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Cell Harvesting Methods Affect Cellular Integrity of Adherent Cells During Apoptosis Detection

GE YAN and THOMAS EFFERTH

Department of Pharmaceutical Biology, Institute of Pharmacy and Biochemistry, Johannes Gutenberg University, Mainz, Germany

Abstract. *Background/Aim: Annexin V and propidium iodide (PI) dual staining is commonly applied in bioscience as a method to detect apoptosis. However, excessive handling of adherent cells may interfere with the integrity of plasma membrane and hence impede the accuracy of this method. Here, we exploited PI uptake as an indicator of cell integrity and investigated how cell harvesting methods and solutions involved in common apoptosis detection techniques affected measurement results. Materials and Methods: Different cell harvesting techniques, staining with PI and flow cytometry were performed. Results: Non-fixed scrapped cells revealed significantly higher fractions of PI-positive staining compared to non-fixed trypsinized cells. In the case of harvesting cells by scrapping, samples stained in binding buffer (68.30±3.55%) showed consistently higher PI-positive staining than samples stained in PBS (36.37±5.90%) in a significant manner (p=0.015). Conclusion: Enzymatic harvesting using 0.25% trypsin instead of mechanical harvesting by rubber scraper caused less damage of cell integrity. Furthermore, the binding buffer used in the apoptosis detection protocol aggravated the existing plasma membrane damage caused by the rubber scraper.*

Apoptosis is one of the modes of cell death involved in both physiological and pathological processes. Membrane phospholipid phosphatidylserine (PS) externalization from the inner membrane to the cell surface is considered a marker for apoptosis. In this context, fluorophore-conjugated annexin V, which reversibly binds to PS with high affinity in a Ca^{+}-dependent manner (1), has been introduced as a reliable method for detecting apoptosis together with dual propidium iodide (PI) staining. Neither annexin V nor PI can penetrate viable cells. Therefore, annexin V- and PI-negative staining is considered as an indicator for vital cells. Upon onset of apoptosis, PS externalization allows binding of annexin V without compromising cell integrity at early stages of apoptosis. Thus, annexin V-positive and PI-negative staining indicates early apoptosis. If the apoptotic process proceeds, cells undergo nuclear condensation, cell shrinkage and loss of plasma membrane integrity, which allows PI to enter cells and bind to DNA. Dual positive staining of annexin V and PI represents late apoptosis or secondary necrosis.

This elegant experimental design reaches its limitation if applied to detect apoptosis in adherent cells, due to inevitable cell membrane damage during harvesting handling regardless of enzymatic or mechanical methods (2, 3). In this study, we investigated how the cell harvesting procedure and solutions applied during apoptosis detection affect cell integrity.

Correspondence to: Prof. Dr. T. Efferth, Department of Pharmaceutical Biology, Institute of Pharmacy and Biochemistry, Johannes Gutenberg University, Staudinger Weg 5, 55128 Mainz, Germany. Tel: +49 61313925751, Fax: +49 61313923752, e-mail: efferth@uni-mainz.de

Key Words: Cell death, cellular technique, flow cytometry, cell harvesting method, adherent cells, apoptosis.

Materials and Methods

Cell line. Human pancreatic neuroendocrine tumor Bon-1 cell line was kindly provided by Prof. Matthias M. Weber (Johannes Gutenberg Medicine University, Germany) and cultured in DMEM/F12 Ham (Gibco, Paisley, UK) medium supplemented with 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Gibco) and incubated at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air.

Propidium iodide uptake. PI was commercially purchased (Sigma-Aldrich, Taufkirchen, Germany) and prepared in PBS (Gibco) at a concentration of 5 mg/ml as stock solution. Aliquots of each 1×10^6 cells/well were seeded into 6-well plates and incubated overnight to allow attachment. The supernatants were collected before harvesting. Cells in each well were rinsed with PBS and harvested *via* two methods, *i.e.* enzymatic or mechanical detachment. As shown in Figure 1, for the enzymatic method, cells in wells 1, 2 and 3 were incubated with 0.25% trypsin-EDTA (Gibco) for 5 min, followed by 10% fetal bovine serum supplemented medium application to neutralize the effects of trypsinization. For the mechanical method, cells in wells 4, 5 and 6 were gently detached by rubber scraper. After harvesting, all cell aliquots were washed with PBS once. Cells from wells 3 and 6 were conceived as positive control by fixing in 1 ml cold 70% ethanol on ice for 1 h. Meanwhile, 10× binding buffer (eBioscience, San Diego,

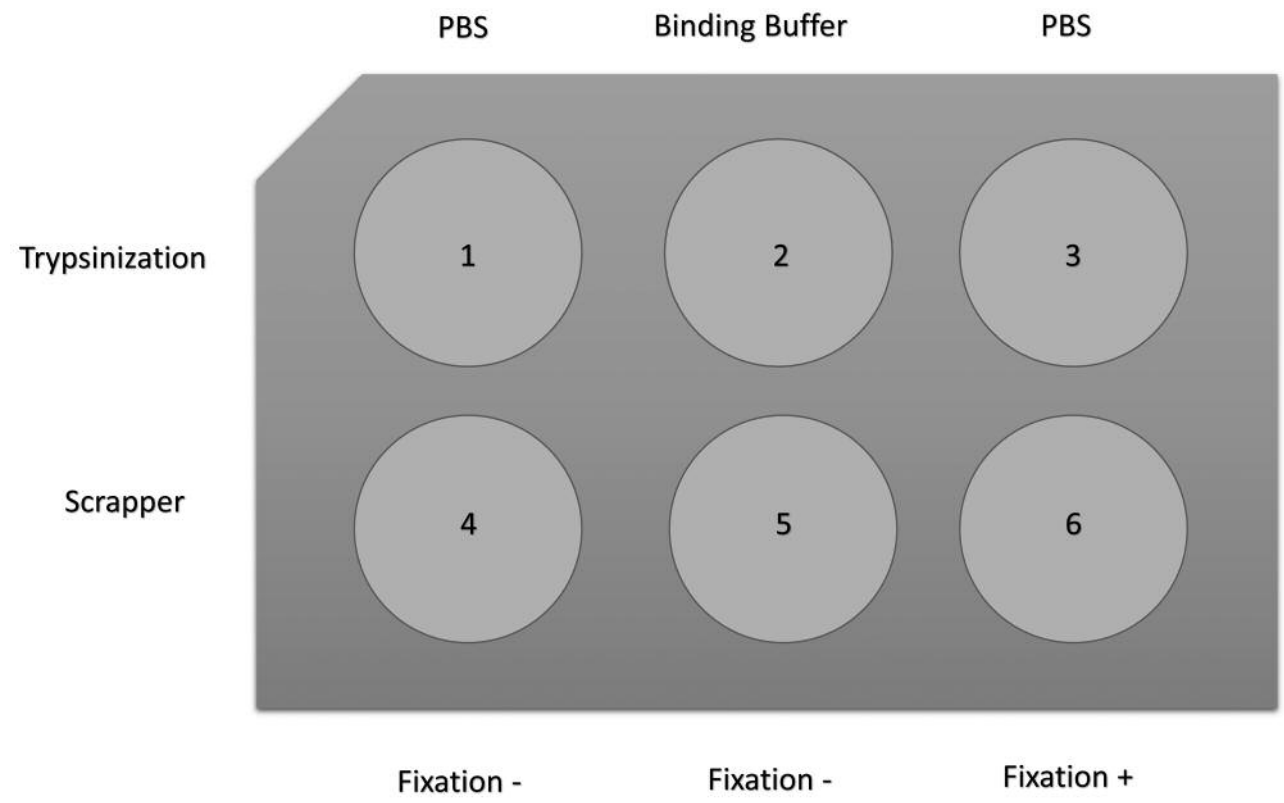

Figure 1. *Experimental setting to investigate detachment methods and applied solutions.*

Table I. *Percentages of PI uptake after cell harvesting.*

Sample treatment		Stain solution	PI Positive±SEM	PI Negative±SEM
Scrapper	Fixation	PBS	93.50±3.01	6.51±3.00
	Non-fixation	PBS	36.37±5.90	63.63±5.90
		Binding Buffer	68.30±3.55	31.70±3.55
Trypsinization	Fixation	PBS	97.93±0.79	2.04±0.80
	Non-fixation	PBS	9.73±3.86	90.27±3.86
		Binding Buffer	6.91±2.50	93.07±2.49

CA, USA) was diluted to 1× with distilled water. Cells from wells 1 and 4 were washed with PBS, while cells in wells 2 and 5 were washed with 1×binding buffer. PI stock solution was further diluted in PBS and 1×binding buffer, respectively, to reach a final concentration of 50 μg/ml. PBS-washed cell pellets (well 1, 3, 4 and 6) were resuspended in 200 μl PBS diluted PI solution, while 1× binding buffer-washed cell pellets (well 2 and 5) were resuspended in 200 μl 1× binding buffer diluted PI solution. Cells were incubated in the dark at room temperature for 20 min. Samples were analyzed by a BD Accuri C6 flow cytometer (Becton Dickinson, Heidelberg, Germany).

Statistical analysis. Data obtained from flow cytometry were further analyzed with FlowJo. The gate for positive PI staining was set according to positive control samples, which underwent fixation. PI staining signals lower than those of the positive controls were considered as negative. The same gate was applied for all repeated samples. Data were presented as mean±SEM of three independent experiments. Two sample *t*-test was applied to calculate significance.

Results

All results are shown in Table I. It was evident that positive control samples which underwent fixation showed ~100% positive PI staining (Figure 2). Interestingly, adherent cells without fixation were more vulnerable to treatment with a rubber scraper, since non-fixed scrapped cells revealed higher fractions of PI-positive staining (36.37±5.90% and

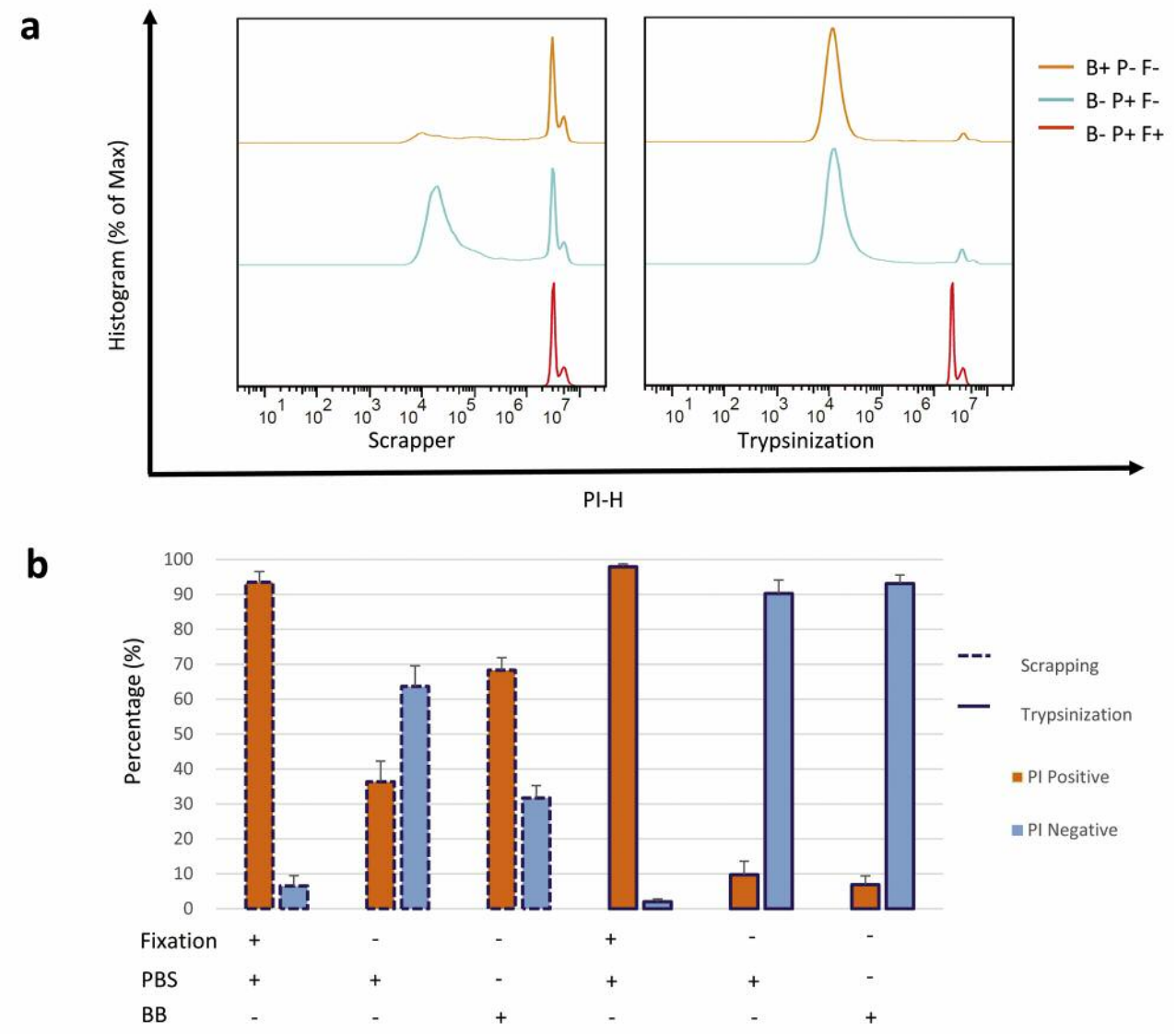

Figure 2. *PI uptake after cell harvesting. (a) DNA histogram of PI-stained cells. PI-H: FL3-H; B: binding buffer; P: PBS; F: fixation. (b) Percentage of positive and negative PI staining after harvesting of cells. BB: Binding buffer.*

68.30%±3.55 with PBS and binding buffer, respectively) compared to non-fixed trypsinized cells (9.73±3.86% and 6.91±2.50%; with PBS and binding buffer, respectively) with a statistically significant difference (p=0.025 and p<0.001; with PBS and binding buffer, respectively). Besides, 90.27±3.86% samples harvested by trypsinization presented negative PI staining, if stained in PBS and similarly 93.07±2.49% in binding buffer indicating that trypsinization preserved the cell membrane integrity in Bon-1 cell line. Surprisingly, in the case of harvesting cells by scrapping, samples stained in binding buffer (68.30±3.55%) showed consistently higher PI-positive staining than samples stained in PBS (36.37±5.90%) and the difference was statistically significant (p=0.015). Obviously, the binding buffer exaggerated the cellular damage caused by scrapping.

Discussion

Apoptosis detection is widely applied in all fields of biosciences. The success and accuracy of apoptosis determination is based on the integrity of the plasma membrane to distinguish real apoptosis from false-positive results caused by cell damage due to excessive or improper experimental handling. Moreover, compromising the cellular

integrity leads to loss of annexin V-positive and viable negatively-stained cells, which is the most interesting cell population. Although negative control samples with dual negative staining for annexin V and PI validated the apoptosis results, our interest was on how to limit false-positive staining.

In general, cells without fixation cannot take up PI due to the impermeability of cell membranes for viability dyes. Here, a small population of naturally dying cells that lost their cellular integrity by default was excluded. However, the application of rubber scraper severely impaired the integrity of plasma membranes especially in combination with binding buffer. This may be caused by disturbance of calcium homeostasis (4, 5) due to overload of Ca^{+} contained in the binding buffer, which was originally intended to ensure calcium availability for proper annexin V binding. The elevated cytosolic calcium concentration has the potential to activate catabolic enzymes, including phospholipases which may lead to accelerated phospholipid degradation and hence increased membrane permeability (6).

This study work compared the enzymatic and mechanical harvesting of adherent cells. Enzymatic harvesting caused milder damage of plasma membranes than rubber scraper-based harvesting based on flow cytometric measurements of PI uptake. Once cells encountered impaired integrity, they were vulnerable to binding buffer conditions. Thus, application of binding buffer deteriorates the compromised plasma integrity and accelerates cell death of already damaged cells.

Conflicts of Interest

The Authors declare that there is no conflict of interest regarding this publication.

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