Lipoprotein Receptors in Acute Myelogenous Leukemia

Failure to Detect Increased Low-Density Lipoprotein (LDL) Receptor Numbers in Cell Membranes despite Increased Cellular LDL Degradation

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The high-affinity degradation of low-density lipoprotein (LDL) is enhanced 3- to 100-fold in leukemic blood cells from patients with acute myelogenous leukemia (AML), suggesting an increased cellular LDL receptor expression. There are, however, inconsistencies regarding the published properties of LDL receptor regulation in AML cells, and previous data on this are indirect. In the present study the aim was to determine whether the LDL receptor number is increased in AML cells. The LDL receptor number was assayed by ligand blot with rabbit ^{125}I -labeled β -very-low-density lipoprotein (β -VLDL) of transferred, SDS-polyacrylamide-gel-electrophoresis-separated AML cell membranes. Samples from 10 patients, six with AML, one with chronic myelogenous leukemia in blast crisis, and three with acute lymphoblastic leukemia, were investigated. The LDL receptor expression was strongly suppressed in all samples to levels lower than that of normal mononuclear cells. This was despite the fact that cells from one patient with AML of M4 subtype had a 50- to 100-fold higher ^{125}I -labeled LDL degradation compared with normal cells. Immunoblots with antibodies against gp330/megalin and the LDL-receptor-related protein (LRP) and ligand blot using ^{125}I -labeled 39-kd receptor-associated protein (RAP) could not detect gp330/megalin or VLDL receptors. The LRP was abundant in AML samples of M4 and M5b subtype, as determined from both RAP ligand blot and immunoblot using an LRP-specific antibody. It is concluded that LDL receptors are suppressed in AML cells. It is possible that the high degradation of ^{125}I -labeled LDL present in type M4 and

M5 AML cells may involve another lipoprotein receptor. (Am J Pathol 1998, 153:1923-1935)

Human cells have receptors for the major cholesterol-carrying lipoprotein in human plasma, low-density lipoprotein (LDL).^{1,2} These LDL receptors transport cholesterol-rich lipoproteins containing apolipoprotein (apo)B or apoE into the cell. Tissues with a high demand for cholesterol, such as the adrenal gland and the corpus luteum, have high receptor numbers.^{3,4} In contrast, LDL receptor expression is strongly suppressed in cells exposed to high LDL levels, such as mononuclear cells within the bloodstream.⁵ When normal cells proliferate, the LDL receptor number is induced,⁶ presumably due to an increased demand for cholesterol. A similar situation might be present in tumor cells. Thus, Ho et al⁷ have shown that freshly isolated leukemic cells from patients with acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML) in blast crisis display a 3- to 100-fold higher specific LDL degradation as compared with mononuclear cells from normal subjects or tumor cells from patients with acute lymphoblastic leukemia (ALL). In that study, five patients with AML and two patients with CML in blast crisis were all found to have increased LDL receptor activity. These findings were later confirmed by Vitols et al⁸ in a large series of patients. They demonstrated that very high LDL degradation was practically always present in samples from CML in blast crisis and AML of subgroups M4 and M5. The cause for this increased specific LDL degradation remains unclear.⁷⁻⁹ Two studies have shown, in two leukemia patients, that the elevated basal specific LDL degra-

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dation in AML cells is not suppressed after incubation with cholesterol and 25-hydroxycholesterol.^{7,9} This fact was recently confirmed in a study on 27 leukemia patients by Tatidis et al.¹⁰ This result is somewhat remarkable considering current concepts of LDL receptor regulation¹ but has been interpreted to be indicative of post-transcriptional regulation of the LDL receptor.⁹

In all previous studies on LDL receptors in leukemia, the cellular high-affinity degradation of ¹²⁵I-labeled LDL has been measured.^{7,11} This is determined from the generation of ¹²⁵I-labeled tyrosine after incubation of cells with ¹²⁵I-labeled LDL at 37°C in the absence and presence of excess unlabeled LDL. The degradation of ¹²⁵I-labeled LDL that can be displaced by unlabeled LDL is then referred to as cellular LDL receptor activity.^{7,11} This functional assay is very robust and has therefore been used extensively to monitor LDL receptor function in cultured cells. However, it is an indirect assay, and in the past years it has been shown that cell surface receptors other than the LDL receptor can also mediate specific, high-affinity degradation of ¹²⁵I-labeled LDL.^{12,13}

We recently found that the gene expression of the LDL receptor and that of the rate-limiting enzyme of cholesterol synthesis, 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, were coordinately suppressed in human renal cell carcinoma.¹⁴ Because of this unexpected finding, we reviewed the studies on LDL receptors in leukemia^{7-10,15-18} and found several observations inconsistent with the current concept of an increased expression of LDL receptors in AML cells (see Discussion). Remarkably, we realized that all studies to date have determined LDL receptor activity by the same indirect LDL degradation assay.

We therefore decided to assay directly the expression of LDL receptors in leukemic cell membranes, with the aim of clarifying whether LDL receptor expression is indeed increased in AML cells. Using an established ligand blot assay,¹⁹⁻²² we found that the expression of LDL receptors in membranes of mononuclear cells from patients with acute leukemia was strongly suppressed, to the same or lower levels than those found in freshly isolated mononuclear cells from normal subjects. The role of the LDL receptor in the abnormal lipoprotein metabolism in AML, and presumably other tumor diseases, therefore requires further attention.

Materials and Methods

Materials

Na¹²⁵I (IMS 30) was obtained from Amersham (Little Chalfont, UK). Bovine serum albumin (fraction V, catalog item A-6003), cholesterol (C-8503), 25-hydroxycholesterol (H-1015), dibutyryl cAMP (D-0227), dimethylsulfoxide (D-3129), erythrosin B (E-7379), Trizma base (T-1503), leupeptin (L-2884), phenylmethylsulfonyl fluoride (P-7626), 1,10-phenanthroline (P-9375), polyoxyethylenesorbitan monolaurate (Tween 20; P-1379), and retinoic acid (RA; R-2625) were from Sigma Chemical Co. (St. Louis, MO). RPMI-1640, fetal calf serum, L-glutamine,

and penicillin/streptomycin were purchased from GIBCO-BRL (Paisley, UK). Dried skim milk was from Semper AB, Stockholm, Sweden. Triton X-100 (number 5629) was from Riedel-de Haen (Seelze, Germany). Bio-Rad protein assay (number 500-0001) and high molecular weight standards (161-0303) were obtained from Bio-Rad Laboratories (Richmond, CA). Tissue culture plates (24 wells, 15-mm diameter) and 175-cm² flasks were from A/S Nunc (Roskilde, Denmark). PD-10 columns were from Pharmacia (Uppsala, Sweden), and nitrocellulose filters (0.45 μ m, type BA 85) were from Schleicher & Schuell (Dassel, Germany). Millex-HA filters were from Millipore (Molsheim, France).

Lipoproteins and Lipoprotein-Deficient Serum

LDLs were isolated from serum of healthy blood donors by sequential ultracentrifugation²³ and labeled with ¹²⁵I (specific activity, 200 to 500 cpm/ng) as described by Langer et al.²⁴ Rabbit β -migrating very-low-density lipoproteins (β -VLDLs) were obtained after ultracentrifugation at $d = 1.006$ g/ml of plasma from cholesterol-fed rabbits and labeled to a specific activity ranging from 400 to 1000 cpm/ng.²⁴ Free ¹²⁵I was removed by gel filtration on a PD-10 column, followed by extensive dialysis against 0.15 mol/L NaCl, 0.01% EDTA, pH 7.5. Before use, labeled LDLs and β -VLDLs were filtered through MILLEX-HA 0.45- μ m and 0.7- μ m filters, respectively. Human lipoprotein-deficient serum (LPDS) was obtained after ultracentrifugation of fresh serum from healthy blood donors at $d = 1.21$ g/ml. LPDS was extensively dialyzed against PBS (140 mmol/L NaCl, 2.7 mmol/L KCl, and 9.5 mmol/L phosphate buffer, pH 7.4) and filtered through a Millex-HA 0.45- μ m filter.

Antibodies and Recombinant 39-kd Receptor-Associated Protein

For Western blot analysis of the LDL-receptor-related protein (LRP), the rabbit polyclonal antiserum R777, previously described by Stefansson et al.,¹² was used (gift of Dr. Dudley Strickland, American Red Cross, Rockville, MD). Monoclonal antibody (MAb) 1H2, specific for human gp330/megalin, was a gift from Dr. Robert McCluskey, Pathology Research Laboratory, Harvard/Massachusetts General Hospital, Charlestown, MA. This antibody cross-reacts with human and mouse gp330/megalin.¹² Goat anti-mouse (GAM; number M-8642) and goat anti-rabbit (GAR; number R-4880) antibodies were obtained from Sigma. The 39-kd receptor-associated protein (RAP) was prepared in *Escherichia coli* as a glutathione-S-transferase (GST)-RAP fusion protein as described by Williams et al.²⁵ To obtain RAP, the GST fusion protein was digested with thrombin (1 μ g/mg fusion protein) while still bound to the glutathione-Sepharose CL4B beads. The supernatant was recovered, and thrombin activity remaining was inhibited by the addition of D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (Calbiochem, La Jolla, CA). The protein was extensively dialyzed against PBS. Antibodies and RAP were labeled

with ^{125}I using the chloramine-T method.²⁶ Free ^{125}I was removed by gel filtration on a PD-10 column. The protein concentration of RAP was determined according to Bradford²⁷ using reagents and protocols supplied by Bio-Rad.

Tissues and Cells

Normal human kidney tissue was obtained from patients undergoing nephrectomy because of renal cell cancer. Bovine adrenal glands were obtained from a local slaughterhouse. Mouse F-9 embryonic carcinoma cells were purchased from the American Type Culture Collection (Rockville, MD). Human mononuclear cells were separated by centrifugation on Lymphoprep (Nyegaard and Co., Oslo, Norway). Normal mononuclear cells were isolated from the buffy coat of two healthy blood donors.

Leukemia Patients

Leukemic mononuclear blood cells were from six patients with AML, one with CML in blast crisis, and three with ALL (Table 1). In one case (patient 1), cells were obtained on two occasions: once at the time of diagnosis (Figure 1) and again 4 months later on relapse of disease. The sample from patient 3 with CML was taken in blast crisis. Cells from all other subjects were obtained at the time of diagnosis. AML subgrouping was made according to the French-American-British (FAB) classification.²⁸ The sampling of leukemic cells was approved by the Ethics Committee at Huddinge University Hospital.

Cellular Degradation of ^{125}I -Labeled LDL

Isolated mononuclear cells were counted using a Bürker chamber. Cell viability, assessed by erythrosin B exclusion, was >90%. Aliquots of 1-ml cell suspensions were seeded in duplicate in 1.75-cm² wells at a density of 3×10^6 cells/ml. Cells were incubated for 4 hours with the indicated concentration of ^{125}I -labeled LDL in 1 ml of RPMI-1640 medium supplemented with L-glutamine, 10% LPDS, and antibiotics (100 IU of penicillin and 100 μg of streptomycin/ml of medium). Incubations were made at

37°C in a humidified atmosphere containing 5% CO₂. The degradation of ^{125}I -labeled LDL was assayed after precipitation of the medium with 10% trichloroacetic acid and subsequent oxidation and removal of free iodide using H₂O₂ and chloroform, respectively, as described by Goldstein et al.¹¹ Blank incubations, in the absence of cells, were performed in parallel, and these values have been subtracted from the data presented. The high-affinity degradation of ^{125}I -labeled LDL (LDL-displaceable or specific degradation) was calculated by subtraction of the degradation in the presence of excess unlabeled LDL from that in the absence of excess unlabeled LDL in duplicate samples. LDL concentration is referred to as protein, determined according to the method described by Lowry et al.²⁹ using bovine serum albumin as standard. ^{125}I -labeled LDL degradation of all mononuclear cells is expressed as nanograms of ^{125}I -labeled LDL degraded per hour per 10⁶ cells. The ^{125}I -labeled LDL degradation of mouse F-9 cells is expressed as nanograms of ^{125}I -labeled LDL degraded per hour per milligram of cell protein.

Stimulation of F-9 Cells with Retinoic Acid plus Dibutyryl cAMP

F-9 cells were cultured in 175-cm² gelatin-coated (0.1%) flasks in RPMI-1640 medium supplemented with glutamine and antibiotics. One group of cells was incubated with 0.1 $\mu\text{mol/L}$ retinoic acid (RA) plus 0.2 $\mu\text{mol/L}$ dibutyryl cAMP (dcAMP) as described by Stefansson et al.¹² After 7 days of incubation, cells were detached by scraping with a rubber policeman and subsequently wash by centrifugation. Specific degradation of ^{125}I -labeled LDL was assayed in parallel in 1.75-cm² wells as described above.

Preparation of Cellular Membranes

Cells (20×10^6 to 50×10^6) were pelleted in an Eppendorf tube by centrifugation after wash in RPMI-1640 medium. After the addition of 200 to 500 μl of lysis buffer (0.5 mol/L NaCl, 0.05% polyoxyethylenesorbitan monolaurate, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 0.02 mg/ml leupeptin, 50 mmol/L Hepes, pH 7.5), cells were homogenized on ice with two 5-second pulses using a polytron (Kinematica, type PT 10/35, Kriens, Luzern, Switzerland) equipped with a mini pestle. Homogenates were rapidly sonicated on ice with two 3-second pulses using a Branson 250 Sonifier (Branson Ultrasonics, Danbury, CT) and thereafter centrifuged at $10,000 \times g$ for 10 minutes at 4°C. The supernatants were rapidly frozen in aliquots and assayed for protein as described for RAP.

SDS-Polyacrylamide Gel Electrophoresis

Separation of Cell and Tissue

Membrane Proteins

Membranes were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)³⁰ under nonreducing con-

Table 1. Data of the Leukemic patients.

Patient	Diagnosis	Sex/age (years)	Sample source	% WBC	% blasts
1	AML M2 (relapse)	F/87	PB	240*	
2	AML M4	M/46	BM	117	39
3	CML in blast crisis	M/63	PB	66	30
4	AML M2	M/57	PB	40	55
5	AML M3	F/48	PB	33	46
6	AML M4	F/66	PB	36	
7	AML M5b	F/75	PB	41	75
8	ALL	F/62	PB	620	70
9	ALL	M/16	PB	26	81
10	ALL	M/38	PB	21	

F, female; M, male; PB, peripheral blood; BM, bone marrow; WBC, white blood cell count ($10^9/\text{ml}$).

*Second sampling.

Figure 1. Absence of detectable LDL receptors in leukemic cells from AML patient 1 (AML subtype M2). Cell and tissue membranes were prepared from mononuclear cells from patient 1, freshly isolated mononuclear cells from a normal subject, and normal human kidney and bovine adrenal glands. The membranes were separated by SDS-PAGE on a 6% gel and thereafter electrotransferred onto a nitrocellulose filter. After incubation of the filter with ^{125}I -labeled β -VLDL (5 $\mu\text{g/ml}$) and subsequent wash as described in Materials and Methods, the filter was exposed to film for 36 hours. M.N., mononuclear.

ditions on gels with the indicated polyacrylamide concentrations. Samples were electrophoresed using a Protean II gel apparatus (Bio-Rad). The separated proteins were transferred onto BA 85 nitrocellulose filters in a Bio-Rad transblot cell overnight as described by Burnette et al.³¹ Molecular weight markers used were human LDL (512 kd) and Bio-Rad high molecular weight standards. The markers were reduced with 2-mercaptoethanol and boiled before loading.

Ligand blot of transferred proteins with rabbit ^{125}I -labeled β -VLDL was performed as previously described.¹⁹ In brief, nitrocellulose filters were incubated with ^{125}I -labeled β -VLDL (5 μg of protein/ml) in an albumin-containing buffer and thereafter washed, dried, and finally exposed onto Cronex film at -70°C for the indicated times, using a Quanta 3 intensifying screen (DuPont, Boston, MA). Quantitation of radioactivity in the indicated bands was made using a Fujix Bio-imaging analyzer (BAS 2000, Fuji Photo Film Co., Tokyo, Japan). Data are expressed as arbitrary units (AU) obtained after background subtraction of irrelevant filter areas.

Ligand and Immunoblots

Nitrocellulose filters were preincubated for 1 hour at room temperature in 3% dry milk in PBS, followed by a 1-hour incubation with 1 $\mu\text{g/ml}$ ^{125}I -labeled RAP in PBS containing 3% dry milk, 0.02% Tween 20, and 5 mmol/L CaCl_2 . Filters were finally washed three times, dried, and exposed to film as described above.

Immunoblots for gp330/megalin and LRP were performed in PBS containing 5% dry milk. After incubation with primary antibody for 1 hour and subsequent washing, a secondary ^{125}I -labeled GAR or GAM antibody was added (2×10^6 cpm/ml) and incubated for an additional hour. After wash, filters were dried and exposed to film as described above.

Results

Our first opportunity to assay LDL receptor expression was in a sample obtained from a patient with AML of the

M2 type. Cell membranes from this patient's leukemic cells were separated by SDS-PAGE. For comparison, cell membranes of fresh mononuclear cells from the peripheral blood of a normal subject and membranes from normal human kidney and bovine adrenal glands were used. Ligand blot using ^{125}I -labeled β -VLDL (Figure 1) showed a strong ~120-kd band in the bovine adrenal sample, representing highly abundant LDL receptors in this tissue. In the sample from normal mononuclear cells, a faint 120-kd band was visible. In addition, two bands of ~500 and >600 kd were seen. Surprisingly, membranes prepared from the leukemic cells showed only two abundant bands of ~500 and >600 kd. No signs of mono- or dimeric forms of the LDL receptor could be detected. The human kidney membranes also showed strong bands of similar but possibly not identical sizes as the leukemic sample. In addition, there were at least three bands around 120 to 160 kd. Thus, the LDL receptor expression in the leukemic tumor cells from this M2 patient was at least as strongly suppressed as in freshly isolated normal mononuclear cells. However, previous data have shown that the degradation of ^{125}I -labeled LDL in leukemic cells from M2 patients may at times (~20%) be within the range observed in normal mononuclear cells.⁸ We therefore decided to assay the specific ^{125}I -labeled LDL degradation of the leukemic cells in the next patient available to make possible a comparison between specific degradation of ^{125}I -labeled LDL and LDL receptor expression as determined by ligand blot.

Patient 2 had AML of the M4 form, a subgroup in which the leukemic cells practically always show an abnormally high ^{125}I -labeled LDL degradation.⁸ The cells from this patient were obtained from a bone marrow sample. We assayed the specific ^{125}I -labeled LDL degradation of this patient's cells at a concentration of 10 $\mu\text{g}/\text{ml}$ ^{125}I -labeled LDL, with and without 150 $\mu\text{g}/\text{ml}$ unlabeled LDL, and compared it with normal mononuclear cells that had been preincubated in LPDS medium for 48 hours. The normal LPDS-preincubated cells had a specific ^{125}I -labeled LDL degradation of 3.9 ng of ^{125}I -labeled LDL/hour/ 10^6 cells, a quantitative value in agreement with previous data on normal derepressed mononuclear cells.⁹ The leukemic cells from patient 2 had a sevenfold higher degradation rate, 28 ng of ^{125}I -labeled LDL/hour/ 10^6 cells.

The remaining leukemic cells from patient 2 and the derepressed normal mononuclear cells were thereafter frozen at -130°C . Cell membranes were prepared from these cells as well as from cell pellets of the same normal subject's mononuclear cells, which had been frozen immediately after their fresh isolation. Cell membranes were separated by SDS-PAGE and subsequently electrotransferred onto a nitrocellulose membrane. Probing with ^{125}I -labeled rabbit β -VLDL (Figure 2) showed a faint, but detectable, 120-kd band in the freshly isolated normal cells (FM1). As expected, 2 days of preincubation of normal cells in LPDS medium resulted in a clearly increased LDL receptor expression (PM1). However, the LDL receptor expression in AML patient 2 was just barely detectable, and quantitation of the 120-kd bands showed an expression that was ~1/10 of the LPDS-preincubated normal mononuclear cells. Thus, it was clear that the

Sample / Patient No.	FM1		PM1		No.2	
^{125}I -LDL degradation	N.D.		3.9		28	
AU of 120 kD bands	3	40	54	291	0	30
μg protein / lane	50	200	50	200	50	200

Figure 2. LDL receptor expression in membranes from normal mononuclear cells and from leukemic cells (patient 2, AML subtype M4) and specific degradation of ^{125}I -labeled LDL by isolated cells. Cell membranes were prepared from a portion of freshly isolated mononuclear cells of a normal subject (FM1). The remaining cells were preincubated in 10% LPDS medium for 48 hours (PM1) before membrane preparation. Cell membranes were separated by SDS-PAGE on a 4% to 8% gel. The separated proteins were electrotransferred onto a nitrocellulose filter that was incubated with ^{125}I -labeled β -VLDL as described. The filter was exposed to film for 7 days. The radioactivity of the 120-kd bands was quantitated in arbitrary units (AU), using a Bio-Imager. The indicated specific ^{125}I -labeled LDL degradation, expressed as ng of ^{125}I -labeled LDL/hour/ 10^6 cells, was determined (see Materials and Methods) on the same occasion in cells from PM1 and patient 2 at a ^{125}I -labeled LDL concentration of 10 $\mu\text{g}/\text{ml}$, with and without excess unlabeled LDL (150 $\mu\text{g}/\text{ml}$).

highly increased specific ^{125}I -labeled LDL degradation of the leukemic cells from this M4 patient was not related to the expression of LDL receptors in the leukemic cell membranes. Instead, the LDL receptors were strongly suppressed to a similar low magnitude as that in normal freshly isolated mononuclear cells (FM1).

As the expression of LDL receptors could not explain the strongly increased specific degradation of ^{125}I -labeled LDL of the leukemic cells of patient 2, we speculated that another lipoprotein receptor may instead be involved. In recent years, a family of lipoprotein receptors has been identified, including the LDL-receptor-related protein (LRP),³² the VLDL receptor,^{33,34} and gp330/megalin.³⁵ It has been shown that gp330/megalin can account for a significant specific degradation of ^{125}I -labeled LDL in mouse F-9 embryonic carcinoma cells.¹² When such cells are incubated with RA and dcAMP (RA+dcAMP) for 7 days, gp330/megalin expression is increased 20-fold.¹² In parallel, the expression of LDL receptors and LRP are reduced, despite a several-fold increase in the specific degradation of ^{125}I -labeled LDL. Thus, an increased expression of gp330/megalin could result in an enhanced LDL degradation despite suppressed LDL receptors.

Figure 3. Absence of gp330/megalin in membranes of leukemic mononuclear cells. Cell membranes from patient 2 were separated by SDS-PAGE on a 4% to 8% gradient gel. As controls, cell membranes from F-9 cells incubated in the absence (F-9) and presence of RA+dcAMP (F-9+RA) were used. The filter was incubated (0.7 $\mu\text{g/ml}$) with mouse MAb 1H2 against rat gp330/megalin. The 1H2 antibody was visualized by incubating the filter with ^{125}I -labeled GAM. The filter was exposed to film for 3 days.

To determine whether gp330/megalin was present in the leukemic cells of patient 2, we again separated cell membranes from this patient with SDS-PAGE. As controls, membranes from F-9 cells incubated in the presence and absence of RA+dcAMP were used. After probing with a MAb directed against gp330/megalin, a 20- to 30-fold increase in the expression of this receptor was found in the F-9 cells incubated with RA+dcAMP (Figure 3). However, gp330/megalin was not detectable in the cell membranes from patient 2.

Another lipoprotein receptor that may be involved in LDL degradation is the VLDL receptor.^{33,34} To evaluate whether VLDL receptors were present in the cell membranes of patient 2, we took advantage of the fact that the VLDL receptor, like gp330/megalin and LRP, binds RAP with high affinity.³⁶ Membranes from the previously used freshly isolated and LPDS-preincubated normal mononuclear cells were separated by SDS-PAGE, together with membranes from patient 2 (Figure 4). Under these conditions, the VLDL receptor has a mobility of 115 to 120 kd.³⁷ After probing with ^{125}I -labeled RAP (Figure 4), no bands were observed in the size range for the VLDL receptor. Very strong bands were found at ~ 500 kd and at ~ 170 kd in all samples; the former should by exclusion represent RAP binding to LRP. Thus, LRP was abundant whereas neither gp330/megalin nor the VLDL receptor could be detected in the leukemic cells from patient 2.

We then wanted to determine whether a suppressed LDL receptor expression, which we had observed in cells from these 2 patients with AML, is a general finding in AML. To answer this question, we chose to proceed using previously freshly frozen cells. The disadvantage with this approach is that it does not allow for the deter-

Figure 4. Binding of ^{125}I -labeled RAP to mononuclear cell membranes from AML patient 2. As reference, freshly isolated normal (FM1) and LPDS-preincubated (PM1) mononuclear cells, described in the legend to Figure 2, were used. Cell membranes were separated by SDS-PAGE on a 4% to 8% gradient gel and transferred onto a nitrocellulose filter. The filter was incubated with 1 $\mu\text{g/ml}$ ^{125}I -labeled RAP, washed, and exposed to film for 3 days. The specific ^{125}I -labeled LDL degradation, expressed as ng of ^{125}I -labeled LDL/hour/ 10^6 cells, was determined as described in the legend to Figure 2. N.D., not determined.

mination of cellular LDL degradation. On the other hand, we could select subjects of particular AML subgroups that have previously been shown to invariably have increased LDL degradation, as well as leukemia types that never show increased LDL degradation, such as ALL.^{7,8} We obtained leukemic cells from five patients with AML, one with CML in blast crisis, and three patients with ALL. All cells had been isolated and thereafter immediately frozen in liquid nitrogen in the presence of 10% dimethylsulfoxide at the time of the patient's first admittance to the hospital. In one case (patient 1), cells were obtained after relapse of disease, before chemotherapy. Leukemic cell membranes were prepared from each sample and subjected to SDS-PAGE, electrotransfer, and subsequent incubation with ^{125}I -labeled β -VLDL (Figure 5). Membranes from the previously used F-9 cells (with and without RA+dcAMP) and from the normal freshly isolated (FM1) and derepressed (PM1) mononuclear cells served as assay controls. In addition, membranes from mononuclear cells from another normal subject were included. These cells had been assayed for specific ^{125}I -labeled LDL degradation, at a concentration of 25 μg of ^{125}I -labeled LDL/ml with and without excess unlabeled LDL (250 $\mu\text{g/ml}$), immediately after their isolation (FM2) and after 48 hours of LPDS preincubation (PM2).

Again, LDL receptors were just barely detectable in freshly isolated normal mononuclear cells (FM1 and FM2). Preincubation in LPDS medium resulted in a clearly detectable 120-kd band in membranes from mononuclear cells of both normal subjects (PM1 and PM2). The LDL receptor expression was slightly reduced in F-9 cells

AU of 120 kD bands	21	136	361	278	29	75	13	16	6	7	0	12	14	14	3	7
¹²⁵ I-LDL degradation	-	3.9	4.7*	11*	0.2	2.3	-	-	-	-	-	28	-	-	-	-
Patient No.							3	4	1	5	6	2	7	8	9	10
Sample / Diagnosis	FM1	PM1	F9	F9+RA	FM2	PM2	CML	M2	M2	M3	M4	M4	M5b	ALL	ALL	ALL
Lane No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16

Figure 5. Suppressed LDL receptor expression in leukemic cell membranes from 10 patients. Cell membranes were prepared from mononuclear cells of six patients with AML of the indicated FAB types, from three patients with ALL, and from one subject with CML in blast crisis (CML). As reference, cell membranes were prepared from freshly isolated normal mononuclear cells (FM1 and FM2) and normal monocytes that had been preincubated for 48 hours in LPDS medium to derepress LDL receptor expression (PM1 and PM2). In addition, cell membranes were prepared from mouse F-9 cells preincubated in the absence (F9) and the presence of RA/dcAMP (F9+RA). Cell membranes were separated by SDS-PAGE on a 6% gel, electrotransferred onto a nitrocellulose filter, and subsequently incubated with ¹²⁵I-labeled β -VLDL as described. The filter was exposed to film for 36 hours. The radioactivity in 120-kD bands was quantitated in arbitrary units (AU), using a Bio-Imager. In samples where the specific ¹²⁵I-labeled LDL degradation was assayed, this was performed in parallel to freezing of cell aliquots for later preparation of membranes for SDS-PAGE. The ¹²⁵I-labeled LDL degradation of mononuclear cells is expressed as ng of ¹²⁵I-labeled LDL/hour/10⁶ cells, and for F-9 cells (asterisk) as ng of ¹²⁵I-labeled LDL/hour/mg of cell protein.

after incubation with RA+dcAMP, although the specific degradation of ¹²⁵I-labeled LDL increased 2.3-fold, in agreement with previous data.¹² In the leukemic samples, LDL receptors (120-kD bands) were only barely detectable in a few samples, and quantitation of the ~120-kD bands in all leukemic cell samples revealed lower expression than found in freshly isolated normal mononuclear cells (FM1 and FM2). In all ALL samples, a clear band was present at ~100 kD; no attempt was made to further characterize this. It could thus be confirmed that the LDL receptor expression in all leukemic samples assayed was strongly suppressed to levels even lower than found in freshly isolated normal mononuclear cells. The whole assay was repeated twice, first using

another batch of ¹²⁵I-labeled β -VLDL and then using human ¹²⁵I-labeled LDL as ligand. When human ¹²⁵I-labeled LDL was used, background levels were increased, but the results were the same in both experiments (not shown).

We then assayed the same series of samples for the presence of gp330/megalin, using the MAb against rat gp330/megalin (Figure 6). To verify the specificity of the assay it was shown that the gp330/megalin expression was increased 20-fold in F-9 cells incubated with RA+dcAMP. No gp330/megalin was detected in normal or malignant mononuclear cells. We proceeded to determine the ability of ¹²⁵I-labeled RAP to bind to the separated cell membranes (Figure 7). As gp330/megalin was

Figure 6. Absence of detectable gp330/megalin protein in cell membranes from normal and leukemic mononuclear cells. All samples used are described in the legend to Figure 5. Cell membranes were separated by SDS-PAGE on a 3% to 4% gradient gel and transferred onto a nitrocellulose filter. The filter was incubated (0.7 μ g/ml) with mouse MAb 1H2 against rat gp330/megalin, which cross-reacts with human gp330/megalin. The mouse MAb was visualized using a GAM ¹²⁵I-labeled antibody (2×10^6 cpm/ml). The blot was exposed to film for 36 hours. The radioactivity in the $\sim$ 500-kD bands was determined with a Bio-Imager.

not present in the samples, all ¹²⁵I-labeled RAP binding to $\sim$ 500-kD region should then presumably, by exclusion, represent binding to LRP. Strong bands were obtained in the $\sim$ 500-kD region, and F-9 cells incubated with RA+dcAMP showed an additional strong band having a clearly slower mobility, obviously due to the binding of ¹²⁵I-labeled RAP to gp330/megalin in that sample. Strong $\sim$ 500-kD bands were present in samples from fresh and derepressed normal mononuclear cells. In the samples from the leukemia patients, there was a wide variation in the expression of the $\sim$ 500-kD band. Low expression was found in ALL and AML of the M2 and M3 subtypes, whereas subtypes M4 and M5b had higher expression.

To confirm the identity of the $\sim$ 500-kD RAP-binding band, we finally analyzed all samples using an anti-LRP antibody (Figure 8). Strong $\sim$ 500-kD bands were seen both in the freshly isolated and in the derepressed normal mononuclear cells (lanes 1, 2, and 6). The expression of this band was somewhat reduced in F-9 cells treated with RA+dcAMP as compared with untreated F-9 cells (lanes 4 and 3, respectively), in agreement with previous re-

sults.¹² There was a wide variation in the expression of this band in the samples of leukemic cells. Relatively low expression was found in ALL and AML of the M2 and M3 subtypes. The highest expression was observed in samples from patients with the M4 and M5b subtype of AML (lanes 12 and 13).

Discussion

Using a direct ligand blot assay, the present study has shown that the expression of LDL receptors in cell membranes from 10 patients with leukemia is suppressed to levels not higher than those observed in cell membranes from freshly isolated normal mononuclear cells. Of the seven patients with myelogenous leukemia, one was CML in blast crisis and two were AML of the M4 form and one of the M5b form, three particular subgroups where cellular LDL degradation is almost invariably elevated.⁸ In one of these M4 patients (patient 2), we had the occasion to assay the specific degradation of ¹²⁵I-labeled

Figure 7. ¹²⁵I-labeled RAP blot of normal and leukemic mononuclear cell membranes. The samples used are described in the legend to Figure 5. Cell membranes were separated by SDS-PAGE on a 3% to 4% gradient gel and transferred onto a nitrocellulose filter. The filter was incubated with 25×10^{-9} mol/L ($1 \mu\text{g/ml}$) ¹²⁵I-labeled RAP and, after wash, exposed to film for 18 hours. The radioactivity of ~500-kD bands was quantitated using a Bio-Imager.

LDL in freshly isolated leukemic cells, using derepressed normal mononuclear cells as reference. It was indeed found that this AML patient had a very high specific degradation rate of ¹²⁵I-labeled LDL, being sevenfold higher than that of derepressed normal mononuclear cells. This corresponds to a 50- to 100-fold higher ¹²⁵I-labeled LDL degradation rate compared with freshly isolated normal mononuclear cells, in which degradation averages ~0.5 ng of ¹²⁵I-labeled LDL/hour/ 10^6 cells when assayed at $25 \mu\text{g/ml}$ ¹²⁵I-labeled LDL.⁸ However, despite this very high specific degradation of ¹²⁵I-labeled LDL, the cell membranes from these tumor cells had an LDL receptor expression that was suppressed to levels not exceeding those found in freshly isolated normal mononuclear cells.

Ho et al⁷ were the first to demonstrate that freshly isolated cells from patients with AML, in contrast to cells from patients with lymphoblastic leukemia, had excessive degradation of ¹²⁵I-labeled LDL. Their findings were made using samples from five patients with AML and two patients with CML in blast crisis. The increased degradation of ¹²⁵I-labeled LDL was interpreted to be due to high-affinity cell surface receptors for LDL, as it was shown to be specific for LDL. However, competition studies using LDL showed a lower affinity than that previously established for this process in normal mononuclear cells.⁷ Thus, when leukemic cells were incubated with $25 \mu\text{g/ml}$ ¹²⁵I-labeled LDL in the presence of 50 and 100

$\mu\text{g/ml}$ unlabeled LDL, the initial degradation was inhibited only by ~20% and 50%, respectively.⁷ Performing a similar experiment on normal mononuclear cells using $15 \mu\text{g/ml}$ ¹²⁵I-labeled LDL in the presence of 15 and 60 $\mu\text{g/ml}$ unlabeled LDL, the same authors reported inhibitions of the initial degradation by ~40% and 75%, respectively.⁵ Furthermore, it was shown that the increased basal degradation of ¹²⁵I-labeled LDL by AML cells of one subject could not be suppressed after the addition of cholesterol and 25-hydroxycholesterol.⁷ These results argue somewhat against the LDL receptor being responsible for the high basal degradation rate of ¹²⁵I-labeled LDL in AML cells. However, on the other hand, it was shown that the high basal specific degradation rate could be strongly induced by preincubating the cells in LPDS medium and that this induction did not occur in the presence of cholesterol and 25-hydroxycholesterol. This would support the involvement of the LDL receptor. However, the quantitative increase in specific ¹²⁵I-labeled LDL degradation was four to five times higher in derepressed leukemic cells⁷ compared with what was observed when normal mononuclear cells were derepressed in the same way.⁵

These results have been reproduced by Vitols et al.⁹ They could confirm that the high basal specific degradation of ¹²⁵I-labeled LDL in cells from an AML patient was not suppressed by cholesterol and 25-hydroxycholesterol, in contrast to normal mononuclear cells. When leu-

Figure 8. Immunoblot for LRP of cell membranes from normal and leukemic mononuclear cells. The samples used are described in the legend to Figure 5. Cell membranes were separated by SDS-PAGE on a 3% to 4% gradient gel and transferred onto a nitrocellulose filter. The filter was incubated (0.8 μ g/ml) with rabbit polyclonal antibody R-777 directed against human LRP. The rabbit antibody was visualized using ¹²⁵I-labeled GAR (2 $\times$ 10⁶ cpm/ml). The picture was generated using a Bio-Imager, and the radioactivity of the ~500-kD bands was quantitated using a Bio-Imager.

kemic cells were derepressed for only 15 hours, the degradation increased from a basal value of 6.7 up to 50.9 ng of ¹²⁵I-labeled LDL/hour/10⁶ cells, whereas normal mononuclear cells, with an initial degradation rate of ~0.5 ng of ¹²⁵I-labeled LDL/hour/10⁶ cells, could increase their degradation to only ~1.6 ng of ¹²⁵I-labeled LDL/hour/10⁶ cells. Thus, the capacity to increase the specific ¹²⁵I-labeled LDL degradation was ~40 times higher in the leukemic cells than in normal mononuclear cells.

Furthermore, Vitols et al⁹ assayed the LDL receptor mRNA levels in cell extracts from the AML patients by solution hybridization. Several noteworthy observations were made. First, the LDL receptor mRNA/¹²⁵I-labeled LDL degradation ratio was 5- to 10-fold higher in normal mononuclear cells than in cells from two AML patients displaying high basal LDL degradation rates. Second, when cells from AML patients were derepressed, there was no clear relation between the increase in ¹²⁵I-labeled LDL degradation and the LDL receptor mRNA level. Third, there was a poor correlation between LDL receptor mRNA levels and ¹²⁵I-labeled LDL degradation, and it was evident that a high basal degradation rate of ¹²⁵I-labeled LDL was not necessarily associated with an increased LDL receptor mRNA level. Fourth, there was no correlation between the mRNA levels for the LDL receptor and HMG CoA reductase in leukemic samples, whereas a significant correlation was obtained using normal

mononuclear cells. Finally, there was an inverse relation between the cellular ¹²⁵I-labeled LDL degradation and the activity of HMG CoA reductase among leukemic samples, in contrast to normal mononuclear cells where a significant positive correlation was found.

The findings of a reduced or absent feedback inhibition of the LDL degradation was recently confirmed by Tatidis et al, who also found that there was no correlation between the free cholesterol in cells and LDL degradation in the material of 27 AML subjects.¹⁰ They did find, however, that there was a strong correlation between cellular LDL degradation and the number of white blood cells in peripheral blood, suggesting that the elevated LDL degradation is linked to growth stimulation.

We believe that the above findings may be in agreement with our present findings of a strongly suppressed LDL receptor expression in cells from patients with AML. The mRNA data of the previous study⁹ also argue against a possible, albeit not very likely, explanation of our data, namely, that the turnover of LDL receptors would be increased 5- to 100-fold in cells from AML patients. We therefore suggest that the high basal specific uptake and degradation of LDL, frequently observed in AML, may involve a steroid-resistant cell surface receptor and not the LDL receptor. The increased uptake of LDL cholesterol by such a structure will then suppress the LDL receptor expression and, in addition, lead to a suppressed activity of HMG CoA reductase as previously

shown.⁹ Whether suppressed LDL receptor expression is frequent in tumors remains an open question. However, in support of this possibility are our previous findings in renal cell carcinoma, where the mRNA levels for the LDL receptor and HMG CoA reductase were coordinately reduced as compared with those of normal kidney.¹⁴

If the LDL receptor would not be involved, what mechanism could then be responsible for the increased basal specific degradation of LDL in AML cells, particularly regarding the finding of an absent binding of ¹²⁵I-labeled β -VLDL or ¹²⁵I-labeled LDL to SDS-PAGE-separated cell membranes? The possibility of a pronounced proteolytic degradation only in our AML samples is not very likely because the large LRP, which should be sensitive to proteolysis, could be readily detected in several samples as, eg, patient 2. If such a proteolytic degradation were present, the LRP abundance would then in fact be highly underestimated.

One possible candidate could be gp330/megalin, as recent studies have shown that this protein can mediate specific degradation of ¹²⁵I-labeled LDL with a several-fold lower affinity as compared with the LDL receptor.¹² This LDL degradation is also resistant to suppression by cholesterol and 25-hydroxycholesterol (M. Rudling, unpublished). However, immunoblots for this protein could not detect the presence of gp330/megalin in malignant or normal mononuclear cells. A second candidate could be the recently discovered VLDL receptor,^{33,34} which can bind and mediate degradation of LDL with a very low affinity.³⁶ However, RAP blots did not detect any protein within the expected size for the VLDL receptor in cell membranes from AML patients.

A third possibility to explain the increased specific LDL degradation in AML would involve LRP. Previous studies by Moestrup et al^{38,39} have indeed shown that this protein is highly expressed in cells of monocytic differentiation, in particular among AML subtypes M4 and M5,³⁹ and LRP expression has actually been proposed as a measure of monocytic differentiation. In contrast, little or no LRP expression is present in cells from patients with plasma cell leukemia, acute or chronic lymphatic leukemia, or hairy cell leukemia³⁹; the latter three forms have been shown to have low cellular specific LDL degradation.^{8,17,18} Using ligand blot with RAP as well as LRP immunoblot, we could confirm a high expression of LRP in cells of monocytic differentiation. The highest LRP expression in the present series was indeed observed in cell membranes from the M5b patient. Low expression was found in cells from patients with ALL, a form of leukemia that has previously been shown not to exhibit any increased specific LDL degradation.^{7,8} It has recently been claimed that LRP may mediate cellular binding and catabolism of LDL *in vitro*, although the biological significance of such a mechanism is yet unknown.¹³

It should be noted that LRP was also abundant in normal mononuclear cells. The expression of LRP may thus be a necessary prerequisite for leukemic cells to take up and degrade LDL at a high rate, but other mechanisms operating via LRP may determine the degree of excessive LDL uptake. Such mechanisms may include the synthesis and secretion of apoE, lipoprotein lipase

(LPL), urokinase, or plasminogen activator inhibitor type I (PAI-I). Studies have shown that apoE and LPL can indeed be expressed in leukemic cells.⁴⁰ ApoE and/or LPL may interact with several lipoproteins, including LDL, thereby facilitating their binding to LRP and subsequent uptake, as has been shown to occur *in vitro*.⁴¹⁻⁴⁵ It may thus be speculated that the high basal, sterol-resistant LDL degradation in AML could be due to the presence of excess of, eg, LPL or apoE interacting with LRP and subsequently LDL. Such mechanisms could also explain why AML cells respond with a highly increased LDL degradation after cholesterol deprivation, as it has been shown that LPL can induce receptor-mediated catabolism of VLDL via the LDL receptor due to the binding of LPL to the LDL receptor.⁴⁶ An involvement of a secreted structure that promotes binding, such as LPL or apoE, would also explain why the ligand blots using β -VLDL or LDL could not visualize any particular structure in the above AML samples. Finally, it should be emphasized that other, not yet described, uptake mechanisms for LDL might operate in AML cells.

It is important to note that the increased LDL uptake in leukemic cells likely occurs *in vivo*. This has been shown after intravenous injection of [¹⁴C]sucrose-labeled LDL into patients with AML.¹⁵ Later isolation of mononuclear cells confirmed a strong relation between the mononuclear cell accumulation of ¹⁴C radioactivity and specific ¹²⁵I-labeled LDL degradation of mononuclear cells assayed *in vitro*. It was also evident that leukemic bone marrow accumulated significant amounts of the injected ¹⁴C radioactivity.¹⁵ The previous finding of an inverse relation between the cellular ¹²⁵I-labeled LDL degradation and the activity of HMG CoA reductase in leukemic samples⁹ also supports that these tumor cells have an increased LDL uptake *in vivo*. Furthermore, leukemic cell catabolism of LDL was strongly and inversely correlated with plasma LDL cholesterol levels.¹⁵ When these LDL-metabolizing tumor cells are eliminated by chemotherapy, plasma LDL cholesterol levels rapidly increase.⁴⁷ AML of the M4 and M5 forms may thus be an important disease prototype, where a lipoprotein uptake mechanism distinct from the LDL receptor may be involved in the significant alteration of plasma LDL cholesterol levels in man. Whether such an uptake mechanism for LDL operates *in vivo* in certain immature normal blood cells within the bone marrow remains to be explored. However, mixes of mononuclear cells obtained from bone marrow samples of normal subjects have similar degradation rates of ¹²⁵I-labeled LDL as cells derived from peripheral blood.⁸ It is also of interest to note that two recent studies^{48,49} have shown that LDL receptors on bone-marrow-derived cells do not significantly contribute to the clearance of plasma LDL or to the plasma lipoprotein pattern in mice after transplantation of normal bone marrow to LDL receptor gene knockout mice.

In summary, we have not been able to confirm that the LDL receptor number is increased in cell membranes from patients with AML of subtypes known to have an enhanced degradation of LDL. This finding, together with data of previous experiments described above, suggest that the LDL receptor is not responsible for the increased

metabolism of LDL in AML cells. Of four presently known receptors within the LDL receptor family, only LRP was significantly expressed in AML cells. Whether LRP or some other not yet defined structure could be of importance for the increased specific uptake and accumulation of LDL in AML cells remains to be elucidated. Additional studies on this specific uptake mechanism are therefore warranted.

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