Annexin V binding assay as a tool to measure apoptosis in differentiated neuronal cells

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Abstract

We describe a rapid and reliable method to quantitate the extent of apoptosis in neuronal cell cultures. Based on their annexin V-affinity, resulting from phosphatidylserine (PS) exposure at the outer leaflet of the plasma membrane, apoptotic cells can be distinguished from annexin V-negative living cells, by using microscopic and flow cytometric procedures. When combined with propidium iodide (PI) the double labeling procedure allows a further distinction of necrotic (annexin V⁺/PI⁺), apoptotic (annexin V⁺/PI⁻) cells. Furthermore, when the cells are incubated with annexin V prior to harvesting, the former cell populations can be separated from cells damaged during isolation (annexin V⁻/PI⁺). In the present paper, we show that the annexin V-binding assay is also applicable to differentiated neuronal cells with fragile neurite outgrowths. © 1998 Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Apoptosis is a programmed, physiological mode of cell death, that plays an important role during embryonic development, the maintenance of tissue homeostasis and the deletion of aberrant cells (Kerr and Harmon, 1991; Lockshin and Zakeri, 1991; Henderson, 1996). Apoptosis is an active process governed by many genes that either promote or block cellular breakdown from within (Nagata, 1997). Several of these genes have been identified in the nervous system and the process of apoptosis has been implicated in many neurodegenerative diseases (Macaya, 1996; Armstrong et al., 1997; Mochizuki et al., 1997). For instance, it has been shown that infantile spinal muscular atrophy is associated with a deletion in the apoptosis inhibitory protein gene (Roy et al., 1995; Xu et al., 1997). In Alzheimer disease, presenilin-2 mutations have been shown to sensitize neural cells to apoptosis induced by trophic factor withdrawal and amyloid beta-peptide (Paradis et al., 1996; Guo et al., 1996, 1997). In order to understand the role of apoptosis in neurodegenerative diseases and to elucidate the underlying molecular mechanisms, it is of importance to reliably detect and quantitate apoptotic cells. To date, scoring of the characteristic changes in nuclear morphology, such as chromatin condensation and fragmentation, is often used to identify apoptotic cells both in vitro and in vivo. A more refined detection system is the TUNEL assay (Wijsman et al., 1993), based on the incorporation of hapten-labeled nucleotides at the sites of nicked DNA. However, the technique is rather cumbersome and does not allow the discrimination between apoptotic and necrotic cells. Recently, a novel apoptosis detection assay has been described based on the measurement of the loss of plasma membrane asymmetry (van Engeland et al., 1998). Under normal physiological conditions, a cell
maintains a strictly asymmetric distribution of phospholipids in the two leaflets of the cellular membranes with phosphatidylserine (PS) facing the cytosolic side (Devaux, 1991, Connor et al., 1992, Higgins, 1994). However, during early apoptosis this membrane asymmetry is rapidly lost without concomitant loss of membrane integrity (Koopman et al., 1994; Homburg et al., 1995; van Engeland et al., 1996, 1997, 1998). This results in the exposure of PS at the outer leaflet of the plasma membrane, which serves a physiological role in the recognition and subsequent removal of the dying cell by means of phagocytosis (Fadok et al., 1992; Savill et al., 1993). This phenomenon can be detected by hapten labeled annexin V, which shows high affinity for PS residues in the presence of millimolar concentrations of Ca\(^{2+}\) (Andree et al., 1990). By simultaneous probing of membrane integrity by means of exclusion of the nuclear dye propidium iodide (PI), apoptotic cells can be discriminated from necrotic cells. These cells become annexin V-positive because of its binding to PS at the cytoplasmic side of the plasma membrane. Although the method was initially developed for the measurement of apoptosis in blood cells, van Engeland et al. (1997) recently showed that the method is also applicable to measure apoptosis in adherent tumor cell cultures. Since differentiated neuronal cells in culture are characterized by fragile neurite outgrowths, it is conceivable that these cells are more susceptible to damage during harvesting procedures, making the intracellular PS sites available for annexin V binding. Such factors could make the annexin V binding assay less suitable for monitoring of apoptosis in these kinds of cell cultures.

The purpose of the present study was to examine the feasibility of the annexin V binding assay to measure apoptosis in differentiated neuronal cell cultures. To this end, apoptosis was induced in differentiated neuronal cell cultures and was monitored both by confocal scanning laser microscopy and flow cytometry.

2. Materials and methods

2.1. Materials

Annexin V-FITC (APOTEST-FITC) was obtained from NeXins Research BV (Rotterdam, the Netherlands). Roscovitine, a kind gift from Dr L. Meyer, Station Biologique, CNRS, Roscoff, France, was dissolved in DMSO.

2.2. Cell lines

The human neuroblastoma cell line CHP-212 (Schlesinger et al., 1976) was cultured in Dulbecco’s modified medium (GIBCO, Paisley, UK), supplemented with 1% glutamine (Serve, Heidelberg, Germany; no. 22942), 10% heat inactivated newborn calf serum (GIBCO, 021-6010M) and 0.1% gentamicin (AUV, Cuyck, The Netherlands).

The human neuroblastoma cell line TR14 (Nuydens et al., 1995; Geerts et al., 1996) was grown in DMEM-F12 medium (GIBCO, Paisley, UK), supplemented with 10% fetal calf serum (GIBCO) and 0.1% gentamicin.

In some experiments, the cells were cultured on eight well glass slides (Nutacon, Schiphol, The Netherlands), precoated with rat tail collagen I.

2.3. Induction of differentiation

TR14 cells were induced to differentiate by seeding the cells at low density in DMEM-F12 supplemented with 30 nM sodium-selenite, 5 µg/ml insulin, 100 µg/ml transferrin, 20 nM progesterone, 100 µM putrescine, 0.1% gentamicin and 1 mM db cAMP (Nuydens et al., 1998). These cells were cultured for 4 days to become differentiated.
2.4. Induction of apoptosis

Apoptosis in CHP-212 cells was induced by incubating the cultures in the presence of 50 μM roscovitine (Schutte et al., 1997). Differentiated TR14 cells were induced to undergo apoptosis using 50 nM okadaic acid (Sigma, Zwijndrecht, The Netherlands) (Nuydens et al., 1998).

2.5. Measurement of PS exposure

Surface exposure of PS by apoptotic cells was measured by adding annexin V-FITC to the culture medium in a final concentration of 3 μg/ml. The cells were subsequently incubated for 3 min at room temperature. Detached cells were collected with the culture supernatant, pelleted by centrifugation and washed twice with culture medium in order to remove excess of annexin V-FITC. The adherent cells were rinsed twice with medium before harvesting. Cells were harvested by mechanical scraping with a rubber police man. Detached and adherent cells were finally pooled and resuspended in culture medium to a final concentration of 1 × 10^6 cells/ml. Prior to flow cytometric analysis, PI was added to a final concentration of 5 μg/ml. Cells grown on glass slides were incubated with 3 μg/ml annexin V-FITC and 5 μg/ml PI for 3 min at RT. The cells were covered with a coverslip and examined by confocal scanning laser microscopy.

2.6. Flow cytometry

For flow cytometric analysis, a FACSort (Becton Dickinson, Sunnyvale, CA) equipped with a single Argon ion laser was used. Excitation was done at 488 nm and the emission filters used were 515-545 BP (green; FITC) and 600 LP (red; PI). A minimum of 10000 cells per sample were analysed and data stored in list mode. Electronic compensation was used to eliminate bleed through of fluorescence.

Data analysis was performed with the standard Lysis and Cellfit software (Becton Dickinson).

2.7. Confocal scanning laser microscopy

Cells grown on glass slides were analysed using the MRC600 confocal scanning laser microscope (BioRad, Hemel Hempstead, UK), equipped with an air-cooled Argon-Krypton mixed gas laser and mounted onto an Axiophot microscope (Zeiss, Oberkochen, Germany). The laser scan microscope was used in the dual parameter setup, according to the manufacturer’s specification, using dual wavelength excitation at 488 and 568 nm. Emission spectra were separated by the standard sets of dichroic mirrors and barrier filters. All scans were recorded in photon counting mode. Differential interference contrast images were collected using normal light illumination. To this end, the laser pathway was blocked and the filter sets were removed.

3. Results

Apoptosis was induced by culturing CHP-212 cells in the presence of the cyclin dependent kinase inhibitor roscovitine (Schutte et al., 1997). After 6 h of induction, cells with the characteristic morphological features of apoptosis became visible (Fig. 1). To quantitate the frequency of apoptotic cells, the cultures were incubated with FITC-labeled annexin V and harvested by
Fig. 3. Comparison of the number of apoptotic cells scored by nuclear morphology and the annexin V binding assay. CHP212 cells were cultured in the presence of 50 μM roscovitine and labeled with annexin V after various periods of time prior to harvesting. The sample was split in two. One part was analyzed using flow cytometry, the other part was cytocentrifuged on glass slides, fixed for 5 min at −20°C in methanol and stained with hematoxylin/eosin. The increase in the number of apoptotic cells, scored on the basis of morphology (solid bars), was paralleled with an increase in the number of annexin V+PI− cells (open bars). After 6 h in the presence of roscovitine, the number of annexin V+PI+ cells (striped bars) starts to increase (a). When the number of apoptotic cells, scored on the basis of morphology, was compared with the total number of annexin V-positive cells, a significant correlation was found (b).

mechanical scraping. This cell suspension was subsequently analyzed by flow cytometry. Fig. 2 shows bivariate annexin V-FITC/PI analyses of such cell suspensions either not subjected to roscovitine (control) or cultured for 6 h in the presence of roscovitine. Apoptotic cells appeared in the annexin V+/PI− fraction, whereas cells damaged by scraping appeared in the annexin V−/PI+ fraction. Undamaged cells remained negative for both parameters. The number of annexin V+/PI− cells agrees well with the number of apoptotic cells scored on the basis of nuclear morphology in hematoxylin/eosin stained cytocentrifuged preparations, except when cells are exposed to roscovitine for prolonged periods of time. The number of annexin V+/PI− cells remain constant, while the number of annexin V+/PI+ cells increased (Fig. 3). The latter fraction comprised the secondary necrotic cells, i.e. apoptotic cells, which lost their membrane integrity.

In contrast to undifferentiated neuronal cells, differentiated cells show neurite outgrowth (Nuydens et al., 1998). For this reason the differentiated cells might be more susceptible to cell damage during the harvesting procedure. In order to investigate the feasibility of the annexin V binding assay to these types of cells, TR14 cells were induced to differentiate in the presence of cAMP. As shown in Fig. 4(a,b), the cells show typical neurite outgrowth after 4 days of induction. When apoptosis is induced using okadaic acid (Nuydens et al., 1998), apoptotic cells can easily be detected using fluorescence microscopy. As shown in Fig. 4(d), apoptotic cells can be readily recognized by an integral membrane staining of FITC-labeled annexin V. Some cells lost their membrane integrity since the condensed chromatin is stained red by the intercalating dye PI. A remarkable finding is that PS exposure, i.e. annexin V-FITC binding, seems to precede the characteristic retraction of the neurites during the process of apoptosis. The total cellular membrane, including the long extensions, was positive for annexin V-FITC.

Parallel cultures were harvested by gently scraping the cells using a rubber policeman and analyzed flow cytometrically. As depicted in Fig. 5, apoptotic cells can clearly be visualized in the bivariate annexin V-FITC/PI analysis. Firstly, undifferentiated TR14 cells were analyzed using annexin V. As shown in Fig. 5(a), ≈35% of the cells are scored vital by the absence of annexin V-binding and propidium uptake (lower left quadrant). The majority of annexin V-positive apoptotic cells still showed an intact cytoplasmic membrane, as indicated by the absence of PI staining. This subpopulation comprised 22% of all cells. As shown in Fig. 5(b), some cells are positive for both annexin V and PI. Next, we analyzed TR14 cells after induction of differentiation. These cultures showed few apoptotic (annexin V+/PI−) and necrotic (annexin V+/PI+) cells. The majority of the cells were either vital (annexin V−/PI−) or damaged during harvesting (annexin V−/PI+). After 12 h in the presence of okadaic acid (Fig. 5c), ≈26% of the cells are scored vital by the
absence of annexin V-binding and PI uptake (Fig. 5c, lower left quadrant). A similar fraction of the cells are damaged during the harvesting procedure, since these cells are PI-positive (Fig. 5c, lower right quadrant). A total of 14% of the cells were only positive for annexin V and excluded PI. This fraction represents the early apoptotic cells. The majority of annexin V-positive cells showed a compromised cytoplasmic membrane as indicated by the PI staining. This subpopulation comprised 37% of all cells and represents the late apoptotic, i.e. secondary necrotic cells.

4. Discussion

A method has been described in which binding of FITC-labeled annexin V was used to detect apoptotic cells in suspension using flow cytometry (Koopman et al., 1994, Homburg et al., 1995). These studies provide evidence that chromatin condensation coincides with exposure of PS residues. Moreover, it was shown that this loss of membrane asymmetry precedes membrane damage.

When applying this assay to adherent cell cultures, in
particular neuronal cells with extensive neurite outgrowth, membrane integrity may be compromised due to cell harvesting. This will make PS, localized at the cytoplasmic side of the plasma membrane accessible for FITC conjugated annexin V. van Engeland et al. (1996), however, recently showed that this approach allows proper quantification of apoptotic cells in adherent cells in culture, when the protocol is adapted. In the present study, we show that even in neuronal cell cultures, we are able to specifically detect vital, damaged and apoptotic cells using flow cytometry as annexin V-/PI-, annexin V-/PI+ and annexin V+/PI- cells, respectively.

That annexin V-positive cells are indeed apoptotic was confirmed by microscopical analysis of parallel cell cultures using chromatin condensation as a marker. A remarkable observation was that PS exposure seems to precede the characteristic retraction of neurites during the process of apoptosis. The method presented here allows a detailed study of cytoskeletal changes in relation to PS exposure at the plasma membrane.

In summary, we have shown the feasibility of the annexin V binding assay to measure apoptosis in differentiated neuronal cells. This approach circumvents the problem of measuring false positives due to membrane damage inflicted upon vital cells during their detachment from the culture flasks by trypsinisation. Furthermore, annexin V labeled cells can be used for multivariate flow cytometric analysis which enables the study of antigen expression or even enzyme activity or Ca²⁺ fluxes during apoptosis. This novel assay may prove important in the further unravelling of the role of apoptosis in neurodegenerative diseases and the underlying molecular mechanisms thereof. Also, this method will allow monitoring of drugs that exert their effect through the induction of apoptosis or by inhibiting this process. The relative ease by which the analysis can be performed allows large scale screening of such biomodulation in neuronal cell cultures.

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