Published in final edited form as:

DNA Repair (Amst). 2006 June 10; 5(6): 731–740. doi:10.1016/j.dnarep.2006.03.009.

γ -H2AX formation in response to interstrand crosslinks requires XPF in human cells

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Abstract

To further define the molecular mechanisms involved in processing interstrand crosslinks, we monitored the formation of phosphorylated histone H2AX (γ -H2AX), which is generated in chromatin near double strand break sites, following DNA damage in normal and repair-deficient human cells. Following treatment with a psoralen derivative and ultraviolet A radiation doses that produce significant numbers of crosslinks, γ -H2AX levels in nucleotide excision repair-deficient XP-A fibroblasts (XP12RO-SV) increased to levels that were twice those observed in normal control GM637 fibroblasts. A partial XPA revertant cell line (XP129) that is proficient in crosslink removal, exhibited reduced γ -H2AX levels that were intermediate between those of GM637 and XP-A cells. XP-F fibroblasts (XP2YO-SV and XP3YO) that are also repair-deficient exhibited γ -H2AX levels below even control fibroblasts following treatment with psoralen and ultraviolet A radiation. Similarly, another crosslinking agent, mitomycin C, did not induce γ -H2AX in XP-F cells, although it did induce equivalent levels of γ -H2AX in XPA and control GM637 cells. Ectopic expression of XPF in XP-F fibroblasts restored γ -H2AX induction following treatment with crosslinking agents. Angelicin, a furocoumarin which forms only monoadducts and not crosslinks following ultraviolet A radiation, as well as ultraviolet C radiation, resulted only in weak induction of γ -H2AX in all cells, suggesting that the double strand breaks observed with psoralen and ultraviolet A treatment result preferentially following crosslink formation. These results indicate that XPF is required to form γ -H2AX and likely double strand breaks in response to interstrand crosslinks in human cells. Furthermore, XPA may be important to allow psoralen interstrand crosslinks to be processed without forming a double strand break intermediate.

Keywords

Crosslinks; Psoralen; Ultraviolet A; Double-strand break; H2AX; XPA; XPF

1. Introduction

DNA interstrand crosslinks (ICLs) that covalently link complementary strands of duplex DNA constitute a unique class of DNA damage that strongly inhibits DNA replication and transcription and is highly cytotoxic [1]. Many commonly used chemotherapeutic drugs such as psoralen, mitomycin C, cisplatin and nitrogen mustards generate ICL. Psoralen plus ultraviolet A (UVA) radiation is commonly used to treat a number of dermatologic conditions, but is associated with an increased risk of squamous cell carcinoma, and possibly

malignant melanoma [2,3]. Psoralen photoadducts are mutagenic, though their role in cutaneous malignancy is still unclear [4-6]. Mechanistically, psoralen intercalates into DNA, and following ultraviolet A (UVA) radiation forms monoadducts with pyrimidine bases initially that with further UVA irradiation may form ICL predominantly at 5' TpA 3' sites [7]. Psoralens are experimentally attractive in that the DNA damage can be precisely and rapidly initiated with UVA, and the relative amounts of monoadducts and ICL can be manipulated by the UVA dosage [8,9].

The processing of ICL has been extensively studied in prokaryotes and yeast and appears to involve the nucleotide excision repair (NER) and recombination pathways, as well as lesion bypass and post-replication repair [1,10,11]. Processing of ICL in mammalian cells appears to be mediated by similarly diverse mechanisms as well as transcription [12,13] and certain mismatch repair proteins [14], although the relative importance of differing pathways and the detailed sequence of events remain to be elucidated [1]. For example, the contribution of NER proteins to ICL repair is unclear. With the exception of cells lacking ERCC1 and XPF proteins, which are extremely sensitive to ICL-forming agents, other NER-deficient cells are only moderately sensitive, suggesting that the NER pathway is not critical to survive ICL formation [15,16]. On the other hand, cells deficient in the XPA protein, which is essential for NER, are defective in ICL removal [17-20], while XPA and another NER protein, XPC, appear to participate in the recognition of ICL associated with triplex-forming oligonucleotides [21,22]. Additionally, the XPF-ERCC1 heterodimer possesses functions independent of its role as a structure-specific 5' endonuclease in NER [23]. The XPF-ERCC1 complex possesses an ICL-dependent 3' to 5' exonuclease activity in the presence of the RPA protein [24] as well as both 5' and 3' endonuclease activities at ICL [25-27]. XPF may also contribute to ssDNA formation at ICL [28], and XPF-ERCC1 can participate in a homology-based recombination process [29,30].

Double strand breaks (DSB) are also generated as a result of ICL processing in mammalian cells [14,16,31]. However, disparate conclusions have been reached regarding the role of XPF and ERCC1 in DSB formation. Rodent cells lacking ERCC1 or XPF have been reported to be deficient in DSB formation in response to psoralen ICL [14,32]. In contrast, ICL resulting from nitrogen mustard or mitomycin C have been reported to induce DSB in *Ercc1*^{-/-} murine embryonic fibroblasts [16,33], and several models of ICL repair place the activity of XPF-ERCC1 downstream of DSB formation [33,34].

The formation of phosphorylated histone H2AX (γ -H2AX) has been widely used as a marker for the presence of DSB in general [35,36], as well as specifically following processing of ICL [32,33]. In the present study, we systematically monitored the formation of γ -H2AX following ICL generated by psoralen or mitomycin C in normal and NER-deficient human fibroblasts. Our results indicate that DSB formation, as reflected by γ -H2AX, requires XPF in human cells that are damaged by ICL. The results also suggest that normal cells may be able to process a subset of psoralen ICL without going through a DSB intermediate and that this process requires XPA.

2. Materials and methods

2.1. Chemicals

The psoralen derivative, 4'-hydroxymethyl-3,4,5'-trimethylpsoralen (HMT), as well as angelicin, and mitomycin C were used as obtained from the manufacturer (Sigma, St. Louis, MO). HMT and angelicin were dissolved in ethanol to form stock solutions that were stored at -20 °C. Aqueous stock solutions of mitomycin C were stored at 4 °C.

2.2. Cells

GM637 (from J. Cleaver) is an SV40-transformed fibroblast cell line from a normal individual that has been shown to be proficient in the removal of psoralen monoadducts and ICL[18,19]. XP12RO-SV and XP2YO-SV (from P. Hanawalt) are SV40-transformed fibroblast cell lines derived from xeroderma pigmentosum complementation group A [37], and group F [38] patients, respectively. XP129 cells (from P. Hanawalt) are a partial revertant of XP12RO-SV [19]. XPF-complemented XP-F cells (from G. Wang), were originally established by stably transfecting XPF cDNA into XP2YO-SV [23]. These cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum at 37 °C. Under these culture conditions, these cell lines had identical rates of proliferation (data not shown). Normal human dermal fibroblasts, NHF-A and NHF-D, were obtained from neonatal foreskin of two normal individuals using standard techniques [39]. A non-transformed XP-F cell line, XP3YO (GM03542), was obtained from the NIGMS Human Genetic Cell Repository (Coriell Institute, Camden, NJ). Non-transformed cells were grown in minimal essential medium (MEM) supplemented with 15% fetal bovine serum, 2× essential amino acids, and non-essential amino acids at 37 °C.

2.3. Treatment of cells

For experiments with UVA, cells were incubated in Hanks balanced salt solution (HBSS) without phenol red and supplemented with 10 μM HMT or angelicin or diluent ethanol for 30 min, then irradiated. The UVA source was a locally designed planar array of six 15 W BL fluorescent lamps (UVP, Upland, CA) that emitted predominantly between 300 and 400 nm, with 98.4% of the irradiance between 320 and 400 nm. A 4.5 mm plate glass filter between the lamps and samples ensured that the irradiance below 320 nm was reduced by at least an additional 97%; the resulting UVA intensity was ~33 W/m². For experiments with ultraviolet C (UVC), cells were washed with HBSS, and irradiated with a germicidal lamp emitting predominantly at 254 nm with an intensity of 0.17 W/m². Both UVA and UVC exposures were measured with an IL1400A photometer equipped with both SEL240 UVC and SEL033 UVA detectors (International Light Inc., Newburyport, MA). For experiments with mitomycin C, cells were incubated in media supplemented with the drug (5 μg/ml) for 1 h at 37 °C. Following all treatments, cells were washed with phosphate-buffered saline (PBS) and allowed to incubate with fresh medium for up to 9 h. γ-Irradiation was performed using a ¹³⁷Cs irradiator (Model 143, J.L. Shepherd and Associates, San Fernando, CA), delivering 3.4 Gy/min and cells were harvested 10 min following irradiation.

2.4. Western immunoblotting

Cells were harvested with a cell scraper, pelleted by centrifugation, and extracted for 20 min on ice in lysis buffer (50 mM Tris pH 7.5, 0.5% (v/v) Nonidet P40, 0.5% (w/v) SDS, 150 mM NaCl, 1 mM EDTA, 0.5% (w/v) sodium deoxycholate supplemented with 1 μg/ml each of leupeptin, pepstatin, and aprotinin, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, and 1 mM sodium orthovanadate). Lysates were thrice sonicated for 10 s on ice, and protein was quantified using the bicinchoninic acid method (Pierce Biotechnology, Rockford, IL). Equivalent amounts of total protein were separated by SDS-15% or 7.5% polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride membranes. After blocking with 5% non-fat milk, the membranes were incubated overnight at 4 °C in buffer containing primary monoclonal antibodies to γ-H2AX (clone JWB301, 1:1000 dilution, Upstate, Lake Placid, NY) or XPF (clone 51, 1:200 dilution, Lab Vision, Fremont CA) or β-actin (clone AC-74, 1:3000 dilution, Sigma, St Louis, MO). Membranes were then incubated in buffers containing horseradish peroxidase-conjugated secondary antibodies to either mouse- or rabbit-IgG and visualized using an enhanced chemiluminescence system (Amersham, Arlington Heights, IL). In some cases, blots were stripped according to the manufacturer's instructions (Pierce Biotechnology), and then reprobed for different proteins and detected as

described above. The intensities of bands were quantified using an Image Analyzer LAS-3000 (Fuji, Tokyo, Japan). γ -H2AX levels were normalized to those of β -actin.

2.5. Immunofluorescence microscopy

Cells grown in chambered slides (Titertek, Naperville, IL) were fixed for 5 min in tris-buffered saline (TBS) supplemented with 2% (w/v) paraformaldehyde. After removing solution, cells were permeabilized for 5 min with 0.5% (v/v) Triton X-100 and 2% (w/v) paraformaldehyde. After washing in TBS, cells were incubated for 1 h with 3% (w/v) bovine serum albumin in TBS, then incubated overnight with fluorescein-conjugated anti- γ -H2AX antibodies (clone JBW301, 1:1000 dilution) in TBS containing 3% (w/v) bovine serum albumin. Following a final wash, cells were mounted with Prolong Gold antifade reagent (Molecular Probes, Eugene, OR) and visualized by brightfield and epifluorescence microscopy (Carl Zeiss, Jena, Germany).

2.6. Flow cytometry

Cells were trypsinized, washed with PBS, and fixed in TBS containing 2.0% (v/v) formaldehyde and 0.5–0.8% (v/v) methanol for 20 min on ice, then washed with 0.1% (w/v) saponin. The cells were then gently resuspended in a permeabilization solution (0.5% saponin, 10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl_2) and incubated with fluorescein-conjugated γ -H2AX antibody (clone JBW301, 1:1000 dilution) for 20 min. Following a final wash, fluorescent cells were analyzed with a FACScan (Becton Dickinson, San Jose, CA). Gating was adjusted to eliminate cells with reduced forward and side-scatter that are characteristic of apoptosis [40].

3. Results

3.1. Psoralen photoadducts strongly induce γ -H2AX in XP-A, but not XP-F cells

GM637, XP12RO-SV and XP2YO-SV cells are SV40-transformed fibroblasts derived, respectively, from a normal individual, and patients with xeroderma pigmentosum complementation groups A and F [37,38]. GM637 but not XP12RO-SV cells are proficient in the removal of psoralen ICL and monoadducts [18,19]. XP12RO-SV and XP2YO-SV cells are deficient in the XPA and XPF NER proteins, respectively, and will henceforth be referred to as XP-A and XP-F cells. When assayed by Western immunoblotting, immunofluorescence, or flow cytometry, γ -H2AX was undetectable in untreated cells, in cells treated with 20 kJ/m² UVA alone, as well as immediately following treatment with both HMT and UVA (Fig. 1A and C, data not shown).

However, by 3 h following treatment with HMT and 20 kJ/m² UVA, Western immunoblotting demonstrated that γ -H2AX levels in NER-deficient XP-A cells were approximately twice those in control GM637 cells (Fig. 1A). Notably, XP-F cells that are also NER-deficient actually exhibited γ -H2AX levels slightly below even those of control GM637 cells. However, only 10 min after γ radiation, XP-F cells formed γ -H2AX as efficiently as GM637 and XP-A cells, indicating that the XP2YO mutation does not otherwise impair the ability of these cells to form γ -H2AX (Fig. 1B). Results following HMT and UVA were confirmed by immunofluorescence. γ -H2AX immunofluorescence intensity was typically punctate with a diffuse background and overall was strongest in XP-A cells, intermediate in GM637 cells and lowest in XP-F cells. γ -H2AX was undetectable in GM637 cells treated with UVA alone (Fig. 1C). Similarly, flow cytometry demonstrated that untreated GM637, XP-A and XP-F cells possessed identical low levels of background fluorescence (Fig. 1D). However, following treatment with HMT and UVA, 68% of XP-A, 60% of GM637 control, and only 18% of XP-F cells exhibited fluorescence above background.

3.2. UVA dose- and time-dependence of γ -H2AX formation due to ICL

To define differences between these cell lines more clearly, we monitored γ -H2AX over 9 h following treatment with HMT and either low (5 kJ/m²) or high (20 kJ/m²) UVA doses (Fig. 2). Because cells irradiated at both doses were processed in parallel and the Western blots were quantified simultaneously, the relative intensities of γ -H2AX bands from 5 kJ/m² (Fig. 2A) and 20 kJ/m² (Fig. 2B) samples may be directly compared. At 5 kJ/m², the absolute number of crosslinks is lower and the relative ratio of monoadducts to crosslinks is higher than at 20 kJ/m² UVA [9,41]. γ -H2AX was barely detectable at 1.5 and 3 h following HMT and UVA treatment, but increased monotonically over 9 h. These results contrast with the almost immediate appearance of γ -H2AX following ionizing radiation [35,42] (Fig. 1) and are consistent with the generation of DSB not as an immediate DNA lesion of HMT photoadducts but rather as an intermediate in response to ICL.

At 3 and 9 h post-UVA, γ -H2AX levels were substantially lower at 5 kJ/m² relative to 20 kJ/m². The differences in γ -H2AX levels among GM637, XP-A and XP-F cell lines that were observed previously at 3 h (Fig. 1) were considerably enhanced by 9 h after UVA. γ -H2AX levels in XP-A cells were twice those observed in control GM637 cells at both UVA doses. However, XP-F cells again exhibited γ -H2AX levels well below those of GM637 control cells. Higher UVA doses (40 or 60 kJ/m²) did not result in further increases in γ -H2AX in all cell lines used (data not shown), suggesting that the γ -H2AX response saturates by these UVA doses.

To determine the contribution of ICL to the γ -H2AX signals observed and confirm that the observed differences between the cell lines were not simply due to monoadducts, we treated cells with HMT and 5 kJ/m² UVA, rinsed away unbound HMT, and then re-irradiated cells with 15 kJ/m² (Fig. 2C). Such splitdose treatments typically achieve a higher proportion of ICL in cells [41]. The γ -H2AX levels observed 3 h post-irradiation in all cells were approximately 50% of those resulting from a single 20 kJ/m² UVA dose (Fig. 2B), but the relative γ -H2AX levels were otherwise comparable, with the highest levels in XP-A cells, and the lowest levels in XP-F cells. These results contrast with those obtained following a single 5 kJ/m² dose which resulted in the same overall level of photoadducts but a much lower proportion of ICL and yet which did not result in significant differences in γ -H2AX levels among cell lines by 3 h post-treatment (Fig. 2A).

As an additional measure of the contribution of ICL to γ -H2AX formation, we measured γ -H2AX levels 3 h following HMT and UVA treatment in XP129 cells which possess a partial reversion of the XPA mutation found in XP12RO-SV cells, restoring proficiency in repair of psoralen ICL but not of monoadducts [19,43] (Fig. 2D). These cells exhibited reduced levels of γ -H2AX relative to XP-A cells, suggesting that XPA-dependent ICL processing in these cells partially eliminates the formation of γ -H2AX.

3.3. γ -H2AX formation following psoralen ICL requires XPF

To further confirm that XPF is necessary to generate γ -H2AX following treatment with HMT, we examined additional cells (Fig. 3). Another XP-F fibroblast cell line (XP3YO) with XPF mutations different from those of XP2YO exhibited γ -H2AX levels that were only 15% of those in normal fibroblasts following treatment with HMT and UVA radiation (Fig. 3A). In addition, XP-F (XP2YO-SV) cells stably transfected with a plasmid encoding wild-type XPF cDNA were examined. In contrast to XP-F cells which had nearly undetectable XPF protein, XPF-corrected cells expressed ~40% of the level of XPF found in control GM637 cells, as previously described [23] (Fig. 3B). Following treatment with HMT and UVA, XP-F cells induced γ -H2AX to only 15% of the level of control GM637, while XPF-complemented XP-F cells induced γ -H2AX levels to 71% of control levels (Fig. 3C). The

slightly lower level of γ -H2AX in XPF-corrected XP-F compared with that of GM637 may be due to the presence of a lower level of functional XPF protein in the cells. These results demonstrate that XPF is necessary and possibly rate-limiting for γ -H2AX formation following ICL.

3.4. Angelicin monoadducts only weakly induce γ -H2AX

To further confirm that differences in γ -H2AX formation following treatment with HMT and UVA are primarily due to ICL and not monoadducts, cells were treated with angelicin and UVA (Fig. 4). Angelicin, a furocoumarin and structural isomer of psoralen, forms only UVA-induced monoadducts but is incapable of forming ICL [44]. In all cells treated with angelicin and 20 kJ/m² UVA, γ -H2AX levels measured by Western immunoblotting were considerably lower than those observed following identical doses of HMT and UVA, and only increased significantly above background in XP-A cells by 9 h following treatment, consistent with minimal ICL and DSB formation following angelicin and UVA treatment. Since angelicin and UVA do not result in ICL, it is possible that the small amount of γ -H2AX observed in NER-deficient XP-A cells at 9 h post-UVA represent DSB formed by collapsed replication forks arrested at unrepaired monoadducts [42,45]. Neither GM637 nor NER-deficient XP-F cells formed appreciable amounts of γ -H2AX following angelicin and UVA treatment.

3.5. Mitomycin C induces γ -H2AX in GM637 and XPA but not XPF cells

To determine if the results obtained with HMT were representative of other ICL-forming agents, the response to mitomycin C was determined. Unlike psoralens, mitomycin C forms both monoadducts and ICL in DNA at purine bases in the absence of UV irradiation, and ICL typically constitute a much lower percentage of the total adducts than can be achieved with psoralens and UVA [1,46]. As seen with HMT and UVA treatment, XP-F cells exhibited significantly lower γ -H2AX levels following mitomycin C treatment than GM637 cells (Fig. 5). Interestingly, in contrast to HMT plus UVA treatment, mitomycin C treatment resulted in γ -H2AX levels that were similar in control GM637 and XP-A cells, assayed by both Western immunoblotting and flow cytometry. XPF-complemented XP-F cells exhibited much higher γ -H2AX levels than XP-F cells (XP2YO-SV) following treatment with mitomycin C (Fig. 3C). As with HMT crosslinks the correction did not entirely restore γ -H2AX induction to normal levels, and may be due to the lower levels of functional XPF in the cells.

3.6. UVC radiation weakly induces γ -H2AX

As further confirmation that the γ -H2AX induced following HMT and UVA as well as mitomycin C were predominantly due to ICL, we monitored γ -H2AX following UVC radiation, which generates predominantly cyclobutane pyrimidine dimers and pyrimidine(6-4)pyrimidone photoproducts in DNA but does not normally induce ICL. As observed with angelicin and UVA, γ -H2AX levels in GM637 control, XP-A and XP-F cells were markedly lower than those observed following HMT and UVA treatment, consistent with a lack of DSB formation (Fig. 6). As noted with angelicin and UVA treatment, while γ -H2AX levels were barely detectable in GM637 and XP-F cells, XP-A cells formed detectable levels by 9 h following UVC, consistent with unrepaired ultraviolet photoproducts that arrest replication leading to DSB [42].

4. Discussion

H2AX is an uncommon histone variant in mammalian cells. Upon exposure of cells to ionizing radiation and a variety of other processes that result in DSB, H2AX proteins residing within megabases of each DSB are rapidly phosphorylated at a carboxy terminal

serine; the phosphorylated variant is denoted as γ -H2AX [35,36]. The functional significance of γ -H2AX formation is still unclear, though it likely represents one of many chromatin modifications in response to DNA damage that facilitate recognition and repair of the damage, and perhaps mediate signaling for other cellular responses [36]. As a practical matter, however, γ -H2AX has been widely used as a surrogate marker for DSB formation [32,33,35,36].

Our result that γ -H2AX forms only after several hours following psoralen and UVA or mitomycin C treatment is consistent with DSB generation following the processing of ICL in human cells. After treatment with both crosslinking agents, γ -H2AX was most abundantly formed in NER-deficient XP-A cells and to a variable extent in GM637 cells, but was barely detectable in XP-F cells that are also NER-deficient. Furthermore, γ -H2AX formation following ICL was substantially restored in an XPF-complemented XP-F cell line. These results suggest that the entire NER pathway is not necessary to generate DSB in cells, and that the XPF protein specifically participates in an NER-independent function that is required and probably rate-limiting for DSB formation in human cells. This conclusion is generally in agreement with prior work, which indicated that the NER functions of XPF are distinct from its role in ICL processing [23,47,48]. Our work in human cells corroborates and complements prior work, all in Chinese hamster ovary cells, demonstrating that ERCC1, which normally is in a heterodimeric complex with XPF, is required for the initial incisions and γ -H2AX formation [32], and that XPF and ERCC1 are required for incisions leading to DSB in an in vitro assay [14]. Our results are also consistent with the finding that XPF loss suppresses sister chromatid exchange following mitomycin C treatment [49], as well as with models in which the ICL-specific 5' and 3' endonuclease activities of the XPF-ERCC1 complex are a relatively early event preceding and required for DSB formation [25,32,50]. Although cell extracts have been reported to make dual incisions 5' to a psoralen ICL, one of which was dependent on XPF, no DSB occurred as a result of this reaction, and thus these types of incisions alone do not explain the dependence of DSB formation on XPF [51].

On the other hand, Chinese hamster ovary cells mutated in XPF, ERCC1 and other NER proteins have been reported to form and remove DSB normally following damage by nitrogen mustard crosslinks [16]. Similarly, persistent γ -H2AX foci have been reported following mitomycin C treatment in *Ercc1*^{-/-} murine embryonic fibroblasts, suggesting that ICL-induced DSB formation is independent of ERCC1, but that ERCC1 is required for resolution of DSB [33]. This work also observed that, following mitomycin C treatment, XP-A murine embryonic fibroblasts formed fewer γ -H2AX foci, which disappeared with wild-type kinetics, suggesting that NER is not required for DSB repair. These results, all in rodent systems, may reflect fundamental differences between rodent and human cells. It is also possible that differences exist in processing specific types of ICL agents or in the assays used to detect DSB. In any case, our results provide strong evidence that human cells require XPF to form ICL-induced DSB. It is unclear whether XPF mediates DSB formation only at replication forks arrested at lesions or if it also can generate DSB independent of replication.

Equally intriguing, XP-A cells formed significantly higher γ -H2AX levels than normal control GM637 cells following HMT and UVA but not mitomycin C treatment. GM637 cells have been reported to rapidly remove psoralen monoadducts, and more slowly remove psoralen ICL with a half-life of 10 h while XP129 cells, partially XPA revertant, are proficient in ICL repair only, and XP-A cells are completely NER-deficient [18,19]. Thus, the lower γ -H2AX levels observed in GM637 and XP129 cells may be due to NER-dependent removal of a large fraction of monoadducts and ICL in GM637 cells and of ICL alone in XP129 cells before replication fork arrest and collapse occur at the sites of DNA lesions. The lack of similar magnitude differences in γ -H2AX formation between GM637 and XP-A cells treated with angelicin and UVA, or with UVC, suggests that differences in

γ -H2AX levels following HMT and UVA are largely due to differences in XPA-dependent ICL processing in these cells. Therefore, NER-proficient GM637 cells may possess alternative, XPA- and likely NER-dependent mechanisms for processing psoralen- but not mitomycin C-induced ICL, and these mechanisms may not generate a DSB intermediate. Some precedent for differential processing of chemically distinct ICL exists. Cisplatin forms a small number of ICL and fails to induce DSB in rodent cells [48]. NER processing of psoralen ICL that depends on XPA and other NER proteins to uncouple one strand of the ICL [21,22], followed by translesion synthesis with a bypass polymerase may be one scenario that could account for reduced DSB formation in GM637 cells relative to XP-A cells [11,20,50,51].

Although neither angelicin nor UVC is capable of forming ICL or directly causing a DSB, both agents as well as HMT treatment followed by low-dose UVA did result in a small, delayed amount of γ -H2AX formation in XP-A cells. Since XP-A cells are completely NER-deficient, it is likely that the low levels of γ -H2AX reflect DSB that occur in those cells that are replicating and whose replication forks are stalled at these lesions [42,45]. The absence of γ -H2AX formation following non-ICL DNA lesions in XP-F cells that are also NER-deficient further suggests that XPF may be important for DSB formation following replication fork arrest at any DNA lesion and not just at ICL.

Acknowledgments

We thank J. Cleaver for providing GM637 cells, P. Hanawalt and A. Ganesan for providing XP12RO-SV, XP129 and XP2YO-SV cells, Gan Wang for providing XPF-complemented XP2YO-SV cells, H. Kataoka for assistance with flow cytometry, and S. Fong for assistance with γ irradiation. We also thank J. Cleaver and members of his lab for initially providing the γ -H2AX antibody, and for helpful discussions, and members of the Oh laboratory and C. Largman for critical reading of the manuscript. This work was supported by NIH grant R01-CA105958.

Abbreviations

DSB	double-strand break
HBSS	Hanks balanced salt solution
HMT	4'-hydroxymethyl-3,4,5'-trimethylpsoralen
ICL	interstrand crosslink
NER	nucleotide excision repair
PBS	phosphate-buffered saline
TBS	tris-buffered saline
UVA	ultraviolet A
UVC	ultraviolet C

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Fig. 1.

HMT photoadducts preferentially induce γ -H2AX in XP-A but not XP-F cells. GM637, XP12RO-SV (XP-A), and XP2YO-SV (XP-F) cells were treated with UVA alone or 10 μ M HMT plus 20 kJ/m² UVA (A, C and D), or with 6 Gy of γ radiation (B). Three hours after UVA and 10 min after γ radiation, cells were assayed. (A) Western immunoblotting of γ -H2AX or β -actin after treatment with UVA alone (left panel) or with HMT and UVA (right panel). γ -H2AX levels were normalized to those of β -actin and plotted; plotted results are the average of three independent experiments. (B) Western immunoblotting of γ -H2AX or β -actin following γ radiation. (C) Epifluorescence microscopy of γ -H2AX in cells treated with UVA alone or UVA and HMT (magnification 400 $\times$). (D) Flow cytometry of γ -H2AX in cells treated with HMT and UVA (bold lines) or their respective untreated controls (plain lines).

Fig. 2.

γ -H2AX induction by HMT-induced ICL is UVA dose- and time-dependent. Cells were treated with 10 μ M HMT and (A) 5 kJ/m², (B) 20 kJ/m² UVA or (C) 5 kJ/m² UVA followed by two rinses in HBSS to remove free HMT, and then immediate re-irradiation with 15 kJ/m² UVA. Western immunoblotting was used to quantify γ -H2AX and β -actin levels initially and at 1.5, 3 and 9 h post-irradiation in (A) and (B) and at 3 h post-irradiation in (C), in GM637 (●), XP12RO-SV (XP-A (■)), and XP2YO-SV (XP-F (▲)) cells. (D) GM637, XP12RO-SV and XP129 cells were treated with 10 μ M HMT and 20 kJ/m² UVA and harvested 3 h post-irradiation. γ -H2AX levels were normalized to those of β -actin and plotted. The plotted results represent the average of two or three independent biological experiments.

Fig. 3.

XPF is required for HMT photoadducts to induce γ -H2AX. (A) Two normal human dermal fibroblast cell lines (NHFA, NHFD) and a non-transformed XP-F fibroblast cell line (XP3YO) cells were treated with 10 μ M HMT and 20 kJ/m^2 UVA. Six hours post-UVA, cells were assayed for γ -H2AX or β -actin by Western immunoblotting. (B) Western immunoblotting of XPF levels in GM637, XP2YO-SV (XP-F) and XPF cDNA-transfected XP2YO-SV (XPF corr.) cells. (C) GM637, XP2YO-SV (XP-F) and XPF cDNA-transfected XP2YO-SV (XPF corr.) cells were treated with 10 μ M HMT and 5 kJ/m^2 UVA, rinsed twice in HBSS to remove free HMT, and then immediately re-irradiated with 15 kJ/m^2 UVA. Six hours post-UVA, cells were harvested for Western immunoblotting of γ -H2AX or β -actin. Plotted results represent the average of two independent biological experiments.

Fig. 4.

Angelicin photoadducts weakly induce γ -H2AX. Cells were treated with 10 μ M angelicin and irradiated with 20 kJ/m² UVA. (A) Western immunoblotting was used to detect and quantify γ -H2AX and β -actin levels initially and at 3 and 9 h post-irradiation in angelicin-treated GM637 control (○), XP-A (□), and XP-F (△) cells. For comparison, γ -H2AX and β -actin levels in GM637 (●), XP-A (■), and XP-F (▲) cells at 3 h following 10 μ M HMT and 20 kJ/m² are also shown. The plotted results represent the average of two independent biological experiments.

Fig. 5. γ -H2AX induction following mitomycin C. GM637 control, XP-A, XP-F (XP2YO-SV) and XPF-complemented XP-F (XP-F corr.) cell lines were treated with mitomycin C for 1 h, and then replaced in normal medium. Nine hours following treatment, cells were analyzed. (A) and (C) Western immunoblotting was used to detect and quantify γ -H2AX and β -actin levels. The plotted results represent the average of two independent biological experiments. (B) Each cell line treated with mitomycin C (bold lines) was compared with its respective untreated control (plain lines) and analyzed by flow cytometry for γ -H2AX.

Fig. 6. γ -H2AX induction following UVC radiation. GM637, XP-A, and XP-F cells were treated with 10 J/m² UVC. Western immunoblotting was used to quantify γ -H2AX and β -actin levels initially and at 1.5, 3 and 9 h post-irradiation in GM637 (●), XP-A (220E;), and XP-F (▲) cells. γ -H2AX levels were normalized to those of β -actin and plotted. The plotted results represent the average of three independent biological experiments.