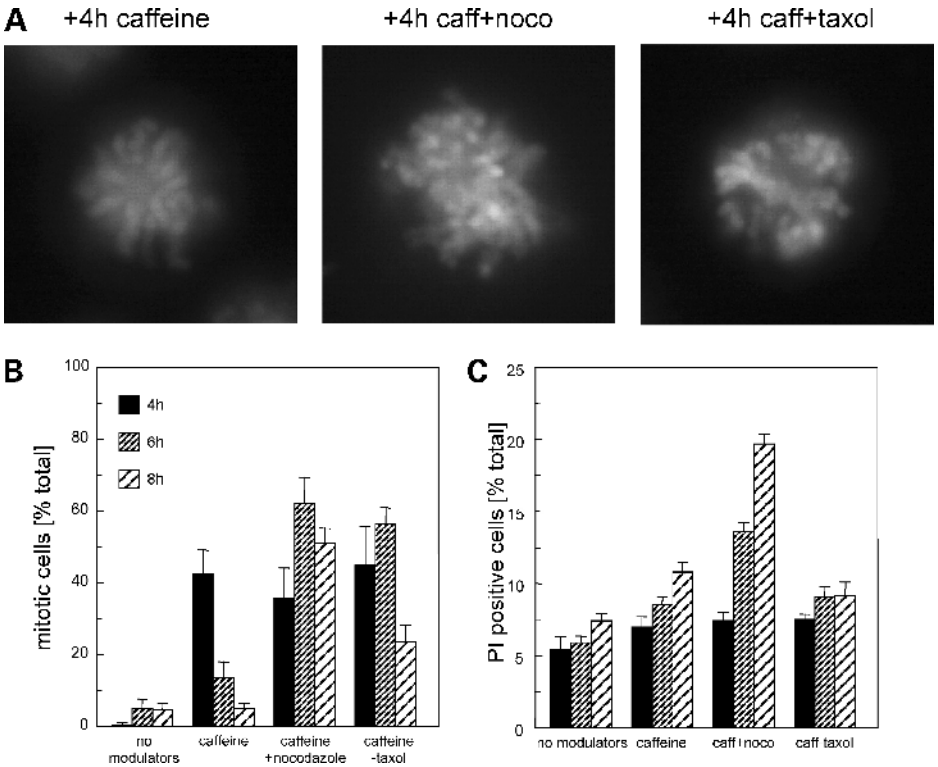

Figure 3. **A,** nuclear morphology of MOLT-4 cells treated with 2 μg/mL melphalan for 18 h and postincubated in the absence or presence of 1 mmol/L caffeine or with a combination of 1 mmol/L caffeine and 50 ng/mL nocodazole (*caff + noco*) as well as 1 mmol/L caffeine and 10 μmol/L taxol (*caff + taxol*). **B** and **C,** changes in the number of mitotic (**B**) and apoptotic (**C**) cells in cell populations of MOLT-4 cells treated with 2 μg/mL melphalan for 18 h and postincubated in the absence or presence of 1 mmol/L caffeine or with a combination of 1 mmol/L caffeine and 50 ng/mL nocodazole as well as 1 mmol/L caffeine and 10 μmol/L taxol.

cells at 8 hours. Postincubation of melphalan treated with caffeine and nocodazole produced a rapid increase of PI-positive cells with no apparent effect for caffeine-taxol combination (Fig. 3C). Microscopic analysis of nuclear morphology revealed only a few cells with apoptotic features among both melphalan-treated cells and melphalan-treated cells postincubated in drug-free medium for 2 or 8 hours. In clear contrast, a substantial fraction of melphalan-treated cells postincubated with caffeine for 8 hours showed characteristic apoptotic cell shrinkage and membrane blebbing (Fig. 4A).

Melphalan-treated cells contained heavily fragmented very high molecular weight DNA (~1,000 kbp) and smaller fragments of ~300 kbp as determined by pulse-field electrophoresis (Fig. 4B). In contrast, no apoptosis-associated internucleosomal DNA laddering was observed in the melphalan-treated cells by 18 hours or for melphalan-treated cells postincubated in drug-free medium for up to 8 hours (Fig. 4C). Interestingly, prolonged incubation of the melphalan-treated cells in drug-free medium led to a decreased DNA fragmentation most probably due to DNA repair. When the melphalan-treated cells were postincubated with caffeine, similar levels of high molecular weight DNA fragmentation were observed for up to 6 hours after addition of caffeine, whereas longer postincubation led to decreased DNA fragmentation. This was accompanied by the appearance of low molecular weight DNA fragments (internucleosomal DNA fragmentation). The cell cycle–specific induction of apoptosis was confirmed by the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay where DNA strand breaks were only observed in the G_2-M region (data not shown).

In parallel, the changes in mitochondrial membrane potential were determined by staining with 3,3′-dihexyloxacarbocyanine iodide, a membrane-specific dye that shows increased affinity to highly polarized membranes (23). Untreated MOLT-4 cells exhibited a high transmembrane potential that remained basically unchanged after 18 hours of melphalan exposure or by 18 hours of melphalan exposure followed by 8 hours of postincubation in drug-free medium. In contrast, postincubation of the G_2-arrested cells with caffeine was accompanied by rapid membrane depolarization (Fig. 5A). Semiquantitative analysis of the flow cytometry data showed that after caffeine addition the fraction of cells with low mitochondrial membrane

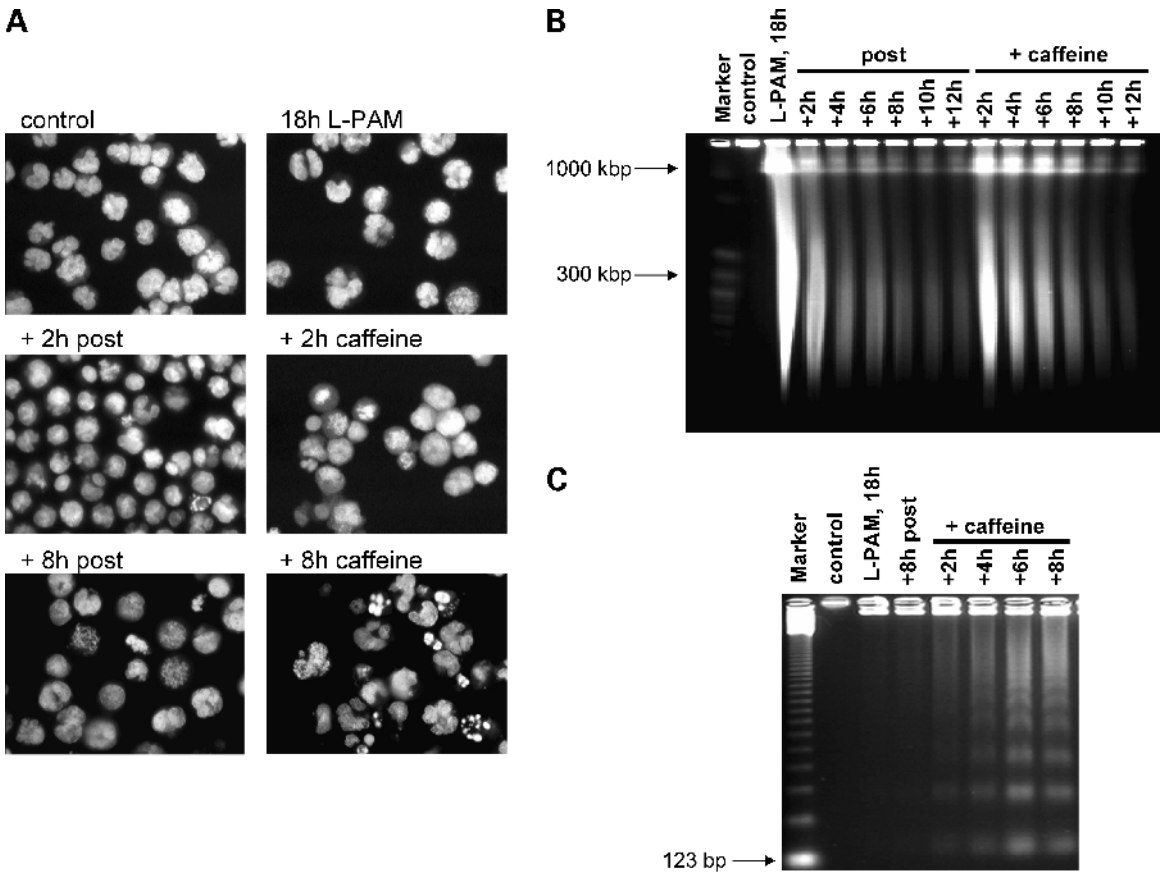

Figure 4. Analysis of nuclear morphology (**A**) and DNA fragmentation (**B** and **C**) and in MOLT-4 cells treated with 2 μg/mL melphalan for 18 h and postincubated in the absence or presence of 1 mmol/L caffeine.

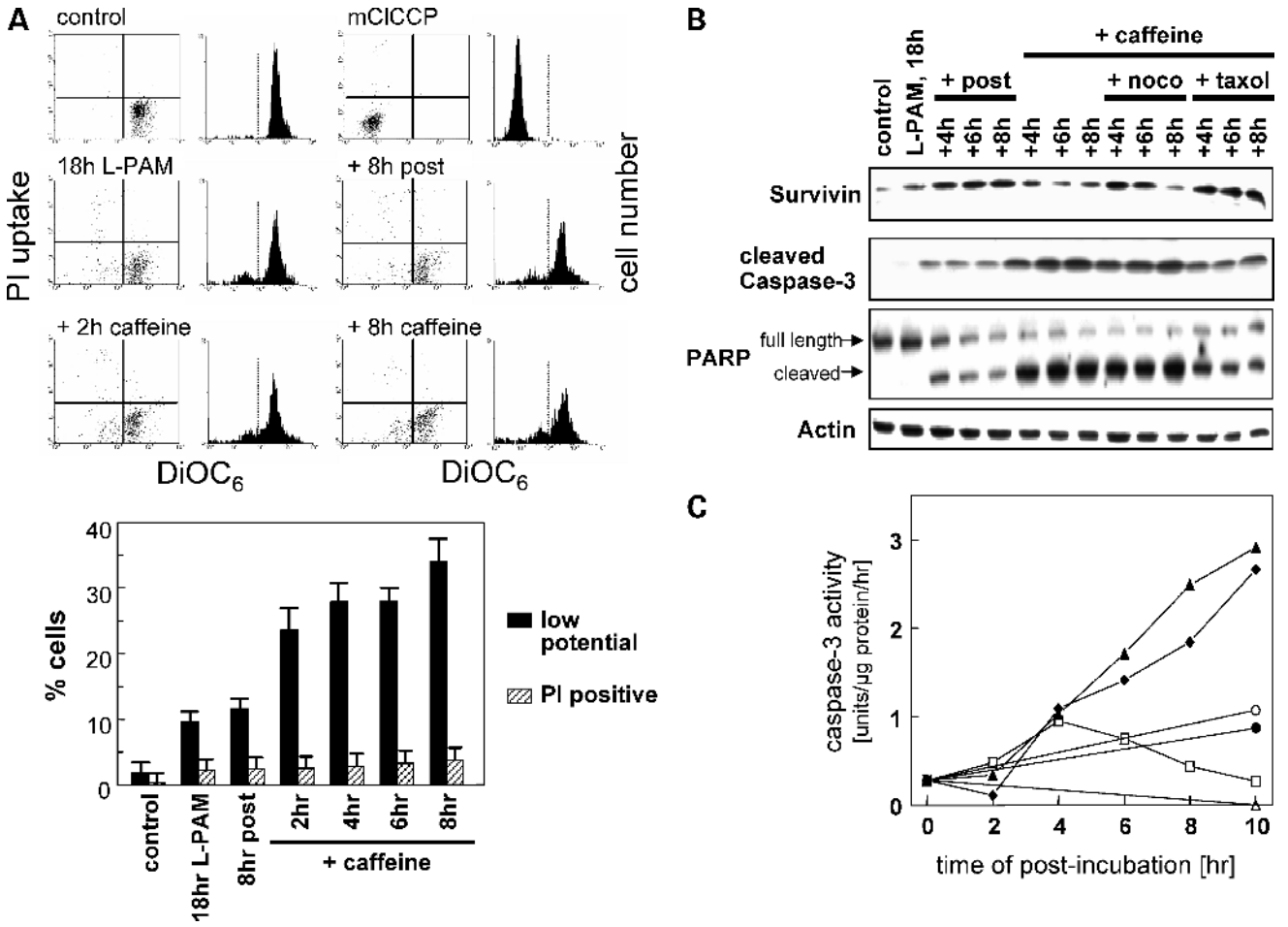

Figure 5. **A,** changes in mitochondrial membrane potential in MOLT-4 cells treated with 2 μg/mL melphalan for 18 h and postincubated in the absence or presence of 1 mmol/L caffeine as determined by 3,3′-dihexyloxacarbocyanine iodide (*DiOC$_6$*) and PI staining and analyzed by flow cytometry. **B,** Western blot analysis of survivin expression, caspase-3 activation, and apoptosis-associated poly(ADP-ribose) polymerase cleavage in MOLT-4 cells. Cells were treated with 2 μg/mL melphalan for 18 h and postincubated in the absence or presence of 1 mmol/L caffeine, combination of 1 mmol/L caffeine and 50 ng/mL nocodazole, or combination of 1 mmol/L caffeine and 10 μmol/L taxol. **C,** caspase-3 activation as determined by an enzymatic activity assay. MOLT-4 cells were treated with 2 μg/mL melphalan for 18 h and postincubated in the absence (●) or presence (▲) of 1 mmol/L caffeine, combination of 1 mmol/L caffeine and 50 ng/mL nocodazole (♦), or combination of 1 mmol/L caffeine and 10 μmol/L taxol (□). Nocodazole (○) and taxol (△) alone had only minor effect on caspase-3 activation in melphalan-treated cells in the absence of caffeine. SD is not shown for clarity but was never higher than 5%.

potential increased ~3-fold compared with cells postincubated in drug-free medium (Fig. 5B). Importantly, fractions of cells that were stained with PI (representing late apoptotic and necrotic cells) were much lower than fraction of cells with depolarized mitochondria. It suggests that within an 8-hour period, changes in the mitochondrial potential were associated with very early stages of cell death induced in melphalan-treated cells postincubated in the absence or presence of caffeine.

Activation of Survivin-Associated Antiapoptotic Signaling in Melphalan-Treated Cells

We showed that both nocodazole and taxol are able to activate the spindle checkpoint in melphalan-treated cells. It is well established that interference with microtubule polymerization, by both stabilizing and destabilizing microtubules, is associated with accumulation of survivin, one of the best characterized proteins of the inhibitor of apoptosis protein family (11). Therefore, we then wanted to compare the effect of nocodazole and taxol on the expression of survivin in melphalan-treated cells.

Western blot analysis showed increased survivin levels in the G_2-arrested melphalan-treated cells (Fig. 5C). Interestingly, nocodazole and taxol affected the survivin levels differently in melphalan-treated cells postincubated with caffeine (Fig. 5D). Checkpoint activation by nocodazole was associated with decreased survivin levels, whereas checkpoint activation by taxol was associated with the accumulation of cells with high survivin levels (Fig. 5C).

Importantly, differences in survivin levels of the melphalan-treated MOLT-4 cells were accompanied by differential activation of the apoptotic cell death pathway. Western blot analysis showed that activated (cleaved) form

of caspase-3 was observed for both melphalan-treated cells and melphalan-treated cells postincubated in drug-free medium for up to 8 hours (Fig. 5C). Addition of caffeine was associated with strong caspase-3 activation and poly(ADP-ribose) polymerase cleavage that was not prevented by nocodazole. In clear contrast, addition of taxol led to decreased caspase-3 activation and decreased poly(ADP-ribose) polymerase cleavage. These results were further confirmed by catalytic activity assays for caspase-3. As shown in Fig. 5D, postincubation of melphalan-treated cells with caffeine was accompanied by rapid activation of caspase-3 that could not be prevented by the addition of nocodazole. In clear contrast, the caffeine-taxol combination was accompanied by decreased activation of caspase-3 in comparison with caffeine alone or the nocodazole-caffeine combination. Together, these results clearly show that the cell death mechanisms associated with enforced mitotic entry of cells with damaged DNA are, at least in part, regulated by microtubule integrity resulting in different effects in the presence of nocodazole and taxol.

Discussion

In this study, we wanted to determine if the cellular sensitivity to DNA damage is altered in situations where both the DNA damage and the spindle checkpoints are activated and to establish how this is related to survivin levels. For these studies, we selected MOLT-4 cells that represent a tumor cell phenotype with high survivin expression and nonfunctional p53 function. MOLT-4 cells were exposed to melphalan, a well-known DNA-damaging agent that very rapidly (<2 hours) induces covalent DNA-DNA cross-links (24). For activation of the spindle checkpoint, we used two different microtubule poisons, nocodazole that inhibits microtubule polymerization and taxol that favors the polymerized form.

Some of the mechanistic aspects of the G_2-M progression and its role in the activation of cellular death pathways following DNA damage are still not elucidated. In particular, the importance of Cdk1 activity has been debated for years. Several studies suggested a possible involvement of Cdk1 activation in the apoptotic process induced by granzyme or by mitogen deprivation (25, 26), whereas other studies proposed that premature Cdk1 activation is required for camptothecin-, etoposide-, and nitrogen mustard-induced apoptosis (27). In contrast, it was shown to be Cdk2 rather than Cdk1 that was required for caspase activation and chromatin condensation during tumor necrosis factor-α–induced and staurosporine-induced apoptosis (28). Interestingly, it has also been proposed that Cdk1 has antiapoptotic functions. Genetic or chemical inactivation of Cdk1 is associated with increased, rather than decreased, cell death following mitoxantrone exposure (29). More recent studies with taxol-treated cells have elegantly proven the important role of Cdk1 in protecting cells during mitosis by activating phosphorylation of survivin (30). Additionally, Cdk1 may inhibit apoptosis by phosphorylation of Bad at Ser^{128} that prevents the association of Bad with Bcl-X_L (31).

The signaling cascades triggered by DNA damage may have at least two functions. They could prevent DNA-damaged cells from entering mitosis until the genotoxic lesions are repaired. Alternatively, survival signaling may be activated to maintain the viability of G_2-arrested cells. In this case, DNA damage checkpoint mechanisms would not only inhibit cell cycle progression but also block the induction of cell death during the prolonged G_2 arrest.

In the present studies, we wanted to clarify which of the different scenarios was operative in melphalan-treated MOLT-4 cells. First, we wanted to establish if triggering of the apoptotic program was comparable between G_2-arrested cells and cells that had entered mitosis with damaged DNA. Our results clearly show that, in our experimental model, cells only started dying after G_2 exit both when this was due to the natural instability of the G_2 DNA damage checkpoint and when the G_2 exit had been provoked by caffeine. Unexpectedly, if the mitotic checkpoint was activated, two different effects could be observed depending on the nature of the agent that was used to

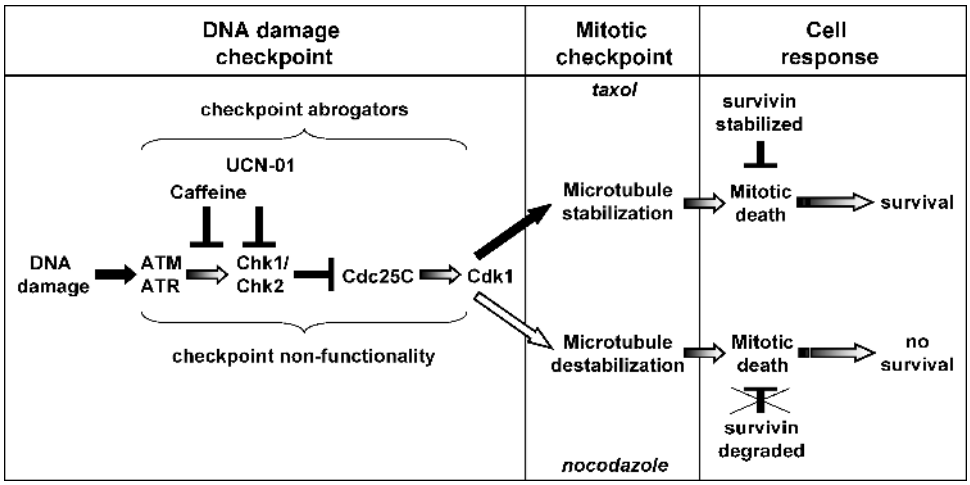

Figure 6. A model for the cellular response of tumor cells progressing through G_2 and M with a damaged genome in the presence of microtubule-stabilizing or microtubule-destabilizing drugs. When cells enter mitosis with damaged DNA due to weak DNA damage checkpoints or to checkpoint abrogation, mitotic cell death is favored if microtubules are destabilized, whereas cell survival may be observed when microtubules are stabilized due to active survivin signaling.

activate the spindle checkpoint. If the microtubules were destabilized by nocodazole, the DNA-damaged cells were arrested in prometaphase with low survivin levels and rapidly entered apoptosis. In striking contrast, if the microtubules were stabilized by taxol, the DNA-damaged cells still arrested in prometaphase but remained viable and very few cells underwent apoptosis. This effect is, most likely, directly mediated by survivin itself given its well-established antiapoptotic functions, although additional mechanisms cannot be excluded. It follows that, depending on the way by which the spindle checkpoint was activated in cells with damaged DNA, cells were either protected from apoptosis, most probably by survivin-associated pathway, or died during mitosis.

From these results, we conclude that Cdk1 plays a dual role in the apoptosis of melphalan-treated cells. On one hand, Cdk1 activation is concomitant with mitotic entry and induction of cell death of the DNA-damaged melphalan-treated cells. On the other hand, Cdk1 is also able to activate survivin-mediated survival signaling that plays a role in protecting DNA-damaged cells during mitosis. Although Cdk1 is able to activate survivin in the presence of both nocodazole and taxol, the protective effect was not observed in the nocodazole-treated cells because survivin became highly unstable when the microtubules were destabilized. In clear contrast, survivin remained stable when the melphalan-treated cells were treated with taxol resulting in microtubule polymerization.

The protein level of survivin changes during the cell cycle and peaks during G_2-M transit (30). Survivin is also stabilized by a wide range of cytotoxic injuries, including Adriamycin, UV irradiation, and taxol, through Cdk1-mediated phosphorylation of Thr^{34} (30). In our work, we showed that survivin levels decrease rapidly in mitotic MOLT-4 cells with depolymerized microtubules, which by itself could have yet another effect on the progression of melphalan-treated cells through mitosis. Survivin directly regulates mitotic functions, such as assembly of mitotic spindle, and is required for the metaphase to anaphase progression (32). Therefore, if survivin is degraded in cells postincubated with nocodazole, the melphalan-treated cells will not be able to progress from metaphase to anaphase and the cells will die during mitosis.

Our results have important pharmacologic implications given the increasing clinical interest in the combinations of cell cycle abrogators and DNA-damaging agents. Furthermore, spindle poisons are frequently used in combination with DNA-damaging agents. If such combinations are given to tumor cells with compromised G_2 checkpoints, the outcome might critically depend on the nature of the microtubule inhibitor. In particular, we would expect microtubule-destabilizing agents, such as the *Vinca* alkaloids, to favor mitotic death (mitotic catastrophe) of the DNA-damaged cells, whereas microtubule-stabilizing agents might favor the survival of a subfraction of cells with genotoxic lesions resulting in increased genetic instability (see our model in Fig. 6). This hypothesis is in full agreement with recent reports on the combined cytotoxicity of γ irradiation and nocodazole in B lymphoma cells that showed enhanced induction of cell death and, even more importantly, complete inhibition of regrowth when irradiation was combined with nocodazole (33).

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