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Differentiation-Promoting Effect of 1-O (2 Methoxy) Hexadecyl Glycerol in Human Colon Cancer Cells

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Alkylglycerols are naturally occurring bioactive ether lipids found in great abundance in the livers of many marine species. In this study, we evaluated the differentiation-promoting potential of a methoxy substituted alkylglycerol—1-O (2 methoxy) hexadecyl glycerol (MHG)—to promote a more benign or differentiated phenotype in human colon cancer cells. Three cell lines with different biological and phenotypic properties were used. They were the moderately differentiated and growth factor–responsive Moser, the growth factor–unresponsive and malignant HT29, and the poorly differentiated and growth factor–unresponsive HCT116. Treatment of these cell lines with MHG resulted in a downmodulation of cellular proliferation, a reduced propensity for anchorage-independent growth, and a reduced capacity in cellular invasion. Induction of the colon-associated and differentiation-related molecule carcinoembryonic antigen was also observed in the three cell lines. Induction of the transformation-sensitive and differentiation-related glycoprotein fibronectin was observed in the HT29 cells. It is concluded that MHG was biologically active and promoted a more benign or differentiated phenotype in these colon cancer cells. Since differentiation-inducing agents may possess chemoprevention properties, the use of MHG and the alkylglycerols in inducing differentiation or in chemoprevention of malignant diseases warrants further investigation. J Cell Physiol 178:173–178, 1999.

From the primitive protozoans to vertebrates, lipids are integral cellular components of living organisms (Kulikov and Muzya, 1997). Lipids play important functional roles in cellular physiology, and the function of a lipid may be determined by its chemical structure (Kulikov and Muzya, 1997). For example, the inositol phospholipids participate in signal transduction, transducing signals from the cell surface towards the cytoplasm and nucleus (Nishizuka, 1984; Berridge, 1984; Daniel et al., 1988; Serhan et al., 1996). Many ether phospholipids are multifunctional. They play important roles in platelet aggregation (Suzuki et al., 1998), inflammatory and immune responses (Orga et al., 1977; Ngwenya and Yamamoto, 1986; Croft et al., 1987; Oh and Jadhav, 1994; Kitchen et al., 1996), cellular proliferation (Brohult, 1960; Bennett and Birnboim, 1997; Rougier et al., 1997), and migration (Bix and Clark, 1998). Other ether lipids of different molecular structures have been shown to be cytotoxic towards cancer cells (Herrmann and Neumann, 1986; Verdonck and van Heugten, 1997; Mollinedo et al., 1997).

Alkylglycerols are naturally occurring ether lipids found in great abundance in the liver oil of many marine species of shark and fish (Hallgren and Larsson, 1962a,b; Bordier et al., 1996). Alkylglycerols are also found in the colostrum and milk of mammals (Hallgren and Larsson, 1962a,b; Orga et al., 1977). Humans have used marine species' liver oil as a diet supplement for a long time. Alkylglycerols have been reported to possess anti-tumor activities in humans, and the administration of alkylglycerols has been shown to decrease the toxic side effects of radiation therapy (Brohult et al., 1977, 1978, 1986). About 20 different types of alkylglycerols exist both in humans and in sharks, and the three most common species are the chimyl, batyl, and selachyl alcohols (Hallgren and Larsson, 1962; Hallgren et al., 1978). Methoxy-substituted alkylglycerols constitute about 2% of alkylglycerols (Hallgren et al., 1978).

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The malignant phenotype is not irreversible. Many differentiation-inducing agents can promote or restore a more normal or benign phenotype to malignant cells (Breitman et al., 1980; Heby, 1981; Langdon et al., 1988; Hoosein et al., 1988; Fatini et al., 1990; Chakrabarty et al., 1990; Varani and Chakrabarty, 1990; Levine and Chakrabarty, 1992). Human colon cancer cells respond to differentiation induction and assume a more normal cellular phenotype (Hoosein et al., 1988; Chakrabarty et al., 1990; Fatini et al., 1990). The induction of a more normal or differentiated phenotype in colon cancer cells is associated with a reduction in cellular proliferation, reduction in anchorage-independent growth and cellular invasion, and an increase in the production of the differentiation-related molecules carcinoembryonic antigen (CEA) and fibronectin (Denk et al., 1972; Niles et al., 1988; Chakrabarty et al., 1989, 1990; Fatini et al., 1990; Huang and Chakrabarty, 1994).

In this study, we evaluated the potential of a methoxy-substituted alkylglycerol—1-O (2 methoxy) hexadecyl glycerol (MHG)—to promote a more differentiated phenotype in human colon cancer cells. In view of the heterogeneity of colon cancer (Brattain et al., 1984), three human colon cancer cell lines with different phenotypic properties were chosen in this study. These were the moderately differentiated and growth factor–responsive Moser (Huang et al., 1992), the growth factor–unresponsive and highly tumorigenic HT29 (Huang et al., 1992; Schroy, 1994), and the growth factor–unresponsive and poorly differentiated HCT116 (Brattain et al., 1984; Huang et al., 1992).

MATERIALS AND METHODS

Reagents

1-O (2-methoxy) hexadecyl glycerol (MHG) was a generous gift from Scandinavian Pharmaceuticals Inc. (Perkasie, PA). MHG was dissolved in a minimal volume of DMSO and diluted to a stock solution of 1 mg/ml in serum-free culture medium. 3-(4,5-dimethyl-thiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and trypan blue dye were purchased from Sigma (St. Louis, MO). Low melting Agarose was purchased from Gibco/BRL (Gaithersburg, MD). Matrigel invasion chambers were purchased from Becton Dickinson (Franklin Lakes, NJ). Anti-human fibronectin polyclonal antibody was purchased from DAKO (Carpinteria, CA).

Cell lines

Human colon cancer cell lines Moser, HT29, and HCT116 cells were cultured in McCoy's 5A medium supplemented with 5% fetal calf serum as previously described (Chakrabarty et al., 1989; Huang and Chakrabarty, 1994). Moser and HCT116 cells were originally obtained from Dr. Michael G. Brattain (Department of Surgery, University of Texas Health Science Center at San Antonio). HT29 cells were purchased from the American Type Culture Collection (Rockville, MD). These cell lines were maintained in 25 cm^2 flasks at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Growth inhibition in monolayer culture

Cells were seeded into 96-well culture plates (200 μl/well at a density of 20,000 cells/ml) and allowed to attach overnight. The medium was then replenished with fresh medium containing different concentrations of MHG. Six replicate determinations for each concentration were performed in one experiment. Cells cultured in medium without MHG served as controls. The cells were then incubated for 3 additional days, and the number of viable cells in each well was estimated by the MTT assay as previously described (Huang et al., 1992). Briefly, 40 μl of MTT (2 mg/ml) solution was added to each well of the culture plates, and the plates were incubated for 3 h at 37°C to allow for the formation of tetrazolium crystals. The solution in each well was then carefully removed with a 25 G needle attached to a vacuum source and the crystals dissolved in 200 μl DMSO. The optical density (OD) of the solution in the wells was then determined by a microplate reader at 570 nm. The % growth inhibition was determined by the formula:

$$\frac{\text{OD untreated cells} - \text{OD treated cells}}{\text{OD untreated cells}} \times 100\%$$

Analyses of CEA and fibronectin

Cells were seeded into 25 cm^2 flasks and allowed to attach overnight. The medium was then replenished with fresh medium (serum-free) containing various concentrations of MHG. Cells cultured in medium without MHG served as controls. The cells were then incubated for 3 additional days at 37°C. Serum-free conditioned medium was then used for the analyses of CEA and fibronectin. Analysis of human CEA was kindly performed by the clinical chemistry laboratory at M.D. Anderson Cancer Center using a human CEA assay kit (Ciba-Corning, Medfield, MA). Analysis of fibronectin was performed by an enzyme-linked immosorbent assay using polyclonal anti-human fibronectin antibody as previously described (Huang and Chakrabarty, 1994). CEA and fibronectin concentrations were normalized to nanograms/10^6 cells.

Anchorage-independent growth and cellular invasion

Cells seeded into 25 cm^2 flasks and treated with MHG as described above but in serum-containing culture medium were used in the anchorage-independent growth and cellular invasion assays as previously described (Chakrabarty et al., 1995).

Anchorage-independent growth. Healthy, viable, untreated control or treated cells (5,000 cells/dish) were seeded into 0.35% soft agarose cloning medium (McCoy's 5A medium supplemented with 10% fetal calf serum) in 60 × 15 mm Petri tissue culture dishes. After 10 days of incubation at 37°C, the colonies were stained with p-iodonitrotetrazolium violet and counted manually with the aid of a magnifying glass.

Invasion of matrigel-coated porous membranes. Healthy, viable control and treated cells were collected, washed with serum-free medium, and then diluted to a concentration of 250,000 cells/ml in the same. A 200 μl volume (50,000 cells) was seeded into

each rehydrated invasion chamber immersed in chemoattractant (NIH3T3 conditioned medium). After 4 days of incubation at 37°C, the bottoms of the chambers were scraped to collect cells that had invaded through the membranes and pooled with cells that had fallen through into the bottom chambers. The total number of invaded cells was then determined by counting with the aid of a hemocytometer. The effect of each treatment with its corresponding control was performed in duplicate chambers. The results presented are an average of two independent experiments.

RESULTS

Since most differentiation-inducing agents have an antiproliferative effect in malignant cells (Denk et al., 1972; Breitman et al., 1980; Heby, 1981; Hoosein et al., 1988; Langdon et al., 1988; Niles et al., 1988; Chakrabarty et al., 1990; Fatini et al., 1990; Varani and Chakrabarty, 1990; Levine and Chakrabarty, 1992), we first determined the antiproliferative effect of MHG on the colon cancer cell lines. MHG inhibited the growth of Moser, HT29, and HCT116 cells to a similar degree (Fig. 1). Maximal inhibition of cell growth (80% inhibition) was observed at 25 μM concentration. Increasing the concentration of MHG did not lead to further increase in growth inhibition (Fig. 1).

The poorly differentiated HCT116 cells produced the least amount of CEA, while the moderately differentiated Moser cells produced the highest amount of CEA and the HT29 cells produced an intermediate amount of CEA (Fig. 2). The induction of differentiation in human colon cancer cells is associated with an upregulation of production of this colon-associated and differentiation-related CEA molecule (Denk et al., 1972; Hoosein et al., 1988; Niles et al., 1988; Chakrabarty et al., 1990; Varani and Chakrabarty, 1990; Levine and Chakrabarty, 1992; Huang et al., 1994). Therefore, we determined the effect of MHG treatment on the production of these molecules. MHG upregulated the production of CEA in all three cell lines (Fig. 2). The moderately differentiated Moser cells produced CEA in the amount of 5 ng/10^6 cells. An increase in CEA production was observed when the cells were treated with either 10, 25, or 50 μM MHG (Fig. 2A). Treatment with 50 μM MHG upregulated CEA production to above 8 ng/10^6 cells. The HT29 cells produced slightly less CEA (4 ng/10^6 cells) than the Moser cells and responded to all concentrations of MHG tested by increasing CEA production (Fig. 2B). Maximal CEA induction (8 ng/10^6 cells) was observed when the cells were treated with 25 μM MHG (Fig. 2B). The poorly differentiated HCT116 cells produced the least amount of CEA (1–2 ng/10^6 cells) (Fig. 2C). Treatment of these cells with 25 μM MHG nevertheless upmodulated CEA production (3–4 ng/10^6 cells) (Fig. 2C).

Compared to normal fibroblasts or other transformed cells, human colon cancer cells produce a relatively low amount of fibronectin (Varani et al., 1991). The induction of differentiation in transformed fibroblasts or colon cancer cells, however, is associated with an upregulation of fibronectin production (Hoosein et al., 1988; Chakrabarty et al., 1989, 1990; Varani and Chakrabarty, 1990; Levine and Chakrabarty, 1992; Huang et al., 1994; Huang and Chakrabarty, 1994; Reynolds et al., in press). Treatment

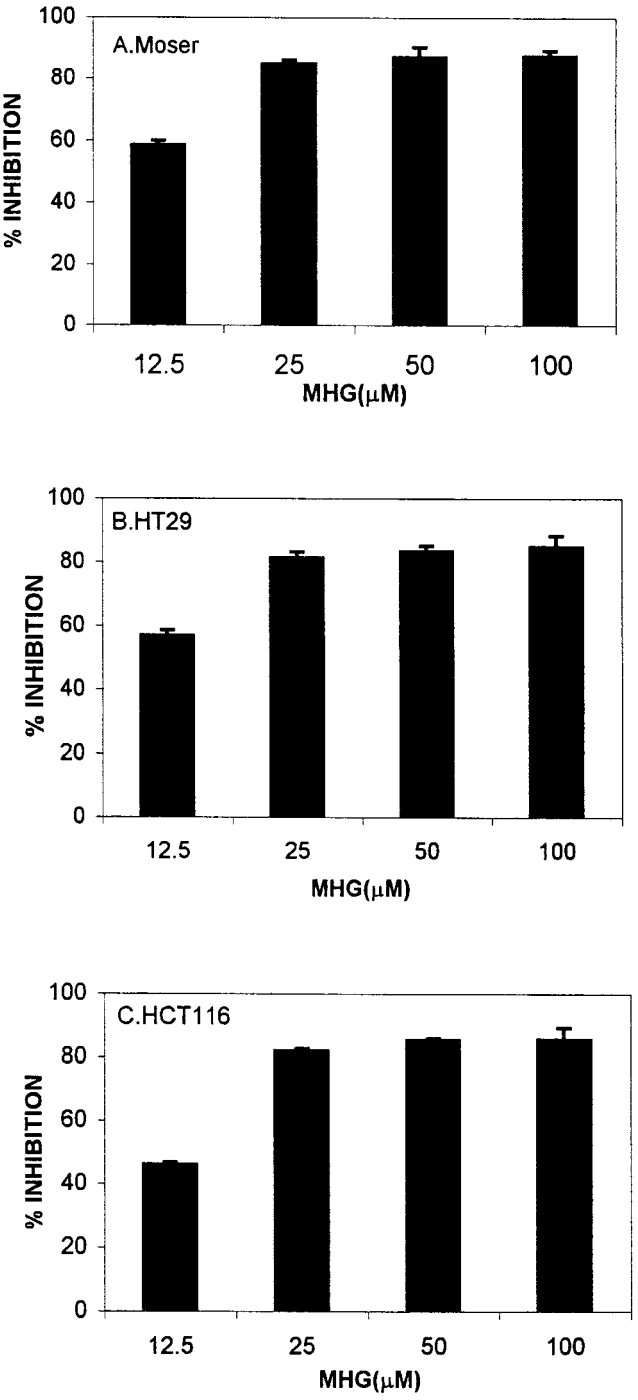

Fig. 1. Growth-inhibitory effect of MHG on human colon cancer cells. Growth inhibition was measured by the MTT assay as described in Materials and Methods. Vertical bars represent the standard error of the means obtained from five independent experiments.

of the HT29 cells with MHG upregulated fibronectin production in a dose-dependent manner (Fig. 3). However, upregulation of fibronectin production was not observed in the Moser and HCT116 cells (not shown).

The effect of MHG treatment on the malignant properties of these cells in terms of anchorage-independent

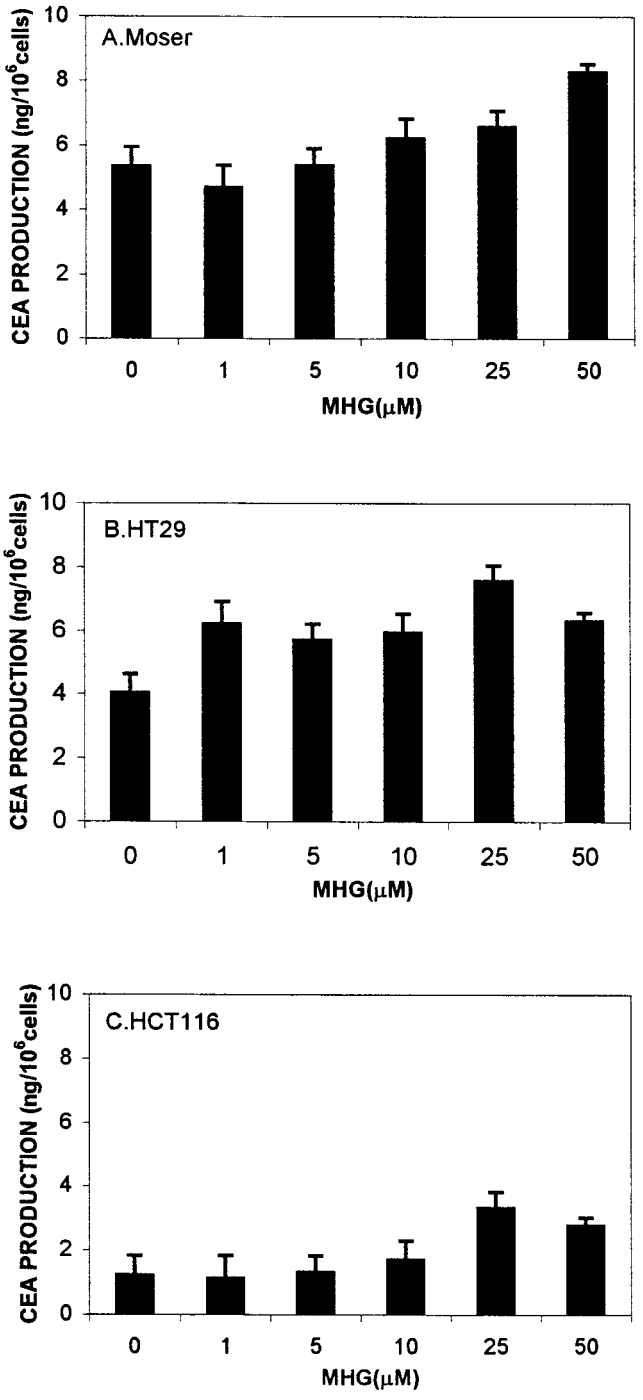

Fig. 2. Induction of CEA production by MHG in human colon cancer cells. CEA secreted into culture medium was quantitated by a human CEA assay kit and the values normalized to ng/10^6 cells as described in Materials and Methods. Vertical bars represent the standard error of the means obtained from four independent experiments.

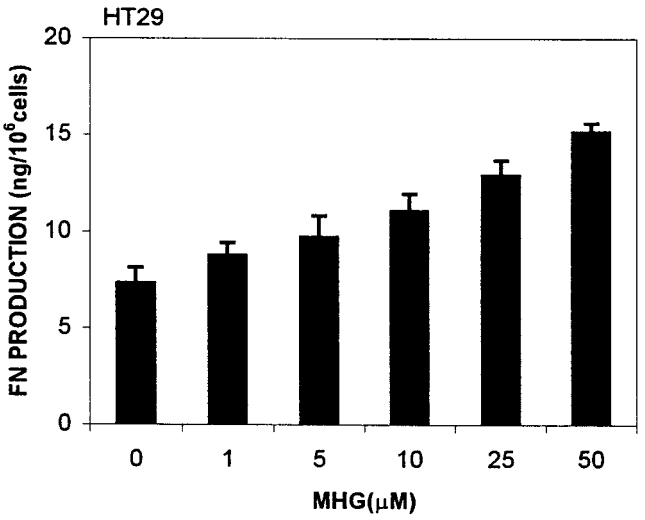

Fig. 3. Induction of fibronectin production by MHG in HT29 cells. Fibronectin secreted into culture medium was quantitated by an immunoassay and the values normalized to ng/10^6 cells as described in Materials and Methods. Each value represents the standard error of the means of three independent experiments.

TABLE 1. Effect of MHG on anchorage-independent growth[1]

Cell lines	Treatment	Number of colonies	% reduction
Moser	None	1,700 ± 63	
	25 μM	1,069 ± 129	37
	50 μM	875 ± 36	49
HT29	None	2,185 ± 152	
	25 μM	1,402 ± 166	36
	50 μM	1,234 ± 81	44
HCT116	None	2,937 ± 279	
	25 μM	1,861 ± 302	37
	50 μM	1,219 ± 169	59

[1]Healthy, viable, control and treated cells (5,000 cells/plate) were seeded into 0.35% soft agarose cloning medium and cultured for 10 days as described in Materials and Methods. The colonies were stained with p-iodonitrotetrazolium violet and counted manually with the aid of a magnifying glass. Each value represents the mean and standard error of three separate experiments.

TABLE 2. Effect of MHG on cellular invasion[1]

Cell lines	Treatment	Number of invasive cells	% reduction
Moser	None	4,976 ± 90	
	25 μM	4,844 ± 220	
	50 μM	3,969 ± 220	19
HT29	None	7,813 ± 442	
	25 μM	5,563 ± 265	29
	50 μM	5,063 ± 266	35
HCT116	None	11,875 ± 354	
	25 μM	9,625 ± 355	19
	50 μM	6,313 ± 265	47

[1]This assay was performed as described in Materials and Methods using matrigel-coated invasion chambers. Duplicate chambers were used in each experiment. The number of invasive cells was determined by counting with a hemocytometer. These results represent the mean and standard error of two independent experiments.

growth and cellular invasion was next determined. The HCT116 cells were found to be most aggressive, while the HT29 cells were intermediately aggressive and the Moser cells least aggressive in terms of these criteria (untreated controls, Tables 1, 2). MHG-treated cells showed a reduced propensity to grow in soft agarose in all three cell lines (Table 1). A similar level of inhibition (36–37%) was observed for all cell lines when treated with 25 μM MHG, and a slightly higher level of inhibition (59%) was achieved for the HCT116 cells when treated with 50 μM MHG in comparison with the Moser (49%) and HT29 (44%) cells (Table 1).

MHG-treated cells showed a reduced ability to invade a matrigel matrix (Table 2). The Moser cells did not respond to treatment with 25 μM MHG, while a slight reduction in cellular invasion was observed for the HT29 and HCT116 cells (Table 2). Treatment of these cell lines with 50 μM MHG reduced the invasive capability of the HCT116, HT29, and Moser cells by 47, 35, and 19% respectively (Table 2).

DISCUSSION

The ability of MHG to promote a more benign or differentiated phenotype was investigated in three human colon cancer cell lines with different phenotypic properties—the Moser, HT29, and HCT116. MHG inhibited the growth of these cell lines with an IC_{50} value that ranged from 11–14 μM. The Moser cell line is responsive to both growth-stimulatory and growth-inhibitory polypeptide growth factors, while the HT29 and HCT116 is not (Huang et al., 1992). Transforming growth factor (TGF) β1 inhibits cellular proliferation, upregulates CEA and fibronectin production in the Moser cells, and induces a more differentiated phenotype in these cells (Chakrabarty et al., 1989, 1990; Huang and Chakrabarty, 1994). Likewise, treatment of Moser cells with the classical differentiation-inducing chemical—retinoic acid—inhibits cell growth and upregulates CEA and fibronectin production with a concurrent induction of a less malignant phenotype (Reynolds et al., in press). Both the HT29 and HCT116 cells do not respond to the growth-inhibitory and differentiation-promoting effect of TGFβ1 (Brattain et al., 1984; Mulder and Brattain, 1989; Huang et al., 1992). HT29 cells also respond to differentiation induction by treatment with retinoic acid, which upregulates the production of both CEA and fibronectin (Reynolds et al., in press), while the responsiveness of the HCT116 cells to retinoic acid has not been characterized. Interestingly, both the Moser and HT29 cells respond to retinoic acid in the uM concentrations (Reynolds et al., in press). These cells, however, respond to other differentiation-inducing agents such as sodium butyrate and difluoromethylornithine in the mM concentrations (Reynolds et al., in press).

MHG stimulated CEA production in all three cell lines. Stimulation of fibronectin production, however, was observed only in the HT29 cells. In this regard, the HT29 cells (unlike Moser cells) responded to both retinoic acid and MHG in terms of the stimulation of CEA and fibronectin production. These differences in responses to different agents may reflect the differences in the mechanisms of action of these agents and/or the phenotypic differences in these cell lines. CEA functions in intercellular adhesion (Benchimol et al., 1989). Its functional role in the differentiation-induction process of colon cancer cells, however, is not known. In noncolon malignant cell lines, stimulation of fibronectin production has also been reported to be tightly associated with the induction of a more differentiated phenotype (Varani and Chakrabarty, 1990; Levine and Chakrabarty, 1992; Huang et al., 1994). The exact functional role of fibronectin in the differentiation-induction process of malignant colon and noncolon cells, however, is also not well understood. Like other differentiation-inducing chemicals (Breitman et al., 1980; Fatini et al., 1990; Heby, 1981; Langdon et al., 1988; Hoosein et al., 1988; Reynolds et al., in press), MHG treatment downmodulated the malignant behavior of these colon cancer cells. Interestingly, MHG appears to be more effective in inhibiting anchorage-independent growth and cellular invasion of the more aggressive HCT116 cells. The reasons behind this are not known.

Carcinogenesis may be viewed as aberrant differentiation, and many differentiation-inducing chemicals possess chemopreventive properties (Hong and Sporn, 1997). Unlike many other differentiation-inducing chemicals, the alkylglycerols constitute a class of ether lipids that naturally occur in living organisms (Hallgren and Larsson, 1962a,b; Bordier et al., 1996). We had shown that MHG, a species of alkylglycerols, was biologically active and promoted a more benign or differentiated phenotype in human colon cancer cells in vitro using uM concentrations. It is likely that these concentrations cannot be achieved in vivo, nor is it desirable. These concentrations of MHG were used in vitro in order to determine its differentiation-promoting effect in a matter of days. Chemoprevention is a long-term process (Hong and Sporn, 1997). It would be of interest to determine the long-term effect of MHG or other species of alkyglycerols using low but non-growth-inhibiting or nontoxic concentrations. These experiments are in progress. Whether the differentiation-promoting effect observed in vitro was coupled with other cellular processes such as cytostasis or apoptosis is not known and requires further studies. The molecular mechanisms of MHG in promoting differentiation are also under investigation. The use of this group of natural substances in differentiation induction of malignant cells or in chemoprevention of malignant diseases has not been thoroughly investigated. Thus, the differentiation-promoting and/or chemoprevention properties of alkylglycerols in colon cancer or other malignant diseases warrants further investigation.

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