Detection of Apoptosis in a Heterogenous Cell Population using Flow Cytometry

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Abstract. Apoptosis induced in human leukemic cells (promyelocytic human leukemic cells HL-60, multidrug-resistant subline HL-60/VCR) and human ovarian carcinoma cells (A2780 and multidrug-resistant subline A2780/ADR) *in vitro* was detected by flow cytometric analysis or DNA electrophoresis. The cytofluorometric techniques utilized, i. e. detection of phosphatidylserine exposed at the outer surface of the plasma membrane , identification of cells with "sub-G_0" DNA content or increased light side scatter (cell internal structure) correlated with the electrophoretic determination of DNA fragmentation ("DNA ladder"). Detection of the 34 kDa mitochondrial protein recognized by the monoclonal antibody Apo2.7 yielded elevated percentages of apoptotic cells, suggesting that this technique detecting both early and late apoptosis in digitonin-fixed cells might not be restricted to the specific detection of programmed cell death.

Key words: Apoptosis — Flow cytometry — "Hypodiploid" DNA content — Annexin V binding — Apo 2.7 monoclonal antibody — Scatter parameters — DNA "ladder"

Introduction

Apoptosis, or programmed cell death (PCD), an active process for removal of redundant or autoreactive immune cells appears as a regulator of key physiological developmental processes involved in embryogenesis, tissue involution or clonal selection in the differentiation of immune system (Bursch et al. 1992; Willey et al. 1980). Failure of neoplastic cells to undergo drug- or radiation-induced apoptosis appears as one of the mechanisms of malignant cell resistance to therapy (Smets 1994, Hickman 1996).

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Apoptosis is characterized by a complex cascade of morphological and biochemical events with cell type- and apoptotic stimulus-dependent variations in the exact sequence of apoptotic alterations. Biochemical alterations of apoptotic cells involve up-regulation of intracellular Ca^{2+} levels, mitochondrial membrane pore transition, increase of intracellular lipid mediator ceramide, high molecular weight DNA fragmentation (to 50–200 kbp fragments), poly (ADP-ribose) polymerase (PARP) proteolytic degradation, exposition of aminophospholipid phosphatidylserine (PS) on the outer surface of the plasma membrane, internucleosomal DNA cleavage (to multiples of 200 bp DNA fragments – "DNA ladder"), nuclear lamin-, topoisomerase I, II, APC protein proteolytic cleavage (Patel et al. 1996; Browne et al 1998) orchestrated by a series of caspases – proteases cleaving their substrate after aspartic acid residue (Cohen 1994).

Morphological alterations of apoptotic cells include cell shrinkage, chromatin condensation, nuclear segmentation and formation of apoptotic bodies. Several biochemical (PS exposition, DNA fragmentation) as well as morphological (cell shrinkage, nuclear segmentation, formation of apoptotic bodies) alterations are reflected by-, and can be assessed with the aid of flow cytometry (FCM) and cytofluorometric techniques. In the present study, we examined the drug- and radiation-induced apoptosis in some human leukemic and carcinoma cell lines characterized by flow cytometric light scatter parameters, exposition of PS on plasma membrane outer surface (Annexin V binding), increased expression of a 34 kDa mitochondrial protein (detected with the monoclonal antibody Apo2.7) and relation between detection of apoptotic cells with "sub-G_0 " DNA content by FCM and apoptosis assessment with the aid of electrophoretic detection of DNA fragmentation.

Materials and Methods

Cell lines The human ovarian carcinoma cell lines A2780 and A2780/ADR were cultured in RPMI 1640 cell culture medium supplemented with 10% fetal calf serum as monolayer cell cultures in humidified 5 0% CO_2 atmosphere Human myeloid leukemia cell line HL-60 and its multidrug-resistant subline HL-60/VCR (with MDR-1 coded Pgp/p170, Marsh et al 1996) obtained from Dr P Ujházy, Roswell Park Cancer Institute, Buffallo, with the consent of Dr M S Center (Kansas State University, Manhattan, KS, USA) were cultured in the same medium as suspension cell culture

Compounds Propidium iodide, staurosporine (SSP) and paclitaxel were from Sigma Chem Co , St Louis, MO, USA Dimethylsulfoxide (DMSO) was from Serva, fluorescence-conjugated Annexin-V-FLUOS was from Boehringer Mannheim, benzamide riboside (BR) (Jayaram et al 1992) was kindly provided by Prof H Jayaram, University of Indiana, School of Medicine, Indianapolis, IN, USA Monoclonal antibody Apo2 7 recognizing the 34 kDa mitochondrial apoptosis-related antigen was from Immunotech, France

Treatment of cells. Cells were incubated with paclitaxel (100 nmol/l) for 1 hour, then washed twice in cell culture medium with subsequent 23 hour cultivation in paclitaxel-free medium Cells were treated in plastic TC-cluster plates at 10^5 cell/ml concentration with BR (10 μmol/l) and SSP (200 nmol/l) or their combination for 24 hours

Radiation exposure was performed with the aid of STABILIPAN X-ray radiotherapy

source (Siemens) equipped with the Thoreus filter, at 300 kV and dose rate of 1 475 Gy min^{-1}

Flow cytometric analysis FACS analysis was performed with the aid of a FACStar (Becton Dickinson, Mountain View, CA) flow cytometer equipped with a 5 W argon ion laser tuned to 488 nm excitation wavelength, according to the instructions of the manufacturer and as described previously (Sedlák et al 1994, 1997, 1998) Data were acquired in CONSORT 30, provided by the manufacturer (Becton Dickinson) and evaluated by WinMDI Version 2 7 software (obtained from Dr J Trotter, Scripps Research Institute, La Jolla, USA)

Flow cytometric detection of apoptosis Apoptosis was detected with flow cytometric analysis as pre-G_0 (hypodiploid) cells in propidium iodide-stained detergent-permeabilized cells, or as cells with phosphatidylserine exposed on the external site of the plasma membrane stained with Annexin-V-FLUOS (Beohringer, Mannheim, Germany) according to Vermes et al (1995) Cell surface- and intracellular expression of the antigen recognized by Apo2 7 monoclonal antibody was performed by immunocytofluorometry according to the previously described procedure (Sedlák et al 1997) on viable or digitonin-fixed cells Digitonin fixation of cells was performed according to the instructions of the monoclonal antibody provider (Immunotech, France)

Gel electrophoresis of fragmented DNA Cells were incubated for indicated time period with BR, SSP or their combination After the treatments, cells were collected and centrifuged at 800 × *g* for 5 min The resulting cell pellets corresponding to 4 10^5 cells were lyzed in 50 μl buffer containing 10 mmol/l Tris, pH 8 0, 10 mmol/l EDTA(Na), 0 5 % Nonidet P40, supplemented with 10 μg proteinase K (Boehringer) and kept for 1 h at 37 °C After inactivation proteinase K (10 min, 70 °C), 5 μg RNAse A (Boehringer, Mannheim, Germany) was added to each sample, for 1 h at 37 °C Finally, the samples were electrophoresed in 2% (w/v) agarose gel and DNA was visualized by staining with ethidium bromide

Results

Detection of apoptosis by flow cytometric measurement of phosphatidylserine (PS) exposition on the outer cell surface

Detection of apoptosis with the aid of Annexin V/FITC binding to the externally exposed PS on the cell membrane of apoptotic human ovarian carcinoma (A2780) cells induced with cytotoxic microtubule-stabilising agent paclitaxel allowed to separate apoptotic and necrotic cells. Apoptotic cells appeared as cells with intact plasma membrane (i. e unstained with propidium iodide) and externally exposed PS (i. e. AnnexinV-FITC stained cells). This technique allowed to demonstrate the sensitivity of human ovarian carcinoma (A2780) cells *in vitro* to paclitaxel- and paclitaxel-radiosensitization to apoptosis and a marked reduction of this apoptosis in multidrug-resistant A2780/ADR cells (Fig. 1)

Flow cytometric detection of Apo2.7 antigen expression

The expression of a 34 kDa mitochondrial protein detected with monoclonal Apo2 7 antibody as a marker for apoptosis was examined in human leukemic HL-60 cells treated with paclitaxel Immunocytofluorometric detection of Apo2.7 binding cells

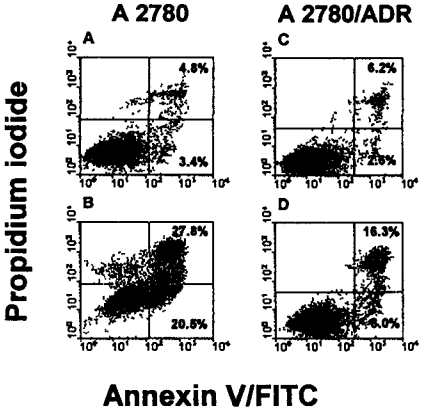

Figure 1. Detection of paclitaxel- and irradiation induced apoptosis in ovarian carcinoma (A2780, A, B) cells and multidrug-resistant subline A2780/ADR (C, D) with the aid of flow cytometric (FCM) measurement of externally exposed PS (Annexin V/FITC binding, FL1) and cell necrosis with propidium iodide staining of non-permeabilized cells, FL2. Abscissa: FL1 – AnnexinV/FITC binding, i.e. PS exposition on the outer surface of cell membrane. Ordinate: FL2 – propidium iodide staining of non-permeabilized cells with impaired cell membrane. Left lower quadrant – viable cells, right lower quadrant – apoptotic cells, right upper quadrant – necrotic cells.

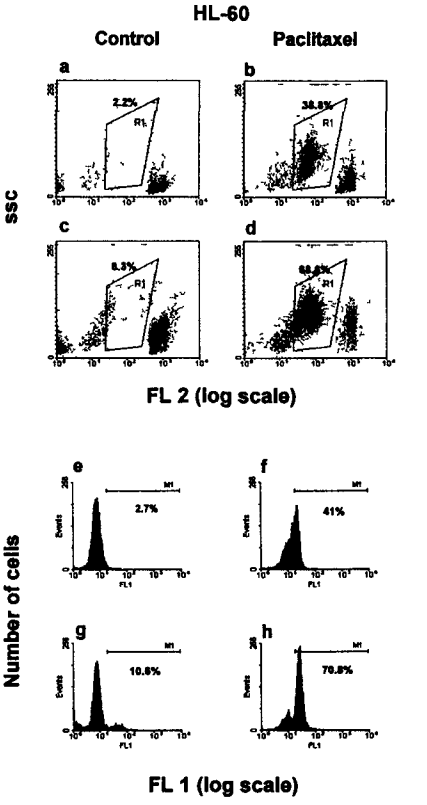

Figure 2. Scatter parameter (SSC – side angle scatter, internal cell structure) vs propidium iodide staining of detergent permeabilized cells, i.e. DNA cell content (*a–d*), and Apo2.7 antigen immunocytofluorometric detection (*e–h*) in HL-60 cells treated with 100 nmol/l paclitaxel for 24 h. Abscissa: (*a–d*) FL2 – propidium iodide staining, i.e. DNA content, (*e–h*): FL1 – binding of Apo2.7 monoclonal antibody to intact (*e, f*) or digitonin-fixed (*g, h*) HL-60 cells. Ordinate: (*a -d*) SSC – side angle scatter, (*e–h*) number of cells. Frames R1 (*a–d*) indicate apoptotic cells identified as cells with hypodiploid DNA content and elevated side angle scatter, markers M1 (*e–h*) indicate Apo2.7 positive cells.

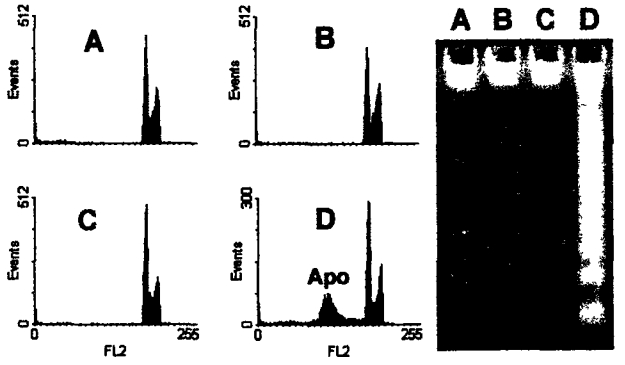

Figure 3. Apoptosis induced in multidrug-resistant human leukemic cells HL-60/VCR (*A*) *in vitro* treated with 10 μmol/l benzamide riboside (*B*), 200 nmol/l staurosporine (*C*), and a combination of BR and SSP (D) for 24 hours detected with the aid of flow cytometry as cells with hypodiploid DNA content and increased internal structure (SSC) or with the aid of agarose DNA electrophoresis ("DNA ladder"). Abscissa: FL2 – propidium iodide staining, i.e. DNA content. Ordinate: number of cells. Apo indicate apoptotic, hypodiploid cells.

revealed aproximately 40% of Apo2.7 positive cells corresponding to the proportion of apoptotic cells detected by other utilized techniques. On the other hand, the elevated proportion (approximately 70%) of digitonin-fixed paclitaxel-treated cells positive for Apo2.7 antigen suggested that this technique for detection of both early and late apoptosis might not be restricted to recognition of apoptotic cells only (Fig. 2).

Flow cytometric detection of cell with "sub-G^0" DNA content in correlation to the detection of DNA degradation with the aid of agarose electrophoresis

Comparison of flow cytometric detection of apoptosis induced in human multidrug-resistant promyelocytic leukemia (HL-60/VCR) treated with inosine monophosphate (IMP) dehydrogenase inhibitor benzamide riboside (BR), protein kinase inhibitor staurosporine (SSP) with electrophoretic analysis of DNA degradation – "DNA ladder" (Fig. 3) showed that only cells treated with both benzamide riboside and staurosporine displayed a similar level of apoptosis detected by both flow cytometry and DNA electrophoresis (Fig. 3).

Discussion

Annexin V binding to phosphatidylserine (PS) on the outer plasma membrane surface of apoptotic cells appears as a tool for evaluation of apoptosis induced with a variety of stimuli in different normal and neoplastic cells. This technique (Boersma et al. 1996; Vermes et al. 1995) based on decreased aminophospholipid translocase

activity and activation of aminophospholid "scramblase" in apoptotic cells was utilized recently to detect e g mercuric compound-induced apoptosis in human monocytes (Insug et al 1997, Shenker et al 1997), dideoxynucleoside analog-induced apoptosis in human T-leukemic cell line (Viora et al 1997), radiation-induced apoptosis in human lymphocyte subsets (Philipe et al 1997) or in hairy cell leukemia patient's cells after 2-chlorodeoxyadenosine treatment (Idink-Mecking et al 1998) Our data showing the exposition of PS in examined drug- and radiation-induced leukemia and carcinoma cell *in vitro* are compatible with the exposition of PS on the outer cell surface as phylogenetically conserved hallmark of early apoptosis (Van den Eijnde et al 1998)

Alterations of light scatter parameters in apoptotic cells reflect the morphological changes characteristic for apoptosis (i e cell shrinkage – decreased forward angle scatter (FAS), nuclear fragmentation, appearance of apoptotic bodies, increased cell granularity – increased right angle scatter) Such alterations of scatter parameters were observed in a variety of apoptotic cells induced by diverse apoptogenic stimuli (Insug et al 1997, Philippe et al 1997, Shenker et al 1997, Viora et al 1997) Our data extend these observations to human promyelocytic leukemia (HL-60) cells induced with benzamide riboside and staurosporine

Detection of a 34 kDa mitochondrial protein recognized by monoclonal antibody Apo 2 7 appears as a relatively new tool for detection of early apoptosis (Koester et al 1997) if increased in digitonin-fixed and permeabilized cells, while cell surface expression of this antigen is a marker of late apoptosis Our data with paclitaxel-induced HL-60 cells suggest that also in our system this mitochondrial protein appears on the cell plasma membrane during late apoptosis However, recent data (Overbeeke et al 1998) suggest that the expression of the antigen detected with the aid of Apo 2 7 monoclonal antibody might not be strictly related to apoptosis In these experiments Annexin V/Propidium iodide flow cytometric assay appeared as the most sensitive test for estimation of apoptosis in cultured neoplastic hematopoietic cells (Overbeeke et al 1998)

Flow cytometry was shown to detect apoptosis (as altered scatter parameters, increased Annexin V binding, and with the aid of TUNEL assay) in samples of organomercury-treated T-cells where DNA electrophoresis failed to demonstrate "DNA ladder" (Shenker et al 1997) Similar data obtained by ourselves in benzamide riboside- and staurosporine induced HL-60 cells show an approximately equivalent sensitivity of DNA electrophoresis and flow cytometric detection of apoptosis in human promyelocytic leukemia cells *in vitro* treated with an IMP dehydrogenase inhibitor, benzamide riboside in combination with a potent protein kinase inhibitor, staurosporine

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