A Mitotic Spindle Requirement for DNA Damage-induced Apoptosis in Chinese Hamster Ovary Cells

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ABSTRACT

Promiscuously reactive electrophilic agents induce DNA and other cellular damage. DNA repair-defective cells, when compared with genetically matched, repair-proficient parental cells, provide a means to distinguish cellular responses triggered by individual genetic lesions from other macromolecular damage. The Chinese hamster ovary (CHO) cell line EM9 is hypersensitive to the alkylating agent ethyl methanesulfonate (EMS) and is unable efficiently to repair DNA single strand breaks in contrast to parental AA8 cells. EM9 was used to examine how CHO cells couple unrepaired DNA strand breaks to loss of viability. Flow cytometry revealed that EMS-treated EM9 cells underwent prolonged cell cycle arrest in G2, followed by entry into mitosis, micronucleation, and apoptosis. EM9 cells synchronized in G1 prior to EMS treatment entered mitosis 24–36 h after release from synchrony, ~12 h after untreated control cells. Mitoses in EMS-treated cells were abnormal, involving multipolar mitotic spindles and elongated and/or incompletely condensed chromosomes. The mitotic spindle poison nocodazole reduced DNA damage-induced apoptosis by >60%, whereas the frequency of micrornucleation was similar in the presence or absence of nocodazole. Flow cytometry revealed that nocodazole-treated cells sustained a second round of DNA replication without intervening mitosis. These results demonstrate that nuclear fragmentation and inappropriate DNA replication are insufficient to trigger apoptosis following DNA strand breakage and demonstrate a requirement for mitotic spindle assembly for this process in CHO cells.

INTRODUCTION

Cell death following exposure to a genotoxin can occur via several routes, including apoptosis, extended or permanent cell cycle arrest, necrosis, and mitotic catastrophe. Such variation can result from differences between cell types, although different modes of cell death can also occur within an individual cell type in response to a single genotoxin (1–3). In some cases, the mechanism by which cell death is triggered is dependent upon the dose of genotoxin used and/or duration of treatment. This may reflect promiscuity with respect to intracellular targets because most genotoxins can damage other macromolecules in addition to DNA, thereby eliciting multiple cell death pathways. For example, free radicals are induced by most genotoxins that arise continuously in cells at a frequency of ~1000 bases/cell/day (11). A number of CHO cell lines have been isolated that exhibit a defect specifically in rejoining SSBs. Most of these mutants harbor mutations within XRCC1, a protein required for binding and stability of DNA ligase III-α polypeptide and for rejoining SSBs induced by DNA base damage (12–17). Here, we have used the XRCC1 mutant cell line EM9 and its genetically matched, repair-proficient parent cell line AA8 to examine how unrejoined SSBs are coupled to loss of viability in CHO cells.

MATERIALS AND METHODS

Cell Growth Conditions, Drug Treatment, and Cell Cycle Synchrony. The CHO cell lines EM9 and AA8 (described previously by Thompson et al. (18) were maintained either as monolayers or in suspension in α-MEM with 10% FBS (Life Sciences, Paisley, United Kingdom). Clonogenic assays were performed in duplicate with 200–300 cells plated per 60-mm dish (Nunc). After 4 h, cells were treated with EMS (1 mg/ml; Sigma Corp.) at 37°C for 1 h. Treated cells were rinsed twice with PBS (Dulbecco's minus Mg2+; Ca2+; Life Technologies, Inc.) and incubated in drug-free medium for 10–14 days to allow formation of macroscopic colonies. Cells were synchronized at the G1–S transition essentially as described by Orren et al. (9). Briefly, cells were incubated for a minimum of 48 h in α-MEM with 0.1% FBS. The low-sodium medium was then replaced with α-MEM containing 10% FBS and 300 μM mimosine (Sigma Corp.), and cells were incubated overnight. Cells were then released into the cell cycle by replacing with normal medium. Synchronized cells were treated with EMS as described above before removal of mimosine-containing medium. Where indicated, the spindle microtubule poison, nocodazole (Sigma Corp.), was added to the fresh medium at a concentration of 20–40 ng/ml.

Flow Cytometry and Microscopy. To determine their DNA content, we fixed 1 × 10⁶ cells (70% methanol/30% PBS) at 4°C for 30 min, washed them twice, and resuspended the cells in PBS (1 ml). When monolayer cultures were used, care was taken to harvest both adherent and floating cell populations. Fixed cells were incubated for 60 min at 37°C with 0.5 mg/ml RNase A (Sigma Corp.). Propidium iodide (Sigma Corp.) was added at a final concentration of 50 μg/ml, and 1 × 10⁶ cells were analyzed by flow cytometry (Becton-Dickinson).

For microscopic investigation of nuclear morphology, cells (1 × 10⁶) were
RESULTS

Unrejoined SSBs Trigger Apoptosis in EM9 CHO Cells. Apoptosis is a genetically regulated mechanism leading to cell death in response to a diversity of stimuli. Although a variety of genotoxins induce apoptosis, the contribution of individual lesions to this response is poorly defined. To examine the relevance of DNA SSBs to apoptosis, mutant EM9 cells and parental AA8 cells were compared for biochemical and morphological hallmarks of apoptosis after treatment with the alkylating agent, EMS. As observed previously (17), treatment of asynchronous populations of AA8 and EM9 cells with 1 mg/ml EMS for 1 h reduced EM9 clonogenicity by >90% and repair-proficient parental AA8 cells by <10% (Fig. 1A). The presence of condensed chromatin in EM9 cells 72 h after EMS treatment suggested that apoptosis contributed to cell death (Fig. 1B). This was supported by the observation of nonrandom DNA degradation in EM9 cells 24 h after EMS treatment (Fig. 1C) and development of sub-G1 DNA content within 72 h as measured by flow cytometry (Fig. 1D).

The occurrence of apoptosis was further indicated by the observed induction of chromatin condensation (Fig. 2A) and nonrandom DNA cleavage (Fig. 2B) in HeLa nuclei incubated with cytosol from EMS-treated EM9 cells. Control cytosol prepared from untreated EM9 cells, or EMS-treated AA8 cells, had little effect on HeLa nuclei. EMS-treated EM9 cytosol also contained more apoptotic caspase activity than did control extracts, as measured by cleavage of HeLa nuclear DNA.

To detect PARP cleavage, nuclei/cells (3 × 10⁶) were lysed in 30 μl of fixative solution containing 0.3 volume of 37% formaldehyde (BDH, Laboratory Supplies, Poole, England), 0.6 volume of 80% glycerol, 0.1 volume of 10× cell extract buffer (see below), and 1 μg/ml Hoechst 33342 or DAPI (Sigma Corp.). Both adherent and suspension fractions of cells for each sample were analyzed for nuclear morphology. Cells to be used for α-tubulin staining were grown directly onto coverslips or, in the case of cells that had detached from coverslips, were centrifuged onto coverslips. Cells were fixed in 70% methanol (−20°C) for at least 30 min. Fixed cells were washed in PBS and then incubated at room temperature for 1 h with the anti-α-tubulin monoclonal antibody, TAT-1 (19), diluted 1:5. After washing with PBS, the cells were stained with secondary antibody conjugated to FITC (DAKO) for an additional h at room temperature. Coverslips were rinsed with PBS, counterstained with DAPI (1 μg/ml), and then mounted onto slides. Samples were analyzed by microscopy (Zeiss) and supporting software (Openlab). Mitotic, apoptotic, and micronucleation indices (see “Results”) are the result of assessing at least 10 individual microscopic fields at ×630.

Preparation of Cytosolic Extracts from CHO Cells and HeLa Cell Nuclei. Cytoplasmic extracts were prepared essentially as described (20). Briefly, suspension cultures of AA8 or EM9 cells (1 × 10³) at a maximum density of 5 × 10⁵ cells/ml were incubated with EMS (1 mg/ml) as described above or left untreated as controls. At various times after the removal of EMS, cells were collected by centrifugation and washed twice in 10 volumes of ice-cold PBS and once in 10 volumes of ice-cold cell extract buffer [CEB: 50 mM PIPES (pH 7.4), 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM DTT, 10 μM cytochalasin B, 1 mM phenylmethylsulfonyl fluoride]. Excess CEB was removed from the cell pellet, which was then transferred to a prechilled Dounce homogenizer (2 ml) and left for 20 min on ice to swell. The cells were then quick frozen in a liquid nitrogen bath and lysed gently while thawing by 15 strokes in a Dounce homogenizer (2 ml) using a tight pestle. Cell lysis was monitored by microscopic examination of an aliquot in the presence of trypan blue. Cell debris was removed by centrifugation at 10,000 × g for 15 min at 4°C. The cleared lysate was carefully transferred to a fresh tube. The protein concentration of extracts prepared in this way was typically 40 mg/ml. The cell lysate was diluted (10–15 mg/ml) in extract dilution buffer [EDB: 10 mM HEPES (pH 7.0), 50 mM NaCl, 2 mM MgCl₂, 5 mM EGTA, 1 mM DTT, 2 mM ATP, 10 mM phosphocreatine, and 50 μg/ml creatine kinase; Boehringer-Mannheim], quick-frozen, and stored in small aliquots under liquid nitrogen. HeLa cell nuclei were isolated as described (20).

Cell-free Reactions and Analysis of Apoptosis in Vitro and in Vivo. Cell-free reactions were performed essentially as described by Martin et al. (20). Each 30-μl reaction comprised 25 μl of cell extract (250–300 μg protein), 4 μl of EDB, and 1 μl of HeLa nuclei prewashed and resuspended in EDB (1 × 10³ nuclei/μl). Reactions were incubated at 37°C for 2 h. At the indicated time points, an aliquot (10 μl) was removed for analysis of PARP protein cleavage or DNA degradation or 1 μl for analysis of chromatin condensation by microscopy (see above). To analyze DNA degradation, 1 × 10⁶ cells/nuclei were added to TE buffer [10 mM Tris-Cl (pH 8.0), 1 mM EDTA] containing 0.5% sodium lauryl sarcosyl and 0.5 mg/ml proteinase K (Sigma Corp.) and incubated for a minimum of 3 h at 50°C. Lysates were then extracted once with an equal volume of aqueous-buffered phenol (Life Technologies, Inc.); nucleic acids were precipitated and resuspended in TE. RNase A was added to 0.1 mg/ml, and incubation continued for 60 min. DNA was separated by agarose gel electrophoresis (1.5% agarose) in the presence of ethidium bromide (0.5 μg/ml).

To detect PARP cleavage, nuclei/cells (3 × 10⁶) were lysed in 30 μl of SDS-PAGE sample buffer [50 mM Tris-Cl (pH 8.0), 2% SDS, 10% glycerol, 0.1% bromphenol blue, and 100 mM DTT] heated to 90°C for 5 min, and polypeptides were separated by SDS-PAGE (7.5%). After electrophoretic transfer, nitrocellulose filters (Hybond C; Amersham Corp., Buckinghamshire, United Kingdom) were immunoblotted and subjected to Enhanced Chemiluminescence as described by the manufacturer (Amersham). Anti-PARP monoclonal antibody (kindly provided by Dr. Said Aoufouchi, Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QL, England, clone A6.4.7; now available from Serotec, clone A6.4.12) was used as primary antibody at 1:100 dilution, followed by incubation with horseradish peroxidase-conjugated secondary antibody at 1:5000 dilution (DAKO).
PARP (Fig. 2C, top panel). As observed in other experimental systems (21–23), PARP cleavage was prevented by the peptide DEVD-CHO, but not by Ac-YVAD-CHO, suggesting the involvement of caspase-3 (Fig. 2C, bottom panel).

G2-M Arrest, Abnormal Mitosis, and Micronucleation Precede SSB-induced Apoptosis in EM9 CHO Cells. Apoptosis can be either cell cycle dependent or cell cycle independent. Because cell cycle dependency may be in part lesion specific, it was of interest to determine which scenario applied to SSBs. Within asynchronous cell populations, EMS-treated EM9 cells arrested with a G2-M (4N) content of DNA within 1h after EMS treatment (see Fig. 1D), before the appearance of cells with sub-G1 DNA content 24–72 h after EMS treatment (Fig. 1, C and D). These results suggest that EMS-treated EM9 cells undergo apoptosis during G2 and/or mitosis, consistent with the involvement of a cell cycle component in SSB-induced cell death. To examine this relationship further, EM9 cells were synchronized in G1 by serum starvation/mimosine (9), mock-treated or EMS-treated for 1 h, and released into cell cycle. Under these conditions, the number of apoptotic EMS-treated cells again increased 24–72 h after release from synchrony (Fig. 3A and B). An analysis of cellular DNA content by flow cytometry and cyclin B1 levels by immunoblotting confirmed that EMS-treated EM9 cells were again present in G2-M during this period, concomitant with the onset of apoptosis (Fig. 3C). Cyclin B1 levels increased in untreated and EMS-treated cells within 15 h, indicating rapid entry into G2-M after release from G1. However, whereas the level of cyclin B1 in untreated cells dropped after this period, consistent with exit from mitosis, the level in EMS-treated cells remained high 15–48 h after release from G1, indicating their presence in G2-M during this period. This interpretation was supported by flow cytometry, which confirmed that EMS-treated cells were present in G2-M up to 48 h after EMS (data summarized in Fig. 3C). That EMS-treated EM9 cells attempted mitosis during this period was indicated by an increase in mitotic chromatin present within multiple nuclei in a single cell, was scored at the indicated times after release from synchrony (Fig. 3, A and B). An analysis of cellular DNA content by flow cytometry and cyclin B1 levels by immunoblotting confirmed that EMS-treated EM9 cells were again present in G2-M during this period, concomitant with the onset of apoptosis (Fig. 3C). Cyclin B1 levels increased in untreated and EMS-treated cells within 15 h, indicating rapid entry into G2-M after release from G1. However, whereas the level of cyclin B1 in untreated cells dropped after this period, consistent with exit from mitosis, the level in EMS-treated cells remained high 15–48 h after release from G1, indicating their presence in G2-M during this period. This interpretation was supported by flow cytometry, which confirmed that EMS-treated cells were present in G2-M up to 48 h after EMS (data summarized in Fig. 3C). That EMS-treated EM9 cells attempted mitosis during this period was indicated by an increase in mitotic chromatin present within multiple nuclei in a single cell, was scored at the indicated times after release from synchrony (Fig. 3, A and B). An analysis of cellular DNA content by flow cytometry and cyclin B1 levels by immunoblotting confirmed that EMS-treated EM9 cells were again present in G2-M during this period, concomitant with the onset of apoptosis (Fig. 3C). Cyclin B1 levels increased in untreated and EMS-treated cells within 15 h, indicating rapid entry into G2-M after release from G1. However, whereas the level of cyclin B1 in untreated cells dropped after this period, consistent with exit from mitosis, the level in EMS-treated cells remained high 15–48 h after release from G1, indicating their presence in G2-M during this period. This interpretation was supported by flow cytometry, which confirmed that EMS-treated cells were present in G2-M up to 48 h after EMS (data summarized in Fig. 3C). That EMS-treated EM9 cells attempted mitosis during this period was indicated by an increase in mitotic chromatin present within multiple nuclei in a single cell, was scored at the indicated times after release from synchrony (Fig. 3, A and B). An analysis of cellular DNA content by flow cytometry and cyclin B1 levels by immunoblotting confirmed that EMS-treated EM9 cells were again present in G2-M during this period, concomitant with the onset of apoptosis (Fig. 3C). Cyclin B1 levels increased in untreated and EMS-treated cells within 15 h, indicating rapid entry into G2-M after release from G1. However, whereas the level of cyclin B1 in untreated cells dropped after this period, consistent with exit from mitosis, the level in EMS-treated cells remained high 15–48 h after release from G1, indicating their presence in G2-M during this period. This interpretation was supported by flow cytometry, which confirmed that EMS-treated cells were present in G2-M up to 48 h after EMS (data summarized in Fig. 3C). That EMS-treated EM9 cells attempted mitosis during this period was indicated by an increase in mitotic
However, the mitoses appeared abnormal, exhibiting incomplete or abnormal chromosome condensation and disorganized chromosome and spindle distribution (Fig. 3E). Furthermore, an increase in the number of micronucleated cells within the EMS-treated population was observed 36–72 h after release from G1 (Fig. 3F), consistent with exit from mitosis in the absence of normal chromosome segregation (1, 2). Micronucleation reflects the formation of discrete nuclear membranes around chromosomes that have not segregated properly and appears to be the result of aberrant mitosis (1).

Assembly of a Mitotic Spindle Is Required for SSB-induced Apoptosis. Abnormal spindle formation and micronucleation was observed previously to precede apoptosis in cisplatin-treated UV41 CHO cells (3). However, the role of these phenomena in the induction of apoptosis was not addressed. To examine whether abnormal mitotic spindle formation activates SSB-induced apoptosis, EMS-treated EM9 cells were prevented from assembling mitotic spindles by the addition of nocodazole. Immunofluorescent analysis of EMS-treated EM9 cells, arrested in G2-M, with anti-α-tubulin antibodies confirmed that nocodazole prevented formation of mitotic spindles (compare Fig. 3E and Fig. 4B, left panels). In addition, nocodazole prevented the defect in chromosome morphology observed previously (compare Fig. 3E and Fig. 4B, right panels). That nocodazole functioned as expected in these experiments was also indicated by the appearance, in asynchronous populations of EM9 cells, of cells with 8N DNA content after incubation for 48–72 h in the presence of this compound (Fig. 4A, right-hand panels). The appearance of octaploid cells results from the completion of a second round of DNA synthesis in cells in which an intervening cell division did not occur. A small subpopulation of 8N cells was also observed in EMS-treated cells in the presence of nocodazole, suggesting that nocodazole relieved SSB-induced apoptosis in these cells. A concomitant decrease in the number of sub-G1 cells was also noted in cells treated with both agents relative to those treated only with EMS (see especially Fig. 4A; 48 h), further suggesting that nocodazole relieved SSB-induced apoptosis. Finally, nocodazole reduced by ∼60% the frequency of apoptotic cells present 48–72 h after EMS treatment, as measured by nuclear morphology (Fig. 4C). These results were observed in four independent experiments. The effect of nocodazole on SSB-induced apoptosis may have been greater than was apparent because nocodazole itself induces some apoptosis over this time, by a different mechanism (24) and may account for some of the residual apoptosis observed in cells treated with both agents.

The results described above strongly suggest that a mitotic spindle was required both for the abnormalities in chromosome condensation/organization visible during mitosis in EMS-treated EM9 cells and for the subsequent onset of apoptosis. In contrast, the overall frequency of micronucleated cells present 48–72 h after EMS treatment was not significantly altered by nocodazole, demonstrating that nuclear fragmentation was not sufficient to trigger apoptosis (Fig. 4D).

DISCUSSION

DNA repair-defective cells provide a useful system by which the response to a genotoxic can be ascribed to a particular DNA lesion, rather than to other macromolecular damage capable of inducing apoptosis (e.g., membrane lipid peroxidation). Here, we have used the DNA repair mutant EM9 to examine how loss of viability is triggered by unrejoined DNA SSBs in CHO cells. Morphological and biochem-
rical analysis of EMS-treated EM9 cells implicated apoptosis in the cell death process, as measured by nuclear morphology, nonrandom DNA cleavage, and caspase activity. In addition to apoptotic EM9 cells, enlarged cells containing micronuclei were observed among the adherent population 36–72 h after EMS treatment. Micronucleation is believed to result when damaged cells escape G2 cell cycle arrest and undergo an aberrant mitosis (1–3, 24, 25). Consistent with this scenario, both asynchronous and G1-synchronized populations of EM9 accumulated in G2-M within 24 h after exposure to EMS and appeared to undergo an aberrant mitosis 24–48 h after EMS treatment (Figs. 3 and 4). The loss of clonogenicity arising from these events is termed mitotic cell death and has been observed in a variety of cell types after exposure to both genotoxins and nongenotoxins, identifying this as a common form of cell death (2, 3, 26). However, despite its widespread occurrence, the molecular mechanisms of mitotic cell death are unknown. There is a paucity of information concerning the relationship between this process and apoptosis, both of which occur in response to DNA damage in HeLa cells and in CHO cells, with apoptosis apparent in the detached population soon after the onset of micronucleation (Refs. 2 and 3 and this study). Both in this study and in a previous study (3), mitotic death was accompanied by the formation of an abnormal mitotic spindle. In the present study, nocodazole reduced SSB-induced apoptosis by 60%, 72 h after EMS treatment, suggesting that the assembly of a mitotic spindle was required to trigger apoptosis in response to single-strand breakage. The effect of nocodazole on SSB-induced apoptosis may have been greater than was apparent, because nocodazole itself induces some apoptosis over this time (~10%) via another mechanism (24) and may thus account for some of the residual apoptosis observed in EMS-treated cells. Although apoptosis was reduced in EMS-treated EM9 cells in the presence of nocodazole, the overall frequency of micronucleation was similar to that present in the absence of nocodazole, suggesting that nuclear fragmentation is insufficient for apoptosis in CHO cells. It has also been suggested that reentry into S-phase or progression through G1 of cells that have not completed cell division can trigger apoptosis (24, 25). However, a subpopulation of EM9 cells that had completed a second round of DNA synthesis was observed in the presence of nocodazole, indicating that inappropriate entry into S-phase is also insufficient to trigger apoptosis. This may reflect the probable absence of functional p53 in CHO cells (27).3

How might a mitotic spindle be required for SSB-induced apoptosis? One possibility is that temporal progression beyond spindle formation is required to trigger apoptosis, which is thus prevented by inhibition of spindle assembly and activation of the mitotic checkpoint. For example, perhaps it is the process of anaphase in the presence of an abnormal spindle or damaged chromosomes that triggers apoptosis. Alternatively, it is possible that the activity of one or more polypeptides that “interface” with the apoptotic machinery is regulated by, or is related to, spindle assembly. Consistent with this notion, the inhibitor-of-apoptosis protein, survivin, has been shown to reside on the mitotic spindle (28). Prolonged G2 nuclear arrest in cells with DNA damage may result in deregulated spindle assembly, thus accounting for the appearance of multipolar spindles, which may inhibit or activate spindle-associated regulators of apoptosis. It is now of interest to identify which, if any, polypeptide components of mitosis are responsible for coupling unpaired DNA damage to apoptosis.

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REFERENCES


3 P. A. Johnson and K. W. Caldecott, unpublished observations.

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