Cell Harvesting Method Influences Results of Apoptosis Analysis by Annexin V Staining

ANNIKA BUNDSCHERER, MANUELA MALSY, RENATE LANGE, PETRA HOFMANN, THOMAS METTERLEIN, BERNHARD M GRAF and MICHAEL GRUBER

Department of Anesthesiology, University of Regensburg, Regensburg, Germany

Abstract. *Background: Annexin V staining is a common tool in apoptosis analysis. However, in adherently growing cell lines, substantial experimental bias could be introduced by membrane damage during the harvesting process. We investigated the influence of three different harvesting methods on the cell membrane integrity of six malignant cell lines. Materials and Methods: Six malignant cell lines were detached enzymatically by standard trypsinization or mechanically by scraping or wash-down by water jet. Membrane damage was measured by annexin V staining. Results: Three out of six cell lines (Mel-Ho, SW480 and PaTu 8988t) were not susceptible to membrane damage long the mothods used here. In HT 29, PANC 1 and A-673 cell lines, a high percentage of cells were stained positively for annexin V after mechanical detachment. These cells would wrongly be declared apoptotic cells. Conclusion: To avoid substantial experimental bias caused by membrane damage, we recommend pre-testing of different harvesting methods before performing apoptosis analysis.*

The annexin V affinity assay is a widely used method for apoptosis analysis, as well as for the discrimination between apoptotic and necrotic cell death. This test is based on the changes in the cell membrane caused by apoptotic processes. Loss of plasma membrane asymmetry is an early step in apoptosis. In viable cells, the membrane phospholipid phosphatidylserine (PS) is located at the inner leaflet of the plasma membrane. During apoptosis PS is externalized and exposed at the cell surface (1). Annexin V is a Ca^{2+}-dependent phospholipid binding protein with high affinity to PS. Since annexin V is not able to penetrate the phospholipid bilayer, vital cells cannot be stained (2). Conjugation of Annexin V to fluorescein isothiocyanate (FITC) allows for apoptosis analysis *via* flow cytometry. To differ between apoptotic and necrotic processes, a vital dye, such as propidium iodide (PI), which can only penetrate compromised cell membrane, can be used for double staining (3). According to the technical data sheet of the FITC annexin V Test (BD Pharming™), this test is recommended for apoptosis analysis in cell lines in suspension. During cell detachment or harvesting of adherent cells, specific membrane damage may occur. Engeland *et al*. give warning of fals positive results due to membrane damage caused by harvesting adherent cells (1). Mechanical cell detachment may influence PS flipping and introduce experimental bias (4). Although annexin V staining is a common tool for apoptosis analysis even in adherent cell lines, only few authors describe the method used for cell harvesting. The aim of this study was to examine the influence of the harvesting methods on results of apoptosis analysis by annexin V staining.

Correspondence to: Dr. Anika Bundscherer, Department of Anesthesiology, University of Regensburg, Franz Josef Strauß Allee 11, 93053 Regensburg, Germany. Tel: +49 9419447801, Fax: +49 9419447802, e-mail: anika.bundscherer@ukr.de

Key Words: Apoptosis, adherent cells, cancer, annexin V staining, cell harvesting.

Materials and Methods

Cell lines. Six cancer cell lines were used in this study: Mel-Ho (melanoma), PANC 1 and PaTu 8988t (pancreatic carcinoma), HT 29 and SW480 (colonic carcinoma) and A-673 (rhabdomyosarcoma). Mel-Ho were kindly contributed by Dr. Christian Hafner, Department of Dermatology, University of Regensburg, HT 29 and SW480 were purchased from the German Collection of Microorganism and Cell Culture (DSMZ, Braunschweig, Germany), pancreatic cell lines were kindly contributed by Professor Dr. Ellenrieder, University of Marburg and A-673 were purchased from CLS-Cell Lines Service, Eppelheim, Germany. Cells were grown in standard growth media, RPMI-1640 (Pan Biotech, Aidenbach, Germany) for Mel-Ho and SW480 and Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, St Gallen, Switzerland) for 8988t, PANC 1, HT 29 and A-673 containing 10% fetal calve serum (FCS, Sigma-Aldrich) and 2 mM L-glutamine (Sigma-Aldrich) supplemented with 5% penicillin plus streptomycin (Sigma Aldrich) (Mel-Ho, HT 29 and SW480), or Myco Zap (Lonza Verviers SPRL, Verviers, Belgium) (8988t and PANC 1). SW480 culture media additionally contained sodium pyruvate (Appli Chem,Darmstadt,

Germany). Cells were cultured at 37˚C in a humified atmosphere with 5% CO_2 and were maintained in monolayer culture. Experiments were performed when cells reached ~80% confluence.

Apoptosis analysis. Cells were seeded in T 12.5 cm^2 cell culture flasks (BD Falcon, Heidelberg, Germany) and allowed to attach overnight. After 24 h attachment time, the supernatant was decanted from the cells to preserve floating cells. Before harvesting, adherent cells were rinsed with warm PBS (Sigma-Aldrich). Three different harvesting methods were used: standard trypsinization, scraping by a rubber policeman, and wash-down by water jet. For enzymatic trypsinization, cells were incubated with 0.125% trypsin-EDTA solution (Sigma-Aldrich) for 1-8 min and detachment was controlled *via* microscopy. For wash-down by water jet, the top of a Pasteur pipette (Brand, Wertheim, Germany) was heated and bented and used with water.

The FITC-Annexin V Apoptosis Detection Kit (BD Bioscience, Heidelberg, Germany) was used according to the manufacturer´s protocol. In brief, floating and harvested cells were mixed, washed twice with cold phosphate buffered saline (PBS) and resuspended in binding buffer at a final density of 10^6 cells/ml. FITC-annexin (5 μl) and PI (5 μl) were added to 100 μL of the cell suspension containing 10^5 cells. The cell suspension was mixed by gently vortexing and then incubated for 15 min at room temperature in the dark. Subsequently, 400 μl of binding buffer were added and cells were analyzed by flow cytometry using FACS Calibur (BD Bioscience, Heidelberg, Germany) and the Cellquest Pro software (BD Bioscience). All tests were performed in duplicates and repeated twice.

Statistical analysis. Results are expressed as the mean±SD. For comparison, the results were positively tested for normal distribution (Kolmogorov-Smirnov test) and homogeneity of variance was analyzed with the Levene test. For comparison between mean values, ANOVA test was used, with Bonferroni test for *post-hoc* analysis for data with homogeneity of variance. For data without homogeneity of variance, Dunnett T3 test was used. Differences were considered statistically significant at $p<0.05$. IBM SPSS Statistics (V 20; IBM New York, NY,USA) and Excel 2010 (Microsoft, Redmond, CA, USA) packages were employed for statistical analysis.

Results

We compared the influence of three harvesting methods (enzymatically by standard trypsinization and mechanically *via* scraping or wash-down by water jet) on the results of apoptosis analysis in six adherently growing cancer cell lines. The results are summarized in Figure 1.

In the Mel-Ho melanoma cell line, the use of different harvesting methods did not affect the number of viable cells as defined by those cells that did not stain positively for annexin V or PI (Figure 1a). A slight but significant difference was detected in the pancreatic cell line PaTu 8988t (Figure 1b).

Enzymatic trypsinization was shown to be the best harvesting method for the four remaining cell lines SW480, HT 29, PANC 1 and A-673, reaching a cell viability >73%±4% in all cell lines (Figure 1c-f). In HT 29, PANC 1 and A-673 more than 49%±3% collected by scraping and more than 56±9% collected by water jet were detected as being apoptotic cells.

After harvesting by trypsinization, cells which stained positively for annexin V ranged from 10%±1% in PaTu 8988t and 26%±4% in A-673 compared to 16%±4% in PaTu 8988t and 78%±3% in A-673 after detachment by scraping.

The number of necrotic cells detected by positive staining only with PI ranged between 1%±0% and 7%±3% in all cell lines tested. The highest rate of necrotic cells was found in HT 29 colon carcinoma cell line, with 5%±3% for trypsinized and washed-down cells and 7%±3% for scraped cells.

Discussion

The aim of this study was to dertermine the influence of different harvesting methods on the cell membrane integrity of cancer cell lines. In three out of six cell lines, mechanical detachment caused cell membrane damage and therefore led to false-positive annexin V staining.

Trypsin is a member of the endopeptidase family, often used for gentle harvesting of adherent cells. Due to its proteolytic activity trypsin facilitates the detachment of cells from cell culture flasks. As calcium plays an essential role in cell adhesion, trypsin is often used in combination with the chelating agent ethylenediaminetetra acetic acid (EDTA). By binding calcium, EDTA disrupts cell adhesion and dissolves the cell layer (5). Harvesting cells by mechanical methods such as scraping may lead to membrane damage. Nevertheless, enzymatic detachment may also cause changes in cell membrane proteins, so mechanical harvesting can be necessary to address specific questions (6). Recently, trypsin was shown to cause substantial metabolite leakage in the SW480 colonic carcinoma cell line. Therefore trypsizination was judged to be an inadequate harvesting method for metobolomic studies (7).

Membrane damage was measured by annexin V staining in the present study. In three out of six cell lines (Mel-Ho, PaTu 8988t and SW480), no or only slight differences in membrane integrity were detected. However, after scraping or wash-down by water jet, more than 49% of HT 29, PANC 1 and A-673 cells stained positively for annexin V and so were detected as apoptotic cells. For this reason, using the mechanical detachment methods for apoptosis analysis in these cell lines would lead to a substantial experimental bias.

The influence of cancer cell characteristics on susceptibility to membrane damage is discussed below. We used five epithelial growing cell lines (Mel-Ho, SW480, HT 29, PaTu 8988t and PANC 1) and one polygonal (A-673) growing cell line for this study. As three of the epithelial cell lines were insensitive to membrane damage while in two cell lines membrane damage occurred, these effects cannot be explained by morphological features alone.

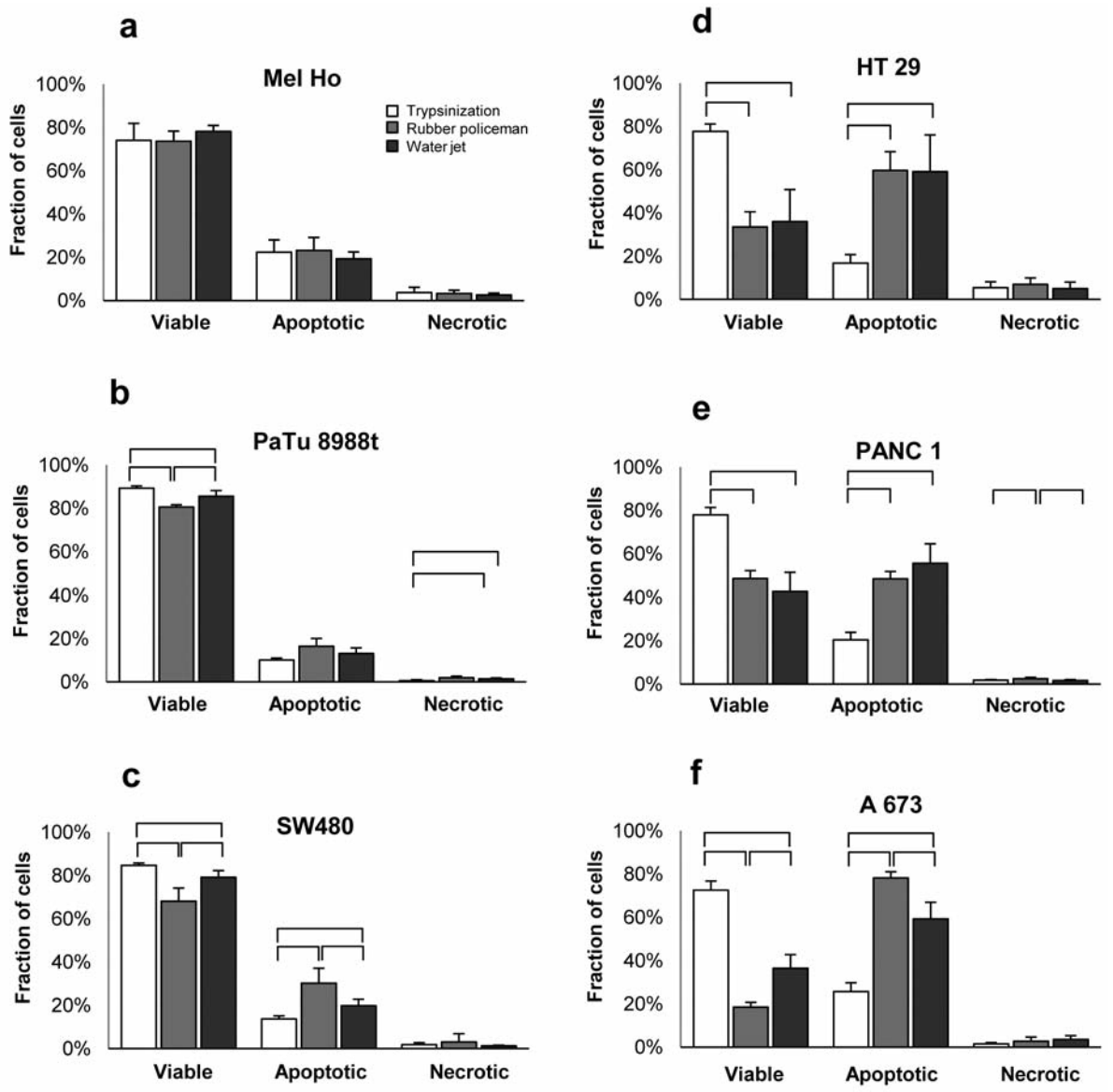

Figure 1. *Influence of trypsinization, scraping by rubber policeman and wash-down by water jet on six carcinoma cell lines. Bars indicate statistical significance at p<0.05.*

Furthermore, we observed HT 29 colonic carcinoma cells and PANC 1 pancreatic carcinoma cells to be highly susceptible to disruption of membrane integrity. However, SW480 colonic carcinoma cells and PaTu 8988t pancreatic carcinoma cells were not affected by cell harvesting methods. This finding indicates that susceptibility to membrane damage may not depend on tumor entity.

Both colonic carcinoma cell lines, HT 29 and SW480, were established from a primary colorectal adenocarcinoma. SW480 was from tumor grade 4 and Dukes' class B (8); the grade of differentiation of HT 29 is not described (9). At 20 h and 22 h respectively, doubling times are comparable in HT 29 and SW480 cell lines. Interestingly, when grown as xenografts in nude mice, these cell lines displayed different histological features. While HT 29 were moderately to well-differentiated, with glandular structures, in the poorly-differentiated SW480 xenografts no tissue structure was detected (9).

The pancreatic cell line PaTu 8988t was established from a liver metastasis of a primary pancreatic adenocarcinoma (10). According to the information of the distributor, after 3-4 days of cultivation the cell population can be distinguished as two

types of cells, one spindle-shaped adherently growing population, and one of rounded cells with loose contact growing on the top of the other population. Furthermore, PaTu 8988t cells grow in loose contacts without membrane outgrowth or junctions. PaTu 8988t cells grown in xenografts exhibited structural characteristics of highly differentiated primary pancreatic adenocarcinoma (10). In contrast, the human cell line PANC 1 was obtained from a pancreatic carcinoma of ductal origin exhibiting a low level of differentiation (11).

The melanoma cell line Mel-Ho was established from a primary tumor of a woman with melanoma. The cells grow adherently with an epithelial-like pattern and the doubling time is about 24 h (according to DSMZ information). For these melanoma cells no detailed information regarding the grade of differentiation or tumorigenity in mice are available. A-673 is a rhabdomyosarcoma cell line established from a muscle of a 15-year-old girl. According to the information of the American Type Culture Collection (ATCC) the morphology of this cell line is polygonal. A-673 cells cause rapidly growing sarcomas in antithymocyte serum-treated mice.

Interestingly, the highly differentiated cell line HT 29 and the poorly differentiated cell line PANC 1 were more susceptible to membrane damage caused by mechanical harvesting *via* scraping or wash-down by water jet. This finding indicates that the loss of membrane integrity by cell harvesting does not depend on the grade of differentiation of the cancer cell line.

Conclusion

In conclusion, resistance to mechanical membrane damage by scraping or wash-down by water jet seems not to depend on the tumor entity or grade of differentiation. However, it is difficult to predict the effect of detachment methods on the membrane integrity of cancer cells. For this reason, we recommend pre-testing to evaluate the most suitable harvesting method for avoiding substantial experimental bias.

Disclosure

The Authors have no conflicts of interest.

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Received May 30, 2013
Revised June 25, 2013
Accepted June 27, 2013