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*Short Report*

# Elemental composition during the apoptotic degradation phase

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**ABSTRACT** Changes in ionic content play a pivotal role in the progression of apoptosis. Electron probe X-ray microanalysis was used to measure the elemental concentrations of monovalent ions ($Na^+$, $Cl^-$, and $K^+$) during the degradation phase of apoptosis. Apoptotic cells have a higher Na content, and a lower Cl and K content. These ionic alterations occurs in cells that have completely disrupted their mitochondrial transmembrane potential.

## Introduction

Apoptosis, or programmed cell death, is an evolutionary conserved mechanism essential for the development and maintenance of tissue homeostasis in multicellular organism. This process results from the action of a genetically encoded suicide program that leads to a series of characteristics morphological and biochemical changes. The apoptotic process can be subdivided at least three different phases:

1) the initiation phase, the pro-apoptotic stimuli trigger activation of the molecular machinery of apoptosis,
2) the committed or effector phase, the molecular executioner machinery is fully activated and
3) the degradation phase, cell acquire the hallmarks of apoptosis including the morphological changes and DNA fragmentation. The morphological changes including cell shrinkage, cytoplasmic blebbing, chromatin condensation and the formation of apoptotic bodies.

Recent work from several groups suggests that changes in ionic content, primarily $K^+$, play a pivotal role in the progression of apoptosis (Bortner *et al.,* 1997; Wang *et al.,* 1999; Nietsch *et al.,* 2000). Also, the disruption of $K^+$ homeostasis constitutes an important step in the apoptotic degradation phase after committed to death has occurred (Dallaporta *et al.*, 1998). In addition, $Cl^-$ has been related with the activation of the DNA fragmentation factor (DFF) endonuclease, involved in DNA degradation into nucleosomal units during apoptosis (Rasola *et al.,* 1999). These findings suggest that transmembrane gradients of monovalent ions play a fundamental role in apoptosis. Based on these data, we decided to determine the alterations of monovalent ions ($Na^+$, $Cl^-$, and $K^+$) in the degradation phase of apoptosis by electron probe X-ray microanalysis (EPXMA). This method is a quantitative electron microscope technique that simultaneously determine the total (free plus bond) concentrations of elements (e.g. Na, Cl and K) in individual cells.

## Material and Methods

The human monoblastoid cell line U937 was used throughout this study. U937 cells were grown in RPMI 160 medium supplemented with 10% FBS, 2mM L-glutamine. Cultures were maintained at 37º C in an atmosphere of 5% $CO_2$ in air. To induce apoptosis, cells were treated with UV light, etoposide (Sigma) and staurosporine (STS) (Sigma). For UV irradiation experiments, cells ($1x10^6$ cell/ml) were exposed to a 302-nm UV transilluminator for 10 min at room temperatures, as previously described (Fernández-Segura *et al.,* 1999a). For exposure to etoposide, drug was added to the culture medium at a final concentration of 20 μg/ml. For STS experiments, the protein kinase inhibitor STS was added to the culture medium at a final concentration of 1 μM. After treatments, cells were incubated at 37º C for different time intervals. Apoptosis was detected by nuclear staining with Hoechst

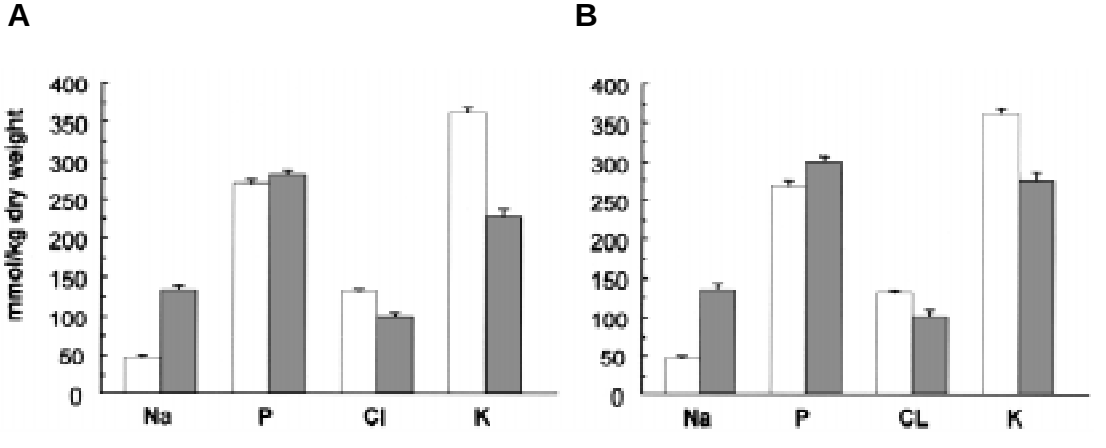


**Fig. 1. Element content (mmol/kg d.w.± SEM) of whole U937 cells under control conditions and during apoptosis induced by UV light irradiation (A) or treatment with etoposide (B).** *After treatment, cells were centrifuged on Millicell inserts, cryofixed with liquid nitrogen, freeze-dried, and examined with a scanning electron microscope. Apoptotic cells appeared smooth and rounded with membrane-bound apoptotic bodies.*

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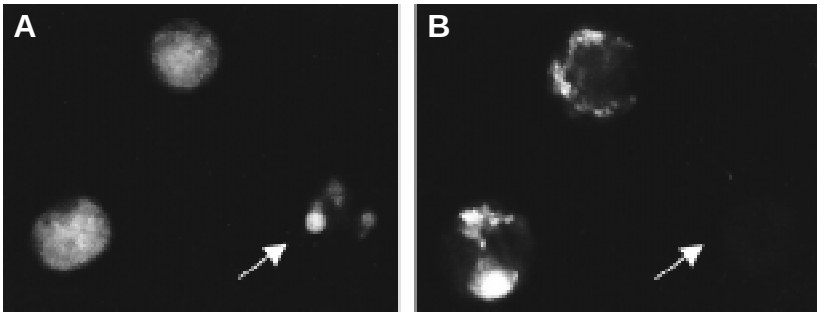


**Fig. 2. Fluorescence microscopy images of nuclei morphology (A) and mitochondrial transmembrane potential of U937 cells after irradiation with UV light.** *Cells were stained with Hoechst 33342 (5 μg/ml) and loaded with rhodamine 123 (50 nM). Note the absence of mitochondrial staining (green) in apoptotic cells (arrows).*
Note: The color version of this figure is available at http://www.ijdb.ehu.es. Follow "Back Issues" link to the *Int. J. Dev. Biol.* 2001 Supplement.

33342. Nuclear staining was done by adding 5 μl/ml of the fluorescent dye to the cell suspension and incubated for 5-10 min at 37º C. Cells were observed in a Leitz Laborlux 12 epiflourescence microscope and images were acquired using a digital camera Leica DC100. To determine changes in the mitochondrial membrane potential ($\Delta\Psi_m$) the rhodamine 123 fluorescent probe (Molecular Probes) was used. Rhodamine 123 is taken up selectively for mitochondria, ad its uptake is dependent on $\Delta\Psi_m$. Cells were loaded with rhodamine 123 (50 nM) for 15 min at 37º C in an atmosphere of 5% $CO_2$, and washed with PBS. Cells were observed in a Leitz Laborlux 12 epifluorescence microscope. For EPXMA, cells under control conditions and after induction of apoptosis were prepared for X-ray microanalysis as previously described (Fernández-Segura *et al.,* 1999b). Briefly, cells were removed from the culture medium and centrifuged into polycarbonate tissue culture plate well inserts (Millipore). After centrifugation, polycarbonate membrane filters were washed with ice-cold distilled water to remove the culture medium, plunge-frozen in liquid nitrogen and freeze-dried using three steps at -100º C, -70º c and -50º C. X-ray microanalysis was done on whole freeze-dried U937 cells in a Philips XL30 scanning electron microscope equipped with an EDAX Si(Li) energy-dispersive X-ray detector. The concentrations of elements in the specimen were obtained with the peak-to-local-background (P/B) ratio method.

## Results

Exposure of U937 to UV irradiation, etoposide, and STS resulted in cell shrinkage, plasma membrane budding and nuclei fragmentation characteristic of apoptotic cell death. To establish whether these morphological changes were associated with alterations of the monovalent ions, we determine Na, Cl, and K content in apoptotic cells by EPXMA. Figure 1 shows the elemental concentration (mmol/kg dry weight) for Na, Cl and K in control and apoptotic cells induced by UV irradiation and etoposide. Apoptotic cells, i.e, shrunken cells with membrane-bound apoptotic bodies were characterized by a reduction in Cl and K content and an increase in comparison with control cells. These elemental changes occurred prior to the loss of plasma membrane integrity as evaluated with trypan blue uptake. To investigate whether the changes in ionic content in apoptotic cells were related with a loss of membrane mitochondrial potential, $\Delta\Psi_m$ was monitored by fluorescence of the cationic lipophilic dye rhodamine 123, a potential-sensitive dye. DNA fragmentation and $\Delta\Psi_m$ were examined simultaneously in individual U937 cells induced to undergo apoptosis. Figure 2 shows that U937 cells with DNA fragmentation display a reduction in rhodamine 123 uptake.

In conclusion, we demonstrated that cells showing typical morphological features of apoptosis were characterized by an alteration of intracellular ion composition, particularly in relation with Na, Cl and K. In addition, these data support that DNA fragmentation, $\Delta\Psi_m$ disruption and changes in monovalent ions are functionally associated.

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## References

BORTNER, C.D., HUGHES F.M., and CIDLOWSKI, J.A. (1997). A primary role for $K^+$ and $Na^+$ efflux in the activation of apoptosis. *J. Biol. Chem.* 272: 32436-32442.

DALLAPORTA, B., HIRSCH, T., SUSUIN, S.A., ZAMZAMI, N., LAROCHETTE, N., BRENNER, C., MARZO, I., and KROEMER, G. (1998). Potassium leakage during apoptotic degradation phase. *J. Immunol.* 160: 5605-5615

FERNÁNDEZ-SEGURA E, CAÑIZARES, F.J., CUBERO, M.A., WARLEY, A., and CAMPOS, A. (1999). Changes in elemental content during apoptotic cell death studied by electron probe X-ray microanalysis. *Exp. Cell Res.* 253: 454-462.

FERNÁNDEZ-SEGURA E, CAÑIZARES, F.J., CUBERO, M.A., CAMPOS, A. and WARLEY, A. (1999). A procedure to prepare cultured cells in suspension for electron probe X-ray microanalysis: application to scanning and transmission electron microscopy. *J. Microsc.* 196: 19-25.

NIETSCH, H.H., ROE, M.W., FIEKERS, J.F., MOORE, A.L., and LIDOFSKY, S.D. (2000). Activation of potassium and chloride channels by tumor necrosis factor α. Role in liver cell death. *J. Biol. Chem.* 275: 20556-20561.

RASOLA, A., FAR, D.F., HOFMAN, P., and ROSSI, B. (1999). Lack of internucleosomal DNA fragmentation is related to $Cl^-$ efflux impairment in hematopoietic cell apoptosis. *FASEB J.* 13: 1711-1723.

WANG, L., XU, D., DAI, W., and LU, L. (1999) An ultraviolet-activated K channel mediates apoptosis of myeloblastic leukemia cells. *J. Biol. Chem.* 274: 3678-3685.