

Radiation-Induced Apoptosis in Thymocytes: pH Sensitization

F. Ojeda^a, I. Skardova^c, M. I. Guarda^a,
C. Maldonado^a, and H. Folch^b

^a Department of Physics and ^b Department of Immunology, Universidad Austral Valdivia, P. O. Box 567, Valdivia, Chile, ^c Department of Internal Medicine, University of Veterinary Medicine, Kosice, Slovak Republic

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Thymocytes were used as a model system to study the effect of microenvironmental pH changes on the radiation-induced apoptosis. We found that the sensitivity of thymocytes toward radiation induced apoptosis is increased by increasing the pH of the incubation medium. The major sensitivity change occurs between pH 7 and 8.

In a given cell suspension the results obtained were similar when the apoptosis evaluation was carried out either by counting the picnotic nuclei, or monitoring the fraction of apoptotic nuclei by flow cytometry; both methods show a radiosensitization when the pH value of incubation media rises from 7 to 8. These results may be important when "*in vitro*" experiments are performed with lymphoid cells, since changes in pH of the media may determine important changes in the results.

Interphase death of lymphocytes is amongst the most sensitive responses of mammals to irradiation (Bac and Alexander, 1967; Anderson, 1976). More recently it has been found that radiation induced interphase death of lymphocytes, at doses below 10 Gy, shows apoptosis features. (Sellins and Cohen, 1987; Ojeda *et al.*, 1992b). Since apoptosis has been recognized to be involved in normal immune functions as well as in some pathologies such as cancer (Stauton and Gaffney, 1995; Kerr *et al.*, 1994) and antigen-induced anergy (Green and Scott, 1994), the study of radiation-induced apoptosis in immunocompetent cells, become an important field of research. Recently, it has been proposed that the membranes participate in radiation-induced apoptosis (Ojeda *et al.*, 1994; Ramakrishnan *et al.*, 1993). Consequently, the mi-

croenvironment of the cells appears to be involved and the pH of the external "milieu" may play a role in this process that results in DNA solubilization. With respect to pH involvement in apoptosis, it has been reported that etoposide induced programmed cell death in human HL-60 cells correlates with intracellular acidification (Barry *et al.*, 1993) and that lovastatin-induced apoptosis in the same cells can be inhibited by intracellular alcalinization (Pérez *et al.*, 1995). On the contrary, experiments carried out in CEM-C7 (a dexamethanonesensitive cell line) indicate that cortisone induces cytoplasmic alcalinization with subsequent DNA fragmentation (Adebodun and Post, 1994). On the other hand, in experiments carried out using Chang liver cells, with a characteristic high capacity to recover from pH gradients, a clear connection between this cellular stress and the apoptotic process can be demonstrated (Sit *et al.*, 1994): Taking these facts into account, and the practical role DNA fragmentation plays in experiments in which lymphoid cells are cultured "*in vitro*", we decided to study the effect of extracellular pH changes in the radiation induced apoptosis of thymocytes.

Thymus cells were obtained from 6–8 weeks old RK mice from our own colony. The animals were sacrificed by an ether overdose, the thymus removed and disrupted in culture medium RPMI-1640. The cell suspension obtained was passed through gauze, washed twice, resuspended in RPMI-1640 and adjusted to 10⁷ cells/ml. Cell samples from the stock suspension were irradiated with different doses, 0.5, 1, and 3 Gy, at a dose rate of 15 cGy/min or kept as non-irradiated control. Irradiation was carried out with an X-Ray source Phillips type 1140, operated at 80 KV, with a 3 mm Pyrex glass and 0.4 mm Cu filter. Cell samples were kept cold during the irradiation procedure in RPMI 1640 at pH 7.0. After irradiation, experimental and non-irradiated control samples were incubated at a concentration of 10⁶ cells/ml in RPMI-1640 medium plus 20 mM Hepes buffer (N-[2-hydroxyethyl]piperazine-N'-[2 ethanesulfonic acid]) supplemented with 5% fetal calf serum at 37 °C in an atmosphere of 95% air and 5% CO₂ (v/v) for 6 hrs, at different pH (6, 7, 8 and 9). At the end of the incubation period the DNA content per nucleus was estimated measuring the fluo-

Reprint requests to Dr. Flavio Ojeda.
Telefax: 56-63-212953.

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Table I. Percentage of intact nuclei in an irradiated or non-irradiated thymus cell suspension after 6 hours of incubation at 37 °C in RPMI-1640 culture medium adjusted at different pH. The determination were done by flow cytometry and each value represents the arithmetic mean of 5 to 7 independent experiments \pm standard deviation.

Dose	pH 6	pH 7	pH 8	pH 9
0 Gy	84.0 \pm 8.0	86.9 \pm 5.8	82.6 \pm 9.3	50.0 \pm 12.4
1 Gy	75.7 \pm 10.1	75.3 \pm 11.9	49.6 \pm 5.0	31.4 \pm 7.6

rescence of individual cells labelled with acridine orange, with a flow cytometer using the 488 nm argon laser line for excitation. DNA labelling was carried out as previously described (Ojeda *et al.*, 1992 a). Briefly, 0.2 ml of acid detergent (0.1% Triton X-100, 0.08 N HCl, 0.15 M NaCl) were added to 0.1 ml of cell suspension followed by 0.6 ml of acridine orange (AO) staining solution 30 s later. Cells showing less DNA content than the lymphocytes in G₀/G₁ phase of the cell cycle were counted as apoptotic according to our previous experience.

In some experiments, the cell suspension stained as described above, was analyzed by a fluorescence microscope to determine the proportion of picnotic nuclei.

The results of the first observation indicate that the pH of the incubation media alone may influence the apoptotic rate process in thymocytes naturally occurring when these cells are kept in *in vitro* conditions. The first line of Table I shows that cells incubated in medium with high pH, suffer higher spontaneous apoptotic change as compared with the thymocytes incubated at physiological pH. When the cultured thymocytes were irradiated beforehand, as shown in the second line of Table I, the effect of irradiation on nuclear integrity, depends on the pH of the culture medium: the cell suspension irradiated with 1 Gy, after 6 hours of incubation shows a significant decrease in non fragmented nuclei when the cells were incubated in alkaline medium (pH 8 and 9) as compared with those irradiated incubated at physiological pH (between 6 and 7).

In order to study the relation between pH and radiation efficiency, thymocytes were X-irradiated with different doses ranging between 0.5 and 3 Gy, and subsequently incubated in culture medium at pH 7 or 8. The percentage of intact nuclei was evaluated, as in the previous experiment, after 6 hours of incubation. As can be seen in Table II, an increase in radiation sensitivity is noticed when the pH of the culture medium is increased in one unit over physiological pH. The comparison of the doses required to obtain equal effects at pH 7 and pH 8 renders a factor of about 2 to 3. Similar results were obtained when in a different set of experiments, the picnotic nuclei, considered as apoptosis indicator, were evaluated by fluorescence microscopy. As shown also in Table II, when the results were expressed as percentage of intact nuclei, in each experimental sample with respect to their control, the results again indicate a pH sensitization which has the same magnitude as described above.

Table II. Percentage of intact nuclei in a thymocyte cell suspension irradiated with different doses and subsequently incubated for 6 hours at pH 7 or 8. The percentage values are always referred to the corresponding non-irradiated control and represent the arithmetic mean of 4 to 6 independent experiments \pm standard deviation. A) determinations done by flow-cytometry and B) determinations done by counting the picnotic nuclei in fluorescence microscopy.

Dose	A)		B)	
	pH 7	pH 8	pH 7	pH 8
0.5 Gy	88.1 \pm 4.8	77.4 \pm 11.0	—	—
1 Gy	81.0 \pm 4.8	54.8 \pm 12.0	69.7 \pm 2.1	50.0 \pm 8.7
3 Gy	60.6 \pm 4.6	38.8 \pm 8.9	44.8 \pm 0.9	29.2 \pm 3.5

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