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# Retinol Esterification Activity Contributes to Retinol Transport in Stellate Cells

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*ABSTRACT.* **The mechanisms of retinol transport and accumulation in hepatic stellate cells (HSC) remain to be elucidated. Our previous studies suggested that retinol esterification activity, particularly lecithin: retinol acyltransferase (LRAT) activity, in liver retinoid metabolism is important to elucidate the relationship between retinol uptake by HSC and the esterification of retinol. In the present study, using a human HSC-like cell line, LI90, we demonstrated that retinol esterification activity of LI90 cells is similar to that of primary cultures of rat HSC and higher than that of a human hepatoma cell line. Further, since progesterone or diphospho-lauroyl-phosphatidylcholine increased retinol esterification activity of LI90 cells, it is likely that LRAT contributes to retinol esterification in LI90. We examined retinol esterification in LI90 cells and clearance of retinol from culture medium. The percentages of both retinol and esterified retinol in LI90 cells increased in a manner dependent on retinol concentration in medium, whereas that of retinol in medium decreased. The percentages of esterified and unesterified retinol in LI90 cells and of retinol in medium were linearly dependent on the logarithm of the initial concentration of retinol in the medium. These results suggest that retinol esterification activity contributes to retinol uptake by HSC and maintenance of non-toxic retinol levels in plasma.**

**Key words:** retinoid/vitamin A/hepatic stellate cell/liver/human

Most retinoid in the body is stored in hepatic stellate cells (HSC), also known as fat-storing cells, Ito cells, or lipocytes. The mechanisms of specific retinoid accumulation in HSC contribute to defending the body from the toxic effects of retinoids, but the transport system of retinol from hepatocytes into HSC remains to be elucidated. This system is important for the maintenance of non-toxic retinol levels in plasma under any vitamin A nutritional status. We reported earlier that retinol added to the medium was transferred into cultured HSC of rat liver and that this transport was not facilitated by retinol binding protein (RBP) (10). We also showed that multivesicular and lamellar bodies are important organelles for retinyl ester formation and for the initial storage of retinoid in lipid droplets in HSC (9, 17). Moreover, the activity of lecithin: retinol acyltransferase (LRAT), the physiological enzyme of retinol esterification in the liver was distributed in hepatic nonparenchymal cells, probably mainly in HSC (11). Based on these results, we suggested that specific transport of retinol into HSC was likely to be dependent on the intracellular esterification of retinol in HSC (9, 10, 11).

To clarify the relationship between retinol uptake by HSC and retinol esterification, we used a new human cell line, LI90, established by Murakami *et al.* that exhibits characteristics comparable with those of HSC from human hepatic mesenchymal tumor (13). In the present study, we first examined the ability of LI90 to esterify retinol, and then studied the relationship between the clearance of retinol from the culture medium and its esterification in cultured cells.

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Abbreviations: HSC, hepatic stellate cell; LRAT, lecithin: retinol acyltransferase; RBP, retinol binding protein; DLPC, diphospholauroyl-phosphatidylcholine; CRBP, cellular-retinol binding protein; FBS, fetal bovine serum.

## *Materials and Methods*

### *Cell Culture*

The cell line LI90 established by Murakami *et al.* from an epithelioid hemangioendothelioma of human liver is well characterized as HSC-like cells that lack the characteristics of endothelial cells or macrophages (12, 13). Moreover, Murakami *et al.* established monoclonal antibodies that reacted specifically with HSC of human liver and cDNA screened with these monoclonal antibodies as probes was encoded as part of human extracellular matrix tenascin (13). In our laboratory, LI90 was maintained in ASF104 medium (Ajinomoto Co., Tokyo, Japan) containing 2% fetal bovine serum (FBS). Methods for isolation, separation, and culture of HSC from rat liver are described elsewhere (7). Cells from HSC-rich fractions were cultured in 24-well plastic culture plates coated with type I collagen in Williams' E medium containing 10% FBS (SC-1). The cell population of SC-1 was heterogeneous, as shown previously (7). We also used 5-times-passaged cells from the HSC-rich fraction (SC-5). SC-5 was cultured in ASF104 containing 2% FBS. In the present study, we used human hepatoma cell line JHH-4 for comparison with the HSC-like cells (2). JHH-4 cells, which have maintained some of the characteristics of hepatocytes, were cultured in serum-free medium ASF104.

After attachment of the cells, the culture medium was changed to serum-free ASF-104, with or without retinol.

### *Fine morphological assay*

Cultured cells were fixed for transmission electron microscopic (TEM) observation in a solution of 2.5% glutaraldehyde in 0.1 M phosphate buffer, adjusted to pH 7.4. After postifixation with 1% $OsO_4$ in 0.1 M phosphate buffer, the specimens were dehydrated in ethanol, and embedded in Epon-Araldite. After sectioning and staining with lead citrate and uranyl acetate, TEM observations were carried out with a JEM-1200EX electron microscope (JEOL, Tokyo, Japan).

### *Assay of ability of cultured cells to esterify retinol*

For this assay, [$^3$H]retinol (DuPont NEN Research Products, Boston, MA, USA) dissolved in ethanol, was added to medium and cultured for 6 h. The reaction was stopped by the addition of ethanol, and retinoids in medium or cultured cells were extracted in hexane. Retinol and retinyl esters were separated on columns of aluminum oxide (14), and radioactivity was determined by liquid scintillation counting.

### *Statistics*

Results are presented as the mean±SD for each treatment group. Statistical comparisons were made between treatment and control groups by unpaired Student's *t* test. A *P* value $\leqq 0.05$ was considered significant.

## *Results*

### *Morphologies of rat HSC, LI90 cells and JHH-4 hepatoma cells*

In phase-contrast microscopic studies, the cell population of primary cultured cells from the HSC-rich fraction (SC-1) was still heterogeneous, but included abundant spindle-shaped cells that contained lipid droplets (Fig. 1A). Five-times-passaged cells from SC-1 became predominantly polygonal and had abundant cytoplasm with well-developed intracytoplasmic filaments (Fig. 1B). LI90 cells were also polygonal, similar to cultured

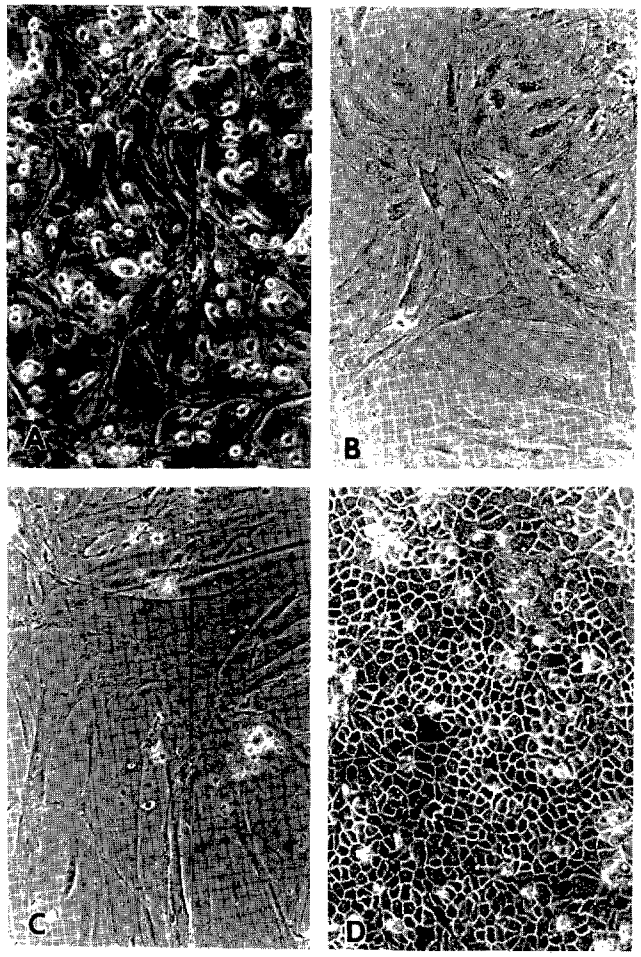


**Fig. 1.** Phase-contrast microscopic views of SC-1, SC-5, LI90 and JHH-4. The cell population of primary cultured cells from the SC-rich fraction (SC-1) was still heterogeneous, but contained abundant spindle-shaped cells that contained lipid droplets (A). Five-times-passaged cells from SC-1 became predominantly polygonal cells that had abundant cytoplasm with well-developed intracytoplasmic filaments (B). LI90 cells were also polygonal, similar to cultured smooth muscle cells (C). Hepatoma cells, JHH-4, had typical epithelial shapes (D).

smooth muscle cells (Fig. 1C). Hepatoma cells, JHH-4, had typical epithelial shapes (Fig. 1D). After 1 week of culture in medium containing $1 \times 10^{-6}$ M retinol, lipid droplets appeared in LI90 cells, and vitamin A spontaneous fluorescence was detected in lipid droplets by fluorescence microscopy (data shown in reference 13).

### *Fine morphologies of LI90 cells cultured with and without retinol*

We examined the fine morphology of LI90 cells cultured with and without retinol by electron microscopy.

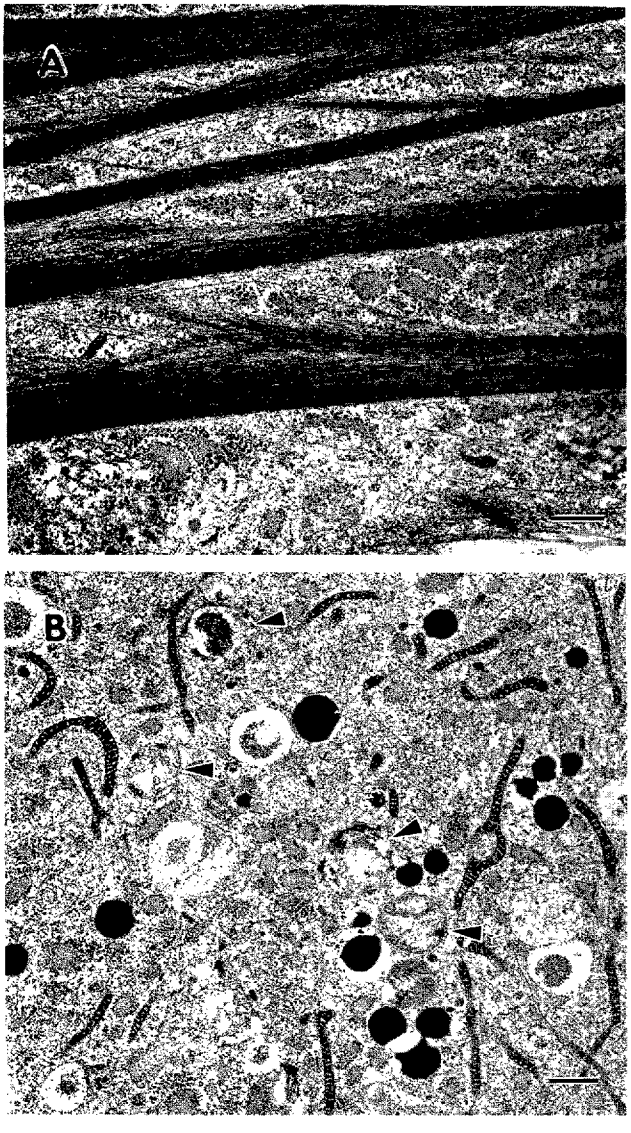


**Fig. 2.** Electron-microscopic views of LI90 cells cultured with and without retinol. LI90 cells contained rich cytoskeletons, particularly as actin bundles (A). After culture with retinol, lipid droplets appeared, with multivesicular bodies (B). Arrowheads, multivesicular bodies. Bar, 1 $\mu$m.

LI90 contained rich cytoskeletons, particularly actin bundles (Fig. 2A). After cell culture with retinol, lipid droplets appeared, most likely containing retinyl esters (Fig. 2B). Multivesicular bodies were rarely observed. We did not observe the lamellar body previously reported in primary cultures of HSC in rat liver (9).

### *Retinol esterification activity of cultured cells*

Retinol esterification activities of rat SC-1 and SC-5 were approximately 10-fold higher than that of human hepatoma JHH-4 ($p<0.0001$) (Fig. 3). The activity of SC-1 was similar to that of SC-5. Progesterone, which increases LRAT activity *in vitro*, increased retinol esterification by SC-1 ($p<0.0001$), but not by SC-5 (Fig. 3).

Retinol esterification activity of LI90 cells cultured with 2% FBS was higher than that of human hepatoma JHH-4 cells cultured with serum free-medium ($p<0.0001$) (Fig. 4A). After preincubation with progesterone 24 h before assay, the retinol esterification activity of LI90 cells significantly increased ($p<0.02$) (Fig. 4B). After preincubation with 0.1 mM concentration of diphospho-lauroyl phosphatidylcholine (DLPC), one of the substrates of LRAT, 24 h before assay, retinol esterification activity of LI90 cells increased ($2.16\pm0.38$ *vs* $5.69\pm0.42$ pmoles/6 h/$10^4$ cells, n=3, $p<0.02$, incubate without DLPC *vs* with DLPC).

### *Retinol uptake and esterification in LI90 cells*

We examined the relationship between retinol uptake

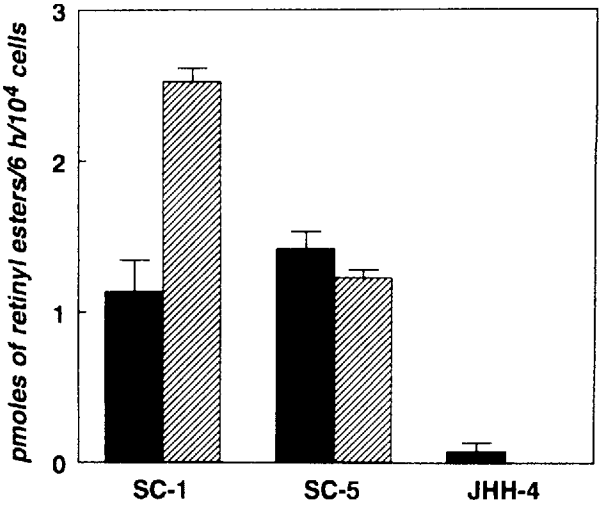


**Fig. 3.** Retinol esterification activities of rat SC-1 and -5 were approximately 10-fold higher than that of human hepatoma cells JHH-4 ($p<0.0001$). Progesterone increased retinol esterification only of SC-1 ($p<0.0001$). ■ Control, ▨ Progesterone

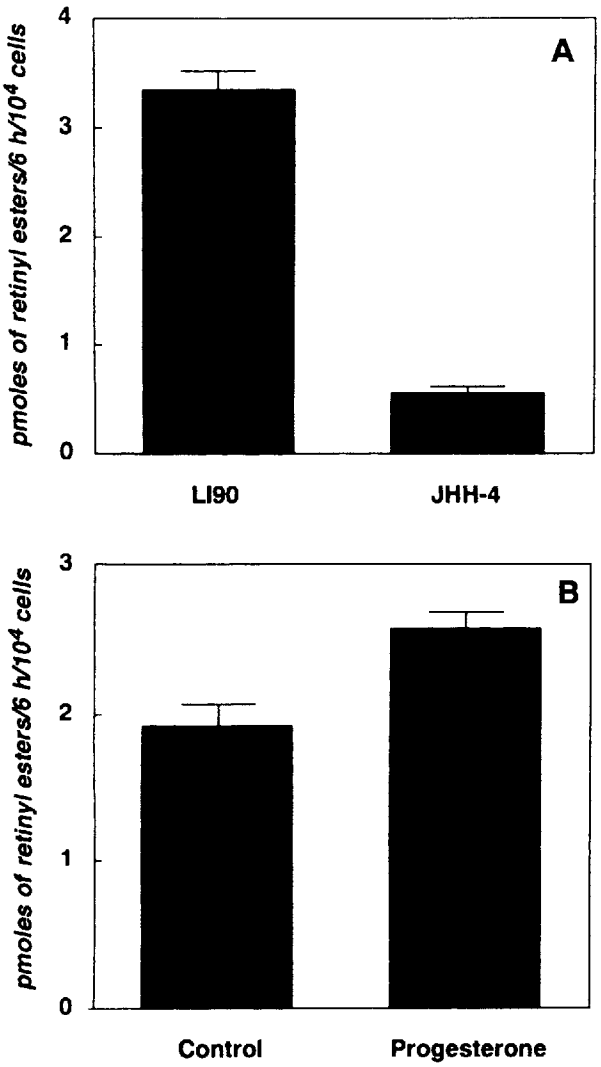


**Fig. 4.** Retinol esterification activity of LI90 cells was approximately 6-fold higher than that of human hepatoma JHH-4 ($p<0.0001$) (A). Progesterone, which was pre-incubated 24 hr before assay, significantly increased the retinol esterification activity of LI90 cells ($p<0.02$) (B).

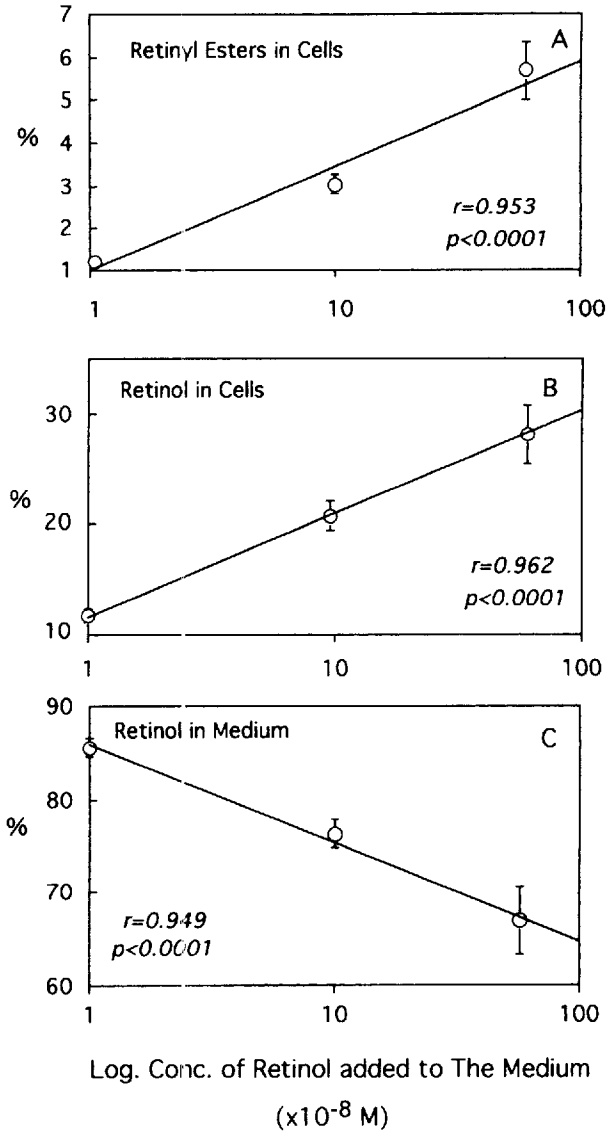


**Fig. 5.** Percent of esterified retinol in LI90 cells relative to the amounts recovered in all four fractions (esterified retinol in cells; free retinol in cells; esterified retinol in medium; and free retinol in medium) increased in proportion to the logarithm of the concentration of [$^3$H]retinol, from $1\times10^{-8}$ M to $5\times10^{-7}$ M (A). The percentage of retinol in cells increased similarly (B), whereas that of retinol in the medium decreased (C).

and esterification in LI90 cells. LI90 cells were cultured with [$^3$H]retinol $1\times10^{-8}$ M, $1\times10^{-7}$ M and $5\times10^{-7}$ M for 6 h, and then the amount of esterified and unesterified retinol in the cells and medium were measured. The percentage of esterified retinol in LI90 cells relative to the amounts recovered in all four fractions (esterified retinol in cells; free retinol in cells; esterified retinol in medium; and free retinol in medium) increased proportional to the logarithm of [$^3$H]retinol concentration, from $1\times10^{-8}$ M to $5\times10^{-7}$ M (Fig. 5A). The percentage of retinol in cells increased similarly (Fig. 5B), whereas that of retinol in medium decreased (Fig. 5C). Esterified retinol in medium was as little as 1%, and was probably released from detached cells.

## *Discussion*

Retinoids are powerful nutritional regulators for maintaining biological homeostasis, and their biological levels are controlled by buffer action of binding proteins in plasma and cytosol. Moreover, plasma level of retinol is regulated by holo-RBP secretion from hepatocytes and

retinol accumulation into HSC, both of which are influenced by vitamin A nutritional status (15).

The mechanisms of specific retinoid accumulation in HSC and retinol transport from hepatocytes into HSC remain to be elucidated. Blomhoff and coworkers suggested that these mechanisms depend on RBP-receptor-mediated endocytosis on HSC (3, 16). Indeed, Båvik *et al.* identified and characterized a retinal pigment epithelial cell membrane receptor for plasma RBP (1), but since this receptor does not appear in the liver, this opens up the possibility of the existence of other types of RBP receptors.

In the present study, we used a new cell line, LI90, for examining the relationship between retinol transport into HSC and retinol esterification activity in HSC. As several cell lines derived from rats or mice have some of the characteristics of HSC, the cell line derived from mice established by Margis and Bolojavic is the only one characterized for retinoid metabolism (6). Most other such cell lines have been characterized for similarities to the cytoskeltons of HSC, but it is unclear what their characteristics are with regard to retinoid metabolism. LI90, established and characterized by Murakami *et al.* from an epitheloid hemangioendothelioma of the human liver (13), show $\alpha$-smooth muscle cell actin filaments in the cytoplasm and produce various connective tissue components, such as collagen types I, III, IV, V, and VI, laminin, and fibronectin. In this study, we showed that LI90 cells maintain a higher retinol esterification activity than that of hepatoma cells and similar in level to that of primary cultures of cells from the HSC-rich fraction of rat nonparenchymal cells. We did not find abundant multivesicular bodies and lamellar bodies observed previously in primary cultures of rat HSC (9). Multivesicular bodies appeared in LI90 cells cultured with retinol. We judged that LI90 cells have some characteristics of retinoid metabolism similar to those of HSC, and are better suited for studies of retinoid metabolism than are primary cultured cells. Because we could not achieve high purification of HSC by our cell separation method, we could not carry out more accurate biochemical studies in comparison with primary cultures of rat HSC. We showed that retinol esterification activity in LI90 cells was increased by progesterone. In rat liver microsomes, progesterone increased LRAT activity, but it decreased acyl CoA: retinol acyltransferase activity (18). Yost *et al.* suggested that retnol associated with cellular-retinol binding protein (CRBP) is specifically "targeted" to the cell membrane site of esterification even when progesterone is present (18). Therefore LRAT is likely to contribute to retinol esterification by LI90 cells. LI90 cells were derived from human liver, and McDonald and Ong detected LRAT activity in human liver (5).

To clarify whether retinol esterification activity contributes to retinol uptake in HSC, we examined retinol esterification in LI90 cells and retinol clearance from culture medium. The percentage of both retinol and esterified retinol in LI90 cells increased in proportion to retinol concentration in medium, whereas that of retinol in medium decreased. The percentage of esterified and unesterified retinol in LI90 cells and of retinol in medium increased proportional to the logarithm of retinol concentration in medium. These results suggest that retinol esterification activity contributes, in part, to retinol uptake by LI90 cells and seems to depend on LRAT activity of LI90 cells. Matsuura and Ross showed that the relationship between LRAT activity induced by retinoic acid and *in vivo* retinol esterification in vitamin A-deficient rats is quite linear (8), and that *in vivo* retinol esterification by rat liver is influenced by at least two factors, LRAT activity of the liver and retinol concentration in the blood. The present study also showed that the retinol concentration in medium strongly influences esterification of retinol in LI90 cells. The increase in percentage of unesterified retinol in LI90 cells was dependent on retinol concentration in medium. We suggest that the cell membrane itself, unidentified receptors of retinol on the cell membrane, and CRBP, contribute to the initial uptake of unesterified retinol by LI90 cells (4), and then the excess retinol in the cells is esterified by LRAT and stored.

In conclusion, human HSC-like cell line LI90 maintains retinol esterification activity, which is probably LRAT activity. Retinol esterification activity seems to contribute not only to retinol storage, but also to retinol uptake into HSC.

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