Preparation of a Complex of Dexamethasone Palmitate–Low Density Lipoprotein and Its Effect on Foam Cell Formation of Murine Peritoneal Macrophages†

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Abstract □ In the early progression of atherosclerosis, LDL migrates in the subendothelial space of the artery and plays an important role in foam cell formations of macrophages. LDL may serve as a carrier of site-specific delivery of drugs to atherosclerotic lesions. In this exploratory study, dexamethasone palmitate (DP) was incorporated in LDL, and an inhibitory effect of this complex on foam cell formations was examined. LDL was isolated from human plasma, and the DP–LDL complex was prepared by incubation in the presence of Celite 545. No degradation nor modification of LDL was observed. The DP/LDL molar ratio of the complex was 35-50:1. Foam cell formations of murine macrophages were induced by incubation with oxidized LDL. When macrophages were pretreated with the DP–LDL complex, accumulation of cholesterol ester in the macrophages induced by oxidized LDL, i.e., an index of foam cell formation, was decreased. These findings indicated that the DP–LDL complex showed similar characteristics to LDL, and the DP–LDL complex inhibited foam cell formations of macrophages in vitro. This study provides the basis for further study of the DP–LDL complex as a drug–carrier complex for treatment of atherosclerosis.

Introduction

In an early stage of progression of atherosclerosis, atherosclerotic plaques are developed by accumulation of foam cells derived from macrophages or fibroblasts.[1] Recent studies indicate that LDL migrates under the epithelium of the vascular smooth muscle and is modified to be incorporated into macrophages in early atherosclerotic lesions.[2−4] In fact, macrophages can be converted to foam cells containing large amounts of cholesterol esters when incubated with modified LDL, mainly oxidized, in vitro.[5,6] Modified LDL and macrophages interact to form foam cell formations at atherosclerotic lesions. Prevention of foam cell formations of macrophages by reducing cholesterol esters accumulated in the foam cells is thought to be antiatherogenic.

It is well known that the process of plaque formation in atherosclerotic lesions possesses a number of features in common with inflammation.[7] Several studies have reported that anti-inflammatory drugs suppress the development of atherosclerosis in New Zealand White rabbits and Watanabe heritable hyperlipidemic rabbits.[8−11] Asai et al. reported that dexamethasone inhibits the formation of foam cells in atherosclerotic lesions.[12]

Plasma lipoproteins are a carrier of not only water-insoluble lipids but also lipophilic compounds such as certain vitamins, hormones, toxins, and others in the circulatory system. Recently, it has been demonstrated that lipoproteins, especially LDL, can be used as a carrier to deliver drugs to specific sites in the body.[13,14] In most studies, LDL is used as a carrier to deliver antineoplastic drugs to cancer cell.[15,16]

In the present study, to assess the feasibility of LDL as a drug delivery system for atherosclerosis, we prepared a complex of dexamethasone palmitate (DP), a steroidal anti-inflammatory drug, and LDL and examined its inhibitory effects on cholesterol ester accumulation in macrophages induced by oxidized LDL.

Materials and Methods

Animal—Male ICR mice were obtained from Japan SLC, Inc. (Hamamatsu, Japan) and used for experiments at 6−8 weeks of age.

Materials—Dexamethasone palmitate (DP) and dexamethasone nonadecanoate were supplied by Green Cross Co. (Osaka, Japan). Celite 545 was purchased from Kanto Chemical Co. Inc. (Tokyo, Japan), dexamethasone and FAT RED 7B from Sigma Chemical Co. (St. Louis, MO), and cholesterol esterase, oxidase, and peroxidase from Toyobo Co. (Tokyo, Japan). All other chemicals were of analytical grade.

Isolation of LDL—LDL (d = 1.019−1.063 g/mL) was isolated by sequential ultracentrifugation (100 000g) from normolipidemic human plasma.[17] The LDL fraction was dialyzed sufficiently against phosphate-buffered saline (PBS, pH 7.4) and then filtered through a MILLEX-HV filter (pore size; 0.45 μm, Millipore Co., Bedford, MA). The LDL fraction was stored at 4 °C under the darkness and used for experiments within a week.

Preparation of the Drug−LDL Complex—The DP−LDL complex was prepared by the method of Seki et al.[18] with minor modification. Briefly, Celite 545 (100 mg) and 1 mL of DP solution (1 mg/mL in MeOH) were agitated by a vortex mixer. The mixture was evaporated under the vacuum for 30 min, and then the residues were pulverized adequately. The residue and 2 mL of LDL suspension (300−400 μg protein/mL) were mixed gently and packed with N_2 gas. The mixture was incubated for 20 h with shaking at 37 °C. After incubation, the mixture was centrifuged at 2000g for 10 min, and the supernatant was dialyzed with PBS. The complex obtained was passed through a MILLEX-HV filter and stored at 4 °C until experiments were performed (<1 week).

Oxidation of LDL and the DP−LDL Complex—Each LDL and DP−LDL complex was diluted to 100 μg protein/mL with PBS and incubated with 5 μM $CuSO_4$ at 37 °C for 20 h. The oxidized LDL (ox-LDL) and the oxidized DP−LDL complex (ox-DP complex) suspensions were dialyzed against PBS. Confirmations of oxidation were obtained by electrophoresis.

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† Abbreviations: LDL, low density lipoprotein; PBS, phosphate buffered saline; T-ch, total cholesterol; F-ch, free cholesterol; CE, cholesterol ester, DP, dexamethasone palmitate; ox-LDL, oxidized LDL; ox-DP complex, oxidized DP−LDL complex.

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Preparation of Macrophage—Peritoneal cells from unstimulated ICR mice were washed with PBS by centrifugation (800*g*). The cells were suspended to 10^6 cells/mL in RPMI1640 medium (Gibco BRL, Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum (Gibco BRL), 50 μM 2-mercaptoethanol, and 10 μg/mL of gentamicin. The cell suspensions were distributed (1 mL each) to wells of a 24-well culture plate (Becton Dickinson, Lincoln Park, NJ), and the plate was incubated for 90 min at 37 °C in a CO_2 incubator. After incubation, nonadherent cells were removed by washing with RPMI 1640 medium, and then the macrophage monolayers were placed in fresh RPMI 1640 medium for 20 h at 37 °C in a CO_2 incubator.

Cholesterol Accumulation Assay in Macrophages—Cholesterol accumulation in macrophages was assayed according to the methods of Miyazaki et al.[19] Macrophage monolayers were incubated with ox-LDL or ox-DP complexes for the periods indicated at 37 °C in a CO_2 incubator. After incubation, the macrophage monolayers were washed twice with RPMI 1640 medium and then PBS. Lipids and proteins were extracted directly from the macrophage monolayers. Briefly, 1 mL of a hexane/2-propanol (3:2) mixture was added to macrophage monolayers in the wells, and the macrophage monolayers were incubated for 30 min at room temperature. The solvent of the mixture was collected and extracted again. The extracts were combined, evaporated under the vacuum, dissolved in 500 μL of EtOH, and used for cholesterol measurements. After lipid extraction, 500 μL of a 0.1 M NaOH solution was added into each well and incubated for 15 min at 37 °C. The extracts were collected, and the extraction was repeated. The combined extracts were used for determination of cell protein.

Measurement of Cellular Cholesterol—The cellular concentration of cholesterol was measured by a fluorometric enzymatic method.[20] Briefly, 200 μL of cellular lipid extract and 2.5 mg of Triton X-100 were mixed and evaporated under the vacuum. The residue was suspended with 200 μL of the enzyme mixture and incubated for 30 min at 37 °C. The enzyme mixture for free cholesterol (F-ch) contained 0.12 units/mL of cholesterol oxidase, 45 units/mL of peroxidase, 0.5 mg/mL of homovanillic acid (Wako Pure Chemical Industries Ltd.), and 2 mg/mL of Triton X-100 in 100 mM sodium phosphate buffer (pH 7.0). The enzyme mixture for total cholesterol (T-ch) contained 0.36 units/mL of cholesterol esterase in the enzyme mixture for F-ch. Following incubation, 2.8 mL of a 0.1 M NaOH was added to stop the reaction, and each fluorescence intensity was measured with excitation at 323 nm and emission at 420 nm using a spectrofluorophotometer (Model FP-770, JASCO, Tokyo, Japan). Cellular cholesterol ester (CE) was determined by subtracting F-ch from T-ch.

Electrophoresis—The electrophoretic mobilities of LDL and DP−LDL complexes were determined by the Pol-E-Film System (Ciba-corning Diagnostics Co., Palo Alto, CA). The samples were loaded at 4.0 μg/well of agarose gel film (Universal Gel/8, Ciba-Corning) and run at 90 V for 40 min. After drying, the gels were stained with FAT RED 7B (Sigma) to visualize lipoprotein bands.

Measurement of LDL and Complex Particle Size—Particle size of LDL and DP−LDL complexes was determined by photon correlation spectroscopy using Coulter N4 plus a submicron particle analyzer (Coulter Co., Miami, FL). All samples were suspended with PBS to 100 μg protein/mL, filtered through an HLC disk (pore size 0.2 μm, Kanto Chemical), and then measured at 20 °C.

HPLC Analysis—DP concentration in the complexes was measured by HPLC as follows: a mixture of samples of the complexes (50 μL) and dexamethasone nonadecanoate (as an internal standard, 50 μL) was extracted by dichloromethane (4 mL). A part of the organic fraction (3 mL) was evaporated under the vacuum at 30 °C. The residue was dissolved in 120 μL of acetonitrile−water (95:5), and a 50 μL of aliqot was injected into the HPLC system (Shimadzu Co., Kyoto, Japan) possessing a 7-μm LiChrosorb RP-18 column (4 × 250 mm, Cica-Merck, Kanto Chemical). The mobile phase was acetonitrile−water (95:5), and flow rate was 2.0 mL/min. The eluate was monitored at 236 nm and quantified on a model C-R6A chromatopac integrator (Shimadzu). The concentrations were determined with resect to a standard curve of DP.

Other Determinations—Proteins were measured by Coomassie Protein Assay Reagent (Pierce Chemical Company, Rockford, IL)[21] using bovine serum albumin as a standard. Each level of cholesterol in LDL or DP−LDL complex suspensions was measured by using commercial kits (Wako Pure Chemical).

Statistical Analysis—Data are expressed as means only or means and SDs ($n = 3$). Statistical analysis was done using nonpaired Student's *t*-test or Fisher's PLSD test. A p value of 0.05 or less was considered a significant difference between the sets of data.

Table 1—Lipids and Proteins Compositions in LDL and the DP–LDL Complex[a]

	LDL	DP–LDL complex
T-ch[b] (mg/100 mL)	103.7 ± 4.18	83.39 ± 2.56*
F-ch[c] (mg/100 mL)	29.84 ± 0.50	23.42 ± 1.02*
CE[d] (mg/100 mL)	73.86 ± 3.71	59.97 ± 2.06*
protein (mg/100 mL)	34.89 ± 1.25	25.99 ± 0.33*
F-ch/T-ch	0.29 ± 0.01	0.28 ± 0.01
T-ch/protein	2.97 ± 0.09	3.21 ± 0.13

[a] Values were mean ± SD ($n = 3$), *: significant difference from LDL, $p < 0.001$. [b] Total cholesterol. [c] Free cholesterol. [d] Cholesterol ester.

Table 2—Particle Size and DP Concentration of the DP–LDL Complex[a]

	particle size (nm)	DP concentration (μg/mL)
LDL	22.43 ± 0.65	–
DP–LDL complex	23.67 ± 0.25	14.19 ± 0.75 [47.60 ± 1.92]

[a] Values were mean ± SD ($n = 3$). Bracketed/values indicate the DP contained in the DP–LDL complex (mol of DP/mol of LDL).

Results

Characteristics of LDL and the DP−LDL Complex—Table 1 shows the concentrations of cholesterols and protein in LDL and the DP−LDL complexes. The T-ch, F-ch, and CE concentrations in the complexes were 83.39, 23.42, and 59.97 mg/100 mL, respectively, and the protein concentration in the DP−LDL complexes was 25.99 mg/100 mL. These values of the DP−LDL complexes were about 75−80% of the corresponding values of LDL, and these percentages were indicated as the yields after the complex preparation. However, the ratios of F-ch/T-ch and T-ch/protein in the DP−LDL complexes were similar to those of LDL (0.28 and 3.21 in the DP−LDL complexes, and 0.29 and 2.97 in LDL, respectively). Furthermore, in the case of oxidized LDL and DP−LDL complexes, these ratios were not different between ox-LDL and ox-DP complexes (data are not shown).

Particle size and DP concentration in the DP−LDL complexes are shown in Table 2. Although several studies have reported that particle size of the drug-LDL complexes is larger than that of LDL,[22,23] the particle size of the DP−LDL complexes in this study (23.67 nm) was not significantly different from that of LDL (22.43 nm). The DP concentration in the DP−LDL complexes was 14.19 μg/mL, and DP contained in the DP−LDL complex was 47.6 mol of DP/mol of LDL (LDL molecular weight of 5.5×10^5 based on protein concentration). The DP concentrations in the complex and those in the LDL particle were not altered by oxidation or dialysis with PBS. DP was not detected in a preparation which was prepared with PBS instead of LDL suspension (data are not shown). Neither aggregation of the DP−LDL complexes nor decrease in DP contained in the DP−LDL complex was recognized after 4 weeks of storage at 4 °C.

LDL is unstable in a buffer after isolation and easily modified by negative ions, air, and other stimuli. To examine whether LDL could be modified by the addition of DP, electrophoresis of the DP−LDL complexes was compared with that of LDL. As shown in Figure 1, electorophoretic mobility and the lipid band of the com-

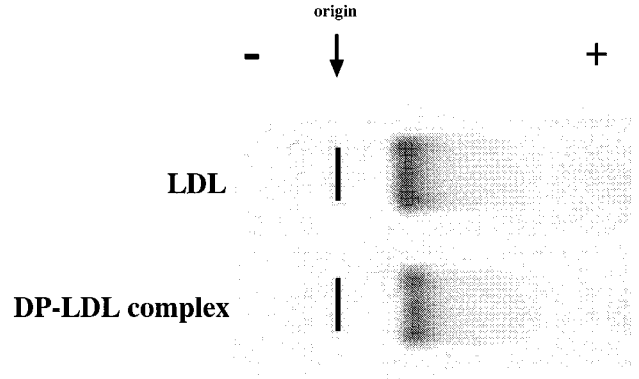

Figure 1—Electrophoretic mobilities of LDL and the DP–LDL complexes. Samples were loaded on agarose gel film at 2 μg/well and run at 90 V for 40 min. The agarose gel film was dried and stained with Fat red 7B.

plexes were similar to those of LDL. Moreover, the concentration of apo-B protein, which is the main protein in the LDL, was over 90% of the total protein concentration in the DP−LDL complexes, and the value was not different from that in LDL (data are not shown). These findings indicate that DP−LDL complexes can be prepared without any transposition and modification.

Cholesterol Accumulation in Macrophages during Incubation with ox-LDL or ox-DP Complex—It is well known that the macrophages are converted to foam cells with lipid droplets consisted of cholesterols during incubation with ox-LDL.

The T-ch and F-ch concentrations in macrophages were increased by incubation with ox-LDL about 2.9- and 2.3-fold of the control value (without ox-LDL), respectively (Figure 2A). The CE concentration in macrophages after incubation with ox-LDL was also increased markedly from 9.2 to 82.4 μg/mg cell protein (Figure 2 B). This increase in CE was associated with conversion of macrophages to foam cells during incubation with ox-LDL. On the other hand, when the macrophages were incubated with ox-DP complexes, CE concentration increased slightly as compared with that with ox-LDL (Figure 2B), while either T-ch or F-ch concentration was increased to the same extent as those in ox-LDL-treated macrophages (Figure 2A).

Effects of the ox-DP Complex on CE Accumulation in Macrophages—Figure 3 shows the changes in cholesterol concentrations in macrophages incubated with ox-LDL alone or ox-LDL together with ox-DP complexes. The concentrations of T-ch, F-ch, and CE in macrophages were increased predominantly by the incubation with ox-LDL alone at 100 μg protein/well rather than those at 50 μg protein/well. The increases in T-ch, F-ch, and CE in macrophages incubated with ox-LDL and ox-DP complexes (total ox-LDL concentration should be 100 μg protein/well) were significantly attenuated as compared with those with ox-LDL alone at 100 μg protein/well. However, the smaller concentration of ox-DP complexes (5 μg protein/well) showed no effects on the increased concentrations of cholesterols in macrophages incubated with ox-LDL at 50 μg protein/well.

Figure 4 shows the changes in cholesterol concentrations in macrophages preincubated with ox-DP complexes at 5 μg protein/well and then incubated with ox-LDL after removing of the ox-DP complexes. Preincubation with ox-DP complexes inhibited the accumulation of T-ch and F-ch in macrophages induced by incubation with ox-LDL. The CE concentration in macrophages preincubated with ox-DP complexes and then incubated with ox-LDL (50.37 μg/mg cell protein) was significantly lower than that in macrophages incubated with ox-LDL alone (83.23 μg/mg cell protein).

Figure 5 shows the changes in cholesterol concentrations in macrophages preincubated with dexamethasone at 0.4 μM in medium and then incubated with ox-LDL after removing of dexamethasone. The CE concentration in macrophages incubated with ox-LDL alone was 62.5 μg/mg cell protein, and the CE concentration in macrophages preincubated with dexamethasone and then incubated with ox-LDL was 39.5 μg/mg cell protein. Therefore, similar to that of the ox-DP complexes, preincubation with dexamethasone inhibited the CE accumulation in macrophages induced by incubation with ox-LDL.

Discussion

Recently, drug−LDL complexes have been examined for use as carriers in site-specific delivery systems.[13−16,22,23] Anticancer drugs have been examined in this drug delivery system. In this study, to study the feasibility of site-specific drug delivery for use in atherosclerosis, the DP−LDL complexes were prepared, and effects of the DP−LDL com-

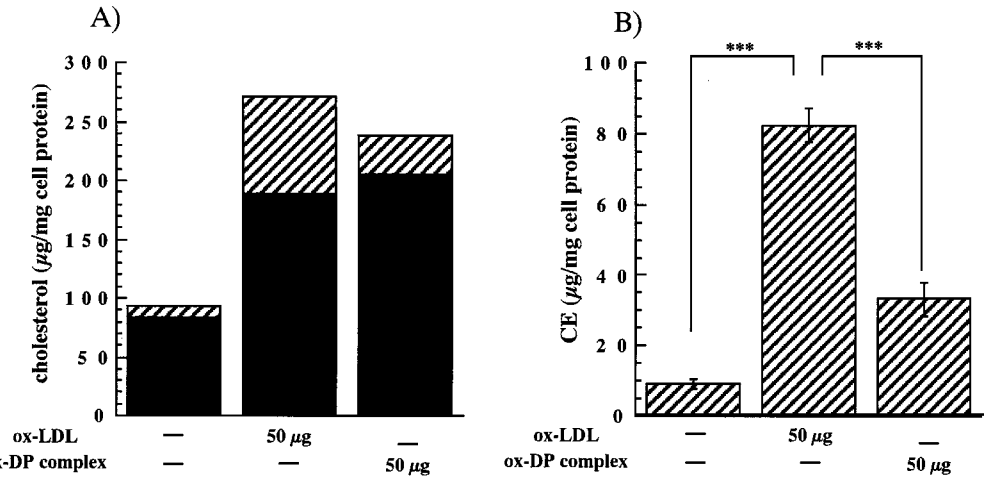

Figure 2—Cholesterol accumulations in macrophages caused by incubation with ox-LDL and the ox-DP complex. Macrophage monolayers were incubated with either ox-LDL or the ox-DP complex (50 μg protein/well, respectively) for 6 h at 37 °C. Final DP concentration in the ox-DP complex medium was 4 μM. After incubation, lipids and protein were extracted, and those levels were measured. In panel A, closed columns and slushed columns are F-ch and CE values, respectively ($n = 3$), and combined columns' heights indicate T-ch values. In panel B, slushed columns show CE values (mean ± SD, $n = 3$). The amount of ox-LDL or the ox-DP complex is indicated under the column as protein contents, and "–" indicates the addition of PBS instead of ox-LDL or the ox-DP complexes. Significant differences between groups were determined using a Fisher's PLSD test and are indicated as *** $p < 0.0001$.

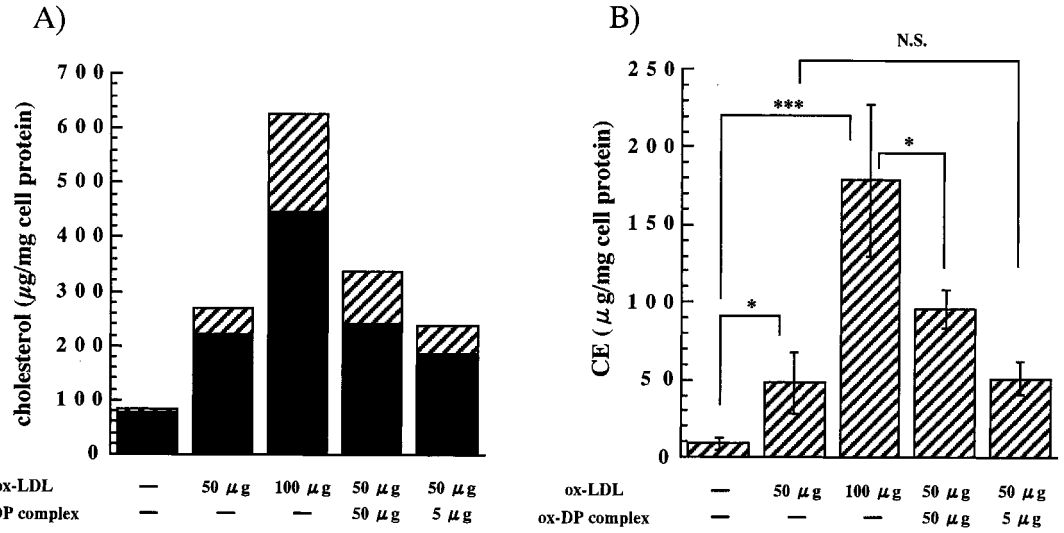

Figure 3—Effect of the ox-DP complex on cholesterol accumulation in macrophages caused by incubation with ox-LDL. Macrophages were incubated with ox-LDL (50 or 100 μg protein/well), or with ox-LDL (50 μg protein/well) and ox-DP complexes (5 or 50 μg protein/well) for 6 h. Final DP concentration in the ox-DP complex medium (5 or 50 μg protein/well) was 0.4 or 4 μM, respectively. Other details are the same as those in Figure 2. *** $p < 0.0001$; * $p < 0.01$. N.S. indicates a difference that is not significant.

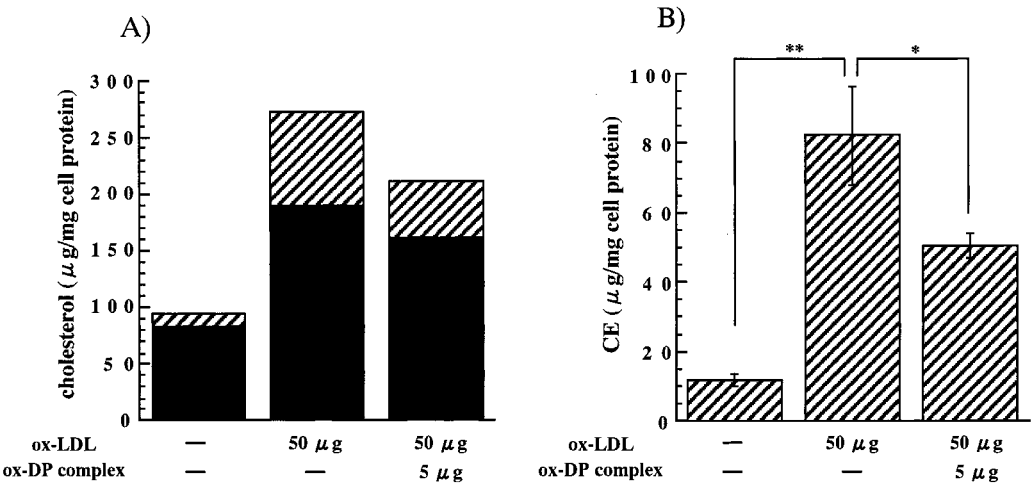

Figure 4—Effect of preincubation with the ox-DP complex on cholesterol accumulation in macrophages caused by incubation with ox-LDL. Macrophages were preincubated with ox-DP complex (5 μg protein/well) for 6 h and then washed with warm medium three times to remove the ox-DP complex. These macrophages were incubated with ox-LDL (50 μg protein/well) for 6 h. Other details are the same as those in Figure 2. ** $p < 0.001$; * $p < 0.01$.

plexes on CE accumulation in macrophages induced by ox-LDL were examined. Because LDL is unstable and easily modified, it was most important to prevent degradation and oxidation of LDL while preparing the DP−LDL complexes. Physiological characteristics and electrophoretic mobilities of the DP−LDL complexes were similar to those of LDL (Table 1 and Figure 1), indicating that the DP−LDL complex can be prepared without degradation, oxidation, and other modifications.

Eley et al.[22] have reported that the particle size (105 nm) of the complex of prednimustine, a steroidal anticancer drug, and LDL is 4 times larger than that of LDL (24.7 nm), and the prednimustine/LDL molar ratio of the complex is 163:1. Lundberg[23] has also demonstrated that the particle size of the prednimustine−LDL complexes is proportional to the concentration of a drug contained in the complex. In this study, however, no significant difference in particle size was observed between the DP−LDL complexes and LDL (Table 2). The reasons for this may be the lower drug concentration contained in our complexes (35−50 mol of DP/mol of LDL) and that the method for complex preparation in our studies was different from that in other studies, in which Celite 545 was not used.

Incubation of macrophages with ox-LDL markedly increased T-ch, F-ch, and especially CE in the cells (Figure 2). Because the accumulation of CE is a good indicator of conversion of macrophages to foam cells,[5,6] the macrophages treated with ox-LDL in this study may be susceptible to foam cell conversion. The accumulation of CE in the macrophages caused by incubation with ox-LDL, however, was significantly attenuated when the macrophages were incubated with ox-LDL containing DP (ox-DP complex). The reduction of CE accumulation may be due to inhibition of CE synthesis or enhancement of CE hydrolysis by DP itself or dexamethasone derived from DP. Asai et al.[12] have reported that dexamethasone can suppress foam cell formations of murine peritoneal macrophages by inhibiting uptake and degradation of lipoproteins.

The T-ch value of macrophages treated with ox-DP complexes was similar to that treated with ox-LDL (Figure 2). This result suggested that ox-DP complexes were incorporated into macrophages by scavenger receptors. Therefore, it is likely that the ox-DP complex can be incorporated into macrophages as well as ox-LDL, and then dexamethasone derived from DP inhibits CE synthesis or enhances CE hydrolysis in macrophages.

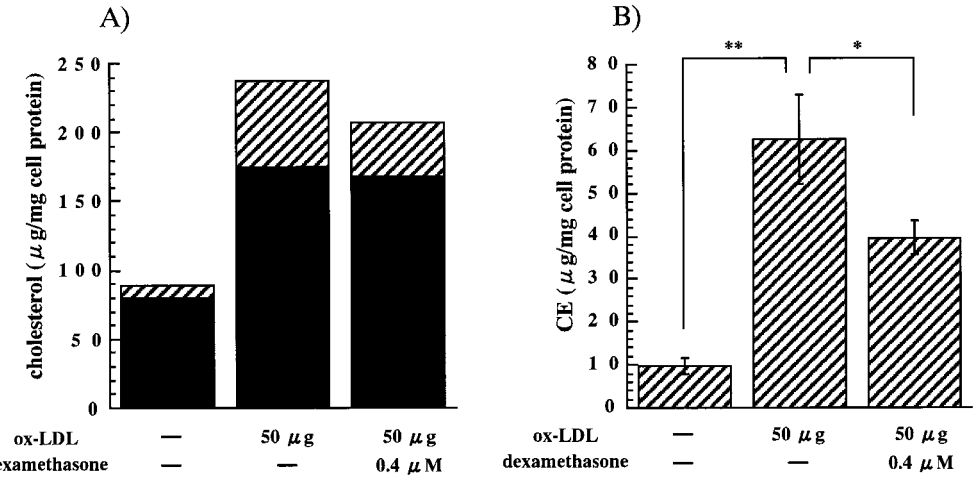

Figure 5—Effect of preincubation with dexamethasone on cholesterol accumulation in macrophages caused by incubation with ox-LDL. Macrophages were preincubated with dexamethasone (0.4 μM) for 6 h and then washed with warm medium three times to remove dexamethasone. These macrophages were incubated with ox-LDL (50 μg protein/well) for 6 h. The amount of ox-LDL (as protein content) or dexamethasone concentration in the well is indicated under the column, respectively. Other details are the same as those in Figure 2. ** $p < 0.001$; * $p < 0.01$.

To confirm the effect of the DP−LDL complex on ox-LDL-induced CE accumulation in macrophages, the cells were incubated with ox-DP complexes together with ox-LDL (Figure 3). Both T-ch and CE contents in macrophages significantly decreased when incubated with 50 μg protein/well of ox-LDL and 50 μg protein/well of ox-DP complexes as compared with those incubated with 100 μg protein/well of ox-LDL alone. This finding indicates that DP or dexamethasone released from ox-DP complexes can reduce the CE accumulation even when the ox-LDL exists simultaneously. However, the lower concentration of ox-DP complexes (5 μg protein/well) showed no effect on the CE accumulation in macrophages incubated with 50 μg protein/well of ox-LDL alone. We considered in this series of experiments that the presence of ox-LDL in 5 μg protein/well of ox-DP complexes is negligible. As shown in Figure 4, we detected a significant attenuation of ox-LDL-induced CE accumulation in macrophages even at 5 μg protein/well (0.4 μM of DP in medium) of the ox-DP complexes, when the cells were incubated with the ox-DP complexes prior to incubation with ox-LDL. Because steroid hormones require a certain period of time to express their effects, the macrophages should be incubated with the ox-DP complexes before incubation with ox-LDL when the concentration of the ox-DP complexes is small. In addition, we examined the effect of free dexamethasone on the CE accumulation in the macrophages induced by ox-LDL. Because free DP was not dissolved in the medium, we used free dexamethasone. The CE accumulation in macrophages incubated with 50 μg protein/well of ox-LDL was significantly inhibited by the pretreatment of the free dexamethasone at 0.4 μM in medium that is the same concentration as that of DP in the ox-DP complexes (Figure 5).

The inhibitory effect of free dexamethasone on the foam cell formation was the same extent as that of the ox-DP complex in the in vitro experiment. This result indicates that ox-DP complexes as well as free dexamethasone can exert a beneficial effect on atherogenic response in vitro. If the DP−LDL complex delivers DP to the atherosclerotic lesions, this complex acts more effectively than free dexamethasone in vivo.

In conclusion, the DP−LDL complex can be prepared by incubation of DP and LDL in the presence of Celite 545, and this complex shows the same lipid composition and electrophoretic mobility as that of LDL. Although this complex is easily oxidized like LDL, the ox-DP complex inhibits CE accumulation in macrophages induced by the ox-LDL. These findings indicate that the DP−LDL complex can be potentially useful as a drug−carrier to atherosclerotic lesions, and reduce foam cell formation in patients with atherosclerosis.

References and Notes

1. Ross, R. The pathogenesis of atherosclerosis:a perspective for the 1990s. *Nature* **1993**, *362*, 801−808.
2. Palinski, W.; Rosenfeld, M. E.; Yla-Herttuala, S.; Guatner, G. C.; Socher, S. S.; Butler, S. W.; Parthasarathy, S.; Carew, T. E.; Steinberg, D.; Witztum, J. L. Low density lipoprotein undergoes oxidative modification in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 1372−1376.
3. Yla-Herttuala, S.; Palinski, W.; Rosenfeld, M. E.; Parthasarathy, S.; Carew, T. E.; Butler, S.; Witztum, J. L.; Steinberg, D. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J. Clin. Invest.* **1989**, *84*, 1086−1095.
4. Witztum, J. L.; Steinberg, D. Role of oxidized low density lipoprotein in atherosclerosis. *J. Clin. Invest.* **1991**, *88*, 1785−1792.
5. Brown, M. S.; Goldstein, J. L.; Krieger, M.; Ho, Y. K.; Anderson, R. G. W. Reversible accumulation of cholesteryl esters in macrophages incubated with acetylated lipoproteins. *J. Cell Biol.* **1979**, *82*, 597−613.
6. Ryu, B. H.; Mao, F. W.; Lou, P.; Gutman, R. L.; Greenspan, P. Cholesteryl ester accumulation in macrophages treated with oxidized low density lipoprotein. *Biosci. Biotech. Biochem.* **1995**, *59*, 1619−1622.
7. Tedgui, A.; Bernard, C. Cytokines, immuno-inflammatory response and atherosclerosis. *Eur. Cytokine Network* **1994**, *5*, 263−270.
8. Bailey, J. M.; Butler, J. Anti-inflammatory drugs in experimental atherosclerosis: 1. Relative potencies for inhibiting plaque formation. *Atherosclerosis* **1979**, *17*, 515−522.
9. Bailey, J. M.; Makheja, A. N.; Butler, J.; Salata, K. Anti-inflammatory drugs in experimental atherosclerosis: 4. Inhibition of atherosclerosis in vivo and thromboxane synthesis and platelet aggregation in vitro. *Atherosclerosis* **1979**, *32*, 195−203.
10. Naito, M.; Yasue, M.; Asai, K.; Yamada, K.; Hayashi, T.; Kuzuya, M.; Funai, C.; Yoshimine, N.; Kuzuya, F. Effects of dexamethasone on experimental atherosclerosis in cholesterol-fed rabbits. *J. Nutr. Sci. Vitaminol.* **1992**, *38*, 255−264.
11. Makheja, A. N.; Bloom, S.; Muesing, R.; Simon, T. M.; Bailey, J. Anti-inflammatory drugs in experimental atherosclerosis: 7. Spontaneous atherosclerosis in WHHL rabbits and inhibition by cortisone acetate. *Atherosclerosis* **1989**, *76*, 155−161.
12. Asai, K.; Funai, C.; Hayashi, T.; Yamada, K.; Naito, M.; Kuzuya, M.; Yoshida, F.; Yoshimine, N.; Kuzuya, F. Dexamethasone-induces suppression of aortic atherosclerosis in cholesterol-fed rabbits. *Arterioscler. Thromb.* **1993**, *13*, 892−899.

13. Tokui, T.; Kuroiwa, C.; Muramatsu, S.; Tokui, Y.; Sasagawa, K.; Ikeda, T.; Komai, T. Plasma lipoproteins as targeting carriers to tumour tissues after administration of a lipophilic agent to mice. *Biopharm. Drug Dispos.* **1995**, *16*, 91−103.
14. Vitols, S.; Soderberg-Reid, K.; Masquelier, M.; Sjostrom, B.; Peterson, C. Low density lipoprotein for delivery of a water-insoluble alkylating agent to malignant cells. *In vivo* and *in vitro* studies of a drug-lipoprotein complex. *Br. J. Cancer* **1990**, *62*, 724−729.
15. Iwanik, M. J.; Shaw, K. V.; Ledwith, B. J.; Yanovich, S.; Shaw, J. M. Preparation and interaction of a Low-Density lipoprotein:Daunomycin complex with P388 leukenic cells. *Cancer Res.* **1984**, *44*, 1206−1215.
16. Shaw, J. M.; Futch, W. S. J.; Schook, L. B. Induction of macrophages anti tumor activity by acetylated low density lipoprotein containing lipophilic muramyl tripeptide. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 6112−6116.
17. Hatch, F.; Lees, R. S. Practical methods for plasma lipoprotein analysis. *Adv. Lipid Res.* **1968**, *6*, 2−63.
18. Seki, J.; Okita, A.; Watanabe, M.; Nakagawa, T.; Honda, K.; Tatewaki, N.; Sugiyama, M. Plasma lipoproteins as drug carriers: Pharmacological activity and disposition of the complex of β-sitosteryl-β-D-glucopyranoside with plasma lipoproteins. *J. Pharm. Sci.* **1985**, *74*, 1259−1264.
19. Miyazaki, A.; Rahim, A. T. M. A.; Ohta, T.; Morino, Y.; Horiuchi, S. High density lipoprotein mediates selective reduction in cholesterol esters from macrophages foam cells. *Biochem. Biophys. Acta* **1992**, *1126*, 73−80.
20. Kunitomo, M.; Yamaguchi, Y.; Matsushima, K.; Bando, Y. Microanalysis of tissue cholesterol using a fluorometric enzymatic methods. *Jpn. J. Clin. Chem.* **1983**, *12*, 117−124.
21. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.
22. Eley, J. G.; Halbert, G. W.; Florence, A. T. Incorporation of prednimustine into low density lipoprotein:activity against P388 cells in tissue culture. *Int. J. Pharm.* **1990**, *65*, 219−224.
23. Lundberg, B. B. Evaluation of methods for complexing prednimustine to low density lipoprotein. *Int. J. Pharm.* **1993**, *99*, 275−283.

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