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STAUROSPORINE-INDUCED APOPTOSIS: ANALYSIS BY DIFFERENT ANNEXIN V ASSAYS

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ABSTRACT

Annexin V assay is a well-known method for the evaluation of cell apoptosis. However, there is a wide range of available kits at the market that are of different quality and price. In this study, staurosporine-induced apoptosis model was used in order to compare low-cost and high-cost Annexin V detection kits from three different producers. Briefly, two human cell lines (KG-1 and NKT) were treated with staurosporine for 1, 3 and 6 hours and then evaluated for the apoptosis induction using 3 different detection kits by flow cytometry. Both cell lines showed significant ($P < 0.05$) increase in cell apoptosis after 3 hours (20 % and 13 %, respectively) and 6 hours (50 % and 20 %, respectively) of incubation with staurosporine. No significant changes in the proportion of apoptotic cells were observed by comparing the data detected using low-cost or high-cost Annexin V assays. In conclusion, tested low-cost Annexin V detection kits could be safely used for an objective and fast flow cytometric assessment of the apoptosis in human or animal cells. However, the efficiency of these kits for the evaluation of apoptosis for example by immunofluorescence microscopy in other cells or specific tissue e.g. embryos could differed. As fluorescence microscopy is less sensitive than flow cytometry, this technique should require an Annexin V detection kit of higher quality that does not have to agree with our findings for flow cytometry.

Key words: cell culture; apoptosis; staurosporine; annexin V assay; flow cytometry

INTRODUCTION

Apoptosis is one of the most fundamental biological processes in mammals that occurs normally during development and aging and maintain homeostasis in multicellular organisms. Apoptosis also occurs as a defence mechanism such as in immune reactions or when cells are damaged by disease or noxious agents (Norbury and Hickson, 2001). Although there are a wide variety of stimuli and conditions, both physiological and pathological, that can trigger apoptosis, not all cells will necessarily die in response to the same stimulus (Elmore, 2007). Apoptosis is typically accompanied by the activation of a class of caspases and widespread biochemical and morphological changes to the cell (Nicholson *et al*., 1997; Porter *et al*., 1997). An important biochemical feature is the expression of cell surface markers that result in the early phagocytic recognition of apoptotic cells by adjacent cells, permitting quick phagocytosis with minimal compromise to the surrounding tissue. This is achieved by the movement of the normal inward-facing phosphatidylserine (PS) of the cell's lipid bilayer to expression on the outer layers of the plasma membrane (Bratton *et al*., 1997).

Annexin V is a recombinant phosphatidylserine--binding protein that interacts strongly and specifically with phosphatidylserine residues and can be used

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for the detection of apoptosis (Van Engeland *et al*., 1998; Arur *et al*., 2003). Annexin V based assay is already known for more than two decades and can be applied both in flow cytometry and in fluorescent microscopy. Koopman *et al*. (1994) were the first to describe a method using extrinsically applied hapten (i.e. FITC or biotin) labeled nnexin V to detect apoptosis. Labeled annexin V binds in the presence of Ca^{2+} to PS residues that are exposed at the outer leaflet of the plasma membrane of apoptotic cells. Annexin V is not able to bind to normal vital cells since the molecule is not able to penetrate the phospholipid bilayer. In dead cells, however, the inner leaflet of the membrane is available for binding of extrinsically applied annexin V, since the integrity of the plasma membrane is lost. To discriminate between dead and apoptotic cells, a membrane impermeable DNA stain, such as propidium iodide (PI) can be added simultaneously to the cell suspension. In this way vital, apoptotic and dead cells can be discriminated on basis of a double-labeling for annexin V and PI, and analyzed by flow cytometry (Van Engeland *et al*., 1998).

Staurosporine is a microbial alkaloid, isolated from *Streptomyces* sp. cultures. Staurosporine has been shown to: inhibit cell cycle progression in a variety of cell lines (Abe *et al*., 1991; Crissman *et al*., 1991; Bruno *et al*., 1992); enhance differentiation of human promyelocytic leukemia cells (Okazaki *et al*., 1988; Okuda *et al*., 1991); inhibit tumor cell invasion (Schwartz *et al*., 1990); and induce morphological changes typical of apoptosis in rat cardiomyocytes (Yue *et al*., 1998) and rat hippocampal neurons (Krohn *et al*., 1998).

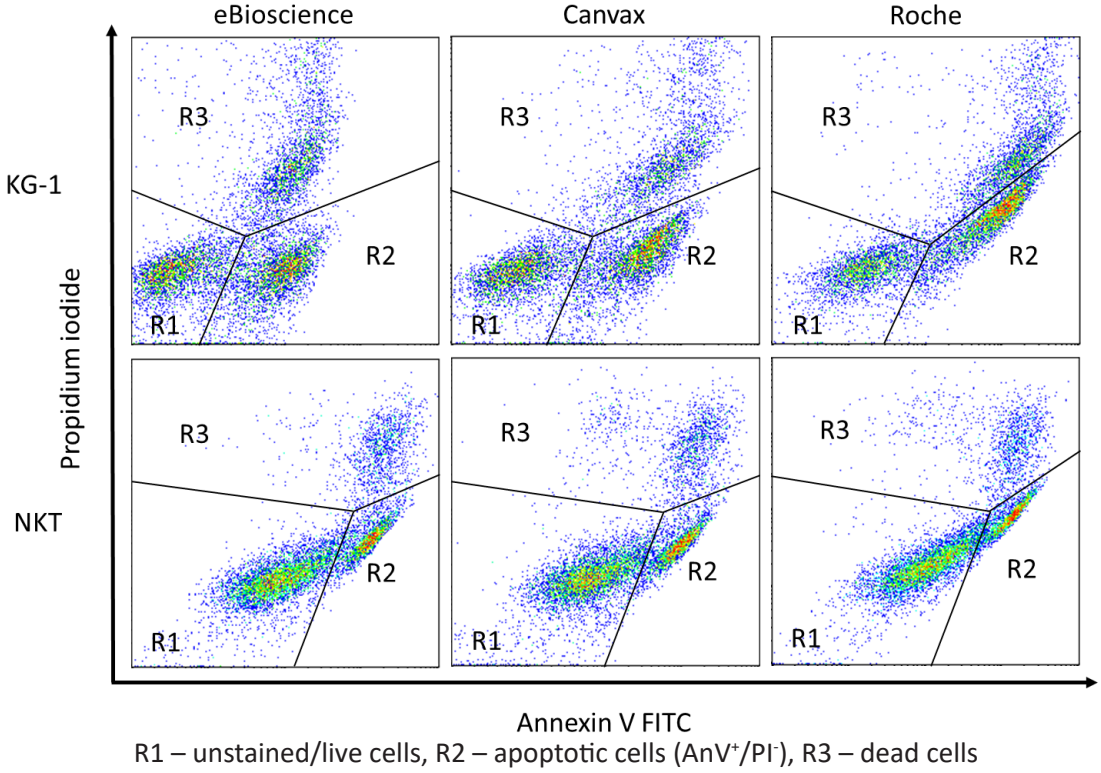

Figure 1. Illustrative dot plots showing evaluation strategy of the proportion of apoptotic cells within the control and staurosporine-induced cells

Many important mechanisms involved in apoptosis have been demonstrated in staurosporine- induced apoptosis models (Xia *et al*., 1995; Jacobsen *et al*., 1996).

At the present there are many Annexin V assay kits available at the market. These kits usually differ in their quality and price. The aim of this study was therefore to compare the quality of Annexin V assay kits of different costs from three different producers by measuring of apoptotic cells using staurosporine-induced apoptosis model and flow cytometry.

MATERIAL AND METHODS

In this study, two types of human cell lines were used in the experiments: non-adherent KG-1 cell line and adherent stromal NK.tert cell line (NKT). Both cell lines were generously provided by Dr. Medhat Shehata (Medical University of Vienna, Austria). Briefly, both cell lines were treated with staurosporine (Santa Cruz Biotechnology, USA) at the concentration of 1 µM for $5x10^5$ cell/ml in specific culture medium: a) in RPMI-1640 medium with 10 % FBS and 1 % antibiotics for KG-1 cells or b) αMEM culture medium (both media from Thermo Fisher Scientific, USA) with 20 % FBS and 1 % antibiotics for NKT cells. Cells were cultured in T25 culture flasks at 37 °C and 5 % CO_2 for 1, 3 and 6 hours. For each time interval the control flasks (cells not treated with staurosporine) were also incubated. The experiment was replicated for three times.

After each time incubation, cells were harvested by centrifugation (KG-1) or using 0.05 % Trypsin-EDTA (NKT) and stained according to the very similar manufacturer's protocols with Annexin V assays obtained from three different producers: Annexin V-FITC Apoptosis Detection Kit (low-cost; Canvax, Spain), Annexin V-FITC Apoptosis Detection Kit (low-cost; eBioscience, Thermo Fisher Scientific, USA) and Annexin-V-FLUOS Staining Kit (high-cost; Roche Slovakia, Slovak Republic). Cells were incubated also with propidium iodide (Molecular Probes, Switzerland) in order to detect dead cells and analysed using a FACSCalibur flow cytometer (BD Biosciences, USA) with cell populations gated to distinguish apoptotic (AnV^+/PI^-) cells (Figure 1). At least 10,000 cells were analysed in each sample.

Obtained results were evaluated using the SigmaPlot software (Systat Software Inc., Germany) with one-way ANOVA (Holm-Sidak method) and expressed as the means ± SEM.

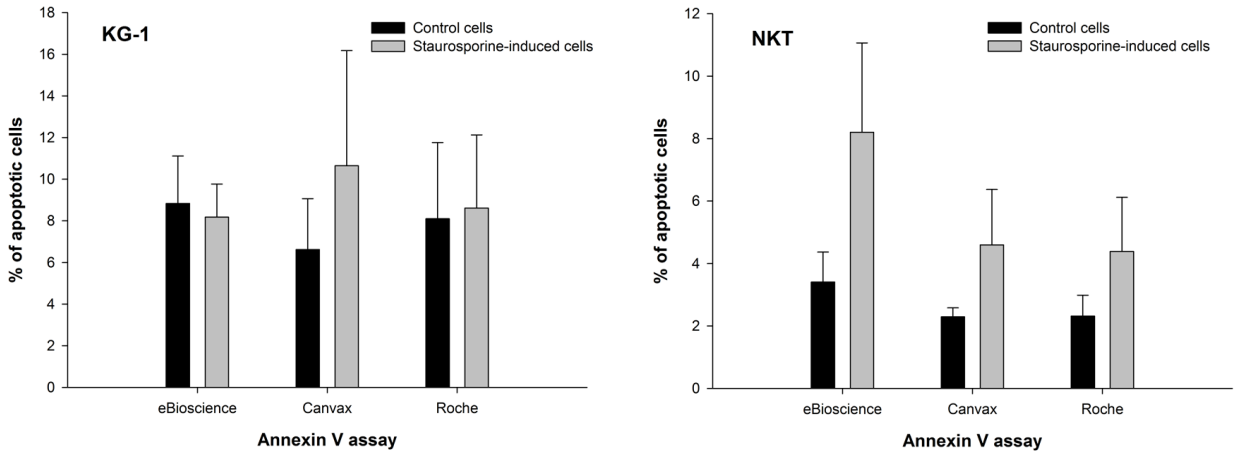

Figure 2. Proportion of apoptotic cells in control and staurosporine-induced cell lines after 1 hour of incubation

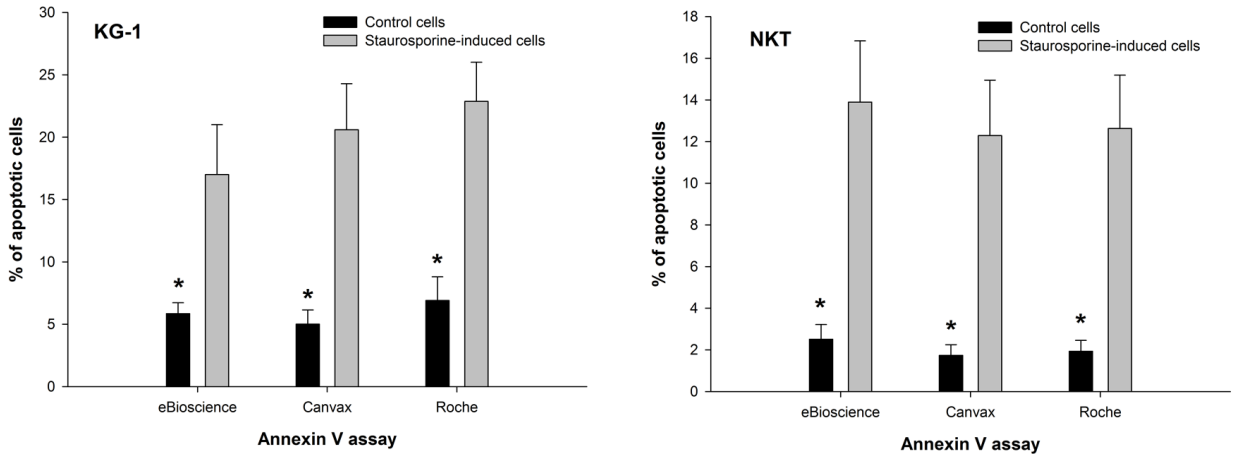

Figure 3. Proportion of apoptotic cells in control and staurosporine-induced cell lines after 3 hours of incubation

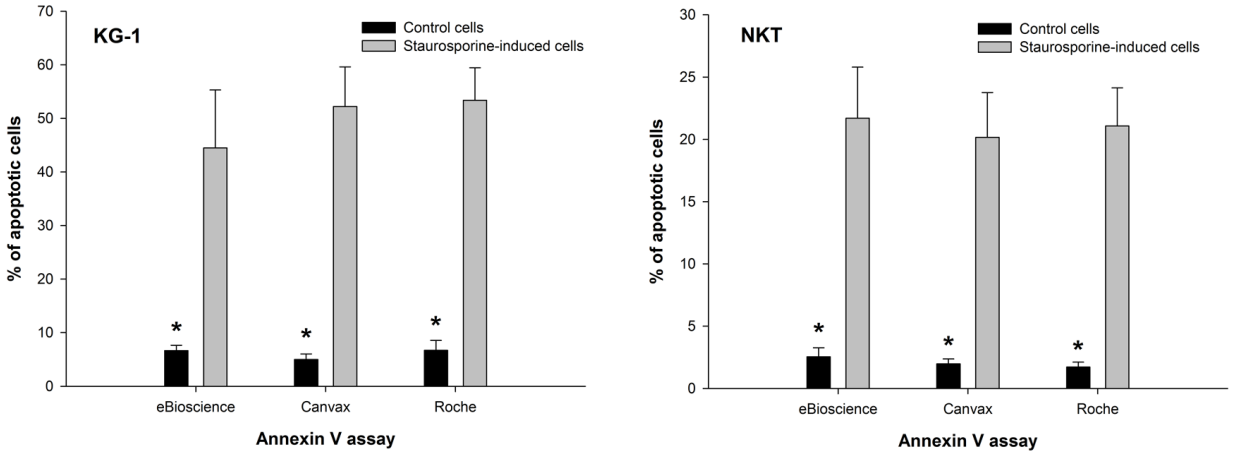

Figure 4. Proportion of apoptotic cells in control and staurosporine-induced cell lines after 6 hours of incubation

RESULTS AND DISCUSSION

In the presented study, cells were incubated with staurosporine for 1, 3 and 6 hours. Flow cytometry did not reveal significant apoptosis induction in KG-1 (about 8 %) or NKT cell line (about 4 % of apoptotic cells) after 1 hour of incubation using any of tested Annexin V assays in comparison to control (uninduced) cells (Figure 2). On the other hand, after 3 hours of incubation staurosporine significantly ($P < 0.05$) induced apoptosis in both cell lines in comparison to control cells (about 20 % in KG-1 and 13% in NKT; Figure 3). However, no significant differences were observed among the used Annexin V assays. The most significant ($P < 0.05$) induction of apoptosis by staurosporine was observed in both cell lines after 6 hours of incubation (about 50 % in KG-1 and 20 % in NKT; Figure 4). Similarly, no significant differences in the percentage of detected apoptotic cells were noticed among the used low-cost or high-cost Annexin V assays.

Obtained results indicated that the efficiency of staurosporine-induced apoptosis in cells strongly depends on the cell type as well as on the incubation time (2-fold increase in the proportion of apoptotic cells when compared KG-1 to NKT cell line and even when compared 3 h to 6 h of incubation; Figure 3 and 4). Antonsson and Persson (2009) observed about 40 % of apoptotic cells using Annexin V assay in non-adherent human leukemic cell line U-937 treated with staurosporine for 24 hours. Belmokhtar *et al*. (2001) noticed distinct ability of staurosporine to trigger apoptosis even in the two different sublines of the same non-adherent cell line L1210 (L1210/S and L1210/0). About 60 % of apoptotic cells were detected in both sublines via Annexin V assay after 3 hours and 12 hours, respectively. Concerning the adherent cells, staurosporine was an effective apoptosis inducer of porcine aortic endothelial cells as determined by Annexin V assay (Kabir *et al*., 2002). In this study, staurosporine treatment for 1 h increased the proportion of apoptotic cells to 33 % of the total cell population as compared with 7 % in control untreated cells. Moreover, after 24 h of incubation with staurosporine the percentage of apoptotic cells increased to 90-95 %. Thus, those studies support our findings about the different effect of staurosporine according to the treatment period and type of treated cells.

CONCLUSION

Staurosporine successfully induced apoptosis in both types of human cell line with different induction efficiency according to the type of cells (adherent or non-adherent) or the time of the incubation. Moreover, this staurosporine-induced apoptosis model definitely proved that the quality of tested low-cost and high-cost Annexin V assays were comparable when used for flow cytometry. At least, the same model could be used for animal cell lines in various biological experiments for an objective and fast assessment of apoptotic cells.

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