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ACID SPHINGOMYELINASE IS ESSENTIAL FOR CHOLESTEROL-REDUCING AGENTS TO POSITIVELY AFFECT ACCUMULATED FREE CHOLESTEROL IN NIEMANN-PICK DISEASE TYPE C FIBROBLASTS

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Abstract
Niemann-Pick disease (NP) is a group of lipid storage disorders that includes three types of diseases. While NP type A (NP-A) and NP type B (NP-B) are caused by a lysosomal acid sphingomyelinase (ASM) deficiency, NP type C (NP-C) is a neurovisceral disorder caused by a deficiency of either the NPC1 or NPC2 protein. This deficiency causes impaired intracellular lipid trafficking, leading to the abundant accumulation of free cholesterol in the lysosome. Thus, NP-C is diagnosed through a filipin test that microscopically detects intracellular free cholesterol in fibroblasts. However, the filipin test was reported to give a positive result even for NP-A and NP-B. Recently, some cholesterol-reducing agents have been reported to be useful candidate for NP-C therapies to reduce accumulated free cholesterol level. In this study, three cholesterol-reducing agents, methyl-β-cyclodextrin, histone deacetylase inhibitors, and thapsigargin, were tested to determine their efficacy in reducing the accumulated free cholesterol level in NP-A and NP-B fibroblasts and drug-induced, ASM-deficient fibroblasts. The results showed that the three agents did not decrease the accumulated free cholesterol level in these cells, showing that ASM is essential for the cholesterol-reducing agents to be effective. This study also suggests that the accumulated free cholesterol of ASM deficient fibroblasts may be originally different from that of NP-C cells.

Key words : filipin staining, Niemann-Pick disease types A and B, Niemann-Pick disease type C, desipramine

Background

Niemann-Pick disease (NP) is a group of lipid storage disorders that are characterized by intracellular and visceral accumulation of sphingomyelin and cholesterol. NP consists of three types of diseases, including NP types A and B (NP-A and NP-B) that are caused by a deficiency of lysosomal acid sphingomyelinase (ASM)[1)].

NP type C (NP-C), a third type of NP, is a rare neurovisceral disorder that is caused by a functional deficiency of either the NPC1 or NPC2 protein[2)]. This deficiency causes impaired intracellular lipid trafficking, leading to the accumulation of free cholesterol in the late endosome/lysosome. Approximately, 95% of NP-C cases are caused by a deficiency of the NPC1 protein. NPC1 is a 13-transmembrane domain protein that resides in the

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limiting membrane of the late endosome/lysosome, whereas NPC2 is a soluble lysosomal protein that can bind cholesterol[3)4)]. Recent studies have shown that NPC1 functionally teams up with NPC2 to transfer free cholesterol from the late endosome/lysosome to the endoplasmic reticulum and plasma membrane[5)]. Thus, NPC1 deficiency share the same pathogenesis and clinical features as NPC2 deficiency.

NP-C is clinically characterized by prominent hepatosplenomegaly and progressive neuronal degeneration showing specific symptoms, including vertical supranuclear gaze palsy and cataplexy[2)]. NP-C includes five clinical subgroups, pre/perinatal, early-infantile, late-infantile, juvenile, and adolescent/adult types, depending on the age of onset[2)]. Because of its broad range of phenotypes, it commonly takes a long time to diagnose NP-C. If a patient is suspected of NP-C, the diagnosis is made by the filipin test using cultured skin fibroblasts. In the filipin test, free cholesterol reacts with the fluorescent filipin, resulting in a strong fluorescent and stable cholesterol-filipin complex that is suitable for in situ detection[2)]. Although the filipin test is a specific diagnostic method for NP-C, it was reported to give a positive result for fibroblasts of NP-A and NP-B patients, who are usually diagnosed on the basis of an ASM enzyme deficiency[6)].

Progressive neurological manifestations have a profound effect on the quality of life in NP-C, however, there is no prophylactic or curative treatment for NP-C. Miglustat administration is the only therapy for existing neurological manifestations[7)]. Recently, some cholesterol-reducing agents have been reported as candidates for NP-C therapy to decrease the accumulated free cholesterol level in NP-C cells. B-cyclodextrin derivatives, which are known as cholesterol-binding agents, have been reported to reduce the accumulated free cholesterol level in NP-C fibroblasts[8)]. Since then, some clinical trials involving β-cyclodextrin have been attempted in NP-C patients and shown significant efficacy of the agent[9)]. Histone deacetylase (HDAC) inhibitors have also been reported as cholesterol-reducing agents in NPC1 deficient fibroblasts[10)]. Thapsigargin, which changes the intracellular calcium distribution, is also known as a cholesterol-reducing agents reducing the accumulated free cholesterol level in NP-C cells[11)].

In this study, cholesterol-reducing agents methyl-β-cyclodextrin, HDAC inhibitors, and thapsigargin, were utilized to attempt to reduce the accumulated free cholesterol level in NP-A and NP-B fibroblasts, which have accumulated free cholesterol, as well as abundant sphingomyelin.

Materials and Methods

Materials

Filipin complex from *Streptomyces filipinensis*, chlorpromazine, progesterone, methyl-β-cyclodextrin, thapsigargin, forskolin, valproic acid, vorinostat, and panobinostat, were obtained from Sigma-Aldrich (St Louis, MO, USA). Desipramine hydrochloride was purchased from Wako (Osaka, Osaka, Japan). Desipramine and chlorpromazine are utilized as functional inhibitors of ASM in this study. Progesterone is known as a reagent to block free cholesterol translocation from lysosomes. Valproic acid, vorinostat, and panobinostat are all included in HDAC inhibitors.

Cell lines

Cultured skin fibroblasts were established from an unaffected individual and three individuals with NP-A, NP-B, and NP-C, respectively. All samples were collected after obtaining written informed consent. Ethical approval was obtained from the Ethics Committee of Akita University, Graduate School of Medicine in Akita, Japan.

Clinical findings of the patients are briefly herein. The NP-A patient, a 1-year-old girl, showed massive hepatosplenomegaly and neurological deterioration during her infancy. Numerous foam cells were found in the bone marrow, suggesting a diagnosis of NP. An enzymatic study of ASM showed 0.25% of control activity in her cultured skin fibroblasts, indicating the diagnosis of NP-A. The diagnosis was confirmed with the mutations observed in the *SMPD1* gene, c.398G>A(p.C133X)/c.398G>A(p.C133X). The NP-B patient, a 47-year-old man, had shown severe hepatosplenomegaly and massive pulmonary infiltration in a chest radiograph since adolescence. NP-B was diagnosed by an enzymatic study of ASM showing 7.7% of control activity in his cultured skin

fibroblasts. Mutations observed in the *SMPD1* gene were c.1480G>T(p.G494C)/ c.1480G>T(p.G494C), confirming the diagnosis of NP-B[12]. The NP-C patient, a 10-year-old girl, showed neurological deterioration and cataplexy in early childhood and was diagnosed with a late infantile type of NP-C by positive filipin staining in her cultured skin fibroblasts[13]. Homozygous mutations, c.2974G<T(p.G992W)/c.2974G<T(p.G992W), were found in the *NPC1* gene, confirming the diagnosis of NP-C.

Cell culture

Cultured skin fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum.

To intensify the uptake of low-density lipoprotein-derived cholesterol, the fibroblasts were cultured in the presence of 10% lipoprotein-deficient serum rather than 10% fetal bovine serum for 72 h[14]. To induce the accumulation of free cholesterol in normal fibroblasts, the cells were incubated with either desipramine (40 μM), chlorpromazine (25 mM), progesterone (1 μg/ml), for 24 h. Then, methyl-β-cyclodextrin, valproic acid, vorinostat, panobinostat, or, thapsigargin were added as a cholesterol-reducing agent to the media of the cells at a concentration of 300 μM, 1 mM, 10 μM, 75 nM, or, 1 μM, respectively, and the cells were incubated for a further 24 h or 48 h.

Filipin staining

The fibroblast cells were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min. For filipin staining to visualizing intracellular free cholesterol, fibroblasts were stained with 300 μg/ml of filipin complex in 1 x PBS for 30 min[2].

The stained cells were observed using a Zeiss LSM 510 META confocal microscope equipped with UV laser.

Amplex® Red cholesterol quantification

The cells were washed two times with 1×PBS to remove residual medium and pelleted by centrifugation at 800 g for 5 min at 4°C. The cells were disrupted by sonication on ice using three 15-S bursts, and cellular homogenates were assayed for protein content by the method proposed by Lowry *el al*. Total and free cholesterol levels were quantified using an Amplex® Red cholesterol assay kit (Molecular Probes Invitrogen Detection Technologies, Paisley, UK). In brief, samples were diluted in a reaction buffer, after which an equivalent volume of Amplex® Red working solution (300 μM Amplex® Red, 2 U/ml cholesterol oxidase, and 2 U/ml horseradish peroxidase) was added ; in addition to these reagents, 0.2 U/ml cholesterol esterase was used in the measurement of the total cholesterol levels. The samples were incubated at 37°C for 30 min, and the fluorescence intensity was measured at excitation and emission wavelengths of 544 and 590 nm, respectively, using the Fluoroscan Ascent system (Thermo Labsystems, Waltham, USA). Total and free cholesterol levels were estimated using cholesterol solutions of known concentrations and normalized to protein content.

Forskolin and ASM deficiency

Forskolin is a chemical that increases intracellular cyclic adenosine 3′,5′-monophosphate (cAMP) by directly activating adenylate cyclase. Since NPC1 is known to be positively regulated by cAMP, forskolin was added into the NP-B fibroblasts to know if activation of NPC1 can reduce the accumulated free cholesterol level in NP-B fibroblasts[15]. NP-B fibroblasts were incubated with forskolin, 150 μM, 300 μM, or 900 μM, for 24 h and the cells were stained with filipin.

Statistical analysis

Data were analyzed using the IBM SPSS Statistics 22.0 software package and are presented as the mean ± standard deviation (SD). Students' unpaired t-test was used to compare the mean differences between 2 groups. A p-value < 0.05 was considered statistically significant.

Results

Filipin staining in NP-A, NP-B, NP-C, and drug-modified normal fibroblasts

Fibroblasts from NP-A, NP-B, and NP-C were grown in the medium with 10% fetal bovine serum for 48 h, and

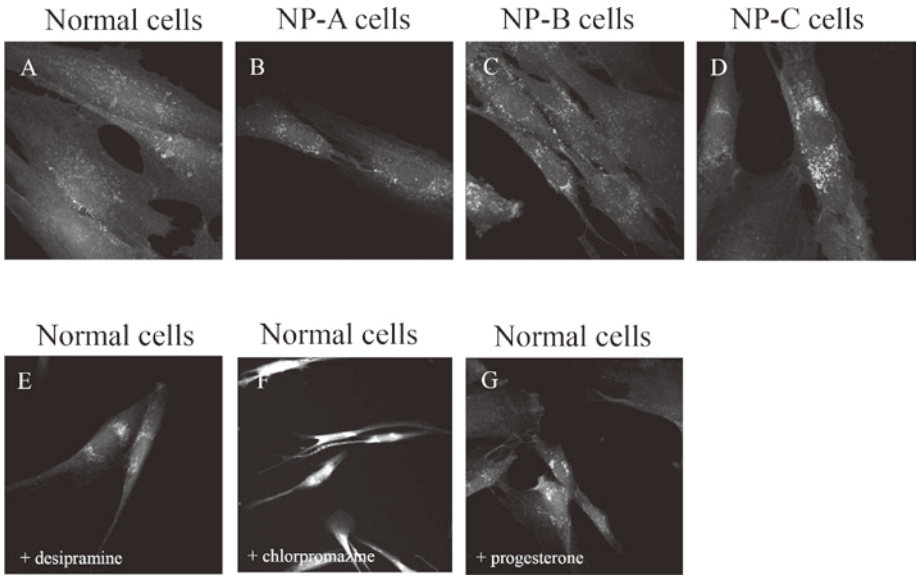

Fig. 1. Filipin staining in NP and drug-modified normal fibroblasts
Fibroblasts from normal, NP-A, NP-B, and NP-C were stained with filipin (A, B, C, and D). Normal, chlorpromazine (25 mM)-incubated, or, progesterone (1 μg/ml)-incubated, fibroblasts was also stained with filipin (E, F, and G).

then the cells were stained with filipin. NP-A and NP-B fibroblasts were weakly but positively stained with filipin, compared with normal fibroblasts (Fig. 1A, B, and C). NP-C fibroblasts were positively stained with filipin (Fig. 1D). A positive filipin test was observed as typical perinuclear staining in these cells. (Fig. 1B, C, and D). Compared with negative staining of filipin in normal fibroblasts, desipramine-, chlorpromazine- or progesterone-incubated fibroblasts were positively stained with filipin (Fig. 1A, E, F, and G).

Effect of methyl-β-cyclodextrin on the accumulated free cholesterol in NP-A, NP-B, NP-C, and drug-modified normal fibroblasts

NP-A, NP-B, and NP-C fibroblasts were grown in the medium with 10% fetal bovine serum for 48 h, and the cells were then incubated with methyl-β-cyclodextrin for 24 h. While the positive staining of filipin completely disappeared after incubation with methyl-β-cyclodextrin in NP-C fibroblasts, methyl-β-cyclodextrin did not reduce the accumulated free cholesterol level in NP-A and NP-B fibroblasts (Fig. 2A, B, and C).

Normal fibroblasts were incubated with desipramine, chlorpromazine, or progesterone for 24 h, and then methyl-β-cyclodextrin was added into the medium and the medium incubated for a further 24 h. While methyl-β-cyclodextrin reduced the accumulated free cholesterol level in progesterone-treated normal fibroblasts, it did not change the accumulated free cholesterol level in desipramine- and chlorpromazine-treated normal fibroblasts (Fig. 2D, E, and F).

HDAC inhibitors and desipramine in NP-C fibroblasts

NP-C fibroblasts was incubated with an HDAC inhibitor, valproic acid, vorinostat, or panobinostat, for 24 h and desipramine was added into the medium and the medium incubated for a further 24 h. HDAC inhibitors reduced the accumulated free cholesterol level in NP-C fibroblasts, while the addition of desipramine caused the accumulation of free cholesterol in NP-C fibroblasts again (Fig. 3).

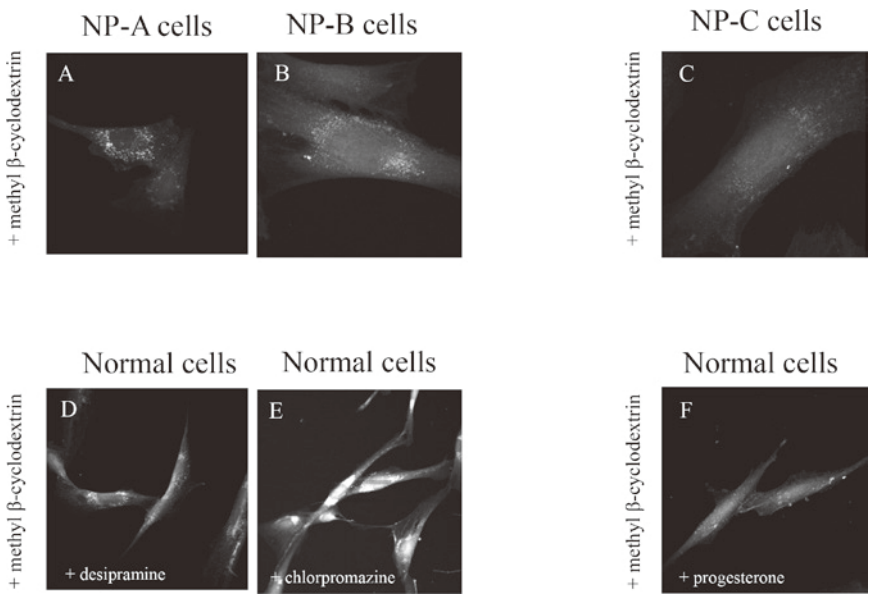

Fig. 2. Effect of methyl-β-cyclodextrin on cholesterol-accumulated various fibroblasts
NP-A, NP-B, and NP-C fibroblasts were treated with methyl-β-cyclodextrin (300 μM) and stained with filipin (A, B, and C). Normal fibroblasts were incubated with desipramine (40 μM), chlorpromazine (25 mM), or progesterone (1 μg/ml) and then methyl-β-cyclodextrin was added into the medium and the medium incubated for a further 24 h. Finally, the cells were stained with filipin (D, E, and F).

Quantification of intracellular free cholesterol in HDAC inhibitor-treated NP-C fibroblasts

NP-C fibroblasts were treated with an HDAC inhibitor, vorinostat or panobinostat, for 24 h, and then the intracellular free cholesterol was quantified by an Amplex® Red cholesterol assay kit. In another line of NP-C fibroblasts, desipramine was added into the medium and the medium incubated for a further 24 h. The intracellular free cholesterol was then quantified. In vorinostat-treated NP-C fibroblasts, desipramine did not significantly affect the intracellular free cholesterol quantity. In the case of panobinostat-treated NP-C fibroblasts, intracellular free cholesterol was significantly higher in the fibroblasts treated with desipramine than in those not treated desipramine (Fig. 4).

Thapsigargin and NP-B and NP-C fibroblasts

NP-B and NP-C fibroblasts were incubated with thapsigargin, which has been reported to reduce the accumulated free cholesterol level in NP-C cells. Thapsigargin moderately reduced the accumulated free cholesterol level in NP-C fibroblasts, but did not change the intracellular free cholesterol level in NP-B fibroblasts (Fig.5).

Activation of NPC1 with forskolin in NP-B fibroblasts

NP-B fibroblasts were treated with forskolin, 150, 300, or 900 μM, for 24 h to activate the function of NPC1 and then stained with filipin. Forskolin treatment did not change the intensity of filipin staining in NP-B fibroblasts (Fig. 6).

Discussion

We first stained various types of NP fibroblasts, normal, NP-A, NP-B, and NP-C, with filipin. NP-C fibroblasts was strongly stained with filipin as expected ; however, NP-A and B fibroblasts were also stained with filipin (Fig. 1). Filipin staining is an established method to diagnose NP-C ; however, positive results are occasionally observed in NP-A and NP-B fibroblasts[6]. To

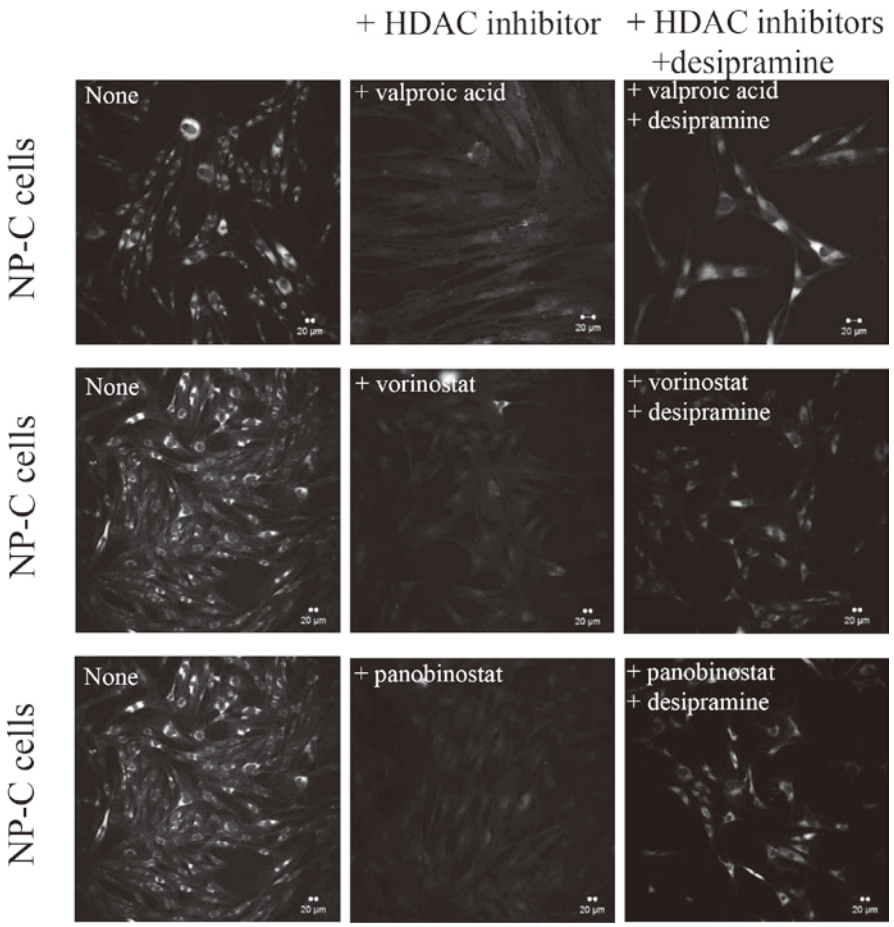

Fig. 3. HDAC inhibitors and desipramine in NP-C fibroblasts
NP-C fibroblasts were incubated with an HDAC inhibitor, valproic acid (1 mM), vorinostat (10 μM), or panobinostat (75 nM), for 24 h and desipramine (40 μM) was added into the medium and the medium incubated for a further 24 h.

know whether ASM deficiency, which is a pathogenesis in NP-A and B, causes the positive filipin staining in normal fibroblasts, ASM deficiency was introduced in normal fibroblasts with desipramine or chlorpromazine. Desipramine and chlorpromazine are known as functional inhibitor of ASM. Together with positive filipin staining in progesterone-induced normal fibroblasts, which has been experimentally used as a model of NP-C, desipramine or chlorpromazine-induced ASM deficient fibroblasts was positively stained with filipin (Fig. 1)[16]. We confirmed that ASM deficiency causes the accumulation of free cholesterol in normal fibroblasts, which can be detected with the filipin test.

NP-A, NP-B, and NP-C are included in the same group of lipid storage disorders that show intracellular and visceral accumulation of sphingomyelin and cholesterol. These diseases share the same clinical characteristics, including prominent hepatosplenomegaly, neurodegenerative clinical course, and specific neurological symptoms. Because it is hard to diagnose with clinical findings, we must be careful when using a filipin test to make a differential diagnosis of NP not to avoid misdiagnosing NP-A and B as NP-C.

In this study, methyl-β-cyclodextrin was shown to reduce the accumulated free cholesterol level in NP-C and progesterone-treated fibroblasts (Fig. 2). However, the

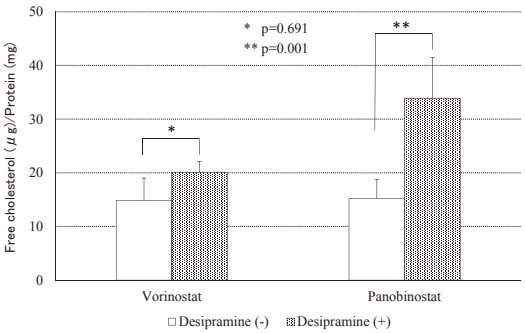

Fig. 4. Quantification of intracellular free cholesterol in NP-C fibroblasts
NP-C fibroblasts were treated with an HDAC inhibitor, vorinostat (10 μM) or panobinostat (75 nM), for 24 h, and then the intracellular free cholesterol was quantified. In another line of NP-C fibroblasts, desipramine (40 μM) was added into the medium and the medium incubated for a further 24 h, and then the intracellular free cholesterol was quantified.

accumulated free cholesterol levels in the NP-A, NP-B, and drug-induced ASM deficient normal fibroblasts were not reduced by methyl-β-cyclodextrin (Fig. 2). These results have never before been reported and we suggest that methyl-β-cyclodextrin may be useful for making a differential diagnosis between NP-A/B and NP-C in the filipin test.

The HDAC inhibitors, valproic acid, vorinostat, and panobinostat, were confirmed to reduce the accumulated free cholesterol levels in NP-C fibroblasts (Fig. 3). However, once ASM deficiency was introduced in NP-C fibroblasts with desipramine, positive filipin staining was again observed in the cells. We confirmed this result by free cholesterol quantification studies using the cells conditioned with each of vorinostat and panobinostat, but valproic acid was not evaluated in this study. The increase of free cholesterol with desipramine was confirmed in the panobinostat-conditioned cells (Fig. 4). Because HDAC inhibitors have been reported to reduce the accumulated free cholesterol levels by intensifying the residual NPC1 function in NP-C cells, our results suggest that ASM deficiency causes the accumulation of free cholesterol through an NPC1-independent mechanism[17].

Thapsigargin, a third cholesterol-reducing agent, was also evaluated in this study (Fig. 5). Thapsigargin was observed to reduce the accumulated free cholesterol level in NP-C fibroblasts, although the free cholesterol did not disappeared completely. By contrast, thapsigargin

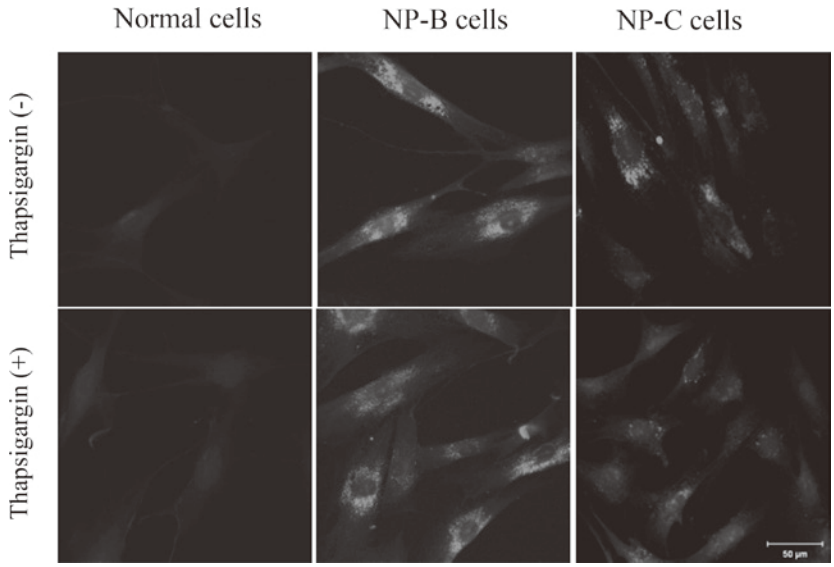

Fig. 5. Thapsigargin and NP-B and NP-C fibroblasts
NP-B and NP-C fibroblasts were incubated with and without thapsigargin (1 μM) for 24 h and stained with filipin.

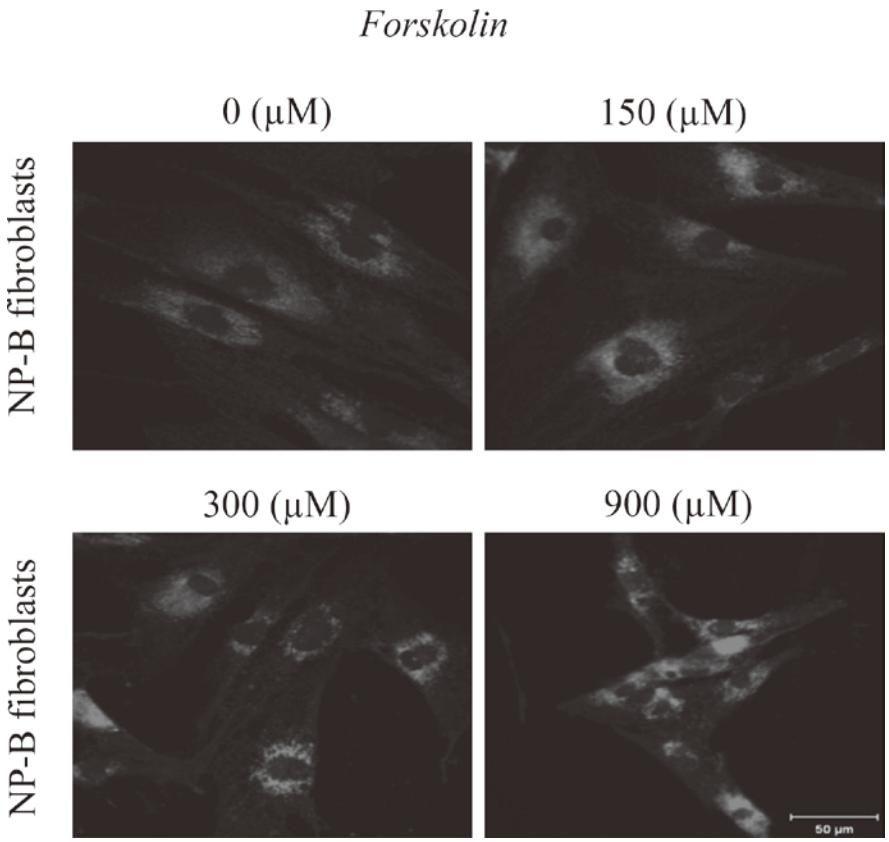

Fig. 6. Activation of NPC1 with forskolin in NP-B fibroblasts
NP-B fibroblasts were treated with forskolin, 150, 300, or 900 μM, for 24 h to activate the function of NPC1 and then stained with filipin.

did not change the accumulated free cholesterol level in NP-B fibroblasts, which has more residual ASM activity than NP-A fibroblasts. The thapsigargin was also shown not to affect the accumulated free cholesterol level in ASM deficient fibroblasts as methyl-β-cyclodextrin and HDAC inhibitors.

The function of NPC1 has been known to be dependent on intracellular cAMP. To know whether activation of NPC1 reduces the accumulated free cholesterol level in NP-B fibroblasts, we treated the NP-B fibroblasts with forskolin, which directly activates adenylate cyclase and increases intracellular cAMP (Fig. 5). In NP-B fibroblasts, which should have functional NPC1, forskolin did not change the intensity of filipin staining at various concentration. These results suggest that ASM deficiency causes the accumulation of free cholesterol through an NPC1-independent mechanism.

Sphingomyelin, an abundantly accumulated lipid in NP-A and B, has unique biological characteristics and interacts strongly with cholesterol to form a stable complex[18,19]. As a result, sphingomyelin and cholesterol have similar subcellular localizations, with both lipids concentrated in the plasma membrane. Sphingomyelin also transiently exists with cholesterol in endosomes and lysosomes. Therefore, metabolism of sphingomyelin has an impact on cholesterol homeostasis. Cholesterol accumulation of in fibroblasts with ASM deficiency could be explained by the co-localization and affinity of sphingomyelin and cholesterol in endosomes and lysosomes.

In summary, three cholesterol-reducing agents,

methyl-β-cyclodextrin, HDAC inhibitors, and thapsigargin, did not decrease the accumulated free cholesterol level in ASM deficient fibroblasts. The results suggest that ASM is essential for the cholesterol-reducing agents to be effective in NP-C cells. This study also suggests that the accumulated free cholesterol of ASM deficient fibroblasts may be originally different from that of NP-C cells.

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