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Influence of cyclosporine on low-density lipoprotein uptake in human lymphocytes

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Abstract

Low-density lipoprotein (LDL) levels are often elevated in renal transplant recipients, and cyclosporine (CsA) therapy in these patients has been implicated. Cardiovascular disease is the major cause of mortality in transplant recipients, and alterations of lipid metabolism represent a common risk factor. The role of CsA on LDL metabolism is still partially defined. The aim of the study was to evaluate the LDL receptor uptake of CsA-transported LDL (CsA-LDL) compared with normal LDL in normal and CsA-treated lymphocytes. Forty-seven healthy unrelated subjects and 6 CsA-treated patients were consecutively enrolled as donors of lymphocytes to measure receptor-mediated LDL metabolism. Normal LDL and CsA-LDL were isolated from blood donors and from patients under CsA immunosuppressive therapy, respectively. Lipoproteins were labeled with a fluorochrome, and LDL receptor uptake was measured by flow cytometry. Normal LDL uptake was 13.95% ± 4.5%, whereas CsA-LDL uptake was 32.47% ± 10.84% ($P < .001$) in healthy lymphocytes. In CsA-treated lymphocytes, normal LDL uptake was 7.48% ± 2.32% vs 12.49% ± 2.44% CsA-LDL ($P < .01$). Lymphocytes of every subject showed at least a 2-fold increased uptake of CsA-LDL vs normal LDL. Our data show that CsA-LDL is internalized more than normal LDL via the LDL receptor in both human healthy and CsA-treated lymphocytes. CsA-treated lymphocytes, in comparison to normal lymphocytes, exhibit a reduced LDL receptor activity.

1. Introduction

Cyclosporine (CsA) is a cornerstone in the immunosuppressive therapy for patients undergoing solid organ transplantation, but side effects of this treatment still present a problem [1]. Hyperlipidemia is a common metabolic disorder in renal transplant recipients [2], and CsA is one of the major factors involved in the impairment of the lipoprotein metabolism.

CsA, as well as other hydrophobic drugs, binds to plasma lipoproteins; in particular, apolipoprotein B lipoproteins can carry about 50% of total plasma CsA [3,4]. In dyslipidemic patients, altered lipoprotein binding is able to modify tissue drug availability and CsA pharmacological activity [5-9].

Although hyperlipidemia in CsA-treated patients is believed to arise from the toxicity CsA exerts in the liver [10], the hypothesis that CsA administration might also impair low-density lipoprotein (LDL) metabolism through modulation of LDL receptor activity has been suggested [11]. However, experiments undertaken to examine this hypothesis, mostly in CsA-treated cells, have led to conflicting results, perhaps because of differing experimental conditions [12-14].

Assessment of LDL binding and uptake by flow cytometry using human lymphocytes, which lack scavenger receptors [15], represents a simple and rapid method to evaluate functional activity of LDL receptor and ligand affinity for LDL particles [16,17].

The aim of the present study was to investigate the receptor-mediated uptake of CsA-transported LDL (CsA-LDL), in comparison to normal LDL, in lymphocytes isolated from a group of healthy volunteers and from 6 renal transplant recipients on CsA immunosuppressive therapy. In

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addition, because lipoprotein binding could be affected by the presence of CsA in the lipoprotein particle, LDL binding was also simultaneously determined in a subset of the 2 groups.

2. Subjects and methods

2.1. Materials

Fetal calf serum and RPMI 1640 with L-glutamine were obtained from GibcoBRL (Life Technologies, Milan, Italy). Bovine serum albumin, 1,1′-dioctadecyl-3,3,3′ ,3′-tetramethyl-indocarbocyanine perchlorate (DiI), dimethyl sulfoxide, penicillin, streptomycin, and lipoprotein-deficient serum (LPDS) were obtained from Sigma (St. Louis, MO). LymphoPrep was from Nycomed Pharma (Oslo, Norway). Millex-GV filters (pore diameter, 0.22 μm) were obtained from Millipore (Molsheim, France). Fifty- and fifteen-milliliter conical tubes and 50- and 100-mL flasks were obtained from Falcon, Becton Dickinson Biosciences, Discovery Labware (Bedford, Mass). All commercially available materials were of the highest grade.

2.2. Subjects

Forty-seven healthy unrelated volunteers were consecutively enrolled as lymphocyte donors from the staff and the students of the Department of Internal Medicine of the University of Turin. The subjects were normolipidemic, without diabetes, hepatic or renal disease, or a family history of coronary heart disease. None of the subjects took drugs affecting lipid metabolism or had thyroid dysfunction as assessed by thyroid-stimulating hormone and T_4 serum levels.

Six renal transplant recipients were recruited as lymphocyte donors from the outpatient ambulatory of the Nephrology and Dialysis Division. Patients selected had more than 24 months of successful transplantation and were on CsA therapy with no prednisone therapy in the last month. All the subjects gave their informed consent.

2.3. Lymphocytes isolation and culture

Blood (15 mL) was collected into lithium-heparin tubes (Vacutainer, Becton Dickinson, Plymouth, UK) and diluted 1:1 with sterile phosphate-buffered saline (PBS), pH 7.4, in a 50-mL conical tube. Lymphocytes were prepared under sterile conditions, using a modified version of the method of Böyum [18] as described by Løhne et al [17]. Briefly, diluted blood was layered upon LymphoPrep and centrifuged at 400*g* for 30 minutes at 20°C. The interface containing the lymphocytes was isolated, and the cells were washed 3 times with sterile PBS and finally with RPMI enriched with L-glutamine (290 mg/L), penicillin (100 000 U/L), streptomycin (100 mg/L), and 10% fetal calf serum. After sedimentation the cell pellet was resuspended in the above solution in a 50-mL flask. The cell suspension was incubated for 72 hours at 37°C in a humidified carbon dioxide incubator (5.0% CO_2) in sterile RPMI with 10% human lipoprotein–depleted serum to obtain maximal receptor expression. Nonadherent cells were harvested and washed twice with cold RPMI/bovine serum albumin (2 g/L). Cells were adjusted to obtain a final concentration of (0.8 to 1) $\times$ 10^6 cells/mL in each tube and used directly in the assay. Viability of the cells was more than 95% as assessed by trypan blue exclusion test.

2.4. Isolation and labeling of LDL

Blood (20 mL) was collected from blood donors by venipuncture in EDTA tubes in the morning, after 12 hours of fasting, and plasma was pooled and immediately separated by low-speed centrifugation at 4°C. All blood donors gave informed consent. LDL (density, 1.019-1.063 g/mL) and LPDS (density, >1.210 g/mL) were isolated and purified by sequential preparative ultracentrifugation as described by Havel et al [19].

LDL was labeled after removing very low-density lipoprotein (density, <1.006 g/mL) and intermediate-density lipoproteins (density, 1.006-1.019 g/mL) by ultracentrifugation of plasma, and cholesterol was measured in the infranate. The infranate was diluted into LPDS to a final concentration of 0.25 mg cholesterol/mL, and DiI dissolved in dimethyl sulfoxide (3 mg/mL) was added. The mixture was incubated at 37°C for 18 hours. LDL labeled with DiI (DiI-LDL) was isolated at a density of 1.063 g/mL through ultracentrifugation at 46 000 rpm in a Beckman 70.1 Rotor for 20 hours at 18°C. Labeled LDL was stored in the dark at 4°C for up to 3 weeks. Purity of each LDL fraction was evaluated by agarose gel electrophoresis.

2.5. Isolation and labeling of CsA-LDL complex

CsA-LDL was isolated from 10-mL EDTA blood of kidney transplant recipients in stable metabolic control under CsA therapy. Plasma was immediately separated by low-speed centrifugation at 37°C and stored at 4°C to prevent any redistribution of CsA. LDL was isolated and labeled with DiI as previously described.

2.6. Labeling efficiency

For each new batch of normal and CsA-LDL, specific activity was measured with a spectrofluorimeter, and the values were extrapolated from a calibration curve. Specific activity ranged from 35 to 50 ng DiI/μg LDL protein. The difference in specific activity between normal and CsA-LDL was less than 12%.

2.7. CsA measurement

CsA concentration in whole blood, plasma, and LDL fraction was measured by Fluorescence Polarization Immuno Assay (FPIA Abbot Laboratories, Abbot Park, IL). CsA concentration in LDL varied between 40 and 50 ng/mg LDL

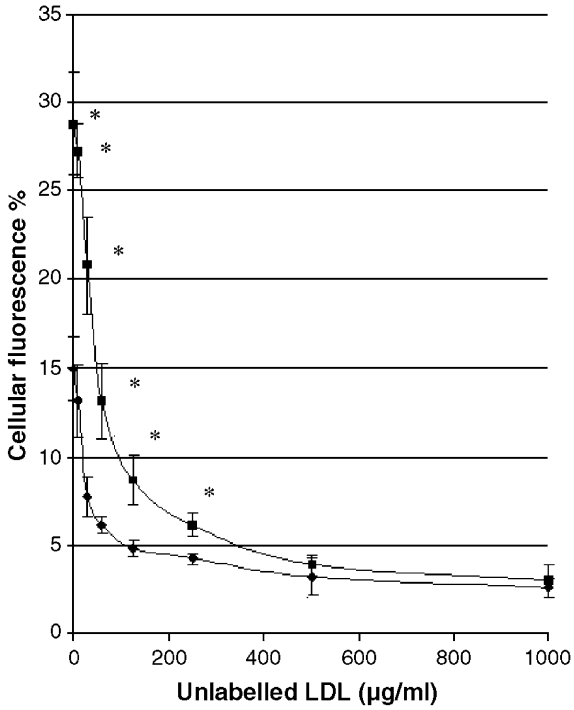

Fig. 1. Competition curve between DiI-labeled LDL (normal LDL, diamonds; CsA-LDL, squares) and unlabeled native LDL. Receptor-mediated LDL uptake of labeled LDL (at a constant concentration of 10 μg/mL) was measured in the presence of increasing concentrations of unlabeled LDL (0, 10, 30, 60, 125, 250, 500, and 1000 μg/mL). Data are the mean ± SD of cellular fluorescence (measure of labeled LDL uptake) measured in lymphocytes isolated from 3 of 47 healthy subjects. Cells were analyzed simultaneously for both labeled LDL. Differences in LDL uptake (normal vs CsA-LDL at the same LDL unlabeled concentration) were evaluated by Student t test. *$P < .05$.

protein. Protein content was measured using the Bio-Rad protein assay.

2.8. LDL uptake assay

Lymphocytes were incubated with normal LDL and CsA-LDL labeled with DiI at a final concentration of 10 μg/mL LDL protein in round-bottomed tubes for 2 hours at 37°C on a rotary shaker in a humidified carbon dioxide incubator (5.0% CO_2). PBMCs were then washed twice and resuspended in PBS and then directly analyzed by flow cytometry. Final cell concentration was (0.8 to 1) × 10^6 cells/mL. Each experiment was performed in duplicate.

2.9. LDL binding assay

Lymphocytes obtained from 6 healthy subjects and 6 CsA-treated patients were incubated in the dark with normal LDL and CsA-LDL labeled with DiI at a final concentration of 10 μg/mL LDL protein in round-bottomed tubes for 2 hours at 4°C, placed on a rotary shaker. Peripheral blood mononuclear cells were washed twice, resuspended in PBS, and then directly analyzed by flow cytometry. Final cell concentration was (0.8 to 1) × 10^6 cells/mL. Each experiment was performed in duplicate.

2.10. Flow cytometry measurements

The uptake of DiI-LDL was measured on a FACScan flow cytometer (Becton-Dickinson, Mountainview, CA) equipped with a 15-mW, 488-nm, air-cooled argon ion laser and linked to a Power Mackintosh G3 computer. A morphological scatter, forward-scatter FSC (cell size), and side-scatter SSC (cell granularity) for each lymphocyte preparation was performed. An acquisition gate was established to exclude cell debris or aggregates as well as to delineate lymphocyte population.

Lymphocytes were checked for purity, before analysis, by measuring the CD45/CD14 expression (Simultest Leucogate, Becton-Dickinson). Lymphocyte-leukocyte ratio was always greater than 98%.

DiI emission (FL2) was measured at 585 ± 21 nm using a band-pass filter from gated cell population. The fluorescence signals from 10 000 cells were routinely collected and analyzed using the Cell Quest Software (Becton-Dickinson).

The number of receptor-positive cells was determined, setting the marker at the highest range of the unstained lymphocytes (autofluorescence) in the acquisition plot (SSC/FL2); in the following acquisition of stained lymphocytes, the fluorescence signals over the marker were expressed as the percentage of positive cells out of the total cells in gated region.

The percentage value obtained (measure in duplicate) reflects the degree of normal or CsA DiI-LDL binding and internalization by lymphocytes through the LDL receptor. The mean intra-assay coefficient value was below 2.0%.

2.11. Statistics

A paired Student t test was applied to compare data. A P value of .01 or less was considered statistically significant.

3. Results

Labeling efficiency was evaluated for every new DiI-LDL batch preparation. Fluorimetric evaluation gave an interbatch difference in fluorescence that was less than 15% for normal LDL and CsA-LDL at the same protein concentration.

A competition curve at 37°C between DiI-LDL (both normal and CsA-LDL) and unlabeled native LDL was performed to examine the influence of CsA-LDL on LDL receptor uptake. Lymphocytes isolated from 3 healthy subjects of the study group were analyzed for receptor-mediated LDL uptake at fixed DiI-LDL (normal and CsA-LDL) concentration (10 μg/mL) and increasing concentrations of unlabeled LDL (0, 10, 30, 60, 125, 250, 500, and 1000 μg/mL) (Fig. 1). In the absence of unlabeled LDL, normal DiI-LDL uptake was 14.94% ± 1.83% and 28.75% ± 2.89% for CsA-DiI-LDL ($P < .01$). The difference was significant up to a concentration of 250 μg/mL of unlabeled LDL. The nonspecific uptake for

Table 1
Physical and metabolic characteristics of the subjects

Variables	Normal subjects	CsA-treated patients
No. of subjects (M/F)	47 (32/15)	6 (4/2)
Age (y)	32 ± 7	52 ± 11
Cholesterol (mg/dL)	176 ± 29	218 ± 35
Triglycerides (mg/dL)	96 ± 18	126 ± 43
LDL cholesterol (mg/dL)	98 ± 16	148 ± 28
HDL cholesterol (mg/dL)	56 ± 11	45 ± 9
Apolipoprotein B (mg/dL)	94 ± 15	117 ± 26
CsA therapy (mg/kg/d)	–	3.75 ± 0.15

Values are mean ± SD.

normal and CsA-DiI-LDL was less than 4% in the presence of more than 50-fold excess of unlabeled LDL.

Table 1 shows the anthropometric and clinical characteristics of the subjects. Table 2 shows the normal and CsA-LDL uptake by the lymphocytes isolated from healthy and CsA-treated subjects. In healthy lymphocytes, the mean ± SD of normal LDL uptake was 13.95% ± 4.5% (range, 5.48%-23.36%), and CsA-LDL uptake was 32.47% ± 10.84% (range, 11.78%-55.23%) ($P < .001$). Lymphocytes of 6 renal transplant recipients showed an LDL uptake of 7.48% ± 2.32% (5.33%-11.80%) for normal LDL and 12.49% ± 2.44% (10.01%-16.42%) for CsA-LDL ($P = .004$).

LDL binding assay, performed at 4°C, measured in 6 healthy subjects was 5.63% ± 1.13% (4.18%-6.15%) for normal LDL and 11.77% ± 2.73% (8.72%-14.42%) for CsA-LDL ($P < .01$). LDL binding assay in lymphocytes from CsA-treated patients was 3.08% ± 0.68% (1.96%-3.88%) for normal LDL and 4.69% ± 1.45% (2.41%-6.44%) for CsA-LDL ($P = .03$).

Normal lymphocytes demonstrated a higher LDL uptake than CsA-lymphocytes (13.95% ± 4.5% vs 7.48% ± 2.32%, $P > .01$ for normal LDL; 32.47% ± 10.84% vs 12.49% ± 2.44%, $P > .01$ for CsA-LDL). Similarly, normal lymphocytes exhibited a higher LDL binding than CsA-lymphocytes (5.63% ± 1.13% vs 3.08% ± 0.68%, $P < .01$ for normal LDL; 11.77% ± 2.73% vs 4.69% ± 1.45%, $P = .03$ for CsA-LDL). Statistical analysis of uptake and binding data in 32 male controls and 4 male patients showed the same significant differences as with the whole group ($P < .01$).

4. Discussion

This study examines the influence of CsA carried by LDL on the uptake of LDL through its own receptor. Our data show that CsA-LDL, in comparison to normal LDL, exhibits a clearly increased uptake by lymphocytes obtained from healthy subjects and from a smaller group of CsA-treated patients. The different behavior of CsA-LDL vs normal LDL in both groups of lymphocytes is also apparent from the evaluation of LDL binding, even if in CsA-treated cells this did not reach statistical significance. These results were confirmed using a competition curve (Fig 1), performed on normal lymphocytes, between DiI-labeled and unlabeled LDL: up to a concentration of 250 μg/mL of unlabeled LDL, receptor-mediated CsA-LDL uptake was increased in comparison to normal LDL.

In the present work, lymphocytes were used as a model for the study of receptor-mediated LDL metabolism. Classical experiments on LDL receptor activity were traditionally performed with ^{125}I-LDL on human skin fibroblasts [20]. Lymphocytes are otherwise now commonly used [16,17,21] because LDL receptor expression is similarly regulated in lymphocytes and hepatic cells [22], and lymphocytes are easy to isolate and to maintain in lipoprotein-free medium and do not seem to possess scavenger receptors [15]. Flow cytometry represents a reliable alternative to the use of radioisotopes in measuring ligand-receptor interaction by means of DiI as fluorescent probe [16,17]. If it does not allow precise determination of surface cell molecules, relative quantitation is sufficient for comparative purpose as in our study [23]. Moreover, incubation at 37°C allows binding and internalization of LDL through its own receptor, whereas incubation at 4°C reflects binding only [24].

Other studies have examined the influence of CsA on cells and LDL behavior. Lopez-Miranda et al [12] observed that CsA-LDL was taken up and degraded by rat fibroblasts at a higher extent than normal LDL, with no effect upon

Table 2
Normal DiI-LDL and CsA DiI-LDL binding and uptake in lymphocytes

	LDL binding		LDL uptake	
	Normal LDL (%)	CsA-LDL (%)	Normal LDL (%)	CsA-LDL (%)
	n = 6		n = 47	
N-lymphocytes	5.63 ± 1.13*	11.77 ± 2.73	13.95 ± 4.5*	32.47 ± 10.84
	n = 6		n = 6	
CsA-lymphocytes	3.08 ± 0.68**	4.69 ± 1.45**	7.48 ± 2.32*,**	12.49 ± 2.44**

Values are mean ± SD. N-lymphocytes indicates lymphocytes isolated from healthy subjects; CsA-lymphocytes, lymphocytes isolated from CsA-treated patients.

* $P < .01$, normal DiI-LDL vs CsA DiI-LDL.

** $P < .01$, N-lymphocytes vs CsA-lymphocytes.

pretreating fibroblasts with CsA. Furthermore, the amount of 3-hydroxy-3-methylglutaryl coenzyme A reductase and LDL receptor proteins measured in vivo after CsA treatment showed no significant variations from the control cells [14]. Measuring the influence of CsA on the activity of LDL receptor by using hepatocyte cultures, al Rayyes et al [13] demonstrated that either native or CsA-incubated LDL exhibited reduced receptor binding in HepG2 cells incubated with a CsA-supplemented medium. This effect was also reversed by statin supplementation [25]. Wasan et al [26] showed that CsA decreases LDL internalization without altering the extent and affinity of its binding to the LDL receptor. CsA did not alter the number of available LDL binding sites, and the association of CsA to the LDL particle did not affect the binding affinity of LDL to its receptor.

In addition to the type of cells used, a difference between our work and those of other authors is the source of CsA-LDL. In studies carried out by other groups, CsA-labeled LDL was obtained by incubating lipoproteins with well-defined amounts of the drug and is probably not exposed to the chemical and physical modifications that are able to influence LDL in subjects under CsA therapy. In vitro CsA-labeled LDL does not exhibit the structural or molecular modifications (oxidation, carbamoylation) [27] that are present in LDL isolated from transplanted patients and perhaps are able to influence the metabolism of lipoproteins.

Our data show that CsA, once transported by LDL, increases both binding and uptake of the lipoproteins by the LDL receptor, whereas the overall effect of CsA on lymphocytes isolated from CsA-treated patients is a significant reduction of LDL catabolism. To estimate whether the number of LDL receptors was decreased with CsA, we measured the number of LDL receptors with a monoclonal antibody (data not shown) in lymphocytes incubated with and without CsA. We observed a significant decrease of LDL receptors in lymphocytes incubated with CsA ($P < .05$).

This decrease of LDL receptor activity as a direct effect of CsA on target cells has been observed by others [26], whereas the increased metabolism of CsA-LDL is a novel finding. Our data were obtained in a large sample of lymphocytes and were previously identified by competition curve. It should be pointed out that the lymphocytes obtained from our healthy subjects showed the same behavior, also with a large degree of variation in the percentage of uptake. The variation was observed both with normal and CsA-LDL and probably is related to the use for the analysis of whole lymphocyte population [28].

Clinical data show that renal transplant recipients are characterized by a slight increase in LDL cholesterol levels and a higher risk of CHD than the general population [29,30]. In this regard, our data support the hypothesis that CsA is involved in the pathogenesis of atherosclerosis in these patients by a modification of the particle enhancing LDL binding and uptake and a reduced LDL receptor activity by the target cells.

These conflicting data could represent 2 different effects of CsA on lipid metabolism: one effect could be because of LDL structure (higher uptake), the second effect could be because of the reduced number of LDL receptors. The second effect seems to prevail as one can observe increased LDL cholesterol levels in transplanted patients under long-term CsA therapy. Hence, CsA-LDL, despite its high affinity for LDL receptor, could be cleared through scavenger receptor because LDL receptor number is reduced. The final picture is similar to the familial hypercholesterolemia.

The apparent dichotomy is not new: increased LDL receptor activity and elevated LDL cholesterol levels were observed in lymphocytes isolated from aged donors [31]. It may also be proposed that the increased CsA-LDL uptake by lymphocytes could favor the atherogenic process because the elevated LDL internalization may be important for the cell's metabolism and immunoglobulin production [32]. Investigating LDL receptor function before and after kidney transplantation could shed light on this phenomenon.

An attractive tool of research would be to evaluate also the behavior of other lipophilic immunosuppressive drugs such as tacrolimus, which have demonstrated beneficial effects on lipid profile in clinical trials [33].

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