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Induction of Urokinase-Type Plasminogen Activator by UV Light in Human Fetal Fibroblasts Is Mediated through a UV-Induced Secreted Protein

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Plasminogen activator was previously shown to be induced by UV light in human cells with low capacity to repair UV-induced DNA lesions. We now show that in human fetal fibroblasts UV light enhanced the two mRNA species coding for the urokinase-type plasminogen activator (uPA) and the tissue-type plasminogen activator, but immunological analysis revealed exclusively uPA activity. Several independent and complementary experiments indicated that induction of uPA was mediated, apparently entirely, through a UV-induced, secreted protein (UVIS) in the growth medium of irradiated cells. First, elevation of uPA mRNA after irradiation was severely blocked by cycloheximide. Second, replacement of conditioned medium in irradiated cells while the rate of plasminogen activator induction was maximal rapidly and completely stopped any further increase in uPA activity. Third, addition of the same removed conditioned medium to nonirradiated cells mimicked UV light in enhancing the level of uPA activity as well as that of uPA mRNA. Fourth, UVIS activity was completely lost by treating the conditioned medium with trypsin but not with nucleases. Kinetic measurements indicated that the accumulation of UVIS rather than the induction of uPA by UVIS conferred the rate-limiting step in the overall process of uPA induction. Both UV light and UVIS acted synergistically with inhibitors of DNA repair for uPA induction. Based on these results, a model is proposed implicating relaxation of DNA torsional stress of an as yet undefined DNA sequence(s) in the induction of UVIS, which is then responsible for activation of the uPA gene.

UV light generates pyrimidine dimers in DNA, arresting DNA replication and elongation of transcription in the living cell. Procaryotic and eucaryotic cells counteract the cytotoxic effect of DNA damage through errorproof repair and also via pathways leading to mutagenesis and, in mammalian cells, to neoplastic transformation (36, 40, 41, 60). The bacterial response to DNA damage is inducible and consists of the expression of a set of functions collectively called SOS (40). A key regulatory role in the SOS response is played by the RecA protein, which when activated, probably by binding to single-stranded regions in the DNA, confers a specific proteolytic activity to the *lexA* gene product, a repressor of many genes, and to the λ phage repressor (39). Self-cleavage of the repressors leads to enhanced transcription of the repressor-relieved genes, resulting in coordinate expression of the SOS functions. These include repair and mutagenic activities, λ phage activation, and enhanced level of the RecA protein (40).

Several activities phenotypically reminiscent of SOS functions are induced by DNA damage in mammalian cells through mechanisms not yet elucidated. These activities include virus reactivation and mutagenesis (6, 57, 58), induction of virus in transformed cells (32), enhancement of postreplication repair (17, 18), and induction of the protease plasminogen activator (PA) (5, 47, 48) and of DNA ligase II, an enzyme implicated in DNA repair (46).

Recently several additional proteins and also mRNA species were reported to be enhanced within a few hours after exposure of human and mouse cells to UV irradiation. The level of the p53 cellular tumor antigen is increased due to posttranslational stabilization (43), the c-*fos* mRNA is elevated (1), and eight abundant proteins of yet undefined function, as well as their encoding mRNAs, are coordinately enhanced (2, 59). This last response could be mimicked by the secreted protein EPIF (extracellular protein synthesis-inducing factor), itself induced by UV light. In mouse cells sensitive to cadmium, UV light activates a quiescent gene coding for the heavy metal-detoxifying protein metallothionein-I, conferring cadmium resistance (38). Gene activation in this case was suggested to result from UV-induced changes of methylation pattern, requiring DNA replication to be permanently fixed. These examples indicate that in mammalian cells UV light increases the level of proteins by multiple mechanisms underlying early and late responses, possibly operating directly and indirectly at the level of gene structure and transcription as well as posttranscriptionally. At least part of these mechanisms may be critical in UV-induced carcinogenesis.

PA is a protease closely associated with malignancy, as indicated by overproduction of PA by tumors and transformed cells (9, 10, 11, 53, 61), coordinate regulation of tumor growth and PA production (13, 42), enhancement of PA synthesis by tumor promoters and physical and chemical carcinogens (20, 48), and the ability of the enzyme to promote tumor metastasis (52). PAs are specific serine proteases that convert the inactive zymogen plasminogen through a single specific proteolytic cleavage into plasmin, a general protease of trypsinlike specificity (19). Two distinct molecular types of human PAs are known, the urokinase-type PA (uPA) and the tissue-type PA (tPA), coded by two separate genes (50, 55). Plasminogen activation functions in blood clot dissolution and has also been implicated in several physiological processes requiring extracellular proteolysis,

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in which PA synthesis appears to be subjected to hormonal modulation (19, 51, 56, 64, 65).

Induction of PA by UV light was detected in human fibroblasts with reduced capacity to repair UV-induced DNA lesions, but not in repair-proficient cells (5, 47). Such a response was found in cells from patients with xeroderma pigmentosum, a cancer-prone disorder with a genetic deficiency in DNA repair, in which it was directly related to the extent of repair deficiency (47). Induction of PA by UV light also occurred in fetal skin fibroblasts. In these cells PA induction was correlated with development-associated changes in DNA excision repair (5). Based on these results it was suggested that PA induction is triggered by persistent DNA damage not efficiently removed by mechanisms of DNA repair (5, 47).

In the present study we have further investigated the pathway by which UV light increases PA in human fetal fibroblasts.

MATERIALS AND METHODS

Materials. GM-0011 fibroblasts were obtained from the Human Genetic Mutant Cell Repository, Camden, N.J. Dulbecco modified Eagle medium was from Gibco Laboratories, Grand Island N.Y. Fetal calf serum (FCS) was purchased from Biological Industries, Kibbutz Beth Haemek, Maale Hagalil, Israel. Human plasminogen was purified from human plasma as described previously (21). Fibrinogen (Sigma Chemical Co., St. Louis, Mo.) was purified (64) and labeled with ^{125}I as described (63). Standard urokinase was obtained from the World Health Organization International Laboratory for Biological Standards, Holly Hill, Hampstead, London. *P*-Aminobenzamidine (Sigma) was coupled to Sepharose 4B (Pharmacia) as described previously (31). Oligo(dT) cellulose (type 7) was purchased from P-L Biochemicals Inc., Milwaukee, Wis. Nitrocellulose (BA85) was from Schleicher & Schuell, Inc., Keene, N.H. [α-^{32}P]dATP (3,000 Ci/mmol) was from Amersham, England. Bovine serum albumin (BSA), Triton X-100, aphidicolin, 1-β-D-arabinofuranosyl cytosine (ara-C), hydroxyurea (HU), 3-aminobenzamide (3AB), caffeine, glutamine, and sodium pyruvate were from Sigma. Novobiocin was from Boehringer Mannheim GmbH, Federal Republic of Germany. All other reagents were of the purest grade available.

Cell cultures. All experiments were performed with GM-0011 skin fibroblasts derived at 8 weeks of gestation and used between passage 10 and 25 of subcultivation. Cells were grown and maintained in Dulbecco modified Eagle medium supplemented with glutamine (4 mM), sodium pyruvate (1 mM), penicillin (2,000 U/ml), streptomycin (100 μg/ml), and 10% heat-inactivated FCS, in an atmosphere of 5% CO_2. For UV irradiation and treatment with inhibitors of DNA repair, cells were seeded at a density of 2×10^5 to 4×10^5 cells per 50-mm petri dish; for treatment with conditioned medium, cells were seeded at 8×10^4 per 30-mm dish; and for RNA preparation cells were seeded into 150-mm dishes at a density of 1.5×10^6 cells (for nonirradiated cells) or 3.5×10^6 cells (for UV irradiation) to reach the same final cell densities. All treatments were performed with logarithmically growing cells 24 h after plating.

Irradiation of cells. Cells were washed twice with phosphate-buffered saline (PBS) and irradiated in a thin layer of PBS at the optimal dose for PA induction, 15 J/m^2, with a Philips lamp (model GI5 T8) at a fluence rate of 1.9 J/m^2 per s, determined with a UVX Radiometer (Ultraviolet Products, Inc., San Gabriel, Calif.). After irradiation cells were treated with fresh medium, either supplemented with FCS or in the absence of serum as specified, and incubated for the indicated times.

Collection of conditioned medium for treatment of cells. UV-irradiated cells and nonirradiated control cells were incubated in the absence of serum for 60 h. Conditioned medium was collected, centrifuged at 4,000 rpm for 15 min to remove cellular debris, and stored at 4°C (sometimes for several weeks) until assayed for its ability to enhance PA in nonirradiated cells.

Treatment of nonirradiated cells with conditioned medium. Medium from nonirradiated cells was removed, and the cells were washed once with medium and treated for the specified time with conditioned medium collected as described above and supplemented as specified with either 2% FCS or BSA (0.5 mg/ml) pretreated for 2 h at pH 2.5 to inactivate contaminating protease inhibitors (65).

Assay of PA. To determine intra- and extracellular levels of PA activity, cells and conditioned medium were collected at different times after initiation of treatment, as described previously (63), and stored at −20°C until assayed. Conditioned medium containing serum was acid-treated prior to the assay to inactivate serum protease inhibitors (65). PA activity was determined in a fibrinolytic assay as previously described in detail (63). Briefly, the assay was performed in Linbro multiwell plates coated with ^{125}I-labeled fibrinogen and containing purified human plasminogen (20 μg/ml). The ability of plasmin, generated from plasminogen by PA, to release ^{125}I-labeled fibrin degradation products was measured. Results are expressed as international units of uPA per milligram of cellular protein in the culture, for both intracellular and secreted enzyme. Protein was determined by the method of Bradford (7).

Preparation and analysis of RNA. To prepare total RNA, cells were scraped into urea-LiCl (3), and the RNA was extracted twice with phenol-chloroform-isoamyl alcohol and once with isoamyl alcohol, precipitated in ethanol, and dissolved in water. Poly(A)-containing RNA was isolated by two successive oligo(dT)-cellulose chromatography steps (4). The probes to identify and quantitate mRNA coding for uPA or tPA were derived from full-length cDNA clones that we constructed for both human enzymes (manuscript in preparation). The cDNA clones coding for uPA or tPA were derived from the HEp-3 carcinoma and the Bowes melanoma cell line, respectively, which produce exclusively uPA and tPA, respectively, at high levels. The uPA probe consisted of a 1,738-base-pair *Sma*I-*Xmn*I fragment. The tPA probe consisted of the 5′ *Eco*RI fragment of 824 base pairs, containing 108 noncoding nucleotides and 716 coding nucleotides. The probes were labeled by nick translation with [^{32}P]dATP (44) to a specific activity of about 3×10^7 to 6×10^7 cpm/μg of DNA. For Northern hybridization analysis, poly(A)-containing RNA was heated at 60°C for 10 min, electrophoresed (10 μg per lane) on 1.5% agarose–6% formaldehyde gel, and blotted into nitrocellulose filter paper as described previously (44). Filters were baked at 80°C for 30 min and then prehybridized for 3 h at 42°C in 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–10× Denhardt solution–0.1% sodium dodecyl sulfate–0.1% PP_i–150 μg of denatured sonicated salmon sperm DNA per ml. ^{32}P-labeled cDNAs were added to 3×10^6 cpm/ml and hybridized overnight at 42°C. Filters were then washed twice in 0.1% sodium dodecyl sulfate at 71°C, dried at room temperature, and exposed to autoradiography. For dot blot analysis (35) the indicated amounts of total RNA were

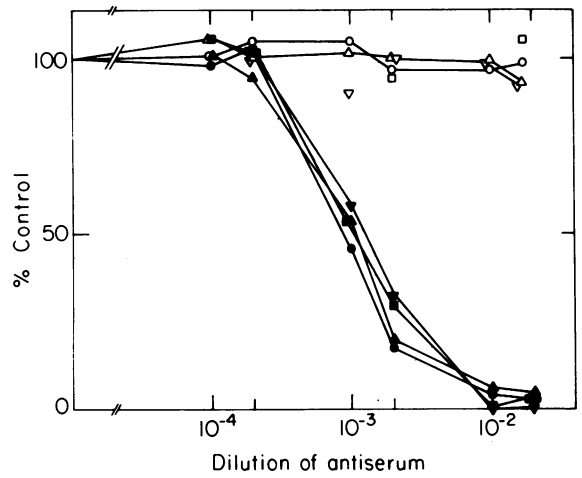

FIG. 1. Immunological analysis of PA activity in irradiated and nonirradiated cells. GM-0011 cells were irradiated as described in Materials and Methods and treated with fresh medium containing 10% FCS. After an incubation period of 60 h, conditioned medium and cellular extract were collected from the irradiated cells and from control nonirradiated cells. Medium and extract samples containing PA activity of 1 to 4 mU of uPA were supplemented with the indicated dilutions of antisera raised against uPA (solid symbols) or Bowes melanoma-derived tPA (open symbols) in a mixture containing PBS, 0.5 or 1% Triton X-100, and 1 mM EDTA and incubated for 30 min at 37°C and then for 1 h at 4°C. At the end of the incubation period the samples were tested for remaining PA activity. Symbols (specific activity in units per milligram of protein shown in parentheses): ●,○, conditioned medium from irradiated cells (15.0); ▲,△, cellular extract from irradiated cells (9.6); ■,□, conditioned medium from control cells (0.13); ▼,▽, cellular extract from control cells (0.05). uPA antiserum completely blocked 4 mU of standard uPA and did not inhibit an equivalent activity of PA derived from Bowes melanoma cells, while the tPA antiserum exhibited an opposite effect (not shown).

dissolved in 50 μl of 6× SSC–7.4% formaldehyde, heated at 65°C for 10 min, and spotted onto a nitrocellulose filter presoaked in 6× SSC for 15 min. The filter was baked, prehybridized and hybridized with the uPA or tPA probe described above, and exposed to autoradiography. For quantitative determination the filters were scanned with a Jr. TLC Plus Densitometer (Helena Laboratories).

RESULTS

UV light enhances uPA and tPA mRNAs but exclusively uPA activity. Exposure of GM-0011 human fetal fibroblasts to UV light was previously shown to enhance the level of PA activity (5). To identify the molecular type of PA enhanced by UV light, we used specific antisera which block the activity of either uPA or tPA (Fig. 1). About 95% of the intra- and extracellular PA activities were quenched by uPA antiserum, while virtually no effect on PA activity was exerted by the tPA antiserum. This result was obtained irrespective of whether the irradiated cells were incubated in the presence or absence of serum. Analysis of the low level of PA activity found in nonirradiated cells revealed the same result. This is consistent also with a previous zymographic analysis of PA activity carried out following electrophoresis in polyacrylamide gels containing casein and plasminogen (5). The absence of active tPA in irradiated GM-0011 cells was somewhat surprising in view of our previous analysis of poly(A)-containing RNA prepared from irradiated and control cells, which indicated that UV light enhanced about 10-fold mRNAs coding for both PA types (49). The analysis was done by Northern hybridization, using synthetic oligonucleotides labeled at the 5′ end with ^{32}P to similar specific activities as specific probes to monitor uPA mRNA and tPA mRNA. We now repeated the same analysis with nick-translated cDNA probes that we constructed for both PA types (Fig. 2). Scanning of the autoradiogram showed that UV light elevated uPA mRNA and tPA mRNA 30- and 25-fold, respectively, essentially confirming the previous result.

Cycloheximide blocks enhancement of PA mRNAs by UV light. Elevation of PA activity after exposure of cells to UV light was slow and reached a maximal intracellular level on day 3 postirradiation (see Fig. 4 and 8). We therefore considered the possibility that it may represent a secondary effect dependent on a primary UV-induced event. We first tested whether enhancement of PA mRNAs after UV irradiation was dependent on protein synthesis by treating the irradiated cells with cycloheximide to inhibit protein synthesis. Total RNA was prepared and subjected to dot blot analysis with cDNA probes coding for uPA and tPA (Fig. 3). It was evident that while cycloheximide had no effect on the low constitutive level of uPA mRNA found in control nonirradiated cells, it inhibited by about 75% the elevation of uPA mRNA above the control level and completely blocked the increase of tPA mRNA measured 60 h postirradiation. This result suggests the involvement of at least one UV-induced protein intermediate in the induction of both mRNA species.

Effect of conditioned medium from irradiated cells on PA induction. We examined whether the putative protein intermediate involved in the induction of PA by UV light was

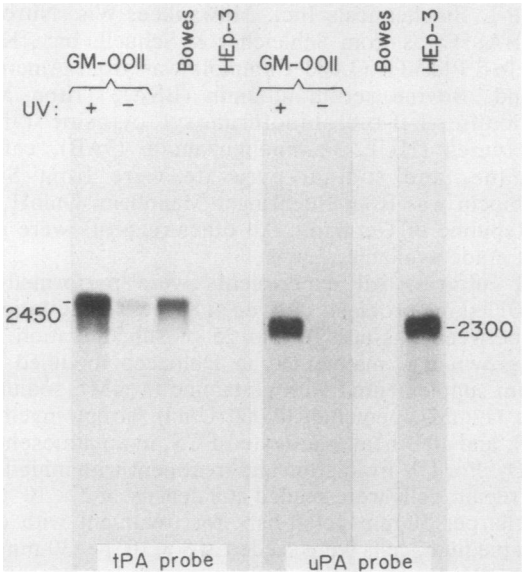

FIG. 2. Northern hybridization analysis of poly(A)-containing RNA from GM-0011 cells exposed to UV light. Cells were irradiated and further incubated for 2 days in the presence of 10% FCS. Poly(A)-containing RNA was prepared from the irradiated cells and from counterpart control cells and subjected to Northern hybridization analysis (10 μg per lane) with nick-translated cDNA probes for uPA mRNA and tPA mRNA. As controls were included poly(A)-containing RNAs (10 μg per lane) prepared from HEp-3 carcinoma and Bowes melanoma cells, which produce exclusively uPA and tPA, respectively. The numbers of bases were calculated according to size markers.

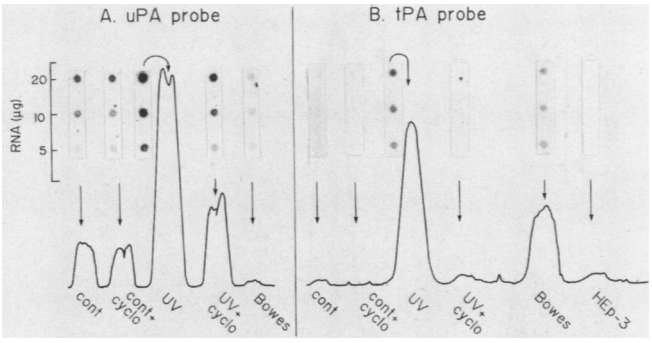

FIG. 3. Effects of cycloheximide on the enhancement of uPA mRNA and tPA mRNA by UV light. Irradiated cells and control (cont) nonirradiated cells were incubated for 60 h postirradiation with 10% FCS. During this time part of the cell cultures were treated with cycloheximide (cyclo) (1 μg/ml). Total RNA was then extracted and subjected to dot blot analysis with uPA cDNA (A) and tPA cDNA (B) as the probes. Total RNA extracted from the Bowes melanoma and HEp-3 carcinoma cells were included as controls. Scan of 10-μg RNA samples is represented.

secreted by the irradiated cells into their growth medium. Conditioned medium was removed 2 days postirradiation and replaced by fresh medium (Fig. 4). In parallel irradiated cells, in which medium was not replaced, intracellular PA activity continued to rise for at least 6 h longer to a maximal level about 25-fold higher than that in control nonirradiated cells, and at the end of day 3 postirradiation it declined somewhat. By contrast, in irradiated cells in which medium was replaced, further elevation of PA activity stopped 3 h after medium replacement, the first time point measured. Activity then dropped sharply, and at the end of day 3 it was equal to the low level found in nonirradiated cells. This result suggests that in the irradiated cells a continuation of PA induction during day 3 postirradiation was completely dependent on an activity secreted during the 2 preceding days.

To complement the experiment described above, we tested whether the conditioned medium removed from irradiated cells contained an activity which could mimic the effect of UV light and enhance PA activity in nonirradiated cells. Nonirradiated cells were treated with conditioned medium collected from either irradiatd (UV-CM) or nonirradiated cells (control-CM) (Fig. 5). At the indicated times the medium was collected and tested for PA activity. UV-CM contained initially a high level of UV-induced PA activity, represented in Fig. 5 as activity at time zero. The exogenously added activity dropped by 40% within 30 min, and after 6 h it reached a minimal level of about one-third of the initial activity. This decline was followed by an elevation of PA activity, and after 31 h of treatment it was about 30% higher than the initial UV-induced activity. No such increase was observed in conditioned medium originally derived from nonirradiated cells. Addition of actinomycin D did not affect the decline of the exogenously added activity, but completely blocked the elevation of PA activity measured thereafter (Fig. 5A). We tested whether the activity which disappeared from the medium could be found associated with the cells, but no activity of that magnitude could be detected. After 30 min of treatment, the activity measured in the

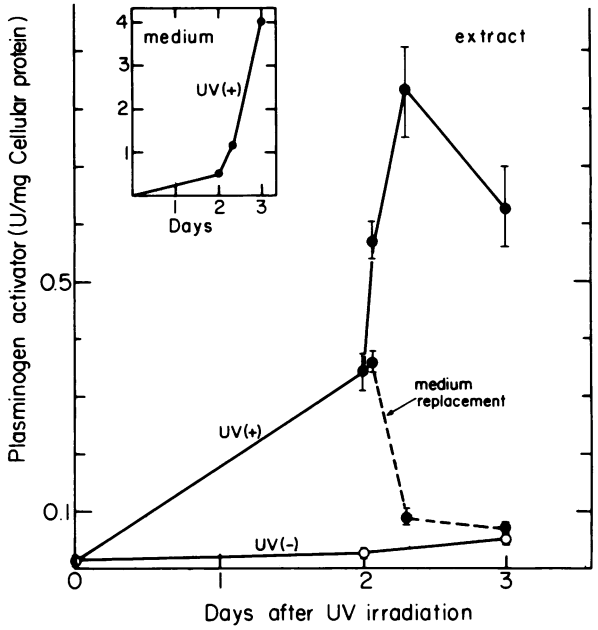

FIG. 4. Effect of replacement of conditioned medium from irradiated cells on continuation of PA induction in the same cells. Two days after exposure of cells to UV irradiation, conditioned medium was removed from part of the cultures and replaced by fresh medium (●---●). These cell cultures, as well as counterpart irradiated cells in which medium was not replaced (●——●), were incubated further. All treatments were performed in the presence of 2% FCS. At the indicated times conditioned medium and cells were collected from the irradiated cells and also from control nonirradiated cells (○) and tested for PA activity.

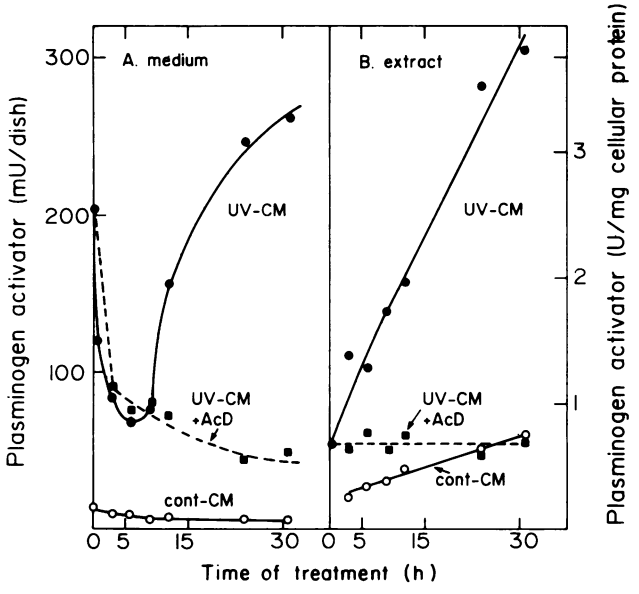

FIG. 5. Effect of conditioned medium from irradiated cells on PA activity in nonirradiated cells. Conditioned medium was collected from irradiated cells (UV-CM) and from nonirradiated cells (cont-CM) in the absence of serum and supplemented with BSA as described in Materials and Methods and subsequently added to nonirradiated cells. Part of each culture was also treated with actinomycin D (AcD; 0.5 μg/ml). At the indicated times medium (A) and cell extracts (B) were collected and tested for PA activity. Activity at time zero represents PA activity initially included in the conditioned medium. As this activity was not produced by the treated nonirradiated cells, PA activity in medium samples (A) was expressed as milliunits of uPA per dish.

cellular extract was about threefold higher than in the control, but it contained only about 5% of the activity which disappeared from the medium during this time (Fig. 5B). However, this initial activity had already increased 3 h after the addition of UV-CM to the nonirradiated cells, it proceeded to rise for the rest of the duration of the experiment, and this increase was completely blocked by actinomycin D. We therefore assumed that the initial PA activity present in UV-CM was rapidly inactivated by a transcription-independent mechanism and that the subsequent elevation represented a newly synthesized PA induced exclusively by conditioned medium derived from irradiated cells. The inhibitory effect of actinomycin D on the level of intracellular PA observed after 3 h of treatment suggested that UV-CM affected the transcription of the uPA gene.

It was reported previously that human fibroblasts rapidly absorb active tPA and, more slowly, uPA through surface receptors and subsequently internalize and inactivate the enzymes (30). Ethanol-fixed cells were shown to bind active tPA efficiently but to inactivate it much more slowly than living cells. We tested whether such a mechanism could account for the fast disappearance of PA activity added exogenously to GM-0011 fibroblasts. Standard uPA and tPA derived from Bowes melanoma cells were added to cells supplemented with fresh medium, and medium samples were taken within 3 h and tested for PA activity. Both PA types disappeared from the medium rapidly with first-order kinetics and about a 40-min half-life. After 3 h a residual activity of about 6% was left in the medium, and no increase was found in the intracellular PA activity compared with control cells (results not shown). We also tested living and ethanol-fixed cells in an agar overlay assay for their ability to retain urokinase-adsorbed activity. After 2 h of incubation, activity was detected exclusively in the fixed cells, indicating that the loss of PA activity in GM-0011 cells followed the binding of active PA to the cell surface, as previously described (30).

To ensure that the elevation in PA activity (Fig. 5) after the disappearance of the exogenous activity was not due to release or reactivation of some cryptic form of this initial activity, we passed the conditioned medium through a benzamidine-Sepharose column to remove uPA (31). In our experiments this treatment removed PA either completely or only partially (Fig. 6). The residual activity can possibly be attributed to the single-chain uPA, which exhibits low affinity to inhibitors (19). In cells treated with UV-CM completely deprived of PA activity by benzamidine treatment, PA activity increased about 17-fold compared with cells treated with control-CM. This experiment indicated that conditioned medium derived from irradiated cells contained uPA-inducing activity, supporting the previous observations; moreover, it excluded the possibility that the inducing activity was PA itself. Immunological analysis of UV-CM-induced PA yielded results identical to those presented for UV-induced PA in Fig. 1; activity was virtually entirely inhibited by uPA antiserum and was not affected by tPA antiserum (not shown).

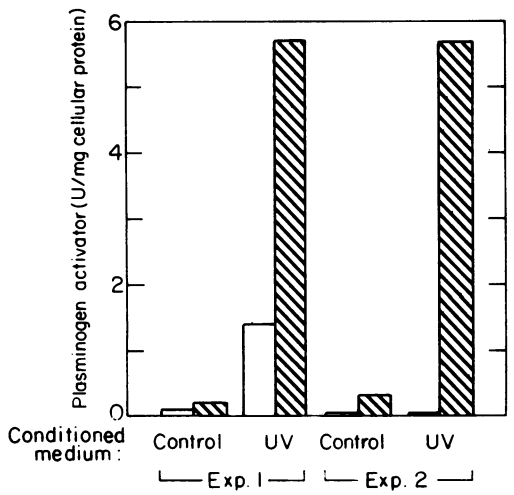

FIG. 6. Effect of benzamidine-treated conditioned medium on PA induction. Conditioned medium from irradiated and nonirradiated cells was collected in the absence of serum and supplemented either with 2% FCS (experiment 1) or with BSA (experiment 2). The medium was then passed through a *p*-aminobenzamidine-Sepharose column and tested for PA activity (open bars). The benzamidine-treated medium was also added to nonirradiated cells for 48 h (experiment 1) or 12 h (experiment 2), collected, and assayed for PA activity (hatched bars).

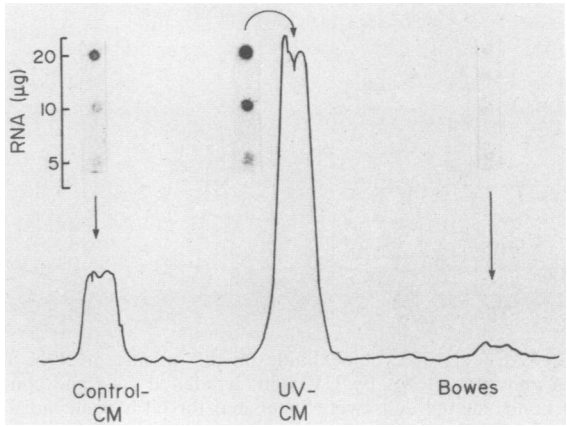

FIG. 7. Effect of conditioned medium from irradiated cells on uPA mRNA accumulation in nonirradiated cells. Conditioned medium was collected from irradiated (UV-CM) and nonirradiated (control-CM) cells in the absence of serum, supplemented with BSA as described in Materials and Methods, and added to nonirradiated cells. After an incubation period of 24 h, total RNA was prepared and tested by dot blot analysis with the uPA cDNA probe. Total RNA from Bowes melanoma cells was used as a negative control. Scan of 20-μg RNA samples is represented.

It was further tested whether UV-CM could enhance the level of mRNA coding for uPA in nonirradiated cells. UV-CM was collected at 60 h postirradiation and added to nonirradiated cells for 24 h. Control cells were treated with conditioned medium derived from nonirradiated cells. Total RNA was extracted and analyzed for uPA mRNA in a dot blot analysis (Fig. 7). Conditioned medium derived from irradiated cells elevated uPA mRNA about fivefold compared with its counterpart control cell level.

Characterization of UVIS. We have shown that UV-CM contained an activity that enhanced the level of uPA activity and of uPA mRNA. We call this activity UVIS for UV-induced, secreted. To test for the chemical nature of UVIS, UV-CM was treated with trypsin, DNase I, and RNase A, all bound to Sepharose. Trypsin completely abolished the ability of the medium to induce PA, while the nucleases were ineffective. UVIS may thus represent the protein intermediate predicted in the experiment with cycloheximide shown in Fig. 3. UVIS activity was nondialyzable, completely stable after heating at 60°C for 30 min, 70% inactivated at 70°C, and

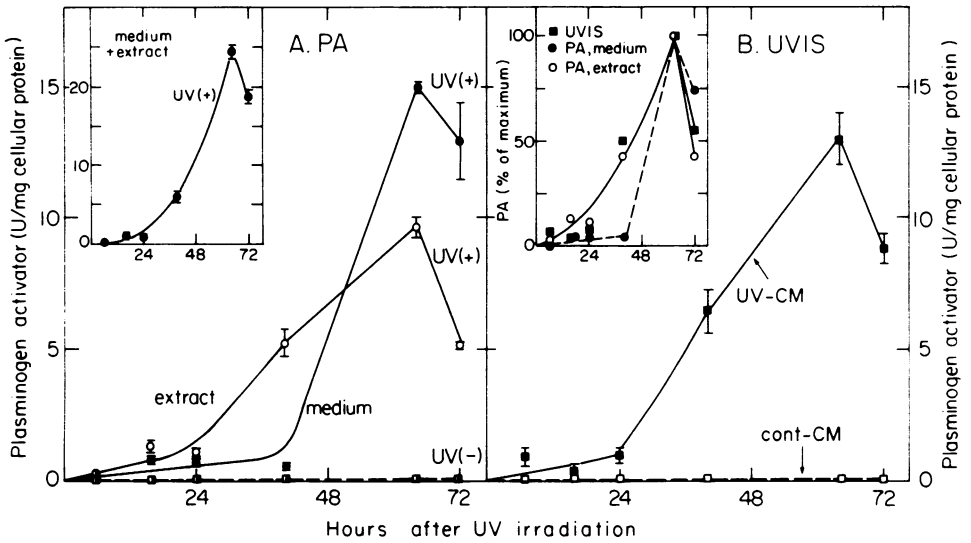

FIG. 8. Kinetics of induction of PA activity and accumulation of UVIS activity after UV irradiation. Cells were irradiated, supplemented with medium without serum, and incubated further. Control nonirradiated cells were treated in parallel. At the indicated times cells and conditioned medium were collected, and samples were tested for UV-induced PA activity (A). The same conditioned medium derived from irradiated cells (UV-CM) and control cells (cont-CM) was supplemented with BSA and added to nonirradiated cells to express UVIS activity. After 24 h of incubation, conditioned medium was collected and tested for UVIS-induced PA activity (B).

entirely lost at 80°C. We noted that it gradually declined when stored at −20°C, whereas at 4°C it was quite stable. We also noted that UVIS was much more active when UVIS-containing conditioned medium was collected in the absence of serum than in its presence. The reason for that is not yet understood. We therefore routinely collected UVIS activity in the absence of serum and supplemented the medium with BSA to avoid adsorption of UVIS to surfaces. We observed, however, that when the conditioned medium was supplemented with serum instead of BSA, PA induction by UVIS was slower.

Kinetics of PA induction and UVIS secretion after UV irradiation. To test whether secretion of UVIS may be a prerequisite for uPA induction by UV light, we compared the kinetics of UVIS accumulation after UV irradiation to that of the elevation of intra- and extracellular PA activity. At different times after exposure to UV light, cells and conditioned medium were collected and tested for PA activity (Fig. 8A). The same medium was also added to nonirradiated cells to express UVIS activity. After an incubation period of 24 h these media were collected and examined for UVIS-induced PA activity (Fig. 8B). This incubation period was selected based on the results depicted in Fig. 5, indicating that PA measured at that time represented a newly synthesized enzyme.

Elevation of intracellular PA activity was first detectable several hours after irradiation; however, during the first day it occurred at a slow rate. Elevation then proceeded at a rate accelerated about fivefold up to 64 hours, when activity reached a maximal value and then declined. Accumulation of PA activity in the medium exhibited a similar pattern, only the slow phase was longer and acceleration started not earlier than 40 h. The decline of PA activity seen in the medium after 72 h was probably due to inactivation of PA by GM-0011 cells, which became apparent when PA synthesis was reduced. Total PA activity, e.g., the sum of the intra- and extracellular activities, increased in an exponential fashion (Fig. 8A, inset). The kinetics of PA induction after UV irradiation was the same whether cells were incubated in the presence of 2 or 10% FCS or in the absence of any serum. It was also seen (Fig. 8B, inset) that accumulation of UVIS activity with time was nearly superimposed on the kinetics of the elevation of intracellular PA (Fig. 8B, inset) and clearly preceded the accumulation of PA in the medium.

Inhibitors of DNA repair act synergistically with UV light and with UVIS for PA induction. It was demonstrated previously that at UV doses up to 30 J/m^2, enhancement of PA activity occurred in cells with low capacity to repair UV-induced DNA lesions, but not in DNA repair-proficient cells (5, 47). It was therefore suggested that PA induction requires persistent DNA damage, which is sufficiently long-lived only in cells which perform DNA repair inefficiently. We speculated that such a putative signal could be produced along the multistep pathway of DNA repair. To find such a signal we used several inhibitors of DNA repair, some of which are known to inhibit specific steps of DNA repair. We predicted that inhibitors which stabilize the putative signal will act

TABLE 1. Effect of inhibitors of DNA repair on PA induction in control and UV-irradiated GM-0011 cells[a]

Drug (concn)	PA level in conditioned medium (U of uPA activity/mg of protein)		Synergism[b]
	Control cells	UV-irradiated cells	
None (control)	0.13	0.34	
Aphidicolin (6 μg/ml)	0.49	2.39	4.0
Ara-C (1 mM)	0.33	4.31	10.0
HU (2 mM)	0.77	8.05	9.3
Novobiocin (1.25 mM)	0.88	5.86	6.0
3AB (8 mM)	0.35	1.30	3.7
Caffeine (3 mM)	1.14	74.66	65.5

[a] Control cells and cells irradiated at 5 J/m^2 were supplemented with fresh medium containing 10% FCS and the specified drugs and incubated for 72 h.

[b] Calculated as the ratio of the activity measured after the combined treatment and the sum of the activities obtained by each of the treatments when given separately. (Activity of nontreated control cells was subtracted from all values prior to calculation).

TABLE 2. Effect of inhibitors of DNA repair on PA induction in GM-0011 cells treated with conditioned medium derived from control and UV-irradiated cells[a]

Drug (concn)	PA level in conditioned medium (U of uPA activity/mg of protein)		Synergism[b]
	Control-CM	UV-CM	
None (control)	0.04	0.89	
Aphidicolin (6 μg/ml)	0.55	11.80	8.7
Ara-C (1 mM)	0.34	6.68	5.8
HU (2 mM)	3.93	26.42	5.6
Novobiocin (1.25 mM)	5.83	19.86	3.0
3AB (8 mM)	1.67	8.98	3.6
Caffeine (3 mM)	25.01	169.38	6.6

[a] Non-irradiated cells were treated for 72 h with control-CM or with UV-CM, both supplemented with 2% FCS.
[b] See Table 1, footnote *b*.

synergistically with UV light for PA induction, whereas those which affect a step past the disappearance of the signal will be ineffective. In this experiment (Table 1) cells were irradiated at a low dose of UV light, which by itself was only slightly effective, and immediately after irradiation the drugs were added in fresh medium supplemented with 10% FCS to mitigate the toxic effects of the drugs. Control nonirradiated cells were treated in parallel. Three days later, when the effect of UV light is usually close to maximal, medium was collected and assayed for PA activity. As predicted, synergistic responses were obtained in the irradiated cells; however, such responses were exhibited by all of the drugs tested, which in addition were found to induce PA when given alone to nonirradiated cells. Caffeine exerted the highest syngergistic effect with UV light, a 65-fold increase in PA activity compared with the sum of the effects of each of the agents given separately. The other five drugs exhibited syngergistic responses in the range of 4- to 10-fold. Caffeine differed from the other inhibitors and from UV light in clearly enhancing the activity of tPA as well as uPA, as determined by immunological and zymographic analyses (not shown).

The same drugs were also tested with UVIS. A clear synergistic response in the range of three- to about ninefold was found in all cases after 3 days of treatment (Table 2), while UVIS activity alone had already passed its maximal effect. Thus, UVIS resembled UV light in that its activity could be potentiated by six different agents affecting DNA repair. It is also evident (Tables 1 and 2) that four of the drugs tested, especially caffeine, were much more effective when tested with the control of UVIS (e.g., when added to conditioned medium derived from nonirradiated cells) than with the control for UV light (e.g., when added to fresh medium). This difference could possibly be accounted for by a synergistic effect of the drugs with a low level of UVIS activity constitutively synthesized in nonirradiated cells. This interpretation is supported by our finding that in several cases when UVIS activity by itself was negligible, it could clearly be visualized through its potentiation by the inhibitors of DNA repair.

DISCUSSION

Several independent but complementary experiments indicate that induction of uPA and possibly also of tPA by UV light is mediated through a UV-induced protein factor secreted into the growth medium of irradiated cells (called UVIS). First, elevation of uPA mRNA and of tPA mRNA by UV light was blocked by cycloheximide, suggesting the involvement of a protein intermediate. Second, removal of conditioned medium from irradiated cells 2 days postirradiation completely stopped any further increase of PA activity, showing that the continuation of PA induction on day 3 was completely dependent on an activity accumulated in the medium during the 2 preceding days. Third, when the same conditioned medium was added to nonirradiated cells, it could mimic UV light and enhance the level of uPA mRNA as well as that of uPA enzymatic activity. Fourth, trypsin, but not nucleases, completely abolished the ability of the medium to enhance PA activity, reinforcing the finding that the secreted activity is of a protein nature.

uPA induction after UV irradiation was largely or entirely mediated via UVIS, as indicated by the dependence of the elevation of uPA mRNA on protein synthesis and of the continuous enhancement of PA on a secreted activity. This conclusion was further supported by the magnitude of the UVIS effect, which was generally comparable to that of UV light, and also by the relative kinetics of PA induction and UVIS secretion after UV irradiation and of PA induction after UVIS treatment. The latter was relatively fast; PA activity was first detectable in the cells after 3 h of treatment and started to accumulate in the medium at a high rate after 6 h. By contrast, in irradiated cells, the increase of intracellular PA activity and the accumulation of UVIS activity were both slow, exhibiting biphasic kinetics which were superimposable. Both activities were elevated at a slow rate during the first day after irradiation and at about a fivefold-accelerated rate during the next 40 h. Accumulation of PA activity in the medium followed that of UVIS activity and reached half its maximal value with about a 9-h delay. Together these results indicate that secretion of UVIS is a prerequisite for uPA induction and that it is likely to be a rate-limiting step in the overall process of uPA induction after UV irradiation. The observation that actinomycin D completely blocked the relatively rapid enhancement of uPA activity by UVIS suggests that UVIS may directly enhance transcription of the uPA gene.

UV light enhanced the levels of the two mRNA species coding for uPA and for tPA to similar extents, yet uPA was the only active PA found in GM-0011 cells and their growth medium after exposure to UV light. We tested whether tPA could be inhibited by a specific inhibitor, but the inhibitory activity found in GM-0011 cells blocked exogenously added uPA more efficiently than it inhibited Bowes melanoma-derived tPA (unpublished results). It is also unlikely that most of the tPA is secreted and then entirely absorbed by the cells, since exogenously added urokinase and tPA were inactivated by GM-0011 with similar half-lives. Possible explanations for the apparent discrepancy are that tPA mRNA, although migrating in the gel like Bowes melanoma-derived tPA, is not efficiently translated, as has recently been reported for primary mouse and rat oocytes (33), that tPA protein is unstable, or that tPA exists in GM-0011 cells as an inactive protein. Whatever the explanation, it appears to hold also for nonirradiated GM-0011 cells, in which a low level of tPA mRNA was usually detected but active tPA could not be found. Because cycloheximide completely blocked induction of tPA mRNA by UV light, we assume that UVIS affects tPA similarly to uPA. However, any conclusion on this issue or on the induction of tPA by UV light must await clarification of tPA metabolism in GM-0011 cells.

We have shown here that six different inhibitors of DNA

repair potentiated the irradiation effect on uPA enhancement. This finding is consistent with previous studies, in which PA induction has been correlated with reduced capacity of cells to perform either excision repair or postreplication repair of UV-induced DNA lesions (5, 47). Together these results imply that long-lived DNA damage is required for PA induction. We speculated that the putative signal could be generated along the multistep pathway of excision repair, consisting of damage recognition, endonucleolytic breakage, excision of the damage-containing oligonucleotide, nucleotide polymerization, and ligation of the new patch into old DNA. It was now hoped to obtain a clue about the signal involved in PA induction by interrupting DNA repair at different steps through the use of inhibitors of DNA repair. Novobiocin was reported to prevent the endonucleolytic step (14, 15, 45), aphidicolin, HU, and ara-C interfere directly or indirectly with DNA polymerization (15, 22, 62, 66, 67), and 3AB was suggested to inhibit the ligation step in DNA repair (16, 23). Caffeine was also tested because it was reported to inhibit postreplication repair in human cells deficient in DNA repair (8).

In spite of their different modes of action, all of the drugs tested were found to have the same two effects on PA induction: they considerably potentiated the effect of UV light and they were also effective when given alone. Both effects were obtained with replicating cells growing in the presence of 10% FCS. Most of the DNA repair inhibitors tested were reported to interfere also with DNA replication, albeit by different mechanisms (14, 26, 34, 37, 45, 54, 66), an effect exerted also by UV irradiation. It is thus possible that some structural form of the DNA resulting from the blockage of DNA replication, e.g., single-strand DNA breaks, single-stranded gaps, and topological changes, may promote PA induction.

In our experiments, aphidicolin, a potent inhibitor of polymerase α, virtually completely inhibited DNA synthesis and yet acted better as a PA inducer when given in combination with UV light then when given alone. In irradiated cells aphidicolin was shown to promote accumulation of single-strand DNA breaks and to enhance cytotoxicity compared with nonirradiated cells (62, 67, 69). As PA induction exhibited the same kinetics after UV irradiation and after combined treatment with UV light and aphidicolin (results not shown), it is suggested that both inductions occur via the same mechanism and that accumulation of single-strand DNA breaks rather then a more general toxic effect underlies the synergistic response. Similarly, HU, ara-C, and 3AB were reported to cause the accumulation of strand breaks in damaged DNA, although 3AB is effective after treatment of cells with alkylating agents (15, 22, 23, 66). Novobiocin, by contrast, was reported to inhibit the formation of DNA breaks after UV irradiation, probably by inhibiting damage recognition (14, 15, 45), a step suggested to require the superhelical topology of the DNA (29). In bacteria, novobiocin inhibits the formation of negative supercoiling by DNA gyrase, a type II topoisomerase catalyzing the formation and removal of DNA supercoils (24). A homologous activity has not yet been demonstrated in eucaryotic cells, and the target enzyme through which novobiocin inhibits excision repair has not yet been identified. If, however, novobiocin affects DNA repair through inhibiting a mammalian gyraselike activity, as proposed previously (25), it could be envisaged to inhibit the supercoiling of those DNA domains which were damaged while being relaxed and also to block the introduction of negative supercoiling into newly repaired domains. Thus, taken together, the results with the

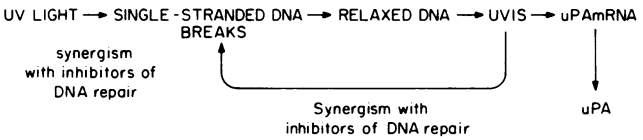

FIG. 9. A model describing uPA induction by UV light. See text for explanation.

inhibitors suggest that changes in the superhelical structure of an as yet undefined DNA sequence(s), whether occurring through inhibition of topoisomeraselike activity or as a consequence of accumulation of DNA strand breaks, may be the putative signal involved in PA induction. This model was originally suggested in a previous study (5), based on the finding that high constitutive levels of PA in human fetal fibroblasts correlated with relaxed topology of the DNA as measured by nucleoid sedimentation (5). Novobiocin-sensitive alterations of DNA topology were recently reported to affect gene expression in mammalian cells (25, 28, 68).

The inhibitors of DNA repair also acted synergistically with UVIS. In view of this result we propose that UVIS, like UV light, introduces single-strand DNA breaks, which can be reversed by activities of DNA repair and may be stabilized by DNA repair inhibitors. According to this model, UVIS acts synergistically with the inhibitors in the same manner described for UV light. It is also tempting to speculate that the breakage activity attributed to UVIS is not random but directed toward specific DNA sequences. Our model thus suggests that the putative long-lived signal in the DNA predicted in previous studies is related to alterations in DNA topology and is involved in PA induction by both UV light and UVIS. As discussed previously, the slow secretion of UVIS appears to provide the rate-limiting step in PA induction by UV light. Hence, we propose that the long-lived signal is necessary for the induction of UVIS by UV light rather than for the induction of PA by UVIS, which occurs at a faster rate. Since we assume that UVIS also triggers the same signal, the model predicts that UVIS exerts a positive feedback on its own synthesis (Fig. 9). Such an autocatalytic effect can account for the acceleration of the rates of both accumulation of UVIS, and induction of PA observed 1 day after UV irradiation; although other explanations, such as occupancy of putative membrane receptors for UVIS and effects due to uPA adsorption to cells, cannot presently be ruled out.

It is unknown whether UVIS can mimic the effects of UV light other than that of PA induction. Recently a protein named EPIF has been described, which is secreted by human fibroblasts after exposure to UV light, mitomycin C, and the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (2, 59), agents also reported to induce PA (20, 48). Like these agents, EPIF itself enhances the level of several abundant proteins and their mRNAs and is suggested to represent a cellular stress response (2). It seems reasonable that induction of PA by DNA-damaging agents other than UV light (48) is also mediated via UVIS activity, and perhaps the induction of UVIS, and through it also that of PA, is part of a general stress response of cells of high death risk (survival of GM-0011 cells irradiated at the optimal dose for PA induction is about 3%; R. Ben-Ishai, unpublished results). UVIS appears to differ from EPIF in that it is more thermostable and more sensitive to storage in the frozen state. XHF1 is another secreted protein induced by UV light

and by EPIF in human fibroblasts, and its relation to UVIS is presently unknown (59). Part of the EPIF response was reported to be elicited also by interleukin 1 (IL-1) (P. Herrlich, P. Angel, H. J. Rahmsdorf, U. Mallick, A. Poting, L. Hieber, C. Lucke-Huhle, and M. Schorpp, Adv. Enzyme Regul., in press). However, UVIS activity was not neutralized by antiserum raised against interleukin 1, nor did interleukin 1 induce PA to a large extent in GM-0011 cells. UVIS may belong to the group of growth factors, some of which have been reported to induce PA (12, 27, 56).

Proteins such as EPIF and UVIS, induced by UV light and capable of activating other genes, could exert significant effects in the multicellular organism. Produced mainly by DNA-damaged cells of low survival, such proteins could transmit the irradiation effects to cells that were not exposed to the irradiation, thereby maintaining and propagating UV-induced biological effects.

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