Carnosic Acid and Promotion of Monocytic Differentiation of HL60-G Cells Initiated by Other Agents

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Background: Carnosic acid is a plant-derived polyphenol food preservative with chemoprotective effects against carcinogens when tested in animals. Recently, we showed that carnosic acid potentiates the effects of 1α,25-dihydroxyvitamin D₃ (1α,25(OH)₂D₃) and of all-trans-retinoic acid (ATRA) on differentiation of human leukemia cells. We now examine the mechanisms associated with carnosic acid-induced enhancement of cell differentiation in subline HL60-G initiated by 1α,25(OH)₂D₃, ATRA, or 12-O-tetradecanoylphorbol-13-acetate (TPA). Methods: We evaluated monocyte differentiation markers (CD11b, CD14, and monocytic serine esterase), cell cycle parameters, and cell proliferation rates after treatment of cells with different agents with or without carnosic acid. We also assessed the abundance of the vitamin D receptor (VDR), retinoid X receptor (RXR)-α, retinoic acid receptor (RAR)-α, and cell cycle-associated proteins by immunoblot analysis (p27, early growth response gene [EGR]-1, and p35Nck5a), the expression of corresponding genes by reverse transcription-polymerase chain reaction (RT-PCR), and the activity of VDR by electrophoretic mobility shift analysis. The two-sided nonparametric Kruskal–Wallis one-way analysis-of-variance test with Dunn’s adjustment was used for statistical analyses. Results: Monocytic differentiation induced by low (1 nM) concentrations of 1α,25(OH)₂D₃, ATRA, or TPA was enhanced by carnosic acid (10 μM), as shown by the increased expression of monocytic serine esterase (P<.001, P<.001, and P = .043, respectively) and of CD11b (P = .008, P = .046, and P = .041, respectively). Increased expression of CD141 was seen only for 1α,25(OH)₂D₃ and ATRA (P = .009 and P = .048, respectively) and also for several cell cycle-associated proteins. Carnosic acid in combination with 1α,25(OH)₂D₃ and ATRA resulted in decreased cell proliferation and blocked the cell cycle transition from G₁ to S phase (P<.05). Carnosic acid alone increased the expression of VDR and RXR-α, but the expression was greatly enhanced in the presence of 1α,25(OH)₂D₃ and ATRA. In combination with TPA, carnosic acid potentiated the expression of VDR and RAR-α. Conclusion: Carnosic acid enhances a program of gene expression consistent with 1α,25(OH)₂D₃-, ATRA-, or TPA-induced monocytic differentiation of HL60-G cells. [J Natl Cancer Inst 2001;93:1224–33]

Myeloid leukemias represent a largely unsolved challenge for chemotherapy of malignant disease. An important advance, however, was the demonstration that patients with a subset of this disease, acute promyelocytic leukemia (APL), can go into remission after treatment with all-trans-retinoic acid (ATRA) (1–3). In this situation, the immature hematopoietic cells are induced to differentiate toward nonproliferating, more mature granulocytes. The genetic defect in APL cells, i.e., a chromosomal translocation that results in a rearrangement of the gene for retinoic acid receptor (RAR)-α, can be compensated by increasing the concentration of ATRA, the physiologic ligand for RAR-α [reviewed in (4)]. Unfortunately, the ATRA-induced remissions of APL are usually followed by a relapse, and other forms of myeloid leukemias also carry a grim prognosis. Different approaches of differentiation therapy need to be evaluated.

Several compounds other than ATRA can induce differentiation of human leukemia cells in vitro, but the translation of these findings to the clinic has so far not been successful. For instance, the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) induces monocyte–macrophage differentiation of HL60 human leukemia cells (5) as well as megakaryocytic differentiation of K562 leukemia cells (6,7); however, this phorbol ester promotes carcinogenesis [e.g., (8,9)], clinical use of TPA cannot be contemplated. Similarly, dimethyl sulfoxide (DMSO) has diverse differentiation-inducing effects on leukemia cells, including morphologic granulocytic differentiation of HL60 cells (10), but it is a well-known chemical solvent unsuitable for internal human use. More promising is the potential use of the natural body metabolite, the vitamin–hormone 1α,25-dihydroxyvitamin D₃ (1α,25(OH)₂D₃), and its chemically modified analogues, which induce monocyte-like differentiation of several human myeloid and myelomonocytic leukemia cell lines but which elicit no general cytotoxicity (11,12). However, even in this case, their clinical use is limited by systemic hypercalcemia resulting from the increased intestinal absorption of calcium and the calcium-mobilizing properties of 1α,25(OH)₂D₃.

One approach to overcoming the problem of vitamin D-induced hypercalcemia, as well as the possible immunosuppressive effects of its analogues, is to combine relatively low doses of 1α,25(OH)₂D₃ with the administration of another agent that augments the differentiation-inducing action of 1α,25(OH)₂D₃ but that does not enhance the levels of circulating calcium and is not immunosuppressive. An example of such an approach is the combination of ketoconazole with 1-deoxy analogues of vitamin D₃, which strongly potentiated the differentiation of HL60 cells in vitro but produced only minor changes in intracellular calcium homeostasis (13). While proof of principle was demonstrated, ketoconazole has substantial toxicity on its own and, so far, has not been introduced as a component of therapy for leukemia.
More recently, some plant-derived dietary antioxidants have been found not only to reduce the risk of cardiovascular disease and cancer in general (14) but also to enhance the differentiation of HL60 cells. Lycopene, a carotenoid present in fruits and vegetables but primarily present in tomatoes, can induce differentiation and can reduce the rate of growth of HL60 cells; however, more remarkably, lycopene cooperates with 1α,25(OH)2D3 to produce these effects (15). Also, carnosic acid, an antioxidant polyphenol derived from the plant rosemary (Rosmarinus officinalis), augments the inhibition of growth and the induction of differentiation of HL60 and U937 leukemia cells by 1α,25(OH)2D3 and ATRA (16). Since carnosic acid is a major component of rosemary extracts that are used widely as additives for the preservation of certain foods [e.g., (17)] and, therefore, appears to be safe for human administration, we investigated the nature and mechanisms of induction of differentiation of HL60 cells by using a combination of carnosic acid and low concentrations of several inducers of monocytic or granulocytic differentiation.

Materials and Methods

Chemicals and antibodies. Carnosic acid was purchased from Alexis Biochemicals (Läufeningen, Switzerland). 1α,25(OH)2D3 was a gift from Dr. Milan Uskokovic (Hoffmann-La Roche Inc., Nutley, NJ). ATRA, TPA, and DMSO were purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions of carnosic acid (10 mM), 1α,25(OH)2D3 (0.25 mM), ATRA (1 mM), and TPA (2 mM) were prepared in absolute ethanol. The antibodies against vitamin D receptor (VDR) (i.e., C-20), retinoid X receptor (RXR)-α (i.e., D-20), cyclophilin D1 (i.e., R-124), early growth response gene (EGR)-1 (i.e., 558), CD5 (i.e., DC17), and p35Nck5a (i.e., C-19) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-cytochrome C (Ab-1) and anti-p27Kip1 antibodies were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA) and BD Biosciences—PharMingen (San Diego, CA), respectively. Phycocerythrin-conjugated anti-CD14 (MY-4-1D) and fluorescein isothiocyanate-conjugated anti-CD11b (MO1-FITC) antibodies were obtained from Coulter Corp. (Miami, FL). Anti-calreticulin antibody (PA3-900) was purchased from Affinity BioReagents Inc. (Golden, CO). Horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) and anti-mouse IgG were obtained from Santa Cruz Biotechnology Inc.

Cell culture and proliferation assay. HL60-G cells (18), a subclone of human promyeloblastic leukemia HL60 cells (19), were cultured routinely at 37 °C in RPMI-1640 medium (Mediatech, Herndon, VA) supplemented with 1% glutamine and 10% heat-inactivated, iron-enriched bovine calf serum (HyClone Laboratories Inc., Logan, UT). The cultures were passaged two or three times weekly to maintain a log-phase growth. If not indicated otherwise, the cells were seeded at 1.0 × 105 cells/mL in 25-cm2 tissue culture flasks and incubated for 96 hours with carnosic acid, 1α,25(OH)2D3, ATRA, TPA, DMSO, or combinations of carnosic acid with the above differentiation inducers. To demonstrate the enhancement of differentiation induced by 1α,25(OH)2D3, or ATRA, we used low (1 nM) concentrations of these inducers, whereas we used 100 nM 1α,25(OH)2D3 or 1 μM ATRA to illustrate the maximal effects of these inducers. Cell growth was estimated by counting the cells with a Coulter counter after dilution in Isoton-II (Coulter Electronics, Hialeah, FL). Cell viability was determined with the trypan blue dye (0.25%) exclusion.

Determination of markers of differentiation. Aliquots of 1 × 106 cells were harvested, washed twice with phosphate-buffered saline (PBS), and suspended in 10 μL 1× PBS. The cell suspensions were incubated for 45 minutes at room temperature with 0.5 μL MY-4-1D-1 and 0.5 μL MO1-FITC (1:20 dilution of the stock antibodies) to analyze the expression of the cell surface markers CD14 and CD11b, respectively. The cells were then washed three times with ice-cold 1× PBS and resuspended in 1 mL of PBS. Two-parameter analysis was performed with the use of a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Isotypic mouse IgG1 was used to set threshold parameters. Monocytic differentiation was also monitored by cytochemical determination of the activity of monocyctic serine esterase (MSE), also known as nonspecific esterase (NSE), as described previously (13).

Cell cycle distribution. The cells (1 × 106) were washed twice with ice-cold 1× PBS and were fixed in 75% ethanol at −20 °C overnight. They were then washed twice with 1× PBS and incubated in 1 mL of a solution containing 1 μg (100 U/mL) ribonuclease (RNase) (BMB, Indianapolis, IN) and 10 μg/mL propidium iodide (Sigma Chemical Co.) for 1 hour at 4 °C in the dark. The cell DNA content was determined with the use of an FACSCalibur flow cytometer, and the cell cycle distribution was analyzed by a ModFit LT computer program (Verity Software House; AMPL Software, Turramurra, Australia), as described previously (20).

Cell extracts and western blotting. The cells (approximately 1 × 107) were washed twice with ice-cold 1× PBS, snap-frozen in liquid N2, and stored at −80 °C. Whole-cell extracts were made by mixing the thawed cell pellets with an extraction buffer, i.e., 20 mM Tris–HCl, 0.25 M sucrose, 10 mM ethylene glycol-O,O′-bis-[2-amino-ethyl]-N,N′,N″,N‴-tetraacetic acid, 2 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.5), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.02% leupeptin, and 10 μg/mL aprotinin, followed by vigorous vortexing for 10 seconds. The extracts were mixed with an equal amount of 3x sodium dodecyl sulfate (SDS) sample buffer (i.e., 150 mM Tris, 30% glycerol, 3% SDS, 1.5 mg/mL bromophenol blue dye, and 100 mM dithiothreitol [DTT]). Equal amounts of extracts (40 μg of protein) were separated with the use of SDS–polyacrylamide gel electrophoresis (PAGE) (12% polyacrylamide gel containing SDS) and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech Inc., Piscataway, NJ). The membranes were blocked with 5% milk in Tris-buffered saline containing 0.1% Tween 20 for 1 hour and blotted for 1 hour with the specified primary antibody and then for 1 hour with a horseradish peroxidase-conjugated secondary antibody. The protein bands were detected with the use of a chemiluminescence assay system (Amersham Pharmacia Biotech Inc.) and visualized on Kodak X-OMAT LS film. The optical density (OD) of each band was quantified with the use of an image quantitator (Molecular Dynamics, Sunnyvale, CA). The strips were stripped according to the manufacturer’s protocol (Amersham Pharmacia Biotech Inc.) and successively reprobed with different antibodies and finally for calreticulin that is present constitutively.

Preparation of nuclear extracts. The nuclear extracts used for gel mobility shift assays were prepared by the procedure described previously (21). All steps were performed at 4 °C. Briefly, 2 × 107 cells were harvested, washed twice with PBS, and resuspended in 0.2 mL of cell extraction buffer (i.e., 10 mM HEPES–KOH [pH 7.9], 1.5 mM MgCl2, 10 mM KC1, 0.5 mM DTT, 0.2 mM PMSF, and 10 μg/mL aprotinin). The cells were kept on ice for 10 minutes, vortexed for 10 seconds, and centrifuged at 4 °C at 16000g for 30 seconds. The pellet was resuspended in 20–40 μL of nuclear extraction buffer (i.e., 20 mM HEPES–KOH [pH 7.9], 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, and 10 μg/mL aprotinin), placed on ice for 20 minutes, and centrifuged at 4 °C at 16000g for 2 minutes. The supernatant was saved as the nuclear extract and stored at −80 °C.

Gel mobility shift assay. The vitamin D response element (VDRE) sequence—oligonucleotide 5′-CTATGGCTTCGGGTAGAGGTCAAGGTTGACTCCGACG-3′—and its complement were synthesized and annealed, and the double-stranded DR3—the response elements are typically composed of two hexamer half-sites organized as directed repeats (DR), and the binding specificity is provided by the spacing between each half-site; following this rule, the consensus VDRE, which has a half-site spacing of three nucleotides (DR3), is called VDRE-DR3—was 5′-end phosphorylated by T4 polynucleotide kinase (Life Technologies Inc. [GIBCO BRL], Rockville, MD). Rockfeller University purchased the [32P]endabeled octamer of 5′-AGaaCA-3′ and, there-
Reverse transcription–polymerase chain reaction (RT–PCR). Total RNA was extracted from 5 × 10^6 cells with the use of the RNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer’s recommended procedure. The RNA concentration was determined spectrophotometrically. For RT–PCR, a 20-μL master mix of RT was prepared as follows: 5 mM MgCl₂, PCR Buffer II, 1 mM deoxyguanosine triphosphate, 1 mM deoxyadenosine triphosphate, 1 mM deoxythymidine triphosphate, 1 mM deoxyuridine triphosphate, 1 μM RNA inhibitor, 2.5 μM random hexamers, a 1-μg sample of RNA, and diethyl pyrocarbonate-treated distilled water. The master mix was incubated in the Perkin-Elmer GeneAmp PCR system 9600 (Roche, Branchburg, NJ) at 42 °C for 15 minutes and then at 99 °C for 5 minutes and at 5 °C for 5 minutes. After RT, 78 μL of PCR master mix containing 2 μM MgCl₂, PCR Buffer II, 2.5 μL of AmpliTaq DNA polymerase, and the following primers was added at a 0.15 μM concentration: EGR-1, upstream primer (5'-AGTAAATGGGACTGCTGTCG-3'), downstream primer (5'-AGGCCCTGATCGCTGAGCCACCC-3'), downstream primer (5'-AGTAAGTGGAGCCTGTGCTG-3'); RXR-α, upstream primer (5'-TCACTTATGACCCCCGTCAG-3'), downstream primer (5'-TCTAGCTTACCTCATTCT-GT-3'); VDR, upstream primer (5'-AGGCCCTTGGAGCAGTGTG-3'), downstream primer (5'-GGTCTCTGATTCCAGGTCAT-3'); 24-hydroxylase, upstream primer (5'-CTCTTGACAACAAACACG-GT-3'), downstream primer (5'-TCCACAGGTTTACATTGTGTG-3'); human osteocalcin (hOC), upstream primer (5'-GAGCCCTCACCTCCTGCGCTATT-3'), downstream primer (5'-GTGAAAGGCCTGATAGCTGTCG-3'); and β-actin, upstream primer (5'-TGGGCTGGCTACCCACCTGTGGCCACGTA-3'), downstream primer (5'-CTAGAAGATGCTGGCGTACAGGTG-3'). The complementary DNAs (cDNAs) in samples were amplified in the GeneAmpPCR System 9600 as follows: 105 seconds at 95 °C as an initial step, followed by 35 cycles of 15 seconds each at 95 °C and 30 seconds each at 60 °C. The PCR products were separated on 2% agarose gel. The intensity of the bands corresponding to VDR, RXR-α, and EGR-1 was measured with the use of the Image QuaNT Program (Molecular Dynamics, Sunnyvale, CA).

**Statistical methods.** All experiments were repeated at least three times (n = 3–8). Measurement of cell number and viability as well as counting of cells stained for MSE (Table 1) in each experiment was carried out in triplicate. The statistical analysis was performed with the GraphPad Prism 3.0 Program (GraphPad Software, San Diego, CA). Data are reported as means ± 95% confidence intervals. Statistically significant differences among the multiple groups (see Figs. 2, 3, 6, and 7 and Table 1) were tested with the use of the nonparametric Kruskal–Wallis one-way-analysis-of-variance test, followed by Dunn’s adjustment for individual groups versus control. Two compounds (A and B) were considered to show enhancement in the particular experiment if the effect of their combination (AB) was larger than the sum of their individual effects (AB ≧ A + B), the data being compared after subtraction of the respective control values from A, B, and AB. Statistically significant differences between AB and A + B were estimated with the use of the nonparametric Wilcoxon matched pairs test. A P value less than .05 was considered to be statistically significant.

**RESULTS**

Enhancement of HL60-G Cell Differentiation

It has already been reported that the plant antioxidant carnosic acid enhances the effects of 1α,25(OH)₂D₃ to induce in wild-type HL60 cells the expression of the CD14 marker of monocytic differentiation as well as the oxidative burst activity and expression of chemotactic peptide receptors (16). In this study, we used a subline of HL60 cells, HL60-G cells established in our laboratory (18). Figs. 1 and 2 illustrate that dual labeling of HL60-G cells with antibodies against CD14 and a general myeloid differentiation marker CD11b can demonstrate increased expression of the CD11b following a 96-hour treatment of these cells with either 1α,25(OH)₂D₃ or ATRA at low concentrations (both at 1 nM), as compared with the control cells (P < .001). The expression of CD14 was increased appreciably in the 1α,25(OH)₂D₃-treated cells (P < .001), but it was minimal in cells treated with ATRA, a finding consistent with the granulocytic lineage of differentiation induced by ATRA (10). TPA at 1 nM caused only a marginal but statistically not significant increase in the expression of CD11b (P = .074) and did not affect the CD14 expression (Fig. 2).

The addition of 10 μM carnosic acid (see inset in Fig. 1 for structure) to this differentiation system did not produce any substantial effect on the expression of the differentiation markers (i.e., CD14, CD11b, and MFE). However, this polyphenol (carnosic acid) augmented the expression of CD11b when the differentiating agent was either 1α,25(OH)₂D₃ (P = .008), ATRA (P = .046), or TPA (P = .041) at a concentration of 1 nM (Figs. 1 and 2). Also, it greatly augmented the effects of 1α,25(OH)₂D₃ on the induction of CD14 (P = .009), while it had no effect on the induction of this marker in the presence of TPA (P = .125). Surprisingly, carnosic acid caused a small but statistically sig-

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**Table 1. Effects of a combination of carnosic acid and other differentiation agents on monocytic differentiation (expression of monocytic serine esterase), viability, and proliferation of HL60-G cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Column A: MSE†</th>
<th>[95% CI] (n = 3)</th>
<th>Column B: viability‡</th>
<th>[95% CI] (n = 4)</th>
<th>Column C: proliferation§</th>
<th>[95% CI] (n = 7)</th>
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<tr>
<td>Control-0 h</td>
<td>ND</td>
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<tr>
<td>Control-96 h</td>
<td>0.3 [0.0 to 0.8]</td>
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<td>10 μM CA</td>
<td>5.3 [0.2 to 10.4]</td>
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<td>1 nM 1α,25(OH)₂D₃</td>
<td>9.5 [3.7 to 15.3]</td>
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<tr>
<td>1 nM 1α,25(OH)₂D₃ + 10 μM CA</td>
<td>65.5 [54.1 to 76.9]</td>
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<tr>
<td>1 nM ATRA</td>
<td>3.3 [0 to 6.9]</td>
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<tr>
<td>1 nM ATRA + 10 μM CA</td>
<td>46.0 [41.2 to 50.8]</td>
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<td>1 nM TPA</td>
<td>1.0 [0 to 3.5]</td>
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<tr>
<td>1 nM TPA + 10 μM CA</td>
<td>19.0 [11.5 to 26.5]</td>
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<tr>
<td>0.125% DMSO</td>
<td>0.3 [0 to 1.7]</td>
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<tr>
<td>0.125% DMSO + 10 μM CA</td>
<td>5.3 [2.4 to 8.2]</td>
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<tr>
<td>100 nM 1α,25(OH)₂D₃</td>
<td>73.0 [63.5 to 82.5]</td>
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<tr>
<td>1 μM ATRA</td>
<td>9.0 [0.2 to 17.8]</td>
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†Data are shown of triplicate measurements made in three to seven independent experiments. See text for details of statistical comparisons between different groups. CI = confidence interval; Control-0 h = untreated HL60-G cells when starting experiment; Control-96 h = untreated HL60-G cells after 96 hours in tissue culture; CA = carnosic acid; 1α,25(OH)₂D₃ = 1α,25-dihydroxyvitamin D₃; ATRA = all-trans-retinoic acid; TPA = 12-0-tetradecanoylphorbol-13-acetate; DMSO = dimethyl sulfoxide; ND = not determined.

‡MSE = 7 monocytic serine esterase, a characteristic marker of monocytic lineage also known as nonspecific esterase, expressed as percentage of positive cells.

§Proliferation was determined by enumeration of the cell numbers in each group of cultures with the use of a Coulter Counter. The values are cells/mL × 10^3.

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significant ($P = .048$) enhancement of CD14 expression induced by ATRA (Figs. 1 and 2). A low concentration of DMSO (0.125%) had no effect on the expression of either CD11b or CD14 (Fig. 2). When carnosic acid was added along with DMSO, a slight but statistically not significant increase in the expression of CD11b was observed ($P = .375$), while the expression of CD14 was not affected.

**Preferential Increase of Monocyte-Specific Markers by Carnosic Acid in Differentiating HL60-G Cells**

The monocytic phenotype is characterized by the high expression of the CD14 marker (22), as exemplified by cells exposed to 1,25-dihydroxyvitamin D$_3$ (D$_3$) (Figs. 1 and 2) and by the expression of the cytoplasmic enzyme MSE (23). In view of the well-established consensus that ATRA induces granulocytic lineage of differentiation in HL60 cells (10), the unexpected synergistic increase in CD14 expression by the carnosic acid–ATRA combination (Figs. 1 and 2) prompted us to further analyze this system. The results showed that there was a marked increase in cells doubly positive for CD14 and CD11b (Fig. 2, B) when carnosic acid was added along with either 1,25(OH)$_2$D$_3$ ($P < .001$) or ATRA ($P = .012$) and in cells positive for MSE ($P < .001$ for both 1,25(OH)$_2$D$_3$ and ATRA, Table 1 column A). A smaller increase in the CD14/CD11b- positive ($P = .039$; Fig. 2, B) and the MSE-positive ($P = .043$; Table 1 column A) cells by the carnosic acid–TPA combination was noted, whereas no augmentation by the polyphenol was seen in the DMSO-treated cells (Fig. 2, B; Table 1 column A). These data indicate that carnosic acid promotes the monocytic phenotype when it is combined with some, although not all, inducers of monocytic differentiation.

**Effects of Carnosic Acid on Survival, Proliferation, and Cell Cycle Traverse of Differentiating Cells**

Carnosic acid at a concentration of 10 μM, either alone or in combination with other differentiating agents, had no detectable cytotoxicity, as determined by microscopic examination of the nuclear morphology of the stained cells (data not shown) or exclusion of the trypan blue dye (Table 1 column B). When added to HL60-G cells for 96 hours, carnosic acid alone only slightly affected cell proliferation (Table 1 column C) compared with untreated control cells, but it statistically significantly reduced the rate of cell growth when it was combined with 10 μM 1,25(OH)$_2$D$_3$ ($P < .001$), ATRA ($P < .001$), or TPA ($P = .008$). However, the enhancing effect of carnosic acid was statistically significant only when it was added together with 1 nM 1,25(OH)$_2$D$_3$ ($P = .015$) or 1 nM ATRA ($P = .025$) (Table 1 column C). In view of the reduced rate of proliferation resulting from the exposure to carnosic acid together with differentiation agents, we examined the parameters of the cell cycle under these conditions. Small but statistically significant blocks of the G$_1$ to S-phase transition manifested by the increase in the G$_0$/G$_1$ and a
decrease in the S-phase cell populations were produced by both 1α,25(OH)₂D₃ (P = .021 and P = .017 for G₀/G₁ and S, respectively) and ATRA (P = .019 and P = .026 for G₀/G₁ and S, respectively), whereas TPA and DMSO were without statistically significant effects at the concentrations used here (Fig. 3, A). It is interesting that, while it had no effect on the cell cycle progression when added alone, carnosic acid enhanced this increase in the G₀/G₁ phase (P = .004) and a decrease in the S phase (P = .027) induced by 1α,25(OH)₂D₃ (Fig. 3, A).

In contrast, carnosic acid did not statistically significantly alter the effects of ATRA (P = .074 and P = .136 for G₀/G₁ and S phases, respectively) or TPA (P = .215 and P = .236 for G₀/G₁ and S phases, respectively) and had no enhancing effect when it was combined with DMSO (P = .455 and P = .332 for G₀/G₁ and S phases, respectively) in these experiments (Fig. 3, A).

Further examination revealed that growth inhibition induced by the combination of carnosic acid and 1α,25(OH)₂D₃ increased considerably with time, leading to a complete growth arrest after 168 hours of treatment (P<.001), without a statistically significant decrease in cell viability (mean [95% confidence intervals] for treated = 96.0% [93.4% to 98.6%] versus control = 98.1% [96.8% to 99.4%] at 168 hours; n = 3) (Fig. 3, B). This effect was accompanied by an increasingly higher G₁/S ratio (Fig. 3, C), signifying a marked G₁- to S-phase block (P<.001 at 168 hours). (For Fig. 3, B, P = .032 and P = .018 at 96 and 120 hours, respectively; for Fig. 3, C, P = .012 and P<.001 at 96 and 120 hours, respectively.).

Consistent with the G₁ block, the cyclin-dependent kinase inhibitor p27/Kip1, which controls the G₁- to S-phase transition in 1α,25(OH)₂D₃-treated HL60 cells (24,25), showed markedly elevated levels in cells treated with the carnosic acid–1α,25(OH)₂D₃ combination. However, in cells treated with the carnosic acid–ATRA, the carnosic acid–TPA, or the carnosic acid–DMSO combinations, the changes in the p27/Kip1 levels were less marked or were not detectable, as shown by representative immunoblots (Fig. 4, A).

Further analysis of the G₁ block was performed by the determination of the steady-state levels of the G₁ cyclins, cyclin D₁ and cyclin E. Previous study of this system showed that, in HL60 cells, as in other systems (26), cyclin D₁ is maximally expressed in mid-G₁, while cyclin E is maximally expressed at the G₁/S boundary (Chen F, Studzinski GP: unpublished data). In the present experiments, the levels of cyclin D₁ and cyclin E in cells treated with 1α,25(OH)₂D₃ paralleled those of p27/Kip1 and the degree of G₁ block (Fig. 4, A). These data suggest that the cell cycle block induced by the combination of 1α,25(OH)₂D₃ and carnosic acid occurs in mid- to late-G₁ phase rather than in G₀ phase. Furthermore, increases in the G₁ cyclins in cells treated with the carnosic acid–ATRA combination suggest that there is a partial G₁ block in these cells, even though it was not statistically
Carnosic acid increased the levels of VDR and RXR-α/heterodimers. We therefore determined the protein abundance of VDR and RXR-α/heterodimers in HL60-G cells, we investigated if products of genes reported to be selectively activated in monocytic differentiation could also be detected. Fig. 4, C, indicates that EGR-1, the expression of which is reported to redirect the development of hematopoietic progenitor cells along the monocyte–macrophage lineage (29,30), and the p35 neuronal Cdk5 activator (p35Nck5a), also specifically associated with the monocytic lineage in hematopoietic
Transcriptional Regulation of VDR, RXR-α, and EGR-1 Abundance by Carnosic Acid

Determination of specific mRNA levels in cell extracts showed that treatment with carnosic acid alone resulted in a statistically significant increase in the expression of VDR (P = .007), RXR-α (P = .011), and EGR-1 (P = .009) genes (Fig. 7), indicating that the increased expression of these genes by carnosic acid occurs at least in part at the transcriptional level. DMSO and 1α,25(OH)2D3 also statistically significantly increased the transcription of VDR (P = .021) and RXR-α (P = .032), respectively, whereas all of the differentiation-inducing agents induced increased expression of the EGR-1 gene (P = .011 to P = .018) (Fig. 7), as was reported previously for the increased expression of EGR-1 by TPA (29). Statistical analysis revealed an enhancement by carnosic acid of the 1α,25(OH)2D3-induced increase in the expression of VDR (P = .034). In addition, carnosic acid produced additive effects with DMSO on VDR expression (P = .097) and with 1α,25(OH)2D3 on EGR-1 expression (P = .076), which were not statistically significant (Fig. 7). However, in general, the cooperation of carnosic acid with differentiation inducers at the mRNA level was less marked than at the protein level, suggesting both transcriptional and post-transcriptional controls.

DISCUSSION

Our results provide potential mechanisms for the enhancement by carnosic acid of 1α,25(OH)2D3- or ATRA-induced monocytic differentiation of HL60-G cells. First, carnosic acid increases the expression of VDR and RXR-α. The heterodimers of VDR–RXR-α thus formed can respond with increased sensitivity to 1α,25(OH)2D3, while RXR-α–RXR-α heterodimers can activate ATRA-responsive genes when ATRA is added instead of 1α,25(OH)2D3. The rationale for this suggestion is based on the previous findings that the increased expression of VDR in various cell types, as for instance by transfection of a VDR expression vector, increases the sensitivity of the cells to 1α,25(OH)2D3 (33). Second, the increased expression of the EGR-1 gene product may, as reported (29,30), direct the differentiation of ATRA-treated HL60 cells toward the monocytic–macrophage lineage and further enhance the 1α,25(OH)2D3-induced monocytic differentiation. Third, the activation of Cdk5 by p35Nck5a, the protein levels of which are also increased by carnosic acid (Fig. 4), also favors the development of the monocytic phenotype, since it has been shown previously that the increased expression of Cdk5 activity in HL60 cells induced to monocytic differentiation by 1α,25(OH)2D3 diverts the phenotype toward the immature granulocyte (29). Considered together, these results suggest that carnosic acid induces a program of gene expression that is characteristic of the monocytic phenotype. Furthermore, this monocytic phenotype appears to be dominant over the granulocytic phenotype in the presence of carnosic acid.

It was reported some time ago that HL60/MR1 cells, a subline of HL60 cells, derived from a transplantable HL60 tumor established in athymic nude mice, differentiate to monocytic rather than to granulocytic cells when they are cultured with ATRA in vitro (34). It is interesting that the cells from this xenotransplant-derived HL60 subline also showed other properties observed by us in carnosic acid-treated HL60 cells; the cells had markedly enhanced sensitivity to ATRA, and the differentiation pathways induced by TPA and DMSO were the same as

cells (31,32), showed increased expression after exposure to carnosic acid (as well as to TPA, as expected in macrophage lineage differentiation) and that carnosic acid enhanced the effects of 1α,25(OH)2D3 or ATRA.
those in the parental HL60 cells. More recently, M2 leukemic blast cells were found to undergo monocyctic differentiation when they were treated with ATRA, which induces functionally active VDR in these cells (35). Thus, it appears that ATRA-induced granulocytic differentiation involves signaling circuits similar to those that are active in monocyctic differentiation; however, in ATRA-treated HL60 cells, there is a lack of an additional input required for the monocyctic form of differentiation. This input may be EGR-1, Cdk5 activated by p35Nck5α, or both of these, mediated by VDR-RXR-α and subjected to electrophoretic mobility shift analysis, indicating the specific nature of the upper band in panel A. Data representative of three similar experiments are shown. Control = untreated HL60 cells at 96 hours. NS or ns = nonspecific band; Abs = antibodies.

The effects of carnosic acid on differentiation induced by low concentrations of TPA or DMSO were less striking. An increase in differentiation resulting from the combination of TPA with carnosic acid was noted by the determination of MSE and CD11b markers, and this increase was statistically significant but was not apparent when CD14 was used as the marker. Conversely, a combination of carnosic acid and DMSO at several concentrations was less than additive (Table 1; A; Fig. 2; and data not shown). Consistent with this finding, there was no detectable enhancement by carnosic acid of VDR or RXR-α protein levels induced by DMSO. The enhancement by carnosic acid of a TPA-induced increase in RAR-α expression was marked, but at present its implications are unclear. In view of the low potential of TPA or DMSO for human use, this situation was not analyzed further; however, it seems that, while differentiation induced by the physiologically occurring compounds 1α,25(OH)2D3 and ATRA is markedly promoted by carnosic acid, differentiation induced by exogenous xenobiotics is at best marginal.

The data also suggest that carnosic acid enhances 1α,25(OH)2D3-induced and probably also ATRA-induced mid- to late-G1-phase cell cycle arrest. The support for this hypothesis is derived from the observation that cyclin D1 levels and cyclin E levels parallel the levels of p27Kip1 (Fig. 4, A), which have been shown previously to be regulating the G1-phase arrest in this system (24,25), and from the knowledge that the maximal expression of cyclin D1 is in mid-G1, while the maximal expression of cyclin E is at the G1/S-phase boundary (26). The increased levels of cyclin D1 in cells that are arresting in G1 phase (Fig. 3) can be explained by a progressive accumulation of cyclin D1, probably just prior to or at the R point (36), where they have maximal expression of cyclin D1. It appears that carnosic acid induces such an arrest in this window of the G1 phase and does not interfere with the normal expression of cyclin D1 or cyclin E. It is also possible that some or all of the other increases in expression in proteins studied here become elevated as a result of the same mechanism, i.e., arrest in mid- to late-G1 phase, already shown to exhibit maximal sensitivity to the differentiation-inducing activity of 1α,25(OH)2D3 (37).

The fulfillment of the promise for the use of 1α,25(OH)2D3 as an agent for differentiation therapy for leukemia is blocked currently by the hypercalcemia attendant on its use in vivo (11). The approaches employed to overcome this limitation have included the use of analogues of 1α,25(OH)2D3 (38–40), also referred to as “deltanoids” [e.g., (41)], or combinations of these deltanoid analogues with chemicals or cytokines to enhance differentiation [e.g., (13)]. For instance, the inhibitors of p38MAP kinase SB203580 and SB202190 were found to markedly potentiate differentiation of HL60 induced by low concentrations of 1α,25(OH)2D3 (42). However, the potential of carnosic acid as a component of differentiation therapy regimen appears to be greater than that of other chemicals because of its anticipated safety and its great potency in enhancing the pro-differentiation of effects of low concentrations of the naturally occurring vitamin/hormone 1α,25(OH)2D3 and the regulator of embryonic development ATRA.

In this study, we have focused on the mechanistic aspects of...
the action of carnosic acid that can identify the effectors of the expression of the monocytic phenotype induced by carnosic acid in combination with natural hormones/morphogens. Whether antioxidant properties of carnosic acid have any relevance to the increased expression of 1,25(OH)2D3-responsive genes and the enhancement of the monocytic phenotype is an open question, and the upstream regulators of the carnosic acid-induced increased abundance of the nuclear receptors VDR and RXRα, the transcription factor EGR-1, or the Cdk5/p35 complex remain to be identified in future studies. However, the identification of these downstream regulators of monocytic differentiation, with its attendant G1 block, should provide new approaches to combined chemoprevention or differentiation therapy for myeloid leukemia.

REFERENCES

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NOTES

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