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New approaches to the study of sphingolipid enriched membrane domains: The use of electron microscopic autoradiography to reveal metabolically tritium labeled sphingolipids in cell cultures

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This paper is the first report on the use of the electron microscopy autoradiography technique to detect metabolically tritium labeled sphingolipids in intact cells in culture. To label cell sphingolipids, human fibroblasts in culture were fed by a 24 hours pulse, repeated 5 times, of 3×10^{-7} M [1-^{3}H]sphingosine. [1-^{3}H]sphingosine was efficently taken up by the cells and very rapidly used for the biosynthesis of complex sphingolipids, including neutral glycolipids, gangliosides, ceramide and sphingomyelin. The treatment with [1-^{3}H]sphingosine did not induce any morphological alteration of cell structures, and well preserved cells, plasma membranes, and intracellular organelles could be observed by microscopy. Ultrathin sections from metabolic radiolabeled cells were coated with autoradiographic emulsion. One to four weeks of exposition resulted in pictures where the location of radioactive sphingolipids was evidenced by the characteristic appearance of silver grains as irregular coiled ribbons of metallic silver. Radioactive sphingolipids were found at the level of the plasma membranes, on the endoplasmic reticulum and inside of cytoplasmic vesicles. Thus, electron microscopy autoradiography is a very useful technique to study sphingolipid-enriched membrane domain organization and biosynthesis.

Keywords: **Electron microscopic autoradiography, sphingolipid domains, tritium, labeling, cell culture**

Abbreviations: **Ganglioside nomenclature is according to Svennerholm [35] and the IUPAC-IUBMB recommendations [36]. GM3, II^3-α-Neu5AcLacCer, α-Neu5Ac-(2-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer; GD3, II^3-(α-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; Cer, ceramide, (2*S*,3*R*,4*E*)-2-(octadecanoyl)amino-1,3-dihydroxy-octadec-4-ene; Sphingosine, C18-sphingosine, (2*S*,3*R*,4*E*)-2-amino-1,3-dihydroxy-[1-^{3}H]octadec-4-ene; Neu5Ac, N-acetylneuraminic acid; PE, phosphatidylethanolamine; SM, sphingomyelin; EMEM, Eagle's minimum essential medium; FCS, fetal calf serum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate buffered saline solution.**

Introduction

Glycosphingolipids were discovered 120 years ago [1], but research on membrane gangliosides has grown rapidly only in the last 30 years. Glycosphingolipid research has been greatly facilitated by the development of high sensitive, high resolution analytical and preparative procedures in the field of chemistry, chromatography, mass spectrometry, NMR spectroscopy, immunochemistry, subcellular and cell membrane fractionations (see references [2] and [3] for an exhaustive presentation on methods for the study of glycosphingolipids and related references).

The first suggestions about a non-random distribution of complex lipids in the cell membranes were presented in the seventies and eighties [4–5], but it was necessary to wait until

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the nineties, following the improvement of membrane fractionation techniques [6–9], to have the first information on the composition of membrane domains, and to know that some of them are enriched in sphingolipids, cholesterol and cell signaling proteins [9–17]. The procedures used to prepare the sphingolipid enriched domains are based on their resistance to solubilization by cold nonionic detergents, to strong alkaline chemical reagents, or to mechanical treatment, and their low buoyant density [6–10]. Since large amounts of cellular material is necessary to carry out the lipid analyses, radiolabeling procedures can be very useful to simplify some analytical aspects [17].

A direct picture of membrane domains enriched in glycosphingolipids has been obtained using immunogold electron microscopy procedures [18–20]. These procedures use colloidal gold conjugated cholera toxin, that specifically binds GM1 ganglioside [18], or anti-ganglioside antibodies and colloidal gold conjugated secondary antibodies [19–20] (see [21] for an exhaustive review). However, control experiments are always necessary to exclude that the glycosphingolipid segregation is mediated by cholera toxin or the antibodies themselves. Moreover, anti-ganglioside antibodies are seldom highly specific, and usually have low affinity for the antigen.

In this paper we show that electron microscopic autoradiography is a very useful technique to investigate the localization and distribution of metabolically radiolabeled cell sphingolipids, revealed by elongated thin ribbons of silver grain deposition that can be clearly seen under the electron microscope. We metabolically radiolabeled all the cell sphingolipids by feeding [1-^{3}H]sphingosine to cultured human fibroblasts [22]. The specific radioactivity of sphingosine fed to cells, 2 Ci-mmole, and the resulting specific radioactivity of [1-^{3}H]sphingosine taken up by the cell after its dilution with the endogenous cell sphingosine, was high enough to allow reasonable autoradiography exposure times, and to give preliminary pictures of the sphingolipid cell trafficking and sphingolipid-enriched domain biogenesis.

Materials and Methods

Materials

The commercial chemicals were the purest available, common solvents were distilled before use, and deionized water, obtained by a MilliQ system (Millipore), was distilled in a glass apparatus. High performance silica gel precoated thin-layer chromatography plates (HPTLC Kieselgel 60, 10×10 cm) were purchased from Merck GmbH; sodium boro[^{3}H]hydride (8 Ci/mmol) from Amersham International; *Vibrio cholerae* sialidase and *Bacillus cereus* sphingomyelinase from Sigma; *Macrobdella decora* ceramide glycanase from Boehringer Mannheim. Sphingosine was prepared from cerebroside as described [23]. [1-^{3}H]Sphingosine (homogeneity over 98%; specific radioactivity, 2 Ci/mmol) was prepared by specific chemical oxidation of the primary hydroxyl group of sphingosine followed by reduction with sodium boro-[^{3}H]hydride [24]. [^{3}H]labeled ceramide, sphingomyelin, phosphatidylethanolamine, lactosylceramide and GD3 ganglioside were extracted from [1-^{3}H]sphingosine-fed cells (see below), purified, characterized and used as chromatographic standards. Human skin fibroblasts were cultured to confluence, as previously described [25–26].

Treatment of cell cultures with [1-^{3}H]sphingosine

[1-^{3}H]Sphingosine was solubilized in an appropriate volume of pre-warmed (37°C) EMEM containing 10% FCS, at a final concentration of 3×10^{-7} M. After removal of the original medium and rapid washing of cells with EMEM, 5 ml of the medium containing the radioactive sphingosine was added to each 100 mm dish and the cells were incubated at 37°C. After 24 hours of incubation, the medium was removed and substituted with fresh medium containing radioactive sphingosine; the treatment with radioactive sphingosine was repeated 5 times. In control experiments cells were incubated two hours with 3×10^{-8} M [1-^{3}H]sphingosine; after the incubation the medium was replaced with 10% FCS-EMEM not containing radioactive sphingosine, and the chase was prolonged 48 hours. At the end of the incubations, cells were washed twice with PBS, scraped off with a rubber policeman, centrifuged at 1000 g for 10 min and subjected to lipid and protein analyses, sphingolipid-enriched fraction preparation or preparation of sections for electron microscopy studies.

Preparation of the detergent-insoluble membrane fraction

The preparation of the detergent-insoluble membrane fraction, which is highly enriched in sphingolipids [12], was carried out according to previously described procedures [6,12]. The detergent-insoluble membrane fraction was prepared by discontinuous sucrose gradient centrifugation of a postnuclear fraction. All the gradient fractions were analyzed for sphingolipid content and enrichment.

Cell lipid extraction and characterization

The cell homogenates, postnuclear supernatants and sucrose gradient fractions obtained after feeding cells [1-^{3}H]sphingosine were subjected to lipid extraction [17]. Lipid extracts were analyzed by TLC, followed by radioactivity imaging (see below). Identity of lipids was assessed by co-migration with standard lipids. The main radioactive lipids were purified by the TLC-blotting technique [27], and characterized by enzymatic and chemical treatments. In particular, gangliosides GM3 and GD3, dissolved in 50 μL of water, were treated at 37°C for 2 hours with 1 mU of *Vibrio cholerae* sialidase, to yield lactosylceramide, and both GM3 and lactosylceramide, respectively. The same GM3 and GD3, dissolved in 500 μL of 25 mM acetate buffer, pH5, 0.075% sodium cholate, were treated at 37°C for 18 h with 1 mU of *Macrobdella decora* ceramide glycanase, to yield ceramide.

Sphingomyelin, dissolved in 30 µL of 100 mM Tris-HCl, pH 7.4, 0.5 mM $MgCl_2$, 0.05% sodium deoxycholate, was treated at 37°C overnight with 11 mU of *Bacillus cereus* sphingomyelinase, to yield ceramide. Phosphatidylethanolamine, formed by recycling of the radioactive ethanolamine produced in the catabolism of sphingosine [22], was characterized following its degradation under alkaline conditions. The reaction mixtures were separated by TLC (see below) and the reaction products identified by chromatographic comparison with standard lipids.

Electron microscopy autoradiography

After metabolic radiolabeling, cells were fixed with 2% glutaraldehyde and scraped from the flask. After washing, the cells were post-fixed with 1% OsO_4, dehydrated with ethanol and embedded in Epon 812. Ultrathin sections were coated with autoradiographic emulsion (IIford L4) and then placed in dry boxes in the dark at 4°C for exposure of the photographic emulsion. At specified intervals the samples were removed from the box and developed in D-19, fixed and post-stained with uranyl acetate and lead citrate [28]. Examination of samples was performed under an electron microscope (Philips CM100, Netherland). After chemical development, the location of radioactive materials was evidenced by the characteristic appearance of silver grains as irregular coiled ribbons of metallic silver.

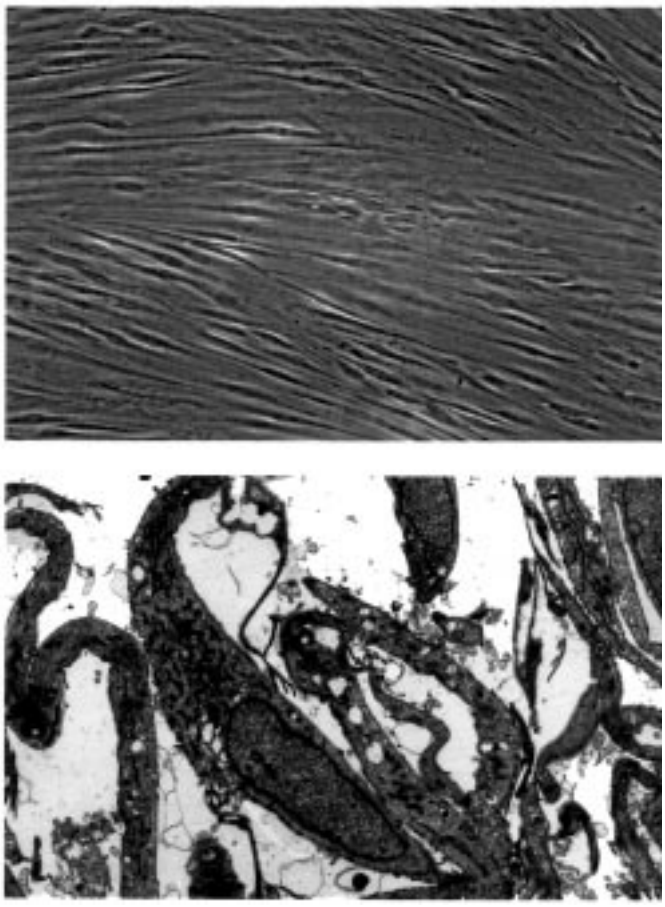

Figure 1. Optic (upper picture) and electron (lower picture) microscopy analysis of cultured human fibroblasts fed 5 times with 3×10^{-7} M [1-^{3}H]sphingosine. Pictures do not reveal any morphological changes. The ultrastructural analysis reveals a good quality of preservation of cellular structures.

Other analytical methods

TLC was performed with the solvent system chloroform/methanol/0.2% aqueous $CaCl_2$, 50:42:11 by vol, to analyze the enzymatic reaction mixtures, and with chloroform/methanol/0.2% aqueous $CaCl_2$, 55:45:10 by vol, to assess the total lipid mixture compositions. Two-dimensional HPTLC was performed to assess the presence of sphingosine, using the following solvent systems: 1st run, chloroform/methanol/0.2% aqueous $CaCl_2$, 55:45:10 by vol; 2nd run, chloroform/methanol/37%NH_4OH, 40:10:1 by vol.

The radioactivity associated with total homogenates, gradient fractions, total lipid extracts and delipidized pellets, was determined by liquid scintillation counting. Radioactivity imaging of TLC plates was performed with a Beta-Imager 2000 instrument (Biospace, Paris). The radioactivity associated with individual lipids was determined with the specific β-vision software provided by the manufacturer (Biospace).

The protein content was determined according to Lowry et al. [29], by the micro BCA kit (Pierce), and as dot spots

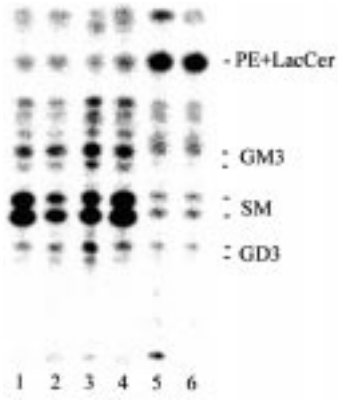

Figure 2. TLC separation of the total lipid mixture from cultured human fibroblasts fed [1-^{3}H]sphingosine by a single (two hour-pulse, 3×10^{-8} M) or a 5 times (24 hour-pulse, 3×10^{-7} M) pulse. Lane 1, homogenate from cells fed 3×10^{-8} M [1-^{3}H]sphingosine; lane 2, homogenate from cells fed 5 times 3×10^{-7} M [1-^{3}H]sphingosine; lane 3, sphingolipid enriched membrane fraction (fraction 5 in Figure 4) from cells fed 3×10^{-8} M [1-^{3}H]sphingosine; lane 4, sphingolipid enriched membrane fraction (fraction 5 in Figure 4) from cells fed 5 times 3×10^{-7} M [1-^{3}H]sphingosine; lane 5, fraction 11 in Figure 4 from cells fed 3×10^{-8} M [1-^{3}H]sphingosine; lane 6, fraction 11 in Figure 4 from cells fed 5 times 3×10^{-7} M [1-^{3}H]sphingosine. Detection by radioactivity imaging; acquisition time, 48 hr.

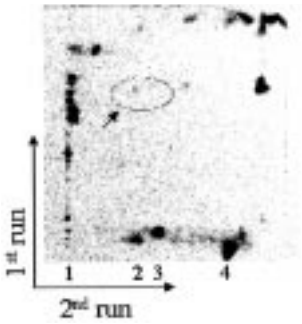

Figure 3. 2D-TLC separation of the total lipid extract from cultured human fibroblasts fed 5 times 3×10^{-7} M [1-^{3}H]sphingosine. Solvent systems: 1st run, chloroform/methanol/0.2% aqueous $CaCl_2$, 55:45:10 by vol; 2nd run, chloroform/methanol/37%NH_4 OH, 40:10:1 by vol. Arrow indicates the TLC area where sphingosine and sphinganine should be localized if present. 1: total radioactive sphingolipid mixture was applied to the left side of the TLC plate. 2, 3 and 4: radioactive sphinganine, sphingosine and ceramide, respectively. Radioactive chromatographic standards were applied on the right side before the 2nd run. Detection by radioactivity imaging; acquisition time, 24 hr.

revealed by Coomassie blue staining, using bovine serum albumin in the presence of sucrose, as the reference standard. The viability of the cells was assessed by the Trypan blue exclusion method.

Results

To perform electron miscroscopy autoradiography analysis of cells containing tritium labeled sphingolipids, we administered [1-^{3}H]sphingosine under different experimental conditions to cells in culture. Feeding conditions were a 24 hr pulse with 3×10^{-7} M [1-^{3}H]sphingosine repeated 5 times and a single 2 hr pulse with 3×10^{-8} M [1-^{3}H]sphingosine. Cells fed 5 times with 3×10^{-7} M [1-^{3}H]sphingosine showed a viability over 90%. The same viability was found in cells fed once with 3×10^{-8} M [1-^{3}H]sphingosine and in untreated control cells. Under both feeding conditions, the use of [1-^{3}H]sphingosine as precursor for sphingolipid biosynthesis did not produce any effect on cell morphology. Figure 1 shows optic and electron microscopic images of fibroblasts fed 5 times 3×10^{-7} M [1-^{3}H] sphingosine. The integrity of the overall cell structure and of all cellular organelles appears well preserved. The typical fibroblast shape and a well preserved cellular structure were evident. Plasma membrane appeared intact and inside the cell, nucleus, mitochondria, endoplasmic reticulum and vacuoles were clearly observed.

[1-^{3}H]Sphingosine was taken up by fibroblasts very quickly, and entered, as expected [22], the biosynthetic pathway of sphingolipids. In addition to sphingolipids, i.e, sphingomyelin, GM3 and GD3 gangliosides, neutral glycosphingolipids and ceramide, radioactive phosphatidylethanolamine was formed, owing to re-cycling of the radioactive ethanolamine derived from sphingosine degradation. The radioactive lipid patterns of fibroblast cells, fed with [1-^{3}H]sphingosine by a single two hour-pulse, (3×10^{-8} M) or five 24 hour-pulses (3×10^{-7} M), as determined by TLC and radioactivity imaging, is reported in Figure 2. For the analysis of free radioactive sphingosine in the total radioactive lipid mixture, it was necessary to perform a 2D-TLC using different solvent systems in the first and second TLC runs. Figure 3 shows that free sphingosine in the total lipid mixture was in trace quantities.

The radioactivity percent distribution was very similar within the lipids extracted from cells subjected to the two different pulse conditions (Figure 2, lanes 1 and 2). Nevertheless, the chosen pulse-chase feeding conditions did not allow introduction of a similar specific radioactivity in the sphingolipids, due to different turnover of each sphingolipid class [17]. In particular, the radioactivity ratio between sphingomyelin and gangliosides was about 2-fold lower than the mass ratio (Table 1).

Radioactivity associated with PE ranged from 6 to 10% of the total lipid associated radioactivity. This confirms that the recycling of sphingosine under the chosen feeding conditions is very high and only a minor portion of sphingosine is phosphorylated and catabolyzed with the production of

Table 1. Protein and lipid content in cultured fibroblasts fed [1-^{3}H]sphingosine.

pulse conditions	*3×10^{-8} M, 2 hr*	*$5 \times (3 \times 10^{-7}$ M, 24 hr)*
μg protein/dish	820 ± 140	820 ± 140
administered dpm/dish	0.85×10^6	30×10^6
associated dpm/mg protein	$0.6 \times 10^6 \pm 0.05 \times 10^6$	$15 \times 10^6 \pm 1.3 \times 10^6$
sphingomyelin, % of radioactive lipids	45 ± 4	43 ± 4
gangliosides, % of radioactive lipids	15 ± 2	14 ± 1.8
others, % of radioactive lipids	34 ± 5	33 ± 4.8
sphingosine, % of radioactive lipids	traces	traces
PE, % of radioactive lipids	6 ± 0.9	10 ± 1.4
sphingomyelin, nmoles/mg protein	20 ± 3.1	20 ± 3.1
gangliosides, nmoles/mg protein	4.0 ± 0.6	4.0 ± 0.6

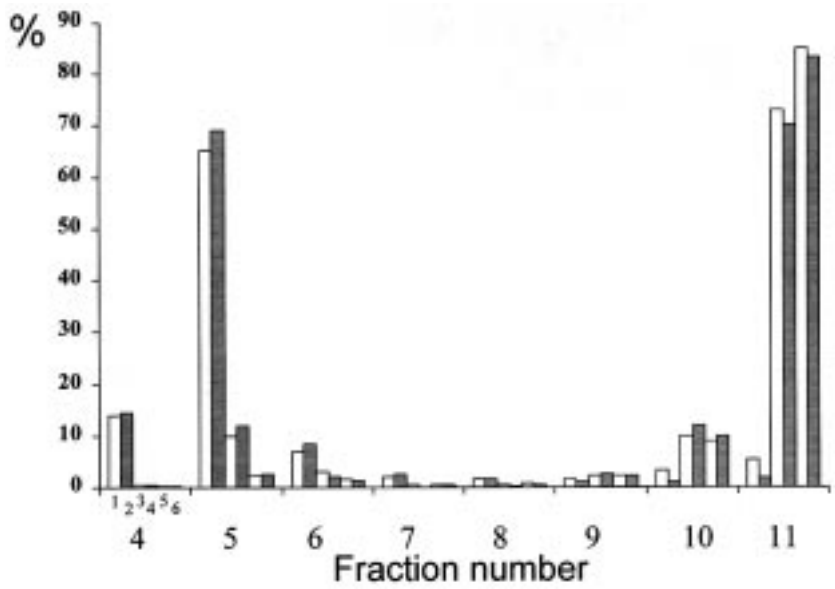

Figure 4. Sucrose gradient fractionation of a postnuclear fraction prepared from fibroblasts homogenized in the presence of detergent. Fraction 1 was collected at the top of the gradient and the pellet-containing fraction 11 at the bottom. Fraction 5 contained the light scattering band located between 5% and 30% sucrose. Fractions 1–3 did not contain radioactivity or proteins. Bar 1, radioactive sphingolipid content from cells fed 5 times 3×10^{-7} M [1-^{3}H]sphingosine; bar 2, radioactive sphingolipid content from cells fed 3×10^{-8} M [1-^{3}H]sphingosine; bar 3, radioactive PE content from cells fed 5 times 3×10^{-7} M [1-^{3}H]sphingosine; bar 4, radioactive PE content from cells fed 3×10^{-8} M [1-^{3}H]sphingosine; bar 5, protein content from cells fed 5 times 3×10^{-7} M [1-^{3}H]sphingosine; bar 6, protein content from cells fed 3×10^{-8} M [1-^{3}H]sphingosine.

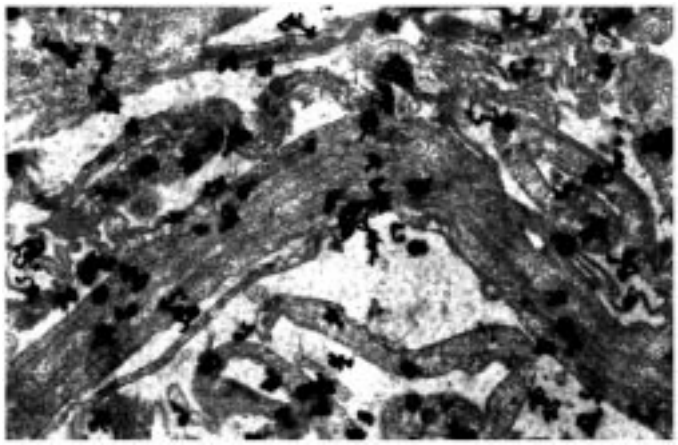

Figure 5. Electron-microscopic autoradiography of cultured human fibroblasts fed 5 times 3×10^{-7} M [1-^{3}H]sphingosine. Silver grains corresponding to tritium labeled lipids are visible inside the cells and at the plasma membranes.

phosphoethanolamine that subsequently enters the metabolic pathway of PE.

Following the two different pulse treatments, cells were used for the preparation of detergent-insoluble membrane fraction. Figure 4 shows that under both pulse conditions, the detergent-insoluble membrane fraction, fraction 5, is highly enriched in sphingolipids, while the majority of PE and of cell proteins were located in the heavier fractions that are at the bottom of sucrose gradient, fractions 10 and 11. The radioactive lipid patterns of fractions 5 and 11 are reported in Figure 2, lanes 3 and 4, and lanes 5 and 6, respectively.

Ultrastructural analysis of ultrathin sections coated with autoradiographic emulsion from cells fed 5 times with 3×10^{-7} M [1-^{3}H]sphingosine showed, after 4 week exposure of photographic emulsion and development, many grains of different sizes originated from the deposition of metallic silver. Many of them were inside the cell body and only a few on plasma membranes (Figure 5). A specific cellular localization of silver grains was clearly obtained with a higher magnification. Many irregular coiled ribbons of metallic silver appeared localized mostly in the endoplasmic reticulum (Figure 6A and 6C), occasionally in nucleus (Figure 6A) and in the

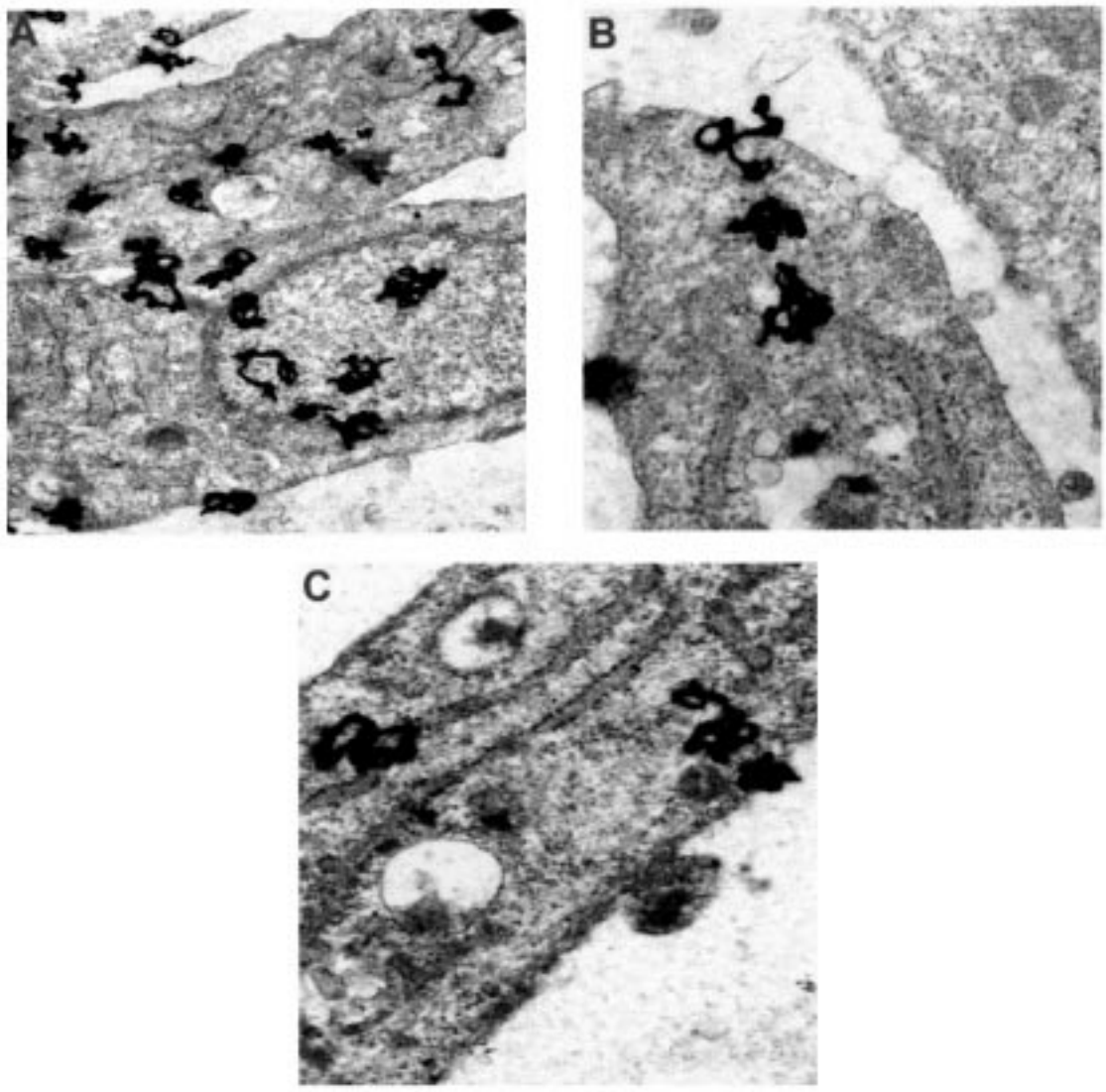

Figure 6. Electron-microscopic autoradiography of cultured human fibroblasts fed 5 times 3×10^{-7} M [1-^{3}H]sphingosine. Higher magnification shows a possible localization of silver grains in correspondence to endoplasmic reticulum and intracytoplasmic vacuoles.

cytoplasmic vesicles apparently moving toward the plasma membrane (Figure 6B).

Discussion

Immunocytochemical approaches using cholera toxin and anti-ganglioside antibodies has allowed observation of ganglioside enriched domains on plasma membrane [18–21]. However, the possible cross-linking effects induced by both cholera toxin and antibodies have raised doubts about the real existence of such domains. Here, we present a new approach for the study of sphingolipid distribution and cellular trafficking through the use of electron microscopic autoradiography, which may offer a substantial contribution to the study of membrane lipid organization.

Electron microscopic autoradiography has been considered a valuable technique that provides exact localization of radioisotope-labeled biological materials in tissue at the cellular and subcellular level while preserving the micro-architecture. Particularly important applications have been those on the processes of macromolecular synthesis and secretion and on the binding site of small molecules such as hormones [30], drugs, toxins and transmitter substances [31]. Very little information is present in the literature concerning lipid metabolism and trafficking [32–33]. However, autoradiographic methods were tedious and difficult. New photographic emulsion consisting of silver bromide grains embedded in a gelatin layer are now available allowing faster and easier autoradiographic analyses. Electrons which are emitted from a radioactive source cause reduction of silver bromide, leading to a deposition of silver grains as elongated thin ribbons that

can be clearly seen under the electron microscope. The advantage of electron microscopy autoradiography is therefore a matter of resolution, providing allocation of silver grains to organelles, which are not recognizable by light microscopy.

In this paper we report on the use of the electron microscopy autoradiography technique to reveal metabolically radiolabeled sphingolipids in the cell. Among the commonly used sphingolipid precursors, sugar, activated sugars, fatty acids and serine have several drawbacks, that make the revealing of glycosphingolipids difficult: 1) they are largely diluted into the cell endogenous pool of the corresponding compounds, this fact resulting in a strong reduction of their original specific radioactivity, 2) they are precursors of many cell components, often present in high concentration, such as glycopolymers, triglycerides and proteins, 3) they are largely catabolyzed to radioactive water that increases the radioactive background. To overcome these problems we used [1-^{3}H]sphingosine which has the following advantages: 1) we synthesized [1-^{3}H]sphingosine [24] in large amounts and with high specific radioactivity; 2) [1-^{3}H]sphingosine is taken up by the cells very rapidly and immediately used for the biosynthesis of all complex sphingolipids, neutral glycolipids, gangliosides, ceramide and sphingomyelin [17,22]; 3) the endogenous cellular content of sphingosine is very low [34], thus, after [1-^{3}H]sphingosine is taken up by the cell, the final specific radioactivity remains quite high; 4) sphingosine is largely recycled, thus the amount of radioactive catabolic phosphoethanolamine used for the biosynthesis of PE is quite low.

The concentration of sphingosine used in our experiments was low and far from the concentration values that are known to modulate cell enzymes [34]. However, before electron microscopic autoradiography analyses, we assessed possible side effects of sphingosine on cells by microscopical and biochemical analysis. Our experimental conditions for cell feeding were the following: a single 2 hr pulse with 3×10^{-8} M [1-^{3}H]sphingosine or a 24 hr pulse with 3×10^{-7} M [1-^{3}H]sphingosine repeated 5 times. The second condition has been chosen to increase the final cell sphingolipid specific radioactivity. Under both conditions, the administration to cells of [1-^{3}H]sphingosine did not induce any morphological alteration of cell structures, and well preserved cells, plasma membranes, and intracellular organelles could be observed (Figure 1). Of course, we found a much higher amount of radioactivity associated with cell sphingolipids administering [1-^{3}H]sphingosine at the higher concentration, but the distribution of radioactivity within sphingolipids (Figure 2) and the distribution of radioactivity within the different fractions obtained by sucrose gradient centrifugation of a detergent-treated cell homogenate (Figure 4) were very similar under both experimental conditions. Thus, for the electron microscopy study, we administered to cells 3×10^{-7} M [1-^{3}H]sphingosine in a 24 hr pulse repeated for 5 days, to yield the highest final cell sphingolipid specific radioactivity.

After one week of exposition, it was already possible to observe several silver grains by electron microscopy autoradiography in ultrathin sections from cultured human fibroblasts submitted to feeding with [1-^{3}H]sphingosine (data not shown). Nevertheless, 4 weeks of exposition yielded clearer pictures (Figures 5 and 6). Silver grains were mostly found inside the cell, whereas only a few could be observed at the level of the plasma membranes. In particular the endoplasmic reticulum and cytoplasmic vesicles appeared frequently labeled by silver grains, as shown in Figure 6. This suggests that the pictures are mainly showing the synthesis of complex sphingolipids from the radioactive sphingosine taken up by the cells, and the subsequent sphingolipid cell trafficking. In some pictures the cell nucleus was also labeled (Figure 6A).

The fact that only a few areas bearing silver grains were seen at the level of the plasma membrane suggests that the picture probably shows only the neobiosynthesized sphingolipids that reach the plasma membrane, where they maintain the same specific radioactivity, not being diluted in the pre-existing membrane components. In fact, we have to recall that the endogenous sphingolipid molar content is about 700–1000-fold higher than that of sphingosine. If neobiosynthesized sphingolipids were diluted with the pre-existing molecules at the level of the plasma membrane, the sphingolipid specific radioactivity would dramatically decrease, thus resulting in the production of fewer silver grains than those produced by the neobiosynthesized sphingolipids localized within the cell.

In conclusion, in this paper we present for the first time the use of an electron microscopy autoradiography procedure to reveal cell tritium labeled sphingolipids. The resulting silver deposition can be clearly observed, but, of course, interpretation of the Figures will require further experiments.

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