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SIALYLTRANSFERASE ACTIVITIES IN CULTURED RAT HEPATOCYTES

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Summary. Previous studies on the age and sex dependency of the ganglioside patterns in rat liver *in vivo* and the concomitant determination of the activities of some enzymes involved in these pathways revealed the prominent role of the sialylation of G_{M3} to G_{D3} in determining the flow to the mono (a)- and polysialo (b)-series, respectively. Here, the influence of hormones on the activities of G_{M3} and G_{D3} synthases in isolated hepatocytes was studied. The combination of several factors (insulin, glucagon, epidermal growth factor, glucocorticoids) was found to be necessary for maintaining *in vivo* activity levels of G_{D3}- but not of G_{M3}-synthase.

Gangliosides are sialic acid-containing glycosphingolipids present in the plasma membrane of a great number of cell types (for review see Ref. 1). Studies of many laboratories indicate that these compounds play an important role in growth control and in the social behaviour of cells during differentiation, development and oncogenic transformation (for review see Ref. 2).

The content and pattern of hepatic gangliosides of normal adult rats are quite constant, but hepatocytes after isolation and transfer to culture dishes reveal remarkable changes (3). One of these alterations is an about six-fold increase of the total amount of lipid-bound sialic acid. Ganglioside biosynthesis takes place in the Golgi apparatus starting with glucosylceramide by sequential addition of sugars, N-acetylaminosugars and sialic acid catalyzed by specific glycosyltransferases. These enzymes have been studied in rat liver Golgi apparatus (4-10). The patterns of gangliosides in whole liver as well as in rat hepatocytes have been described (11-13). In primary cultures of rat hepatocytes the pattern of gangliosides is known to change with the time of cultivation and following hormone treatment (3). G_{M3} is a key intermediate of ganglioside biosynthesis. It constitutes a branchpoint of the monosialo (a)- and the polysialo (b)-glycosphingolipid pathways (14). The conversion of G_{M3} to G_{D3} has been identified as the step regulating the usage of these pathways.

Abbreviations: EGF, epidermal growth factor; PBS, phosphate-buffered saline; G_{D3}, $II^3(Neu5NAc)_2$-lactosylceramide; G_{M3}, $II^3Neu5NAc$-lactosylceramide.

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To further investigate the mechanisms participating in this regulation, the response of the activities of the enzymes G_{M3} synthase (E.C. 2.4.99.-) and G_{D3} synthase (E.C.2.4.99.8) to various hormones were measured in primary culture of rat hepatocytes that were kept in chemically defined media.

MATERIALS AND METHODS

William E medium was obtained from Flow laboratories, Mechenheim, FRG. Insulin, glucagon, EGF, dexamethasone, CMP-Neu5Ac and lactosylceramide were from Sigma, Deisenhofen, FRG. Collagenase Type II (Worthington) was from Seromed, Berlin, FRG; CMP-N-acetyl-[4,5,6,7,8,9- ^{14}C]neuraminic acid (262 mCi/mmol) was obtained from Amersham Buchler, Braunschweig, FRG. Ganglioside G_{M3} was from Pallmann KG, München, FRG; Sephadex G 25 was from Pharmacia, Freiburg, FRG. All other chemicals and organic solvents were of analytical grade and purchased from Merck, Darmstadt or Roth, Karlsruhe, FRG.

Fed female Wistar rats weighing 180 - 200 g were used. The livers were removed aseptically under Nembutal anaesthesia and the hepatocytes obtained by a modification (15) of the method of Seglen (16). After a 25 min perfusion with 40 mg collagenase/ 100 ml medium, the liver was removed from the perfusion system, the capsule opened, and the cells were gently shaken and suspended in washing buffer (Krebs-Henseleit, containing 5.5 mM glucose). After filtering through a nylon filter (79 μm mesh) the cells were washed three times by centrifugation at 15 g for 2 min. Cells were used when the viability was 90% or greater as determined by the exclusion of trypan blue. Hepatocytes were plated at the density of 3 x 10^6 cells per culture on 60 mm plastic culture dishes coated with rat tail collagen prepared according to Michalopoulos and Pitot (17) in a William E medium containing 0.2% (v/v) fatty acid-poor bovine serum albumin, streptomycin (100 μg/ml) and penicillin (100 U/ml). This enriched medium will henceforth be addressed as "modified William E medium". Insulin (10 μg/ml), glucagon (10 μg/ml), EGF (10 ng/ml), and dexamethasone (10^{-7} M) were added as indicated. Cells were incubated in a humidified atmosphere (air/CO_2, 95/5%) at 37°C. The cells were allowed to attach to the dishes for 4 h. The spent medium together with nonadherent material was aspirated and replaced by the same medium containing the supplements. The medium was changed every 24 h for the remainder of the experiment. At the times indicated, the medium was decanted, the cells were washed three times with PBS and quickly frozen in liquid nitrogen.

The sialyltransferases were measured as described by Busam and Decker (8) with several modificiations. 0.3 ml of ice-cold medium containing 0.5 M sucrose, 1% dextran (mass/vol), and 37.5 mM Tris-maleate buffer, pH 6.75, per dish was layered on top of the frozen cells. The cell suspension was then transferred in a Potter-Elvehjem homogenizer and homogenized with 10 strokes. Protein was determined by the method of Lowry (18). Sialyltransferase assays contained 200 μg of homogenate protein in a total volume of 50 μl and substrate, detergent and buffer. Specifically, the incubation mixture for G_{M3} synthase contained 0.5 mM lactosylceramide (GL_2), 75 μg Triton X-100, 150 mM sodium cacodylate buffer, pH 5.7, 4.0 mM CMP-Neu5Ac and 600 000 cpm CMP-[^{14}C]Neu5Ac. The incubation was run at 37°C for 45 min. The G_{D3} synthase assay was performed as follows: 0.5 mM G_{M3}, 1.0 mM CMP-Neu5Ac, 200 000 cpm CMP-[^{14}C]Neu5Ac, 150 mM sodium cacodylate buffer, pH 5.8, and 75 μg Triton X-100. The reaction proceeded for 30 min at 37°C. The assay procedure including appropriate blanks (without lipid acceptor) and product separation were as described previously (8).

RESULTS AND DISCUSSION

In order to study the metabolism of gangliosides in cultured hepatocytes the G_{M3} and G_{D3} synthase activity had to be maintained at levels close to those found *in vivo*.

Table 1. G_{D3} synthase activity of rat hepatocytes

Cells		Medium			
		A	**B**	**C**	**D**
Fresh			475 ± 67		
Cultured	24 h	223 ± 12	423 ± 9	350 ± 29	406 ± 83
	48 h	245 ± 45	230 ± 37	393 ± 77	438 ± 78
	72 h	253 ± 19	240 ± 21	237 ± 66	490 ± 47
	96 h	240 ± 30	253 ± 12	300 ± 14	540 ± 67

Results are expressed as pmol x h^{-1} x (mg protein)$^{-1}$ and represent the mean value ± S.D. of 9 (fresh cells) and 3 (cultured cells) independent determinations.
Medium **A**: modified William E medium + insulin (10 μg/ml); Medium **B**: modified William E medium + insulin (10 μg/ml) + glucagon (10 μg/ml); Medium **C**: modified William E medium + insulin (10 μg/ml) + glucagon (10 μg/ml) + EGF (10 ng/ml); Medium **D**: modified William E medium + insulin (10 μg/ml) + glucagon (10 μg/ml) + EGF (10 ng/ml) + dexamethasone (10^{-7} M).

Cultivation of rat hepatocytes in Leibovitz L 15 and Williams E medium with serum supplement (data not shown) resulted in very low activity of G_{D3} synthase. After 24 h in culture, the activity was about 30% of the level found in freshly isolated hepatocytes. Even when serum-supplemented medium was used as a plating medium only, activity did not exceed 50% for the remainder of the experiment. Some cell types, especially epithelial cells, cannot be cultured in serum-containing medium. This may be due to a deficiency in components essential for survival

Table 2. G_{M3} synthase activity of rat hepatocytes

Cells		Medium	
		C	**D**
Fresh		341 ± 31	
Cultured	24 h	310 ± 59	182 ± 47
	48 h	633 ± 17	252 ± 31
	72 h	547 ± 86	245 ± 16
	96 h	727 ± 57	327 ± 63

Results are expressed as pmol x h^{-1} x (mg protein)$^{-1}$ and represent the mean value ± S.D. of 6 (fresh cells) and 3 (cultured cells) independent determinations. For medium designations see Table 1.

and proliferation or the presence of inhibitors or toxic factors in serum (19, 20). Serum inhibits the expression of liver specific functions, and was found to be cytostatic to parenchymal cells (21). After few days in culture some features of fetal liver became evident (22). Tables 1 and 2 show the effect of various hormonally defined media on G_{D3} and G_{M3} synthase activity of cultured hepatocytes. The enzyme activity was determined in fresh hepatocytes and after 24, 48, 72, and 96 h in culture. The G_{D3} and G_{M3} synthase activities in freshly isolated hepatocytes are compatible with results of Schüz-Henninger et al. (14) and Senn et al. (23). The hepatocytes formed a confluent monolayer 24 h after plating and could be maintained in culture during the experiments in all media used. In Medium A the G_{D3} synthase activity was between 50 and 57% of the level found in freshly isolated hepatocytes. Although addition of insulin and glucagon (Medium B), or EGF, insulin and glucagon (Medium C) delayed the fall of G_{D3} synthase activity, no difference was observed between these three media after 72 h in culture, and enzyme activity was between 50 and 63% of the activity of fresh hepatocytes. However, addition of 10^{-7} M dexamethasone together with EGF, insulin, and glucagon (Medium D) resulted in a G_{D3} synthase activity close to that found in fresh hepatocytes.

Dexamethasone revealed remarkable changes also in the G_{M3} synthase activity (Table 2). G_{M3} is the primary ganglioside of hepatocytes from which all other gangliosides can be synthesized. Without dexamethasone G_{M3} synthase activity in primary culture of rat hepatocytes rose during the experiment and after four days in culture was about two times that of fresh hepatocytes. However, in the same medium with dexamethasone, the G_{M3} synthase level was close to the activity in fresh cells.

A general involvement of glycolipids in growth control has been indicated by many observations of enhanced glycolipid synthesis as cultured cells reach confluency and "contact inhibition" of growth occurs (for review see Ref.24). It is well established that EGF is a potent growth-promoting factor of rat hepatocytes especially in combination with insulin and glucagon both *in vivo* (25, 26) and in primary culture (27). In cultured neonatal rat hepatocytes, a physiological concentration of EGF in the presence of insulin and dexamethasone is sufficient to induce their entry into S phase and mitosis (28). Adult liver parenchymal cells capable of expressing differentiated function have been maintained for a few days in primary culture, but upon adaptation to culture conditions, cells lose many specific functions and after short time show phenotypic characteristics of fetal liver (22, 29). Dexamethasone when continuously present in the medium maintains the characteristic adult pattern of cultured hepatocytes in absence of serum for longer periods of time (30). Phillips et al. (3) observed that dexamethasone delayed the increase of total lipid-bound sialic acid in primary cultures of rat hepatocytes.

Although the mechanism responsible for the observed differences is as yet unclear, we were able to maintain the G_{M3} and G_{D3} synthase activity in the modified serum-free William E

medium containing EGF, insulin, glucagon, and dexamethasone at levels close to those found *in vivo*. The system appears suitable for investigations of the ganglioside metabolism in primary cultures of rat hepatocytes.

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