DEVELOPMENTAL CHANGES IN GANGLIOSIDES DURING MYOGENESIS OF A RAT MYOBLAST CELL LINE AND ITS DRUG RESISTANT VARIANTS

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SUMMARY: Cloned cells of a myoblast line show the presence of GM_3, GM_2, GM_1 and GD_{1a} gangliosides. The amount of GM_3, GM_2 and GM_1 gangliosides does not vary significantly during the differentiation of myoblasts to myotubes. However, the concentration of GD_{1a} transiently increases almost 3-fold just prior to the fusion of myoblasts and returns to the basal levels in the myotubes. Mutant myoblasts selected for 5-azacytidine resistance and unable to fuse produce only GM_3 and traces of GM_2. We conclude that GD_{1a} probably participates in the fusion process through yet unknown mechanism.

INTRODUCTION: It is now well established that most extraneural types of cells in culture synthesize gangliosides (1,2). The function of these compounds, however, remains enigmatic. Some gangliosides, such as GM_1, have been shown to act as receptors for various bacterial toxins (3,4), but the biological role of others is unknown. The localization of the gangliosides in cellular membranes (4,5) suggests, however, that they may be involved in several membrane-mediated phenomena, such as cell-cell recognition (7,8) contact inhibition (8,9) control of growth (10,11) and cellular morphology (11,12).

The cytodifferentiation of nucleated myotubes *in vitro* has been found to occur via aggregation and fusion of mononucleated myoblasts, both in primary cultures (13,14) and in established myoblast cell lines (15). Since differentiation of myoblasts of necessity, must involve alteration of membrane components of the cells destined to fuse, and since gangliosides have been implicated in cell contact and growth processes (7-12) we decided to follow the changes in gangliosides during myoblast differentiation. To give physiological meaning to these changes, we also investigated ganglioside patterns of several myoblast variants and particularly of a class of 5-azacytidine resistant mutants which are deficient in fusion and are unable to differentiate. In the following account we present data to show that just before myotube formation in all fusion-competent myoblasts the concentration of one of the digangliosides (16), GD_{1a} increases significantly, while mutants that are unable to fuse show extremely simple pattern of ganglioside distribution.

Abbreviations: GLC, gas-liquid chromatography; HGPRTase, hypoxanthine-guanine phosphoribosyl transferase; SDS, sodium dodecyl sulfate; TLC, thin layer chromatography.

MATERIALS AND METHODS: Cloned lines, derived from Yaffe's rat myoblast cell line, L6 (15) were used. L6-9/1 and H9 were independent, cloned lines isolated from L6, and were functionally and morphologically "wild-type" with respect to their fusion and drug resistant properties. AG-2 was an 8-azaguanine resistant mutant, lacking the enzyme HGPRTase (17), but retaining its fusion-competence, like the wild-type. D3 was one of a representative class of 5-azacytidine resistant mutants (18) which were morphologically altered (extremely elongated bipolar shape compared to the spindle-shaped wild-type myoblasts) and unable to fuse or differentiate in other ways (Fig. 1). The mutant cell lines had been maintained in continuous culture in the absence of the appropriate drug for over two years (generation time, 18-20 hours) and had retained their drug resistant phenotypes. The various cell lines were routinely maintained at 37° in monolayer cultures in Dulbecco-modified Eagle's medium supplemented with 10% horse serum in Falcon tissue culture flasks. Incubation atmosphere was 5% CO_2 and 95% air. Large scale cell preparations were made in roller bottles. Fusion index (a measure of differentiation) was determined in cultures according to the procedure of Morris and Cole (19).

Cells were labelled with D-[1-^{14}C]-galactose (60mCi/mmole) by growth for 48 hours prior to harvesting in medium containing 5μCi of galactose per 10 ml. At different stages of differentiation (Figs. 1 and 2), cells were harvested by scraping with a rubber policeman in citrate-buffered saline, sedimented, and stored as pellets at -20° pending ganglioside analysis.

Gangliosides were extracted and purified according to procedures described by Laine et al. (20). They were separated by TLC on silica gel G by two successive developments with chloroform-methanol-2.5 N NH_4OH (60:35:8, v/v; ref. 25). The gangliosides were visualized autoradiographically by exposing the TLC plates to "Kodak No-Screen X-ray film" and visually by the use of Svennerholm's resorinol reagent. To measure radioactivity in gangliosides the separated bands were scraped directly into scintillation counting vials, dispersed in 1 ml of absolute alcohol and counted in Aquasol. Preparative thin layer chromatography of gangliosides were carried out on 500 micron thick silica Gel H thin-layer plates prewashed by running in methanol-concentrated HCl (9:1, v/v). The stoichiometric analysis of the carbohydrate moieties of separated gangliosides was performed (20) after derivatization of 0-methyl-glycosides to trimethylsilyl ethers by GLC.

For the isolation of surface membranes from myoblasts, the method of Scher and Barland (21) as modified by Carlsen and Till (22) was used. The purity of the isolated membranes was checked by electron microscopy. The membrane preparations were dissolved in a small amount of buffer containing 0.5 mM Tris-HCl, pH 8.0, 0.02 M β-mercaptoethanol, 1% SDS and 20% glycerol at 37°. Aliquots of this solution were used for SDS polyacrylamide gel electrophoresis (23). Protein concentrations were measured by the method of Lowry et al. (24).

RESULTS AND DISCUSSION: The only gangliosides present in significant amounts in the wild type cell lines, H_9 and L6-9/1, are GM_3, GM_2, GM_1 and GD_{1a}. In accordance with the findings of workers on whole skeletal muscle (26,27), GM_3 is also the major ganglioside in myoblasts followed by GM_2. Although we have not quantitated the proportion of the gangliosides, all clones and mutants which are capable of forming myotubes, invariably show the presence of all of these 4 gangliosides. For instance, an 8-azaguanine resistant mutant of myoblasts which lacks the enzyme HGPRTase (AG-2) but is able to fuse and differentiate invariably yields the same ganglioside distribution pattern as seen with wild-type clones. Clonal variations in ganglioside patterns exhibited by some types of cells in culture (8) apparently are uncommon or non-existent in myoblast

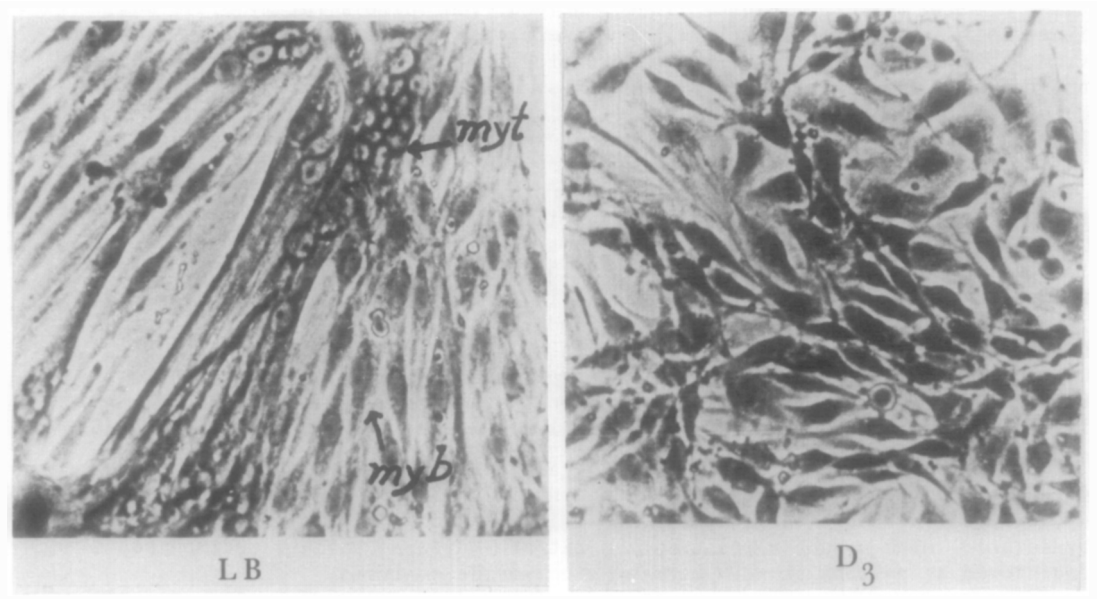

Fig. 1. Morphological characteristics of the wild-type (L6-9/1) and a non-fusing 5-azacytidine resistant (AZC^R) variant after 6 days in culture. Myt, myotubes showing clusters of nuclei (stage 3 of differentiation, described in the text); myb, myoblasts in arrayed patterns (stage 2 of differentiation) prior to fusion.

cell lines. In contrast to the results obtained with wild type cells, 5-azacytidine resistant mutant D_3 cells which are unable to fuse and propagate only as myoblasts (Fig. 1), show the presence of GM_3 virtually as the sole ganglioside. Traces of GM_2 are present but complex gangliosides are missing from the cells (Table 1); other independently isolated non-fusing AZC^R mutants (18) qualitatively show the presence only of GM_3. This pattern is invariant in cells cultured for various lengths of time and at different densities. In contrast to the changes observed in gangliosides we have been unable to find any qualitative variations in the glycoprotein pattern of the isolated membranes from fusion-incompetent AZC^R cells and fusion-competent wild type cells using electrophoresis in polyacrylamide gels. The observations made above raise the possibility that complex gangliosides may be involved either in the fusion of myoblasts per se or in the recognition of other fusion-competent myoblasts during myogenesis. If this were so, one would expect to find variations in ganglioside patterns as the myoblasts fuse to form myotubes. There are three stages in the development of myotubes in culture: the first stage is the 'confluent' stage at day 2 after plating when the myoblasts divide and rapidly form a sheet of

TABLE 1

DISTRIBUTION OF ^{14}C-LABEL AMONG THE GANGLIOSIDES OF WILD-TYPE (H_9) AND A 5-AZACYTIDINE RESISTANT MUTANT (D_3) DERIVED FROM IT

Ganglioside	cpm and percent of total radioactivity in gangliosides			
	H_9 cells		D_3 cells	
	cpm/10 mg cell protein	% of total	cpm/10 mg cell protein	% of total
GM_3	22,642	74	19,000	98.8
GM_2	5,782	18.9	230	1.2
GM_1	367	1.2	0	0
GD_{1a}	1,805	5.9	0	0

About 7-9 x 10^5 cells/10 cm tissue culture dish were plated and allowed to grow overnight. The medium was then aspirated and replaced with one containing 5 μCi of ^{14}C-galactose/10 ml. Cells were grown for further 48 hours after which the gangliosides were isolated and quantitated as described in the text.

cells on the plate; on days 3-5 the myoblasts reach the second stage, when the cells arrange themselves in longitudinal arrays; subsequently, at stage three, the arrayed cells begin to fuse (Fig. 1), and myotube formation is essentially complete by day 8-10 (Fig. 2). Cells were harvested at each of these stages and the distribution of radioactivity measured in the four gangliosides. The results are plotted as percentage values in Fig. 2 along with the fusion index curves which essentially show the time of progression of fusion in cultures from which samples were taken for ganglioside analysis. During differentiation GM_3 + GM_2 decrease from 90 percent to about 80 percent of total gangliosides. Values of GM_1 change hardly at all. However, there is a large increase at stage 2 (arrayed) in the quantity of GD_{1a} (relative increase of about 360%) followed by a drop to stage 1 (confluent) values immediately after fusion (Fig. 2). Although we have presented data only from wild type H_9 clone in Fig. 2, results similar to these were found with clone L6-9/1 and AG-2. The percentage distribution of radioactivity in the four gangliosides may vary somewhat from experiment to experiment but very significant increases in GD_{1a} levels at stage 2 only were always found in each fusing clone. Myotubes (stage 3) in each of the 3 clones showed the same quantity of GD_{1a} as in confluent myoblasts (stage 1). It thus seems possible that GD_{1a} has some role to play in the fusion process.

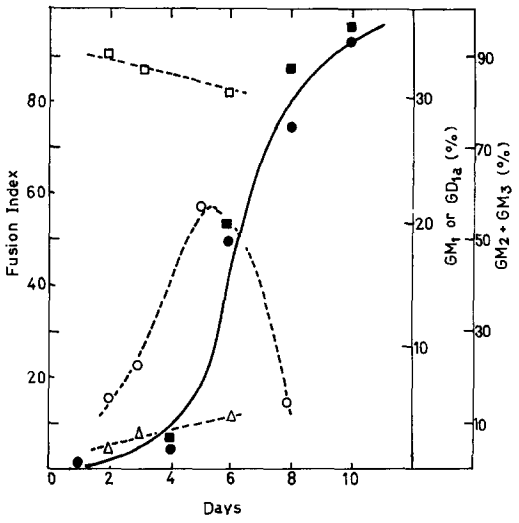

Fig. 2. Fusion index and the distribution of gangliosides at various stages of differentiation in wild-type H9. Symbols ■ and ● refer to fusion indices obtained at two different times. The values for gangliosides (□, GM_2 + GM_3; O, GD_{1a}; Δ, GM_1) are plotted as percentages of the total gangliosides.

The data presented above are reminiscent of the findings of Yogeeswaran and Hakomori (9) with mouse 3T3 fibroblasts. These authors found that out of several gangliosides only GD_{1a} increases at early stages of cell contact ('touching' stage) in culture and quickly returns to its normal levels in contact-inhibited cells. Furthermore, this increase is not discernible in transformed 3T3 lines suggesting some role for GD_{1a} in growth control and in the phenomenon of contact inhibition.

The most pressing question that is not answered by our findings is whether increase in GD_{1a} triggers fusion in myoblasts or it merely is a chemical expression of proximal apposition of two myoblasts in an arrayed configuration. The fusion asynchrony of our cell lines makes the task of interpretation difficult, although it is quite clear that enhancement of GD_{1a} synthesis is not a post-fusion event. It seems doubtful that GD_{1a}, or for that matter, other gangliosides are involved in the primary cell-cell recognition process because the consensus is that the gangliosides are buried in the glycocalyx of glycoproteins (8) and as such would not be accessible from outside. However, fusion requires the direct apposition of the two membranes at the level of the lipid bilayers which could be accomplished by protein clustering brought about by cell-cell recognition process (29). Gangliosides, thus exposed, may serve as the 'second line' of recognition in the plane of the lipid bilayer, and it is possible that, in the case of myoblasts, GD_{1a} may play this role. It is also possible that

high local concentrations of GD_{1a} may destabilize the lipid bilayer, as has been demonstrated for artifical bilayers (30) and in this way enhance fusion of two myoblast membranes.

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