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SHORT COMMUNICATION

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Comparison of the uptake of retinoids 13-cis-retinoic acid and Ro 13-6298 delivered to HL-60 cells by serum albumin or low-density lipoprotein

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Abstract Retinoids, a class of polyisoprenoids including retinol and retinoic acid, regulate and control diverse physiological functions via their cell-differentiating and morphogenic potential. In the present study we showed that the extracellular concentration of retinoid-binding proteins such as albumin limits the amount of retinoid entering the human promyelocytic leukemia cell line HL-60. These cells accumulate 5–10 times more retinoid when delivered free in solution than when bound to either albumin or low-density lipoprotein (LDL). Moreover, the effect of protein binding is concentration-dependent, with a higher concentration of binding protein corresponding to a lower level of cellular uptake. Furthermore, the uptake of the ester derivative is higher than that of the acidic retinoid. These observations suggest that (a) the cellular uptake of both retinoids occurs via the free form of the ligand in solution, with the free concentration of ligand decreasing as the carrier-protein concentration increases, and (b) according to a passive mechanism, the ester derivative, unionized and lipophilic, enters the cells more easily than does the acidic derivative.

Key words Retinoids · Protein binding · HL-60 cells · Uptake

Introduction

The retinoids, a class of polyisoprenoids including retinol and retinoic acid, regulate and control diverse physiological functions via their cell-differentiating and morphogenic potential. Recent reports have shown the interest of retinoids as anticancer agents, with remission induction being observed in patients with acute promyelocytic leukemia and high response rates being reported for patients with cervical cancer and metastatic squamous skin cancer [7].

Synthetic retinoid analogues are mainly acidic derivatives or the corresponding esters. Because these compounds are practically water-insoluble, plasma proteins act as carriers in the bloodstream. The plasma proteins involved are mainly albumin for the acidic derivatives and lipoproteins for the neutral or ester derivatives [8]. In a series of retinoids we have distinguished two retinoid derivatives that differ in their distribution on plasma proteins: Ro 4-3780, or 13-cis-retinoic acid, and Ro 13-6298, or ethyl *p*-[(*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8,-tetramethyl-2-naphthyl)-1-propenyl]benzoat (Fig. 1). The association constants to albumin were 122,100 M^{-1} for 13-cis-retinoic acid versus 5,280 M^{-1} for Ro 13-6298, and the association constants to low-density lipoproteins (LDL) were 6.14×10^6 M^{-1} for 13-cis-retinoic acid versus 147×10^6 M^{-1} for Ro 13-6298 [9]. Accordingly, 13-cis-retinoic acid was mainly albumin-

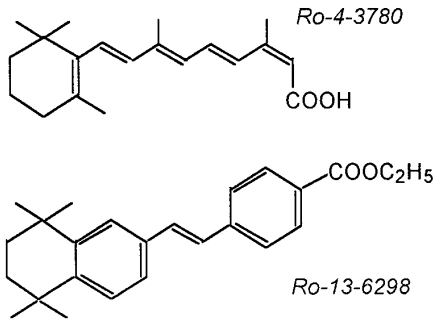

Fig. 1 Chemical structures of 13-cis-retinoic acid and Ro 13-6298

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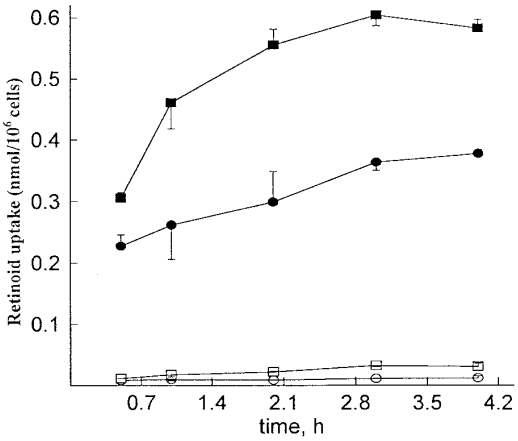

Fig. 2 Time course of 13-cis-retinoic acid (*black circles*) and Ro 13-6298 (*black squares*) uptake by HL-60 cells. Results are expressed in nmol/10^6 cells as mean values ± SD for 6 experiments. Cells were incubated at 37 °C in the absence (*black circles*) or presence (*white circles*) of 300 µ*M* albumin for 13-cis-retinoic acid or in the absence (*black squares*) or presence (*white squares*) of 33 µ*M* LDL for Ro 13-6298. The initial concentration of retinoid was 4 µ*M* for 13-cis-retinoic acid and 2.8 µ*M* for Ro 13-6298

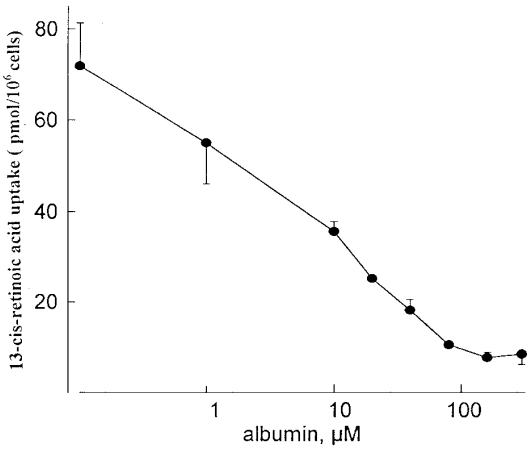

Fig. 3 Uptake of 13-cis-retinoic acid by HL-60 cells as a function of albumin concentration. Results are expressed in pmol/10^6 cells as mean values ± SD for 6 experiments. Cells were incubated for 2 h at 37 °C in the presence of increasing concentrations of albumin. The initial concentration of 13-cis-retinoic acid was 1.25 µ*M* for each albumin concentration

bound in plasma, i.e., to 80% (total plasma binding 99%), whereas Ro 13-6298 was essentially lipoprotein-bound, with the fraction bound to LDL amounting to 70% (total plasma binding 99.6%). Thus, in the present study we investigated the effect of the main carrier protein, of 13-cis-retinoic acid, albumin, and that of LDL, the carrier of Ro 13-6298, on their uptake by HL-60 cells.

Materials and methods

Materials

All chemicals were of analytical grade. Labeled [^{14}C]-Ro 13-6298 (54.97 Ci/mol, 99.1% pure) and [^{14}C]-13-cis-retinoic acid (28.38 Ci/mol, 98.8% pure) were supplied by Roche (Basel, Switzerland). Albumin (A1887) was purchased from Sigma (France). The LDLs were isolated by sequential ultracentrifugal flotation of serum at increasing density [3]. The isolated lipoprotein fraction was dialyzed overnight against buffer (ph 7.4; 120 m*M* NaCl, 5 m*M* KCl, 2.5 m*M* $CaCl_2$, 1*mM* $MgSO_4$, 20 m*M* TRIS) to remove excess salt and then diluted to the desired concentration with buffer. The molecular weight of the LDL was assumed to be 3×10^6 Da (1 µ*M* = 3 mg/ml).

Cell culture

Promyelocytic leukemia cell (HL-60) was purchased from the American Tissue Culture Collection (ATCC 240, Rockville, Md., USA). This cell line was maintained in RPMI-1640 containing no glucose (Gibco, BRL, France) but supplemented with 10% heat-inactivated fetal calf serum, 100 U penicillin, and 50 µg streptomycin. Cells were grown at 5×10^5 cells/ml in 150-cm^2 flasks (Costar, France) and were maintained and incubated at 37 °C in a humidified incubator containing 5% CO_2 in the exponential phase of growth by subculturing every 4 days. Cells were counted with a hemocytometer, and viability was assessed by trypan blue exclusion.

Uptake assay

Cultured cells were washed by three cycles at 500 *g* for 5 min in serum-free RPMI medium and resuspended at 10^6 cells/ml. The cells were incubated for 3 h at 37 °C with labeled ligand in the presence or absence of different concentrations of albumin or LDL. The ligand concentration ratio was the same for all of the protein concentrations, and the 13-cis-retinoic acid and Ro 13-6298 concentrations were 1.25 µ*M*. After incubation, cells were centrifuged at 500 *g*/min and washed twice with ice-cold buffer and ligand uptake was assessed by the amount of cell-associated radioactivity. Each experiment was repeated five to six times and results were expressed as mean values ± SD.

Results

Time course of retinoid uptake by HL-60 cells

The levels of cellular uptake of 13-cis-retinoic acid and Ro 13-6280 are depicted in Fig. 2. The uptake was studied with or without the relevant binding protein: 300 µ*M* albumin for 13-cis-retinoic acid (4.1 µ*M*) or 0.33 µ*M* LDL for Ro 13-6280 (2.8 µ*M*). For both retinoids the extracellular binding to either albumin or LDL dramatically decreased the cellular uptake. The uptake of 13-cis-retinoic acid, free or albumin-bound, and of LDL-bound Ro 13-6298 appeared to reach a plateau after approximately 2 h, whereas the uptake of free Ro 13-6298 was more progressive, reaching peak levels at 3 h. A comparison of the uptake of free retinoids (delivered without protein) revealed that the cellular uptake of the ester Ro 13-6298 was higher than that of the acidic 13-cis-retinoic acid, although the concentration gradient (initial concentration of retinoid) was greater for 13-cis-retinoic acid.

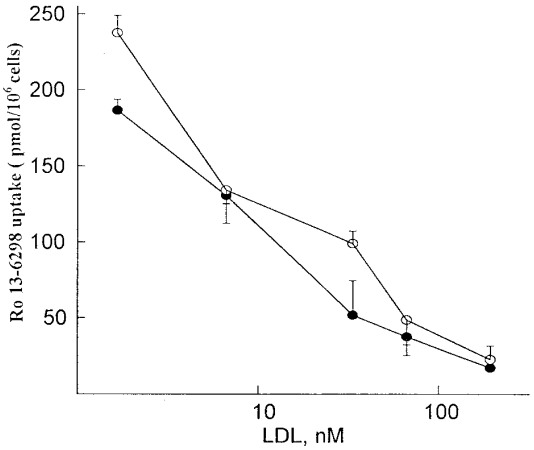

Fig. 4 Uptake of Ro 13-6298 by HL-60 cells as a function of LDL concentration. Results are expressed in pmol/10^6 cells as mean values ± SD for 6 experiments. Cells were incubated for 2 h at 37 °C in the presence of increasing concentrations of LDL. The initial concentration of 13-cis-retinoic acid was 1.25 μ*M* for each LDL concentration (*white circles* Mevinolin-treated cells with increased numbers of LDL membrane receptors)

Effect of albumin on HL-60 uptake of 13-cis-retinoic acid

Albumin concentrations varied from 0.1 to 300 μ*M*, resulting in 13-cis-retinoic acid binding of 2%, 11%, 55%, 71%, 83%, 91%, 95%, and 98.5%. As shown in Fig. 3, increasing amounts of albumin continuously decreased the cellular uptake of 13-cis-retinoic acid, indicating that this uptake was decreased because of 13-cis-retinoic acid albumin binding.

Effect of LDL on HL-60 uptake of Ro 13-6298

We tested the effect of increasing amounts of LDL on the uptake of Ro 13-6298 by two HL-60 populations, HL-60 cells grown as described above or HL-60 cells preincubated 48 h before the experiment with mevinolin (2 μ*M*), a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (CoA) reductase, to increase the number of LDL receptors. LDL concentrations varied from 1.66 to 200 n*M*, resulting in Ro 13-6298 binding of 20%, 50%, 83%, 91%, and 97%. As shown in Fig. 4, there was a continuous decrease in Ro 13-6298 uptake when the LDL concentration was increased, indicating that LDL binding of Ro 13-6298 decreased its availability. The uptake seen in HL-60 mevinolin-treated cells was slightly higher than that observed in the cells grown under standard conditions. This difference was marginally significant ($P = 0.06$).

Discussion

After their absorption from the gastrointestinal tract, retinoids are transported by plasma proteins from their site of resorption to the sites of action. Because of their lipophilicity, retinoid esters are mainly lipoprotein-bound, whereas acidic derivatives are mainly albumin-bound [8, 9]. In the present study we showed that the extracellular concentration of retinoid-binding proteins such as albumin limits the amount of retinoid entering the human promyelocytic leukemia cell line HL-60. We thought that this cell line would be particularly well suited for this study because it is the prototype cell line for retinoic acid-induced differentiation of myeloid leukemia cells [4].

The results of this study demonstrate the following points. First, HL-60 cells accumulate 5–10 times more retinoid when delivered free in solution than when bound to either albumin or LDL as shown by the kinetics experiments. Second, the effect of protein binding is concentration-dependent, with a higher concentration of binding protein corresponding to a lower level of cellular uptake. Third, the uptake of the ester derivative is higher than that of the acidic retinoid. These observations suggest that (a) the cellular uptake of both retinoids occurs via the free form of the ligand in solution, with the free concentration of ligand decreasing as the carrier-protein concentration increases, and (b) according to a passive mechanism, the ester derivative, unionized and lipophilic, enters the cells more easily than does the acidic derivative. A similar conclusion was reached in in vitro studies on the effect of the retinol-binding protein on the delivery of retinol to keratinocytes [2].

As previously reported, the incubation of HL-60 cells in the presence of a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase for 48 h leads to an increased number of LDL receptors on the cells and, hence, to an enhanced uptake (2-fold increase) of LDL [10]. Because the uptake of LDL-bound ligands is thought to occur via the LDL-receptor pathway [5], an increase in the number of LDL membrane receptors should result in an increase in the delivery of LDL-bound ligand. In the present study we observed only a slight increase in the cellular uptake of Ro 13-6298, indicating that the ligand delivery via the LDL-receptor pathway is low as compared with the passive diffusion of the free ligand. Similarly, it was observed that the uptake of etretinate by cultured human fibroblasts via LDL delivery was essentially due to a passive exchange with the plasma membrane [1]. In that experiment and the present study the interaction between the lipoprotein and the ligand was spontaneous and fully reversible. In contrast, experiments dealing with incorporation of ligands in the lipoprotein structure using retinyl ester [10] or *N*-trifluoroacetyladriamycin-14-valerate [5] or ellipticine ester derivatives [6] have shown that the cellular uptake of lipoprotein-ligand complex occurs via the LDL-receptor pathway.

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