# Impact of Different Low-Density Lipoprotein (LDL) Receptor Mutations on the Ability of LDL to Support Lymphocyte Proliferation

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**Based on the demand for cholesterol for membrane formation, we determined the ability of low-density lipoprotein (LDL) to support proliferation in lymphocytes bearing different LDL receptor mutations, which were treated "in vitro" with lovastatin to inhibit endogenous cholesterol synthesis. Peripheral lymphocytes were isolated from two patients with homozygous familial hypercholesterolemia (FH), one homozygote for the mutation N804K ($FH_{Colmenar}$) in exon 17, herein described for the first time, and a compound heterozygote carrying the mutations D280G and G528V, which determine a transport-defective biochemical phenotype. Flow cytometric analysis with 1,1′-dioctadecyl-3,3,3,3′-tetramethylindocarbocyanineperchlorate (DiI)-LDL showed normal LDL binding but defective internalization in lymphocytes from case 1, whereas in lymphocytes from case 2 both LDL binding and internalization were affected. Studies with mitogen-stimulated lymphocytes demonstrated that despite the different phenotype, the ability of LDL to support proliferation was impaired in both cases to a similar extent. These results indicate that internalization of the LDL particle is required for expression of the mitogenic effect of LDL.**


THE LOW-DENSITY LIPOPROTEIN (LDL) receptor mediates the specific cellular uptake of apolipoprotein B-100–containing lipoproteins, mainly LDL. Thereby, LDL receptor activity determines the clearance rate of plasma LDL and consequently plasma LDL levels.[1] Mutations in the LDL receptor gene cause the autosomal codominant disease, familial hypercholesterolemia (FH).[2] Heterozygous FH (FH) individuals with one defective gene have markedly elevated plasma cholesterol that is frequently associated with premature coronary heart disease.[2,3] Homozygous FH patients are more severely affected, and without intensive cholesterol-lowering treatment such as LDL apheresis, they rarely reach the age of maturity.[3] A large number of mutations of the LDL receptor gene (deletions, insertions, and nonsense and missense mutations) have been characterized at the DNA level. Five classes of functional defects were identified[3-6] that affect the synthesis, intracellular transport, ligand-binding properties, internalization, and recycling of the receptor. According to the molecular defect, absolute LDL internalization by cells through the LDL receptor is reduced in all five classes, whereas LDL binding to cells is diminished in all except class 4 mutations.[3,4] This allows the functional distinction of class 4 from the other phenotypes.

Normal LDL receptors are required for the efficient provision of LDL cholesterol to cells.[7] This has been clearly shown in proliferating cells, which have increased demands for cholesterol for membrane formation. Upon inhibition of cholesterol synthesis, for example, with hepatic hydroxymethyl glutaryl coenzyme A reductase inhibitors, cells depend on lipoproteins for normal growth. Thus, whereas LDL efficiently reverses lovastatin-mediated suppression of proliferation in normal lymphocytes, this lipoprotein is ineffective in LDL receptor–negative cells.[8] This approach has been used to measure the functional utilization of cholesterol by cells rather than merely the binding and uptake.[8,9] However, the effect of different LDL receptor mutations on the ability of LDL to support cell proliferation has not been studied. The effect of LDL on cells bearing a noninternalizable LDL receptor (class 4 mutation) is particularly interesting because some actions of LDL may be mediated by signal transduction and may not require lipoprotein internalization.[10] On the other hand, in addition to the LDL receptor, other receptors may contribute to the uptake of lipoprotein cholesterol, an aspect that has not been evaluated in circulating lymphocytes.

In the present investigation, we studied LDL receptor activity by flow cytometry and LDL cholesterol utilization for cell growth in lymphocytes from two homozygous FH patients with different LDL receptor mutations affecting LDL binding or internalization. We found that the ability of LDL to support proliferation was impaired in both cases to a similar extent, which indicates that internalization of the LDL particle is required for expression of the mitogenic effect of LDL.

## SUBJECTS AND METHODS

### *Patients*

Clinical characteristics of the patients have been reported previously.[11,12] In brief, case 1 is a 15-year-old boy diagnosed with homozygous FH at the age of 6 months, with serum cholesterol concentrations varying from 850 to 1,000 mg/dL. At the age of 8 years, the patient had multiple xanthomas and corneal arcus with no clinical evidence of atherosclerotic disease, and he entered a program of LDL apheresis with dextran sulfate columns (Liposorber LA-15; Kanegafuchi Chemical Industry, Osaka, Japan). The evolution of the plasma lipids and clinical condition during the LDL apheresis treatment period has been reported elsewhere.[11] Currently, LDL apheresis is being performed biweekly and LDL cholesterol levels before each treatment are within the range of 315 to 439 mg/dL.

Case 2 is a 33-year-old woman clinically diagnosed at the age of 7 years, when she had cutaneous and tendinous xanthomas on the elbows,

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knees, and Achilles tendon and severe hypercholesterolemia. At the age of 27, the patient had mild aortic stenosis without significant lesions in the coronary arteries, and entered the LDL apheresis treatment. The effect of this treatment on plasma lipids is reported elsewhere.[11,12] During the period the present investigations were performed, LDL cholesterol plasma concentrations before each apheresis session were within the range of 361 to 487 mg/dL. Molecular analysis of the LDL receptor gene showed that this patient is a compound heterozygote, harboring the mutations D280G (exon 6) and G528V (exon 11), which are mapped to the LDL-binding region and epidermal growth factor–precursor homology region, respectively.[13]

### *LDL Isolation and Labeling*

LDL was isolated from either a pool of sera from normolipidemic individuals or from patient no. 2 by single vertical-spin ultracentrifugation (VTi 50 rotor; Beckman Instruments, Palo Alto, CA).[14] In the latter case, the starting material was retained in the dextran sulfate affinity columns used for LDL apheresis treatment. No difference in mitogenic activity was detected between these two LDL preparations. The LDL fractions were exhaustively dialyzed against saline (0.15 mol/L NaCl), sterilized by filtering, and stored in the dark at 4°C. When indicated, LDL was labeled with the fluorescent probe 1,1′-dioctadecyl-3,3,3,3′-tetramethylindocarbocyanineperchlorate ([Dil] Molecular Probes, Leiden, The Netherlands) at a final ratio of 300 mg Dil/mg LDL protein as previously described.[15,16] The extent of Dil incorporation into LDL was assessed by fluorometric measurements on a Perkin-Elmer (Norwalk, CT) spectrofluorometer set at excitation and emission wavelengths of 565 and 575 nm, respectively. The Dil-LDL standard curve was obtained after extracting Dil with chloroform as described by Stephan and Yurachek.[17]

### *Assay of LDL Receptor Activity in Peripheral Blood Mononuclear Cells*

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by density-gradient centrifugation over Lympho-Prep (Nycomed, Oslo, Norway; density 1.077).[18,19] Cells at the interphase were collected, washed twice, and resuspended in culture medium. To upregulate LDL receptors, PBMCs ($0.5 \times 10^6$ mL) were cultured in a cholesterol-deficient medium (RPMI 1640 containing 10% lipoprotein-deficient fetal bovine serum [LPDS] and 2 mmol/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin as preservatives) for 5 days at 37°C in a humidified incubator with 5% $CO_2$. After incubation, the viability of cells in suspension determined by Trypan blue exclusion was greater than 90% to 95% in all experiments. After the upregulation period, nonadherent cells (peripheral blood lymphocytes [PBLs]) were removed, washed once, and resuspended in RPMI 10% LPDS medium ($1 \times 10^6$ cells/mL) with increasing concentrations of Dil-LDL, and incubated for 2 hours at 4°C or 37°C for binding and uptake assays, respectively.[20-22] For each concentration, the nonspecific binding or uptake was obtained by incubation with a 50-fold excess of nonlabeled LDL. After the incubation period, cells were washed with phosphate-buffered saline (PBS) and fixed with PBS/2% paraformaldehyde, and finally washed and resuspended in 1 mL PBS for the subsequent flow cytometric analysis.

Samples were analyzed on a FACScan (Becton Dickinson, Heidelberg, Germany) equipped with an argon ion laser that emits at 488 nm and LYSYS II software (Becton Dickinson). In each analysis, 10,000 cells were analyzed with logarithmic amplification of the fluorescence intensity. Lymphocytes were gated by forward- and side-scatter characteristics. All results are expressed in terms of the median intensity of fluorescence (MIF) in arbitrary units (AUF) after subtracting the autofluorescence obtained from cells incubated in the absence of Dil-LDL.[23]

### *Lymphocyte Proliferation Assay*

Cell incubations were performed according to the method of Cuthbert et al[7,8] with slight modifications. PBMCs were isolated as before, resuspended in RPMI 1640 medium containing 10% LPDS and 2.5 μg/mL phytohemagglutinin ([PHA] Wellcome Reagents, Beckenham, UK), and distributed in sterile 96-well plates (Multiscreen-HV; Millipore, Bedford, MA) at a rate of $0.2 \times 10^6$ cells/0.225 mL/well. Cultures were further supplemented with or without 0.5 μmol/L lovastatin (generously provided by Dr J. González Esteban, Merck Sharp and Dohme, Madrid, Spain) dissolved in dimethyl sulfoxide (final concentration per well, 0.05%) to block cellular cholesterol synthesis, and increasing concentrations of LDL. After 72 hours of incubation at 37°C in a humidified incubator with 5% $CO_2$, cell proliferation was assessed by [$^3$H]-thymidine incorporation into DNA. For this, the cultures were supplemented with 10 mmol/L 5-fluordeoxyuridine (Sigma Chemical, St Louis, MO), and 1 hour later, the cells received an 18-hour pulse with 0.5 mCi [$^3$H-methyl]-thymidine (5 Ci/mmol; Amersham Ibérica, Madrid, Spain). Cells were harvested onto filters using the Multiscreen filtration system (Millipore), and finally, radioactivity was counted (LS 3800; Beckman Instruments).

### *Molecular Analysis of the LDL Receptor Gene*

Polymerase chain reaction (PCR) amplification of genomic DNA, single-strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis, and sequence analysis were performed as previously described.[24] The sequence change was verified through analysis of both DNA strands using the corresponding oligonucleotide primers and the Sequenase Version 2 kit (USB, La Jolla, CA).

### *Other Analytical Methods*

Total cholesterol and triglyceride levels were measured enzymatically (Menarini Diagnostici, Florence, Italy); high-density lipoprotein cholesterol was determined after plasma precipitation with the dextran sulfate/$MgCl_2$ reagent,[25] and LDL cholesterol was estimated by the Friedewald formula.[26] The apolipoprotein A-I level was measured by nephelometry (Beckman Instruments) with a World Health Organization standard.

## RESULTS

To determine the biochemical defect in two patients clinically diagnosed with homozygous FH who were being treated with LDL apheresis in our clinic, we first undertook binding and internalization studies with Dil-labeled LDL in lymphocytes. For this, PBLs were isolated and cultured for 5 days in a cholesterol-deficient medium to maximally stimulate LDL receptor,[20] and then incubated with Dil-LDL and analyzed by flow cytometry. Cells from the two patients and three normolipemic controls were tested in parallel. The specific LDL-binding activity of cells from case 2 was highly reduced, in accordance with the molecular defect in this patient (class 2 LDL receptor mutation).[13] In contrast, lymphocytes from case 1 efficiently bound LDL (maximal binding [$B_{max}$], 83% of control), with a $K_d$ similar to that of the controls (1.7 and 1.5 μg/mL, respectively). On the other hand, lymphocytes from the two FH patients had a very low activity to internalize LDL as indicated by the respective $B_{max}$ values, which were less than 10% of the control values. These results were compatible with the clinical homozygous FH phenotype of case 1, with a defect affecting primarily the internalization of LDL (Fig 1A and B).

To determine the molecular basis of the LDL receptor mutation of case 1, we analyzed his genomic DNA by SSCP and

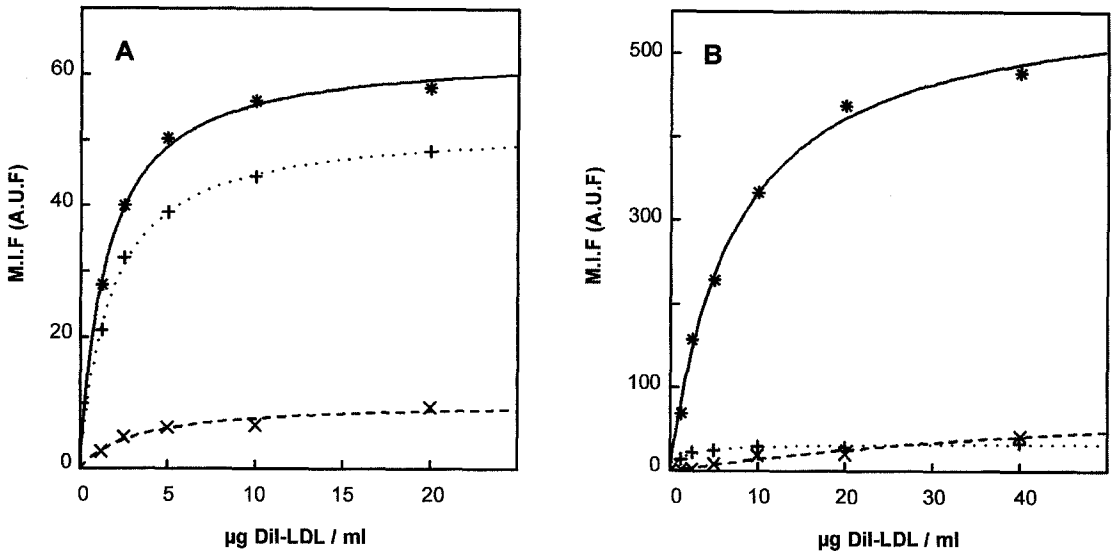


**Fig 1. Surface binding of Dil-labeled LDL at 4°C (A) and cell association of Dil-labeled LDL at 37°C (B) in upregulated lymphocytes from control subjects and homozygous FH patients with the N804K ($FH_{Colmenar}$) and D280G/G528V mutations. PBMCs were incubated for 5 days in lipoprotein-deficient medium to upregulate LDL receptor activity, and then with the indicated amounts of Dil-LDL for 2 hours at 4°C (A) or 37°C (B). Specific binding and uptake were calculated as the difference between total and nonspecific values obtained in the presence of a 50-fold excess of unlabeled LDL. Control (----), FH homozygote carrying the $FH_{Colmenar}$ mutation (·····), and FH compound heterozygote carrying mutations D280G and G528V (- - - -). Control values correspond to the mean ± SEM for 3 normolipidemic subjects; values for HFH patients are the mean of quadruplicates for a representative experiment from 2 showing similar results.**

DNA sequencing of both strands. The mutation was detected after amplification of a 230–base pair fragment of exon 17[24]; no other abnormally migrating bands were detected in any of the other exons of the LDL receptor gene. DNA sequence analysis showed that the patient was a homozygote for a cytosine to adenine transversion in exon 17, which is expected to result in an asparagine to lysine change in amino acid residue 804 ($FH_{Colmenar}$). This change does not create or eliminate a restriction enzyme cutting site, and therefore, the mutation was traced in the family by SSCP analysis. The two parents were heterozygous for the N804K ($FH_{Colmenar}$) mutation.

To evaluate the impact of these LDL receptor mutations on cell physiology, we measured [$^3$H]thymidine incorporation into DNA in PHA-stimulated PBMCs cultured in a cholesterol-deficient medium.[7-9] For simplicity, results from only one control subject are shown in Fig 2. In the absence of lovastatin, lymphocytes from both the controls and HFH patients proliferated normally, and the addition of LDL to the medium did not affect this parameter (Fig 2A). In the presence of 0.5 mmol/L lovastatin, [$^3$H]thymidine incorporation into DNA was markedly inhibited (>95%) in the three cases. Supplementing the medium with LDL reversed this inhibition in the control lymphocytes; however, in LDL receptor–deficient lymphocytes, LDL was ineffective in restoring cell growth. It is worth mentioning that the lack of response to LDL was similar in the two patients despite different LDL receptor defects. The inhibition of DNA synthesis by lovastatin was efficiently reversed by mevalonate in both control and LDL receptor–deficient lymphocytes, indicating that the effect of lovastatin was specific (Fig 2B). These results demonstrate that either the absence of LDL receptors in the cell membrane or the inability of the LDL receptor to be internalized hampered the provision of LDL cholesterol to lymphocytes that is required for cell growth.

## DISCUSSION

In the present study, we have determined the impact of two LDL receptor phenotypes on the ability of LDL to support cell proliferation. PBLs were taken from two patients clinically diagnosed with homozygous FH. The patients are unrelated and are being treated with dextran sulfate LDL apheresis in our hospital.[11] One of the patients (case 2) is a compound heterozygote carrying two different point mutations in the LDL receptor gene (D280G and G528V).[13] Case 1 is homozygous for a mutation in exon 17 (N804K), herein described for the first time ($FH_{Colmenar}$).

Lymphocytes from case 2 showed a decrease of both LDL binding and internalization activities, in accordance with her molecular defect.[13] The mutation G528V in exon 11 produces a class 2a phenotype (transport-defective, no processing), which corresponds to the lack of receptor binding activity.[27] The other mutation in exon 6—D280G—results in a class 2b phenotype (transport-defective, slow processing)[6]; this type of defect is associated with a detectable but markedly reduced binding activity (2% to 25% of normal LDL receptor activity).[27] This compound heterozygote condition may explain the residual binding activity observed in cells from case 2 ($B_{max}$, 15% of the control).

Lymphocytes from case 1 expressed a practically normal LDL binding activity, with a $B_{max}$ of 83% of the control value and a $K_d$ of 1.7 µg/mL indicating high-affinity kinetics, while LDL internalization was greatly impaired. These results demonstrate that case 1 had an internalization defect, class 4 pheno-

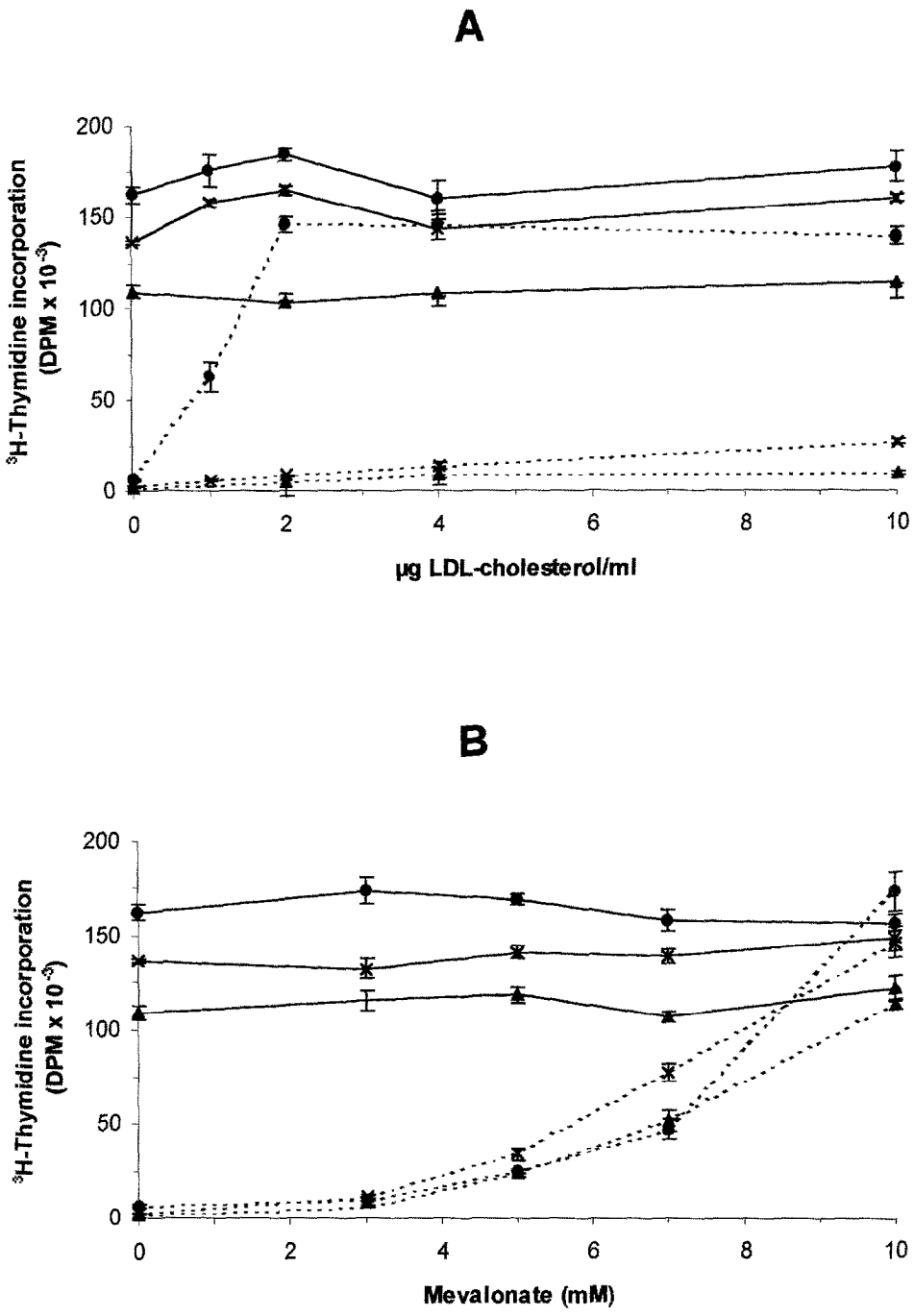


**Fig 2. LDL receptor function in lymphocytes from a control subject and patients with homozygous FH carrying the $FH_{Colmenar}$ and D280G/G528V mutations. $^3$H-thymidine incorporation into DNA was evaluated in PHA-stimulated lymphocytes incubated for 72 hours in lipoprotein-deficient medium in the absence (----) or presence of 0.5 µmol/L lovastatin (·····) and increasing concentrations of LDL cholesterol (A) or mevalonate (B). Control (●); FH homozygote carrying $FH_{Colmenar}$ (×); FH compound heterozygote carrying mutations D280G and G528V (▲). Mean ± SEM of quadruplicates from a representative experiment of 3 showing similar results.**

type, and suggest the presence of a mutation(s) in the cytoplasmic domain of the LDL receptor gene.[28] The molecular studies showed that the patient was homozygous for a point mutation in exon 17, which consisted of a cytosine to adenine transversion that is expected to result in an asparagine to lysine change in amino acid residue 804. This mutation cosegregates with hypercholesterolemia in the family members. Since the transversion N804K does not create or eliminate a restriction site in the LDL receptor gene, screening for the $FH_{Colmenar}$ mutation in newly identified FH patients is currently performed by PCR-SSCP.

The majority of mutations of the LDL receptor gene identified in FH patients affect the LDL binding domain.[5,29] The N804K mutation described herein belongs to a small group of point mutations affecting the tetrameric sequence NPVY that is totally conserved in LDL receptors from many species.[30] A variant of this sequence, NPxY, is present in multiple copies in the cytoplasmic tail of the GP330 protein[31] and the LDL receptor–related protein[32] and as a single copy in other cell surface receptors.[30] The sequence NPVY is the signal for directing the LDL receptor to coated pits,[30] and mutations in this sequence produce LDL receptor proteins that reach the cell

surface and bind the lipoprotein normally but fail to cluster in coated pits, so the LDL is not efficiently internalized and fails to reach its normal site of degradation in lysosomes.[28] This is known to occur in patients carrying the mutation Y807C.[24,33] Thus, the mutation N804K described here can be causally related to the LDL internalization defect and consequently the hypercholesterolemia observed in case 1.

Cuthbert et al[7,8] were pioneers in observing that the proliferative response to LDL was impaired in LDL receptor–deficient lymphocytes compared against controls, an approach that has been used for the diagnosis of FH. Koivisto et al[9] used this method in a comparative study of the function of LDL receptors in FH heterozygotes, and found that lymphocytes from patients with the $FH_{Espoo}$ allele (a deletion of exon 15 of the LDL receptor gene) had a growth rate intermediate between the rate in lymphocytes from healthy subjects and patients with the $FH_{Helsinki}$ gene (a major deletion of exons 16 through 18), concluding that $FH_{Espoo}$ deranges LDL receptor function only partly.[9] Therefore, we decided to examine LDL receptor function in lymphocytes from our FH homozygotes, who carried mutations affecting different exons and domains of the LDL receptor. We observed that LDL was ineffective in restoring cell growth in lymphocytes from the two FH patients, which indicates that both the absence of LDL receptors in the plasma membrane and the inability to internalize them hamper the ability of LDL to support cell proliferation. In a previous study, we found that the LDL fractional catabolic rates were very similar in these two patients ($0.052 \pm 0.003$ and $0.049 \pm 0.005$ pools per day, respectively).[11] This indicates that either homozygosity for $FH_{Colmenar}$ or compound heterozygosity for D280G/G528V result in a similar severe reduction of LDL clearance in vivo, in accordance with the impairment of LDL cholesterol utilization observed in proliferating lymphocytes.

The present results demonstrate that internalization of the LDL particle is required for the cell growth–supporting effect of LDL, which underlines the importance of the LDL receptor for the provision of lipoprotein cholesterol to proliferating cells. It has been recently reported that LDL exerts effects in cells other than merely the provision of cholesterol, for example, the stimulation of expression of the insulin-like growth factor-1 receptor gene.[34] Whether this particular effect is mediated by the LDL receptor and/or coupled to LDL internalization is presently unknown. Cells carrying the $FH_{Colmenar}$ mutation could be a useful tool to examine this issue.

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