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Uptake of [^{3}H]Vitamin D_3 from Low and High Density Lipoproteins by Cultured Human Fibroblasts (42270)

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Abstract. The plasma distribution and cellular uptake of [^{3}H]vitamin D_3 was studied *in vitro* using cultured human fibroblasts. Incubation of [^{3}H]vitamin D_3 (cholecalciferol) with plasma followed by sequential ultracentrifugal fractionation of the lipoproteins indicated that 2–4% of the radioactivity associated with the very low density lipoprotein (VLDL), 12% with low density lipoprotien (LDL), and approximately 60% with the high density lipoprotein (HDL). The remaining radioactivity, 25%, was associated with the sedimented plasma fractions. By comparison, an average of 86% of the radioactivity from [^{3}H]1,25-dihydroxycholecalciferol associated with the sedimented plasma fractions. The uptake of [^{3}H]vitamin D_3 from plasma, LDL, or HDL was studied in cultured human cells; uptake by normal fibroblasts was greatest from LDL and least from plasma. The cellular association of vitamin D_3 was time, concentration, and temperature dependent. At a concentration of 50 μg LDL/ml of medium, the uptake of [^{3}H]vitamin D_3 from LDL at 37°C was rapid and reached a maximum at approximately 4 hr; it was slower from HDL but continued to increase slowly up to 24 hr. The significance of these *in vitro* findings is uncertain since much of the vitamin D_3 absorbed from the intestine reportedly associates with chylomicrons and is rapidly taken up by the liver.

Although the metabolism of vitamin D has been extensively investigated and reviewed (1–4) there has been much speculation on the circulation of vitamin D from the sites of formation or absorption to target tissues (1). Current information suggests that, like most steroids, vitamin D and its metabolites are bound to protein during plasma transport. A number of ligand binding assays for this vitamin have been reported during the last decade, but most have been limited to the hydroxylated forms of the vitamin (5). The major protein carrier for the D metabolites is vitamin D binding protein (DBP), which is an α-globulin and has been well-characterized; it is now known to be identical to serum group specific component (GC or GC-globulin), which has been studied in the past as a genetic marker (6–14).

Instillation of [^{14}C]vitamin D_3 into the rat duodenum resulted in the rapid incorporation of the label into the chylomicrons with subsequent recovery in the liver (15). Early studies (11, 2) had indicated that vitamin D had much less affinity for DBP than the hydroxylated form; however, recent studies using disequilibrium conditions have indicated that D_3 has only a threefold less ability than 25-OH-D_3 in displacing [^{3}H]25-OH-D_3 from DBP (16). It was of interest to determine whether another carrier might be important in the plasma distribution of inactive vitamin D_3. The purposes of this study were to determine the distribution of [^{3}H]vitamin D_3 added *in vitro* to plasma, to compare its distribution with that of 1,25-dihydroxycholecalciferol, and to determine whether lipoproteins might be involved in the association of vitamin D_3 with cells *in vitro*.

Materials and Methods. *Cells.* Normal skin fibroblasts (GM969) and skin fibroblasts from a patient with homozygous familial hypercholesterolemia (FH) (GM1915, Human Genetic Mutant Cell Repository, Camden, N.J.) were grown in a 5% CO_2 atmosphere in basal medium with Earle's salts (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (GIBCO). For experiments, cells were plated into 60-mm petri dishes as previously described (17). Cell stocks were used between passage 15 and 19. Twenty-four hours prior to use, growth medium was replaced with lipoprotein-free medium.

Preparation of lipoprotein fractions. After ultracentrifugal separation and removal of very low density lipoprotein (VLDL), LDL was isolated from human plasma in the presence of 0.02% sodium azide, 0.01% merthiolate, 0.15% phenylmethylsulfonylfluoride

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(PMSF), and 0.2% soybean trypsin inhibitor by differential density preparative ultracentrifugation between density (*d*) 1.006 and 1.063 g/ml (18). After removal of LDL, the infranate was adjusted to *d* 1.20 with solid KBr for the isolation of HDL. The isolated lipoproteins were dialyzed extensively against phosphate-buffered saline (0.15 *M* NaCl/0.01 *M* sodium phosphate; PBS) at pH 7.4. Samples were filter-sterilized and stored at 4°C until use. Purity of LDL and HDL was determined by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (19). Protein concentrations were determined by the method of Lowry (20).

Addition of [^{3}H]vitamin D_3 or [^{3}H]-dihydroxyvitamin D to plasma or lipoprotein samples. [1α,2α(n)^{3}H]Vitamin D_3, sp act 25 Ci/mmole (Amersham, Arlington Heights, Ill.), had a radiochemical purity of 96.6% as determined by thin-layer chromatography. [26,27 *methyl* ^{3}H]1α,25-Dihydroxyvitamin D, sp act 170 Ci/mmole (Amersham), had a radiochemical purity of 94.4%. One to five microcuries (40–200 pmole) [^{3}H]vitamin D_3 were added to 1 ml toluene:ethanol (9:1) in a sterile foil-covered glass vial containing a single layer of glass beads; in comparison experiments 3–10 pmole [^{3}H]dihydroxyvitamin D were used. The solvent was dried under nitrogen; 10 ml plasma or 2 to 4 ml lipoprotein (15–30 mg protein) was added to the vial and incubated at 37°C for 4 hr and then held at 4°C overnight. The lipoprotein fractions were separated from the plasma by differential density ultracentrifugation as described above. In other experiments, the labeled plasma was divided into three portions and adjusted to densities 1.006, 1.06, or 1.20 g/ml and centrifugal at 40,000 rpm for 24 hr for isolation of VLDL, VLDL plus LDL, or VLDL plus LDL plus HDL, respectively.

In experiments where [^{3}H]vitamin D_3 was incorporated into LDL or HDL, the samples were chromatographed on a 75 × 1.5-cm Sepharose 6B column in phosphate-buffered saline to verify the association of vitamin D_3 with the lipoprotein. Coincidence of the A_{280} profile and the ^{3}H radioactivity confirmed the association. LDL or HDL eluted in the included volume; specific activities before and after chromatography were comparable. There was minimal solubility of [^{3}H]vitamin D_3 in saline alone. Calculations based on an average LDL molecular weight of 2.5×10^6 indicated that an average of two [^{3}H]vitamin D_3 molecules were present per particle. Based on an average HDL molecular weight of 3.5×10^5, less than one [^{3}H]vitamin D_3 molecule was present per particle for most experiments.

Before each experiment, the purity of the [^{3}H]vitamin D_3 was checked by thin-layer chromatography, using unlabeled D_3 as a standard. Samples were spotted on silica gel TLC sheets containing fluorescent indicator and chromatographed in chloroform:ethanol (9:1). The location of vitamin D_3 was determined by fluorescence under 254-nm light. Sheets were covered with clear tape and cut into strips (2 × 0.5 cm) for counting in Econofluor-2 (New England Nuclear, Boston, Mass.).

LDL and HDL were radiolabeled with ^{125}I by a modification of the iodine monochloride technique (21).

Uptake by cells. The cellular uptake of [^{3}H]vitamin D_3 and [^{125}I]lipoprotein was determined in a modification of the cell uptake assay of Goldstein and Brown (22) using either labeled LDL, HDL, or plasma. Cells were incubated for the indicated times at 37°C or 4°C in basal medium containing the labeled material. Fetal bovine serum was omitted from the incubation medium. After incubation, medium was removed and the cell monolayers were washed five times with cold PBS. Cells were hydrolyzed with 2 ml of 0.1 *N* NaOH. Aliquots were taken for protein determinations and the remaining suspension was used for quantitation of the ^{125}I radioactivity by a Model 5500 Gamma Counter and of the ^{3}H radioactivity in Aquasol (New England Nuclear) or Dimilume (Packard, Downers Grove, Ill.) by a scintillation spectrometer. Efficiency of tritium counting was 39%. Significance of difference was calculated by Student's *t* test (23).

Results. Sequential ultracentrifugation and fractionation of duplicate samples of plasma (10 ml) incubated with 1 μCi [^{3}H]vitamin D_3 in three trials resulted in an average of 2, 11, and 62% of the radioactivity appearing in the VLDL, LDL, and HDL fractions, respectively. In two separate trials, plasma samples were divided into three portions and adjusted with solid KBr to densities 1.006, 1.063, or 1.20 g/ml; a single 24-hr centrifugation was then performed. The *d* = 1.006 sample (VLDL only)

contained an average of 4% of the radioactivity, the $d = 1.06$ sample (VLDL plus LDL) contained 16%, and the $d = 1.20$ sample (VLDL, LDL plus HDL) contained 72% of the radioactivity. In each case, the majority of the remaining counts were located at the bottom of the centrifuge tube. In addition, two subsequent centrifugation experiments compared the distribution of radiolabeled vitamin D_3 and dihydroxyvitamin D in plasma. As in the previous experiments, the majority of the radioactivity from [^{3}H]D_3, an average of 69%, associated with the lipoproteins; however, an average of only 14% (range 7–19%) of the tritium radioactivity from the dihydroxyvitamin D-incubated plasma was associated with the lipoproteins.

Isolated HDL and LDL which were incubated separately *in vitro* with [^{3}H]vitamin D_3 exhibited the same SDS–PAGE and Sepharose 6B elution patterns as native HDL and LDL, indicating that the procedure did not result in obvious proteolysis or breakdown of the lipoproteins. Also, the thin layer chromatograms of the radioactive material extracted from labeled HDL and LDL showed no change in the R_f, indicating no alteration in [^{3}H]vitamin D_3 during the experimental procedures.

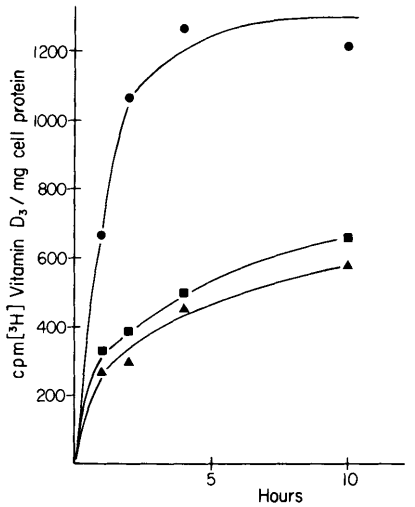

FIG. 1. Normal skin fibroblasts were incubated at 37°C for 2 hr with medium containing 2.5×10^3 cpm [^{3}H]vitamin D_3 per ml to determine cellular uptake of D_3 from plasma (▲), LDL (●), or HDL (■). The medium contained 50 μg LDL protein/ml, 93 μg HDL/ml, or 697 μg plasma protein/ml.

TABLE I. CELLULAR UPTAKE OF [^{125}I]LDL AND [^{125}I]HDL BY NORMAL AND FH FIBROBLASTS AT 37°C

Time of incubation (hr)	μg [^{125}I]LDL/mg cell protein[a]		μg [^{125}I]HDL/mg cell protein	
	Normal cells	FH cells	Normal cells	FH cells
0.5	1400[b]	770	250	340
1	1450	820	260	290
2	2210	890	390	310
6	3970	1390	440	380

[a] Incubation medium contained 50 μg [^{125}I]LDL or 90 μg [^{125}I]HDL/ml to correspond to concentrations in [^{3}H]D_3 experiments.

[b] Values are averages of duplicates in a typical experiment of four such experiments. The average variation about the mean value was 9%. Values represent total binding and uptake.

A comparison of uptake by normal fibroblasts from plasma, LDL, or HDL with each medium containing the same amount of [^{3}H]vitamin D_3 showed much greater cellular uptake when the D_3 was associated with LDL ($t = 8.7$ for LDL vs plasma, $P < 0.001$) (Fig. 1). The uptake from HDL was slightly higher than from plasma ($t = 2.6$ for HDL vs plasma, $P < 0.025$).

LDL is known to be taken up by normal cells via a receptor-mediated pathway. As has been shown by many other researchers (22, 24, 25), LDL receptor-positive normal human skin fibroblasts bind and take up [^{125}I]LDL with high affinity, while cells from patients with homozygous familial hypercholesterolemia (FH) are receptor-negative and incorporate LDL only nonspecifically. HDL uptake by fibroblasts is not receptor-mediated and, as seen in Table I, it was taken up by both cell lines in a nonspecific manner. [^{125}I]LDL incorporation increased in both normal cells and FH cells with time but to a much lesser extent in the receptor-negative cells (Table I). Radioactivity associated with [^{125}I]HDL was approximately the same as FH and normal cells and increased only slightly over the 6 hr. Association of unlabeled vitamin D_3 with the ^{125}I-labeled LDL or HDL did not alter the cellular uptake of these lipoproteins.

Table II shows the results of experiments in

TABLE II. CELLULAR UPTAKE OF [^{3}H]VITAMIN D_3 ASSOCIATED WITH LDL OR HDL[a]

Time	[^{3}H]Vitamin D_3-LDL (cpm/mg cell protein)		[^{3}H]Vitamin D_3-HDL (cpm/mg cell protein)	
	N	FH	N	FH
10 min	[b]590 ± 130[c]	520 ± 120	240 ± 20	240 ± 10
30 min	790 ± 60	670 ± 80	310 ± 20	330 ± 10
1 hr	800 ± 90	900 ± 70	410 ± 50	360 ± 10
2 hr	930 ± 90	920 ± 70	500 ± 90	410 ± 20
4 hr	1120 ± 60	1280 ± 120	510 ± 90	510 ± 50
6 hr	1060 ± 50	1150 ± 80	670 ± 50	560 ± 60
24 hr	1230 ± 60	1140 ± 80	770 ± 160	720 ± 120

[a] Normal and FH skin fibroblasts were incubated at 37°C for the times indicated in media containing 50 μg LDL protein/ml or 90 μg HDL protein/ml. In each experiment, the [^{3}H]vitamin D_3 in the HDL medium as calculated and adjusted to be the same as that in the LDL medium, approximately 3500–3800 cpm/ml medium. Efficiency of counting was 39%.

[b] Each value is based on the mean of eight samples in four experiments, except the 10 min and the HDL 6- and 24-hr values, which are means of four samples in two experiments.

[c] Standard error of the mean.

which [^{3}H]vitamin D_3 uptake was compared in normal vs FH cells. No significant difference between normal and FH cells occurred in the time-dependent uptake of [^{3}H]vitamin D_3 associated with LDL, even though high-affinity uptake of LDL occurred only in receptor-positive cells (Table I). As indicated in Fig. 1, the uptake of [^{3}H]vitamin D_3 from HDL was much lower ($t = 14.8$, $P < 0.001$); there was no difference in the uptake from HDL between the two cell lines (Table II). In these experiments, the D_3 had been incorporated *in vitro* into the separated fractions. Similar uptake experiments were performed with LDL and HDL isolated from plasma incubated with [^{3}H]vitamin D_3, and, again, no differences were noted between the two cell lines. The pattern of uptake differed between LDL and HDL, as there was little or no increase in ^{3}H radioactivity with LDL after 4-hr incubation while the radioactivity associated with the HDL continued to increase gradually over the 24-hr period. Even so, the amount of D_3 uptake from HDL was much less than from LDL.

Data in Table III indicate that the transfer of D_3 from HDL into cells was temperature dependent ($t = 2.94$, $P < 0.05$ for both normal and FH cells). Even at 4°C some [^{3}H]vitamin D_3 apparently associated with cells. [^{125}I]HDL uptake was determined as a control in these experiments; while the ratio of ^{3}H/^{125}I associated with HDL in the medium was only 0.23, this ratio was, on the average, 0.44 for both lines at 4°C and 0.63 at 37°C.

As shown in Table IV, uptake of [^{3}H]-vitamin D_3 from LDL was also temperature

TABLE III. TEMPERATURE-DEPENDENT CELLULAR UPTAKE OF [^{3}H]VITAMIN D_3 FROM HDL

	cpm [^{3}H]vitamin D_3/mg cell protein			
	10 min	30 min	60 min	120 min
Normal cells (4°C)	210 ± 20[a]	210 ± 20	230 ± 10	240 ± 20
Normal cells (37°C)	250 ± 30	310 ± 30	340 ± 60	430 ± 50
FH cells (4°C)	170 ± 10	210 ± 20	210 ± 20	240 ± 10
FH cells (37°C)	250 ± 10	320 ± 20	330 ± 20	440 ± 30

[a] Each value, ±SD, is based on the average of triplicate plates in a representative experiment. The medium contained 100 μg HDL/ml and approximately 3×10^5 cpm/ml.

TABLE IV. TEMPERATURE-DEPENDENT CELLULAR UPTAKE OF [^{3}H]VITAMIN D_3 FROM LDL

	cpm [^{3}H]vitamin D_3/mg cell protein			
	30 min	60 min	120 min	240 min
Normal cells (4°C)	870 ± 90	820 ± 70	940 ± 50	1210 ± 0
Normal cells (37°C)	1900 ± 140	2240 ± 150	2560 ± 120	4010 ± 80
FH cells (4°C)	700 ± 20	880 ± 90	1140 ± 60	1200 ± 30
GH cells (37°C)	1880 ± 20	2400 ± 140	2740 ± 270	3650 ± 20

Note. Each value is the average ±SD of duplicate determinations in a representative experiment of two such experiments. The medium contained approximately 5.8×10^5 cpm/ml.

dependent with no significant difference occurring between the two cell lines. In the [^{125}I]LDL control for this experiment, the uptake of ^{125}I radioactivity differed at 37°C for the two lines (data not shown, see Table I for representative data). The [^{125}I]LDL uptake at 4°C was much lower and did not differ between the normal and FH cells. This has been shown previously by numerous investigators (17, 22, 26, 27).

The uptake of [^{3}H]vitamin D_3 is dependent upon the concentration of labeled LDL in the medium (Table V). A larger proportion of the vitamin D_3 moved into the cells at lower concentrations, as shown by the ratio of ^{3}H to ^{125}I counts. Similar results were seen in concentration-dependent studies with HDL (Table VI).

Discussion. Vitamin D_3 (cholecalciferol) is produced in the skin by the photochemical conversion of 7-dehydrocholesterol. Presumably, it then diffuses into the blood. Bouillon and Van Baelen (28) have suggested that since DBP circulates in large molar excess, very little vitamin D would remain unbound or free and that tissue distribution may be largely controlled by DBP. The liver D_3 may be inactivated and excreted into bile (29) or may undergo hydroxylation to 25-hydroxycholecalciferol. Vitamin D_3 which is absorbed from the intestines rapidly appears in the chylomicrons and is transported to the liver for hydroxylation (15, 30). Further hydroxylation occurs in the kidney. Small amounts of 25-hydroxylase activity are thought to be present in extrahepatic sites (31).

In the present *in vitro* study, vitamin D_3 associated mostly with the lipoproteins in plasma, especially with HDL. These findings are in contrast to those reported by Kida and Goodman (32), but agree with earlier conclu-

TABLE V. CONCENTRATION-DEPENDENT CELLULAR UPTAKE OF [^{3}H]VITAMIN D_3 FROM LOW DENSITY LIPOPROTEIN

μg LDL/ml medium (hr)	[^{125}I]cpm/mg cell protein		[^{3}H]cpm/mg cell protein	
	FH cells	Normal cells	FH cells	Normal cells
5	2400	3700	800 (0.30)[a]	900 (0.20)
15	5100	9500	1100 (0.20)	1400 (0.10)
25	9900	14000	1500 (0.20)	1700 (0.10)
50	16000	20000	1800 (0.10)	1800 (0.10)

[a] Numbers in parentheses are the ratios of ^{3}H cpm to ^{125}I cpm. Values are the averages of duplicate determinations in a typical experiment. There were 1.1×10^4 cpm/ml in the 50 μg [^{3}H]LDL medium/ml and 1.5×10^6 cpm/ml in the 50 μg [^{125}I]LDL medium/ml (ratio = 0.01). The incubation was for 2 hr at 37°.

TABLE VI. CONCENTRATION-DEPENDENT CELLULAR UPTAKE OF [^{3}H]VITAMIN D_3 FROM HIGH DENSITY LIPOPROTEIN

μg HDL/ml medium	[^{125}I]cpm/mg cell protein		[^{3}H]cpm/mg cell protein	
	FH cells	Normal cells	FH cells	Normal cells
5	1700	1800	1000 (0.60)[a]	900 (0.50)
15	5500	4500	1800 (0.30)	1400 (0.30)
25	8000	7100	1800 (0.20)	1900 (0.30)
50	11700	11200	2800 (0.20)	2800 (0.20)

[a] Values in parentheses are the ratios of ^{3}H cpm to ^{125}I cpm. Values are the averages of duplicate determinations. In this experiment, there were 8.6×10^5 cpm/ml in the 50 μg [^{125}I]HDL medium and 2.3×10^4 cpm/ml in the 50 μg [^{3}H]HDL medium (ratio = 0.03). The incubation was for 2 hr at 37°C.

sions that D_3 associates with lipoproteins after intestinal absorption (1, 33). The relevance of these *in vitro* findings to *in vivo* events is not clear, but two reports from *in vivo* studies in rats have stated that duodenally instilled vitamin D_3 associated with chylomicrons in the lymph and transferred *in vitro* (34) and *in vivo* (35) to the $d > 1.006$ g/ml fractions of lymph or plasma. In hepatectomized rats approximately one-half of the injected chylomicrons labeled with [^{3}H]vitamin D_3 were recovered after 30 min in the $d > 1.21$ g/ml fraction. Hence, *in vivo* vitamin D_3 in the chylomicron apparently is taken up by the liver or it transfers to DBP.

Many of the hydrophobic compounds transported by HDL and LDL exchange or transfer from lipoprotein directly to cell membranes (36). The LDL particle also delivers its components to cells *in toto* when it binds to the cell membrane receptor and is internalized. When equal concentrations of [^{3}H]vitamin D_3 were present in LDL, HDL, or plasma, cellular uptake was greatest from LDL; the ratio of ^{3}H/^{125}I, however, indicated that D_3 movement did not parallel [^{125}I]LDL uptake by the cells. Likewise, the ^{3}H/^{125}I ratio in HDL indicated that greater movement of the [^{3}H]vitamin D_3 into cells occurred compared to [^{125}I]HDL. The receptor-positive cells did not accumulate more [^{3}H]vitamin D_3 than the receptor-negative cells, and it is unclear why more [^{3}H]vitamin D_3 associated with cells which were incubated with LDL compared to HDL. The incubation medium contained the same amount of [^{3}H]vitamin D_3, but the number of HDL particles was greater than the number of LDL particles. Little is presently understood about the nonspecific association of LDL and HDL with cell membranes. From the data in Table I on the uptake of [^{125}I]LDL and [^{125}I]HDL by FH cells it appears that the nonspecific association of LDL with cultured cells is much greater than that of HDL, despite the higher concentration of HDL in the medium.

It was not possible to quantitate the total (labeled and unlabeled) vitamin D_3 present in the cultured cells because of detection limits and therefore it is not known whether the total cellular content of the vitamin increased. It is clear that *in vitro* the LDL receptor pathway is not essential for the delivery of vitamin D_3 to cells. Similar observations have been noted with other labeled compounds associated with LDL. It has been shown that the LDL receptor pathway is not necessary for the uptake of benzo[*a*]pyrene into cultured cells (27). However, a difference in cellular accumulation of [^{3}H]cholesterol occurred in normal vs FH cells (17), indicating the importance of the LDL receptor-mediated pathway in cholesterol delivery. Also, in contrast to D_3, vitamin E uptake into cells from LDL appears to be dependent upon the LDL pathway (37).

It seems evident that the individual components of lipoproteins can transfer from (and perhaps into) the lipoprotein particles independently of each other. Accumulation by cells *in vitro* depends upon time and temperature of incubation and the concentration of the lipoprotein as well as concentration of the labeled hydrophobic compound within the lipoprotein. Transfer of lipid in the absence of an intact LDL receptor pathway may be a mechanism to ensure the delivery of essential lipid components to cells.

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