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# Analysis of relationship between cell cycle stage and apoptosis induction in K562 cells by sedimentation field-flow fractionation

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ABSTRACT

Recently, sedimentation field-flow fractionation (SdFFF) was used to study the specific kinetics of diosgenin-induced apoptosis in K562 cells. Here, we propose a new SdFFF cell separation application in the field of cancer research concerning the correlation between induction of a biological event (i.e. apoptosis) and cell status (i.e. cell cycle position). SdFFF isolated subpopulations depending on the cell cycle position allowing the study of apoptosis kinetics and extent. Results showed that cells in G0/G1 phases (F3 cells) underwent significant and earlier apoptosis than cells in the active part of the cell cycle (S/G2/M phases). Results shed light on the correlation between differences in apoptosis kinetics and cell cycle stage when exposure to the inducer began. SdFFF monitoring and size measurement also led to the description of different subpopulations demonstrating complex variations in density between fractions associated with differences in biological processes.

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## 1. Introduction

Within the field-flow fractionation (FFF) superfamily, sedimentation FFF (SdFFF) could be described with flow-FFF, hollow fiber-FFF, hybrid-FFF, magnetic FFF or DEP-FFF [1–13], as one of the most useful methods for cell separation and sorting [14–23]. SdFFF allows the macroscale preparation of functional cell populations for analytical and preparative applications [16–23]. Similar to many other cell separation techniques (centrifugation, elutriation, microfluidic devices, etc.), the principle of cell separation is based on physical criteria such as size and density [11,22,24–27]. The fundamental principle of FFF is based on the differential elution of species submitted to the combined action of: (1) a parabolic profile generated by flowing a mobile phase through a ribbon-like capillary channel and (2) an external field applied perpendicularly to the flow direction [28–30]. In SdFFF, a multigravitational external field is generated by rotation of the separation channel in a rotor basket, constituting one of the most complex devices used in FFF separation [25,26,29]. SdFFF could be defined as a gentle, non-invasive and tagless method. These advantages are based on the drastic limitation of cell-solid phase interaction by the use of: (1) a specific separation device: empty ribbon-like channel without stationary phase and (2) a device setup allowing the "Hyperlayer" elution mode, a size/density driven separation mechanism. Since the report of Caldwell et al. [14] on mammalian cells, FFF, SdFFF and related technologies have been used in many biological fields such as hematology, microorganism analysis, biochemistry/biotechnology and molecular biology, neurology and cancer research [4,20,22,31–43,7,44–48]. Over several years, we have studied the use of SdFFF in cancer research to study chemical induced apoptosis or differentiation in cancer cell lines. Different aspects have been evaluated including: (1) monitoring of the biological event [17,49–51]; (2) cell sorting of specific subpopulations such as pre-apoptotic [17], or differentiated cells [49] which can then be further used as models; (3) kinetics of the biological event using both the monitoring and cell separation capacities of SdFFF [19,21]. Cell sorting of specific phenotypes or immature cells from complex cancer cell populations such as neuroblastomas have been also performed [16,41].

Diosgenin is a well-known steroidal saponin, which can be found in several plant species. It has been described to have various effects *in vivo* [52–54] and *in vitro*, such as inducing megakaryocytic differentiation of human erythroleukemia cells at 10 μM, or exerting antiproliferative and pro-apoptotic actions (40 μM) on rheumatoid arthritis synoviocytes or cancer cell lines [17,19,21,49–51,55–63]. In a recent work, we used SdFFF to explore the specific biphasic

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apoptosis kinetics in K562 cells (erythroleukemic cell line) after diosgenin exposure [19,64]. The association of washout (removal of the chemical inducer from the culture medium) and early SdFFF "Hyperlayer" elution effectively separated different apoptotic stages in the same population. These results suggested different sensitivities to apoptosis induction, and we hypothesized that apoptosis kinetics could depend on the cell cycle position when diosgenin exposure started [19].

The goal of this work was to examine the possible relationship between cell cycle position and apoptosis kinetics. To achieve this goal, SdFFF was used to sort subpopulations from crude K562 cells to analyse the cell cycle before any apoptosis induction, leading to an immediate cell cycle status distribution. At the same time, to avoid any distortion between cell cycle analysis and apoptosis induction, fractions were subcultured and incubated for 6 h with diosgenin. Then, early apoptosis was monitored (SdFFF) and quantified by flow cytometry.

As previously described [20], results showed the capacity of SdFFF to sort cells depending on their position in the cell cycle, leading to the preparation of different subpopulations with regards to kinetics and the extent of apoptosis. These results led to a better understanding of the mechanism of diosgenin-induced apoptosis.

## 2. Materials and methods

### 2.1. Cell line, cell culture and treatment

The K562 human erythroleukemia cell line was provided by Dr I. Dusanter-Fourt (INSERM U567-CNRS UMR8104, Paris, France). Cells were cultured in RPMI-1640 medium (Gibco, Invitrogen, Paisley, Scotland) supplemented with 10% fetal calf serum (Gibco), 1% sodium pyruvate, 1% HEPES [N-(2-hydroxy-ethyl)piperazine-N′-2-ethansulfonic acid] (Gibco), 100 μg/mL streptomycin and 100 U/mL penicillin. Cultures were maintained in a humidified atmosphere with 5% $CO_2$ at 37 °C. Cells were seeded at $2 \times 10^5$ cells/mL in 75 $cm^2$ tissue culture flasks (Sarstedt, Marnay, France) and allowed to grow in culture medium for 24 h prior to SdFFF elution. Cells were harvested, counted by trypan blue dye exclusion method and resuspended in PBS (Gibco) at $2.5 \times 10^6$ cells/mL before SdFFF analysis.

### 2.2. SdFFF device, cell elution conditions and subpopulation preparation

SdFFF separation device used in this study derived from those previously described and schematized [17,19]. The apparatus was composed of two 938 mm × 40 mm × 2 mm polystyrene plates, separated by a mylar® spacer in which the channel was carved. Channel dimensions were 818 mm × 12 mm × 0.175 mm with two 50 mm V-shaped ends. The measured total void volume (channel volume + connecting tubing + injection and detection device) was 1792 ± 2.00 μL ($n = 6$). Void volume was calculated after injection and retention time determination of a non-retained compound (0.10 g/L of benzoic acid, UV detection at 254 nm). The channel rotor axis distance was measured at $r = 14.82$ cm. A Waters 515 programmable HPLC pump (Waters Associates, Milford, MA, USA) was used to pump the sterile mobile phase. Sample injections were done by means of a Rheodyne® 7125i chromatographic injector (Rheodyne, Cotati, CA, USA). The elution signal was recorded at 254 nm by means of a Waters 486 Tunable Absorbance Detector (Waters Associates) and a M1111 (100 mV input) acquisition system (Keithley, Metrabyte, Tauton, MA, USA) operated at 4 Hz connected to a PC computer. A M71B4 Carpanelli engine (Bologna, Italy) associated with a Mininvert 370 pilot unit (Richard Systems, Les Ullis, France), controlled the rotating speed of the centrifuge baskets. Sedimentation fields were expressed in units of gravity, $1\,g = 980\,cm/s^2$, and calculated as previously described [37]. Cleaning and decontamination procedures have been previously described [26].

The optimal elution conditions ("Hyperlayer" mode) were determined experimentally and were: flow injection through the accumulation wall of 100 μL K562 cell suspension ($2.5 \times 10^6$ cells/mL); flow rate: 0.80 mL/min; mobile phase: sterile PBS, pH 7.4 (Gibco); external multi-gravitational field strength: 8.00 ± 0.01 *g* (219.7 ± 0.1 rpm).

Fig. 1A summarizes the protocol used to prepare the different studied subpopulations. After 24 h incubation, K562 cells ($2.5 \times 10^6$ cells/mL in sterile PBS pH 7.4) were eluted (Fig. 1B) resulting in the separation of four cell fractions collected and designated as follows: (1) TP (total peak) (elution time 3 min 30 s to 6 min 50 s); and (2) Fn (fraction number) with F1 (3 min 30 s to 4 min 20 s), F2: (4 min 25 s to 5 min 20 s) and F3 (5 min 30 s to 6 min 50 s). To obtain a sufficient quantity of cells for cell cycle studies, or culture and later apoptosis induction, monitoring and quantification, successive SdFFF cumulative fraction collections were performed (12–16).

Cell cycle was immediately analyzed after SdFFF elution (Fig. 1). Otherwise, control and SdFFF eluted cells were incubated for 6 h with diosgenin in order to induce apoptosis which was monitored (SdFFF elution) and quantified (flow cytometry) (Fig. 1).

### 2.3. Cell cycle analysis

After SdFFF sorting, cells (fractions and control, Fig. 1) were fixed and permeabilized in 70% ethanol in phosphate-buffered saline (PBS) at −20 °C overnight, washed in PBS, treated with RNase (40 U/μL, Boehringer Mannheim, Meylan, France) for 1 h at room temperature and stained with propidium iodide (PI) (50 μg/mL). Flow cytometry experiments were carried out using a FACS Vantage Diva SE (Becton Dickinson, USA) and were performed as previously described [61]. Data were analyzed with the Cell Quest Software (Becton Dickinson, USA).

### 2.4. Coulter counter

A 256 channel Multisizer II Coulter Counter (Beckman Coulter, Fullerton, CA) was used to determine the mean cell population diameter. Cells: crude population or SdFFF collected fraction were diluted in Isoton® to a final volume of 15 mL. The counting conditions were: 500 μL sample volume, cumulating three successive assays. Results are displayed as the mean ± S.D. for three different experiments.

### 2.5. Apoptosis induction and quantification

As described in Fig. 1, control and SdFFF eluted cells were cultured in 24 well plates for 6 h at a density of $1.5 \times 10^5$ cells/well with 1 mL culture medium/well, in a humidified atmosphere with 5% $CO_2$ at 37 °C. Cells were incubated (treated cells) or not (control cells) with 40 μM diosgenin ([25R]-5α-spiroten-3β-ol) (Sigma–Aldrich, Saint-Quentin Fallavier, France). The same amount of vehicle (<0.1% ethanol) was added to control cells. As previously described, early apoptosis, which is the most significant stage to follow apoptosis kinetics [19], was quantified by an ANNEXIN V-FITC Kit (Beckman Coulter, Paris, France). This assay determines the cell surface appearance of phosphatidylserines (PS), negatively charged phospholipids usually located in the inner leaflet of the plasma membrane. In the early phase of apoptosis, cell membrane integrity is maintained but cells lose the asymmetry of their membrane phospholipids. PS become exposed at the cell surface and form one of the specific signals for recognition and removal of apoptotic cells by macrophages. The ANNEXIN V-FITC Kit is an apoptosis kit based on the binding properties of Annexin V to PS and the DNA-

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