Cell Death and Differentiation (2004) 11, 1153–1154

www.nature.com/cdd

Letter to the Editor

A novel method for the combined flow cytometric analysis of cell cycle and cytochrome *c* release

Cell Death and Differentiation (2004) **11,** 1153–1154. doi:10.1038/sj.cdd.4401480
Published online 23 July 2004

Dear Editor,

Most anticancer drugs exert their effects by the induction of apoptosis and/or interfering with cell cycle progression.[1] Often these drugs give rise to specific patterns of cell death and cell cycle arrest that vary according to the drug used and the molecular status of the target cell. A prominent example for such differences is the p53 status. The human colorectal cancer cell line HCT116 harboring wild-type p53 is sensitive to 5-fluorouracil (5-FU), while HCT p53 $-/-$ cells, generated by somatic homologous recombination, are resistant to the effects of 5-FU.[2] In contrast, treating these cells with adriamycin (doxorubicin) yields the opposite effect. This example highlights how tumor cell responses are a function of the presence or absence of particular molecular functions and can in turn vary dependent on the drug used. Cell death and cell cycle are often affected at the same time. A method that allows to determine the cellular responses to a given drug with regard to apoptosis and cell cycle could therefore provide important information on the molecular mechanisms of drug cytotoxicity and sensitivity of individual tumor samples. It also offers the opportunity to clearly distinguish between the loss of cell cycle phases due to apoptosis, which might be misinterpreted as cell cycle arrest, and genuine cell cycle effects of the antitumor drug. Furthermore, the method could serve in the elucidation of combination drug therapies by matching the apoptosis/cell cycle profiles of two or more drugs in order to achieve the largest synergistic effects, for example, a drug that drives cells into a particular cell cycle phase could be combined with a drug that mainly elicits cell death in that segment of the cell cycle.

Flow cytometry can rapidly and quantitatively analyze the characteristics and responses of individual cells and is easily automatable. Therefore, as a well-established and routine method, it is suited for the analysis of individual patient material in order to arrive at optimized personalized antitumor drug therapies in the clinical setting or to aid the identification of novel compounds and combination treatment by high-throughput screening methods. Here, we present a novel approach for the simultaneous assessment of apoptosis and cell cycle based on mitochondrial cytochrome *c* release combined with propidium iodide (PI) staining and its application in the analysis of drug-induced cytotoxicity in tumor cell lines.

We used three different cell lines in our studies: Jurkat cells (human leukemic T cells), H9 cells (human T-lymphocyte cell line) and HCT116 cells (human colon cancer cells) that were grown in their normal growth media (RPMI1640 and McCoys, respectively, that contained 10% FCS and 1% penicillin/streptomycin). These cells were seeded in 24-well plates in 1 ml medium, treated with various antitumor drugs including etoposide (30 μg/ml; Sigma, St Louis, MS, USA), cytarabine (Ara-C) (10 μg/ml; Sigma, St Louis, MS, USA) and 5-FU (400 μM; Sigma, St Louis, MS, USA) and harvested 6–24 h post-treatment. Cells growing in suspension were added directly to 2 ml of the washing buffer, while adherent cells (HCT116 cells) were harvested by treatment with 1% trypsin/0.05% EDTA (Biochrom, Berlin, Germany). The washing buffer contained Hanks-buffered saline (Invitrogen, Carlsbad, CA, USA), 0.1% sodium azide (Sigma, St Louis, MS, USA) and 10% bovine serum albumin fraction V (Sigma, St Louis, MS, USA). Following centrifugation at 300 $\times$ *g*, the cells were fixed in 4% paraformaldehyde for 20 min at 4°C and subsequently rinsed with washing buffer. After recentrifugation, the cell pellet was resuspended in 1 ml GM buffer containing 1.1 g/l glucose, 8 g/l NaCl, 0.4 g/l KCl, 0.39 g/l Na_2HPO_4 and KH_2PO4 supplemented with 1 μg/ml digitonin (Sigma, St Louis, MS, USA) from a 50 mg/ml stock solution stored at -20°C. Digitonin is used to render cells accessible to the cytochrome *c* antibody and PI. The cell suspension was vortexed at low speed and incubated for 2 min on ice, after which the cells were washed in GM buffer without digitonin and centrifuged. Subsequently, the cells were blocked in 55 μl phosphate-buffered saline (PBS) containing mouse $IgG_{1\kappa}$-purified immunoglobulin (Sigma, St Louis, MS, USA) for 5 min at room temperature. Anti-cytochrome *c* antibody (7H8.2C12, BD Pharmingen, San Diego, CA, USA) was then added as a 1 : 20 dilution (in PBS) of the stock supplied by the manufacturer. The cells were incubated for 20 min at 4°C with the cytochrome *c* antibody and then washed once in washing buffer. An isotype-matched immunoglobulin IgG_{2b} (DAKO, Glostrup, Denmark) was used as control. The cytochrome *c* antibody was detected by a goat $F(ab')_2$ anti-mouse IgG_{2b}-FITC-conjugated secondary antibody (Southern Biotechnology Associates, Birmingham, AL, USA), applied as a 1:20 dilution of the commercial stock solution.

The specificity of the cytochrome *c* antibody (7H8.2C12, BD Pharmingen, San Diego, CA, USA) has been tested by immunocytochemical staining revealing that the antibody we used could not detect the cytosolic cytochrome *c* after apoptosis induction as demonstrated by Stahnke *et al*.[3] This is in contrast to another cytochrome *c* antibody that is able to bind to mitochondrial as well as cytosolic cytochrome *c* after stimulation ruling out cellular cytochrome *c* loss during the

apoptotic process or by the permeabilization procedure.[3,4] Therefore, the reduction in cytochrome *c* signal as detected by our antibody (7H8.2C12, BD Pharmingen, San Diego, CA, USA) reflects mitochondrial cytochrome *c* release and early-onset of apoptosis.[3] The cytochrome *c* staining procedure was followed by treatment with PI. Cells were washed in washing buffer following the incubation with the secondary antibody and subsequently treated with 200 μl solution S (PBS buffer freshly supplemented with 20 μg/ml RNase A (Roche Diagnostics, Mannheim, Germany) and 15 μg/ml PI from a 1 mg/ml stock solution (Sigma, St Louis, MS, USA)). After vortexing at low speed, the cells were incubated for 15 min at 37°C and then kept on ice until flow cytometric measurement. Cell analysis was performed by flow cytometry on a FACSCalibur Cytometer (Becton Dickinson) equipped with a 488 nm argon and a 650 nm red diode laser.

Figure 1 shows examples of simultaneous measurement of DNA content and cytochrome *c* in different cell lines following treatment with various drugs. The measured cytochrome *c* signal (*y*-axis) is plotted against the PI signal (*x*-axis). Gating on the morphologically unaltered cells allows the detection of early apoptotic events upon stimulation, demonstrated by decrease of the cytochrome *c* signal (cytochrome *c* lo). Despite the mitochondrial cytochrome *c* release, cells at the early onset of apoptosis maintain their preapoptotic DNA content and appear as a cell population with lower *y*-value (cytochrome *c*) and unchanged *x*-value (PI) below the original population that defines a particular cell cycle phase as indicated by the labels (G1, S, G2). In conclusion, the cytochrome *c versus* PI plot allows to identify the cell cycle phase cells are dying from, by following the path of cells after drug treatment along the cytochrome *c* axis.

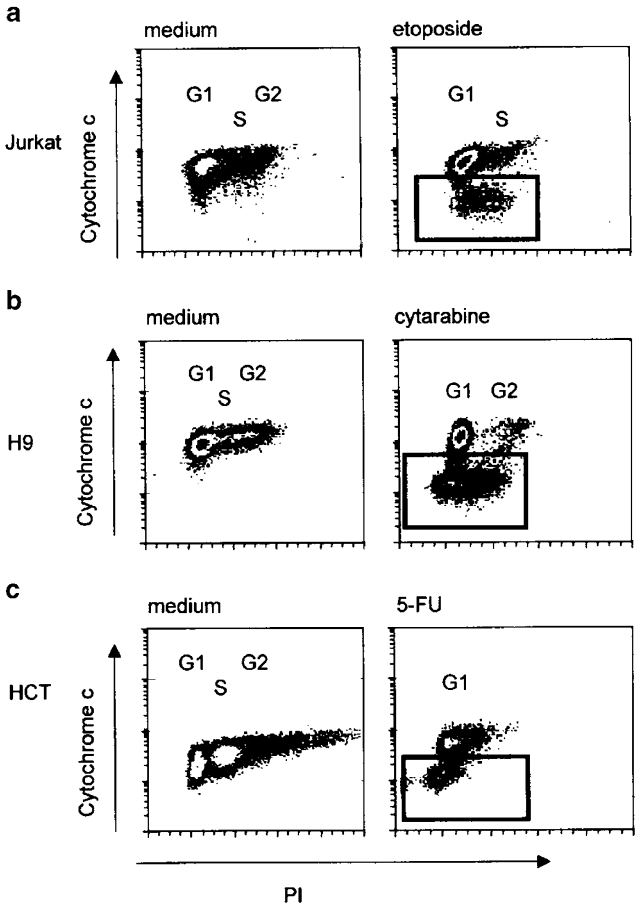

Figure 1 Jurkat (**a**), H9 (**b**) and HCT116 (**c**) cells were treated with etoposide, cytarabine and 5-FU, respectively. Carrier-treated controls are labeled 'medium'. The dot density plots depict the DNA content (*x*-axis, lin-scale) *vs* cytochrome *c* signal (*y*-axis, log-scale) of cells treated with cytotoxic drugs as indicated. The decreased cytochrome *c* signal marks the onset of apoptosis in the labeled cell cycle phases

Thus, Jurkat cells treated with etoposide undergo apoptosis mainly from G1- and S-phase as indicated by the reduced cytochrome *c* signal in these cell cycle phases (Figure 1a). Another pattern of apoptosis induction is observed in H9 cells treated with the nucleotide analogue cytarabine (Figure 1b). The analysis of cytarabine-treated H9 cells revealed that all cells in S-phase respond with cytochrome *c* release and consequently undergo apoptosis. Cells in G2-phase are also triggering apoptosis upon treatment, but to a lesser extent. The third example shows that HCT116 cells treated with 5-FU give rise to apoptosis after 24 h only out of G1-phase (Figure 1c). In contrast to Jurkat and H9 cells, HCT116 harbor wild-type p53 known to induce G1 arrest upon cytotoxic drug treatment or irradiation. Thus, differential patterns of apoptosis induction related to cell cycle phases can be detected by simultaneous measurement of cytochrome *c* release and DNA content.

Measuring cytochrome *c* release and cell cycle has the advantage of detecting very early apoptotic and mitochondrial processes over an Annexin-V/cell cycle combination. Chemotherapeutic drugs used in antitumor treatments frequently exert their effects by triggering the mitochondrial apoptotic machinery,[5] and resistance to those drugs is often afforded by defects in this system.[6] Our method is able to uncover such mitochondrial resistance mechanisms and, therefore, represents a valuable technical advancement. The method may prove useful in identifying the apoptosis-sensitive cell cycle phase for a given tumor sample/anticancer drug combination. It offers the opportunity to design personalized drug regimens and to identify new combined treatment modalities.

Acknowledgements

We thank Gabi Aggeler for her excellent technical assistance. The study was supported by grants from the Deutsche Forschungsgemeinschaft and Bundesministerium für Bildung und Forschung.

A Mohr[1], RM Zwacka[2], K-M Debatin[1] and K Stahnke[1]*

[1] University Children's Hospital, University of Ulm, Ulm 89075, Germany
[2] Division of Gene Therapy, University of Ulm, Ulm 89081, Germany
* Corresponding author: A Mohr, University Children's Hospital Ulm, Prittwitzstr. 43, Ulm 89073, Germany; E-Mail: andrea.mohr@medizin.uni-ulm.de

1. Darzynkiewicz Z *et al.* (1981) Cytometry 4: 279–286
2. Bunz F *et al.* (1999) J. Clin. Invest. 104: 263–269
3. Stahnke K *et al.* (2004) Apoptosis 4: 457–465
4. Renz A *et al.* (2001) Blood 98: 1542–1548
5. Herr I and Debatin K-M (2001) Blood 98: 2603–2614
6. Kim CN *et al.* (1997) Cancer Res. 57: 3115–3120