Production of inducible nitric oxide is required for monocytic differentiation of U937 cells induced by vitamin E-succinate

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SUMMARY

Many putative differentiating agents arrest cell growth prior to progression of the cell through differentiation. Vitamin E-succinate is known to be a potent modulator of haematopoietic differentiation as well as an inhibitor of cell growth in vitro and in vivo. In this study, we examined whether vitamin E-succinate could modulate the monocytic differentiation of U937 human monoblasts. Treatment with vitamin E-succinate for 1-4 days inhibited the proliferation of U937 cells. Vitamin E-succinate also induced monocytic differentiation as indicated by the increase in nitro blue tetrazolium reduction activity, and the expression of monocyte specific cell surface antigen, CD11c, and integrins α5 and β1. The monocytic differentiation of U937 cells was also induced when the cells were cultured in fibronectin-coated wells. Monocytic differentiation was enhanced when the cells were treated with both vitamin E-succinate and fibronectin, suggesting that vitamin E-succinate and fibronectin synergistically act on monocytic differentiation of U937 cells. During monocytic differentiation of U937 cells induced by vitamin E-succinate and/or fibronectin, nitric oxide was detected in supernatants. The production of nitric oxide was not detected when monocytic differentiation of U937 cells was induced by phorbol-12 myristate 13-acetate, a well known inducer of macrophage-like cell differentiation. Vitamin E-succinate and/or fibronectin induced monocytic differentiation was blocked by the treatment of nitric oxide synthase inhibitor, N-G-monomethyl-L-arginine. In contrast, treatment of cells with sodium nitroprusside, a chemical nitric oxide donor, stimulated monocytic differentiation of U937 cells at an early time point. Taken together, these results suggest that nitric oxide is an important intermediator at an early stage of vitamin E-succinate and/or fibronectin-induced monocytic differentiation of U937 cells.

Key words: Vitamin E-succinate, Monocytic differentiation, Nitric oxide

INTRODUCTION

Vitamin E-succinate (VES) is a modulator of haematopoietic differentiation as well as a potent inhibitor of cell growth in vitro and in vivo (Turley et al., 1992; Charpentier et al., 1993; Moriguchi et al., 1993; Kelloff et al., 1994; Turley et al., 1997). The exact mechanism of VES action in growth inhibition and differentiation is unknown. Cell growth and differentiation are regulated and delicately balanced by the activities of growth stimulators and suppressors (Rosalès and Juliano, 1996). In many cases, differentiation of cells accompanies decrease or cessation in their active proliferation. Mononuclear phagocytes originate in the bone marrow where progenitor cells differentiate into monoblast, promonocyte, and monocyte stages. Human monoblastic leukemia U937 cells are capable of differentiation into monocytes and macrophages by various agents such as phorbol-12 myristate 13-acetate (PMA), 1,25-dihydroxyvitamin D3 (VD3), and retinoic acid (RA). Upon induction of terminal differentiation, U937 cells express functional differentiation markers, acquire a monocyte/macrophage-like cell morphology, and arrest cell growth in the G0/G1 phase of the cell cycle (Einat et al., 1985; Kim et al., 1991; Oeberg et al., 1993). Recently, it has been reported that certain cells have been shown to be a target and source of nitric oxide (NO), and that NO inhibits the growth and induces the differentiation of certain cell types including keratinocyte and leukemia cell lines (Magrinat et al., 1992; Shami et al., 1995; Arany et al., 1996). Human monocyte-like cell line U937, derived from a patient with generalized histiocytic lymphoma, can differentiate into mature macrophages following induction with PMA (Nilsson et al., 1980; Minta and Pambun, 1985; Pucillo et al., 1993). In HL60 cells it was found that VES induces monocytic differentiation through increasing the binding of cells to fibronectin (FN). It was suggested that FN might play an important role in negative regulation of the survival of monocytes through its interaction with integrin α5, which is selectively up-regulated during monocytic differentiation (Turley et al., 1992, 1995; Terui et al., 1996). Since it was thought that VES and NO have a very similar function in growth inhibition and differentiation of haematopoietic cells,
we examined whether VES and NO have any effect on monocytic differentiation of U937 promonocytes.

MATERIALS AND METHODS

Cell culture

U937 is a myelomonocytic cell line derived from a patient with histiocytic lymphoma and Raw 264.7 is an Abelson leukemia virus-transformed murine macrophage cell line. These cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 100 i.u./ml penicillin, and 50 μg/ml streptomycin (Gibco BRL, Gaithersburg, MD, USA) at 37°C in a humidified 5% CO₂ incubator. Cell viability was assessed by trypan blue exclusion staining.

Preparation of FN-coated wells

For the assay of cell adhesion to FN, FN-coated wells were prepared as described previously (Guan and Hynes, 1990). Briefly, flat-bottomed 96-well plates (Corning, New York, NY, USA) were coated with 1 μg/100 μl of FN (Promega, Madison, WI, USA) for 2 hours at 37°C. The plates were rinsed with PBS and incubated at 37°C with 100 μl of 1% BSA for 1-2 hours. The plates were rinsed again and 100 μl of 5×10⁵/ml cell suspension in culture medium was added to each coated well. After another 30 minutes incubation at 37°C, nonadherent cells were removed by gentle washing twice with HBSS/10 mM Hepes, and attached cells were used for experiments. As a negative control, a coated well with BSA only was used.

Proliferation assay

Cells were cultured at a density of 5×10⁵ cells/ml in 96-well flat-bottomed tissue culture plates (100 μl/well) in the absence or presence of VES (10 μg/ml), vehicle (0.1% ethanol and 5 μg/ml succinic acid) or PMA (32 nM, Sigma) for 1-4 days. The vehicle was used in every experimental protocol and never varied by >5% from the untreated control. Cells were also cultured on FN-coated wells or incubated with VES followed by culture on FN-coated wells. Cultured cells were pulsed with 1 μCi of [³²H]thymidine (84 Ci/mmol; Amersham, UK) during the last 8 hours of culture, harvested onto glass fiber filters, and radioactivity was measured using a liquid scintillation counter (TriCarb 1500, Packard, USA).

NBT reduction assay

NBT reduction assay was carried out as reported by Collins et al. (1980). Cells (1×10⁶) treated with VES and/or surface FN were pelleted, washed twice with RPMI-1640 medium, and resuspended in 2 ml of RPMI medium containing 0.1% NBT and 100 ng/ml PMA. This cell suspension was incubated for 20 minutes at 37°C and Wright-Giemza staining was then performed on cytopsin preparations of cell suspensions (Cytospin3, Shandon, UK). The percentage of cells containing intracellular blue-black formazan deposits was determined under light microscopy on a minimum of 200 cells for each experiment. In some experiment, cells were also treated with L-NMMA (2 mM) as a competitive NOS inhibitor or SNP (2 mM) as a chemical donor of NO in the same conditions as above.

Immunofluorescence and confocal microscopy

The expression and subcellular localization of cell surface antigens CD11c and CD14 were assessed using indirect immunofluorescence staining and laser scanning confocal microscopy. Cells were prepared with a Cytospin3 and dried for 10 minutes. The slides were fixed for 10 minutes in methanol:acetone (1:1) and blocked for 10 minutes in 5% normal goat serum in PBS. The cells were incubated with mouse anti-human CD11c (Leu M5) or CD14 (Leu M3) which were purchased from Becton-Dickinson (Mountain View, CA, USA) for 30 minutes at room temperature. The binding of primary antibody was detected by using a FITC-conjugated goat anti-mouse-IgG (Sigma). Nuclei were counterstained with propidium iodide (50 μg/ml). Immunofluorescence was analyzed by laser scanning confocal microscopy, using a Carl Zeiss 410 confocal microscope (Axiovert 100; Lens 3× 40/0.75; laser line=488, 543).

Western blot analysis

Cells were lysed in 10 mM Tris-HCl, pH 7.4, containing 1% sodium dodecyl sulfate (SDS), 10 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 10 μg/ml pepstatin A, and 2 mM phenanthroline. The lysate was sonicated 2 seconds to decrease viscosity. Proteins (40 μg) were separated by electrophoresis on a 7.5%-polyacrylamide gel containing 0.1% SDS. The resolved proteins were transferred onto a nitrocellulose membrane (Schleicher and Schuell, Keene, NH, Germany), and the membrane was blocked for 1 hour in Tris-buffered saline (TBST; 10 mM Tris, 0.1 M NaCl, and 0.1% Tween-20, pH 7.5) containing 5% non-fat dry milk (blocking buffer). The membrane sheets were incubated with rabbit anti-mouse iNOS (Santa Cruz, CA, USA), mouse anti-human integrin α5 (Endogen, Boston, MA, USA), or rabbit anti-human integrin β1 (Chemicon, Temecula, CA, USA) for 1 hour, washed three times with TBST, incubated with secondary antibodies conjugated with horseradish peroxidase (Sigma), and visualized using an enhanced chemiluminescence detection kit (Amersham).

Measurement of nitrite

NO synthesis in cell cultures was measured by a microplate assay as described by Green et al. (1982). Briefly, cells (5×10⁵/ml) were either untreated or treated with VES, LPS/IFN-γ (10 ng/ml, 5 unit/ml, respectively), or PMA, or cultured on FN-coated wells. In the case of a combination of VES with surface FN or LPS with IFN-γ, cells were preincubated on FN-coated well or LPS, for 6 hours, respectively. The treated cells were cultured for 48 hours. 100 μl of conditioned medium was collected and incubated with an equal volume of the Griess reagent (1% sulfanilamide/0.1% N-(1-naphthyl)-ethylendiamine dihydrochloride/2.5% H₃PO₄) at room temperature for 10 minutes. The absorbance at 540 nm was determined in a Titertek Multiskan (Flow Laboratories, North Ryde, Australia). NO₂- was determined using sodium nitrite as standard.

Electrophoretic mobility shift assay

The consensus oligonucleotide for probing of nuclear factor kappa B (NF-κB; Promega) is as follows: NF-κB 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ 3′-CTA ACT CCC CTG AAA GGG TCC G-5′. The probe was prepared by labeling with [³²P]ATP (3,000 Ci/mmol; Amersham) using a T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA) and used for an electrophoretic mobility shift assay. Nuclear extracts of cells were prepared according to the method of Schreiber et al. (1989) and 5 μg samples were incubated with a labeled oligonucleotide probe (2×10⁶ cpm) in a 15 μl of reaction mixture containing 20 mM Hepes, pH 7.9, 1 mM EDTA, 60 mM KCl, 12% glycerol, 1 mM dithiothreitol, and 4 μg poly(dI- dC) - poly(dI-dC) at 27°C for 30 minutes. The reaction products were analyzed by 5% TBE-acrylamide gel electrophoresis and autoradiography.

Statistics

Statistical analyses for data were done using Student’s t-test.

RESULTS

Inhibition of U937 cell growth

Because a loss of proliferation accompanies the differentiation process in many cell types, we investigated whether VES modulates proliferation of U937 cells, a human promonocyte...
cell line. VES significantly inhibited the growth of U937, in a dose-dependent manner (1-10 μg/ml, data not shown). Tritiated thymidine incorporation studies showed that VES (10 μg/ml) inhibited cell growth up to 86% and 95% in cells cultured for 3 and 4 days, respectively (Fig. 1A). The inhibition of cell growth was very similar to that induced by PMA which acts as an inducer of differentiation in promonocytes used as a positive control (Minta and Pambrun, 1985; Pucillo et al., 1993). The appropriate vehicle control (0.1% ethanol and 5 μg/ml succinic acid) did not affect U937 cell proliferation (data not shown). In addition to VES, interaction of U937 cells with coated FN also decreased thymidine incorporation by 47% and 89% at day 3 and 4 of the culture, respectively.

**Induction of monocytic differentiation**

We next examined whether the ability of growth inhibition by VES and/or FN is related to the monocytic differentiation of U937 cells. Functional differentiation of promonocytes into monocytes/macrophages was quantified by the ability of U937 cells. VES and/or FN is related to the monocytic differentiation of U937 cells. Induction of monocytic differentiation

**Fig. 1.** Proliferation and differentiation of U937 cells. (A) The effect of VES and/or FN on proliferation of U937 cells. Cells cultured for the indicated periods were pulsed with 1 μCi of [3H]thymidine during the last 8 hours of culture, and harvested. Radioactivity of the incorporated [3H]thymidine was measured by liquid scintillation counting. Data represent the mean of three independent experiments and the difference was significant at P<0.05 by Student's t-test. (B) Induction of differentiation by VES and/or FN. Treated cells were pelleted, washed, and incubated with 0.1% nitro blue tetrazolium and 100 ng/ml PMA in RPMI 1640 medium for 20 minutes at 37°C. Wright-Giemza stains were then performed on cytospin preparations of cell suspensions. The percentages of NBT-positive cells were determined as compared to untreated control cells. Points and bars are means ± s.d. for three cultures.

**NO and iNOS are induced by VES and/or FN in U937 cells**

NO inhibits proliferation of certain cell types including keratinocytes and leukemia cells (Magrinat et al., 1992; Brysk et al., 1995; Shami et al., 1995). Conversely, factors that stimulate cell proliferation inhibit NO production (Heck et al., 1992). We, therefore, examined whether differentiation of U937 cells by VES and/or FN could support NO production and expression of iNOS. When the cells were treated with VES and FN for 24-48 hours, NO was detected in the cell supernatants of U937 cells. Production of NO by VES and FN was much greater than that induced by well known NO inducers, e.g. LPS/IFN-γ (Butz et al., 1994; Adler et al., 1995). These inductions of NO were blocked by the treatment of L-NMMA, an inhibitor of NOS (Fig. 4A). The pattern of NO production was similar to that of iNOS expression which is determined by western blot analysis (Fig. 4B). The expression pattern of 135 kDa protein was also correlated to that of integrins α5 and β1 expression (Figs 3 and 4B). As shown in
Fig. 4C, activity of NF-κB analyzed by EMSA was significantly increased by the treatment of VES and/or FN in U937 cells. This further confirmed the NO induction by these treatments, because activation of NF-κB is crucial for the increase in iNOS gene expression.

**DISCUSSION**

In the current study, we examined the effect of VES and FN on the proliferation and differentiation of U937 cells. VES or FN inhibited cell growth up to 95% in cells cultured for 4 days.

**NO induces NBT reduction activity during monocytic differentiation**

To study the role of NO production by VES and/or FN in monocytic cell differentiation, the NBT reduction activity of cells was analyzed after treatment of NOS inhibitor (L-NMMA) or a chemical donor of NO (SNP), with VES and/or FN, or PMA. Treatment of L-NMMA almost completely inhibited the NBT reduction activity that is induced by VES and/or FN. However, no inhibition of NBT reduction activity was observed in cells treated with PMA (Fig. 5A). Treatment of cells with SNP remarkably generated NO (Fig. 5B) that is related to the increase in NBT reduction activity induced by VES and/or FN at day 1 of the culture. However, SNP treatment decreased PMA-induced NBT reduction activity and also decreased VES- and/or FN-induced NBT reduction activity after day 2 of culture (Fig. 5C). This is accompanied by an increase in apoptotic cell death (data not shown). This finding suggests that VES- and/or FN-induced NO plays an important role in monocytic cell differentiation in vitro but not for terminal differentiation of cells.

**Fig. 2.** Expression of cell surface antigen CD11c on human promonocytic cell line U937. Cells were untreated (A), treated with VES (B), cultured on FN-coated well (C) or treated with VES following culture on FN-coated well for 6 hours (D). Cells were analyzed by using indirect immunofluorescence staining to observe CD11c, and the level and subcellular localization of cell surface antigen, CD11c were assessed by laser scanning confocal microscopy as described in Materials and Methods. For counter staining, propidium iodide solution (50 μg/ml in PBS) was used.

**Fig. 3.** Expression of integrin α5 and β1 subunits in U937 and Raw 264.7 cells. Cells were untreated (CON), treated with VES, cultured in FN-coated wells (FN), or treated with VES following culture in FN-coated wells for 6 hours (FN/VES). Integrins α5 and β1 were analyzed by western blotting using specific antibodies.
As with other negative regulators of cell growth, inhibition of proliferation by VES or FN was accompanied by induction of the monocytic differentiation of U937 which was assessed by increased NBT reduction activity, and increased CD11c and integrin α5β1 expression. However, the kinetics of NBT reduction activity induced by VES or FN was quite different from that induced by PMA as a well known inducer of monocyte/macrophage differentiation. Although induction of differentiation by VES or FN was less effective than that induced by PMA, a combination of VES and FN remarkably increased functional differentiation of promonocytic leukemia cells.

Monocytic differentiation is associated with the expression of the monocyte/macrophage lineage-specific antigens CD11c and CD 14 (Dudley et al., 1989; Oeberg et al., 1993; Turley et al., 1996). VES and/or FN induced significant amounts of CD11c expression on the cell surface. Monocyte interaction with proteins of the extracellular matrix is regulated by expression of specific cell-surface receptors which is dependent on the differentiation state (Bevilacqua et al., 1981; Bianco, 1983). It has been suggested that integrin α5β1, a receptor for FN, plays an important role during monocyte/macrophage differentiation (Ferreira et al., 1991; Pucillo et al., 1993; Terui et al., 1996). We showed in this study that expression of integrins α5 and β1 was markedly increased by treatment of VES in U937 promonocye cells, while it was unchanged in the terminally differentiated Raw 264.7 mouse macrophage cells. Although the U937 cells cultured on FN-coated wells expressed less integrin α5 and β1 than did the VES-treated cells, a combination of VES and FN synergistically increased expression of α5 and β1. However, these treatments had little effect on the morphology of U937. Taken together the results indicate that VES is an inducer of monocytic differentiation through adhesion to FN.

Haematopoietic cell growth and differentiation depend on a complex interplay between progenitor cells and other cells present in the bone marrow microenvironment (e.g. endothelial cells, macrophages, fibroblasts and fat cells). Many soluble factors secreted by these cells play key roles in normal and malignant cell development. Of these factors, NO derived from several sources can modulate cellular differentiation and alter gene expression (Magrinat et al., 1992). The low level of NO production by human monocyte and human peritoneal macrophages has been generally found under numerous different culture conditions and after stimulation with numerous different agents (Denis, 1991, 1994; Weinberg et al., 1995). In this experiment, we observed that significant amounts of NO were released during the VES- and/or FN-induced monocytic differentiation of U937 but not during PMA-induced terminal differentiation. Moreover, VES- and/or FN-induced monocytic differentiation was reversed almost completely by a competitive NOS inhibitor, while differentiation was augmented by a chemical donor of NO at an early time point of treatment (day 1). However, the role of NO in terminal differentiation of U937 cells induced by PMA was controversial. This is due to the observation that a competitive NOS inhibitor had no effect on PMA-induced differentiation, whereas SNP significantly decreased PMA-induced NBT reduction activity and increased apoptotic cell death. These results suggest that VES induces production of NO which is required for an early stage of monocytic differentiation. However, in the later stage of differentiation by PMA, signalling events other than NO are required to obtain full differentiation. This idea was supported by the observation by others that the steady state levels of iNOS mRNA and NO generation in monocytes and macrophages activated by various agents depend on the stage of mononuclear phagocyte differentiation in monocytes and monocyte-derived macrophages (Shami et al., 1995; Jungi et al., 1996). NO modulates various functions of human mononuclear phagocytes, i.e. NO generation is required for the antitumoral effect of monocytes/macrophages. According to Dugas et al. (1996), VD3- and RA/VD3-treated U937 cells induces NO generation which leads to a decrease in the growth arrest and
subsequent differentiation of leukemic cells. Our present results also indicate that VES and/or FN induces iNOS protein expression as well as NO production. Induction of iNOS expression by these treatments is further supported by the increased NF-κB activity. VES thus appears to induce NO through the activation of NF-κB which results in an increase in iNOS expression, and stimulation of monocytic differentiation of U937 cells.

A number of lines of evidence have suggested that apoptosis is also a major physiological mechanism of terminal differentiation of haematopoietic cells such as monocytes and granulocytes (Mangan et al., 1991; Savill et al., 1989). NO induces apoptosis in thymocytes, peripheral T cells, myeloid cells and neurons (Sandau et al., 1997; Sciortari et al., 1997; Williams and Smith, 1993; Xie et al., 1997). Endogenously generated or exogenously supplied NO induces apoptotic cell death in terminally differentiated Raw 264.7 macrophages (Shimaoka et al., 1995; Messmer et al., 1996; von Knethen and Brune, 1997). The data obtained in this study support our conclusion that generation of NO is required for an early stage of monocytic differentiation induced by VES but not for terminal differentiation induced by PMA. The evidence suggests that a competitive NOS inhibitor had no effect on PMA-induced differentiation, whereas spontaneously released NO significantly decreased PMA-induced NBT reduction activity and also decreased VES- and/or FN-induced NBT reduction activity after day 2 of culture. With the appearance of apoptotic cell death, decreases in the NBT reduction activity of PMA-treated and VES- and/or FN-treated cells after day 2 of culture indicate that NO induces apoptosis as in terminally differentiated Raw 264.7 macrophage cells. In summary our study has shown that VES inhibits U937 cell growth and induces monocytic differentiation of U937 cells by increasing the expression of monocyte specific surface antigen, CD11c, and integrins α5 and β1. VES also induces NO, a potent antitumoral mediator, and iNOS expression during the induction of monocytic differentiation. The monocytic differentiation of U937 cells by VES is negatively or positively regulated by an NOS inhibitor or a chemical donor of NO, respectively. Furthermore, there is a functional synergy between VES and FN. Thus, we conclude that NO production is one of the signalling pathways required for VES- and/or FN-induced differentiation of U937 promonocytic leukemia cells.

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