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Expression of a Smaller Lecithin:Retinol Acyl Transferase Transcript and Reduced Retinol Esterification in MCF-7 Cells

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Retinyl ester concentration is regulated by retinoic acid (RA) through an autoregulatory loop, which acts on lecithin:retinol acyltransferase (LRAT). We tested whether retinol esterification activity is downregulated in human mammary carcinoma cells and whether LRAT expression is RAR-regulated. Normal human mammary epithelial (HMEC) cells expressed a retinoid-upregulated 5-kb LRAT transcript and synthesized retinyl esters from ^{3}H-retinol. Human carcinoma MCF-7 cells failed to express the 5-kb LRAT transcript and to synthesize retinyl esters. Instead, they expressed a 2.7-kb LRAT transcript. Both transcripts were upregulated by RA. Stable expression of the dominant-negative RARα403 blunted the up-regulation of LRAT mRNA by RA. We conclude that retinol esterification is decreased in MCF-7 vs normal mammary cells; that these cancer cells express a shorter (2.7 kb) LRAT transcript, and that retinoid receptors are involved in the regulation of LRAT-mediated retinyl ester synthesis by RA.

Key Words: **LRAT; RAR; breast cancer; retinyl esters.**

Vitamin A and its derivatives, the retinoids, are critical regulators of cell growth and differentiation. Retinoic acid is routinely used in differentiation therapy of acute promyelocytic leukemia (APL) (1) and has been shown to be effective in chemoprevention trials (2, 3). Therefore, we investigated retinyl ester synthesis in normal and neoplastic human breast epithelial cells.

A schematic representation of the flow of metabolic events responsible for the synthesis and degradation of retinoic acid and its precursors (retinol and retinyl esters) is shown in Fig. 1. The parent compound retinol functions as a substrate for different families of enzymes that drive either its esterification or oxidation pathway. The microsomal lecithin:retinol acyltransferase (LRAT) converts retinol to retinyl esters, the storage form of vitamin A. The cytosolic alcohol dehydrogenases (ADHs) and/or microsomal short chain dehydrogenases/reductases (SCD/R) catalyze its conversion to retinal. Retinaldehyde dehydrogenases (RALDHs) further irreversibly oxidize retinal to retinoic acid (RA). RA is in turn converted to its derivatives (4-hydroxy and 4-oxo RA) by oxidation via CYP450s (Fig. 1). Retinoic acid acts through two members of the nuclear receptor superfamily, the retinoic acid receptors (RARα,β,γ) and the retinoid X receptors (RXRα,β,γ). Ligand activated receptors recognize retinoic acid response elements (RAREs) in the promoter region of RA regulated genes, thereby controlling transcription. A complex homeostatic mechanism, which works as a sensor for retinoid status, controls the fate of intracellular retinol. Any accumulation of RA prevents retinol from being oxidized and causes it to be sequestered into the esterification pathway (4). In human keratinocytes, RA treatment causes a 50% reduction in the conversion of retinol to RA and induces LRAT activity (4). LRAT activation is observed upon treatment with a synthetic activator of RARs and this activation does not occur when either transcription or translation is blocked (4). These data implicate RARs in RA induction of LRAT.

MATERIALS AND METHODS

Infection of MCF-7 cells with retroviral vectors. The retroviral vector LXRARα403SN was a gift from Dr. S. J. Collins (Fred Hutchinson Cancer Research Center, Seattle, WA). In this construct,

Abbreviations used: PBS, phosphate-buffered saline; RAR, retinoic acid receptor; LRAT, lecithin:retinol acyltransferase; RA, all-*trans* retinoic acid; DMSO, dimethyl sulfoxide; RALDH, retinal dehydrogenase; ADH, alcohol dehydrogenase; SCD/R, short chain dehydrogenase-reductase.

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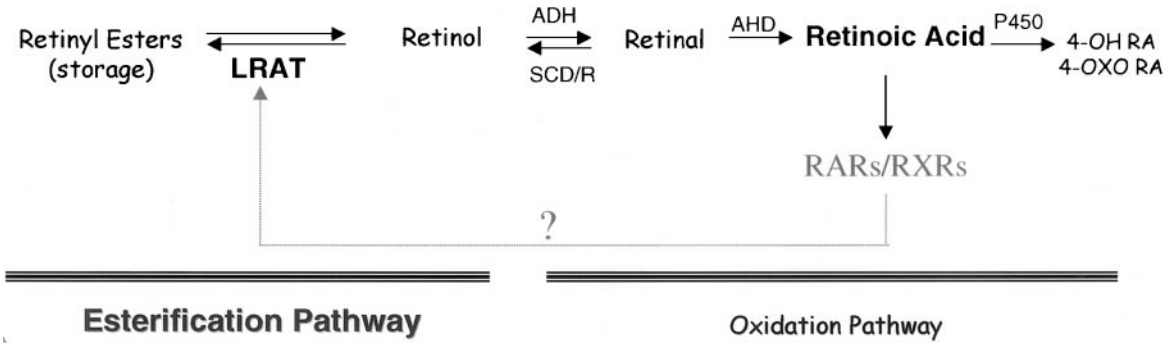

FIG. 1. Feedback regulation of lecitan:retinol acyltransferase (LRAT) expression by retinoic acid (RA). Metabolic flow and interconversions of natural retinoids are shown. Note that the oxidation of retinal to retinoic acid is irreversible, as are the subsequent oxidative steps from retinoic acid to 4-hydroxy- and 4-oxo-retinoic acid.

a truncated RARα gene is inserted into the retroviral vector LXSN upstream of the neomycin resistance gene. Cells were seeded at 50% confluency in 100-mm dishes. After 24 h, they were infected with the LXSN or the LXRARαSN retroviral constructs in the presence of 4 μg/ml Polybrene. After overnight incubation, the medium was replaced and cells were grown for 36–48 h before G418 (1 mg/ml) was added. G418-resistant cells were isolated. Cells were maintained in complete DMEM with 10% FBS, in 5% CO_2 atmosphere at 37°C.

Northern blot analyses. Human mammary HMEC, LXSN-MCF-7 and LXRARα403SN-MCF-7 cells were grown in MEGM (mammary epithelial cell growth medium) (Clonetics, San Diego, CA) and DMEM with 10% FBS, respectively. When the cultures reached 95% confluence the cells were harvested and seeded in 10-cm-diameter tissue culture dishes at 2×10^6 cells/dish. After 24 h, the medium was removed and replaced with 2% FBS DMEM with 1 μM retinoids (RA and retinol) or vehicle (DMSO) for LXSN-MCF-7 and LXRARα403SN-MCF-7 cells. HMEC cells were treated in similar ways, except that they were grown in MEGM. Isolation of polyA$^+$ mRNA was performed with a Micro-Fast Track mRNA Isolation Kit (Invitrogen, Carlsbad, CA). mRNA was fractionated on a 1% agarose gel and blotted overnight onto Nytran membranes (Schleicher and Schuell, Keene, NH) using a Turboblotter Transfer System (Schleicher and Schuell). The membranes were hybridized with the probes (1.5×10^6 DPM/ml) using Hybrosol I solution according to the manufacturer's manual (Intergen Co., Purchase, NY). The probes were labeled with [α-^{32}P]dCTP using a random primer labeling method by Lofstrand Labs Ltd (Gaithersburg, MD). hRARα cDNA was used to verify RARα403 over expression (Fig. 3C). GAPDH cDNA was used as quantitative control. A specific probe for human LRAT gene was generated by RT-PCR as specified below.

RT-PCR. Total RNA was isolated from MCF-7 cells with a RNeasy Mini Kit (Qiagen, Chatsworth, CA). PCR primers for LRAT bracketed bases 2178-2582 (405 bp PCR product) of the human sequence (GenBank Accession No. AF071510). The primer sequences were 5′-^{2178}cggggcaatcagtcaatgataatc^{2201}-3′ and 5′-^{2582}gtttcagacagtggtttttgctgc^{2559}-3′. Reverse transcription of 1 μg of total RNA to cDNA and subsequent amplification were performed using Gene Amp RNA PCR kit (Perkin Elmer-Roche, Branchburg, NJ) according to the manufacturer's protocol. PCR products were fractionated on a 1.3% agarose gel, purified, and TA cloned (TOPOTA Cloning, Invitrogen). The specificity of the PCR product was verified by its digestion with DdeI (New England Biolabs Inc., Beverly, MA). This restriction endonuclease introduced a single cut, thus originating two pieces: 91 and 314 bp long as expected.

HPLC. Human mammary HMEC or MCF-7 cells were seeded at 1.5×10^6 and let grow for 24 h in DMEM containing 2% FBS, prior to the addition of ^{3}H-retinol (1 μCi/ml at 30 nM). After 6 h incubation, cells were rinsed once in PBS 1 μM retinol and twice in ice-cold PBS and scraped in ethanol, spun at 14,000*g* and the supernatant was used for further studies. Aliquots of the extracts were counted for radioactivity determination and analyzed by reverse phase HPLC on a Resolve C-18, 5 μm (3.9×300 mm) column (Waters Co., Milford, MA) to determine the extent of retinol esterification. The mobile phase consisted of acetonitrile, dichloromethane, methanol, and 1-octanol (90:15:10:0.1 concentration) plus 0.01% butylated hydroxytoluene, according to the procedure of Barua *et al.* (5) at a flow rate of 1 ml/min.

RESULTS

Expression of the 5-kb (HMEC) and 2.7-kb (MCF-7) LRAT transcripts is upregulated by retinoids. Northern blot analysis of mRNA from HMEC cells shows a main LRAT transcript of 5.0 kb (Fig. 2). This transcript is stimulated by RA (2.2-fold) and also by retinol, albeit to a lesser extent (1.8-fold), possibly because it requires oxidation to RA. In sharp contrast, human breast cancer MCF-7 cells fail to show the 5.0-kb transcript (even after prolonged exposure). Instead, they express a much shorter transcript (2.7 kb) (Figs. 3A and 3B), not detectable in HMEC cells (even after much longer exposure). Interestingly, RA also stimulates this shorter transcript by 3.1-fold (Figs. 3A and 3B).

LRAT transcription is RAR-dependent. We utilized LXRARα403SN, a retroviral construct with dominant negative activity over all RARs, to obtain high expression of the truncated receptor RARα403 in MCF-7 cells and test the concept that RARs may be involved in the observed RA-induced LRAT transcript expression in these cells. Figure 3C verifies RARα403 mRNA overexpression in MCF-7 cells transfected with the LXRARα403SN retroviral construct. The size of the dominant-negative transcript is 4.7 kb as expected (6).

Figures 3A and 3B clearly shows that the RARα403 dominant negative blunts the expression as well as the induction by RA of LRAT mRNA in MCF-7 cells, demonstrating that RA-induced stimulation is RAR mediated.

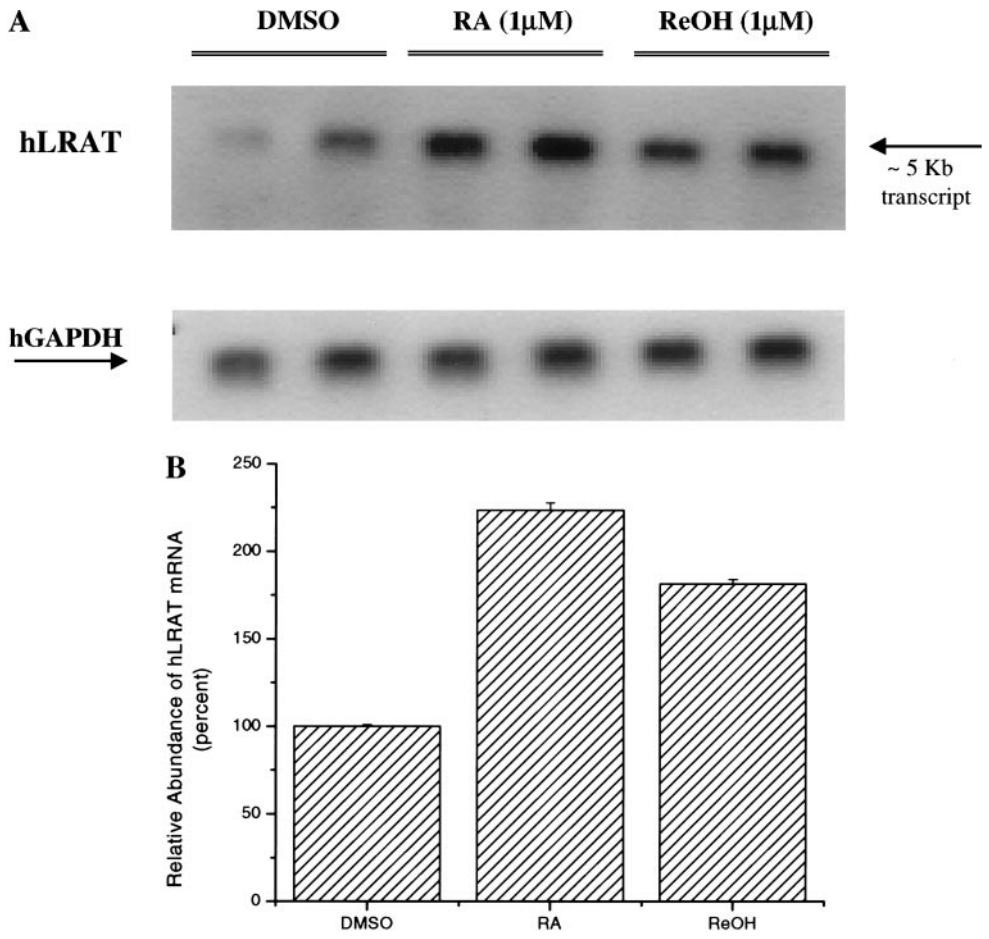

FIG. 2. (A) Northern blot analysis of LRAT transcripts (polyA mRNA) in HMEC cells at 24 h of treatment with RA (10^{-6} M), retinol (10^{-6} M), or solvent (DMSO) control. Hybridizing probe was prepared as specified under Materials and Methods. (B) Densitometric analysis of results shown in A.

MCF-7 cells esterify ^{3}H-retinol to a lesser extent than HMEC. Because of the different size transcript for LRAT, we were interested to test possible differences in retinol esterification between HMEC and MCF-7 cells. Figure 4 demonstrates a marked (about 80%) reduction in the ability of MCF-7 cells to esterify ^{3}H-retinol in both control LXSN- and in LXRARα403SN-expressing cells. Similar results were obtained at at 6 (Fig. 4) and 24 h (not shown).

DISCUSSION

Our previous work, as well as that of other investigators have shown a marked drop in the ability of cancer cells to accumulate retinyl esters. A 99% reduction in retinyl ester content of both transplanted, as well as chemically induced primary rat liver hepatocellular carcinomas compared to the host liver tissue was observed (7). This was attributed to the neoplastic state of the cell (rather than its proliferative state) on the basis of analysis of regenerating liver, which showed significant levels of retinyl esters. A reduction in retinyl esters was also found in human hepatic tumors (8) and rat intestinal adenocarcinomas (9), as well as in cultured head and neck and skin carcinoma cells (10, 11) and in rat breast cancer cells (12). On the basis of these studies we have proposed the concept that certain carcinoma cells loose the dependence on retinoids and develop an adaptive phenotype (13, 14) no longer requiring retinoids.

It was, therefore, of interest to determine whether the synthetic enzyme LRAT was affected by the neoplastic state. The LRAT gene has been recently cloned from human retinal pigment epithelial cells (15). We utilized a 405 bp DNA fragment corresponding to position 2178-2582 near the end of the 3′-UTR nucleotide sequence as a probe; this fragment, as reported by Ruiz *et al.* (15), hybridizes exclusively to the 5-kb transcript, thus eliminating the lower molecular weight transcripts, detectable with the full length cDNA probe. Strikingly, however, mRNA from the MCF-7 cells showed mainly a band of 2.7 kb. It remains to be established whether this shorter

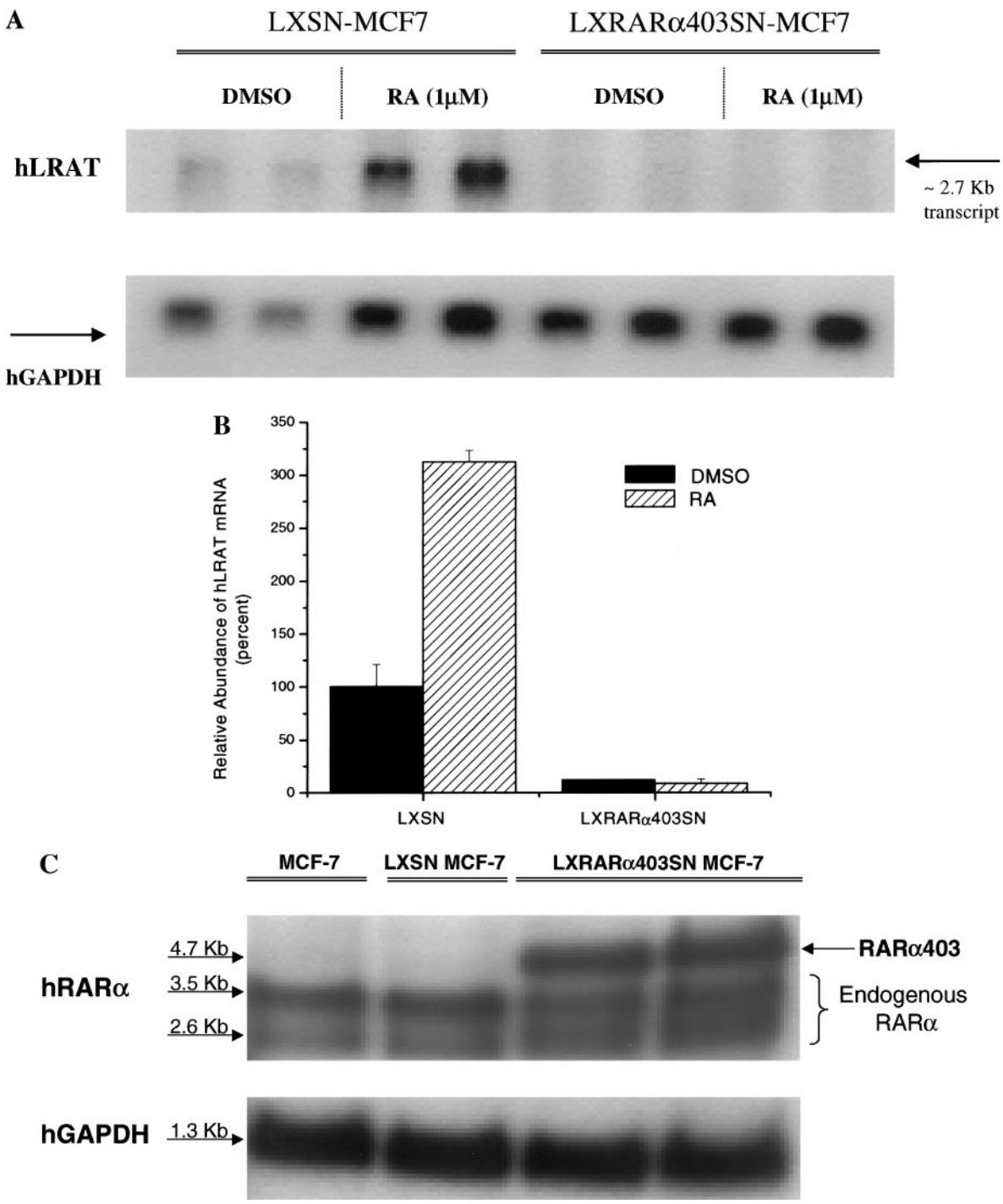

FIG. 3. (A) Northern blot analysis of LRAT transcripts (polyA mRNA) in human breast cancer MCF-7 (control LXSN- and RARα403) cells at 24 h of treatment with RA (10^{-6} M) or solvent control. (B) Densitometric analysis of results shown in A. (C) Overexpression of RARα403 mRNA in LXRARα403SN MCF-7 cells.

transcript results from a mutated sequence of the gene and/or alternative splicing. It is of interest that both the 5-kb and the 2.7-kb transcripts are RA-inducible. Further, overexpression of the dominant negative RARα403 construct blunted the RA induction of the transcript in MCF-7 cells.

Finally, the finding that retinyl ester synthesis is decreased by approximately 80% in MCF-7 cells, compared to normal HMEC cells, suggests that the shorter transcript is less able to generate a functional LRAT protein. Further experimentation is focused on determining the molecular alterations responsible for these changes.

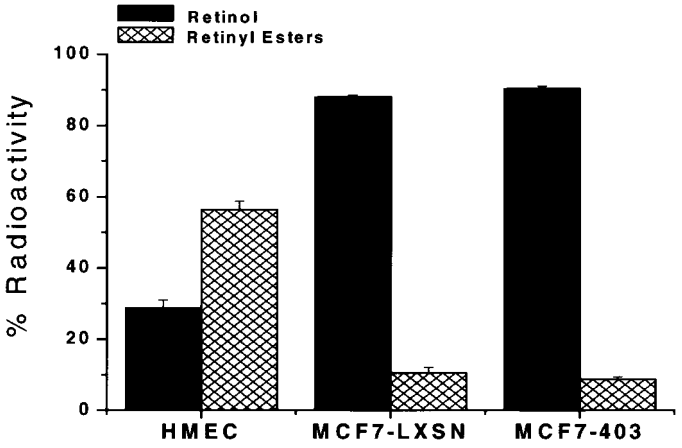

FIG. 4. Esterification of ^{3}H-retinol by cultured HMEC and MCF-7 cells (control LXSN- and RARα403). Retinol and retinyl esters were separated by reverse phase HPLC analysis as specified under Materials and Methods.

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