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Influence of Particle Size on the Distributions of Liposomes to Atherosclerotic Lesions in Mice

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ABSTRACT In order to confirm the efficacy of liposomes as a drug carrier for atherosclerotic therapy, the influence of particle size on the distribution of liposomes to atherosclerotic lesions in mice was investigated. In brief, liposomes of three different particle sizes (500, 200, and 70 nm) were prepared, and the uptake of liposomes by the macrophages and foam cells in vitro and the biodistributions of liposomes administered intravenously to atherogenic mice in vivo were examined. The uptake by the macrophages and foam cells increased with the increase in particle size. Although the elimination rate from the blood circulation and the hepatic and splenic distribution increased with the increase in particle size in atherogenic mice, the aortic distribution was independent of the particle size. The aortic distribution of 200 nm liposomes was the highest in comparison with the other sizes. Surprisingly, the aortic distribution of liposomes in vivo did not correspond with the uptake by macrophages and foam cells in vitro. These results suggest that there is an optimal size for the distribution of liposomes to atherosclerotic lesions.

KEYWORDS Liposomes, Particle size, Drug carrier, Macrophages and foam cells, Atherogenic mice

INTRODUCTION

In the progressive process of atherosclerosis, the atherosclerotic lesions develop by accumulation of foam cells derived from macrophages by uptake of oxidized low density lipoprotein (LDL) (Ross, 1993). Consequently, in the atherosclerotic lesions, the macrophages and foam cells accumulate an excess of cholesterol ester (CE) and richly present in the subendothelial space (Yla-Herttuala et al., 1989; Witztum & Steinberg, 1991; Ryu et al., 1995). The progressive process of atherosclerosis is similar to that of chronic inflammation (Tedgui & Bernard, 1994). Several studies have reported that the anti-inflammatory drugs inhibit the development of atherosclerosis in vivo (Makheja et al., 1989; Naito et al., 1992; Asai et al., 1993; Van Put et al., 1995; Tauchi et al., 2001). The use of drug carriers has been proposed as a

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technique to deliver these drugs efficiently to the macrophages and foam cells in the atherosclerotic lesions. Therefore, the detailed evaluation of the pharmacokinetic properties of the drug carrier chosen is important.

The anti-inflammatory drug–LDL complex selectively accumulates in atherosclerotic lesions and effectively inhibits the development of atherosclerosis in atherogenic mice (Tauchi et al., 2001). Unfortunately, because LDL is a biogenic component, it is very difficult to obtain in significant quantities. Thus, an artificial drug carrier is needed to deliver anti-inflammatory drugs to atherosclerotic lesions. Recently, artificial drug carriers such as liposomes and nanospheres have been used widely for therapeutic applications because of their ability to alter the pharmacokinetics and reduce the toxicity of the associated drugs. In particular, liposomes have attracted great interest as a potential drug carrier system because they are easy to prepare, their particle size can be altered easily, and lipid formulations and surface modification are possible. The liposomes accumulate in atherosclerotic lesions (Hodis et al., 1991). However, there is little information about the efficacy of liposomes as drug carriers for atherosclerotic therapy. Because "long blood circulation," "increase of vascular wall crossing," and "increase of uptake by macrophages and foam cells" are required for efficient delivery of anti-inflammatory drugs to atherosclerotic lesions, the particle size of the liposomes may be an important factor in the delivery of anti-atherosclerotic drugs such as anti-inflammatory drugs to atherosclerotic lesions.

In the present study, the uptake of liposomes by macrophages and foam cells in vitro and the biodistribution of liposomes administered intravenously to atherogenic mice in vivo were investigated to evaluate the influence of particle size.

MATERIALS AND METHODS

The investigation protocol conforms to the Guiding Principles for the Care and Use of Experimental Animals in Hokkaido Pharmaceutical University (published in 1988). All procedures with human subjects were conducted in accordance with the Declaration of Helsinki and informed consent was obtained from all volunteers.

Materials

Egg yolk phosphatidylcholine (PC) was purchased from NOF Co. (Tokyo, Japan) and cholesterol (CH) from Wako Pure Chemicals Co., Ltd. (Osaka, Japan). Dicetylphosphate (DCP) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). [^{3}H]Cholesterylhexadecylether ([^{3}H]CHE) was purchased from NEN Life Science Products, Inc. (Boston, MA, USA). All other reagents were of analytical grade and commercially available.

Preparation of Liposomes

Liposomes were prepared by modification of a conventional lipid film hydration method (Bangham et al., 1965; Funato et al., 1992). Briefly, PC, CH, and DCP in a lipid molar ratio of 7/2/1 were dissolved along with [^{3}H]CHE as a no-exchangeable lipid phase marker (Pool et al., 1982; Derksen et al., 1987) in chloroform/methanol (9/1), followed by evaporation to obtain a thin lipid film. The lipid film was completely hydrated by phosphate buffered saline (PBS, pH 7.4) to obtain liposomes. Lipsomes were extruded through polycarbonate filters with pore sizes of 600, 200, and 100 nm (Nuclepore, CA, USA) five times each. The particle sizes of the liposomes were determined by photon correlation spectroscopy using a Coulter N4 plus a submicron particle analyzer (Coulter Co., Miami, FL). The mean particle sizes of liposomes were 524, 218, and 69.4 nm after extrusion.

Preparation of Oxidized LDL

The low density lipoprotein (LDL) (d = 1.019–1.063 g/mL) was isolated by sequential ultra-centrifugation (100,000 × g) from the plasma of healthy volunteers (Hatch & Less, 1968). The low density lipoprotein (LDL) fraction was dialyzed against PBS (pH 7.4) at 4°C, and then concentrated to 1 mg protein/mL using Centriprep 30 (Millipore Co., Bedford, MA). The oxidized LDL was prepared by incubation with 5 μM $CuSO_4$ for 20 h at 37°C (Tauchi et al., 1999). Oxidation of LDL was confirmed by electrophoresis.

Preparation of Macrophages and Foam Cells

The peritoneal cells from unstimulated mice were suspended at a concentration of 10^6 cells/mL in RPMI

1640 medium (Gibco, BRL, Life Technologies, Rockville, MD). One mL samples of the cell suspension were transferred to the wells of 24-well culture plates (Becton Dickinson, Lincoln Park, NJ), and the plates were incubated for 90 min at 37°C in 5% CO_2. After incubation, non-adherent cells were removed by washing with RPMI 1640 medium, and then the macrophage monolayer was placed in fresh RPMI1640 medium for 20 h at 37 °C in 5% CO_2. The foam cells were induced from the macrophage monolayer by incubation with oxidized LDL (50 μg protein/well) for 20 h at 37°C in 5% CO_2. After incubation, the foam cell monolayer was washed with RPMI 1640 medium to remove the oxidized LDL. The foam cell formation was checked by measuring the cellular CE level. The cellular CE levels of the macrophages and foam cells determined by the methods described previously (Chono et al., 2005) were 15 ± 2 and 125 ± 10 μg/mg cell protein, respectively.

In Vitro Uptake Experiment by Macrophages and Foam Cells

Liposomes were added to macrophages and foam cells, and then the cells were incubated for 1 h at 37°C in 5% CO_2. The concentration of liposomes in medium was 186 nmol total lipid/mL. After incubation, the medium was removed and the cells were washed with RPMI 1640 medium. The cells were extracted with 1 mL 0.1 M NaOH solution. One hundred microliters of the extract and 0.9 mL Hionic-Fluor (Packard BioSci. Co., Meriden, CT, USA) were mixed and stored overnight. The radioactivity in the samples was then determined by scintillation counting. The cell protein level was determined by Coomassie Protein Assay reagent (Pierce Chemical Company, Rockford, IL, USA) using bovine serum albumin as a standard (Branford, 1976).

Preparation of the Normal and Atherogenic Mice

Male ICR mice were obtained from Japan SLC, Inc. (Hamamatsu, Japan), and used for experiments at 6 weeks of age. The normal and atherogenic mice were prepared by the method of Yamaguchi et al. (1993). In brief, the normal and atherogenic mice were fed a normal diet or atherogenic diet containing 10% linoleic acid and 1.5% cholesterol, respectively. Each mouse was given the diet (6 g/day) for 14 weeks. The serum cholesterol levels and aortic CE levels of the mice were determined by the methods described previously (Tauchi et al., 2003). The serum cholesterol levels of normal and atherogenic mice were 165 ± 7 and 294 ± 23 mg/dL, respectively. The aortic CE levels of normal and atherogenic mice were 0.28 ± 0.03 and 0.95 ± 0.08 μg/mg aorta, respectively. The hepatic and splenic weights of atherogenic mice were approximately 1.5 times those of normal mice.

Biodistribution and Pharmacokinetic Analysis

Liposomes were administered intravenously through a tail vein to normal and atherogenic mice. The injected volume was 2.5 mL/kg with the dose corresponding to 46.5 μmol total lipid/kg. At indicated time points after administration, blood was sampled from the jugular vein under ether anesthesia. The aorta, liver, and spleen were collected after blood sampling. An artery from the heart to the diaphragm was chosen as the aorta. These tissues were washed with PBS, blotted dry, and weighed. The radioactivities in blood, aorta, liver, and spleen were assayed as described previously (Kawakami et al., 2001). Ten microliters of blood, aorta, and a small amount of liver and spleen were digested with 1 mL Soluene-350 (Packard BioSci. Co.) by incubating overnight at 45°C. Following digestion, 0.2 mL isopropanol, 0.2 mL 30% hydroperoxide, 0.1 mL 5 M HCl, and 5 mL Hionic-Fluor were added. The samples were stored overnight and the radioactivity of the samples was determined by scintillation counting.

The time-courses of the liposome concentration in blood were analyzed using Eq. (1) with the nonlinear squares program, MULTI (Yamaoka et al., 1981). The Damping Gauss Newton method was chosen as an algorithm, and the inverse of the blood concentration was used as a weight. The pharmacokinetic parameters were calculated as follows:

$$C_b = C_0 \, EXP(-k_e t) \quad (1)$$

$$AUC = C_0/k_e \quad (2)$$

$$MRT = 1/k_e \quad (3)$$

$$CL_{tot} = D_{iv}/AUC \quad (4)$$

$$t_{1/2} = 0.693/k_e \quad (5)$$

$$V_d = D_{iv}/C_0 \quad (6)$$

where k_e is the elimination rate constant, C_0 is the corresponding zero-time blood concentration, t is time, and D_{iv} is the intravenous dose. The AUC, MRT, CL_{tot}, $t_{1/2}$, and V_d are the area under the blood concentration-time curve, the mean residence time, the total body clearance, the half-life, and the distribution volume, respectively.

The aortic clearance (CL_a), hepatic clearance (CL_h), and splenic clearance (CL_s) were calculated as follows:

$$CL_a = X(t)_a / AUC\ (0 \rightarrow t) \quad (7)$$

$$CL_h = X(t)_h / AUC\ (0 \rightarrow t) \quad (8)$$

$$CL_s = X(t)_s / AUC\ (0 \rightarrow t) \quad (9)$$

where $X(t)_a$, $X(t)_h$, and $X(t)_s$ are the aortic distribution, hepatic distribution, and splenic distribution at time t, respectively. AUC(0→t) is the area under the blood concentration-time curve from time 0 to t.

RESULTS

Uptake of Liposomes by Macrophages and Foam Cells

The uptake of liposomes by macrophages and foam cells was examined. The uptake of liposomes by these cells is shown in Fig. 1. The uptake of liposomes was particle size-dependent in both types of cells. In the case of particle sizes of 200 and 500 nm, the uptake by macrophages was higher than that by foam cells.

Biodistribution and Pharmacokinetics of Liposomes

The biodistribution of liposomes administered intravenously to normal and atherogenic mice was examined. The time-courses of blood concentration of liposomes are shown in Fig. 2. In each particle size, the time course of the blood concentration of liposomes in atherogenic mice was similar to that in normal mice. The elimination rate of liposomes from blood increased with the increase in the particle size in both types of animal.

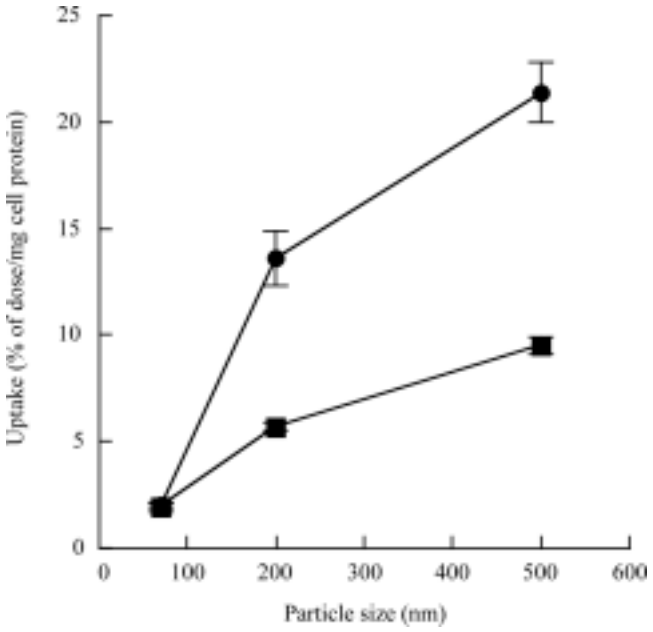

FIGURE 1 **The Uptake of Liposomes by Macrophages and Foam Cells. [^{3}H]CHE Labeled Liposomes (186 nmol Total Lipid/mL in Medium) Were Added to These Cells, and Then the Cells Were Incubated for 1 h at 37°C. After incubation, Cell Extracts Were Collected. Radioactivity in the Cell Extract Was Determined by Scintillation Counting. Cell Protein Was Also Determined As Described in Materials and Methods. Each Value Represents the Mean ±S.E. of Four Experiments. Symbols; (•), Macrophages; (■), Foam Cells.**

The tissue distribution of liposomes is shown in Fig. 3. In each particle size, the aortic distribution of liposomes in atherogenic mice increased in comparison with that in normal mice, although the hepatic and splenic distribution of liposomes in atherogenic mice was lower than that in normal mice. In normal mice, the aortic distribution of liposomes was decreased with the increase in the particle size. On the other hand, in atherogenic mice, the aortic distribution of liposomes with a particle size of 200 nm was the highest in comparison with the other sizes. The hepatic and splenic distribution of liposomes increased with the increase in the particle size in both types of animal.

The pharmacokinetic parameters of the liposomes are summarized in Fig. 4. The AUC, MRT, CL_{tot}, $t_{1/2}$, V_d, CL_h, and CL_s were size-dependent in both types of animal. The CL_a in atherogenic mice was size-independent, although the CL_a in normal mice was size-dependent. In brief, the aortic distribution and

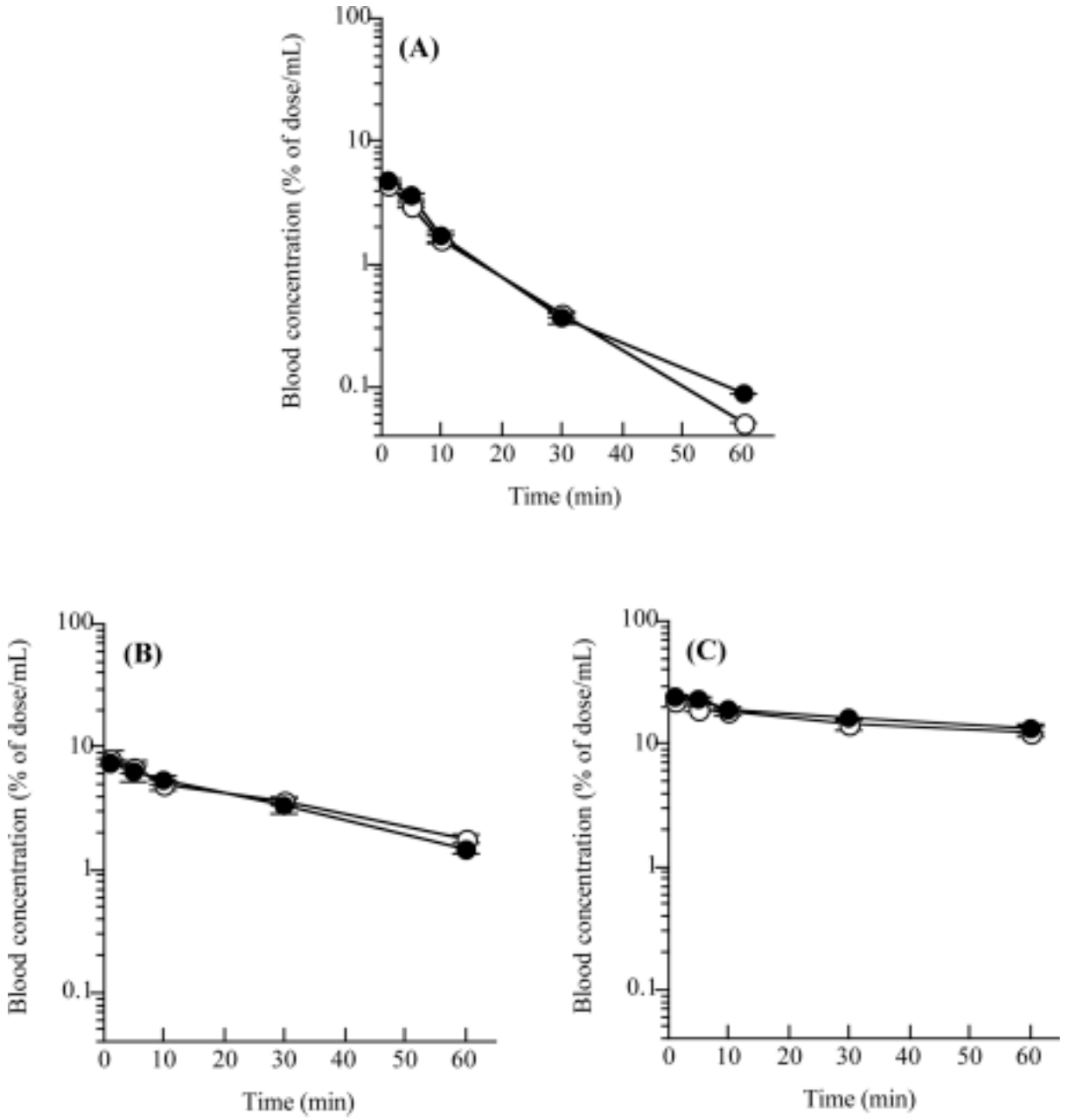

FIGURE 2 **The Time-Courses of the Blood Concentration of Liposomes in Normal and Atherogenic Mice. [^{3}H]CHE Labeled Liposomes (46.5 μmol Total Lipid/kg) Were Administered Intravenously to Mice. At Each Time Point, Blood Was Collected. Radioactivity in the Blood Was Determined by Scintillation Counting. The Particle Sizes of the Liposomes Were (A), 500 nm; (B), 200 nm; (C), 70 nm. Each Value Represents the Mean ± S.E. of Four Experiments. Symbols: (○), Normal Mice; (•), Atherogenic Mice.**

systemic distribution of liposomes in atherogenic mice were, respectively, size-independent and-dependent.

Relationship Between Aortic Distribution and Uptake In Vitro

The relationship between the aortic distribution in atherogenic mice and uptake by macrophages and foam cells in vitro was evaluated. The relationship between the CL_a in atherogenic mice and uptake by these cells in vitro are shown in Fig. 5. The CL_a did not correspond to the uptake by both types of cell in vitro.

Relationship Between Biodistribution and Blood Circulation

The relationship between the biodistribution and blood circulation in atherogenic mice was evaluated.

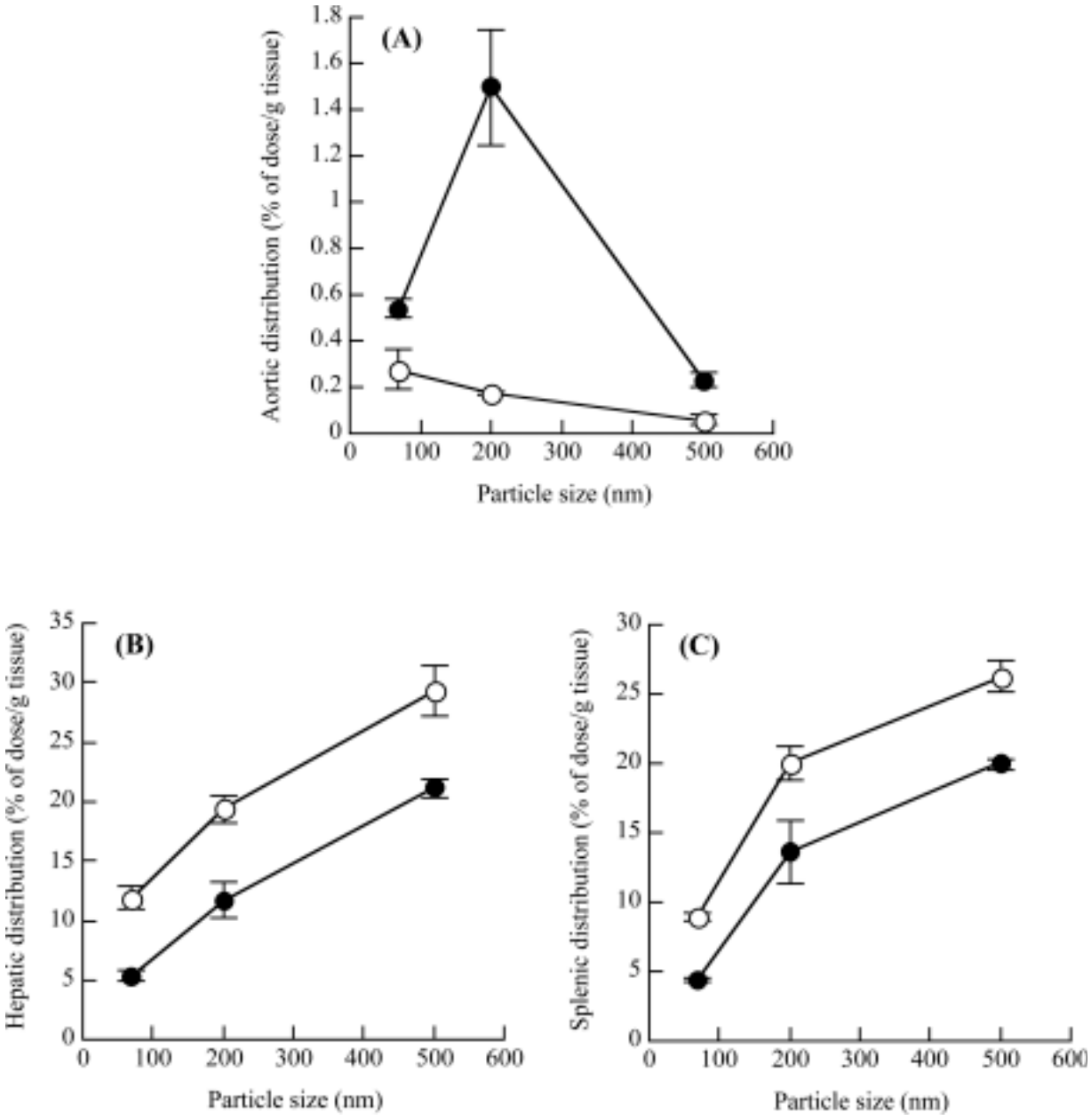

FIGURE 3 **The Tissue Distribution of Liposomes in Normal and Atherogenic Mice. [^{3}H]CHE Labeled Liposomes (46.5 μmol Total Lipid/kg) Were Administered Intravenously to Mice. At 1 h After Administration, Aorta, Liver, and Spleen Were Collected. Radioactivity in the Aorta, Liver, and Spleen Was Determined by Scintillation Counting. The Tissue Distribution of Liposomes Was (A), Aorta; (B), Liver; (C), Spleen. Each Value Represents the Mean ± S.E. of Four Experiments. Symbols: (○), Normal Mice; (•), Atherogenic Mice.**

The relationship between the tissue clearance and MRT in atherogenic mice is shown in Fig. 6. Although the CL_h and CL_s decreased with the increase in the MRT, the CL_a did not correspond to the MRT.

DISCUSSION

Liposomes accumulate in atherosclerotic lesions in experimental atherosclerosis (Hodis et al., 1991). However, influence of the particle size of liposomes on the accumulation in the atherosclerotic lesions has not been examined until now. Therefore, in this study, the influence of the particle sizes of liposomes on the biodistribution in atherogenic mice was examined to obtain information to achieve efficient drug delivery to atherosclerotic lesions. In addition, the biodistribution of liposomes in atherogenic mice was compared with that in normal mice.

Initially, liposomes of three different particle sizes (500, 200, and 70 nm) were prepared, and the uptake of

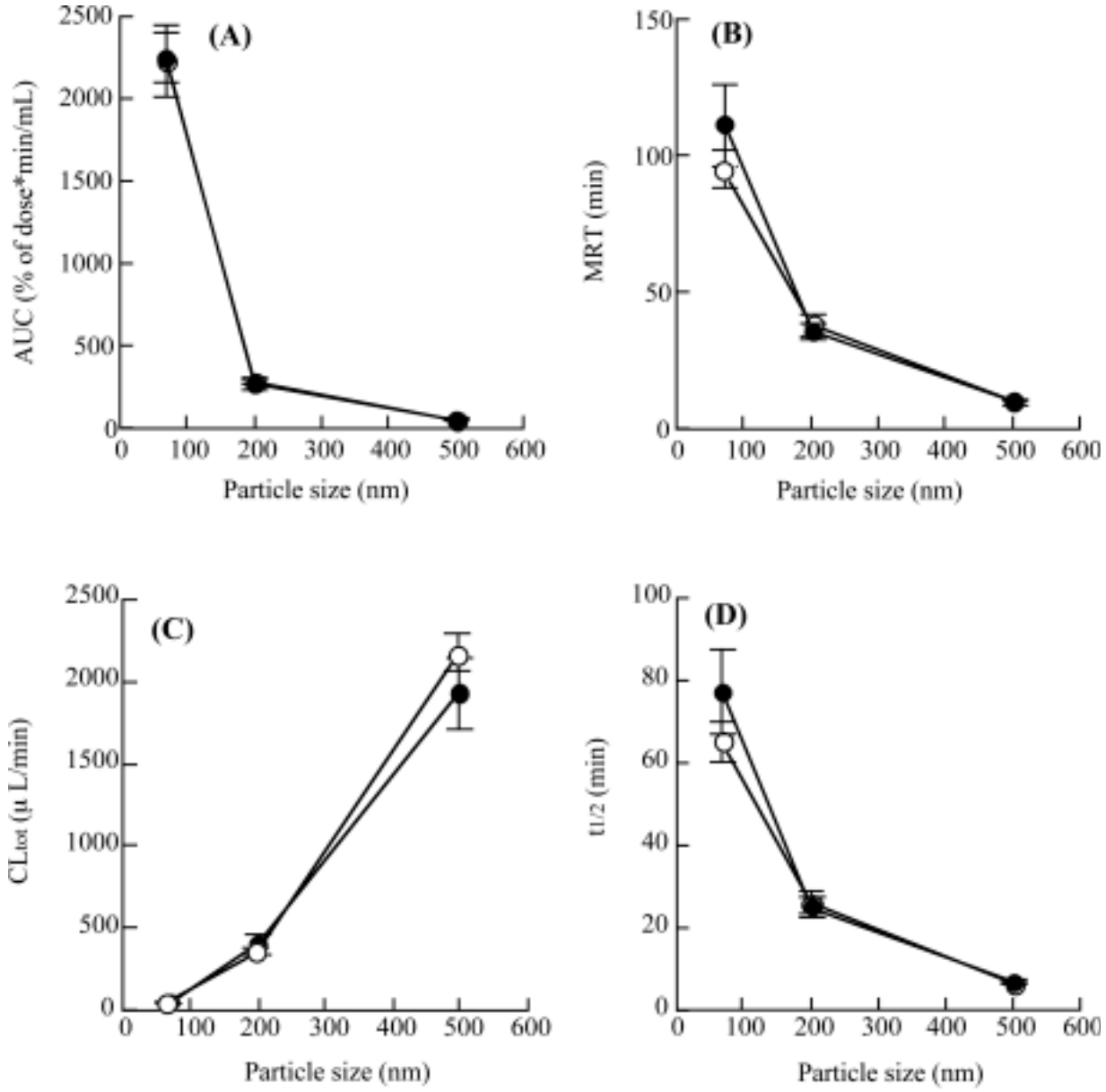

FIGURE 4 **The Pharmacokinetic Parameters of Liposomes in the Normal and Atherogenic Mice. [^{3}H]CHE Labeled Liposomes (46.5 μmol Total Lipid/kg) Were Administered Intravenously to Mice. Blood Was Collected at Each Time Point. At 1 h After Administration, the Aorta, Liver, and Spleen Were Collected. Radioactivity in the Blood and Each Tissue Was Assayed. The Pharmacokinetic Parameters Were Calculated as Described in Materials and Methods. The Pharmacokinetic Parameters Were (A), AUC; (B), MRT; (C), CL_{tot}; (D), $t_{1/2}$; (E), V_d; (F), CL_a; (G), CL_h; (H), CL_s. Each Value Represents the Mean ± S.E. of Four Experiments. Symbols: (○), Normal Mice; (•), Atherogenic Mice.**

liposomes by macrophages and foam cells as target cells in vitro was investigated. The uptake of liposomes by macrophages was size-dependent (Fig. 1) as reported previously (Hsu & Juliano, 1982). The uptake by foam cells was also size-dependent (Fig. 1). Although there is no report about the uptake of liposomes by foam cells, it was clear that the uptake of liposomes by foam cells was size-dependent over the range 70–500 nm. In each particle size, the uptake of liposomes by foam cells tended to be lower than that by macrophages (Fig. 1A and B). These results suggest that the uptake capacity of liposomes by foam cells diminishes following excessive cellular CE accumulation. Although the uptake mechanism of liposomes by macrophages and foam cells was not investigated in this study, according to a previous report (Harashima et al., 1994), it is supposed that the uptake of liposomes is mediated by phagocytosis (500 nm and 200 nm) and endocytosis (70 nm).

Secondly, the biodistribution of liposomes administered intravenously to atherogenic mice and normal

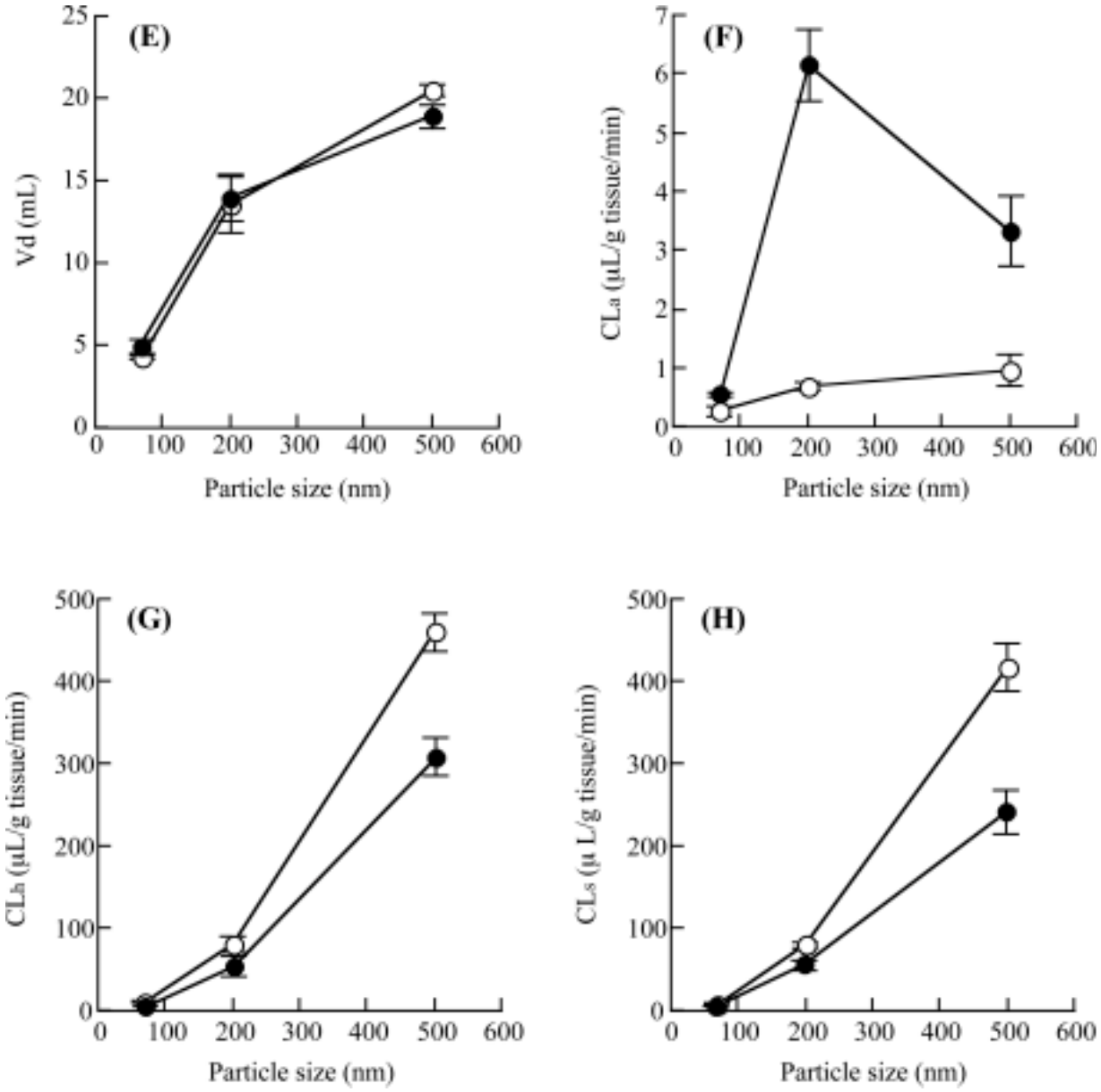

FIGURE 4 **(Continued).**

mice was investigated to evaluate the influence of the particle size of liposomes. The elimination rates of liposomes of each particle size from the blood circulation in the atherogenic mice were similar to those in normal mice (Fig. 2). This indicates that the blood concentration of liposomes is unaffected by the serum cholesterol level. The hepatic and splenic distributions in both types of mice were size-dependent (Fig. 3B and 3C). This agrees with some earlier reports (Harashima et al., 1994, 1996; Houng et al., 1998, 1999). The hepatic and splenic distributions per gram tissue weight of the atherogenic mice were reduced approximately two-thirds in comparison with the normal mice (Fig. 3B and 3C). However, the hepatic and splenic distribution per whole tissue weight of the atherogenic mice was similar to that in the normal mice, because the hepatic and splenic weight of the athrogenic mice was approximately 1.5 times that of the normal mice. Thus, the elimination rate of liposomes from blood in atherogenic mice appears to be similar to that in normal mice. These data indicate that the systemic distribution of liposomes in atherogenic mice is similar to that in normal mice. The hepatic and splenic distribution of liposomes in the normal and atherogenic mice corresponded to the uptake by macrophages in vitro (Figs 1A, 3B, and 3C).

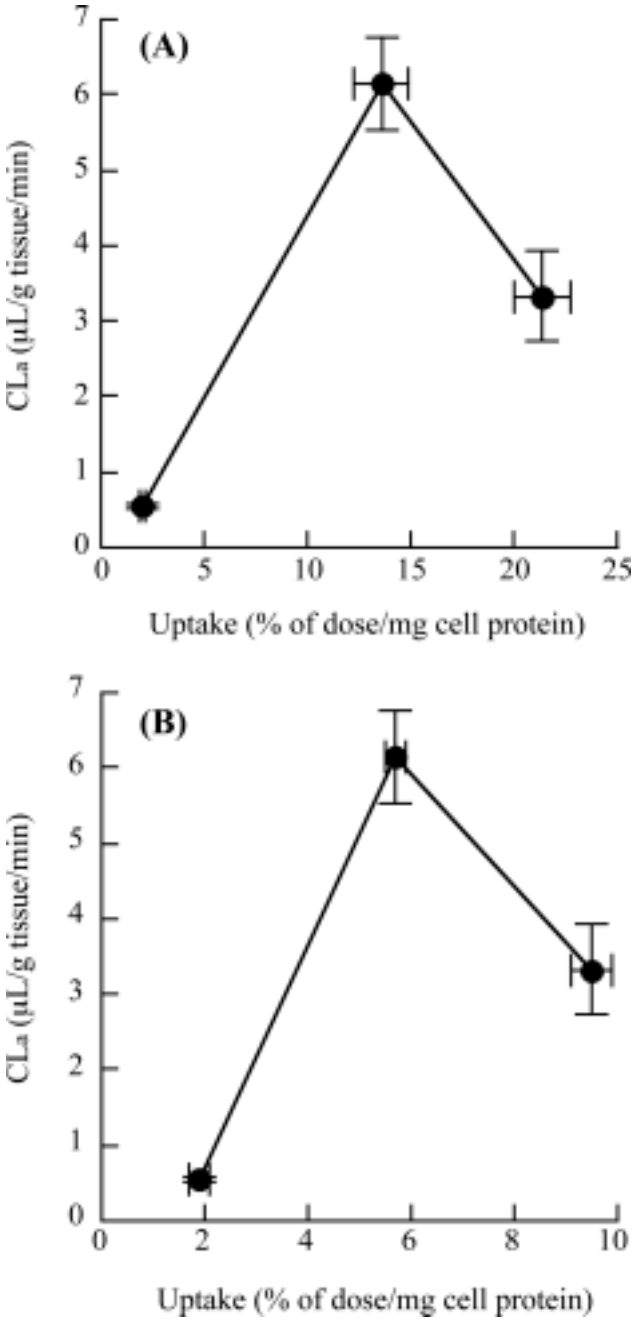

FIGURE 5 **The Relationship Between the CL_a in Atherogenic Mice and Uptake by Macrophages and Foam Cells In Vitro. The CL_a in Atherogenic Mice Shown in Fig. 4(F) and the Uptake by Macrophages and Foam Cells Shown in Fig. 1 Were Plotted. The Relationships Were (A), CL_a and Uptake by Macrophages; (B), CL_a and Uptake by Foam Cells.**

These results indicate that the hepatic and splenic distributions are influenced by the size-dependent uptake by Kupffer cells and splenic macrophages. The aortic distribution of liposomes of each particle size in the atherogenic mice was higher than that in the normal mice (Fig. 3A). In the case of atherosclerotic lesions, the tight junctions among vascular endothelial cells are loose (Ross, 1999). The aortic distribution of liposomes in the atherogenic mice may be increased by enhanced vascular permeability. The aortic distribution of 200 nm liposomes was the highest in comparison with the other sizes in the atherogenic mice (Fig. 3A). The AUC, MRT, CL_{tot}, $t_{1/2}$, V_d, CL_h, and CL_s in atherogenic mice were size-dependent, althought the CL_a was size-independent (Fig. 4). The atherosclerotic lesion is a very fine tissue although it is similar to that in the reticuloendothelial system. These pharmacokinetic parameters indicate that the aortic distribution of liposomes does not influence the systemic biodistribution in atherogenic mice. Surprisingly, the aortic distribution of liposomes in the atherogenic mice did not correspond to the uptake by the macrophages and foam cells in vitro (Fig. 5). These results suggest that there is an optimal size for the delivery of liposomes to atherogenic lesions. The CL_a did not correspond to the MRT in the atherogenic mice (Fig. 6A). This indicates that the blood circulation does not influence the distribution of liposomes to atherosclerotic lesions in the atherogenic mice. Based on this study, we considered the aortic distribution of liposomes in the atherogenic mice is as follows: The 500 nm liposomes cannot cross the vascular wall because they are too large and, therefore, the aortic distribution is low. The 200 nm liposomes cross the vascular wall and are taken up by the macrophages and foam cells in the atherosclerotic lesions and, therefore, the aortic distribution is high. The 70 nm liposomes can cross the vascular wall, but they are not taken up by the macrophages and foam cells in the atherosclerotic lesions and, therefore, the aortic distribution is low. The CL_a of 200 nm liposomes was the highest in comparison with liposomes of other sizes in the atherogenic mice (Fig. 4F). This study shows that the CL_a of 200 nm liposomes is based on the high uptake rate by the macrophages and foam cells in the atherosclerotic lesions.

In conclusion, in this study, the biodistribution of liposomes administered intravenously to atherogenic mice was investigated to evaluate the influence of the particle sizes of liposomes, and we showed that 200 nm liposomes were distributed to a greater extent than the other sizes in the atheroscleritic lesions. The high distribution of the 200 nm liposomes in the atherosclerotic lesions may be due to the uptake by the macrophages and foam cells. We except that 200 nm liposomes will be useful as drug carriers to deliver anti-atherosclerotic drugs such as anti-inflammatories

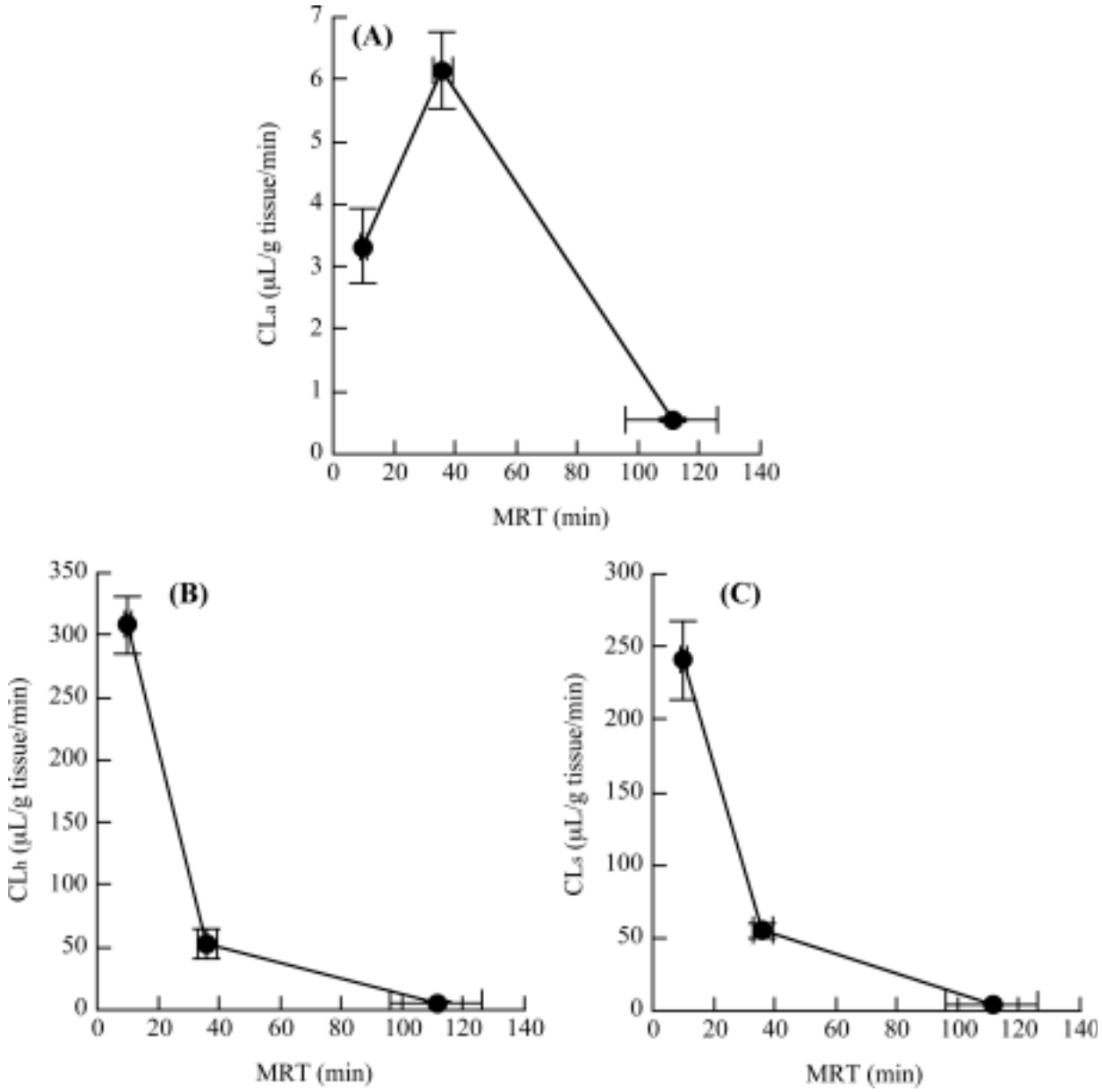

FIGURE 6 **The Relationship Between the Tissue Clearance and MRT in Atherogenic Mice. The CL_a, CL_h, and CL_s in Atherogenic Mice and the MRT Shown in Fig. 4 were Plotted. The Relationships Were (A), CL_a and MRT; (B), CL_h and MRT; (C), CL_s and MRT.**

(Bailey & Buther, 1973; Bailey et al., 1979; Makheja et al., 1989; Naito et al., 1992; Asai et al., 1993), ACAT inhibitors (Kelley et al., 1988), lactoferrin (Kajiwara et al., 1994), and HMG-CoA reductase inhibitors (Paoletti et al., 1997). In the future, further evaluation of liposomes containing anti-atherosclerotic drugs for atherosclerotic therapy should be very interesting.

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