ATR/Chk1 pathway is essential for resumption of DNA synthesis and cell survival in UV-irradiated XP variant cells

Emmanuelle Despras1, Fayza Daboussi1, Olivier Hyrien2, Kathrin Marheineke2 and Patricia L. Kannouche1,*

1Centre Nationale de la Recherche Scientifique (CNRS) UMR8200, Laboratoire Stabilité Génétique et Oncogénèse, Université Paris-Sud, Institut Gustave Roussy, 39 rue Camille Desmoulins, 94800 Villejuif, France and 2Centre Nationale de la Recherche Scientifique (CNRS) UMR8541, Laboratoire de Génétique Moléculaire, Ecole Normale Supérieure, 46 rue d’Ulm, Paris, France

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DNA polymerase eta (polh) performs translesion synthesis past ultraviolet (UV) photoproducts and is deficient in cancer-prone xeroderma pigmentosum variant (XP-V) syndrome. The slight sensitivity of XP-V cells to UV is dramatically enhanced by low concentrations of caffeine. So far, the biological explanation for this feature remains elusive. Using DNA combing, we showed that translesion synthesis defect leads to a strong reduction in the number of active replication forks and a high proportion of stalled forks in human cells, which contrasts with budding yeast. Moreover, extensive regions of single-strand DNA are formed during replication in irradiated XP-V cells, leading to an over-activation of ATR/Chk1 pathway after low UVC doses. Addition of a low concentration of caffeine post-irradiation, although inefficient to restore S-phase progression, significantly decreases Chk1 activation and abrogates DNA synthesis in XP-V cells. While inhibition of Chk1 activity by UCN-01 prevents UVC-induced S-phase delay in wild-type cells, it aggravates replication defect in XP-V cells by increasing fork stalling. Consequently, UCN-01 sensitizes XP-V cells to UVC as caffeine does. Our findings indicate that polh acts at stalled forks to resume their progression, preventing the requirement for efficient replication checkpoint after low UVC doses. In the absence of polh, Chk1 kinase becomes essential for replication resumption by alternative pathways, via fork stabilization.

INTRODUCTION

DNA helix deforming lesions, like the pyrimidine dimers formed by ultraviolet (UV), block replication fork progression. To allow completion of DNA synthesis, specialized DNA polymerases can transiently replace replicative polymerases and perform limited incorporation of nucleotides in the vicinity of the damage. This process is called translesion synthesis (TLS) (1). Although potentially mutagenic (2), TLS is important for genetic stability as highlighted by the cancer-prone xeroderma pigmentosum variant (XP-V) syndrome. This hereditary disease is characterized by a high sensitivity to sunlight and a markedly increased risk of skin cancers. XP-V cells harbor a mutation in the gene encoding DNA polymerase eta (polh), a specialized polymerase able to accurately bypass the most abundant photoproduct, the TT cyclobutane pyrimidine dimer (TT-CPD) (3,4). XP-V cells present a defect of replication of UV-damaged DNA and an increased mutagenesis upon UVC exposure but efficiently repair photoproducts (5–8). In the absence of polh, lesion bypass by alternative error-prone polymerases may account for higher mutation rates (9).

Despite the crucial role of polh in the replication of UV-induced lesions, XP-V cells only exhibit a slight sensitivity to UV. Addition of low doses of caffeine after irradiation greatly enhances UV sensitivity although the mechanism of action of caffeine remains unclear (10). Caffeine is a pleiotropic agent known to inhibit the phosphatidylinositol 3-kinase-related kinases (PIKK) among which are key factors of checkpoint signaling (11,12). After UV exposure,
ataxia-telangiectasia-mutated and Rad3-related (ATR) kinase is activated through interaction of its partner ATRIP with single-stranded DNA (ssDNA) generated at stalled forks and coated with replication protein A (RPA) [for review, see (13)]. Among several targets, ATR activates checkpoint kinase I (Chk1) that controls S-phase progression through inactivating phosphorylation of Cdc25A phosphatase (14). Moreover, ATR signaling seems required for replication fork stabilization (15,16). As it was shown that several targets of ATR were more phosphorylated following UVC exposure in the absence of polh (17–19), we asked whether these mechanisms could be involved in XP-V phenotype.

Whether TLS acts at stalled fork or is a post-replicative mechanism is still a matter of debate. For instance, inactivation of TLS in a nucleotide excision repair (NER)-deficient Saccharomyces cerevisiae strain has no effect on fork blockage but further increases the extent of gaps formed behind the fork (20). In contrast, deletion of POLH impairs fork restart in irradiated chicken cells (21). Using DNA molecular combing, we studied the implication of polh in fork progression after UVC in human cells. We showed that the stronger inhibition of DNA synthesis observed in XP-V cells after UVC is characterized by a higher reduction of active replication forks and a greater number of stalled forks. Moreover, XP-V cells accumulated ssDNA regions after UVC exposure and presented an activation of ATR checkpoint pathway at lower doses than polh-expressing cells. Inhibition of Chk1 kinase activity aggravates fork stalling and S-phase arrest in XP-V cells and, as a consequence, sensitized XP-V cells to UVC, as previously observed with caffeine. Our data demonstrate that polh is required for replication restart of stalled forks in human cells. In its absence, Chk1 kinase activity is crucial for resumption of DNA synthesis, presumably via stabilization of stalled forks.

RESULTS

XP-V cells accumulate in S-phase after UVC because of a reduced number of active forks and an elevated level of fork stalling

It was previously shown that XP-V cells displayed a stronger S-phase delay than wild-type cells after exposition at 2 J/m² (22,23). To better understand the cellular mechanism leading to this delay, we carried out a set of experiments.

First, we showed that stable complementation of XP-V immortalized fibroblasts with POLH cDNA restored S-phase progression of cells irradiated in or outside S-phase (Supplementary Material, Fig. S1A). Then, we checked if this increased delay was due to S-phase slow-down or arrest. Flow cytometry analysis of XP-V (XP30ROpolhDNA) or complemented (XP30ROpolh) cells pulse-labeled with bromodeoxyuridine (BrdU) showed that UVC induced a dose-dependent decrease of BrdU incorporation that was more important and persistent in the absence of polh (Fig. 1A). This reduction was attenuated in cells entering S-phase 6 h after UVC exposure, likely because these cells partially repaired photoproducts in G1 (indicated by arrows in Supplementary Material, Fig. S1B). XP30ROpolh cells transiently accumulated in S-phase after 2 and 7 J/m² (Fig. 1B). Accumulation was prolonged in XP30ROpolhDNA cells, especially after 7 J/m² where it was mostly caused by S-phase arrest. Similar results were found in XP-V CTag cells compared with wild-type MRC5 cells (data not shown).

To unravel if the greater reduction of DNA synthesis observed in irradiated XP-V cells was attributed to reduced origin firing, higher fork stalling and/or reduced fork velocity, we used DNA molecular combing. This stretching technique combined with immunofluorescence allows statistical analysis of replication dynamics on the level of single DNA fibers labeled with modified nucleotides (24). Successive pulses of iododeoxyuridine (IdU) and chlorodeoxyuridine (CldU) allow visualizing replication initiation sites and fork elongation (Fig. 1C). Fork density was more strongly decreased in CTag cells than in MRC5 cells with a diminution of 75% and 51%, respectively, 6 h after 7 J/m² (Fig. 1D, left panel). This result was confirmed with a single BrdU pulse (Supplementary Material, Fig. S2). XP30RO cells also presented a higher and persistent decrease of fork density after UVC than XP30ROpolhDNA cells (Supplementary Material, Fig. S3). We did not observe a significant increase of inter-origin spacing (data not shown). This suggests that firing of whole origin clusters was inhibited after UVC. However, we cannot exclude a bias in this measurement as, unlike fork density, inter-origin spacing depends on fiber length distribution. The proportion of asymmetric replication bubbles, defined as bubbles where one of the sister forks failed to incorporate the second label, gives an estimation of fork blockage (25–27). It increased upon UVC exposure, which suggests that asymmetric bubbles indeed revealed forks stalled at photoproducts (Fig. 1D, right panel). This effect was stronger in XP-V cells than in wild-type cells (2.9- and 1.7-fold in CTag and MRC5 cells, respectively). Similarly, the number of forks that failed to resume elongation 30 min after UVC was higher in CTag than in MRC5 cells (data not shown). Mean fork velocity was slightly but significantly increased in both cell lines after UVC (data not shown).

We conclude that consequences of UVC on replication dynamics, i.e. decreased number of active forks and increased fork stalling, were greatly enhanced in the absence of polh. We then asked whether this effect was the cause or the consequence of an increased S-phase checkpoint activity.

Persistent fork stalling correlates with extensive ssDNA regions in irradiated XP-V cells

ssDNA was shown to accumulate at forks stalled at UVC-induced lesions, which may constitute the signal for ATR activation (20,28). We then asked if the increased number of stalled forks observed in XP-V cells after UVC was correlated with increased formation of ssDNA. To visualize ssDNA in fixed cells, we detected BrdU incorporated in whole DNA in non-denaturing conditions, as previously described (29). BrdU foci were formed after 10 J/m² in XP-V cells and co-localized with RPA70, the 70 kDa subunit of RPA (Fig. 2A). This confirmed that BrdU foci were sites containing ssDNA. Moreover, the number of cells containing BrdU foci increased in a UVC dose-dependent manner (data not shown). We excluded the possibility that ssDNA foci were formed by NER because they were observed
in XP-A fibroblasts (data not shown). ssDNA foci were detected in cells presenting a dotted pattern of proliferating cell nuclear antigen (PCNA), suggesting that they formed in S-phase cells (Fig. 2B, left panel). To simultaneously detect ssDNA and replication foci, cells were cultivated in the presence of IdU for 48 h, irradiated and S-phase cells were pulse-labeled with CldU prior to fixation. IdU was first detected in non-denaturing conditions, then CldU was stained after DNA denaturation. We showed that ssDNA-positive cells were also positive for CldU (Fig. 2B, right panel). This confirms that ssDNA foci were only formed during S-phase after UVC irradiation and may reveal sites of perturbed DNA replication.

We then compared the kinetics of ssDNA foci formation after 2 J/m² in XP30ROpol and XP30ROpcDNA cells. Cells were counted according to their ssDNA patterns: no, weak or bright nuclear staining (Fig. 2C). Cells with bright pattern accumulated in the absence of polη, with a peak at 9 h, whereas weak pattern slightly increased in polη-expressing cells. This suggests that ssDNA formed transiently after UVC in normal cells but accumulate in the absence of polη. More than 95% of ssDNA-positive cells were in S-phase at early time points in both cell lines. However, 24 h after irradiation, 30% of ssDNA-positive XP-V cells failed to incorporate CldU (data not shown). These cells were probably arrested in S but we cannot rule out the possibility that ssDNA persisted in G2. Similar results were obtained in CTag versus MRC5 cells (data not shown). In accordance with the presence of extensive ssDNA regions, the 32 kDa subunit of RPA (RPA32) became resistant to detergent extraction and hyper-phosphorylated in irradiated XP-V cells (Fig. 2D). Moreover, mono-ubiquitinated PCNA (Ub-PCNA) was only slightly induced after 2 J/m² in both cell lines but accumulated in insoluble fraction 24 h after UVC exposure in XP30ROpcDNA cells. Given the already described implication of Ub-PCNA at arrested forks (30), this result confirmed that XP-V cells experienced prolonged fork stalling.

As BrdU staining, immunological detection of UVC-induced DNA lesions with commercial antibodies requires DNA denaturation. However, in ssDNA-positive XP-V cells, we also detected CPDs in non-denatured DNA (Fig. 2E). This staining was not a side effect of BrdU incorporation because CPDs were also detected in the absence of BrdU (data not shown). ssDNA-positive cells may then contain unreplicated lesions. It was shown that XP-V cells present higher levels of histone H2AX phosphorylation (γH2AX) after UVC (19). We showed that these bright γH2AX cells were also positive for ssDNA (Fig. 2E). This

Figure 1. Reduced number of active forks and higher level of stalling lead to a persistent S-phase delay in UVC-irradiated XP-V cells. (A and B) XP30ROpol and XP30ROpcDNA cells were irradiated at 2 or 7 J/m² and incubated the indicated times. Cells were pulsed-labeled with BrdU prior to harvesting and analyzed by flow cytometry. (A) Mean BrdU fluorescence of BrdU-positive cells (percentage of the corresponding mock-irradiated sample). (B) Evolution of the number of cells in S-phase. Left, middle and right panels show total, BrdU-positive and BrdU-negative (i.e. non-incorporating cells with a DNA content between 2 and 4 N) S-phase cells, respectively. Values are expressed as a percentage of total cells. (C) DNA combing procedure. Upper panel: cells were sequentially labeled with successive pulses of IdU and CldU 6 h after irradiation at 7 J/m². IdU, CldU and total DNA were detected by immunofluorescence on combed DNA. Lower panel: example of a combed DNA molecule (red) with several IdU and CldU tracks (blue and green, respectively). Initiation (O) and termination (T) sites are indicated. Asymmetric replication bubbles were defined as bubbles where one of the sister forks did not incorporate the second label. (D) Replication dynamics in MRC5 and CTag cells 6 h after 7 J/m². Values are the mean of two independent experiments. Left panel: fork density was determined as the number of forks per Mb of S-phase DNA measured on random fields. Percentage of mock-irradiated cells are indicated on top. More than 200 Mb were measured per condition. Right panel: proportion of asymmetric bubbles. UVC/mock ratios are indicated on top. At least 150 intact bubbles per condition were analyzed.
Figure 2. Single-strand DNA accumulates in UVC-irradiated XP-V cells during S-phase. (A) XP30RO cells were cultivated for 48 h in the presence of BrdU. Cells were fixed 4 h after irradiation at 10 J/m². Co-detection of BrdU (in red) and RPA70 (in green) was done in non-denaturing conditions. Magnification ×100. (B) Left panel: co-detection of BrdU (in red) and PCNA (in green) was done in the same conditions as in (A). Magnification ×100. Right panel: CTag cells were cultivated for 48 h in the presence of IdU. Cells were irradiated at 10 J/m² and incubated for 4 h. Prior to fixation, replicating cells were stained with CldU. IdU was detected in non-denaturing conditions (in green, ssDNA) before CldU detection in denaturing conditions (in red, S-phase cells). Magnification ×60. (C) XP30ROpol and XP30ROpcDNA cells were labeled with BrdU for 48 h, irradiated at 2 J/m² and fixed at different times after UVC. BrdU was stained in non-denaturing conditions in order to detect ssDNA. Left panel: three patterns were observed according to the level of nuclear ssDNA foci (no, weak or bright). Right panel: repartition of ssDNA-positive cells expressed as a percentage of total cells. Values are the mean ± SD of three independent experiments (200 cells were counted per condition and per experiment). (D) At various times after 2 J/m², cells were fractionated and insoluble and soluble fractions were analyzed by western blotting. The lower migrating band in PCNA blot corresponds to the mono-ubiquinated form of PCNA (Ub-PCNA). Antibody against RPA32 also detects hyperphosphorylated forms of the protein (HP-RPA32). Lamin A/C and GAPDH were blotted as controls for insoluble and soluble fractions, respectively. (E) CTag and MRC5 cells were stained with BrdU for 48 h, irradiated at 2 J/m² and fixed after 6 h. BrdU was detected in non-denaturing conditions simultaneously with cyclobutane pyrimidine dimers (CPD) or histone H2AX phosphorylated on serine 219 (γH2AX) (magnification ×100).
suggests that prolonged stalling at UVC-induced lesions in the absence of POLH leads to fork destabilization.

Altogether, these experiments showed that persistent fork stalling observed in irradiated polh-deficient cells was associated with the formation of extensive ssDNA regions that may constitute a signal for ATR activation.

**ATR checkpoint pathway is over-activated following exposure of XP-V cells to low doses of UVC**

In the context of this study, it was important to evaluate the effects of a low dose of UVC on the ATR pathway. We analyzed the amount of Chk1 kinase phosphorylated on serine 345 (pChk1 S345) as it reflects its level of activation (31). XP-V cells presented a strong phosphorylation of Chk1 after 2 J/m² which persisted for 24 h whereas amounts of pChk1 S345 remained low in polh-expressing cells (Fig. 3A). Chk1 overphosphorylation was a direct consequence of polh defect, as it was also observed in MRC5 cells where polh amount was transiently reduced with siRNAs (Fig. 3B). pChk1 S345 amounts were greatly reduced in XP30ROpolh and XP30ROpcDNA cells when ATR expression was down-regulated (Fig. 3C), confirming that Chk1 overphosphorylation in XP-V cells was indeed dependent on ATR. No recruitment of ATR, Claspin and Rad18 on nuclear structures was observed after 2 J/m², suggesting that basal levels on chromatin are sufficient for checkpoint activation and TLS regulation at this low dose (Supplementary Material, Fig. S4). We asked whether over-activation of ATR pathway was required for XP-V cells survival after UVC.

**XP-V cells are highly sensitive to UVC after ATR/Chk1 pathway inhibition**

ATR inhibitor caffeine sensitizes XP-V cells to UVC (10). However, caffeine is a pleiotropic agent. It also targets Ataxia telangiectasia mutated (ATM) and may impact other mechanisms beside those of cell cycle control [for a review, see (32)]. To test if XP-V sensitization by caffeine results from the inhibition of ATR pathway, we targeted Chk1 using two approaches. Down-regulation of Chk1 expression with siRNAs sensitized XP-V cells to UVC, whereas complemented cells transfected with unspecific siRNAs (siNT) or siChk1 presented similar survival curves (Fig. 4A). However, as Chk1 is involved in cell proliferation even in the absence of exogenous stress (33,34), transfection with siChk1 decreased cell growth (data not shown). Moreover, Chk1 may be involved in PCNA monoubiquitination independently of its kinase activity, suggesting that its down-regulation could affect TLS (35). We then chose to chemically inhibit Chk1 kinase activity using 7-hydroxystaurosporine (UCN-01) which displays an IC50 of 10 nM toward Chk1 (36,37). The concentrations we used did not impede the growth of unirradiated cells (data not shown). Addition of 25 nM UCN-01 post-irradiation greatly decreased XP-V cell survival (Fig. 4B and C). The increase of sensitivity was similar to that obtained with 0.38 mM caffeine. Conversely, wild-type and complemented XP-V cells were not sensitized by these treatments. Similar results were obtained with 10 nM UCN-01 (data not shown) and with another Chk1 inhibitor, SB-218078 (Supplementary Material, Fig. S5). Finally, 25 nM of UCN-01 and 0.38 mM caffeine also sensitized to the same extent MRC5 cells where the amount of polh was transiently reduced without affecting cells transfected with siNT (Fig. 4D). This shows that the effect of UCN-01 on XP-V cell sensitivity is a direct consequence of polh defect. Taken together, our results provide evidence for a crucial role of Chk1 kinase in cell survival after UVC irradiation in the absence of polh.

**Inhibition of ATR/Chk1 pathway exacerbates XP-V cell replication defect after UVC**

To study the effect of Chk1 inhibition on cell cycle after UVC, XP30ROpcDNA and XP30ROpolh cells were incubated with 25 nM UCN-01 post-irradiation (2 J/m²), pulse-labeled with BrdU and analyzed by flow cytometry (Supplementary Material, Fig. S5).
Material, Fig. S6). As expected, UCN-01 alleviated the UVC-induced DNA synthesis inhibition in XP30RO polh cells and the concomitant S-phase delay (Fig. 5A and B). Similar effects were observed at early times after irradiation in XP30ROpcDNA cells. However, UCN-01 failed to restore DNA synthesis 24 and 32 h after UVC exposure (Fig. 5A). Moreover, UCN-01 aggravated XP-V phenotype by increasing the number of cells totally arrested in S-phase (Fig. 5B). This effect was dependent on UCN-01 concentration (data not shown).

The concentration of caffeine we used in our experiments (0.38 mM) is low and does not efficiently inhibit ATR activity in vitro (12). However, we showed that this concentration was sufficient to decrease the strong pChk1 S345 levels observed in XP-V cells after all UVC doses tested and in normal cells after 20 J/m² (Fig. 5C and Supplementary Material, Fig. S7). Hyperphosphorylation of RPA32 was not affected by caffeine (data not shown). Flow cytometry showed that addition of 0.38 mM caffeine post-irradiation failed to abrogate UVC-induced S-phase delay in both cell lines, indicating that residual pChk1 S345 amounts observed at this concentration were sufficient for checkpoint activity. However, caffeine increased the number of irradiated XP-V cells arrested in S-phase (Fig. 5D). Our results strongly suggest that caffeine and UCN-01 inhibit a Chk1-dependent pathway crucial for XP-V cells but that may be independent of Chk1 involvement in regulation of S-phase progression.

UCN-01 increased the number of stalled forks in irradiated XP-V cells

To clarify this point, XP30ROpcDNA and XP30ROpolh cells were grown for 6 h after 7 J/m² in the presence or not of 25 nM UCN-01. Quantification of IdU/CldU incorporation by flow cytometry showed that UCN-01 failed to restore DNA synthesis levels in XP30ROpcDNA cells as early as 6 h after 7 J/m² (Fig. 6A).

Analysis of replication dynamics by DNA combing confirmed the results obtained in CTag versus MRC5 cells, i.e. a stronger decrease of fork density (Fig. 6B) and a higher proportion of asymmetric bubbles (Fig. 6C) in irradiated XP-V cells. However, in this experiment, mean fork velocity remained unchanged after UVC (Supplementary Material, Fig. S8). In mock-irradiated cells, addition of UCN-01 increased the number of active forks and decreased fork velocity, in agreement with recent studies (33,34). Fork density was similar in XP30ROpolh cells treated with UCN-01 irrespective of irradiation, showing that the decrease of DNA synthesis observed after UVC exposure in the presence of polh is indeed the consequence of replication checkpoint. However, fork density remained low in irradiated XP30ROpcDNA cells despite UCN-01 addition (Fig. 6B). UCN-01 alone slightly increased the number of asymmetric bubbles, to the same extent in both cell lines. In addition, UCN-01 treatment post-irradiation further increased fork stalling in XP30ROpcDNA cells without significantly affecting XP30ROpolh cells (Fig. 6C). Taken together, these data confirm that addition of UCN-01 post-irradiation aggravated replication defect of XP-V through increased fork stalling.

DISCUSSION

It is quite clear that polh plays a key role in the replication of UV-damaged DNA and its inactivation results in the cancer-prone XP variant syndrome. However, two essential questions need to be resolved: to what extent does polh defect affect origin firing and fork progression in human cells and why are XP-V cells specifically sensitized to UV by low
Concentrations of caffeine? For the first time, progression of in vivo-labeled replication forks was measured using DNA combing in irradiated XP-V cells. We showed that XP-V cells presented a higher number of stalled forks, indicating that UVC lesions are bypassed at the fork in human cells. We also found that, in the absence of polh, cells accumulate ssDNA regions during S-phase, presumably at UVC-induced lesions, suggesting that backup pathways are slow. Concomitantly, ATR pathway is over-activated. Chk1 plays an essential role for fork restart in XP-V cells and inhibition of its kinase activity by UCN-01 strongly sensitizes them to UVC, as previously observed with caffeine.

Consequences of polh deficiency on replication of UV-damaged DNA in human cells

Although many current models envisage TLS acting at the replication fork to allow DNA bypass with minimal arrest (39), recent evidence suggests that TLS is predominantly post-replicative (20), as formerly postulated by Lehman et al. (6). In yeast, inactivation of TLS does not further increase fork stalling (20), while, in chicken cells, inactivation of POLH does (21), indicating that the mechanisms used to overcome replication blocks may differ significantly among eukaryotes. Here, we clearly observed that polh deficiency leads to a higher number of asymmetric replication bubbles in human cells, demonstrating that polh is required for replication restart at UVC-stalled forks. In accordance to our findings, replication in human cell-free extracts of a plasmid bearing a TT-CPD in the leading strand showed extensive uncoupling of leading and lagging strands in the absence of polh but no evidence of repriming, the small size of the substrate being the limitation of these experiments (40,41). As DNA combing resolution is limited to 1–2 kb and therefore preclude the visualization of internal discontinuities, we cannot rule out the possibility that polh also participates in a post-replicative mechanism, whether to fill in gaps resulting from replication reinitiation beyond the lesion or to resolve termination between the arrested fork and the fork emanating from the most proximal origin.

Furthermore, we showed that addition of UCN-01 post-UVC increased S-phase arrest and fork blockage without fully restoring origin activation when polh is defective. This indicated that the decrease of DNA synthesis observed in indicates XP-V cells is the consequence of both inhibition of origin firing and physical blockage of fork progression. In contrast, in normal cells, fork arrests are rapidly resolved by efficient TLS and decrease of fork density is mostly due to checkpoint activity, in agreement with what was observed in cancerous HeLa cells (42,43). A model of replication dynamics in wild-type and XP-V cells is depicted in Fig. 6D.

ATR/Chk1 pathway is over-activated in XP-V cells

Enhanced ATR signaling in irradiated XP-V cells has already been reported (17). However, authors irradiated cells at 15 J/m², which is a toxic dose for XP-V cells, and analyzed the phosphorylation state of Chk1 shortly after irradiation (<8 h). Our study revealed that XP-V cells display an...
increased amount of pChk1 S345 during at least 24 h after only 2 J/m², consistent with our cell cycle analysis. Moreover, we demonstrated that strong levels of pChk1 S345 in XP-V cells were a direct consequence of polh defect and were indeed mediated by ATR. A growing body of evidence suggests that primed RPA-coated ssDNA is the signal for ATR checkpoint activation (28,44,45). Generation of excessive regions of ssDNA in irradiated XP-V cells was postulated from alkaline sucrose gradient experiments but, to our knowledge, never directly observed. We have established the ssDNA accumulation during S-phase in fixed XP-V cells after 2 J/m². Concomitantly, RPA was recruited to nuclear structures. Our results suggest that, in the absence of polh, persistent stalling of replicative polymerase at photoproducts generates extensive ssDNA tracks, leading to sustained checkpoint activation.

Hyperphosphorylated forms of RPA32 became evident 6 h after 2 J/m² in XP-V cells. Hyperphosphorylation of RPA32 after UV was shown to depend on DNA-PKcs, a member of the PIKK family that is recruited at double-strand break (DSB) ends to initiate non-homologous end joining (18). RPA32 hyperphosphorylation may then reveal formation of DSBs at collapsed forks, as it was already proposed (19). This is consistent with the strong phosphorylation of histone H2AX on serine 219 we observed in the fraction of irradiated XP-V cells presenting ssDNA foci.

**Why are XP-V cells sensitized to UVC by low concentrations of caffeine?**

UVC sensitization of XP-V cells by caffeine is known for some time (10). However, the precise mechanism remained unclear. Here, we showed that 0.38 mM caffeine or 25 nM UCN-01 sensitized XP-V cells to UVC with similar reduction in survival without affecting polh-expressing cells. This is a rather low dose of caffeine compared with what is usually used in literature (28,33,42). Addition of 0.38 mM caffeine did not abrogate UVC-induced S-phase slow-down, whereas addition of 25 nM UCN-01 did. However, this concentration was sufficient to decrease Chk1 phosphorylation in XP-V cells and to increase the number of S-phase arrested cells. Taken together, our results strongly suggest that caffeine sensitizes XP-V cells through inhibition of a Chk1-dependent pathway distinct from regulation of S-phase progression. We infer that this pathway is involved in maintaining fork stability.

In agreement with a role of the ATR pathway in the regulation of replication, UCN-01 and caffeine increase fork density, which is compensated by a concomitant decrease of fork velocity [our results and (33,34,46)]. Thus, after irradiation, the probability to encounter a UVC lesion should be similar than in untreated cells (Fig. 6D). Notably, checkpoint

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**Figure 6.** UCN-01 increases fork stalling in XP-V cells after UVC irradiation. XP30ROpolh and XP30ROpcDNA cells were irradiated at 7 J/m² and incubated in the presence or not of 25 nM UCN-01 for 6 h. Replication forks were then labeled as described in Fig. 1C. (A) IdU/CldU incorporation was quantified by flow cytometry and expressed as a percentage of the value obtained in untreated cells (i.e. no UVC and no UCN-01). Percentages are indicated on top. (B) Fork density was determined by DNA combing. Percentages of untreated cells values are indicated on top. (C) Proportion of asymmetric bubbles. At least 50 intact bubbles were counted per condition. (D) Model of replication dynamics in UV-irradiated XP-V and normal cells. On the top: in wild-type cells (WT), fork stalling only slightly increases after UVC because of efficient bypass by polh. Decrease of fork density is mainly the consequence of ATR checkpoint activation. In XP-V cells, both checkpoint and persistent fork blockage (resolved by slow alternative pathways) contribute to decrease fork density. Consequently, the resumption of DNA synthesis is delayed. On the bottom: inhibition of ATR checkpoint increases origin firing but decreases fork velocity. While in WT cells blocking lesions are still efficiently bypassed by polh, in XP-V cells the lack of checkpoint-dependent stabilization of stalled forks prevents usage of alternative pathways, leading to the inability to resume replication, fork collapse and cell death. Blue rectangle, IdU; green rectangle, CldU; black rectangle, DNA replicated before labeling; red triangles, UVC-induced lesions.
inhibition in normal cells did not increase significantly fork stalling and cell death after low doses of UVC. This shows that checkpoint activity is dispensable in the presence of efficient bypass by polθ and low amounts of pyrimidine dimers. This also confirms that TLS can occur in the absence of an intact checkpoint, as shown in yeast (47). On the contrary, we observed an increase of fork stalling in irradiated XP-V cells upon Chk1 inhibition. Since it was shown that replisome components dissociate from stalled forks if ATR signaling is impaired (15,16), inhibition of ATR/Chk1 pathway may prevent usage of alternative specialized polymerases to bypass photoproducts. On the other hand, UCN-01 and caffeine may directly inhibit alternative fork rescue pathways. As a matter of fact, it was suggested that resolution of UVC-induced fork blockage partly relies on homologous recombination (HR) in XP-V cells (48) and ATR pathway is thought to regulate HR through phosphorylation of various substrates as Rad51 or Werner and Bloom helicases (49–51).

In conclusion, in this study, we have highlighted the importance of Chk1 kinase in human cells deficient in polθ after UVC exposure. Further investigations are needed to determine the alternative pathways that take place at stalled forks to tolerate DNA damage and allow the slow resumption of DNA synthesis in irradiated XP-V cells at the detriment of genetic stability.

MATERIALS AND METHODS

Cell culture

Wild-type (MRC5-V1), XP-V (CTag, XP30RO, XP30ROpcDNA) or XP-V stably expressing POLH cDNA [XP30RO polθ, (8)] SV-40 immortalized fibroblasts were maintained in Minimal Eagle Medium (MEM; Gibco) supplemented with 10% fetal calf serum (FCS), l-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin under 5% CO2. As around 20% of XP30RO cells are tetraploid, XP30ROpcDNA cells, which carry empty pcDNA vector (Invitrogen) used for complementation, were selected to present a proportion of tetraploid cells close to that of XP30ROpolθ cells. They behave as XP30RO parental cells (this article and data not shown). XP30ROpolθ and XP30ROpcDNA cells were grown in the presence of 100 μg/ml zeocin (Invitrogen).

Treatments

For UVC irradiation (254 nm), cells were washed in preheated phosphate buffer saline (PBS) and irradiated without any medium at a fluency of 0.65 J/m²/s. Stock solution of UCN-01 (Sigma), SB-218078 (Calbiochem) and caffeine (Sigma) was prepared at 10, 12.5 in Dimethyl Sulfoxide (DMSO) and 38 mM in water, respectively. When indicated, drugs were added in culture medium just after UVC exposure after appropriate dilution in complete medium.

siRNA transfection

siRNAs were used to transiently down-regulate the expression of POLH (5'-GAAGUAUGUCCAGAUCCU-3', Eurogentec), ATR (SMART pool, Dharmacon) and Chk1 (5'-GCG UGCCGUAGACUGUCC-3', Eurogentec) genes. Cells were transfected with 30 nm of siRNAs using Interferin (Polyplus) according to the manufacturer's instructions, and were treated 48 h after transfection.

Proliferation assay

Cells were plated at 2 × 10⁵ per well of a six-well plate 24 h before UVC exposure. In the case of siRNA transfection, cells were transfected 24 h after seeding (1.5 × 10⁵ cells per well) and incubated 48 h before irradiation. Cells were incubated for 72 h and counted with trypan blue using a neubauer haemocytometer.

Western blot

For analysis of phosphorylated proteins, cells were scrapped directly in NaCl; EDTA; Tris, Nonidet (NETN) buffer [150 mM NaCl, 1 mM EDTA, 50 mM Tris pH 7.5, 0.5% NP-40, anti-proteases (Complete Mini EDTA free, Roche), anti-phosphatases (PhosSTOP, Roche)] and incubated 30 min on ice. Extracts were sonicated 15 s at amplitude 30%. For cell fractionation, cells were scrapped in cold PBS and centrifuged. Pellet was resuspended in one volume of CytoSkleton (CSK) 100 buffer (100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 10 mM PIPES pH 6.8, 1 mM Ethyleneglycol tetra-acetic Acid (EGTA), 0.2% Triton × 100, anti-proteases) and incubated 15 min on ice. Sample was centrifuged at 7000 r.p.m. for 5 min at 4°C. Supernatant was kept as ‘soluble protein fraction’. Pellet was resuspended in one volume of lysis buffer (50 mM Tris pH 7.5, 20 mM NaCl, 10 mM MgCl₂, 0.1% SDS, anti-proteases), lysed 10 min on a wheel at room temperature to give the ‘insoluble protein fraction’. For whole cell extracts (WCE), cells were directly lysed in two volumes of lysis buffer as described above. Aliquots were kept for Bradford quantification (Biorad). Laemmli 5× was added and proteins were denatured 10 min at 90°C. The equivalent of 20 μg of proteins per samples was separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Membranes were blotted with antibodies directed the following proteins: ATR (Santa Cruz), β-catenin (BD Biosciences), Chk1 (Santa Cruz), pChk1 S345 (Cell Signaling), Claspin (Bethyl), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Abcam), lamin A/C (Santa Cruz), PCNA (Santa Cruz), polθ (Abcam), Rad18 (ab57447, Abcam), RPA32 (Calbiochem) and γ-tubulin (Sigma).

Flow cytometry

To study cell cycle distribution, cells were treated, incubated for the indicated times and 10 μM BrdU was added to culture medium 10 min prior to harvesting. Alternatively, to follow S-phase progression, S-phase cells were pulse-labeled with 10 μM BrdÚ for 10 min. Cells were then washed thoroughly, treated and incubated in fresh medium for the indicated times. Cells were trypsinized, washed in PBS and fixed in 80% ice-cold ethanol overnight at −20°C. The day of analysis, samples were incubated in 15 mM pepsin for 20 min at 37°C. DNA was denaturated in 2 N HCl for 20 min at room temperature. The pellet was washed in Bu
buffer (0.1% FCS, 0.1% Tween 20, 0.1 m Hepes in PBS), and was incubated with 1/50 mouse anti-BrdU antibody (DAKO) in Bu buffer for 45 min. After centrifugation, pellet was resuspended and incubated with 1/50 anti-mouse-Fluorescein isothiocyanate (FITC) antibody (AbCys). Total DNA was stained in 25 μg/ml propidium iodide and 50 μg/μl RNase (Sigma) in PBS for 30 min at room temperature. Samples were analyzed on a FACScalibur using the CellQuest Pro software (BD Sciences). 20,000 cells were counted for each sample.

Detection of ssDNA in fixed cells

We used a protocol previously described (29). Briefly, cells were labeled with 100 μM BrdU for two doubling times, washed and irradiated. Cells were fixed in 4% paraformaldehyde containing 0.2% Triton × 100. BrdU was stained in non-denaturing conditions which enable to detect only free BrdU or BrdU incorporated in ssDNA. For simultaneous detection of ssDNA and replication foci, cells were cultivated for 48 h in the presence of 10 μM IdU (Sigma). S-phase cells were labeled with 100 μM CldU (Sigma) for 30 min prior to fixation. Coverslips were blocked in 3% BSA buffer and incubated at room temperature. Washes were made in PBS, unless otherwise indicated. Coverslips were mounted in mounting medium (Dako) supplemented with 4',6'-diamidino-2-phenylindole (DAPI) (Sigma). ssDNA (BrdU) staining: (i) 1/50 mouse anti-BrdU antibody (Becton Dickinson) for 1 h; (ii) 1/1000 anti-mouse Alexa Fluor 488 (all Alexa Fluor antibodies from Molecular Probes) for 30 min. ssDNA/protein co-detection: (i) 1/50 rat anti-BrdU antibody (Abcys) + 1/2000 RPA70 (Oncogene), 1/500 PCNA (Santa Cruz), 1/1000 CPD (Cosmo Bio) or 1/10,000 γH2AX (Upstate); (ii) 1/1000 anti-rat AlexaFluor 594 + 1/1000 anti-mouse Alexa Fluor 488. As untreated SV-40 immortalized fibroblasts displayed few bright γH2AX foci, γH2AX staining was acquired with very short time of exposure to minimize this background. Simultaneous detection of ssDNA and replication foci: (i) IdU was stained as indicated for BrdU, with the addition of 15 min wash in high salt buffer (200 mM NaCl, 0.2% Tween 20, 0.2% NP-40 in PBS) after primary antibody incubation; (ii) antibodies were fixed in 4% paraformaldehyde for 15 min and DNA was denatured in 1.5 M HCl in PBS for 30 min; (iii) CldU was detected with 1/50 rat anti-BrdU antibody and 1/1000 anti-rat Alexa Fluor 594.

DNA molecular combing

Cells were successively labeled for 20 min with 100 μM IdU and 100 μM CldU at 37°C and incubated for 10 min at 37°C in complete medium containing 1 mM thymidine (Sigma) before trypsination. Preparation of DNA and combing on silanized glass coverslips was done as already described using agarose plugs containing 20,000 cells per condition (52). Combed DNA was denatured in 0.5 M NaOH for 20 min or in 50% formamide for 5 min at 80°C. Coverslips were blocked in blocking buffer [1% blocking reagent (Roche), 0.05% Tween 20 in PBS] overnight at 4°C in a humid chamber. For modified nucleotides, antibodies were diluted in blocking buffer and for total DNA in 1% BSA buffer (1% BSA, 0.1% Tween 20 in PBS). If not indicated otherwise, coverslips were washed three times in PBS between each antibody. Staining protocol was performed as follows: (i) CldU detection: 1/20 rat anti-BrdU 1 h at room temperature, 1/50 chicken anti-rat Alexa Fluor 488 20 min at 37°C, 1/25 goat anti-chicken Alexa Fluor 488 20 min at 37°C; (ii) IdU detection: 1/5 mouse anti-BrdU 1 h at room temperature, 6 min in 0.5 M NaCl, 20 mM Tris, 0.5% Tween 20, two washes in PBS, 1/200 rabbit anti-mouse Alexa Fluor 350 20 min at 37°C, 1/25 goat anti-rabbit Alexa Fluor 350 20 min at 37°C; (iii) detection of total DNA: blockage in 1% BSA buffer, 1/25 mouse anti-guanosine (Argene) 45 min at 37°C, 1/25 goat anti-mouse Alexa Fluor 594 30 min at 37°C. Coverslips were mounted in phenylenediamine and stored at −20°C before analysis. Coverslips were scanned with an Olympus IX 81 or a Nikon Ti inverted microscope connected to a CoolSNAP HQ CCD camera (Photometrics) run by MetaMorph version 6.3r7 (Molecular Devices). Fluorescent signals were measured with ImageJ software (Rasband, 1997–2007). For fork density determination [number of forks/S-phase DNA length (Mb)], number of forks and total DNA length were determined on random fields. DNA length was corrected with the proportion of S-phase cells measured by flow cytometry on the same samples (see Supplementary Table for raw data). Asymmetric replication bubbles were defined as bubbles where one of the forks failed to incorporate CldU. Total DNA was used to exclude broken bubbles. For fork velocity determination, merged forks and broken signals were excluded. The values of fork velocities were plotted as a frequency distribution. Statistical analysis was done with a Mann–Whitney test.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. none declared

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