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In Vitro Effects of Glycosphingolipids on Human Tumor Cell Proliferation (41031)[1]

PAMELA M. KIMBALL, LISA HAMMONDS, JOHN M. McKIBBIN, MICHAEL G. BRATTAIN, GREGORY GLOVER, AND MARYLA WEBB

Department of Biochemistry, University of Alabama in Birmingham, University Station, Birmingham, Alabama 35294

Abstract. A drug-resistant subpopulation of cells (HTFU) was previously isolated from the human colonic carcinoma cell line HT29. The cell lines varied significantly in *in vitro* growth patterns with HTFU cells showing contact inhibition while HT29 cells demonstrated uncontrolled growth (10). The malignant cell lines were grown in the presence of various concentrations of blood group A fucolipid, $Lewis_b$, Forssman hapten, and ceramide trihexoside for 2 weeks. Although HTFU cells demonstrated an equal or enhanced sensitivity to the lipids relative to HT29 cells, the cell lines responded in similar fashion to each lipid. Treatment with Forssman and ceramide trihexoside induced a temporary inhibition of cell proliferation, however, continued exposure to these lipids became stimulatory. Exposure to $Lewis_b$ and blood group A fucolipids caused a permanent retardation of cell proliferation.

A growing body of evidence suggests that cell surface carbohydrates may play some part in the control of cell division. Reagents which bind or degrade carbohydrates such as lectins, periodate, and glycosidases tend to stimulate mitotic division. Tumor cells or fibroblasts transformed with oncogenic viruses often have lower amounts of gangliosides and fucolipids and larger amounts of the shorter carbohydrate chain glycolipids such as lactosylceramide. A deficiency of glycosyl transferase activity for the biosynthesis of these substances is indicated from several studies. This subject has been reviewed (1–4). The blood group ABO and Lewis fucolipids are of interest because they are generally present in tissues which are capable of rapid proliferation and generally absent in tissues which do not undergo cell division or do so at lower rates. The blood groups A and B fucolipids may be found in normal intestinal tissues but often not in adjacent adenocarcinoma tissue. In view of these relationships it may be postulated that such glycolipids may function in control or suppression of growth in cells capable of rapid proliferation. Results of experiments by others on the effects of added globoside and gangliosides on growth of transformed fibroblasts give support to this view (5–8). This paper describes the effects of some complex glycolipids on the growth of tumor cells in culture.

Materials and Methods. *Cell lines.* The human colonic carcinoma cell line HT29 was kindly provided by Dr. Jorgan Fogh (Sloan Kettering Institute, N.Y.). The characteristics of the HT29 line have been reported (9). Exposure of HT29 cells to toxic concentrations of 5-fluorouracil resulted in the isolation of a drug-resistant cell population which was designated HTFU (10). The two malignant cell lines differed with respect to *in vitro* and *in vivo* growth characteristics. Of particular interest in this study was the variation in *in vitro* proliferative ability: HTFU cells showed contact inhibition; HT29 cells demonstrated uncontrolled proliferation.

Lipid isolation. The glycolipids were isolated from human and canine intestine by extraction with ethanol:ether (3:1), followed by column chromatography of these extracts on silicic acid and florisil, solvent partition, dialysis, and preparative thin-

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layer chromatography as described previously (11). Blood group A fucolipid from dog intestine (GalNAc(α1 → 3) {Fucα1 → 2}Galβ1 → 4 GlcNAc β1 → 3 Galβ1 → 4 Glc → Ceramide) and $Lewis_b$ fucolipid from human intestine (Fuc (α1 → 2) Galβ1 → 3 {Fuc α1 → 4} GlcNAc β1 → 3 Gal β1 → 4 Glc → Cermide) were identified by sugar analysis (12), permethylation analysis of native and defucosylated lipid (4, 12), and by mass spectrometry (13). Ceramide trihexoside (Galα1 → 4 Gal β1 → 4 Glc → Ceramide) and Forssman glycolipid (GalNAcα1 → 3 GalNAcβ1 → 3 Galα1 → 4 Galβ1 → 4 Glc → Ceramide) were isolated from canine intestine and identified by sugar analysis, permethylation analysis, and mobility on thin-layer chromatography. Aliquots of stock solutions of the glycolipids in chloroform:methanol (1:1) were evaporated to dryness in flasks under reduced pressure. Cell culture medium was then added and left overnight for uptake of lipid into the medium. All of the glycolipids used are easily water soluble except the ceramide trihexoside. Presumably the lipids bind to the calf serum proteins of the medium and are transferred to the cultured cell plasma membranes (5, 14).

Growth curves. Cultures were maintained at 37° in 5% CO_2 in air in McCoy's enriched media (GIBCO) and antibotics (6.4 μg/ml gentamicin, 90 μg/ml penicillin, 90 μg/ml streptomycin). Cells were inoculated into 35-mm petri dishes (Corning Glass Works, Corning, N.Y.) at a concentration of 50,000 cells/dish. After 2 days in culture, the old media were removed and were replaced with media containing various concentrations of blood group A fucolipid, $Lewis_b$ fucolipid (Le_b), Forssman glycolipid (Fors), or ceramide trihexoside (CTH). All subsequent daily media changes contained appropriate concentrations of lipids. Control growth curves were performed in which cells were grown without added lipid. At regular intervals, duplicate petri dishes of control and lipid-treated cells were subcultured with 0.25% trypsin (GIBCO) and 0.2% EDTA in tissue culture medium. Cell counts were performed with hemocytometer chambers and viability was assessed by trypan blue exclusion (10).

Growth in semisolid media and tumorgenicity. HT29 cells grown in the presence or absence of A fucolipid were cultured in agarose by the procedure we have previously reported (10, 15). Briefly, cells (2×10^4) were suspended in a final concentration of 0.27% agarose (Pharmacia Fine Chemicals, Piscataway, N.J.). One milliliter of this suspension was layered over a base layer of 0.5% agarose in 35-mm petri dishes and incubated in a humid atmosphere at 37° with 5% CO_2. After 2 days incubation, 1 ml of tissue culture medium containing appropriate lipid concentrations was applied to each agarose plate to provide sustenance and prevent drying of the agarose. Cultures were terminated after 4 weeks and the number of colonies were determined by microscopic examination. A colony consisted of 20 or more cells. Appropriate controls were counted initially to determine the number, if any, of colony-sized aggregates. The aggregates were deducted from the final colony count.

HT29 cells were grown in the absence or presence of 1.0 μM A fucolipid for 2 weeks. Cells were harvested and suspended in tissue culture medium at a concentration of 2×10^6 cells/0.1 ml. A 0.1-ml volume was injected sc into the flanks of genetically athymic "nude" mice. Animals were observed daily for signs of tumor formation.

Results. *Human tumor cell exposure to CTH, Fors, Le_b fucolipid, and A fucolipid.* HT29 and HTFU cells were exposed to three concentrations (serial 10-fold dilutions) of each lipid for a minimum of 14 days. Preliminary experiments showed that the contact-inhibitable HTFU cells were appreciably more sensitive to Le_b, CTH, and Fors than were HT29 cells. For this reason the maximum glycolipid concentration to which HTFU cells were exposed was one-tenth that of the HT29 cells. HT29 and HTFU cells showed an equal sensitivity to blood group A fucolipid.

The presence of $Lewis_b$ fucolipid caused a significant concentration-dependent inhibition in the growth of both cell lines. Le_b-exposed HT29 cultures showed a reduction in cell number relative to the controls for the duration of the growth curve, but the cells were viable by trypan blue exclusion

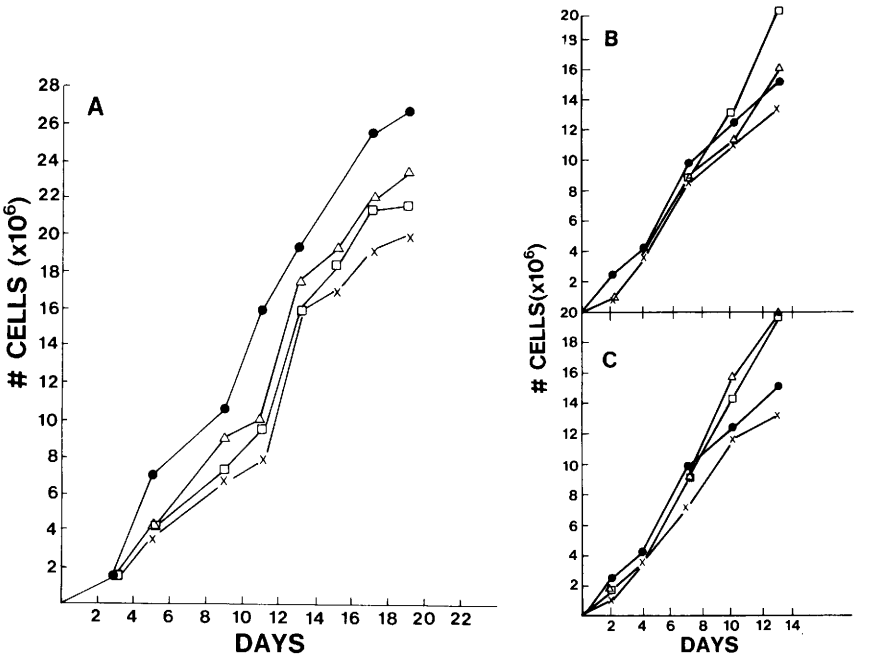

FIG. 1. Growth curves of HT29 cells grown in the presence of glycolipids: (A) Le_b; (B) CTH; (C) Fors. Cells were grown in the absence of lipid (●) or in the presence of 11.0 μM (X), 1.1 μM (□), 0.11 μM (△). The average standard deviations for cells grown in Le_b, CTH, and Fors were 6.1, 0.2, and 0.2%, respectively.

(Fig. 1A). Exposure of HTFU cells to the minimum concentrations of Le_b (0.011 μM) resulted in retardation of cell growth and a reduction in the final saturation density (Fig. 2A). Higher Le_b concentrations caused HTFU cells to detach from the petri dishes at particular cell densities. Detached cells did not exclude trypan blue. The experiment was repeated several times with equivalent results.

The two cell lines responded similarly in the presence of CTH and Forssman glycolipid. Cell proliferation was temporarily retarded but continued exposure to the lower concentrations of these lipids (1.1 and 0.11 μM) appeared to stimulate growth in HT29 cultures (Figs. 1B and C). Similar results were obtained in HTFU cultures as continued lipid exposure allowed the treated cells to exceed the saturation density maintained by the control group (Figs. 2B and C). Lipid-treated HTFU cells failed to demonstrate contact inhibition during the 14-day growth curve. Repetition of the experiments yielded equivalent results.

The proliferative responses of HT29 and HTFU cells to blood group A fucolipid were similar to those observed following their exposure to Le_b. HT29 cells showed permanent concentration-dependent inhibition of cell proliferation (Fig. 3A). Cells were viable as assessed by trypan blue exclusion. Similarly, the proliferative ability of HTFU cells was retarded in the presence of fucolipid (Fig. 3B). The final saturation density of HTFU cells was dependent on the lipid concentration.

Continued exposure to glycolipids. HT29 cells were divided into three experimental groups: Groups I and II cells were grown in the basal medium for 11 days; Group III cells were grown in the presence of 11.0 μM Forssman glycolipid during this period. Cells from each group were subsequently cultured at an inoculum of 50,000 cells/dish. Groups II and III were grown in the presence of 11.0 μM Forssman glycolipid, and Group I was continued on the basal medium. After 5 days in culture, Groups II and III cells showed a 30% reduction in cell

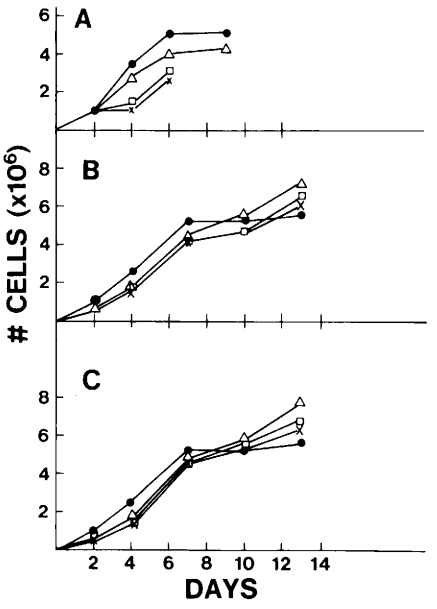

FIG. 2. Growth curves of HTFU cells grown in the presence of glycolipids: (A) Le_b; (B) CTH; (C) Fors. Cells were grown in the absence of lipid (●) or in the presence of 1.1 μM (X), 0.11 μM (□), 0.011 μM (△). The average standard deviations for cells grown in Le_b, CTH, and Fors were 7.8, 1.0, and 2.7%, respectively.

number relative to the control cells of Group I. This suggested that the stimulation of cell proliferation observed in the growth curves (Figs. 1B and C, 2B and C) was not caused by cellular adaptation to the lipid.

The long-term effect of blood group A fucolipid on cell proliferation was similarly determined for HT29 and HTFU cells. Equivalent results were obtained from each cell line. After 5 days incubation, Groups II and III cells showed approximately 25% reduction in cell number relative to the control cells (Group I). This suggested that the presence of fucolipid produced a permanent inhibition in the replication rate of the cells.

Growth in semisolid media and tumorigenicity. Growth in semisolid media is frequently used as criteria for anchorage independence (10, 15). HT29 cells were cultured in the presence of 1.10 μM A fucolipid for 2 weeks prior to plating into agarose. Control cultures were grown in the absence of lipid. Following 4 weeks incubation in agarose, controls demonstrated a plating efficiency of 68% while lipid-treated cells had a plating efficiency of 49%. Tumors were palpable after 7 days and ex-

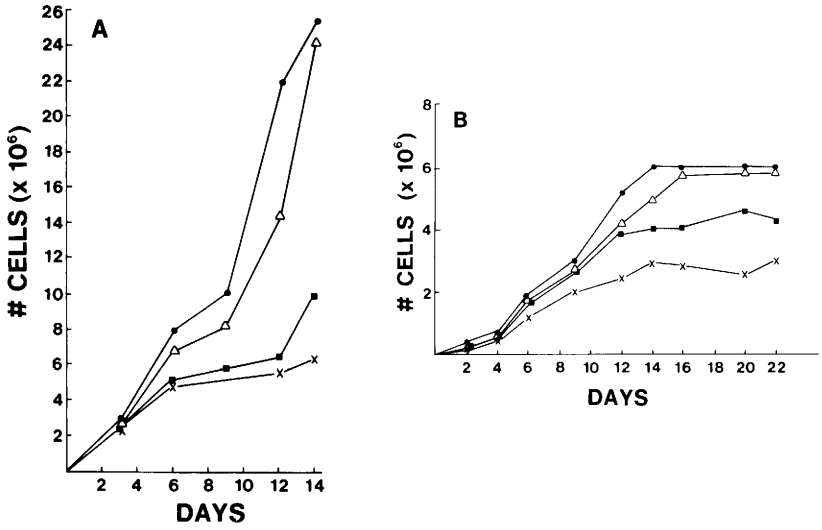

FIG. 3. Growth curves of HT29 cells (A) and HTFU cells (B) grown in the presence of blood group A fucolipid at concentrations of 11.0 μM (X), 1.1 μM (■), 0.11 μM (△). Control cells were grown in the absence of lipid (●). The average standard deviations for HT29 and HTFU growth curves are 7.7 and 2.8%, respectively.

ceeded a diameter of 1 cm after 25 days in all of the animals injected with control cells. The tumor growth rate of HT29 cells was similar to what we previously reported (10). After 25 days tumor formation was apparent in one out of five animals injected with lipid-treated cells; however, after 2 to 4 months tumors formed in three of the remaining animals. Tumor growth proceeded at approximately half the rate observed in tumor formation from control cells.

Exposure of murine tumor cells to lipids. Cell line No. 26 was grown in the presence of Forssman glycolipid or CTH for 10 days. Initial experiments demonstrated that a minimal lipid concentration of 0.11 μM was necessary to produce any effects on cell proliferation. As summarized in Table I, CTH retarded cell proliferation throughout the 10-day period but Forssman glycolipid appeared to increase the rate of cell growth relative to the control.

Discussion. Growth of HTFU and HT29 cells was inhibited by either Le_b or A blood group glycolipids at a concentration of 11.0 μM although for HTFU cells, only 1.1 μM Le_b lipid was necessary to produce comparable growth suppression. The actual concentration of available glycolipid needed for these effects may be much lower than the total added to the medium since most of it probably remains bound to the serum proteins. The sensitivity of these lines to the Le_b fucolipid is of interest since the Le_a, Le_b, and iso Le_a fucolipids have been isolated from intestinal adenocarcinoma tissue (16, 17). Blood group A or B substances were not detected in these adenocarcinomas although the donors were of these blood groups. Siddiqui *et al.* (18) found marked reductions in the blood group ABO activity of the glycolipid fraction from human colonic carcinoma tissue as compared to adjacent normal tissue. A decrease in ABO antigenic activity in gastrointestinal carcinoma tissue has been recognized (19, 20). We have isolated glycolipids from 16 human small intestines and found that the Le_a or Le_b fucolipids are usually the dominant fucolipids present in noncancerous tissue and may be isolated from an adult intestine in amounts exceeding 100 mg. From our data it is doubtful that there is an increase in these two substances in tumor tissue over normal tissue but the copresence of Le_a and Le_b (16) in adult tissue, and the presence of iso Le_a (X hapten) (17) has not occurred in our series in sufficient amounts for isolation.

TABLE I. EFFECTS OF LIPIDS ON MURINE TUMOR CELL PROLIFERATION

Culture	5 Days (%)	10 Days (%)
1. Control	100	100
2. Fors exposed	87	138
3. CTH exposed	56	46

Note. A murine colonic carcinoma cell line which does not show contact inhibition was grown in the presence of 0.11 μM CTH or Forssman glycolipid. After 5 or 10 days exposure, cells were subcultured and counted. Data are expressed as the percentage of cells found in lipid-treated cultures relative to the number of cells found in control cultures.

Previous studies on growth inhibition of transformed fibroblasts with globoside (5) and gangliosides (6–8) have demonstrated uptake of these lipids into the cells and into the plasma membrane. However, the concentrations of lipids used in these studies were 20- to 800-fold greater than the largest amounts of fucolipids used in our work and uptake of the latter into the tumor cell plasma membrane needs to be established. The entry of fucolipids into human erythrocyte membranes has been demonstrated by the *in vitro* conversion of blood group O cells to blood group A and blood group Le_{a-b-} to blood groups Le_{a+} and Le_{b+} by incubation with dilute solutions of the appropriate fucolipids (21).

CTH and Fors were included in this study as a comparative reference for the effects of the blood group fucolipids. CTH and Fors had less effect on cell proliferation than did an equal concentration of fucolipids. The eventual stimulatory effects of the glycolipids on cell proliferation were not investigated although the effects did not appear to be a function of cellular adaptation. The presence of blood group fucolipids produced a permanent retardation in the cellular replication rate of both cell lines and also appeared to influence cell density phenomena. Although we do not

know the biological significance of these effects, it is interesting that fucolipid treatment eliminated or retarded the tumor-forming ability of HT29 cells injected into nude mice. The intercalation of fucolipids into the plasma membrane (19) may produce changes in membrane fluidity, recognition potential, composition, or antigenicity. These changes may decrease the interactions among malignant cells or between normal and malignant cells.

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