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Ultraviolet exposure of thymocytes: selective inhibition of apoptosis

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Abstract.
Purpose: To evaluate selective effects of ultraviolet (UV) irradiation on spontaneous and induced apoptosis in freshly extracted mice thymocytes.
Materials and methods: Cells were exposed to UV radiation with emission peaks of 365 nm (UVA) exposures of 1620–10 200 J m^{-2}, of 312 nm (UVB) exposures of 34–1620 J m^{-2} or of 254 nm (UVC) exposures of 1.5–1620 J m^{-2}, and incubated for 5.5 h with or without hydrocortisone, phorbol-12-myristate-13-acetate or anti-Fas antibody. Additionally, cells were irradiated with gamma-rays (5 Gy) before UVB exposure (408 J m^{-2}) at different times. Apoptosis was quantified by DNA fragmentation.
Results: Up to an irradiation of 5000 J m^{-2}, UVA exposure did not show any effect on thymocyte apoptosis, while at 10 200 J m^{-2} irradiation, considerable DNA fragmentation was observed. In contrast, UVB and UVC irradiation clearly inhibited natural and cortisone-induced apoptosis. Moreover, UVB inhibited apoptosis triggered by phorbol-12-myristate-13-acetate and gamma-irradiation, but not by anti-Fas antibody.
Conclusions: The response of mouse thymocytes in culture to UV irradiation strongly depends on the wavelength used. It is suggested that either a survival or an apoptotic pathway occurs depending on the physiological state of the cell, spectral composition of the UV light and cell type. The possible involvement of extracellular signal-regulated kinase and stress-activated protein kinase/c-Jun N-terminal kinase in the apoptotic pathway is discussed.

1. Introduction

The ultraviolet (UV) part of the solar spectrum is responsible for a variety of phenomena that directly affect biological organisms. Among these, it has been demonstrated that UV light is involved in premature ageing, exacerbation of infectious disease (Chapman *et al.* 1995, Fisher 1996) and skin cancer (Whittaker 1996). Skin cancer may be considered as the most severe consequence attributed to the immunosuppressive effect of UV light (Kripke 1994). It has been proposed that UV light-induced apoptosis participates in the pathogenesis of this disease. In this context, the Fas-pathway of apoptosis has been suggested to be involved in the mechanism of UV-induced malignancy (Caricchio *et al.* 1998, Schwarz *et al.* 1998, Hill *et al.* 1999).

Finally, UV light has been shown to trigger the apoptotic sensitivity of lymphoid cell lines (Godar and Luca 1995, Godar 1996, Caricchio *et al.* 1998), which play an important role in the homeostasis of the immune system.

Most of the studies on UV induction of apoptosis have been carried out in lymphoid cells lines, but it has also been described that activated peripheral lymphocytes are triggered to undergo apoptosis by UV exposure (Bazar and Deeg 1992). The susceptibility of resting lymphocytes to UV-induced apoptosis is still not resolved. Bazar and Deeg (1992) reported that lymphocytes are not triggered to undergo apoptosis by UV, while Yaron *et al.* (1996) showed apoptotic DNA fragmentation in resting lymphocytes exposed to UV light but always at a lower level than the DNA fragmentation seen in activated lymphocytes under similar experimental conditions. According to Lonskaya *et al.* (1997), UVC induces apoptosis in rat thymocytes at low dose ranges (<20 J m^{-2}), while higher doses showed an inhibitory effect. On the other hand, low doses of UVA can induce either a fast or a delayed apoptosis in thymocytes (Lonskaya *et al.* 2001).

Freshly extracted thymocytes are highly sensitive to the induction of apoptosis by chemical and physical agents, leading to increased DNA fragmentation in about 5 h (Ojeda *et al.* 1990, 1992a, b, Haertel *et al.* 1998). These facts suggest that this model system can be a sensitive cellular target for the study of UV-induced apoptosis. The present results show that only UVA at high doses induces apoptosis in murine thymocytes. In contrast, UVB and UVC inhibit natural apoptosis as well as apoptosis induced by hydrocortisone, phorbol-12-myristate-13-acetate (PMA) or gamma-irradiation. Only anti-Fas antibody-induced apoptosis remains unaltered by UVB exposure.

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2. Materials and methods

2.1. *Cells*

Thymus cells were obtained from 6–8-week-old Rockefeller male mice bred at the Immunology Department, Universidad Austral de Chile, Valdivia, Chile. Animal usage was approved by El Comitė de Bioėtica 'Uso de animales en la Investigación' of the university. The animals were sacrificed by ether overdose and the thymus cells were extracted and teased in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 0.2% bovine serum albumin. The cell suspension was filtered through thin cotton layers to remove the remaining cell clusters and was adjusted to 5×10^6 cells ml^{-1}. All steps were performed in an iced water bath.

2.2. *UV sources and exposure*

- UVA source (emission peak at 365 nm): a UVGL-58 Mineralight lamp (Upland, CA, USA) irradiated cells at a distance of 14 cm. The irradiance at the sample level was 6.6 W m^{-2}, which was determined with a UVA dosimeter (Cole Parmer, Vernon Hills, IL, USA).
- UVB source (emission peak at 312 nm): a Cole Parmer lamp (9815 series) irradiated cells at a distance of 14 cm. The irradiance at the sample level was 6.8 W m^{-2}, which was determined with a spectroradiometer SUV 100 (Biospherical Instruments, San Diego, CA, USA).
- UVC source (emission peak at 254 nm): a germicidal lamp (General Electric, Fairfield, CT, USA) irradiated cells at a distance of 44 cm. The irradiance at the sample level was 0.3 W m^{-2} (UVC dosimeter, Cole Parmer). Emission spectra and the emission peak of UVA, UVB and UVC were determined with the spectroradiometer SUV (figure 1). UV exposure was carried out in Petri dishes with a 2-mm-deep cell suspension. Control and experimental samples were cooled on an iced water bath during UV exposure.

2.3. *Induction of apoptosis*

The following chemicals and procedure were used as apoptosis inductors: hydrocortisone (Sigma, St Louis, MO, USA) at a final concentration of 10^{-6} M, 20 ng ml^{-1} PMA (Sigma) or 1 μg ml^{-1} anti-Fas antibody (15400D, PharMingen, San Diego, CA, USA). In addition, thymocyte suspensions were irradiated with a dose of 5 Gy (0.6 Gy min^{-1}) with a Co^{60} machine from the Hospital Base of Valdivia. During irradiation procedures, the experimental samples and their controls were maintained in an iced water bath.

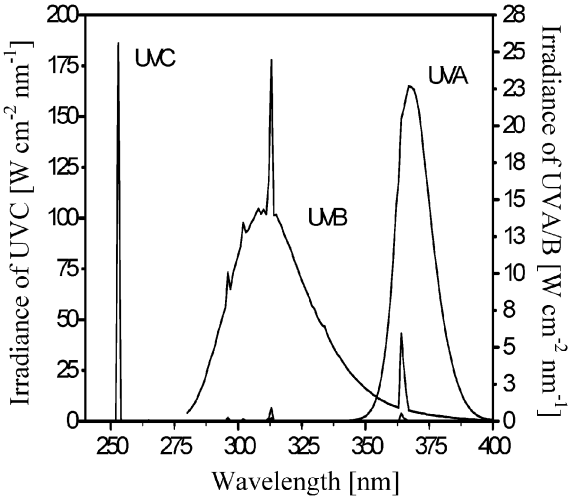

Figure 1. Emission spectra of the UVA, UVB and UVC lamps used. See the text for details of measurements.

In all cases, cells were exposed to UV radiation and immediately after irradiation the chemical inductors of apoptosis were added and incubated at 37°C. The incubation was carried out in humidified atmosphere with 5% CO_2 for 5.5 h. In some experiments, gamma-irradiated cells were exposed to UVB for 1 min (total dose = 408 J m^{-2}) at various times during the post-irradiation incubation period and then allowed to complete the period.

2.4. *Quantification of apoptosis*

Apoptosis was quantified through DNA fragmentation (Ojeda *et al.* 1992b) as proposed by Kizaki *et al.* (1990). Briefly, 1 ml sample containing 5×10^6 cells were centrifuged and resuspended in lysis buffer containing 0.5% Triton X-100, 5 mM Tris-HCl (pH 7.4) and 1 mM ethylene diamine tetraacetic acid for 20 min on ice. The lysate was centrifuged for 20 min at 16 000*g* to separate intact from fragmented DNA. After 1 min at 250 W sonication (500 W Ultrasonic processor, Cole-Parmer), the DNA contents of both the sediment and the supernatant were measured by fluorescence of 4′,6-diamidino-2-phenylindole (DAPI) (Sigma). This was done by adding 10–100 μl of the sample to 2 ml reagent (100 ng ml^{-1} DAPI in 10 mM Tris, pH 7.4, containing 100 mM NaCl), and the fluorescence intensity was determined at 454 nm (excitation at 362 nm) with a Kontron SFM 25 spectrofluorometer (Kontron, Germany). The percentage of fragmented DNA was calculated from the ratio of the DNA content in the supernatant and the total DNA in the lysate.

To rule out the occurrence of necrotic death because of UV light exposure, the intactness of the cell membrane was assessed microscopically by the

trypan blue exclusion test (Romeis 1928). Additionally, the presence of pycnotic cell nuclei labelled with acridine orange (Darzynkiewicz *et al.* 1979) was observed with a fluorescence microscope.

2.5. *Quantification of Extracellular signal-regulated kinases*

Control and UVB irradiated thymocytes were analysed for the presence of activated ERK1 and ERK2 by protein immunoblot analysis according to Bommhardt *et al.* (2000).

3. Results

In freshly extracted murine thymocytes, the number of apoptotic cells increased steadily with incubation time (figure 2). After approximately 20 h of incubation, almost the entire cell population showed apoptotic features. In comparison with this spontaneous apoptosis, thymocytes exposed to apoptosis inducing agents (e.g. X-irradiation with 3 Gy (figure 2, inset) showed an enhanced apoptotic rate. This induced apoptosis became detectable approximately 2 h after treatment and could be quantified reliably until the number of apoptotic cells reached a saturation level (about 10 h after treatment with 3 Gy). As shown in figure 2, PMA, UVA ($10\,200\,J\,m^{-2}$) and hydrocortisone significantly increased the apoptotic rate in thymocytes, while UVB ($408\,J\,m^{-2}$) had the opposite effect. UVB-irradiated cells were protected from apoptosis, since no enhanced DNA fragmentation could be detected in comparison with the values in freshly extracted cells. After 5.5 h, the apoptotic rate induced by hydrocortisone was significantly higher than the rates of PMA and UVA. In all experiments, the number of necrotic cells measured with Trypan blue test was less than 10% (data not shown).

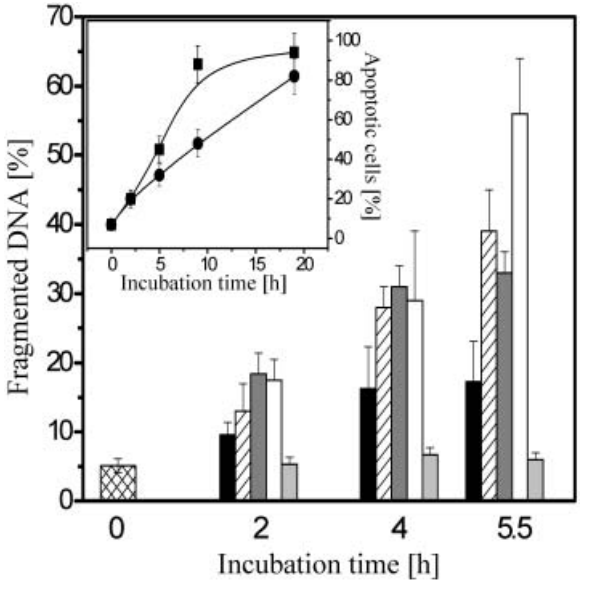

Figure 2. Time course of apoptosis in freshly extracted murine thymocytes. The percentage of DNA fragmentation was determined spectrometrically by 4′,6-diamidino-2-phenylindole (DAPI) fluorescence after 0, 2, 4 and 5.5 h of incubation. DNA fragmentation was determined in freshly extracted thymocytes (crossed bar, 0 h), in untreated cells (black bars), in cells treated with phorbol-12-myristate-13-acetate (PMA) (striped bars, $20\,ng\,ml^{-1}$), in cells irradiated with UVA (grey bars, $10\,200\,J\,m^{-2}$), in cells treated with hydrocortisone (white bars, 1 μM) and in cells irradiated with UVB (light grey bars, $408\,J\,m^{-2}$). Means and standard deviations were calculated from four or more experiments. The inset shows a representative example of the complete time course of apoptosis in untreated thymocytes (circles) and in cells exposed to X-irradiation (3 Gy, squares). In this plot the percentage of apoptotic cells was determined by flow cytometry as described by Haertel *et al.* (1998).

Figure 3 summarizes the effects of UVB ($408\,J\,m^{-2}$) on apoptosis, induced by hydrocortisone, PMA, gamma-irradiation and anti-Fas antibody, 5.5 h after induction. Evidently, UVB inhibited spontaneous apoptosis (cf. with figure 2) as well as the enhanced apoptosis induced by hydrocortisone, PMA or gamma-irradiation. In contrast, UVB had no effect on anti-Fas antibody-induced apoptosis.

The inhibitory effects of UVB and UVC on DNA fragmentation depend on the applied doses. Figure 4

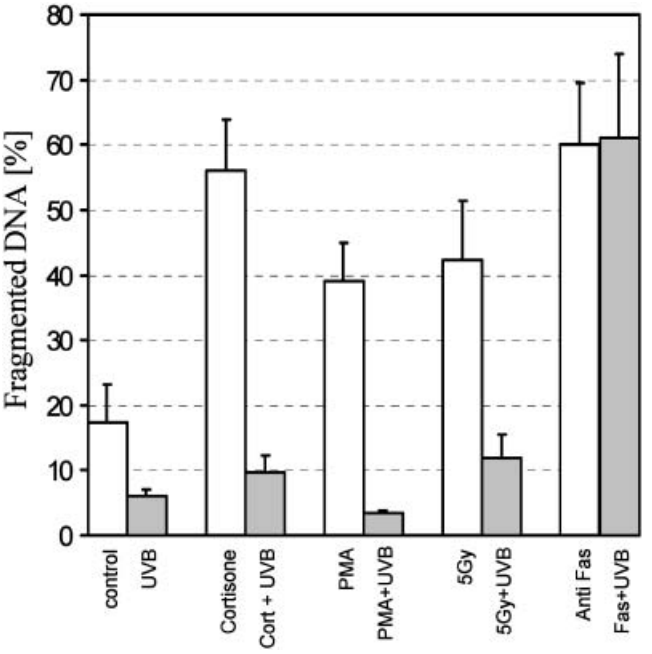

Figure 3. Effect of UVB irradiation ($408\,J\,m^{-2}$) on apoptosis induced by different chemical agents or by gamma-irradiation measured at 5.5 h after treatment. Means and standard deviations were calculated from four or more experiments.

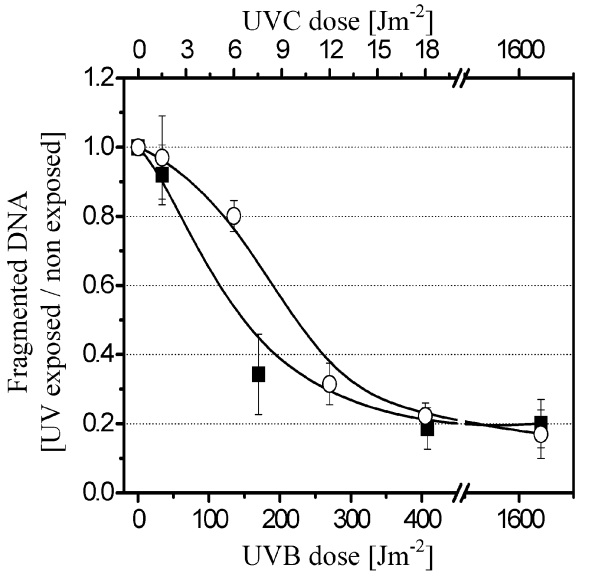

Figure 4. Dose-dependent effect of UVB (squares) and UVC (circles) irradiation on apoptosis induced by hydrocortisone (1 μM). Data points were calculated by the ratio of the percentage of fragmented DNA in samples exposed to UVB or UVC and treated with cortisone and the fragmented DNA in non-exposed samples but treated with cortisone measured at 5.5 h. Means and standard deviations were calculated from four or more experiments.

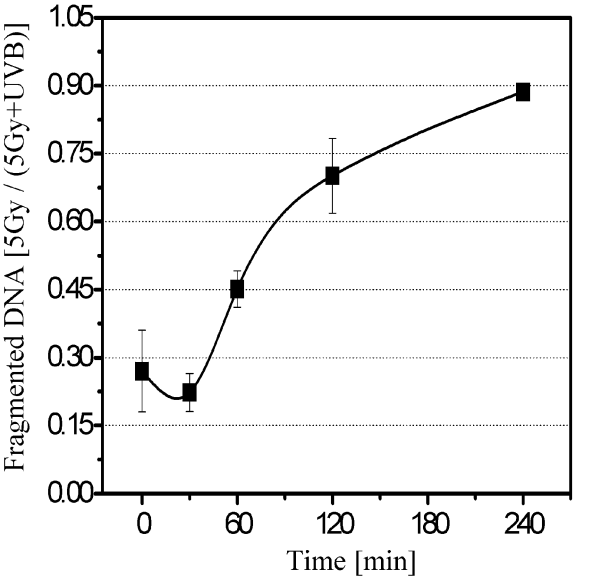

Figure 5. Cell suspensions were irradiated with 5 Gy gamma-irradiation followed by exposure to 408 J m^{-2} UVB at times ranging from 0 to 4 h between both irradiations. After 5.5 h of incubation at 37°C (see the Materials and methods), fragmented DNA was determined. Data represent the ratio of fragmented DNA in UVB-exposed samples and non-ultraviolet light-exposed samples. Means and standard deviations were calculated from four or more experiments.

shows this dose-dependent effect on hydrocortisone-induced apoptosis (1 μM). The exposure dose at which half the maximal effect was seen (D_{50}) was estimated to be around 120 J m^{-2} for UVB and at around 9 J m^{-2} for UVC. Thus, the protection of thymocytes from hydrocortisone-induced apoptosis obtained with UVC proved to be around 10 times more efficient than UVB. Both irradiation sources finally reduced hydrocortisone-induced DNA fragmentation to 0.2 ± 0.07 (UVB at 1620 J m^{-2}) and 0.16 ± 0.07 (UVC at 1620 J m^{-2}), which are not statistically different.

To test if the inhibitory effect of UVB is an early event in radiation-induced apoptosis, thymocytes were irradiated with gamma-rays (5 Gy) and exposed to UVB at different times after gamma-irradiation during the incubation period. Figure 5 shows that UVB irradiation, applied as long as 30 min after triggering apoptosis with gamma-irradiation (5 Gy), still had the same effect on apoptosis as the irradiation with UVB applied before gamma-irradiation. Protective effects of UVB irradiation could be detected as long as about 2 h after gamma-irradiation.

The viability of the cells at the end of the incubation, as measured by trypan blue exclusion test, ranged from 85 to 90%, including control samples.

4. Discussion

The results indicate that glucocorticoid-induced apoptosis in thymocytes is inhibited by exposure to UVB or UVC. The D_{50} UV inhibitory exposure doses for cortisone-induced apoptosis were 120 and 9 J m^{-2} for UVB and UVC, respectively. This effect was observed for both UV types up to 1600 J m^{-2}. The present findings contrast with those of Lonskaya *et al.* (1997), which show that irradiation of thymocytes with doses higher than 20 J m^{-2} UVC inhibits cortisone-triggered apoptosis and that exposure doses less than 20 J m^{-2} induce apoptosis by themselves. For UVA-induced apoptosis, the present studies show that exposure doses as high as 5000 J m^{-2} produce no detectable effect, whereas exposure to a dose of 10 200 J m^{-2} consistently induces apoptosis. This result differs from those of Lonskaya *et al.* (2001), in which UVA at doses ranging between 1 and 100 J m^{-2} induced apoptosis in thymocytes.

The only paper dealing with thymocyte apoptosis induced by UV *in vitro* describes that UV light can induce DNA fragmentation in transgenic thymocytes caspase 9 (–/–) (Hakem *et al.* 1998). Caspase 9-deficient thymocytes are resistant to cortisone but sensitive to anti-Fas antibody, therefore caspase 9 appears to be a requirement for apoptosis induction by cortisone, in a similar way than caspase 8 is

involved in anti-Fas apoptosis induction (Hakem *et al.* 1998). When these results are compared with the present finding dealing with the inhibition by UVB of apoptosis induced by hydrocortisone, gamma-irradiation and PMA but not by anti-Fas antibody, it appears that caspase 9 is involved in the survival pathway activated by UV exposure in normal thymocytes.

Upon UV exposure of primary cultures of peripheral lymphocytes, it has been reported that activated lymphocytes undergo UVB-induced apoptosis, while resting lymphocytes seem to be either refractive (Bazar and Deeg 1992) or more resistant than the activated ones (Yaron *et al.* 1996). These results suggest that there might be two different pathways to react to the UV challenge: one leading to apoptosis and the other to survival where the fate of the cell would be dependent on its activation state. This idea is in line with a recent report proposing a two pathway model triggered by UVC irradiation (Kitagawa *et al.* 2002): UVC-induced activation of SAPK/JNK, leading to an apoptotic response and the alternative pathway involving ERK activation that leads to survival. According to this model, the fate of the cell would depend on the relative degree of activation of each pathway. In this regard, note that in 2B4.11 cells (Fas negative), anti-CD3 protects the cells from glucocorticoid-induced apoptosis through a pathway involving ERK activation (Jamieson and Yamamoto 2000). Additionally, mitogen-activated protein kinase 1, a precursor in the ERK activation pathway, inhibited glucocorticoid-induced apoptosis in primary T-cells. If this model applies to thymocytes, exposure of these cells to UVB or UVC would strongly stimulate the ERK survival pathway. In a preliminary set of experiments using the present system, an increase of phosphorylation in ERK 1 was detected from 0.23 in control cells to 0.70 in the irradiated sample. In the case of ERK 2, this increase goes from 0.12 to 0.46 in irradiated cells.

Since UVB exposure does not interfere with anti-Fas-induced apoptosis, it is possible that Fas receptor activation induces a pro-apoptotic signal which is too strong to be counteracted by the UVB-induced survival pathway. Otherwise, the lack of a cross-talk between the anti-Fas pro-apoptotic and the UVB anti-apoptotic pathways would also explain the results. A more detailed study of the UVB/UVC-induced activation of ERK1/2 should lead to a better understanding of these processes in the future.

Acknowledgements

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