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A PLANT STEROID, DIOSGENIN, A NEW MEGAKARYOCYTIC DIFFERENTIATION INDUCER OF HEL CELLS

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SUMMARY. We investigated the effect of plant steroids 5α-spirosten-3β-ol (diosgenin), 5α-spirostan-3β-ol (tigogenin) and 5α-spirostan-3β-ol-12-one (hecogenin) on the human erythroleukemia cell line (HEL TIB 180) and found that diosgenin addition to HEL cell cultures induces morphological and biochemical changes characteristic for megakaryocyte cells. Diosgenin-treated cells exhibit, at the ultrastructural level, increases in size in cytoplasmic and nuclear complexity. At the biochemical level, we demonstrated that diosgenin-treated HEL cells increased glycoprotein Ib (GpIb) expression as previously described in the megakaryocytic differentiation of HEL cells induced by nanomolar dose phorbol myristate acetate treatment. © 1995 Academic Press, Inc.

HEL cells are a triphenotypic cell line constitutively expressing an erythroid phenotype but also expressing antigens of the other lineages (1-3). These cells increase their erythroid phenotype after stimulation with agents such as δ-aminolevulinic acid (1). Similarly, an increase in macrophage phenotype occurs after micromolar dose phorbol myristate acetate (PMA) treatment (2). Inducible changes in megakaryocyte phenotype have also been reported after nanomolar dose PMA (3), dimethylsulfoxide (4), retinoic acid (5), or 1α,25-dihydroxy vitamin D3 (5) treatments. We hypothesized that HEL cells would be an excellent model to test for new differentiating agents. The present report describes megakaryocytic differentiation of HEL cells induced by the plant steroid (5α-spirosten-3β-ol : diosgenin) in comparison with nanomolar dose PMA megakaryocytic induced differentiation (3), assessed by cellular morphology, endomitotic process and glycoprotein Ib (GpIb) expression.

MATERIALS AND METHODS

Materials. PMA, plant steroïds and colchicine were obtained from Sigma Chemical Co, St Louis, Mo. HEL cells (TIB 180) were kindly provided by P[r] Cartron (INSERM U76).

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Cell culture and treatment. HEL cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) heat inactivated (56°C, 30 min) fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere containing 5% (v/v) CO_2. Cells were seeded at 1.5×10^5 cells/ml and grown in the presence or absence of diosgenin (10 nM to 16 µM) or PMA (1.6 nM). These compounds were dissolved in ethanol. The final concentration of solvent was below 0.1 % (v/v) which did not cause morphological changes (data not shown). Every days, for 5 days, cell viability and cellular morphology were examined by 1 % trypan blue exclusion using an hemocytometer. Cells with changed size were numerated.

Cellular morphology. Morphological characterization was performed after 48 and 72 hours of cultivation, in presence or absence of diosgenin (8 µM) and in presence or absence of PMA (1.6 nM), using light-microscopy on May-Grunwald-Giemsa stained preparations. Electron-microscopy was performed on ultrathin uranyl acetate and lead citrate coloured HEL sections. For electron microscopy, cells were fixed in 0.5 % 0.1 M phosphate buffered glutaraldehyde for 10 min and washed three times with 0.1 M phosphate buffer before fixation in 1 % 0.1 M phosphate buffered osmium tetroxide for 30 min at room temperature. Cells were dehydrated in graded alcohol to propylene oxide and infiltrated in graded concentrations of epon/propylene oxide. Cells were pelleted in microfuge tubes and embedded in pure epon (60°C, 48 hours). Areas of interest were identified on 1 µm sections after toluidine blue staining. Ultrathin sections (60 nm) were stained with uranyl acetate and lead citrate and examined by an electron microscope (CM 10 Philips).

Endomitotic process. Cells were seeded at 1.5×10^5 cells/ml in the presence or absence of diosgenin (8 µM) and in the presence or absence of PMA (1.6 nM). After 48 and 72 hours, an aliquot of each culture was treated by colchicine (2.5 mg/ml) for 3 hours. Mitotic index (percentage of cells blocked at metaphase stage) and the percentage of cells containing one or two or four and more nuclei was determined in each culture by light microscopic evaluation of May-Grunwald-Giemsa stained preparations.

Glycoprotein Ib expression. Antibodies (Ig G anti-human GpIb and FITC conjugated anti-IgG) were obtained from Dako (Denmark). Each antibody was used at a dilution of 1:50. After 72 hours incubation, diosgenin (8 µM) or PMA (1.6 nM) untreated or treated cells were suspended at 10^6 cells/ml in phosphate buffered saline (PBS). 10 µl of each suspension was deposed on a slide, dried at 37°C for 1 hour and fixed by 4% paraformaldehyde in PBS for 30 min. After blocking Fcγ receptors with 4% bovin serum albumin (30 min), fixed cells were submitted to indirect immunofluorescence reaction and slides were mounted in a Vectashield mounting medium (Vector laboratories) to reduce photobleaching. Cells were observed with a confocal laser Nikon microscope (Compu Add 333, Tracor Noran Instruments) equipped with a krypton-argon mixed gas laser. To quantify fluorescence intensities, FITC-labeled cells were excited by a 488 nm wavelength light beam using the Odissey system and the levels of immunoreactivity were scanned by the Image 1 analyser system. Values were expressed in arbitrary units. Laser confocal views were obtained by averaging 256 live images (Z-series with 1 µm series). Microphotographs were taken from the monitor with a Polaroid Quickprint video printer using 100 Asa film.

RESULTS

Cell culture and treatment. Cells were treated by diosgenin at a range of concentrations (10 nM to 10 µM) as described in Materials and Methods. After different times of culture, cell viability and cellular morphology were examined. No difference was observed with control cells excepted at a concentration of 10 µM where proliferation ceased and a large number of cells with increased size was observed (data not shown). After these results, a new range of concentrations (2 µM to 16 µM) was used (data not shown). At the concentration of 16 µM and after 72 hours of cultivation, cell proliferation was 10-fold reduced than at the concentration of 2 µM. Simultaneously, number of cells with large size

was 3-fold increased. A concentration of 8 μM was selected for further studies because this concentration resulted in a slight proliferation and size changes in a large number of cells. Slight proliferation and increased of cellular size are characteristic of a megakaryocytic differentiation. We studied morphological change using light-microscopy and electron-microscopy, endomitotic process and GpIb expression using confocal laser microscopy after diosgenin treatment (8 μM). PMA (1.6 nM) treatment was used as a control of megakaryocytic differentiation for each study (cell morphology, endomitotic process, GpIb expression).

Cell morphology. After 48 hours incubation, May-Grunwald-Giemsa stained preparations of untreated cells showed numerous cells with a high nucleus/cytoplasm ratio and a relatively simple nuclear structure. Some (less<2%) larger cells had a megakaryocytic-like aspect with a low nucleus/cytoplasm ratio and a multilobulated nucleus as previously described (3).

Diosgenin treated cultures (fig 1) exhibit a change in cellular size (cells with increased size are seen but normal cells are also present), a change in nuclear aspect (cells with increased size presented a multilobulated nucleus) and mitosis are less numerous than in control culture.

After 72 hr incubation of treated and untreated cells, the same characteristics were observed.

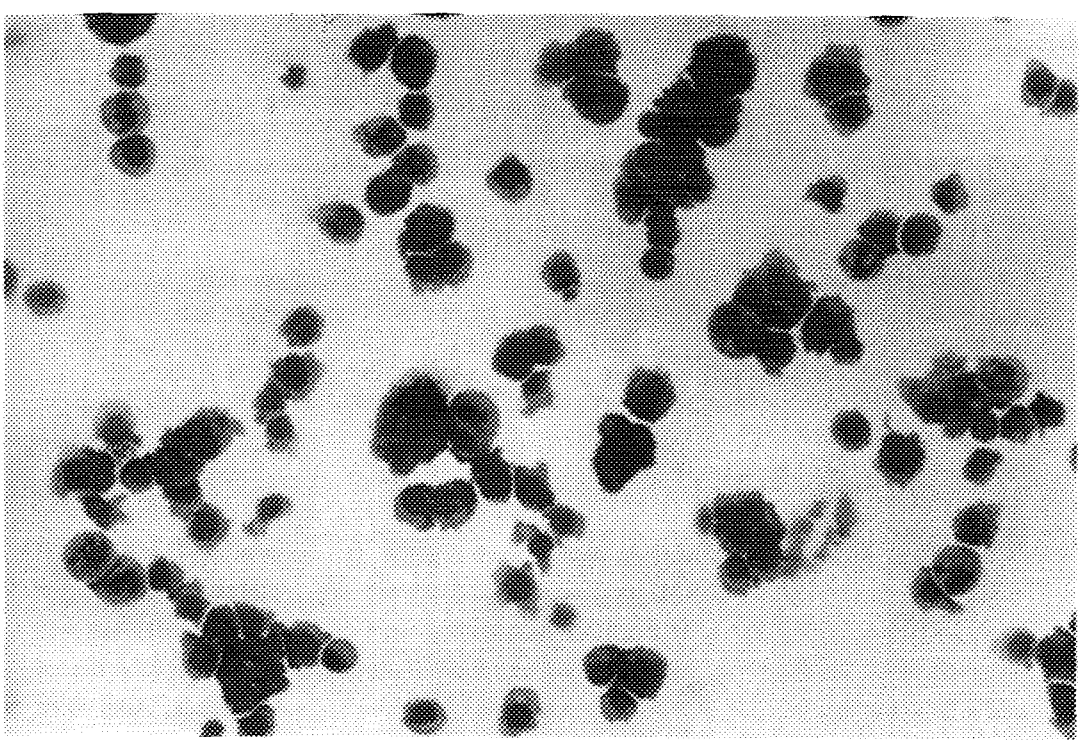

Fig 1. Morphology of 48-hour diosgenin-treated HEL cells. Light microscopy of May-Grunwald-Giemsa-stained diosgenin (8 μM)-treated HEL cells preparation (magnification × 380).

Ultrastructural studies were performed to examine cytoplasmic changes. After 48 hours, untreated culture exhibited mononucleated cells with high nucleus/cytoplasm ratio. In the cytoplasm, a slight mitochondrial number was observed.

Diosgenin or PMA treated cells showed the same variations in cellular size and nuclear multilobulation than in light microscopy study. Cytoplasmic content was changed : an apparent increase of mitochondrial number but also Golgi cisterns and α-granule-like structures was observed (fig 2). After 72 hours incubation, the same characteristics were observed in all treated cells (data not shown).

Endomitotic process. Megakaryocytic differentiation is characterized by cytoplasmic changes and by an increase in DNA content (6). Endomitotic process result in cells having 2,4,8 and more nuclei (7). Colchicine is an inhibitor of mitosis which blocks cells at the metaphase stage (8-10). Diosgenin, PMA treated and untreated HEL cell mitotic activity was verified by colchicine treatment as described in Materials and Methods. Results (tables 1 and 2) showed an equivalent mitotic index in all cultured cells. Simultaneously, the percentage of

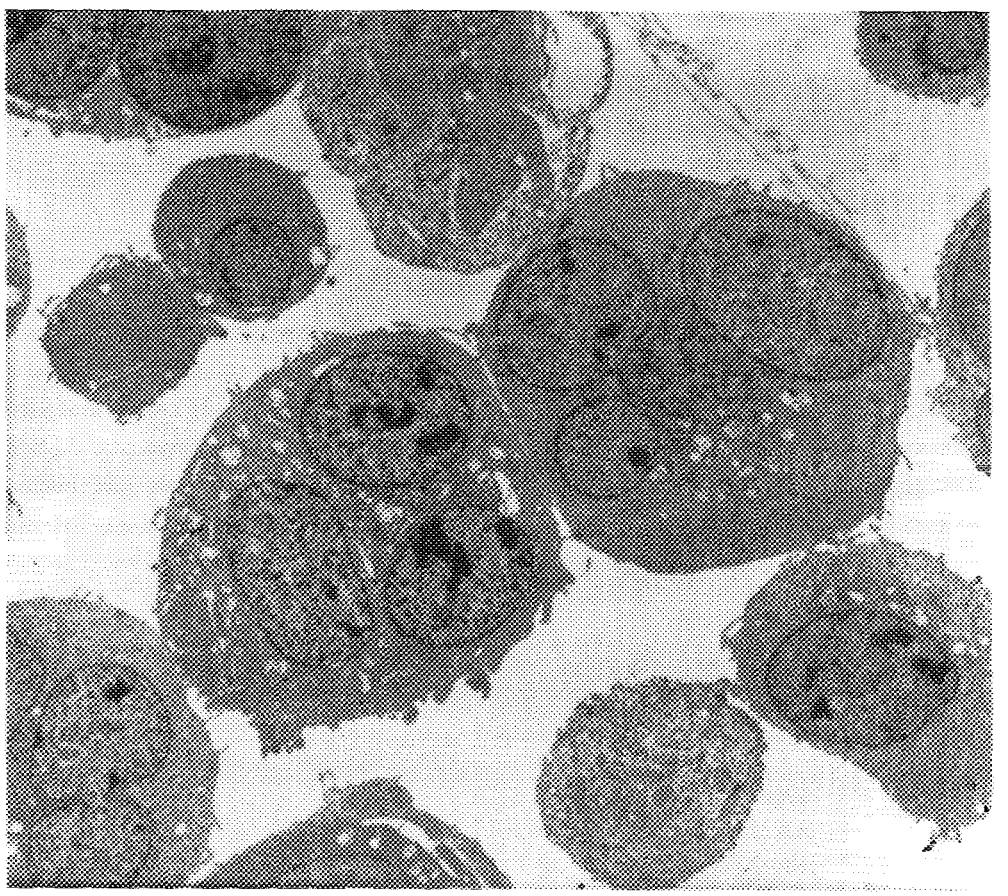

Fig 2. Ultrastructure of 48-hour diosgenin (8 µM)-treated HEL cells. (magnification × 1650). Note marked nuclear lobulation and increase in cytoplasmic content and cytoplasmic organelles.

Table 1

Mitotic index and percentage of cells with 1, 2, 4 and more nuclei after 48 hr cultivation of untreated (control) and diosgenin- or PMA-treated HEL cells

	control	HEL +Diosgenin	HEL+PMA
mitotic index	4	5	4
cells (1 nucleus)	99	76	69
cells (2 nuclei)	1	15	21
cells (4 and more nuclei)	0	9	10

The values shown represent the mean percentage of two experiments.

cells with four and more nuclei increased in all treated cells. The increase was more pronounced with PMA than with diosgenin treatment.

Gplb expression. Long and al.(3) described an increased expression of Gplb during HEL megakaryocytic PMA induced differentiation. They noted that Gplb expression and other megakaryocytic markers were increased after three days of culture. Confocal laser microscopy showed a very slight expression in control cells and a 3-fold increase in Gplb expression in diosgenin treated cells (fig 3). Gplb expression was increased 4-fold in PMA treated cells (data not shown) and these results were in agreement with those of Long and al.(3) which described a 6-fold increase in Gplb expression by HEL in the presence of PMA with a different technique (solid-phase radioimmunoassay).

DISCUSSION

HEL cells have a mixed hematopoietic cell phenotype. These cells expressed erythroid characteristics but also antigens of the other lineages. They can be differentiated in the megakaryocytic phenotype by nanomolar dose PMA treatment (3). After diosgenin treatment, we observed an increased degree of commitment to the megakaryocytic lineage; HEL cells showed a marked increase in cell size, cytoplasmic content and nuclear complexity. Endomitotic process was demonstrated by the increase in cell containing four and

Table 2

Mitotic index and percentage of cells with 1, 2, 4 and more nuclei after 72 hr cultivation of untreated (control) and diosgenin- or PMA-treated HEL cells

	control	HEL+Diosgenin	HEL+PMA
mitotic index	4	6	5
cells (1 nucleus)	99	79	68
cells (2 nuclei)	1	8	10
cells (4 and more nuclei)	0	13	22

The values shown represent the mean percentage of two experiments.

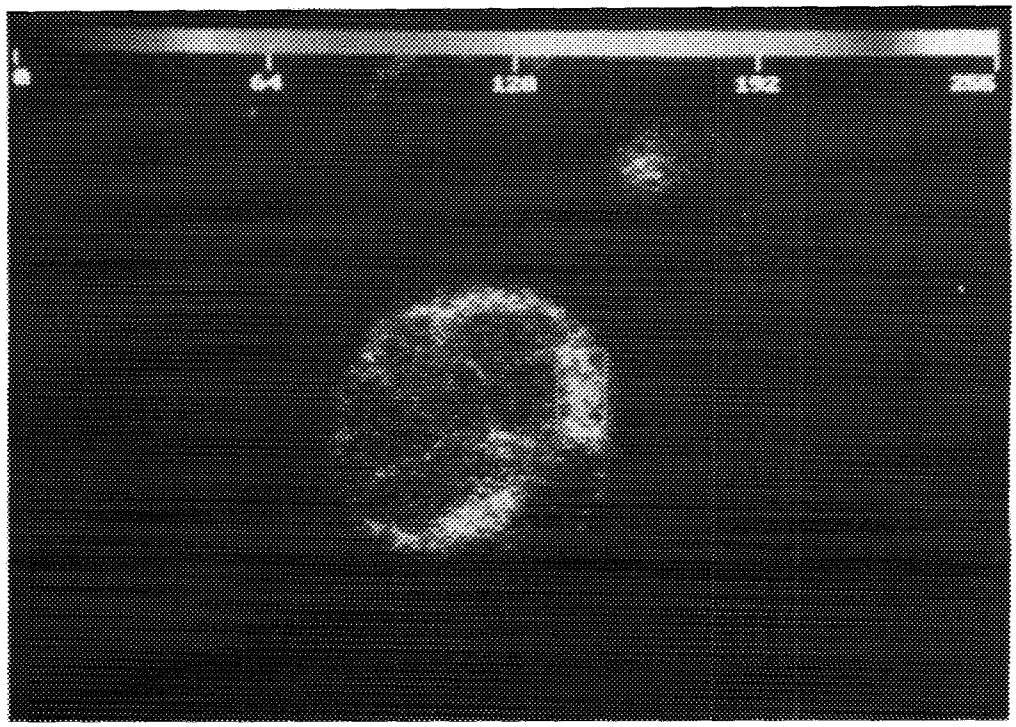

Fig 3. Gplb expression of 72-hour diosgenin-treated HEL cells. (magnification × 400).
Cytoplasmic fluorescence was 3-fold increased over that in control cell.

more nuclei. Gplb expression was significantly increased (3-fold) after diosgenin treatment. For all studies, nanomolar dose PMA megakaryocytic induced differentiation was used as a positive control. After these results, we tested two other plant steroids (5α-spirostan-3β-ol : tigogenin and 5α-spirostan 3β-ol-12-one : hecogenin). These compounds exhibited a very slight activity (data not shown) but studies were limited by difficulties to dissolve these compounds in ethanol. Recently, bile acids were shown to differentiate HL 60 cells (11). We are currently studying other same family compounds on HEL cells and on other cell lines.

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