Ultraviolet Radiation-induced Apoptosis Is Mediated by Activation of CD-95 (Fas/APO-1)*

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Exposure to ultraviolet light (UV) can induce apoptosis in mammalian cells. The mechanism by which UV radiation engages the suicide apparatus is unclear. Here we demonstrate that UV radiation can activate the Fas pathway via receptor aggregation and subsequent recruitment of the death effector molecule FADD/MORT1. UV radiation-induced apoptosis was inhibited by both a dominant negative version of FADD (FADD-DN) and the caspase inhibitor CrmA. Thus, activation of the Fas pathway represents a physiologic mechanism by which UV radiation triggers apoptosis.

The ability of enucleated cells to mount a UV response has led to the suggestion that membrane or cytosolic events likely mediate the response (8). UV exposure for example induces rapid tyrosine phosphorylation of the EGF receptor, suggesting a prominent role for membrane initiated events in the UV response (9). Additionally, it has recently been demonstrated that UV-irradiation results in clustering and subsequent activation of the EGF, IL-1, and TNF receptors, and this contributes to the activation of the JNK cascade observed within minutes of exposure to UV radiation (10). Given this, we hypothesized that UV-irradiation may induce apoptosis by activation of cell surface death receptors, the prototypic example being CD-95.

MATERIALS AND METHODS

Cell Lines and Culture Conditions—The MCF7 and BJAB stably transfected cell lines used in this study have been described previously (11). The cells were maintained in RPMI 1640 containing 10% heat-inactivated fetal calf serum, 1% nonessential amino acids, 1% L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C in an atmosphere of 5% CO2. The transfected MCF7 cell lines were grown in the presence of 0.5 mg/ml G418 sulfate (Life Technologies, Inc.), and the transfected BJAB cell lines were grown in 3 mg/ml G418 sulfate.

Irradiations—Freshly seeded cells were irradiated in at room temperature without growth media with a germicidal UV light (254 nm). The fluence of the UV light source was measured prior to each experiment with a UVX radiometer (UVP, Inc.). Following irradiation, the cells were supplied with media and incubated for the indicated time.

Morphological Analysis—Apoptotic morphology was assessed by using DNA-staining dyes. For propidium iodide (Sigma) staining, MCF7 cells were plated at a density of 5 × 10^4 cells onto chamber slides (Nunc, Inc.). Following irradiation, both treated and control slides were incubated for 48 h prior to staining. Slides were rinsed 2 times with PBS, fixed in 4% paraformaldehyde at room temperature for 30 min, rinsed 3 times with PBS, and stained at room temperature for 10 min in a 100 µg/ml solution of propidium iodide in PBS. After staining, the slides were rinsed 2 times with PBS, blotted dry, and mounted using Vectashield mounting medium (Vector Laboratories). BJAB cells (1–2 × 10^6) were stained using Hoechst 33342 (Sigma). After a 48-h incubation, both control and irradiated cells were rinsed 2 times with PBS, stained at room temperature for 30 min in a 0.8 mg/ml solution of Hoechst 33342 in PBS, rinsed 2 times with PBS, and wet mounted using 20 µl of the cell suspension. Slides were examined immediately after staining using a Leitz Laborlux S microscope.

Quantitative Analysis—Apoptotic cells were quantitated based on

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¶ The abbreviations used are: IL, interleukin; TNF-α, tumor necrosis factor α; EGF, epidermal growth factor; FADD-DN, dominant negative version of FADD; PBS, phosphate-buffered saline; PFGE, pulse field gel electrophoresis.
nuclear morphology using fluorescence microscopy, and the percentage of apoptotic cells was calculated. A minimum of 100 cells were counted for each sample, and each experiment was done in triplicate.

**Pulse Field Gel Electrophoresis (PFGE)**—Logarithmically growing cells were irradiated with 10 joules of UV radiation and incubated at 37 °C. Cells were washed once in PBS and resuspended at $2 \times 10^7$ cells/ml. They were then mixed with an equal volume of a 1.4% suspension of low melting point agarose (Bio-Rad). The mixture was poured into a mold and allowed to solidify. The agar blocks were treated for 24 h at 56 °C with lysis solution (100 mM EDTA, 10 mM Tris, 20 mM NaCl) containing proteinase K (1 mg/ml) and Sarkosyl (1%), after which lysis solution was replaced with storage buffer (50 mM EDTA, 10 mM Tris).

**PFGE** was performed using a CHEF Mapper (Bio-Rad) at 10 °C in the 2-state mode with linear ramping. State 1 was 30 h with a 120° included angle, 1.9 V/cm, and a switching interval of 90 s. State 2 was 51 h with an included angle of 120°, 1.9 V/cm, and a switching interval of 40 min. The gel was then stained with ethidium bromide (5 μg/ml) and photographed.

**CD-95 Cross-linking and Immunoprecipitation**—15 million BJAB cells were mock irradiated or treated with 15 or 30 joules of UV irradiation and incubated for 20 min. Cells were pelleted and washed twice in PBS and resuspended in 2 mL of lysis buffer (50 mM Tris, 150 mM NaCl, 0.1% Tween 20, 10 mM EDTA, 1 mM DTT) containing protease inhibitors (Complete, Roche Applied Science). The cells were lysed by sonication, and the lysate was clarified by centrifugation at 13,000 g for 15 min. The supernatant was mixed with 10 μg of a Fas-specific rabbit polyclonal antibody (Santa Cruz) and incubated at 4 °C for 1 h. The immunoprecipitate was then washed three times with ice-cold PBS and resuspended in 100 μl of SDS sample buffer. Western blot analysis of the immunoprecipitate was done using a Fas-specific rabbit polyclonal antibody (Santa Cruz).

**FIG. 1.** Fas overexpression results in increased sensitivity to UV-induced apoptosis in a time- and dose-dependent manner. MCF-7 or MCF-7-Fas cells were UV irradiated and stained for nuclear morphology for determination of apoptosis. At least 100 cells were counted to determine apoptotic counts, and each point represents an average of three experiments. **A,** MCF-7 or MCF-7-Fas cells were irradiated with 10 joules, and the percentage of cells undergoing apoptosis was determined by nuclear staining at 3, 6, 12, 24, 36, and 48 h after treatment. B, identical cultures were treated with various doses of UV radiation, and the percentage of cells undergoing apoptosis was determined by nuclear staining after 48 h. MCF-7-Fas cells are represented by open squares, and MCF-7 cells are represented by open circles.

**FIG. 2.** UV irradiation results in Fas-receptor multimerization. A, diagrammatic representation of the experimental design for panel B. The basic principle is that, when antibody is limiting, more molecules of the Fas-receptor will be immunoprecipitated if aggregation occurs after UV-treatment. In contrast, when antibody is not limiting, equal amounts of Fas-receptor should be immunoprecipitated. B, BJAB cells that were unirradiated controls or irradiated with 15 and 30 joules were treated with the cross-linking reagent 3,3'-dithiobis(sulfosuccinimidyl propionate) and lysed for immunoprecipitation using a Fas-specific antibody (Apol) as described above under antibody (Ab) limiting conditions (top panel) or antibody excess conditions (bottom panel). Western blot analysis of the immunoprecipitate was done using a Fas-specific rabbit polyclonal antibody (Santa Cruz). C, control and UV-irradiated BJAB cells (30 joules) were fixed with 4% paraformaldehyde and stained for indirect immunofluorescence using a Fas-specific antibody as described above. Cells were viewed and photographed using a confocal microscope with a × 60 objective.
Fig. 3. UV irradiation-dependent co-immunoprecipitation of FADD with Fas-receptor. BJAB cells were UV irradiated at 15 and 30 joules or left untreated. 20 min after irradiation, the cells were treated with a cross-linker and lysed, and the extract was used to immunoprecipitate (IP) the Fas-receptor as described under "Materials and Methods." Half the immunoprecipitate was used for Western blot analysis to detect the presence of FADD using a rabbit polyclonal antiserum (top panel), and the other half was used to detect the presence of Fas (bottom panel) as described above.

ammonium acetate for 10 min. The cells were pelleted and washed twice in PBS and lysed using 500 μl of lysis buffer (20 mM Tris (pH 7.4), 140 mM NaCl, 10% glycerol, 1% Triton X-100, and 2 mM EDTA) containing a protease inhibitor mixture (Boehringer Mannheim) on ice for 30 min with agitation. The lysates were then used for immunoprecipitations in the presence of an anti-Fas antibody (Apo-1, Kamiya Labs, CA) at 0.5 μg/ml (antibody limiting) or 10 μg/ml (antibody excess). Immune complexes were precipitated using protein A-Sepharose (Pharmacia Biotech Inc.) and washed three times in lysis buffer. The precipitate was resuspended in Laemmli buffer, boiled for 5 min, and resolved on SDS-polyacrylamide gel electrophoresis. The resolved samples were transferred onto a polyvinylidene difluoride (Millipore Corp.) membrane for Western blot analysis. The presence of CD-95 or associated molecules was then detected using the appropriate primary and secondary antibodies followed by detection using chemiluminescence (Pierce).

Analysis of CD-95 Using Immunofluorescence—BJAB cells were UV irradiated with 30 joules or were mock-irradiated. After 20 min, they were fixed with 4% paraformaldehyde and stained for immunofluorescence using an anti-CD-95 antibody (Transduction Laboratories) followed by an fluorescein isothiocyanate-conjugated secondary antibody. Fas-receptor specific immunostaining was visualized and photographed using a Bio-Rad MRC-1000 confocal microscope with a × 60 objective.

RESULTS AND DISCUSSION

Ligand dependent activation of CD-95 requires multimerization though ligand independent activation can occur upon oligomerization following overexpression (12). To test the hypothesis that UV-induced apoptosis used components of the CD-95 death pathway, we analyzed MCF-7 breast carcinoma cells and its transfected derivative line MCF-7-Fas, which constitutively expresses approximately 5-fold more CD-95 receptor (12). MCF-7-Fas cells were significantly more sensitive to UV-induced apoptosis in a time- and dose-dependent manner (Fig. 1, A and B). To examine if UV irradiation induced CD-95 multimerization, we utilized an immunoprecipitation protocol (Fig. 2, A and B) employing limiting antibody conditions that would preferentially immunoprecipitate oligomerized receptor. At limiting antibody concentrations, there appeared to be a linear relationship between the amount of CD-95 immunoprecipitated and the UV dose. As expected, this relationship was not observed under conditions of antibody excess (Fig. 2B). UV-induced CD-95 multimerization was not a cell line-specific phenomenon as it was also observed in both BJAB and Jurkat cells (data not shown). Indirect immunofluorescence experiments using CD-95-specific antibodies were also performed to visualize CD-95 oligomerization upon UV irradiation (Fig. 2C). In the absence of treatment, CD-95 was visualized heterogeneously on the cell surface. Upon treatment with UV, CD-95 immunoreactivity was detected as very large, brightly staining aggregates.

To investigate if UV irradiation-mediated multimerization of CD-95 resulted in recruitment of the adapter molecule FADD,
co-immunoprecipitation experiments were performed (Fig. 3). Immunoprecipitation of CD-95 followed by immunoblotting using a FADD-specific antiserum, revealed a UV dose-dependent increase in the association of FADD with CD-95. This phenomenon was observed in Jurkat (data not shown) and BJAB cells.

FADD serves as a conduit for death signals from other death receptors including TNFR1 and DR3 (13). A dominant negative version of FADD (FADD-DN) that lacks the death effector domain and is therefore unable to recruit FLICE inhibits cell death induced by all three receptors (CD95, TNFR1, and DR3). If UV irradiation was inducing apoptosis by activating death receptors, then the observed cell death should be inhibited by FADD-DN. Indeed, cells expressing FADD-DN (MCF7-FADD-DN) were significantly more resistant to UV-induced apoptosis than their vector-transfected counterparts (Fig. 4, A-C). Forty-eight h following UV irradiation, vector-transfected MCF-7 cells had morphological features typical of apoptosis (Fig. 4A, top panel), including cytoplasmic shrinkage and nuclear condensation. The ability of FADD-DN to protect from UV-induced apoptosis was also observed with BJAB cells (Fig. 4D). In contrast to differences seen with UV irradiation, the sensitivity to apoptosis of CD-95-overexpressing cell lines, as well as FADD-DN-expressing cells, did not differ significantly from control cell lines upon γ irradiation (Fig. 4E). These results indicate that CD-95 and FADD are not involved in γ radiation-induced apoptosis.

Consistent with the involvement of the FADD-FLICE axis in UV irradiation-induced cell death was finding the CrmA, a poxvirus-encoded serpin that preferentially inhibits FLICE-blocked UV-induced cell death. Expression of CrmA, but not an active site mutant, CrmA-mut (11), resulted in attenuation of UV-induced apoptosis as determined by inhibition of DNA fragmentation (Fig. 5).

The results presented here for the first time delineate the pathway involved in UV-induced apoptosis. UV-mediated oligomerization of CD-95 and likely other death receptors is the initiating event that triggers the downstream FADD-FLICE death effector pathway. The mechanism by which UV irradiation induces receptor oligomerization remains unclear. We speculate, however, that energy transfer to cell surface receptors may induce conformational changes that allow for oligomerization and engagement of the downstream signaling machinery.

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FIG. 5. Expression of the caspase inhibitor CrmA results in inhibition of UV-induced apoptosis. BJAB cells expressing the pox-virus-derived inhibitor of caspase, CrmA, or its inactive point mutant, CrmA-mut, were treated with 10 joules of UV irradiation, and after 24 h, PFGE was performed on the respective genomic DNA.