Receptor-mediated internalization of high density lipoprotein by rat sinusoidal liver cells: identification of a nonlysosomal endocytic pathway by fluorescence-labeled ligand

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Abstract Rat sinusoidal liver cells possess the surface receptor for high density lipoprotein (HDL) (Murakami, M., S. Horiuchi, K. Takata, and Y. Morino. 1987. *J. Biochem. (Tokyo)* **101:** 729–741). The present study was undertaken to determine whether cell surface-bound HDL underwent subsequent endocytic internalization by using ^{125}I-labeled HDL and fluorescein isothiocyanate-labeled HDL (FITC-HDL). The cell-associated radioactivity obtained by a 40-min incubation with ^{125}I-labeled HDL at 37°C was released into the medium as acid-precipitable forms upon further incubation at 37°C. When further incubated at 0°C instead of 37°C, however, this release was significantly reduced. A similar phenomenon was observed after the cell-associated ligands had been treated with trypsin. The cell-associated ligands obtained after a 1-hr incubation with ^{125}I-labeled HDL at 0°C were largely counted for by those bound to the outer surface of the cells, thus suggesting that HDL is internalized into cells at 37°C but not at 0°C. Moreover, when cells were incubated with FITC-HDL at 0°C, the cell-associated ligands were found in a pH 7.2 ± 0.1 compartment, whereas when incubated at 37°C, its microenvironmental pH became much more acidic, exhibiting pH 6.2 ± 0.1. Furthermore, this value returned to 7.1 ± 0.1 upon treatment with carbonylcyanide *m*-chlorophenylhydrazone known to dissipate the total protonomotive force. These results suggest, therefore, that the internalization process does follow receptor-mediated binding of HDL in rat sinusoidal liver cells. This notion was also supported by fluorescence microscopic observations. — **Takata, K., S. Horiuchi, A. T. M. A. Rahim, and Y. Morino.** Receptor-mediated internalization of high density lipoprotein by rat sinusoidal liver cells: identification of a nonlysosomal endocytic pathway by fluorescence-labeled ligand. *J. Lipid Res.* 1988. **29:** 1117–1126.

Supplementary key words HDL receptors • receptor-mediated endocytosis • nonlysosomal acidic compartment • fluorescein pH probe

Evidence for the protective role of HDL in an atherogenic process is twofold: *i*) plasma HDL levels are epidemiologically correlated inversely with the incidence of coronary artery disease (1), and *ii*) HDL serves in vitro as an acceptor of unesterified cholesterol efflux from foam cells (2)—cholesteryl ester-laden macrophages found in atherosclerotic lesions—by a mechanism identical or similar to that of "reverse cholesterol transport" (3, 4). The specificity of interaction of HDL with macrophages has been studied with the membrane-associated binding sites for HDL (the HDL receptor) as with other cells such as fibroblasts, arterial smooth cells, endothelial cells, hepatocytes, and cells of steroidogenic tissues (5–10). The molecular masses of HDL receptors so far demonstrated by ligand blotting experiments were 110 kDa in human fibroblasts (11), 90 kDa in hepatocytes (12, 13), and 120 kDa in human placenta (14). However, pathophysiological function of these receptors at the molecular level has not been fully elucidated. One of the controversial points on the HDL pathway is whether HDL bound to its macrophage surface receptor is internalized into the cells, or rather, shows reversible extracellular binding without internalization. Oram, Johnson, and Brown (15) recently showed that cholesterol-loaded mouse peritoneal macrophages exhibited specific binding for HDL, but cell surface-bound HDL particles were not internalized. However, morphological approaches by Schmitz et al. (16) using the same cells revealed that HDL-colloidal gold complexes were internalized and delivered to endosomes, followed by subsequent retroendocytosis.

In an earlier study (17) we demonstrated that rat sinusoidal liver cells express specific binding sites for HDL. Incubation of these cells with ^{125}I-labeled HDL did not lead to intracellular degradation of the ligand. From the effect of trypsin treatment of cell-associated ^{125}I-labeled HDL, it was suggested that cell surface-bound

Abbreviations: HDL, high density lipoprotein; FITC, fluorescein isothiocyanate; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; TCA, trichloroacetic acid; PBS, phosphate-buffered saline; kDa, kilodalton(s).

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HDL was subjected to internalization, followed by intracellular routing through a nonlysosomal pathway.

To provide more solid evidence for this notion, the present study was undertaken to measure the change in microenvironmental pH of HDL during its interaction with sinusoidal liver cells by using FITC-labeled HDL. The results support the post-binding internalization model.

MATERIALS AND METHODS

Chemicals

FITC and collagenase were purchased from Wako Chemical Co. (Osaka, Japan). Heparin-agarose gel was from Pharmacia and Bolton-Hunter reagent (^{125}I) was from New England Nuclear. Other chemicals were of the best grade available from commercial sources.

Preparation of HDL and its derivatization

HDL was isolated ultracentrifugally at d 1.063–1.21 g/ml from fresh human plasma (18) and passed through heparin-agarose affinity chromatography to remove a trace amount of apolipoproteins E and B (19) as described previously (17, 20). HDL was iodinated with ^{125}I with Bolton-Hunter reagent to a specific radioactivity of 610 cpm/ng of protein according to the manufacturer's instructions.

To prepare FITC-HDL, the coupling reaction was performed in 0.1 M bicarbonate buffer (pH 9.5) with HDL FITC ratio of ~100:1 on a weight basis. In a typical experiment, to 1.0 ml of HDL solution (4–5 mg/ml) was added 50 μl of FITC solution (1 mg/ml) which was dissolved in 0.1 M bicarbonate buffer (pH 9.5). After 3 hr incubation at 4°C with occasional stirring on a Vortex mixer, unbound FITC was removed by gel filtration on a Sephadex G-50 column in 50 mM Tris-HCl (pH 8.0) and 0.15 M NaCl, followed by dialysis overnight at 4°C against 0.15 M NaCl and 1 mM EDTA (pH 7.4). The stoichiometric incorporation of FITC into HDL was determined according to the method of Jobbagy and Kiraly (21) and protein concentration by the method of Lowry et al. (22). On average, the conjugate containing 2.8–4.3 mol of FITC per mol of HDL was used for experiments. FITC-HDL was labeled with ^{125}I with Bolton-Hunter reagent as described above to a specific radioactivity of 440 cpm/ng of protein. Although the labeling efficiency of FITC-HDL with Bolton-Hunter reagent was reduced by about 20% when compared with unmodified HDL, significant radioiodination with the reagent was achieved.

Experiments with ^{125}I-labeled HDL

Sinusoidal liver cells were prepared from male Wistar rats by the collagenase perfusion method (23) and suspended in Eagle's essential medium containing 3% bovine serum albumin buffered with 20 mM 2-(4-hydroxyethyl)-1-piperazineethanesulfonic acid to pH 7.4 (buffer A).

The binding assay was carried out as described previously (17, 20, 23). Briefly, a reaction mixture contained, in a total volume of 0.1 ml of buffer A, 1.0×10^6 cells and 2.2 μg/ml of ^{125}I-labeled HDL in the presence of unlabeled FITC-HDL or HDL. After 1 hr incubation on ice with several intervals on a Vortex mixer, 1.0 ml of ice-cold buffer A was added to each tube, followed by centrifugation at 12,800 *g* for 25 sec at 4°C. The supernatant was discarded and the pelleted cells were resuspended in 1.0 ml of ice-cold buffer A. Cells were then washed twice more to determine the cell-associated radioactivity.

To examine the effect of unlabeled HDL on cell surface-bound ^{125}I-labeled HDL, 4.0×10^7 cells were preincubated with 3.4 μg/ml of ^{125}I-labeled HDL on ice for 40 min in 4 ml of buffer A. Cells were then washed with 50 ml of ice-cold buffer A, resuspended in 4 ml of buffer A, and divided into two equal portions. Each half was incubated on ice with or without 0.3 mg/ml of unlabeled HDL. Aliquots (0.2 ml) were withdrawn at different times and transferred to polyethylene tubes, followed by centrifugation at 12,800 *g* for 30 sec. The supernatant was used to determine the TCA-precipitable and TCA-soluble radioactivity. The cell pellets were used to determine the cell-associated radioactivity as described previously (17).

To examine the post-binding events of HDL, 4.5×10^7 cells were preincubated with 2.3 μg/ml of ^{125}I-labeled HDL for 40 min at 37°C in 8 ml of buffer A, washed with 50 ml of ice-cold buffer A, and then with 50 ml of ice-cold PBS. Cells resuspended in 8 ml of PBS were divided into two equal portions. One was incubated at 0°C for 20 min (portion A) and the other was incubated in parallel in the presence of 0.4% trypsin (portion B). Both portions A and B were washed with 50 ml of ice-cold buffer A and resuspended in 4 ml of buffer A containing 0.3 mg/ml of unlabeled HDL. Each portion was further divided into two equal portions which were then incubated at 0°C and 37°C, respectively. Aliquots (0.2 ml) were withdrawn at various times for determination of cell-associated radioactivity and TCA-precipitable radioactivity in the incubation medium as described above.

Binding and subsequent endocytic behavior of ^{125}I-labeled FITC-HDL were determined in exactly the same way as described above except that ^{125}I-labeled HDL was replaced by ^{125}I-labeled FITC-HDL.

Experiments with FITC-HDL

Cells (8×10^6/ml) were preincubated on ice for 1 hr with 120 μg/ml of FITC-HDL in 2.0 ml of buffer A. For binding studies, 1.0 ml of the cell suspension was withdrawn and centrifuged at 800 *g* for 3 min at 4°C. Cells were washed three times with 1.0 ml of ice-cold PBS, resuspended in 1 ml of PBS, and used for the experi-

ments. The remaining half of the cell suspension was used for uptake studies; cells were further incubated at 37°C for 30 min, washed three times with 1 ml of PBS and resuspended in 1 ml of PBS, followed by fluorescence spectroscopic analyses as described below.

To modulate intracellular proton gradients by CCCP and NH_4Cl, cells were treated with FITC-HDL at 37°C under conditions identical to those for uptake studies. After the initial fluorescence spectrum was measured, 10 mM CCCP in ethanol or 100 mM NH_4Cl in PBS was added to the cell suspension to the final concentration of 10 μM and 10 mM, respectively, and incubated at 0°C. At the indicated times, cell suspensions were mixed well and placed immediately for fluorescence intensity measurements (see Fig. 6B). As a control experiment, cells were incubated with FITC-HDL at 0°C for 1 hr under conditions identical to those for the binding study. After the initial fluorescence intensity was measured, CCCP or NH_4Cl was added to the cell suspension and the fluorescence spectrum was measured at various times in the same way (see Fig. 6C).

Prior to these experiments, we confirmed that the fluorescence intensity of FITC-HDL as well as its 490/450 nm ratio in a cell-free system were affected neither by CCCP, NH_4Cl, nor ethanol, in which CCCP was dissolved at the concentrations used for these experiments. We also confirmed that the fluorescence spectra of FITC-HDL measured at 4°C were indistinguishable from those measured at 37°C.

Fluorescence spectroscopy

All fluorescence measurements were done with a Hitachi 850 spectrofluorometer interfaced to a microcomputer. Temperature was maintained at 4°C with a Eleya digital Uni-Ace UA-100 circulating water bath. Cells were placed in a 1-cm path-length cuvette and the fluorescence intensity at 520 nm was measured separately for the excitation wavelengths of 490 nm and 450 nm. Three time-averaged readings with a 5-sec signal integrator were recorded. Before each reading cells were mixed with a Pasteur pipette to avoid sedimentation of the cells in the cuvette so that no systemic change of the fluorescence signal was observed. Unless otherwise specified, each measurement was the average of duplicate samples. Cellular autofluorescence was subtracted from the sample fluorescence spectrum in each experiment by measuring the fluorescence intensities of cells that had not been exposed to FITC-HDL under incubation conditions identical for uptake and binding studies, respectively.

To construct a standard curve for calibrating pH gradient, each tube containing 8×10^6 cells and 120 μg/ml of FITC-HDL in 1.0 ml of buffer A was incubated for 30 min at 37°C in the same way as that described above for uptake studies. Cells in each tube were washed three times with 1 ml of PBS and resuspended in 1 ml of 0.1 M sodium acetate (pH 4.0–6.5) or 0.1 M sodium phosphate buffer (pH 6.0–8.2). To the cell suspension was added CCCP to a final concentration of 10 μM. After 1 hr equilibration on ice, the intensity of each pH point was determined as described above. As indicated by Ohkuma and Poole (24), the ratio of intensities at two particular excitation wavelengths with a fixed emission wavelength is a more reliable pH index than the absolute intensity. This ratio is independent of fluorescein concentration, ionic strength of the medium, or specific anions or cations (24, 25); the ratio is also unaffected by photobleaching (25). However, fluorescence sensitivity to environmental factors may not be excluded (26). The necessity for in situ calibration of pH-sensitive probes has been amply demonstrated and discussed in detail (27, 28). Therefore, in each set of experiments, we constructed a cell-associated FITC-HDL standard curve for 490/450 nm ratios at different pH values in which the internal pH was equilibrated by an external pH determined either by 0.1 M sodium phosphate and 10 μM CCCP or 0.1 M sodium acetate and 10 μM CCCP.

Fluorescence microscopy

For fluorescence microscopy, 1×10^7 cells in 1.0 ml of buffer A were incubated with 120 μg/ml of FITC-HDL either at 0°C for 1 hr (for the binding study) or at 37°C for 30 min (for the uptake study). Cells were then washed three times with 1.0 ml of PBS, suspended, and fixed with 2% formalin in PBS for 10–15 min at room temperature. After extensive washing with PBS, cells were resuspended in 0.2 ml of PBS. A drop of the cell suspension was placed on a glass slide, sealed with a cover slip, and observed under a fluorescence microscope (Nikon RFL) using glycerol-PBS as the light passage between the microscopic lens and the cover slip.

RESULTS

Interaction of ^{125}I-labeled HDL with rat sinusoidal liver cells

Our previous study has shown that incubation of ^{125}I-labeled HDL with rat sinusoidal liver cells resulted in an increase in cell-associated radioactivity, but subsequent intracellular degradation of the ligand did not take place (17). To further elaborate the post-binding behavior of HDL, the cells were incubated with ^{125}I-labeled HDL for 37°C for 40 min, replaced with unlabeled HDL, and subjected to further incubation at 37°C. Amounts of the cell-associated ^{125}I-labeled HDL decreased with time, and this decrease was accompanied by a concurrent increase in the radioactivity in the medium as TCA-precipitable forms (**Fig. 1A**). Thus, HDL seemed to be released intact after

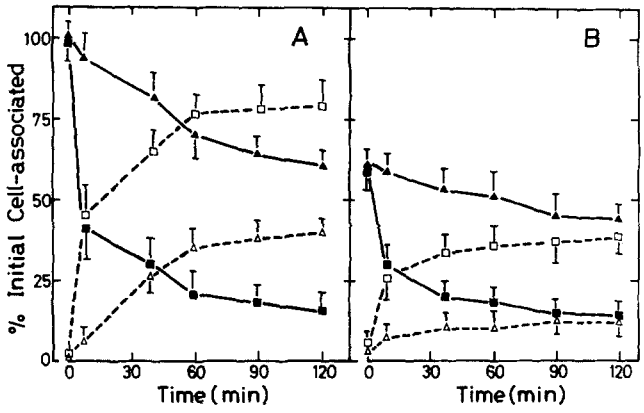

Fig. 1. Interaction of ^{125}I-labeled HDL with rat sinusoidal liver cells. Cells (4.5×10^7) incubated for 40 min at 37°C with 2.3 μg/ml of ^{125}I-labeled HDL in 8 ml of buffer A were washed and resuspended in 8 ml of PBS and divided into two equal portions. One portion was incubated at 0°C for 20 min (portion A) and the other was incubated in parallel with 0.4% trypsin (portion B). Both portions A and B were washed and resuspended in buffer A containing 0.3 mg/ml of unlabeled HDL. (Panel A) Portion A was further divided into equal portions. One half was incubated at 37°C and time aliquots were withdrawn to determine the cell-associated radioactivity (■) and TCA-precipitable radioactivity in the extracellular medium (□). The other half was incubated at 0°C and the cell-associated radioactivity (▲) and TCA-precipitable radioactivity (△) were determined. (Panel B) Portion B was similarly divided into equal portions and each half was incubated at 37°C and 0°C. The cell-associated radioactivity at 37°C (■) and at 0°C (▲) as well as TCA-precipitable radioactivity in the medium at 37°C (□) and 0°C (△) were determined in the same way as described above. More than 95% of the radioactivity released into the medium was TCA-precipitable. The cell-associated radioactivity determined immediately after the addition of unlabeled HDL was taken as a zero-time value. The zero-time value of portion A was 3.6 ng/10^6 cells and each experimental point in A and B was expressed as percent of this value. Each value represents the mean of two parallel incubations and the vertical bar shows the range.

the interaction with these cells. However, when further incubation was performed at 0°C instead of 37°C, the decrease in the cell-associated radioactivity as well as the increase in TCA-precipitable radioactivity in the medium were suppressed to a greater extent, suggesting a temperature-dependent nature of the release phenomenon. In order to determine whether or not the outer surface-bound HDL is responsible for this release, cells incubated with ^{125}I-labeled HDL at 37°C were treated with trypsin to remove trypsin-accessible outer surface-bound ligands and further incubated at 0°C and 37°C, respectively. As shown in Fig. 1B, trypsin treatment reduced cell-associated HDL by 40–50%. Further incubation at 37°C following trypsin treatment resulted in a similar time-dependent release of cell-associated radioactivity into the medium, whereas no detectable release was observed after further incubation at 0°C.

We next determined the location of ^{125}I-labeled HDL when the cells were incubated with ^{125}I-labeled HDL at 0°C and replaced with unlabeled HDL, followed by further incubation at 0°C. Significant amounts of cell-associated ^{125}I-labeled HDL were released into the medium, whereas the similar experiment without unlabeled HDL did not lead to the release of the bound ligand (**Fig. 2**). Thus, ^{125}I-labeled HDL associated with the cells by a 40-min incubation at 37°C might be located in a different place after a 40-min incubation at 0°C, the former in an HDL-unreplaceable compartment and the latter in a ligand-replaceable compartment, probably at the outer surface of plasma membranes. Results of these experiments using ^{125}I-labeled HDL (Figs. 1 and 2) seem to favor the internalization of receptor-bound HDL.

Microenvironmental pH and internalization of FITC-HDL

The measurement of cellular microenvironmental pH with FITC-labeled ligands is one of the reliable choices for determining whether cell surface-bound ligands are internalized into the cell or not (24, 29). We first examined whether FITC-HDL would behave like unlabeled HDL in terms of surface binding and subsequent endocytic events. FITC-HDL was as effective as unlabeled HDL in competition for the binding of ^{125}I-labeled HDL to the cells (**Fig. 3**). When ^{125}I-labeled FITC-HDL was incubated with cells at 37°C and the increase in TCA-soluble radioactivity released into the medium was mea-

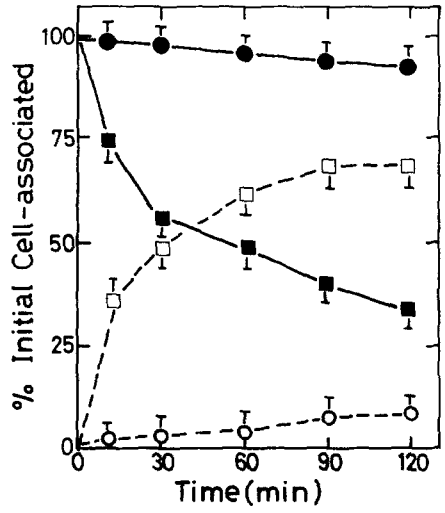

Fig. 2. Effect of unlabeled HDL on cell surface-bound ^{125}I-labeled HDL. A reaction mixture contained 4.0×10^7 cells and 3.4 μg/ml of ^{125}I-labeled HDL in 4 ml of buffer A. After incubation on ice for 40 min, cells were washed and resuspended in 4 ml of buffer A. One half of the cell suspension was further incubated on ice with 0.3 mg/ml of unlabeled HDL and time aliquots were taken to determine cell-associated radioactivity (■) and TCA-precipitable radioactivity of the extracellular medium (□). The other half was incubated on ice without unlabeled ligand and the cell-associated radioactivity (●) and TCA-precipitable radioactivity in the medium (○) were similarly determined. More than 98% of the radioactivity released into the incubation medium was TCA-precipitable. The 100% value for the initial cell-associated ^{125}I-labeled HDL was 0.52 ng/10^6 cells. Each value represents the mean of two parallel incubations and the bar shows the range.

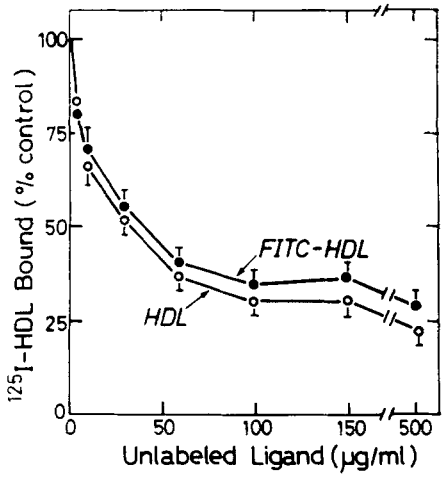

Fig. 3. Effect of FITC-HDL on ^{125}I-labeled HDL binding. Cells (1.0×10^6) were incubated for 1 hr at 4°C with 2.2 μg/ml of ^{125}I-labeled HDL in 0.1 ml of buffer A at the indicated concentrations of unlabeled FITC-HDL (●) or HDL (○). Cells were washed and amounts of the cell-bound ligands were determined as described under Materials and Methods. The 100% value was 0.81 μg/10^6 cells. Each value represents the mean of duplicate experiments with the bar showing the range.

sured, no significant intracellular degradation was observed (data not shown). Furthermore, when the binding and subsequent endocytic behavior were examined with ^{125}I-labeled FITC-HDL in an experimental system similar to that of Fig. 1, the cell-associated ^{125}I-labeled FITC-HDL was released into the medium upon incubation at 37°C to a greater degree when compared to the incubation at 0°C (**Fig. 4**), a finding consistent with the endocytic behavior of ^{125}I-labeled HDL shown in Fig. 1. Thus, the use of FITC-HDL as a ligand for the HDL receptor was verified; chemical modification of HDL with FITC had no effect on the recognition of the ligand by the HDL receptor on the post-binding behavior.

FITC is known to be a good pH indicator because of alterations in the fluorescence intensity that accompany the titration of the carboxyl group. The shift in its excitation spectrum provides the specific pH measurement (24, 30). **Fig. 5** shows typical excitation spectra of FITC-HDL under pH values ranging from 4.0 to 10.5. The standard calibration curve was obtained by incubating FITC-HDL with cells in the presence of CCCP as described under Materials and Methods. The ratio of the fluorescence intensities at 490 nm to those at 450 nm was plotted and its pH-dependent calibration curve is shown in **Fig. 6A.** Cells were incubated with FITC-HDL and the microenvironmental pH of FITC-HDL was measured from this pH-dependent standard curve. Upon incubation at 0°C with the fluorescent ligand, pH was estimated to be 7.2 ± 0.1, a value slightly lower than that of the extracellular medium (Fig. 6A). However, when cells were incubated with the ligand at 37°C for 10 min and washed with ligand-free medium to remove unbound ligands, the microenvironmental pH of the cell-associated FITC-HDL was 6.2 ± 0.1, a value much more acidic than that obtained after incubation at 0°C (Fig. 6A). Thus, it is likely that by incubation of FITC-HDL at 37°C but not at 0°C, HDL-receptor complexes were internalized into the cells and exposed to an acidic compartment.

The above prediction was further tested by weak bases such as NH_4Cl or protonophore CCCP which are known to lead to a breakdown of the pH gradient across the membranes (30–36). Cells were incubated with FITC-HDL for 30 min at 37°C, washed with ligand-free medium, and resuspended in the buffer A in the same way as shown in Fig. 6A. From the fluorescence measurement, microenvironmental pH of the cell-associated FITC-HDL was estimated to be 6.2. When CCCP was added to this cell suspension, the fluorescence ratio of 490/450 nm was shifted to 4.3–4.5, a value corresponding to pH 7.0–7.2 (Fig. 6B). Although less effective, a similar change was seen with ammonium chloride, one of the lysosomotropic amines. In the absence of these reagents, no change in pH occurred during the incubation (Fig. 6B). By contrast, when a similar experiment was done with cells that had been preincubated with FITC-HDL at 0°C for 1 hr, there was no change in the 490/450 nm ratio of FITC-HDL (Fig. 6C). The significant increase in the 490/450 nm ratio of cell-associated ligands obtained by the addition of CCCP or NH_4Cl and incubation at 37°C

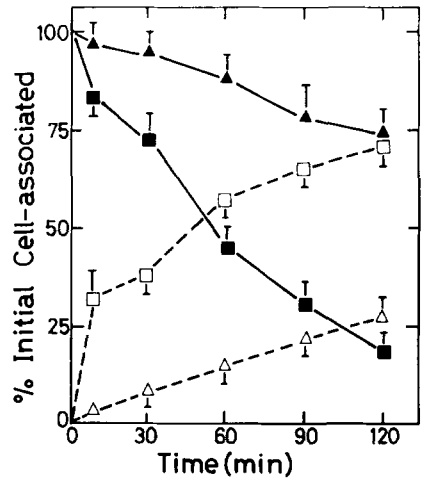

Fig. 4. Endocytic behavior of ^{125}I-labeled FITC-HDL. Cells (2.2×10^7) were preincubated for 40 min at 37°C with 2.7 μg/ml of ^{125}I-labeled FITC-HDL in 4 ml of buffer A, washed and resuspended in 4 ml of buffer A containing 0.25 mg/ml of unlabeled FITC-HDL. One half of the cell suspension was further incubated at 37°C and time aliquots were taken to determine the cell-associated (■) and TCA-precipitable radioactivity (□). The other half was incubated on ice and the cell-associated (▲) and TCA-precipitable radioactivity (△) were determined in the similar manner. More than 85% of the radioactivity released into the incubation medium was TCA-precipitable. The 100% value for the initial cell-associated ^{125}I-labeled FITC-HDL was 4.4 ng/10^6 cells. Each value represents the mean of two parallel incubations and the bar shows the range.

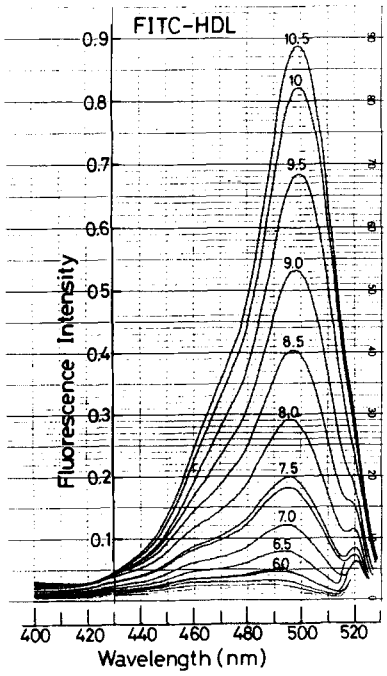

Fig. 5. pH-Dependent fluorescence spectrum of FITC-HDL. Each tube contained 10 μg of FITC-HDL in 1.0 ml of 0.1 M buffer solution (sodium acetate; pH 4.0–5.5, sodium phosphate; pH 5.5–7.5, Tris-HCl; pH 7.5–9.0, sodium carbonate; pH 9.0–10.5). The fluorescence spectrum was measured with each sample as described under Materials and Methods. The number on the curve shows pH.

but not at 0°C strongly suggested the importance of the protonomotive force in maintaining the acidity of the FITC-HDL-containing vesicles. These results taken together provide a strong support to the notion that receptor-mediated recognition of HDL by sinusoidal liver cells is followed by internalization of cell-bound HDL and intracellular routing by a nonlysosomal pathway.

Microscopic observation of FITC-HDL

Finally fluorescence microscopic observation showed that incubation of the cells with FITC-HDL at 0°C resulted in a specific but diffusely distributed fluorescence along the cell surface, whereas upon incubation at 37°C under parallel conditions, denser fluorescent grains were visible around the cell periphery (**Fig. 7**), suggesting that cell surface-bound FITC-HDL was internalized at 37°C but not at 0°C. The result of this morphological observation supports our contention obtained from biochemical approaches described above.

DISCUSSION

Results obtained from the present study have provided supporting evidence for the contention that HDL bound to its surface receptor on rat sinusoidal liver cells was internalized into the cells.

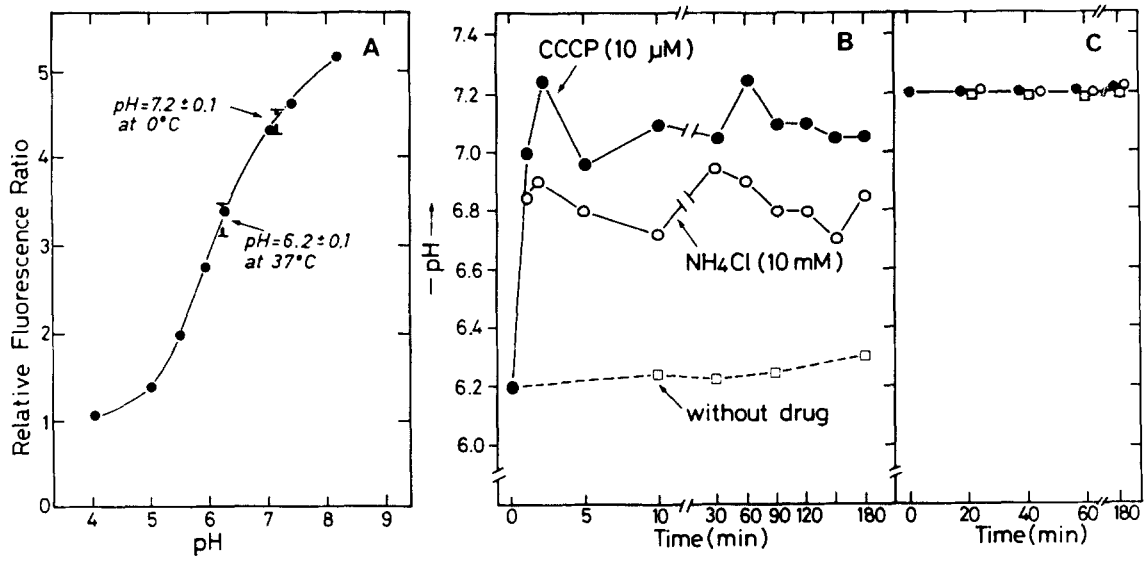

Fig. 6. Panel A: Microenvironmental pH in cellular incubation of FITC-HDL at 37°C and 0°C. Cells were incubated with FITC-HDL in the presence of CCCP at various pH values as described under Materials and Methods. The standard calibration curve was obtained by plotting the fluorescence ratio of 490/450 nm against pH (● — ●). Cells were incubated with FITC-HDL for 30 min either at 0°C or at 37°C, washed with PBS, and the fluorescence pattern was measured with each sample and the 490/450 nm ratio was determined. The estimated pH values are the mean values of quadruplicate experiments with bars denoting the range. Panel B: Effect of CCCP and ammonium chloride on the microenvironmental pH of FITC-HDL. After preincubation on ice for 1 hr with 120 μg/ml of FITC-HDL in buffer A, cells were incubated at 37°C for 30 min, washed, and resuspended in PBS. Portions of the cell suspension were incubated on ice in the absence (□) or presence of 10 μM CCCP (●) or 10 mM NH_4Cl (○) and the change in pH was monitored as described under Materials and Methods. Panel C: After 1 hr incubation with FITC-HDL at 0°C, the cells were washed and resuspended in PBS. Portions of the cell suspension were incubated in the absence (□) or presence of CCCP (●) or NH_4Cl (○) and the 490/450 nm ratio was similarly determined from each fluorescence spectrum. The data in B and C are typical of the results obtained in four experiments.

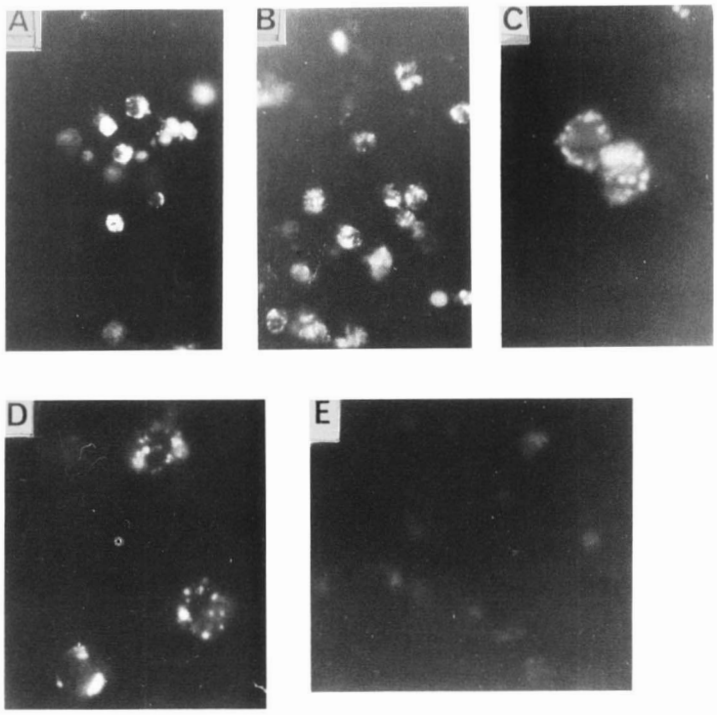

Fig. 7. Fluorescence micrographs of sinusoidal liver cells after incubation with FITC-HDL. Cells were incubated at 37°C for 30 min (A–D) or 0°C for 60 min (E) with 0.12 mg of FITC-HDL in 1 ml of buffer A. After washing with ligand-free PBS, cells were fixed with 2% formalin and processed for fluorescence microscopy as described under Materials and Methods. A, B, and E, ×260; C and D, ×1040.

To determine whether the ligand in question is internalized into the cell or not, effect of protease treatment on cell-associated ligands is one of the conventional choices. Oram et al. (15) using ^{125}I-labeled HDL and mouse peritoneal macrophages concluded from the trypsin-insensitive nature of cell-associated HDL that cell surface-bound HDL particles did not undergo internalization, thereby proposing a mechanism by which the HDL receptor only mediates extracellular binding to its ligand in a reversible manner (extracellular binding model). On the other hand, based on our two findings obtained from in vitro experiments using ^{125}I-labeled HDL and rat sinusoidal liver cells that incubation of these cells with ^{125}I-labeled HDL at 37°C did not result in intracellular degradation of the ligand, and that about 40% of the cell-associated ^{125}I-labeled HDL particles were insensitive to proteolytic digestion, we proposed that receptor-HDL complexes undergo internalization and subsequent intracellular delivery by a nonlysosomal pathway (internalization model). Obviously, these two models are not mutually exclusive because even in the latter model, extracellular reversible biding could occur between HDL and its surface receptor. Thus, the critical question on this issue should be whether or not quantitative amounts of surface-bound HDL particles do undergo internalization. Time-dependent decrease in the cell-associated ^{125}I-labeled HDL at 37°C was greater than that observed at 0°C and this decrease was quantitatively accounted for by a concomitant increase in TCA-precipitable ^{125}I-labeled HDL in the extracellular medium (Fig. 1A). This release phenomenon was observed even after the cell-associated ^{125}I-labeled HDL had been treated with trypsin (Fig. 1B). When cells were preincubated with ^{125}I-labeled HDL at 0°C and subjected to further incubation with unlabeled HDL at the same temperature, more than 60% of the cell-associated ligands exhibited a time-dependent release into the medium (Fig. 2). From these results it is likely that the difference observed between 37°C results and 0°C results may reflect the ligands that were endocytosed into the cells and released back into the extracellular medium (see Fig. 1). However, other experimental approaches would be desirable to obtain more solid evidence for this conclusion. Therefore, we employed FITC-HDL as a pH probe.

Since its introduction by Ohkuma and Poole (24), the pH-dependent alteration in the fluorescence intensity as well as the excitation spectrum of FITC-labeled ligands have been used to estimate the pH inside lysosomes and endosomes after fluorescein-conjugated macromolecules are directed to these organelles (30, 35). Several laboratories have shown that the intralysosomal environment in intact cells is maintained at a pH of 4.6–5.0 (37, 38). In

a lysosome-oriented ligand undergoing receptor-mediated endocytosis, ligand–receptor complexes are endocytosed via coated pits to form coated vesicles and delivered to endosomes, and finally routed to lysosomes. Because endocytosis is a series of these continuous reactions, it is difficult to determine with accuracy how the acidity of intravesicular pH increases during the endocytic process. However, using FITC-transferrin (29) and asialoorsomucoid (38), the pH of the endosomes was reported to be 5.0–5.8, between the pH of lysosomes and of extracellular fluid.

When FITC-HDL was incubated with sinusoidal liver cells at 0°C or 37°C, a marked change in fluorescence intensity was observed. At 0°C the ratio of 490/450 nm corresponded to a pH range of 7.1–7.3 (Fig. 6A) which is very close to the external pH of the buffer, indicating that most cell-associated FITC-HDL particles were represented by ligands bound to the extracellular phase of surface membranes. In contrast, a significant shift in pH to an acidic region (pH 6.1–6.3) was observed when cells were incubated at 37°C. In a similar experiment using membrane fractions obtained from the sinusoidal liver cells, the 490/450 nm ratio of membrane-bound FITC-HDL obtained by 37°C incubation did not differ from that obtained by 0°C incubation (data not shown). Thus, it is likely that cell surface-bound FITC-HDL particles were internalized and then exposed to an acidic environment during incubation at 37°C.

Weak bases such as NH_4Cl have been shown to accumulate in lysosomes and endosomes as a result of equilibrium distribution and hence raise their intravesicular pH (31, 33, 34). A similar phenomenon was observed in the present study. The acidic pH of FITC-HDL returned almost to the external pH by the addition of CCCP or NH_4Cl; in both cases the pH sensed by the marker increased quite rapidly within 1–2 min and remained more or less constant during the presence of these compounds (Fig. 6B). However, these compounds had no effect on the microenvironmental pH of FITC-HDL (pH 7.2) bound to the cells during incubation at 0°C (Fig. 6C). Since the cell-associated HDL obtained by 0°C incubation comprises mainly HDL bound to outer surface membranes (Fig. 2), the proton gradient may be involved in the post-binding events of HDL with these cells which occurred at 37°C but not at 0°C, a finding that gives positive support for our internalization model.

Fukuda et al.[2] employed a morphological approach to examine the post-binding events of HDL in rat peritoneal macrophages. Using a monovalent conjugate of HDL and horseradish peroxidase, it was shown that HDL bound to the coated pits of the surface membranes was internalized to form coated vesicles and delivered through endosomes to the transreticular elements of the Golgi apparatus, followed by retroendocytosis back to the extracellular medium. A parallel biochemical study using ^{125}I-labeled HDL revealed that 50% of the cell-associated HDL particles were resistant to trypsin treatment (S. Horiuchi, unpublished results). Moreover, incubation of FITC-HDL with these macrophages at 37°C but not at 0°C resulted in a significant decrease in its microenvironmental pH, an observation virtually similar to that seen in the present study using sinusoidal liver cells. Thus, it seems likely that the internalization mechanism might be operative in the post-binding fate of some, if not all, of HDL particles that were bound to their surface receptors of macrophages or macrophage-derived cells.

It becomes increasingly clear that HDL interaction with its membrane-associated receptor results in selective cholesterol transfer; amounts of HDL cholesterol transferred to the cells are disproportionately higher than those of HDL apolipoprotein (10, 17, 39, 40). This selective cholesterol transfer was suggested to occur without internalization of cell surface-bound HDL (41). In the light of the present finding, however, it is also possible to expect that the endocytic pathway of HDL via a nonlysosomal route might contribute, to some functional extent, to the cholesterol transfer across surface membranes of sinusoidal liver cells and probably of macrophages. It seems important, therefore, to determine whether the endocytosis–resecretion pathway of HDL is a main post-binding fate of surface-bound HDL or, alternatively, a simple supplemental route to back up cholesterol transfer by an "extracellular binding" mechanism.

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