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Involvement of DNA replication in ultraviolet-induced apoptosis of mammalian cells

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Abstract Exposure of cells to ultraviolet (UV) light damages the genome and the persistence of DNA lesions triggers apoptosis in mammalian cells. RNA transcription blockage by DNA damage is believed to be implicated in signaling for UV-induced apoptosis, but the role played by DNA replication in this process is still unclear. To address this point, we have employed the DNA polymerase inhibitor aphidicolin in UV-irradiated wild-type and *XPB*-mutated Chinese hamster ovary cells. The data obtained with synchronized cells indicate that induction of apoptosis by UV light is independent of the cell cycle phase. Nevertheless, cells treated with aphidicolin after UV exposure showed a significant prevention of apoptosis induction when compared to proliferating cells. These results were observed in both DNA-repair proficient and deficient cells, indicating that the prevention of apoptosis by aphidicolin is independent of the cells' ability to repair the photolesions caused by UV. Taken together, these data suggest that replication of damaged DNA also leads to critical events signaling for UV-induced cell death.

Keywords DNA replication · Apoptosis · Ultraviolet · Aphidicolin

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Introduction

Ultraviolet light (UV-C, 254 nm) induces helix-distorting lesions in DNA, such as pyrimidine [6–4] pyrimidone photoproducts (6–4 PPs) and cyclobutane pyrimidine dimers (CPDs) [1]. The presence of these photolesions in the double-helix produces diverse biological responses in mammalian cells, such as blockage of DNA replication and RNA transcription, chromosomal breakage, DNA recombination, mutations and, eventually, cell death by apoptosis [2]. The latter event is characterized by being a particular type of cell death, genetically controlled and with several specific characteristics, such as caspase activation, chromatin and cytoplasm condensation, nuclear and DNA fragmentation, externalization of phosphatidylserine residues, culminating

with cellular membrane convolution generating apoptotic bodies [3, 4].

In eukaryotic cells, these UV-induced DNA lesions are normally removed by nucleotide excision repair (NER). The NER pathway is composed of two sub-pathways: transcription coupled repair (TCR), that discriminates lesions present in the transcribed strand of active genes, and global genomic repair (GGR), that removes lesions from the rest of the genome [5, 6]. There are several human disorders related to defective NER proteins (TCR-NER, GGR-NER or both), which affect individual cancer proneness and even organism development (e.g. xeroderma pigmentosum, Cockayne's syndrome and trichothiodystrophy [7]). NER-deficient cells are hypersensitive to UV light exposure [5] but immediate removal of photolesions is known to prevent apoptosis, indicating that unrepaired DNA lesions are the main cause of UV-induced apoptosis in mammalian cells [8, 9].

Another well-known mechanism of DNA repair is photoreactivation, which is performed by photolyases, enzymes that specifically remove UV-induced lesions (CPDs or 6–4 PPs) from the double-helix, using the energy of visible light [10]. Although genes presenting similarity to photolyases have been found in rats and humans cells, there is evidence that photoreactivation does not occur in placental mammals [11]. The presence of proteins belonging to the photolyase/blue light receptor family in these mammals is in fact related to the maintenance of circadian rhythms [12].

The presence of CPDs in UV-irradiated cells has been shown to be one of the initial signals for the commitment to the apoptotic program [8, 9]. However, downstream signals responsible for the induction of the biological responses that follow the formation of UV photoproducts are far from being completely understood. Several proteins responsible for genome stability have their function affected by UV photolesions, including poly(ADP-ribose)polymerase-1 (PARP-1) [13], DNA repair and DNA replication machineries and DNA translesion polymerases [14, 15].

In this context, the relation between UV-induced apoptosis and replication of damaged DNA is not a consensus. While some data point to a lack of correlation between UV-induced cell killing and the replication of damaged DNA [16], recent reports point to the opposite [17, 18]. Mckay et al. [17], working with primary human fibroblasts, showed that UV-induced apoptosis is associated with S-phase progression, and Dunkern and Kaina [18] showed that in Chinese Hamster Ovary (CHO) cells UV-induced apoptosis is dependent of cell proliferation and DNA breaks that arise when the DNA replication machinery is blocked by a photolesion.

In this work, the role of DNA synthesis of damaged templates in UV-induced apoptosis was further investigated. To address this matter, apoptotic rates were analyzed in both proliferating and synchronized populations of CHO cells. Replication inhibition was achieved by the use of aphidicolin, a mycotoxin that specifically inhibits DNA replication in eukaryotes by inhibiting the binding of 2′-deoxynucleotides–5′-triphosphates (dNTPs) to the DNA polymerases α and δ of these organisms [19]. The data indicate that the commitment to the apoptotic process is similar in cells that were UV-irradiated in different phases of the cell cycle; however, progression of the DNA replicative S phase is necessary for the commitment to cellular suicide. These results were obtained in DNA-repair-proficient fibroblasts (CHO-9) and in DNA-repair-deficient fibroblasts (CHO 27.1). Results obtained with CHO cells expressing a marsupial photolyase gene (CHO*phr* and CHO-XPB*phr*, [9]), brought evidence that during the first 24 h after exposure of the cells to UV irradiation there are critical events leading to a commitment to death. Since cells treated with aphidicolin were able to prevent the induction of apoptosis, we conclude that the inhibition of damaged DNA replication is somehow blocking upstream apoptotic signals. The reduction of apoptosis by aphidicolin is not linked to transcription blockage, since this drug does not interfere with RNA synthesis in either non-irradiated or UV-irradiated cells. Taken together, the data indicate that replication of CPDs and 6–4 PPs is necessary to trigger apoptosis in mammalian cells, independently of the cells' ability to remove the UV photolesions from the genome.

Materials and methods

Cell culture

Wild-type (CHO-9) and XPB-mutated (CHO 27.1) cells were routinely grown in DMEM (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal calf serum (FBS; Cultilab, Campinas, SP, Brazil) and 1% antibiotic-antimycotic (Invitrogen Corporation), at 37°C in a humidified 5% CO_2 atmosphere. These cell lines were kindly provided by Dr. MZ Zdzienicka (Leiden University, The Netherlands). CHO cell lines stably expressing the rat kangaroo CPD-photolyase (PHR) were generated as previously reported [9]. Hygromycin-B (200 μg/ml, Invitrogen Corporation) was added to the culture medium for selection of cells carrying the exogenous gene.

DNA synthesis quantification

Approximately 4.0×10^4 cells were plated in 35 mm Petri dishes and treated with different concentrations of aphidicolin (Biomedicals Inc., Ohio, USA) for at least 24 h. After this period, cells were incubated in a medium containing ^{3}H-methyl-thymidine (4.0 μCi/ml, specific activity of 89.0 Ci/mmol, Amersham-Pharmacia Biotech, USA) for 15 min. Cells were then washed once with phosphate-

buffered saline (PBS), once with 5% trichloroacetic acid and twice with hydrated alcohol, before 0.3 M NaOH was added. Part of this cell lysate (20% of the total volume) was applied on Whatman 17 paper, which was again washed once with 5% trichloroacetic acid, twice with hydrated ethanol and once with acetone. Radioactivity was measured with a liquid scintillation spectrometer (Beckman LS 7000). The other part of the lysate (80% of the total volume) was used for absorbance reading at 260 nm (Hitachi U-200 spectrophotometer), for data normalization. The ratio between radioactivity and absorbance expresses the amount of ^{3}H-methylthymidine incorporated by the cells during DNA synthesis.

RNA synthesis quantification

RNA synthesis was determined based on a method described previously [20]. Approximately 4.0×10^4 cells were plated in 35 mm Petri dishes and treated with aphidicolin (0.1 μg/ml) for 24 h. After this period, cells were incubated in a medium containing 3% dialyzed fetal calf serum and [5-^{3}H] uridine (4.0 μCi/ml, specific activity of 27.0 Ci/mmol, Amersham-Pharmacia Biotech) for 30 min. Cells were then harvested and separated into two samples. In one of the samples, cells were lysed (NaCl 0.3 M; Tris-HCl pH 8.0 20 mM; EDTA 2 mM; SDS 1% and Proteinase K 200 μg/ml) and then transferred to Whatman 17 paper and washed twice with 15% trichloroacetic acid and hydrated ethanol for 30 min, for radioactivity measurement. The second sample was used to determine the absorbance at 260 nm, for data normalization. The ratio between radioactivity and absorbance expresses the RNA synthesis in these cells.

Cell cycle synchronization

Cell cycle synchronization was achieved using a modification of the method described by Spadari et al. [19] Approximately 6.0×10^4 exponentially growing cells were plated (60 mm Petri dishes) in culture medium. After 24 h the culture medium was replaced, and aphidicolin (0.1 μg/ml) was added and left for 24 h. Cells were then washed with PBS and kept in fresh medium for 16 h. After this period, aphidicolin (0.1 μg/ml) was added again for 8 h, and cells were then released. Samples for cytometric analysis were harvested at different times after aphidicolin release.

UV irradiation and flow cytometry analysis

Approximately 1.0×10^5 cells were plated in 60 mm Petri dishes 24 h before UV irradiation. Cells were washed twice with pre-warmed PBS and irradiated by a low-pressure germicidal lamp (UV light emitting mainly at 254 nm, with a dose rate of 1.00 J/m^2/s). Cells were kept in presence or absence of aphidicolin for 48 or 72 h. After this period, both adherent and detached cell populations were collected and centrifuged at 1.500 rpm for 10 min. Pelleted cells were lysed with 500 μl of a hypotonic fluorochrome solution (50 μg/ml PI in 0.1% sodium citrate plus 0.1% Triton X-100), and incubated at least 30 min on ice in the dark. Then, the samples were transferred to microtubes, and PI fluorescence was measured by flow cytometry (FACScalibur, Becton Dickinson, San Jose, CA). Results were obtained as percentage of subdiploid nuclei (CellQuest Software, Becton Dickinson, CA), which represents the apoptotic cells [21].

Cell survival

Approximately five hundred cells were seeded into 60 mm Petri dishes, 8 to 10 h before UV irradiation. One week later colonies were fixed with 10% formaldehyde and stained with 1% violet crystal. Colonies with the minimal number of 15 cells were scored. Survival was expressed in relation to the untreated control. Values are given as the mean of three independent experiments.

Photoreactivation experiments

When indicated, photoreactivation was performed on CHO*phr* or XPB*phr* cell lines for a period of 2 h in PBS (0 h or 24 h after UV irradiation). Single cell layers were illuminated 10 cm over fluorescent lights (two daylight lamps, Philips 15 W; emission, 400–700 nm). After photoreactivation cells were maintained in complete medium until flow cytometry analysis.

Morphological analysis of apoptosis and fluorescence microscopy

Approximately 3.5×10^4 cells were plated in 35 mm Petri dishes. 24 h after plating, cells were washed twice with pre-warmed PBS and UV-irradiated at indicated doses. Where indicated, the cells were placed into medium containing aphidicolin (0.1 μg/ml) immediately after UV irradiation. Cells were harvested 48 h after UV treatment, centrifuged, and resuspended in 20 μl of PBS. Then, 2 μl of AO/EB solution (100 μg/ml acridine orange, AO; 100 μg/ml ethidium bromide, EB, in PBS) were added [21]. The cells were analyzed under a fluorescence microscope (Leica DM LB) using a fluorescein filter and a 40X objective.

Results

Effect of aphidicolin on DNA replication and transcription

Aphidicolin is a mycotoxin extracted from cultured filtrates of fungi that specifically inhibits DNA replication by

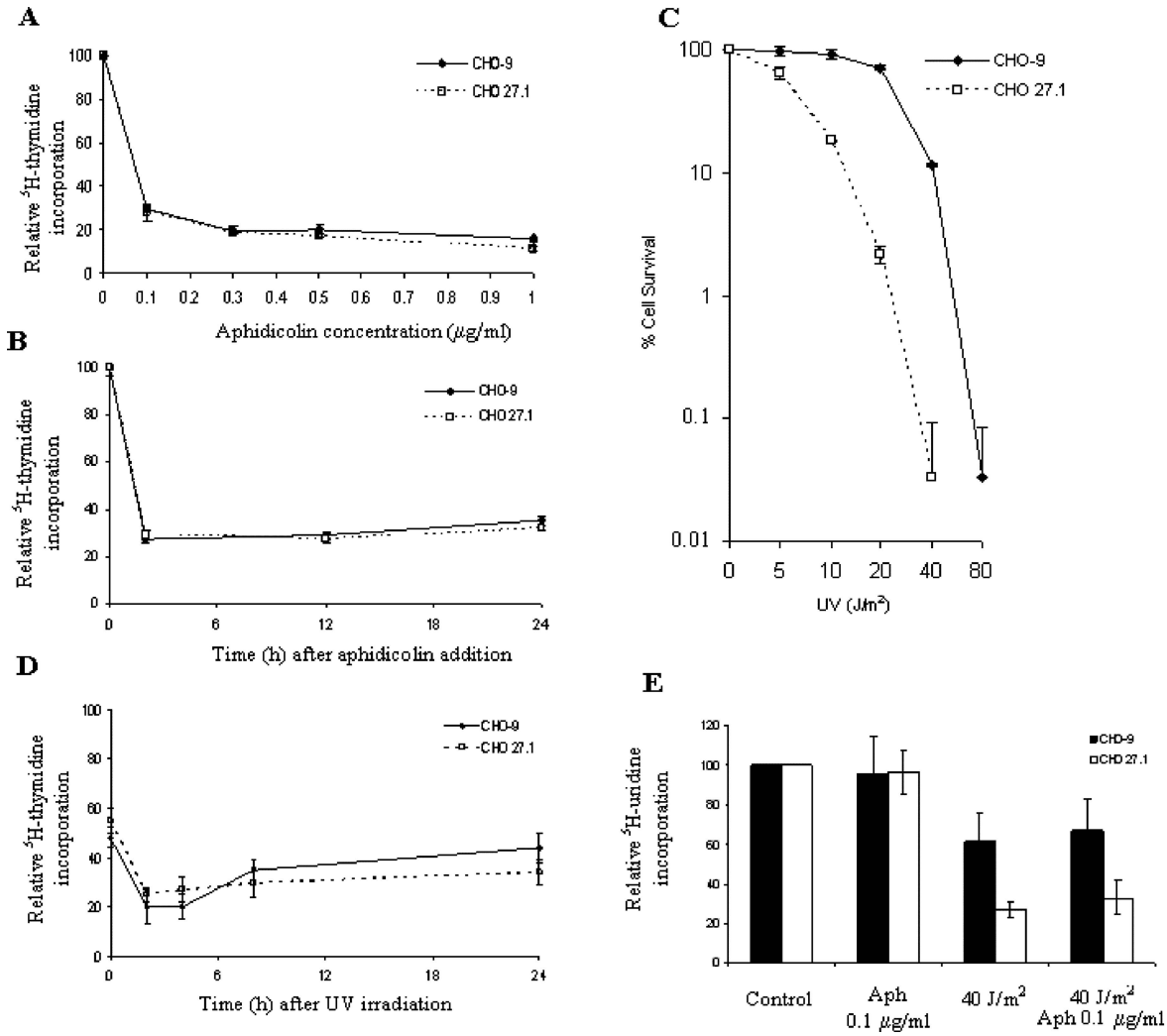

Fig. 1 UV cell survival and aphidicolin and UV effects in DNA and RNA synthesis. (A) Aphidicolin-dose response curve in DNA synthesis: cells were maintained at the indicated concentrations of aphidicolin for 24 h and then pulse-labeled with ^{3}H-methyl-thymidine for 15 min before harvesting. (B) Kinetics of replication arrest induced by aphidicolin: cells were incubated with 0.1 μg/ml of aphidicolin for the indicated periods of time, before DNA labeling with ^{3}H-methyl-thymidine for 15 min. (C) Cell survival: cells were plated at low density, UV-irradiated at the indicated doses and cultivated for seven days before scoring surviving colonies, as described in Material and methods. (D) DNA synthesis inhibition by UV light: irradiated cells (20 J/m^2 for CHO 27.1 and 40 J/m^2 for CHO-9) were pulse labeled with ^{3}H-methyl-thymidine at the indicated times. For figures (A) through (D) CHO-9 (–◆–) and CHO 27.1 (···□···) cell lines. (E) Effects of aphidicolin and UV irradiation on RNA synthesis: cells were treated as indicated, and transcription rates on UV-irradiated samples were measured 8 h after exposure to UV (in Aph. 0.1 μg/ml, cells were kept with aphidicolin during this period). The cells were RNA-labeled with [5- ^{3}H]-uridine for 1 h, CHO-9 (black bars) and 27.1 (open bars) cell lines. DNA and RNA synthesis was determined as described in Materials and methods and the data are presented in relation to control cells

interacting with the DNA polymerases of eukaryotes. We first set out to assure that this drug was efficient in the cells used. CHO-9 and 27.1 cells were kept in different concentrations of aphidicolin and their replication was measured by ^{3}H-methyl-thymidine incorporation. Figure 1(A) represents the relative DNA synthesis of both cells lines where it demonstrates that aphidicolin inhibits DNA replication for over 70% at the concentration of 0.1 μg/ml. It is important to emphasize that higher concentrations of aphidicolin induce apoptosis in these cells, so that the concentration of 0.1 μg/ml was used in the other experiments of this work. The kinetics of replication arrest induced by aphidicolin was also examined (Fig. 1(B)): within a period of 2 h of incubation, maximum DNA synthesis-inhibition activity was already observed. In Fig. 1(C), a colony survival curve is presented for both cells. As expected, the DNA repair deficient cells are more sensitive to UV, and doses chosen for this work were those that induce comparable levels of cytotoxicity for both cell lines, between 2 and 10% survival (40 J/m^2 for CHO-9 and 20 J/m^2 for CHO 27.1). As shown in Fig. 1(D), these UV doses promote an immediate response inhibiting DNA synthesis, with a maximum of 80% inhibition observed 2 h after

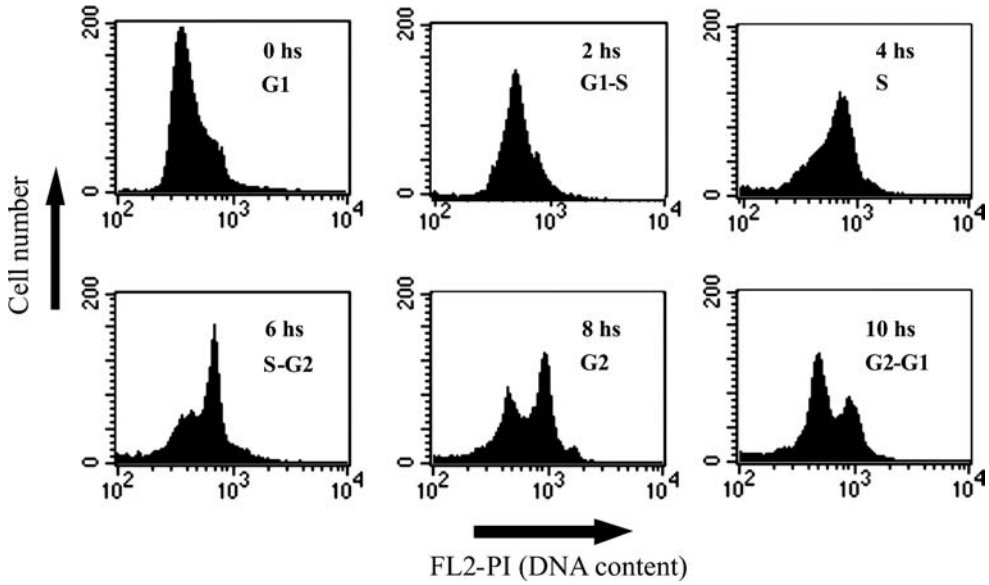

Fig. 2 Synchronization of CHO-9 cells by a double block with aphidicolin. Exponentially growing cells were cultivated for 24 h with aphidicolin, released in fresh normal medium without aphidicolin for 16 h, when aphidicolin was added again for a period of 8 h. Cells where then released in fresh medium without aphidicolin. The figures represent cytometry histograms collected at the times indicated after the last aphidicolin block (modified from [24]). Relative PI fluorescence (DNA content) is represented on the *x*-axis (FL2-PI), and cell number is represented on the *y*-axis (Events)

irradiation. However, both cells recover DNA synthesis 24 h later, indicating they are able to replicate the UV-damaged DNA.

Since blockage of RNA synthesis is often linked to apoptosis induction by UV light, the effects of aphidicolin on RNA transcription were investigated (Fig. 1(E)). Cells were kept with 0.1 μg/ml aphidicolin for 24 h, and the transcription rates measured by [5-^{3}H]-uridine incorporation. The results indicate that aphidicolin does not affect the transcription rates. Similar results were observed for UV-irradiated cells. The data confirm that UV irradiation causes the blockage of RNA synthesis and that the effect is stronger in DNA-repair-deficient cells; however, treatment with aphidicolin presented no additional effect in the UV-induced inhibition of transcription.

Influence of cell cycle on UV-induced apoptosis

To examine whether there is any cell cycle-dependent variation of apoptosis induction by UV light, cells were submitted to a double block of DNA replication inhibition with aphidicolin. Figure 2 represents the histograms generated from flow cytometry analysis after cell synchronization. Immediately after aphidicolin release, most of the cells are synchronized in G1 phase, although a small part of the cells is blocked in S phase. Later, there is a clear peak movement to the right (G2 phase, up to 8 h), indicating that most of the cells are synchronized throughout the cell cycle. Cells were then UV-irradiated at different times after aphidicolin. In these experiments, half of the samples were used to check cell synchronization and the other half was irradiated (40 J/m^2), in order to have most of the cell population at specific cell cycle phases (G_1, S and G_2/M). Table 1 shows that the cells were equally induced to apoptosis, regardless of the cell cycle phase they were at the moment of UV-irradiation.

Effect of damaged DNA replication on UV-induced apoptosis

To address the participation of damaged DNA replication in UV-induced apoptosis, we investigated cells that were either proliferating or kept with aphidicolin (0.1 μg/ml) after UV exposure. These experiments were initially performed with the DNA-repair-proficient CHO-9 cell line (Fig.

Table 1 Apoptosis in cell-cycle phase-specific UV irradiated CHO-9 cells

Cell cycle phase	% of UV-induced apoptosis
G_1	41 ($\pm$4)
S	42 ($\pm$4)
G_2/M	45 ($\pm$6)

Cells were synchronized by a double-block of aphidicolin (see Materials and Methods). At specific times (0 h for G_1, 2 h for S, and 6 h for G_2) after aphidicolin release, cells were UV-irradiated (40 J/m^2) and 48 h later the nuclei were isolated and stained with PI for analysis by flow cytometry. Sub-G1 cells were considered as apoptotic cells. In a parallel experiment cells were checked for synchronization (as in Fig. 2). Each value represents the mean ($\pm$ standard error) of three independent experiments.

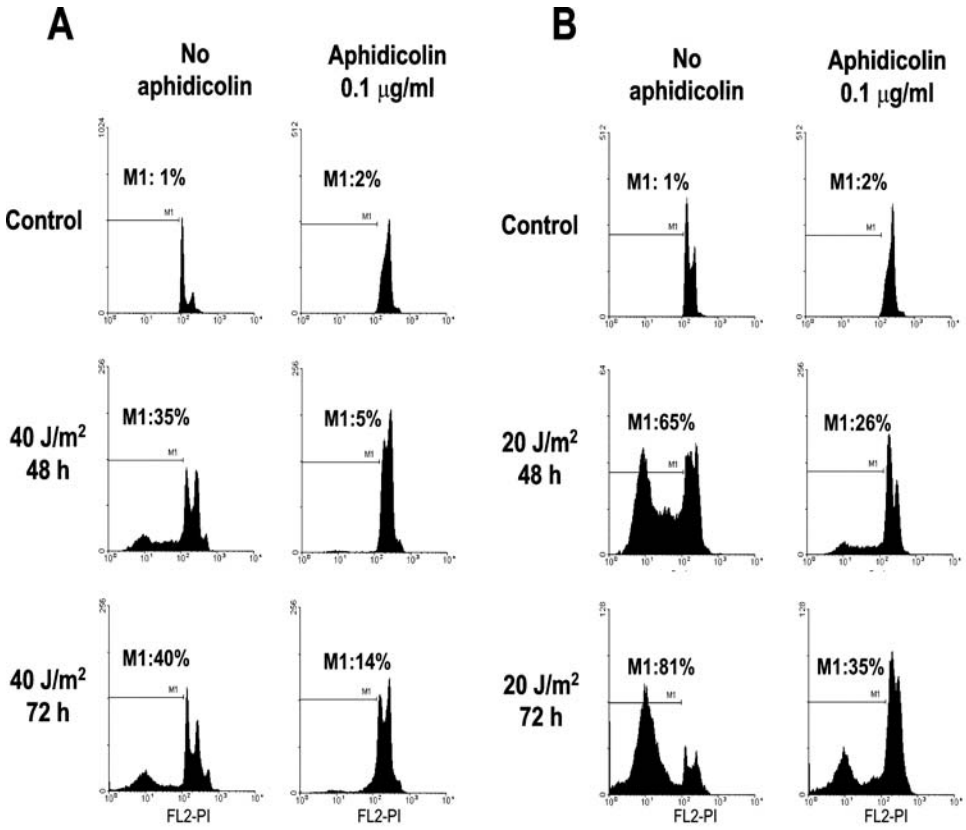

Fig. 3 UV-induced apoptosis is prevented by DNA synthesis inhibition with aphidicolin. Approximately 1.0×10^5 cells were irradiated, at the indicated doses, and cultivated with or without aphidicolin after UV-irradiation. CHO-9 (A) and CHO 27.1 (B) had their nuclei isolated and stained with PI for flow cytometry analysis 48 and 72 h after UV exposure. The percentage of apoptotic cells (sub-G_1, M1) is indicated each histogram. Relative PI fluorescence (DNA content) is represented on the *x*-axis (FL2-PI), and cell number is represented on the *y*-axis (Events)

3(A)). It is important to emphasize that as dead cells normally detach after UV-irradiation, both detached and adherent cells are included in the apoptosis experiments. Analysis of cells treated with aphidicolin demonstrates that the concentration used was not toxic, but when added after UV-irradiation, it protects cells from apoptosis, as it can be observed by the simultaneous reduction of the frequency of apoptotic cells (sub-G1) and increase of cells in G1, S and G2 phases of the cell cycle. Strikingly, this phenomenon is also observed in the DNA-repair-deficient XPB-mutated cells (Fig. 3(B)), which are unable to remove photoproducts from their genome. Therefore, inhibition of damaged DNA replication can avoid apoptosis, regardless of the removal of lesions from the genome, demonstrating that the encounter of the DNA replication machinery with a UV photoproduct is somehow an active and fundamental event for apoptosis induction after exposure to UV light. It is important to mention that aphidicolin not only inhibited apoptosis for 48 h but sustained this protection for 72 h, demonstrating that it is not only delaying apoptosis but indeed preventing it for longer periods.

To further understand the importance and the relation between replication of damaged DNA and persistence of unrepaired UV photoproducts in the apoptosis-triggering process, we have repeated these experiments with CHO*phr* cells, which express a marsupial CPD-photolyase, and are able to remove CPDs, when exposed to light. As it has been previously reported (8, 9), photorepair immediately after UV irradiation efficiently prevents cells from entering apoptosis (Fig. 4). However, if photoreactivation is only performed 24 h after UV exposure there is no reduction of apoptosis. Thus, after this period of time, critical events responsible for UV-induced cell death have already occurred and the cells are already committed to apoptosis. In despite to this, cells which had their replication inhibited by aphidicolin showed a remarkable prevention of apoptosis up to 48 h after UV irradiation, independently of photorepair. Similar experiments were performed with CHO-XPB*phr* cells, which, although being defective in repair of UV photolesions by NER, are proficient in photoreactivation of CPDs lesions by photolyase. Also in this cell line photoreactivation of lethal damage was efficient only if performed immediately after

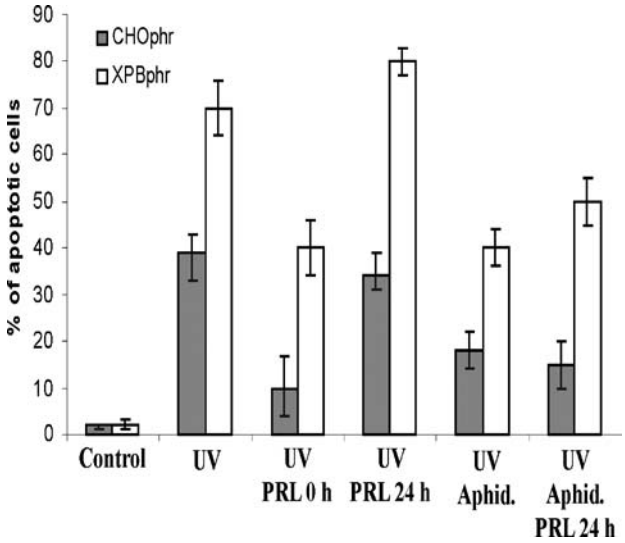

Fig. 4 UV-induced apoptosis is prevented by photoreactivation and DNA synthesis inhibition with aphidicolin. CHOphr (gray bars) and CHO-XPBphr (open bars) cells were irradiated (40 J/m^2 and 20 J/m^2, respectively), kept in the dark or submitted to photoreactivating light (PRL) conditions immediately (0 h) or after 24 h, as shown. Samples cultivated with aphidicolin (0.1 μg/ml) were kept with the drug for the entire period after UV-irradiation. The nuclei were isolated for apoptosis analysis 48 h after UV exposure. Apoptosis was measured as the percentage of sub-G_1 population, as described in Materials and methods

UV irradiation (Fig. 4), not contributing to prevent apoptosis when performed 24 h later. However, when these cells had their replication inhibited by aphidicolin during the post-UV incubation period, a considerable reduction of apoptosis was also observed, independently of lesion removal by photorepair.

Taken together, these results confirm that, although the presence of UV photoproducts acts as an initial signal to UV-induced apoptosis, replication of damaged DNA is necessary to trigger this death pathway, regardless of the cell ability to perform DNA repair.

Morphological analysis of UV-induced apoptosis

To further confirm the role of damaged DNA synthesis in UV-induced apoptosis, CHO-9 and 27-1 cells were UV-irradiated (40 and 20 J/m^2, respectively) and treated with aphidicolin (0.1 μg/ml), followed by determination of apoptosis using vital staining and fluorescence microscopy, a method that discriminates between live, necrotic or apoptotic cells. Apoptotic cells are identified by their highly condensed nuclei, which are positive for both acridine orange and propidium iodide, therefore staining red. Live cells are only positive for acridine orange, therefore staining green. The results are shown in Fig. 5. Basically, no necrotic cells were observed in these experiments. Confirming the data with flow cytometry, the frequency of apoptosis was reduced in cells incubated in the presence of aphidicolin after UV irradiation, in both cell lines employed. These results indicate that inhibition of DNA synthesis is not only protecting the cell from the induction of nuclear DNA fragmentation, but it is also avoiding membrane permeabilization and the consequent cell condensation, another classical end-point hallmark of apoptosis.

Discussion

Although it is widely accepted that UV-C irradiation triggers the apoptotic pathway in mammalian cells, the molecular mechanisms behind this phenomenon are not completely understood. Several lines of evidence point to the presence of photolesions on the double-helix as a starting point for this event. For instance, NER-deficient cells are hypersensitive to the deleterious effects of UV light, namely due to the increased apoptosis induced by this physical agent. Photoreactivation of CPDs and 6–4 PPs in xeroderma pigmentosum cells also leads to prevention of apoptosis, demonstrating that both lesions are initial signals to the cell death program in these cells [22, 23]. Moreover, the removal of CPDs by photoreactivation reverts most of the lethal effects of UV light, when cells are illuminated immediately after irradiation [8, 9, 22]. In the present work, we used the DNA synthesis inhibitor aphidicolin to investigate the role played by the replication of photolesions in the induction of apoptosis.

Upon cell synchronization, we were unable to detect differences in the apoptotic levels of cells irradiated at moments when most of the population was in G_1, S or G_2 phases (Table 1). In these experiments, independently of the cell cycle phase in which cells were irradiated, they have achieved the G_1/S phase border during the 48 h post-UV incubation period, as cells were kept without aphidicolin. In contrast, when cells were treated with aphidicolin during the post-UV incubation period, the frequency of apoptotic cells was greatly reduced, when compared to proliferating cells (Figs. 3–5). This probably reflects the importance of the encounter of the replicative machinery with a photolesion in the signaling process for the induction of apoptosis by UV light. The prevention of apoptosis observed in cells that had their replication inhibited clearly indicates that replication of UV-damaged DNA triggers critical events required for the commitment to the apoptotic process. Apoptosis induced by UV is often linked to unrepaired DNA base damage that signals downstream stress pathways, mainly stalled transcription complexes and/or a loss of transcription efficiency (for a recent review see [24]). In this sense, it was necessary to verify that treatment with aphidicolin had no influence on RNA transcription (Fig. 1(E)). Moreover, inhibition of DNA synthesis also prevented apoptosis in NER-deficient XPB cells, which are unable to remove lesions from the genome. UV induced

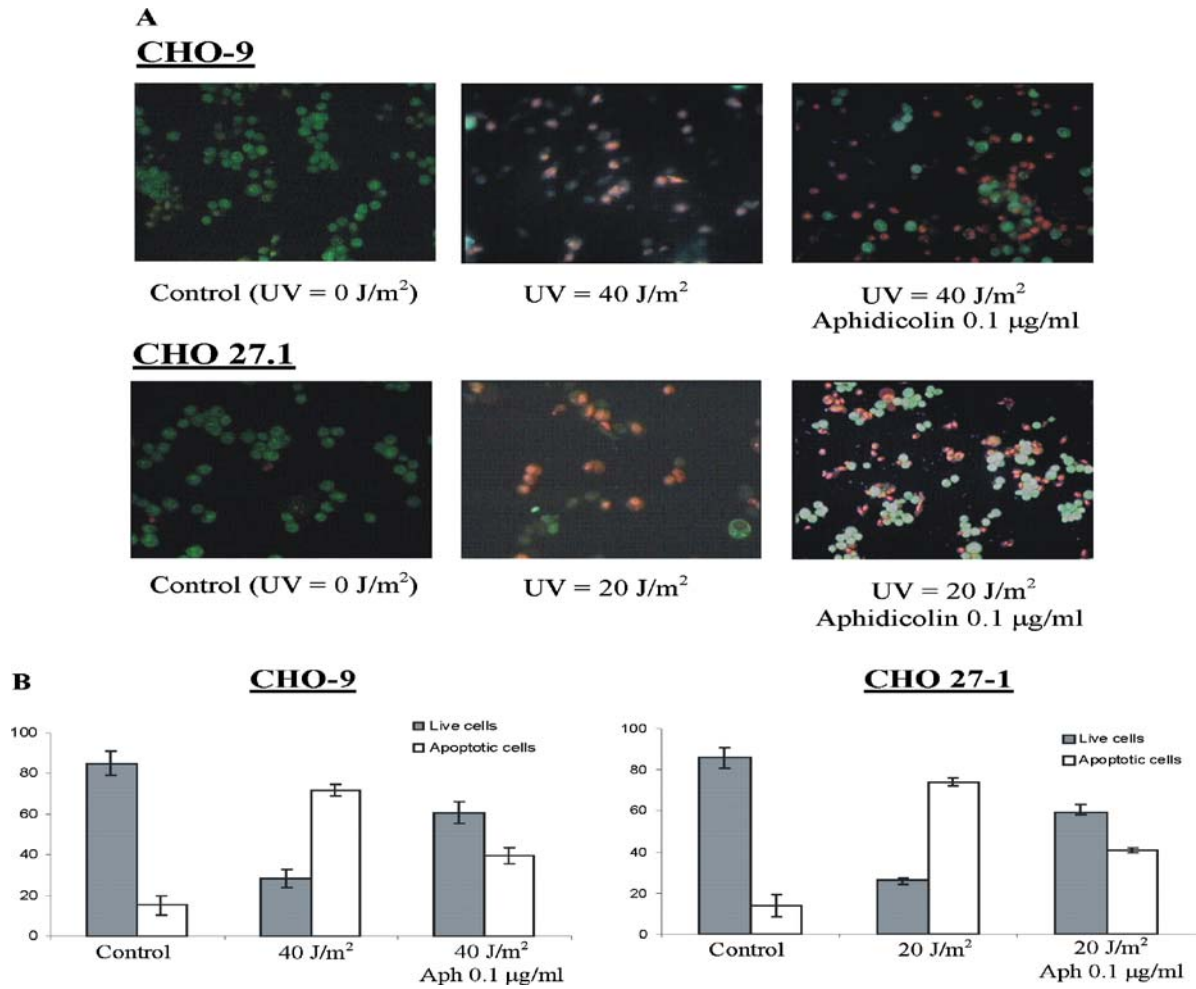

Fig. 5 Morphological analysis of apoptosis in CHO-9 and 27.1 cells treated with aphidicolin. (A) Morphological aspect of live (green) and apoptotic (orange) cells 48 h after UV exposure in the different treatments (as indicated). (B) 1000 to 1500 cells were counted for each condition represented in the graph: gray bars correspond to living cells and open bars correspond to apoptotic cells. The cells were analyzed under a fluorescence microscope (Leica DM LB) using a fluorescein filter and a 40X objective

DNA lesions are known inhibitors of RNA polymerase II [25], and there is a clear association on the transcription blockage after UV irradiation and apoptosis induction. This is clear with the high levels of apoptosis in cells which are defective in the removal of lesions in transcribed genes (TCR), such as CS and XPA, and low levels of apoptosis in cells defective in GGR only, such as XPC cell lines [26]. Similarly, we reported that photorepair reduces UV-induced apoptosis, as well as recovers RNA transcription in CHO cells [9]. On the other hand, the data presented here indicate that DNA synthesis through a damaged template is also a requirement for this type of cellular suicide mechanism.

Working with human primary DNA-repair-deficient fibroblasts a decrease of UV-induced apoptosis is observed in XPC cells maintained in confluence [27], which is also in agreement with a possible role of DNA replication of damaged templates in the commitment to cell death by apoptosis. Moreover, the induction of apoptosis in primary human cells was shown to require S-phase progression after UV irradiation [17]. Therefore, although active transcription coupled repair efficiently prevents apoptosis induction, the replication of damaged DNA may work as an additional signal to the apoptotic response. To reconcile these informations, it has been proposed (Ljungman and Lane [24]) that when transcription occurs during the S-phase, the presence of DNA damage may increase the possibility of collision of the RNA and DNA polymerases. These collisions can lead to the breakdown of replication forks, a potential signal triggering apoptosis (see below).

The results with XPB-mutated cells exclude the possibility that replication inhibition prevents cellular suicide by a passive process, simply granting more time for the repair machinery to perform the excision of damaged sites. In fact, these observations elicit a more exciting and complex mechanism, where the encounter of the replication machinery with photoproducts may be acting as an active signal during

UV-induced apoptosis. In the experiments presented here, it was not possible to discriminate which replication-related event is involved in apoptosis signaling: (i) the stalling of the replication complex by a helix-distorting lesion or (ii) the bypass of these UV-induced photoproducts. In agreement to the first hypothesis, Dunkern and Kaina [18], also working with CHO cells, pointed out that during the replication of photolesions these may be converted into double-strand breaks (DSBs). Recently, Halicka et al. [28] demonstrated that during UV-induced apoptosis there is a great induction of histone H2AX phosphorylation (γH2AX). Interestingly, this phosphorylation occurred mainly in S phase of irradiated cells and correlates with the induction of DSBs in these samples. Moreover, replication inhibition by aphidicolin was able to prevent the appearance of γH2AX in S-phase cells. Since other histones have already been shown to participate in the mechanisms of signal transmission from the nucleus to the mitochondria, after DNA-damage by ionizing radiation [29], the phosphorylation of H2AX might also work as a late signal released from the nucleus after DNA damage, capable of triggering apoptosis. The resulting scenario would then be: (1) formation of UV photoproducts on the double-helix; (2) stalling of the DNA replication machinery at sites containing the unrepaired lesion; (3) generation of DSBs by these stalled replication forks (collision with transcription machinery could increase DSBs at this point); and (4) phosphorylation of H2AX acting as a signal to the mitochondrial death pathway. Experiments to confirm this hypothesis are already in progress.

Hammond et al. [30] recently published a comparison between hypoxia, hydroxyurea and aphidicolin-induced DNA synthesis arrest. The authors claim that cells treated with high doses of aphidicolin present, simultaneously with rapid replication arrest, formation of DNA strand breaks. This phenomenon might explain the cytotoxic effects observed with aphidicolin concentrations higher than 1 μg/ml (not depicted here). However, the concentration used in most of this work had no adverse effects on the cells (Fig. 3), which excludes the possibility that the drug treatment induces the formation of double strand breaks by itself.

Conclusion

The present report demonstrates that, despite the fact that cell cycle phase is not a preponderant factor in the induction of apoptosis by UV light, progression through the DNA replicative S phase can represent a crucial step of the process. The observation of this phenomenon also in cells that are unable to remove UV photoproducts provide evidence that this is not just a matter of granting more time to the DNA repair machinery to act. The mechanisms underlying this phenomenon remain partially unknown, and new approaches will be necessary to clarify the processes which translate photolesions in the double-helix into suicidal responses of the cell.

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