DNA Replication Is Required To Elicit Cellular Responses to Psoralen-Induced DNA Interstrand Cross-Links

YASSMINE M. N. AKKARI,* RAYNARD L. BATEMAN, CAROL A. REIFSTECK, SUSAN B. OLSON, AND MARKUS GROMPE

Department of Molecular and Medical Genetics, Oregon Health Sciences University, Portland, Oregon 97201

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Following introduction of DNA interstrand cross-links (ICLs), mammalian cells display chromosome breakage or cell cycle delay with a 4N DNA content. To further understand the nature of the delay, previously described as a G2/M arrest, we developed a protocol to generate ICLs during specific intervals of the cell cycle. Synchronous populations of G1, S, and G2 cells were treated with photoactivated 4′-hydroxymethyl-4,5,8-trimethylpsoralen (HMT) and scored for normal passage into mitosis. In contrast to what was found for ionizing radiation, ICLs introduced during G2 did not result in a G2/M arrest, mitotic arrest, or chromosome breakage. Rather, subsequent passage through S phase was required to trigger both chromosome breakage and arrest in the next cell cycle. Similarly, ICLs introduced during G1 did not cause a G1/S arrest. We conclude that DNA replication is required to elicit the cellular responses of cell cycle arrest and genic instability after psoralen-induced ICLs. In primary human fibroblasts, the 4N DNA content cell cycle arrest triggered by ICLs was long lasting but reversible. Kinetic analysis suggested that these cells could remove up to ~2,500 ICLs/genome at an average rate of 11 ICLs/genome/h.

Many clinically important chemotherapeutic chemicals can induce DNA interstrand cross-links (ICLs). These include mitomycin C (MMC), diepoxybutane, nitrogen and sulfur mustards, cisplatin, and photoactivated psoralens (21). ICLs pose a particular challenge to DNA repair systems since they involve both strands of DNA and cannot, therefore, be repaired using the redundant information in the complementary strand.

In Escherichia coli and yeast, the repair of ICLs involves the sequential action of several repair pathways. In E. coli, genetic and biochemical studies pointed to a recombinational-in cisional repair (4, 13) in addition to a pathway involving a DNA glycosylase (28). In yeast, unlike in E. coli, double-strand breaks occur in response to ICLs. The repair of these breaks is dependent on the presence of RAD52 (homologous recombin ation repair) and RAD2 (excision repair) but not on RAD6 (mutagenic repair) (5, 12, 19).

Much less is known about the biology of cross-link repair in mammalian cells. Many of the studies in this field have focused on Fanconi anemia (FA) cells because of their sensitivity to cross-linking agents. The FA cellular phenotype is manifested as increased chromosome breakage (1) and a marked cell cycle delay with a 4N DNA content after introduction of ICLs (16). This delay has been also described as a G2/M checkpoint. It scored for normal progression and not at the G2/M boundary. We found that passage through S phase was necessary for these lesions to induce a cellular response regardless of whether the damage was introduced pre- or post-DNA replication. An estimate of the ICL repair rate in human cells and a model for the cellular recognition of ICLs are presented.
MATERIALS AND METHODS

Cells and media. Normal primary diploid fibroblasts were derived from human neonate foreskin samples. Two different cell lines from unrelated individuals (PD743.F and PD744.F) were used for all experiments and yielded similar and consistent results. Only PD743.F is described in this paper. Cells under passage 8 were employed for all the experiments described. Cells were maintained in α-modified Eagle medium (GIBCO/BRL) with 20% fetal calf serum (FCS) (Summit, Fort Collins, Colo.), 1% glutamine (GIBCO/BRL), and 0.1% penicillin-streptomycin (GIBCO/BRL) at 37°C and 5% CO₂.

Cell treatment and denaturation-renaturation gel electrophoresis. Cells were seeded at 3,000 cells/cm² and allowed to recover for 24 h before treatment. They were then preincubated in HMT (Sigma) in Hank’s balanced salt solution (HBSS; GIBCO/BRL) in the dark for 10 min and then irradiated for 20 min using a transilluminator (Ultra-Lum, Paramount, Calif.) with fixed wavelength (365 nm) (UVA) and at maximum intensity. Subsequently, cells were washed twice with HBSS at 15-min intervals and reincubated for an additional 30 min. Following treatment, cells were allowed to recover at 37°C in complete medium. In all cases, control cells were irradiated with UVA but without any drug. The dose of UVA was 10 to 11 mW/cm². DNA for denaturation-denaturation gel electrophoresis was isolated as described by Vos and Hanawalt (30). DNA samples were denatured in 0.4 N NaOH at 55°C for 10 min and then loaded onto the gel. The gel was probed with the human 28S rRNA gene, yielding a 17-kb band (24). Autoradiogram band intensities were measured using a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). Several experiments were performed and gave similar results. If DNA is produced randomly into the genome, the fraction of denaturable DNA molecules (those without ICLs) corresponds to the zero fraction (ICL₀) of a Poisson distribution. We used this relationship to calculate the number of ICLs per genome from the measured percentage of non-denatured DNA (30).

Ionizing radiation was administered by exposing cells to various doses (5, 10, and 20 Gy) using a 300 Gy source.

Cell growth assays. Cells were seeded at 3,000 cells/cm² and treated with the appropriate drug. At various time intervals, cells were harvested by trypsinization, resuspended in 1 ml of phosphate-buffered saline (PBS; GIBCO/BRL), diluted in Isoton II balanced electrolyte solution, and counted in a Coulter Counter model 21, using the Coulter Multisizer AccuComp software (version 1.19). Trypan blue exclusion was used to determine that all counted cells were viable. For the clonogenic assay, cells were seeded at 1,000 cells/100-mm-diameter plate and treated with HMT and UVA. Following recovery, clones were stained with methylene blue and counted.

BrdU labeling. Cells were plated at 3,000 cells/cm² and treated with different concentrations of HMT as described above. Before each time point, the cells were incubated with 20 µg of bromodeoxyuridine (BrdU; Sigma)/ml for 24 h and then fixed with 60% ethanol–95% formaldehyde–4% glacial acetic acid for 2 min. The cells were then washed three times with PBS for 2 min each and denatured in 0.07 N NaOH for 5 min. After three washes for 2 min each, cells were then fixed with 60% ethanol–9% formaldehyde–4% glacial acetic acid for 2 min. DNA samples were denatured in 0.4 N NaOH at 55°C for 10 min and then loaded onto the gel. The gel was probed with the human 28S rRNA gene, yielding a 17-kb DNA band (24). Autoradiogram band intensities were measured using a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). Several experiments were performed and gave similar results. If DNA is produced randomly into the genome, the fraction of denaturable DNA molecules (those without ICLs) corresponds to the zero fraction (ICL₀) of a Poisson distribution. We used this relationship to calculate the number of ICLs per genome from the measured percentage of non-denatured DNA (30).

RESULTS

Rationale for the assay. Quantitative estimates of the number of ICLs which are compatible with repair and cell survival of primary human cells have not been previously reported. We therefore wanted to establish a protocol that would provide a quantifiable level of ICLs as well as cell survival. Following treatment with MMC, diepoxbutyrate or HMT plus UVA irradiation (365 nm), we measured ICLs by denaturation-renaturation gel electrophoresis (30) and determined cell survival. Only HMT and UVA provided a measurable level of DNA cross-links without death of >50% of cells. We found that a second UVA irradiation step (see Materials and Methods) was able to saturate the conversion of psoralen monoaducts into ICLs (Fig. 1A). Indeed, further in vitro UVA irradiation of DNA samples isolated from cells treated with our HMT-plus-UVA protocol did not produce additional cross-links on denaturing gels (data not shown). The quantitation of ICLs induced by different doses of HMT plus UVA showed a direct correlation between an increase in the HMT dose and an increase in the number of cross-links formed (Fig. 1B). It is important to mention that when HMT doses of <1 ng/ml were used and less than 6,000 ICLs/genome were present, the assay was at its detection limit (∼1% alkali-resistant DNA). Therefore, the numbers of cross-links measured in such samples were only approximate and were based on the assumption that the number of ICLs was proportional to the HMT dose.

Cell growth in response to ICLs. In contrast to ICLs induced by UVA alone, HMT-plus-UVA-induced ICLs caused a long growth arrest or delay (Fig. 2A). However, wild-type cells were able to recover from up to 0.3 ng of HMT/ml plus UVA after 8 ± 1 days. Upon resumption of growth the cells had no detectable cytogenetic abnormalities such as translocations or aneuploidy (200 metaphases examined; data not shown). Higher doses (>1 ng/ml or >6,000 ICLs) induced a “permanent” growth arrest (>20 days) but did not kill the cells, as
indicated with trypan blue labeling. At even higher doses (>10 ng/ml or >26,000 ICLs/genome), cell death occurred within a few days. Importantly, the kinetics of recovery from low doses of HMT was not consistent with clonal expansion of a small subpopulation of cells but rather suggested a relatively homogeneous response of most of the cells in the population. This was confirmed by performing a time course of BrdU incorporation after the introduction of ICLs. Consistent with the measurement of cell numbers, the BrdU labeling index dropped dramatically within 2 days after ICL treatment. The index increased sharply to ~30% at the same time as the cell number increased (Fig. 2B). Given that the average time to complete one cycle for human primary fibroblasts is ~24 h, this indicated that the reinitiation of growth reflected nearly simultaneous recovery of most cells in the population and not clonal survival.

In addition, a clonogenic survival assay was performed and showed that ~60% of cells treated with 0.3 ng/ml indeed recovered and formed clones (Fig. 2C). Together, these data suggest that normal human fibroblasts have the capacity to remove approximately 2,500 ICLs/genome at an average rate of ~11 ICLs/h (20). The BrdU labeling remained at 0 for the cells treated with 3 ng of HMT/ml plus UVA, thus further demonstrating that the constant cell number observed in these samples did not reflect a balance of continuing cell division and cell death.

### Cell cycle response to psoralen-induced ICLs

We next investigated the cell cycle stage at which HMT-plus-UVA-treated cells were arrested (Fig. 3). FACS analysis 24 h after treatment revealed that doses that produce more than 16,000 ICLs/genome (>3 ng of HMT/ml) resulted in an S-phase arrest (with a prominent shoulder in early S), followed by cell death.
probably by apoptosis, as evidenced by the presence of a sub-
G1 peak a few days later (data not shown). Doses that pro-
duced 6,000 to 16,000 ICLs/genome (1 to 3 ng of HMT/ml)
caused the cells to arrest with an obvious intermediate DNA
content, i.e., in S phase. Cells did not recover from the arrest
within 15 days. At lower levels (less than approximately 2,500
ICLs/genome), however, cells arrested with an apparent 4N
DNA content and after a prolonged arrest (~8 days) resumed
cycling. However, flow cytometry profiles showed that a shoul-
der of partially replicated DNA was present even at doses that
resulted in a G2/M arrest (Fig. 3). Together with the obvious
intermediate DNA content seen at higher doses, this observa-
tion suggested that the cells were also arrested in S phase
(albeit with near-4N DNA content) and not in G2/M.

DNA replication is required to cause cell cycle arrest after
ICLs. To determine whether ICLs are preferentially processed
in G2, where fully formed sister chromatids are available as
undamaged templates for recombinational repair, a synchron-
ous population of G2 cells was treated with either HMT plus
UVa or gamma irradiation (10 Gy). Seven hours later, most
HMT-plus-UVa-treated cells had entered G1 (Fig. 4). In contrast
to gamma-irradiated cells, which were immediately
arrested in G2, where fully formed sister chromatids are available as
undamaged templates for recombinational repair, a synchro-

FIG. 4. Synchronization and treatment of PD743.F cells in G2. (Left) cells
were synchronized in G2 and treated with 3 ng of HMT/ml (generating 16,000
ICLs/genome). (Right) Seven hours after treatment, most cells have divided and
entered G1. The table below shows the corresponding percentages of cells in G1,
G2, and S phases.

One potential explanation for the lack of G2/M arrest after
introduction of ICLs during G2 is a very rapid and complete
repair of the lesions during this phase of the cell cycle. Alter-
natively, cells could be resistant to the introduction of HMT-
plus-UVa-induced ICLs during this phase of the cell cycle. To
exclude these possibilities, we again treated cells with agents
that induced ICLs in G2 and asked whether they could pass
through the second mitosis after ICL induction (Table 2). Indeed,
although cells traversed a normal first mitosis, passage
through the second mitosis was blocked. Therefore, complete
and rapid repair and lack of ICL formation during G2 could
not explain the normal mitosis after ICL induction. Addition-
ally, aberrant mitoses with chromosome breakage (see below)
were observed following treatment with caffeine to override
the block. FACS analysis further confirmed that the cells were
arrested in S phase (intermediate DNA content) after success-
fully completing the first mitosis (data not shown). Together,
these results showed that wild-type cells recognized the pres-
ence of ICLs in the context of DNA replication and that
passage through S phase was required for triggering a cell cycle
arrest.

ICL-induced chromosome breakage requires prior DNA
replication. Since chromosomal breakage is a common pheno-
type observed in cells treated with DNA cross-linking agents
(2), the results described above prompted us to look for chro-
mosomal aberrations in the mitosis following the introduc-
tion of ICLs in S phase compared to G2 phase. Consistent with
our previous findings, cytogenetic evaluations of metaphase
spreads of samples treated in G2 revealed the absence of chro-
mosomal breakage in the first mitosis even at the highest HMT
doses. In contrast, extensive chromosomal breakage resulted

| Samples | Treatment with 3 ng/ml in G2 | 7 hours following treatment with 3 ng/ml in G2 |
|---------|-------------------------------|-----------------------------------------------|
| G0G1    | 9%                            | 64%                                           |
| G2M     | 86%                           | 18%                                           |
| S       | 5%                            | 18%                                           |

### Table 1. Mitotic index and chromosome breakage analyses after treatment in G2

| Sample treatment | No. of mitoses/1,000 cells | No. of breaks/cell |
|------------------|---------------------------|--------------------|
| 0 ng/ml          | 60                        | 0.1                |
| 0.3 ng/ml        | 56                        | 0.1                |
| 3 ng/ml          | 51                        | 0.3                |
| Gamma irradiation| 1                         | >8                 |
| 0 ng/ml + caffeine| 72                       | 1.1                |
| 0.3 ng/ml + caffeine| 73                     | 1.8                |
| 3 ng/ml + caffeine| 59                       | 1.7                |
| Gamma irradiation + caffeine| 49                | 7.9                |

* Cells were treated in G2 either with different doses of HMT (values with
units of nanograms per milliliter) or with gamma irradiation at 10 Gy and then
incubated with or without caffeine (2 mM) following treatment.

* Very few cells were observed in mitosis.
when gamma-irradiated cells were treated with caffeine to overcome the G2/M checkpoint (Table 1). In addition, cells treated with HMT plus UVA in S phase arrested immediately and, in the presence of caffeine, showed extensive chromosome breakage. Moreover, the second mitosis was blocked, and chromosomal breaks were observed when cells were treated with caffeine subsequent to the first mitosis (Table 2).

**Cellular responses to ICLs induced during G1**. It is well known that a G2/S checkpoint is activated and the initiation of S phase is prevented when mammalian cells are subjected to high doses of gamma irradiation in G1 (17). In contrast, FACS analysis of primary fibroblasts treated with HMT plus UVA in G1 and then released into the cell cycle did not reveal any delay at the G1/S boundary (Fig. 5A). Furthermore, quantitative denaturation-renaturation gel electrophoresis of DNA samples isolated from cells retained in G1 indicated the absence of any strand incision near the cross-link even after several days. The number of measured ICLs remained constant during the length of the experiment (~14,700, ~15,100, and ~15,700 ICLs/genome on days 1, 2, and 5, respectively). Additionally, cells retained in G1 and treated with agents inducing ~16,000 ICLs/genome were healthy even 2 weeks after treatment, whereas cells treated in the proliferating cell cycle showed marked cell death (Fig. 5B). The latter observation suggests that cell death, a third cellular response to ICLs, also requires passage through S phase to be elicited.

**DISCUSSION**

The biology of cellular responses to ICLs is of relevance not only to cancer chemotherapy but also to the genetic disease FA and to the DNA repair field in general. Unfortunately, little is known about this process in mammalian cells. In this study, we sought to understand the cell cycle kinetics of ICL repair. We chose to work with primary fibroblasts with intact cell cycle checkpoints and a cross-linking regimen which permitted a pulse-like introduction of the damage and which was compatible with cell survival. This was possible with protocols using HMT and saturating UVA. We were able to generate ICLs during specific cell cycle intervals and to determine whether primary human cells responded differently to ICLs introduced during G1, S, or G2.

**ICLs do not trigger an immediate G2/M arrest or chromosome breakage.** For bacteria and yeast, genetic experiments have suggested an important role for homologous recombination in ICL repair (12). Thus, we hypothesized that the G2/M delay observed in mammalian cells after treatment with cross-linking agents represents a checkpoint which allows time for the delay observed in mammalian cells after treatment with cross-linking agents (12). Although they interpreted their findings as a G2/M rather than a late S-phase delay, their results indicate that this phenomenon is not exclusive to HMT plus UVA as a cross-linking agent.

Our present data cannot conclusively distinguish between an S-phase checkpoint and a passive mechanical block to replication presented by ICLs. Both interpretations, however, are compatible with the fact that the structure of an ICL makes it an obvious obstacle to replication.

**ICLs do not trigger a G1/S arrest.** Previous reports have indicated that ICLs in actively transcribed genes are preferentially repaired compared to transcriptionally silent loci (31). This observation implies the repair of DNA cross-links during the G1 phase of the cell cycle, when housekeeping genes are transcribed. Additionally, if DNA double-strand breaks are structural intermediates in ICL repair, they would induce a p53-mediated G1/S cell cycle delay (18). We therefore sought to determine whether HMT-plus-UVA treatment during G1 could generate a G1/S delay similar to that observed after ionizing radiation. Our results showed that even doses of HMT plus UVA which caused a long-lasting (>8-day) mitotic arrest with 4N DNA content caused no G1/S delay. Furthermore, no ICL incision was detected in cells held in G1 by serum starvation. These results suggest that efficient repair of ICLs does not occur during the G1 phase of the cell cycle of fibroblasts.

**Rate of ICL repair in wild-type human fibroblasts.** Our study is the first to report an estimate of the number of ICLs which can be removed by primary mammalian cells. Wild-type human fibroblasts were able to recover from ICL damage and reenter the cell cycle without chromosomal abnormalities. In multiple independent experiments, asynchronous fibroblasts from different individuals showed remarkable consistency in the time of reentry into the cell cycle after ICL treatment. In all cases, it took 8 ± 1 days after the introduction of ~2,500 ICLs/genome. Therefore, under the assumption of a constant repair rate, it can be estimated that ~11 ICLs/genome/h are removed.

Previously, by using MMC and various transformed cell lines, others have reported much faster rates of ICL repair (8, 30, 33). However, in those studies, only the initial incision of the ICL was measured and only short-term assays performed at 48 h after ICL induction were used (3). Moreover, long-term cell survival was not reported, and our data indicate that the very high number of ICLs induced in those studies would have been incompatible with prolonged cell survival.

**Conclusions for mammalian ICL recognition and repair.** The introduction of psoralen-induced ICLs during G1 resulted neither in a prolonged G1/S delay nor in any incision of these ICLs. Additionally, those ICLs generated during G1 also did not delay the subsequent mitosis or result in chromosomal breakage. Therefore, our data indicate that ICLs may not be sensed and hence not repaired in either G1 or G2 in human primary fibroblasts. Rather, DNA replication appears to be required to induce cell cycle arrest and/or chromosomal breakage in response to ICLs. Our results are consistent with earlier work performed with plants (7). In those studies, it was shown that all cytogenetic abnormalities, seen after treatment with
nitrogen mustard, were due to so-called DNA misreplication. The lack of an immediate G2/M arrest following ICLs suggests that mammalian cells, and perhaps all eukaryotes, may not utilize the undamaged sister chromatid as a template for ICL repair. It is important to mention, however, that although these conclusions may be true for all cross-linking agents, our experiments were only performed using HMT plus UVA.

Two main possibilities for the repair of ICLs exist. First, the removal of ICLs during the next S phase may involve deletion of the lesion followed by religation, as was suggested for FA cells. This process would always result in the loss of genetic information and may be mutagenic (6). Alternatively, it is conceivable that ICL repair involves interchromosomal mitotic recombination (repair by gene conversion) rather than recombination between sister chromatids. The existence of such a pathway has recently been documented for double-strand break repair (23).

DNA replication is required to trigger the classic cellular responses to ICLs including both chromosome breakage and arrest with 4N DNA content. We therefore propose a model in which at least the initial steps of mammalian ICL recognition and repair occur exclusively in S phase (Fig. 6).

FIG. 5. (A) FACS analysis of PD743.F cells treated in G1 after serum starvation with either HMT plus UVA or ionizing radiation. Following treatment, cells were released into the cell cycle by serum addition and analyzed at three different time points. Untreated cells progress into the cell cycle as observed after 24 h and 40 h. Cells treated with HMT at 0.3 ng/ml (generating ~2,500 ICLs/genome) also showed progress into S phase after 24 h, and by 40 h cells were arrested with a 4N DNA content. In contrast, cells treated with 5 and 10 Gy did not enter the cell cycle within these time points. (B) Comparison of cell viability in response to HMT-plus-UVA treatment of cycling cells (a) to that for cells retained in G1 by serum starvation (b). Approximately 2 weeks following treatment with 3 ng of HMT/ml (generating ~16,000 ICLs/genome), cycling cells show a higher degree of cell death than cells retained in G1 by serum starvation.
Caffeine is added to the cells. As a result of an aberrant mitosis, chromosome DNA triggers a cell cycle arrest, whereby cells do not enter mitosis unless reduced prior to replication, DNA synthesis stalls at the lesion. This unreplicated mitosis Artefact is not present.

FIG. 6. A model for the cellular response to ICLs. When ICLs are introduced prior to replication, DNA synthesis stalls at the lesion. This unreplicated cross-linking event is not present.

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