Different Susceptibility of Malignant versus Nonmalignant Human T Cells Toward Ultraviolet A-1 Radiation-Induced Apoptosis

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Ultraviolet (UV) A-1 (340–400 nm) radiation is highly effective in inducing apoptosis in skin-infiltrating T cells and thereby exerts beneficial effects in patients with T cell-mediated skin diseases. In this *in vitro* study, we report that malignant and normal T cells differ in their susceptibility toward UVA-1 radiation-induced apoptosis. Dose–response studies revealed that malignant CD4$^+$ T cells isolated from a patient with adult T cell leukemia and Sezary's syndrome as well as malignant T cell lines exhibited a significantly higher susceptibility toward UVA-1 radiation-induced apoptosis 4 h (early apoptosis) and 24 h (late apoptosis) after exposure than normal, CD4$^+$ T cells. This difference was specific for UVA-1 irradiation because it was not detected when apoptosis was induced in these cells through exposure to UVB radiation or stimulation with cell-permeable ceramides. It has been shown that UVA-1 radiation-induced T cell apoptosis is initiated through the generation of singlet oxygen. This is in agreement with the present observation that stimulation of unirradiated cells with a singlet oxygen-generating system induced apoptosis in malignant cells to a greater extent than in normal cells. Moreover, downregulation of FAS surface expression in malignant T cells was associated with the inhibition of UVA-1 radiation/singlet oxygen-induced apoptosis in these cells. It was thus of great interest to learn that addition of the caspase inhibitor Z-VADfmk decreased and interferon-γ stimulation, which is known to upregulate caspase levels including caspase-3, increased the sensitivity of T cells toward UVA-1 radiation-induced apoptosis. Furthermore, malignant T cells had significantly higher procaspase-3 levels when compared with normal cells. These studies indicate that the susceptibility of human T cells toward UVA-1 radiation-induced apoptosis is related to the availability of caspases such as caspase-3 and that strategies directed at upregulating caspase levels will increase the efficacy of UVA-1 phototherapy.

Key words: singlet oxygen/caspase 3/ultraviolet A-1/apoptosis.
J Invest Dermatol 122:477–483, 2004

Ultraviolet A-1 (UVA-1; 340–400 nm) radiation phototherapy was first used a decade ago for the treatment of patients with acute, severe exacerbation of atopic dermatitis (Krutmann *et al*, 1992). Since then, its indication spectrum has been gradually extended and now includes other T cell-mediated skin diseases such as cutaneous T cell lymphoma as well (Krutmann *et al*, 2001; Krutmann and Morita, 2003). This development has been fostered by mechanistic studies that were conducted in atopic dermatitis patients undergoing UVA-1 phototherapy. UVA-1 irradiation of lesional atopic skin was found to be associated with depletion of skin-infiltrating CD4$^+$ T cells, a concomitant downregulation of the *in situ* expression of T helper cell-derived cytokines and the subsequent clearance of eczema (Grewe *et al*, 1994, 1998). Further *in vivo* studies revealed that T cell depletion was preceded by the induction of apoptosis in skin-infiltrating T cells and it is therefore now generally

accepted that induction of T cell apoptosis is the basic mechanism of action of UVA-1 phototherapy (Morita *et al*, 1997).

The signaling pathways involved in UVA-1 radiation-induced T cell apoptosis have only partially been characterized. *In vitro*, UVA-1 radiation was shown to induce apoptosis in CD4$^+$ T cells by a mechanism that was initiated through the generation of singlet oxygen and subsequently involved the FAS/FASL system (Morita *et al*, 1997). It should be noted that UVA-1 radiation caused apoptosis in human T cells in a biphasic manner with the induction of early apoptosis after only 1 to 4 h after exposure through a pathway that is independent of *de novo* protein synthesis (preprogrammed cell death) and the induction of late apoptosis after 16 to 24 h, which requires protein synthesis (Godar, 1999a).

In patients with atopic dermatitis, optimal therapeutic responses seem to require relatively high doses of UVA-1 radiation, that is, single doses up to 130 J per cm^2 (Dittmar *et al*, 2001; Krutmann *et al*, 2001). In contrast, equivalent clinical responses could be observed in patients with cutaneous T cell lymphoma (mycosis fungoides), regardless of whether 130 J per cm^2 or less than half of this dose, 60 J

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per cm^2 were used (Plettenberg *et al*, 1999). We therefore speculated that malignant T cells might be more sensitive to UVA-1 irradiation compared with normal, nonmalignant T cells. We were particularly interested in testing this hypothesis because the existence of T cell populations with different UVA-1 susceptibilities, besides the obvious clinical implications, would provide a unique experimental system to further study the molecular pathways involved in UVA-1 radiation-induced apoptosis.

In this *in vitro* study we confirm our hypothesis and demonstrate for the first time that the sensitivity of human T cells toward UVA-1 radiation-induced apoptosis is defined at the level of caspase-3 availability.

Results

To test the hypothesis that malignant T cells are more susceptible to UVA-1 radiation-induced apoptosis than normal cells, CD4$^+$ T cells that were freshly isolated from a patient with adult T cell leukemia, from a patient with Sezary's syndrome, and from healthy human volunteers were exposed *in vitro* to UVA-1 radiation. Dose–response experiments revealed that 24 h after irradiation, significantly more malignant than normal cells were apoptotic (Fig 1*a*; p<0.05). A similar difference between normal and malig-

nant T cell lines was also observed when normal cells were compared with malignant cells from five different CD4$^+$ T cell lines (Fig 1*b*; p<0.05). This difference could also be detected when malignant (MOLT4) cells and normal cells were analyzed 4 h after exposure (Fig 1*c*; p<0.05). Note that analysis at 4 h only revealed T cell apoptosis, which is due to early protein synthesis-independent mechanisms, whereas measurements at 24 h after exposure detected both early and late T cell apoptosis (Morita *et al*, 1997; Godar, 1999a). In all future experiments, measurements were therefore routinely carried out 24 h after irradiation.

To assess whether the observed differences in UVA-1 radiation-induced apoptosis were due to differences in T cell activation, normal T cells were stimulated for 16 h with 1 μg of the T cell mitogen concanavalin A ("activated T cells") or left unstimulated ("resting T cells"). Concanavalin A stimulation slightly increased the percentage of apoptotic T cells in unirradiated cells by about 5% (Fig 1*d*). Upon UVA-1 irradiation, however, no significant differences in the percentage of apoptotic cells was detected between resting and concanavalin A-stimulated cells.

To study the specificity of this difference, in the following experiments, T cell apoptosis was induced in these cells either through UVB irradiation or stimulation with cell permeable C2- and C6-ceramides. In marked contrast to UVA-1 irradiation, neither UVB irradiation nor stimulation
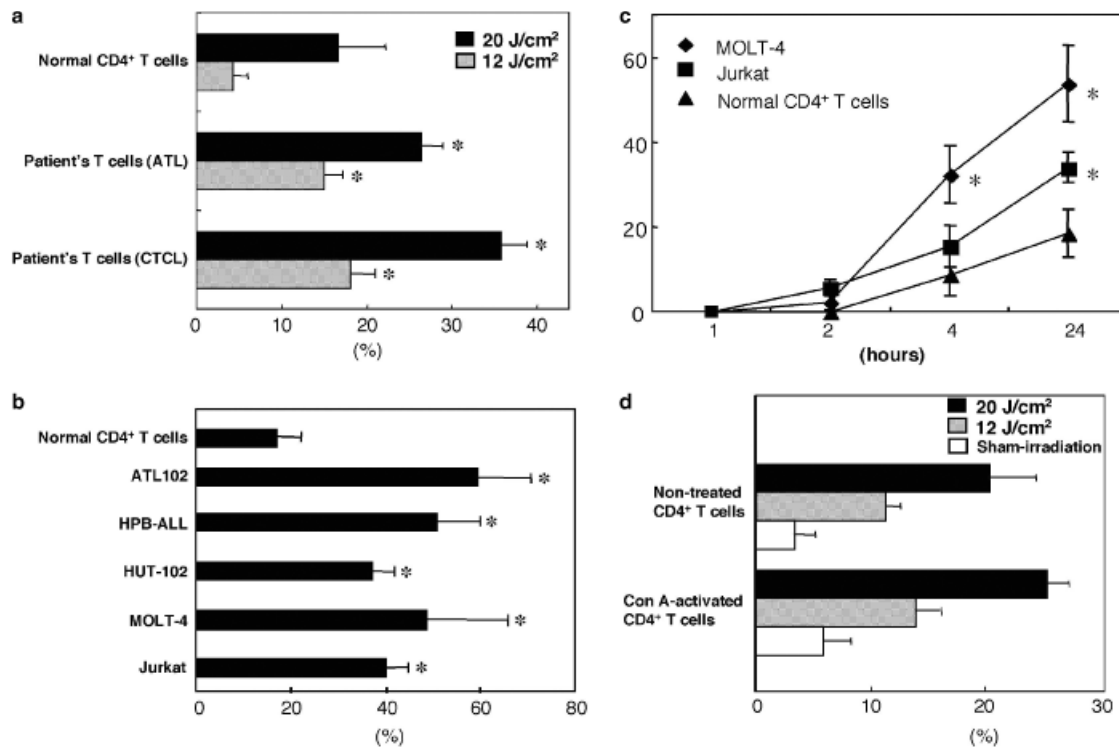

Figure 1
Malignant T cells have a higher sensitivity than normal T cells toward UVA-1 radiation-induced apoptosis. (*a*) Freshly isolated malignant T cells from adult T cell leukemia and cutaneous T cell lymphoma (Sezary's syndrome) show a higher susceptibility toward UVA-1 radiation-induced apoptosis compared with normal CD4$^+$ T cells. (*b*) Malignant T cell lines are significantly more susceptible toward UVA-1 (20 J per cm^2) radiation-induced apoptosis compared with normal CD4$^+$ T cells. (*c*) Time kinetics of UVA-1-induced apoptosis in normal and malignant cells. All cells were irradiated with 20 J per cm^2 of UVA-1 and subjected to FACS analysis at the indicated time points. The rate of apoptosis in the irradiated cells was calculated by subtracting the rate of sham-irradiated controls from the rate of irradiated cells. (*d*) Nontreated and concanavalin A-activated (16 h, 1 μg) normal T cells do not differ in their susceptibility toward UVA-1 radiation-induced apoptosis. In each experiment, the percentage of apoptotic cells within the sham-irradiated control cells was always less than 10% and did not differ between the different cell populations. Data are expressed as percentage annexin V-positive cells 24 h after irradiation and represent the mean ± SEM of three independent experiments. *p<0.05.

with C2- or C6-ceramides induced apoptosis in malignant cells at different rates than those obtained for normal, nonmalignant cells (Fig 2a,b).

UVA-1 radiation-induced apoptosis in normal, nonmalignant T cells can be mimicked in unirradiated cells through stimulation with singlet oxygen, which was generated through thermal decomposition of the water-soluble endoperoxide $NDPO_2$ (Morita *et al*, 1997). In this study we have observed that treatment of cells with $NDPO_2$-induced apoptosis in malignant cells to a greater extent than in normal, nonmalignant cells (Fig 3; $p < 0.05$). This difference was comparable to that observed after UVA-1 irradiation.

In previous studies UVA-1 radiation-induced apoptosis in normal, nonmalignant T cells was found to be mediated through the FAS/FASL system (Morita *et al*, 1997). To assess the role of FAS signaling in UVA-1 radiation-induced apoptosis in malignant T cells, malignant cells from three different Jurkat cell lines were analyzed. Wild-type Jurkat cells had abundant cell surface FAS expression (Fig 4, *left panel*). In contrast, C5 cells that had been generated through repetitive FAS antibody treatment expressed less FAS molecules on their surface and upon treatment with

anti-FAS antibody did not undergo apoptosis (Caricchio *et al*, 1998). Finally, R1 cells, which were generated through repetitive stimulation of Jurkat cells with FAS antibody and FASL transfectant, had almost no FAS surface expression and as a consequence were completely resistant to FAS antibody or FASL-induced apoptosis (Caricchio *et al*, 1998). Upon exposure to UVA-1 radiation, both C5 and R1 cells exhibited significantly lower apoptosis rates compared with wild-type Jurkat cells (Fig 4, *right panel;* $p < 0.05$).

In the following experiments we asked whether differences in FAS signaling would account for the different susceptibility of normal versus malignant T cells toward UVA-1 radiation-induced apoptosis. FACS analysis of FAS surface expression in freshly isolated cells as well as the cell lines employed in this study, however, did not reveal any correlation between the level of FAS surface expression and the susceptibility toward UVA-1 radiation-induced apoptosis (data not shown). This is in contrast to the data obtained with the three Jurkat cell lines shown above (Fig 4) and indicated the possibility that signaling elements of the FAS/FASL pathway downstream from FAS surface expression could determine the susceptibility of T cells toward UVA-1 radiation. Accordingly, normal and malignant cells significantly differed when compared for caspase-3 availability. As is shown in Fig 5a, malignant cells, which were highly susceptible to UVA-1 radiation-induced apoptosis, expressed higher procaspase-3 levels compared with normal T cells. Even more important, UVA-1 radiation-induced T cell apoptosis was significantly reduced in malignant T cells upon treatment of cells with Z-VADfmk, which has been shown to prevent T cell apoptosis mainly through inhibition of caspases including caspase-3 (Fig 5b; $p < 0.05$). Also, R1 cells, which were previously shown to be resistant toward UVA-1 radiation-induced apoptosis (Fig 4) and thus different
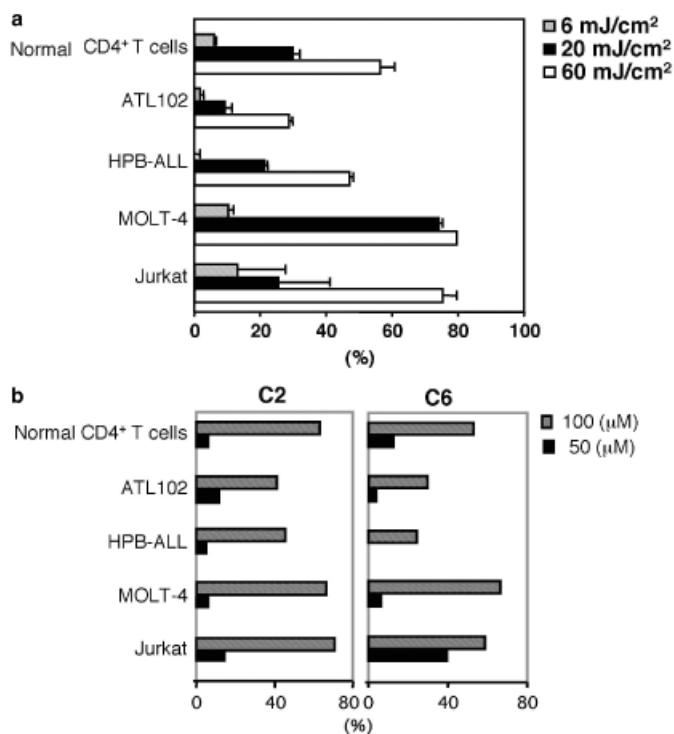
Figure 2
Malignant T cells are exquisitely sensitive to UVA-1, but not to UVB radiation and ceramide stimulation- induced apoptosis. (*a*) UVB radiation induces similar rates of apoptosis in malignant and normal T cells. The percentage of apoptotic cells within the sham-irradiated control cells was always less than 10% and did not differ between the different cell populations. Data represent percentage of annexin V-positive cells 24 h after irradiation and are expressed as mean values ± SEM of three independent experiments. (*b*) Ceramide treatment induces similar rates of apoptosis in malignant and normal T cells. For ceramide treatment, C2 and C6 ceramides were dissolved in ethanol and added to the culture medium (50 and 100 μM). After 24 h, cells were washed and subjected to annexin V analysis. Data represent one of three essentially identical experiments and are depicted as percentages of annexin V-positive cells.
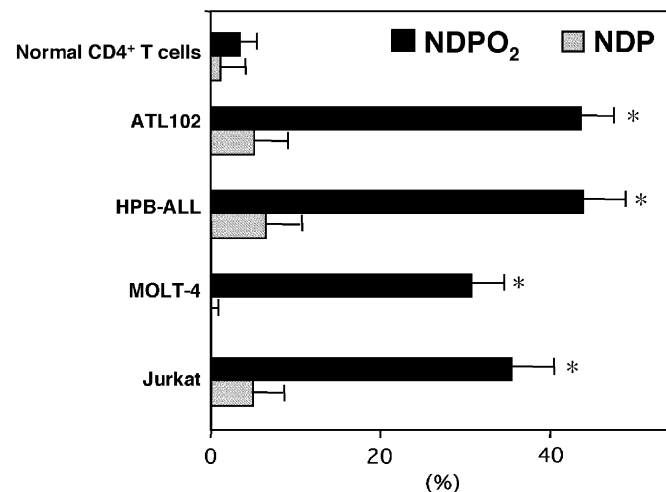
Figure 3
Malignant T cells are more susceptible than normal T cells to singlet oxygen-induced apoptosis. Singlet oxygen induces higher apoptosis rates in malignant T cells compared with normal T cells. *$p < 0.05$ Singlet oxygen was generated through thermal decomposition of the endoperoxide $NDPO_2$. T cells were incubated with $NDPO_2$ (20 mM) or NDP (20 mM). After 24 h, the percentage of apoptotic cells was determined as described under Materials and Methods. Data are expressed as percentages of annexin V-positive cells and represent mean values ± SEM of three independent experiments.
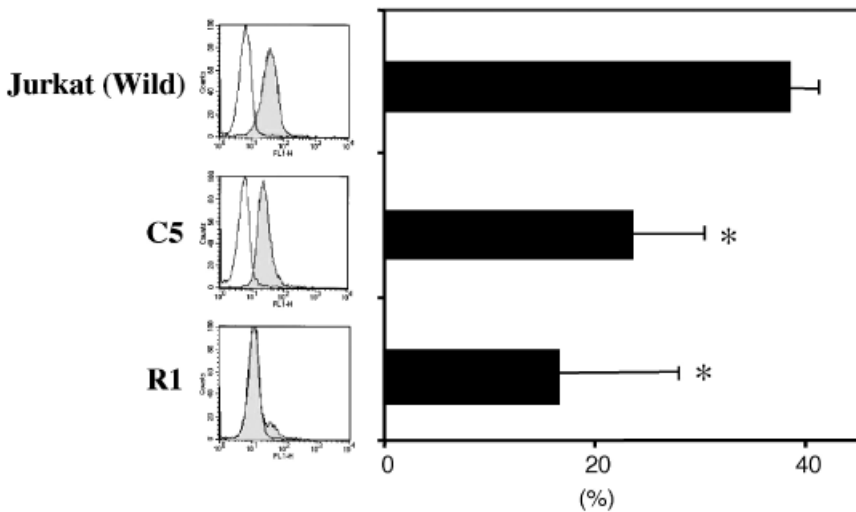
Figure 4
Involvement of FAS in UVA-1 radiation-induced apoptosis in Jurkat cells. The FACS histograms in the *left panel* depict FAS surface expression in three different Jurkat cell lines. Wild-type Jurkat cells have abundant FAS expression on the surface. The C5 cell line shows less FAS expression and upon treatment with anti-FAS antibody does not undergo apoptosis (Caricchio *et al*, 1998; data not shown). The R1 cell line shows almost no FAS expression on the surface expression and as a result is resistant to FAS antibody and FASL treatment (data not shown). To assess the susceptibility of these cells toward UVA-1 radiation-induced apoptosis, cells from each Jurkat line were exposed to 20 J per cm^2 UVA-1, and 24 h later, the percentage of annexin V-positive cells was determined by FACS analysis as described under Materials and Methods (*right panel*). The percentage of apoptotic cells within the sham-irradiated control cells was always less than 10% and did not differ between the different cell populations. Data represent mean values ± SEM of three independent experiments. *$p < 0.05$.

from other malignant T cells such as wild-type Jurkat cells, also expressed very low procaspase-3 levels (Fig 5*a*). Levels of caspases including caspase-3 can be upregulated in human cells upon stimulation with recombinant human IFN-γ (Dai and Krantz, 1999). In this study, IFN-γ stimulation significantly increased the susceptibility of R1 cells toward UVA-1 radiation-induced apoptosis and this increase was significantly reduced by addition of Z-VADfmk (Fig 5*c*; $p < 0.05$).

Discussion

In this study, malignant T cells showed a higher susceptibility toward UVA-1 radiation-induced apoptosis compared with normal CD4$^+$ T cells. This difference could not be attributed to differences in T cell activation, because activation of normal T cells with the mitogen concanavalin did not significantly increase the sensitivity of these cells toward UVA-1 radiation-induced apoptosis. This *in vitro* observation provides an explanation for the clinical experience that in patients with cutaneous T cell lymphoma (mycosis fungoides), equally good therapeutic responses were observed regardless of whether single doses of 130 J per cm^2 or less than half of this dose, that is, 60 J per cm^2 were employed, although in patients with atopic dermatitis, which show skin infiltrates consisting of nonmalignant T cells, the high-dose regimen is required to achieve optimal results (Dittmar *et al*, 2001; Krutmann *et al*, 2001). As a consequence, the high-dose regimen is no longer used for the UVA-1 phototherapy of CTCL patients in our department as well as in an ongoing international multicenter trial that compares UVA-1 phototherapy with PUVA for this indication.

Comparative studies with CD4$^+$ T cells from five healthy donors revealed some interindividual differences in the susceptibility of these cells toward UVA-1 radiation-induced apoptosis (data not shown). These differences, however, were not significant (chi-square test for goodness of fit), whereas significant differences could be detected between normal cells from each of the five donors and the malignant

cells tested in this study ($p < 0.05$). UVA-1 radiation-induced T cell apoptosis is thought to be mediated through the generation of singlet oxygen (Morita *et al*, 1997; Godar, 1999a). Future studies will therefore have to test the hypothesis that interindividual differences in the antioxidant status of skin-infiltrating T cells are of relevance for the susceptibility of their donors toward UVA-1 radiation phototherapy.

The increased susceptibility of malignant T cells was found to be relatively specific, because a difference between malignant and normal cells could only be detected if apoptosis was induced by irradiating cells with UVA-1, but not if apoptosis was induced through UVB irradiation or stimulation with apoptogenic doses of cell-permeable ceramides. This observation further supports the concept initially developed by Godar (1999b) that UVA-1 and UVB radiation induce T cell apoptosis through fundamentally different mechanisms. It also implies that ceramides, which have been shown to mediate UVA-1 radiation-induced biologic effects in human epithelial cells (Grether-Beck *et al*, 2000), are unlikely to be involved as second messenger in UVA-1 radiation-induced T cell apoptosis. From a clinical point of view one could therefore argue that UVA-1 radiation represents the UV region of choice for the treatment of patients with cutaneous T cell lymphoma. In this regard it is important that UVA-1 radiation-induced T cell apoptosis is mediated through the generation of singlet oxygen (Morita *et al*, 1997; Godar, 1999a). This is in line with the present observation that induction of apoptosis in T cells through stimulation with a singlet oxygen-generating system revealed significant differences between normal and malignant T cells, which were essentially identical to those observed after UVA-1 irradiation of cells. Singlet oxygen generation may therefore represent a therapeutic principle for the management of malignant T cell diseases.

An increased susceptibility of malignant (proliferating cells) compared with nonmalignant cells (quiescent cells) has also been observed if tumor cells and normal cells are exposed to ionizing radiation (Kaplan, 1981). In this regard the "Law of Bergonié and Tribondeau" states that (1) proliferating cells are more susceptible than nonproliferating
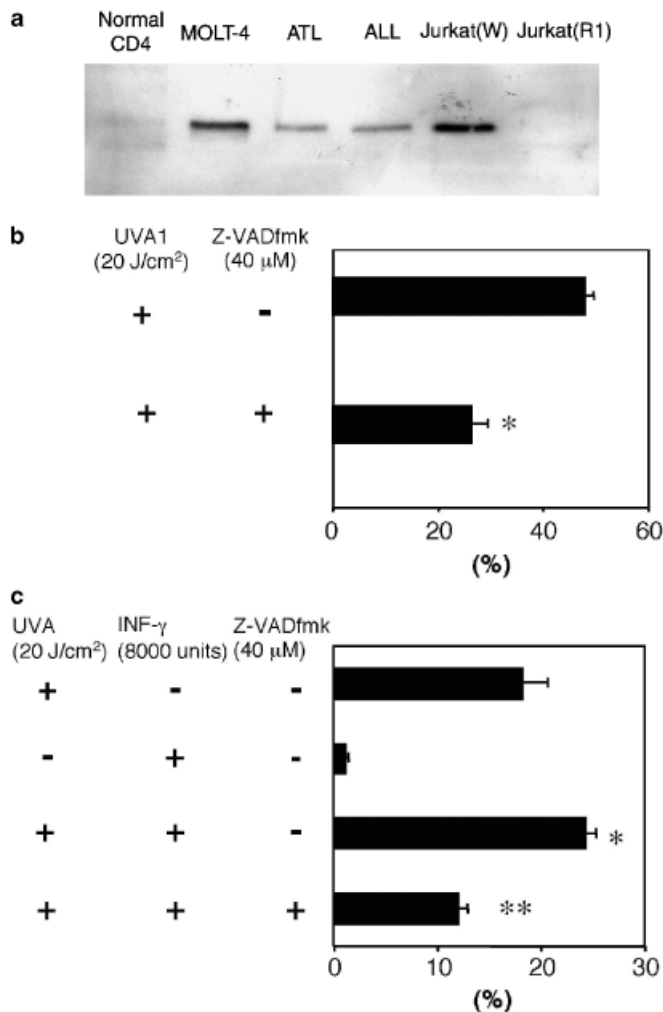
Figure 5
Relevance of caspase-3 levels for the sensitivity of T cells towards UVA-1 radiation-induced apoptosis. (*a*) Western blot analysis of procaspase-3 levels in normal and malignant T cells. Ten-microgram lysates of unirradiated cells were equally loaded to each lane. Data represent one of three essentially identical experiments. (*b*) UVA-1 radiation-induced apoptosis in Jurkat cells (wild type) is inhibited by the caspase inhibitor Z-VADfmk. Cells were incubated with 40 μM Z-VADfmk for 1 h at 37°C. The cells were then washed with PBS and irradiated with UVA-1 (20 J per cm²). After 24 h, the cells were harvested and the percentage of annexin V-positive cells was assessed by FACS analysis. The percentage of apoptotic cells within the sham-irradiated control cells was always less than 10% and did not differ between the different cell populations. Data are expressed as mean values ± SEM of three independent experiments. *p<0.05. IFN-γ treatment enhances UVA-1 radiation-induced apoptosis in FAS-depleted Jurkat cells (R1). *p<0.05. For IFN-γ treatment, cells were incubated with 8000 units of recombinant human IFN-γ 6 h before UVA-1 irradiation. The enhancement of UVA-1 radiation-induced apoptosis by IFN-γ was significantly inhibited through addition of Z-VADfmk. Data represent mean values ± SEM of the percentage of annexin V-positive cells, which were analyzed 24 h after irradiation, of three independent experiments. **p<0.01.

cells and that (2) morphologically and physiologically undifferentiated cells are more susceptible than differentiated cells. It should be noted, however, that not for all cell types differences in the susceptibility toward ionizing radiation can be explained by these two rules (Haber and Rothstein, 1969).

In this study, UVA-1 radiation-induced T cell apoptosis was shown to involve caspase, in particular caspase-3,

activation. Accordingly, UVA-1 radiation-induced apoptosis was significantly inhibited through addition of the caspase inhibitor Z-VADfmk. In addition, T cells with a low sensitivity toward UVA-1 radiation-induced apoptosis such as normal, nonmalignant cells as well as R1 cells expressed lower levels of procaspase-3 compared with T cells that were characterized by a higher susceptibility toward UVA-1 radiation-induced apoptosis, that is, MOLT4, adult T cell leukemia, ALL, and wild-type Jurkat cells. Finally, treatment of R1 cells with IFN-γ significantly increased the sensitivity of these cells toward UVA-1 radiation-induced apoptosis, and this increase was significantly inhibited through addition of Z-VADfmk, indicating that the IFN-γ effect was mainly due to upregulation of caspase levels, as has previously been reported to be the case for human cells (Dai and Krantz, 1999). Based on these observations we would like to propose that caspases such as caspase-3 represent molecular sensors for the susceptibility of T cells toward UVA-1 radiation/singlet oxygen-induced apoptosis. It should be noted that the caspase inhibitor employed in the present study is not specific for caspase-3 and that IFN-γ treatment, in addition to caspase-3, also induces upregulation and activation of caspases-8 and -9. It is therefore possible that the availability of other caspases at least partially controls UVA-1 radiation/singlet oxygen-induced T cell apoptosis as well.

To the best of our knowledge, this is the first report to suggest a critical role for caspases in defining the susceptibility of pathogenetically relevant T cells toward a phototherapeutic regimen. The present observation that stimulation of T cells with IFN-γ, and possibly also IFN-α (Thyrell *et al*, 2002), significantly enhances UVA-1 radiation-induced T cell apoptosis indicates that strategies directed at upregulating caspase levels will increase the efficacy of UVA-1 phototherapy and might prompt further interest in combination regimens of these cytokines with UVA-1 phototherapy for atopic dermatitis and cutaneous T cell lymphoma.

Materials and Methods

Isolated cells and cell lines CD4+ T cells were prepared from a patient with adult T cell leukemia or from a patient with a leukemic form of a cutaneous T cell lymphoma (Sezary's syndrome) or from five different healthy human volunteers (normal, nonmalignant T cells) by isolating peripheral blood mononuclear cells from the venous blood of these individuals using Ficoll-Paque density gradient (Amersham Pharmacia Biotech, Uppsala, Sweden) centrifugation as previously described (Krutmann *et al*, 1990). CD4+ T cells were positively selected by magnetic cell sorting using VarioMACS sorting device (Milteny Biotec, Bergisch-Gladbach, Germany). The purity of CD4+ cells was greater than 95%, as was assessed by FACS analysis using the anti-CD4 mAb MT310 (DakoCytomation A/S, Copenhagen, Denmark) and a FACScan

flow cytometer (BD Immunocytometry Systems, Mountain View, CA) as described below. Freshly isolated cells were UV irradiated immediately after isolation. Malignant T cell lines used in this study included HUT 102, HPB-ALL, and ATL 102, which were all provided by S. Iida (Graduate School of Medical Sciences, Nagoya City University, Nagoya, Japan), as well as Jurkat and MOLT4, which were obtained from the Riken cell bank (Tsukuba, Japan). FASL-transfectant, variant FAS-expressing Jurkat cell lines (given by R. Caricchio, University of Pennsylvania, Philadelphia, PA) were employed for the study (Caricchio *et al*, 1998). These cell lines were either FAS-antibody- or FASL-transfectant-resistant. They expressed less or almost no FAS on the surface. All cells were cultured in RPMI 1640 (Sigma Chemical Co.) supplemented with 10% heat inactivated fetal calf serum and washed once in phosphate-buffered saline (PBS) before exposure to UV radiation.

***In vitro* UV irradiation** Cultured or freshly isolated cells were harvested and resuspended in PBS in 6-cm tissue culture dishes (Becton Dickinson Labware, Franklin Lakes, NJ). Lids were removed and cells (5×10^5/mL) were exposed to UVA-1 radiation from a partial-body UVA-1 irradiation device (Sellamed 2000 system, Dr. Sellmaier, Sellas GmbH, Gevelsberg, Germany) as previously described (Morita and Krutmann, 2000). The emission was filtered with a UVA-1 filter (Sellas GmbH) and an infrared absorbing filter UG1 (Schott, Mainz, Germany) and consisted exclusively of wavelengths in the range of 340 to 450 nm. The UVA-1 irradiance was measured with a IL 1700 research radiometer (International Light, Newburyport, MA) and a IL SED 038 detector (International Light) and found to be 50 mW per cm^2 at a tube to target distance of 30 cm. For UVB irradiation, UVB Torex FL20S lamps (Toshiba, Tokyo, Japan) were used, which emit their energy primarily in the range of 290 to 320 nm with a peak emission at 305 nm. The UVB output of these lamps was measured with an UVR-3036/S2 radiometer and an UVB detector (Clinical Supply, Gifuhashima, Japan) and found to be 0.4 mW per cm^2 at a tube-to-target distance of 25 cm. Control cells were sham irradiated; that is, they were treated identically, however, without exposure to UVA-1 or UVB radiation. In these cells, the rate of apoptosis was always less than 10% and did not differ from that observed in malignant or normal cells before irradiation. After exposure to either UVB or UVA-1 radiation, cells were kept in their culture medium at 37°C at 5% CO$_2$ for the indicated periods before further analysis.

Singlet oxygen generation Singlet oxygen was generated by thermal decomposition of the endoperoxide of the disodium salt of 3,3′-(1,4-naphthylidene)dipropionate (NDPO$_2$), 1 mM in PBS, for 1 h in the dark at 37°C yielding excited singlet molecular oxygen and 3,3′-(1,4-naphthylidene)dipropionate (NDP). This singlet oxygen generating system previously proved to be well suited for use in T cell cultures (Morita *et al*, 1997), because it is water-soluble and nontoxic up to 40 mM for 1 h of incubation. Infrared emission of singlet oxygen was measured with a liquid-nitrogen-cooled germanium photodiode detector (Model EO-817 L, North Coast Scientific Co., Santa Rosa, CA) as described. The rate of singlet oxygen generation was monitored by the formation of NDP. Fifteen minutes after addition of 1 mM NDPO$_2$ the rate of singlet oxygen generation was 3 μM per min. As controls, cells were stimulated with NDP, which had been generated through thermal decomposition from the same batch of NDPO$_2$ used in these experiments.

Detection of apoptosis The number of apoptotic T cells was determined by FACS analysis as the percentage of annexin V$^+$ cells. During the process of apoptosis, cells display phosphatidyl serine at the cell surface membrane. This very early event in T cell apoptosis can be detected in T cells undergoing early protein synthesis-independent as well as late protein synthesis-dependent apoptosis and was therefore a suitable biologic endpoint for monitoring T cell apoptosis in the present study (Godar, 1999a). In brief, irradiated cells were harvested at the indicated time points,

washed once with PBS, and incubated with FITC-labeled annexin V (MBL, Nagoya, Japan) for 15 min at room temperature. Cells were then washed again and resuspended in PBS at a concentration of 2×10^6 cells per mL and immediately subjected to FACS analysis as described below. For each variable, 10^4 cells were analyzed. Data are given as percentage annexin V-positive cells.

Immunofluorescence flow cytometry Cells (5×10^5) were washed twice in PBS, resuspended, and incubated with FITC-conjugated anti-human CD95 mAb UB2 (MBL, Nagoya, Japan) or a FITC-conjugated isotype control (MBL) for 30 min at 4°C. The cells were washed twice in PBS and subsequently analyzed using a FACScan flow cytometer (BD Immunocytometry Systems). For each variable, 10^4 cells were analyzed. Data are given as histograms of fluorescence intensity versus cell number.

Western blot analysis Cells were harvested in lysis buffer containing 0.3% Triton-X (Katayama Chemical, Osaka, Japan), 1 μg per mL leupeptin (Sigma Chemical Co.), and 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.). Cells were homogenized by repetitively passing them through a 27-gauge needle syringe. The protein concentration of each sample was determined according to bicinchoninic acid protein assay using a commercially available kit (micro BCA protein assay, Pierce, Rockford, IL). Equal aliquots of the protein samples (10 μg/sample) were subjected to 15% gels for SDS-PAGE and then electro-transferred to polyvinylidivinylidene difluoride membranes (Millipore Co., Bedford, MA). Nonspecific binding was blocked through overnight incubation of membranes in 5% skim milk, 0.05% Tween 20 in Tris-buffered saline, pH 7.5. The following day the membranes were incubated with the anti-caspase-3 mAb CPP32 (MBL) for 2 h at room temperature. Bound primary antibody was reacted with anti-mouse peroxidase-conjugated IgG (DakoCyto-mation A/S) for 1 h at room temperature, and chemiluminescence was detected (ECL, Amersham Pharmacia Biotech, Buckingham-shire, England). The blot was exposed to hyperfilm (Amersham Pharmacia Biotech).

Statistical analysis Data analysis was performed by using the two-tailed Student's *t* test to determine the difference between values obtained in the indicated cells and in controls.

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