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Alterations in the content and physiological role of sphingomyelin in plasma membranes of cells cultured in three-dimensional matrix

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Abstract The three-dimensional (3D) cell culture approach offers a means to study cells under conditions that mimic an in vivo environment, thus avoiding the limitations imposed by the conventional two-dimensional (2D) monolayer cell cultures. By using this approach we demonstrated significant differences in the plasma membrane phospholipid composition and susceptibility to oxidation in cells cultured in three-dimensional environment compared to conventional monolayer cultures. The plasma membrane sphingomyelin (SM), which is a functionally active membrane phospholipid, was markedly increased in plasma membranes of 3D cells. To analyze the mechanisms underlying SM accumulation, we determined the activities of sphingolipid-metabolizing enzymes like neutral sphingomyelinase and ceramidase, which are also related to cellular redox homeostasis and to oxidative stress. Fibroblasts cultured in three-dimensional environment showed different redox potential and lower lipid susceptibility to oxidative damage compared to monolayer cells. The relative content of unsaturated fatty acids, which serve as targets of oxidative attack, was observed to be higher in major phospholipids, such as phosphatidylcholine and phosphatidylethanolamine, in plasma membranes of 3D cells. The possibility that the higher level of SM, might be responsible for the lower degree of oxidation of 3D phospholipids was tested by selective reduction of SM through treatment with exogenous sphingomyelinase. The results showed that the decrease of plasma membrane SM was accompanied by an increase of the lipid peroxides in both 2D and 3D cells. We presume that culturing as a monolayer is stressful for the cells and leads to activation of certain stress-related enzymes, resulting in reduction of the SM level. Our results show that the lower content of plasma membrane SM in cells cultured as a monolayer renders the phospholipid molecules more susceptible to oxidative stress.

Keywords Sphingomyelin · Lipid peroxidation · Phospholipids · Three-dimensional cell culture

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Introduction

Our current understanding of the cellular membrane lipid organization and functions is based mainly on studies using conventional monolayer, two-dimensional (2D) cell cultures. Increasingly, researchers are recognizing the limitations of these 2D cell cultures and an alternative three-dimensional (3D) cell culture approach, offering a means to study cells under conditions that reproduce an in vivo environment, has been established [1, 2]. Evidence has emerged that cell culturing under such conditions induces changes in a number of signaling pathways controlling cell metabolism and behavior [3, 4].

To mimic in vivo-like conditions, we have cultured cells in three-dimensional environment [5]. In these experimental settings cells interact with a pliable fibrillar meshwork of extracellular proteins, organize specific adhesive contacts, and demonstrate changes in their morphology, proliferation, and migration [6].

We focused our interest particularly on sphingomyelin (SM) because sphingolipids are known to play a key role in receptor-mediated signal transduction pathways, acting as second messengers in processes like proliferation, apoptosis, and inflammation [7, 8]. Sphingolipids are basically colocalized with cholesterol in specialized raft-microdomains, which contain a variety of enzymes participating in sphingolipid metabolism, such as sphingomyelinase (SMase) and ceramidase [9]. Recent evidence suggests that reactive oxygen species (ROS) are involved in regulation of the sphingolipid metabolism affecting mainly SMase and ceramidase [for review see 10]. In addition, some sphingolipids have been reported to influence cellular redox homeostasis [10].

Among its multiple structural and functional roles, SM has also been recognized as an inhibitor of the oxidation of unsaturated phosphatidylcholine and cholesterol in lipoproteins and cell membranes [11, 12]. Although the mechanism of the protective antioxidant function of SM is still unclear, there is increasing evidence, confirming this vitally important role of SM which is the second most abundant plasma membrane phospholipid.

Our investigations showed that enzymes like neutral sphingomyelinase and ceramidase, which are basically associated with cellular response to environmental stress, were activated in the cells cultured as a monolayer. The present studies confirm the possibility that the higher content of plasma membrane SM could have a certain protective role against oxidative damage of the membrane lipids despite of the presence of more unsaturated fatty acids in the membrane phospholipids of 3D fibroblasts. Thus, we suggest that cell culturing as a monolayer occurs as a more stressful process for the cells and is accompanied by changes in the SM metabolism and signaling, which make the plasma membrane lipids more susceptible to oxidative damage and possibly to any type of environmental stress.

Materials and methods

Cell culture

Fibroblast cell line GD25β1 was obtained as described elsewhere [13]. The cells were grown in DMEM and enriched with 10% fetal bovine serum (FSB) with addition of a mixture of antibiotics-antimycotics: Penicillin (100 U/ml), Streptomycin (100 μg/ml), and Amphotericin B (0.25 μg ml). Cells were cultured at 37°C, in a humidified atmosphere supplemented with 5% CO_2. To obtain monolayer (2D) cell cultures fibroblasts were seeded at a concentration of 0.6×10^5 cells per cm^2 and cultured for 24 h. At this time a 100% confluent monolayer of cells was formed with cell density of $1.0 \times 10^5 \pm 0.35$ cells per cm^2. Three-dimensional (3D) cell cultures were obtained as described elsewhere with slight modifications [5]. Briefly, the cells were seeded at the same density and allowed to produce their own matrix by culturing them for five consecutive days under the conditions described above. The total cell density in the 3D culture was $8.7 \times 10^5 \pm 0.35$ cells per cm^2, corresponding to about $1.1 \times 10^5 \pm 0.22$ cells per cm^2/layer.

Isolation of plasma membrane fractions

Plasma membranes were obtained according to the procedure described elsewhere [14] involving differential centrifugation. Briefly, the post-nuclear supernatant was loaded on a discontinuous sucrose gradient and centrifuged at $100{,}000 \times g$ for 2.5 h. The plasma membrane fraction was obtained at a density of 45% (w/v), suspended in ice-cold 100 mM Tris buffer, pH 7.4 and used immediately for lipid analysis.

Lipid extraction and analysis

Lipid extraction was performed with chloroform/methanol according to the method of Bligh and Dyer [15]. The organic phase obtained after extraction was concentrated and analyzed by thin layer chromatography. The phospholipid fractions were separated on silica gel G 60 plates in a solvent system containing chloroform/methanol/2-propanol/triethylamine/0.25% KCl (30:9:25:18:6 v/v) [16]. The location of the separate fractions was determined either by spraying the plates with 2′,7′-dichlorofluorescein or by iodine staining. The spots were scraped and quantified by determination of the inorganic phosphorus [17]. Cholesterol content was assayed by gas chromatography using a medium polarity RTX-65 capillary column (0.32 mm internal diameter, length 30 m, thickness 0.25 μm). Calibration was achieved by a weighted standard of cholesterol.

Fatty acid analysis

The phospholipid extracts were saponified with 0.5 N methanolic KOH and methylated with boron trifluoride–methanol complex (Merck) [18]. The fatty acid methyl esters were extracted with hexane and separated by gas chromatography on a capillary column coated with Supelcowax 10-bound phase 9 (i.d. 0.32 mm, length 30 m, film

thickness 0.25 μm); (Supelco, Bellafonte, PA) fitted in a Perichrome (France) gas chromatograph. Quantification was referred to an internal standard of heptadecanoic methyl ester.

Sphingomyelinase activity assay

Sphingomyelinase activity was determined by the method of Nikolova-Karakashian et al. [19] with minor modifications. Briefly, the cells were scraped from the dishes and centrifuged at 300×*g* for 5 min. They were lysed in 0.2% Triton X-100 in 100 mM Tris pH 7.4 buffer supplemented with 25 μM genestein for 10 min on ice. The lysed cells were homogenized with three passes through a 25-gauge needle and 10 μl aliquots were taken for protein assay. NBD-sphingomyelin was added to the lysates to a final concentration 20 μM and incubations were performed for 10 min at 4°C. Aliquots of this mixture containing 0.1 mg protein and 3 μM substrate were added to 5 mM $MgCl_2$, 10 mM Tris pH 7.4 to a final volume of 0.3 ml. All buffers contained 0.2% Triton X-100. After incubation for 1 h at 37°C the reaction was stopped by addition of 1 ml chloroform–methanol 2:1 (v/v). The samples were evaporated and separated in a system containing diethyl ether: methanol (99:1v/v) and the spots corresponding to ceramide were scraped and eluted. After addition of hexane the fluorescence of the samples was measured at 455 (excitation) and 530 nm (emission).

Sphingomyelin synthase assay

Sphingomyelin synthase was determined by the procedure described by Tefesse et al. [20] with modifications. Briefly, the cells were scraped in PBS containing protease inhibitors and cellular plasma membranes were isolated as described above. The incubation mixture contained 20 mM Tris pH 7.4, 0.3 mg membrane protein, 50 μg NBD-ceramide, and 5 μg PC to final volume of 500 μl. After incubation for 2 h at 37°C the reaction was stopped with 1 ml chloroform–methanol 2:1 (v/v). The lipids were separated in a system containing diethyl ether: methanol (99:1v/v) and the spots corresponding to sphingomyelin was scraped and eluted. Hexane was added to the samples and fluorescence was measured at 455 (excitation) and 530 nm (emission).

Ceramidase assay

Ceramidase activity was determined by the method of Nikolova-Karakashian et al. [19] with minor modifications. Briefly, the cells were scraped from the dishes and centrifuged at 300×*g* for 5 min. They were lysed in 0.2% Triton X-100 in 100 mM Tris pH 7.4 buffer supplemented with 25 μM sodium vanadate for 10 min on ice. The lysed cells were homogenized with three passes through a 25-gauge needle and 10 μl aliquots were taken for protein assay. NBD-ceramide was added to the lysates to a final concentration 20 μM and incubations were performed for 10 min at 4°C. Aliquots of this mixture containing 0.1 mg protein and 3 μM substrate were added to 0.5 M acetate buffer pH 4.5 to a final volume of 0.3 ml. All buffers contained 0.2% Triton X-100. After incubation for 1 h at 37°C the reaction was stopped by addition of 5 ml hexane and 4 ml 10% citric acid. The samples were evaporated, dissolved in hexane and NBD—dodecanoic acid was quantified after addition of hexane and determination of the fluorescence intensity (excitation 455 and emission 530 nm).

Cell viability assay

Cell viability was determined by tetrazolium salt measurement (MTT assay), involving assessment of the succinate dehydrogenase-induced conversion of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide into formazan crystals [21]. Formation of formazan was measured at 570 nm.

Measurement of reactive oxygen species (ROS)

The generation of ROS was assessed by spectrofluorimetric analysis, using 2′-7′-dichlorodihydrofluorescein diacetate, a non-polar compound which reacts with ROS to produce the highly fluorescent dihydrofluorescein. The cells were incubated with 10 μM dichlorodihydrofluorescein at 37°C for 30 min. The cells were washed three times with warm PBS to remove the unincorporated dye, scraped in 2 ml PBS and fluorescence was measured at 485 (excitation beam) and 525 nm (emission beam) [22, 23]. The level of ROS is presented as measured fluorescence intensity per mg protein.

Measurement of the reduced glutathione (GSH)

The content of GSH was determined spectrophotometrically according to the procedure described by Ellman, 1959 [24] with slight modifications, using cell lysate obtained from 2D and 3D cells in 0.25 M Tris, 20 mM EDTA, pH 8.2. Briefly, this procedure involved centrifugation at 2500×*g* and mixing supernatant aliquots with 10 mM 5,5′-dithiobis-2-nitrobenzoic acid. The absorbtion was measured at 412 nm. The obtained values are expressed as nmol GSH per mg protein.

Lipid peroxidation (LPO) assay

Lipid peroxidation was determined by the procedure described by Carini et al. [22]. The lipid peroxidation was assessed by measuring the loss of *cis*- parinaric acid (PNA) fluorescence. The cells were incubated with 10 μM PNA at 37°C for 30 min. The media was removed and cells were washed three times with warm PBS to remove the unincorporated dye. The cells were scraped in 2 ml PBS and fluorescence was measured at 502 (excitation beam) and 520 nm (emission beam). The level of lipid peroxidation was determined as fluorescence intensity per mg protein.

Treatment of cells with exogenous sphingomyelinase

Degradation of sphingomyelin was performed using 2 U/ml sphingomyelinase, added to the incubation medium for 30 min at 37°C [14]. Cell permeability to trypan blue was not altered as a result of sphingomyelinase treatment of cells. The partially delipidated cells thus obtained were divided into two groups: the first one was used for incubation with H_2O_2 and the second one—for isolation of plasma membranes, which were subjected to lipid analysis.

Protein determination

The content of protein was determined according to Bradford [25].

Statistical analysis

Statistical processing of the data was made by one-way analysis of variance (ANOVA), using InStat software.

Results

The phospholipid (PL) composition of plasma membranes isolated from GD25β1 cells cultured either as a monolayer or in three-dimensional environment is shown in Table 1. Apparently, the content of almost all PL fractions was different in 2D compared to 3D cells. Especially the level of sphingomyelin (SM) was higher by about 50% in plasma membranes of cells cultured in 3D conditions. In addition, the level of the other choline-containing phospholipid—phosphatidylcholine (PC) was significantly reduced, whereas the aminophospholipids phosphatidylethanolamine (PE) and phosphatidylserine were increased (Table 1).

We focused our interest particularly on the augmentation of SM in 3D cells due to its significant role in maintenance of the structure and functional activity of the raft-microdomains, which are considered as cellular signaling platforms.

Table 1 Phospholipid composition of plasma membranes isolated from fibroblasts cultured as a monolayer (2D) and in three-dimensional matrix (3D) (mol%)

Phospholipid	2D	3D
Sphingomyelin	10.00 ± 0.97	15.45 ± 1.12*
Phosphatidylcholine	42.91 ± 2.56	30.73 ± 2.07*
Phosphatidylserine	8.02 ± 0.82	15.66 ± 0.95*
Phosphatidylinositol	12.25 ± 1.71	8.95 ± 1.32**
Phosphatidylethanolamine	17.54 ± 1.07	22.19 ± 1.22*
Phosphatidylglycerol	7.65 ± 1.05	7.38 ± 1.15
Lysophosphatidylcholine	1.74 ± 0.49	3.26 ± 0.75**

Results are means ± SD of three separate experiments

*$P < 0.001$; ** $P < 0.01$

Also, we have previously reported differences in the cell signaling between 2D and 3D cells [3]. To investigate the mechanisms underlying the elevation of SM in plasma membranes of 3D cells, we analyzed the activities of the three SM-metabolizing enzymes that could be responsible for its accumulation, involving neutral sphingomyelinase (nSMase), sphingomyelin synthase, and ceramidase. The results showed that the activity of nSMase was lower whereas SM synthase was more active in 3D compared to 2D cells (Fig. 1). Also, a decrease of ceramidase activity was observed in 3D cells. The observed changes in the sphingolipid-metabolizing enzymes were in good accordance with the alterations of SM in plasma membranes of 3D cells compared to 2D, which will be discussed below.

Since nSMase and ceramidase are basically related to cellular responses to environmental stress [26] and SM signaling is sensitive to lipid peroxidation (10), we analyzed the differences in the susceptibility of 2D and 3D cells to oxidative stress. We chose to study the cellular response particularly to oxidative attack, because sphingolipids play an important role in maintenance of the

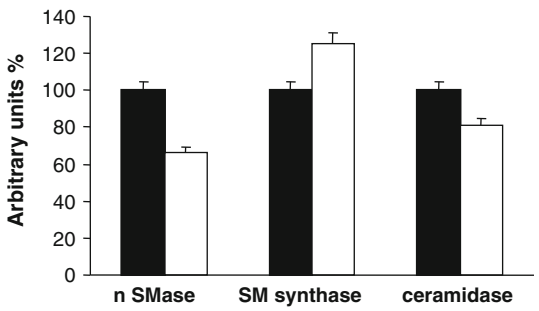

Fig. 1 Alterations of enzyme activities (as indicated in the figure), participating in sphingolipid metabolism in cells cultured as monolayer (2D) (*black bars*) and in three-dimensional matrix (3D) (*white bars*). Results are given as means ± SD of three separate experiments. The observed differences between 2D and 3D cells for each enzyme activity were statistically significant at $P < 0.001$

Table 2 Levels of ROS and GSH in fibroblasts cultured as monolayer (2D) and in three-dimensional matrix

	2D		3D	
	Control	H_2O_2	Control	H_2O_2
ROS	225 ± 17	1024 ± 69*	147 ± 15	546 ± 25*
GSH	676.5 ± 31	112.3 ± 12*	897.1 ± 44	187.6 ± 23*

ROS reactve oxygen species, *GSH* reduced glutathione

ROS are expressed as fluorescence intensity per mg protein and GSH as nmol per mg protein

Values are means ± SD of four separate determinations

* $P < 0.001$

cellular redox homeostasis [10]. In addition, SM has been reported to function as an antioxidant under definite conditions [11] and since its content was significantly different in 2D and 3D fibroblasts (Table 1), we analyzed its role in the susceptibility of the plasma membrane lipids to oxidative damage in the studied cells.

The oxidative cellular status was estimated by determination of the ROS and glutathione level (Table 2) as a characteristic of the oxidant/antioxidant cell balance [27]. Apparently, the content of ROS was lower by about 40% and GSH was higher by about 30% in 3D compared to 2D cells. The level of lipid peroxides was higher in the cells cultured as a monolayer compared to "matrix" cells (Fig. 2a).

Further studies were focused on the response of 2D and 3D cells, which as mentioned above had different redox potential (Table 2), to exogenous oxidative stress induced by incubation with H_2O_2. The results demonstrated a lower degree of lipid peroxidation in 3D, compared to 2D fibroblasts (Fig. 2a). In addition, the levels of ROS and GSH were also significantly altered in both 2D and 3D cells as a result of H_2O_2 treatment (Table 2). It should be noted that the level of ROS was increased about 4.5-fold in 2D cells and 3.7-fold in 3D cells as a result of H_2O_2 treatment. The reduction of the GSH content (expressed per mg protein) induced by the oxidative attack was 6-fold in 2D and 4.8-fold in 3D cells.

Since SM has been recognized as a protector of lipids against oxidative damage under definite conditions [11, 12], we presumed that its elevated content in plasma membranes of 3D fibroblasts could be responsible for the lower degree of lipid peroxidation in these cells. To test this hypothesis, we analyzed the antioxidant role of SM through its selective degradation by treatment of 2D and 3D cells with exogenous SMase. As a result of this partial delipidation, the level of SM was reduced by 37% in 2D and by 41% in 3D cells. The cells with reduced content of SM were used for estimation of the degree of lipid peroxidation (Fig. 2b). The results showed that SM reduction was accompanied by an increase in the lipid peroxidation in both 2D and 3D fibroblasts, which confirmed its eventual protective effect against phospholipid oxidation. However, despite of the decreased SM level, the content of lipid peroxides was still lower in 3D compared to 2D cells (Fig. 2b).

Another possible reason for the lower susceptibility to oxidation of the phospholipid molecules in 3D cells could be the degree of unsaturation of their acyl chains, the latter occurring as targets of lipid peroxidation. The analysis of the fatty acid composition of the major plasma membrane phospholipids (PC and PE) revealed marked differences between 2D and 3D cells (Table 3). Unexpectedly, the major plasma membrane phospholipids in 3D cells contained higher levels of unsaturated fatty acids, which are illustrated by the values of the saturated/unsaturated fatty acids ratio. These results show that despite the higher content of unsaturated fatty acids in plasma membranes of 3D fibroblasts, the measured level of lipid peroxides was lower, compared to 2D cells (Fig. 2a).

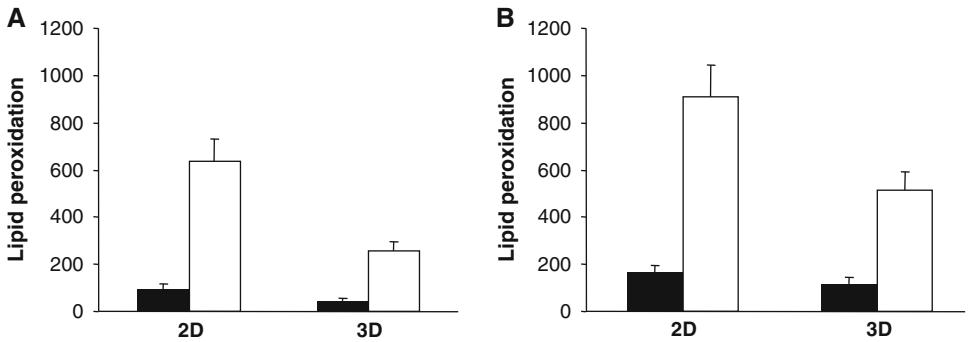

Fig. 2 Formation of lipid peroxides in fibroblasts cultured as a monolayer (2D) and in three-dimensional matrix (3D). **a** intact cells; **b** cells incubated with sphingomyelinase. Untreated cells—*black bars*, cells treated with hydrogen peroxide—*white bars*. Results are expressed as fluorescence intensity per mg protein. Values are means ± SD (*error bars*) of four determinations. Statistical significance is calculated by comparison of 2D with 3D cells for each separate treatment ($P < 0.001$)

Table 3 Levels of saturated (SAT), monounsaturated (MONO), and polyunsaturated (PU) fatty acids in phospholipids of plasma membranes of cells cultured as monolayer (2D) or in three-dimensional matrix (3D)

Fatty acids	PC		PE	
	2D	3D	2D	3D
SAT	60.21	54.68*	40.47	36.45**
MONO	12.84	17.00*	55.15	55.26
PU	26.95	28.32	4.38	8.29*
SAT/UNSAT	1.51	1.20*	0.68	0.57**

Results are expressed as mol%

PC phosphatidylcholine, *PE* phosphatidylethanolamine, *UNSAT* unsaturated fatty acids

Values are means ± SD of four separate experiments

* $P < 0.001$; ** $P < 0.01$

Discussion

The development of 3D cell cultures has been stimulated by tissue engineering studies aiming at creating living tissue equivalents, needed for regenerative medicine and suitable for tissue replacement. Several research groups have demonstrated that under definite conditions, fibroblasts can produce in vitro a thin, three-dimensional sheet of ECM material [1, 28]. These cell-produced matrices present a unique possibility for in vitro studies of cell developing in a more natural environment such as ECM and not on solid substrate [5, 6, 28].

Our studies revealed marked differences in the plasma membrane phospholipid composition (Table 1) that are probably a result of specific interactions of 2D and 3D fibroblasts with the surrounding environment, involving alterations in the exchange of lipid precursors and cholesterol with the extracellular matrix. Such lipid alterations could affect certain membrane-associated processes like formation and stability of plasma membrane microdomains as well as signal transduction. Since SM is a major component of the plasma membrane raft-domains, the physiological significance of the differences observed in the SM content in plasma membranes of cells cultured as a monolayer and in three-dimensional matrix, is of particular interest.

To analyze the possible mechanisms underlying the differences in the SM content, we monitored three enzyme activities which could be responsible for SM accumulation: nSMase, SM synthase, and ceramidase. SMases are enzymes that hydrolyze SM thus producing ceramide and phosphocholine, and are implicated in signal transduction and cell responses to various stimuli. It should be noted, that the activity of nSMase was lower in cells cultured in three-dimensional conditions (Fig. 1), which might be one possible reason for the higher level of SM in their plasma membranes. In addition, SM synthase was more active in 3D cells, which could also contribute to SM accumulation. It is quite likely that growing on a solid substrate is stressful for the "monolayer" cells and might induce activation of stress-related enzymes like nSMase and ceramidase [10], both of them being more active in 2D cells (Fig. 1). The differences observed between 2D and 3D cells in the sphingolipid-metabolizing enzymes are closely related to variations in the content of physiologically important sphingolipids like SM and ceramide. Sphingolipid metabolites are recognized as essential components of signal transduction and are implicated in various stress responses [10, 29–31]. Ceramide, a key metabolite in the catabolism and anabolism of SM, has been shown to play an important role in the processes of apoptosis, cell cycle arrest, and differentiation [29]. Our previous studies showed an insignificant increase (about 10%) in the ceramide content in plasma membranes of 3D cells [12] which could be due to the lower ceramidase activity reported in the present study (Fig. 1).

Since cellular redox potential is regarded as a regulator of SMases by some authors [for review see 10], we analyzed the response of the cells with different level of SM, ceramide, and SM-metabolizing activities to oxidative attack. Oxidative stress has been reported to affect sphingolipid metabolism, leading to generation of sphingolipid metabolites which participate in transmembrane signaling [10]. On the other hand, there is evidence suggesting that certain sphingolipids, such as ceramide, are able to induce oxidative stress through activation of NADPH oxidase [32] and/or downregulation of antioxidant enzymes [33].

The observation that two stress-related enzymes, such as nSMase and ceramidase, were more active in "monolayer" cells suggests that these conditions are more stressful for the cells. The increased nSMase activity is likely to contribute to the reduction of plasma membrane SM in 2D cells, which also exhibited a higher rate of lipid peroxidation (Fig. 2a). Since SM has been reported to protect cholesterol against oxidative damage in model membranes [34], we tested the possibility that this sphingolipid could also prevent the oxidation of plasma membrane phospholipids, since lipid peroxidation was lower in the SM-enriched 3D cells (Fig. 2a). To analyze this hypothesis we selectively manipulated the content of SM in 2D and 3D fibroblasts by treatment with exogenous SMase. The obtained results showed that SM reduction was accompanied by an increase of the rate of lipid peroxidation (Fig. 2b), making the protective role of the SM quite possible. On the other hand, ceramide accumulation has been suggested to induce additional oxidation of the membrane phospholipids [10, 35, 36]. However, we think that this option is less probable, because lipid peroxidation was lower in plasma membranes of 3D fibroblasts, in

which we reported a slight increase of the ceramide level in our previous studies [12].

It should also be noted that the major plasma membrane phospholipids, PC and PE, contained more unsaturated fatty acids in 3D compared to 2D fibroblasts, which could be a prerequisite for a higher degree of lipid peroxidation in the "matrix" cells. Most elevated in 3D cells was arachidonic acid, which was higher by 210 and 145% in PC and PE, respectively (data not shown). However, a higher content of lipid peroxides was measured in 2D cells which contained lipids with lower degree of fatty acid unsaturation, implying that there are certain factors in 3D cells which protect the phospholipid acyl chains against oxidative damage. Our data support the presumption, that the level of plasma membrane SM in 3D cells plays a protective role by preventing the phospholipid fatty acids from oxidative attack. Although the mechanism of this process is still unclear, there are several hypotheses to shed more light on the antioxidant functions of SM. Oborina and Yappert [37] suggest that the ability of SM to form inter- as well as intra-molecular hydrogen bonds is related to lower ability of the free radicals to penetrate the membrane bilayer. In addition, sphingolipids like SM and ceramide are known to make membranes more rigid which could also hinder the processes of oxidative attack of the lipid molecules [11].

Thus, the current studies show that monolayer culturing might represent a stressful growth environment in which the cells need to adapt to rather unusual conditions like solid substrate and lack of natural medium such as the extracellular matrix. The differences in the levels of ROS and GSH in 2D and 3D fibroblasts imply that the culturing conditions affect the cellular redox potential and thus influence the response reactions to environmental stress. We presume that SM is likely to play a protective role against lipid peroxidation and that the environmental stress, including oxidative stress, induces activation of stress-related enzymes like nSMase, leading to degradation of SM, which is accompanied by an elevated susceptibility of the plasma membrane lipids to oxidative damage.

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