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ORIGINAL ARTICLE

**Vania T. M. Hungria · Maria C. Latrilha
Debora G. Rodrigues · Sergio P. Bydlowski
Carlos S. Chiattone · Raul C. Maranhão**

Metabolism of a cholesterol-rich microemulsion (LDE) in patients with multiple myeloma and a preliminary clinical study of LDE as a drug vehicle for the treatment of the disease

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Abstract *Purpose*: Previously we have shown that cholesterol-rich microemulsions that bind to LDL receptors have the ability to concentrate in acute myeloid leukemia cells and in ovarian and breast carcinomas. Thus, LDE may be used as a vehicle for drugs directed against neoplastic cells. Indeed, we subsequently showed that when carmustine is associated with LDE the toxicity of the drug is significantly reduced in patients with advanced cancers. The aim of the present study was to verify whether LDE may be taken up by multiple myeloma cells and whether patients with multiple myeloma respond to treatment with LDE associated with carmustine. *Methods*: A total of 131 consecutive volunteer patients with recently diagnosed multiple myeloma classified as clinical stage IIIA had their plasma lipid profile determined. LDE plasma kinetics were performed in 14 of them. Cell uptake of LDE and the cytotoxicity of carmustine associated with the emulsion were evaluated in a multiple myeloma cell line. A pharmacokinetic study of LDE-carmustine was performed in three patients. Finally, an exploratory clinical study of LDE-carmustine (carmustine dose 180 mg/m^2 body surface every 4 weeks) was performed in seven untreated multiple myeloma patients. *Results*: LDL cholesterol was lower in the 131 multiple myeloma patients than in healthy controls and the fractional clearance rate (FCR, in units per minute) in the 14 multiple myeloma patients was twice that in 14 paired healthy control subjects. Moreover, entry of LDE into multiple myeloma cells was shown to be mediated by LDL receptors. Taken together, these findings indicate that LDE may target multiple myeloma. The exploratory clinical study showed that gammaglobulin decreased by 10-70% (mean 36%) after three cycles and by 25-75% (mean 44%) after six cycles. Furthermore, there was amelioration of symptoms in all patients. Cholesterol concentrations increased after treatment, suggesting that the treatment resulted in at least partial destruction of neoplastic cells with receptor upregulation. Side effects of the treatment were negligible. *Conclusions*: Because it targets multiple myeloma and, when associated with an antineoplastic agent, produces therapeutic responses in patients with fewer side effects, LDE has the potential for use as a drug vehicle in the treatment of the disease.

Keywords Emulsions · Cancer chemotherapy · Multiple myeloma · Carmustine · Cholesterol · Low-density lipoprotein (LDL) receptors · Drug targeting

V. T. M. Hungria · C. S. Chiattone
Hematology and Hemotherapy Section, Santa Casa Medical School, São Paulo, Brazil

M. C. Latrilha · D. G. Rodrigues · R. C. Maranhão (✉)
Lipid Metabolism Laboratory, Heart Institute (INCOR), University of São Paulo Medical School Hospital, Av. Dr. Eneas de Carvalho Aguiar 44, 1 subsolo, 05403-000 São Paulo, Brazil
Tel.: +55-11-30688794
Fax: +55-11-30695574

S. P. Bydlowski
Department of Hematology, University of São Paulo Medical School Hospital, São Paulo, Brazil

Introduction

Neoplastic disease may have pronounced effects on the regulation of plasma lipid metabolism that lead to changes in the plasma concentrations of lipids and apolipoproteins. It has increasingly become recognized that these changes may bear important clues for the understanding of the pathophysiology, and even for the prognosis and treatment, of neoplastic diseases. In this regard, patients with acute myeloid leukemia develop lower plasma levels of low-density lipoprotein (LDL), the lipoprotein that carries most of the cholesterol present in the plasma [45].

LDL is derived from very low-density lipoprotein (VLDL), the triglyceride-rich lipoprotein that is synthesized by the liver. After entering the blood stream, VLDL is acted upon by lipoprotein lipase on the endothelial surface of capillary vessels. Triglycerides are broken down to glycerol and free fatty acids that are absorbed by muscle and adipose tissue where they are

reesterified and stored as a source of energy for the organism. Thus, the original VLDL particles progressively become more depleted in triglycerides and more dense. LDL is the final degradation product of VLDL. After prolonged (2-3 days) circulation in the blood, LDL is captured by specific cell membrane receptors that internalize the lipoprotein into vesicles [6, 7]. These vesicles fuse with lysosomes, dehydrate and finally disrupt, releasing to the cytosol their contents of lipids and degraded protein of the LDL particles. LDL cholesterol delivered to the cell participates in several processes, such as membrane synthesis.

The LDL plasma fraction is composed of quasi-spherical particles with diameters in the range 25-30 nm. The lipoprotein particles are constituted of a core of cholesteryl esters and residual triglycerides surrounded by a monolayer of phospholipids with small amounts of free cholesterol. A single, large molecular weight protein, apolipoprotein (apo) B100, is attached to the particle surface. ApoB100 is the ligand to the LDL receptor.

A seminal study by Ho et al. [22] showed that overexpression of LDL receptors occurs in acute myeloid leukemia cells. Because LDL uptake by the leukemic cells is increased, there is increased removal of the lipoprotein from the plasma, resulting in reduced total and LDL cholesterol plasma levels [45]. This is a unique mechanism for selective drug targeting and a great number of studies have focused on the use of LDL as a carrier to direct chemotherapeutic agents to leukemic and other neoplastic cells. LDL receptor overexpression and the concentration of LDL in neoplastic tissues is in fact a widespread phenomenon that has been documented not only in acute myeloid leukemia but also in several neoplastic diseases including breast cancer [12], bronchogenic carcinoma [17], gallbladder cancer [43], glioma [25], metastatic prostate carcinoma [20] and myeloproliferative diseases [14].

Native LDL is difficult to isolate from human plasma and drug incorporation into native LDL is also difficult, so that its use in routine cancer treatment is unlikely. However, we have shown that artificially made emulsions (LDE) with a lipidic structure roughly resembling that of LDL particles, as described above, but made without protein, are taken up by LDL receptors [30]. In contact with plasma, LDE acquires apoE and other apolipoproteins from native lipoproteins. ApoE can also be recognized by LDL receptors allowing binding of LDE to the receptors.

Relative to native LDL, LDE is more rapidly removed from the plasma [21] because it binds to the receptor through apoE, and not through apoB100 as does native LDL. The affinity of apoE for the receptor is severalfold greater than that of apoB100 [24]. Consequently, the emulsion particles are even more efficiently removed by the receptors than native LDL. Compared with native LDL, the shorter testing period, the uniformity of the preparation and the possibility of using a single preparation in a great number of subjects are advantages of the LDE model that facilitate the performance of plasma kinetic studies. To take advantage of these properties, LDE has been used as a surrogate for native LDL to investigate the process of LDL removal from the plasma [30, 31, 38]. This strategy is similar to the use of triglyceride-rich emulsions to diagnose alterations in the metabolism of chylomicrons, the lipoproteins that carry in the circulation the dietary fats absorbed by the intestine, in several disease states [4, 5, 32, 44] and to evaluate the effects of drugs upon this metabolism [39, 40, 42].

Subsequent studies have shown that after intravenous injection into patients with acute myeloid leukemia, LDE concentrates in the malignant cells in a similar manner to native LDL. Concentration of the emulsion into neoplastic tissues has also been documented in patients with mammary [15] and ovarian [1] carcinoma. The uptake of LDE by these malignant tissues is, respectively, fivefold and tenfold greater than that of the corresponding normal organ.

In contrast to native LDL, LDE is suitable for large-scale industrial production and may conceivably be the ideal vehicle to deliver drugs to neoplastic tissues by the LDL receptor pathway. Recently, studies on the stability, cytotoxicity and toxicity to animals of the lipophilic chemotherapeutic agent carmustine associated with LDE have been performed [34]. Carmustine was chosen to start these studies because it easily and substantially associates with the emulsion. In a dose-escalating pilot study in 42 patients with advanced cancers, it has been shown that doses of carmustine associated with LDE much larger than the doses used in routine chemotherapy are well tolerated [34].

This study was designed to verify the suitability of the use of LDE as a drug vehicle in multiple myeloma. To this end, we measured the LDL cholesterol levels in a large cohort of recently diagnosed untreated patients with the disease to definitively establish whether or not LDL cholesterol is decreased in multiple myeloma. In some of these patients, we then determined the plasma kinetics of the LDE used, as in previous studies, as a surrogate for native lipoprotein. With this strategy, the ''increased LDL plasma removal-low plasma LDL cholesterol'' pattern that is characteristic of patients with neoplasias with pronounced LDL receptor upregulation was confirmed in multiple myeloma patients. Furthermore, the entry of LDE via the LDL receptor was documented in a multiple myeloma cell line. Those observations, together with the results of our previous clinical study with LDE-carmustine [34], prompted us to test LDE as a drug vehicle in a exploratory pilot study in patients with multiple myeloma.

Materials and methods

Patients

The patients were selected from the outpatient clinics of the Hematology and Hemotherapy Section of the Santa Casa Medical School Hospital, in the city of São Paulo, Brazil. They were allocated to the three protocols outlined below.

Lipid profile evaluation A total of 131 consecutive untreated patients with multiple myeloma had their plasma lipid profile determined. Of these patients, 68 were female and 63 male; their ages ranged from 36 to 82 years (mean ± SD 58 ± 12 years). All were classified as clinical stage IIIA [9]. As defined by the clinical stage criteria, none of the patients had creatinine > 2.0 mg/dl. This is an important issue, because renal failure may elicit an altered lipid profile [41]. The patients also did not have concomitant metabolic diseases or hepatic dysfunction, as evaluated by clinical and laboratory criteria. The lipid profile of the 52 of patients who achieved either partial or total hematological remission (defined as a reduction in the electrophoresis gammaglobulin peak by 50-75% and > 75%, respectively) was again determined during the remission period for comparison with pretreatment values. The patients with multiple myeloma were compared with 110 healthy subjects, 64 male and 46 female, aged 36-82 years (58.1 ± 12.4 years).

Determination of LDE plasma kinetics From the group of 131 untreated patients, 14 volunteers were selected to participate in a study for the determination of LDE plasma kinetics. Eight were male and six female; their ages ranged from 38 to 70 years (mean ± SD 57 ± 11 years). Their body mass index (BMI) ranged from 18 to 27 kg/m^2 (24 ± 3 kg/m^2). This group of patients was compared with a control group of 14 healthy subjects from the laboratory database paired for age, sex and BMI. In one of these patients, LDE plasma kinetics were again determined after six treatment cycles with carmustine associated with LDE performed as described below.

Pilot study of the treatment of patients with LDE-carmustine Seven untreated patients with multiple myeloma were studied. Four were male and three female; their ages ranged from 46 to 78 years (63 ± 11 years). All had stage IIIA disease.

Plasma lipid determinations

Commercial enzymatic methods were used for the determination of total cholesterol (Boehringer-Mannheim, Penzberg, Germany) and triglycerides (Abbott Laboratories, Abbott Park, Ill.). HDL cholesterol was determined by the same method used for total cholesterol after lipoprotein precipitation with magnesium phosphotungstate. VLDL cholesterol and LDL cholesterol were calculated using the formula of Friedewald et al. [11].

LDE preparation

LDE was prepared from a lipid mixture composed of 40 mg cholesteryl oleate, 20 mg egg phosphatidylcholine, 1 mg triolein and 0.5 mg cholesterol. ^{3}H-Cholesteryl oleate was added to the mixture. The LDE microemulsion was prepared by emulsification of lipids by prolonged ultrasonic irradiation in aqueous medium followed by a two-step ultracentrifugation of the crude emulsion with density adjustment by the addition of KBr according to the method of Ginsburg et al. [13] as modified by Maranhão et al. [29, 31]. LDE was dialyzed against saline solution and passed through a 0.22-μm filter for injection into the patients.

LDE and LDE-carmustine uptake by multiple myeloma cells

The experiment was performed with a human myeloma cell line (RPMI-8226, American Type Tissue Collection). The cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS) and antibiotics (penicillin/streptomycin) at 37°C in a humidified incubator under an atmosphere containing 5% (v/v) CO_2. Prior to the experiments, the cells were plated in 5-mm dishes (10^5 cells/dish) for 24 h. The next day, the medium was replaced by medium containing 10% lipoprotein-deficient serum. On the third day, subconfluent cells were allocated to incubation experiments performed in triplicate over 4 h at 37°C with increasing amounts (0.05 to 0.5 mg/ml) of LDE total lipid labeled with ^{3}H-cholesteryl oleate ether. The cells were then washed three times with cold phosphate-buffered saline (PBS) plus bovine serum albumin and twice with PBS at 37°C, transferred to Eppendorf tubes and centrifuged for 15 min at 14,000 rpm. The supernatants were removed and NaOH 0.5 *M* was added to disrupt the cell pellets under vortex mixing. Radioactivity was measured in liquid scintillation (PPO/DM-POPOP/Triton X-100/toluene 5 g/0.5 g/333 ml/667 ml) vials using a Packard 1600 TR spectrometer (Meridien, Ct.) and protein concentrations were determined by the method of Lowry et al. [28].

The experiment to determine the uptake of LDE-carmustine by multiple myeloma cells was performed as described above, but with increasing amounts of LDE labeled with ^{3}H-cholesteryl oleate ether associated with increasing amounts of ^{14}C-carmustine (0.0625 to 0.375 mg/ml). ^{14}C-Carmustine was purchased from Moravek Chemicals (Brea, Calif.).

Competition between LDE and LDL for uptake by multiple myeloma cells

This experiment was performed with RPMI-8226 cells as described above, but with the addition of increasing amounts of native human LDL to each culture dish. The concentration of LDE labeled with ^{3}H-cholesteryl oleate ether was constant at 0.2 mg/ml in each dish. The native LDL concentrations ranged from 0.05 to 0.4 mg/ml.

Uptake by myeloma cells of LDE with a fluorescent label

A fluorescent cholesteryl ester analog (cholesteryl BODIPY) was purchased from Molecular Probes (Eugene, Ore.). Fluorescent LDE was prepared by adding the fluorescent probe (2.24 μmol) to the LDE lipid mixture. After sonication, fluorescent LDE was purified as described for LDE. The RPMI-8226 cell line was used in these experiments. Cells were cultured in a six-well plate (6×10^4 cells per well). After 24 h, the medium was changed and fluorescent LDE (12.5 μg/ml) was added. After 2 h incubation, cell internalization of the fluorescent LDE was visualized under fluorescence microscopy.

Cytotoxicity to human myeloma cells

The RPMI-8226 cells used in the assays were in the mid-logarithmic growth phase. The cells were harvested from the culture and distributed into 96-well culture plates. After 24 h incubation, LDE-carmustine and commercial carmustine were added to the wells in quadruplicate. We used final carmustine concentrations of 0.01, 0.1, 1, 10 and 100 μg/ml. The cells were left in the incubator for a further 24 h and at the end of this period the medium was removed and the numbers of living tumor cells were determined by the colorimetric MTT assay. The cell viability of each well was expressed as the "survival index" according to the following equation:

$$\text{Survival index (in\%)} = \frac{\text{absorbance of test} - \text{absorbance of blank}}{\text{absorbance of control} - \text{absorbance of blank}} \times 100$$

The 50% inhibitory concentration (IC_{50}) was determined as the drug concentration required to inhibit cell growth by 50%.

Determination of LDE plasma kinetics

The participants had fasted for 12 h at the beginning of the test at approximately 9 a.m., but they were allowed two standard meals during the study, at approximately 12:30 p.m. and 7 p.m. LDE (5-6 mg, 500 μl) containing 37 kBq ^{14}C-cholesteryl oleate was intravenously injected as a bolus. Plasma samples were collected over 24 h at 5 min and 1, 2, 4, 6, 8, 12 and 24 h. Aliquots (1.5 ml) of blood

plasma were extracted with chloroform/methanol (2:1, v/v), and the solvent phase was transferred to counting vials and dried under a nitrogen stream. Radioactivity was quantified in a scintillation solution (PPO/dimethyl POPOP/Triton X-100/toluene, 5 g/0.5 g/333 ml/667 ml) using a Packard Tricarb 2100TR spectrometer.

Calculation of LDE FCR

Plasma FCR of the LDE ^{14}C-cholesteryl oleate of the emulsion was calculated according to the method described by Matthews [35], where a_1, a_2, b_1 and b_2 were estimated from biexponential curves obtained from the remaining radioactivity found in plasma after injection, fitted by the least squares procedure, as $y = (a_1 \cdot e^{-b_1 t}) + (a_2 \cdot e^{-b_2 t})$ where y represents the plasma radioactivity decay.

Plasma kinetics of carmustine associated with LDE

^{14}C-Carmustine was associated with LDE and injected into three cancer patients. The plasma kinetics of LDE-^{14}C-carmustine were determined as described above for LDE labeled with ^{14}C-cholesteryl oleate.

Pharmacokinetic parameters were calculated using a multicompartmental model using a computer and software from PK Solutions (Ashland, Ohio). The log plasma concentration versus time curves were fitted by biexponential equations and the half-lives ($t_{1/2}$) calculated by dividing 0.693 by the rate constant for each phase. The total AUC was calculated using the linear trapezoidal method with extrapolation to infinity. Total plasma clearance was calculated by dividing the dose by the AUC. The volume of distribution at steady state was estimated graphically from trapezoidal total area measurements.

LDE association with carmustine

Carmustine was associated with LDE by co-sonication of the drug with the emulsion (1:3, w/w) for 5 min at 27°C using the flat tip (1 cm) of a Branson cell disrupter model 450 (Danbury, Ct.) at an output of 20 W and under a nitrogen stream. The rate of association of carmustine with LDE under these conditions and the stability of the carmustine-LDE complex have been previously reported [34].

Clinical pilot study

LDE-carmustine at carmustine dose of 180 mg/m^2 body surface was given as a 2-h intravenous infusion to the seven patients with multiple myeloma every 4 weeks. The flask containing the solution was protected from light. During the treatment period, the patients were evaluated every week, when histories were taken, physical examination carried out and complete blood counts and differential counts were determined. Gammaglobulin, transaminases, alkaline phosphatase, total bilirubin, urea and creatinine were determined at least monthly. The protocol design required that patients would be immediately removed from the study upon diagnosis of disease progression, so that careful weekly clinical monitoring was performed. The patients would also be dropped from the study on the appearance of grade 3 or 4 clinical or laboratory toxicity. The data recorded in this study are those obtained monthly during the week preceding LDE-carmustine administration.

Ethical and radiological protection considerations

All the experimental protocols involving subjects were approved by the Ethics Committee of the University of São Paulo Medical School Hospital and by the National Council of Health of the Brazilian Ministry of Health. Informed consent was obtained from each participant. The safety of the radioactive dose in the experiments in which labeled lipids and drug were intravenously injected into the patients was ensured according to the regulations of the International Commission on Radiological Protection (ICRP), as described in our previous study [32].

Statistical analysis

The differences in the plasma lipids were evaluated using Student's *t*-test, and the Mann-Whitney test was used for FCR data analysis. Differences with $P < 0.05$ were considered statistically significant.

Results

Plasma lipid profile

Table 1 shows the plasma lipid data from the 131 untreated patients with multiple myeloma and the healthy controls. Total, LDL ($P < 0.0001$) and HDL ($P < 0.0005$) cholesterol values of the patients were significantly lower than those of the controls. Triglycerides and VLDL cholesterol, however, were not different between the two groups. In the 52 patients who had their lipid profile determined before and after treatment, total and LDL cholesterol levels significantly rose, assuming intermediate values between those of the untreated patients and the controls. Of note is the fact that HDL cholesterol and triglycerides were unchanged during hematological remission compared with pretreatment levels.

Table 1 Concentration of plasma lipids (in mg/dl) determined in 131 untreated multiple myeloma patients, in 52 patients before and after treatment and in 110 healthy control subjects (expressed as means ± SD)

	Untreated	Multiple myeloma patients		Healthy controls	*P* value[a]
		Pretreatment	Posttreatment		
Cholesterol					
Total	149 ± 54	41 ± 50*	180 ± 48*	203 ± 39	< 0.0001
LDL	88 ± 43	79 ± 39*	116 ± 38*	132 ± 35	< 0.0001
HDL	36 ± 18	35 ± 18	38 ± 13	48 ± 13	< 0.0005
VLDL	25 ± 12	23 ± 11	25 ± 12	23 ± 11	NS
Triglycerides	123 ± 62	125 ± 64	130 ± 76	118 ± 56	NS

*$P < 0.01$, posttreatment vs pretreatment values
[a]Pretreatment vs control values

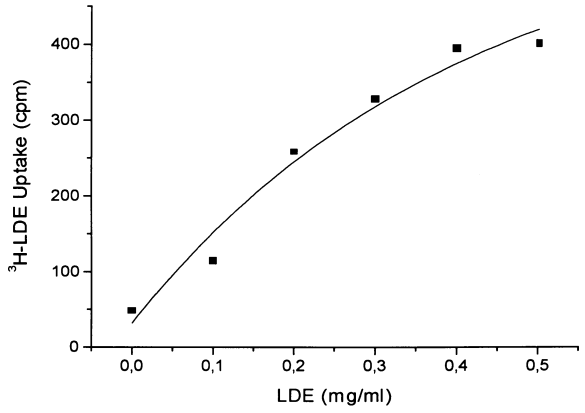

Fig. 1 Uptake of ^{3}H-cholesteryl ether-labeled LDE by cultivated RPMI-8226 multiple myeloma cells. Cells were incubated for 4 h with LDE (0.05-0.5 mg/ml) labeled with ^{3}H-cholesteryl oleate ether. The cell pellet was washed and centrifuged, and the radioactivity was measured in liquid scintillation vials

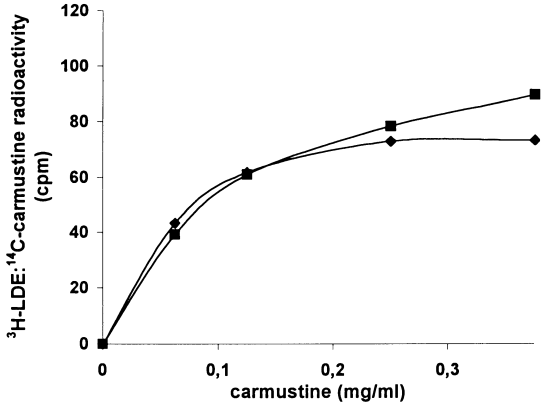

Fig. 2 Uptake of ^{3}H-cholesteryl ether-labeled LDE-^{14}C-carmustine by cultivated RPMI-8226 multiple myeloma cells. Cells were incubated with LDE-carmustine (0.0625-0.375 mg/ml carmustine) labeled with ^{3}H-cholesteryl oleate ether (■) and ^{14}C-carmustine (♦). The cell pellet was washed and centrifuged, and the radioactivity was measured in liquid scintillation vials

LDE and LDE-carmustine uptake by neoplastic cells and competition with LDL

^{3}H-Cholesteryl ether-labeled LDE incubated in increasing amounts with cultivated multiple myeloma cells was taken up by the cells in a typical dose-saturating manner (Fig. 1). RPMI-8226 cells incubated with increasing amounts of LDE-carmustine labeled with both ^{3}H-cholesteryl ether and ^{14}C-carmustine showed a proportional increase in the uptake of both labels (Fig. 2). The addition of increasing amounts of native LDL to incubates of multiple myeloma cells progressively decreased the uptake by the cells of labeled LDE (Fig. 3). In Fig. 4 the micrographs show the internalization of fluorescently labeled LDE into multiple myeloma cells.

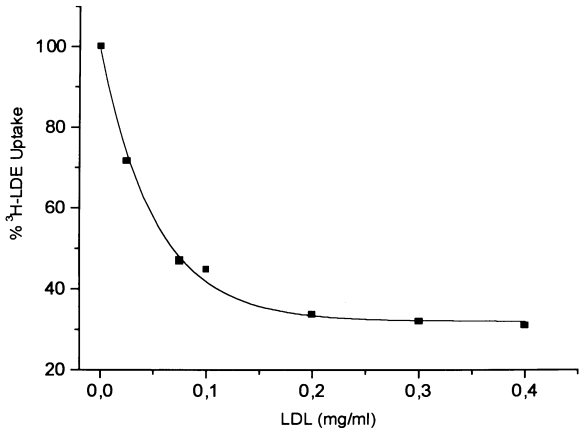

Fig. 3 Competition between native LDL and ^{3}H-cholesteryl ether-labeled LDE for uptake by cultivated RPMI-8226 multiple myeloma cells. Cells were incubated with increasing amounts of native human LDL. The concentration of LDE labeled with ^{3}H-cholesteryl ether was constant corresponding to 0.2 mg/ml in each dish. Native LDL concentrations ranged from 0.05 to 0.4 mg/ml

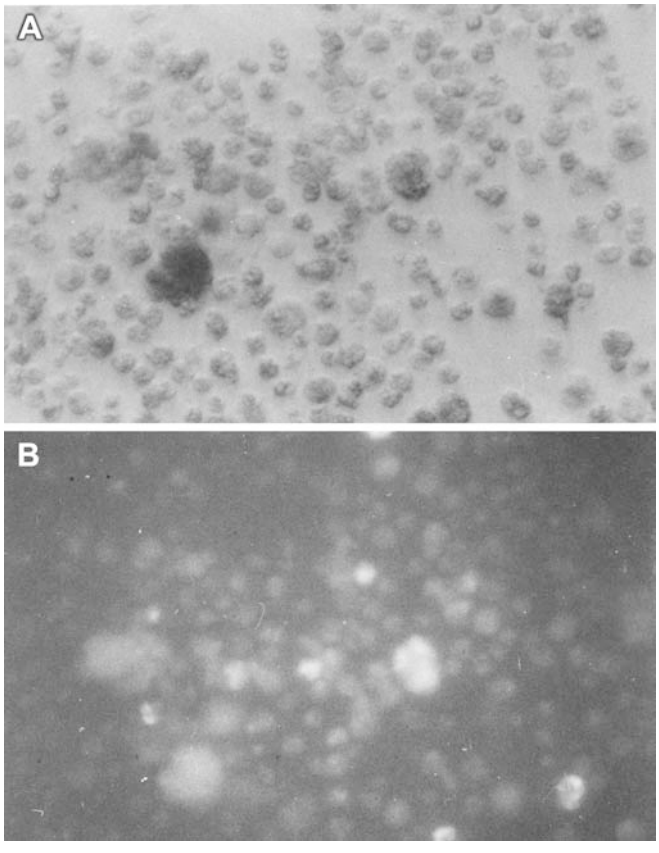

Fig. 4A, B Internalization of fluorescently labeled LDE into multiple myeloma cells. LDE was labeled with a fluorescent cholesteryl ester analog. RPMI-8226 cells were incubated with fluorescent LDE (12.5 μg/ml) for 2 h. Internalization of fluorescent LDE was visualized by fluorescence microscopy. **A** Light microscopy; **B** fluorescence microscopy of the same section

Cytotoxicity to human myeloma cells

Figure 5 shows the results of the experiments in which RPMI-8226 cells were incubated with carmustine alone

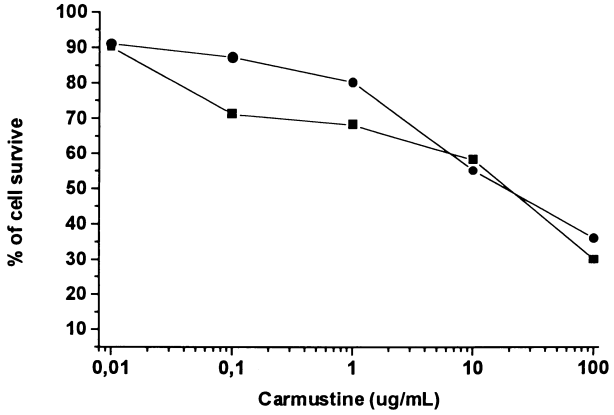

Fig. 5 Cytotoxicity of free carmustine and carmustine associated with LDE. Survival of RPMI-8226 myeloma multiple cells with increasing amounts of LDE-carmustine (●) and carmustine (■). Cells were incubated for 24 h at 37°C. The percentage cell survival was determined by the MTT assay. The data presented are the means ± SEM of three experiments performed in quadruplicate

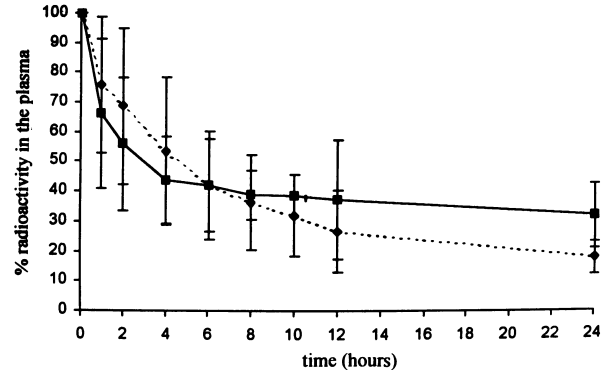

Fig. 6 Decay curve of a ^{3}H-cholesteryl ester emulsion obtained from 14 patients with multiple myeloma (*dotted line*) and 14 controls (*continuous line*). The labeled emulsion was intravenously injected as a bolus and plasma samples were taken over 24 h for radioactive counting in scintillation vials

and associated with LDE. The dose-response curves of the free drug were similar to those obtained with LDE-carmustine (carmustine $IC_{50} = 19.9$ μg/ml, LDE-carmustine $IC_{50} = 18.9$ μg/ml). Therefore, the cell killing capacity of the drug was preserved when associated with LDE.

Plasma kinetics of LDE

Figure 6 shows the mean decay curves of the emulsion ^{3}H-cholesteryl esters obtained from 14 multiple myeloma patients and 14 controls in whom the emulsion plasma kinetics were determined. During the first 6 h, the emulsion mean plasma decay was slightly slower in the multiple myeloma patients than in the controls, but from 6 h onwards the decay became faster until the final (24-h) radioactivity measurement, and at this time-point the patients retained in the plasma much less radioactivity from the emulsion than the controls.

Table 2 shows the data on age, plasma lipids, BMI and FCR of the emulsion radioactive label of the 14 patients and their controls. The emulsion FCR in the multiple myeloma patients was twice that in the control subjects ($P < 0.05$). Total, LDL and HDL cholesterol concentrations were lower in the patients than in the controls. In the case of LDL cholesterol, the values were roughly half those in the controls.

Plasma kinetics of carmustine associated with LDE

Figure 7 shows the average decay curve of ^{14}C-carmustine associated with LDE obtained from three multiple myeloma patients. The decay curve is in the same range as those for LDE shown in Fig. 6. Table 3 summarizes the pharmacokinetic parameters of carmustine associated with LDE.

Clinical pilot study of LDE-carmustine

A total of 40 treatment cycles in the seven patients were analyzed (minimum four, maximum six cycles per patient). LDE-carmustine showed no or minimal toxicity in all cycles. In all cycles, myelotoxicity, as documented by platelet, leukocyte and red blood cell counts, was grade 0. Arterial hypertension, fever, dyspnea, alopecia, and hepatic, renal and gastrointestinal toxicities were also grade 0. All patients reported a reduction in pain, and in all of them the hemoglobin concentration consistently increased during LDE-carmustine treatment.

Figure 8 shows the monthly evolution of the gammaglobulin peak in the plasma protein electrophoresis profile. After three cycles, gammaglobulin had decreased by 10-70% (mean 36%) and after six cycles by 25-75% (mean 44%) in relation to pretreatment values. Plasma cholesterol concentrations consistently increased in all patients during treatment (Fig. 9).

Table 2 Individual characteristics, plasma lipids (mg/dl) and fractional clearance rates (FCR, in h^{-1}) of the LDE radioactive label in 14 multiple myeloma patients and in 14 controls

	Age (years)	BMI (kg/m^2)	Cholesterol				Triglycerides	LDE FCR
			Total	LDL	HDL	VLDL		
Patients	57 ± 10	24 ± 3	113 ± 47*	65 ± 36*	27 ± 10*	22 ± 12	97 ± 57	0.0865 ± 0.0642*
Controls	56 ± 13	26 ± 3	204 ± 47	135 ± 35	44 ± 12	25 ± 15	126 ± 77	0.0389 ± 0.0321

*$P < 0.001$

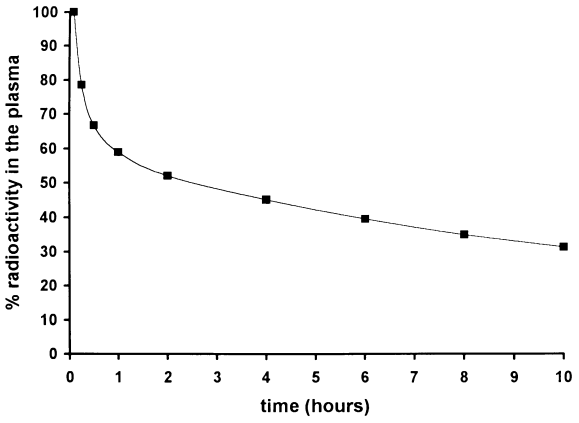

Fig. 7 Plasma decay curve of the ^{14}C-carmustine. The labeled ^{14}C-carmustine was associated with LDE and injected as a bolus into three cancer patients. Plasma samples were taken over 10 h for radioactive counting in scintillation vials. The curve is the mean of the data obtained from the three patients

Discussion

The ''increased LDL clearance-decreased LDL cholesterol' pattern was found in multiple myeloma patients. Uptake of LDE by multiple myeloma cells in culture was shown and confirmed to be mediated by the LDL receptors. Taken together, these results strongly suggest that in multiple myeloma upregulation of LDL receptors occurs, and these findings provide the mechanistic basis for the use of LDE to direct chemotherapeutic agents against multiple myeloma cells.

Low LDL cholesterol concentrations were documented in a large cohort of 131 untreated, newly diagnosed patients with multiple myeloma. In fact, this is a somewhat controversial issue in the literature: in two studies [10, 18] each enrolling approximately 40 multiple myeloma patients, LDL cholesterol was found to be decreased, but in 21 patients enrolled in a third study the levels were not different from those of the controls [27].

Regarding the other lipoprotein fractions, our finding of low HDL cholesterol is in accordance with that of the study by Hachem et al. [18]. Both fast triglycerides and VLDL cholesterol mirror the VLDL plasma levels and the fact that they were unchanged in the patients is also in agreement with the findings of the above-mentioned studies [36, 37]. Therefore, it can be stated that multiple myeloma is associated with low LDL and HDL fractions whereas VLDL is unchanged. We have recently shown that chylomicron metabolism is also altered in this disease [23].

In the 14 multiple myeloma patients in whom removal of LDE from the plasma was evaluated, the FCR of the emulsion radioactive label was greater than in the healthy control subjects. Considering the association between LDE and native LDL, this finding may explain why the plasma total and LDL-cholesterol levels were diminished in these patients. The adequacy of LDE as a tool to test in vivo the mechanisms of LDL removal from the plasma has been demonstrated in previous clinical studies, wherein the plasma kinetic results obtained with LDE were as expected for native LDL. In this respect, removal of LDE occurs more slowly in patients with familial hypercholesterolemia [33], in whom LDL receptor function is defective, and is increased in untreated patients with acute myeloid leukemia due to LDL receptor upregulation [29, 31]. Conversely, after disease remission achieved by chemotherapy, LDE removal was decreased and LDL cholesterol concentrations were increased. Recently, we have been able to show that LDE FCR is negatively correlated with age [38]. This also corroborates the validity of LDE in mimicking the behavior of the native lipoprotein because in several studies it has been shown that LDL plasma removal progressively diminishes with age due to the age-related decrease in LDL receptor expression [16, 36].

The plasma concentration of LDL is determined by the balance between the rate of lipoprotein production and the rate of removal from the plasma. The status of LDL receptor function is the major determinant of both LDL removal and LDL concentration in the plasma [6]. Therefore, diminution of LDL plasma concentration in the multiple myeloma patients can be ascribed to the increased clearance of the lipoprotein, as documented by the increased LDE FCR. LDE plasma clearance was increased in multiple myeloma patients, indicating that LDL receptor function is increased in this disease at an intensity sufficient to lower LDL plasma concentration. Increased LDE FCR is conceivably a consequence of LDL receptor upregulation that is a widespread phenomenon in neoplasias. Increased LDL receptor activity may result from the increased need for cholesterol and other lipids for membrane construction, resulting from uncontrolled proliferation of the neoplastic cells, and elicits increased LDL removal from the plasma with consequent lower total and LDL cholesterol plasma concentrations. Therefore, in the present study, the association of low total and LDL cholesterol plasma concentrations with accelerated removal of LDE from the plasma strongly suggests that multiple myeloma

Table 3 Pharmacokinetic parameters of carmustine following bolus infusion of ^{14}C-carmustine associated with LDE as measured in three patients

Patient	Dose (mg/m^2)	$t_{1/2}$ (min)	$t_{1/2}$ (h)	Vss (ml/kg)	AUC (mg/min/ml)	CL (ml/min/kg)
1	7.8	37.5	33.7	61.9	13.4	1.5
2	6.8	24.9	21.6	31.4	17.2	1.2
3	6.9	84.2	45.7	29.2	38.7	0.5
Mean	7.2	48.9	33.7	40.9	23.1	1.1

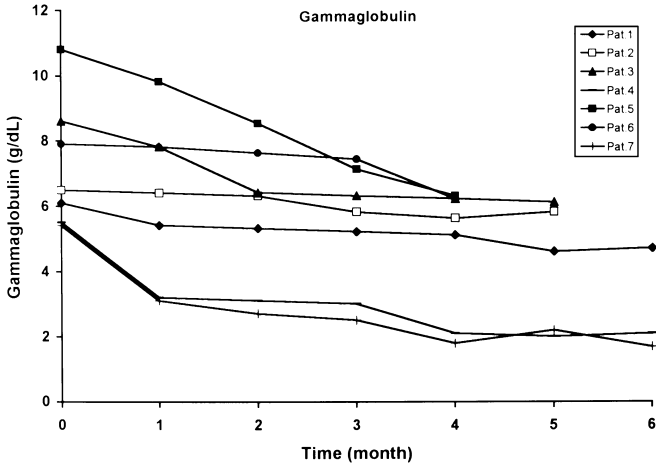

Fig. 8 Monthly evolution of gammaglobulin peak in the plasma protein electrophoresis profile under the LDE-carmustine treatment. Determinations were performed a few days before the monthly administration of the LDE-carmustine dose

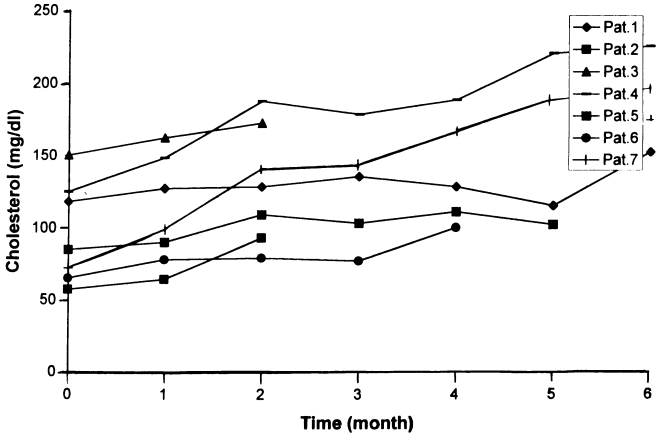

Fig. 9 Monthly evolution of the total cholesterol concentration under the LDE-carmustine treatment. Determinations were performed a few days before the monthly administration of the LDE-carmustine dose

cells, by expressing increased LDL receptor activity, may take up LDE at a greater rate. This possibility is further supported by the fact that the myeloma patient in whom LDE kinetics were also determined after remission showed a diminution in the emulsion plasma clearance concomitant with increased total and LDL cholesterol concentrations. Taken together, our results suggest that multiple myeloma cells take up LDE, and by extension native LDL, in amounts sufficiently large to affect the plasma clearance of the particles and plasma cholesterol concentration.

It is appealing to compare the current multiple myeloma data with those for acute myeloid leukemia found our previous study [29]. In multiple myeloma patients the removal of the emulsion from the plasma was twice that in control subjects, resulting in a 33% reduction in the plasma pool of LDL cholesterol. The impact of acute myelocytic leukemia was somewhat greater than that of multiple myeloma: a threefold greater LDE removal from the plasma resulted in a 47% reduction in the LDL-cholesterol concentration. In multiple myeloma cells, LDL receptor function was increased in the range 3- to 100-fold. Therefore, it can be inferred that LDL function is also markedly increased in multiple myeloma. It is also noteworthy that HDL cholesterol in multiple myeloma shows the same pattern as in acute myelocytic leukemia: HDL levels are low at the start of treatment and, in contrast with LDL cholesterol, do not increase during remission.

In this study we also showed that a cultivated multiple myeloma cell line takes up LDE in a dose-saturating manner that is characteristic of LDL. Indeed, the experiments investigating competition between LDE and native LDL clearly showed that LDL interferes with LDE binding to myeloma cells, indicating that both share the same receptor mechanisms that capture the particles in this neoplastic lineage. Finally, LDE internalization into these cells was demonstrated visually by fluorescence microscopy.

The fact that the internalization rates of the drug were similar to those of the emulsion strongly supports the assertion that carmustine is indeed delivered to the multiple myeloma cells by LDE. Similar to the results of experiments with mucoepidermoid lung carcinoma cells (NCI-H292) described in our previous report [34], the cytotoxicity of carmustine against the cultivated multiple myeloma cell lineage was not diminished by association with the emulsion. These experiments make clear that LDE, while directing carmustine to multiple myeloma cells, is able to preserve the pharmacological activity of the drug.

Because free carmustine quickly disappears from the plasma [26], the finding of the plasma decay curve of carmustine associated with LDE being similar to that of the emulsion cholesteryl oleate suggests that the drug tends to remain associated with the emulsion while in the plasma compartment. Indeed, the pharmacokinetic data in Table 3 show that carmustine associated with LDE has a significantly greater half-life and severalfold smaller clearance than those reported in the literature for free carmustine [26].

Supported by the results discussed above, we went on to investigate in a preliminary manner the possible role of LDE as a drug carrier in multiple myeloma. Our recent clinical study [34] established the safety of a first preparation of a chemotherapeutic agent associated with LDE. The association of carmustine with LDE is stable and the drug does not lose its therapeutic effectiveness as assayed in neoplastic cells. The animal toxicity studies showed a remarkable tolerability of the complex, much greater than the commercial carmustine formulation. Administered to patients with advanced solid cancers, LDE-carmustine was practically free of important side effects up to the dose tested, i.e. 350 mg/m^2 every 4 weeks, a dose much higher than those used in routine chemotherapy.

Although substantial progress has been made, multiple myeloma treatment is still largely unsatisfactory. Formerly, in the 1960s, the survival time of the patients was less than 1 year. Treatment with alkylating agents alone or in combination with other drugs has increased the survival time to 3 years [3, 37]. Complete remissions are unusual and it is doubtful whether any patients with multiple myeloma are cured with conventional chemotherapy. High-dose chemotherapy followed by autologous bone marrow peripheral blood stem cell transplantation may further increase the survival time to 5 years [2, 8, 19]. However, this treatment is restricted by the clinical condition of the patient since the disease most frequently occurs in those above 60 years of age.

Carmustine has been used in chemotherapeutic schemes for multiple myeloma treatment, although only as a second-line drug. We therefore sought to determine whether LDE-carmustine could be used in untreated multiple myeloma patients with no indication for bone marrow transplantation. The 180 mg/m^2 every 4 weeks dosage corresponds to the carmustine dose used in conventional chemotherapy. Although a previous dose-escalating study in patients with solid cancers had shown that much higher doses could be employed, we adopted the 180 mg/m^2 level because the disease compromises the bone marrow, where the main toxicity of carmustine occurs. The results, however, showed that the preparation, while also eliciting no side effects in other organs and tissues, did not produce myelotoxicity.

In all patients a marked fall in plasma gammaglobulin, the main disease evolution marker, occurred. The average gammaglobulin fall of 44% observed is compatible with currently used treatments. It is worthwhile pointing out that corticosteroids, that are used as adjuvants to chemotherapeutic agents in multiple myeloma and have malignant cell killing action, were not used here. The increase in plasma cholesterol that occurred in all patients may indicate destruction of multiple myeloma cells which would diminish the population of cells with more avidity for the emulsion resulting in a reduction in plasma removal and a consequent decrease in cholesterol concentration.

In conclusion, our results suggest that LDE may concentrate in multiple myeloma cells due to increased LDL receptor function. In a subsequent clinical pilot study of LDE-carmustine, it was shown that the preparation has therapeutic activity with virtually no side effects. This is interesting because carmustine is not a first-line drug in the treatment of this disease and the potential for the administration of larger doses allowed by the low toxicity [34] was not explored. Thus, LDE has the potential to be used as a vehicle for chemotherapeutic agents in the treatment of multiple myeloma.

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