Suppression of Postmitochondrial Signaling and Delayed Response to UV-induced Nuclear Apoptosis in HeLa Cells

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Activation of postmitochondrial pathways by UV irradiation was examined using mouse lymphoma 3SB and human leukemic Jurkat cells and two human carcinoma cell lines (HeLa and MCF-7). Exposure of 3SB and Jurkat cells resulted in large amounts of cytochrome c and apoptosis-inducing factor (AIF) being released into the cytosol, and a clear laddering pattern of DNA fragments was observed within 3 h of incubation after irradiation. Simultaneously, activation of caspase-9 and its downstream caspases was detected. HeLa and MCF-7 cells also showed extensive release of mitochondrial factors and caspase-9 activation at 4 to 6 h after exposure, but apoptotic nuclear changes appeared much later. Compared with 3SB and Jurkat cells, these carcinoma cell lines exhibited reduced activation of caspase-9-like proteolytic activity by UV radiation, and levels of caspase-3-like activity in HeLa cells were extremely low, similar to those in caspase-3-deficient MCF-7 cells. These results suggest that the delayed response to UV-induced nuclear apoptosis in HeLa cells is due to a reduced activation of the caspase cascade downstream of cytochrome c release and suppression of caspase-3 activity.

Key words: HeLa cells — UV — Apoptosis — Cytochrome c — Caspase

Tumor cells show a wide variation in their radiosensitivity among different tissue sources. Thymocytes and most hematopoietic cell lines are known to exhibit interphase cell death that occurs before the next division, irrespective of the stage in the cell cycle, and they quickly display a typical morphology characteristic of apoptosis. Recently, it has been reported that many human tumor cell lines which generally undergo necrotic death also show a delayed apoptosis following exposure to UV or ionizing radiation. Since the delayed form of apoptosis is more commonly associated with non-hematopoietic cells, an understanding of its control might be useful to improve the clinical results of tumor radiotherapy.

Apoptosis is an active process of selective cell killing for homeostasis of multicellular organisms during embryogenesis and tissue remodeling. This form of cell death is also essential for the elimination of cells damaged by various external stimuli. During the process of apoptosis, various proteins that are normally sequestered in the intermembrane space of mitochondria, such as cytochrome c and apoptosis-inducing factor (AIF), are released into the cytosol. Once released, AIF is imported into the nucleus and induces chromatin condensation and large-scale (~50 kbp) DNA fragmentation. In a series of biochemical studies, the apoptotic protease activating factors (Apafs) were purified from HeLa cytosolic supernatant and Apaf-1, Apaf-2 and Apaf-3 were identified as the human homologs of Ced-4, cytochrome c and caspase-9, respectively. Cytochrome c in the cytosol binds to Apaf-1 and triggers the formation of oligomeric complexes containing cytochrome c, Apaf-1, and caspase-9, that activate pro-caspase-9. The activated caspase-9 then initiates a caspase cascade involving the downstream executioners, caspase-3, -6 and -7. Since it has been reported that fibroblasts derived from the cytochrome c, Apaf-1 or caspase-9 knockout mouse embryos are resistant to cell death induced by UV irradiation, this insult is considered to preferentially trigger the production of postmitochondrial signals leading to apoptosis through activation of caspase-9.

The sensitivity to external stimuli and the mode of apoptotic cell death are cell type- and stimulus-specific, indicating the existence of regulators that modulate the individual steps of apoptosis pathways. It has been reported that members of the inhibitors of apoptosis (IAP) family of proteins suppress apoptosis by direct inhibition of caspases and heat shock proteins (HSPs), which are induced in response to environmental stress, functioning to modulate the progress of apoptosis. However, the cell-type dependence of apoptotic pathways has not been well analyzed in cultured mammalian cells. In this study, we therefore examined the activation of postmitochondrial signals during UV-induced apoptosis using two kinds of cell lines, mouse lymphoma and human leukemia cells, which undergo rapid apoptosis, and human carcinoma cell lines which are known to be characterized by delayed apoptosis. The results demonstrate that there is

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wide variation in the processes of apoptotic cell death between the two cell types and suggest that the delayed apoptosis in UV-irradiated HeLa cells is due to a reduced activation of the caspase cascade downstream of cytochrome c release and suppression of caspase-3 activity.

**MATERIALS AND METHODS**

**Reagents** Anti-cytochrome c (monoclonal clone 7H8.2C12) and anti-caspase-6 (monoclonal clone B93-4) antibodies were purchased from Pharmingen (San Diego, CA). Anti-caspase-3 polyclonal antibodies, caspase-3 (H-277) and cleaved caspase-3 (D175), were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling Technology (Beverly, MA), respectively. Anti-caspase-7 (monoclonal clone 4G2) and anti-caspase-9 (monoclonal clone 5B4) antibodies were from MBL (Nagoya), and the anti-cytochrome oxidase subunit IV (COX IV) (monoclonal clone 20E8-C12) antibody was purchased from Molecular Probes (Eugene, OR). To generate antibodies against human AIF, we immunized rabbits on the basis of the amino-acid sequence reported previously,12 and purified the antiserum using affinity membrane-blotted recombinant human AIF protein (amino acids 1–873). The AIF antibody was stored in phosphate-buffered saline (PBS) containing 0.01% NaN₃ and 1% bovine serum albumin. Protein concentrations were determined using an assay kit containing the Bradford dye reagent (BioRad, Hercules, CA). The peptides 7-amino-4-methylcoumarin (AMC), N-acetyl-Asp-Glu-Val-Asp-α-(4-methylcoumaryl-7-amide) (DEVD-MCA), and N-acetyl-Leu-Glu-His-Asp-MCA (LEHD-MCA) were obtained from the Peptide Institute (Osaka). Benzoyloxycarbonyl-methylcoumaryl-7-amide) (DEVD-MCA), and N-acetyl-Asp-Glu-Val-Asp-(4-α-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 10 mM PMSF, 100 µg/ml each of aprotinin, leupeptin, and pepstatin) was synthesized on the basis of the amino-acid sequence reported previously.12,13 and used for the precipitation of DNA, as described previously.17 DNA samples were loaded onto 2% agarose gels for electrophoresis and the gels were stained with 0.5 µg/ml ethidium bromide solution.

**Preparation of cytosolic and mitochondrial fractions** Cells were harvested, washed twice with ice-cold PBS, resuspended in ice-cold transport buffer (20 mM HEPES pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM glycochelidiaminetetracetic acid (EGTA), 2 mM dithiothreitol (DTT), 1 µg/ml each of aprotinin, leupeptin, and pepstatin) containing 100 µg/ml (for 3SB and Jurkat cells) or 300 µg/ml (for HeLa and MCF-7 cells) digitonin and allowed to permeabilize at 37°C for 5 min. After centrifugation at 14 000 g for 15 min at 4°C, the supernatant and pellet were stored at −80°C, and used as the cytosolic and mitochondrial fractions for western blot analysis. Contamination of mitochondria in cytosolic extracts was monitored with COX IV.

**Western blot analysis** Cell lysates were prepared as described previously,27 electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Immobilon-P membranes (Millipore, Bedford, MA). Western blotting was performed with the enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL).

**Measurement of caspase activity** A total of 1×10³ cells were harvested and washed twice with ice-cold PBS, and resuspended in 50 µl of KPM buffer (50 mM KCl, 50 mM piperazine-N,N'-bis(2-ethanesulfonic acid) pH 7.0, 10 mM EGTA pH 7.0, 2 mM MgCl₂, 20 µM cytochlasin B, 10 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml each of chymostatin, leupeptin, antipain, and pepstatin). The cells were frozen in liquid nitrogen followed by thawing, then they were disrupted using a microtubule homogenizer, and then quickly frozen by immersion in liquid nitrogen. This step was repeated five times. The resulting homogenates were centrifuged at 14 000 rpm for 15 min at 4°C to remove cell debris and the supernatant was stored at −80°C for caspase fluorometric protease assays. Using 96-well plates, 190 µl aliquots of ICE standard buffer (100 mM HEPES pH 7.5, 10% sucrose, 0.1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), 10 mM DTT, 1 mM PMSF, 1 µg/ml each of chymostatin, leupeptin, antipain, and pepstatin) containing 100 µM of DEVD-MCA, LEHD-MCA were incubated at 37°C for 10 min and 10 µl aliquots of cell extract (4 µg/ml proteins) were added to the substrate mixture. The formation of fluorescent aminomethylcoumarin (AMC) was

**Analysis of DNA fragmentation** A total of 1.5×10⁶ cells were washed twice with ice-cold PBS and lysed in 100 µl of cell lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM EDTA pH 8.0 and 0.5% Triton X-100) at 4°C for 10 min. After centrifugation at 16 000 rpm, the supernatant was used for the precipitation of DNA, as described previously.27 DNA samples were loaded onto 2% agarose gels for electrophoresis and the gels were stained with 0.5 µg/ml ethidium bromide solution.

**Cell culture and apoptosis assay** HeLa (human cervical carcinoma) and MCF-7 (human breast carcinoma) cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). Human leukemic Jurkat cells and the mouse thymic lymphoma 3SB cell line were grown in suspension in RPMI 1640 and DMEM, respectively, both supplemented with 10% FBS. For analysis of UV-induced apoptosis, actively growing cells were exposed to germicidal lamps (UV-C) at a dose rate of 1.2 J/m²·s. Immediately after irradiation or after various periods of post-irradiation incubation, the cells were fixed in 1% glutaraldehyde at 4°C overnight, washed with PBS, and resuspended in PBS containing 1 µg/ml DAPI (4′,6-diamidino-2-phenylindole). To measure the apoptosis frequency, 1000 or more stained cells were counted under a Zeiss Axiovert 135M fluorescent microscope. Experiments were performed in triplicate.
assessed with excitation at 355 nm and measurement at an emission wavelength of 460 nm, using a Fluoroskan Ascent and a linked data analysis system (Labsystems, Helsinki, Finland). Experiments were performed in triplicate.

RESULTS

UV causes rapid or delayed apoptosis depending on the cell type In a previous study, we demonstrated 3SB, a mouse thymic lymphoma cell line that expresses wild-type

Fig. 1. Induction of apoptosis by UV radiation. A, fluorescence microscope photographs of 3SB, Jurkat, HeLa and MCF-7 cells. After irradiation with 20 J/m² of UV, the cells were incubated for the indicated time periods and stained with DAPI. B, time course of the appearance of apoptotic cells after UV irradiation with 20 J/m². The frequency of apoptotic cells was calculated as the percentage of cells with abnormal nuclear morphology for 3SB ( ), Jurkat ( ■ ), HeLa ( ○ ) and MCF-7 ( □ ) cell lines. Each point represents the mean±SD from three independent experiments. C, electrophoretic patterns of fragmented DNA from UV-irradiated cells.
p53 protein, to exhibit apoptosis shortly after exposure to X-rays or UV radiation. In this study, we stained the cells with DAPI and examined changes in nuclear morphology under a fluorescence microscope. As shown in Fig. 1A, condensed chromatin and fragmented nuclei could be seen in 12.8% of the 3SB cells 3 h after exposure to 20 J/m² UV. The percentage of 3SB cells exhibiting nuclear change increased and reached 47.7% at 6 h after exposure, almost all the apoptotic cells showing extensive nuclear condensation and multiple apoptotic bodies containing nuclear fragments (Fig. 1A). Similar rapid appearance of apoptotic cells was observed with the human leukemic cell line, Jurkat (Fig. 1B), despite its defect in functional p53.29) At 3 and 6 h after exposure to UV radiation, 24.0% and 55.6% of Jurkat cells, respectively, displayed clear apoptotic morphological changes in nuclei (Fig. 1, A and B). As expected, when irradiated with 20 J/m² UV and incubated for 3 h, a ladder-like fragmentation pattern of DNA could be detected in both 3SB and Jurkat cell lines by agarose gel electrophoresis, and the proportion of oligonucleosomal DNA fragments increased with increase in post-irradiation incubation (Fig. 1C). In contrast, two human carcinoma cell lines, HeLa and MCF-7, underwent apoptosis much more slowly (Fig. 1B). In fact, less than 6.0% of the cells exhibited nuclear changes characteristic of apoptosis when incubated for 6 h after UV exposure. After 12 or 24 h of post-irradiation incubation, the percentages of apoptotic cells induced by 20 J/m² UV were 27.0% and 64.4% for HeLa and 15.4% and 50.2% for MCF-7 cells, respectively, major changes in dying cells being chromatin condensation and incomplete nuclear fragmentation (Fig. 1A). Such observations are consistent

![Fig. 2. Induction of cytochrome c and AIF release by UV radiation. After the indicated periods of incubation subsequent to UV irradiation (20 J/m²), cytosolic extracts and mitochondria fractions were prepared from UV-irradiated cells and subjected to western blot analysis, using antibodies specific for cytochrome c (A) or human AIF (B).](image-url)
with the finding that these carcinoma cells started to exhibit blurred oligonucleosomal DNA fragmentation at 12 h post-irradiation (Fig. 1C).

**Both types of cells undergo relatively early release of mitochondrial factors after UV irradiation** It has been established that mitochondria play a major role in apoptosis induced by various external stimuli. They have been shown to initiate apoptosis by releasing cytochrome c and AIF, which binds to Apaf-1 and activates downstream caspases and translocates to the nucleus and stimulates chromatin condensation and large-scale DNA fragmentation, respectively. These findings have led to the speculation that delayed apoptosis could be triggered by slow release of these mitochondrial factors in response to UV irradiation. To assess this possibility, we therefore examined by western blotting the levels of cytochrome c and AIF in cytosolic and mitochondria fractions prepared from cells after irradiation with 20 J/m² UV. Fig. 2A shows the changes in cytochrome c after exposure. At 2 h following UV exposure, extensive cytochrome c translocation from the mitochondrial into cytosolic fraction was observed in 3SB and Jurkat cells, just prior to the appearance of apoptotic cells. Relatively early release of cytochrome c was also detected in UV-irradiated HeLa and MCF-7 cell lines (within 6 h after exposure), compared with their delayed expression of the apoptotic phenotype. Similar UV-induced translocation of AIF from mitochondria to cytosol was observed in these cell lines (Fig. 2B).

**Delayed apoptosis corresponds to a reduced activity of caspases in UV-irradiated cells** Previous studies have shown that pro-caspase-9 with a long prodomain is acti-

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**Fig. 3.** Activation of caspases after exposure to UV radiation. A, at the indicated time periods of incubation after UV irradiation (20 J/m²), whole cell extracts were prepared from UV-irradiated cells and subjected to western blot analysis, using anti-caspase-6, -caspase-7 and -caspase-9 antibodies. B, in the case of western blot analysis of caspase-3, extracts from HeLa and Jurkat cells were electrophoresed and analyzed using two antibodies, Caspase-3 (H-277) and Cleaved Caspase-3 (D175). A non-specific band (asterisk) was detected with the anti-caspase-3 (H-277) antibody.
activated in response to cytochrome c release\(^{16,17}\) and caspase-9 in turn activates downstream caspases with short prodomains, such as caspase-3, caspase-6 and caspase-7.\(^{10,18}\) Thus, we next examined activation of these caspases in UV-irradiated cells. As shown in Fig. 3A, cleaved products corresponding to active subunits of caspase-9 and -7 appeared at 2 h after exposure to UV in Jurkat cells. Mature 37 kDa (p37) and 35 kDa (p35) fragments of caspase-9\(^{14,20}\) were also detected in HeLa and MCF-7 cells at 4 to 6 h after exposure (Fig. 3A). A significant decrease in levels of pro-caspase-6 was observed in Jurkat, HeLa and MCF-7 cells incubated for 6 h or more after UV irradiation. The time course of appearance of these cleaved products and the change in pro-caspase-6 levels in 3SB cells were the same as in Jurkat cells (data not shown).

The most intensively studied effector caspase is caspase-3. Using two kinds of anti-caspase-3 polyclonal antibodies, we analyzed the effects of UV irradiation on 32 kDa pro-caspase-3 processing, which involves a

![Fig. 4](image-url)
sequential proteolytic cleavage to produce 20 kDa (p20) and 19 kDa (p19) intermediates and the mature 17 kDa (p17) form of the enzyme. At 3 h after UV exposure, we observed a clear p17 band corresponding to the mature cleavage products in addition to p20 and p19 bands in Jurkat cells (Fig. 3B). However, conversion of caspase-3 to its mature form was not detected in HeLa cells even when incubated for 12 h after exposure, although a thin band of p17 was detected in this cell line after 24 h of post-irradiation incubation (Fig. 3B). In the case of MCF-7 cells, we could not detect any band corresponding to pro-caspase-3 (Fig. 3B). This may be a result of homozygous deletion of the caspase-3 gene (CASP-3). To further confirm the cleaved caspases to be active, we examined DEVD-MCA or LEHD-MCA cleavage to compare the induction of caspase-3- or caspase-9-like proteolytic activity, respectively, in response to UV irradiation among the four cell lines. 3SB and Jurkat cells exhibited rapid induction of DEVD-MCA cleaving activity within 6 h of post-irradiation incubation, but HeLa and MCF-7 cells exhibited little cleavage even with incubation for 48 h after exposure (Fig. 4A). Striking induction of LEHD-MCA cleaving activity was also detected in 3SB and Jurkat cells 3 to 6 h after exposure to UV radiation (Fig. 4A). In contrast, this proteolytic activity in HeLa and MCF-7 cells slowly started to increase at 6 h after UV irradiation, reached a low value at 12 h and then decreased thereafter (Fig. 4A). As reported previously, the induction of DEVD-MCA-cleaving activity by treatment with tumor necrosis factor (TNF) or staurosporine was extremely low in MCF-7 cells. Surprisingly, in the case of HeLa cells, UV irradiation with 20 J/m² was not sufficient to activate caspase-3 undergoing DEVD-MCA cleavage, and the activation of LEHD-MCA specific cleavage by UV exposure was also relatively low compared with the 3SB and Jurkat cases (Fig. 4A). Similar low levels of caspase-3-like activity have been observed in HeLa cells when injected with heart cytochrome c. Furthermore, whereas in UV-irradiated HeLa and MCF-7 cells, cytochrome c release from mitochondria was detected much earlier than the expression of apoptotic phenotypes, these alterations and activation of caspases could be induced by UV exposure in 3SB and Jurkat cells at almost the same time as cytochrome c release. These results indicate that mitochondrial cytochrome c release is an early event in common in the apoptosis process in these hematopoietic and non-hematopoietic cell lines, and suggest that delayed response to UV-induced nuclear apoptosis cell in HeLa cells is due to their reduced activation of the downstream executioner, caspase-3. To confirm the existence of caspase-independent delayed apoptosis, we examined the effect of a caspase inhibitor on the induction of DNA fragmentation by UV exposure (20 J/m²). As seen in Fig. 4B, a broad spectrum caspase inhibitor, Z-VAD-FMK, completely blocked DNA fragmentation in 3SB and Jurkat cells but did not suppress the delayed production of oligonucleosomal DNA fragments in HeLa and MCF-7 cells. These data suggest that the delayed form of apoptosis in human carcinoma cells occurs in a caspase-independent pathway.

**DISCUSSION**

The results obtained in this study demonstrate that apoptotic cell death in HeLa and MCF-7 cells is delayed following exposure to 20 J/m². The delayed form of apoptosis has been observed commonly in non-hematopoietic cells irradiated with UV radiation. For example, Chigancas et al. reported that apoptotic HeLa cells were evident at 24 or 48 h after UV irradiation but photoreactivation immediately after the exposure caused a clear reduction of apoptosis. Recently, McLlroy et al. found Jurkat cells exposed to DNA-damaging agents such as UV radiation and anticancer drugs to undergo rapid apoptosis accompanied by caspase-3 activation and DNA fragmentation. These results suggest that UV irradiation initiates apoptosis signal transduction through UV-induced DNA damage, but the time course and pattern of the expression of apoptotic phenotypes vary markedly depending on the cell type. Since UV radiation is thought to release cytochrome c in coordination with caspase activation leading to the expression of various apoptotic phenotypes, the delayed apoptosis apparent in HeLa and MCF-7 cells could be a result of defects in their caspase cascade. In fact, these human carcinoma cell lines exhibited reduced activation of caspase-9-like and caspase-3-like proteolytic activities by UV radiation, compared with 3SB and Jurkat cells undergoing rapid apoptosis.

The caspase cascade in the mitochondrial pathway is known to be suppressed by IAP proteins, which inactivate some members through direct interaction via the baculoviral IAP repeat (BIR) domain. XIAP, c-IAP 1 and c-IAP 2, all of which contain 3 copies of BIR, have been shown to bind to caspase-3, -7 and -9 and inhibit their proteolytic activity. Since overexpression of these proteins suppresses apoptosis induced by various stresses, the reduced activation of caspases observed in UV-irradiated HeLa and MCF-7 cells may be derived from a high IAP content in the cells. To assess this possibility, we therefore examined by western blotting the levels of XIAP, c-IAP 2 and survivin expressed in HeLa and MCF-7 cells after irradiation with UV radiation. However, values for whole-cell extracts proved constant over the 6 h post-irradiation periods (data not shown), being similar in all four cell types (data not shown). Usually, HSP proteins are constitutively expressed in mammalian cell lines and their accumulation is known to take more than 6 h after exposure to hyperthermia or radiation. We therefore speculate that there are other inhibitors suppressing the activation of caspases
in these human carcinoma cell lines. We are now analyzing molecules associated with apoptosomes derived from UV-irradiated cell extracts in an attempt to identify such factors.

Consistent with previous reports, HeLa cells underwent apoptotic nuclear changes during prolonged periods of incubation after UV irradiation, while dying cells showed mainly abnormal diffuse chromatin condensation in the nucleus. Similar observations were made in UV-irradiated MCF-7 cells. Since AIF is released from mitochondria to the cytosol in response to UV irradiation and irradiated MCF-7 cells. Since AIF is released from mitochondria to the cytosol in response to UV irradiation and is known to cause such peripheral chromatin condensation, this mitochondrial factor that acts in a caspase-independent fashion may be involved in inducing the delayed form of apoptosis in these human carcinoma cells. Surprisingly, the levels of caspase-3-like activity in HeLa cells were found to be extremely low, similar to those in caspase-3-deficient MCF-7 cells, but a ladder-like fragmentation pattern of DNA was detected in these cell lines, in addition to smear-type DNA fragments, by agarose gel electrophoresis 24 and 48 h after exposure to UV radiation. Although caspase-3 is a central downstream effector of apoptosis, activating a specific DNase (CAD/DFF-40) which cleaves chromosomal DNA into oligonucleosomal fragments recently identified endonuclease G (endoG) as a factor capable of inducing oligonucleosomal DNA fragmentation of DNA in mouse fibroblasts lacking CAD/DFF-40. By genetic screening of Caenorhabditis elegans, Parrish et al. also isolated a cps-6 gene encoding a homologue of human mitochondrial endoG. Their data indicate that endoG released from mitochondria into the cytosol may serve as a supplementing nuclease, causing genomic DNA fragmentation in a caspase-independent manner.

Whatever the underlying mechanisms, our results clearly show that HeLa cells exhibit a delayed form of apoptosis when irradiated with 20 J/m² of UV radiation and this apoptosis response is associated with reduced activation of postmitochondrial signaling downstream of cytochrome c release and suppression of caspase 3 activity. Such a delayed response to radiation-induced apoptosis is known to be a common feature in most human carcinoma cell lines. Therefore, identification of the key molecule delaying and suppressing the activation of cytochrome c-mediated caspases could facilitate the search for targets for effective anticancer therapy.

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