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Increased cyclooxygenase-2 and thromboxane synthase expression is implicated in diosgenin-induced megakaryocytic differentiation in human erythroleukemia cells

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ABSTRACT

Differentiation induction as a therapeutic strategy has, so far, the greatest impact in hematopoietic malignancies, most notably leukemia. Diosgenin is a very interesting natural product because, depending on the specific dose used, its biological effect is very different in HEL (human erythroleukemia) cells. For example, at 10 μM, diosgenin induced megakaryocytic differentiation, in contrast to 40 μM diosgenin, which induced apoptosis in HEL cells previously demonstrated using sedimentation field-flow fractionation (SdFFF). The goal of this work focused on the correlation between cyclooxygenase-2 (COX-2) and thromboxane synthase (TxS) and megakaryocytic differentiation induced by diosgenin in HEL cells. Furthermore, the technique of SdFFF, having been validated in our models, was used in this new study as an analytical tool that provided us with more or less enriched differentiated cell fractions that could then be used for further analyses of enzyme protein expression and activity for the first time. In our study, we showed the implication of COX-2 and TxS in diosgenin-induced megakaryocytic differentiation in HEL cells. Furthermore, we showed that the analytical technique of SdFFF may be used as a tool to confirm our results as a function of the degree of cell differentiation.

Current therapies used to treat cancer are highly toxic and often nonspecific. A potentially less toxic approach involves the application of agents that can induce cancer cell differentiation, referred to as differentiation therapy [1]. This approach is based on the assumption that cancer cells exhibit aberrant patterns of differentiation and that appropriate treatment can induce tumor cell reprogramming, resulting in a loss of proliferative capacity and induction of terminal differentiation [1]. Differentiation induction as a therapeutic strategy has, so far, the greatest impact in hematopoietic malignancies, most notably leukemia [2]. Differentiating agents (chemicals or natural compounds) generally activate or reactivate hematopoietic signaling pathways and transcription factors that allow leukemic cells to differentiate [3].

The HEL (human erythroleukemia)[1] cell line was established from the peripheral blood of a patient with erythroleukemia. HEL cells constitutively express an erythroid phenotype characterized by glycophorin A (GpA) expression but also express antigens of other lineages [4,5]. Megakaryocytic differentiation from diploid progenitors (2 N cells) is characterized by progressive polyploidization (e.g., acquisition of DNA content greater than 2 N) [6] and acquisition of megakaryocytic markers such as the GpIIb–IIIa (CD41-CD61) integrin and the GpIb-V–IX complex [7]. Normal mature megakaryocytes are hyperploid, and their DNA contents can range widely from 4 N to 128 N with an average DNA ploidy of approximately 16 N [6].

Diosgenin [(25*R*)-5-spirosten-3β-ol] is a steroidal saponin that can be found in several plant species, particularly in fenugreek seeds (*Trigonella foenum graecum*) and wild yam roots (*Dioscorea villosa*). Diosgenin has recently been shown to exert antiproliferative and proapoptotic actions on rheumatoid arthritis synoviocytes [8] or on cancer cells in vitro [9–12] and in vivo [13]. Previously,

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[1] *Abbreviations used:* HEL, human erythroleukemia; GpA, glycophorin A; Diosgenin, (25*R*)-5-spirosten-3β-ol; SdFFF, sedimentation field-flow fractionation; NF-κB, nuclear factor-kappa B; MAPK, mitogen-activated protein kinase; COX-2, cyclooxygenase-2; PGE_2, prostaglandin E_2; TxS, thromboxane synthase; Hepes, *N*-(2-hydroxy-ethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; PVDF, polyvinylidene fluoride; mPGES, microsomal PGE_2 synthase; EIA, enzyme immunoassay; PBS, phosphate-buffered saline; PI, propidium iodide; FACS, fluorescence-activated cell sorter; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; SD, standard deviation; ANOVA, analysis of variance; TP, total peak; AML, acute myelogenous leukaemia; TPO, thrombopoietin; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; PKC, protein kinase C; PMA, phorbol myristate acetate.

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Beneytout and coworkers [14] demonstrated that diosgenin induced megakaryocytic differentiation of HEL cells with increased cell size, nuclear complexity, and GpIb expression. Recently, especially by using sedimentation field-flow fractionation (SdFFF) cell sorting, we demonstrated that diosgenin-differentiated cells showed nuclear polyploidization and increased expression of platelet marker CD41 associated with a decrease in GpA [15,16]. Furthermore, we showed that nuclear factor–kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs), especially ERK, were implicated in this mechanism [17].

Diosgenin is a very interesting natural product because, depending on the specific dose used, its biological effect is very different in HEL cells. For example, at 10 μM, diosgenin induced megakaryocytic differentiation, in contrast to 40 μM diosgenin, which induced apoptosis in HEL cells [18]. This was demonstrated using SdFFF, which allowed us to validate the use of this technique so as to rapidly detect biological processes.

We previously demonstrated that 40 μM diosgenin induced apoptosis, in HEL cells, with increased cyclooxygenase-2 (COX-2) expression and prostaglandin E_2 (PGE_2) production [11]. However, this arachidonic acid metabolism pathway was not studied in differentiating conditions (10 μM diosgenin). The goal of the current work focused on the correlation between COX-2 and thromboxane synthase (TxS) and megakaryocytic differentiation induced by diosgenin in HEL cells. Furthermore, with the technique of SdFFF having been validated in our models [15,16,18], this method was used in the current study as an analytical tool that provided us with more or less enriched differentiated cell fractions that could then be used for further analyses of enzyme protein expression and activity for the first time.

Here we demonstrate for the first time that 10 μM diosgenin (differentiating conditions) increased COX-2 and TxS expression and activities in HEL cells. This observation was confirmed using SdFFF in enriched populations of differentiated cells versus undifferentiated cells. Furthermore, the involvement of COX-2 and TxS in diosgenin-induced differentiation in HEL cells was analyzed not only using specific inhibitors of each metabolic pathway but also using cotreatments with PGE_2 or TxA_2 agonists and diosgenin.

Materials and methods

Cell line, cell culture, treatment, and light microscopy

The HEL cell line was kindly provided by J. P. Cartron (INSERM U76, Paris, France). Cells were seeded at 10^5 cells/ml in 75 cm^2 tissue culture flasks, grown in RPMI 1640 medium (Gibco BRL, Cergy-Pontoise, France) supplemented with 10% fetal calf serum (Gibco BRL), 1% sodium pyruvate, 1% Hepes [*N*-(2-hydroxy-ethyl)piperazine-*N*′-2-ethanesulfonic acid], 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL). Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were allowed to grow for 24 h in culture medium prior to exposure or not to 10 μM diosgenin (Sigma–Aldrich, Saint Quentin Fallavier, France). A stock solution of 10^{-2} M diosgenin was prepared in ethanol and diluted in culture medium to give the appropriate final concentration. The same amount of vehicle (= 0.1% ethanol) was added to control cells. Cell viability was determined by the trypan blue dye exclusion method. For light microscopy, after treatment, cultured cells were examined under phase contrast microscopy (400×) and pictures were taken with an image acquisition system (Nikon, Champigny sur Marne, France).

When pharmacological inhibitors of COX-2 (NS-398) or TxS (BM 567) (Cayman Chemical, SpiBio, Massy, France) were used, cells were pretreated with 5 μM NS-398 or 10 μM BM 567 24 h before adding 10 μM diosgenin for 96 h. For U0126 (ERK inhibitor) treatment (Calbiochem, La Jolla, CA, USA), cells were pretreated with 20 μM U0126 for 3 h before adding 10 μM diosgenin for 96 h.

To analyze the effects of agonists of PGE_2 (sulprostone) or TxA_2 (U46619) and the effects of exogenous PGE_2 and TxB_2 (Cayman Chemical), cells were cotreated simultaneously with 10 μM diosgenin and 100 nM sulprostone, 1 μM U46619, 100 nM PGE_2, or 1 μM TxB_2 for 96 h.

Protein expression analysis after treatments

HEL cells were washed and lysed in RIPA lysis buffer (50 mM Hepes [pH 7.5], 150 mM NaCl, 1% deoxycholate, 1% NP-40, 0.1% sodium dodecyl sulfate [SDS], and 20 μg/ml aprotinin) containing protease inhibitors (Complete Mini, Roche Diagnostics, Meylan, France). Western blot was performed as described previously [9]. Briefly, proteins (20–60 μg) were separated by electrophoresis on SDS–polyacrylamide gels, transferred to polyvinylidene fluoride (PVDF) membranes (Amersham Pharmacia Biotech, Saclay, France), and probed with respective antibodies against COX-1, microsomal PGE_2 synthase (mPGES), TxS (Cayman Chemical), and COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; TEBU, Le Perray en Yvelines, France). After incubation with secondary antibodies (Dako, Trappes, France), blots were developed using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech) and immediately exposed to X-ray film. Membranes were then reblotted with monoclonal anti-β-actin antibody (Sigma–Aldrich). Western blots were analyzed by densitometry (Kodak 1D3.5 scientific imaging system), and protein expression was normalized to β-actin.

Assay of prostanoid production

HEL cells were pretreated or not by NS-398 (COX-2 inhibitor) or BM 567 (TxS inhibitor) before the addition of diosgenin. PGE_2 and TxB_2 concentrations in the medium were measured by an enzyme immunoassay (EIA) kit according to the manufacturer's instructions (Cayman Chemical) and were normalized with respect to the number of viable cells present in the particular culture at the time of sampling or with respect to the amount of protein extracted to each culture condition.

SdFFF, cell elution, and subculture conditions

SdFFF separation devices used in this study were derived from those described and schematized previously [19,20]. The apparatus was composed of two 938 × 40 × 2-mm polystyrene plates separated by a Mylar spacer in which the channel was carved. Channel dimensions were 818 × 12 × 0.175 mm with two 50 mm V-shaped ends. The measured total void volume (channel volume + connecting tubing + injection and detection device) was 1790 ± 2 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 an M1111 (100 mV input) acquisition device (Keithley, Metrabyte, Tauton, MA, USA) operated at 2 Hz connected to a Macintosh computer. An 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, where 1 g = 980 cm/s^2, and were calculated as described previously [21].