Vol. 186, No. 1, 1992
July 15, 1992

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 543-548

GROWTH INHIBITION OF LEUKEMIA CELL LINE CEM-C1 BY FARNESOL: EFFECTS OF PHOSPHATIDYLCHOLINE AND DIACYLGLYCEROL

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Received May 27, 1992

Acute leukemia cells of the established line CEM-C1 were treated during growth in serum-free medium with various concentrations of *trans-trans* farnesol. At concentrations ranging from 9.0 to 31.5 μM, farnesol inhibited growth of these cells without causing cell lysis. This effect was preceded by very rapid inhibition of choline incorporation in cellular lipid fraction. The growth inhibitory effect was prevented to a large extent by incubation with phosphatidylcholine or diacylglycerol.

For the last several years, post-translational modifications of proteins by endogenous isoprenoids have been receiving much attention. Much less information exists in the literature concerning the effects of exogenous isoprenoids added to the growing cells. Among the few studies is the report by Bard et al. (1) demonstrating inhibition of growth and fluidization of the cell membranes of Saccharomyces cerevisiae and Candida albicans by geraniol-containing liposomes. This treatment also resulted in a decrease in the transition temperature of phosphatidylcholine vesicles and in changes in permeability to erythritol. More recently, studies by Schoff et al. (2) demonstrated cytostatic effects of geraniol on two murine lines, leukemia P388 and melanoma B16. In the *in vivo* experiments included in their study geraniol, when added to the diet fed to mice previously inoculated with suspensions of P388 cells, considerably increased the survival time of the animals.

It has been demonstrated long ago by Christophe and Popjak (3) that geraniol and farnesol phosphates may be dephosphorylated and then oxidized, respectively, to geranoic and farnesoic acids. The possibility was raised that farnesoic acid which inhibits mevalonic acid kinase might serve as a controlling factor in the flow of mevalonate products into

0006-291X/92 $4.00

various biosynthetic pathways (4). Starting from this assumption we have examined the effects of exogenous farnesol on growth of human T-cell leukemia line CEM-C1. Unexpectedly, the earliest effect which preceded growth inhibition of these cells by farnesol was a marked reduction in the incorporation of labeled choline into cellular lipids. This effect was reversible by phosphatidylcholine and diacylglycerol.

MATERIALS AND METHODS

Cell cultures and media: The cultures of CEM-C1 and CEM-C7 cells were given to us by Dr. E.B. Thompson from the University of Texas Medical Branch at Galveston. The promyelocytic leukemia line HL-60 was obtained from the American Type Culture Collection in Rockville, MD. The cells were propagated routinely in the RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin (100 units/ml) and streptomycin (100 μg/ml). For the experiments, the cells were incubated in the serum-free Iscove's medium. Both media were purchased from GIBCO BRL, Gaithersburg MD. Fetal bovine serum was purchased from Hyclone, Logan, UT.

Chemicals: *Trans-trans* farnesol (98% pure) was purchased from Aldrich Chemical Co., Milwaukee, WI; nerolidol, geraniol, squalene, dolichols, phosphatidylcholine and 1,2-dioleoyl-rac-glycerol (diacylglycerol) were purchased from Sigma Chemical Co, St. Louis, MO. All radiolabeled compounds including [methyl-^{3}H] thymidine (48 mCi/mmol), [2-^{14}C] acetic acid (55 mCi/mmol), [methyl-^{3}H] choline chloride (85 mCi/mmol) and L-[4,5-^{3}H(N)] leucine (53 mCi/mmol) were purchased from NEN, Boston, MS.

Cell growth experiments: In all experiments the cells were preincubated for 24 hours in Iscove's medium without addition of isoprenoids. The compounds were then added and the cells incubated from the initial density of 1 x 10^5 cells/ml in plastic wells (2.5 ml of cell suspension/well) for the additional one, two and three days. The number of cells in each well was determined by electronic counting using Coulter Model B counter.

Labeling of cells with radioisotopes: Incorporation of acetate into cellular lipids was measured by incubating the cell suspensions in the presence of [^{14}C] acetate (1.5 μCi/ml) for various time intervals. The incorporation was terminated by adding 1 mM unlabeled acetate, immersing the cell suspension in an iced bath followed by centrifugation at 500 x g for five minutes. The cells were washed twice with 0.85% NaCl containing 1 mM sodium acetate, pelleted again and the cellular lipids were extracted by the method of Bligh and Dyer (5). The lipid extracts were saponified at 80° C in 15% KOH in 50% ethanol for one hour and the non-saponifiable lipids extracted with hexane. The hexane extracts were dried in scintillation vials, 10 ml of Packard Opti-fluor was added to each vial and the sample radioactivity was measured in Packard model 2200CA scintillation analyzer. Incorporation of [^{3}H]-methylcholine was determined by a similar procedure. In this case 1 mM choline chloride in 0.85% NaCl was used for stopping the incorporation and washing the cells. Also, the saponification step was omitted. Thymidine incorporation was measured using 1.5 μCi of [methyl-^{3}H] thymidine per ml of cell suspension. The incorporation of label was determined in the trichloroacetic acid-precipitable fraction. The incorporation of L-[4,5-^{3}H(N)] leucine was measured in both the whole cells and in the trichloroacetic acid precipitable cell fraction using 1.5 μCi of choline per ml of cell suspension.

RESULTS

The results of a preliminary experiment examining the effects of five isoprenoids on growth of CEM-C1 cells are shown in Table I. It is evident that after 72 hours of

Table I

Effects of five isoprenoids on growth of CEM-C1 cells

Compound	Concentration (μM)	Growth after 72 hours (percent control)
Farnesol (*trans-trans*)	22.5	68
	31.5	45
Nerolidol (mixed isomers)	9.0	91
	45.0	71
Geraniol	22.5	102
	45.0	108
Dolichols (mixture C_{80}-C_{105})	22.5*	106
	45.0*	109
Squalene	22.5	106
	45.0	110

The cells were preincubated in serum-free Iscove's medium for 24 hours. The cells were then incubated in the presence of the indicated concentrations of individual isoprenoids and counted after 72 hours of incubation.
*Calculated based on M.W. of C_{90}.

incubation only farnesol had a distinctly inhibitory effect on proliferation of these cells. Based on the analysis of cell size distribution there was no evidence of cell destruction by farnesol at concentrations used in this experiment. At concentrations of 40 μM or higher considerable cell fragmentation was observed both microscopically and by cell volume distribution in Coulter Channelyzer. To assess the growth inhibitory effects in more detail, the cells were counted up to 72 hours of incubation. The results (Fig. 1) show that farnesol effect was related to the inhibition of cell proliferation rather than cell lysis. The relationship between concentration of farnesol and growth of cells shown in Fig. 2 indicates good linear relationship between farnesol concentration and growth inhibition. In order to obtain some insight into the temporal sequence of biochemical events preceding growth inhibition, CEM-C1 cells were pulsed with several compounds after relatively short incubation (½, 1½, and 3 hours) in the presence of 22.5 μM farnesol, followed by one hour pulse with radiolabeled choline, thymidine, acetate or leucine. The incorporation of the label was measured in the total lipid fraction (choline), trichloroacetic acid precipitable fraction (thymidine and leucine), non-saponifiable lipid fraction (acetate) and total unbroken cells (leucine). The results (Fig. 3) showed a very marked inhibition of choline incorporation into cellular lipids that was evident almost immediately. In contrast, the incorporation of other intermediates was either delayed or was much smaller. It should be noted that the inhibition of choline incorporation by farnesol was enhanced by

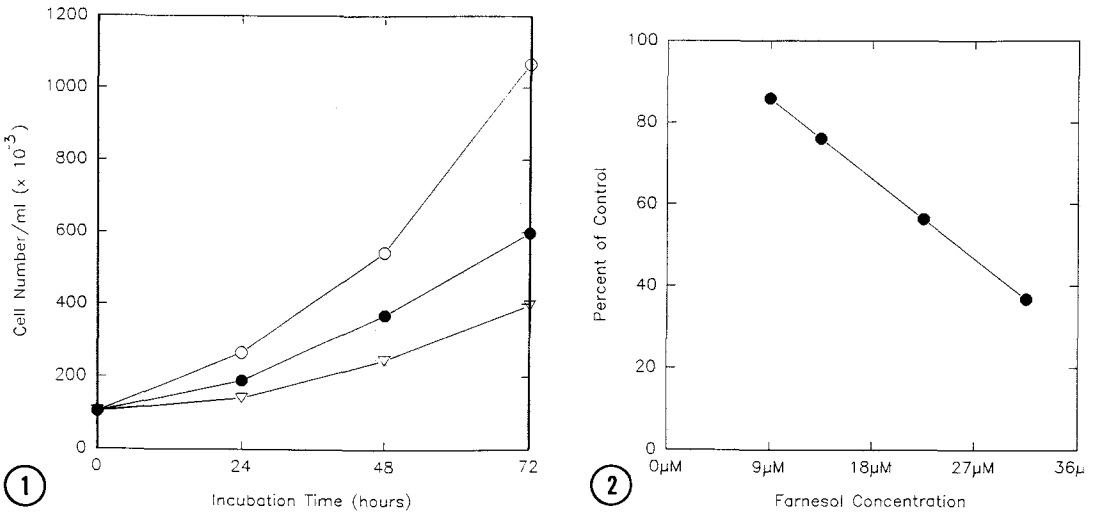

Fig. 1. Growth inhibition of CEM-C1 cells by farnesol. Cells preincubated for 24 hours in serum-free Iscove's medium were treated with either 22.5 μM farnesol (●) or 31.5 μM farnesol (▽); control = ○. Cells were counted after 24, 48, and 72 hours of incubation. Standard deviation based on triplicate cultures were less than 5.5% of the mean.

Fig. 2. Concentration dependence of growth inhibition of CEM-C1 cells by farnesol after 72 hours of incubation. Growth conditions the same as in Fig. 1. The slope was calculated by linear regression. The correlation coefficient = 0.999.

preincubating cells in Iscove's medium for 3 days prior to the addition of farnesol (data not shown). Growth inhibition by farnesol was examined also in the glucocorticoid sensitive line CEM-C7 and human promyelocytic line HL-60 (Fig. 4). The CEM-C7 cells were somewhat more resistant and the HL-60 cells were more sensitive to farnesol than the CEM-C1 line. Further experiments with other cell lines, both grown in suspension and anchorage-dependent did not reveal any consistent pattern of resistance or sensitivity.

Since most cells in culture require choline for synthesis of choline phospholipids (6), the remarkable inhibition of choline incorporation indicated that farnesol might interfere with one or more steps in the biosynthetic sequence leading to phosphatidylcholine. This was suggested further by the results of another experiment in which cells were grown for 72 hours in 22.5 μM farnesol and increasing levels of phosphatidylcholine. As shown in Fig. 5a, phosphatidylcholine greatly diminished growth inhibition by farnesol. Similar increase in resistance to farnesol toxicity was seen with diacylglycerol (Fig. 5b) although the results of the experiment were complicated by the apparent stimulation of growth by diacylglycerol alone (about 20% above control, data not shown).

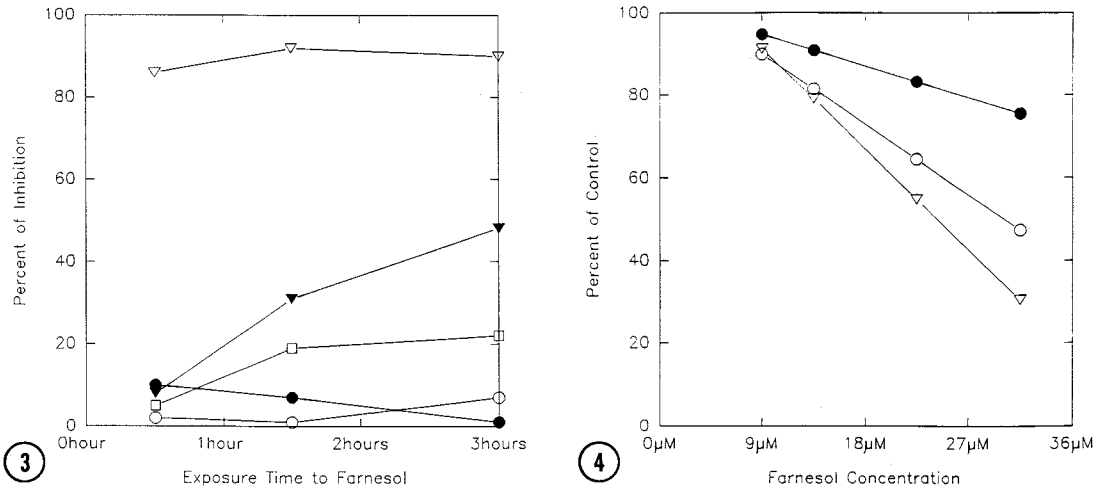

Fig. 3. Effect of farnesol on incorporation of metabolic precursors. CEM-C1 cells preincubated in Iscove's medium for 24 hours were incubated in the presence of 22.5 μM farnesol for the indicated time intervals. At each point they were pulsed for one hour with the following precursors: [methyl-^{3}H] thymidine (▼), [2^{14}C] acetic acid (□), [methyl-^{3}H] choline chloride (▽) and L-[4,5-^{3}H(N)] leucine (whole cells ●, TCA precipitate ○). Labeling was terminated and the cells extracted as described in Materials and Methods. Each point is a mean of three cultures. The SD was less than 15% of the mean.

Fig. 4. Comparison of three cell lines grown in suspension cultures in their sensitivity to farnesol after 48 hours of incubation. The cell lines included CEM-C1 cells (○), CEM-C7 cells (●), and HL-60 cells (▽). Growth conditions were the same as in Fig. 1. The slopes were calculated by linear regression with the correlation coefficients of 0.990, 0.931 and 0.962 respectively.

DISCUSSION

The results of these experiments do not support the idea that farnesol-induced inhibition of cell growth is the primary result of interference with biosynthesis of mevalonate products. As shown in Fig. 3 acetate incorporation into non-saponifiable lipids was affected by farnesol to a much lesser extent than the incorporation of choline. Although, it is possible that uptake of farnesol might ultimately lead to increases in membrane fluidity, similar to those observed in fungi treated with geraniol, and thus cause cell death, it is more probable that farnesol affects primarily the biosynthesis of phosphatidylcholine via Kennedy pathway (7). This idea is particularly interesting in context of the results from the group of Vance (8) indicating that diacylglycerol plays a role in the translocation of CTP:choline phosphate cytidylytransferase from the cytosol where it is inactive to the membrane fraction where this enzyme assumes its active form. Recent data by Slack et al. (9) show that such activation may be brought about also by the addition of exogenous phospholipase C which generates diacylglycerol. Thus it appears that

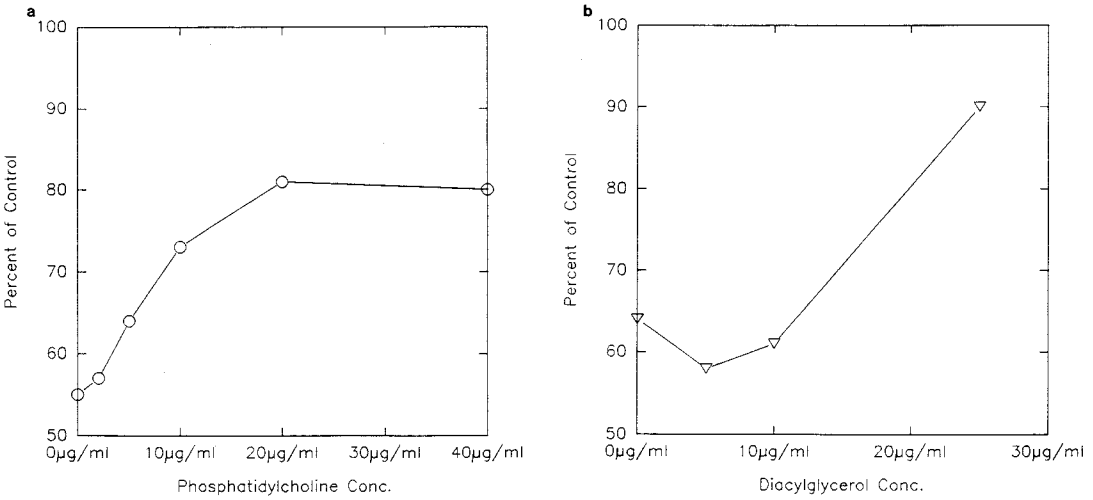

Fig. 5a. Partial reversal of growth inhibition by farnesol by co-incubation of CEM-C1 cells with phosphatidylcholine. Incubation conditions the same as described for Fig. 1. The percent of control was determined using the means of three cultures. The SD was less than 6% of the mean.

Fig. 5b. Partial reversal of growth inhibition by farnesol by co-incubation of CEM-C1 cells with diacylglycerol. Incubation conditions the same as described for Fig. 1. The percent of control was calculated using the means of triplicate cultures with SD less than 6.5% of the mean.

elucidation of the role of farnesol in the biosynthetic process of the major constituent of plasma membrane is of considerable potential value.

ACKNOWLEDGMENTS

This work was supported by the grant CA08315 from the National Cancer Institute and by the Department of Veterans Affairs. We thank Dr. E.B. Thompson for providing us with CEM-C1 and CEM-C7 cells. Excellent typing of the manuscript by Ms. Joyce Capps is gratefully acknowledged.

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