Prolonged Cell Cycle Response of HeLa Cells to Low-Level Alkylation Exposure

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Abstract

Alkylation chemotherapy has been a long-standing treatment protocol for human neoplasia. N-methyl-N-nitro-N-nitrosoguanidine (MNNG) is a direct-acting monofunctional alkylator. Temozolomide is a clinical chemotherapeutic equivalent requiring metabolic breakdown to the alkylating agent. Both chemicals have similar mechanistic efficacy against DNA mismatch repair–proficient tumor cells that lack expression of methylguanine methyltransferase. Clinically relevant concentrations of both agents affect replicating cells only after the first cell cycle. This phenomenon has been attributed to replication fork arrest at unrepaired O6-methyldeoxyguanine lesions mispaired with thymine during the first replication cycle. Here, we show, by several different approaches, that MNNG-treated tumor cells do not arrest within the second cell cycle. Instead, the population slowly traverses through mitosis without cytokinesis into a third cell cycle. The peak of both ssDNA and dsDNA breaks occurs at the height of the long mitotic phase. The majority of the population emerges from mitosis as multinucleated cells that subsequently undergo cell death. However, a very small proportion of cells, <1:45,000, survive to form new colonies. Taken together, these results indicate that multinucleation within the third cell cycle, rather than replication fork arrest within the second cell cycle, is the primary trigger for cell death. Importantly, multinucleation and cell death are consistently avoided by a small percentage of the population that continues to divide. This information should prove clinically relevant for the future design of enhanced cancer chemotherapeutics. [Cancer Res 2009;69(15):6307–14]

Introduction

N-methyl-N-nitro-N-nitrosoguanidine (MNNG) produces several different alkylated DNA lesions, the majority of which are repaired efficiently by the base excision repair pathway and are of low mutagenic potential (1–3). One exception is the O6-methyldeoxyguanine (O6-meG) modification that is repaired by methylguanine methyltransferase (MGMT). The MGMT suicide mechanism covalently transfers the O6-methyl group to an active site residue, thereby rendering the enzyme useless after a single repair reaction (4). If left un repaired, O6-meG is frequently mispaired with thymine by replicating polymerases, resulting in an A → G mutation if not corrected before the succeeding replication cycle (5, 6). DNA mismatch repair (MMR)–proficient cells that lack MGMT expression show significantly enhanced sensitivity to alkylating agents by decreased colony survival and increased G → A mutation frequency of surviving cells (5–7). MMR-proficient cells exposed to low concentrations of monofunctional alkylating agents have been reported to undergo G2 arrest within the second cell cycle and subsequent apoptosis (8–10). Conversely, cells that lack MMR and MGMT activity are highly tolerant to alkylating damage (decreased G2 arrest and increased colony survival), with surviving cells sustaining a high mutation frequency. This is likely due to the combined effects of increased cell survival and absence of both MMR and MGMT repair activities (11–14). From this, it is apparent that the MMR pathway is required for O6-meG–induced damage signaling but not for the actual repair of this DNA lesion.

The phenomenon of second cell cycle arrest and subsequent apoptosis after exposure to low concentrations of monofunctional alkylating agents has been attributed to the recruitment of MMR proteins to O6-meG:T mispairs formed during the first S phase (15, 16). Two different models have been proposed for triggering of a DNA damage response and cell cycle arrest within the second G2 phase. One model involves direct activation of the ATR signaling pathway by these complexes (16, 17). This model has been substantiated by the generation of knock-in mice harboring separation-of-function mutations in the MMR pathway (18, 19). Another model describes an indirect triggering of the DNA damage signaling pathway by futile rounds of mismatch excision repair opposite the damaged nucleotide (8, 20). Persistent excision intermediates would lead to replication fork arrest that, in turn, would trigger the ATR damage signaling cascade (21). Because fairly broad concentration ranges of monofunctional alkylators such as MNNG have been reported to induce this phenomenon within different cell lines, the terminology of low-level MNNG is defined to be within a concentration range that does not induce immediate cell cycle arrest but eventually induces cell death as evidenced by the colony survival assay (13, 22). The extended length of time between initial low-level alkylation exposure and subsequent onset of death of the tumor cell population provides significant challenges for understanding cellular mechanisms contributing to this process. Delayed cell cycle arrest and DNA damage and repair events contributing to cell death by exposure to temozolomide, a clinical chemotherapeutic agent, have been reported to be mechanistically identical to tumor cell lines exposed to low-level MNNG (23). Therefore, exposure of tumor cells to direct-acting alkylators, such as MNNG, has become a useful tool to decipher mechanisms that limit chemotherapeutic efficacy of alkylating drugs such as temozolomide. Major limitations of treatment protocols involving alkylating agents include toxicity to...
the patient, development of tumor resistance, and chemotherapy-induced secondary tumors (24–27).

Here, we have investigated the long-term consequences of low-level MNNG exposure to HeLa MR cells that are MMR proficient and lack expression of MGMT. A methodical examination of late temporal events within the MNNG-treated tumor cell population shows that G2 arrest within the second cell cycle, as has been generally accepted, does not occur to any measurable extent despite that activation of the ATR signaling pathway does occur during this part of the cell cycle (10, 15, 28). Rather, the cell population continues to slowly traverse through the subsequent mitotic phase, with the majority of mitotic cells exhibiting aberrant centrosomes and increased strand breakage. After completion of mitosis, multinucleated cells predominated. The majority of this population eventually succumbs to cell death over the next few days. However, once this crisis has passed, multiple overlapping clones of mononucleated cells that have escaped cell death continue to divide to form growing colonies.

Materials and Methods

Cell lines. HeLa MR cells (MGMT−) were grown in DMEM/Ham’s F12 50/50 mix (DMEM/F12; Invitrogen) + 10% fetal bovine serum (Atlanta Biologicals, Inc.) at 37 °C in a 5% CO2 humidified atmosphere. HeLa MR cells were a kind gift from Dr. Sankar Mitra (University of Texas, Galveston, TX).

Chemicals and reagents. MNNG, etoposide, camptothecin, and thymidine were all purchased from Sigma. Z-Val-Ala-Asp(Ome)-fluoromethylketone (Z-VAD) was purchased from Bachem. 4’,6-Diamidino-2-phenylindole (DAPI) was purchased from Molecular Probes. Antibodies against caspase-8 and phospho-CDC2 (Y15) were from Cell Signaling Technology, antibody against CDC2 was from Santa Cruz Biotechnology, antibody against replication protein A (RPA) was from Calbiochem, antibody against phospho–histone H3 was from Millipore, antibody against phospho-Chk1 (S345) was from Cell Signaling Technology, antibody against Chk1 was from Bethyl, antibody against phospho–histone H2AX was from Abcam, anti–poly(ADP-ribose) polymerase (PARP) was from BD Pharmingen, and antibodies against α-tubulin and γ-tubulin were from Sigma. Secondary antibodies Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 546 goat anti-mouse IgG were purchased from Molecular Probes.

Cell cycle synchronization, DNA damage treatment, and inhibition of apoptosis. Cell cycle synchronization into late G1–early S by double thymidine block (DTB) has been described previously (7, 29). For MNNG treatment, 0.2 μmol/L MNNG was added to medium immediately after release from DTB. Z-VAD (50 nmol/L) was added to cells starting 24 h after release from DTB to inhibit apoptosis. Cells were treated with etoposide (500 nmol/L) for 20 h or camptothecin (1 μmol/L) for 20 h and Z-VAD (50 nmol/L) was added to cells starting 24 h after treatment, 0.2 mol/L thymidine block (DTB) has been described previously (7, 29). For MNNG synchronization, cells were trypsinized, pelleted by centrifugation (600 × g for 5 min), and resuspended in 500 μL PBS. Ice-cold 70% ethanol (4.5 mL) was slowly added to each cell suspension while gently vortexing to inhibit clumping. Before flow analysis, cells were repelleted, rinsed with PBS, and incubated for 30 min in staining solution containing 0.1% Triton X-100, 0.2 mg/mL RNase A, and 20 μg/mL propidium iodide. Monitoring of cell cycle phase distribution was accomplished by using a Beckman/Coulter EPICS Elite flow cytometer, using 10,000 cells per time point, as described previously (30). These 96-h experiments were repeated thrice in duplicate.

Protein isolation and immunoblot analysis. Whole cells, nuclear lysates, and chromatin cross-linked proteins were isolated as described previously (7). After determination of protein concentrations (Bio-Rad), supernatants were stored at −80 °C. DNA concentrations of chromatin cross-linked experiments were determined by 260/280 absorbance ratio, as described previously (7). For immunoblots, equal protein concentrations of whole-cell or nuclear extracts, or protein extracted from equal DNA concentrations (chromatin-protein cross-link experiments), were resuspended in SDS sample buffer and separated by denaturing SDS-PAGE. Transfer to polyvinylidene difluoride membranes and immunoblot analyses were performed as described previously (7). Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL) following the manufacturer’s directions (ECL solution; Amersham Pharmacia Biotech, Inc.) via exposure to X-ray film. Chemiluminescence quantification of each protein band was measured using the Alpha Innotech FluoroChem HD2.

Indirect immunofluorescence and microscopy. For indirect immunofluorescence detection by microscopy, HeLa MR cells were plated onto glass coverslips at a density of 20,000 per coverslip. After synchronization and MNNG treatment, cells were fixed at indicated times with either 4% paraformaldehyde (pH 7.5) or ice-cold methanol for 15 min, depending on the antibody to be used, per manufacturer’s protocol. Images were acquired using a Nikon TE2000U fluorescence microscope equipped with a Photometrics CoolSnap EZ Monochrome digital camera system and NIS Elements Basic Research software package.

Comet assays. dsDNA breaks within cells synchronized by DTB ± MNNG were measured by neutral comet assay, as described by Olive and colleagues (31), and ssDNA breaks were measured by alkaline comet assay as described by Singh and colleagues (32), with modifications as described below.

For neutral comet assay, cell suspensions were mixed with 0.75% low-melting point agarose, layered onto the microscope slides precoated with 1% normal agarose, and allowed to solidify. A final layer of 0.75% low-melting point agarose was then added. The slides were immersed in cold lysis solution [2.5 mol/L NaCl, 100 mmol/L EDTA, 10 mmol/L Tris (pH 10), with 1% Triton X-100 added just before use] for 1 h at 4 °C in the dark. Slides were rinsed thrice in Tris-borate EDTA (TBE) buffer [90 mmol/L Tris, 90 mmol/L boric acid, 1 mmol/L EDTA (pH 8.3)] and incubated for 30 min in the dark. Electrophoresis was carried out in fresh TBE buffer at 25 V for 30 min. The alkaline comet assay was performed similar to the neutral comet assay, except that after lysis the slides were transferred to an electrophoresis tank containing ice-cold alkaline solution [300 mmol/L NaOH, 1 mmol/L EDTA (pH > 13)] and incubated for 20 min in the dark. Electrophoresis was carried out for 25 min at 300 mA. The slides were then placed into neutralizing solution [0.4 mol/L Tris-HCl (pH 7.5)] for 10 min and stained with SYBR Green (Trevigen). Comets were scored using a Nikon epifluorescence microscope at ×200 magnification. Fifty cells were analyzed per slide using Comet Assay Software 5.5F (Kinetic Imaging). The parameters used to evaluate DNA damage were Olive tail moment, a measurement of tail length and fluorescent intensity of DNA within the tail, and percentage of tail DNA, a measurement of amount of DNA migrated into the tail and expressed as a percentage of DNA present in the comet head. Each time point of the experiment was repeated at least twice.

Results

We have investigated low-level MNNG-induced events occurring after the first cell cycle to better understand mechanisms within the cell contributing toward cell death. HeLa MR cells synchronized to late G1–early S phase by DTB were released into medium containing 0.2 μmol/L MNNG, allowing HeLa MR cells to traverse the first cell cycle in a normal fashion, after which the cells begin to exhibit several effects of alkylation treatment.

Initially, we examined phosphorylation patterns of RPA, as this information can be used to distinguish between synchronized cells undergoing mitosis or a DNA damage response. Figure 1A depicts two separate experimental time lines of the phosphorylation status of RPA after MNNG treatment of synchronized HeLa MR cells. The upper immunoblot is of RPA cross-linked to chromatin at several time points up to 48 hours after release from synchrony. Both MNNG-treated and untreated cells exhibit the mitotic phosphorylation form of RPA (form 3, shown in RPA phosphorylation forms box) at 6 hours, indicating similar synchronous traversal of the first cell cycle. Cells not exposed to MNNG lose synchrony after 8 hours.
therefore, the second mitotic phase of untreated cells is undetectable (7). MNNG-treated cells seem to undergo a delayed and very prolonged mitotic phase starting at 24 hours after treatment, as evidenced by the predominance of the mitotic RPA form. This form is in addition to the hyperphosphorylated form (form 5) of RPA bound to the chromatin at 32 to 48 hours, indicating a DNA damage response as well (33). We then performed an experiment using equal amounts of protein from whole-cell lysates were immunoblotted for RPA-p34. The +Z lanes indicate that cells were treated with Z-VAD at 24 h after DTB release. RPA phosphorylation controls (right) are 0 h for nonphosphorylated RPA (forms 1 and 2). Both 8 h (nocodazole arrested) and mitotic shake indicate the first mitotic phase after DTB release (mitotic RPA; form 3). Camptothecin and etoposide treatment produced hyperphosphorylated RPA (form 5). S phase contains phosphorylated RPA that is neither mitotic nor hyperphosphorylated (form 2). A, cell cycle analysis by flow cytometry of DNA content. HeLa cells were synchronized by DTB and treated with 0.2 μmol/L MNNG. Whole cells were fixed with ethanol for flow cytometry at indicated times. The bottom panel of the upper flow diagrams depict cells treated with Z-VAD at 24 h after DTB. Bottom, an enlargement of the 72- to 96-h time points. C, cell cycle analysis by phase-specific phosphorylated proteins. Equal amounts of protein from whole-cell lysates harvested at the indicated times were immunoblotted for phospho-CDC2 (Tyr15; G2 phase) and phospho-H3 (mitotic phase). Loading control was α-tubulin.

**Figure 1.** A, phosphorylated RPA within DTB HeLa MR cells over time. Cells were synchronized by DTB and released into medium with or without 0.2 μmol/L MNNG (0 h) and then harvested at indicated times. Top immunoblot, cells were exposed to 1% formalin and cross-linked chromatin was purified at each time point. Equal amounts of cross-linked chromatin were immunoblotted with antibody to RPA-p34. Bottom immunoblot, cells were harvested at each time point and equal amounts of protein from whole-cell lysates were immunoblotted for RPA-p34. The +Z lanes indicate that cells were treated with Z-VAD at 24 h after DTB release. RPA phosphorylation controls (right) are 0 h for nonphosphorylated RPA (forms 1 and 2). Both 8 h (nocodazole arrested) and mitotic shake indicate the first mitotic phase after DTB release (mitotic RPA; form 3). Camptothecin and etoposide treatment produced hyperphosphorylated RPA (form 5). S phase contains phosphorylated RPA that is neither mitotic nor hyperphosphorylated (form 2). B, cell cycle analysis by flow cytometry of DNA content. HeLa cells were synchronized by DTB and treated with 0.2 μmol/L MNNG. Whole cells were fixed with ethanol for flow cytometry at indicated times. The bottom panel of the upper flow diagrams depict cells treated with Z-VAD at 24 h after DTB. Bottom, an enlargement of the 72- to 96-h time points. C, cell cycle analysis by phase-specific phosphorylated proteins. Equal amounts of protein from whole-cell lysates harvested at the indicated times were immunoblotted for phospho-CDC2 (Tyr15; G2 phase) and phospho-H3 (mitotic phase). Loading control was α-tubulin.

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discovered that careful fixation of the whole cell (slowly adding ethanol to carefully dispersed cell pellets) before flow cytometry inhibits mechanical degradation of the chemically damaged nuclei. By this method, we were able to show that MNNG-treated HeLa cells seem to undergo a very prolonged traversal of the second G2-M phase of the third cell cycle (Table 1; Fig. 1B), with a portion of the population cycling further (note 84- to 96-hour time points). This MNNG-induced slow traversal of the cell cycle is unaltered by the addition of Z-VAD to inhibit apoptosis. Flow analysis also indicates that the bulk of the population does not undergo cytokinesis at the end of the second cell cycle. The largest peak throughout 48 to 96 hours is of cells containing 4N DNA, which is not distinguishable by flow analysis between G2 or M or G1 phase of cells failing to undergo cytokinesis during mitosis. Table 1 shows the percent of cells in each phase of the second and third cell cycles containing 4N→8N DNA. Cells containing sub-G1 (apoptotic) DNA do not appear until 72 to 96 hours and do not exceed 23% of the population. The smallest number of cells contain from 2N up to 4N DNA, which is likely a mixture of cells that have halted within the second cell cycle and cells that are dividing normally during this period of time.

We then investigated phosphorylation of CDC2 (also known as CDK1) and of histone H3 to determine if there is biochemical evidence for MNNG-induced cell cycle arrest in G2 or M of the second cell cycle. Dephosphorylation of Tyr15 of the CDK1/cyclin B1 complex triggers entrance into mitosis (34), whereas H3 is only phosphorylated from prophase through anaphase of mitosis (35). The sequential phosphorylation/dephosphorylation of these two proteins indicates that G2 phase (pCDC2) of the second cell cycle has started by 24 hours, peaks at 48 hours, and is almost complete by 72 hours, whereas the mitotic phase (pH3) begins at 48 hours, peaks at 60 to 72 hours, and is almost complete by 96 hours (Fig. 1C). Inhibition of apoptosis with Z-VAD does not alter these biochemical cell cycle events, in agreement with flow analysis and RPA phosphorylation events. Z-VAD does inhibit the apoptotic process beginning at 72 hours after MNNG treatment, evident by

<table>
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<tr>
<th>2nd cell cycle</th>
<th>24 h</th>
<th>2nd + 3rd cell cycle*</th>
<th>48 h</th>
<th>48 h + Z</th>
<th>60 h</th>
<th>60 h + Z</th>
<th>72 h</th>
<th>72 h + Z</th>
<th>84 h</th>
<th>84 h + Z</th>
<th>96 h</th>
<th>96 h + Z</th>
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<tr>
<td>G1 (%)</td>
<td>11</td>
<td>(G2-M-G1)</td>
<td>95</td>
<td>96</td>
<td>96</td>
<td>94</td>
<td>86</td>
<td>85</td>
<td>69</td>
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<td>S (%)</td>
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<td>2</td>
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<td>27</td>
<td>21</td>
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<td>31</td>
</tr>
<tr>
<td>G2 (%)</td>
<td>10</td>
<td>G2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>9</td>
<td>5</td>
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</tbody>
</table>

*Percent of cells containing 4N-8N DNA in each phase of second and third cell cycle.

Figure 2. A, effect of Z-VAD treatment on synchronized HeLa MR cells. Cells were plated in triplicate at 8 × 10⁵/100-mm plate, synchronized by DTB, and treated with 0.2 µmol/L MNNG (0 h) and with Z-VAD at 25 h as indicated. At each indicated time, plates were rinsed in PBS and cells were trypsinized and counted using a Coulter Z2. Columns, mean number of cells per plate; bars, SD. Paired t tests were performed between untreated and Z-VAD–treated cells at each time point. Bar graph and statistics were achieved using Prism GraphPad software. B, phosphorylation of Chk1 over time within synchronized cells. Equal amounts of protein from whole-cell lysates harvested at the indicated times were immunoblotted for total Chk1 and phospho-Chk1 (S345). Bar graph, quantification of chemiluminescent immunoblot signals using Alpha Innotech FluoroChem HD2 imaging system and Prism GraphPad software.
immunoblot analyses of PARP and caspase cleavage products (Supplementary Fig. S1). Flow analysis indicates that up to 23% of the cells at 84 to 96 hours contain sub-G₁ DNA. This is also substantiated by the number of cells still adhered to plates at each time point (Fig. 2A). Although a significantly increased number of cells in Z-VAD–treated plates occur only at 72 and 96 hours, there are consistently more cells in each Z-VAD plate beginning at 60 hours after MNNG (35 hours after Z-VAD) treatment. Phosphorylation of Chk1 by ATR has been hypothesized to trigger the second cell cycle G₂ arrest and subsequent apoptosis after MNNG treatment (15, 16); therefore, we investigated the phosphorylation of Chk1 over time. Chk1 is highly phosphorylated through 48 hours but begins to decrease at 60 hours and is almost completely gone by 96 hours regardless of treatment with Z-VAD (Fig. 2B). This decrease of phospho-Chk1 is in synchrony with cells leaving G₂ phase of the second cell cycle (Fig. 1A–C).

Table 2. DTB HeLa MR + 0.2 μmol/L MNNG mitotic and multinucleated % of population

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Total percent mitotic</th>
<th>Normal mitotic</th>
<th>Abnormal mitotic</th>
<th>Total percent multinucleated</th>
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<tr>
<td>72</td>
<td>26.2%</td>
<td>5.9%</td>
<td>17.1%</td>
<td>21.6%</td>
</tr>
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</table>

*Undetermined normal or abnormal mitosis (3.2%) from total of 963 cells.

The above results led us to investigate the fraction of the MNNG-treated population undergoing mitosis over this time period. Microscopic visualization of pH3-stained cells confirmed that the percent of mitotic cells peaked at 72 hours (~ 20%) with or without Z-VAD (Fig. 3A). We have previously treated HeLa cells with nocodazole to arrest in prophase, released, and observed pH3-stained cells for up to 2 hours.1 We were unable to determine what percent of the MNNG-treated population entered or completed mitosis between 24 and 96 hours, a 72-hour time period. However, this microscopic evaluation revealed an increasing population of multinucleated cells as mitotic cells decreased over time. An equivalent number of cells were either in mitosis or multinucleated at 72 hours, indicating a crucial time point after MNNG treatment for evidence of abnormal mitosis preceding multinucleation (36). Indeed, we observed approximately thrice the number of abnormal mitotic cells compared with cells undergoing normal mitosis at 72 hours (Table 2; Fig. 3B). The abnormal mitotic cells contained increased numbers of centrosomes, and the multinucleated cells contained numerous nuclear lobes. In contrast, untreated HeLa cells completely lacked abnormal mitotic or multinucleated cells, with <5% of the population undergoing normal mitosis.

We then asked if increased DNA breaks preceded the abnormal mitotic phase, as strand breaks during the second replication phase have been hypothesized to be the proximate cause of cell death after low-level MNNG treatment (8, 15). Figure 4A instead shows a surprisingly close correlation between the height of mitotic phase (pH3) and elevation of γH2AX (dsDNA breaks) within the

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1 Unpublished results.
MNNG-treated cells. To determine if the increased γH2AX was cell cycle related or induced by actual strand breaks within individual cells, we performed comet assays after MNNG treatment. Figure 4B shows that both ssDNA and dsDNA breaks are in highest abundance at 72 hours by measurements of both Olive tail moment and percentage of tail DNA. Although ~40% of the population is either mitotic or multinucleated at this time (Fig. 3A), multinucleated cells increase to ~50% of the population by 96 hours, which has less strand breaks. In addition, G2 phase of the cell population is highest at 48 hours, with lower strand breaks (Figs. 1C and 2F). Taken together, these results point toward increased DNA strand breaks occurring during abnormal mitosis. Surprisingly, both ssDNA and dsDNA breaks are decreased by 96 hours. This may simply indicate that living cells at 96 hours have either sustained less or repaired more of the previously occurring DNA damage.

As a whole, immunoblot, flow, and microscopic analyses indicate that low-level MNNG treatment of this neoplastic cell line does not induce a generalized G2 arrest within the second cell cycle nor replication fork arrest during the preceding DNA synthesis phase. Instead, the majority of the population continues to survive through G2 and mitosis into the third cell cycle up to 96 hours after treatment.

These studies have shown that MNNG-treated cells survive even beyond 96 hours. Therefore, we examined cell survival using our current plating protocol rather than the standard colony survival assay (300 cells/60-mm plate) that we have previously determined to result in 100% cell death after 0.2 µmol/L MNNG (7). The current protocol results in $1.4 \times 10^7$ synchronized cells to be exposed to MNNG treatment. From this number, we have repeatedly observed that at least 300 cells per 150-mm plate continue to grow into overlapping colonies within 2 weeks of MNNG treatment. Therefore, ~1:46,000 of the original MNNG-treated cells seem to survive the treatment protocol. Standard colony survival assays would not detect this low percentage of surviving cells. Survival may also depend on closer cell proximity. We are currently
investigating biochemical and genetic alterations contributing to the survival of these colonies.

Discussion

Our current studies show that low-level MNNG-treated tumor cells are not undergoing a detectable amount of second cell cycle G2 arrest, as has been previously reported (9, 16). These cells are instead undergoing a prolonged G2 and mitotic phase, with a significant proportion of the population surviving into the third cell cycle by 96 hours after treatment. During this time, a very small subset continues to cycle normally. This population-wide traversal beyond mitosis is evident by several parameters, including mitotic RPA and flow cytometric profiles, as well as cell phase–specific markers for G2 (pCDC2) and mitotic phase (pH3). We have also shown that phospho-Chk1 is increased during G2, in agreement with reports of others (15, 16). However, here, we show that Chk1 is decreased to background levels as the cell population moves into mitosis and beyond. Although the onset of apoptotic cell death is apparent by apoptotic cleavage products and decreased cell numbers as the cells traverse G2 into the mitotic phase, multiple indicators show this to be a low-level ongoing event up to 96 hours beyond treatment. Most significantly, at 72 hours, mitotic and multinucleated populations are equal, after which the mitotic fraction decreases as the multinucleated fraction increases. From this, we hypothesize that cell death of the MNNG-treated tumor cell population occurs primarily after cells have become multinucleated within the third cell cycle. Our results indicate that cell death does not occur to a measurable extent during S or G2 of the second cell cycle despite previous evidence by several conventional biomarkers (10, 15–17, 21, 28, 30, 37). Therefore, DNA strand breaks caused by replication fork collapse during S phase leading to cell death in G2 are an unlikely mechanism of cell death after low-level alkylation exposure. It is important to note that our current results are not in disagreement with previous investigations. Here, we have shown a very protracted G2 phase as well as activation of the ATR DNA damage signaling pathway and subsequent onset of apoptotic indicators, all of which are generally attributed to cell cycle arrest and apoptosis of the entire population. Conventional flow cytometry of ethanol-fixed nuclei shows that DNA content moves only from second phase G2-M into sub-G1, also indicating population-wide apoptosis (15, 30). We now believe that this type of flow cytometry profile reflects the alkylation-damaged nuclei undergoing artifactual (mechanical) disintegration during the flow cytometric process. Thus, by replacing conventional flow cytometric procedures and extending the experimental timeline, we have observed that there is consistent population-wide survival of MNNG-treated cells beyond G2 of the second cell cycle. These results indicate that after low-level alkylation damage, tumor cells undergo a transient second cell cycle pause rather than permanent arrest and cell death. Although HeLa cells do not contain active p53, it should be noted that the majority of human neoplasms either do not contain active p53 or contain other mutations that inactivate DNA damage checkpoint genes (38).

Some fraction of the MNNG-treated tumor cell population likely undergoes mitotic catastrophe, as the prolonged mitotic phase is significantly impaired by ssDNA and dsDNA breaks and the presence of multiple centrosomes (34, 36, 39). It has been reported that cells with damaged DNA that undergo activation of pCDC2 (pCDK1) enter mitosis but develop highly aberrant mitotic figures and enter the ensuing G1 as highly multinucleated and nonviable (36). Indeed, we have found that the majority of cells complete the second mitotic phase without undergoing cytokinesis (cell division) but instead become multinucleated with highly abnormal, multi-lobed nuclei containing 4N DNA. These cells eventually die without further replication, although a fraction of the population gains up to 8N DNA, indicating traversal through S phase. The remaining long-term surviving cells are much fewer in number and do not seem multinucleated (studies ongoing). These studies should help to refine targeting of future chemotherapy regimens. Ensuring that tumor cells do not escape mitotic catastrophe after alkylation treatment could be a viable direction to examine more closely.

Disclosure of Potential Conflicts of Interest

The authors declare that they have no competing financial interests.

Acknowledgments

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