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Decrease of Cholesterol in Mouse Melanoma Causes Secretion of Lysosomal Enzymes

Akihiro Michihara*, Ken Toda, Michihisa Suenobu, Kenji Akasaki and Hiroshi Tsuji
Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, Fukuyama, Hiroshima 729-0292, Japan

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We examined the change in the subcellular distribution of a lysosomal enzyme, β-glucuronidase (β-G), caused by decreased cholesterol levels in mouse melanoma cells using an HMG-CoA reductase inhibitor, lovastatin and lipoprotein-deficient serum (LDS). There was a decrease in the cholesterol content of the cells and increased secretion of the mature form of β-G located in lysosomes, as documented by Percoll density gradient fractionation, digitonin permeabilization and immunoprecipitation. Furthermore, another lysosomal enzyme, cathepsin H, was found to be released in the medium from cells treated with lovastatin. Both the precursor and mature forms of cathepsin H were detected in the medium of treated cells. Next, when cells were treated with LDS without lovastatin, concomitantly with the decrease in the levels of cholesterol and β-G activity in the cells, β-G activity in the medium increased. Also, the ratio of β-G (3.2-fold) released in the medium from cells treated with Dulbecco's modified Eagle medium (D-MEM) containing lovastatin and LDS was higher than that (2.3-fold) on treatment with D-MEM containing LDS without lovastatin. From these results, it was suggested that the exocytosis of mature enzymes from lysosomes into the medium or mis-sorting of the lysosomal precursor forms to the medium was caused by the lovastatin- and/or LDS-induced decrease in the cholesterol content of the cells, although the mechanism of secretion by lysosomal enzymes differed somewhat.

Key words: β-glucuronidase, cathepsin H, exocytosis, lysosome, melanoma, mis-sorting, mouse.

INTRODUCTION

The term lipid rafts refers to putative membrane microdomains with a different composition from surrounding regions of the membrane. It is thought that lipid rafts are enriched in cholesterol, glycosphingolipids, sphingomyelin, phospholipids with long, unsaturated acyl chains, glycosylphosphatidylinositol (GPI)-linked proteins and at least some membrane-spanning proteins (*1*). Lipid rafts are found in the plasma membrane and in the membranes of the Golgi apparatus and of endosomes containing molecules involved in endosomal recycling pathways (*2–4*).

Different types of invaginations occur at the plasma membrane to mediate membrane endocytosis (*2*). Many surface receptors are internalized into coated pits by a clathrin-dependent pathway. Distinct clathrin-independent, raft-dependent internalization pathways occur in caveolae and non-coated invaginations, but both are dynamin-independent and require different small GTPases. Both pathways lead to the endosomal compartment, while caveolae fuse with another sorting compartment known as the caveosome. The relationship between the early endosomal compartment and the caveosome is not yet understood. Lipid raft-dependent pathways invariably require cholesterol, and sometimes the coat protein caveolin 1, and dynamin, a large GTPase implicated in pinching off the emerging membrane buds (*5*).

Cholesterol is normally found mainly in the plasma membrane, Golgi apparatus and early or recycling endosomes (*2*). Cholesterol is not uniformly distributed within the endosomal–lysosomal system. It is abundant within early endosomes and recycling endosomes and relatively scarce in late endosomes and lysosomes (*6–12*). In late endosomes, large amounts of internal vesicles termed multivesicular bodies (MVBs) can accumulate. The internal membranes of MVBs contain cholesterol-rich membranes (*13*). Lipid rafts are segregated from the pathway that leads to lysosomal degradation (*14*). It has been postulated that in individuals with lysosomal storage diseases, lipid rafts accumulate in late endosomes and lysosomes (*15*). Support for this hypothesis comes from overexpression of proteolipid protein, which is routed to late endosomes and lysosomes. Concomitantly, cholesterol accumulates in these compartments as well, accompanied by mistrafficking of raft components (*16*). Based on these data, it was suggested that cholesterol is present in the plasma membrane and in the membranes of the Golgi apparatus, of early endosomes, of late endosomes, and of lysosomes, although the relative amounts of cholesterol differ somewhat.

*To whom correspondence should be addressed: Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, Fukuyama, Hiroshima 729-0292, Japan.
E-mail: mitihara@fupharm.fukuyama-u.ac.jp

As described above, cholesterol has a key role in organizing lipid rafts, which serve as platforms for cell signalling, protein organization and sorting (including endocytosis) (*17*). Cholesterol homoeostasis is tightly controlled by spontaneous efflux, synthesis in the endoplasmic reticulum (ER) and receptor-mediated uptake of lipoproteins (*18, 19*). The rate-limiting enzyme in cholesterol's synthesis is 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which can be inhibited by lovastatin (*20*). Therefore, lovastatin is used as a cholesterol-lowering drug (*21*). The effects of lovastatin increase the synthesis of low-density lipoprotein (LDL) receptor and thereby increase the uptake of LDL cholesterol from the plasma membrane. Other groups reported that lovastatin increases surface LDL receptor expression by decreasing the rate of receptor internalization in proliferating lymphocytes, in contrast to the effects on liver cells (*22*). Also, it was reported that Rab5 and Rab7 proteins, which are involved in the regulation of the endocytic pathway, were significantly increased in FRTL-5 thyroid cells treated with lovastatin, although the amounts of Rab5 and Rab7 bound in the early and late endosomes, respectively, were decreased as compared with those in untreated cells (*23*). Furthermore, it was reported that cholesterol is required for the rapid endocytosis, macropinocytosis, viral escape and infection of adenovirus type 2 (*17*). These findings suggested the possibility that the rate of internalization or the endocytic pathway in cells other than liver cells was affected by the lovastatin-induced decrease in cholesterol in cells.

After lysosomal enzymes, which are present at the final stage in the endocytic pathway, are synthesized, they are bound to mannose 6-phosphate receptors in the trans-Golgi, where the complexes are diverted from the secretory pathway and then transported predominantly to late endosomes (*24, 25*). In some cell types, the pathways for the biosynthesis and the endocytosis of the lysosomal enzymes partially converge in the early endocytic structures (*26, 27*). The enzymes are transported to lysosomes *via* compartments (Golgi apparatus, early or late endosomes and lysosomes) containing cholesterol. In the present study, we investigated the change in the subcellular distribution of a lysosomal enzyme caused by decreasing the level of cholesterol in mouse melanoma.

MATERIALS AND METHODS

Materials—Male Wistar rats weighing 200 g, male ddy mice at 8 weeks of age and male rabbits weighing 1.5 kg were obtained from Shimazu Experimental Animals (Kyoto, Japan). Mouse melanoma (B16F10) cells were kindly provided by Dr J. T. August (Johns Hopkins University). Lovastatin was kindly provided by Merck. Lipoprotein-deficient serum (LDS) was obtained from Sigma. Microcon YM-10 was obtained from Millipore. Affigel10 was obtained from Bio-Rad. Percoll and the ECL western blotting detection kit were from Amersham Pharmacia Biotech (Tokyo, Japan). Specific anti-rat mevalonate pyrophosphate decarboxylase (MPD) IgG (*28*), phenobalbital-inducible cytochrome P-450 (CYP2B1, a microsomal marker) IgG (*29*), β-G IgG (*30*) and cathepsin H IgG (*31*) were prepared as in previous studies. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG goat IgG was bought from O.E.M. Concepts, Inc. (Toms River, NJ, USA). Eagle's essential medium was obtained from GIBCO. Complete Mini (tablets containing protease inhibitors) was purchased from Roche. The Cholesterol E-test Wako was from Wako (Japan). All other chemicals were of reagent grade, and purchased from various commercial sources.

Cultured B16F10—B16F10 cells were diluted to 3×10^6 per 60 mm tissue culture dish with Dulbecco's modified Eagle medium (D-MEM) containing 10% fetal bovine serum (FBS), and then incubated in humidified air containing 5% CO_2 at 37°C for 24 h. In some experiments, the cells were shifted to D-MEM in the presence of 10% LDS (from which the cholesterol had been removed), and then various concentrations of lovastatin (final concentration, 0–30 μM) were added for various periods of time ranging from 0 to 48 h.

Cell Fractionation by Percoll Density Gradient Centrifugation—Cells incubated in 60 mm tissue culture dishes were washed several times in cold PBS, then in a cold isotonic sucrose solution (0.25 M sucrose, 1 mM EDTA, 1 μM pepstatin A, 1 μM leupeptin, 1 μM PMSF, complete Mini and 10 mM Tris–HCl buffer, pH 7.3), and removed from the dish using a rubber policeman. About 3×10^6 cells in 1.5 ml of the sucrose solution were homogenized using Cell Disruption Bombs (Central Scientific Commerce, Inc.), and then centrifuged at 650 g for 5 min. The post-nuclear supernatant (PNS; 1.0 mg/ml) was diluted with Percoll to a final concentration of 30% and centrifuged at 25,000 rpm for 30 min in a Beckman 70.1 Ti rotor. Following centrifugation, the gradients were divided into 18×0.5 ml fractions by downward displacement. The densities of the gradient fractions were obtained from the refractive indices.

Cell Permeabilization—Cell permeabilization was carried out using digitonin according to the method of Michihara *et al*. (*32*) Lovastatin-treated or untreated B16F10 cells were incubated in 1.5 ml of KHM buffer [20 mM phosphate buffer (pH 7.2), 110 mM KOAc and 2 mM MgOAc] in the presence of digitonin (40 μg/ml) for 10 min at 4°C, and the cells were homogenized with 1.5 ml of cold isotonic sucrose solution containing 1% Triton X-100. β-G activity in the cells (M/O; membrane/organelle fraction) and medium (cytosolic fraction) was measured and expressed as a percentage of the total β-G activity.

Conventional Cell Fractionation—Cell fractionation was carried out according to the method of de Duve *et al*. (*33*). After lovastatin-treated or untreated B16F10 cells were homogenized with 1.5 ml of isotonic sucrose solution using Cell Disruption Bombs, the homogenate was centrifuged at 650 g for 5 min. The PNS was centrifuged at 106,000 g for 1 h. The supernatant was designated as the cytosolic fraction. β-G activity in the PNS containing 1% Triton X-100 and in the cytosolic fraction was measured. β-G activity in M/O was calculated from the β-G activity in the PNS containing

1% Triton X-100 and in the cytosolic fraction, and was expressed as a percentage of the total.

Enzyme Assays—β-G, APDE I (alkaline phosphodiesterase I) and LDH (lactate dehydrogenase) were assayed as described by Robins *et al*., Ikehara *et al*. and Abei.(*34–36*), respectively.

Protein Determination—Protein levels were measured by the method of Lowry *et al*. (*37*) using bovine serum albumin (BSA) as the standard.

Gel Electrophoresis—Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on 12% slab gels according to Laemmli (*38*).

Immunoblot Procedures—Proteins in an SDS-slab gel were transferred to a nylon membrane by electrophoresis using a modified version of the procedure of Towbin *et al*. (*39*). Immunoreactive bands were visualized using an ECL western blotting detection kit. Densities of the immunopositive bands were quantified with an Intelligent Quantifier (Bio Image).

Immunoprecipitation—Medium of B16F10 cells cultured in the absence or presence of lovastatin was applied to a 50 μl column of anti-rat β-G IgG-Affigel10 (2 mg of β-G IgG/ml of packed gel) equilibrated with PBS. The anti-rat β-G IgG-Affigel10 was washed with PBS until the absorbance at 280 nm dropped to the baseline level. β-G bound to the column was eluted with 500 μl of 6 M Urea. Five hundred microlitres of the eluted fraction was concentrated to 100 μl using Microcon YM-10.

Medium of B16F10 cells cultured in the absence or presence of lovastatin was applied to a 50 μl column of anti-rat cathepsin H IgG-Affigel10 (1 mg of cathepsin H IgG/ml of packed gel) equilibrated with PBS. The anti-rat cathepsin H IgG-Affigel10 was washed with PBS until the absorbance at 280 nm dropped to the baseline level. The immunocomplex beads containing cathepsin H and anti-rat cathepsin H IgG-Affigel10 were suspended in 50 μl of sample buffer for electrophoresis containing 5% β-mercaptoethanol. The mixtures were boiled in a hot bath for 3 min. The supernatants collected by centrifugation were resolved by SDS–PAGE.

Statistics—Statistical analysis was carried out using Student's *t*-test. Data are presented as the mean ± SD.

Cholesterol Content of Cells—Two hundred microlitres of PNS was mixed with 5 ml of Folch extract [chloroform (*2*):methanol (*1*)], and the mixture was incubated for 10 min at 37°C with shaking. After the mixture was centrifuged at 3000 rpm for 10 min, 3 ml of the supernatant was evaporated dry by boiling at 100°C and then it was dissolved in 200 μl of isopropylalchol containing 1% Triton X-100. The cholesterol content of the solution was determined using the Cholesterol E-test Wako.

RESULTS

The Effect of Lovastatin And LDS on Mouse Melanoma Cells—One of the first steps in the biosynthesis of cholesterol from acetic acid is catalysed by mevalonate pyrophosphate decarboxylase (MPD) (*32*). When the amount of cholesterol in cells is reduced by lovastatin, the levels of enzymes involved in the synthesis of cholesterol, including MPD, in cells are increased by a feedback mechanism. Therefore, the increase in the level of MPD and decrease in the cholesterol content of cells were measured to examine the maximum effect by lovastatin. At first, the dose-dependency of the effect of lovastatin on the level of MPD in the cytosolic fraction and the cholesterol content of the cells was examined. As described in 'Materials and Methods', the cytosolic fraction (to detect MPD) and the PNS (to detect the cholesterol content) were prepared from B16F10 cells incubated for 24 h with various concentrations of lovastatin in medium containing 10% FBS or 10% LDS. Compared with the level of MPD in B16F10 cells incubated for 24 h in medium containing FBS taken as 1, the level of MPD was markedly increased by treatment with 10 μM lovastatin (Fig. 1, A and B). The cholesterol content was also significantly decreased by treatment with 10 μM lovastatin (Fig. 1C). Next, the time-course of the effect of 10 μM lovastatin on the level of MPD and the cholesterol content was examined. The level of MPD was most markedly increased at 24 h (Fig. 1, D and E) and the cholesterol content was also significantly decreased at 24 h (Fig. 1F). These results indicate that the lovastatin-treatment conditions causing the maximal increase of MPD were 24 h of treatment with 10 μM lovastatin. From these findings, it was established that the cholesterol level in cells was reduced by the treatment conditions described above.

Rate of Survival of Mouse Melanoma Cells Treated with Lovastatin—It was previously reported that the treatment of cells with lovastatin caused apoptosis (*40*). Therefore, to estimate the rate of survival of lovastatin-treated cells, the LDH activity in the medium of lovastatin-treated or untreated cells was measured. The proportion of LDH (4%) in the medium of cells treated with 10 μM lovastatin for 24 h was similar to that in the medium of untreated cells (Fig. 2). These results indicated that almost all lovastatin-treated cells survived, and did not undergo apoptosis.

Lovastatin-Induced Change of Subcellular Distribution of β-glucuronidase in Mouse Melanoma as Shown by Percoll Density Gradient Centrifugation—To examine the change in the subcellular distribution of lysosomal enzymes caused by lovastatin, we first examined the subcellular distribution of marker enzymes in mouse melanoma cells by using Percoll density gradient centrifugation. As shown in Fig. 3A, the major peaks of β-G (a lysosomal marker enzyme) and APDE I (a plasma membrane marker enzyme) appeared in fractions 16 and 3, respectively. When the subcellular distribution of β-G was compared between B16F10 cells cultured in the absence and presence of 10 μM lovastatin for 24 h, the β-G activity in the lysosomal fractions (fractions 15–18), *i.e*. the highest density fractions on the Percoll gradient was found to have disappeared in the cells treated with lovastatin as compared with the level in untreated cells (Fig. 3B). These results suggested the possibility that lovastatin caused the disruption of lysosomes.

The Lack of Disruption of Lysosomes by Lovastatin—The above experimental results suggested the possibility that lovastatin caused the disruption of lysosomes. To determine whether the disruption of lysosomes by lovastatin occurs in the living cells, we examined the β-G activity in the cytosolic fraction obtained from

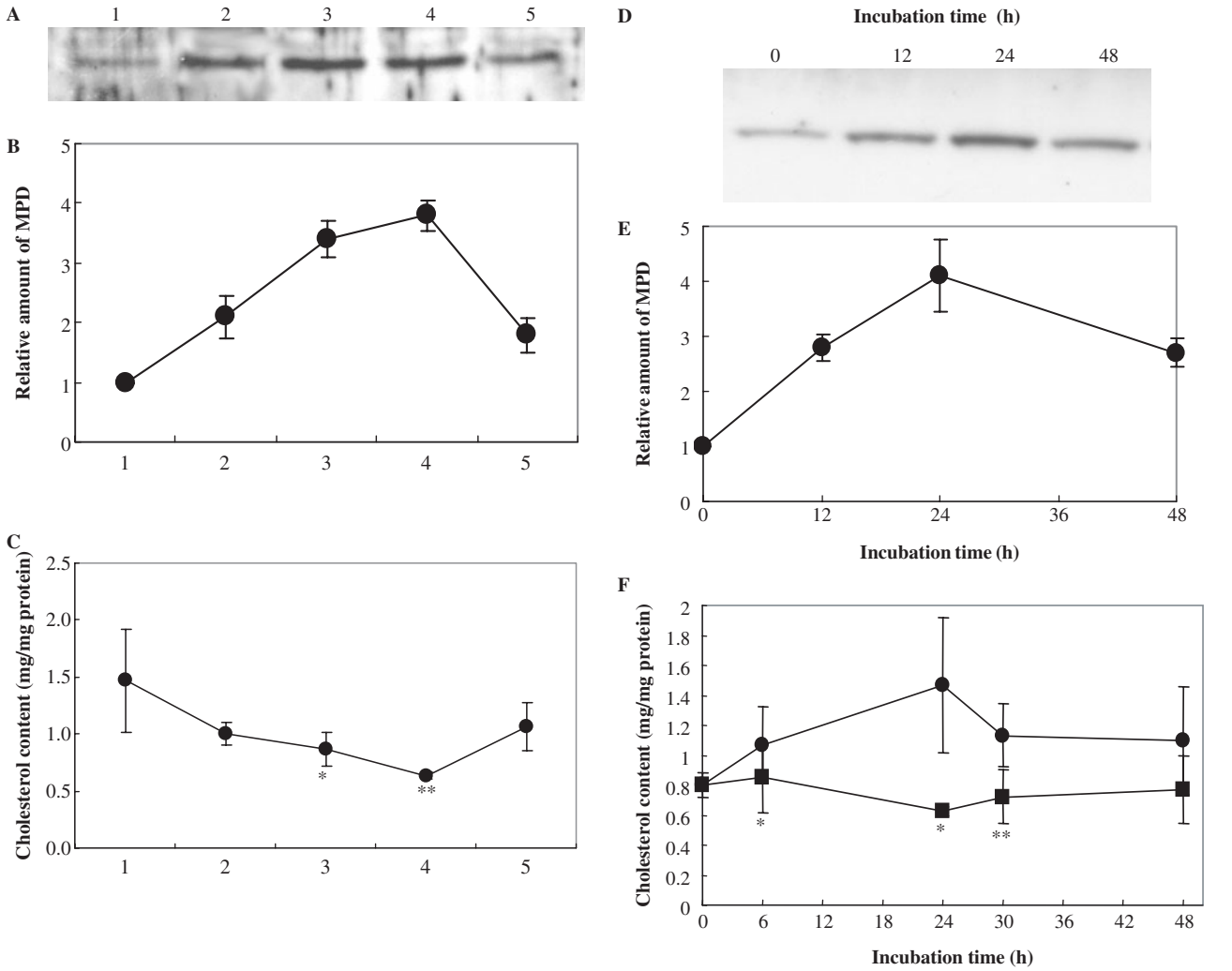

Fig. 1. **Time-course and dose-dependency of the effect of lovastatin on the level of MPD in the cytosolic fraction and cholesterol content of the cells.** (A) Using conventional cell fractionation, cytosolic fractions were prepared from B16F10 cells incubated for 24 h with various concentrations of lovastatin in medium containing 10% FBS or 10% LDS. Each cytosolic fraction (10 μg) was subjected to immunoblotting using an anti-rat-MPD antiserum. Lane 1, cells incubated in medium containing FBS; lane 2, cells incubated in medium containing LDS; lane 3, cells incubated in medium containing LDS and 5 μM lovastatin; lane 4, cells incubated in medium containing LDS and 10 μM lovastatin; lane 5, cells incubated in medium containing LDS and 30 μM lovastatin. (B) Signals of MPD in A were measured using Intelligent Quantifier. The level of MPD in the cytosolic fraction prepared from B16F10 cells incubated for 24 h in the medium containing FBS was taken as 1. Data are the means of three experiments, and the variation in values was less than 5%. (C) Cholesterol content was measured under the same conditions as in A as described in 'Materials and Methods'. Data are the means of three experiments. Significantly different: $^{*}P<0.05$. (D) Cytosolic fractions (10 μg) were prepared from B16F10 cells incubated with or without 10 μM lovastatin for the indicated periods of time. (E) Signals of MPD in D were measured using Intelligent Quantifier. The level of MPD in the cytosolic fraction prepared from B16F10 cells incubated in medium containing FBS was taken as 1. Data are the means of three experiments. (F) Cholesterol content was measured using B16F10 cells incubated with (■) or without (●) 10 μM lovastatin for the indicated periods of time as described in 'Materials and Methods'. Data are the means of three experiments. Significantly different: $^{*}P<0.05$, $^{**}P<0.01$.

digitonin-permeabilized B16F10 cells. Digitonin treatment (40 μg/ml for 10 min) of mouse melanoma has been reported to permeabilize the plasma membrane reversibly, leaving subcellular organelles intact (*32*). Therefore, after cells were incubated in the presence or absence of lovastatin, β-G activity in the permeabilized cells (M/O fraction; membrane/organelle fraction) or medium (cytosolic fraction) was examined under the same conditions as described above. As shown in Fig. 4A, the proportion of β-G activity in the cytosolic fraction and M/O fraction from untreated and lovastatin-treated cells were 4 and 96%, respectively, in both groups of cells. These results indicate that the disappearance of β-G activity in the lysosomal fractions on Percoll density gradient fractionation of B16F10 cells treated with lovastatin was not due to the disruption of lysosomes in the living cells. Next, in order to establish whether the disruption of lysosomes on homogenization or centrifugation during cell fractionation was caused by lovastatin, the level of β-G activity in the cytosolic fraction obtained

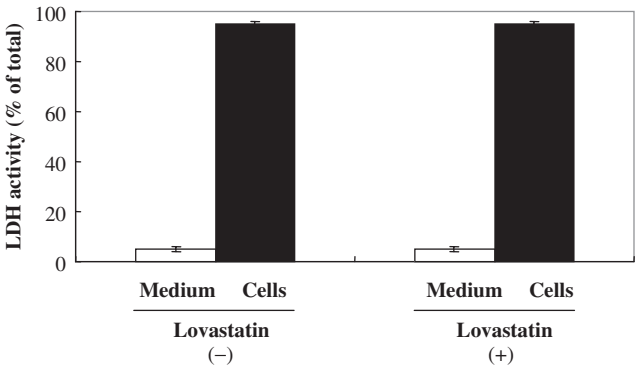

Fig. 2. **LDH activity in the medium of B16F10 cells.** B16F10 cells were cultured in the presence or absence of 10 μM lovastatin for 24 h. Thereafter, the cells were homogenized with 1.5 ml of isotonic sucrose solution containing 1% Triton X-100 and centrifuged at 106,000 g for 1 h. LDH activity in the supernatant (■) and medium (□) was measured and expressed as a percentage of the total. Data are the means of three experiments.

by the conventional method of cell fractionation was measured. As shown in Fig. 4B, the proportion of β-G activity in the cytosolic fraction and M/O fractions from untreated cells were 13 and 87%, while that in the same fractions from lovastatin-treated cells were 15 and 85%, respectively. These results indicate that the disappearance of β-G activity in the lysosomal fractions on Percoll density gradient fractionation of B16F10 cells treated with lovastatin was not due to the disruption of lysosomes caused before or during the cell fractionation. From these results, it was concluded that lovastatin did not cause the disruption of lysosomes.

Release of β-G from Lysosomes to the Medium Induced by Lovastatin—To clarify the effects of lovastatin on the total amount of β-G in cells, we compared the specific activity of β-G between lovastatin-treated and -untreated B16F10 cells. As shown in Table 1, the total activity and specific activity of β-G in lovastatin-treated cells were significantly lower than those in untreated cells. These results suggested the possibility that the decrease in the total activity of β-G in lovastatin-treated cells resulted from the release of β-G into the medium from lysosomes. Therefore, the β-G activity was measured in the medium and in B16F10 cells cultured in the absence or presence of 10 μM lovastatin for 24 h. As shown in Fig. 5A, the total activity of β-G was significantly higher in the medium of B16F10 cells treated with lovastatin than in the medium of untreated cells. The total activity of β-G contained in both the medium and B16F10 cells treated with lovastatin was similar to that of non-treated B16F10 cells. It was previously reported that β-G was located in the ER and lysosomes (*30*). Therefore, to establish whether the decrease in the total activity of β-G in cells treated with lovastatin was due to a decrease in lysosomes or microsomes, the MPL fraction containing lysosomes, microsomes and cytosol was isolated by conventional cell fractionation. The β-G level in each of these fractions in the B16F10 cells treated with lovastatin was significantly reduced as compared with that in untreated cells. However, the degree of the decrease in the amount of β-G was significantly greater in the MPL fraction than microsomal fraction. These results indicated that lovastatin caused the release of β-G to the medium from lysosomes rather than microsomes. During conventional cell fractionation, as described above, the leakage of β-G to the cytosol from lysosomes or microsomes was caused by homogenization or centrifugation. Thus, it was suggested that the proportion of β-G that leaked into the cytosol depended on the proportion of β-G in lysosomes versus microsomes.

Effect of Lovastatin on Protein in the Endoplasmic Reticulum—We examined changes in the amount of protein located in the ER by immunoblotting, in order to investigate whether the observed phenomenon (release of β-G induced by lovastatin) is a lysosomal protein-specific event. As shown in Fig. 6, the amount of CYP2B1, a marker enzyme of the ER, in cells treated with lovastatin was similar to that in non-treated cells, when bands detected by immunoblotting were quantitatively measured by Interigent Quantifier. From these results, it was suggested that lovastatin did not change the amount of protein in the ER. Therefore, it was suggested that the release of β-G to the medium by lovastatin was a lysosomal protein-specific event.

Immunoprecipitation of β-G in Medium—To further establish that β-G was released into the medium from lysosomes in B16F10 cells treated with lovastatin, we performed an immunoblot analysis after the immunoprecipitation of β-G in the medium. At first, to establish whether anti-rat β-G IgG-Affigel10 reacted with β-G released to the medium from B16F10 cells treated with lovastatin, immunoprecipitation of β-G from the medium was performed using anti-rat β-G IgG-Affigel10, Affigel10 and nonimmuno-rabbit IgG-Affigel10 (Fig. 7A). The amount of β-G activity in the supernatant obtained by centrifugation after immunoprecipitation using Affigel10 and nonimmuno-rabbit IgG-Affigel10 was similar to the amount in the medium of B16F10 cells treated with lovastatin. In contrast, when the β-G activity in the supernatant obtained by centrifugation after immunoprecipitation using anti-rat β-G IgG-Affigel10 was measured, it was found to have disappeared, though the fraction eluted with 6 M urea had β-G activity. These results demonstrated that anti-rat β-G IgG-Affigel10 reacted with β-G in the medium of B16F10 cells. Next, when the molecular weight of β-G in mouse and rat liver lysosomes was measured by immunoblotting, β-G in mouse was detected as a single band of 72 kDa (Fig. 7B, lane 1). Also, the molecular weight of β-G in the MPL fraction of B16F10 cells obtained by cell fractionation was similar to that in lysosomes of mouse liver (Fig. 7B, lanes 3 and 4). From these results, it was found that the molecular weight of β-G in lysosomes of B16F10 cells corresponded to that in lysosomes of mouse liver. Furthermore, β-G eluted with 6 M urea after immunoprecipitation with anti-rat β-G IgG-Affigel10 from the medium of B16F10 cells cultured in the absence or presence of lovastatin was detected by immunoblot analysis. The molecular weight of the eluted β-G was similar to that in the lysosomes of mouse liver, but β-G was not detected in the urea-eluted fraction from non-treated cells (Fig. 7B,

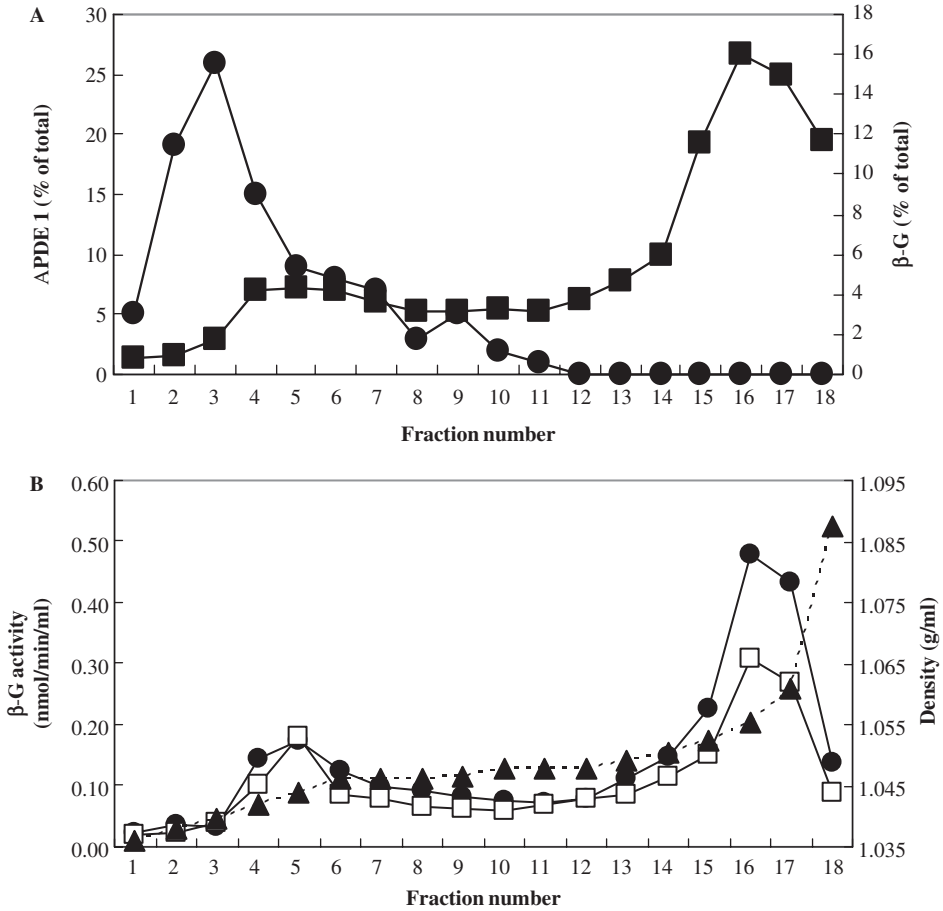

Fig. 3. **Effects of lovastatin on subcellular distribution of β-G.** B16F10 cells were homogenized and centrifuged at 650 g for 5 min. The PNS was centrifuged on a Percoll gradient. The gradient was divided into 18 fractions by displacement in a downward direction. (A) The distribution of alkaline phosphodiesterase I (APDE I; plasma membrane marker enzyme; ●) and β-glucuronidase (β-G; lysosomal marker enzyme; ■) is shown. β-G and APDE I activity were expressed as a percentage of the total. Data are the means of three experiments. (B) B16F10 cells were cultured in the presence (□) or absence (●) of 10 μM lovastatin for 24 h. Thereafter, the cells were homogenized in an isotonic sucrose solution and centrifuged at 650 g for 5 min. The PNS was centrifuged on a Percoll density gradient, giving 18 fractions of 0.5 ml each. The β-G activity in each fraction and the density (▲) were measured. Data are the means of three experiments.

lanes 5–7). Therefore, it was found that treatment of mouse melanoma cells with lovastatin caused the release of β-G to the medium from lysosomes.

Time-Course of Release of β-G from Lysosomes to Medium—The time-course of the release of β-G into the medium from B16F10 cells treated with lovastatin was examined. The total activity of β-G released in the medium in response to lovastatin treatment was significantly increased by 3 h as compared with that in the medium of non-treated cells (Fig. 8A). The total activity of β-G and total amount of protein in B16F10 cells treated with lovastatin were significantly reduced at 12 and 3 h, respectively, as compared with those in non-treated cells (Fig. 8, B and C). From these results, it was clear that the release of β-G into the medium from lysosomes by lovastatin was time-dependent.

Immunoprecipitation of Cathepsin H in Medium—To further characterize the release of lysosomal enzymes to the medium, an immunoblot analysis of cathepsin H in the medium of B16F10 cells treated with lovastatin was attempted. However, as D-MEM supplemented with FBS or LDS contained large amounts of albumin, the electrophoretic pattern of bands obtained using medium containing FBS or LDS was complicated, and cathepsin H could not be detected by immunoblotting (data not shown). Thus, we attempted to perform immunoprecipitation for cathepsin H in the medium and cells treated with D-MEM containing LDS in the presence or absence of lovastatin. As shown in Fig. 9A, bands of 41 and 28 kDa in the medium of B16F10 cells treated with lovastatin were detected by immunoprecipitation using anti-rat cathepsin H IgG-Affigel10. Cathepsin H, a lysosomal enzyme, was initially synthesized as a proenzyme of 41 kDa (*41*). The proenzyme was subsequently processed, becoming a mature enzyme of 28 kDa located in lysosomes. Therefore, both the mature and precursor forms of cathepsin H were released into the medium of B16F10 cells treated with lovastatin. When quantitative measurements of the two forms were made in the medium and cells treated with lovastatin,

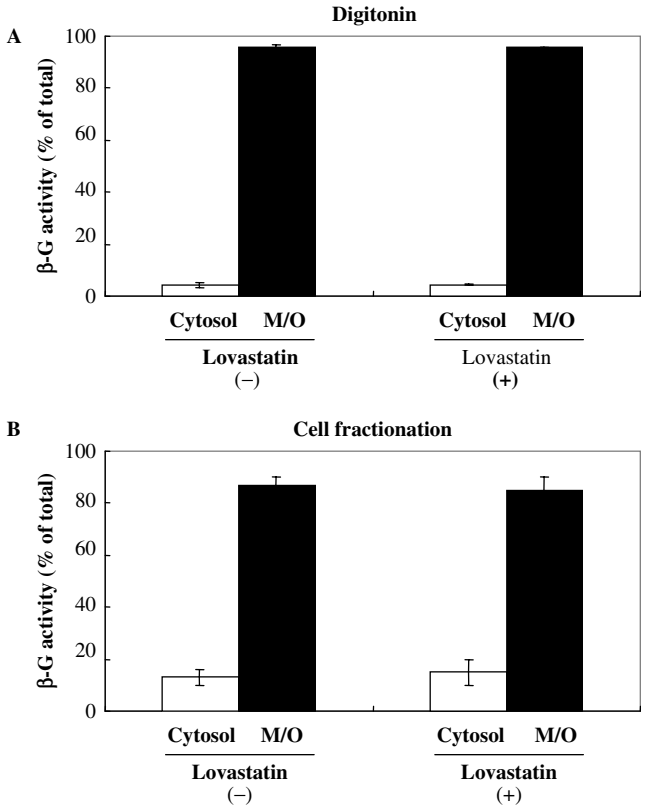

Fig. 4. **β-G activity in the cytosolic fraction obtained from permeabilized cells or conventional cell fractionation.** (A) Lovastatin-treated or untreated B16F10 cells were incubated in 1.5 ml of KHM buffer [20 mM phosphate buffer (pH 7.2), 110 mM KOAc, and 2 mM MgOAc] in the presence of digitonin (40 μg/ml) for 10 min at 4°C, and the cells were homogenized with 1.5 ml of isotonic sucrose solution containing 1% Triton X-100. β-G activity in the cells (M/O; membrane/organelle fraction) and medium (cytosolic fraction) was measured and expressed as a percentage of the total. (B) After lovastatin-treated or untreated B16F10 cells were homogenized with 1.5 ml of isotonic sucrose solution, the homogenate was centrifuged at 650 g for 5 min. The PNS was centrifuged at 106,000 g for 1 h. The supernatant was designated as the cytosolic fraction. β-G activity in the PNS containing 1% Triton X-100 and cytosolic fraction was measured. β-G activity in the M/O was calculated from the β-G activity in the PNS containing 1% Triton X-100 and in the cytosolic fraction, and was expressed as a percentage of the total. Data are the means of three experiments.

Table 1. **Comparison of specific activity of β-G between lovastatin-treated and untreated cell.**

	Activity (nmol/min/ml)	Protein (mg/ml)	Specific activity (nmol/min/mg)
Untreated cells	2.8 ± 0.3	1.4 ± 0.2	2.0 ± 0.1
Lovastatin-treated cells	1.5 ± 0.2	1.1 ± 0.1	1.4 ± 0.1*

Significantly different: $*P < 0.001$ ($n = 3$).

both forms were found to be present at higher levels in the medium of cells treated with lovastatin than in the medium of untreated cells (Fig. 9B). Also, it indicated that levels of both the mature and precursor forms of cathepsin H were significantly lower in the cells treated

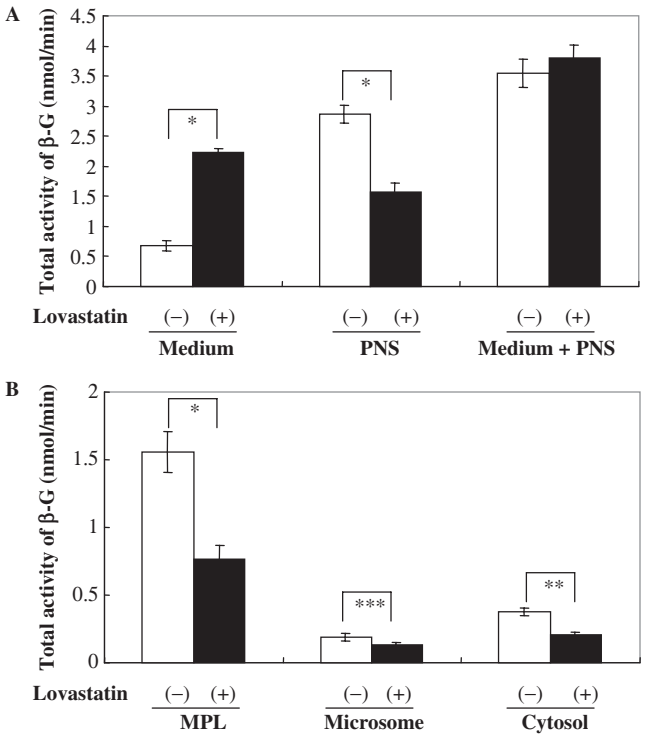

Fig. 5. **β-G activity in the medium and each fraction obtained by conventional cell fractionation.** B16F10 cells were cultured in the presence or absence of 10 μM lovastatin for 24 h. After lovastatin-treated or untreated B16F10 cells were homogenized with 1.5 ml of isotonic sucrose solution, the homogenate was centrifuged at 650 g for 5 min. The obtained supernatant (PNS) was centrifuged at 20,000 g for 20 min. The new supernatant was then centrifuged at 106,000 g for 1 h. This last supernatant was designated the cytosolic fraction. Also, the precipitates obtained at 20,000 g and 106,000 g were suspended in homogenization buffer containing 1% Triton X-100 and were designated as the MPL fraction (fraction containing lysosomes) and microsomal fraction, respectively. β-G activity in the medium, PNS, MPL fraction, microsomal fraction and cytosolic fraction was expressed as the total activity of β-G in each fraction. Data are the means of three experiments. Significantly different: $*P < 0.005$, $**P < 0.01$, $***P < 0.05$.

with lovastatin than in the untreated cells. Furthermore, no significant difference was found in the amount of precursor form of cathepsin H contained in both the medium and B16F10 cells between the treatment with lovastatin and that without lovastatin. Also, the amount of mature cathepsin H contained in both the medium and B16F10 cells treated with lovastatin was similar to that in non-treated B16F10 cells. From these results, it was concluded that both the exocytosis of mature enzymes from lysosomes to the medium and mis-sorting of the precursor forms were caused by the decrease in the amount of cholesterol in the cells. Also, a difference in the ratio of the precursor and mature forms released into the medium was observed depending on the particular lysosomal enzyme.

Release of β-G to the Medium Induced by LDS—In order to clarify whether the observed effect (release of β-G) is directly related to the decrease in cholesterol or

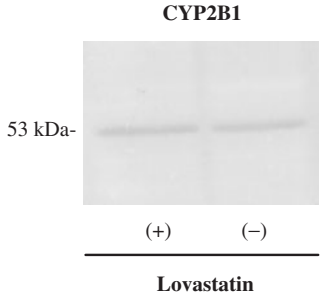

Fig. 6. **Immunoblot analysis of CYP2B1.** B16F10 cells were cultured in the presence (+) or absence (–) of 10 μM lovastatin for 24 h. After lovastatin-treated or untreated cells were homogenized with 1.5 ml of isotonic sucrose solution, the homogenate was centrifuged at 650 g for 5 min. Immunoblot analysis of CYP2B1 was carried out using the supernatant (30 μg; PNS). Bands were measured by Interigent Quantifier.

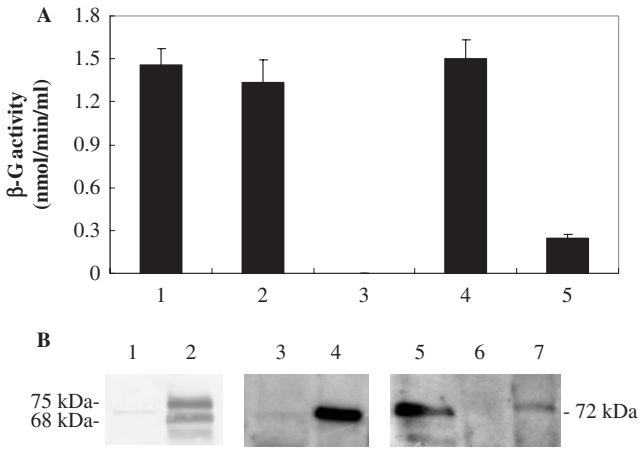

Fig. 7. **Immunoprecipitation of β-G in the medium.** (A) After B16F10 cells were cultured in the presence of 10 μM lovastatin for 24 h, immunoprecipitation of β-G in the medium was carried out. Medium was added to Affigel10 only, anti-rat β-G antibody-Affigel10 or nonimmuno-rabbit IgG Affigel10 equilibrated with PBS and rotated at 4°C for 24 h prior to centrifugation at 3000 g for 5 min. β-G activity in the supernatant was measured. Lane 1, medium; lane 2, Affigel10 only; lane 3, anti-rat β-G antibody-Affigel10; lane 4, nonimmuno-rabbit IgG-Affigel10; lane 5, fraction eluted from anti-rat β-G antibody-Affigel10. (B) Immunoblot analysis using anti-rat β-G antibody was performed using the lysosomes of mouse liver (lane 1, 0.5 μg; lane 4, 5 μg; lane 5, 5 μg), lysosomes of rat liver (lane 2, 0.5 μg), mouse melanoma MPL fraction (lane 3, 20 μg) or fraction eluted with urea after anti-rat β-G antibody-Affigel10 was incubated with β-G in the medium of B16F10 cells cultured in the absence (lane 6, 50 μl) or presence (lane 7, 50 μl) of lovastatin.

the side-effects of lovastatin, we carried out a comparison of β-G activity released in to medium from cells treated with FBS or LDS. As shown in Fig. 10, the total activity of β-G was significantly higher in the medium of B16F10 cells treated with LDS than that of cells treated with FBS. The total activity of β-G was significantly decreased in cells treated with LDS as compared with those treated with FBS. The total activity of β-G contained in both the medium and B16F10 cells treated with LDS was similar to that on treatment with FBS. As described above,

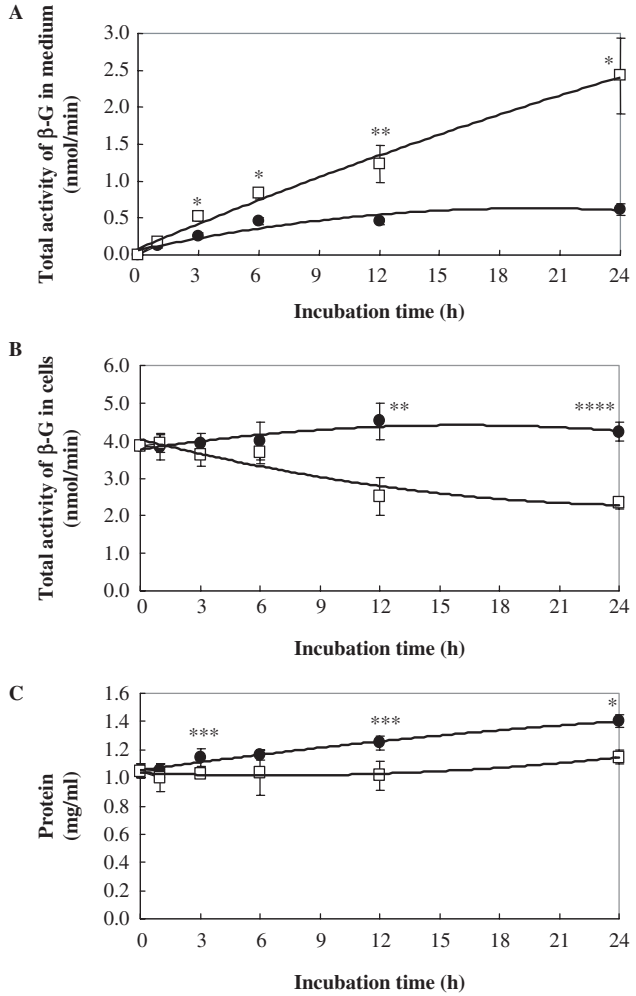

Fig. 8. **Time-course of the effect of lovastatin on the level of β-G.** β-G activity in the medium and in B16F10 cells incubated with (□) and without (●) 10 μM of lovastatin for the indicated periods of time were measured. Also, total protein in the cells was measured. Data are the means of three experiments. (A) Total activity of β-G in medium; (B) total activity of β-G in cells; (C) total protein in cells. Significantly different: $^{*}P<0.005$, $^{**}P<0.01$, $^{***}P<0.05$, $^{****}P<0.0005$.

cholesterol decreased in cells treated with D-MEM containing LDS without lovastatin (Fig. 1C); therefore, this indicated that cells treated with D-MEM containing LDS caused the release of β-G into the medium, concomitantly with the decrease of cholesterol. Also, the ratio of β-G (3.2-fold) released in the medium from cells treated with D-MEM containing lovastatin and LDS (Fig. 5A) was higher than that from cells (2.3-fold) treated with D-MEM containing LDS without lovastatin (Fig. 10). From these results, it was suggested that the secretion of β-G involved both the decrease in the amount of cholesterol in the cells and the side-effects of lovastatin.

DISCUSSION

From the decrease of β-G activity in the lysosomal fraction on Percoll density gradient fractionation, the

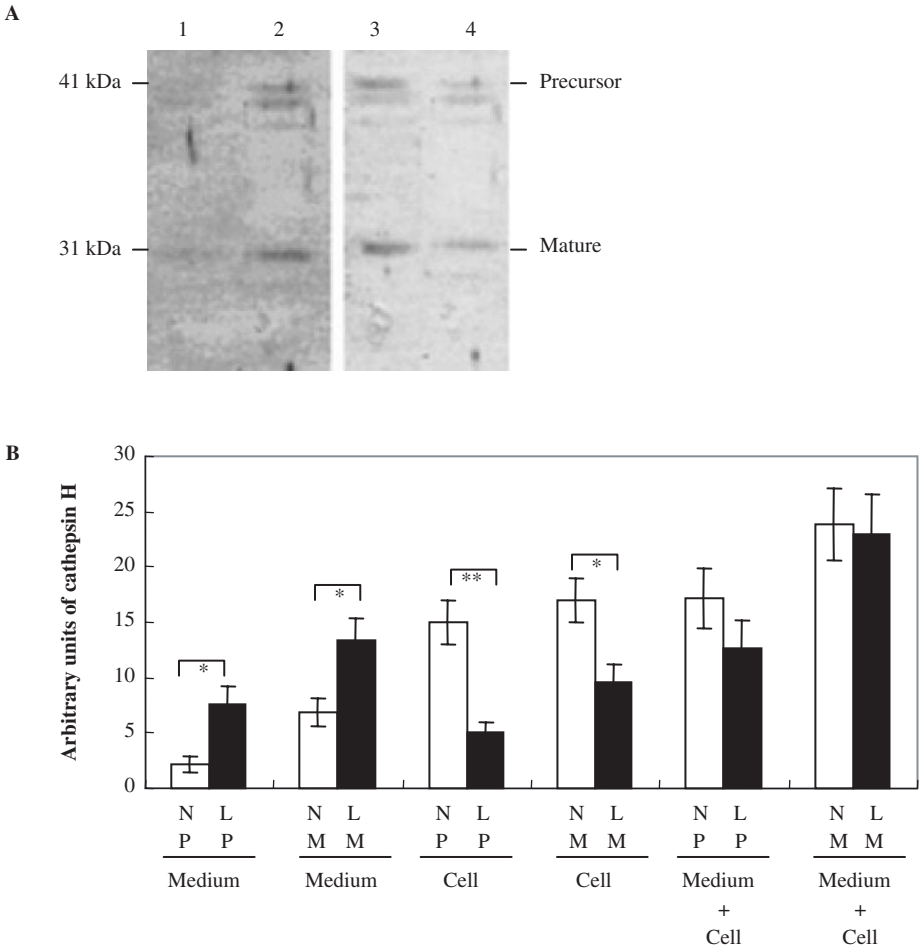

Fig. 9. **Immunoprecipitation of cathepsin H in the medium and cells.** (A) After B16F10 cells were cultured in the presence or absence of 10 μM lovastatin for 24 h, immunoprecipitation of cathepsin H in the medium and cells was carried out. Medium or cells were added to anti-rat cathepsin H antibody-Affigel10 equilibrated with PBS and rotated at 4°C for 24 h. The immunoblot analysis was carried out as described in 'Materials & Methods'. Lane 1, medium in the absence of 10 μM lovastatin; lane 2, medium in the presence of 10 μM lovastatin; lane 3, cells in the absence of 10 μM lovastatin; lane 4, cells in the presence of 10 μM lovastatin. (B) Signals for cathepsin H in A were measured using Intelligent Quantifier. N, no treatment (□); L, lovastatin-treatment (■); P, precursor form; M, mature form. Data are the means of three experiments. Significantly different: $^{*}P < 0.005$, $^{**}P < 0.001$.

increase of β-G activity in the medium and the detection of the mature form of β-G in the medium by immunoprecipitation, we found that the mature form of β-G located in lysosomes was released into the medium with a decrease in the amount of cholesterol in the cells when mouse melanoma was treated with lovastatin and LDS. Namely, the lysosomal exocytosis was caused by the decrease in the cholesterol content of the cells. Furthermore, a quantitative immunoblot analysis of cathepsin H by immunoprecipitation carried out using medium and cells treated with lovastatin indicated that the mature and precursor forms of cathepsin H were released into the medium. From these results, it was suggested that mis-sorting of the precursor forms of lysosomal enzymes was caused by a decrease in the amount of cholesterol in the cells. Therefore, cholesterol may be an important factor in the transport of lysosomal enzymes from the Golgi apparatus to lysosomes *via* late endosomes. In fact, the cholesterol present in the compartment was involved in the transport of lysosomal enzymes and a loss of cholesterol inhibited the endocytic pathway (*2, 6–23*).

The difference in the ratio of precursor and mature lysosomal enzymes released into the medium may be due to separate routes of secretion and lysosomal exocytosis or to altered rates of interaction with intracellular molecules that regulate secretion. Others have found that monesin (*42*) and cynate (*43*) stimulate the secretion of the precursor of cathepsin D but not of β-hexosaminidase from fibroblasts. Also, similar differential intracellular transport rates have been noted for membrane (*44*) and secretory (*45*) proteins. It was reported that the transport of β-G (*46*) and cathepsin H (*47*) to lysosomes was regulated by mannose-6-phosphate receptor (M6PR). If the transport of β-G and cathepsin H by M6PR from the Golgi apparatus to late endosomes or M6PR-dependent endocytosis was inhibited by the depletion of cholesterol, the increase in the precursor form of lysosome enzymes in medium was caused by mis-sorting of lysosome enzymes and inhibition of M6PR-dependent

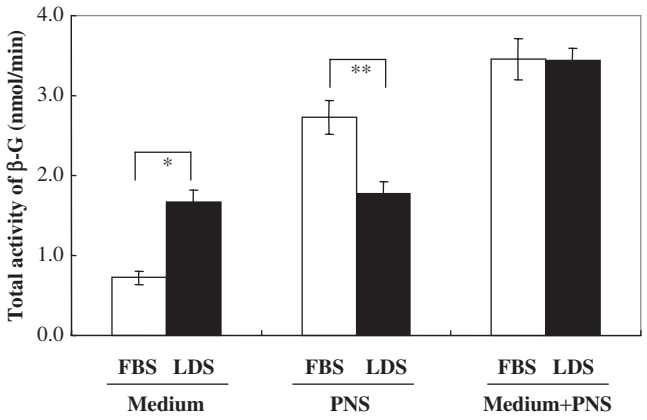

Fig. 10. **Release of β-G activity into medium by LDS.** B16F10 cells were cultured for 24 h with D-MEM containing 10% FBS (□) or 10% LDS (■). After FBS- or LDS-treated cells were homogenized with 1.5 ml of isotonic sucrose solution, the homogenate was centrifuged at 650 g for 5 min. β-G activity in the supernatant (PNS) and medium was expressed as the total activity of β-G. Data are the means of three experiments. Significantly different: $^{*}P<0.001$, $^{**}P<0.005$.

endocytosis. Also, it was reported that the processing of β-G or transport of newly synthesized β-G to lysosomes was slower than that of cathepsin H (*41, 48*). Therefore, the difference in the ratio of precursor and mature lysosomal enzymes released into the medium may be caused by the difference in the rate of processing or transport of lysosome enzymes.

Recent experiments with macrophages demonstrated a striking effect of ammonium chloride and other amines; a much greater and more rapid secretion of the mature form only, the precursor form only, or both forms of lysosomal enzyme, although the ratio of mature and precursor forms was reflected by species or cell-type differences (*49–52*). Some of these effects of ammonia may be related to the known accumulation of ammonia and other amines in lysosomes (*53, 54*) and in other acidic organelles (*55*), with an accompanying increase in organellar pH (*56*). On the other hand, the process of lysosomal exocytosis is considered to be essential for the repair of large wounds in plasma membranes (*57*). Other groups reported that this may occur in various physiological situations during mechanical stress in the gut, muscle or skin (*58–60*). It was also reported that calcium flows into the cell due to a disruption of the plasma membrane or the effects of drugs such as the ionophore ionomycin, and an increase in cytosolic calcium could possibly trigger the exocytosis of peripheral lysosomes, as shown in fibroblasts and kidney epithelial cells, and might influence the behaviour of the lysosomal compartment (*61–63*). As shown in those reports, little is known about the mechanism causing lysosomal exocytosis, except for the effect of an increase of calcium in the cytosol. In the present study, we found that lovastatin and/or LDS causes the release of lysosomal enzymes to the medium from lysosomes. Lovastatin is a hydrophobic, not basic drug. Therefore, we concluded that the increase in pH of acidic organelles as well as the ammonium chloride was not involved in the release by lovastatin. Lovastatin is a potent competitive inhibitor of the enzyme HMG-CoA reductase, which catalyses the rate-limiting conversion of HMG-CoA to mevalonate in the pathway producing cholesterol (*1*). Concomitantly with the decrease in the amount of cholesterol in cells treated with lovastatin and/or LDS, a decrease may also occur in the plasma membrane, as cholesterol is a major constituent of the plasma membrane. Therefore, lysosomal exocytosis may promote the rapid repair of wounds in the plasma membrane ruptured by a loss of cholesterol. Rao *et al*. (*64*) reported that the increase of calcium in the cytosol triggers a synaptotagmin-regulated process, which results in the fusion of lysosomes with the plasma membrane. If this occurs, lysosomal membrane lipids could be used for the rapid repair of the plasma membrane. The promotion of secretory granules along the secretory route was also discussed as a cause of the mis-sorting of the precursor form. Membranes in the Golgi apparatus contain lipid rafts (containing cholesterol). When a decrease of cholesterol in the plasma membrane occurs, supply of cholesterol from the Golgi apparatus to plasma membrane takes place along the secretory route, resulting in the lysosomal precursor form being released from the Golgi to medium. If this occurs, lipids in the Golgi apparatus could be also used for the rapid repair of wounds in the plasma membrane. Further study is necessary to understand the mechanism behind the secretion of lysosomal enzymes on lysosomal exocytosis and mis-sorting caused by the decrease in the amount of cholesterol in the cells.

REFERENCES

1. Nichols, B. (2003) Caveosomes and endocytosis of lipid rafts. *J. Cell Sci.* **116**, 4707–4714
2. Helms, J.B. and Zurzolo, C. (2004) Lipids as targeting signals: lipid rafts and intracellular trafficking. *Traffic* **5**, 247–254
3. Nichols, B.J. and Lippincott-Schwartz, J. (2001) Endocytosis without clathrin coats. *Trends Cell Biol.* **11**, 406–412
4. Nabi, I.R. and Le, P.U. (2003) Caveolae/raft-dependent endocytosis. *J. Cell Biol.* **161**, 673–677
5. van Deurs, B., Roepstorff, K., Hommelgaard, A.M., and Sandvig, K. (2003) Caveolae: anchored, multifunctional platforms in the lipid ocean. *Trends Cell Biol.* **13**, 92–100
6. Brotherus, J. and Renkonen, O. (1977) Subcellular distributions of lipids in cultured BHK cells: evidence for the enrichment of lysobisphosphatidic acid and neutral lipids in lysosomes. *J. Lipid Res.* **18**, 191–202
7. Hornick, C.A., Hui, D.Y., and DeLamatre, J.G. (1997) A role for retrosomes in intracellular cholesterol transport from endosomes to the plasma membrane. *Am. J. Physiol.* **273**, 1075–1081
8. Mukherjee, S., Zha, X., Tabas, I., and Maxfield, F.R. (1998) Cholesterol distribution in living cells: fluorescence imaging using dehydroergosterol as a fluorescent cholesterol analog. *Biophys. J.* **75**, 1915–1925
9. Gagescu, R., Demaurex, N., Parton, R.G., Hunziker, W., Huber, L.A., and Gruenberg, J. (2000) The recycling endosome of Madin-Darby canine kidney cells is a mildly acidic compartment rich in raft components. *Mol. Biol. Cell* **11**, 2775–2791
10. Kobayashi, T., Yamaji-Hasegawa, A., and Kiyokawa, E. (2001) Lipid domains in the endocytic pathway. *Semin. Cell Dev. Biol.* **12**, 173–182
11. Lusa, S., Blom, T.S., Eskelinen, E.L. *et al.* (2001) Depletion of rafts in late endocytic membranes is controlled by

NPC1-dependent recycling of cholesterol to the plasma membrane. *J. Cell Sci.* **114**, 1893–1900
12. Nichols, B.J., Kenworthy, A.K., Polishchuk, R.S., Lodge, R., Roberts, T.H., Hirschberg, K., Phair, R.D., and Lippincott-Schwartz, J. (2001) Rapid cycling of lipid raft markers between the cell surface and Golgi complex. *J. Cell Biol.* **153**, 529–541
13. Mobius, W., van Donselaar, E., Ohno-Iwashita, Y., Shimada, Y., Heijnen, H.F., Slot, J.W., and Geuze, H.J. (2003) Recycling compartments and the internal vesicles of multivesicular bodies harbor most of the cholesterol found in the endocytic pathway. *Traffic* **4**, 222–231
14. Kobayashi, T., Stang, E., Fang, K.S., de Moerloose, P., Parton, R.G., and Gruenberg, J. (1998) A lipid associated with the antiphospholipid syndrome regulates endosome structure and function. *Nature* **392**, 193–197
15. Simons, K. and Gruenberg, J. (2000) Jamming the endosomal system: lipid rafts and lysosomal storage diseases. *Trends Cell Biol.* **10**, 459–462
16. Pagano, R.E. (2003) Endocytic trafficking of glycosphingolipids in sphingolipid storage diseases. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **358**, 885–891
17. Imelli, N., Meier, O., Boucke, K., Hemmi, S., and Greber, U.F. (2004) Cholesterol is required for endocytosis and endosomal escape of adenovirus type 2. *J. Virol.* **78**, 3089–3098
18. Maxfield, F.R. and Wustner, D. (2002) Intracellular cholesterol transport. *J. Clin. Invest.* **110**, 891–898
19. Simons, K. and Ikonen, E. (2000) How cells handle cholesterol. *Science* **290**, 1721–1726
20. Tobert, J.A. (2001) Lovastatin and beyond: the history of the HMG-CoA reductase inhibitors. *Nat. Rev. Drug Discov.* **2**, 517–526
21. McKenney, J.M. (1988) Lovastatin: a new cholesterol-lowering agent. *Clin. Pharm.* **7**, 21–36
22. Chan, P.C., Lafreniere, R., and Parsons, H.G. (1997) Lovastatin increases surface low density lipoprotein receptor expression by retarding the receptor internalization rate in proliferating lymphocytes. *Biochem. Biophys. Res. Commun.* **235**, 117–122
23. Laezza, C., Bucci, C., Santillo, M., Bruni, C.B., and Bifulco, M. (1998) Control of Rab5 and Rab7 expression by the isoprenoid pathway. *Biochem. Biophys. Res. Commun.* **248**, 469–472
24. Kornfeld, S. and Mellman, I. (1989) The biogenesis of lysosomes. *Annu. Rev. Cell Biol.* **5**, 483–525
25. Peters, C. and von Figura, K. (1994) Biogenesis of lysosomal membranes. *Febs Lett.* **346**, 108–114
26. Ludwig, T., Griffiths, G., and Hoflack, B. (1991) Distribution of newly synthesized lysosomal enzymes in the endocytic pathway of normal rat kidney cells. *J. Cell Biol.* **115**, 1561–1572
27. Rijnboutt, S., Stoorvogel, W., Geuze, H.J., and Strous, G.J. (1992) Identification of subcellular compartments involved in biosynthetic processing of cathepsin D. *J. Biol. Chem.* **26**, 15665–15672
28. Michihara, A., Sawamura, M., Nara, Y., Ikeda, K., and Yamori, Y. (1997) Purification and characterization of two mevalonate pyrophosphate decarboxylases from rat liver: a novel molecular species of 37 kDa. *J. Biochem. (Tokyo)* **122**, 647–654
29. Tsuji, H. and Akasaki, K. (1994) Identification and characterization of lysosomal enzymes involved in the proteolysis of phenobarbital-inducible cytochrome P450. *Biol. Pharm. Bull.* **17**, 568–571
30. Himeno, M., Nishimura, Y., Tsuji, H., and Kato, K. (1976) Purification and characterization of microsomal and lysosomal beta-glucuronidase from rat liver by use of immunoaffinity chromatography. *Eur. J. Biochem.* **70**, 349–359
31. Yokota, S., Tsuji, H., and Kato, K. (1986) Immunocytochemical localization of cathepsin H in rat kidney. Light and electron microscopic study. *Histochemistry* **85**, 223–230
32. Michihara, A., Akasaki, K., Yamori, Y., and Tsuji, H. (2003) Subcellular distribution of mouse mevalonate pyrophosphate decarboxylase. *Biol. Pharm. Bull.* **26**, 579–584
33. de Duve, C., Pressman, B.C., Gianetto, R., Wattiaux, R., and Appelmans, F. (1955) Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem. J.* **60**, 604–617
34. Robins, E., Hirsch, H.E., and Emmons, S.S. (1968) Glycosidases in the nervous system. I. Assay, some properties, and distribution of beta-galactosidase, beta-glucoronidase, and beta-glucosidase. *J. Biol. Chem.* **243**, 4246–4252
35. Ikehara, Y., Mansho, K., Takahashi, K., and Kato, K. (1978) Purification and characterization of alkaline phosphatase from plasma membranes of rat ascites hepatoma. *J. Biochem.* **83**, 1471–1483
36. Abei, H. (1974) *Methods of Enzymatic Analysis,* Vol. 2, ed. By Bergmeyer, H.U., Verlag Chemie, Wienheim, pp. 673–684
37. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275
38. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
39. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354
40. Shellman, Y.G., Ribble, D., Miller, L., Gendall, J., Vanbuskirk, K., Kelly, D., Norris, D.A., and Dellavalle, R.P. (2005) Lovastatin-induced apoptosis in human melanoma cell lines. *Melanoma Res.* **15**, 83–89
41. Nishimura, Y. and Kato, K. (1987) Intracellular transport and processing of lysosomal cathepsin H. *Biochem. Biophys. Res. Commun.* **148**, 329–334
42. Pohlmann, R., Kruger, S., Hasilik, A., and von Figura, K. (1984) Effect of monensin on intracellular transport and receptor-mediated endocytosis of lysosomal enzymes. *Biochem. J.* **217**, 649–658
43. Gumbiner, B. and Kelly, R.B. (1982) Two distinct intracellular pathways transport secretory and membrane glycoproteins to the surface of pituitary tumor cells. *Cell* **28**, 51–59
44. Fitting, T. and Kabat, D. (1982) Evidence for a glycoprotein "signal" involved in transport between subcellular organelles. Two membrane glycoproteins encoded by murine leukemia virus reach the cell surface at different rates. *J. Biol. Chem.* **257**, 14011–14017
45. Strous, G.J. and Lodish, H.F. (1980) Intracellular transport of secretory and membrane proteins in hepatoma cells infected by vesicular stomatitis virus. *Cell* **22**, 709–717
46. Ludwig, T., Munier-Lehmann, H., Bauer, U., Hollinshead, M., Ovitt, C., Lobel, P., and Hoflack, B. (1994) Differential sorting of lysosomal enzymes in mannose 6-phosphate receptor-deficient fibroblasts. *Embo. J.* **13**, 3430–3437
47. Nishimura, Y., Kawabata, T., Yano, S., and Kato, K. (1990) Inhibition of intracellular sorting and processing of lysosomal cathepsins H and L at reduced temperature in primary cultures of rat hepatocytes. *Arch. Biochem. Biophys.* **283**, 458–463
48. Lazzarino, D. and Gabel, C.A. (1990) Beta-Glucuronidase is transported slowly to lysosomes in BW5147 mouse lymphoma cells: evidence that the prelysosomal enzyme is not restricted to the endoplasmic reticulum. *Arch. Biochem. Biophys.* **282**, 100–109

49. Riches, D.W. and Stanworth, D. (1980) Primary amines induce selective release of lysosomal enzymes from mouse macrophages. *Biochem. J.* **188**, 933–936
50. Riches, D.W., Watkins, J.L., and Stanworth, D.R. (1983) Biochemical differences in the mechanism of macrophage lysosomal exocytosis initiated by zymosan particles and weak bases. *Biochem. J.* **212**, 869–874
51. Jessup, W., Leoni, P., Bodmer, J.L., and Dean, R.T. (1982) The effect of weak bases on lysosomal enzyme secretion by mononuclear phagocytes. *Biochem. Pharmacol.* **31**, 2657–2662
52. Brown, J.A., Novak, E.K., and Swank, R.T. (1985) Effects of ammonia on processing and secretion of precursor and mature lysosomal enzyme from macrophages of normal and pale ear mice: evidence for two distinct pathways. *J. Cell Biol.* **100**, 1894–1904
53. de Duve, C., de Barsy, T., Poole, B., Trouet, A., Tulkens, P., and Van Hoof, F. (1974) Commentary. Lysosomotropic agents. *Biochem. Pharmacol.* **23**, 2495–2531
54. Ohkuma, S. and Poole, B. (1981) Cytoplasmic vacuolation of mouse peritoneal macrophages and the uptake into lysosomes of weakly basic substances. *J. Cell Biol.* **90**, 656–664
55. Maxfield, F.R. (1982) Weak bases and ionophores rapidly and reversibly raise the pH of endocytic vesicles in cultured mouse fibroblasts. *J. Cell Biol.* **95**, 676–681
56. Dean, R.T., Jessup, W., and Roberts, C.R. (1984) Effects of exogenous amines on mammalian cells, with particular reference to membrane flow. *Biochem. J.* **217**, 27–40
57. Reddy, A., Caler, E.V., and Andrews, N.W. (2001) Plasma membrane repair is mediated by Ca(2+)-regulated exocytosis of lysosomes. *Cell* **106**, 157–169
58. McNeil, P.L. and Ito, S. (1989) Gastrointestinal cell plasma membrane wounding and resealing in vivo. *Gastroenterology* **96**, 1238–1248
59. McNeil, P.L. and Khakee, R. (1992) Disruptions of muscle fiber plasma membranes. Role in exercise-induced damage. *Am. J. Pathol.* **140**, 1097–1109
60. McNeil, P.L. and Steinhardt, R.A. (1997) Loss, restoration, and maintenance of plasma membrane integrity. *J. Cell Biol.* **137**, 1–4
61. Jaiswal, J.K., Andrews, N.W., and Simon, S.M. (2002) Membrane proximal lysosomes are the major vesicles responsible for calcium-dependent exocytosis in nonsecretory cells. *J. Cell Biol.* **159**, 625–635
62. Tapper, H., Furuya, W., and Grinstein, S. (2002) Localized exocytosis of primary (lysosomal) granules during phagocytosis: role of Ca2+-dependent tyrosine phosphorylation and microtubules. *J. Immunol.* **168**, 5287–5296
63. Jans, R., Sartor, M., Jadot, M., and Poumay, Y. (2004) Calcium entry into keratinocytes induces exocytosis of lysosomes. *Arch. Dermatol. Res.* **296**, 30–41
64. Rao, S.K., Huynh, C., Proux-Gillardeaux, V., Galli, T., and Andrews, N.W. (2004) Identification of SNAREs involved in synaptotagmin VII-regulated lysosomal exocytosis. *J. Biol. Chem.* **279**, 20471–20479