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Content of Dolichol and Retinol in Isolated Rat Non-Parenchymal Liver Cells

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The liver sinusoids, that are considered as a functional unit, harbour four types of sinusoidal cells (Ito, Kupffer, endothelial and pit cells). Dolichol content has been determined in many tissues and subcellular compartments, alteration has been reported in many types of liver injury, but until now no data are available on its content in every type of sinusoidal non-parenchymal liver cells. Dolichol and retinol metabolism might intersect in their traffic in biological membranes. Intercellular as well as intracellular exchange of retinoids is an essential element of important processes occurring in liver cells. It has been suggested that the role of dolichol, besides being a carrier of oligosaccharides in the biosynthesis of *N*-linked glycoproteins, may be to modify membrane fluidity and permeability, and facilitate fusion of membranes. Dolichol in the membrane is intercalated between the two halves of the phospholipid bilayer, but its exact disposition is not known and the movement and distribution of retinoid in membranes may vary with the geometry of the membranes. Therefore the aim of this study is to obtain a global understanding of the sinusoidal system regarding dolichol and retinol content in each type of isolated rat liver sinusoidal cell, in normal conditions and after vitamin A administration. The information that can be drawn from the present results is that with normal vitamin A status of the animal, the dolichol content is almost uniform in all liver cells. After vitamin A supplementation, a great increase of dolichol, together with the known increase of retinol, can be measured only in a subpopulation of the Ito cells, the Ito-1 subfraction. Therefore in the cells that are present in the hepatic sinusoid, different pools of dolichol may have separate functions. Because retinol traffic among cells, membranes and plasma still remains to be fully understood, roles of dolichol in the exchange of vitamin A among sinusoidal liver cells are discussed.

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KEY WORDS — dolichol; liver non-parenchymal cells; stellate liver cells; vitamin A

ABBREVIATIONS — HCs, hepatocytes; KCs, Kupffer cells; NPLCs, non-parenchymal liver cells; SECs, sinusoidal endothelial cells

INTRODUCTION

Dolichols, synthesized in the endoplasmic reticulum and, in part, in peroxisomes, are α-saturated polyisoprenoid alcohols widely distributed within cells in phosphorylated form, or as fatty acid esters or as free alcohol.[1,2] The role of dolichol, besides being a carrier of oligosaccharides in the biosynthesis of *N*-linked glycoproteins may be by way of its long isoprenoid chain, to modify membrane fluidity, permeability and fusion.[3–6]

The liver sinusoids harbour four types of sinusoidal cells: endothelial (SECs), Kupffer (KCs), Ito cells (perisinusoidal, stellate, or fat storing cells) and pit cells. The parenchymal cells account for about 90 per cent of the liver mass and represent about 65 per cent of the cell number. The SECs, KCs and the Ito cells represent about 60, 30, and 10 per cent respectively of the non-parenchymal liver cells (NPLCs); pit cells are less than 1 per cent in number.[7,8] Pit cells are large granular lymphocytes, that have only recently been described and represent a liver-associated natural killer population (NK), that differ from peripheral blood NK cells.[9] Pit cells constitute a very small proportion of sinusoidal cells and will not be

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considered in the present work. SECs (endothelial cells), situated between the space of Disse and the sinusoidal lumen, act as a semi-accessible barrier with their typical fenestrae arranged in 'sieve plates'. Their fenestrae play a role in the exchange of fluid, lipoproteins and solutes between blood and hepatocytes. The diameter and number of fenestrae, that have rings of cytoskeletal proteins and measure approximately 150–175 nm, can be influenced by a variety of agents.[10] SECs have been reported to have increasingly diverse functions: endocytosis, a scavenger function in clearing hyaluronan, denaturating collagen and procollagen from blood, efficient endocytosis of lipoproteins particles;[7] a wide variety of protein production[11] and prostanoid secretion has recently been reported by Hashimoto *et al.*[12]

Kupffer cells (KCs), represent the largest population of macrophages in the body, constituting up to 90 per cent of mononuclear phagocyte systems in direct contact with the blood stream. Several functions have been reported for these cells, including endocytosis, antigen processing, elimination of circulating IgG–immune complexes, secretion of cytokines and cytotoxicity. Recently a role of Kupffer cells in the differentiation of the hepatic NK was reported.[11,13]

Ito cells, located in the space of Disse, are a heterogeneous class of cells, that with their dendritic processes extend either over the parenchymal cells or around the endothelial cells. A characteristic of these cells is the presence of lipid droplets filled with vitamin A in their cytoplasm. These cells have two main functions: the storage of vitamin A in the lipid droplets and fibrogenesis.[14,15] As pericytes, they influence also the sinusoidal blood flow.[16] Two subfractions of Ito cells (Ito-1 and Ito-2) have been recently isolated.[17,18]

Retinol traffic between cells, membranes and plasma still remains to be fully elucidated.[19] Intercellular as well as intracellular traffic of retinoid is an essential element of very important processes occurring in liver cells. A role of dolichol in the exchange of vitamin A between sinusoidal liver cells has been suggested.[18]

Because the liver sinusoids can be regarded as a functional unit,[20] to obtain a global understanding of the system, it is necessary firstly to acquire knowledge about all the individual cells of the sinusoids. Dolichol content has been determined in many tissues and subcellular compartments. Alteration has been reported in many types of liver injury, e.g. after ethanol, drugs, toxins or pesticides[1,21–23] but until now no data are available on its content in all the non-parenchymal liver cells, a prerequisite for these studies.

Therefore we have undertaken a systematic study of dolichol and retinol content in isolated rat liver sinusoidal cells, in normal conditions and after vitamin A administration. Part of these data has recently been reported:[18] in this note the content of dolichol in the other sinusoidal cells, not reported in the literature, is included.

MATERIALS AND METHODS

Wistar male rats, weighing approximately 350–400 g were used (Morini, Italy, 7–15 days of acclimatization). The animals were fed a standard diet in pellets (Italiana Mangimi), and allowed free access to water, in an environment at constant temperature (22°C) and humidity (55 per cent), light and dark cycle of 12 h. The official Italian regulation N. 116/92 for the care and use of laboratory animals was followed.

One lot of rats was given during the 3 days before killing, by oral gavage, retinyl palmitate (Sigma), emulsified in 1 ml of tocopherol-stripped corn-oil (ICN Biochemicals), for a total of 400,000 IU per rat. Another pair-fed control group received an equal amount of corn oil at the same time by gavage.

Cell Isolation Procedures

The liver of rats, under pentothal sodium (Abbott) anaesthesia (50 mg kg^{-1}, i.p.), was linearly perfused *in situ*, following essentially the method of Berry and Friend.[24] The vena porta was cannulated and first the liver was preperfused with a medium containing 0·2 μM EDTA, without collagenase to remove all blood from the tissue (500 ml). Subsequently, a linear perfusion was performed for 15–20 min with perfusion medium containing 0·5 per cent collagenase (type IV, Sigma, EC 3.4.24.3) and 1 mM $CaCl_2$, at 37°C (200 ml). The paste-like liver was incubated in 30 ml of medium containing collagenase, by gentle shaking, for 20 min. Then it was filtered through nylon gauze (100 μm) to discard undigested tissue. The total liver cells (TOTCs) were suspended in a volume of 70 ml of Hanks' solution with 5 mM glucose and 5 mM L-methionine and parenchymal cells were harvested from the sinusoidal cells by sedimentation at 50 *g* for 25 s at 4°C. The sediment of hepatocytes was washed once. The supernatant

was centrifuged at 600 *g* for 4 min at 4°C and the pellet of NPLCs was washed repeatedly in the same buffer. The pellets of NPLCs were resuspended in 5 ml of buffer and layered on the top of a density-gradient of 1·5 ml each of 16, 12, 8 and 6 per cent arabinogalactan (Larcoll, Sigma, L0650) in buffer. This gradient was spun for 25 min at 50,000 *g* in a Spinco SW 27 rotor at 20°C, without the acceleration and deceleration program. Four layers were obtained. The layer of cells at the interface between the buffer at the top of gradient and the 6 per cent Larcoll layer contains vitamin A-repleted fat-storing cells (called Ito-1 fraction). The top of the 8 per cent layer contains vitamin A-poor fat-storing cells (called Ito-2 fraction). Ito cell identification was done as previously described.[18] The tops of the 12 and 16 per cent layers are enriched with KCs and SECs respectively. Separately they were gently centrifuged to remove the Larcoll. The purity of KCs and SECs was further enhanced by selective adherence. The pellets were resuspended in 1 ml of D-MEM (Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham, Sigma, D-8900) and seeded on two 35 mm diameter uncoated plastic tissue culture dishes (Corning 35, NY) and maintained under an atmosphere of 5 per cent CO_2 in air at 37°C for 40 min. KCs selectively adhered and formed a monolayer, while on such surfaces SECs do not attach. The monolayer of KCs adherent to dishes was removed with a pipette and washed before further analyses. The purity of KCs was established by immunocytochemistry by using two monoclonal mouse anti-human macrophages, CD68, clone KP1 and clone PG-M1 (DAKO LTD, UK). The KCs were more than 90 per cent pure, except for some SECs and cell debris.

The non-adherent cells containing SECs, removed by gentle washing, were further incubated with 0·4 per cent pronase, (w/v) (*Streptomyces griseus*, Sigma type XIV, E.C. 3,4,24.31) at 37°C for 60 min, with gentle shaking, to remove parenchymal cell debris: purified SECs were pelleted and washed.[25] Purity of SECs was assessed by demonstrating negative staining for KCs: SECs were around 70 per cent pure, major contaminants were KCs and unidentifiable cells.

Retinol and Dolichol Analysis

These analyses were carried out as already described:[18] in brief, to aliquots of 0·5 ml of liver cell preparations resuspended in water, were added 0·5 ml of 0·25 per cent pyrogallol in ethanol and 0·25 ml of 60 per cent KOH. After saponification for 45 min at 85°C, the cooled samples were extracted three times with seven volumes of ethanol/*n*-hexane (2/5 v/v). The pooled extracts were washed twice with equal volumes of 5 per cent acetic acid and dried under nitrogen. Each extract was dissolved in 1·0 ml of methanol and analysed by HPLC (Waters Model 510). All steps were done in the dark as much as possible. The analytical column used for HPLC was a Waters μ-Bondapak C18, 300 mm × 3·9 mm, with a precolumn insert Guard-Pak μBondapak C18. Detection was carried out with a Waters Model 991 detector.

For retinol analysis, 100 μl of extract was injected onto the column and separations were achieved by isocratic elution using methanol–water (95:5, v/v) as a mobile phase at a flow rate of 1·0 ml min^{-1} and the effluent was monitored at 325 nm over 20 min. All runs were performed at ambient temperature. Retinol was identified by comparison of retention time with that of a standard (from 1 to 4 μg (10 μl)$^{-1}$ all trans retinol, Sigma) injected separately onto the same column.

For dolichol analysis, 800 μl of extract was injected onto the column and the separation was achieved using as mobile phase methanol/propan-2-ol containing 20 mM ortho-phosphoric acid (all HPLC grade, BDH). In a first phase of chromatography, methanol/propan-2-ol (98:2) was used for a 6 min period. Then the switch setting was changed, and a linear gradient was run for another 2 min to attain methanol/propan-2-ol (45:55); thereafter the conditions were maintained for another 30–32 min at this value throughout the analysis. The low-rate was 1·5 ml min^{-1} and the effluent was monitored at 206 nm, according to Adair and Keller.[26]

The peak areas of sample (isoprenologs 16–21) were calculated by an automatic Waters integration program and the concentration in the sample calculated using standards of dolichol (2 μg (10 μl)$^{-1}$, C80–C105 from porcine liver, Sigma D4511). Data are expressed as μg for retinol and ng of dolichol per mg of protein. Protein was determined by the method of Hartree[27] with bovine serum albumin as standard.

Statistical Analysis

Values shown in Table 1 represent means ± SD. All groups of data were evaluated by analysis of variance (ANOVA). A difference was considered significant at $P < 0{\cdot}001$.

Table 1. Content of total dolichol and retinol in rat liver cells

	Dolichol		Retinol	
	At normal vitamin A status	After vitamin A supplementation	At normal vitamin A status	After vitamin A supplementation
ITO-1	209 ± 12	711 ± 30*	12·1 ± 2·6	175·8 ± 30·8*
ITO-2	193 ± 4	197 ± 10	4·6 ± 0·1	69·2 ± 11·8*
KCs	211 ± 24	182 ± 26	4·5 ± 1·0	13·6 ± 2·1
SECs	226 ± 39	153 ± 24	4·2 ± 1·0	13·0 ± 3·8
HCs	221 ± 22	184 ± 19	1·0 ± 0·1	5·3 ± 1·1†
TOT Cs	188 ± 21	135 ± 20	1·4 ± 0·3	8·5 ± 0·8†

Values expressed as ng mg^{-1} protein for dolichol and μg mg^{-1} protein for retinol ±SD.
* $P < 0{\cdot}001$ compared with all the other values.
† $P < 0{\cdot}001$ compared with the values obtained without vitamin A supplementation.

Unless otherwise indicated, all experiments were repeated at least three times, in duplicate.

RESULTS AND DISCUSSION

Data for dolichol and vitamin A content in NPLCs and in HCs before and after vitamin A administration are reported in Table 1.

Without vitamin A supplementation to the rats, the total dolichol contents in the liver cell populations seem to have homogeneous behaviour. Besides HCs and Ito cells, SECs and KCs also have a measurable content of retinol, in agreement with Wake[28] who reported about 4 per cent of total vitamin A in KCs. Similar data are reported by Geerts and Wisse[29] that give 75 per cent in Ito cells, 21 per cent in parenchymal cells, 3 per cent in SECs and 1 per cent in KCs, but differ from the much lower values of Blomhoff[30] and Blaner *et al.*[31] Phagocytic activity of KCs depends on the liver perfusion: during the isolation procedure an increase of activity with an accumulation of retinol may occur. A comparison with the retinol contents that are reported in the literature is nevertheless difficult to make. First of all, in other papers the content of retinoids are not reported for all cell types. Other differences are the different number of cells of each type, as well as differences in the size of the cells, and differences in the diets. In fact the amount and distribution of retinoids in the liver cells depends on the vitamin A status of the animal and on the function of liver with respect to retinol metabolism, while plasma levels generally tend to remain relatively constant.[32] Nevertheless our data are in the same range as many of the available data.

After vitamin A supplementation, the content of vitamin A increases in all cells. The increase in hepatocytes is in agreement with the uptake of retinoids from chylomicron remnants, through the apo-B receptor. Retinol is then transferred to the Ito cells for holding.[19] The great increase in Ito cells is in agreement with their role: at low reserves of retinol, the major fraction of retinol is in the mass of the hepatocytes, but during vitamin A overload Ito cells function as depots. In this situation, the increase of retinol in SECs and KCs also is not surprising, because both types of cells have receptors for lipoproteins, that transport carotenoids in plasma. Their role in retinol storage or metabolism however is a matter of controversy.[7,31] Worthy of note is the behaviour of dolichol after vitamin A supplementation, that once more confirms Ito cells heterogeneity. In fact only in Ito-1 subfraction, the main storage depot of vitamin A, does dolichol increase with the increase of vitamin A. In the other types of cells the dolichol content does not change significantly after vitamin A supplementation.

In Table 2 the percentage chain length distribution of the dolichols in the different liver cells is reported. The distribution of the isoprene residues is in agreement with the species specificity of the dolichols. In rat 18 and 19 residues appear as major derivatives. The increase after vitamin A supplementation of Dol-18 and Dol-19 with a parallel decrease of Dol-17 observed in all cell types, but especially in KCs, might mean a protective role of retinol on isoprenoid chain catabolism or peroxidation, in agreement with the production of oxygen free radicals by KCs.

Vitamin A and dolichol share, besides their similar hydrophobic characteristics, many metabolic pathways and functions. In plasma they are transported by lipoprotein; after entering HCs they are mainly cytoplasmic.[19,33,34] Both of them in the liver are present chiefly as esters. Both are involved in the large number of covalent post-transitional modifications of proteins that control their

Table 2. Isoprenologue percentage distribution of dolichols in the liver cells

	Dol-16	Dol-17	Dol-18	Dol-19	Dol-20	Dol-21
At normal vitamin A status						
ITO-1 cells	4·8	12·1	39·2	24·1	14·4	5·2
ITO-2 cells	2·7	16·7	40·0	26·8	10·1	3·6
KCs	4·2	19·8	36·2	25·5	10·1	3·7
SECs	5·2	19·4	37·7	26·7	8·2	2·7
HCs	5·1	13·8	36·6	30·7	10·5	2·8
TOT Cs	4·8	14·5	36·6	30·6	10·6	3·0
After vitamin A supplementation						
ITO-1 cells	3·4	15·4	43·9	25·2	8·8	3·2
ITO-2 cells	4·1	10·0	46·4	26·0	10·2	3·3
KCs	1·0	12·5*	42·2*	33·8*	8·0	3·0
SECs	1·7	11·7*	44·2*	30·2*	10·1	2·1
HCs	4·6	13·3	35·7	31·3	10·6	3·6
TOT Cs	3·6	13·9	37·7	31·4	10·4	2·8

* $P < 0{\cdot}001$ compared with the values obtained without vitamin A.

activities, such as glycosyl-transfer reactions. Retinol, besides retinoylation of proteins,[35] causes phosphorylation and dephosphorylation in nuclear proteins and histones, altering nucleosome conformation and chromatin activity. Dolichol, on the other hand, may be involved in isoprenylation of Ras proteins, that play a central role in the regulation of signals involved in the cell cycle.[36] A role in apoptosis has been reported independently for retinoic acid and dolichol.[37,38]

More interestingly, dolichol and retinol metabolism might intersect in their role and traffic in biological membranes. It has been suggested that the dolichol content significantly alters the structure and dynamics of lipid motion, facilitating fusion of membranes.[3,6,39] With respect to retinoids, their movement and distribution in the membranes may vary with the geometry of the membranes, e.g. with the bilayers' radius of curvature, while a gavage of excess of vitamin A has been reported to alter the trafficking of glycoproteins through the Golgi apparatus.[40,41]

The production of extracellular matrix components, considered to be synthesized by Ito cells, SECs and parenchymal cells, seems to occur in liver injury especially by activation of Ito cells, while they lose vitamin A and in the meantime acquire a phenotype resembling myofibroblast-like cells, currently considered as mainly responsible for fibrosis.[42]

Alteration in the interchange of vitamin A between HCs and Ito cells in liver injury has been suggested.[43] On the basis of these observations, it has recently been hypothesized that the role of dolichols in Ito-1 cells might be for storage of retinol in the lipid droplets, or for accompanying this lipid across the hydrophobic double layer of the membrane by flip-flop.[18] The fact that dolichol does not increase in HCs, SECs and KCs but only in Ito-1 cells after vitamin A supplementation, is in agreement with this proposal. Therefore in the cells that are present in the hepatic sinusoid, different pools of dolichol might have separate functions.

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