Resistance to alkyl-lysosphospholipid-induced apoptosis due to downregulated sphingomyelin synthase 1 expression with consequent sphingomyelin- and cholesterol-deficiency in lipid rafts

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The ALP (alkyl-lysosphospholipid) edelfosine (1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine; Et-18-OCH3) induces apoptosis in S49 mouse lymphoma cells. To this end, ALP is internalized by lipid raft-dependent endocytosis and inhibits phosphatidylcholine synthesis. A variant cell-line, S49ΔK, which is resistant to ALP, was shown previously to be unable to internalize ALP via this lipid raft pathway. The reason for this uptake failure is not understood. In the present study, we show that S49ΔK cells are unable to synthesize SM (sphingomyelin) due to downregulated SMS1 (SM synthase 1) expression. In parental S49 cells, resistance to ALP could be mimicked by small interfering RNA-induced SMS1 suppression, resulting in SM deficiency and blockage of raft-dependent internalization of ALP and induction of apoptosis. Similar results were obtained by treatment of the cells with myriocin/ISP-1, an inhibitor of general sphingolipid synthesis, or with U18666A, a cholesterol homeostasis perturbing agent. U18666A is known to inhibit Niemann–Pick C1 protein-dependent vesicular transport of cholesterol from endosomal compartments to the trans-Golgi network and the plasma membrane. U18666A reduced cholesterol partitioning in detergent-resistant lipid rafts and inhibited SM synthesis in S49 cells, causing ALP resistance similar to that observed in S49ΔK cells. The results are explained by the strong physical interaction between (newly synthesized) SM and available cholesterol at the Golgi, where they facilitate lipid raft formation. We propose that ALP internalization by lipid-raft-dependent endocytosis represents the retrograde route of a constitutive SMS1- and lipid-raft-dependent membrane vesicular recycling process.

Key words: apoptosis resistance, cholesterol, edelfosine, lipid raft, sphingomyelin synthase (SMS).

INTRODUCTION

The synthetic ALP (alkyl-lysosphospholipid), edelfosine (1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine; Et-18-OCH3) is the prototype of a group of new clinically used anticancer agents [1–4]. These unnatural phospholipids with a single alkyl-chain insert readily into the lipid-bilayer of the plasma membrane and, because they are poorly degraded by lipases [5], they accumulate and disturb lipid-metabolism in the cell. For example, ALP inhibits the activity of CTP:phosphocholine cytidylyltransferase, the rate-determining enzyme for PC (phosphatidylcholine) biosynthesis [6]. We have reported previously [7,8] that this cytidylyltransferase inhibition is a direct trigger for apoptosis in S49 lymphoma and HeLa carcinoma cells. Since cytidylyltransferase is active inside the cell (ER (endoplasmic reticulum) and nucleus) [9], ALP needs to be internalized to inhibit the enzyme. We found that ALP accumulates preferentially in a detergent-resistant sphingolipid- and cholesterol-rich membrane fraction that is representative of microdomains known as lipid rafts [7]. ALP is then internalized by lipid raft- and dynamin-dependent, clathrin-independent endocytosis [7,8]. In an ALP-resistant S49 variant cell-line, S49ΔK, selected by continuous culturing of S49 cells in the presence of ALP, we found no raft-dependent ALP internalization and, consequently, no disturbance of PC metabolism and no initiation of apoptosis [7]. The mechanism behind this uptake failure and consequent cellular resistance to ALP is unknown.

As a first approach to elucidate why lipid rafts in S49ΔK cells are unable to mediate internalization of ALP, we questioned if the lipid rafts in the resistant cells have a different lipid composition compared with the parental S49 cells. This question is the more relevant, since we have shown previously [7] that the artificial breakdown of plasma membrane SM (sphingomyelin) by an exogenous (bacterial) sphingomyelinase, or the extraction of cholesterol, prevented ALP internalization and apoptosis. We therefore focused on the analysis of the sphingolipids, as these are major lipid raft constituents that, in association with cholesterol, determine the detergent insolubility [10]. It is well known that SM and cholesterol show tight, mutual interaction [11,12] and that cellular SM levels therefore co-regulate cholesterol homeostasis [12,13]. Conversely, it is less clear whether changes in membrane cholesterol may also affect the levels and/or synthesis of SM [13]. In the present study, in order to disturb cholesterol homeostasis, we used the amphiphilic drug U18666A, a well-established inhibitor of NPC1 (Niemann–Pick C) protein-mediated vesicular transport of cholesterol from endosomal compartments to the trans-Golgi network and the plasma membrane [14–17].

Abbreviations used: 2D-TLC, two-dimensional TLC; ALP, alkyl-lysosphospholipid; C6-NBD-ceramide, N-(6-[(7-nitro-benz-2-oxa-1,3-diaz-o-4-yl)amino]caproyl)-ceramide; C12:0-SM, lauroyl sphingomyelin; edelfosine, 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine; ER, endoplasmic reticulum; exoSM, exogenous C12:0-sphingomyelin; FasL, Fas ligand; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GlcCer, glucosylceramide; HRP, horseradish peroxidase; HPRT, hypoxanthine guanine phosphoribosyl transferase; LacCer, lactosylceramide; NPC1, Niemann–Pick C1; PC, phosphatidylcholine; PE, phosphatidylethanolamine; RT, reverse transcriptase; sRNA, small interfering RNA; S49ΔK, ALP-resistant S49 cells; S49mock, mock-transduced S49 cells; SM, sphingomyelin; SMS, SM synthase.

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In the present study, we show that the S49<sup>AR</sup> cells are unable to synthesize SM due to downregulated SMS1 (sphingomyelin synthase) expression. This lack of SM is especially apparent in the detergent-resistant membrane fraction, together with a decrease in cholesterol. Conversely, disturbance of cholesterol homeostasis, or siRNA (small interfering RNA)-induced downregulation of SMS1 directly, prevented SM synthesis in parental S49 cells. We show in both cell-line variants that the lack of SM production is linked directly with aborted ALP uptake and apoptosis resistance.

**EXPERIMENTAL**

**Materials**

The ALP edelfosine and U18666A (3-β-[2-(diethylamino)-ethoxyl]androst-5-en-17-one) were purchased from BioMol. [3H]ALP ([1H]Et-18-OCH<sub>3</sub>; 58 Ci/mmoll) was synthesized by Moravek Biochemicals. [α<sup>32</sup>P]Choline chloride (58 mCi/mmoll), [α<sup>32</sup>P]-L-serine (54 mCi/mmoll) and [3H]1-sphingosine were synthesized by Mr P. Weber (DSM, Delft, The Netherlands). C12:0-SM (lauroyl sphingomyelin; N-Lauroyl-d-erythro-sphingosyolphosphorylcholine) was from Avanti Polar Lipids. C<sub>6</sub>-NBD-ceramide (N-6-{[7-nitro-benz-2-oxa-1,3-diazoyl]amino[caproyl]-}ceramide) was from Molecular Probes. D609 (tricyclo decan-9-yl-xanthogenate) was from Calbiochem. Myricorin/IP-1 was from Sigma. Soluble recombinant human FasL (Fas ligand), APO-1L, was from Alexis. Reagents containing the siRNA oligonucleotide directed against SMS1 were transfected into the human embryonic kidney 293T [expressing the large T-antigen of SV40 (simian virus 40)] cell-derived packaging cell-line Phoenix Amphi, using FuGene<sup>®</sup> transfection reagent, according to the manufacturer’s instructions (Roche Molecular Biochemicals). Transfected cells were selected with 1 µg/ml puromycin (Invitrogen). Virus-containing supernatants were harvested after 2–5 days and stored at −80°C until further use. S49 cells were seeded on dishes coated with RetroNectin (Roche Molecular Biochemicals) and transduced with 1 ml of virus-containing supernatant per 0.5 × 10<sup>6</sup> cells. Supernatants were removed after overnight incubation, and cells were cultured in fresh medium. The selection of transduced cells began 48 h after transduction, and selected cells were grown in the presence of 200 µg/ml puromycin.

**ALP uptake and apoptosis assay**

Cells were grown to a density of 1 × 10<sup>6</sup>/ml and ALP (edelfosine) was added at the effective apoptotic concentration of 15 µM, supplemented with 0.2 µCi of [3H]ALP/ml. At given time points, samples were taken, put on ice for 2 min and then washed three times with ice-cold PBS. Samples were lysed in 0.1 M NaOH prior to liquid scintillation counting.

Apoptosis was induced with 15 µM ALP or 500 ng/ml FasL for 6 h. Cells were washed in PBS and lysed overnight at 4°C in 0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X-100 and 50 µg/ml propidium iodide, according to Nicoletti et al. [20]. Fluorescence intensity of propidium iodide-stained nuclei was determined on a FACSscan (Becton Dickinson), and data were analysed using Lysis software.

**Lipid analysis**

Cells, grown to a confluence of 10<sup>6</sup> cells/ml, were incubated with [1<sup>4</sup>C]methyl-choline chloride (1 µCi/ml), [1<sup>4</sup>C]phosphoglycerol (1 µCi/ml), [1<sup>4</sup>C]3-L-serine (0.4 µCi/ml) or [3H]cholesterol (0.02 µCi/ml), where indicated. At given time points, aliquots of cells were taken, washed and resuspended in 200 µl PBS. Lipids were extracted with chloroform/methanol (1:2, v/v) and phase separation was induced using 1 M NaCl. The organic phase was washed in a solution of methanol/water (47:49:3 by vol.) and separated by one-dimensional silica TLC, using chloroform/methanol/acetate acid/water (60:30:8:5 by vol.) or, in the case of sphenosine labelling, using chloroform/methanol/0.2% CaCl<sub>2</sub> (60:40:9 by vol.). 2D-TLC (two-dimensional TLC) was performed using 2 × chloroform/methanol/NH<sub>4</sub>OH (60:60:5 by vol.), with intermittent drying, in the first dimension, followed by 1 × chloroform/methanol/acetate acid/water (50:30:8:4, by vol.) in the second dimension. Radioactive lipids were visualized and quantified using a Fuji BAS 2000 TR Phospholmager and identified using internal standards, which were visualized by iodine staining. Tritiated lipids were visualized after dipping the TLC plate in 12.5% (w/v) diphenylxazole dissolved in...
diethyl ether, drying and subsequent autoradiography. Unlabelled lipids (e.g., C_{16:0}-SM) were visualized by spraying the plate with sulfuric acid, followed by heating (charring) in an oven at 180°C for 30 min.

Mass sphingolipid analyses were conducted by liquid chromatography, electrospray ionization tandem MS as described by Merrill et al. [21].

**In vitro SMS assay**

SM synthesis was performed *in vitro* (1 h, 37°C) on a crude membrane preparation from HeLa cells, using C_{6}-NBD-ceramide (60 nM) as a substrate [22]. To this end, cells were homogenized by passing them through a 27.5 gauge needle five times. Nuclei were spun down at 300 g for 10 min and, from the supernatant, crude membranes were obtained by centrifugation at 40,000 rev./min in an airfuge for 1 h. Lipids were extracted and separated by TLC (see above).

**Isolation of lipid rafts**

A lipid raft fraction was prepared by detergent extraction of cells and sucrose-gradient centrifugation, essentially as described previously [23]. In brief, 2 × 10^8 cells were solubilized into 1 ml of an ice-cold buffer consisting of 25 mM Mes, 150 mM NaCl and 1% (v/v) Triton X-100, and homogenized using a Dounce homogenizer. The extract was centrifuged on a discontinuous sucrose gradient at 39,000 rev./min in a SW41 rotor for 20 h at 4°C, followed by the manual collection of 11 1.0 ml fractions from the top of the gradient.

### RESULTS

**S49^{AR} cells show a defect in the synthesis of SM**

Previously, we reported [7] that the synthetic ether-lipid, edelosine, induces apoptosis in S49 lymphoma cells in a dose- and time-dependent fashion. The onset of apoptosis in these cells was relatively fast, and already apparent after 3 h [7]. An ALP-resistant variant cell-line, S49^{AR}, generated by culturing S49 cells in the presence of ALP and continuous selection of surviving cells, did not undergo apoptosis mediated by ALP. S49^{AR} cells were not derived from a single-cell clone with a single genetic mutation, but are a population of cells in which the sensitivity to ALP is suppressed in a reversible manner: after 4–5 weeks of culturing but are a population of cells in which the sensitivity to ALP is suppressed in a reversible manner: after 4–5 weeks of culturing and regain the phenotypic properties (to be described below) of the parental S49 cells (results not shown). We demonstrated that, in order to induce apoptosis, ALP needs to be internalized by endocytosis via lipid rafts [7,8]. This particular process was disrupted in the S49^{AR} cells, although ALP still remained associated with the detergent-resistant lipid raft fraction from these cells [7]. As the uptake of ALP could be prevented by the treatment of cells with cholesterol-sequestering agents or the breakdown of SM [7], we addressed the question whether the difference in ALP sensitivity of the cells could be related to a different lipid composition of their lipid rafts, which may impede internalization. We focused on analysing the sphingolipids, as being major lipid raft constituents [10]. Importantly, we found that S49^{AR} cells were deficient in SM (Figure 1 and Table 1). Radiolabelling of the cells with the SM precursors [14^C]methyl-choline chloride or [14^C]3-L-serine, followed by lipid analysis by 2D-TLC separation, revealed two typical SM spots in S49 cells, which were absent in S49^{AR} cells (Figure 1A). These two SM spots correspond respectively to a (major) pool with relatively short acyl-chains (16 C-atoms) and a (minor) SM pool with long acyl-chains (predominantly C_{24:1}) and containing a C_{16}-dihydro-(sphinganine) species (Table 1) [24]. No other conspicuous differences in lipid profile between the two cell lines were seen on these 2D-TLC plates.

The lack of SM synthesis in S49^{AR} cells was also reflected in SM mass levels in these cells, as determined by MS (Table 1). Results are means ± S.D. for triplicates.

**Figure 1** S49^{AR} cells are devoid of SM synthesis

(A) S49 and S49^{AR} cells were labelled overnight with the precursors [14^C]methyl-choline or [14^C]3-L-serine. Lipids were extracted and separated by 2D-TLC. The location of SM (typically two spots) is marked by a white dotted box. (B) S49 and S49^{AR} cells were labelled with [3H]-sphingosine for the times indicated. (Sphingo)lipids were extracted and separated by one-dimensional TLC. The location of SM and other sphingolipids, ceramide (Cer), GlcCer and LacCer is indicated. PE is a catabolic end-product of sphingosine degradation.

<table>
<thead>
<tr>
<th>SM type</th>
<th>S49 cells (pmol/10^6 cells)</th>
<th>S49^{AR} cells (pmol/10^6 cells)</th>
<th>Fetal calf serum (pmol/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{16:0}</td>
<td>101.6 ± 8.4</td>
<td>20.3 ± 1.4</td>
<td>615.9 ± 128.4</td>
</tr>
<tr>
<td>C_{16:0}-DH*</td>
<td>6.9 ± 0.1</td>
<td>1.8 ± 0.4</td>
<td>77.8 ± 17.4</td>
</tr>
<tr>
<td>C_{18:0}</td>
<td>12.3 ± 2.3</td>
<td>18.0 ± 0.1</td>
<td>115.1 ± 24.0</td>
</tr>
<tr>
<td>C_{20:0}</td>
<td>6.9 ± 0.6</td>
<td>1.7 ± 0.3</td>
<td>146.5 ± 32.1</td>
</tr>
<tr>
<td>C_{22:0}</td>
<td>3.7 ± 0.2</td>
<td>2.1 ± 0.1</td>
<td>162.9 ± 16.0</td>
</tr>
<tr>
<td>C_{24:1}</td>
<td>121.3</td>
<td>27.7</td>
<td>1154.2</td>
</tr>
<tr>
<td>Sum others†</td>
<td></td>
<td></td>
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</tbody>
</table>

* C_{16:0}-DH, sphingomyelin containing an N-palmitoyl-dihydro-sphingosine moiety.
† C_{16:0}, C_{18:0}, C_{20:0}, C_{22:0} and C_{24:1}, in amounts less than 1.9 and 0.8 pmol/10^6 cells for S49 and S49^{AR} cells respectively, and less than 60.1 pmol/mg of protein for fetal calf serum.

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**Table 1** SM mass levels in S49 and S49^{AR} cells and in the fetal calf serum used in cell culture

Sms in lipid extracts were assayed by liquid chromatography, electrospray ionization tandem MS [21]. Results are means ± S.D. for triplicates.
to approx. one-fifth in the S49AR cells. Given the almost complete lack of SM synthesis in S49AR cells (see also Figures 1B, 2 and 3), this residual mass SM has most likely been taken up by the cells directly from the fetal calf serum in the culture medium, as this serum is also rich in C16:0-SM (Table 1).

To visualize the synthesis of other sphingolipids in the cells, we radio-labelled the cells with a different metabolic precursor, [3H]-sphingosine. Figure 1(B) shows the time-dependent synthesis of sphingolipids in the two cell-lines. It is clear that, over the 4 h time period, S49AR cells were unable to synthesize SM from sphingosine, while S49 cells were able to do so (predominantly the SM pool with C16 acyl-chain length), evidently via the intermediate ceramide. No profound differences in the synthesis of GlcCer (glucosylerceramide) or LacCer (lactosylceramide) were seen between the two cell-lines. The spot of PE (phosphatidylethanolamine) represents a metabolite from sphingolipid degradation, via sphingosine-1-phosphate lyase-mediated phosphatidylethanolamine production, as shown and discussed previously [25].

We next isolated the detergent-resistant lipid raft fractions from [3H]-sphingosine-labelled cells, using sucrose-gradient centrifugation. Figure 2 shows that, in S49 cells, SM is concentrated in the lipid raft fractions (fractions 3–5), as expected, whereas SM was absent from the raft fractions from S49AR cells. Again, no clear differences in the other lipids were observed between the two cell-lines. GlcCer, LacCer and ceramide were normally present in the lipid raft fractions of both cell-lines. Taken together, the data indicate that S49AR cells show a specific defect in the synthesis of the lipid raft component SM, but not of other (sphingo)lipids. Furthermore, given the type of SM precursors used, and the unimpeded production of the precursor ceramide (Figures 1B and 2), it is apparent that the defect in SM production lies in the final biosynthetic step, catalysed by SMS.

S49AR cells do not express SMS, whereas S49 cells only express SMS1

In order to investigate the mechanism underlying the defective SM synthesis in S49AR cells, we analysed the expression of SMS1 and SMS2, the two recently identified isozymes capable of executing the final enzymatic step of SM synthesis [26,27]. RT (reverse transcriptase)-PCR analysis revealed SMS1 expression in S49 but not in S49AR cells (Figure 3A). SMS2 was not detectable at all in either cell-line (transcripts were not visible after amplification up to 45 cycles of PCR). This result was verified by real-time PCR, which showed that, compared with S49 cells, SMS1 expression in

S49AR cells was reduced approx. 50-fold (Figure 3B). SMS2 was not expressed in either cell type (no detectable transcript after 50 PCR cycles, whereas it was readily detected in cDNA made from mouse embryo cells that were used as a positive control; results not shown). Thus, the lack of SMS expression in S49AR cells directly explains the defect in SM synthesis in the S49AR cells.

Downregulation of SMS1 expression by siRNA blocks SM synthesis and prevents ALP uptake and the induction of apoptosis in S49 cells

We demonstrated previously [7] that S49AR cells are resistant to ALP because, unlike the parental S49 cells, they are unable to internalize ALP via lipid rafts. Since S49AR cells lack SMS expression and SM synthesis, we first tried to introduce the SMS1 gene into S49AR cells. This, however, resulted in the loss of viability of SMS1-transfected cells (both in S49 and in S49AR cells; results not shown). We next downregulated SMS1 expression in S49 cells by stable transduction of siRNA against SMS1, yielding so-called S49AR(SMS1) cells, to see if reduced SM synthesis would lead to impaired ALP internalization, resulting in a phenotype similar to S49AR cells. SMS1 mRNA levels in S49AR(SMS1) cells were reduced 6-fold, as revealed by real-time PCR (Figure 3B). Figure 3(C) confirms that these SMS1-downregulated cells show significantly reduced SM synthesis, down to one-tenth of the level observed in mock-transduced S49 (S49mock) control cells, whereas little or no changes were seen in the synthesis of the other sphingolipids. Thus, by inhibiting SMS1 gene expression specifically, we can block the synthesis of SM in S49 cells to a similar low level as in S49AR cells.
decrease the incorporation of [14C]3-L-serine precursor into [14C]-
reduced ALP-induced apoptosis compared with parental S49 cells or S49mock cells. Cells were
10^3 cells and are means ± S.D. value was within the size of the symbol). (C) Similar to S49mock cells, S49siSMS1 cells show
myriocin/ISP-1 treatment (S49 cells) abrogates
ALP uptake and apoptosis induction, similar to S49AR cells.

We next tested whether the inhibition of SM synthesis would lead to reduced ALP uptake. Figure 4(A) shows that, indeed, the time-dependent uptake of [3H]ALP by S49siSMS1 cells was decreased to a similar extent as observed in S49AR cells, whereas S49 and S49mock cells showed the same high uptake-kinetics as reported previously [7]. The synthesis of SM is therefore essential for ALP internalization in these cells.

The production of SM can also be inhibited indirectly by preventing the synthesis of the SM1 substrate ceramide, using myriocin/ISP-1, an inhibitor of serine-palmitoyltransferase [28], the initiating enzyme of the sphingolipid biosynthetic pathway. Treatment with 5 μM myriocin/ISP-1 for 5 h, was found to decrease the incorporation of [14C]-3-L-serine precursor into [14C]-SM of S49 cells by 63 % (results not shown). Figure 4(B) shows that myriocin/ISP-1 inhibited the uptake of [3H]ALP by S49 cells depending on the time of pre-incubation (1 or 16 h), to values that were intermediate between those of S49 and S49AR cells.

We next tested the reduced uptake of ALP in SMS1-down-regulated S49siSMS1 cells would abrogate apoptosis induction in these cells, in a similar manner to the S49AR cells. Figure 4(C) shows that, indeed, the level of apoptosis induced by ALP in these SMS1-deficient cells dropped from 55 % (S49 and S49mock cells) to 15 %. The sphingolipid synthesis inhibitor myriocin/ISP-1 (5 μM, pre-incubated for 2h) inhibited ALP-induced apoptosis in S49 cells, measured after 6 h, by 31 ± 6 % (n = 4). We note, however, that the inhibitor tended to induce apoptosis by itself when longer pre-incubation times were used.

To answer the question of whether the reduced ALP uptake in the SMS1-deficient cells was due to the lack of SM activity or, as such, the lack of SM in plasma membrane lipid rafts, we loaded the S49AR cells with exogenous SM. In our experience, the most efficient way to do this was to let the cells take up C_{12,0}-SM, which worked better than SM with longer acyl-chains, via the serum lipoproteins in the culture medium. To this end, C_{12,0}-SM was injected into the complete culture medium at final concentration of 20 μM (see Experimental section). After 3 days of culturing in this medium, cells were washed and their lipid rafts were isolated. Figure 5(A) shows that the exoSM (exogenous C_{12,0}-SM) was incorporated in the S49AR detergent-resistant raft fractions to at least similar (or higher) proportions as the endogenous C_{16,0}-SM in rafts from S49 cells. However, this exoSM loading of S49AR cells did not result in more [3H]ALP uptake by these cells (Figure 5B), nor did it affect the resistance of these cells to ALP-induced apoptosis (Figure 5C).

Collectively, these results suggest strongly that SM synthesis, rather than a mere SM accumulation in lipid rafts at the plasma membrane, is required for ALP internalization to induce apoptosis.

**SM deficiency in lipid rafts is accompanied by reduced cholesterol content**

Paradoxically, we demonstrated in previous work [7,8] that lipid raft SM hydrolysis by exogenous (bacterial) sphingomyelinase prevented ALP internalization and apoptosis induction. The same happened when lipid rafts were disrupted by cholesterol-sequestering agents such as methyl-β-cyclodextrin. Furthermore, it is well known that SM tightly associates with cholesterol [11,12], which favours lipid raft formation and causes detergent insolubility of these membrane microdomains [29]. We therefore questioned whether the lack of SM synthesis in S49AR cells would be accompanied by decreased cholesterol content in the lipid raft fractions. To answer this question, we incubated S49 and S49AR cells with exogenous [3H]cholesterol overnight to label the various subcellular compartments to equilibrium, and then determined the distribution of [3H]cholesterol among the sucrose gradient fractions of Triton X-100-solubilized cell membranes. Figure 6A shows that radiolabelled cholesterol is enriched in the detergent-insoluble lipid raft fractions, and that cholesterol partitioning to these lipid raft fractions is higher in the S49 cells than in the S49AR cells.

Thus detergent-insoluble lipid rafts from S49AR cells are not only deficient in SM, but also have reduced cholesterol content. These results are consistent with the notion that the levels of SM and cholesterol in lipid rafts are mutually interdependent, conceivably due to the well known tight physical interaction between these two lipids [11,12].

**SM synthesis and apoptosis sensitivity depend on cholesterol homeostasis**

SMS1 is located in the Golgi apparatus [26], where its two enzymatic products, SM and diacylglycerol, play an important role in new lipid raft formation and secretory vesicle formation at the trans-Golgi network for transport to the plasma membrane (see Discussion). SMS catalyses the production of SM and
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Figure 5  Exogenous SM incorporates into lipid rafts of S49AR cells, but does not facilitate ALP uptake and apoptosis induction in these cells

(A) S49AR cells were cultured in the presence of exogenous C12:0-SM (exoSM; 20 µM, added to the culture medium for 3 days). Cells were then washed and detergent-insoluble lipid raft fractions (R) were isolated and combined, and separated from non-lipid-raft fractions (NR, combined). Lipids were extracted, separated by TLC and visualized by charring with sulfuric acid. Positions of phospholipids are indicated. Note that C12:0-SM has become enriched in lipid rafts (R) from S49AR cells, and migrates on TLC slightly lower than (more hydrophobic) endogenous C16:0-SM (compare with S49 rafts). LPC, lyso PC.

(B) Uptake of [3H]ALP (at 2 h) by S49AR cells cultured with (grey bars) or without exogenous C12:0-SM (exoSM) (white bars) in comparison with S49 cells (black bars). Experimental conditions were the same as described in the legend for Figure 4. Results are means ± S.D. for three separate experiments performed in triplicate.

(C) ALP-induced apoptosis in S49 cells (black bars) and in S49AR cells that were cultured with (grey bars) or without exogenous C12:0-SM (exoSM) (white bars). Cells were stimulated with 15 µM edelfosine for 6 h and apoptosis (nuclear fragmentation) was determined by FACScan analysis. Results are means ± S.D. for four separate experiments performed in triplicate.

diacylglycerol from ceramide and PC, but can also catalyse the reverse reaction [30], resulting in an equilibrium. Given the strong physical association of SM with cholesterol, one would expect that the availability of cholesterol at the site of SM synthesis influences this enzymatic equilibrium and stabilizes SM formation through its sequestration in a (transient) SM–cholesterol complex, thus facilitating/driving SM production and new lipid raft formation. We reasoned that, when cholesterol is deficient at this SMS1 location, SM and diacylglycerol can be more easily converted back into PC and ceramide, the net result being that SM production is inhibited. To test this hypothesis, we used U18666A to disturb NPC1-mediated vesicular transport of cholesterol out of the endosomal compartments [14,15] to the trans-Golgi network and lipid rafts [17], and we determined SM production and cholesterol partitioning in the lipid raft fractions. So, S49 cells were allowed to take up and process exogenous [3H]cholesterol in the presence or absence of U18666A. As shown in Figure 6(B), cells that received U18666A showed less [3H]cholesterol partitioning in the lipid raft fractions compared with the control cells. In fact, the altered cholesterol profile from the U18666A-treated cells looked very similar to that from the S49AR cells (compare the graphs of Figures 6A and 6B).

We next tested how this disturbance of cholesterol homeostasis affected SM synthesis. Figure 7(A) shows that the time-dependent synthesis of SM in S49 cells is inhibited by about 80% in the presence of U18666A. This inhibition of SM synthesis was indirect, via cholesterol, as confirmed by a control experiment shown in Figure 7(B). In this experiment, we incubated crude cellular membranes with fluorescently NBD-labelled ceramide as a substrate for endogenous SMS. In the in vitro assay, U18666A did not inhibit SM synthesis, whereas D609, a known SMS inhibitor [31,32], did (Figure 7B). Collectively, the results suggest that SM synthesis is blocked indirectly through U18666A-mediated disturbance of intracellular cholesterol homeostasis.

Since U18666A not only disturbs cholesterol homeostasis, but also abrogates SM synthesis concomitantly, we next tested

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whether this inhibitor would prevent ALP uptake and apoptosis induction, in a similar manner to the SMS1-deficient cells. Figure 8(A) shows the time-dependent uptake of \[^{3}H\]ALP by S49 and S49\(^{AR}\) cells. In the presence of U18666A, the uptake by S49 cells decreased to the level of S49\(^{AR}\) cells. Furthermore, U18666A treatment blocked ALP-induced apoptosis in S49 cells in a dose-dependent manner, whereas the drug did not induce apoptosis by itself (Figure 8B). As an additional control, U18666A had no effect on the induction of apoptosis by an entirely different inducer, FasL (Figure 8B), indicating that the drug specifically blocked the ALP uptake, but exerted no specific effect on apoptotic signalling steps.

We reported previously [7,8], that ALP induces apoptosis by, primarily, lipid-raft-mediated endocytic uptake and subsequent inhibition of PC synthesis inside the cell. Since U18666A appeared to compromise ‘normal’ lipid raft formation (both SM synthesis and cholesterol incorporation in the detergent-resistant lipid raft fraction were inhibited), and inhibits ALP internalization, one would expect that U18666A would also prevent PC inhibition. Figure 8(C) shows that ALP-induced PC inhibition is, indeed, alleviated by U18666A. Thus it appears that U18666A, through disturbing cholesterol homeostasis and decreased SM synthesis, which disables cholesterol–SM association in lipid rafts, prevents ALP from reaching its intracellular target, cytidylyltransferase in the ER.

**DISCUSSION**

In the present study, we have shown that S49 lymphoma cells express only one of the two known mammalian SMS isotypes, SMS1, and that the activity of this enzyme is required for ALP internalization and ALP-induced apoptosis in these cells. To initiate apoptotic signalling, ALP has to be internalized by endocytosis via lipid rafts to inhibit the rate-determining enzyme, CTP:phosphocholine cytidylyltransferase, of PC synthesis in the

**Figure 7** Disturbance of cholesterol homeostasis by U18666A indirectly inhibits SM biosynthesis.

(A) S49 cells were labelled for the times indicated with \[^{14}C\]methyl-choline, in the absence or presence of U18666A (2 \(\mu\)g/ml; pre-incubated for 4 h). Lipids were extracted and separated by TLC. Positions of phospholipids are indicated. SM appears typically as two spots (see main body text and the legend for Figure 1). LPC, lyso PC. (B) D609 (50 \(\mu\)g/ml), but not U18666A (2 \(\mu\)g/ml), inhibits the synthesis of (NBD-conjugated) SM, when added to crude membranes with NBD-ceramide as a substrate.

**Figure 8** U18666A inhibits ALP uptake and ALP-induced apoptosis, and alleviates ALP-mediated inhibition of PC synthesis.

(A) S49 cells (●, ○) and S49\(^{AR}\) cells (□, ■) were treated without (●, □) or with (○, ■) 2 \(\mu\)g/ml U18666A for 4 hours and then incubated with \[^{3}H\]ALP for the indicated times. Uptake of \[^{3}H\]ALP was measured as described in the Experimental section. (B) Induction of apoptosis in S49 cells by 15 \(\mu\)M ALP (●) or 500ng/ml FasL. (□) as a function of the concentration of U18666A. (C) S49 cells were labelled with \[^{14}C\]methyl-choline for 4 h, in the presence or absence of 15 \(\mu\)g/ml U18666A, as indicated. Radiolabelled PC was determined by TLC analysis (upper panel), and quantified by phosphoimaging (lower panel). Results are the means ± S.D. for three experiments. Where no error bar is observed, this means that the S.D. falls within the size of the symbol.

ER [7,8]. In S49\(^{AR}\) cells, this raft-dependent process is blocked [7]. We have demonstrated by using three different radiolabelled precursors, \[^{14}C\]methyl-choline, \[^{14}C\]3-L-serine and \[^{3}H\]1-sphingosine, that the S49\(^{AR}\) cells do not synthesize SM. The lack of SM in these cells, confirmed by MS, was due to the absence of SMS expression as determined by PCR. Furthermore, siRNA-induced downregulation of SMS1 expression in the parental S49 cells prevented SM production, ALP uptake and apoptosis induction, in a similar manner to the S49\(^{AR}\) cells. Similar results were obtained when the S49 cells were treated with U18666A, which disturbs cholesterol homeostasis and, in this way, inhibits SM synthesis indirectly. That this inhibition by U18666A is indeed indirect, was verified by the fact that the compound did not affect SMS activity in a cell-free system (isolated membranes), whereas D609, a known SMS inhibitor [31,32], blocked this activity \textit{in vitro}. D609 (at 30 \(\mu\)g/ml) also blocked the synthesis of SM \textit{in vivo} (but notably inhibited the biosynthesis of other lipids to some moderate extent as well) and prevented ALP-induced apoptosis in S49 cells (results not shown). Indirect inhibition of SM production by myriocin/ISP-1, an inhibitor of serine palmitoyltransferase, an early enzymatic step in sphingolipid synthesis, likewise inhibited ALP uptake and apoptosis induction in these cells. The results obtained by gene silencing of SMS1 could thus be mimicked by pharmacological inhibition of the enzyme directly, or of the entire biosynthetic pathway of sphingolipids.
Precisely how S49 cells become resistant to ALP upon continuous culturing in the presence of this ether-lipid remains unknown. Our results suggest a mechanism that includes transcriptional downregulation of SMS1. Possible involvement of stress mediators such as heat-shock proteins or lipid (possibly ALP)-sensitive transcription factors may be considered in future studies. To escape ALP toxicity, the cells apparently shut off their lipid-raft-mediated endocytic uptake, by downregulating the synthesis of the key lipid raft component SM (but not of other sphingolipids, as shown in Figures 1B and 2). This SM synthesis occurs in the Golgi, the location of SMS1 [26], and must somehow be linked to endocytic uptake of ALP at a spatially distant site, the plasma membrane. An appealing physico-chemical linker of these two spatio-temporal processes is the dynamic, and at any time transient (SM containing) lipid raft that is newly assembled in the trans-Golgi network, is subject to vesicular trafficking to the plasma membrane [10,33] and from there mediates ALP internalization through endosomes [7,8,33]. In this concept, ALP internalization by lipid raft-dependent endocytosis may thus be viewed as part of a constitutive SMS1-dependent membrane recycling process.

In the Golgi apparatus, SMS1 and its two enzymatic products, SM and diacylglycerol, play a major role in microdomain (lipid raft) formation and vesicle biogenesis at the trans-Golgi network [13,34–36] respectively. Diacylglycerol represents a major cue for protein kinase D (PKD1) recruitment to trans-Golgi membranes [37]. This PKD1 regulates the fission of transport vesicles on their way to the plasma membrane [34]. Inhibition of SMS1-mediated diacylglycerol production was shown to block this process [37]. In the Golgi, newly synthesized SM is assumed to seek association with cholesterol in nascent lipid rafts [10,38]. Cholesterol partitioning in new lipid rafts was shown to occur before it reaches the plasma membrane [39], and is required for the formation of secretory vesicles from the trans-Golgi network [33,40]. Cholesterol availability at this site was shown to be regulated by the NPC1 protein [17]. In NPC1-knockout cells, cholesterol transport from endosomal compartments to the trans-Golgi network is blocked, and this defect can, in normal cells, be mimicked by the compound U18666A [14,15]. Treatment of S49 cells with this inhibitor of cholesterol homoeostasis indeed reduced cholesterol distribution in lipid rafts and inhibited SM production indirectly. The latter may be tentatively explained by insufficient stabilization of newly synthesized SM through the absence of cholesterol at this site (see also reasoning in Results section). In turn, we speculate that this deficient SM synthesis with consequent lack of SM–cholesterol assembly in new lipid rafts might be a causal link to inhibition of ALP uptake and apoptosis induction, like in the S49Δ cells.

The blockage of constitutive SM synthesis (and of concomitant production of diacylglycerol), as we effectuated in various ways (siRNA of SMS1, myristic/IPSP-1, D609) is likely to inhibit anterograde vesicular trafficking, as noted previously [34,36]. The mere accumulation of SM in plasma membrane rafts, by supplying SM-deficient S49Δ cells with exogenous SM, failed to facilitate ALP internalization and apoptosis induction (Figure 5). Likewise, loading of the plasma membrane with exogenous (cycloextrin-formulated) cholesterol did not facilitate ALP uptake and apoptosis (results not shown; [18]). Our results are therefore consistent with the possibility that constitutive biosynthesis of SM, in concert with recruited (endogenous) cholesterol, directs the assembly of SM-containing lipid rafts at the trans-Golgi network and further translocation to the plasma membrane, and that this dynamic and constitutive process is essential for the raft-dependent internalization of ALP. Collectively, these results support the idea that ALP internalization is carried by the retro-grade route of constitutive lipid raft–vesicular cycling that may exist between the trans-Golgi, the plasma membrane and the endosomal compartments [10,33,41–44]. This hypothesis and the role of SMS1 in this particular recycling process and ALP sensitivity needs to be verified further in future studies.

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