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Caspase-9 is the upstream caspase activated by 8-methoxypsoralen and ultraviolet-A radiation treatment of Jurkat T leukemia cells and normal T lymphocytes

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A B S T R A C T

Background and Objectives. A combination of 8-methoxypsoralen and ultraviolet-A radiation (PUVA) is used for the treatment of T cell-mediated disorders, including chronic graft-versus-host disease. The mechanisms of action of this therapy, referred to as extracorporeal phototherapy, have not been fully elucidated. PUVA is known to induce apoptosis in T lymphocytes collected by apheresis, however scarce information is available concerning the apoptotic pathways activated by PUVA.

Design and Methods. We used Jurkat human T leukemia cells and normal T lymphocytes to analyze the PUVA-triggered caspase activation pattern by means of immunoblot analysis, *in vitro* caspase activity assays, and selective caspase inhibitors coupled to flow cytometric analysis.

Results. PUVA treatment induced activation of apical caspases-9 and -8, and of effector caspases-3 and -7 in Jurkat cells and human T lymphocytes. While activation of caspase-9 occurred as early as 1 h after PUVA treatment of Jurkat cells, procaspase-8 cleavage was delayed and was detected 6 h after the exposure. Also in normal T lymphocytes, cleavage of caspase-8 was subsequent to activation of caspase-9. PUVA-dependent proteolytic cleavage of procaspase-8 was blocked by inhibitors selective for either caspase-9 or -3. Moreover, procaspase-8 was cleaved *in vitro* by activated caspase-3, which gave rise to proteolytic fragments equivalent to those generated *in vivo*.

Interpretation and Conclusions. Activation of caspase-8 in PUVA-treated Jurkat cells and normal T lymphocytes is secondary to up-regulation of caspase-9. Overall, our results identify caspase-9 as the critical upstream caspase initiating apoptosis by PUVA in Jurkat T-cells and human T lymphocytes.

Key words: apoptosis, psoralen, apical caspases, effector caspases, PI3K/Akt.

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Extracorporeal phototherapy (ECP) is a treatment in which leukocytes obtained by apheresis are exposed to 8-methoxypsoralen (8-MOP) and ultraviolet (UV)-A radiation and then the treated cells are reinfused into the patient. The combination of 8-MOP and UV-A irradiation, referred to as PUVA, is a useful therapeutic approach in the treatment of skin diseases such as cutaneous T-cell lymphomas¹ and other T cell-mediated immune disorders, including chronic graft-versus-host disease (GVHD).²

8-MOP is a naturally occurring inert chemical that can be photoactivated by UV-A to become a powerful photosensitizing agent. 8-MOP intercalates between nucleic acid base pairs. UV-A activates the intercalated complex, resulting in the formation of psoralen monoadducts.³ The psoralen monoadducts lead to cross-links in

DNA strands that are believed to be the primary cause of PUVA-induced cell killing.⁴

Even though the mechanisms of action of PUVA treatment are still elusive, available evidence suggests it induces apoptosis in Jurkat T cells⁵ and lymphocytes.⁶ However, scarce information is available concerning the mechanisms underlying the apoptotic cell death effected by PUVA.

A mechanism central to apoptosis is activation of a family of cysteine proteases referred to as caspases. Caspases are synthesized as proenzymes (holoenzymes) which are activated by cleavage. Caspases-2,-8,-9, and -10, termed apical caspases, are usually the first to be stimulated in the apoptotic process and they then activate effector caspases (namely caspase-3, -6, and -7).⁷

Among the apical caspases, caspase-9 is activated in response to internal insults

such as DNA damage, whereas caspases-8 and -10 are effectors of the death-receptor-mediated apoptotic signaling pathway, initiated, for example, by CD95.⁷ However, there are reports showing that DNA-damaging agents induce CD95 ligand (CD95L) expression and CD95 receptor-ligand interactions, which lead to activation of caspase-8.⁸

It is well known that UV radiation has multiple cellular targets that trigger different apoptosis cascades: one leads to release of mitochondrial cytochrome c and activation of caspase-9,⁹ while the other causes ligand-dependent and -independent clustering or activation of membrane death-receptors thus resulting in the activation of caspase-8.¹⁰

It has been shown that PUVA treatment of Jurkat T cells causes mitochondrial dysfunction, a decrease in mitochondrial transmembrane potential ($\Delta\psi_m$), and release of cytochrome c⁵ but it is not known whether this results in subsequent activation of caspase-9. Furthermore, in mouse epidermal cells PUVA activates effector caspase-3.¹¹ However, a comprehensive investigation of the caspase activation pattern induced by PUVA treatment is lacking.

Here, we have investigated the caspase activation pattern of PUVA-treated Jurkat T cells and T lymphocytes from healthy donors. We show that PUVA treatment activates caspases-9, -8, -7, and -3. However, activation of caspase-8 was later than that of caspase-9, and it was completely inhibited by pre-treating Jurkat cells with inhibitors selective for either caspase-9 or caspase-3. Taken together, these findings suggest that, in Jurkat T cells and normal T lymphocytes, activation of caspase-8 by PUVA is secondary to activation of caspases-9 and -3.

Design and Methods

Materials

Bovine serum albumin (BSA), normal goat serum (NGS), monoclonal antibody to β -tubulin, and peroxidase-conjugated anti-mouse or anti-rabbit IgG were from Sigma, St. Louis, MO, USA. The COMPLETE Protease Inhibitor Cocktail, and the Lumi-Light^{Plus} enhanced chemiluminescence (ECL) detection kit were from Roche Applied Science, Milan, Italy. The ApoAlert Caspase-3 fluorometric assay kit was from BD Biosciences Clontech (Palo Alto, CA, USA). The Caspase-7 fluorometric assay kit was from Cell Signaling Technology (Beverly, MA, USA). Activated human recombinant caspase-3, Z-VAD-FMK (a broad inhibitor of effector caspases), Z-DEVD-FMK (a caspase-3 selective inhibitor), VEID-CHO (a caspase-6 selective inhibitor), Z-IETD-FMK (a caspase-8 selective inhibitor), and Z-LEHD-FMK (a caspase-9 selective inhibitor), CHAPS, Wortmannin, SH-6,

and PD98059 were from Calbiochem (La Jolla, CA, USA). Monoclonal antibody to human procaspase-9 (clone B40) was from BD Transduction Laboratories (San Diego, CA, USA). The following antibodies were from Cell Signaling Technology: mouse monoclonal to human caspase-8 (clone 1C12, #9746), rabbit polyclonals to caspase-3 (#9662), caspase-6 (#9762), caspase-7 (#9492), caspase-10 (#9752), poly (ADP-ribose) polymerase (PARP, #9542), total Akt (#9272), and to Ser 473 phosphorylated Akt (p-Akt).

Cell culture

Human Jurkat T lymphoblastoid cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (complete medium). Caspase inhibitors (final concentration: 100 μ M) were added to the cells 1 h prior to PUVA treatment, from a 25 mM stock in dimethyl sulfoxide (DMSO).

Isolation and liquid culture of normal T lymphocytes

Peripheral blood from normal donors was collected in heparin tubes after informed consent. T cells were isolated by negative selection. Briefly, lymphocytes from normal donors were obtained by Ficoll-Hypaque gradient separation. Non-T-cells were labeled with a cocktail of biotinylated monoclonal antibodies, followed by anti-biotin magnetic microbeads (Pan T cell Isolation Kit II, Miltenyi Biotec, Bergish Gladbach, Germany). Non-T cells were subsequently eliminated with MACS columns and Vario MACS equipment (Miltenyi). Purification (>95%) was checked by flow cytometry utilizing a fluorescein isothiocyanate (FITC)-conjugated CD3 monoclonal antibody (Beckman-Coulter Immunology, Miami, FL, USA). Two milliliters of the cell suspension were seeded in Petri dishes. Untreated and PUVA-treated T cells were resuspended at a concentration of 5×10^6 /mL cells in complete medium containing phytohemagglutinin (0.5 mg/mL).

PUVA treatment

Cells seeded in Petri dishes (10^6 /mL) were incubated with 8-MOP (200 ng/mL, Gerotz Pharmazeutika, Vienna, Austria) in serum-free medium for 5 min at room temperature and then exposed to 2.0 J/cm² of UV-A light. UV-A irradiation was performed by the PUVA Combi Light irradiator (DERMAT BVBA, Heverlee, Belgium). After treatment cells were incubated in complete medium at 37°C for the indicated times.

Flow cytometric analysis

For sub-G1 (apoptotic cells) peak analysis, cells were fixed with 70% cold (4°C) ethanol for 1h and subsequently stained with propidium iodide (PI, DNA-Prep kit, Beckman-Coulter Immunology). The subdiploid DNA

content was analyzed and calculated using an Epics XL flow cytometer with the appropriate software (Beckman-Coulter Immunology). At least 10,000 events were acquired.

Measurement of caspase-3 and caspase-7 activities

These activities were measured using fluorimetric assays according to the manufacturer's instructions, using DEVD-7-amino-4-trifluorethyl coumarin (DEVD-AFC) as the substrate for caspase 3 and Ac-DEVD-AFC as the substrate for caspase 7, as reported elsewhere.¹²

Preparation of cell homogenates and immunoblot analysis

Cells, washed twice in PBS containing the COMPLETE Protease Inhibitor Cocktail, were then lysed at $\sim 10^7$ /mL in boiling electrophoresis sample buffer containing the protease inhibitor cocktail. Protein (80 μ g/lane), separated on SDS-polyacrylamide gels, was transferred to nitrocellulose sheets. The sheets were saturated in PBS containing 5% NGS and 4% BSA for 60 min at 37°C (blocking buffer), then incubated overnight at 4°C in blocking buffer containing the primary antibody. After four washes in PBS containing 0.1% Tween-20, they were incubated for 30 min at room temperature with peroxidase-conjugated secondary antibody, diluted 1:5,000 in PBS-Tween-20, and washed as above. Bands were visualized by the ECL method. To ensure equal loading, blots were always first probed with an antibody to β -tubulin, then stripped and re-probed. All the blots are representative of three separate experiments.

In vitro caspase-8 cleavage

Jurkat cells were lysed in 25 mM Hepes-NaOH, pH 7.4, 10% sucrose, 0.1% CHAPS, 2 mM EDTA, 5 mM DTT. Cell lysates were spun down for 3 min in a microfuge, and a protein assay was performed on the supernatant. Fifty nanograms of activated caspase-3 were added to 50 μ g of protein and incubation proceeded for 3 h at 37°C. The caspase-9 inhibitor was present at 100 nM (from a 1 mM stock solution in DMSO).¹³

Results

PUVA induces apoptosis in Jurkat T cells and normal T lymphocytes

We first investigated whether PUVA treatment induced apoptotic cell death of Jurkat cells. Flow cytometric analysis of PI-stained samples showed that after 6 h of treatment there was a significant increase in the number of apoptotic cells, evaluated as a sub-

Table 1. Percentage of apoptotic cells in response to PUVA treatment. Jurkat T cells and normal T lymphocytes were subjected to PUVA and the analysis of PI-stained samples by flow cytometry was carried out at different times after treatment. Results are the mean of three different experiments \pm SD. An asterisk indicates a statistically significant difference ($p < 0.01$).

Time elapsed from treatment	Control (no treatment)	PUVA treatment
Jurkat cells 0 h	1.0 \pm 1.0	–
Jurkat cells 1 h	1.2 \pm 1.1	1.5 \pm 1.0
Jurkat cells 3 h	1.9 \pm 1.4	2.9 \pm 1.7
Jurkat cells 6 h	2.7 \pm 1.6	28.2 \pm 4.6
Jurkat cells 12 h	4.9 \pm 2.8	45.5* \pm 6.6
Jurkat cells 24 h	5.8 \pm 3.6	59.2* \pm 7.9
T lymphocytes 0 h	1.4 \pm 0.8	–
T lymphocytes 24 h	2.7 \pm 1.3	15.5* \pm 2.9
T lymphocytes 36 h	3.5 \pm 1.8	43.3* \pm 7.2
T lymphocytes 48 h	3.2 \pm 1.6	61.3* \pm 8.8

diploid peak (Table 1). The number of apoptotic cells progressively increased by 12 h and 24 h after PUVA treatment. In contrast, in untreated cells the percentage of apoptotic cells increased only very slightly (Table 1). We then analyzed PUVA-treated human T lymphocytes for apoptosis. As shown in Table 1, a significant increase in the number of apoptotic cells began to appear 24 h after treatment. Subsequently, the percentage of apoptotic cells rose dramatically at 36 and 48 h. As a control, we evaluated whether treatment with either methoxypsoralen or UV-A alone could induce apoptotic cell death. However, no significant increase in the amount of apoptotic cells was measured in Jurkat cells or T lymphocytes (*not shown*).

Activation of caspases and cleavage of PARP in response to PUVA treatment

We first investigated the possible activation of apical caspases (caspases-9, -8, and -10) in Jurkat T cells. Cleavage of procaspase-9 was detected as early as 1 h after treatment (Figure 1). Since we employed an antibody that only recognizes procaspase-9, we saw a decrease in the intensity of the band corresponding to the holoenzyme but not to its proteolytic fragments. Cleavage of procaspase-8 into the characteristic 43/41-kDa proteolytic fragments was apparent 6 h after treatment and was accompanied by a decrease in the amount of the holoenzyme. Thereafter, at 12 h after PUVA exposure the cleavage was much more pronounced. In contrast, caspase-10 was not cleaved even in samples taken 24 h after treatment.

We then investigated effector caspases (caspases-3, -7, and -6) in Jurkat T cells (Figure 2). Caspase-3 was cleaved: the 24-kDa proteolytic fragment of caspase-3

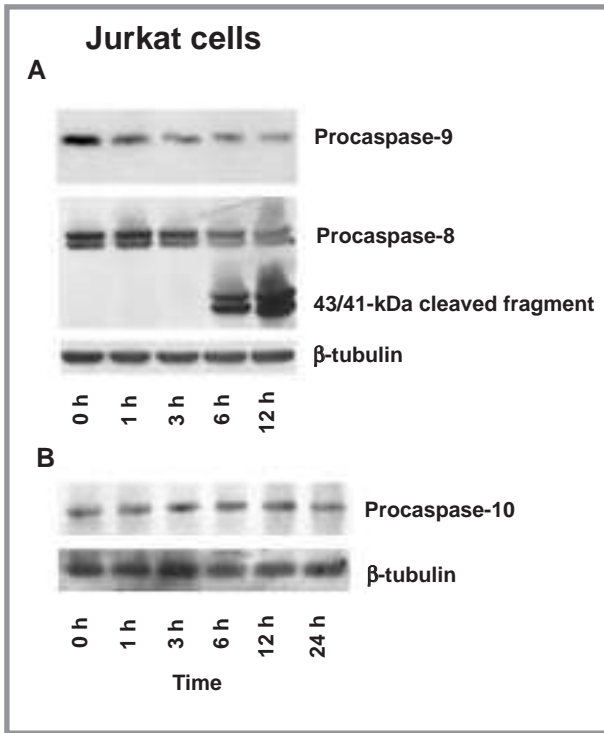


Figure 1. PUVA treatment of Jurkat cells results in proteolytic cleavage of procaspases-9 and -8. Samples at different times after PUVA exposure were analyzed by immunoblotting for procaspases -9, -8, and -10.

was already apparent 6 h after treatment and its amount dramatically increased at 12 h. Cleavage of caspase-3 was almost completely blocked if the cells had been incubated, prior to PUVA exposure, with a caspase-9 selective inhibitor (Z-LEHD-FMK). An inhibitor of caspase-8 (Z-IETD-FMK) also reduced proteolytic activation of caspase-3, even though it was not as effective as Z-LEHD-FMK. The time course of proteolytic cleavage of caspase-7 was similar to that of caspase-3, and its cleavage was inhibited by both Z-LEHD-FMK and Z-IETD-FMK, with the latter being less effective. In contrast, we did not detect proteolysis of caspase-6, even when we studied samples as late as 24 h after treatment. Cleavage of the caspase-3 and -7 substrate, PARP, was evident 6 h after treatment. PARP proteolysis was completely inhibited by pre-incubation with the caspase-9 inhibitor, while the caspase-8 inhibitor was less effective.

As far as T lymphocytes from healthy donors were concerned, a decrease in the amount of procaspase-9 began to appear 6 h after PUVA treatment (Figure 3). In contrast, cleavage of procaspase-8 was a much later phenomenon, because we detected it at 24 h. Caspase-10 was not cleaved. The cleavage of effector caspases-3 and -7 was seen at 24 h and increased at 48 h. Caspase-6 was not cleaved (Figure 3).

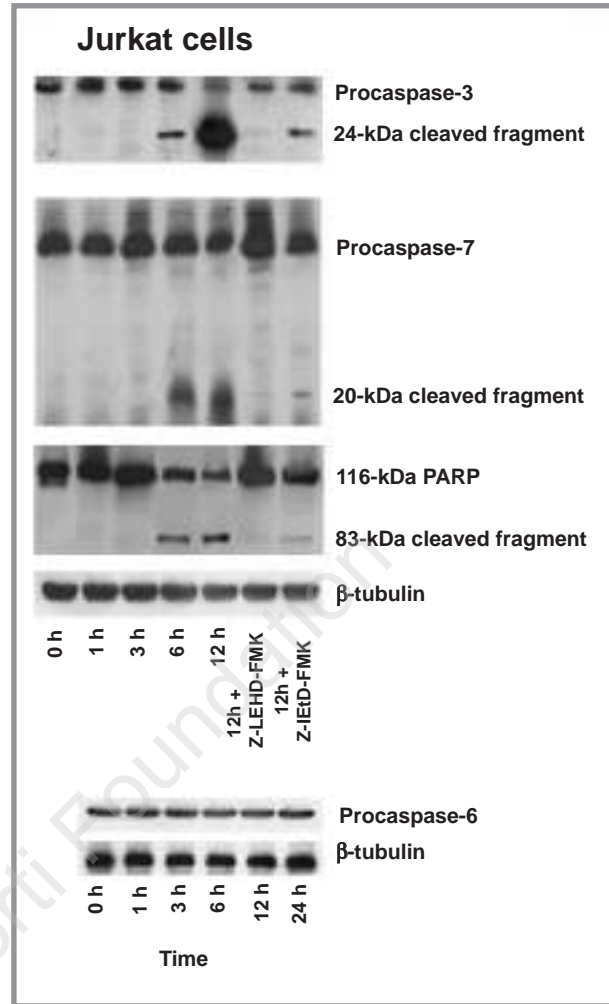


Figure 2. Procaspases -3 and -7, and PARP are cleaved in Jurkat cells in response to PUVA exposure. Samples at different times after PUVA treatment were analyzed by immunoblotting for procaspases -3, -6, -7, and for PARP. The caspase inhibitors (Z-LEHD-FMK, Z-IETD-FMK) had been present at 100 μ M for 1 h prior to PUVA treatment.

Fluorimetric measurement of caspase-3 and -7 activities in Jurkat cells

Activation of the two effector caspases, caspase-3 and -7, was also studied by *in vitro* enzymatic assays. As shown in Figure 4, massive activation of caspase-3 was detected 12 h after PUVA treatment of Jurkat T cells. The activation was markedly reduced by a Z-LEHD-FMK and, to a lesser extent, by Z-IETD-FMK. A dramatic increase in caspase-7 activity was also evident after 12 h. Caspase-7 activity could be inhibited by caspase-9 and caspase-8 inhibitors, although the latter were less effective. Overall, the results obtained by *in vitro* fluorimetric assays were in good agreement with the findings provided by immunoblots.

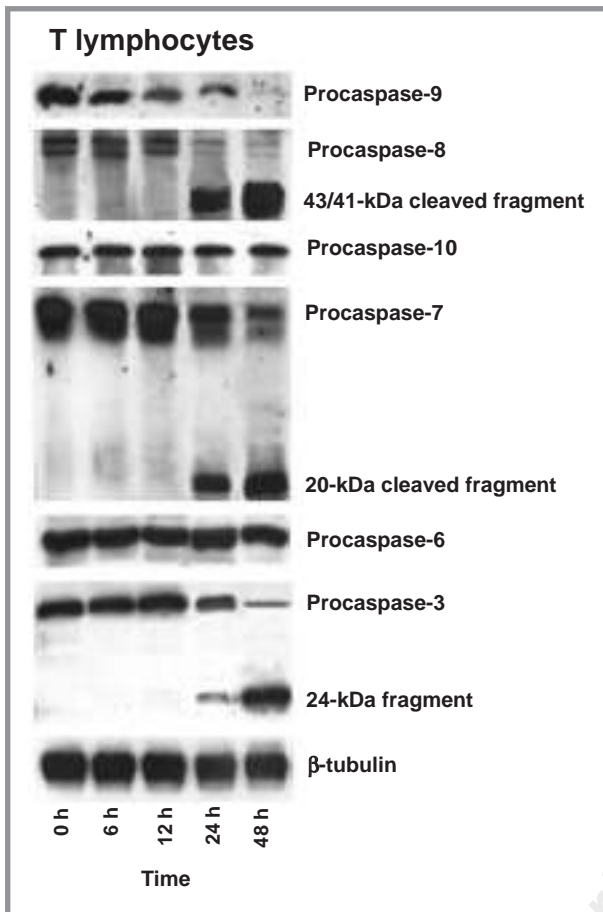


Figure 3. Procaspases -9, -8, -3, and -7 are cleaved in normal T lymphocytes in response to PUVA treatment. Samples from healthy donors at different times after PUVA exposure were analyzed by immunoblotting.

Effect of caspase inhibitors on PUVA-induced apoptosis of Jurkat cells

To evaluate the relevance of caspase activation in the induction of apoptosis, we used peptide inhibitors which are known to be selective for the activated caspases. As shown in Figure 5, flow cytometric analysis of PI-stained samples showed that Z-VAD-FMK (a broad inhibitor of effector caspases) almost completely blocked apoptotic cell death. An inhibitor selective for caspase-3 (Z-DEVD-FMK) also markedly reduced the number of cells undergoing apoptotic cell death. VEID-CHO (an inhibitor of caspase-6, which is not activated in response to PUVA exposure) did not affect the percentage of apoptotic cells at all. This result confirmed the selectivity of the inhibitors. Since no selective inhibitors for caspase-7 are available, we could not evaluate the relative contribution of this effector caspase to the apoptotic process.

As far as apical caspases were concerned, an inhibitor of caspase-8 (Z-IETD-FMK) reduced the num-

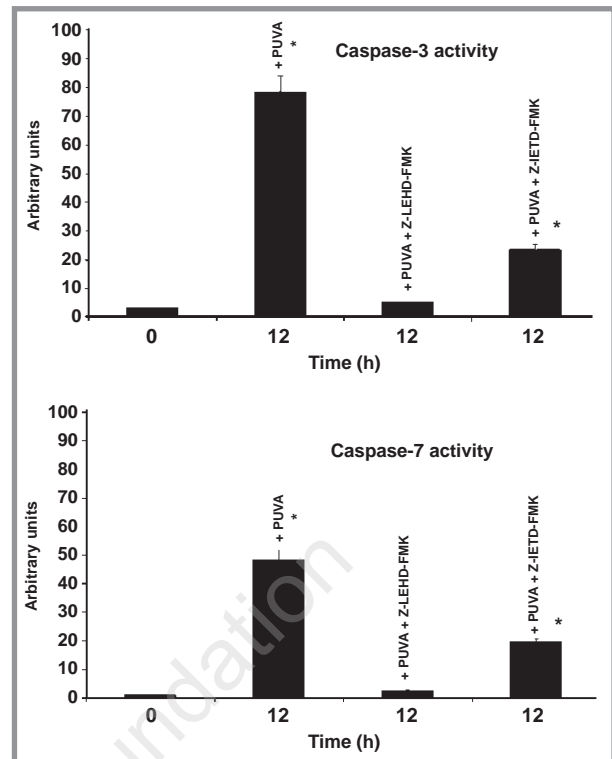


Figure 4. *In vitro* activity of caspases -3 and -7 is increased in response to PUVA. Jurkat T cells were treated with PUVA as indicated in Materials and Methods. The caspase inhibitors (100 μ M) had been present for 1 h prior to treatment. Results are the mean of three different experiments \pm SD. The asterisks indicate statistically significant differences ($p < 0.01$) in comparison with untreated cells (time 0 h).

ber of apoptotic cells, while an inhibitor of caspase-9 (Z-LEHD-FMK) almost completely prevented apoptotic cell death (Figure 5).

Inhibition of caspase-9 or -3 blocks activation of caspase-8

Since it has been shown that PUVA treatment of lymphocytes results in increased expression of CD95/CD95L,¹⁴ the cleavage of caspase-8 could be due to direct activation of the death-receptor pathway. However, it could also be secondary to a caspase-3-linked cleavage, as reported, for example, in UV-induced apoptosis of human keratinocytes.¹³ Our preliminary experiments, performed by flow cytometry, had shown that neither CD95 nor CD95L surface expression increased after PUVA treatment of Jurkat T cells (*data not presented*). However, to explore this possibility further, we first used selective caspase inhibitors. As shown in Figure 6A, when Jurkat T cells had been pre-treated with a caspase-9 inhibitor (Z-

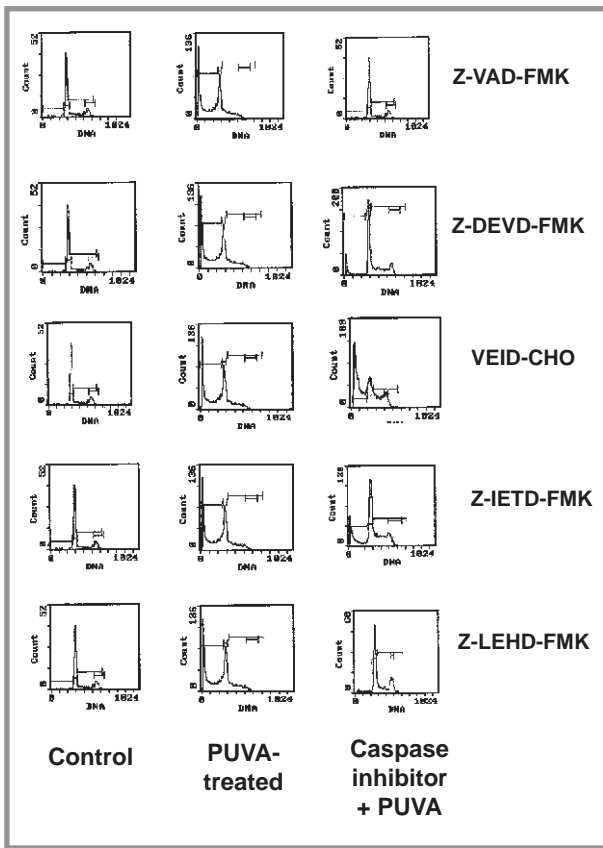


Figure 5. Caspase peptide inhibitors prevent PUVA-induced apoptotic cell death. Jurkat T cells were incubated for 1 h with the indicated caspase inhibitors (100 μ M), subjected to PUVA, then incubated for 12 h. At the end of the incubation flow cytometric analysis of PI staining was carried out. The addition of an equal amount of carrier solvent (DMSO) had no effect (*not shown*). For each condition, a representative histogram of three different experiments is shown.

LEHD-FMK) or a caspase-3 inhibitor (Z-DEVD-FMK), no proteolytic cleavage of caspase-8 could be detected by immunoblotting performed on samples 12 h after PUVA treatment. These results pointed to the likelihood that, in our system, activation of caspase-8 is secondary to the activation of effector caspase-3 which is in turn activated by caspase-9.

***In vitro* cleavage of caspase-8 by activated caspase-3**

As further proof that caspase-8 could be cleaved by caspase-3, we performed experiments on cell extracts of Jurkat T cells. The addition of activated human recombinant caspase-3 resulted in the cleavage of procaspase-8, as demonstrated by immunoblot analysis showing a marked reduction of the holoenzyme and appearance of the 43/41- kDa cleaved fragments (Figure 6B). The cleavage could not be consequent to

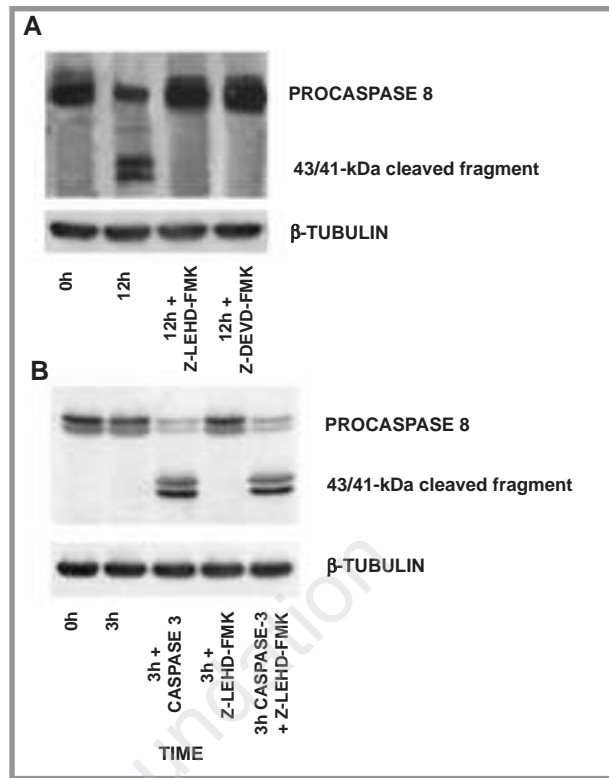


Figure 6. Procaspase-8 is cleaved in a caspase-9- and -3-dependent manner following treatment with PUVA. **A:** Immunoblot analysis for *in vivo* cleavage of procaspase-8. Jurkat cell samples were incubated for 1 h with the indicated caspase inhibitors (100 μ M), subjected to PUVA, then further incubated for 12 h. Blots are representative of three separate experiments. **B:** *In vitro* cleavage of procaspase-8 by active human recombinant caspase-3. Jurkat T cell extracts (50 μ g of protein) were incubated at 37°C for 3 h in the presence of 50 ng of activated caspase-3. Z-LEHD-FMK (100 nm) had been pre-incubated with the samples for 30 min prior to addition of caspase-3. The incubation was stopped by adding electrophoresis sample buffer.

caspase-9 activation, because the presence of a caspase-9 inhibitor (Z-LEHD-FMK) during the *in vitro* assay did not prevent cleavage of procaspase-8 by activated caspase 3.

Inhibition of the phosphoinositide 3 kinase (PI3K)/Akt cascade potentiates PUVA-induced apoptosis

Since the therapeutic effect of PUVA treatment could somehow be related to the induction of apoptosis,¹⁵ it might be desirable to enhance the number of apoptotic cells in response to PUVA. The PI3K/Akt signaling network is extremely important as it has a powerful anti-apoptotic effect.¹⁶ We, therefore, decided to investigate whether inhibition of the PI3K/Akt pathway could result in a higher percentage of apoptotic cells following PUVA exposure.

To inhibit the PI3K/Akt signaling network we used

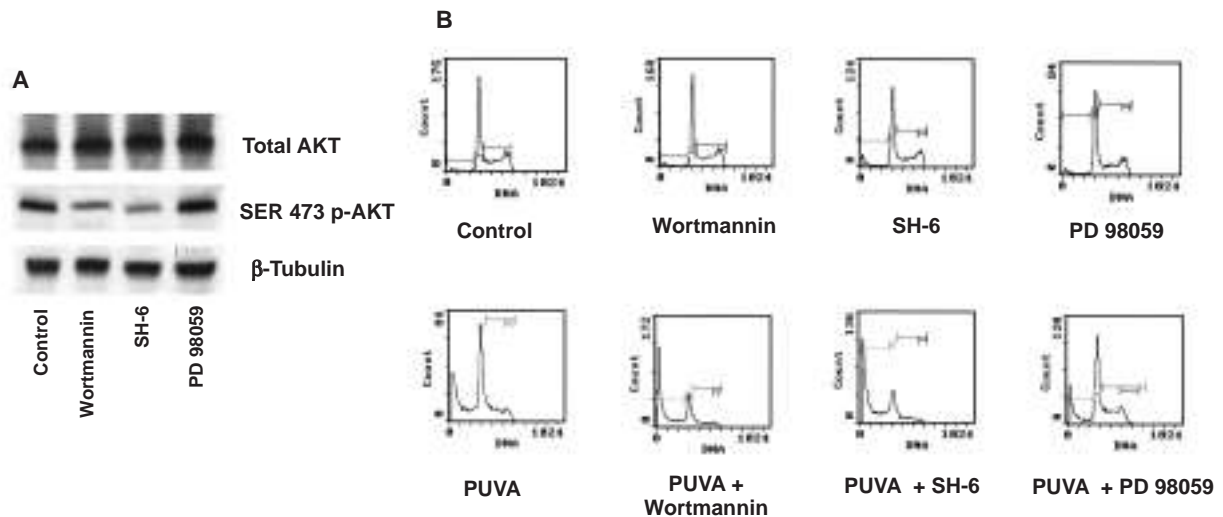


Figure 7. Inhibitors of the PI3K/Akt signaling pathway enhance PUVA sensitivity of Jurkat T cells. **A:** Immunoblot analysis for total Akt and Ser 473 p-Akt. Cells were incubated for 2 h with Wortmannin (300 nM), SH-6 (10 μ M), or PD98059 (50 μ M). **B:** Flow cytometric analysis for PI staining of samples treated with the inhibitors alone or exposed to inhibitors followed by PUVA treatment. Samples were analyzed 12 h after exposure to PUVA. For each condition, a representative histogram of three different experiments is shown.

either Wortmannin, a pharmacological inhibitor of PI3K, or SH-6, which is selective for Akt.¹² Moreover, as a control, we employed PD98059, an inhibitor of the extracellular-regulated kinase (ERK) pathway, which is also important for cell survival.¹⁷ As shown in Figure 7, immunoblot analysis with an antibody to total Akt revealed that the inhibitors did not affect the expression of Akt. However, when the analysis was performed with an antibody to Ser 473 p-Akt (an active form of the enzyme), we detected a decrease in the amount of this p-Akt form in response to either Wortmannin or SH-6, but not to PD98059. Flow cytometric analysis revealed that incubation with either Wortmannin or SH-6 significantly increased the percentage of apoptotic Jurkat T cells in response to PUVA treatment, whereas PD98059 was not effective. Wortmannin or SH-6 *per se* only very slightly increased the percentage of apoptotic cells (Figure 7).

Discussion

ECP treatment is primarily utilized for the therapy of cutaneous T-cell lymphoma, even though it has also been shown to be of clinical benefit in the treatment of other T-cell mediated conditions, including chronic GVHD and autoimmune disorders. Although many aspects of PUVA therapy have been studied quite extensively, the molecular mechanisms by which it kills cells have not been fully elucidated. Hence, we reasoned that it would be important to gain more insight into the mechanisms of cell death mediated by PUVA, because

they might provide a way to modulate the effectiveness of PUVA as well as its clinical outcome.

Our results showed that PUVA treatment of Jurkat cells and normal T lymphocytes induces activation of the apical caspases -9 and -8, as well as of the effector caspases -3 and -7. In contrast, apical caspase-10 and effector caspase-6 are not activated. Our time course study showed caspase-9 to be the first caspase to be activated, this activation occurring as early as 1 h after treatment in Jurkat cells, whereas in normal T lymphocytes it was detected after 6 h. Caspase-9 activation is consistent with the occurrence of mitochondrial dysfunction reported by others in PUVA-treated Jurkat cells.⁵ In Jurkat cells, apical caspase-8, and effector caspases-3, and -7 showed activation after 6 h. This delay might be consistent with apoptosome complex formation consequent to mitochondrial damage,¹⁸ occurring prior to full activation of the caspase cascade. Also in normal T lymphocytes, activation of caspases-8, -3, and -7 was delayed in comparison with activation of caspase-9. Our unpublished results showed that neither UV-A nor 8-MOP, if used separately, activated caspases in Jurkat cells or normal T lymphocytes, consistently with the lack of apoptosis induction. The dramatic inhibition of PUVA-induced apoptosis by Z-LEHD-FMK strongly suggested that caspase-9 was the dominant upstream caspase in PUVA-treated Jurkat T cell apoptosis. The inhibition caused by Z-LEHD-FMK and by Z-VAD-FMK (which has a wider inhibition pattern) were similar, while Z-IETD-FMK, a specific inhibitor of caspase-8, was less effective. Preincubation of Jurkat cells with Z-DEVD-FMK, an inhibitor of caspase-3, resulted in inhibition of PUVA-induced

apoptosis. However, some cells underwent apoptosis, thus indicating that also caspase-7 is important for the execution phase of apoptosis in this system. The fundamental role of caspase-9 in triggering Jurkat cell apoptosis in response to PUVA was also demonstrated by activity assays for caspases-3 and -7. Indeed, Z-DEVD-FMK almost completely prevented activation of these caspases, whereas Z-IETD-FMK was less effective.

Interestingly, the activation of caspase-8 by PUVA in Jurkat cells was completely blocked by either Z-LEHD-FMK (caspase-9 specific) or Z-DEVD-FMK (caspase-3 specific). Therefore, one possible explanation is that caspase-8 activation seen after PUVA exposure was not due to death receptor activation but rather resulted from activation of other caspases that are dependent on caspase-9, such as caspase-3. In this connection, it is important to emphasize that procaspase-8 has a potential caspase-3 cleavage site at position DEAD398₊, and cleavage at this site would generate fragments with a molecular weight in good agreement with the sizes of known caspase-8 cleaved products, such as the 43/41 kDa fragments.¹⁹ To explore this possibility, we determined whether procaspase-8 present in Jurkat T cell extracts could be cleaved *in vitro* by human recombinant catalytically active caspase-3. A marked cleavage of procaspase-8 into the 43/41-kDa fragments was observed after 3 hours of incubation. Importantly, in this cell-free system the cleavage was not prevented by Z-LEHD-FMK caspase-9 inhibitor. Therefore, our data are in good agreement with the findings of Sitailo *et al.*¹³ who demonstrated that, in UV-induced apoptosis of human keratinocytes, activation of caspase-8 is directly or indirectly dependent on caspase-3 which, in turn, has been activated by upstream caspase-9. However, these authors did not prove that activated caspase-3 could cleave procaspase-8 *in vitro*, whereas we have done so.

We also established that inhibitors of the PI3K/Akt

survival pathway markedly increased Jurkat T-cell sensitivity to PUVA. Interestingly, Jurkat T cells are known to display sizable levels of active (phosphorylated) Akt,²⁰ hence this signaling pathway seemed an attractive target to increase the efficacy of PUVA treatment.

In conclusion, we feel that we have identified some of the critical mechanisms underlying PUVA-induced apoptosis in Jurkat T cells and normal T lymphocytes. In bone marrow transplantation 30–60% of allogeneic transplants are affected by chronic GVHD.²¹ The effectiveness of PUVA treatment in cutaneous chronic GVHD has been demonstrated and this type of treatment is effective in patients resistant to conventional immunosuppressive therapy²² or who tolerate other treatments poorly.²³

In addition, even though the relative contribution of apoptosis to the therapeutic effect of PUVA is still unclear,^{24–25} we feel that it might be very important to have tools which can increase the number of apoptotic cells in response to this treatment, thus possibly enhancing the tolerogenic response. The sensitizing drugs might be easily administered to the removed lymphocytes and then washed out prior to re-infusion. This would not result in systemic toxicity by the inhibitors and could improve the therapeutic outcome.

Further studies on the apoptosis signaling networks activated by PUVA carried out on leukopheresed lymphocytes of treated patients should increase our knowledge in this field.²⁶

AMM: conception and design of the experiments, drafting of the article; AC: conception and design of the experiments; PLT: conception and design of the experiments, drafting of the article; AMB: conception and design of the experiments; CT: critical revision of the article for important intellectual concept; FR: conception and design of the experiments; FF: conception and design of the experiments; RC: final approval of the version to be published. The authors reported no potential conflicts of interest.

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**AMM and AC contributed equally to this work.*

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