Resistance to alkyl-lysophospholipid-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-lysophospholipid) 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, S49^{AR}, 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 S49^{AR} cells are unable to synthesize SM (sphingomyelin) due to down-regulated 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 S49^{AR} 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-lysophospholipid), edelfosine (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; Et-18-OCH₃) is the prototype of a group of new clinically used anti-cancer 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 cytidyltransferase, the rate-determining enzyme for PC (phosphatidylcholine) biosynthesis [6]. We have reported previously [7,8] that this cytidyltransferase inhibition is a direct trigger for apoptosis in S49 lymphoma and HeLa carcinoma cells. Since cytidyltransferase 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, S49^{AR}, selected by continuous culturing of S49 cells in the presence of ALP, we found no raft-dependent ALP internal-

ization 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 S49^{AR} 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-lysophospholipid; C₆-NBD-ceramide, *N*-{6-[(7-nitro-benz-2-oxa-1,3-diazo-4-yl)amino]caproyl}-ceramide; C_{12:0}-SM, lauroyl sphingomyelin; edelfosine, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; ER, endoplasmic reticulum; exoSM, exogenous C_{12:0}-sphingomyelin; FasL, Fas ligand; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GlcCer, glucosylceramide; HRPT, hypoxanthine guanine phosphoribosyl transferase 1; LacCer, lactosylceramide; NPC1, Niemann–Pick C1; PC, phosphatidylcholine; PE, phosphatidylethanolamine; RT, reverse transcriptase; siRNA, small interfering RNA; S49^{AR}, ALP-resistant S49 cells; S49^{mock}, mock-transduced S49 cells; SM, sphingomyelin; SMS, SM synthase.

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In the present study, we show that the S49^{AR} 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 abrogated ALP uptake and apoptosis resistance.

EXPERIMENTAL

Materials

The ALP edelfosine and U18666A (3- β -[2-(diethylamino)-ethoxy]androst-5-en-17-one) were purchased from BioMol. [³H]ALP ([³H]Et-18-OCH₃; 58 Ci/mmol) was synthesized by Moravsek Biochemicals. [¹⁴C]Methyl-choline chloride (58 mCi/mmol), [¹⁴C]3-L-serine (54 mCi/mmol) and [³H]1 α ,2 α (*n*)-cholesterol (49 Ci/mmol) were purchased from Amersham Biosciences. [³H]1-sphingosine was synthesized by Mr P. Weber (DSM, Delft, The Netherlands). C_{12:0}-SM (lauroyl sphingomyelin; *N*-Lauroyl-D-erythro-sphingosylphosphorylcholine) was from Avanti Polar Lipids. C₆-NBD-ceramide (*N*-{6-[(7-nitro-benz-2-oxa-1,3-diazo-4-yl)amino]caproyl}-ceramide) was from Molecular Probes. D609 (tricyclodecan-9-yl-xanthogenate) was from Calbiochem. Myriocin/ISP-1 was from Sigma. Soluble recombinant human FasL (Fas ligand), APO-1L, was from Alexis. Reagents for lipid extraction and subsequent analyses, as well as Silica 60 TLC plates (20 × 20 cm) were from Merck.

Cells and culture conditions

Mouse S49 lymphoma cells were grown in Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 8% (v/v) fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin at 37 °C in a 5% CO₂/95% air atmosphere. S49^{AR} cells were obtained through two rounds of selection, by growth of S49 cells in the presence of 15 μ M edelfosine for 72 h, followed by the plating of the cells in semi-solid medium and the isolation of colonies of surviving cells [18]. The selected S49^{AR} cells could be grown continuously in 15 μ M edelfosine with a doubling time of 12 h, similar to that of the parental S49 cells. S49^{AR} cells maintained resistance by treatment with ALP every 2 weeks. All experiments with S49^{AR} cells were performed with cells grown without the selection agent for at least 3 days.

Loading of S49^{AR} cells with exogenous C_{12:0}-SM was performed by injecting the ethanol-dissolved lipid into the complete culture medium (final concentration 20 μ M) and culturing the cells in this medium for 3 days.

PCR and retroviral transduction

In order to amplify SMS1 and SMS2 by reverse transcriptase PCR, the following primers were used: forward SMS1 primer 5'-CC-GATGGCAGCTTCAGCATC-3'; reverse SMS1 primer 5'-AGC-GCCGAGGAGAATACTC-3'; forward SMS2 primer 5'-GTTG-CATGGAATGGTACC-3'; reverse SMS2 primer 5'-CTCA-CATCCT GTGCGGAGAC-3'. As a control, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was amplified using forward primer 5'-TGCACCACCTGCTTAG-3' and reverse primer 5'-GG-ATGCAGGGATGATGTTTC-3'. For real-time PCR, we used the forward SMS1 primer: 5'-TGGCATGCATTTCAACTGTTC-3'; and the reverse SMS1 primer: 5'-AGCTTCATTATCTCCGCA-CTTG-3'. The amplification was related to the HRPT (hypoxan-

thine guanine phosphoribosyl transferase 1) gene, using the forward primer: 5'-CTGGTGAAAAGGACCTCTCG-3'; and the reverse primer: 5'-TGAAGTACTCATTATAGTCAAGGGCA-3'. In order to silence the expression of SMS1, the following primers were used: sense, 5'-GATCCCCGCATGGGAGTTGATTT-AGATTCAAGAGATCTAAATCAACTCCCATGCTTTTTTGGAA-3'; and antisense, 5'-AGCTTTTCCAAAAAGCATGGGA-GTTGATTGA GATCTCTTGAATCTAAATCAACTCCCATGCGGG-3'.

For retroviral gene transduction, cDNAs were cloned into the retroviral vector pRETRO-SUPER [19]. Empty vector or vector 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 Ampho, using FuGeneTM6 transfection reagent, according to the manufacturer's instructions (Roche Molecular Biochemicals). Transfected cells were selected with 1 μ g/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 × 10⁶ 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 μ g/ml puromycin.

ALP uptake and apoptosis assay

Cells were grown to a density of 1 × 10⁶/ml and ALP (edelfosine) was added at the effective apoptotic concentration of 15 μ M, supplemented with 0.2 μ Ci 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 μ M 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 μ g/ml propidium iodide, according to Nicoletti et al. [20]. Fluorescence intensity of propidium iodide-stained nuclei was determined on a FACScan (Becton Dickinson), and data were analysed using Lysis software.

Lipid analysis

Cells, grown to a confluence of 10⁶ cells/ml, were incubated with [¹⁴C]methyl-choline chloride (1 μ Ci/ml), [³H]sphingosine (1 μ Ci/ml), [¹⁴C]3-L-serine (0.4 μ Ci/ml) or [³H]cholesterol (0.02 μ Ci/ml), where indicated. At given time points, aliquots of cells were taken, washed and resuspended in 200 μ l 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/chloroform (47:49:3 by vol.) and separated by one-dimensional silica TLC, using chloroform/methanol/acetic acid/water (60:30:8:5 by vol.) or, in the case of sphingosine labelling, using chloroform/methanol/0.2% CaCl₂ (60:40:9 by vol.). 2D-TLC (two-dimensional TLC) was performed using 2 × chloroform/methanol/NH₄OH (60:60:5, by vol.), with intermittent drying, in the first dimension, followed by 1 × chloroform/methanol/acetic acid/water (50:30:8:4, by vol.) in the second dimension. Radioactive lipids were visualized and quantified using a Fuji BAS 2000 TR PhosphorImager 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) diphenyloxazole dissolved in

diethylether, drying and subsequent autoradiography. Unlabelled lipids (e.g. C_{12:0}-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 C₆-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, edelfosine, 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 without ALP, the cells gradually become resensitized to the lipid 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 [¹⁴C]methyl-choline chloride or [¹⁴C]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

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

(A) S49 and S49^{AR} cells were labelled overnight with the precursors [¹⁴C]methyl-choline or [¹⁴C]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 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 $\pm$ S.D. for triplicates.

SM type	S49 cells (pmol/10 ⁶ cells)	S49 ^{AR} cells (pmol/10 ⁶ cells)	Fetal calf serum (pmol/mg of protein)
C _{16:0}	101.6 $\pm$ 8.4	20.3 $\pm$ 1.4	651.9 $\pm$ 128.4
C _{16:0} -DH*	6.9 $\pm$ 0.1	1.8 $\pm$ 0.4	77.8 $\pm$ 17.4
C _{18:0}	2.3 $\pm$ 0.2	1.8 $\pm$ 0.1	115.1 $\pm$ 24.0
C _{24:1}	6.8 $\pm$ 0.6	1.7 $\pm$ 0.3	146.5 $\pm$ 32.1
Sum others†	3.7 $\pm$ 0.2	2.1 $\pm$ 0.1	162.9 $\pm$ 16.0
Total	121.3	27.7	1154.2

* C_{16:0}-DH, sphingomyelin containing an *N*-palmitoyl-dihydrosphingosine moiety.

† C_{20:0}, C_{22:0}, C_{24:0}, C_{26:0} and C_{26:1} in amounts less than 1.9 and 0.8 pmol/10⁶ cells for S49 and S49^{AR} cells respectively, and less than 60.1 pmol/mg of protein for fetal calf serum.

containing a C₁₆-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). S49 cells contain mainly the C_{16:0}-SM species, which is reduced

Figure 2 Comparison of sphingolipid distribution of detergent-resistant lipid raft fractions isolated from S49 and S49^{AR} cells

Lipid raft fractions (3 and 4) from S49^{AR} cells, but not from parental S49 cells, are devoid of SM, while the distribution of the other sphingolipids shows no profound differences between the two cell-lines. Cells were labelled for 4 h with [³H]-sphingosine. Triton X-100-insoluble lipid rafts were isolated on sucrose density gradients, and lipids were extracted and separated by TLC. T, total lysate extract. Positions of lipids are indicated as described in the legend for Figure 1(B).

to approx. one-fifth in the S49^{AR} cells. Given the almost complete lack of SM synthesis in S49^{AR} 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 C_{16:0}-SM (Table 1).

To visualize the synthesis of other sphingolipids in the cells, we radiolabelled the cells with a different metabolic precursor, [³H]-1-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, S49^{AR} 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 (glucosylceramide) 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 phosphoethanolamine production, as shown and discussed previously [25].

We next isolated the detergent-resistant lipid raft fractions from [³H]-1-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 is absent from the raft fractions from S49^{AR} 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 S49^{AR} 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.

S49^{AR} cells do not express SMS, whereas S49 cells only express SMS1

In order to investigate the mechanism underlying the defective SM synthesis in S49^{AR} 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 S49^{AR} 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

Figure 3 Expression of SMS1 and SMS2 in S49 and S49^{AR} cells

(A) RT-PCR for gene transcription of *SMS1* and *SMS2* on cDNA generated from S49 (S) and S49^{AR} (R) cells. Primer sets were used for GAPDH (loading control), *SMS1* and *SMS2*. (B) Real-time PCR analysis of *SMS1* in S49, S49^{AR}, S49^{mock} and S49^{siSMS1} cells. Gene transcription is expressed as a percentage of that in S49 cells, with the expression of the HRPT gene serving as an internal standard. (C) S49^{siSMS1} cells are almost devoid of SM biosynthesis, while the synthesis of other (sphingo)lipids is unaffected. Sphingolipids were labelled with [³H]-sphingosine for 4 h. Lipids were extracted and separated by TLC. Positions of lipids are indicated in the legend for Figure 1(B). So, sphingosine.

S49^{AR} 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 S49^{AR} cells directly explains the defect in SM synthesis in the S49^{AR} 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 S49^{AR} cells are resistant to ALP because, unlike the parental S49 cells, they are unable to internalize ALP via lipid rafts. Since S49^{AR} cells lack SMS expression and SM synthesis, we first tried to introduce the *SMS1* gene into S49^{AR} cells. This, however, resulted in the loss of viability of *SMS1*-transfected cells (both in S49 and in S49^{AR} cells; results not shown). We next downregulated *SMS1* expression in S49 cells by stable transduction of siRNA against *SMS1*, yielding so-called S49^{siSMS1} cells, to see if reduced SM synthesis would lead to impaired ALP internalization, resulting in a phenotype similar to S49^{AR} cells. *SMS1* mRNA levels in S49^{siSMS1} 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 (S49^{mock}) 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 S49^{AR} cells.

Figure 4 Inhibition of SM synthesis, through siRNA-induced SMS1 silencing (S49^{siSMS1} cells) or myriocin/ISP-1 treatment (S49 cells) abrogates ALP uptake and apoptosis induction, similar to S49^{AR} cells

(A) Uptake of [³H]ALP in S49 (●), S49^{mock} (○), S49^{siSMS1} (□) and S49^{AR} cells (■) followed over time. (B) Time-dependent uptake of [³H]ALP in S49 (●) cells that were pre-incubated with 5 μM myriocin/ISP-1 for 1 h (△) or 16 h (▲), to inhibit sphingolipid biosynthesis, compared with S49^{siSMS1} cells (■). Results are presented as [³H]ALP radioactivity (dpm) bound per 10³ cells and are means ± S.D. for four experiments (the absence of error bars indicates that the S.D. value was within the size of the symbol). (C) Similar to S49^{AR} cells, S49^{siSMS1} cells show reduced ALP-induced apoptosis compared with parental S49 cells or S49^{mock} cells. Cells were stimulated with 15 μM edelfosine for 6 h, and apoptosis (nuclear fragmentation) was determined by FACS analysis.

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 S49^{siSMS1} cells was decreased to a similar extent as observed in S49^{AR} cells, whereas S49 and S49^{mock} 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 SMS1 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 S49^{AR} cells.

We next tested if the reduced uptake of ALP in SMS1-down-regulated S49^{siSMS1} cells would abrogate apoptosis induction in these cells, in a similar manner to the S49^{AR} cells. Figure 4(C) shows that, indeed, the level of apoptosis induced by ALP in

these SMS1-deficient cells dropped from 55 % (S49 and S49^{mock} cells) to 15 %. The sphingolipid synthesis inhibitor myriocin/ISP-1 (5 μM, pre-incubated for 2 h) 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 SMS activity or, as such, the lack of SM in plasma membrane lipid rafts, we loaded the S49^{AR} 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 S49^{AR} 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 S49^{AR} 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 S49^{AR} cells would be accompanied by decreased cholesterol content in the lipid raft fraction. To answer this question, we incubated S49 and S49^{AR} 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 S49^{AR} cells.

Thus detergent-insoluble lipid rafts from S49^{AR} 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

Figure 5 Exogenous SM incorporates into lipid rafts of S49^{AR} cells, but does not facilitate ALP uptake and apoptosis induction in these cells

(A) S49^{AR} cells were cultured in the presence of exogenous C_{12:0}-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 C_{12:0}-SM has become enriched in lipid rafts (R) from S49^{AR} cells, and migrates on TLC slightly lower than (more hydrophobic) endogenous C_{16:0}-SM (compare with S49 rafts). LPC, lyso PC. (B) Uptake of [^3H]ALP (at 2 h) by S49^{AR} cells cultured with (grey bars) or without exogenous C_{12:0}-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 $\pm$ S.D. for three separate experiments performed in triplicate. (C) ALP-induced apoptosis in S49 cells (black bars) and in S49^{AR} cells that were cultured with (grey bars) or without exogenous C_{12:0}-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 $\pm$ 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

Figure 6 Cholesterol levels are decreased in lipid raft fractions from S49^{AR} cells and U18666A-treated S49 cells

(A) Cholesterol profiles of gradient fractions from S49 (●) and S49^{AR} (□) cells. Cells were incubated overnight with [^3H]cholesterol (0.02 $\mu\text{Ci}/\text{ml}$; trace amount dissolved in ethanol injected into the serum-containing culture medium), followed by Triton X-100 solubilization and sucrose-gradient centrifugation. [^3H]cholesterol was quantified by liquid scintillation counting. (B) U18666A treatment of S49 cells causes redistribution of cholesterol out of the raft fractions, yielding a profile similar to S49^{AR} cells (compare with A). S49 cells were incubated with [^3H]cholesterol as above. Prior to solubilization, cells were treated with 2 $\mu\text{g}/\text{ml}$ U18666A (○) or without (●) for 4 h. [^3H]cholesterol in the gradient fractions was separated on TLC and was visualized by autoradiography (lower panel) and was quantified by liquid scintillation counting (upper panel), expressed as percent of total cholesterol on the gradient.

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 S49^{AR} 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

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 μ 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 μ g/ml), but not U18666A (2 μ g/ml), inhibits the synthesis of (NBD-conjugated) SM, when added to crude membranes with NBD-ceramide as a substrate.

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, cytidyltransferase 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 cytidyltransferase, of PC synthesis in the

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 μ 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 μ M ALP (●) or 500ng/ml FasL (□) as a function of the concentration of U18666A. (○) Represent cells that received no ALP or FasL. Apoptosis was measured by nuclear fragmentation, using FACScan analysis. (C) S49 cells were labelled with 1 μ Ci/ml of [14 C]methyl-choline for 4 h, in the presence or absence of 15 μ M ALP and/or 2 μ g/ml U18666A, as indicated. Radiolabelled PC was determined by TLC analysis (upper panel), and quantified by phosphorimaging (lower panel). Results are the means $\pm$ 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 *in vitro*. D609 (at 30 μ g/ml) also blocked the synthesis of SM *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 homeostasis 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 S49^{AR} cells.

The blockage of constitutive SM synthesis (and of concomitant production of diacylglycerol), as we effectuated in various ways (siRNA of SMS1, myriocin/ISP-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 S49^{AR} cells with exogenous SM, failed to facilitate ALP internalization and apoptosis induction (Figure 5). Likewise, loading of the plasma membrane with exogenous (cyclodextrin-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.

We thank Alfred Merrill, Elaine Wang and Samuel Kelley (Georgia Institute of Technology, Atlanta, GA, U.S.A.) for conducting the SM analyses by MS using the Sphingolipid Analysis Core funded by NIH (National Institutes of Health) GM069338 as part of the Lipid MAPS Consortium. We thank Jannie Borst, Nullin Divecha, Jon Halstead and Menno van Lummel (Netherlands Cancer Institute, Amsterdam, The Netherlands) for useful discussions. Catelijne Stortelers is thanked for advice on real-time PCR. This study was funded by the Dutch Cancer Society, grant NKI 2005-3377.

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Received 2 August 2006/13 October 2006; accepted 19 October 2006

Published as BJ Immediate Publication 19 October 2006, doi:10.1042/BJ20061178