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Detection and quantification of apoptosis in transiently transfected adherent cells

Patrice Lassus* and Urszula Hibner

Institut de Génétique Moléculaire, CNRS UMR 5535, 1919 route de Mende, 34293 Montpellier Cedex 5, France

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ABSTRACT

Apoptosis is a highly conserved form of cell death present in all eukaryotic cell types and controlled by multiple genes. Several methods have been developed to quantify apoptosis, but none is adapted for all cell types. It is particularly difficult to reliably assay apoptosis of adherent cells. We describe a new, rapid and reliable flow cytometric method which can be used for quantifiying apoptosis in a sub-population of transiently transfected adherent cells. This technique is based on the detection of transfected cells and the apoptotic sub-population by immunofluorescence and Annexin-V labelling, respectively.

Apoptosis, or programmed cell death, is an active process that is fundamental to the development and homeostasis of multicellular organisms (1,2). An apoptotic cell undergoes multiple morphological changes, including membrane blebbing, cell shrinkage, chromosomal condensation and DNA fragmentation (3). In the early stages of apoptosis, phosphatidylserine is translocated from the inner to the outer layer of the cytoplasmic membrane, resulting in the exposure of this phospholipid at the external cell surface (4).

Several methods have been developed for the quantitative detection of apoptotic cells, including techniques based on the labelling of oligonucleosomal fragments, specific for apoptosis, by fluorescent dyes [TUNEL assay (5), Hoechst dye and propidium iodide labelling (6)]. An alternative technique is based on the labelling of apoptotic cells by a Ca^{2+}-dependent phosphatidylserine-binding protein termed Annexin-V (4).

Numerous genes are implicated in the regulation of apoptosis (7–11). Since many of the studies concerning the control of programmed cell death are based on transient transfection assays, it is of the utmost importance to establish reliable quantitative methods for measuring apoptosis within a transfected sub-population of cells (12,13). This has proved difficult for adherent cell types. The use of fluorescence microscopy to detect transfected cells and quantify apoptosis within this population by Hoechst or TUNEL assay is inappropriate since apoptotic cells tend to detach from the dish. Furthermore, the characteristic apoptotic peak detectable by flow cytometry after propidium iodide labelling, representative of a population with less than 2N DNA, rarely appears in these cells. Rather, the apoptotic population is indistinguishable from cellular debris, rendering quantification impossible.

We have developed an alternative method for quantification of apoptosis in a sub-population of transiently transfected adherent cells. The cells are co-transfected with a gene of interest and a plasmid encoding a truncated rat CD2 surface antigen in the 3:1 ratio. The assay is based on flow cytometry and combines labelling of apoptotic cells with Annexin-V-FITC and detection of CD2 + transfected cells. We have opted for the use of a cell surface marker (CD2) rather than the green fluorescent protein (GFP) for detection of transfected cells, because we found it difficult to eliminate the overlap of the GFP fluorescence emission into the FL3-H (red fluorescence) channel.

To validate the method, we used a fibroblastic cell line 10.1 (14). We have previously shown that these cells readily undergo apoptosis in the absence of serum (15). The apoptotic response is enhanced by overexpression of the tumour suppressor p53 gene (15) and delayed by the anti-apoptotic protein bcl-2.

150 000 cells were seeded in a 6 cm diameter dish and grown in DMEM-glutamax supplemented with 10% FCS. The cells were transfected with 0.75 µg of a plasmid eoncoding a rat CD2 antigen lacking the intracytoplasmic domain together with 2.25 µg of either a control, empty vector (pCDNA3) or a plasmid carrying either pro-apoptotic p53 or anti-apoptotic bcl-2 gene. LipofectAMINE Reagent (Gibco BRL) (3 µl per transfection) was used according to the manufacturer's instructions. Following transfection the cells were cultured in DMEM-10% FCS for 24 h, rinsed once with PBS and cultured for additional 16 h in serum-free DMEM. Forty hours after transfection, floating cells were collected and adherent cells were detached with trypsin. The cells were pooled, centrifuged at 250 *g* for 5 min at room temperature, rinsed with PBS, transferred to an Eppendorf tube and centrifuged as above. The cells were then carefully resuspended with a cut-off yellow tip in 100 µl of binding buffer (10mM HEPES pH 7.4; 140 mM NaCl; 5mM $CaCl_2$). An aliquot of 2.5 µl of Annexin-V-FLUOS (Boehringer Mannheim) was added and the incubation carried out for 20 min in the dark at room temperature. The cells were centrifuged and the pellet was resuspended with a cut-off yellow tip in 30 µl of binding buffer. The cells were then fixed by adding 200 µl of 3.7% formaldehyde in PBS (formaldehyde 37%; Sigma) for 10 min. After centrifugation, the fixed cells were rinsed once with PBS and incubated for 1.5 h at room temperature with biotinyled anti-rat CD2mAB OX-34 (Cedarlane) diluted 1:30 in a 100 µl final volume PBS . The cells were centrifuged, rinsed with PBS and incubated for 30 min with streptavidin-Tri Color (Calatg) diluted 1:100 in 100 µl PBS. Cells were rinsed in PBS and resuspended in 400 µl of PBS. Flow cytometry analysis was carried out on a Becton Dickinson FACScan. Quantification of transfected cells was determined on the FL-3 channel and percentage of apoptotic cells on the FL-1 channel.

As shown in Figure 1, this method allows visualisation of four distinct cell sub-populations, readily separating transfected from non-transfected cells and apoptotic from live cells. Comparison of

*To whom correspondence should be addressed. Tel: +33 467 61 36 55; Fax: +33 467 04 02 31; Email: lassus@jones.cnrs-mop.fr

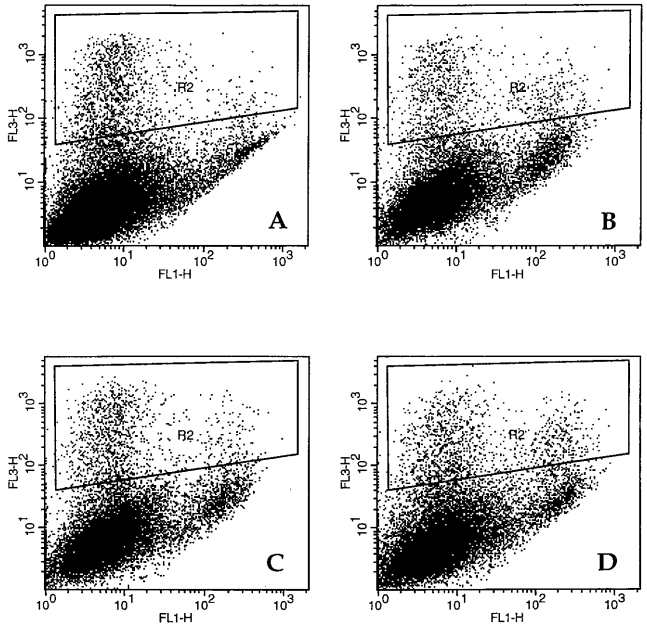

Figure 1. Bi-dimensional analysis of apoptosis in transfected cells by flow cytometry. The *y* axis (FL-3H) represents Tri-Color fluorescence intensity (CD2 positive cells). The *x* axis represents FITC fluorescence intensity (Annexin-V positive cells). Cells were transfected with CD2 + empty pCDNA3.1 vector (**A** and **B**); CD2 + full-length human bcl-2 (**C**); CD2 + full-length wild type human p53 (**D**). Cells were cultured in the presence (A) or absence (B, C and D) of serum. Transfected cells are on the upper half of the panel (R2 region) and apoptotic cells are on the right half of the panel.

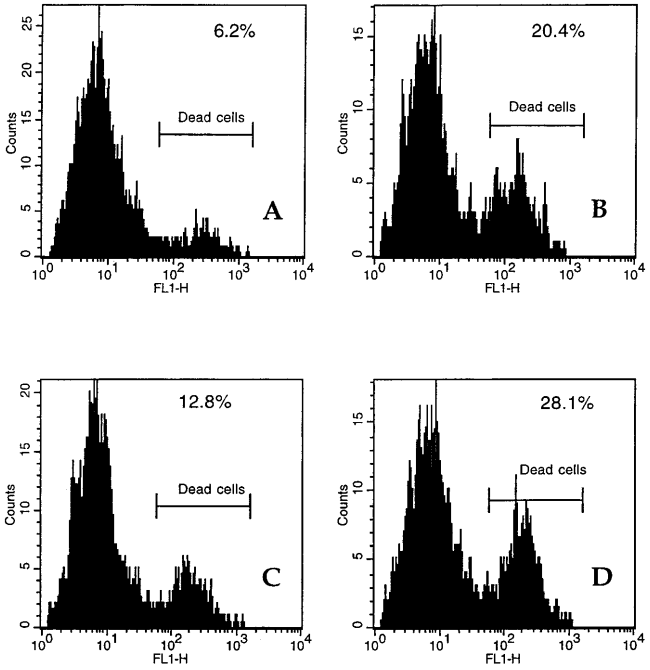

Figure 2. Quantification of apoptosis on the transfected cells population. Transfected cells were gated by region R2 (determinate in Fig. 1). The FITC intensity (Annexin-V positive cells) of this population is represented by histograms. (**A**–**D**) represent the same experiments as described in Figure 1. As expected, the percentage of apoptotic cells increases when cultured in the absence of serum (A: 6.2% versus B: 20.4%). Apoptosis induced by serum starvation is delayed by overexpression of bcl-2 (B: 20.4% versus C: 12.8%) and enhanced by forced expression of p53 (B: 20.4% versus D: 28.1%).

cell cultured in the presence (Fig. 1A) or absence of serum (Fig. 1B) shows that the population of apoptotic cells increases under the conditions of starvation. Among the transfected cells, apoptosis decreases specifically when bcl-2 is expressed (Fig. 1C) while the forced expression of p53 enhances the apoptotic response (Fig. 1D).

The quantification of apoptosis in the sub-population of transfected cells is represented in Figure 2. The Tri-Color positive cells (CD2+) were gated with the R2 region and the percentage of apoptotic cells was analysed on FL-1 histogram. As expected, the peak of dead cells varies as a function of the culture conditions and the expression of the transgene.

We have successfully used the same method to quantitate apoptosis in transfected sub-populations of several adherent cell lines, including primary mouse fibroblasts (MEFs), immortalized mouse fibroblasts (NIH 3T3, Swiss 3T3), REF 52 rat fibroblasts, hamster CCL39 fibroblasts and HeLa, a human epithelial cell line (data not shown). The method thus appears generally applicable for study of adherent cell lines.

Moreover, it would appear that Annexin V staining is an early event in a vast majority of apoptotic cells and our method is thus especially sensitive for detection of early engagement into apoptosis. At later stages of apoptosis the appearance in culture of cells having undergone fragmentation or secondary necrosis is likely to render the analysis more difficult. However, this limitation is also true for all the alternative methods of apoptosis quantification.

In conclusion, we have developed an efficient and rapid method to study the effect of transiently expressed proteins on the apoptotic process. Although this method has been specifically designed for analysing apoptosis of adherents cells, it can be also adapted for non-adherent cell types.

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