Effect of Specific Binding of High Density Lipoprotein to Eel Hepatocytes on Their Secretion of Lipoprotein

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Key words: high-density lipoprotein/hepatocytes/eel/receptor/lipoprotein/fluorescent lipophilic dye

ABSTRACT. Specific binding of eel serum high-density lipoprotein (HDL) to eel hepatocytes was demonstrated by using a synthesized fluorescent lipophilic dye. HDL binding was inhibited by the addition of unlabeled HDL. The binding of HDL to the hepatocytes was saturated at concentrations over 100 μg HDL protein/ml and Kd value was 20 μg HDL protein/ml. A fluorescent photomicrograph of the cultured eel hepatocytes which were incubated with the dye showed the bright, circumferential plasma membranes stained with the dye. ¹²⁵I-HDL was incorporated into the acid insoluble- and soluble-fractions of the cultured hepatocytes during incubation at 28°C for 1 h. There are three remarkable characteristics of the effect of HDL on the cultured hepatocytes. One is that the addition of HDL to the hepatocytes induced the efflux of cholesterol, triacylglycerol, and phospholipid from the hepatocytes. The second characteristic is that the efflux of the intracellular lipids was carried out with very-low-density-like or chylomicron-like lipoprotein secreted by the hepatocytes. The third characteristic is that HDL specifically stimulated the synthesis of the lipoprotein and had no effect on the synthesis of intracellular proteins and the secreted proteins except for the lipoprotein.

We reported that cultured eel hepatocytes secreted only one kind of lipoprotein (1). The secreted lipoprotein has some unique characteristics, which are not observed in mammalian lipoproteins. Its main apoproteins consist of apo A and B and the main lipid is triacylglycerol. Since its density is between chylomicron and very-low-density lipoprotein (VLDL), we call it VLDL-(or chylomicron-) like lipoprotein. Although the main composition of eel serum lipoproteins is high-density lipoprotein (HDL), the cultured hepatocytes do not secrete HDL. In another previous report we investigated the effect of serum HDL on the synthesis of the lipoprotein in the cultured hepatocytes (2). Serum HDL had a stimulatory effect on the synthesis of the secreted lipoprotein. We further investigated the effect of HDL on the synthesis and secretion of the lipoprotein by cultured eel hepatocytes.

HDL is thought to mediate the process of reverse cholesterol transport from peripheral tissues to liver where it is catabolized. Cholesterol efflux by HDL has been recognized in some cultured cells such as epithelial cells of intestine (3, 4) and fibroblasts (5). In these findings, HDL binding protein, or HDL receptor, on the plasma membrane of the cells always takes part in the process of reverse cholesterol transport, though the exact function of HDL receptor is still unclear (6-8).

We investigated whether eel serum HDL could bind to eel hepatocytes specifically, and mediate the process of reverse cholesterol transport. Using HDL labeled with a fluorescent dye, specific binding of the HDL to the hepatocytes was investigated. It was found that the specific binding of HDL to the hepatocytes induced an efflux of cholesterol, phospholipid, and triacylglycerol from the cells. Furthermore, the efflux of intracellular lipids was carried out by a VLDL-(or chylomicron-) like lipoprotein, not by HDL which took part in the cholesterol efflux in mammalian cells (3-5).
MATERIALS AND METHODS

Materials. Na\(^{125}\)I (585 MBq/\mu g iodine) and L-[\(^{14}\)C] leucine (11.5 GBq/mmol) were obtained from Amersham Japan. Aniline, 1-bromopentadecane, N,N-dimethylformamide, 4N-dimethylpyridinium iodide, and piperidine for synthesis of N,N-dipentadecylaminostyrylpyridinium iodide (di-15-ASP) were purchased from Nakarai Tesque, Tokyo Kasei Kogyo Co., Wako Pure Chemical Ind., Aldrich, and Wako Pure Chemical Ind., respectively. Collagenase for dispersion of eel hepatocytes and insulin from bovine pancreas were obtained from Wako Pure Chemical Ind. Williams’ medium E and fetal bovine serum were from Flow Laboratories Inc. and Gibco BRL, respectively. Dextran sulfate cellulose was a kind gift from Kanegafuchi Chemical Co. Other chemicals were obtained from Wako Pure Chemical Ind. or Nakarai Tesque.

Eels. Cultured eels (Anguilla japonica) weighing 200–250 g were purchased from Sueyoshi Co. in Kagoshima. They were starved for several days before experiments.

Fractionation of lipoproteins. The HDL was isolated from eel serum by density gradient ultracentrifugation (9). An equal amount of 0.75% NaCl was laid over the eel serum containing KBr (0.4 g/ml) in a centrifuge tube and the tube was centrifuged at 50,000 rpm for 20 h in a 70Ti rotor using an Optima L-80 model centrifuge (Beckman). After centrifugation, 40 fractions were collected from the bottom of the tube with a peristaltic pump and a fraction collector. HDL with densities between 1.1458 and 1.1200 g/ml was collected. Its apoproteins consisted of apo A-I and A-II as shown in Fig. 1-A.

The lipoprotein secreted by cultured eel hepatocytes was also isolated by density gradient ultracentrifugation as described previously (1).

Synthesis of N,N-dipentadecylaminostyrylpyridinium iodide (di-15-ASP) and the labelling of HDL with di-15-ASP. Di-15-ASP was synthesized according to the method of Corsetti et al. (10) and synthesized di-15-ASP was identified by \(^1\)H-NMR and infrared absorption spectrum. The labelling of HDL with di-15-ASP was also carried out by the method of

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**Fig. 1.** SDS-PAGE of the eel serum HDL and the excitation and emission spectra of the HDL labeled with N,N-dipentadecylaminostyrylpyridinium iodide (di-15-ASP). (A) The HDL was isolated by density gradient ultracentrifugation and 10 μg protein of the HDL was applied to a 5–17.5% acrylamide gradient slab gel. (B) The isolated HDL was labeled with di-15-ASP as described in "Materials and Methods". The excitation (monitored at 555 nm, I) and emission (excited at 470 nm, II) spectra of 5.4 μg protein of di-15-ASP-HDL/ml PBS was measured at room temperature.
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Corsetti et al. (10). HDL (0.5 mg protein/ml) was incubated with 75 \( \mu \)g di-15-ASP in 137 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl\(_2\), 3.3 mM NaH\(_2\)PO\(_4\), 5.6 mM glucose, and 20 mM HEPES (pH 7.4) buffer containing 0.12% bovine serum albumin (BSA) at room temperature for 3 h under the dark. After incubation, the density of the solution was adjusted to 1.200 g/ml with KBr and the solution was centrifuged at 50,000 rpm for 20 h at 15°C. The top layer of di-15-ASP-HDL was recovered with a Pasteur pipette and dialyzed against 0.9% NaCl—0.01% EDTA (pH 7). Free di-15-ASP in di-15-ASP-HDL was further removed by a dextran sulfate cellulose column (1 ml bed vol.) preliminarily equilibrated with phosphate-buffered saline (PBS). Free di-15-ASP was adsorbed on the column and di-15-ASP-HDL was passed through the column. Figure 1-B shows the excitation (monitored at 555 nm) and emission (excited at 470 nm) spectra of di-15-ASP-HDL (5.4 \( \mu \)g HDL protein/ml PBS) at room temperature. Fluorescence intensity and spectra of di-15-ASP-HDL were not changed for at least one month. The excitation and emission spectra was measured by a spectrofluorometer (Nihon Bunko FP-777).

Iodination of HDL. Iodination of HDL was carried out according to the method of Fielding et al. (11). HDL of 1 mg protein in 1 ml of 150 mM NaCl-0.24 mM EDTA (pH 7.4) was mixed with 10 \( \mu \)l of Na\(^{125}\)I (37 MBq), 300 \( \mu \)l of 1 M glycine-NaOH buffer (pH 10.0), and 210 \( \mu \)l of 1.32 mM iodine monochloride and the mixture was kept in ice water for 5 min. The mixture was then applied to a Sephadex G-50 column (1.5 x 10 cm) equilibrated with 150 mM NaCl-0.24 mM EDTA (pH 7.4). \(^{125}\)I-HDL was eluted and dialyzed against the same buffer. \(^{125}\)I-HDL was obtained with specific activity of 188 cpm/ng protein and the radioactivity recovered in a fraction of \(^{125}\)I-HDL precipitated by 5% trichloroacetic acid (TCA) was 97% of total radioactivity. Radioactivity was measured by a \( \gamma \)-ray scintillation counter (Packard 500C).

Preparation of freshly isolated hepatocytes and primary culture of eel hepatocytes. Isolated eel hepatocytes were prepared by collagenase digestion of a perfused liver as reported previously (12).

Isolated hepatocytes of 2 x 10\(^7\) and 10\(^7\) were inoculated into 7 ml and 2 ml of culture medium in a 10- and 6-cm plastic dish (Falcon), respectively. Plastic dishes were precoated with fibronectin isolated from horse serum by affinity chromatography using gelatin-Sepharose (13, 14). The culture medium consisted of Williams’ medium E (WE medium) containing 23 mM NaHCO\(_3\), 5% fetal bovine serum (FBS) and 0.16 \( \mu \)g insulin as described previously (1, 15). Hepatocytes were cultured at 28°C under saturated humidity in 5% CO\(_2\) in an air atmosphere.

Incubation of di-15-ASP-HDL with freshly isolated hepatocytes. Freshly isolated hepatocytes of 2 x 10\(^7\) cells/ml were incubated with di-15-ASP-HDL (25–200 \( \mu \)g protein/ml) with or without unlabeled HDL in WE medium at 0°C for 2 h under the dark. After incubation, the hepatocytes were washed three times with cold PBS and suspended with 0.5 ml of cold PBS. The cell suspension was kept in ice water and filtered with nylon gauze before analysis by a flow cytometer.

Incorporation of \(^{125}\)I-HDL into the cultured hepatocytes. Eel hepatocytes in a 6-cm dish were cultured in 2 ml WE medium for 9 days. On day 10 they were washed twice with 2 ml of FBS- and insulin-free WE medium and \(^{125}\)I-HDL and FBS- and insulin-free WE medium were added to hepatocytes. After incubation at 28°C for 1 h, hepatocytes were washed three times with 0.5 ml PBS and 1 ml of 0.1 N NaOH was added to the cells. The lysed cells were placed a glass tube and the dish was washed with 0.5 ml of 0.1 N NaOH. The washing solution was transferred to the same test tube and 0.4 ml of 50% TCA was added. After centrifugation at 3,000 rpm for 10 min, the precipitate was washed twice with 0.5 ml of 5% TCA. The radioactivity of the combined supernatant was determined as an acid soluble-fraction. The precipitate was dissolved with 1 ml of 0.1 N NaOH for protein assay and radioactivity measurement as an acid insoluble-fraction.

Incorporation of \(^{14}\)C-leucine into lipoprotein secreted by cultured hepatocytes and extraction of lipid from the hepatocytes. Hepatocytes in a 10-cm dish were cultured for 5 days in WE medium. On day 6 the medium was removed and the cells were washed three times with 2 ml of PBS. Hepatocytes were then incubated with or without HDL (2.6 mg protein /ml) and \(^{14}\)C-leucine (55.5 KBq/dish) in 7 ml of FBS- and insulin-free WE medium. After incubation at 28°C for 24 h, the medium was recovered and hepatocytes were washed with 3 ml of PBS. The medium and PBS washing the cells were combined and the combined was centrifuged at 3,000 rpm for 5 min. The supernatant was applied to a Sephadex G-25 column (1.6 x 15 cm) equilibrated with PBS. Protein fractions were pooled as extracellular proteins and 6.8 g KBr was dissolved in the 17 ml of the pooled protein fraction for lipoprotein fractionation.

After hepatocytes were washed with PBS, 0.75 ml of methanol was added and the cells were scraped with a silicon rubber policeman. The dish was washed twice with 0.65 ml of methanol, and 0.63 ml of chloroform and 0.5 ml of water were added to the combined methanol and scraped cells in a glass tube. After shaking up to 2 h, the glass tube was centrifuged at 3,000 rpm for 10 min. The supernatant was transferred to another glass tube and the precipitate was reextracted with 2.38 ml of chloroform-methanol-water (1 : 2 : 0.8). After centrifugation, the supernatant was combined. The precipitate was dried under N\(_2\) stream and dissolved in 2 ml of 0.1 N NaOH for radioactivity measurement and protein assay as intracellular protein. Chloroform (1.25 ml) and water (1.25 ml) were added to the combined supernatant and the mixture was centrifuged. The chloroform layer was washed and evaporated to dryness under N\(_2\) stream. Lipids extracted from hepatocytes were dissolved with 200 \( \mu \)l of 5% Triton X-100 and used for analysis of lipids. Radioactivity was measured by a liquid scintillation counter (Aloka LSC-3500).

Protein and lipid assay. Protein was assayed by the method of Bradford (16) in the range of 0.1–1.5 mg/ml and by the method of Smith et al. (17) in the range of 1–20 \( \mu \)g/ml. Tri-
acetylglucerol, phospholipid, total and free cholesterol contents of lipoproteins and lipids extracted from hepatocytes were determined by means of enzymatic kits (Kyowa Medex) known as Determiner TG-S555, Determiner PL, Determiner TC-555, and Determiner FC-555, respectively.

SDS-PAGE and autoradiography. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out by the method of Laemmli (18). 14C-Apoproteins of the lipoprotein secreted by cultured hepatocytes were resolved by electrophoresis on 5–17.5% acrylamide gradient slab gels. After electrophoresis, the gels were dried and the radioactivity of resolved proteins on the gels were determined by a Fujix BAS1000 Bio-imaging Analyzer (Fuji Photo Film Co.). 14C-Methylated proteins (Amersham Japan) were used as molecular weight markers. They were myosin (200,000), phosphorylase b (97,400), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and lysozyme (14,300).

Proteins on gel were stained in 0.25% (w/v) Coomassie Brilliant Blue R-250 in 45% ethanol-10% glacial acetic acid and destained with 25.5% ethanol-8% glacial acetic acid. Calibration proteins for molecular weight in the range of 14,000 to 94,000 (Pharmacia) were used.

Flow cytometry. The isolated hepatocytes stained by di-15-ASP-HDL were analyzed with an Epics Elite flow cytometer (Coulter Electronics) equipped with an argon-ion laser. Excitation was 500 mV at 488 nm. Optimum signal to noise ratio for collection of the di-15-ASP-HDL fluorescence was obtained using two filters (550 DL and 600 DL). Fluorescence channel number was determined by subtracting the mean fluorescence channel number of unstained cells from that of stained cells.

Microscopy. Fluorescence microscopy of cultured eel hepatocytes on cover slips was performed by an epifluorescence microscope (Olympus BH2-RFC) with a filter of BP490 for excitation. Eel hepatocytes of 106 cells were cultured in a 3.5 cm plastic dish (Celldesk LF, Sumitomo Bakelite Co.) in which a plastic cover slip with low fluorescence was laid. The hepatocytes were cultured in 1.5 ml WE medium and on day 3 they were washed twice with cold WE medium. Fresh cold medium and di-15-ASP-HDL (25 µg protein/ml) were then added to the hepatocytes and the cells were incubated at 0°C for 2 h under the dark. After incubation, hepatocytes were washed with cold PBS and the cells on a plastic cover slip was kept in ice water until microscopic examination.

**RESULTS**

**The binding of di-15-ASP-HDL to freshly isolated hepatocytes.** When di-15-ASP-HDL was incubated at the concentration of 100 µg protein/ml with freshly isolated hepatocytes at 0°C for 2 h, the fluorescence of the hepatocytes increased by one order than that of unstained hepatocytes as shown in Fig. 2. This increase of fluorescence disappeared by addition of unlabeled HDL of 800 µg protein/ml to the incubation mixture.
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of di-15-ASP-HDL was saturated at concentrations over 100 µg HDL protein/ml and Kd was about 20 µg HDL protein/ml.

**Fluorescence microscopy of cultured hepatocytes incubated with di-15-ASP-HDL.** Cultured hepatocytes were incubated with 25 µg di-15-ASP-HDL protein/ml at 0°C for 2 h. After incubation, a cover slip was taken from a dish and the hepatocytes on it were observed by an epifluorescence microscope. A fluorescent photomicrograph of the hepatocytes demonstrates bright, circumferential plasma-membranes stained with di-15-ASP-HDL (Fig. 4). These results strongly suggest the presence of specific binding sites for HDL on the plasma membranes of eel hepatocytes.

**Incorporation of 125I-HDL by cultured hepatocytes.** 125I-HDL was linearly incorporated into the acid insoluble- and soluble-fractions of the cultured hepatocytes dependent on concentrations indicated (Fig. 5). Though the amount of 125I-HDL in the acid soluble fraction, which was about 7% of the total incorporated amount, was relative low compared with that in the acid insoluble-fraction, the appearance of the radioactivity in the acid soluble fraction shows that 125I-HDL was incorporated into cultured eel hepatocytes and decomposed within the cells during 1 h. In the presence of an excess of unlabeled HDL, the incorporation of 125I-HDL was extremely inhibited as shown in Fig. 5.

**Effect of HDL on the synthesis of lipoprotein by cultured hepatocytes.** The hepatocytes were incubated with 14C-leucine and serum HDL at concentration of 2.6 mg protein/ml in FBS- and insulin-free WE medium at 28°C for 24 h. The incorporation of 14C-leucine into the total secreted and the intracellular proteins in the presence of HDL were almost the same as those in the absence of HDL (Table I). The lipoprotein in the secreted proteins was fractionated by density gradient ultracentrifugation and pooled from fractions 37 to 40 shown in Fig. 6. Lipoprotein synthesis by cultured hepatocytes in the presence of HDL was 143% of that synthesized by the cells in the absence of HDL. Serum HDL had a specifically stimulatory effect on the synthesis of the lipoprotein in cultured hepatocytes. It was re-

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**Fig. 4.** Fluorescent photomicrogram of the cultured hepatocytes incubated with di-15-ASP-HDL. The hepatocytes of 10⁶ cells/dish were cultured in 1.5 ml of WE medium for 2 days. On day 3 the cells were washed twice with cold fresh WE medium and incubated with 25 µg di-15-ASP-HDL protein/ml at 0°C for 2 h in the dark. The cells were washed with cold PBS after incubation and the cells on a plastic coverslip were examined with an epifluorescence microscope (Olympus BH2-RFC), Magnification, × 624

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Fig. 5. Incorporation of $^{125}$I-HDL into the cultured hepatocytes. The hepatocytes of $10^7$ cells/dish (6 cm) were cultured for 8 days in 2 ml of WE medium at 28°C and then washed with FBS- and insulin-free WE medium twice. The hepatocytes were incubated with $^{125}$I-HDL at the indicated concentration in FBS- and insulin-free WE medium at 28°C for 1 h. When the cells were incubated in the presence of cold HDL, its concentration was 100 times higher than that of $^{125}$I-HDL. After incubation, the cells were divided into the acid insoluble and soluble fractions as described in "Materials and Methods" and radioactivities of both fractions were measured. Specific activity of $^{125}$I-HDL was 188 cpm/ng HDL protein. (△) and (●), the acid insoluble fraction in the absence and the presence of cold HDL; (○) and (▲), the acid soluble fraction in the absence and the presence of cold HDL.

The hepatocytes synthesized only one kind of lipoprotein with or without serum HDL (Fig. 6A and B). The apolipoproteins of both secreted lipoproteins contained apo A and B (Fig. 6a and b). The density of the secreted lipoprotein was below 1.05 g/ml. The addition of serum HDL to the cultured hepatocytes did not induce HDL synthesis.

Effects of HDL on rate of lipoprotein secretion and lipid composition of secreted lipoprotein and intracellular lipids. The lipid and protein contents of the secreted lipoprotein fractions shown in Fig. 6 were assayed. It was found that the rate of the secretion of the lipoprotein by the cultured hepatocytes incubated with HDL was twice that by the hepatocytes incubated without HDL. The amount of the secreted lipoprotein of the former during 24 h incubation was 317 μg lipoprotein/mg cell protein, while that of the latter was 160 μg lipoprotein/mg cell protein (Table II-A). Characterization of the composition of the lipoprotein secreted in the presence of HDL was as follows; the ratios of triacylglycerol, free cholesterol, cholesterol ester, phospholipid, and protein were 53.9, 5.0, 6.2, 21.6, and 13.3%, respectively (Table II-A). The synthesized lipoprotein by the hepatocytes with or without HDL was assumed to be the same lipoprotein as reported previously (1), judging from the lipid and apoprotein compositions described above. Among the lipids transported from the hepatocytes, quantitatively triacylglycerol was the one most transported due to the lipid composition of the secreted lipoprotein. However, the rates of the cholesterol ester and free cholesterol efflux were increased by HDL 5-fold and 3.7-fold, respectively, than those in the hepatocytes incubated without HDL. The rates of phospholipid and triacylglycerol efflux were also stimulated by HDL 3-fold and 1.7-fold, respectively (Table II-A). These results indicated that HDL stimulated the efflux of cholesterol, triacylglycerol, and phospholipid from the hepatocytes. It is known that the primary effect of HDL in mammalian cells is cholesterol efflux from cells (3-5). However, in eel hepatocytes HDL seems to stimulate the efflux of not only cholesterol but also phospholipid and triacylglycerol from eel hepatocytes.

Addition of HDL increased the intracellular cholesteryl ester and phospholipid, but did not change or only slightly decreased the intracellular free-fatty acids, triacylglycerol, and free cholesterol as shown in Table II-B.

**DISCUSSION**

Although the function of HDL receptor is still unclear (6-8), its presence on plasma membranes of cells...
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Fig. 6. Lipoprotein fractionation of the secreted proteins by density gradient ultracentrifugation and SDS-PAGE of the fractionated lipoprotein. The hepatocytes of $2 \times 10^7$ cells/dish (10 cm) were incubated with $^3$H-leucine (55.5 KBq/dish) and serum HDL (2.6 mg HDL protein/ml) in FBS- and insulin-free WE medium at 28°C for 24 h. The secreted proteins in the medium were then separated by a Sephadex G-25 column (1.6 × 15 cm) and fractionated by density gradient ultracentrifugation.

A, Lipoprotein fractionation of the secreted proteins of control. In control the hepatocytes were incubated in the absence of serum HDL. B, Lipoprotein fractionation of the secreted proteins obtained from the medium in which the hepatocytes were incubated in the presence of serum HDL. Longitudinal bars indicate S.D. for three experiments. Horizontal bars in Figures A and B represent the position in which serum HDL is fractionated. a, Autoradiogram of the secreted lipoprotein fractionated as shown in A. The lyophilized secreted lipoprotein (15 μg protein) which was pooled from fractions 37 to 40 was applied to a 5–17.5% acrylamide gradient slab gel. b, Autoradiogram of the secreted lipoprotein fractionated as shown in B. The lyophilized secreted lipoprotein (15 μg protein) which was pooled from fractions 37 to 40 was applied to a 5–17.5% acrylamide gradient slab gel.

such as hepatocytes (19, 20, 21–23), epithelial cells of small intestine (3, 4), and platelets (24) has been confirmed and the cloning of the HDL receptor protein was reported (25). Apo A-I, which is a main component of HDL apoproteins, has an important role for binding to HDL receptor protein and for cholesterol efflux (3, 4,
6). Therefore, in the investigation on HDL receptors, apoproteins composition of the HDL is critical. We used the HDL which consisted of apo A-I and A-II and had no other apoproteins corresponding to apo B and E. Apo B is a main apoprotein of low-density lipoprotein (LDL) and a ligand for LDL receptor (7). Apo E can also bind LDL receptor (7).

We demonstrated the specific binding of serum HDL to eel hepatocytes by using di-15-ASP-HDL. Freshly isolated hepatocytes bound to di-15-ASP-HDL which was incubated with the cells at 0°C for 2 h under the dark and the fluorescence of the cells bound to it could be detected by a flow cytometer. Its intensity was higher than that of the control cells incubated without di-15-ASP-HDL. The binding was inhibited by unlabeled HDL. The binding of di-15-ASP-HDL to the hepatocytes was saturated at concentrations over 100 µg HDL protein/ml and Kd was 20 µg HDL protein/ml. The Kd value of 20 µg HDL protein/ml was almost the same as those of rat liver (19, 20, 23, 26, 27) and epithelial cells of rat intestine (3, 4), which were 10 to 50 µg/ml. Furthermore, when cultured eel hepatocytes were incubated with di-15-ASP-HDL at 0°C for 2 h under the dark and observed by an epifluorescence microscope, the bright, circumferential plasma membranes stained with di-15-ASP-HDL were observed.

The rate of incorporation of 125I-HDL into the cultured hepatocytes was dependent on its concentration in the range of 1.25 to 5 µg/ml at 28°C for 1 h incubation. Its incorporation was inhibited by the addition of unlabeled HDL. Since about 7% of the total incorporation of 125I-HDL was determined in the acid soluble fraction, it was assumed that 125I-HDL was taken into the hepatocytes, probably by a receptor-mediated endocytosis. There was another result to support the incorporation of HDL into the hepatocytes. HDL affected intracellular lipid contents. The contents of intracellular cholesteryl ester and phospholipid were increased by HDL, but those of free fatty acids, triacylglycerol, and free cholesterol were not changed or only slightly decreased. We assumed that the increase of cholesteryl ester and phospholipid in the cells was due to the increase of HDL by the cells, since phospholipid and cholesteryl ester are the main lipids of eel serum HDL, the compositions of which are 32 and 14%, respectively, as reported previously (1).

There were three remarkable characteristics of the effects of HDL on cultured eel hepatocytes. The first was that the addition of HDL to the hepatocytes induced the efflux of cholesterol, triacylglycerol, and phospholipid from the hepatocytes. It is known in mammalian cells that HDL stimulates only the cholesterol efflux from cells (3-5). In the eel hepatocytes, by contrast, HDL stimulated the rate of efflux of four kinds of lipids, free cholesterol, cholesteryl ester, triacylglycerol, and phospholipid, from the hepatocytes. The second characteristic was that the efflux of the intracellular lipids was carried out with only one kind of secreted lipoprotein. The secreted lipoprotein was identified as a VLDL- (or chylomicron-) like lipoprotein as reported previously (1, 28), judging from its apoproteins and lipid compositions. The secreted lipoprotein contained apo A and B as its main protein components and its main lipid was triacylglycerol. The rate of secretion of the lipoprotein by the hepatocytes incubated with HDL was twice that by the hepatocytes incubated without HDL. The amount of secreted lipoprotein of the former during 24 h incubation was 317 µg lipoprotein/mg cell protein, while that of the latter was 160 µg lipoprotein/mg cell protein. Although triacylglycerol was the intracellular lipid that was most transported due to the lipid composition of the secreted lipoprotein, the rates of cholesterol ester and free cholesterol efflux were increased by HDL 5-fold and 3.7-fold, respectively, than those in hepatocytes incubated without HDL. The HDL used in these experiments consisted of apo A-I.

**Table II. Lipid composition and rate of secretion of lipoprotein (A) and lipid contents in cultured hepatocytes (B).**

<table>
<thead>
<tr>
<th>A</th>
<th>Secreted Lipoprotein (µg/mg cell. prot.)</th>
<th>Control</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Condition</td>
<td>TG</td>
<td>FC</td>
<td>CE</td>
</tr>
<tr>
<td>Control</td>
<td>101.7±29.8</td>
<td>4.32±1.58</td>
<td>3.77±1.62</td>
</tr>
<tr>
<td>HDL</td>
<td>170.9±33.3</td>
<td>15.92±3.67*</td>
<td>19.64±7.81**</td>
</tr>
<tr>
<td>B</td>
<td>FFA</td>
<td>TG</td>
<td>FC</td>
</tr>
<tr>
<td>Condition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.95±0.49</td>
<td>72.09±15.06</td>
<td>24.16±4.95</td>
</tr>
<tr>
<td>HDL</td>
<td>1.76±0.05</td>
<td>70.71±3.29</td>
<td>22.39±0.85</td>
</tr>
</tbody>
</table>

TG, FC, CE, PL, and FFA represent triacylglycerol, free cholesterol, cholesterol ester, phospholipid, and free fatty acids, respectively. The values were mean±S.D. for three experiments.

* Significantly different from the control value. P<0.01.
** Significantly different from the control value. P<0.05.
and A-II. Herold et al. (4) reported the role of apoproteins A-I and A-II, and demonstrated using liposome containing only apo A-I or apo A-I and A-II that apo A-I and A-II stimulated free cholesterol and cholesteryl ester efflux, respectively. These reports were consistent with the effect of HDL on cholesterol efflux in the eel hepatocytes. Therefore, in eel hepatocytes HDL had a stimulatory effect on cholesterol efflux as in mammalian cells (3-5), but at the same time HDL stimulated the phospholipid and tracylglycerol efflux. This seemed to be due to the VLDL- (or chylomicron-) like lipoprotein triggering an efflux of intracellular lipids. In mammalian cultured cells such as small intestinal crypt cells (3, 4) and fibroblasts (5), HDL is resorbed with intracellular cholesterol following endocytosis after binding of HDL to the cells (3, 4, 8, 26, 27, 29), or HDL with intracellular cholesterol is released from the specific binding proteins of plasma membrane of the cells following cellular signal induction such as the activation of phospholipase D and protein kinase C (4-7, 24). In these cells HDL is the only lipoprotein to participate in the efflux of cholesterol.

The third characteristic is that HDL specifically stimulated the incorporation of 14C-leucine into the secreted lipoprotein and had no effect on the incorporation into intracellular proteins and secreted proteins except for the lipoprotein. Furthermore, HDL did not induce the synthesis of HDL, but stimulated the synthesis of only one kind of lipoprotein, a VLDL- (or chylomicron-) like lipoprotein.

These characteristics of the effects of HDL described above suggest the novel role of HDL in the transport of lipids. Though there are two hypotheses on the role of HDL for cholesterol efflux, namely HDL-retroendocytosis (3, 4, 8, 26, 27, 29) and cellular signal transducing by HDL (4-7, 24), neither hypotheses seems to be applicable to the role of eel serum HDL, since HDL in eel hepatocytes seems only to provide the initial step for the transport of lipids and HDL itself seems not to participate in transporting intracellular lipids. Instead, a VLDL- (or chylomicron-) like lipoprotein transports the intracellular lipids in eel hepatocytes.

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(Received for publication, June 5, 1995
and in revised form, June 20, 1995)