NEW BIOMEDICAL TECHNOLOGIES

Enhancement of Liver Cell Proliferation by GM_3 Ganglioside

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In experiments on rats subjected to partial hepatectomy and experimentally induced hepatitis it is shown that GM_3 ganglioside of equine erythrocytes can enhance liver cell proliferation. The effect was also observed in experiments on a primary hepatocyte culture *in vitro*; moreover, enhancement of cell proliferation did not depend on the type of sialic acid residues.

Key words: *equine erythrocyte-derived GM_3 ganglioside; hepatocytes; cell proliferation*

Gangliosides are sialic acid residue-containing glycosphingolipids of the cell surface. They participate in cell-cell, cell-virus, and cell-molecule interactions and in the functioning of the plasma membrane. An example of role of gangliosides in plasma membrane functioning is equine erythrocyte GM_3 ganglioside-induced modulation of autophosphorylation of certain growth factor receptors, a process which leads to the inhibition of cell proliferation [6]. In the present study we show that GM_3 ganglioside can enhance cell proliferation both *in vivo* and *in vitro*.

MATERIALS AND METHODS

The following products were used in the experiments: acetonitrile (Lichrosolv, Merck); commercial preparations of total phospholipids derived from bovine brain and equine erythrocytes; egg yolk phosphatidylcholine; bovine brain phosphatidylcholine and gangliosides; Total Erythrocyte Lipid preparation (Biolek, Kharkov). Products used in the work with cell cultures were purchased from Flow, Inc. All reagents were of fine or superfine grade.

Thin-layer chromatography of gangliosides was performed on Kieselgel 60 glass slides (Merck) using solvent composed of chloroform, methanol, and 0.2% $CaCl_2$ solution at a v/v ratio of 60:35:8. Gangliosides were detected using resorcin/orcin reagent.

Analytical and preparative high-performance liquid chromatography (HPLC) was performed on Gilson apparatus using columns with silicagel modified with aminopropyl groups as described by us earlier [9].

GM_3-NeuGc ganglioside was obtained from equine erythrocytes and purified by silicagel column chromatography [4]. According to the results of HPLC, the preparation was of 97% or greater purity. The ganglioside contained no admixtures of phospholipids or peptides, as judged from analysis using specific Molybdenum Blue (Sigma) reagents and fluorescamine.

GM_3-NeuAc ganglioside was obtained by treating natural GM_3-NeuGc (582 mg) with a 1 M

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solution of tetramethylammonium in 90% n-butanol (100 ml) at 100°C for 4 hours [12] with modifications. After hydrolysis the reaction mixture was evaporated to dry ness, dissolved in water (200 ml), and subjected to chromatography on a Lichroprep RP18 column (3×7 cm, 0.040-0.063 mm, Merck). Deacylated GM_3, a less hydrophobic by-product, was eluted with 150 ml of methanol-water mixture (80:20 v/v). The desired product, GM_3-$NeuNH_2$, was eluted with 150 ml of methanol. Following evaporation, the substance was dissolved in 150 ml of methanol/$NaHCO_3$ 4% aquerous solution (1:1 v/v) and acetylated by adding 0.2 ml acetic anhydride every 30 min. The reaction was conducted under the control of repeated assays for the presence of initial product using thin-layer chromatography. After completion of the reaction, the mixture was diluted with water in a v/v ratio of 1:1. GM_3-NeuAc was extracted using a Lichroprep RP18 column (3×5 cm). The column was washed with water, and the desired substance eluted with methanol and evaporated to a volume of 2-4 ml. The ganglioside was precipitated with acetone (1:10), filtrated, and dried. The final output was about 340 mg (58%).

In the experiments on the effect of lipids on hepatocyte proliferation *in vivo*, aqueous solutions of gangliosides (6 mg/ml) and aqueous suspensions of phospholipids (15 mg/ml) were used. The work was carried out on 3-6-month-old male Wistar rats.

Hepatectomy was performed as described earlier [8]. Lipids were injected intraperitoneally in a dose of 0.2 ml/100 g body weight, immediately postoperation and 10 hours later. Rats were decapitated 22 hours after the operation.

Hepatitis was induced by local hyperthermia [1]. GM_3-NeuGc was administered every 12 hours as described above. Rats were decapitated 24, 48, 72, and 96 hours postoperation.

An hour before decapitation animals were injected intraperitoneally with ^{3}H-thymidine (6 Ci/mmol, 80 μCi/100 g body weight). The specific radioactivity of liver cell DNA was recorded as described previously [2].

In the study of the GM_3-NeuGc- and GM_3-NeuAc-mediated effect on hepatocyte proliferation *in vitro*, both compounds were first purified by preparative HPLC on a Zorbax-NH_2 column. The resultant products showed single peaks in the analytic chromatography assay and individual spots in thin-layer chromatography with detection by resorcin/orcin reagents and 5% H_2SO_4 solution in ethanol.

Gangliosides were dissolved in water to the concentration of 1.5 mg/ml. Following filtration through 0.22-μ filters (Millipore) the ganglioside content was calculated from the amount of neuraminic acid and the gangliosides were added to the culture medium in final concentrations of 3, 7.5, 15, 30, and 60 μg/ml.

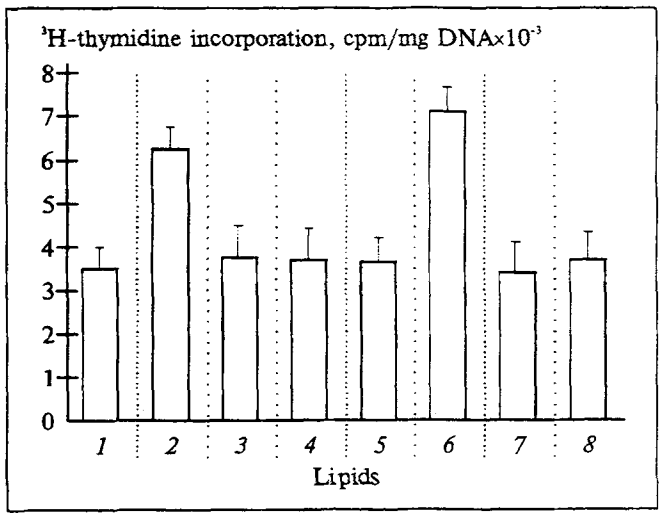

Fig. 1. Effect of lipids on proliferative activity of liver cells in rats with partial hepatectomy. *1*) control (no lipids administered); *2*) Total Erythrocyte Lipids; *3*) bovine brain phospholipids; *4*) bovine brain phosphatidylcholine; *5*) egg yolk phosphatidylcholine; *6*) GM_3–NeuGc; *7*) equine erythrocyte phospholipids; *8*) bovine brain gangliosides. Here and in Fig. 2: results are presented as ($M \pm m$) of two independent experiments, five rats per experiment.

Hepatocytes for primary cultures were isolated by perfusion of 2-day-old pig liver with collagenase (Sigma) solution as described earlier [11]. The cells were placed in 7.1-cm^2 dishes precoated with collagen (4.5×10^5 cells per dish) and cultured in L-15 medium supplemented with 10% fetal serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C. Twenty-four hours later the cell-conditioned medium was temporarily aspirated, and the

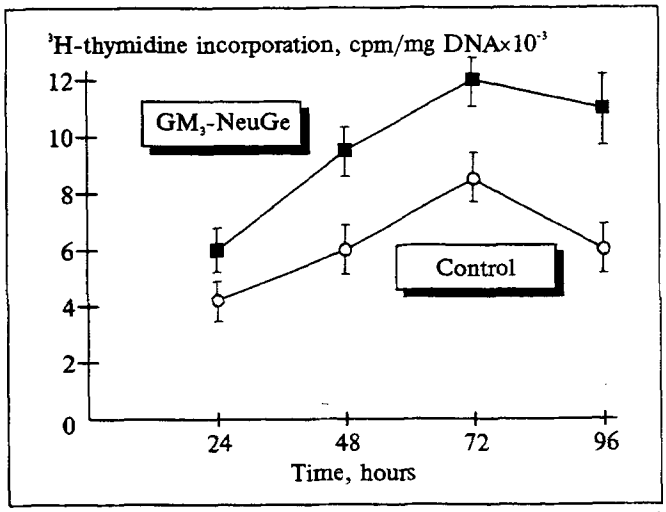

Fig. 2. Efect of GM_3–NeuGc on proliferative activity of liver cells after experimentally induced hepatitis.

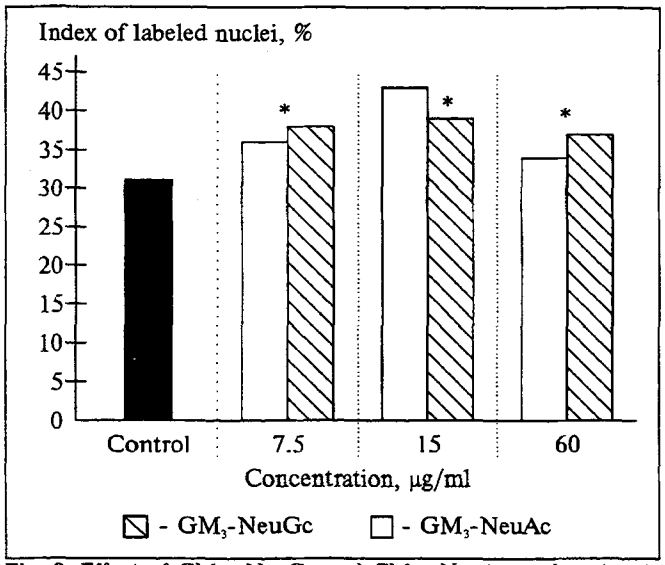

Fig. 3. Effect of GM_3—NeuGc and GM_3—NeuAc on hepatocyte proliferation in a primary culture. The results are presented as the mean percentage of labeled cells calculated from 4 independent experiments, 3 dishes per experiment. *: results reliably differ from the control ($p<0.05$).

cells were treated with ganglioside solution for 3 hours, after which growth medium was added again. The proliferative response was measured by histoautoradiography following pulse labeling with ^{3}H-thymidine (6 Ci/mmol, final concentration 1 μCi/ml), with subsequent fixation as described earlier [3].

RESULTS

Our experiments studied the effect of lipids on liver cell proliferation. Partially hepatectomized rats injected with Total Erythrocyte Lipids preparation showed an increased radioactivity incorporation into the liver cell DNA (Fig. 1). Since bovine phosphatidylcholines and lipids produced practically no effect on cell proliferation (Fig. 1), we assumed the proliferation-enhancing effect to be connected with GM_3 ganglioside found in the erythrocyte-derived lipid complex.

Administration of purified GM_3-NeuGc to partially hepatectomized rats led to a twofold increase of DNA radioactive labeling as compared to the control. The effect of GM_3 was specific, since total bovine gangliosides containing GM_1, GD_{1a}, GD_{1b}, GT_{1b}, and GQ exerted no effect on liver cell proliferation. A GM_3-mediated effect was observed 22 hours postoperation, thus coinciding in time with the maximal incorporation of the radioactive label following partial hepatectomy in the control group that received no preparations. On the other hand, erythrocyte-derived lipids depleted of GM_3 ganglioside had no influence on liver cell proliferation.

A GM_3-induced increase of hepatocyte proliferative activity was also shown in the rats with experimentally induced hepatitis (Fig. 2). The dynamics of radioactive label incorporation was similar to that in the control but surpassed it during all periods of observation. The nature of GM_3-induced enhancement of cell proliferation was not found to be dose-dependent within the dose range tested (1-6 mg/100 g body weight).

Thus, our results show that equine erythrocyte-derived GM_3-NeuGc ganglioside can enhance liver cell proliferation *in vivo*. However, in several reports it has been shown that the gangliosides can inhibit cell proliferation. For instance, exogenous GM_3 inhibits the growth of DNA cells cultured in medium containing a single growth factor - fibroblast growth factor (FGF) [5]. GM_3 suppresses the proliferation of 3T3 mouse cells, this being related to the inhibition of platelet-derived growth factor (PDGF) receptor phosphorylation [6]. Diminished proliferative activity of cells of the human carcinoma line A431, which are characterized by high-level expression of epidermal growth factor (EGF) receptors, also correlates with the specific inhibition of receptor phosphorylation after the addition of GM_3 but not other gangliosides [7].

To confirm our results obtained *in vivo* and to elucidate the possible role of the type of sialic acid residues, we conducted a study of the effect of GM_3 on hepatocyte proliferation in primary *in vitro* culture. Both GM_3-NeuGc and GM_3-NeuAc proved to enhance proliferative activity (Fig. 3). The effect was observed whether a final ganglioside concentration of 60 μg/ml culture medium was used (the dose used earlier by other workers) or whether concentrations of 7.5 and 15 μg/ml culture medium were used.

The enhancing effect of equine erythrocyte-derived GM_3 on cell proliferation may be explained by the differences in the cell types used by us and other investigators. Hepatocyte proliferation appears to be induced and governed by a specific growth factor, namely, hepatocyte growth factor (HGF), which is fundamentally different from growth factors studied earlier (FGF, PDGF, and EGF) [10]. Moreover, HGF receptor phosphorylation may be influenced by GM_3 ganglioside in a somewhat different way. However, the fine structure of equine erythrocyte-derived GM_3 ganglioside, namely, specific features of ceramide structure, seem to be of crucial importance in determining whether GM_3 has a positive or negative effect on cell proliferation.

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