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QUARTERLY

Human fibroblasts in culture metabolize differently exogenous G_{M3} ganglioside species containing C18 and C20 sphingosine*°

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Preparation of radioactive G_{M3} species containing isotopically labeled C18 sphingosine or C20 sphingosine is reported and their use for studying some aspects of the sphingolipid biosynthesis in cells is discussed.

Human fibroblasts in culture that have only C18 sphingolipids and G_{M3} as the major gangliosides, were fed with the two radioactive G_{M3} species. The radioactive gangliosides were taken up by the cells and metabolized. The analyses of the radioactivity metabolic fate, in this model provides the following information.

***i* – About 70–80% of the total catabolic sphingosine is re-cycled for biosynthesis of complex sphingolipids.**

***ii* – A small amount of the catabolic C20 sphingosine was re-cycled for biosynthesis of C20 sphingolipids, thus yielding complex lipids that are not naturally present in fibroblast cells.**

***iii* – A regulatory step in the biosynthesis of sphingolipid species differring long chain base content, C18 or C20 sphingosine, is in some way involved in the first steps**

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Abbreviations: ganglioside nomenclature is according to Svennerholm [29] and the IUPAC-IUB recommendations [30]. G_{M3}, II^3Neu5AcLacCer, α-Neu5Ac-(2-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer; G_{D3}, II^3(Neu5Ac)$_2$LacCer, α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer; LacCer, β-Gal-(1-4)-β-Glc-(1-1)-Cer; GlcCer, β-Glc-(1-1)-Cer; Neu5Ac, *N*-acetylneuraminic acid; Cer, ceramide; Sph, sphingosine, (2*S*,3*R*,4*E*)-2-amino-1,3-dihydroxy-octadec/eicos-4-ene; [3-^{3}H*(C18 sphingosine)*]G_{M3}, α-Neu5Ac-(2-3)-β-Gal-(1-4)-β-Glc-(1-1)-{(2*S*,3*R*,4*E*)-2-(octadecanoyl)-amino-3-hydroxy-[3-^{3}H]octadec-4-ene}; [3-^{3}H*(C20 sphingosine)*]G_{M3}, α-Neu5Ac-(2-3)-β-Gal-(1-4)-β-Glc-(1-1)-{(2*S*,3*R*,4*E*)-2-(octadecanoyl)-amino-3-hydroxy-[3-^{3}H]eicos-4-ene}; EMEM, Eagle's minimum essential medium; FCS, fetal calf serum.

of sphingolipid biosynthesis, and thus plays a decisive role in the availability of the long chain bases.

Gangliosides, sialic acid containing glycosphingolipids that are normal components of the plasma membranes [1], are biosynthesized in the Golgi apparatus. The ganglioside species containing C18 and C20 sphingosine are both components of neuronal cells where they undergo marked quantitative changes during development of the nervous system [2–7]. Biosynthesis of C18 and C20 sphinganine is the first step of sphingolipid biosynthesis. It is then followed by N-acylation of sphinganines to dihydroceramides that are, in turn, oxidized to ceramides [8]. Furthermore, it has been suggested that the availability of C18 and C20 sphinganine, present in cells as free molecules in a ratio similar to that of C18 and C20 sphingolipids [7, 9], is related to biosynthesis of the ganglioside species containing C18 and C20 sphingosine. Thus the C18 and C20 sphinganine distribution could be regulated through the enzymatic properties of 3-ketosphinganine synthase (EC 2.3.1.50) towards palmitic and stearic acids, which are, together with serine, the respective precursors of C18 sphinganine and C20 sphinganine [10].

There is evidence that exogenously added gangliosides are taken up by the cells by endocytosis, degraded in the lysosomes, and the catabolites formed, of sugar and lipid nature, are partly re-cycled for biosynthetic purposes [11–14]. Human fibroblasts contain G_{M3} and G_{D3} gangliosides, the two molecular species containing C18 sphingosine. The present work was undertaken with the aim to follow the metabolic processing of an unnatural ganglioside species containing C20 sphingosine by these cells and to verify whether the unnatural availability of C20 sphingosine is sufficient to yield complex sphingolipids containing C20 sphingosine. The experimental model we adopted consisted of cultured human skin fibroblasts fed with G_{M3} ganglioside containing radioactive C18 or C20 sphingosine. Basing on this model it has been confirmed that the availability of C18 and C20 sphingosine is one of the regulatory steps in biosynthesis of complex sphingolipid species differing in the sphingosine content.

MATERIALS AND METHODS

Ganglioside G_{M3} was extracted from bovine brain, purified to over 99% purity and characterized [15]. All the reference lipids were available in the laboratory.

Preparation of [3-^{3}H(C18 sphingosine)]-G_{M3} and [3-^{3}H(C20 sphingosine)]G_{M3}. These two compounds were prepared by the dichloro-dicyano-benzoquinone/sodium boro[^{3}H]hydride and reversed phase HPLC purification procedures [16] that yield the isotopic tritium labeled *erythro* species. The scheme of the procedure is shown in Fig. 1.

Twenty milligrams of G_{M3} was dissolved in 10 ml of chloroform/methanol (2:1, v/v), and mixed with 10 ml of a solution of Triton X-100 in the same solvent (60 mg/ml). The solvent was evaporated and the residue dissolved in 10 ml of a dichloro-dicyano-benzoquinone solution in sodium dehydrated toluene (60 mg/ml). The mixture was maintained at 37°C for 40 h under constant stirring in a screw-capped tube. The solvent was then evaporated under vacuum at 37°C, and the dark brown residue suspended in 10 ml of cold acetonitrile and sonicated in an ultrasonic water bath. After centrifugation at 12000 × *g*, the supernatant, which contained Triton X-100 and dichloro-dicyano-benzoquinone, was discarded. The treatment was repeated four times to obtain a white pellet. The oxidized G_{M3}, 3-*keto*-G_{M3}, was separated from the unreacted G_{M3} on a silica gel 100 column (100 cm × 2 cm), equilibrated and eluted with a mixture of chloroform/methanol/water (60:35:3, by vol.). This 3-*keto*-G_{M3}, dissolved in 5 ml of propanol/water (7:3, v/v), was treated, at room temperature for 30 min and

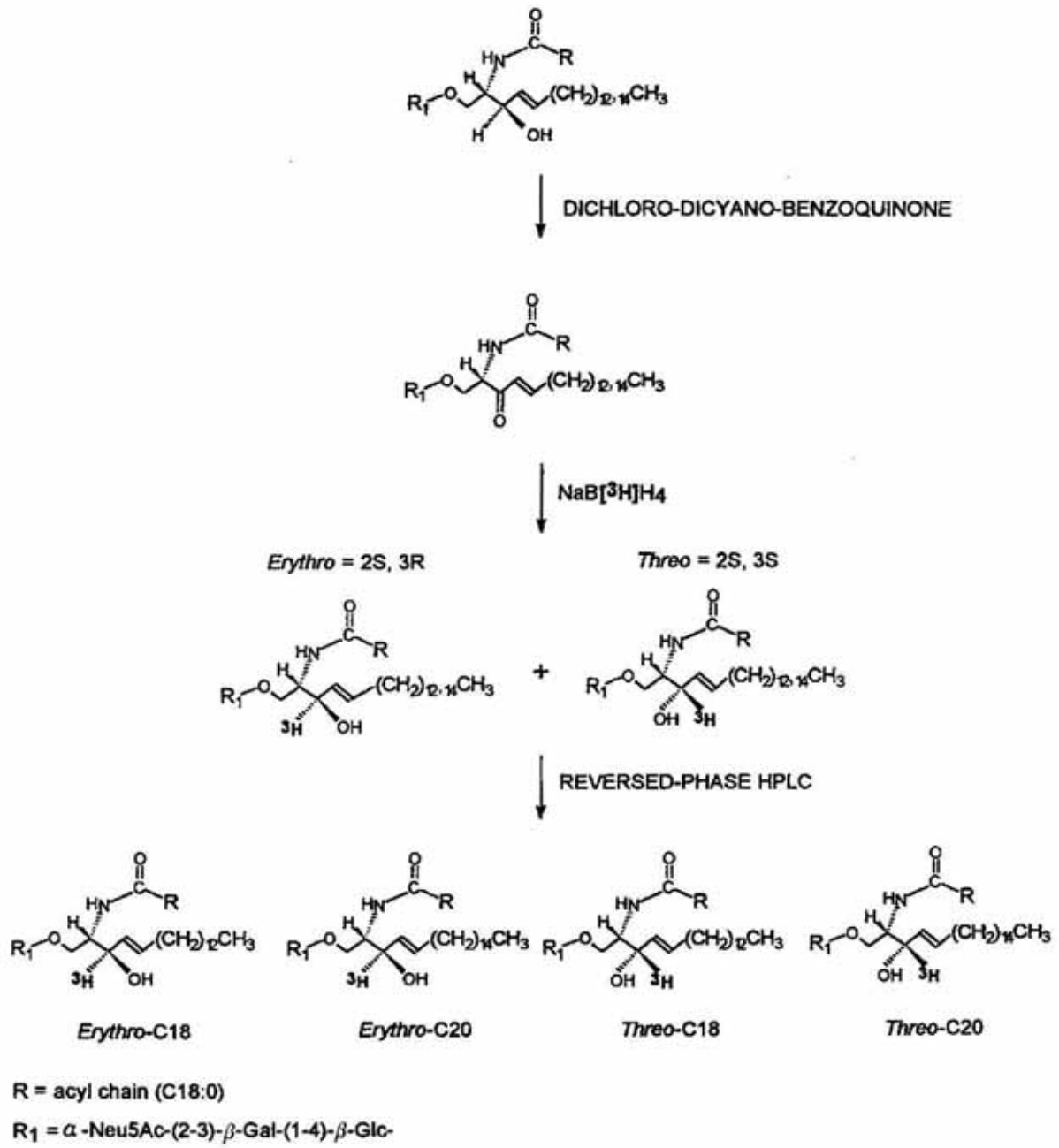

Figure 1. Scheme of the procedure for the preparation of [3-^{3}H*(C18 sphingosine)*]G_{M3} and [3-^{3}H*(C20 sphingosine)*]G_{M3}.

under stirring, with 100 mCi of solid [^{3}H]$NaBH_4$, (8 Ci/mmol, Amersham Int.); then 2 mg of $NaBH_4$ was added and the reaction continued for 30 min. The mixture was concentrated almost to dryness, diluted with redistilled water, dialyzed and dried. The residue was subjected to reversed phase HPLC for separation of the four G_{M3} molecular species containing C18 sphingosine and C20 sphingosine in the *erythro* and *threo* configurations, the latter being by-products of the reduction reaction. For chromatographic separation: 2.5 cm × 25 cm LiChrosphere reversed phase RP18-column (Merck, Germany) was used

equilibrated and eluted with the solvent system: acetonitrile/5 mM phosphate buffer, pH 7.0 (60:40, by vol.) at a flow rate of 10 ml/min. All the chromatographic procedural steps – sample preparation, loading into the injector-loop and chromatographic separation – were performed at 45°C. The elution profile was monitored by a flow-through detection of both the UV absorbance at 195 nm and the radioactivity content. The purified [3-^{3}H*(C18 sphingosine)*]G_{M3} and [3-^{3}H*(C20 sphingosine)*]G_{M3}, with a specific radioactivity of 2 Ci/mmol, were stored (50 μCi/ml) at 4°C in propanol/water (7:3, v/v).

Culture of fibroblasts. Human skin fibroblasts were obtained by the punch technique from healty subjects. Subcultures for experiments, at confluency, were prepared and used as described by Leroy *et al.* [17].

Treatment of cultured fibroblasts with radioactive gangliosides. Radioactive G_{M3}, dissolved in propan-1-ol/water (7:3, v/v) was pipetted into a sterile tube and dried under a stream of nitrogen. The residue was dissolved in an appropriate volume of pre-warmed (37°C) EMEM to obtain a final ganglioside concentration of 3×10^{-7} M. Five milliliters of this mixture was added to each 75 cm^2 flask, after accurate removal of the original culture medium. After 5 h incubation, the radioactive medium was removed and the flasks washed repeatedly, first with EMEM solution, then with 10% FCS-containing medium for 30 min, and finally the cells were incubated for 3 or 7 days with 10 ml of fresh unlabeled 10% FCS-EMEM. At the end of the chase time the flasks were rinsed three times with Hank's solution, and the cells harvested in water (2 ml) by scraping with a rubber scraper.

Analysis of cell associated radioactivity. Lyophilized cells were subjected to lipid extraction and fractionation [18], and analyzed for radioactivity content and distribution.

Analysis of cell medium tritiated water. Tritiated water [19] formed during metabolic processing of the gangliosides was determined as follows. One milliliter of the cell culture medium was dialyzed overnight against 2 ml of water. The dialysate was distilled at 100°C and the distillation product, containing tritiated water was counted. Control experiments were performed by incubating the radioactive gangliosides with the cell medium in the absence of cells.

Other analytical procedures. TLC was performed on high performance silica gel thin-layer chromatographic plates with the following solvent systems: a) chloroform/methanol/0.2% aqueous $CaCl_2$ (55:45:10, by vol.) tc assess composition of aqueous phase (gangliosides); b) chloroform/methanol (2:1, by vol.) and, after plate drying, chloroform/methanol/water (110:40:6, by vol.) to analyze the composition of the organic phase (G_{M3}, neutral sphingolipids and sphingomyelin).

The radioactivity present in the cell medium, that associated with total cells, and with the organic and aqueous phases obtained by lipid extraction and fractionation, was determined by liquid scintillation counting. Radioactivity of individual lipids separated by TLC was determined by radiochromatoscanning (Digital Autoradiograph, Berthold, Germany).

The protein content was determined [20] with bovine serum albumin as the reference standard.

Cell gangliosides were assayed by densitometric quantification after TLC separation [21], using pure gangliosides as the reference standards.

RESULTS AND DISCUSSION

C18 sphingosine and C20 sphingosine are the main long chain base components of gangliosides in the nervous system [22]. Of all the complex sphingolipids, gangliosides of the nervous system are the sole compounds that contain the C20 sphingosine species [23–25]. The C20 sphingosine is not present or present in a very scant amount in the extra-nervous system. In *in vitro* and *in vivo* systems [2–6, 23–25] the C18 ganglioside content increases

during cell differentiation and remains constant during cell aging. On the other hand, only very small amounts of C20 gangliosides are present at the beginning of development but, during differentiation and aging their amounts increase quite markedly, though they still form a minor part of the ganglioside mixture.

The *de novo* biosynthesis of a sphingolipid requires biosynthesis of sphinganine [8], its acylation and conversion of dihydroceramide to ceramide which, in turn, becomes available for the subsequent biosynthetic steps, i.e., in the case of gangliosides, for sequential glycosylation. Moreover, the sphingolipid catabolism yields sphingosine that becomes partly degraded and partly re-cycled [19, 26, 27]. Therefore, although no information is available on direct use of sphingosine or on the necessity of its preliminary reduction to sphinganine in ceramide biosynthesis, it is clear that both the catabolic sphingosine and biosynthesized sphinganine contribute to the long chain base pool necessary to maintain the appropriate sphingolipid turnover. Thus sphinganine biosynthesis provides not only the bases needed to increase the sphingolipid cell content but also those needed to replace the catabolic sphingosines that undergo degradation.

The different distribution of the C18 and C20 ganglioside species, together with the increase in sphingomyelin content during cell development [28], require a precise modulation of the sphingolipid metabolic processing. In this regard, we found that a predetermined distribution of C18 and C20 sphinganine plays a key role in sphingolipid biosynthesis modulation [7], and, in confirmation of this, we showed that the C18/C20 sphinganine ratio is very similar to that of the C18/C20 sphingolipids in neuronal cells during differentiation and aging in culture [28]. More recently [10] we reported that the enzyme 3-ketosphinganine synthase modulates the distribution of C18 and C20 sphingolipids by changing its kinetics properties towards palmitic and stearic acids, the precursors of C18 and C20 sphinganine.

To study the effect of the long chain bases on biosynthesis of sphingolipid species we provided cells that do not contain C20 sphingolipids with C20 sphingosine. Gangliosides administered to the cells in culture are taken up by the cells and enter the ganglioside metabolic pathway [12–14] producing catabolic sphingosine that is re-cycled for the biosynthesis of complex sphingolipids. Thus, human fibroblasts in culture, that do not contain C20 sphingolipids, C20 sphingosine and C20 sphinganine, were fed with G_{M3} containing radioactive C20 sphingosine, [3-^{3}H*(C20 sphingosine)*]G_{M3}, and the metabolic fate of radioactive C20 sphingosine was compared with that of radioactive C18 sphingosine after feeding the cells with G_{M3} containing radioactive C18 sphingosine, [3-^{3}H*(C18 sphingosine)*]G_{M3}.

The amount of radioactive G_{M3} added to the cells in culture was very small and during the pulse-chase time it entered the cells, modifying the original endogenous sphingolipids content by 2–5%. Analysis of the distribution of radioactivity within the ganglioside catabolites and the biosynthesized compounds derived from the catabolite recycling was carried out after a 3 or 7 day-chase. The data are reported in Table 1.

After feeding of fibroblasts with [3-^{3}H*(C18 sphingosine)*]G_{M3}, the cell associated radioactivity was carried, in addition to G_{M3}, also by LacCer, GlcCer, Cer, G_{D3} and sphingomyelin. Radioactive sphingosine was detected in traces. Substantial amounts of tritiated water, the final catabolite of radioactive sphingosine, were also found in the medium. It should be noted that radioactive sphingomyelin can solely be produced by a biosynthetic process which re-cycles radioactive sphingosine liberated during exogenous G_{M3} degradation [26–27]. On the other hand, radioactive LacCer, GlcCer and Cer could derive from both the catabolism of a taken up and processed [3-^{3}H*(C18 sphingosine)*]G_{M3} and biosynthesis by re-cycling of released sphingosine.

Table 1. Distribution of radioactivity in cultured fibroblasts fed with [3-^{3}H(C18- sphingosine)]G_{M3} and [3-^{3}H(C20 sphingosine)]G_{M3}.

The ganglioside concentration was 3×10^{-7} M; the pulse time was 5 h and was followed by 3 or 7 day-chase. The radioactivity linked to individual substances has been expressed as percent of the total cell radioactivity, this accounting also for that carried by cell tritiated water released into the cell medium; 3–4% of the total cell radioactivity was distributed within the non-characterized TLC bands. Data are means of 4 experiments with two cell lines; S.D. ±20%. G_{M3} biosynthesized by re-cycling of sphingosine has been calculated on the basis of radioactive G_{D3} and of the endogenous G_{M3}/G_{D3} molar ratio.

	[3-^{3}H*(C18 sphingosine)*]G_{M3}		[3-^{3}H*(C20 sphingosine)*]G_{M3}	
	3 days	7 days	3 days	7 days
	% of total cell associated [^{3}H]G_{M3}			
G_{M3}(total)	48.6	37.0	45.1	29.0
G_{D3}	2.0	3.8	0.8	1.1
LacCer	3.9	5.3	1.3	1.5
GlcCer	2.2	2.1	1.4	1.1
Cer	3.0	1.8	0.8	0.5
SM[a]	25.0	26.5	4.5	2.9
LCB[b]	traces	traces	traces	traces
Water	10.9	20.0	43.3	62.2
Total	95.6	96.5	97.2	98.3
Biosynthesized G_{M3}	6.1	11.6	2.4	3.3
G_{M3} metabolized	57.5	74.6	57.3	73.3
	% of total radioactive metabolites			
G_{M3}	10.6	15.6	4.2	4.5
G_{D3}	3.5	5.1	1.4	1.5
LacCer	6.8	7.1	2.3	2.0
GlcCer	3.8	2.8	2.4	1.5
Cer	5.2	2.4	1.4	0.7
SM	43.5	35.5	7.9	3.9
LCB	traces	traces	traces	traces
Water	19.0	26.8	75.5	84.8
	d.p.m./mg cell protein		d.p.m./mg cell protein	
Cell radioactivity	129150	106000	148000	142000

[a]SM, sphingomyelin; LCB, long chain base.

We previously [19] demonstrated that LacCer, GlcCer and Cer result primarily from a biosynthetic process by re-cycling of liberated sphingosine.

As the first approximation, looking at the amount of metabolic radioactive water, we concluded that the extent of the sphingosine re-cycling process is high. Our results suggest that 70–80% of the sphingosine formed by sphingolipid catabolism is re-cycled for the biosynthesis of complex sphingolipids.

The [3-^{3}H*(C20 sphingosine)*]G_{M3} was largely catabolized. The extent of the re-cycling process of C20 sphingosine appears to be very limited, as proved by the simultaneous presence of a high amount of tritiated water. Nevertheless, a small amount was re-cycled for the biosynthesis of G_{D3} and sphingomyelin. Both radioactive G_{D3} and sphingomyelin contain C20 sphingosine; in fact any metabolic transformation of C20 sphingosine to C18 sphingosine will lead to the loss of radioactivity, tritium being at position 3 of the molecule. The finding that fibroblasts provided with unnatural C20 sphingosine can biosynthesize C20 sphingolipids suggests that C20 sphingosine can be a substrate for the enzyme systems in the cells yielding only C18 sphingolip-

ids. This confirms that the biosynthesis of sphingolipid species differing in the long chain base content, C18 or C20 sphingosine, is in part related to the first steps of sphingolipid biosynthesis, i.e., those steps that determine the availability of long chain bases of different length of the hydrophobic chain [7, 9, 10].

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