Evidence that Singlet Oxygen-induced Human T Helper Cell Apoptosis Is the Basic Mechanism of Ultraviolet-A Radiation Phototherapy

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Summary

Ultraviolet A (UVA) irradiation is effectively used to treat patients with atopic dermatitis and other T cell mediated, inflammatory skin diseases. In the present study, successful phototherapy of atopic dermatitis was found to result from UVA radiation-induced apoptosis in skin-infiltrating T helper cells, leading to T cell depletion from eczematous skin. In vitro, UVA radiation-induced human T helper cell apoptosis was mediated through the FAS/FAS-ligand system, which was activated in irradiated T cells as a consequence of singlet oxygen generation. These studies demonstrate that singlet oxygen is a potent trigger for the induction of human T cell apoptosis. They also identify singlet oxygen generation as a fundamental mechanism of action operative in phototherapy.

Materials and Methods

UVA Phototherapy. Five patients with atopic dermatitis as defined by Hanifin and Rajka (9) were enrolled after informed consent was obtained. All patients had extensive atopic dermatitis (total clinical score greater than 40; reference 10). Patients were hospitalized for UVA phototherapy. Patients had not been treated with any systemic or topical agent 4 wk before start of UVA phototherapy. For phototherapy, the patient’s whole body was exposed to 130 J/cm² UVA1 radiation from UVASUN 30,000 BIOMED (Mutzhas, Munich, Germany), as previously described.
In Vivo Ultraviolet A Irradiation. T cells were harvested and resuspended in RPMI1640 medium without phenol red (Biochrom, Berlin, Germany) in 12 well flat-bottom tissue culture plates (Becton-Dickinson, Heidelberg, Germany). Lids were removed and cells (5 × 10^6/ml) were exposed to UVA radiation from a UVASUN 5000 BIOMED irradiation device (Mutzhas) as described previously (16). Subsequently, cells were collected by centrifugation, resuspended in complete medium, and cultured in the presence of mouse anti-human FAS mAb ZB4 (mIgG1; Dako Diagnostika, Hamburg, Germany) or an isotype control antibody (mIgG1; Sigma Chemicals, Deisenhofen, Germany). The ZB4 antibody was previously shown to prevent FAS/FASL-induced T cell apoptosis by binding to FAS molecules (13). Maximal effects were exhibited if the antibodies were added to cells 1 hour before UVA irradiation.

In Vitro Detection of Apoptosis. For the TUNEL assay, the in situ cell death detection kit from Boehringer Mannheim (Mannheim, Germany) was used. Cells were washed and analyzed by flow cytometry using a FACScan (Becton-Dickinson) counting 1 × 10^4 cells per sample. Incubation of dUTP without TdT served as the negative control population and data are given as % positive (apoptotic) cells.

To examine DNA fragmentation, cells were collected and resuspended in 10 mM Tris HCl (pH 7.5), 1 mM EDTA, 0.15 M NaCl, 1% SDS, and 0.2 mg/ml proteinase K and 0.5 mg/ml of RNAase (Sigma). After 1 h incubation at 65°C DNA was extracted with phenol and chloroform, precipitated with ethanol, and dissolved in 10 mM Tris HCL (pH 7.5) containing 1 mM EDTA. Extracted DNA was loaded in a 2% agarose gel and visualized by staining with ethidium bromide.

Immunofluorescence Flow Cytometry. FAS and FASL surface expression was assessed by immunofluorescence flow cytometry using anti-FAS mAb DX2 (mIgG1; Pharmingen, Hamburg, Germany) or anti-FASL mAb 33 (mIgG1; Dianova, Hamburg, Germany) as described (16).

Chemical Treatments and Singlet Oxygen Generation. All chemicals were purchased from Sigma except for sodium azide (MERCK, Darmstadt, Germany). Sodium azide (50 mM in PBS) was only present during irradiation of cells. For irradiation in the presence of high water, deuterium oxide (99.9 atom % D) was used in a final concentration of 90% in PBS (14–16).

Results

In all patients, UVA phototherapy led to a significant improvement of skin symptoms, as assessed by a clinical scoring system (total score before UVA phototherapy: 65.4 ± 6.2; total score after UVA phototherapy: 18.2 ± 3.4; P < 0.001) (10). Skin specimens were analyzed for apoptotic cells using the TdT-mediated dUTP labeling (TUNEL) assay, followed by a confocal microscope. The ratio of apoptotic cells was assessed. In atopic-specific human T helper cells, in vitro UVA radiation (Fig. 1A) or an isotype-matched anti-CD4 mAb (data not shown). After 10 exposures, the total number of intradermally located, CD4^+ T cells had been significantly diminished, and most of the remaining cells showed signs of apoptosis (Fig. 2). No apoptotic cells were detected in the epidermal compartment (data not shown).

In atopic-specific human T helper cells, in vitro UVA irradiation induced apoptosis (Fig. 3, see also Figs. 5, 6). Significant apoptosis was already detectable 4 h after exposure, reaching a maximum 24 h after irradiation with 30 J/cm^2 UVA radiation (Fig. 3 and data not shown).

Before UVA radiation exposure, FASL molecules were not present on the cell surface (Fig. 4), but significant FASL surface expression was detected in UVA-irradiated cells already 4 h after exposure. Ultraviolet A radiation-induced surface FASL expression was dose-dependent and maximal upon exposure of cells to 30 J/cm^2 UVA. In contrast to FASL, FAS surface expression remained essentially unaltered upon UVA irradiation (Fig. 4).

Addition of the blocking anti-FAS antibody ZB4 (13), but not of equivalent concentrations of an isotype control antibody (Fig. 5) or an isotype-matched anti-CD4 mAb (data not shown), significantly lowered UVA radiation-induced human T helper cell apoptosis (Fig. 5).

In the following experiments, reagents capable of quenching (sodium azide) or enhancing (deuterium oxide) singlet oxygen effects were assessed for their capacity to modulate UVA radiation-induced human T helper cell apoptosis (14–16). Irradiation of cells in the presence of sodium azide
significantly inhibited UVA radiation-induced FASL surface expression as well as apoptosis in human T helper cells, whereas irradiation of cells in the presence of deuterium oxide resulted in a slight, but consistent increase in the percentage of FASL expressing as well as apoptotic cells (Fig. 6).

We next assessed whether UVA radiation-induced apoptosis could be mimicked by stimulating unirradiated human T helper cells with singlet oxygen. Singlet oxygen was generated by thermal decomposition of NDPO₂ (17). As shown in Fig. 7, singlet oxygen increased FASL surface expression in unirradiated T cells to an extent similar to that observed in UVA-irradiated cells. Similar to FASL surface expression, singlet oxygen generation also induced apoptosis in unirradiated cells (Fig. 7). NDPO₂-induced FASL surface expression as well as apoptosis were significantly enhanced, if T cells were stimulated in the presence of deuterium oxide.

Figure 1. Qualitative analysis of UVA phototherapy induced apoptosis in CD4⁺ cells present in lesional skin of a patient with atopic dermatitis. Biopsy specimens were obtained from lesional skin (flexural creases of the left elbow) of a patient with atopic dermatitis before (A) and after one (B), two (C) and three (D) exposures to UVA radiation and analyzed for apoptotic (green fluorescence) and CD4⁺ (red fluorescence) cells as described in Materials and Methods. All photographs show dermis.
UVA Radiation Phototherapy and Singlet Oxygen-induced T Cell Apoptosis

Discussion

Successful UVA phototherapy was previously found to downregulate lesional expression of the T helper cell-derived cytokine IFN-γ in atopic dermatitis (17). Interferon-γ production by intradermal T helper cells is thought to be a major cause for the generation and maintenance of eczema in atopic dermatitis patients (4, 7, 18, 19). In the present study, phototherapy led to induction of apoptosis in intradermal T helper cells, subsequent depletion of T cells from lesional atopic skin and concomitant improvement of clinical symptoms. Taken together these results indicate that the therapeutic effectiveness of UVA phototherapy for atopic dermatitis results from induction of T helper cell apoptosis. At least under in vitro conditions, human T helper cell apoptosis may be induced by short wavelength UVB radiation as well (20). Depletion of T cells from human skin via induction of apoptosis may thus represent a general mechanism relevant for phototherapy of inflammatory skin diseases.

Because of its physical properties, UVA radiation applied during phototherapy can reach the lower levels of the human dermis (5). Under conditions closely resembling the therapeutic situation, in vitro UVA irradiation induced apoptosis in atopen-specific human T helper cells indicating that phototherapy-induced apoptosis in intradermal T helper cells resulted from direct effects.

Atopen-specific CD4+ T cells employed in the present study constitutively expressed FAS antigen, as has been shown to be the case for activated human T cells (21). Unirradiated T cells did not express significant FASL surface levels, but abundant FASL expression was detected 4 h after UVA radiation and was further increased 16 h after exposure. FAS/FASL interaction may cause autocrine suicide in FAS-expressing T cells (22–24). Ultraviolet A radiation-induced FASL expression was of functional relevance, because interference with FAS/FASL interaction through addition of a blocking anti-FAS antibody effectively prevented UVA radiation-induced human T cell apoptosis. In this regard, UVA radiation-induced human T helper cell apoptosis resembled T cell apoptosis induced by anti-CD3 antibody, phorbol ester plus calcium ionophore, staphylococcal enterotoxin superantigen and cytotoxic drugs (22–26).

Figure 4. FAS and FASL expression in UVA-irradiated T helper cells. T cells were exposed to increasing doses of UVA radiation (0–30 J/cm²). 4 (A) and 16 (B) h after exposure, cells were analyzed for FAS and FASL surface expression by FACS analysis as described in Materials and Methods. Data are given as histograms of cell number versus fluorescence intensity and represent one of five essentially identical experiments.

Figure 5. UVA radiation and NDPO₂ induced T cell apoptosis. T cells were preincubated in the presence of anti-Fas antibody ZB4 (1 µg/ml) (open bar) or an isotype control antibody (solid bar) for 1 h at 37°C. Cells were then exposed to UVA radiation (30 J/cm²) or NDPO₂ (15 mM). After 4 h, the percentage of apoptotic cells was determined using the TUNEL method as described in Materials and Methods. Data represent mean ± SD of six experiments.
The present study indicates that increased FASL surface expression is important for UVA radiation-induced T cell apoptosis. Recently, it has been demonstrated that the generation of singlet oxygen is a primary mediator in UVA radiation-induced biological effects including increased expression of cell surface molecules (14–16). The capacity of sodium azide to suppress, of deuterium oxide to enhance, and of NDPO₂ to mimic UVA radiation-induced FASL expression and apoptosis indicated a prominent role for singlet oxygen in this system. Singlet oxygen-induced T cell apoptosis involved the FAS/FASL system, because blocking anti-FAS antibodies effectively inhibited human T helper cell apoptosis which was induced by UVA irradiation or stimulation with a singlet oxygen generating system. These studies demonstrate that singlet oxygen, by virtue of its capacity to activate the FAS/FASL system, serves an important role in control of human T cell apoptosis. This conclusion is supported by previous studies indicating that in human T cells FAS-mediated apoptosis was related to the generation of reactive oxygen species and dependent on the thiol-status of T cells (27). Similarly, the thiol-status of human cells was shown to control UVA radiation-induced, singlet oxygen-mediated gene expression (28, 29).

Singlet oxygen is produced by a variety of biological systems, and is a significant biochemical intermediate in several biological processes (30). The present observation that singlet oxygen can induce human T cell apoptosis has added a previously unrecognized biological activity of great importance to the list of biological effects, which were found to be mediated by singlet oxygen. Our studies also indicate that generation of singlet oxygen within human skin may constitute a therapeutic principle underlying phototherapy of inflammatory skin disease.
References


