Sphingomyelinase treatment of low density lipoprotein and cultured cells results in enhanced processing of LDL which can be modulated by sphingomyelin

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Abstract The addition of neutral sphingomyelinase from *S. aureus* to the medium of rat intestinal epithelial cell cultures (IEC-6) containing added human low density lipoprotein (LDL) resulted in two- to fivefold increases in LDL uptake and degradation. This overall effect was shown to be the combined result of sphingomyelinase activity on the composition of the LDL particle and a separate action directly on the cells when native LDL was incubated with sphingomyelinase from *S. aureus* followed by removal of the sphingomyelinase. Analysis of sphingomyelinase-treated LDL showed that >95% of the sphingomyelin (SM) was hydrolyzed, but no changes were observed in all the other components of the LDL particle. This modified LDL particle (SM(-)LDL) was also bound and degraded at higher rates than control LDL in a variety of cell lines, e.g., HepG2, GM-43, and CHO-K1 cells. No evidence of increased aggregation of SM(-)LDL could be observed. The increased processing of SM(-)LDL was due to enhanced affinity to LDL receptors and not to an increase in LDL receptor number. When sphingomyelinase from *S. aureus* was added to the medium of IEC-6 or GM-43 cells, which were processing SM(-)LDL, further increases in SM(-)LDL processing were observed, which were primarily due to greatly enhanced cellular degradation of SM(-)LDL, with little change in receptor binding and cell association. Since there was little sphingomyelin remaining in SM(-)LDL, it was assumed that the action of sphingomyelinase on the cells resulted in the enhanced degradation. In support of this concept, previous addition of sphingomyelin to cells growing in lipoprotein-deficient medium followed by addition of SM(-)LDL greatly inhibited the degradation of the apolipoprotein of SM(-)LDL. On the other hand, addition of sphingomyelin concomitantly with SM(-)LDL did not inhibit degradation. These results are interpreted to indicate that there may be two pathways for cellular processing of sphingomyelin, one of which may be a determinant in the lysosomal processing of the apolipoprotein of LDL. In support of this concept, addition of desipramine, an inhibitor of lysosomal sphingomyelinase, to IEC-6 cells in culture greatly inhibited the degradation of ^{125}I-labeled LDL without affecting the receptor binding and cell association. Overall, these results suggest that sphingomyelin may play a modulatory role in cellular cholesterol homeostasis by regulating uptake of LDL as well as LDL processing.—**Gupta, A. K., and H. Rudney.** Sphingomyelinase treatment of low density lipoprotein and cultured cells results in enhanced processing of LDL which can be modulated by sphingomyelin. *J. Lipid Res.* 1992. **33:** 1741–1752.

Supplementary key words cultured cells • LDL receptors • LDL processing • sphingomyelin • sphingomyelinase

The sequence of reactions involved in the production, turnover, and transport of sphingomyelin have generated much interest as products of its metabolism, e.g., sphingosine and ceramide, appear to function as signalling molecules. (For reviews, see references 1–5). One of the key enzymes involved in regulating the level of sphingomyelin in a cell is sphingomyelinase (SMase) which is located in the plasma membrane and the lysosomes. The lysosomal enzyme has a pH optimum of 4.5–5.5 while that in the plasma membrane exhibits a neutral pH optimum and requires Mg^{2+}. It has long been known that there is a close relationship between the levels of cholesterol and sphingomyelin in cellular membranes, especially the plasma membrane (6, 7). Studies have been performed on the effects of disruption of this relationship on cholesterol metabolism by adding exogenous sphingomyelinase from bacterial sources to cells in culture (8, 9). This treatment caused hydrolysis of plasma membrane sphingomyelin with the resultant relocation of cholesterol from the plasma membrane to the cell interior, as evidenced by increased acyl-CoA:cholesterol acyl transferase activity and

Abbreviations: FCS, fetal calf serum; CS, calf serum; LPDS, lipoprotein-deficient serum; LDL, low density lipoprotein; SM(-)LDL, sphingomyelin-treated LDL passed through a Sephadex G-200 column; SMase, sphingomyelinase; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; HMG, hydroxymethylglutaryl; TCA, trichloroacetic acid; PBS, phosphate-buffered saline.

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cholesteryl ester mass. These effects were also accomplished by down-regulation of cholesterol synthesis, which was shown to be the result of inhibition of HMG-CoA reductase (9).

These observations showing links between sphingomyelin content of plasma membranes and cholesterol metabolism have been supported by earlier reports that described the effects of supplementation of sphingomyelin to fibroblast cultures. Addition of sphingomyelin to fibroblasts caused stimulation of HMG-CoA reductase activity and cholesterol synthesis, and the binding and uptake of low density lipoprotein was inhibited (10, 11). It has also been shown that LDL uptake inhibited endogenous sphingomyelin synthesis (12).

In this study, we examined the effect of depletion of plasma membrane and cellular content of sphingomyelin on the binding, uptake, and degradation of low density lipoproteins. Decreases in the levels of plasma membrane and cellular sphingomyelin were effected by exogenous addition of neutral sphingomyelinase from *S. aureus*. Our observations reported here show that the addition of sphingomyelinase to the medium of cells that are processing LDL results in a several-fold increase in the rate of LDL processing. This effect is shown to be due to *a)* modulation of the composition of the LDL particle leading to increased affinity for the LDL receptor, and *b)* a direct effect on the cell resulting in increased degradation of LDL and protein. Further observations are reported that indicate different pathways for the uptake of sphingomyelin by cells, and that further suggest that the level of cellular sphingomyelin may be a modulatory factor in the rate of cellular LDL processing.

EXPERIMENTAL PROCEDURES

Materials

(RS)-3-[^{14}C]HMG-CoA (57 mCi/mmol), (RS)-5-[^{3}H]mevalonolactone (24 Ci/mmol) were obtained from DuPont-New England Nuclear Corp. Sodium [^{125}I]iodide (17 Ci/mg) was purchased from ICN pharmaceuticals. Neutral sphingomyelinase from *S. aureus* was obtained from the Sigma Chemical Company. Cell culture supplies were obtained either from M. A. Biological Associates or Grand Island Biological Co. Human serum was obtained from normal donors. All other chemicals were obtained from local sources and were of the highest purity grade available. Desipramine, sphingomyelin, and dextran sulfate were obtained from Sigma Chemical Co.

Cell cultures

Rat intestinal epithelial cells (IEC-6 cells, CRL 1592), obtained from American Type Culture Collection (ATCC) were grown as monolayers as described previously (13, 14). On day 0, 1×10^5 cells were seeded in 35-mm, 6-well plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal calf serum (FCS) or calf serum (CS). Prior to the addition of SMase or other agents, cells were incubated for 24–48 h in medium supplemented with lipoprotein-deficient serum protein (2 mg/ml) as described in the pertinent legends to the figures or tables. All experiments were conducted using cells in the late logarithmic phase of growth. None of the treatment conditions had any significant effect on the growth of the cells during the time course of these studies.

Human hepatoblastoma cells (HepG2) obtained from American Type Culture Collection (HB-8065) were maintained as monolayers in DMEM medium supplemented with 10% FCS or CS (v/v) as described previously (9, 14). For experiments, cells were seeded at 5×10^4 cells into Petri dishes (35 × 15 mm, 6-well plates) in DMEM with 10% CS or FCS (v/v). Prior to the addition of other agents or SMase, cells were incubated for 24–48 h with DMEM medium supplemented with lipoprotein-deficient serum protein (4 mg/ml). Human skin fibroblasts obtained from Human Mutant Genetic Repository (GM-0043) or (GM-2000) were maintained on DMEM medium supplemented with 10% FCS or CS (v/v). For experiments, cells were seeded at 1×10^5 cells into Petri dishes (35 × 15 mm; 6-well plates) in DMEM medium supplemented with 10% CS or FCS (v/v). Prior to the addition of other agents or SMase, cells were incubated for 24–48 h with DMEM medium supplemented with lipoprotein-deficient serum protein (4 mg/ml). Chinese hamster ovary cells wild type (CHO-K1) obtained from ATCC were maintained in F-12 medium supplemented with 10% FCS. For experiments, cells were seeded at 1×10^5 cells into 6-well plates in F-12 medium containing 10% FCS. Prior to the addition of the SMase or other agent, the cells were incubated for 24–48 h with F-12 medium supplemented with LPDS (4 mg protein/ml).

Isolation, iodination, and processing of low density lipoprotein

Lipoprotein-deficient fetal bovine serum ($d \geq 1.215$ g/ml) was isolated by ultracentrifugation of fetal bovine serum after adjusting the density with KBr (15). Human LDL ($d \geq 1.019$–1.063 g/ml) was similarly obtained from normal human serum. The concentration of LDL is expressed in terms of its protein content (μg/ml). Preparation of ^{125}I-labeled LDL and processing was studied essentially as described by Drevon et al. (16) and Goldstein, Basu, and Brown (17). A typical preparation had a specific activity of 110–250 dpm per ng protein. For the studies on binding and degradation of LDL, cells were cultured as described in the legends to the figures. On day 4, ^{125}I-labeled LDL (25–250 μg/ml) was added. After the indicated time of incubation, medium was collected for determination of LDL degradation by cells. The cells were washed twice with PBS containing bovine serum albumin (BSA)

(2 mg/ml) at 4°C and twice more with PBS at 4°C. To measure the amount of LDL bound to receptors, 2 ml HEPES buffer (50 mM, pH 7.5) containing dextran sulfate (10 mg/ml) was added. After 2 h incubation at 4°C, the buffer was removed. The content of radioactivity released in the buffer provided a measure of receptor binding. The cells were then digested with 2.0 ml sodium hydroxide (0.1 N). After 24 h, the digests were collected and measured for cell-associated and internalized radiolabel. To determine the amount of degraded LDL, 2.0 ml of 20% TCA was added to the previously collected medium (2.0 ml) which was vortexed and incubated at 4°C for 1 h followed by centrifugation. The amount of ^{125}I-radiolabel in the TCA-soluble fraction was measured after treatment with silver nitrate (5%) as described previously (16, 17). To determine whether there was any protease activity as a result of SMase treatment, the release of free ^{125}I after SMase treatment was measured. ^{125}I-labeled LDL medium without cells was incubated in presence and absence of SMase. After 6 h incubation, the medium was removed and processed as described above.

To determine the binding of ^{125}I-labeled LDL to receptors, the cells were incubated for 6 h at 37°C with or without SMase. They were then cooled to 4°C. The medium was removed after 1 h incubation and fresh LPDS medium containing indicated concentrations of ^{125}I-labeled LDL with or without excess unlabeled LDL (100-fold) was added. After 2–4 h incubation, the medium was removed and cells were washed 3 times with 2 ml of ice-cold PBS containing 2 mg/ml BSA. The cells were rinsed twice with 2 ml PBS, and digested with 1 ml 0.1 N sodium hydroxide, and radiolabel was measured. The difference in radioactivity after dilution with excess unlabeled LDL represented specific binding.

To determine the phospholipid content of LDL, the total lipids were extracted and separated via preparative thin-layer chromatography with chloroform–methanol–acetic acid–water 50:25:8:4 as the developing solvent. Individual phospholipids were visualized by iodine vapor staining. Spots corresponding to standard phosphatidylcholine and sphingomyelin were removed and phospholipids were extracted twice with 2 ml chloroform–methanol (2:1) and the phosphorus content was assayed by the method of Bartlett (18). The content of LDL free and esterified cholesterol was determined by the enzymatic method of Carlson and Goldfarb (19). Triglyceride content of LDL was determined using a commercial kit from Sigma Chemical Co. LDL protein was measured by the method of Schacterle and Pollack (20) using bovine serum albumin as standard.

Preparation of SM(-)LDL

To prepare SM(-)LDL, 10 mg ^{125}I-labeled LDL (2 mg/ml) was incubated with neutral SMase from *S. aureus* (1 u/ml) in PBS containing 1 mM EDTA at 37°C. As a control, ^{125}I-labeled LDL (10 mg, 2 mg/ml) was incubated without SMase in PBS-EDTA at 37°C. After 4 h incubation, the incubation mixture was cooled to 4°C. SMase was separated from LDL by passing the incubation mixture on a Sephadex G-200 column (2 × 35 cm). The column was eluted with PBS-ETDA. Fractions of 3.0 ml were collected at a flow rate of 30 ml per h. Absorbance at 280 nm was measured which represented the protein. A 50-μl aliquot from each fraction was assayed for neutral sphingomyelinase activity as described previously (21). After separation, it was found that 0.6 mu of SMase activity was present in each 100 μg of SM(-)LDL.

RESULTS

When neutral SMase from *S. aureus* was added to the LDL-containing medium of IEC-6 cell cultures, we observed a two- to fivefold increase in the amount of LDL that was taken up and degraded. A representative experiment displaying the optimal conditions for this effect is shown in **Fig. 1.** The addition of 100 mu/ml of SMase to medium containing different amounts of ^{125}I-labeled LDL during a 4-h incubation period showed an approximate fourfold increase in the total overall processing of LDL. There were similar increments in the amounts of

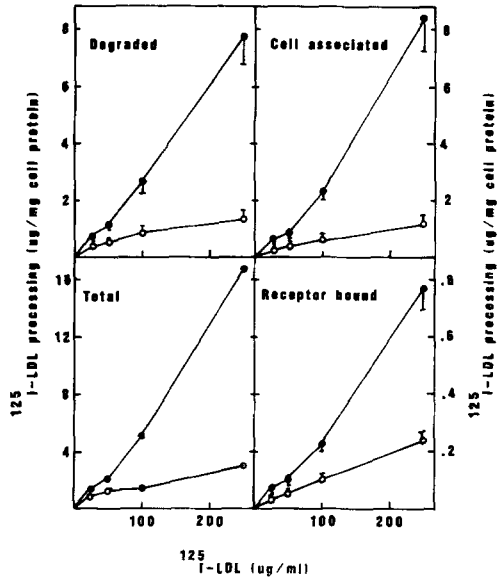

Fig. 1. Effect of sphingomyelinase on ^{125}I-labeled LDL processing in IEC-6 cells. IEC-6 cells were seeded (day 0) at 1 × 10^5 cells (6-well plates) in 2 ml DMEM medium supplemented with 5% (v/v) fetal total calf serum (FCS) medium. The cells were refed 2 ml DMEM medium containing lipoprotein-deficient serum (LPDS medium; protein 2 mg/ml) on days 3 and 4. On day 4, sphingomyelinase (SMase; 100 mu/ml) was added. After 1 h pretreatment with SMase, ^{125}I-labeled LDL was added at the indicated concentrations. After an additional 6 h incubation, cells and medium were harvested for the determination of the ^{125}I-labeled LDL that was receptor-bound, cell-associated, and degraded. The values represent the mean and SE of triplicate determinations.

LDL that were receptor-bound, cell-associated, and cell-degraded. Saturation of the above parameters was not observed even with levels of 250 μg/ml of ^{125}I-labeled LDL, presumably due to the greatly increased rate of overall processing of LDL induced by SMase addition to the medium.

These observations raised the question whether the enhanced processing of LDL was due to the action of SMase on the LDL particle, or on the cells, or both. To determine this point, native LDL was incubated with *S. aureus* SMase followed by purification of the LDL via passage through a Sephadex G-200 column as described in Experimental Procedures. When this treated LDL was added to the medium of IEC-6 cells as described in the legend to Fig. 1, we observed a major augmentation in the overall processing rate as evidenced by corresponding increases in the amounts of LDL that were cell-associated and degraded (**Fig. 2**). This result showed that the effect of the SMase action was exerted primarily on the LDL particle. We then examined the binding to LDL receptors and degradation of normal and modified ^{125}I-labeled LDL under similar conditions in four cell lines of varied origin, e.g., intestinal epithelial cells (IEC-6), human skin fibroblasts (GM-43), human hepatoma cells (HepG2) and Chinese hamster ovary cells (CHO-K1). The results showed increased binding at 4°C and degradation at 37°C of the modified LDL in all cell lines tested; the extent of the increment varied depending on the cell line (**Fig. 3**). Evidence that the increased binding and affinity of the SMase-treated LDL is specific to the LDL receptor was obtained with LDL receptor-negative fibroblasts from familial hypercholesterolemic patients (GM-2000). Incubation of these cells with SMase and ^{125}I-labeled

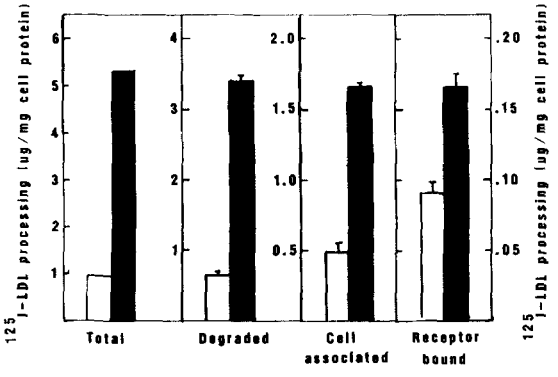

Fig. 2. Processing of native ^{125}I-labeled LDL (n-LDL) and SM(-) ^{125}I-labeled LDL by IEC-6 cells. IEC-6 cells were grown for 3 days as described in the legend to Fig. 1. On day 4, fresh LPDS medium was fed with native or SM(-) ^{125}I-labeled LDL (100 μg/ml). After 6 h incubation at 37°C, the cells and medium were harvested for the determination of receptor binding cell association and degradation of labeled LDL. The light bars represent native ^{125}I-labeled LDL and the dark bars SM(-) ^{125}I-labeled LDL. Total represents the sum of binding, cell-associated, and degraded labeled lipoprotein. The values represent the mean and SE of triplicate determinations.

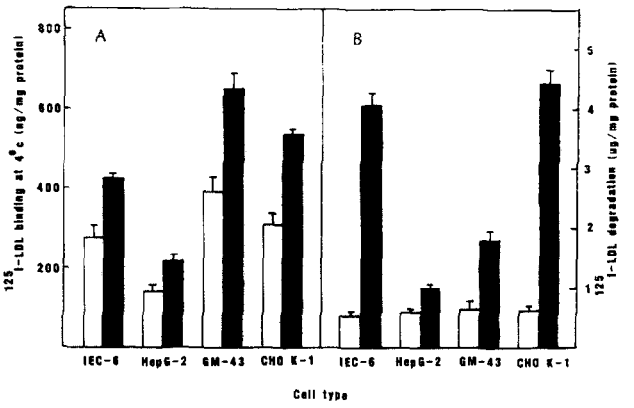

Fig. 3. Binding (panel A) and degradation (panel B) of native and SM(-) ^{125}I-labeled LDL in IEC-6, CHO-K1, HepG2, and GM-43 cell lines. For binding determinations, all cell types were grown for 3 days. On day 4, cells were refed LPDS medium. After 6 h in LPDS medium, the cells were cooled to 4°C and 30 min later the medium was removed and fresh medium containing normal and SM(-) ^{125}I-labeled LDL (100 μg/ml) was added. After an additional 2-h incubation at 4°C, the medium was removed. The cell monolayer was washed with PBS containing BSA (2 mg/ml) and PBS minus BSA. The radiolabel was measured after digesting the cells with 0.1 N sodium hydroxide (1 ml). For determination of LDL degradation, cells were grown for 3 days as described above. On day 4, cells were fed fresh LPDS medium. ^{125}I-labeled LDL (100 μg/ml) or SM(-) ^{125}I-labeled LDL were added and incubation was continued at 37°C for 6 h. Cells and medium were harvested for determination of LDL degradation. The light bars represent native ^{125}I-labeled LDL and the dark bars SM(-) ^{125}I-labeled LDL. The data represent the mean and SE of triplicate determination.

LDL revealed no change in the processing of LDL (data not shown).

The nature of the changes induced in the structure and composition of the LDL particle after treatment with SMase from *S. aureus* was then determined. Purification of the SMase-treated LDL via passage through a Sephadex G-200 column, as described in Experimental Procedures, revealed that small amounts of SMase adhered to the LDL. Under the conditions of treatment and separation adopted, it was found that approximately 0.6 mu of SMase activity adhered to each 100 μg of LDL. Addition of SMase equivalent to that remaining after elution of the LDL to control LDL had no effect on the uptake of LDL (data not shown).

Analysis of the purified SM(-)LDL (**Table 1**) showed that more than 95% of the sphingomyelin content had been hydrolyzed, but the phospholipid, triglyceride, cholesterol, and cholesteryl ester content were unaffected. No evidence of protease activity on the LDL could be detected as evidenced by the absence of TCA-soluble ^{125}I-labeled protein after SMase treatment. Comparison of the electrophoretic properties of SM(-)LDL and native LDL under denaturing as well as nondenaturing conditions revealed no observable change (data not shown).

In order to determine whether any aggregation of LDL occurred during the formation of SM(-)LDL, LDL was

TABLE 1. Effect of sphingomyelinase treatment on the lipid composition of LDL

Lipid	No Treatment	SMase Treatment
	μg/mg LDL protein	
Sphingomyelin	199 ± 18.8	7.7 ± 2.7
Phosphatidylcholine	426.4 ± 15.2	433 ± 1.7
Cholesteryl esters	2072 ± 107	2201 ± 154
Free cholesterol	879 ± 40	944 ± 43
Triglycerides	27.1 ± 2.7	27.3 ± 2.2

Low density lipoprotein (2 mg/ml) was incubated with or without neutral sphingomyelinase from *S. aureus* (1 u/ml) in PBS-EDTA at 37°C. After 4 h incubation, total lipid was extracted with chloroform–methanol 2:1. Sphingomyelin and phosphatidylcholine from total lipid were measured after thin-layer chromatography. The data represent the mean and SE of triplicate determinations.

incubated with SMase from *S. aureus* under the conditions adopted for modification of the LDL particles, and the increase in turbidity at 430 nm was determined. Similar methods have been used to measure the extent of self aggregation of LDL resulting from modification of the phospholipid content (21, 22). We could observe no increase in absorption with the SM(-)LDL particle, indicating that aggregation had not occurred under our conditions (**Fig. 4**). Aggregation was readily observable after rapid vortexing for 10 sec. Analysis of the processing of the vortexed SM(-)LDL showed that these particles displayed increased binding affinity and cell association but no increase in degradation could be observed (**Fig. 5**). The possibility that the observed enhanced processing of SM(-)LDL was the result of an increase in the number of

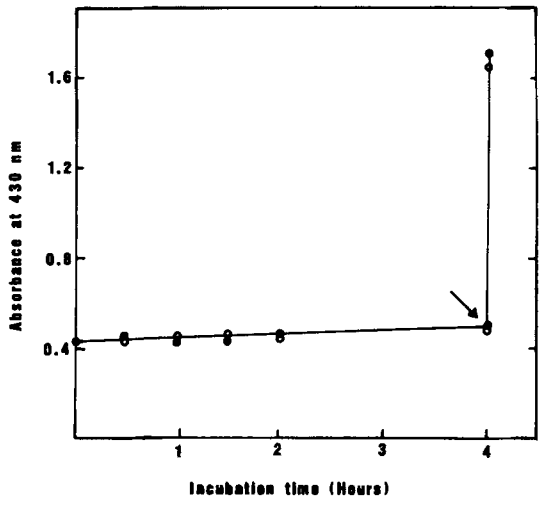

Fig. 4. Effect of sphingomyelinase on the turbidity of LDL. LDL (2 mg/ml) was incubated with or without neutral sphingomyelinase (1 u/ml) at 37°C in phosphate-buffered saline containing 1 mM EDTA. At each indicated time point, aliquots from duplicate samples were removed to measure the absorption of light at 430 nm (A 430 nm). The arrow indicates the time of vortexing. The samples were vortexed at full speed for 20 sec. All values shown are mean of duplicate samples which varied by less than 7%.

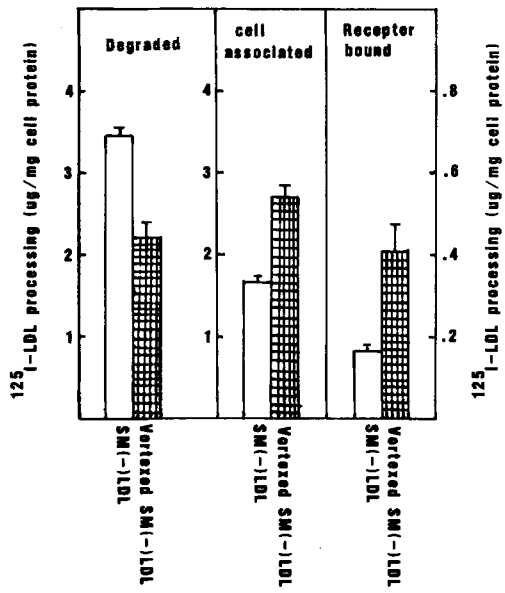

Fig. 5. Processing of SM(-)LDL and vortexed SM(-)LDL in IEC-6 cells. IEC-6 cells were grown for 3 days as described in the legend to Fig. 1. On day 4, cells were fed fresh LPDS medium and SM(-) ^{125}I-labeled LDL (100 μg/ml) or vortexed SM ^{125}I-labeled LDL (100 μg/ml) were added (vortexed at full speed for 30 sec). The cells and medium were harvested after an additional 6 h of incubation for the determination of receptor binding, cell association, and degradation. Values represent the mean and SE of triplicate determinations.

LDL receptors during the 6-h incubation period was investigated in the following manner. Normal and SM(-)LDL were incubated with cells for 6 h, and the cells were then cooled to 4°C. The medium was removed and fresh medium containing native ^{125}I-labeled SM(-)LDL was added and the binding to LDL receptors at 4°C was measured as described in Experimental Procedures. The results of a representative experiment with CHO-K1 and IEC-6 cells are shown in **Fig. 6.** In the case of IEC-6 cells, a minor decrease in binding of native LDL to LDL receptors was observed, whereas in CHO cells a more significant decrease was observed. These results indicate that continued exposure to SM-LDL for 6 h did not result in any increase in the level of LDL receptors; actually small decreases were observed. Thus, when cells are preincubated with SM(-)LDL, the increased processing observed with modified LDL was actually due to increased affinity to the LDL receptor, and not to the generation of a larger number of receptors.

Taken together, the results of all of the foregoing experiments allow the reasonable conclusion that the major change in the SM(-)LDL is the almost complete removal of SM from the phospholipid portion. Under our conditions, we could not detect gross physical changes, e.g., aggregation. The removal of sphingomyelin results in increased affinity for the LDL receptor and greatly enhanced processing of LDL.

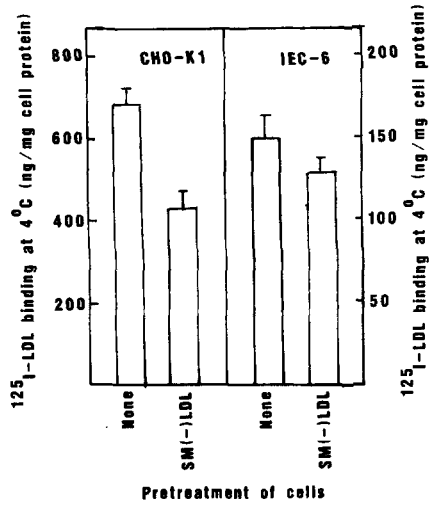

Fig. 6. Binding of native ^{125}I-labeled LDL in IEC-6 and CHO-K1 cells pretreated with or without SM(-)LDL. CHO-K1 and IEC-6 cells were grown for 3 days. On day 4, cells were fed LPDS medium with or without SM(-)LDL (100 μg/ml). After 6 h incubation, cells were cooled at 4°C. Thirty min later the medium was removed and cells were washed with cold PBS, and fresh LPDS medium containing ^{125}I-labeled LDL (100 μg/ml) was added. After 2 h incubation at 4°C, the medium was removed. The cells were washed twice with 2 ml of PBS containing 2 mg/ml BSA and twice with 2 ml of PBS minus BSA. The cells were finally digested with 1 ml of 0.1 N NaOH. Radiolabel and protein content of the cell digests were measured as previously described. Values represent the mean and SE of triplicate determinations.

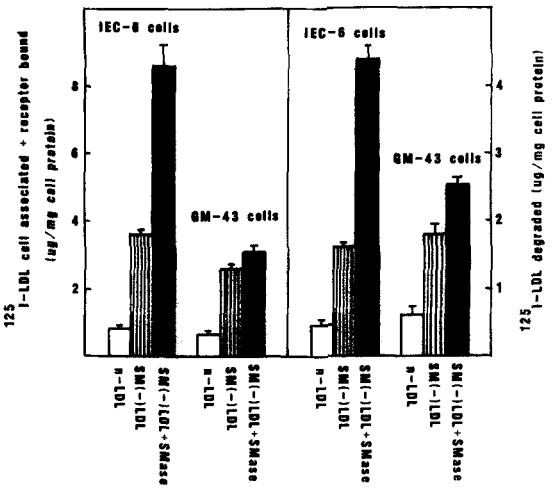

Fig. 7. Effect of SMase on the processing of native and SM(-)LDL in IEC-6 and GM-43 cells. Cells were grown for 3 days. On day 4, cells were fed fresh LPDS medium with or without SMase (100 mu/ml). After 30 min preincubation, native ^{125}I-labeled LDL (100 μg/ml) or SM(-) ^{125}I-labeled LDL (100 μg/ml) was added. After an additional 6 h incubation, the medium and cells were harvested for determination of ^{125}I-labeled LDL processing. The data represent the mean and SE of triplicate determinations.

In the course of the foregoing investigations, we observed that adding SMase to the medium of cells that were processing native LDL generally resulted in processing rates that appeared to be greater than that which would be expected solely on the basis of depletion of the SM content of the LDL particle. To determine whether SMase addition also caused cellular changes that would affect the uptake of LDL independently of the depletion of sphingomyelin in the LDL particle, we performed the following experiments. SM(-)LDL was purified and incubated with cells in the presence of SMase added to the medium. As the SM(-)LDL was essentially free of sphingomyelin, any observable effects of SMase addition to the medium could reasonably be attributed to the action of SMase on the cells. The results of a typical experiment (**Fig. 7**) clearly show that the presence of SMase in the medium greatly enhanced the uptake and processing of modified LDL. Further examination of this effect showed that it did not appear to be due to increased binding of the SM(-)LDL at 4°C because of the following observations with representative experiments. IEC-6 cells were incubated with and without SMase from *S. aureus* in the medium for 6 h, then the cells were cooled to 4°C, removed from the SMase-containing medium, and the binding of ^{125}I-labeled native and ^{125}I-labeled SM(-)LDL was determined. No major changes in the binding of SM(-)LDL could be observed (**Fig. 8**), indicating that SMase treatment of the cells did not affect the binding affinity of SM(-)LDL or the number of LDL receptors. Thus, the increased rate of degradation of LDL observed after SMase addition (Fig. 7) appears to be unrelated to changes in the binding affinity or number of LDL receptors, but appears to be due to other effects of SMase action directly on the cells.

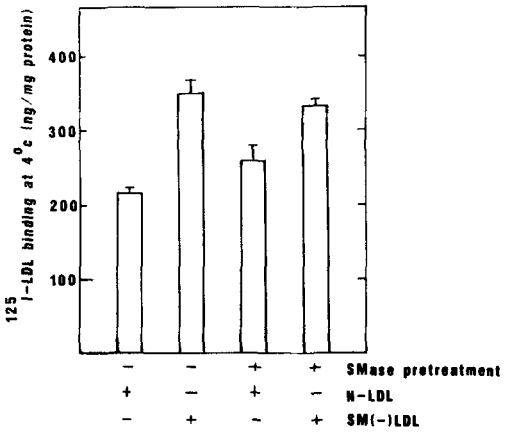

Fig. 8. Binding of native and SM(-)LDL in IEC cells treated with or without SMase. IEC-6 cells were grown for 3 days as described in the legend to the Fig. 1. On day 4, fresh LPDS with or without SMase (100 mu/ml) was fed. After 6 h incubation, the cells were cooled to 4°C. After 30 min incubation the medium was removed and cells were washed. Fresh LPDS medium containing native or SM(-) ^{125}I-labeled LDL (100 μg/ml) was added and incubation was continued for 2 h at 4°C. The medium was removed and cells were processed as described in the legend to Fig. 6. The data represent the mean and SE of triplicate determinations.

As one of the effects of the presence of *S. aureus* SMase in the medium of cells in culture is to greatly reduce the level of cellular sphingomyelin (9), the foregoing results raised the possibility that the level of cellular sphingomyelin was a determinant in regulating the rates of degradation of the apoprotein of endocytosed LDL. We therefore examined the effect of increasing the level of cellular sphingomyelin on the processing of normal and SM(-)LDL. IEC-6 cells were incubated for 0.5 h in lipoprotein-deficient medium containing various amounts of sphingomyelin, then ^{125}I-labeled SM(-)LDL was added and the processing of the LDL was determined. The results of a representative experiment shown in **Fig. 9** reveal that receptor binding and cell association of LDL were not significantly changed; on the other hand, there was a marked inhibition of the degradation of LDL with increasing concentrations of sphingomyelin added to the medium. Similar results were obtained with fibroblasts and HepG2 cells (data not shown). Addition of ceramide to the medium had no effect. Another mechanism to increase levels of cellular sphingomyelin would be via inhibition of lysosomal sphingomyelinase activity. We treated cells with desipramine, a known inhibitor of lysosomal SMase activity (23), and determined the effect on LDL processing. As shown in **Fig. 10,** desipramine greatly inhibited LDL degradation, with a minor increase in the level of LDL that was cell-associated and receptor-bound. These data mimic those displayed in Fig. 9, which show the effect of adding sphingomyelin to cells. These observations are in agreement with the concept that the level of cellular SM may also be a regulatory posttranslational determinant in the overall processing of LDL.

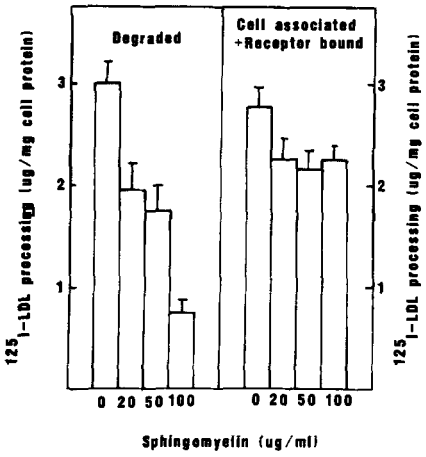

Fig. 9. Effect of sphingomyelin added directly to the medium on the processing of SM(-)LDL in IEC-6 cells. IEC-6 cells were grown for 3 days as described in the legend to Fig. 1. On day 4, cells were fed fresh LPDS medium with or without indicated concentration of sphingomyelin. Sphingomyelin was added as an ethanolic solution. Control dishes received an equivalent amount of ethanol. The final concentration of ethanol never exceeded 1%. After 30 min incubation, SM(-) ^{125}I-labeled LDL (100 μg/ml) was added. The cells and medium were harvested after an additional 6 h incubation for the processing of ^{125}I-labeled LDL. The data represent the mean and SE of triplicate determinations.

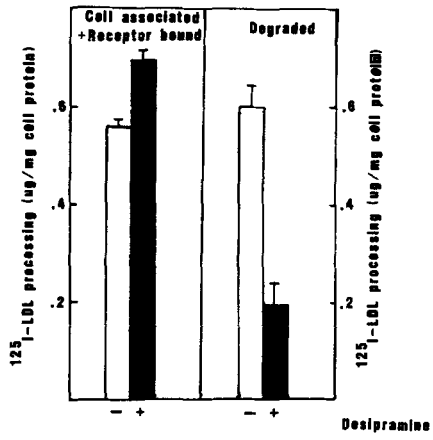

Fig. 10. Effect of desipramine on ^{125}I-labeled LDL processing in IEC-6 cells. IEC-6 cells were grown for 3 days as described in the legend to the Fig. 1. On day 4, cells were fed fresh LPDS medium with or without desipramine (10 μM). After 1 h incubation, ^{125}I-labeled LDL (100 μg/ml) was added. Medium and cells were harvested for the determination of LDL processing after an additional 6 h incubation. Cell-associated LDL in these experiments is the sum of receptor-bound and internalized, undegraded LDL. The data represent the mean of the triplicate determinations and SE.

Coincident with these experiments, we examined the effect of adding sphingomyelin back to the medium of IEC-6 cell cultures that were processing SM(-)LDL. Surprisingly, and contrary to expectations based on the previous observations, we observed that there was little difference between the binding and degradation of SM(-)LDL in the presence and absence of sphingomyelin. A representative experiment is shown in **Fig. 11.** Previous tests (data not shown) had shown that sphingomyelin added

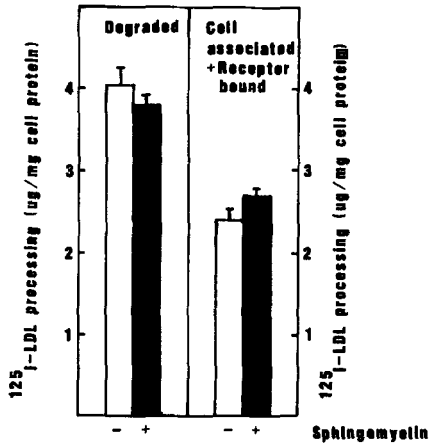

Fig. 11. The processing of LDL preincubated with sphingomyelin in IEC-6 cells. IEC-6 cells were grown for 3 days as described in the legend to Fig. 1. On day 4, cells were fed medium containing SM(-) ^{125}I-labeled LDL (100 μg/ml) or SM(-) ^{125}I-labeled LDL (100 μg/ml) that had been preincubated with sphingomyelin (100 μg/ml) for 4 h at 37°C. After 6 h incubation with cells, medium and cells were harvested for determination of LDL processing. The data represent the mean and SE of triplicate determinations.

back to SM(-)LDL in the same ratios as in the experimental conditions was bound to the LDL. In agreement with this we observed that the uptake of labeled sphingomyelin in the presence and absence of SM(-)LDL was enhanced in the presence of SM(-)LDL (**Table 2**). Thus sphingomyelin uptake per se could not account for the results obtained, rather the mode of administration of sphingomyelin was an important factor. Further discussion of the significance of these data is in the following section.

DISCUSSION

There are many observations showing that modification of LDL by various procedures and agents, e.g., phospholipase-C, Cu^{2+} oxidation, acylation, or growth hormones (24–27) causes major alteration in the binding of the modified LDL particle to the physiological LDL receptor. In all of these instances the modified LDL particles no longer bind to the normal LDL receptor but bind instead to scavenger receptors (28) or are processed via non-receptor-linked uptake. Some of these changes may be due to charge alterations, e.g., acetylation of LDL or to changes in LDL size or conformation so that normal binding to the LDL receptor is abolished. Modification of LDL by proteases (29), on the other hand, results in a particle with enhanced binding to the physiological receptor.

This study arose from previous observations in our laboratory (9) and those of others (8) that addition of SMase from *S. aureus* to cells in culture resulted in depletion of plasma membrane sphingomyelin, followed by movement of cholesterol to the cell interior, as evidenced by a rise in cholesterol esterification. Additionally, cholesterol synthesis from acetate was inhibited which we found (9) was localized at the HMG-CoA reductase step.

TABLE 2. Uptake of [^{14}C]sphingomyelin by IEC-6 cells in the presence and absence of SM(-)LDL

	[^{14}C]sphingomyelin Associated with Cells	
[^{14}C]Sphingomyelin Added	Without SM(-)LDL	With SM(-)LDL (100 μg/ml)
μg/dish	μg/mg protein	
25	0.897 ± 0.092	0.880 ± 0.055
50	1.074 ± 0.121	1.336 ± 0.035
100	2.049 ± 0.127	2.463 ± 0.122

Cells were grown for 3 days as described in the legend to Fig. 1. On day 4, cells were fed fresh LPDS medium; [^{14}C]sphingomyelin preincubated (for 4 h at 37°C) with SM(-)LDL (100 μg/ml) at indicated concentrations was added to one group of dishes. An incubation was carried out with sphingomyelin alone as control. After additional 6 h incubation, the medium was removed, the cells were washed with PBS, and the radiolabel in the cells was measured after extraction of total lipids. The values represent the mean and SE of triplicate determinations.

Kudchodkar, Albers, and Bierman (10) and Gatt and Bierman (11) observed that the addition of sonically dispersed positively charged liposomes containing sphingomyelin to cultures of skin fibroblasts suppressed the binding and utilization of LDL, and stimulated cholesterol synthesis and HMG-CoA reductase activity. In view of these latter observations we asked whether depletion of plasma membrane/cellular sphingomyelin by exogenous neutral SMase from *S. aureus* would have an opposing effect, i.e., stimulation of the processing of LDL. When SMase was added together with ^{125}I-labeled LDL to IEC-6 cells in culture, a two- to fivefold enhancement in overall processing of LDL was observed, which was a reflection of increases in binding, cell association, and degradation parameters of LDL processing (Fig. 1). On the other hand, when SMase was added to the medium of IEC-6 cells in culture and incubated for 6 h, then cooled to 4°C to compare the binding of ^{125}I-labeled LDL with untreated control cultures, we observed no significant change (Fig. 8). Thus treatment of cells with SMase per se did not increase the number of LDL receptors or their binding affinity to LDL.

LDL was treated with SMase and purified so that it was essentially free of SMase (SM(-)LDL). We observed that SM(-)LDL when added to IEC-6 cells in culture was processed at higher rates (1.5- to 5-fold) than untreated LDL (Fig. 2). We found that this was due to a greatly enhanced binding affinity to the LDL receptor (Fig. 3) in a variety of cell lines but no increase in the number of receptors (Figs. 6, 8). LDL receptor-negative human skin fibroblasts from a familial homozygous hypercholesterolemic subject (GM-2000) were completely unresponsive to the addition of SMase from *S. aureus* in the medium with respect to nonreceptor-linked processing (data not shown).

What changes occurred in the LDL particle as a result of SMase action? Analysis of the major components of the SM(-)LDL particle revealed almost complete disappearance of sphingomyelin, but no significant changes in the levels of phospholipid, free cholesterol, cholesteryl, ester or triglyceride (Table 1). We could detect no indication of protease action by SMase as evidenced by the complete lack of ^{125}I-labeled acid-soluble radioactivity when ^{125}I-labeled LDL was subjected to SMase treatment (data not shown). After purification of SM(-)LDL on a Sephadex G-200 column, we could detect a small residue of SMase activity in the SM(-)LDL (0.6 mu SMase per 100 μg of SM(-)LDL); however, control experiments showed that addition of SMase to native LDL equivalent to the amounts of residual SMase bound to SM(-)LDL after purification had no effect on the processing of LDL (data not shown). It has been shown that when LDL is aggregated there is increased uptake and processing of LDL via LDL receptors (22). Under our conditions we observed no increase in absorbance of SM(-)LDL at 430 nm (Fig. 4), indicating that no aggregation had occurred. It has

been observed that a short period of vigorous vortexing of LDL preparations resulted in aggregation and increased processing by macrophages (22). We observed that aggregation could be readily induced in SM(-)LDL by rapid vortexing for 10 sec (Fig. 4). Comparison of the LDL processing parameters of the vortexed and nonvortexed SM(-)LDL revealed that vortexing resulted in increased binding and cell association, but degradation was decreased or remained the same. In other experiments (data not shown) we found that addition of SMase to native LDL that was aggregated by vortexing greatly increased the processing of this LDL. From these observations, it appears that aggregation, insofar as our methodology could detect it, was not a factor in the enhanced processing of SM(-)LDL.

Taken together, all of the foregoing observations provide evidence that complete removal of sphingomyelin from an LDL particle, causes a change in the particle such that its binding affinity for LDL receptors in a large variety of cells is greatly enhanced, thereby leading to increased processing of LDL. Our results suggest, furthermore, that the level of sphingomyelin in LDL appears to be a significant determinant in the rates of cellular processing of this lipoprotein. We tried restoring sphingomyelin to SM(-)LDL to determine whether the SM(-)LDL particle could be reconstituted back to the native form. Although sphingomyelin was readily reassociated with SM(-)LDL, cells did not process it as the native form (vide infra). Thus the change in LDL structure that must have occurred as a result of loss of sphingomyelin via SMase was not restored by re-addition of sphingomyelin. The exact nature of the change induced in the LDL particle by loss of sphingomyelin remains to be elucidated with more sensitive methods, e.g., the application of monoclonal antibodies to specific areas of the apoB polypeptide after SMase treatment than were currently used by us.[2]

We examined whether treatment of cells with exogenous SMase also exerted a direct effect on the cells that was independent of the observed effects of SMase on the composition of the LDL particle. We raised this question because we noted that, although incubation of cells with SM(-)LDL alone showed increased rates of processing, they were still less than the rates of processing obtained when LDL and SMase were incubated together (data not shown). Our experiments showing that incubation of SM(-)LDL with SMase from *S. aureus* greatly increased the rate of processing of the SM(-)LDL indicated that the SMase was also exerting an extra effect on the cell to enhance the processing of the modified LDL (Fig. 7). This interpretation seems reasonable as there was very little sphingomyelin left in the SM(-)LDL particle. We have previously shown that incubation of IEC-6 cells with SMase resulted in a major decrease in cellular sphingomyelin levels, which most probably also involved changes in the sphingomyelin:cholesterol ratio in all cellular membrane entities (9). As shown in Fig. 7, incubation of IEC-6 cells with SM(-)LDL and 100 mu/ml of SMase from *S. aureus* resulted in increased processing that was primarily due to an enhanced rate of degradation of the SM(-)LDL. There were no significant changes in binding at 4°C of ^{125}I-labeled LDL in CHO-K1 and HepG2 cells that were preincubated at 37°C for 6 h with SMase from *S. aureus,* thereby indicating that SMase did not affect the binding affinity of LDL or the number of LDL receptors. This result is in agreement with similar data obtained when IEC-6 cells were incubated with SMase.

In order to observe the effects of SMase on cellular degradation of LDL, it is necessary that the cells be in contact with SMase. We have found that when IEC-6 cells are incubated with SMase under the conditions outlined in Fig. 1, and then washed with fresh medium and reincubated with medium containing ^{125}I-labeled LDL, no increases in processing of LDL could be observed (data not shown). This would suggest that very little SMase is incorporated by the cells; in fact, it has been observed (30) that resynthesis of plasma membrane sphingomyelin occurs within 30 min after removal of SMase from the medium.

The foregoing results show that direct SMase treatment of cells in culture leads to enhanced degradation of LDL and suggest that the level of cellular sphingomyelin is a determinant in the rate of LDL degradation. In support of this possibility we found that when IEC-6 cells are incubated for a short time with various amounts of sphingomyelin added to the lipoprotein-deficient medium followed by the addition of native ^{125}I-labeled LDL or SM(-)LDL, LDL degradation was greatly inhibited, with little effect on receptor binding or cell association (Fig. 9). If the inhibition of LDL apolipoprotein degradation is related to cellular levels of sphingomyelin, it might be expected that increasing the level of cellular sphingomyelin by inhibiting lysosomal sphingomyelinase would induce the same effect as sphingomyelin addition. In agreement with this concept we found (Fig. 10) that desipramine, an

[2]During the course of this work, Chatterjee reported in an abstract (42) that treatment of human skin fibroblasts with human urinary SMase stimulated LDL processing by virtue of increased cell surface binding, internalization, and degradation. Sphingomyelin was stated to inhibit LDL receptor activity. This report did not distinguish between effects of SMase on the LDL particle or the cell. Our observations reported here show that the inhibitory effect of sphingomyelin is primarily exerted on the degradation of LDL. Since submission of this report, Xu and Tabas (21) reported that treatment of LDL with neutral SMase enhanced by 2- to 6-fold the LDL uptake by macrophages via LDL receptor-mediated endocytosis. The enhancement was specific for sphingomyelin hydrolysis, and was accompanied by up to 10-fold increase in the cholesteryl ester content of the macrophages. Xu and Tabas also observed that treatment of LDL with SMase caused the formation of fused or aggregated spherical particles. We did not observe this effect under our conditions, which may be due to undetermined differences in experimental procedures. We could, however, confirm the changes in aggregation after vortexing.

known inhibitor of lysosomal sphingomyelinase, when added to IEC-6 cells in culture greatly inhibited LDL apolipoprotein degradation. These data are in agreement with the suggestion that cellular levels of sphingomyelin can modulate the degradation of endocytosed LDL. Addition of ceramide had no observable effect on LDL processing. Our results are analogous to those obtained by Bierman and collaborators (10, 11) who observed that addition of liposomes of sphingomyelin made positively charged by the addition of octadecylamine (stearylamine) to cultures of fibroblasts inhibited the binding and degradation of LDL. The observations of these workers, however, are more difficult to interpret because of the presence of stearylamine which is a lysosomotropic agent known to inhibit LDL-induced cholesteryl ester formation and the postlysosomal transport of cholesterol (31).

When preincubation of sphingomyelin with cells was omitted and sphingomyelin was added concomitantly to native or SM(-)LDL, we were surprised to observe that there were no changes in the uptake, binding, and degradation of either form of LDL. Further examination of the uptake of sphingomyelin by the cells showed sphingomyelin was readily incorporated into cells regardless of whether or not SM(-)LDL was present in the medium. In fact, there was enhanced uptake in the presence of SM(-)LDL. These observations suggest that simply restoring sphingomyelin to media containing SM(-)LDL does not result in reversion of SM(-)LDL to the native LDL form; furthermore, the data also suggest that sphingomyelin when taken up by a cell in lipoprotein-deficient medium is apparently processed differently than sphingomyelin bound to LDL. This interpretation seems reasonable as uptake of sphingomyelin from lipoprotein-deficient medium results in marked inhibition of proteolysis of subsequently added LDL, while, on the other hand, concomitant addition of LDL plus sphingomyelin does not result in inhibition of LDL degradation. This result differs from that of Bierman et al. (10, 11) but the observations of these investigators could be accounted for by the effect of stearylamine that was present in their liposomal preparations of sphingomyelin. It has been shown (32) that the cellular uptake of sphingomyelin is associated with the constituents of serum and, after incorporation, is conveyed to the lysosomes for degradation. Thus the greater uptake in the presence of LDL would be expected.

Recent reports show that there are several pathways by which cells take up and process sphingomyelin. Quinn and Allen (33) showed that there are two separate pools of sphingomyelin in BHK cells. A recent report from Levade et al. (34) provides evidence for different pathways of uptake and degradation of sphingomyelin in lymphoblastoma cells. One pathway involved uptake of sphingomyelins in the presence of lipoproteins via apoB/E receptor-linked endocytosis followed by degradation specifically via lysosomal SMase. Other sphingomyelins were taken up via another route that involved an LDL receptor-independent pathway and subsequent degradation by a nonlysosomal SMase. In the absence of serum all sphingomyelins were taken up by the latter pathway. The authors suggest that lipoproteins play a significant role in the cellular metabolic routing of sphingomyelin in lymphoblastoid cells. Our experiments were carried out with intestinal epithelial cells, and lipoprotein-deficient serum plus or minus LDL was always present. As lysosomes are a major site of proteolysis of LDL apolipoproteins, it seems reasonable to speculate that lysosomal sphingomyelin levels may modulate lysosomal protein-degrading systems. The mechanism of this effect remains to be elucidated, but a target for further investigation would be the relationship of sphingomyelin levels in lysosomes to retention of cholesterol in these organelles (35), and possible membrane alterations leading to changes in degradative activity.

There also exists evidence from clinical and physiological studies that indicates that sphingomyelin levels are related to serum and cellular cholesterol homeostasis. It is now well known that genetic defects in sphingomyelin degradation, e.g., Niemann-Pick disease, results in disturbance in cholesterol metabolism (36–39). Additional reports from clinical studies (40, 41) show that increased levels of plasma LDL in homozygous and heterozygous familial hypercholesterolemic subjects is also accompanied by elevations in the percentage of sphingomyelin in LDL. Increased levels of sphingomyelin are also found in fibroblasts from these subjects. It is not known whether this is a significant factor in the turnover of plasma LDL of heterozygotes. In this connection it is worthy of note that in hypercholesterolemic rabbits the ratio of sphingomyelin to phosphatidylcholine increased fivefold in very low density lipoproteins (43). In addition, the turnover of the very low density lipoprotein from hypercholesterolemic rabbits was greatly delayed compared to normal (44). Billheimer and Gaylor (45) have shown that sterol carrier protein-mediated exchange of cholesterol between liposomes of varied composition is inhibited by sphingomyelin.

The observations we have reported here extend and support the conclusion that the often observed close connection between the levels of sphingomyelin and cholesterol in cell membranes and organelles plays an important role in many aspects of cellular cholesterol trafficking and metabolism. Further investigations of the connection between sphingomyelin levels and cellular cholesterol trafficking and homeostasis should provide new insight into the regulatory elements of this complex process that involve many levels of cellular activity.

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