Carnosol Delays Chemotherapy-Induced DNA Fragmentation and Morphological Changes Associated With Apoptosis in Leukemic Cells

Susan J. Zunino and David H. Storms
United States Department of Agriculture, Agricultural Research Service, Western Human Nutrition Research Center, University of California, Davis, USA

Carnosol, from the herb rosemary, has been shown to induce apoptotic cell death in high-risk pre-B acute lymphoblastic leukemia (ALL). In the present study, carnosol was tested for its ability to sensitize leukemia cells to chemotherapeutic agents. Carnosol reduced the percentage of cell death in the pre-B ALL lines SEM, RS4;11, and REH when combined with cytarabine, methotrexate, or vincristine compared to the chemotherapeutic agents alone. Analysis of DNA strand breaks by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling showed that carnosol delayed DNA cleavage in the cells when combined with chemotherapeutic drugs. Co-treatment of the cells with carnosol and chemotherapeutic drugs did not reduce mitochondrial membrane depolarization compared to the drug treatment alone. Time course analysis of caspase-3 activation by flow cytometry showed co-treatment with carnosol and drugs increased the activation of caspase-3 above that observed for the chemotherapeutic drugs alone. A lower percentage of caspase-3 positive cells progressed to an apoptotic phenotype when co-treated with carnosol and the chemotherapeutic drugs compared to drugs alone. These data show that carnosol blocks the terminal apoptotic events induced by chemotherapeutic drugs and suggest that increased dietary intake of carnosol may potentially decrease the effectiveness of some standard chemotherapy treatments used for leukemia.

INTRODUCTION
Carnosol is a plant-derived phenolic antioxidant found in the herb rosemary. The structure of carnosol is shown in Fig. 1. Carnosol has also been shown to downregulate the anti-apoptotic protein Bcl-2 and induce significant apoptotic cell death in high-risk and other B-lymphoid acute lymphoblastic leukemia (ALL)-derived lines (5).

Chromosomal abnormalities in the ALL-1 gene (also known as MLL, HRX, and HTRX1) on chromosome 11 are frequently involved in childhood ALL. The chromosomal translocation t(4;11)(q21;q23) is found in greater than 60–85% of infants (6,7) diagnosed with ALL, and the presence of this chromosomal abnormality is strongly associated with a poor prognosis. Commonly used chemotherapeutics for treating high-risk ALL include prednisone, doxorubicin, cytarabine (AraC), methotrexate (MTX), and vincristine (8).

The plant-derived polyphenols genistein, emodin, curcumin, and silymarin, as well as polyphenols found in green tea, have shown chemosensitizing and radiosensitizing effects in a variety of cancer cells (9). Dose-dependent differences in sensitizing activity have been observed in vitro. For example, pretreatment with 30 \( \mu \)M resveratrol was reported to sensitize a number of cancer cells including neuroblastoma, glioblastoma, breast carcinoma, prostate carcinoma, REH B-cell leukemia, and Jurkat T leukemia cells to the chemotherapeutic drugs doxorubicin, AraC, paclitaxel, MTX, and 5-fluorouracil (10). However, Ahmad et al. (11) reported that low-dose resveratrol (4–8 \( \mu \)M) inhibited vincristine- or daunorubicin-induced apoptosis in human HL60 myeloid leukemia cells. Some of these compounds have protective action in vivo against chemotherapy-induced tissue toxicity. Curcumin prevented adriamycin-induced nephro and myocardial toxicity in rats (12,13). Silymarin also prevented anthracycline-mediated toxicity in rat cardiomyocytes (14).

We have shown carnosol induced apoptosis in t(4;11) and other ALL-derived lines (5). We hypothesized that carnosol could be a potential candidate for increasing the efficacy of conventional chemotherapeutic agents used in the treatment of high-risk ALL. To test this hypothesis, apoptosis was analyzed in ALL cell lines treated with different concentrations of carnosol and the chemotherapeutic agents AraC, MTX, and vincristine.
MATERIALS AND METHODS

Cell Culture and Reagents

SEM and RS4;11 are established cell lines from patients diagnosed with pre-B cell acute lymphoblastic leukemia (ALL) containing the chromosomal translocation t(4;11)(q21;q23) (15,16). The REH cell line (pre-B cell ALL without the translocation) was obtained from American Type Culture Collection (Manassas, VA). All cell lines were maintained at 37°C, 5% CO₂ in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma), 50 IU/ml penicillin, 50 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 1 mM sodium pyruvate, and 2 mM l-glutamine (Invitrogen). For each experiment, the cells were split to a density of 1.0 × 10⁶/ml before treatment.

As a control for the dissolving medium used for each chemical, an equivalent amount of the specific diluent was always added to a control cell population in every experiment (designated untreated). Carnosol was purchased from Alexis Corp. (Lausen, Switzerland) and dissolved in dimethyl sulfoxide (Sigma, St. Louis, MO). Cytarabine (AraC), MTX, and vincristine were purchased from Sigma. JC-1 dye (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetrathiylbenzimidazolylcarbocyanine iodide) was purchased from Molecular Probes (Eugene, OR).

Analysis of Cell Death

Analyses were performed on a FACS Canto fluorescence-activated cell sorter (FACS) using FACSDiva software (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Cells were plated in 96-well microtiter plates and treated with diluents (controls) or combinations of carnosol and AraC, MTX, or vincristine for 48 h. Cell death was measured by lysing the cells in a hypotonic solution containing 1 mg/ml sodium citrate, 0.1% Triton X-100, and 50 µg/ml propidium iodide (Sigma) and analyzing the resultant nuclei by FACS as previously described (17). The extent of cell death (%) was determined by measuring the fraction of nuclei that contained subdiploid DNA content. Fifteen thousand events were collected for each sample analyzed for subdiploid nuclei.

DNA Fragmentation

DNA fragmentation in the cells was evaluated using the ApoDirect apoptosis kit, a single step stain based on the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay, according to the manufacturer’s recommendations (BD Biosciences, Mountain View, CA). The cells were cotreated for 48 h with the different chemical combinations and then fixed and permeabilized with 70% ethanol at –20°C. The DNA was labeled, and the cells were analyzed by FACS. Analysis gates were set according to manufacturer’s recommendations to exclude aggregated cells and examine the apoptotic populations in different phases of the cell cycle.

Analysis of Mitochondrial Dysfunction

The mitochondria-selective lipophilic dye JC-1 was used to measure changes in mitochondrial membrane potential as previously described (17). Cells were plated in 96-well microtiter plates and cotreated with carnosol and AraC, MTX, or vincristine. Measurements of mitochondrial dysfunction were performed on cells treated for 24 h. Ten thousand events were collected for each sample stained with JC-1. All analyses of whole cells were performed using appropriate scatter gates to exclude cellular debris and aggregated cells.

Analyses of Caspase-3 Activation

Activation of caspase-3 was determined using the active caspase-3 antibody apoptosis kit (BD Biosciences) according to the manufacturer’s recommendations. The cells were plated in 24-well plates and treated with diluent or carnosol plus or minus the chemotherapeutic drug. The activation of caspase-3 was analyzed by FACS at 4, 8, 12, and 24 h after treatment, and 10,000 events were collected for each sample. Forward and orthogonal light scatters were used to gate on the cell populations displaying a viable versus apoptotic morphology (5,18,19) to determine the progression of cells with active caspase-3 toward an apoptotic phenotype. Ten thousand events were collected for each sample.

Statistical Analysis

All statistical analyses were performed with GraphPad software (GraphPad Software, Inc., San Diego, CA), and the data were displayed as arithmetic means ± SEM. P values were obtained using 2-way analysis of variance (ANOVA) with Bonferroni post-tests (confidence interval of 95%) for evaluation of the significance of differences between treatment and control groups.

RESULTS

In the present study, the cell lines SEM and RS4;11 that were derived from patients with high-risk t(4;11) ALL and REH B-ALL cells without the translocation were used to examine whether carnosol could augment the apoptotic activities of the chemotherapeutic agents AraC, MTX, or vincristine, which are used in the treatment protocols for high-risk leukemia (8). We previously found that carnosol at concentrations of 3
FIG. 2. Cotreatment with carnosol reduces cell death induced by cytarabine (AraC), methotrexate (MTX), and vincristine in ALL-derived cells. Cells were co-treated with 0 (white bar), 3 (hatched bar), or 6 µg/ml (black bar) carnosol and AraC (0.1 or 1 µg/ml), MTX (1.0 or 10 µg/ml), or vincristine (0.6 or 2.0 µg/ml). After 48 h, the cells were lysed in hypotonic buffer containing propidium iodide, and the resulting nuclei were analyzed by FACS. The extent of cell death (%) was determined by measuring the fraction of nuclei that contained subdiploid DNA content for A: SEM; B: RS4;11; and C: REH cells. Asterisks represent a statistically significant difference of $P < 0.05$ compared to the cells treated with chemotherapeutic agent alone. The data represent three separate experiments.

and 6 µg/ml (approximately 9 and 18 µM, respectively) induced significant apoptosis in these leukemia cells, but not in normal peripheral blood mononuclear cells (5). Therefore, these concentrations were used to evaluate the chemosensitizing effects of carnosol. The leukemia cells were cotreated with 0, 3, or 6 µg/ml carnosol and AraC (0.1 or 1.0 µg/ml), MTX (1 or 10 µg/ml), or vincristine (0.6 or 2.0 µg/ml). Cell death was assessed after 48 h by PI staining of nuclei and evaluation of loss of DNA (subdiploid population) by FACS (Fig. 2). By this method, a significant reduction in cell death was observed in cells co-treated with carnosol and chemotherapeutic drugs compared to cells treated with the chemotherapeutic agents alone. The t(4;11) ALL line SEM showed a significant reduction in cell death after co-treatment with both 3 and 6 µg/ml carnosol and both concentrations of AraC or vincristine as well as after cotreatment with 6 µg/ml carnosol plus 10 µg/ml MTX ($P < 0.05$). RS4;11 showed the greatest reduction in death at an AraC concentration of 1 µg/ml and MTX concentrations of 1 and 10 µg/ml when combined with either 3 or 6 µg/ml carnosol. RS4;11 was less sensitive to vincristine and had a slight, but statistically significant, decrease in cell death when these cells were cotreated with 3 µg/ml carnosol and 0.6 or 2.0 µg/ml vincristine ($P < 0.05$). The non-t(4;11) line REH showed a decrease in cell death after co-treatment with 3 and 6 µg/ml carnosol in combination with both concentrations of AraC, MTX, or vincristine compared to cells treated with chemotherapeutic agents alone.

To further analyze the effect of carnosol on chemotherapy-induced DNA fragmentation, the cells were co-treated with 1.0 µg/ml AraC, 10 µg/ml MTX, or 2.0 µg/ml vincristine in the presence or absence of 6 µg/ml carnosol. After 24 and 48 h, DNA strand breaks were labeled by TUNEL, and the cells were analyzed by FACS. Although DNA strand breaks were observed at 24 h (data not shown), the greatest inhibitory effect of carnosol was observed at 48 h. By 48 h, there was a distinct decrease in the percentage of RS4;11 cells with fragmented DNA after cotreatment with carnosol plus AraC or MTX compared to cells treated with drug alone (Fig. 3). The percent of SEM and REH cells with DNA strand breaks was not significantly decreased after co-treatment with carnosol plus drugs compared to cells treated...
FIG. 3. Percentage of cells with DNA strand breaks in the presence or absence of carnosol and chemotherapeutic drugs. Cells were co-treated with 0 (white bars) or 6 μg/ml carnosol (black bars) and AraC (1 μg/ml), MTX; (10 μg/ml), or vincristine (2 μg/ml) for 48 h. Cells were fixed, permeabilized, and labeled with FITC-dUTP using the TUNEL assay. Asterisks represent a statistically significant difference of \( P < 0.05 \) compared to the cells treated with chemotherapeutic agent alone. The data represent 3 separate experiments.

Carnosol and Leukemic Cells

Mitochondrial membrane depolarization is an early step in apoptosis and precedes apoptosis-induced DNA fragmentation and changes in cellular morphology (20). Because a decrease in cell death and DNA strand breaks was observed when cells were treated with carnosol and the chemotherapeutic drugs, we analyzed cells at an earlier time point to determine whether this reduction was due to inhibition of early events in the apoptotic process. The cells were co-treated for 24 h with 0 or 6 μg/ml carnosol and 1 μg/ml AraC, 10 μg/ml MTX, or 2 μg/ml vincristine and then stained with the mitochondrial-selective dye JC-1. Co-treatment of the cells with carnosol and chemotherapeutics did not reduce the amount of mitochondrial membrane depolarization compared to the chemotherapeutic agents alone (Fig. 5). Mitochondrial membrane depolarization was actually increased in RS4;11 cells treated with carnosol plus AraC, MTX, or vincristine, and in REH cells treated with carnosol plus MTX (\( P < 0.05 \)).

Activation of caspase-3 is an early to intermediate event during apoptosis that is necessary to induce DNA fragmentation and changes in cellular morphology. Caspase-3 mediates the cleavage of the majority of cytoplasmic and nuclear proteins, including those responsible for maintaining cytoskeletal structure (21). To determine whether carnosol co-treatment inhibited the activation of caspase-3, a time course was performed over a 24-h period to compare the activation status of caspase-3 between the different treatment groups. The cells were co-treated with 0 or 6 μg/ml carnosol and 1 μg/ml AraC, 10 μg/ml MTX, or 2 μg/ml vincristine for 4, 8, 12, and 24 h, stained for active caspase-3, and analyzed by FACS (Fig. 6). A statistically significant increase in the activation of caspase-3 was observed at 8 and 12 h after treatment for SEM and REH cells co-treated with carnosol and the three chemotherapeutic drugs and at 12 h for RS4;11 treated with AraC. By 24 h, the increase in activated caspase-3 was maintained for SEM co-treated with carnosol plus vincristine, RS4;11 co-treated with carnosol plus AraC, and REH cells co-treated with carnosol plus MTX. Carnosol treatment alone also induced activation of caspase-3. These data show that co-treatment with carnosol does not inhibit chemotherapy-induced mitochondrial depolarization or caspase-3 activation, both early apoptotic events that precede later stage DNA fragmentation and changes in morphology.

Apoptotic cells display decreased forward light scatter properties and increased orthogonal scatter due to changes in complexity of the cell membrane, that is, membrane blebbing (5,18,19). We used forward and orthogonal scatter parameters to examine the localization patterns of activated caspase-3 population of cells at the 24 h time point. Cells that displayed a “viable” or “apoptotic” phenotype as defined by size and complexity were gated, and the percentage of cells with activated caspase-3 was determined for each population (Fig. 7A). SEM cells cotreated with carnosol and chemotherapeutic agents had increased numbers of activated caspase-3 positive cells within the viable cell population compared to cells treated with drug alone (Fig. 7B; \( P < 0.05 \)). RS4;11 cells showed an increase in caspase-3-positive cells when cotreated with AraC and carnosol, but not with MTX or vincristine. REH cells did not show a statistically significant increase in caspase-3 positive cells in the viable cell populations when co-treated with carnosol and drugs compared to drugs alone.

**DISCUSSION**

Apoptotic cells exhibit a variety of morphological changes resulting in membrane blebbing, DNA fragmentation, chromatin...
condensation, and nuclear or cytosolic formation of apoptotic bodies that are eventually phagocytosed (22). DNA fragmentation occurs in the execution phase of the apoptotic pathway and is generally involved in the reorganization of the chromatin before the formation of apoptotic bodies. Formation of high molecular weight fragments of approximately 700, 300, and 50 Kbp correlates with the early chromatin alterations displayed in preapoptotic cells (23,24). DNA fragmentation during apoptosis is mediated by at least 2 endonucleases, endonuclease G (Endo G) and the DNA fragmentation factor (DFF), also known as caspase-activated DNase (CAD) (25). Endo G is compartmentalized in the mitochondrial intermembrane space and is released along with other apoptogenic factors from the mitochondria after an apoptotic stimulus. Endo G translocates to the nucleus together with apoptosis-inducing factor to initiate DNA cleavage (26). DFF/CAD is activated by caspase-3 mediated cleavage of the cytosolic inhibitor DFF45/inhibitor of CAD. Furthermore, caspase-3 cleaves and inactivates proteins involved in DNA repair and DNA damage signaling, such as poly(ADP-ribose) polymerase (PARP), the homologous recombination Rad51, the mismatch repair protein MutL protein homolog 1 (MLH1), and the DNA-dependent protein kinase ataxia telangiectasia mutated (ATM) (27–30).

Carnosol delayed chemotherapy-induced DNA fragmentation and structural changes associated with the formation of apoptotic bodies. Our data suggests that the apoptotic process is delayed by carnosol at the endonuclease level because earlier apoptotic events (i.e., mitochondrial depolarization and caspase-3 activation) induced by carnosol in combination with chemotherapeutics were unhindered and even enhanced by co-treatment. Curcumin, a polyphenol from turmeric, was also shown to activate caspase-3 in Jurkat T cell leukemia, but was proposed to block the activity of DFF by binding to the active site of the endonuclease (31). However, carnosol co-treatment also delayed cell shrinkage and changes in membrane structure of SEM leukemia cells when combined with all three chemotherapeutic drugs and RS4;11 when co-treated with AraC. Mouse embryonic fibroblasts and T lymphocytes from caspase-3 knockout mice and MCF-7 breast carcinoma cells with loss of caspase-3 showed defects in cellular shrinkage and membrane blebbing in response to apoptotic stimuli (32,33). Caspase-3 can cleave and inactivate many cytoskeletal proteins, including gelsolin, fodrin,
and Gas2, cleavage of which results in the architectural changes during apoptosis (21,34,35). Therefore, it is possible that carnosol, although inducing greater cleavage of caspase-3 to the active form when combined with the chemotherapeutic agents, may compromise the enzymatic activity of caspase-3, resulting in the delay of DNA fragmentation and maintenance of cellular morphology in some cells. Alternatively, carnosol may increase caspase-3 activity, but block other proteins involved in reorganization of the cytoskeletal structure. Many phenolic compounds have been shown to inhibit protein kinases in signal transduction pathways (36). Caspase-3 cleavage can activate kinases, such as Rho-associated kinase 1 and p21-activated kinase 2, which induce apoptotic morphology, and carnosol could potentially inhibit the activity of one or more of these enzymes (27,32).

Carnosol is an abietane diterpenoid that is highly lipophilic (Fig. 1). Acetylation or methylation of the hydroxyl groups of several diterpenoids, including carnosol, was shown to reduce

FIG. 5. Co-treatment with carnosol does not reduce mitochondrial membrane depolarization induced by AraC, MTX, or vincristine. Cells were co-treated with 0 (white bars) or 6 µg/ml carnosol (black bars) plus either diluent (control), AraC (1 µg/ml), MTX (10 µg/ml), or vincristine (2 µg/ml) for 24 h, stained with JC-1, and analyzed by FACS. Asterisks indicate statistically significant differences of \( P < 0.05 \) compared to cells treated with chemotherapeutic agent alone. The data represent three separate experiments.

FIG. 6. Caspase-3 activation is increased in cells co-treated with carnosol and AraC, MTX, or vincristine. A: SEM; B: RS4:11; and C: REH cells were co-treated with 0 (open squares) or 6 µg/ml carnosol (black squares) and 1 µg/ml AraC, 10 µg/ml MTX, or 2 µg/ml vincristine for 4, 8, 12, and 24 h. Cells were permeabilized, stained with FITC-conjugated anti-caspase-3 antibody, and analyzed by FACS. Asterisks indicate statistically significant differences of \( P < 0.05 \) compared to cells treated with chemotherapeutic agent alone. The data represent three separate experiments.
FIG. 7. The number of caspase-3 positive cells still displaying a viable phenotype is increased after co-treatment for 24 h with carnosol and chemotherapeutic drugs compared to chemotherapeutic agents alone. Fig. 7A: Forward and orthogonal light scatter parameters were used to delineate the cell populations that exhibited a viable phenotype (V) versus apoptotic morphology (A). Fig. 7B: Forward and orthogonal light scatter parameters were used to gate on the viable cell populations expressing active caspase-3 after co-treatment with 0 (white bars) or 6 µg/ml carnosol (black bars) and 1 µg/ml AraC, 10 µg/ml MTX, or 2 µg/ml vincristine. The data are from three separate experiments.

cytotoxic activity of these compounds against P388 murine leukemia cells (37). However, conversion of the hydroxyl groups on the C ring to o-quinone either maintained or increased cytotoxicity of the molecule. These data indicate the C ring containing the hydroxyl groups (that can be oxidized to o-quinone) is important for cytolytic function of carnosol in the murine leukemia cells, and may be responsible for induction of apoptosis in the human cell lines. The inhibitory effect on DNA fragmentation and changes in apoptosis-induced morphology may be related to the ability of carnosol, as well as other diterpenes, to incorporate into the cellular lipid membrane and decrease membrane fluidity (38). Signal transduction pathways that rely on membrane fluidity for receptor-mediated activation signals have been shown to be inhibited by carnosol (39).
The data described in this article suggest caution in the use of carnosol in conjunction with some standard chemotherapeutic agents. Increased dietary intake of carnosol as a supplement or from food may interfere with the antileukemia activity of certain chemotherapeutic drugs. However, because carnosol alone has been shown to kill these leukemia cells in vitro, further evaluation of the mechanism of action and efficacy is warranted in vivo.

ACKNOWLEDGMENT

This study was supported by the United States Department of Agriculture, Agricultural Research Service CRIS Project 5306-51530-013-00D.

REFERENCES


