CHARACTERIZATION OF APOPTOSIS IN PROSTATIC ANDROGEN-INDEPENDENT CELL LINES.

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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 cytotoxic drugs. It's established that prostatic regression after androgen withdrawal occurs by apoptosis. Results from experiments using cytotoxic agents in human hormone independent prostate cancer are scarce, raising the question whether the relative drug insensitivity 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 neoplastic 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% CO2 in multiship plates with 12 wells, 105 cells/well, with culture media, DMEM (FLOW Lab), supplemented with 4% penicillin-streptomycin (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, 12, 100 µM. After 8 hours incubation, cells were detached to analyze apoptosis, detected by three methods: DAPI staining, to assess nuclear morphological changes, flow cytometry, and DNA internucleosomal fragmentation in agarose gel electrophoresis.

Apoptosis staining: Cells are counted (0,5 x 106) by hemocytometer, washed in PBS, and resuspended in 50 µl of PBS to make a smear. Air dried slides are fixed in methanol at -20º for 15-20 minutes. Air dried, stained with DAPI (Serva) for 20 minutes at room temperature and darkeness, 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(105) were centrifuged at 1000 rpm for 5 min., and washed three times in PBS. Pellet was resuspended in 450 µl PBS and 25 µl 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 106 cells were washed in PBS and resuspended in 50 µl Tris borate EDTA pH: 8. (Merck), 2,4 µl Nonidet P 40 (SIGMA). Then 2,4 µl RNase -1/100, 1mg/ml (SIGMA) were added to each sample prior to incubation at 37º for 2 hours. 10 µl proteases K (Boehringer Mannheim) were added and incubation at 37º continued overnight. Samples were heated to 65º, and 20 µl agarose were mixed with each sample before loading them into the dry wells of a 2% agarose gel in TAE 1x (Merck). Molecular weight marker was loaded 4,2 µl marker (Amresco E-261), 8,µl water and 0,25 bromophenol blue in 10 µl 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 staining 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, within 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 amongst 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 differences 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 treated PC-3 cells was 25,5%, and 4,5% in control group. In DU 145 treated cells, the percentage of sub G1 cells was 14,8 %, and 7,1 % in control group.

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

DISCUSSION: Observations that many types of cells undergo apoptotic cell death, an active process, have received renewed attention. An understanding that tumor cell population dynamics depends upon changes in the balance of cell loss and gain has raised the possibility of pharmacological 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 electrophoresis describe continuous exposure for 48-72 hours to 10 mM 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 accumulation 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. We demonstrated a ladder pattern for PC3 cell line treated with 100 μM 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 phenotypically low level or activity of a endonuclease, or even a topographical difference in chromatin structure that make it inaccessible to an endonuclease have been described. (2,7)

CONCLUSIONS: We have been able to characterize 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 100 μM 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-789/93 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