

Photorepair Prevents Ultraviolet-induced Apoptosis in Human Cells Expressing the Marsupial Photolyase Gene¹

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ABSTRACT

Photolyase absorbs blue light and employs the energy to remove UV-induced DNA damage, cyclobutane pyrimidine dimers, or pyrimidine pyrimidone (6–4) lesions. These enzymes have been found in many living organisms ranging from bacteria to aplacental mammals, but their photoreactivation effect, such as survival increase of UV-irradiated cells by light-illumination, has not been identified in placental mammals, including humans. Therefore, we introduced a photolyase gene derived from the marsupial rat kangaroo, *Potorous tridactylus*, into HeLa cells and established the first human cell line capable of photorepairing UV-induced pyrimidine dimers. Several clones were found to increase cell survival after UV irradiation when illuminated by fluorescent light. The induction of apoptosis by UV irradiation was investigated in these photoreactivation-proficient cells. Several typical features of the programmed cell death, such as internucleosomal DNA degradation, presence of subdiploid cells, loss of membrane integrity, and chromosomal condensation, were found to be induced by UV in the HeLa cells, but they can be reduced by photorepair. This implicates that cyclobutane pyrimidine dimers cause UV-induced apoptosis in human cells.

INTRODUCTION

Cells from multicellular organisms have the capacity to trigger an autonomous cell death program during embryonic development, immune response, and tissue homeostasis and after induction of DNA damage. This programmed cell death is genetically controlled and leads to particular characteristic changes termed apoptosis (1, 2). Deregulation in apoptosis can either result in abnormal cell growth, such as that seen in some forms of cancer and autoimmune diseases, or death, as in cases of neurodegenerative disease. Some features of apoptosis in mammalian cells include caspase activation, nuclear fragmentation, chromatin and cytoplasm condensation, DNA fragmentation, cellular membrane convolution generating apoptotic bodies, and externalization of phosphatidylserine residues (3–5).

Apoptosis can be induced by UV light (UV, 254 nm) irradiation, and this is probably related to the blockage action of DNA lesions in RNA polymerase II-transcribed genes (6, 7). These initial events signal for a series of changes in the cell that may lead to death. After UV irradiation, there is an accumulation of p53 protein, which seems to be a key mediator of the apoptosis process (8). Cells that are deficient in p53 have decreased levels of apoptosis by UV (9). Cells that have accumulated p53 may simply stop the cell cycle to allow time for DNA repair or start the cell death program, with the classical end points of apoptosis (9, 10).

One of the initial signals for apoptosis in UV-irradiated cells seems to

be the appearance of CPDs³ because the elimination of these lesions by photoreactivation in marsupial (11, 12) and fish (13) cells interferes with the induction of apoptosis. Photoreactivation is a known mechanism of DNA repair performed by photolyases, which remove specifically UV-induced lesions, such as CPD or (6–4) PP. These enzymes contain chromophores capable of capturing photons of blue light and a mechanism for converting this electronic excitation in CPD and (6–4) PP removal (14). Although (6–4) PP photolyases were discovered only recently in a few organisms (15), the CPD photolyase has been found in prokaryotes, lower and higher eukaryotes, but their existence in placental mammals is contested. Many research groups have found evidence for the lack of DNA photoreactivation in human cells (16, 17). The photolyase/blue light receptor genes have been found in mouse and human cells (18); however, their function may be related to the maintenance of circadian rhythms (19–21).

In the perspective of understanding the relationship between apoptosis and DNA damage repair, this work describes human HeLa cell clones expressing the marsupial *phr* gene. The data demonstrate that the product of this marsupial gene works independently in human cells, without the need of any cofactor. Photoreactivation immediately after UV irradiation increased cell survival in cells expressing the *phr* gene by preventing the initiation of the apoptotic program. Taken together, the data indicate that CPDs are the main initial signal for UV-induced apoptosis, which can be prevented if the damage is promptly repaired.

MATERIALS AND METHODS

Cell Culture. HeLa cell line is derived from the cervical adenocarcinoma of a 31-year-old Negro female (ATCC CCL2). HeLa cells were routinely grown in DMEM (Life Technologies, Inc.) supplemented with 10% FCS and antibiotics, at 37°C in a humidified 5% CO₂ atmosphere.

Cell Transfection. The plasmids used for cotransfection were pCY4B, which has the photolyase gene of *Potorous tridactylus* (rat kangaroo) under the control of a chicken β -actin promoter, and the pSAJ3, which has the hygromycin resistance gene (22). PCY4B was a generous gift from Dr. Junnichi Miyazaki (Osaka University, Japan) to Dr. Akira Yasui. After the standard transfection methodology (23), 5 μ g of DNA were added to DMEM medium with lipofectin (Life Technologies, Inc.), 1 mg/ml, without serum and antibiotics. Two days later, 200 μ g/ml hygromycin B (Life Technologies, Inc.) were added to the medium for clone selection.

Cell Survival. Approximately 1500 cells were plated in 60-mm Petri dishes 14–16 h before the UV irradiation. This procedure is necessary for cell adhesion and to assure the irradiation of a majority of isolated cells. Cells were washed twice with prewarmed PBS and irradiated with a low-pressure germicidal lamp (254 nm). Photoreactivation was performed for 2 h in PBS, except when indicated otherwise. Single cell layers were illuminated 10 cm over fluorescent lights (two daylight lamps, Philips 15 W; emission, 400–700 nm). The cells were kept at 37°C. After treatment, cells were maintained in complete medium for 15–25 days and then fixed with 10% formaldehyde and stained with 1% violet crystal. Colonies with the minimal number of 15 cells

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³ The abbreviations used are: CPD, cyclobutane pyrimidine dimer; (6–4) PP, (6–4) photoproduct; *phr*, photolyase gene; ESS, endonuclease-sensitive site; PI, propidium iodide; AO, acridine orange; EB, ethidium bromide; PRL, photoreactivating light.

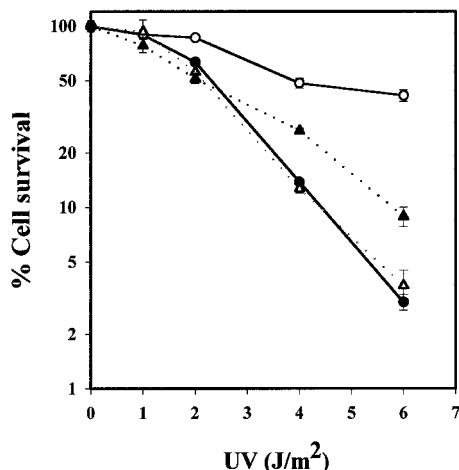


Fig. 1. Photoreactivation in human cells. Low density cultures of HeLa parental cells (▲, △) and HeLa-PHR2 cells (●, ○) were UV-irradiated with the indicated doses and then maintained in the dark (▲, ●) or under fluorescent light illumination (△, ○). Each experiment was performed in triplicate.

were scored. The survival values were obtained as the ratio of the number of colonies from irradiated cells:nonirradiated cells.

DNA Extraction. Cells were plated in semiconfluence (~10⁶ cells/plate), and 48 h after the UV treatment, cells were harvested and genomic DNA was extracted according to the method described by Tilly and Hsueh (24). Briefly, pelleted cells were resuspended in 300 μl of buffer A [0.1 M NaCl, 10 mM EDTA, 0.3 M Tris-HCl, 0.2 M sucrose (pH 8.0)] and incubated at 65°C for 30 min in the presence of 18 μl of 10% SDS. Then, 50 μl of 8 M potassium acetate were added, and samples were incubated on ice for 60 min before centrifugation (7500 rpm; 10 min). The supernatant was then extracted with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) followed by chloroform:isoamyl alcohol (24:1). Finally, the aqueous phase was ethanol-precipitated. After 1 h of RNase treatment (500 μg/ml), the samples were submitted to electrophoresis in a 2% agarose gel with EB (1 μg/ml) for 15 h at 10V.

Measurement of UV ESS. Cells were grown in complete medium containing [³H]methyl-thymidine (0.5 μCi/ml) for 48 h. Nuclei were prepared with 0.5% Triton X-100, 0.1 M NaCl, and 10 mM EDTA, washed twice with PBS, and incubated in NET buffer (100 mM NaCl, 10 mM Tris-HCl, and 10 mM EDTA; Ref. 25) with and without T4-endonuclease V for 30 min at 37°C. Molecular weights of untreated and treated DNA were determined by alkaline sucrose gradient sedimentation, as described before (26). From these values, the number of ESSs, expressed per 10⁷ daltons, which corresponds to the number of CPD, was calculated.

Fluorescence Microscopy. Cells were harvested 24 h after UV treatment, centrifuged, and resuspended in 20 μl of PBS. Two μl of AO/EB solution (one part of 100 μg/ml AO in PBS, one part of 100 μg/ml EB in PBS; Ref. 27) was added. The cells were analyzed in a fluorescence microscope (Leica DM LB) using a fluorescein filter and a 60× objective.

Flow Cytometry Analysis of Individual Nuclei. Cells were trypsinized 24 h after the UV treatment and centrifuged at 1500 rpm for 5 min. Pelleted cells (0.5 × 10⁶ to 1.0 × 10⁶ 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 (27). Then, samples were transferred to microtubes, and PI fluorescence was read by flow cytometry (FACScalibur, Becton Dickinson). Results were obtained as the percentage of subdiploid nuclei, which represent the apoptotic cells.

RESULTS

Selection of Photoreactivation-positive Human Clones. A plasmid containing the *phr* gene from marsupial cells, under the control of the chicken β-actin promoter, was cotransfected with a plasmid conferring hygromycin resistance into HeLa cells. Forty hygromycin-resistant clones were screened for UV survival followed by photoreactivation by exposure to fluorescent light. After a first screening, the clone named HeLa-PHR2 showed a better survival increase after photoreactivation and was chosen for further analysis.

An UV survival curve for HeLa-PHR2 cells is shown in Fig. 1. There is a clear recovery of the cell ability to form colonies when exposed to photoreactivation light, a fact not observed in control cells. Therefore, the data demonstrate that the CPD-specific photolyase from the marsupial is active in the human cell environment and that the CPD removal by the marsupial photolyase can protect cells, at least partially, from UV light. The rate of photoreactivation depended on the duration of light exposure, achieving maximal levels in 1 h (Fig. 2A). This is in agreement with that observed for marsupial cells in similar condition (11). The efficiency of photoreactivation decreased progressively with time and practically disappeared when cells were exposed to fluorescent light 8 h after the UV treatment (Fig. 2B). Thus, irreversible events must have occurred during this period as a consequence of DNA damage, committing the cells to death.

CPD Removal by Photorepair in HeLa-PHR2 Cells. The number of CPDs after UV irradiation was determined by employing T4-endonuclease and alkaline sucrose gradients. This enzyme recognizes and nicks the DNA at the CPD site, and the reduction of the DNA molecular weight is detected by alkaline sucrose gradients. Thus, the number of ESSs corresponds to the number of CPDs in the cell genome. The data are shown in Table 1. In UV-irradiated HeLa-PHR2 cells, there was a 32% reduction of ESSs in cells exposed to light when compared to those maintained in the dark. This number corresponds to CPD removal in these cells caused by photoreactivation. No change in the number of ESSs was observed in UV-irradiated HeLa wild-type cells when they were maintained in the dark or light conditions.

Photorepair of UV-induced DNA Fragmentation by Apoptosis in HeLa-PHR2 Cells. DNA damage by UV was shown to induce a delayed apoptosis in mammalian cells when compared to immediate

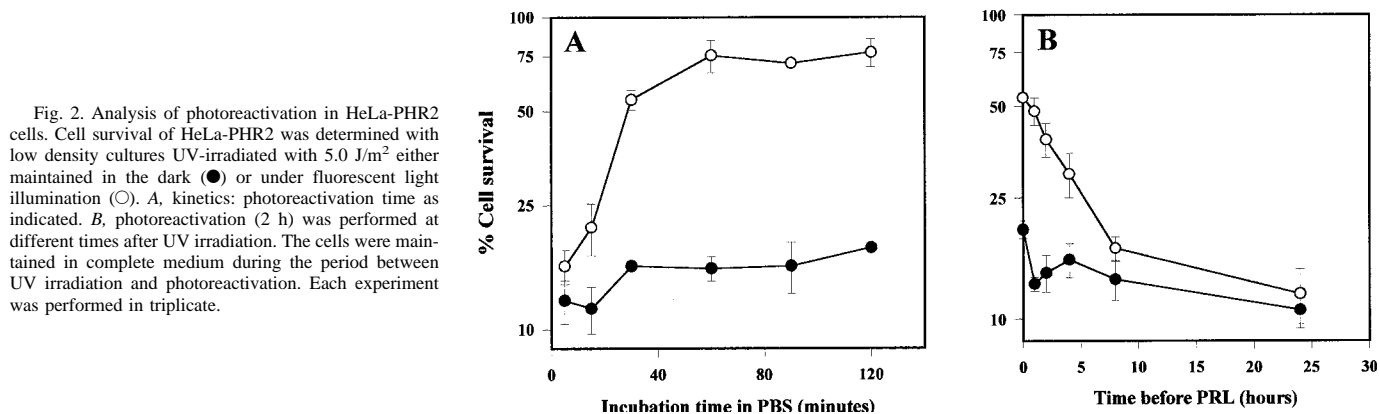


Fig. 2. Analysis of photoreactivation in HeLa-PHR2 cells. Cell survival of HeLa-PHR2 was determined with low density cultures UV-irradiated with 5.0 J/m² either maintained in the dark (●) or under fluorescent light illumination (○). A, kinetics: photoreactivation time as indicated. B, photoreactivation (2 h) was performed at different times after UV irradiation. The cells were maintained in complete medium during the period between UV irradiation and photoreactivation. Each experiment was performed in triplicate.

Table 1 Photorepair of ESSs in HeLa and HeLa-PHR2 cells^a

UV (J/m ²)	Treatment	ESS	
		HeLa cells	HeLa-PHR2 cells
0		0.010 ± 0.007	0.012 ± 0.008
5.0	2 h dark	0.149 ± 0.020	0.138 ± 0.016 ^b
5.0	2 h PRL	0.143 ± 0.006	0.094 ± 0.020 ^b

^a The ESSs were determined in nuclei by sedimentation in alkaline sucrose gradients, as described in "Material and Methods." Values are mean ± SD.

^b These values are statistically different. $P < 0.1$, Student *t* test.

apoptosis induced by UVA (320–400 nm) (28). The induction of DNA internucleosomal cleavage, a hallmark of apoptosis, in UV-irradiated HeLa cells submitted or not to photoreactivation, was investigated by agarose gel electrophoresis. Representative results of these experiments are shown in Fig. 3. There is a clear induction of the DNA ladder, the result of DNA cleavage, in UV-irradiated HeLa cells. Exposure of the parental HeLa cells to photoreactivation light does not change the intensity of the DNA ladder (Fig. 3A). However, for HeLa PHR2 cells, there is a clear reduction of the internucleosomal DNA degradation (Fig. 3B), indicating that photorepair can prevent the UV-induced apoptosis in this cell line.

In marsupial cells, the ability to reduce the level of apoptosis by light decreases rapidly after UV, such that there is no effect if photoreactivation is performed only 24 h after irradiation (11). HeLa cells present a faster apoptotic response as far as DNA ladder formation is analyzed (a clear DNA ladder is observed 24–48 h after UV, compared to 96 h necessary for marsupial cells), so that one would expect that the commitment to apoptosis would also occur earlier than in the human cells. In Fig. 4, it is observed that PRL effect on apoptosis decreases with time in that it is minimal at 8 h after UV irradiation. Later, 24 h after UV, no significant photoreactivation of the DNA ladder is detected. Thus, the signals for triggering apoptosis are probably activated progressively in the cell population, and during this short period of time (8 h), most of the cells have exceeded the commitment point for cell death.

Flow cytometry analysis using PI staining was performed to quantify apoptotic cells after UV irradiation. By using this technique, apoptotic nuclei are identified in the subdiploid region (sub-G₁) of the cell cycle histograms (27). In Fig. 5, it is shown that UV-irradiated HeLa and HeLa-PHR2 cells, maintained in culture for 24 h, have their nuclei concentrated in the subdiploid region of the cell cycle histogram (M1 region). Photoreactivation of HeLa-PHR2 cells immediately after UV reduced the relative number of sub-G₁ nuclei after UV irradiation, which was not observed in the parental cell line.

Photorepair of UV-induced Apoptotic Morphology. Morphological assessment of cell death was investigated using the AO/EB staining for fluorescence microscopy. AO is a vital dye that will stain both live and dead cells. EB will stain only cells that have lost membrane integrity. Live cells will appear uniformly green, whereas late apoptotic cells will stain orange with dots in the nuclei as a consequence of chromatin condensation. Necrotic cells will also stain

orange, but these have a nuclear morphology resembling that of viable cells, with no condensed chromatin (27). These different morphological features were analyzed, and the frequency of apoptotic cells was determined. Basically, no necrotic cells were observed in UV-irradiated cells. When cells were maintained in the dark, the relative number of apoptotic cells increased with time after UV irradiation (10 J/m²). There was a clear reduction of apoptotic cells when they were photoreactivated immediately after UV (Fig. 6). In HeLa cells that do not express the marsupial photolyase, there was no reduction of apoptotic cells after exposure to PRL (data not shown).

DISCUSSION

The presence of photolyase activity in human cells is a matter of controversy, with several evidences for the lack of this enzyme in placental mammals (17, 29). The results presented in this paper confirm the inability of HeLa parental cells to recover from UV irradiation by light illumination. By employing a heterologous gene, the marsupial photolyase gene, a human cell line derived from HeLa cells, was obtained that is able to partially remove CPDs and increase resistance to UV irradiation in the presence of light. This increased survival is dependent on the time of light exposure, reaching a maximum in 1 h of photoreactivation. Moreover, if photoreactivation is not performed immediately after UV irradiation, the cell survival is compromised, indicating that CPD lesions are being processed and irreversible events are committing the cells to death. Examples of successful trials with microinjection of photolyase enzyme from *Escherichia coli* and yeast in mammalian cells were already described, indicating the viability of such heterologous systems (30, 31). Our system benefits from the close proximity between marsupial and placental animals and demonstrates that the activity of the marsupial photolyase does not depend on factors intrinsic of the marsupial cells because it works similarly in human cells.

Recent data indicate that there are at least two types of photolyases, one specific for CPD (CPD photolyase) and another specific for pyrimidine (6–4) PP photoproducts [(6–4) photolyase; Refs. 15 and 32]. Human genes with high sequence homology to the photolyase/blue light photoreceptor family have been reported (19, 20). Despite several similarities with photolyases already studied, *e.g.*, FAD and pterin cofactor, evidence for photolyase activity to repair CPD is lacking. The same lack of activity was verified with (6–4) photolyases (33). These genes seem to function as blue-light receptors in humans, suggesting a divergence of photolyase genes early in evolution (15). More recently, work with mice mutated at these photolyase-homologous genes has indicated that these proteins are essential for the maintenance of free-running periodicity of locomotion activity (18). Therefore, the human nucleotide excision repair seems to be the main mechanism responsible for removing UV damages, such as CPD and (6–4) PP (34–36). Interestingly, the genes involved in nucleotide excision repair are also involved in other cellular processes, such as transcription and apoptosis (37–39).

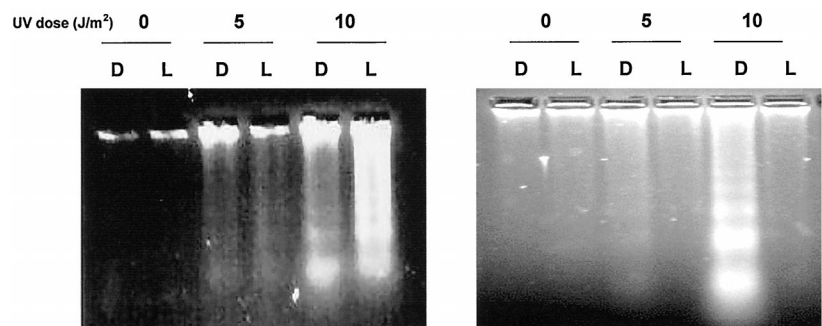


Fig. 3. Photorepair of apoptosis in HeLa-PHR2 cells. DNA analysis was performed in HeLa parental (left) and HeLa-PHR2 cells (right) after UV irradiation with the indicated doses. Cells were maintained in the dark (D) or under light exposure (L) and harvested for DNA extraction 48 h after UV irradiation.

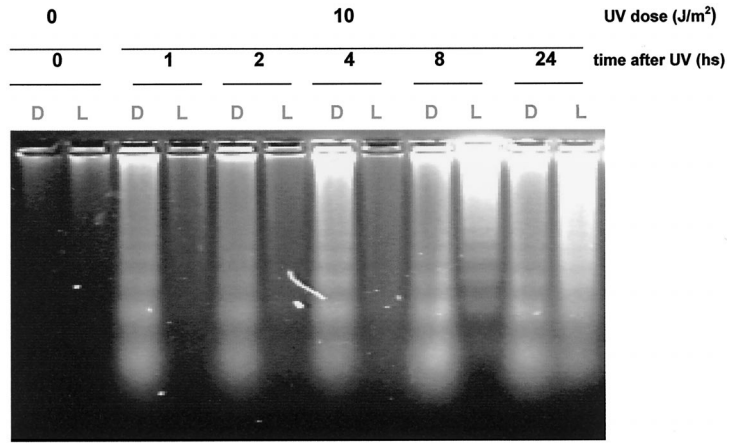


Fig. 4. Decreased photorepair of apoptosis in HeLa-PHR2 cells with time after UV irradiation. DNA degradation by apoptosis was examined in cells exposed to PRL (L) or maintained in the dark (D) for 2 h at different times after UV irradiation (10 J/m²). Cells were harvested, and DNA analysis was performed by agarose electrophoresis 48 h after UV irradiation.

The HeLa-PHR2 cells provide an efficient system to understand how human cells behave after UV irradiation in terms of events necessary for apoptosis. In this work, it is shown that HeLa cells may enter in the apoptosis process of cell death because a DNA ladder, generated by internucleosomal DNA fragmentation, can be observed 48 h after UV irradiation. The DNA ladder is highly reduced in cells that were submitted to photoreactivation and morphological changes typical of apoptotic cell death attributable to loss of membrane integrity, chromatin condensation, and nuclear fragmentation are also photorepaired in UV-irradiated HeLa-PHR2 cells. These data confirm recent observations in marsupial (11, 12) and fish (13), indicating that in human cells, photoreactivatable lesions are also the first signal to trigger apoptosis. These lesions are most likely to be CPD because the photolyase gene used in these experiments is involved specifically in the removal of these lesions (29). Therefore, the data implicate CPD as primarily responsible for UV-induced apoptosis.

Recently, reduction of UVB-induced apoptosis by photorepair in human cells was also reported (40). In those experiments, the photolyase enzyme from the cyanobacterium *Anacystis nidulans* was transiently introduced in HeLa cells by liposomes (called photosomes), and exposure to PRL reduces both apoptosis after UVB irradiation and the induced cleavage of PARP and caspase-3. Although the photolyase used was from a prokaryote, our conclusions are consistent

with Kulms *et al.* (40), indicating that nuclear signals (triggered by CPD) are responsible for most of the apoptotic response.

Early events of the apoptotic pathway must be triggered by the mechanisms that monitor CPD in the cell genome, so that their removal by photoreactivation prevents the initiation of the apoptosis program. The preferential repair of active genes is associated with the RNA transcription by polymerase II, which is stalled by DNA damage (37). The interruption of RNA polymerase II by DNA lesions has been proposed to be the main alarm signal that triggers a pathway leading to apoptosis (6, 7). Studies with human cells that have deficiencies in preferential DNA repair (41) have shown that there is a strong correlation between apoptosis and the inhibition of RNA transcription. This blockage of transcription would also be responsible for the p53 accumulation (38, 41), a protein that is certainly implicated in the early events of apoptosis. In this work, it is demonstrated that CPDs are the main kind of DNA damage that would obstruct RNA polymerase II leading to the cascade of events that result in cell death.

Another possible mechanism for the induction of apoptosis by UV can involve the preferential DNA repair *per se*. This can generate DNA strand breaks that are recognized by nick sensors that would start the whole apoptotic process. This is the case of PARP and p53 protein because both can bind to and are activated by DNA single

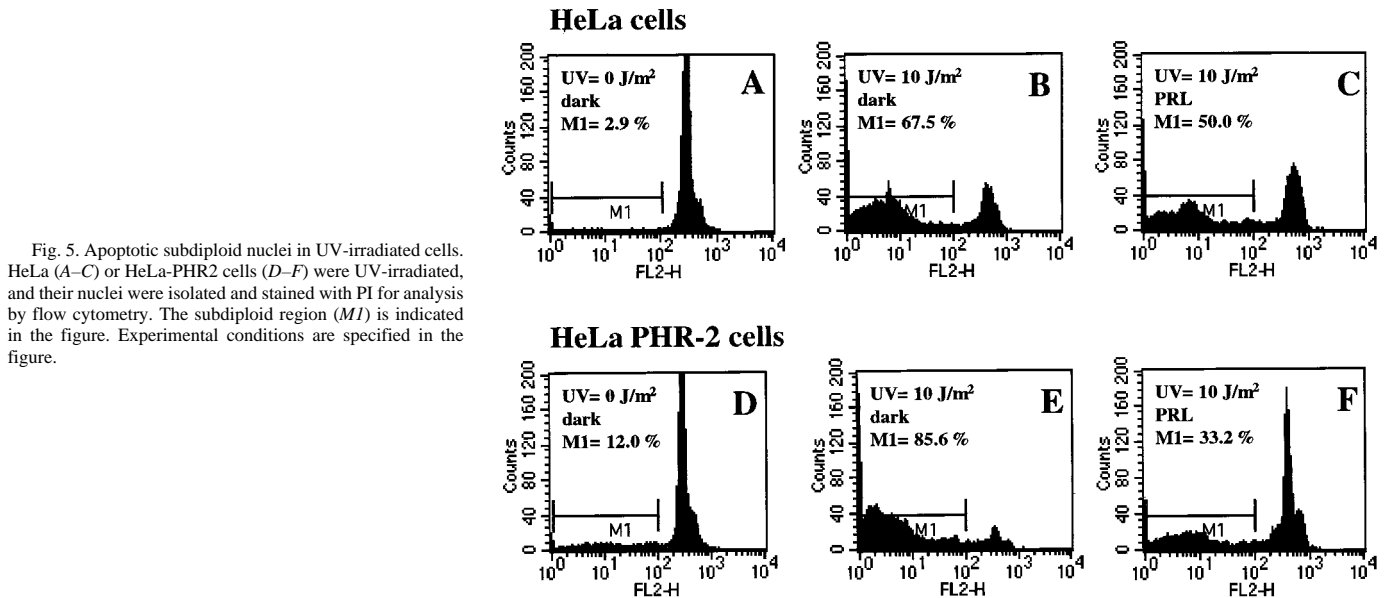
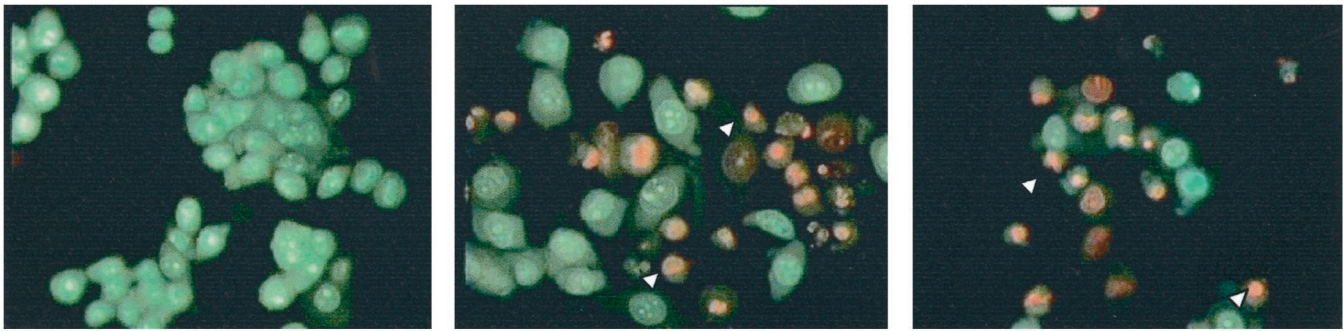


Fig. 5. Apoptotic subdiploid nuclei in UV-irradiated cells. HeLa (A–C) or HeLa-PHR2 cells (D–F) were UV-irradiated, and their nuclei were isolated and stained with PI for analysis by flow cytometry. The subdiploid region (M1) is indicated in the figure. Experimental conditions are specified in the figure.

A

HeLa cells

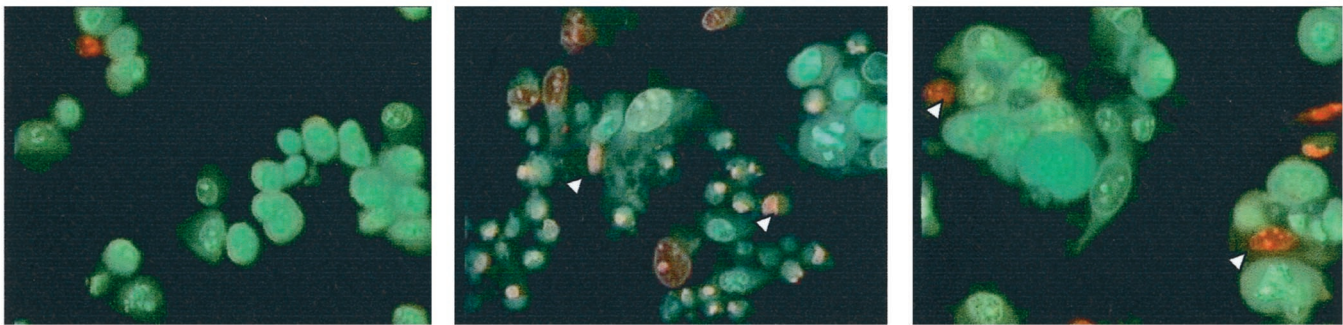


Control (UV = 0 J/m²)

UV = 10 J/m² dark

UV = 10 J/m² PRL

HeLa PHR-2 cells



Control (UV = 0 J/m²)

UV = 10 J/m² dark

UV = 10 J/m² PRL

B

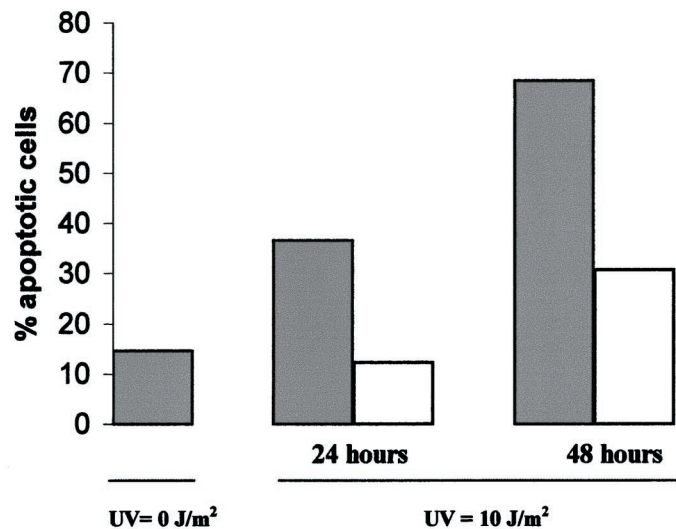


Fig. 6. Apoptotic morphological changes are reduced after photoreactivation in HeLa-PHR2 cells. *A*, morphological aspect of live (green) and apoptotic cells (orange; arrows) 48 h after the respective treatments. *B*, 1000 to 1500 cells were counted for each condition represented in the graphic, where gray bars correspond to samples maintained in the dark and open bars correspond to those exposed to PRL.

strand breaks, albeit in different manners (42, 43). Moreover, it has been shown that p53 binds to XPB and XPD proteins, two components of the TFIIH complex, which participates directly in RNA transcription and DNA repair, and they may be components of the apoptotic pathway (44). Recent studies reveal the possibility of PARP to be directly involved in p53 regulation, binding in some of its

specific domains and altering its DNA-binding function (45). Therefore, RNA polymerase blockage by CPD could signal directly for apoptotic pathway, but also it could call for preferential DNA repair action, promoting DNA strand breaks that would act as sensors for other alarm molecules in the cell, such as p53 and/or PARP. These two models fit in with the results presented here, which indicate that

elimination of CPD by photolyase can halt the signal responsible for induction of cell death.

It should be pointed out that the HeLa cell line is a cervical adenocarcinoma, transformed by the human papillomavirus. It is known that the papilloma virus E6 protein, in association with an additional cellular factor termed E6AP, can complex with p53 and promote its degradation by the ubiquitination system (46). This phenomenon can impair the p53 functions. One could argue simply that the apoptotic pathway observed in this work is independent of p53. However, the p53 protein levels in the cell lines used may still be enough for the apoptotic responses. Similar observations have been described revealing that p53-induced cell cycle (G_1) arrest can be abrogated after p53 degradation, but apoptosis remains working through a different p53-dependent pathway (47, 48). Moreover, it has been shown that UVB induces apoptotic response in HeLa cells (40), and treatment of HeLa cells with sodium arsenite induces an accumulation of p53 protein, which might be activated in response to DNA damage (49). Thus, the UV-induced apoptotic pathway described in this work can still be dependent on low levels of p53 of HeLa cells, which would be enough for the activation of the other events leading to cell death.

In conclusion, the heterologous activity of the marsupial photolyase in human cells demonstrated that CPDs are among the initial signals for the induction of apoptosis by UV. Human cells have many of the death "effectors" known, although their mechanisms of action and temporal activity are not completely understood. Thus, human photoreactivating cells may represent a powerful tool for these investigations, providing important information for the comprehension of the cascade of events that are initiated in UV-damaged cells.

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