Uptake of retinyl ester in HL-60 cells via the low-density-lipoprotein-receptor pathway

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Newly absorbed retinol is transported in association with chylomicrons and their remnants. In addition, after intake of high doses of retinol, significant amounts are also found in low-density lipoprotein (LDL). As both chylomicron remnants and LDL may be taken up by cells via the LDL receptor, and retinoids inhibit proliferation of some leukaemic cells, we have studied the uptake of retinol in leukaemic cells via the LDL-receptor pathway. HL-60 cells contain saturable binding sites for LDL. The binding of LDL to its receptor has a dissociation constant of about 3.2×10^{-9} M, and the number of receptors per cell was calculated to be about 2700. Uptake of ^{125}I -LDL by HL-60 cells was increased 2-fold by preincubating the cells with mevinolin. The presence of specific receptors for LDL on HL-60 cells was further confirmed by the finding that exogenous LDL cholesterol was able to up-regulate the ACAT (acyl-CoA: cholesterol acyltransferase) activity of HL-60 cells. We then tested the uptake of retinyl ester in leukaemic cells via the LDL-receptor pathway. HL-60 cells were incubated with LDL or chylomicron remnants labelled with [^3H]retinyl palmitate. Uptake of retinyl ester associated with both LDL and chylomicron remnants was observed. Furthermore, the presence of excess LDL decreased the uptake by 75–100%, supporting the hypothesis that the uptake of retinyl ester occurred via the LDL receptor in HL-60 cells.

INTRODUCTION

Retinoids are highly effective in preventing experimentally induced cancers in animal models [1] and they have been shown to promote differentiation of a variety of cell types *in vitro* [2]. Furthermore, retinoids have been reported to differentiate leukaemic blast cells isolated from patients with acute myeloid leukaemia (AML) [3]. A probable effect on leukaemic cells *in vivo* has also been reported [4].

AML in children is a rare, but serious, disease. In spite of progress in remission induction rates as result of more aggressive cytostatic therapy, the majority of children will still relapse and die of their disease [5]. Since 1981, 20 Norwegian children with AML have been treated according to a protocol combining cytostatics and high doses of retinol after the induction of remission [6]. Of these, 18 children are still alive, whereas two children died soon after admission. Of the 18, 13 children are still in their first remission, with a mean relapse free survival of 36 months [6]. On the basis of the encouraging results from this protocol, we decided it was worthwhile to study the mechanism of uptake of retinol in leukaemic cells.

After absorption of retinol by the intestinal enterocytes, retinol is transported in chylomicrons and their remnants mainly to the liver, the main retinol-storage organ [7,8]. Retinol-binding protein (RBP) is, under normal conditions, responsible for transport of retinol between the liver-storage and extrahepatic tissues [9]. However, when large doses of retinol are taken by mouth, a significant amount of retinol is transported in plasma as retinyl ester in low-density lipoproteins (LDLs) [10].

In the present study we have examined the uptake of retinyl ester via the LDL-receptor pathway in the human promyelocytic cell line HL-60. These cells are highly sensitive to differentiation-induction by retinoids [11].

MATERIALS AND METHODS

Chemicals

All-*trans*-retinoic acid and all-*trans*-retinol were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Garamycin was from Schering Corp. (Kenilworth, NJ, U.S.A.). Fetal-calf serum (FCS) and RPMI-1640 medium were obtained from Gibco Limited (Paisley, Renfrewshire, Scotland, U.K.). May-Grünwald- and Giemsa-stain solutions were from E. Merck (Darmstadt, Germany). Na^{125}I was purchased from New England Nuclear (Boston, MA, U.S.A.). [^{14}C]Oleic acid was from Amersham, and TLC-Ready Foils were from Schleissner und Scheull, Dassel, Germany. Mevinolin was kindly provided by Merck Sharp and Dohme, Rahway, NJ, U.S.A.

Cell-culture techniques

The HL-60 cell line was purchased from the American Type Culture Collection (A.T.C.C.) (Rockville, MD, U.S.A.) and cultivated in RPMI-1640 medium with 10% (v/v) heat-inactivated FCS (unless otherwise stated) and 0.1 mg of Garamycin/ml in a humidified atmosphere of CO_2/air (1:19) at 37 °C. Cells were cultured in plastic tissue-culture flasks and maintained with a seeding density of 4×10^5 cells/ml twice weekly. The cells were counted in a Bürkner chamber, and viability was assessed by the exclusion of 0.1% Trypan Blue. Cellular

Abbreviations used: LDL, low-density lipoprotein; ACAT, acyl-CoA: cholesterol acyltransferase; AML, acute myeloid leukaemia; RBP, retinol-binding protein; FCS, fetal-calf serum; NBT, Nitroblue Tetrazolium; LDS, lipoprotein-deficient serum; PBS, phosphate-buffered saline (0.02 M-phosphate/0.15 M-NaCl, pH 7.4).

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morphology was evaluated after staining with May-Grünwald Giemsa. Differentiation was assessed by the capacity of HL-60 cells to reduce Nitroblue Tetrazolium (NBT) [12].

Lipoprotein-deficient FCS (LDS)

The lipoprotein-deficient fraction of fetal-calf serum (LDS) was prepared by a single centrifugation of 214 000 g for 48 h at 4–10 °C after density adjustment to 1.215 g/ml with NaBr. The LDS was dialysed for at least 24 h at 4 °C against three changes of at least 50 vol. of phosphate-buffered saline (PBS), pH 7.4, containing 1 mM-EDTA.

Lipoproteins

Human LDL [density (ρ) 1.019–1.063 g/ml] was prepared from 100 ml of serum from a healthy donor as described by Havel *et al.* [13]. Briefly, LDL was prepared by ultracentrifugation (40 000 rev./min for 20 h) in a Sorvall ultracentrifuge (OTD 55B with a TFT 70.38 rotor) using solid NaBr for density adjustment. The isolated fraction was dialysed for at least 36 h at 4 °C against three changes of at least 50 vol. of PBS containing 1 mM-EDTA.

Radioiodination of LDL was done by the iodine monochloride (ICl) procedure, a method modified from that of McFarlane [14]. A portion (100 μ l) of an ICl solution (33 mM) was incubated with 20 μ l of Na¹²⁵I and 1 ml of LDL in a 0.4 M-glycine buffer, pH 10. A portion (1 ml) of the final solution contained 3–6 mg of LDL protein/ml. The labelled LDL was dialysed at least 10 times against 1 litre volumes of PBS at 4 °C.

Our LDL preparation was analysed by SDS/polyacrylamide-gel electrophoresis and shown to contain almost exclusively apolipoprotein B (M_r 500 000). Since each LDL particle contains only one apolipoprotein B molecule [24], we calculated the molar concentration from the protein determinations.

Chylomicrons were isolated from intestinal lymph collected from male Wistar rats as described by Drevon [15]. Remnants were prepared from the chylomicrons by incubation of 2 ml of lymph with 3 ml of 20% (w/v) fatty-acid-free albumin in PBS, pH 7.4, and 3 ml of post-heparin plasma. The post-heparin plasma was prepared from rats (200 g) which had received 30 units of heparin 10 min before isolation. During this incubation, 40–50% of the triacylglycerol was hydrolysed. After incubation, the chylomicron remnants ($\rho < 1.019$ g/ml) were isolated by ultracentrifugation [13], and dialysed against three changes of 50 vol. of PBS at 4 °C.

Chylomicron remnants or LDL were labelled with [³H]retinyl palmitate as described previously for [³H]-cholesteryl oleate [16]. Two portions of 50 μ l (0.42 μ mol) of [³H]retinyl palmitate in acetone were added to 0.5 ml of a human plasma fraction ($\rho > 1.25$ g/ml) containing the cholesteryl ester-transfer protein. After evaporation of the acetone with N₂, the solution was incubated at 37 °C for 10 min. Then 2.5 nmol of lipoprotein was added, and this mixture was incubated for 6–7 h at 37 °C. The lipoproteins were reisolated by ultracentrifugation and dialysed as described above.

Binding and uptake assays

Cells were grown as described above. At the start of the experiment the cells were washed three times in a serum-free RPMI-1640 medium and resuspended at

10⁷ cells per ml at 4 °C. Cell viability was assessed by Trypan Blue dye exclusion and found to be never less than 95%. The cells were incubated with labelled ligand with or without the addition of 50-fold molar excess of unlabelled LDL to determine specific binding. Specific binding is defined as total bound ligand minus non-specifically bound. After incubation at 4 °C, the cells were washed three times in ice-cold PBS, and bound LDL was assessed by the amount of cell-associated radioactivity. The same procedure as for the binding assays was followed in the uptake assays, except that the temperature was maintained at 37 °C during the experiments.

Assay for cholesteryl ester formation

Cells were incubated for 24 h with 10% (v/v) LDS. Thereafter, half of the cells were reincubated with the addition of 0.1 μ M-LDL for another 24 h. The other cells were allowed to continue in culture with 10% LDS only. At 48 h after the start of the experiments, 100 μ M [¹⁴C]-oleic acid was added to all tissue cultures [17]. The cells were then incubated with the radiolabelled oleic acid for 24 h. After having washed the cells 3 times with PBS, extraction was done with chloroform/methanol (2:1, v/v). Finally the extract was evaporated under N₂, redissolved in hexane (0.1 ml) and analysed on a TLC foil as described [17].

Chemical analysis

Protein was determined by the method of Lowry *et al.* [18], with bovine serum albumin as standard.

RESULTS

Binding of ¹²⁵I-LDL to HL-60 cells

The time course of binding of ¹²⁵I-LDL to HL-60 cells at 4 °C is presented in Fig. 1 as an average for three experiments. Binding was complete after 120 min and remained constant upon further incubation. Addition of a 30-fold molar excess of unlabelled LDL decreased the binding of ¹²⁵I-labelled LDL with an average of 43.6% (45.3, 53.3 and 32.3%) after 240 min, suggesting a saturable binding site for LDL. Fig. 1(b) shows the specific binding of ¹²⁵I-labelled LDL to HL-60 cells as the difference between ¹²⁵I-LDL bound in the absence and in the presence of 30-fold unlabelled LDL.

Competition for binding of ¹²⁵I-LDL by unlabelled LDL

We have tested the effect of increasing amounts of unlabelled LDL on the binding of ¹²⁵I-LDL to HL-60 cells. HL-60 cells were incubated with various concentrations of LDL at 4 °C for 2 h, and cell-associated radioactivity was determined. As seen from Fig. 2, the amount of radioactivity bound to HL-60 cells was decreased by adding to the incubation mixture increasing amounts of unlabelled LDL. Similar results were obtained in four experiments.

These results were further analysed by using the Scatchard equation [19], as shown in Fig. 3. The binding of LDL to its receptor has a dissociation constant of about 3.2×10^{-9} M, and the number of receptors per cell is about 2700.

Uptake of ¹²⁵I-LDL by HL-60 cells

Fig. 4(a) presents the average time course of uptake of ¹²⁵I-LDL at 37 °C by HL-60 cells in three experiments.

Fig. 1. Binding of ^{125}I -LDL to HL-60 cells

(a) The time course of binding of ^{125}I -LDL (8.0 nM) to HL-60 cells (10×10^6 cells/ml) was measured at 4 °C. Cells were incubated in the absence (○) or presence (●) of a 30-fold molar excess of unlabelled LDL. (b) Specific binding of ^{125}I -labelled LDL (■) is presented as the difference between ^{125}I -LDL bound in the absence and in the presence of a 30-fold molar excess of unlabelled LDL. The results are an average for three experiments. Binding of ^{125}I -LDL was determined as described in the Materials and methods section.

Fig. 2. Displacement of binding of radioactive LDL by increasing amounts of unlabelled LDL

HL-60 cells (10×10^6 cells/ml) were incubated with ^{125}I -LDL (0.7 nM) and various concentrations of unlabelled LDL at 4 °C for 2 h, and cell-associated radioactivity was determined. The Figure shows the results from one representative experiment.

Radioactive LDL was incubated with HL-60 cells in the absence or in the presence of 30-fold unlabelled LDL. During the first 1 h of incubation, increasing amounts of radioactivity were taken up by the cells. From 60–240 min, only a slight increase in cell-associated radio-

Fig. 3. Scatchard analysis of LDL binding to HL-60 cells

The results presented in Fig. 2 are plotted as described by Scatchard [19].

Fig. 4. Uptake of ^{125}I -LDL by HL-60 cells

(a) The time course of uptake of ^{125}I -LDL (7.1 nM) at 37 °C by HL-60 cells (1×10^7 cells/ml) is presented. Radioactive LDL was incubated with HL-60 cells in the absence (○) or in the presence (●) of a 30-fold molar excess of unlabelled LDL. (b) The specific amount of cell-associated LDL is presented as the difference between ^{125}I -LDL uptake in the absence and in the presence of a 30-fold molar excess of unlabelled LDL (■).

activity was observed. This is most likely due to recycling of occupied receptors to the cell surface. Upon addition of a 30-fold molar excess of unlabelled LDL, the average decrease in cell-associated radioactivity was 59.7% (54.3, 54.7 and 70.0%). The specific amount of cell-associated ^{125}I -LDL is shown in Fig 4(b). Only negligible trichloro-

acetic acid-soluble degradation products were detected during the 240 min incubation period.

Regulation of LDL receptors by mevinolin

To test whether the LDL receptors on HL-60 cells are subjected to metabolic regulation, we preincubated the cells with mevinolin ($1\ \mu\text{M}$) for 2 days. Mevinolin is an inhibitor of the rate-limiting enzyme in the synthesis of cholesterol, 3-hydroxy-3-methylglutaryl-CoA reductase [20]. The specific uptake of radiolabelled LDL was then determined in mevinolin-treated cells and in control cells. At all timepoints studied, the uptake in mevinolin treated cells was increased about 2-fold. After 240 min, mevinolin-treated cells accumulated, in an average of three experiments, 240% (170, 310 and 190%) the amount of radioactivity accumulated by control cells, suggesting an up regulation of LDL receptors by mevinolin.

Acyl-CoA:cholesterol acyltransferase (ACAT) activity in HL-60 cells

Another, more indirect, method to measure uptake via LDL receptors, is to measure the activity of the enzyme responsible for esterification of cholesterol with long-chain fatty acids, ACAT. It was demonstrated by Goldstein *et al.* [21] that uptake of excess cholesterol via the LDL receptor will activate ACAT.

After preincubation with LDL, the average increase in ACAT activity was 430% (280, 340, 450 and 650%), compared with the controls, in four experiments. These results show that exogenous LDL cholesterol increases ACAT activity in HL-60 cells, supporting the evidence of receptors for LDL on these cells.

Does induction of differentiation by retinoids affect the uptake of LDL?

The expression of a number of cell-surface proteins is changed when HL-60 cells are induced to differentiate by retinoids [22]. When cells were preincubated with $3.0\ \mu\text{M}$ -retinol or $1\ \mu\text{M}$ -retinoic acid for 5 days, about 30 and 70% respectively of the cells differentiated to more mature cells, as judged by their ability to reduce NBT. The specific uptake of LDL at 37°C was then assessed in these cells. This experiment showed that induction of differentiation by retinoids does not affect the specific uptake of LDL by HL-60 cells.

Uptake of [^3H]retinyl palmitate-labelled LDL

We then wanted to test to what extent growth-inhibiting factors such as retinoids could be taken up by leukaemic cells via the LDL receptor. Radioactive retinyl palmitate was incorporated into LDL as described in the Materials and methods section. The cells were incubated at 37°C with LDL labelled with [^3H]retinyl ester in the absence or in the presence of a 15-fold molar excess of unlabelled LDL. As shown in Fig. 5, [^3H]retinyl palmitate-labelled LDL was efficiently taken up by the HL-60 cells (average of three experiments). Furthermore, after 180 min, the average uptake was decreased 61.7% (81.0, 56.2 and 48.0%) in the presence of excess LDL, indicating uptake of retinyl palmitate via the LDL receptor.

Uptake of [^3H]retinyl palmitate-labelled chylomicron remnants via the LDL receptor

Newly absorbed retinol is transported from the

Fig. 5. Uptake of [^3H]retinyl palmitate-labelled LDL

HL-60 cells (1×10^7 cells/ml) were incubated at 37°C with LDL (144 nM) labelled with [^3H]retinyl ester (10^5 c.p.m./mg of LDL protein) in the absence (●) or in the presence (○) of a 15-fold molar excess of unlabelled LDL. Cell-associated radioactivity was assessed after different periods of time. The Figure represents the mean for three experiments.

Fig. 6. Uptake of [^3H]retinyl palmitate-labelled chylomicron remnants

HL-60 cells (1×10^7 cells/ml) were incubated at 37°C with chylomicron remnants ($74\ \mu\text{g}/\text{ml}$) labelled with [^3H]retinyl ester (5.8×10^6 c.p.m./mg of protein) in the absence (●) or in the presence (○) of an excess of unlabelled LDL ($1.9\ \mu\text{M}$). Cell-associated radioactivity was assessed after different periods of time. The Figure represents the mean result for three experiments.

intestine to the liver in association with chylomicrons and their remnants. It is shown in, e.g., fibroblasts that chylomicron remnants may be taken up by the LDL receptor [23]. We therefore tested the ability of HL-60 cells to take up [^3H]retinyl palmitate-labelled chylomicron remnants.

HL-60 cells were incubated with radioactive

chylomicron remnants in the absence or in the presence of excess unlabelled LDL ($1.9 \mu\text{M}$). As shown in Fig. 6, the uptake of radioactive chylomicron remnants was comparable with the uptake of retinyl ester-labelled LDL (Fig. 5). The average uptake in three experiments was diminished 66.9% (62.9, 69.9 and 68.0%) when an excess of unlabelled LDL was added. The results indicate that retinoids also may enter the promyelocytic leukaemia cells via the LDL receptor as a part of the chylomicron remnants.

DISCUSSION

In the present study we showed that the human promyelocytic-leukaemic-cell line HL-60 contains saturable, high-affinity, binding sites for LDL. Furthermore, we have demonstrated that retinyl ester in LDL or chylomicron remnants may enter the leukaemic cells through the LDL-receptor pathway.

Mevinolin is a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase [20], the rate-limiting enzyme in cholesterol synthesis. This fungal metabolite leads to increased numbers of LDL receptors on fibroblasts and thereby to an increased uptake of LDL [24]. Our results show that preincubation of HL-60 cells for 2 days in the presence of mevinolin increases the specific uptake of LDL 2-fold. This mevinolin-mediated elevation of LDL receptors may be of importance in increasing the uptake of growth-inhibiting agents, like retinol, in leukaemic cells.

Cells cultured in LDS synthesize sufficient cholesterol for growth, but do not synthesize excess cholesterol. When cells have taken up LDL by the receptor route, cholesterol liberated from LDL activates ACAT. The ability to stimulate this reaction is dependent on its ability to enter cells via receptor-mediated endocytosis [21]. Our results show that LDL cholesterol leads to increased ACAT activity, supporting the existence of LDL receptors on HL-60 cells.

Ho *et al.* [25] found saturation curves for freshly isolated leukaemic cells similar to the saturation curves for receptor-mediated ^{125}I -LDL uptake and degradation in human fibroblasts. The rate of uptake, however, was found to be 3–100-fold higher in blood mononuclear cells from patients with acute myeloid leukaemia compared with healthy subjects. Later, Vitols *et al.* [26] performed a more extensive study on receptor activity in leukaemic cells. The highest number of receptors was found in leukaemic cells with features of monocytes. Two of the patients in their study had a promyelocytic leukaemia (FAB M3), and their cells had relatively low degradation rates. The HL-60 cell line was originally isolated from a patient with promyelocytic leukaemia [12]. Thus, HL-60 cells resemble the M3 according to the FAB classification and have relatively few receptors for LDL compared with other myeloid leukaemic cells.

Florén *et al.* [23] reported that chylomicron remnants, like LDL, were internalized by skin fibroblasts via the LDL pathway. This may occur because chylomicrons contain apolipoproteins E, which are recognized by the LDL receptors, as has been shown by Hui *et al.* [27]. Wilson *et al.* [10], and later Berr & Kern [28], showed that retinyl ester mainly appears in chylomicrons and their remnants 5–6 h after ingestion of retinol. However, they also found a small, but significant, increase in LDL

retinyl ester concentration at 6 and 12 h after feeding. We have found that retinyl ester in chylomicron remnants and LDL are both taken up via the LDL receptor in HL-60 cells.

The children in the Norwegian AML study [6] were given $52 \mu\text{mol}$ (50000 i.u.) of retinol (as retinyl palmitate in oil)/day per m^2 . This protocol has improved the prognosis for these children. We suggest that this is due to the retinol medication. The present study shows that retinol may enter the leukaemic cells in chylomicron remnants or in LDL. In a parallel study [29] we have shown that retinol taken up via the LDL-receptor pathway leads to decreased proliferation and induction of differentiation in myeloid leukaemic cells.

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