CHARACTERIZATION OF APOPTOSIS IN PROSTATIC ANDROGEN-INDEPENDENT CELL LINES.

Mercedes SALIDO, Antonio LOPEZ, José APARICIO, Josefa LARRAN, José VILCHES.
Department of Cell Biology. School of Medicine. University of Cadiz.

Prostate carcinoma is the second leading cause of cancer death in men. While more than 80% of the tumors initially respond to hormonal intervention, most relapse and are then refractory to further hormonal manipulation and citotoxic drugs. It's stablished that prostatic regression after androgen withdrawal ocurrs by apoptosis. Results from experiments using cytotoxic agents in human hormone independent prostate cancer are scarce, raising the question whether the relative drug insensivity of advanced prostate cancer is due to the loss of the potential to undergo apoptosis.(1,2,3,4,5)

We tested whether etoposide, a topoisomerase II inhibitor, could induce apoptosis in two prostatic cancer cell lines: PC-3, a p53 deficient cell line with biological characteristic of prostatic adenocarcinoma derived from a bone metastasis, and DU 145 a cell line with androgenic receptors, but described as androgen unresponsive, derived from a lymph metastasis, in order to get an "in vitro" model to test different anticancer drugs to induce and, eventually, revert apoptosis in prostatic cancer.

MATERIAL AND METHODS: Two neoplasic prostatic androgen-independent cell lines were used:PC-3 (Nuclear Iberica) and Du 145, (AmericanType Culture Collection).Cells were cultured in our laboratory at 37º under humidified atmosphere with 95% air and 5% CO_2 in multidish plates with 12 wells, 10^6 cells/well, with culture media, DMEM (FLOW Lab), supplemented with 4% penicilin-streptomicin (FLOW Lab), 0,4% gentamicin (GIBCO), 5% FCS (SERVA), for 24 hours to achieve adherence to the plate. Treatment groups were established: control group, with culture medium, and etoposide, doses 1,2 μM, 12 μM and 100 μM. After 8 hours incubation, cells were detached to analize apoptosis, detected by three methods: DAPI staining, to assess nuclear morphological changes, flow cytometry, and DNA internucleosomal fragmentation in agarose gel electrophoresis.

Apoptosis staining: Detached cells are counted (0,5 x $10^{6)}$ by hemocytometer, washed in PBS, and resuspended in 50 μl of PBS to make a smear. Air dried slides are fixed in metanol at -20º for 15-20 minutes, air dried, stained with DAPI (Serva) for 20 minutes at room temperature and darkness, and mounted with antifadging media -O-phenilendiamine- (Sigma) and preserved in darkness and -20º until examination. Percentages of apoptosis are calculated as apoptotic cells/non apoptotic cells.

Flow cytometry: Cells(10^5) were centrifuged at 1000 rpm for 5 min., and washed three times in PBS. Pellet was resuspended in 425 μlPBS and 25μl propidium iodide, 50 μl of NP-40 in 1% PBS were added, prior to citometric analysis. (Epics XL Coulter)

DNA fragmentation.: DNA fragmentation was monitored by a gel electrophoresis method. Briefly, samples of 10^6 cells were washed in PBS and resuspended in 50 μl Tris borate EDTA -pH: 8- (Merck), 2,4 μl Nonidet P 40 (SIGMA). Then 2μl RNAsa -1/100, 1mg/ml-(SIGMA)were added to each sample prior to incubation at 37º for 2 hours. 10μl proteinase K((Boehringer Manheim) were added and incubation at 37º continued overnight. Samples were heated to 65º, and 20 μl agarose were mixed with each samplebefore loading them into the dry wells of a 2% agarose gel in TAE 1x (Merck). Mollecular weight marker was loaded ,4μl marker (Amresco E-261), 8μl water and 0,25 bromophenol blue in 10μl agarose 1%. (Pronadisa). The gels were run at 70 V until the marker dye had migrated 3-4 cm, and then at 15 V overnight.DNA was visualized by stanining in ethidium bromide and destaining in water.

RESULTS

Apoptosis staining (fluorescent DAPI): The morphology of etoposide treated and control cells analyzed in cell extensions revealed that nuclei of control cells were round and consisted of blue fluorescent staining (fluorescence range 300-400 nm), with one or two nucleolus. In treatment groups, whithin 8 hours of exposure to etoposide, apoptotic cells appeared with chromatin condensation either to the periphery of the nuclear membrane or in clumps within the cell. The amount of condensed chromatin and apoptotic bodies varied markedly between some cells indicating different stages of apoptosis. The percentage of apoptosis in PC-3 cells treated with etoposide 100 μm for 8 hours reached 85% (over 200 nuclei) and 10% in control group. In DU 145, 100 μm etoposide treated cells 42% of apoptosis was found, and 16% in control group. No significative diferences were found between control group and groups treated with etoposide 1,2 μM and 12 μM.

Cell cycle distribution by Flow cytometry : Apoptotic cells appeared in both cell lines with DNA content lower than G1 at dose 100 μM and 8 hours of treatment. We could also observe an accumulation of cells in G1, especially in Du 145. No evidence of apoptosis was found at lower doses. The percentage apoptotic cells in trated PC-3 cells was 25,8%, and 4,5 % in control group. In Du 145 trated cells, the percentage of sub G_1 cells was 14,8 %, and 7,1 % in control group.

DNA fragmentation.: DNA internucleosomal fragmentation was observed in PC-3 cells treated with 100μM etoposide, but not with 1,2 μM and 12 μM. No internucleosomal fragmentation was found in DU 145 at any doses.

DISCUSION: Observations that many types of cells undergo apoptotic cell death, an active process, have received renewed attention. An understanding that tumor cell population dinamics depends upon changes in the balance of cell loss and gain has raised the possibility of pharmacoloogical intervention to increase cell loss by apoptosis. Many anticancer drugs initiate apoptosis, and it is possible that the failure of certain cancer cells to engage in apoptosis may explain the inherent drug resistance of many tumors.(2,6,7,8,9)

Our treated cells exhibited the typical nuclear morphology described for apoptosis, after 8 hours of treatment with 100 μM etoposide in PC 3 and even in DU 145 cell line which has no internucleosomal fragmentation demonstrable by electrophoresis. Previous studies that quantify apoptosis in PC3 by means of agarose gel electroforesis describe continuous exposure for 48-72 hours to 10mM tenoposide or 4μg/ml etoposide to obtain DNA fragmentation.(1,2,5,7,8,10). FCM results are concordant with the typical

features described for apoptosis: a detectable increase in fluorescence corresponding to less than G1 DNA content in both cell lines, and also an acummulation of cells in G1 area, especially in DU145 ,as described previously, especially for etoposide-induced apoptosis.(1,7,11,12) Several studies support microscopic examination to be one of the most reliable parameters for the detection of apoptosis due to the lack of a DNA ladder formation in apoptotic models, and the difficulty to recognize apoptotic cells by flow cytometry when DNA cleavage apparently does not reach the internucleosomal level. We could observe that percentages of apoptosis assessed by FCM are lower than those obtained by direct nuclei counting in cells stained with DAPI.(12) Therefore, in our case, even though FCM is an useful tool to characterizate apoptosis, quantitatively, direct cell counting is much more reliable. We demonstrated a ladder pattern for PC3 cell line treated with 100μM etoposide, but not with lower doses. In DU 145, and in consonance with previous data in our review, that describe "resistance" to the production of internucleosomal ladders, DNA internucleosomal fragmentation was not observed with any of the doses used, even when morphological and citometric features of apoptosis were present. The reasons for the resistance of DU 145 cell nuclei to fragmentate their DNA are unclear, and a phenotipically low level or activity of an endonuclease, or even a topographical difference in chromatin structure that make it inaccesible to an endonuclease have been described. (2,7)

CONCLUSIONS: We have been able to characterizate drug induced apoptosis in the prostatic androgen independent cell lines PC3 and DU 145, by the three classical apoptotic detection methods: morphological, biochemical, and cytometric. We found etoposide 100μM treatment for 8 hours adequate to induce apoptotic changes in these cells, a lower dose than previously used in our bibliographical review, so as the drug exposure time. To our purpose results obtained after morphological quantification of nuclear changes are more reliable and precise than those obtained by flow cytometry. Work supported by a SAF-763/93 grant by CICYT.

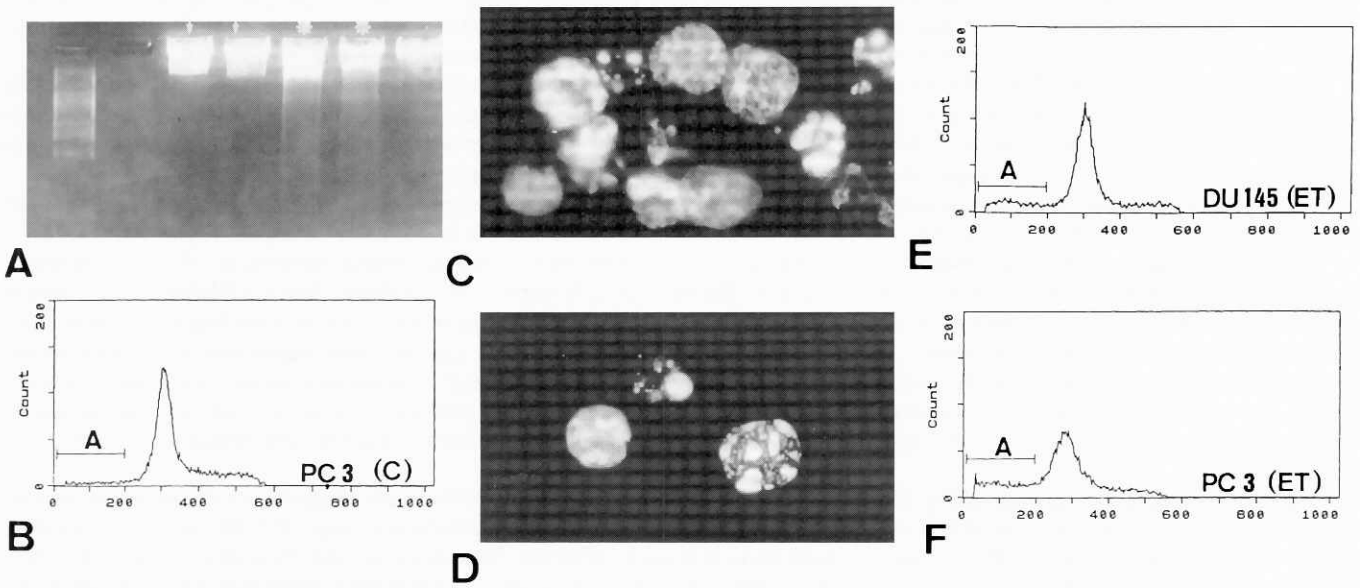

Fig 1: a. Internucleosomal DNA fragmentation (*PC3, Du145);b. FCM for control cells (A:apoptotic cells);c.d. Nuclear morphology:apoptotic bodies; e.f. FCM for etoposide treated cells.

REFERENCES

1.- Borner, M.M., Myers, C.E., Santor, O., Sei, .Y, Toko, .T, Trepel.J., Schneider, E. (1995). Drug induced apoptosis is not necessarially dependent on macromolecular synthesis or proliferation in the p53 negative human prostate cancer cell line PC-3. Cancer Res. 55: 2122-2128.

2.- Oberhamer, .F, Wilson, J.,, Dive, C., Morris, I.D., Hickman, J.A., Wakeling, A.G., Walker, P.R., Sikorska, M.(1993). Apoptotic death in epithelial cells: cleavage of DNA prior to or in the abcense of internucleosomal fragmentation. EMBO J. 12(9): 3679-3684.

3.- Kiprianou, N., English, H.F., Isaacs, J.T.(1990) Programmed cell death during regression of PC-82 human prostate cancer following androgen ablation. Cancer Res. 50: 3748-3753.

4.- Schwartzman, R.A., Cidlowski, J.A.(1993). Apoptosis: The biochemistry and molecular biology of programmed cell death. Endocrine Review. 14: 133-151.

5.- Stewart, B.W.(1994). Mechanisms of apoptosis. Integration of genetic, biochemical and cellular indicators. J Natl Cancer Inst. 86 (17): 1286-12966.- Gerschenson, L.E., Rottello, R.J. (1992). Apoptosis: a different type of cell death. FASEB J. 6: 2450-2455.

6.- Gerschenson, L.E., Rptello, R.J. (1992). Apoptosis: A different type of cell death. FASEB J.6:2450-2455.

7.- Catchpoole, D.R., Stewart, B.W.(1995) Formation of apoptotic bodies is associated with internucleosomal DNA fragmentation during drug induced apoptosis. Exp Cell Res. 216: 169-177.

8.- Berchem, G.J., Bosseler, M., Sugars, L.Y., Voeller, H.J., Zeitlin, S., Gelmann, E.P.(1995). Androgens induce resistance to bcl-2 mediated apoptosisin LNCaP prostate cancer cells. Cancer Res. 55: 735-738

9.- Barry, M.A., Reynolds, J.A., Eastman, A.(1993). Etoposide induced apoptosis in human HL60 cells is associated with intracellular acidification. Cancer Res. 53: 2394-2357.

10.- Earnshaw, W.C.(1995). Nuclear changes in apoptosis. Current Opinion in Cell Biology. 7:337-343.

11.- O'Connor, P.M., Jackmann,J. (1996). Synchronization of mammalian cells. In *Cell Cycle matherials and methods.* (Ed. M.Pagano) Springer Verlag, ,Heidelberg, pp 63-74.

12.- Zamai, L., Falcieri, E., Marhofka, G., Vitale, M.(1996). Supravital exposure to propidium iodide identifies apoptotic cells in the abcense of nucleosomal DNA fragmentation. Cytometry. 23: 303-311.