Ganglioside GM2 is substrate for a sialidase in MDCK cells

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Abstract GM1 ganglioside carrying a fluorescent fatty acid in substitution of the natural one, has been administered to cultured Madin-Darby canine kidney (MDCK) cells for different pulse times (0.5–24 h), and its metabolic fate was followed. The fluorescent GM2, asialo-GM2, asialo-GM1 and ceramide were the only detectable metabolites. The complete absence of fluorescent GM3 is consistent with the presence in these cells of a sialidase working on GM1 and GM2 gangliosides. After treatment of the cells with chloroquine the fluorescent GM1 remained essentially undegraded, indicating a catabolic processing at lysosomal level.

Key words: Madin-Darby canine kidney cell; Asialo-monosialoganglioside 2; Sialidase; Fluorescent ganglioside; *N*-Lissamine rhodamine

1. Introduction

The monosialogangliosides GM1 and GM2 are known to be resistant to the action of most sialidases [1–3]. The occurrence of a sialidase activity on GM2 was reported in Tay-Sachs patients [4,5] and in different rat [6,7] and mouse [8] tissues. However, in all the reported cases the ganglioside GM2 was preferentially converted into GM3, GM2 → asialo-GM2 being a minor pathway.

Recently, Riboni et al. [9] have established that the major degradation pathway of GM1 in the neurotumoral cell line Neuro2a is: GM1 → GM2 → asialo-GM2 → Lac-Cer → Glc-Cer → Cer.

During a study on ganglioside transport in polarized Madin-Darby canine kidney (MDCK) cells, we observed that the fluorescent GM2 derived from administered GM1 was completely converted to asialo-GM2. In the meantime asialo-GM1 was a minor catabolite, indicating that in this cell line the predominant degradative pathway is also determined by a sialidase acting on ganglioside GM2.

2. Materials and methods

2.1. Chemicals

Minimum essential medium with Earl's salts (MEM), fetal bovine serum (FBS; heat inactivated before use) and glutamine were from Life Technology (Gibco, Milan, Italy). *Arthrobacter ureafaciens* sialidase (EC 3.2.1.18) was from Calbiochem (Inalco, Milan, Italy), bovine testes β-galactosidase (EC 3.2.1.23) was from Boehringer (Mannheim, Germany) and recombinant β-*N*-acetyl-hexosaminidase (EC 3.2.1.52) was from Biolabs (Celbio, Pero, Italy). Solvents and miscellaneous reagents, TLC aluminium sheets silica gel 60 were from Merck (Darmstadt, Germany). *N*-Lissamine rhodaminyl-(12-aminododecanoyl) GM1 and GM2 (C12-LRh-GM1 and C12-LRh-GM2) were synthesized as reported for sulforhodamine GM1 and sulforhodamine GM2 [10]. Standard fluorescent asialo-GM1 and asialo-GM2 were prepared from the corresponding gangliosides by *A. ureafaciens* sialidase treatment [11]. Glucosyl-ceramide and ceramide, both containing the LRh fluorophore, have been obtained following the general synthetic scheme reported for the LRh-sulfatide [12]. LRh-lactosyl-ceramide was prepared from the corresponding fluorescent asialo-GM2 by β-*N*-acetyl-hexosaminidase treatment. Typically, in a final volume of 100 μl, 50 pmol of asialo-GM2 were incubated at 37°C for 4 h in the presence of 10 mU of enzyme, 0.29 mM Na-taurodeoxycholate in 10 mM sodium-citrate buffer, pH 4.2. All the above analogues have also been prepared containing the fluorophore BODIPY. In this case the lyso-derivative of sphingolipid has been reacylated by means of 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*S*-indacene-3-dodecanoic acid (BODIPY®FLC12, Molecular Probes, Eugene, OR, USA) activated as *N*-hydroxysuccinimide ester according to Marchesini et al. [12].

2.2. Cell culture and incubation with C12-LRh-GM1

Madin-Darby canine kidney (MDCK) cells were cultured in MEM containing 10% FBS and 4 mM glutamine. Confluent cells (1–1.5 mg of total protein) were pulsed for 0.5–24 h in MEM containing 1% FBS, 4 mM glutamine and 5 μM C12-LRh-GM1. At the end of the pulse cells were scraped off the plates, centrifuged and lyophilized.

2.3. Analysis of the fluorescent lipids

Fluorescent lipids were extracted from lyophilized cells with chloroform/methanol (60:40 by volume). Aliquots were taken to quantify cell-associated fluorescence. Fluorescent lipids were separated on TLC (aluminium sheets silica gel 60) using ethyl acetate/butanol/chloroform/methanol/0.25% KCl (25:25:25:16:9 by volume). Individual spots were scraped off the plates, extracted from the gel in chloroform/methanol/water (5:5:1 by volume) and dried under a nitrogen stream. Their fluorescence intensities were read in chloroform/methanol (6:4 by volume) on a Jasco FP770 spectrofluorometer using excitation and emission wavelengths of 565 and 575 nm, respectively.

2.4. Identification of the metabolites LRh-asialo-GM1 and LRh-asialo-GM2

The extracts obtained from cells incubated for 24 h in the presence of LRh-GM1 were analyzed for fluorescent lipids content. After TLC separation, the spots co-migrating with standard LRh-asialo-GM1 and LRh-asialo-GM2 were isolated, subjected to mild acid hydrolysis [9] and treated either with β-galactosidase or with β-hexosaminidase [13].

3. Results

When MDCK cells were pulsed with C12-LRh-GM1, the GM1 uptake as well as the production of fluorescent metabolites were time-dependent. The only fluorescent ganglioside present at any time was C12-LRh-GM2 (Fig. 1 and Table 1); no C12-LRh-GM3 was detectable even at incubation times longer than 24 h. After incubation with C12-LRh-GM2, again no GM3 was present within an 8-h pulse (Table 1).

Among the non-gangliosidic metabolites, ceramide and two other fluorescent compounds were detected. The first detect-

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Abbreviations: Ganglioside nomenclature follows the suggestions of Svennerholm (Adv. Exp. Med. Biol. (1980) 125, 11–12) and the IUPAC-IUB recommendations (Lipids (1977) 12, 455–468); Cer, ceramide; Glc-Cer, glucosyl-ceramide; Lac-Cer, lactosyl-ceramide; LRh, lissamine rhodamine

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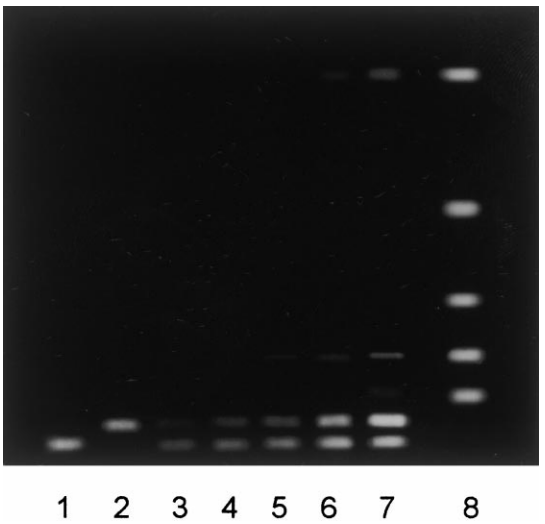

Fig. 1. MDCK cells were pulsed with C12-LRh-GM1 (5 μM) for 0.5, 1, 4, 8 and 24 h (lanes 3, 4, 5, 6 and 7, respectively). TLC solvent system: ethyl acetate/butanol/chloroform/methanol/0.25% KCl (25:25:25:16:9 by volume). Lane 1, standard LRh-GM1; lane 2, standard LRh-GM2; lane 8, standard LRh-asialo-GM1, LRh-asialo-GM2, LRh-LacCer, LRh-GLcCer and LRh-Cer (from bottom to top).

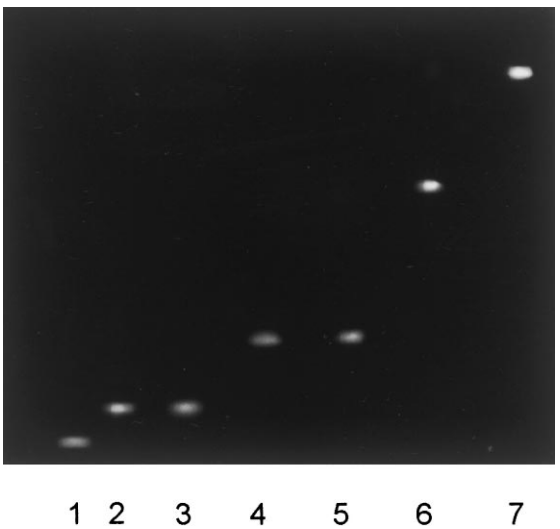

Fig. 2. β-Hexosaminidase treatment of the asialo-GM2 produced by MDCK cells. Zero time (lane 3) and after 4 h (lane 4). TLC solvent system as in Fig. 1. Lane 1, standard LRh-asialo-GM1; lane 2, standard LRh-asialo-GM2; lane 5, standard LRh-LacCer; lane 6, standard LRh-GlcCer; lane 7, standard LRh-Cer.

able product, comigrating on TLC with fluorescent standard asialo-GM2, gave rise to fluorescent Lac-Cer when subjected to β-hexosaminidase treatment (Fig. 2). The minor one, detectable in traces at 24 h pulse, comigrating with standard fluorescent asialo-GM1, was transformed into asialo-GM2 by action of β-galactosidase (Fig. 3). Mild acid hydrolysis generated Lac-Cer and Glc-Cer from asialo-GM2; Lac-Cer, Glc-Cer and asialo-GM2 from asialo-GM1 (Fig. 4). Ceramide was the major compound present at 24 h pulse.

When MDCK cells were incubated with fluorescent C12-LRh-GM1 in the presence of 50 μM chloroquine, no fluorescent metabolites were present into the cells.

Pulse experiments using BODIPY-GM1 gave results qualitatively very close to those obtained with LRh-GM1 (data not shown).

4. Discussion

In this study we have administered fluorescent monosialoganglioside (i.e. *N*-lissamine rhodamine and BODIPY containing GM1) to MDCK cells and studied its intracellular metabolism. Already at 0.5 h pulse GM2 is produced, but

Table 1
Quantitative analysis of metabolites produced after administration of C12-LRh-GM1 or C12-LRh-GM2 to MDCK cells

	Administered ganglioside					
	C12-LRh-GM1					C12-LRh-GM2
Pulse (h)	0.5	1	4	8	24	8
Total cell-associated fluorescence	0.482	0.537	0.949	1.193	2.088	0.749
	0.518	0.575	1.013	1.374	2.338	0.851
Total metabolites	0.028	0.055	0.286	0.639	1.602	0.015
	0.046	0.093	0.355	0.765	2.013	0.029
Gangliosides						
GM2	0.028	0.055	0.253	0.556	1.392	0.734
	0.046	0.093	0.313	0.668	1.777	0.822
GM3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Other lipids						
Asialo-GM1	N.D.	N.D.	N.D.	N.D.	0.019	N.D.
	N.D.	N.D.	N.D.	N.D.	0.023	N.D.
Asialo-GM2	N.D.	N.D.	0.033	0.042	0.092	0.015
	N.D.	N.D.	0.042	0.048	0.100	0.029
Cer	N.D.	N.D.	N.D.	0.041	0.099	N.D.
	N.D.	N.D.	N.D.	0.049	0.113	N.D.

Individual fluorescent spots, separated by TLC, were scraped off the plates, extracted with chloroform/methanol/water (5:5:1 by volume) and spectrofluorometrically quantified. Total and individual metabolites are expressed as nmol/mg of protein. Data are from two independent experiments.

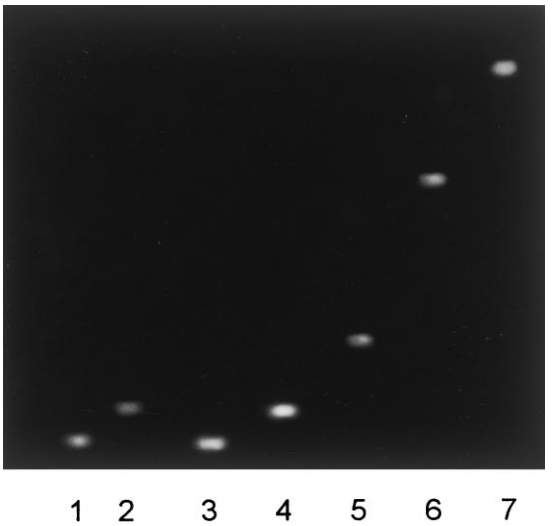

Fig. 3. β-Galactosidase treatment of the asialo-GM2 produced by MDCK cells. Zero time (lane 3) and after 4 h (lane 4). TLC solvent system as in Fig. 1. Lane 1, standard LRh-asialo-GM1; lane 2, standard LRh-asialo-GM2; lane 5, standard LRh-LacCer; lane 6, standard LRh-GlcCer; lane 7, standard LRh-Cer.

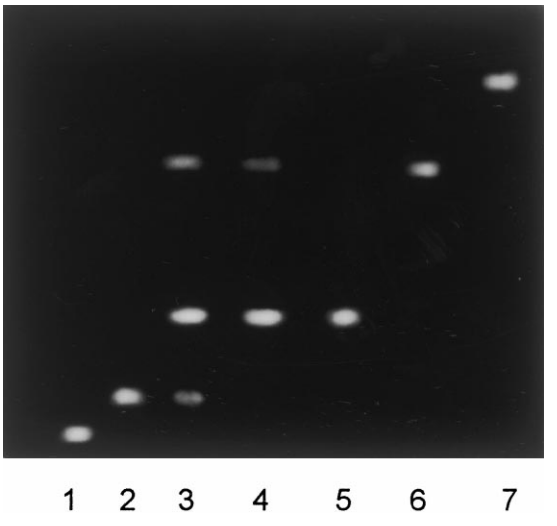

Fig. 4. TLC analysis of C12-LRh-asialo-GM1 (lane 3) and C12-LRh-asialo-GM2 (lane 4) isolated from MDCK cells and subjected to mild acid hydrolysis. TLC solvent system as in Fig. 1. Lane 1, standard LRh-asialo-GM1; lane 2, standard LRh-asialo-GM2; lane 5, standard LRh-LacCer; lane 6, standard LRh-GlcCer; lane 7, standard LRh-Cer.

no asialo-GM1 is detectable. GM2 is mainly degraded to asialo-GM2. At 24 h pulse, when GM2 represents 87% of total cell fluorescence, asialo-GM1 is detectable as minor component whereas GM3 is undetectable. Therefore the predominant pathway of GM1 degradation in MDCK cells appears to be the following: GM1 → GM2 → asialo-GM2 → → → Cer. The pathway through asialo-GM1 is a minor one, whereas the degradation pathway via GM3, which is the major degradation pathway in normal mammalian cells [3], is silent in these cells. No fluorescent Lac-Cer and Glc-Cer were detected after administration of either C12-LRh-GM1 or C12-BODIPY-GM1, even at the shortest time investigated, thus indicating their rapid conversion to ceramide.

Although the degradation routes of GM1 observed in MDCK cells closely resemble those described in Neuro2a cells [9], it should be noticed that our results have been obtained using analogues of GM1 ganglioside carrying bulky fluorophores which could alter the flow rates of the ganglioside molecules along the different degradation pathways.

Sialidase activity toward GM1 and GM2 is presumably located at lysosomal level, being abolished under conditions which are known to block the activity of lysosomal enzymes.

The conclusion of the present investigation is that MDCK cells present a sialidase activity on GM1 and GM2 gangliosides as do the neurotumoral Neuro2a cells [9] and PC12 cells, another cell type of tumoral nature (our data, not published). The hypothesis [9] that the expression of this sialidase activity could be linked to the tumoral nature of the cell-line, seems now more probable, but still remains to be demonstrated.

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