A novel rapid apoptosis assay based on thiol redox status

M. E. Skindersø, L. D. Johansen, S. Kjærulff; ChemoMetec A/S, Allerod, Denmark

Introduction

Apoptosis is a highly regulated process characterised by a number of distinct biochemical and morphological changes such as externalization of phosphatidylserine, activation of caspases, chromatin condensation and DNA fragmentation. It has long been known that treatments which either directly or indirectly induce oxidative stress also possess the ability to induce apoptosis. More recently the role of redox status alterations in apoptosis induction has been recognized. Cellular redox potential is largely determined by reduced glutathione (GSH) which accounts for more than 90 % of cellular non-protein thiols , whereas the oxidized form, glutathione disulfide (GSSG), normally represents <2% of the total glutathione pool. The concentration of GSH has been found to decrease upon induction of apoptosis, also when using non-oxidative apoptogenic agents, due to extrusion of GSH.

We have developed assays for determining the level and distribution of thiols in a cell population. The assays are extremely fast and easy and are useful not only for investigating the cellular redox state but also for assaying apoptosis. In the assays, the cells to be investigated are stained using a thiol-reactive probe; VitaBright-43 or VitaBright-48, which immediately reacts with intracellular thiols, forming a fluorescent compound. We found that though both thiol probes could be used to detect changes in the level of free thiols correlating well with apoptotic markers, other properties such as detection of early versus late apoptosis and staining kinetics differed among the probes. We have used our image cytometer; the NucleoCounter NC-3000 for the assays used in this study.

Methods and Results

Jurkat cells were grown at 37 °C, 5 % CO2 in FCS supplemented RPMI and apoptosis was induced by adding camptothecin (CPT) at different concentrations and time intervals. Using two novel thiol reactive agents (VitaBright-43 and VitaBright-48) and a well established GSH probe; monochlorobimane, we investigated whether changes in the level of free thiols can be used as an apoptotic marker. Upon addition to cells the probes permeate the cell membrane and react with intracellular thiols, causing cellular fluorescence. Cytometric quantification of the cell fluorescence (without washing) can then be used to determine the population's cellular thiol level at the single cell level. Apoptotic traits such as phosphatidylserine externalisation, caspase activity and mitochondrial potential were investigated at different time points after induction of apoptosis and correlated to changes detected using the thiol probes. The data shown are representative for three identical experiments.

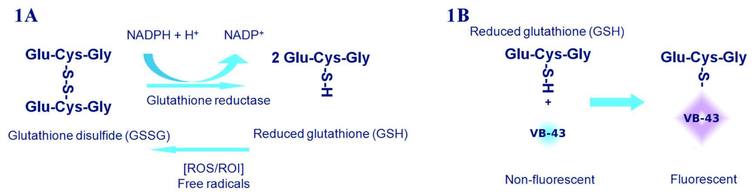

Fig. 1A. Glutathione redox cycle. The tripeptide glutathione exists in a reduced state (GSH) and an oxidized state; glutathione disulfide (GSSG). In the reduced state the thiol group of cysteine is able to donate a reducing equivalent (H++ e-) to unstable molecules such as free radicals. The thereby formed GSSG can be regenerated to GSH by the enzyme glutathione reductase. 1B. GSH readily reacts with the non-fluorescent thiol reactive probe e.g. VitaBright-43 forming a fluorescent product. Quantifying the fluorescence intensity of the stained cell population are then used to determine the cellular thiol level at the single cell level, providing information about health and redox state.

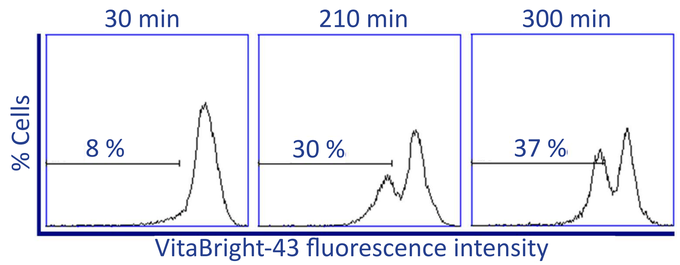

Fig. 2. The vitality assay using the NucleoCounter NC-3000™. Fluorescence intensity of VitaBright-43 stained cells. Propidium iodide positive (nonviable) cells have been excluded from the histogram. Over time after treatment with CPT cause a decrease in fluorescence intensity in a subpopulation over time. The marker line is used to divide the population into high and low fluorescence intensity subpopulations to enable exact quantification of the changes in fluorescence intensity and hence thiol level caused by CPT treatment.

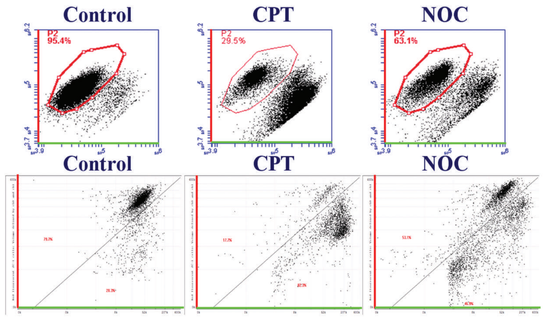

Fig. 3. Changes in the mitochondrial potential (Ψmit) due to CPT or NOC exposure were detected using the JC-1 stain which fluoresces green (X-axis) in apoptotic cells (i.e. cells with a collapsed Ψmit) and red (Y-axis) in healthy cells. First panel shows results obtained using a flow cytometer (Accuri) while the NucleoCounter NC-3000 has been used in the second panel.

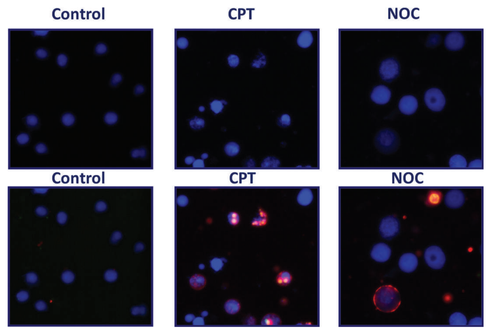

Fig. 4. Externalisation of phosphatidylserine. Cells were stained with Annexin V-AlexaFluor 594 conjugate, SYTOX green and VitaBright-48. Nonviable cells (appearing green), and apoptotic cells (appearing red) have a lower VitaBright-48 fluorescence intensity than viable, healthy cells (appearing blue only). The first panel shows VitaBright-48 staining only while the second panel is a super-imposition showing the staining with all three stains. Images shown were captured using an Olympus microscope and in-house built software. Stained cells were also analysed using the NucleoCounter NC-3000™ in order to obtain quantitative data. This showed that 12%, 47% and 33% of control, CPT treated, and NOC treated cells respectively, were Annexin V positive and SYTOX green negative.

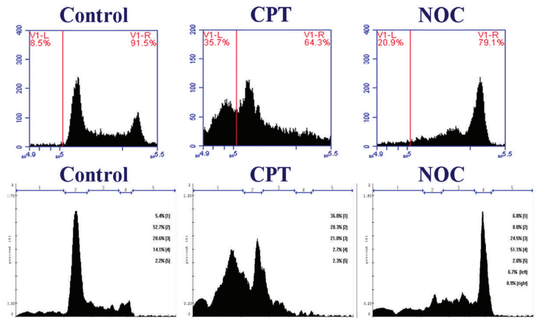

Fig. 5. Cell cycle profile of Jurkat cells either treated with 10 µM CPT or 1 µM or left untreated. Both CPT and NOC affect the cell cycle profile, but only CPT causes DNA fragmentation. First panel shows DNA histograms obtained using a flow cytometer (Accuri), while the NucleoCounter NC-3000™ has been used in the second panel.

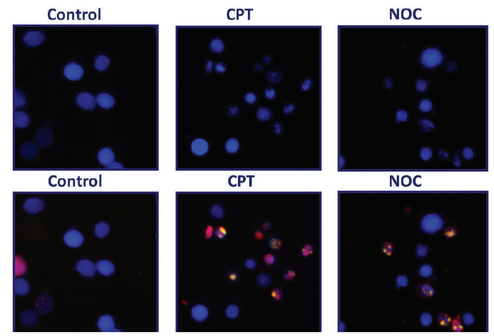

Fig. 6. Poly-caspase activity measured with FLICA. Cells were incubated with a sulforhodamine-labeled inhibitor of caspases (SR-FLICA), SYTOX green and VitaBright-48. Nonviable cells (appearing green), and apoptotic cells (appearing red) have a lower VitaBright-48 fluorescence intensity than viable, healthy cells (appearing blue only). The first panel shows VitaBright-48 staining only while the second panel is a super-imposition showing the staining with all three stains. Images shown were captured using an Olympus microscope and in-house built software. Stained cells were also analysed using the NucleoCounter NC-3000™ in order to obtain quantitative data. This showed that 4 %, 33 % and 18 % of control, CPT treated, and NOC treated cells respectively, had a high level of caspase activity.

Conclusions

As documented by this study, the decrease in reduced thiols measured using VitaBright-43 or monochlorobimane correlates well with other known assays for early apoptosis. Measuring the thiol level using VitaBright-48 may also be used, but this probe detects changes in the thiol level (and hence apoptosis) later than VitaBright-43 and monochlorobimane. When using either VitaBright-43 or VitaBright-48 the cells immediately reach maximal fluorescence intensity after addition of the probe, while monochlorobimane needs careful timing. Combined staining with an impermeable stain such as propidium iodide enables exclusion of nonviable cells. As we show here it is also possible to perform multiplexing by adding a third stain e.g. annexin V with a far red fluorescent label. In this case cells should be stained with the annexin V-probe according to manufacturer's recommendations first and then added the thiol probe shortly before analysis.

All three thiol probes provide a very rapid, easy and reliable way of assaying apoptosis by either flow or image cytometry as no washing or incubation steps are required. Results are also easy to interpret; presenting the data as a histogram showing the fluorescence intensity of all viable cells enables division of cells into subpopulations depending on their thiol level as shown in figure 2; apoptotic cells exhibit low fluorescence intensity while healthy cells have a high intensity score. Based on this study we suggest expanding the list of biochemical changes which may be evaluated for detection of apoptosis with measurement of the level of free thiols e.g. using VitaBright-43.