Vol. 215, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 4, 1995 Pages 199-204

BINDING OF VITAMIN D TO LOW-DENSITY-LIPOPROTEIN (LDL) AND LDL RECEPTOR-MEDIATED PATHWAY INTO CELLS

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Received August 28, 1995

Summary: The present study was undertaken to identify serum components other than vitamin D-binding proteins that bind to 1,25(OH)$_2$ D$_3$, and its analog. The binding rate of 1,25(OH)$_2$ D$_3$, 22-oxa-1,25(OH)$_2$D$_3$ (OCT) or 25(OH) D$_3$ to total lipoprotein(TLP) represented 16.7%, 4.65%, and 3.11% of total counts added, respectively. Polyacrylamide gel electrophoresis of the TLP revealed that 1,25(OH)$_2$ D$_3$ and OCT were associated with LDL. The binding studies of OCT-bound LDL to the fibroblasts showed specific pathway to the cells mediated by LDL-receptor. These findings may have important implications in understanding the mechanisms of the diverse biological actions of 1,25(OH)$_2$ D$_3$ and in designing a novel delivery system for vitamin D analogs.

Since 1,25-dihydroxy cholecalciferol (1,25-(OH)$_2$ D$_3$) has a potent antiproliferative and differentiation-inducing activity (1,2) as well as repressive activity of parathyroid hormone related protein gene, 1,25-(OH)$_2$D$_3$ is expected to have potential as a therapeutic agent for malignancy-associated hypercalcemia. However, 1,25-(OH)$_2$D$_3$ itself is not suitable for clinical application because of its potent hypercalcemic activity. In recent years various vitamin D$_3$ analogs have been developed in an attempt to separate the classic calcemic activity from the nonclassic actions on cellular proliferation and differentiation in cancer cells (3). Among the analogs of 1,25-(OH)$_2$D$_3$, 22-oxa-1,25(OH)$_2$D$_3$ (OCT) has been shown to inhibit cancer cell growth *in vitro* and *in*

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0006-291X/95 $12.00

vivo more potently than the parent compound and yet have little calcemic activity (4,5). Based on the observations that the affinity of OCT for plasma vitamin D-binding protein (DBP) is approximately 1/260-1/580 of that of the parent compound (6,7) and that OCT is rapidly cleared from the circulation (7,8), it is thought that the differences in their pharmacokinetics are at least in part caused by the differences in their affinity for DBP and may cause the differences in their biological activity. Besides the pharmacokinetic differences, alternative reasons for the nonclassic activity of OCT may be the requirement of specific carrier proteins or the delivery of the sterol to various target tissues. The present study was undertaken to identify the carrier proteins in human serum other than DBP that bind to vitamin D analogs and the delivery system to the cells.

Materials and Methods

Chemicals-: OCT and [2ß -^{3}H]OCT (3.2TBq/mmol) were synthesized in Chugai laboratory as described previously (9). 1,25-$(OH)_2D_3$ was obtained from Philips Duphar Co. (Amsterdam, The Netherlands), and 1,25-$(OH)_2$ [26,27-*methyl* -^{3}H] D_3 (6.55TBq/mmol) and 25-(OH) [26,27-methyl-^{3}H] D_3 (6.55TBq/mmol) were purchased from Amersham (Buckinghamshire, U.K.). Monoclonal anti-human apo B100 antibody-coupled sepharose 4B gels (apo B100 gels) were kindly provided by Japan Immunoresearch Laboratories(10). Lysine-coupled sepharose 4B gels (Lysine gels) were purchased from Pharmacia Biotech Co.(Uppsala, Sweden). Normal human fibroblasts were obtained from a normolipidemic subject. LDL-receptor negative fibroblasts were kindly provided by Dr. Y. Miyake(11).

Binding of OCT to serum lipoproteins: Serum was obtained from normal volunteers after a 12-hour fast. Each 100 µl of the serum was incubated with [^{3}H]-OCT, 1,25-$(OH)_2$[^{3}H]-D_3 and 25(OH)[^{3}H]-D_3 at 37°C for 15 min at the concentrations of 1nM, 20nM, and 200nM. After the incubations, total lipoprotein (TLP) fractions were separated by ultracentrifugation to a density less than 1.21 g/ml. Lipoproteins of TLP fractions were displayed on a 2-16% polyacrylamide gradient gel electrophoresis (GGE) without SDS as described previously(12). After electrophoresis, the gels were subjected to autoradiography by analysis with a Fuji BAS 2000 Bio-image analyzer system(Fuji Photo Film Co Ltd. Tokyo)(13). Apo B100 gels were used to separate the apo B100-containing lipoproteins from the serum according to the method of Nakajima et al.(10). Thirty µl of serum, which had been incubated with radiolabeled vitamin D analogs at the concentrations of 100 pM, 1nM, and 20 nM at 37°C for 15 min, was added to 300 µl of apo B100 gel. The gel mixtures were incubated for 1 hr and centrifuged at 5000 rpm for 5 min in microcentrifuge MRX-150 (Tomy,Tokyo); 300 µl of the supernatant was taken to count the radioactivities, and the gels were washed three times with cold phosphate buffered saline (PBS) and were counted. Lysine gels were used to separate Lp(a) from LDL fraction according to the method of Snyder et al.(14).

Binding of OCT bound-LDL to fibroblasts: Fibroblasts were plated in 60-mm plastic Petri dishes in Minimum Essential Medium(MEM) containing 10% fetal bovine serum at $7.5X10^4$ cells for use 7 days later. At 48 hr before the experiment, the cells were washed twice with cold PBS followed by the addition

of MEM containing 10% lipoprotein deficient serum. The medium on the cells was replaced with the medium containing [^{3}H]-OCT bound LDL. The incubations were conducted for 0,1,2,5 hours at 37°C at the concentration of 10 μg/ml of [^{3}H]-OCT bound-LDL with or without a 50 fold molar excess of unlabeled LDL or HDL. After incubations, the cells were washed 5 times with ice-cold PBS containing 0.5% BSA , twice with ice-cold PBS and then dissolved by incubating them with 1 ml of 0.1N NaOH for 30 min. The dissolved cells were removed into scintillation vials. To wash-out the surface bound radioactivity, the cells were incubated with 50 mM NaCl / 10mM HEPES (pH 7.4) containing 4mg/ml dextran sulfate (DS) for 1 hr after 5hr-incubations with [^{3}H]-OCT bound LDL at 37°C.

Results

When normal human serum was incubated at 37°C with vitamin D analogs, the binding rate of each analog to TLP is as shown in Table 1. The binding rates of 1,25(OH)D3, OCT, and 25(OH)D3 were constant at concentrations between 1nM and 200 nM and were between 15.8 and 17.2 %, between 4.65 and 4.98%, and between 2.36 and 3.11% of the total counts added, respectively. The remaining radioactive analogs were recovered in the bottom fraction with a density more than 1.21 g/ml. Figure 1 shows autoradiography of GGE of the TLP and revealed a visible single band on each lane, of which Rf corresponded to purified LDL displayed in the far left lane of the protein staining gels of Fig.1. The binding study with use of apo B100 gels showed that binding

Table 1. Binding of OCT, 1,25(OH)$_2$ D3, and 25(OH) D3 to total lipoprotein fraction or apo B100-containing lipoproteins

	TLP-bound	ApoB100 LP-bound
OCT (1nM)	4.65 ± 0.62	5.78 ± 0.88
1.25(OH)$_2$D3 (1nM)	16.7 ± 0.65	13.4 ± 0.49
25(OH) D3 (1nM)	3.11 ± 0.27	5.33 ± 0.44
	% of total counts	
	Mean ± SE(n=3)	

Each 100 μl of the serum was incubated with [^{3}H]-OCT, 1,25-(OH)$_2$[^{3}H]-D3 and 25(OH)[^{3}H]-D3 at 37°C for 15 min at the concentrations of 1nM, 20nM, and 200nM. After the incubations, total lipoprotein (TLP) fractions were separated by ultracentrifugation to a density less than 1.21 g/ml. Apo B100 gels were used to separate the apo B100-containing lipoproteins (apo B100 LP) from the serum. The serum, which had been incubated with radiolabeled vitamin D analogs at the concentrations of 100 pM, 1nM, and 20 nM at 37°C for 15 min, was added to apo B100 gels. The binding rates represent the percentage of the total radioactivities added to the serum. The results obtained from the incubations with vitamin D analogs at the concentration of 1 nM were shown.

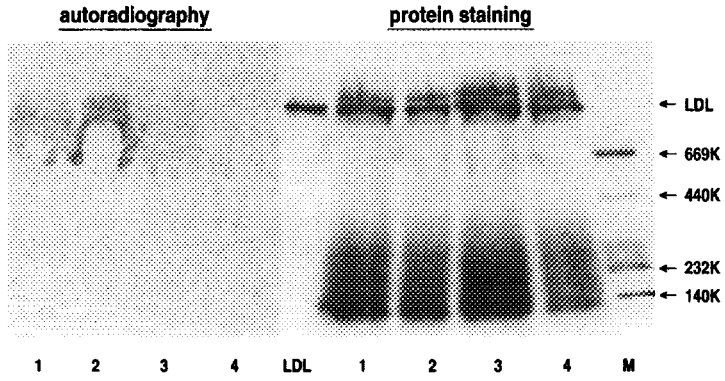

Fig.1. Autoradiography and protein staining of vitamin D analogs-bound lipoproteins displayed by polyacrylamide gel electrophoresis.
Each 100 μl of the serum was incubated with ^{3}H-OCT, 1,25-(OH)$_2$[^{3}H]-D$_3$ and 25(OH)[^{3}H]-D$_3$ at 37°C for 15 min. After the incubations, total lipoprotein (TLP) fractions were separated and were displayed on a 2-16% polyacrylamide gradient gel electrophoresis without SDS. After electrophoresis, the gels were subjected to autoradiography by analysis with a Fuji BAS 2000 Bio-image analyzer system.
1: TLP incubated with [^{3}H]-OCT, 2, TLP incubated with 1,25-(OH)$_2$[^{3}H]-D$_3$, 3,4: TLP incubated with 25(OH)[^{3}H]-D$_3$, M: molecular weight marker, LDL: LDL separated from control serum to the density between 1.019 and 1.063 g/ml.

rates of the analogs were basically coincident with the binding rate with TLP, as shown in Table 1, suggesting that the binding lipoprotein in the TLP is apo B100-containing lipoprotein. The study of Lysine gels was performed to separate Lp(a) from LDL fractions, and revealed that only 0.031% of OCT and 0.035% of 1,25(OH)$_2$D$_3$ were recovered in Lp(a) fraction.

When [^{3}H]-OCT-bound LDL was incubated with skin fibroblasts derived from a normal subject, a time-dependent increase in cellular uptake of [^{3}H]-OCT was observed, which was inhibited by excess LDL, but not by HDL. In contrast, LDL-R-negative fibroblasts revealed little uptake of [^{3}H]-OCT-bound LDL, as shown in the left panel of Fig.2. In order to determine whether [^{3}H]-OCT-bound LDL associated with fibroblasts was on the surface or inside the cells, the fibroblasts were washed with DS. As shown in the right panel of Fig.2, about 90% of total count associated with the cells was recovered inside the cells.

Discussion

The results of our present study indicate clearly that vitamin D analogs bind solely to LDL among lipoproteins, though the binding rate of each analog was

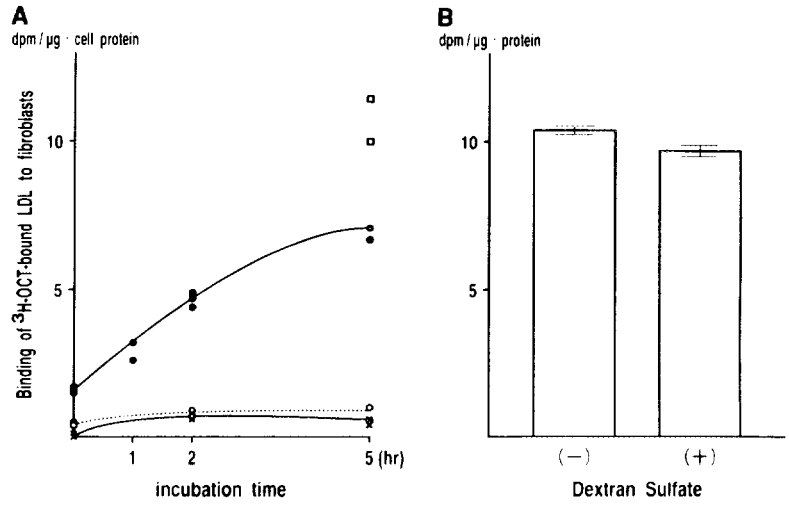

Fig 2. Binding of [^{3}H]-OCT bound-LDL to fibroblasts.
At 48 hr before the experiment, the fibroblasts were washed twice with cold PBS followed by the addition of MEM containing 10% lipoprotein deficient serum. After addition of [^{3}H]-OCT bound-LDL, the incubations were conducted for 0,1,2,5 hours at 37°C with or without a 50-fold molar excess of unlabeled LDL or HDL. Panel A) showed the time course of binding of [^{3}H]-OCT bound-LDL to the cells. Control fibroblasts were incubated with [^{3}H]-OCT bound-LDL with (-X-) or without unlabeled LDL (-●-), or with HDL (-□-). LDL receptor negative fibroblasts were incubated with [^{3}H]-OCT bound-LDL(-o-). The experiments were conducted with duplicate or triplicate dishes. To wash-out the surface bound radioactivity, the cells were incubated with dextran sulfate for 1 hr after 5hr-incubations with [^{3}H]-OCT bound LDL at 37°C. Panel B) showed the results of triplicate dishes for each. The vertical bars are mean ± standard error.

not so high, between 5 and 16 % of the total radioactivity added. When lipoproteins are separated by ultracentrifugation, some of the surface-bound components such as apolipoprotein E or A-I are dissociated from the particles by the effect of centrifugal gravity and/or high salt concentration. However, the study of apo apo B100 gels showed coincidental results with ultracentrifugal separation, suggesting that the low binding rate was not an artifact of ultracentrifugation and that the binding lipoproteins were apo B100-containing lipoproteins. There are three kinds of lipoproteins containing apo B100 in human serum, VLDL, LDL, and Lp(a). The result from GGE excluded the possibility of the binding to VLDL, and the study of Lysine gels revealed that the binding to Lp(a) was negligible. Okano et al. (6) and Kobayashi et al. (8) raised the possibility that OCT and 1,25(OH)$_2$D$_3$ bind to chylomicrons as well as LDL . Our study was conducted using human serum after 12-hr fasting, by which time it is usually free from chylomicrons. The half life of chylomicrons is very short,

between 5 and 15 min (15), and the fractional catabolic rate of LDL has been reported to be 0.45 day^{-1}(16). Thus LDL may play a major role as a carrier protein of vitamin D analogs in addition to DBP. Another important result of our study is that vitamin D-bound LDL is transported into the cells through LDL receptor. This evidence suggests that, as a result of their low binding to DBP, OCT is transported at low concentration in blood by LDL and enters the cells, LDL-receptor mediated pathway. Ho et al. reported that malignant cells express high levels of LDL receptor activity(17). Endo et al. also showed that OCT is incorporated into cancer cells effectively despite its rapid clearance from the circulation(18). Taken together, it is suggested that LDL receptor pathway may play a role in the entrance of OCT into the cells expressing high levels of LDL-receptor such as certain malignant cells. These findings may have important implications in understanding the mechanisms of the diverse biological actions of $1,25(OH)_2 D_3$, and in designing a delivery system for the analogs.

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