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Study of diosgenin-induced apoptosis kinetics in K562 cells by Sedimentation Field Flow Fractionation

J. Bertrand[a], B. Liagre[b], G. Bégaud-Grimaud[c], M.O. Jauberteau[a], P. Cardot[c], J.L. Beneytout[b], S. Battu[c,*]

[a] Laboratoire d'Immmunologie, EA 3842, "Homéostasie Cellulaire et Pathologies", Université de Limoges, Faculté de Médecine, 2 rue du Docteur Marcland, 87025 Limoges Cedex, France
[b] Laboratoire de Biochimie, EA 4021 "Biomolécules et Thérapies Anti-tumorales", Université de Limoges, Faculté de Pharmacie, 2 rue du Dr Marcland, 87025 Limoges Cedex, France
[c] Laboratoire de Chimie Analytique et Bromatologie, EA 3842, "Homéostasie Cellulaire et Pathologies", Université de Limoges, Faculté de Pharmacie, 2 rue du Docteur Marcland, 87025 Limoges Cedex, France

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ABSTRACT

Recently, the use of SdFFF in cancer research has been studied in order to better understand major phenomena implicated in cancer development and therapy: apoptosis and differentiation. In this report, we used SdFFF as a monitoring and cell separation tool to study the kinetics of apoptosis. Incubation of K562 cells with diosgenin, used as cellular model, led to a surprising apoptotic process occurring in two phases (after 24 and 48 h incubation), associated with specific p-ERK expression. Based on the capacity to sort apoptotic cells, results showed that SdFFF cell separation was an effective analytical tool to obtain different subpopulations regardless of the kinetics and extent of apoptosis. Results also showed that, after proper biological calibration of elution profiles, SdFFF can be used to monitor either the induction or the kinetics of a biological event.

1. Introduction

Conceptualized and developed from the late 1960s by Giddings [1], Field Flow Fractionation (FFF) is described as one of the most versatile separation techniques [1–3]. The fundamental principle 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 [1–3]. 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 [2,4,5]. Among members of the FFF family, SdFFF appears as an effective method for the separation of cells by using the "Hyperlayer" elution mode [2,3,6–14]. In few minutes, SdFFF sorts viable cells which can be cultured for further uses without specific labeling of any kind [4,5,7,12,15–19]. As SdFFF only takes advantages of biophysical cell properties (size, density, shape, rigidity), it could be described as a tagless method [4,5,7,12,15–19]. Since the work of Caldwell et al. [7] 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 [16,17,19–38]. Over several years, we have studied the use of SdFFF in cancer research, in particular to study chemical apoptosis or differentiation induction in cancer cell lines. Different aspects can be evaluated including (1) monitoring of the biological event [15,18,39,40]; (2) cell sorting of specific subpopulations such as pre-apoptotic [40], or differentiated cells [39] which can be used as models; (3) kinetics of the biological event using both the monitoring and cell separation capacities of SdFFF; and finally (4) cell sorting of specific phenotypes from complex cancer cell populations such as neuroblastomas [41].

Cancer progression is a complex, multi-step process associated with genetic changes. One of them leads to a disturbed balance between cell division and growth on one hand, and programmed cell death (apoptosis) on the other hand. Apoptosis is an important process in a wide variety of different biological systems, including normal cell turnover, the immune system, embryonic development, metamorphosis and also in chemical-induced cell death [42,43]. In fact, the development and study of new therapeutic agents to stop tumors expansion and induce apoptosis are one of the main themes developed in cancer research. Diosgenin is a steroidal saponin, which can be found in several plant species, and particularly in fenugreek or wild yam roots. This plant steroid has been thoroughly described to have various effects *in vivo* [44–46] and *in vitro*. Diosgenin induces megakaryocytic differentiation of HEL cells, a human

* Corresponding author. Tel.: +33 5 5543 5979; fax: +33 5 5543 5858.
E-mail address: serge.battu@unilim.fr (S. Battu).

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erythroleukemia cell line [47]. Moreover, diosgenin has been shown to exert antiproliferative and pro-apoptotic actions on rheumatoid arthritis synoviocytes [48] or on cancer cell lines [15,18,49–53].

Recently, a study demonstrated that diosgenin induced apoptosis in K562 and HEL cells (two erythroleukemic cell lines) with two different kinetic profiles [54]. In fact, HEL cells were recruited to undergo to significant apoptosis after 24 h diosgenin treatment [18,49], as has been shown in osteosarcoma cells [50]. In contrast, K562 cells seemed to undergo apoptosis in two times after 24 and 48 h diosgenin treatment [54]. Previous studies showed that ERK (extracellular signal-regulated kinase) activation was implicated in mitosis regulation [55] and was necessary for proliferation and apoptosis inhibition [56]. In this way, ERK inhibition during diosgenin treatment of HEL cells seemed to be correlated with apoptosis activation and proliferation inhibition [54]. Surprisingly, ERK activation was also shown during diosgenin treatment in K562 cells [54], and the link between diosgenin induced apoptosis and ERK activation in K562 cells was not established.

The goal of this work was to elucidate the specific apoptotic process during diosgenin treatment in K562 cells. To achieve this goal, we used SdFFF which has been exploited in cancer research namely for (1) cell sorting of specific subpopulations such as pre-apoptotic cells [39,40,57]; and (2) kinetic studies by the association of cell sorting with a washout process [57]. Thus, K562 cells were incubated for a short period with diosgenin before washout of the diosgenin and SdFFF elution. The collected fractions were then subcultured for 4, 24 and 48 h without diosgenin. This gave us a biological tool to study diosgenin-induced apoptosis in different subpopulations, without the continuous influence of inducer.

Based on the capacity of SdFFF to sort pre-apoptotic cells [40], results showed that the association of washout with cell separation appeared as an effective analytical tool to obtain different subpopulations regardless of the kinetics and extend of 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 humified atmosphere with 5% CO_2 at 37 °C. Cells were seeded at 2×10^5 cells/mL in 75 cm^2 tissue culture flasks (Sarstedt, Marnay, France) and allowed to grow for 24 h in culture medium prior to exposure or not to 40 μM diosgenin ([25*R*]-5α-spiroten-3β-ol) (Sigma–Aldrich, Saint-Quentin Fallavier, France)). The same amount of vehicle (<0.1% ethanol) was added to control cells. After 12 h, cells were harvested, counted by trypan blue dye exclusion method and resuspended in PBS (Gibco) at 2.5×10^6 cells/mL before SdFFF analysis.

2.2. SdFFF device and cell elution conditions

SdFFF separation device used in this study (Fig. 1) derived from those previously described and schematized [40,58]. 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 an unretained 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 pilot unit Mininvert 370 (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 [25]. Cleaning and decontamination procedures have been previously described [4].

The optimal elution conditions ("Hyperlayer" mode) were determined experimentally and were: flow injection through the accumulation wall of 100 μL of K562 cell suspension (2.5×10^6 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).

2.3. Subpopulation preparation: washout, SdFFF elution and fraction collection

Fig. 2 summarizes the protocol used to prepare the different studied subpopulations. K562 cells were seeded at 2×10^5 cells/mL and treated or not (control) with 40 μM diosgenin. After 12 h incubation, an aliquot of treated cells was used as a positive control for apoptosis induction (K562 Dios cells). The remaining of treated cells underwent washout (WO) in order to eliminate diosgenin. Cells were centrifuged for 10 min at 1300 rpm (300 × *g*). The pellet was washed twice with sterile PBS pH 7.4. Aliquots of washed cells (2×10^5 cells/mL) were subcultured in fresh culture media without diosgenin, and corresponded to the K562 WO population. The remaining washed cells were suspended in sterile PBS pH 7.4 (2.5×10^6 cells/mL) prior to SdFFF elution which allowed the preparation of four cell fractions collected and designated as follows: TP:elution time—4 min 25 s to 8 min 30 s and F*n*: F1:elution time—4 min 25 s to 5 min 30 s, F2:elution time—5 min 40 s to 6 min 50 s, F3:elution time—7 min 5 s to 8 min 30 s. To obtain a sufficient quantity of cells for culture and later SdFFF elution, successive SdFFF cumulative fraction collections were performed [8–10]. Fractions were subcultured in the absence of diosgenin for 4, 24 and 48 h. As shown in Fig. 2, different populations were obtained: control, K562 Dios, K562 WO, F1, F2 and F3 which could be characterized by fractogram monitoring, apoptosis and proliferation studies.

2.4. Apoptosis quantification

After SdFFF, apoptosis was quantified by "cell death" enzyme-linked immunosorbent assay (ELISA) (Cell Death Detection ELISA^{PLUS}, Roche Diagnostics, Meylan, France). This method differentiates between late apoptotic and necrotic cells. This ELISA method simultaneously uses two monoclonal antibodies against DNA and histones to allow the specific detection of mono- and oligo-nucleosomes, characteristic of apoptosis in the cytoplasmic fractions. Cytosol extracts were obtained according to the manufacturer's protocol and apoptosis was measured as previously described [50]. Moreover, apoptosis was quantified by an ANNEXIN V-FITC Kit (Beckman Coulter, Paris, France). Apoptosis can be detected and measured by many parameters. One of these parameters is the cell surface appearance of phosphatidylserine (PS), a negatively charged phospholipid usually located in the inner leaflet

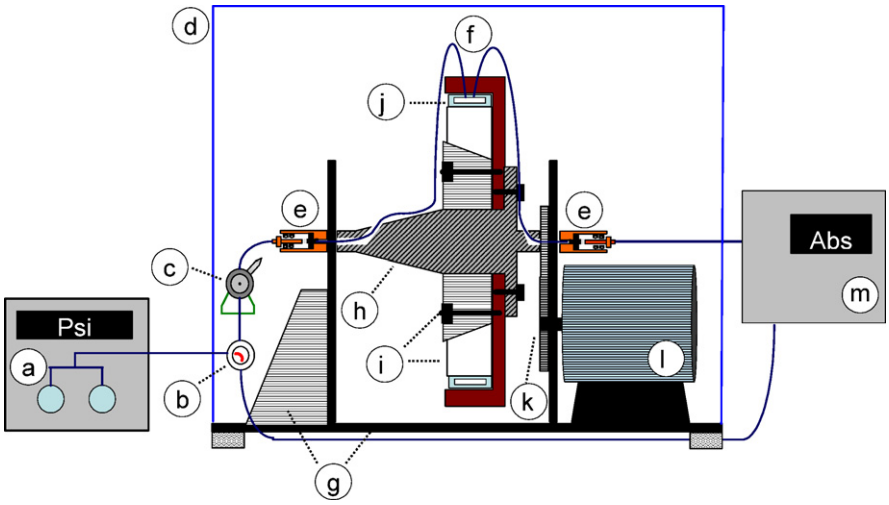

Fig. 1. Transversal cut of an SdFFF system: (a) HPLC chromatographic pump; (b) switching valve; (c) HPLC sample injection valve; (d) security system; (e) inlet and outlet rotating seals; (f) inlet and outlet peak tubing; (g) static device; (h) rotor; (i) channel wall sealing system; (j) channel, channel walls; (k) Teflon gear wheels; (l) electric motor and speed control device; (m) HPLC UV–vis detector and signal acquisition system.

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-intercalating capabilities of propidium iodide (PI). At the appropriate time, samples were prepared according to the manufacturer's protocol and analyzed by flow cytometry. Ten thousand cells were acquired, and data were analyzed using the computer program WinMDI (Version 2.8).

2.5. BrdU incorporation

BrdU (5-bromo-2′-deoxyuridine) is a DNA base analogue in which the methyl group of thymidine is replaced with a bromide. BrdU is incorporated into DNA during replication. Consequently, its cell labeling is proportional to DNA synthesis during the S phase. After SdFFF analysis, cells were treated with 50 μM BrdU (Sigma) during 48 h, fixed and permeabilized with IntraStain (DakoCytomation, Trappes, France). DNA was denatured in 1N HCl for 1 h at room temperature, cells were washed in PBS and stained with BrdU mouse monoclonal antibody (IIB5, Santa Cruz, Tebu Bio, Le Perray

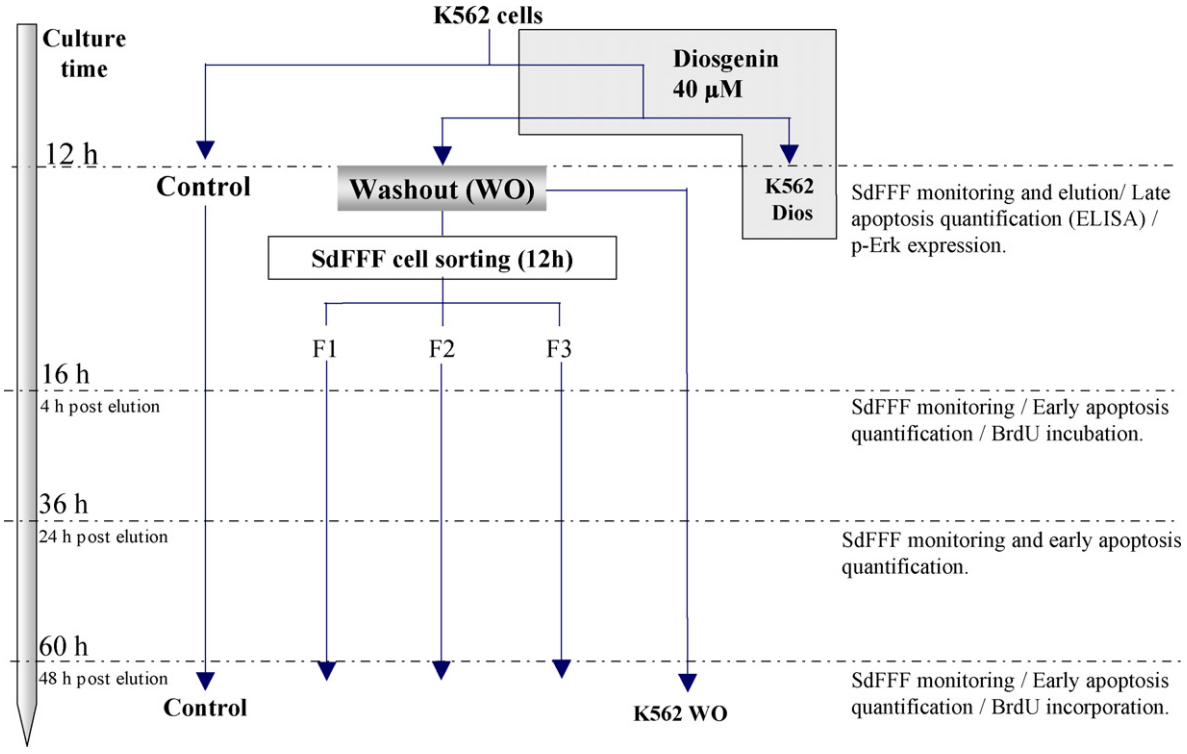

Fig. 2. Preparation of different K562 cell subpopulations. K562 populations were prepared ranging from control (never exposed to diosgenin) to K562 Dios (continuously exposed to diosgenin). As described in Section 2.3, washout and SdFFF elution (12 h) eliminated diosgenin, allowing cell apoptosis to proceed without continuous influence from inducer. Incubation time, 0–60 h, was counted from the start of incubation with 40 μM diosgenin.

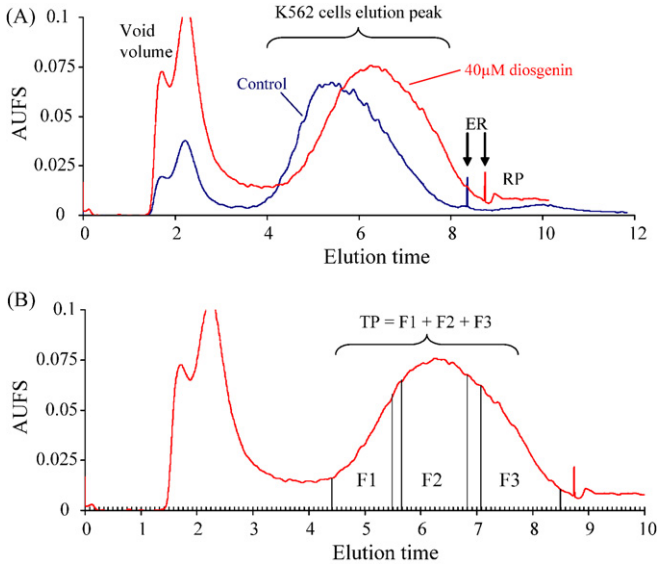

Fig. 3. Representative fractograms of K562 cells. (A) Comparison of elution profiles of K562 cells incubated alone (control) or with 40 μM diosgenin. (B) SdFFF fractograms and fraction collection for 40 μM diosgenin treated cells with: TP (4 min 25 s to 8 min 30 s) and F1 (4 min 25 s to 5 min 30 s)–F2 (5 min 40 s to 6 min 50 s)–F3 (7 min 5 s to 8 min 30 s). Elution conditions: channel thickness: 175 μm; flow injection of 100 μL cell suspension (2.5×10^6 cells/mL); spectrophotometric detection at $\lambda = 254$ nm; flow rate: 0.80 mL/min and external multi-gravitational field: 8.00 ± 0.01 g. ER corresponds to the End of channel Rotation: at this moment, the mean externally applied field strength is equal to zero gravity, thus RP, a residual signal, corresponds to Release Peak of reversible cell-accumulation wall sticking.

En Yvelines, France) at a 1:100 dilution in Versene/SVF 10% for 30 min at 4 °C, allowed by FITC secondary antibody (Santa Cruz) at a 1:200 dilution in Versene/10% SVF for 30 min at 4 °C and then analyzed by flow cytometry. Ten thousand cells were acquired and data were analyzed using the computer program WinMDI (Version 2.8).

2.6. p-ERK expression

p-ERK expression (active phosphorylated form of ERK) was quantified by flow cytometry. After SdFFF analysis, cells were washed with PBS, fixed and permeabilized with IntraStain (Dako-Cytomation). Then, cells were stained with 1:100 human p-ERK mouse monoclonal antibody (E-4, Santa Cruz) in Versene/10% SVF for 30 min at 4 °C, and then with 1:200 FITC secondary antibody (Santa Cruz) in Versene/10% SVF for 30 min at 4 °C, followed by flow cytometry analysis. Ten thousand cells were acquired and data were analyzed using the computer program WinMDI (version 2.8).

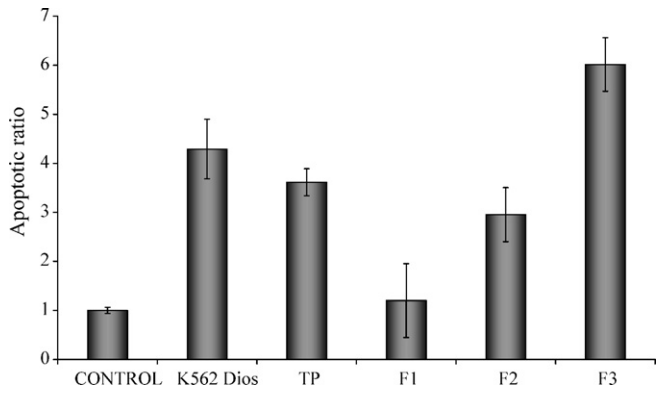

Fig. 4. Comparison of K562 cell apoptotic ratios. Subpopulations were obtained as described in Fig. 2 after 12 h incubation with 40 μM diosgenin. Apoptosis was quantified by "Cell Death" Enzyme-Linked Immunosorbent Assay (Cell Death Detection ELISA^{PLUS}, Roche Diagnostics). This method differentiates late apoptotic from necrotic cells by specific detection of mono- and oligo-nucleosomes in the cytoplasmic fraction, which is characteristic of apoptosis. Results were expressed as a mean apoptotic ratio with the control population apoptotic ratio equal to 1 ($n = 3 \pm$ S.D., all values are significantly different: $p < 0.01$, with the exception of control versus F1).

2.7. Statistical analysis

The median and standard deviation (S.D.) were calculated using Excel software (Microsoft, Version 2002). Statistical analysis of differences was carried out by ANOVA test using Statview (Version 5.0). A p-value of less than 0.05 (Fisher's PLSD test) was considered to indicate significance.

3. Results and discussion

3.1. SdFFF elution

As described [4,15–18,25,39,40], SdFFF is a simple, fast, economical and effective method for cell separation. SdFFF takes into account and respects the principal cellular biological properties such as (1) functional integrity [16,17,25,39]; (2) short and long term viability without induction of additional apoptosis [15,18,40,59]; (3) maturation and differentiation stages which are essential for undifferentiated cell sorting (stem cells, progenitors) [17,59]. As

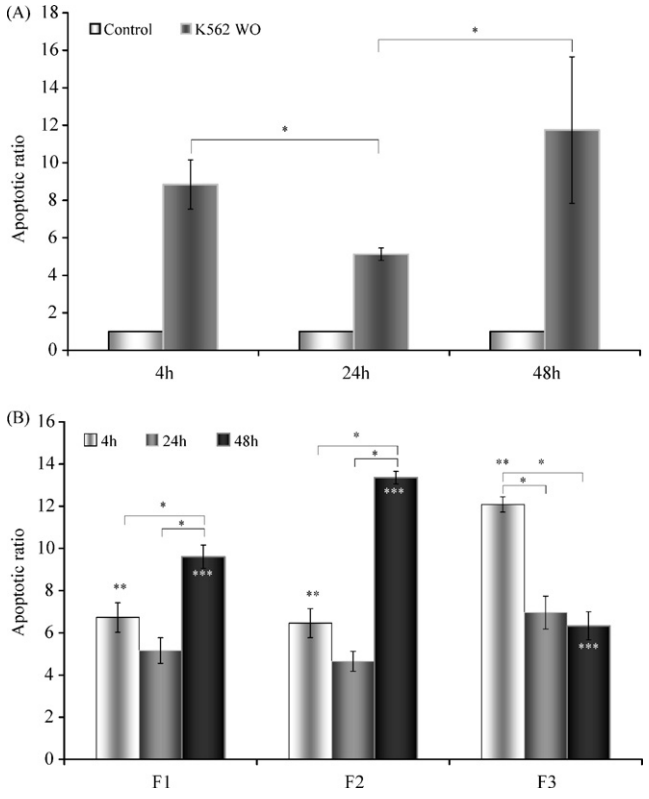

Fig. 5. Kinetics of early phase apoptosis. Subpopulations were obtained as described in Fig. 2 after 12 h incubation with 40 μM diosgenin. After SdFFF separation, cells were subcultured and an ANNEXIN V-FITC kit was used at the appropriate time. This early phase of apoptosis was detected and quantified by flow cytometry by detecting the cell surface appearance of phosphatidylserines which become exposed during apoptosis. Results were expressed as a mean apoptotic ratio with the control population apoptotic ratio equal to 1 ($n = 3 \pm$ S.D., $p < 0.05$. *Significant values between incubation times. **,***Significant values between F1 and F2 versus F3 after 4 and 48 h incubation).

SdFFF only takes advantages of biophysical cell properties (size, density, shape, rigidity), this tagless method [19] provides an advantage over FACS or MACS (fluorescent or magnetic activated cell sorting) when labels do not exist or when they could interfere with further cell uses (culture, transplantation) or when they could induce differentiation. Otherwise, the off-line association of SdFFF and FACS constitutes a very effective method to characterize subpopulations [16,39].

Recently, SdFFF was used to elucidate some of the most important phenomena involved in cancer therapy which are apoptosis and differentiation. Until now, four aspects have been explored: (1) monitoring of biological events induction [15,18,39,40]; (2) cell sorting of specific subpopulations such as pre-apoptotic [40], or differentiated cells [39] which could be used as models; (3) kinetics of biological events using both the monitoring and cell separation capacities of SdFFF [57]; and (4) the phenotypic relationship in complex cancer cell populations [41].

Because SdFFF provides enriched, sterile, viable and functional pre-apoptotic cells [40], it could be used in association with washout to investigate the particular mechanism and kinetics of diosgenin-induced apoptosis in K562 cells population.

To achieve this goal, device setup and elution conditions (see Section 2) were optimized to reduce harmful cell/channel interactions by working in the "Hyperlayer" elution mode. In this mode, cells are focused, away from the accumulation wall, in a thin layer corresponding to their equilibrium position where the external field is exactly balanced by hydrodynamic forces [2,3,6–14]. "Hyperlayer" elution mode predicts a size/density dependent cell elution order [2,3,6–14]. At equivalent density, large particles generate more lift forces and are focused in faster streamlines to be eluted first. On the other hand, gravitational forces increase with particle density. Denser particles are focused near the accumulation wall to be eluted last. Cell shape and rigidity also influence cell elution order [12]. The different average velocities, and the retention order of different species are compared by means of the observed retention ratio R_{obs} [13]. As previously described [4,6,7,10,18,25,39], the external field and flow rate dependence of R_{obs} is one of the most important parameters to confirm the "Hyperlayer" elution mode. Under the "Hyperlayer" elution mode, at constant field, the increase in flow rate induces an increase in R_{obs}. At constant flow, the increase in field decreases R_{obs}. If the external field can be sufficiently increased, or flow rate sufficiently decreased to offset lift forces, cells are confined to a very thin layer and in contact with the accumulation wall. This elution mode is described as "steric" [2,3,6–12,14,13] and it may be considered as a limit case of the "Hyperlayer" mode. By driving species in contact with the accumulation wall, the "Steric" elution mode enhances cell/channel wall interactions which lead to channel poisoning with harmful consequences on cell integrity, viability and recovery. Actually, cell/channel wall interactions could induce cell differentiation and/or cell death which interfere in the study of processes such as apoptosis.

Fig. 3A and B displays representative fractograms obtained for control and treated cells. In each case, two major peaks were observed, the first corresponding to the unretained species (void volume peak, $R_{obs} \approx 1$), the second corresponding specifically to the cell population with $R_{obs} = 0.382 \pm 0.012$ ($n > 9$) for control, and $R_{obs} = 0.352 \pm 0.011$ ($n = 9$) for treated cells. As described [18,39], if the absolute value of R_{obs} depends on condition and culture time, we observed that R_{obs} varied from 0.320 (12.00 g external field strength and 0.8 mL/min as flow rate) to 0.413 (7.00 g external field strength and 1.2 mL/min as flow rate) for control cells. Besides the field or flow dependence of R_{obs}, the "Hyperlayer" elution mode description also predicted that samples were not in close contact with the accumulation wall. By using the following equation [14,17],

$$R = \frac{6s}{\omega} \quad (1)$$

in which R is the retention ratio (R_{obs} values), ω is the channel thickness (175 μm), and s is the distance of the center of the focused zone from the channel wall [14], s was 11.05 μm. The mean cell diameter was 13.05 ± 0.28 μm ($n > 3$, Coulter Counter®). Thus, cell radii were less than the approximate average cell elevation value: $r = 6.53 < s = 11.05$ μm. Finally, after total cell elution, when the external field was turned off (mean external field = 0.00 g), we observed a residual signal (RP, Fig. 3A) corresponding to the reversible particle release from the accumulation wall.

These results confirms that K562 cells are eluted in "Hyperlayer" mode, as described for many other cell lines [2,3,5–12,14–18,25,39,40,13].

3.2. SdFFF monitoring of apoptosis, fraction collection and subpopulation sorting

As previously described [15,18,40], the decrease in R_{obs} between control and treated cells (Fig. 3A) is proof of apoptosis induction by diosgenin. As described for other cell lines [15,18,40], this shifting could be observed as early as 6 h incubation (data not shown). This confirms the value of SdFFF in monitoring induction of biological events. As described [15,18,40], early steps of apoptosis induction were associated with an increase in mean cell diameter. In the case of K562 cells, the mean Δdiameter was + 1.06 ± 0.15 μm ($n = 4$). Diosgenin induced apoptosis led to major size/density changes which have been monitored by SdFFF in many cell models [15,18,40]. According to the "Hyperlayer" elution mode description [2,3,6–12,14,13], as size and density are first order parameters to determine particle elution, an increased particle size correlated with increased retention time should indicate a large increase in density. A 175 μm thick channel appeared to be more effective to take into account a density increase in the cell population, allowing pre-apoptotic sub-population separation respecting functional integrity, viability, sterility and biological processes [40].

As described [4,60–62], even the most apparently homogenous cultured cell lines can only be described through biophysical (size, density, shape) and biological (metabolic activity, cell cycle position . . .) matrices of polydispersity. Thus, the necessary Hyperlayer elution of such populations led to broad peaks composed of the juxtaposition of the multiple/different subpopulations. Optimal

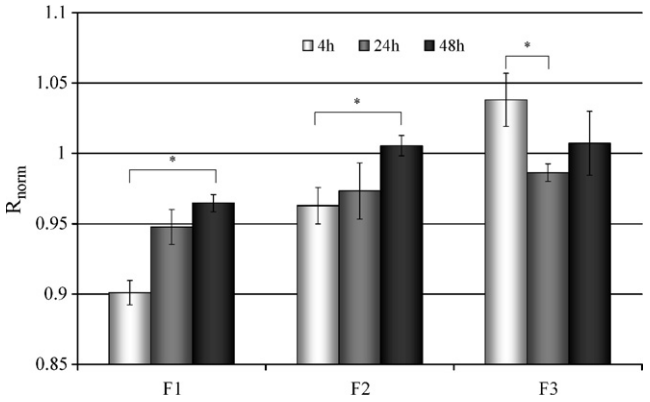

Fig. 6. SdFFF monitoring of apoptosis in subpopulations. Subpopulations (Fn and K562 WO cells) were obtained and analyzed by SdFFF after 4, 24 and 48 h subculture as described in Fig. 2. Elution conditions were described in Fig. 3. R_{norm}, defined as the ratio of R_{obs} for Fn versus R_{obs} for K562 WO (apoptotic reference), was calculated for each fraction as a function of subculture time. Results were expressed as a mean $R_{norm} \pm$ S.D. ($n = 3$, $^{*}p < 0.05$).

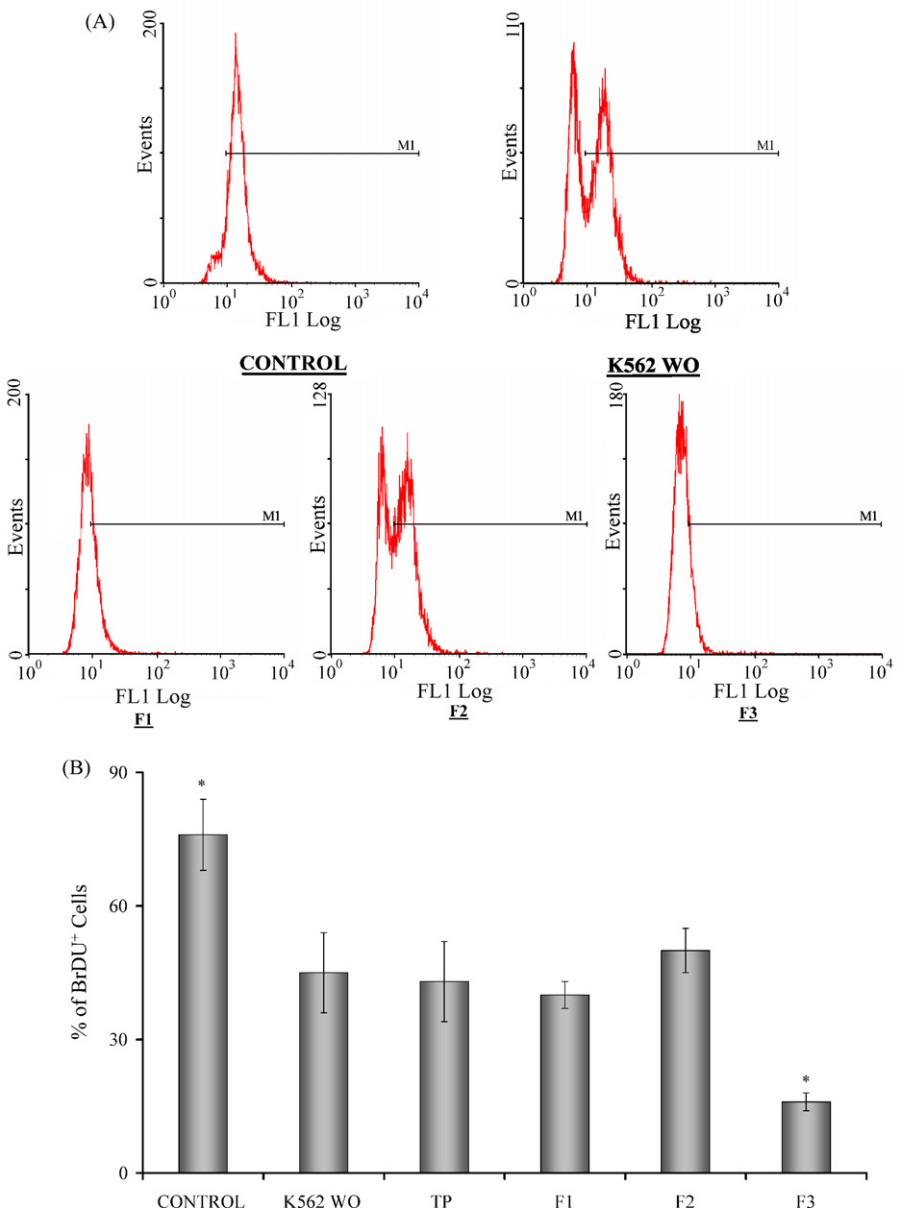

Fig. 7. Cell proliferation. Subpopulations were obtained as described in Fig. 2 after 12 h incubation with 40 μM diosgenin. After SdFFF analysis, cells were subcultured with 50 μM BrdU during 48 h. BrdU (5-bromo-2′-deoxyuridine) is a DNA base analogue incorporated into DNA during replication. Consequently, cell labeling is proportional to DNA synthesis during S phase. Cells incorporating BrDU were detected by flow cytometry (A). (B) Results were expressed as a mean percentage of $BrDU^+$ cells as a function of subpopulation and incubation time ($n = 3 \pm$ S.D., control and F3 are significantly different from all other population with $p < 0.01$).

elution is a balance between a sufficient retention time to collect subpopulations of interest and reduced cell trapping and plate height. If fast and resolutive separations can be performed by using high flow rate and field strength [63,64], gentle conditions using low flow rate and external field (0.4–1.0 mL/min, 10–50 *g*) should be selected to avoid mechanical cell injury [7,25,41,57,59].

Nevertheless, an effective biophysical cell separation does not give any information concerning effective biological isolation. Optimal elution conditions are achieved when external field strength/flow rate balance leads to an effective subpopulation isolation and characterization. To determine if cell sorting of the different K562 sub-populations (regarding apoptosis) was effectively achieved, cell fractions were collected (Fig. 3B) as follows: (1) the total peak fraction (TP) which corresponded to the total population eluted; and (2) peak fractions 1, 2 and 3 (F*n*) which are time-dependent collected fractions of the retained peak profile.

Then a ELISA assay (Cell Death Detection $ELISA^{PLUS}$) was performed on the different sub-populations to specifically quantify the late apoptotic process (Fig. 4). The increase in apoptotic ratio confirmed that diosgenin induced K562 cell apoptosis after 12 h incubation (K562 Dios versus control, Fig. 4) [54]. We also observed that SdFFF elution did not significantly modify apoptosis in populations (K562 Dios cells versus TP, Fig. 4). Finally, results showed that SdFFF was able to isolate apoptotic cells as previously described in osteosarcoma cells [40]. Fig. 4 shows different apoptosis levels in the fractions: while fraction F1 expressed an apoptosis level similar to the control population, F3 contained highly apoptotic cells.

Afterwards, cell culture, apoptosis and proliferation assays were performed on collected fractions, after 4, 24 and 48 h subculture (Fig. 2), to better understand the kinetics of apoptosis induction in this particular model K562-diosgenin.

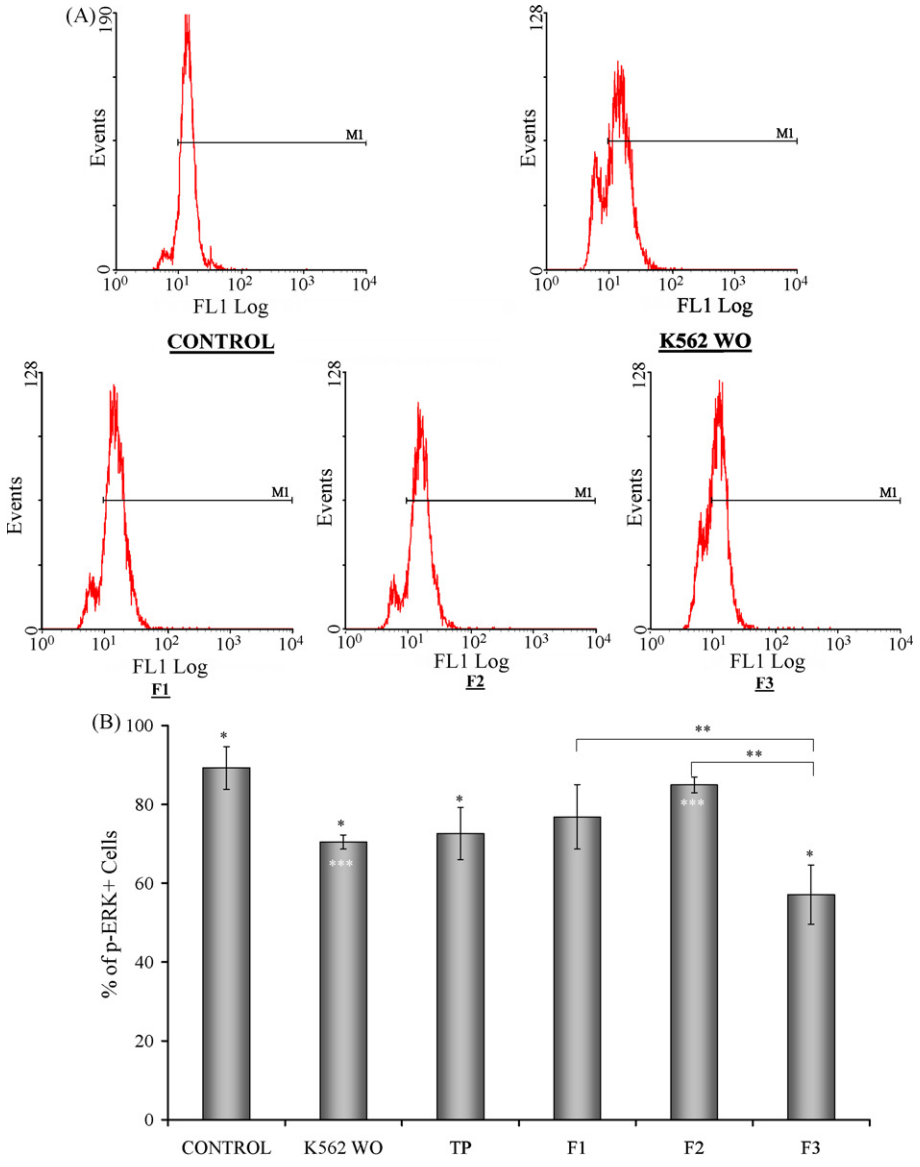

Fig. 8. p-ERK expression. Subpopulations were obtained as described in Fig. 2 after 12 h incubation with 40 μM diosgenin. After SdFFF analysis, cells were stained with human p-ERK mouse monoclonal antibody. p-ERK expression was analyzed by flow cytometry (A). (B) Results were expressed as a mean percentage of p-ERK$^+$ cells as a function of subpopulation ($n = 4$, ± S.D., $p < 0.05$. significant values between populations are indicated by *, ** or ***).

3.3. Study of apoptosis kinetics

To better understand the kinetics of apoptosis in K562 cells, we studied the early phase of apoptosis. This parameter appeared more sensitive than the later stage to describe cell population dynamics. This early phase of apoptosis can be detected and quantified by flow cytometry by detecting the appearance of phosphatidylserines which become exposed on the cell surface during apoptosis.

In cell lines such as HEL or 1547, diosgenin strongly induced apoptosis after 24 h incubation which rapidly decreased after 48 h leading to massive cell death of the whole population [18,49,50,52,54]. In agreement with previous results measured by ELISA [54], Fig. 5A showed that early apoptosis levels in K562 WO cells were still high throughout the entire subculture time (4–48 h). These results demonstrated that the whole K562 population did not undergo into apoptosis at the same time, or with the same kinetics after diosgenin treatment. They also demonstrated that diosgenin was necessary for induction but not to maintain apoptosis.

Then, WO/SdFFF cell separation associated with flow cytometry detection of early phase of apoptosis appeared to be an effective analytical tool to obtain different subpopulations regardless of the kinetics and extend of apoptosis without the continuous influence of the inducer.

After 4 h subculture, Fig. 5B showed that the profile of early apoptosis was quite similar to that obtained for late apoptosis (Fig. 4). Thus, we observed high levels of apoptosis in F3 compared to F1 and F2 (Fig. 5B). F3 corresponds to the major portion of apoptotic cells (early and late stages) in the K562 population. After 24 and 48 h, we observed a decrease in the percentage of early apoptotic cells in F3. Concerning F1 and F2, we observed a similar low amount of apoptosis after 4 and 24 h subculture (Fig. 5B). However, in contrast to F3, these fractions exhibited a large increase in apoptosis after 48 h subculture (Fig. 5B). This process was particularly pronounced in F2 in which the apoptotic ratio appears to be about three times higher than for the 24 h subculture, leading to the a higher rate of apoptosis in the K562 subpopulation.

As described in Fig. 2, the K562 WO population could be considered to be a combination of F1, F2 and F3. Then, after 4 h subculture, the apoptotic level in WO population could be linked to F3 apoptosis (Fig. 5). After 24 h subculture, the lower apoptotic ratio in each fraction (Fig. 5B) explained the apoptosis decrease in K562 WO population (Fig. 5A). After 48 h subculture, the apoptosis increase observed in the K562 WO population could be due to apoptosis observed in F1 and F2 (Fig. 5A).

In order to monitor the kinetics of apoptosis, subpopulations were also eluted by SdFFF after 4, 24 and 48 h subculture. Elution profiles were then compared to those of K562 WO cells used as a reference. To better understand retention changes during apoptosis, R_{obs} values were compared and normalized to those of K562 WO cells.

The normalized retention ratio was defined:

$$R_{norm} = \frac{R_{obs} \text{ for F}n}{R_{obs} \text{ for K562 WO}} \quad (2)$$

Fig. 6 shows changes in R_{norm}. After 4 h incubation, R_{norm} values only reflected the respective position of each fraction in the elution profile from which they were collected (Fig. 3B). For example, F1 which expressed the lowest apoptotic ratio (Fig. 5B) showed the lowest R_{norm}. Nevertheless, interesting results only appeared after 24 and 48 h subculture. R_{norm} in F3 tended to decrease (Fig. 6) which it could be associated to apoptotic ratio decrease in this fraction (Fig. 5B). In contrast, as for apoptotic ratio, R_{norm} tended to increase for F1 and F2. F2 tends to a similar level as that observed in F3. The normalized retention ratio could be compared to the results obtained for apoptosis quantification by ELISA or flow cytometry, leading to similar kinetic profiles (Figs. 4, 5B and 6), with the difference that SdFFF takes into account the elution of either the residual nonapoptotic and the whole apoptotic populations (early and late stages). Then, as previously described for differentiation [57], SdFFF was also able to monitor apoptosis kinetics in different subpopulations (Fig. 6).

The apoptotic ratio showed that 12 h incubation with 40 μM diosgenin was enough to induce apoptosis in K562 cells. While one subpopulation quickly underwent apoptosis (cells eluted in F3) and was responsible for apoptosis until 4 h subculture, others were concerned by a later process leading to major apoptosis after 48 h subculture (in particular cells eluted in F2). These different subpopulation kinetics could explain the particular apoptotic profile in K562 cells.

3.4. Cell proliferation and p-ERK expression

The difference between cells eluted in F3 and those eluted in F1 or in F2 could possibly be explained by differences in cell proliferation stages.

BrdU is a DNA base analogue in which the methyl group of thymidine is replaced with a bromide. BrdU is incorporated into DNA during replication. Consequently, its cell labeling is proportional to DNA synthesis during the S phase, and then is proportional to proliferation. Thus, BrdU incorporation assay determine if F1 or F2 cells must proliferate before undergoes apoptosis. Many studies demonstrated that ERK activation (phosphorylation of ERK, p-ERK) is necessary in proliferation and apoptosis inhibition [56]. Surprisingly previous results have shown ERK activation after 12 h incubation with 40 μM diosgenin [54].

Fig. 7A shows representative cytograms of BrDU incorporation by the different subpopulations. Nearly 75% of control K562 cells incorporated BrDU (Fig. 7B). K562 WO cells exhibited a decreased percentage of $BrDU^+$ cells (Fig. 7B) confirming that 40 μM diosgenin inhibited the proliferation of K562 cells [54]. Nevertheless, our results showed p-ERK expression in a large portion of K562 WO cells (≈70%) in comparison to control cells (≈90%) (Fig. 8B). As shown in Fig. 8, and according to previously published results [54], the maintenance of a high level of ERK expression in the treated population was also associated with an increase in labeling intensity (Fig. 8A).

Again, SdFFF analysis (Figs. 7B and 8B) enabled us to detect differences between fractions, and results could be compared to apoptosis kinetics (Figs. 4 and 5B). F1 and F2, involved in later apoptosis processes, had proliferation and p-ERK expression levels similar to WO and TP population. In contrast, F3, which underwent significant and early apoptosis, had the lowest proliferation rate and p-ERK expression levels.

Thus, subpopulation separation could explain the apparently contradictory observation between apoptosis induction and ERK activation showed in the whole population (K562 WO).

4. Conclusion

In this study, we propose a new SdFFF cell separation application in the field of cancer research concerning the study of apoptosis kinetics after exposure to an inducing agent. The association of washout, early SdFFF separation and analytical cell characterization methods such as ELISA assay (late phase of apoptosis), flow cytometry (early phases of apoptosis) and cell proliferation (BrdU incorporation, p-ERK expression), was necessary to achieve this goal. Results confirmed the effective separation of different apoptotic stages in the same population. Each subpopulation could then be used as a model to study the specific mechanisms and kinetics of diosgenin induced apoptosis in K562 cells. While cells eluted in F3 quickly (4 h) underwent to an important apoptosis correlated with low cell proliferation (low BrdU incorporation and p-ERK expression), cells eluted in the first part of fractogram, in particular in F2, seemed to proliferate (high BrdU incorporation and p-ERK expression) before undergoing late and strong apoptosis after 48 h subculture. From a biological point of view, these results explain previous observations of apoptosis occurring in two phases (after 24 and 48 h incubation), and associated with a surprising p-ERK expression level in the treated population. This result also suggests different sensitivities to apoptosis induction. We might hypothesize that before entering apoptosis, cells eluted in F1 and F2 seemed to need to finish the cell cycle in which they progressed (p-ERK expression) and pass through mitosis, allowing BrdU incorporation. In this case, did the apoptosis kinetics depend on the cell cycle position when diosgenin exposure started? A complete study establishing the relationship between cell cycle position and apoptosis kinetics lies beyond the aim of this work, nevertheless, SdFFF cell separation would be very useful to explore this phenomena in future studies.

Finally, this study also showed, after calibration of elution profiles according to apoptotic ratios, that SdFFF elution could be used not only to monitor induction but also the kinetics of a biological event in a cell population.

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References

[1] J.C. Giddings, Sep. Sci. 1 (1966) 123.
[2] J.C. Giddings, Science 260 (1993) 1456.
[3] J.C. Giddings, M.E. in, K. Schimpf, J.C. Caldwell, Giddings (Eds.), Field-Flow Fractionation Handbook, John Wiley & Sons, Inc., New York, 2000, p. 3.
[4] S. Battu, J. Cook-Moreau, P.J.P. Cardot, J. Liq. Chromatogr. Relat. Technol. 25 (2002) 2193.
[5] T. Chianea, N.E. Assidjo, P.J.P. Cardot, Talanta 51 (2000) 835.
[6] K.D. Caldwell, T.T. Nguyen, T.M. Murray, M.N. Myers, J.C. Giddings, Sep. Sci. Technol. 14 (1979) 935.
[7] K.D. Caldwell, Z.Q. Cheng, P. Hradecky, J.C. Giddings, Cell Biophys. 6 (1984) 233.
[8] K.D. Caldwell, in: M.E. Schimpf, K.D. Caldwell, J.C. Giddings (Eds.), Field-flow Fractionation Handbook, John Wiley & Sons, Inc., New York, 2000, p. 79.
[9] M. Martin, P.S. Williams, in: F. Dondi, G. Guiochon (Eds.), Theoretical Advancement in Chromatography and Related Separation Techniques, Kluwer, Dordrecht, 1992, p. 513.
[10] J. Plockova, F. Matulik, J. Chmelik, J. Chromatogr. A. 955 (2002) 95.
[11] M.R. Schure, K.D. Caldwell, J.C. Giddings, Anal. Chem. 58 (1986) 1509.
[12] X. Tong, K.D. Caldwell, J. Chromatogr. B. 674 (1995) 39.
[13] P.S. Williams, S. Lee, J.C. Giddings, Chem. Eng. Commun. 130 (1994) 143.
[14] J. Chmelik, J. Chromatogr. A. 845 (1999) 285.
[15] C. Corbière, S. Battu, B. Liagre, P.J.P. Cardot, J.L. Beneytout, J. Chromatogr. B. 808 (2004) 255.
[16] L. Guglielmi, S. Battu, M. Le Bert, J.L. Faucher, P.J.P. Cardot, Y. Denizot, Anal. Chem. 76 (2004) 1580.
[17] C. Lautrette, P.J.P. Cardot, C. Vermot-Desroche, J. Wijdenes, M.O. Jauberteau, S. Battu, J. Chromatogr. B. 791 (2003) 149.
[18] D.Y. Leger, B. Liagre, P.J.P. Cardot, J.L. Beneytout, S. Battu, Anal. Biochem. 335 (2004) 267.
[19] P. Reschiglian, A. Zattoni, B. Roda, E. Michelini, A. Roda, Trends Biotechnol. 23 (2005) 475.
[20] S. Li, J. Nickels, A.F. Palmer, Biomaterials 26 (2005) 3759.
[21] M. Andersson, K. Fromell, E. Gullberg, P. Artursson, K.D. Caldwell, Anal. Chem. 77 (2005) 5488.
[22] C.I.J. Andersson, C. Arfvidsson, P.T. Kallio, K.-G. Wahlund, L. Buelow, Biotechnol. Lett. 25 (2003) 1499.
[23] C. Arfvidsson, G. Wahlund Karl, Anal. Biochem. 313 (2003) 76.
[24] Z. Chen, A. Chauhan, J. Colloid Interface Sci. 285 (2005) 834.
[25] S. Battu, W. Elyaman, J. Hugon, P.J.P. Cardot, Biochim. Biophys. Acta 1528 (2001) 89.
[26] L. Farmakis, A. Koliadima, Biotechnol. Prog. ACS ASAP (2005).
[27] M.T. Garcia, R. Sanz, M.T. Galceran, L. Puignou, Biotechnol. Prog. 22 (2006) 847.
[28] P. Gascoyne, J. Satayavivad, M. Ruchirawat, Acta Trop. 89 (2004) 357.
[29] Y. Huang, J. Yang, X.B. Wang, F.F. Becker, P.R. Gascoyne, J. Hematho. Stem Cell 8 (1999) 481.
[30] D. Kang, M.H. Moon, Anal. Chem. 77 (2005) 4207.
[31] H. Lee, S.K.R. Williams, K.L. Wahl, N.B. Valentine, Anal. Chem. 75 (2003) 2746.
[32] J. Luo, M. Leeman, A. Ballagi, A. Elfwing, Z. Su, J.-C. Janson, K.-G. Wahlund, J. Chromatogr. A. 1120 (2006) 158.
[33] P. Reschiglian, A. Zattoni, B. Roda, L. Cinque, D. Melucci, B.R. Min, M.H. Moon, J. Chromatogr. A. 985 (2003) 519.
[34] A. Roda, D. Parisi, M. Guardigli, A. Zattoni, P. Reschiglian, Anal. Chem. 78 (2006) 1085.
[35] R. Sanz, P. Cardot, S. Battu, M.T. Galceran, Anal. Chem. 74 (2002) 4496.
[36] G. Yohannes, M. Sneck, S.J.O. Varjo, M. Jussila, S.K. Wiedmer, P.T. Kovanen, K. Oeoerni, M.-L. Riekkola, Anal. Biochem. 354 (2006) 255.
[37] J. Yang, Y. Huang, X.-B. Wang, F.F. Becker, P.R.C. Gascoyne, Anal. Chem. 71 (1999) 911.
[38] X.B. Wang, J. Yang, Y. Huang, J. Vykoukal, F.F. Becker, P.R. Gascoyne, Anal. Chem. 72 (2000) 832.
[39] D.Y. Leger, S. Battu, B. Liagre, J.L. Beneytout, P.J.P. Cardot, Anal. Biochem. 355 (2006) 19.
[40] G. Bégaud-Grimaud, S. Battu, B. Liagre, D.Y. Léger, J.L. Beneytout, P.J.P. Cardot, J. Chromatogr. A. 1128 (2006) 194.
[41] G. Bégaud-Grimaud, S. Battu, P. Lazcoz, J.S. Castresana, M.O. Jauberteau, P.J.P. Cardot, Int. J. Oncol. 31 (2007) 883.
[42] D.R. Green, J.C. Reed, Science 281 (1998) 1309.
[43] D.R. Green, G.I. Evan, Cancer Cell. 1 (2002) 19.
[44] K. Higdon, A. Scott, M. Tucci, H. Benghuzzi, A. Tsao, A. Puckett, Z. Cason, J. Hughes, Biomed. Sci. Instrum. 37 (2001) 281.
[45] J. Turchan, C.B. Pocernich, C. Gairola, A. Chauhan, G. Schifitto, D.A. Butterfield, S. Buch, O. Narayan, A. Sinai, J. Geiger, J.R. Berger, H. Elford, A. Nath, Neurology 60 (2003) 307.
[46] J. Raju, J.M. Patlolla, M.V. Swamy, C.V. Rao, Cancer Epidemiol. Biomarkers Prev. 13 (2004) 1392.
[47] J.L. Beneytout, C. Nappez, M.J. Leboutet, G. Malinvaud, Biochem. Biophys. Res. Commun. 207 (1995) 398.
[48] B. Liagre, P. Vergne-Salle, C. Corbiere, J.L. Charissoux, J.L. Beneytout, Arthritis Res. Ther. 6 (2004) 373.
[49] D.Y. Leger, B. Liagre, C. Corbiere, J. Cook-Moreau, J.-L. Beneytout, Int. J. Oncol. 25 (2004) 555.
[50] S. Moalic, B. Liagre, C. Corbiere, A. Bianchi, M. Dauca, K. Bordji, J.L. Beneytout, FEBS Lett. 506 (2001) 225.
[51] C. Corbière, B. Liagre, F. Terro, J.L. Beneytout, Cell Res. 14 (2004) 188.
[52] C. Corbiere, B. Liagre, A. Bianchi, K. Bordji, M. Dauca, P. Netter, J.L. Beneytout, Int. J. Oncol. 22 (2003) 899.
[53] J. Raju, R.P. Bird, Cancer Lett. 255 (2007) 194.
[54] B. Liagre, J. Bertrand, D.Y. Leger, J.L. Beneytout, Int. J. Mol. Med. 16 (2005) 1095.
[55] G.L. Johnson, R. Lapadat, Science 298 (2002) 1911.
[56] W. Woessmann, N.F. Mivechi, Exp Cell Res. 264 (2001) 193.
[57] D.Y. Leger, S. Battu, B. Liagre, P.J.P. Cardot, J.L. Beneytout, J. Chromatogr. A. 1157 (2007) 309.
[58] X. Morelon, S. Battu, C. Salesse, G. Begaud-Grimaud, D. Clédat, P.J.P. Cardot, J. Chromatogr. A. 1093 (2005) 147.
[59] I. Comte, S. Battu, M. Mathonnet, B. Bessette, F. Lalloue, P. Cardot, C. Ayer-Le Lievre, J. Chromatogr. B. 843 (2006) 175.
[60] P.J.P. Cardot, Y. Denizot, S. Battu, in: J. Cazes (Ed.), Encyclopedia of Chromatography, M. Dekker Inc., New York, 2005, p. 282.
[61] P.J.P. Cardot, T. Chianea, S. Battu, in: J. Cazes (Ed.), Encyclopedia of Chromatography, M. Dekker Inc., New York, 2001, p. 742.
[62] N.E. Assidjo, T. Chianea, I. Clarot, M.F. Dreyfuss, P.J.P. Cardot, J. Chromatogr. Sci. 37 (1999) 229.
[63] J.C. Giddings, X. Chen, K.G. Wahlund, M.N. Myers, Anal. Chem. 59 (1987) 1957.
[64] T. Koch, J.C. Giddings, Anal. Chem. 58 (1986) 994.