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HL-60 CELLS BECOME RESISTANT TOWARDS ANTITUMOR ETHER-LINKED PHOSPHOLIPIDS FOLLOWING DIFFERENTIATION INTO A GRANULOCYTIC FORM

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1-O-Alkyl-2-O-methyl-sn-glycero-3-phosphocholine (alkylmethoxy-GPC) exerts a highly selective cytotoxic activity towards a variety of tumor cells that is not seen in normal cells. Human promyelocytic leukemia (HL-60) cells are particularly sensitive to this cytotoxic action. In this report we show that when HL-60 cells are differentiated into a granulocytic form by dimethylsulfoxide (Me_2SO) they become resistant toward the cytotoxic effects of alkylmethoxy-GPC. Also, after short-term exposures of the HL-60 cells to alkylmethoxy-GPC, the uptake of [methyl-^{3}H]choline is inhibited in the undifferentiated cells, but not in those differentiated with Me_2SO. Thus, cellular choline uptake appears to be a useful index for assessing the susceptibility of cells to the cytotoxic effects of antitumor phospholipids. [^{3}H]Alkylmethoxy-GPC is poorly metabolized by both cell populations as is evident by the trace quantities of labeled metabolites formed; also, alkylmethoxyglycerols do not exert any cytotoxic activity toward undifferentiated cells. These results demonstrate that differences in the cytotoxic response of sensitive (undifferentiated) and resistant (differentiated) cells to alkylmethoxy-GPC are not due to differences in their ability to metabolize alkylmethoxy-GPC or to a phospholipase C-generated toxic metabolite. Instead the data support our earlier hypothesis that the antitumor action of alkylmethoxy-GPC is, at least in part, caused by an impaired transport of small molecules across the membrane of sensitive cells. © 1988 Academic Press, Inc.

Extensive studies have clearly shown that a number of unnatural ether-linked phospholipids possess highly selective antineoplastic activity (1). The 2-O-methyl-substituted analog of platelet activating factor, 1-O-alkyl-2-O-methyl-sn-glycero-3-phosphocholine (alkylmethoxy-GPC), exhibits direct antiproliferative and cytotoxic activity towards a variety of tumor cells in culture (2,3), whereas normal cells such as human polymorphonuclear neutrophils are highly resistant (4). Human promyelocytic leukemia (HL-60) cells are particularly sensitive to the lethal effects of alkylmethoxy-GPC (4,5), although resistant lines of cancer cells have also been described (3,6). Also of interest is that sub-cytotoxic levels of alkylmethoxy-GPC can induce differentiation of HL-60 cells into granulocytes and monocytes (7).

A number of investigations with sensitive cells have indicated alkylmethoxy-GPC perturbs membrane structures (8-10), modifies phospholipid

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metabolism (11-14), and inhibits nutrient transport (14). Moreover, the fact that alkylmethoxy-GPC inhibits protein kinase C activity (13,15-17) and alters cellular protein phosphorylation patterns (13,18) suggests a stimulus-response coupling mechanism might be involved in the response of sensitive cells to the methoxy analog.

Our studies show that when HL-60 cells are differentiated into granulocytes with dimethyl sulfoxide (Me_2SO) they become resistant toward the cytotoxic action of alkylmethoxy-GPC and, therefore, exhibit a response similar to what is seen in normal cells. Also, the inhibition of [^{3}H]choline uptake observed only in the undifferentiated cells treated with the methoxy analog supports the use of choline uptake as an index for measuring the degree of the cytotoxic response elicited by the antitumor phospholipid agents.

MATERIALS AND METHODS

[^{3}H]Alkylmethoxy-GPC (1-[^{3}H]alkyl-2-O-methyl-sn-glycero-3-phosphocholine), containing mixed C-16 and C-18 chain lengths, was purchased from Amersham, Arlington Heights, IL. It was 95% radiopure based on thin-layer chromatography on Silica Gel H layers developed in chloroform/methanol/acetic acid/water;(50/30/8/4, v/v) and was used without further purification. [Methyl-^{3}H]choline chloride was purchased from Dupont (New England Nuclear), Boston, MA. We obtained unlabeled alkylmethoxy-GPC (1-O-octadecyl-2-O-methyl-sn-glycero-3-phosphocholine), choline chloride, formyl-Nle-Leu-Phe-Nle-Tyr-Leu, Silica Gels G and H, and the lipid chromatography standards from Sigma Chemical Co., St. Louis, MO. The 1-octadec-9'-10'-enyl-2,3-diacetyl-sn-glycerol was a gift from Western Chemical Industries, Vancouver, British Columbia. Other commercial products included Vitride [$NaAlH_2(OCH_3CH_2OCH_3)_2$] from Eastman Organic Chemicals, Rochester, NY, Optifluor aqueous counting scintillant from Packard Instrument Company, Downers Grove, IL, and cell culture media from Grand Island Biological Co., Grand Island, NY.

1-Alkyl-2-O-methyl-sn-glycerol, prepared by Vitride reduction (19) of alkylmethoxy-GPC, was purified by chromatography on Silica Gel G layers developed in chloroform/methanol (98/2, v/v). The product migrated close to that of a 1-octadec-9'-10'-enyl-2-acetyl-glycerol standard (20) on Silica Gel G chromatoplates developed in hexane/ether/methanol/acetic acid (70/30/5/1, v/v).

Cell Culture: HL-60 cells from the American Type Culture Collection (Rockville, MD) were propagated at 37°C in a humidified 5% CO_2/95% air atmosphere. The culture density was maintained between 2 x 10^5 and 2 x 10^6 cells/ml by transferring the cells every 3-4 days to fresh RPMI-1640 medium containing 10% fetal bovine serum (heat-inactivated), penicillin (100 units/ml), streptomycin (100 μg/ml), and L-glutamine (4 mM). The doubling time for these cultures was 25-30 h. Cells were induced to differentiate by adding Me_2SO (1.3%) to the cultures (21) and the extent of differentiation assessed by reduction of nitroblue tetrazolium (NBT) dye (22). For the NBT reduction assays, cells were suspended in a Hepes-buffered salt solution (23) at 37°C and exposed to 5 μg/ml cytochalasin B for 10 min followed by 1 μM formyl-Nle-Leu-Phe-Nle-Tyr-Leu for 15 min. The proportion of differentiated cells determined by this method typically increased from approximately 5% before induction to 65-75% after a 5-day exposure to Me_2SO. Trypan-blue dye (0.1%) exclusion (24) revealed greater than 95% cell viability at the start of all experiments. Cells were counted in a hemacytometer; adherent cells were suspended by repeated pipeting immediately before enumeration.

Cytotoxicity Assays: Undifferentiated and Me_2SO-differentiated HL-60 cells were removed from their culture media by centrifugation (700xg for 5 min), washed once, and suspended to 1.0 x 10^6 cells/ml in fresh media

containing fetal bovine serum. The suspensions (1 ml) were placed into 16-mm well culture dishes for treatment with alkylmethoxy-GPC; the concentration of the alkymethoxy-GPC solvent (ethanol) in the incubation did not exceed 1% and had no apparent effects. The IC_{50} value was defined as the concentration of alkylmethoxy-GPC that reduced the viable cell number by one-half during a 24-h period (5).

[^{3}H]Alkylmethoxy-GPC Labeling: Undifferentiated and Me_2SO-differentiated HL-60 cells were removed from the cultures, washed, and suspended to 2.0 x 10^6 cells/ml in fresh media containing fetal bovine serum; 1-ml aliquots were then placed in 16-mm culture wells. [^{3}H]Alkylmethoxy-GPC (0.1-0.2 μCi) plus unlabeled alkylmethoxy-GPC (0.25 - 15.0 μM) were added to the cells in 10 μl ethanol. After a 4-h or 20-h labeling period, the suspensions were centrifuged (11,500xg for 30 s) and the pelleted cells washed three times with phosphate-buffered saline (PBS). The washed cells were suspended in PBS and samples were removed for determination of cell number and radioactivity. For analyses of [^{3}H]alkylmethoxy-GPC metabolites, cells, including the media, were extracted by a modified Bligh and Dyer (25) procedure, with the methanol containing 2% acetic acid. Recovery of tritium in the lipid extracts (84$\pm$3%) was the same for both differentiated and undifferentiated cultures and for incubations with cell-free culture medium.

Phospholipids were separated on Silica Gel H layers developed in chloroform/methanol/acetic acid/water (50/30/8/4, v/v) and neutral lipids were resolved on Silica Gel G layers developed in hexane/ether/methanol/acetic acid (70/30/5/1, v/v). Specific areas of the chromatograms corresponding to lipid standards were removed and the distribution of tritium determined by liquid scintillation spectrometry.

Protein was measured by the procedure of Lowry, et al. (26), with bovine serum albumin as the standard.

[Methyl-^{3}H]choline Uptake: Undifferentiated and Me_2SO-differentiated HL-60 cells were removed from the cultures, washed, and suspended in (1.0 x 10^6 cells/ml) in fresh media containing fetal bovine serum. Equal portions of cells were incubated for 4 h with either 12.5 μM alkylmethoxy-GPC or 0.625% ethanol (control). The suspensions were centrifuged (700xg for 5 min), washed twice in Hank's balanced salt solution, and suspended (2.0 x 10^6 cells/ml) in choline-free RPMI-1640 medium buffered with 20 mM Hepes (pH 7.3). The cells were preincubated at 37°C for 10 min and then [methyl-^{3}H]choline (final concentration, 10 μM; sp. act. 0.2 Ci/mmol) was added to initiate the uptake measurements. Aliquots were removed at 5-min intervals, centrifuged (11,500xg for 30 s), and the pelleted cells washed twice with PBS containing 0.1 mM choline chloride. The washed cells were suspended in PBS and the radioactivity measured; blank values, obtained from identical incubations done in an ice bath were subtracted from each of the experimental values.

RESULTS AND DISCUSSION

Our results show that the increased resistance of HL-60 cells to the cytotoxic effects of alkylmethoxy-GPC is clearly related to the degree of cellular differentiation as seen from the correlation of the recovery of viable cells following a 24-h exposure to alkylmethoxy-GPC with the capacity of the cells to reduce NBT (Fig. 1). Maximum resistance of the HL-60 cells toward alkylmethoxy-GPC cytotoxicity occurred at 5 d after exposure to Me_2SO. A 24-h exposure of undifferentiated cells to alkylmethoxy-GPC in the presence of 10% fetal bovine serum yielded a dose-dependent cytotoxic response with an IC_{50} value of 6 μM (Fig. 2), whereas the IC_{50} value in the Me_2SO-differentiated HL-60 granulocytes was 30 μM. At this high concentration of alkylmethoxy-GPC, greater than 90% of the undifferentiated HL-60 cells were lysed.

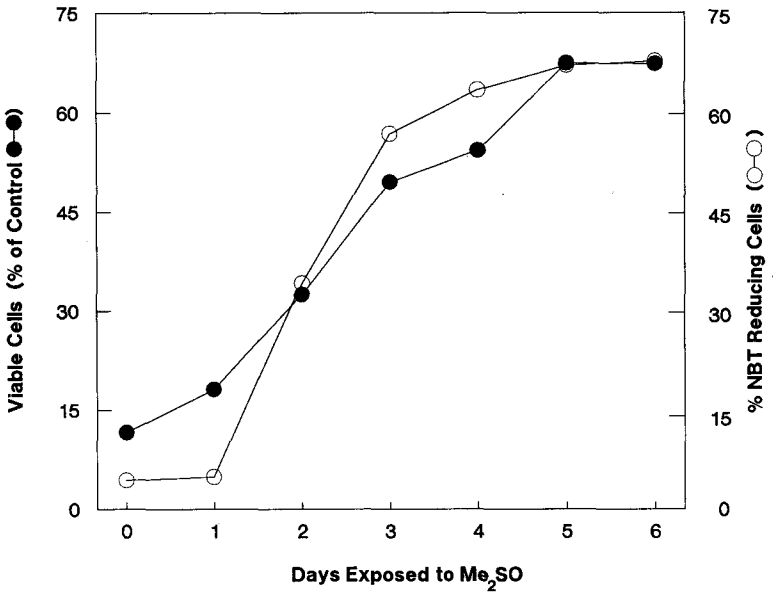

Fig. 1 Development of resistance of HL-60 cells toward alkylmethoxy-GPC-mediated cytotoxicity as a function of differentiation to a granulocytic form by Me_2SO. Cells were removed from culture 1-6 d following the addition of Me_2SO for assay of NBT reduction and cytotoxicity of 25 μM alkylmethoxy-GPC. Each value corresponds to the average of duplicate determinations and is representative of three separate experiments.

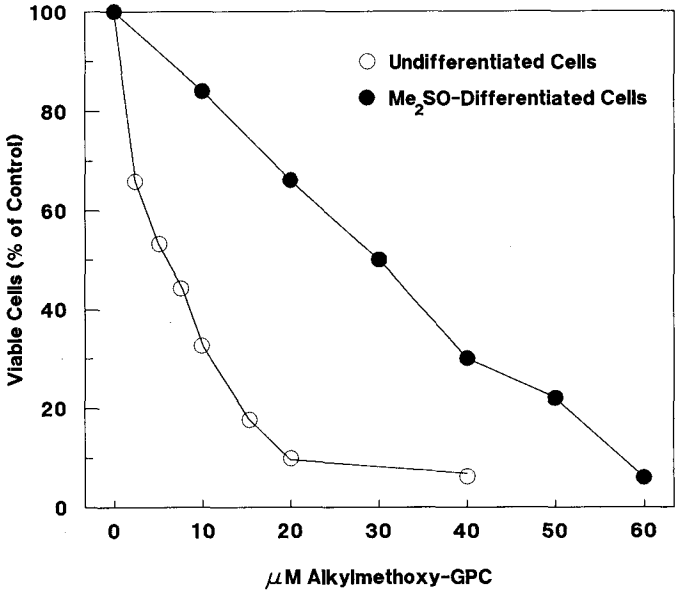

Fig. 2 Cytotoxicity of alkylmethoxy-GPC toward undifferentiated and Me_2SO-differentiated HL-60 cells. Each value corresponds to the average of duplicate determinations and is representative of three separate experiments.

Several reports indicate that the sequestration of ether-linked phospholipids in cell surface membranes is an important contributing factor in the cytotoxic mechanism (9,14,27). We found Me_2SO-differentiated HL-60 granulocytes treated with [3H]alkylmethoxy-GPC (12.5 μM) for 4 h contained smaller amounts ($p<0.05$, based on paired experimental values) of the cytotoxic phospholipid (791 $\pm$ 249 pmol/10^6 cells, n=6) than the undifferentiated HL-60 cells (1547 $\pm$ 480 pmol/10^6 cells, n=8); under these experimental conditions no change in cell viability was apparent. The Me_2SO-differentiated HL-60 cells also contained less tritium when incubated with low concentrations (0.5 μM) of [^{3}H]alkylmethoxy-GPC for either 4 or 20 h. Although statistically significant ($p<0.01$), differences between the undifferentiated and differentiated cells were less apparent for each of the conditions tested when labeling was expressed on either a protein or phospholipid basis since these constituents are considerably lower in the differentiated HL-60 granulocytes in comparison to the undifferentiated cells (23). These results are consistent with those reported earlier for comparisons between polymorphonuclear neutrophils and HL-60 cells (27).

Hoffman, et al. (14,27) reported alkylmethoxy-GPC is poorly metabolized regardless of the cytotoxic response of cells to this phospholipid and also that it is not a substrate for the alkyl monooxygenase that cleaves the ether bond. In addition, they (27) found the activities of the cleavage enzyme in different cell types did not correlate with the cytotoxic response. The current work clearly shows that Me_2SO-induced differentiation does not alter the limited ability of HL-60 cells to metabolize alkylmethoxy-GPC. After an 18-h incubation of HL-60 cell suspensions with either cytotoxic (15 μM) or sub-cytotoxic (0.5 μM) levels of [3H]alkylmethoxy-GPC, greater than 93% of the tritium found in lipid extracts remained with the parent compound in both the undifferentiated and differentiated cells. This result further supports the notion that differences in the metabolism of the antitumor phospholipid between sensitive and resistant cells do not contribute to its highly selective antitumor response.

As detected in earlier experiments (27), labeled 1-alkyl-2-acyl-sn-glycerophosphocholine was formed to a limited extent (<1% of tritium) in both the undifferentiated and Me_2SO-differentiated cells; it presumably orginates after oxidation of the 2-O-methyl moiety and a subsequent acylation step. Trace amounts of labeled 1-alkyl-2-O-methylglycerol also were found in our experiments and could have been formed via a phospholipase C (27) or through the combined actions of a phospholipase D and a phosphohydrolase. Based on indirect evidence, Fleer et al. (28-29) proposed a phospholipase C-dependent cleavage of the methoxy analog is required for its cytotoxic activity. This is an attractive hypothesis since 1-alkyl-2-O-methyl-glycerol has previously been identified as a minor alkylmethoxy-GPC metabolite in HL-60,

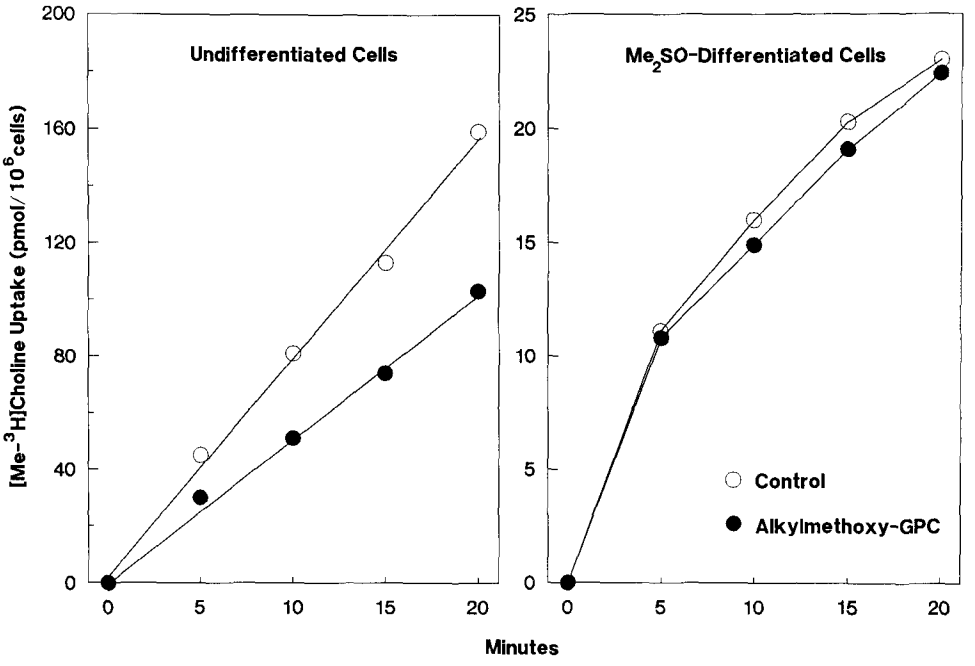

Fig. 3 Effect of alkylmethoxy-GPC on [methyl-^{3}H]choline uptake by undifferentiated and differentiated (granulocytic form) HL-60 cells. These values are representative of two separate experiments.

PMN (27), MDCK, K562 (30), Ehrlich ascites (31) and mouse MO_4 fibrocarcinoma cells (32), and it is known to inhibit protein kinase C in vitro (32). However, in our experiments 1-alkyl-2-O-methyl-glycerol (up to 25 μM) was not cytotoxic toward the undifferentiated HL-60 cells. We believe these data rule out any role for a phospholipase C activity as a factor in the cytotoxic mechanism of the antitumor ether-linked phospholipids.

Inhibition of cell transport processes appears to be a general and early phenomenon observed in sensitive cells exposed to alkylmethoxy-GPC (14). In our studies, treatment of undifferentiated HL-60 cells for 4 h with 12.5 μM of the methoxy analog inhibited the initial rate of [methyl-^{3}H]choline rate by 35% (Fig. 3) without affecting the proportion of cells that excluded Trypan-blue dye. After differentiation of the HL-60 cells into the granulocytic form, [methyl-^{3}H]choline was taken up at a significantly lower rate than the undifferentiated cells, but the uptake was unaffected by the methoxy analog (12.5 μM).

Results from our studies demonstrate that the chemical-induction of differentiation in HL-60 cells to a granulocytic form causes them to become resistant towards the cytotoxic action of alkylmethoxy-GPC. Therefore, since comparisons can be made between sensitive and resistant cells of the same cell line, the HL-60 system described in this report should be an extremely useful

model for future investigations of the detailed molecular mechanism(s) responsible for the selective cytotoxic action of alkylmethoxy-GPC. In addition, inhibition of the uptake of [^{3}H]choline observed in the undifferentiated cells treated with alkylmethoxy-GPC and the lack of inhibition in the differentiated cells further supports our earlier concept that indicates the antitumor action of alkylmethoxy-GPC is, at least in part, due to an impaired transport of molecules across the plasma membrane of sensitive cells.

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