Skin Pharmacology and Physiology

LRAT Overexpression Diminishes Intracellular Levels of Biologically Active Retinoids and Reduces Retinoid Antitumor Efficacy in the Murine Melanoma B16F10 Cell Line

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Abstract

Background/Aim: Vitamin A (all-*trans*-retinol, ATRol) serves as a precursor for all-*trans*-retinoic acid (ATRA), a ligand for the retinoic acid receptor (RAR), representing a potent regulator for many physiological processes. While murine melanoma cells are highly sensitive to retinoid treatment, human melanoma cells have developed still unidentified mechanisms that mediate cellular retinoid resistance. One of the key retinoid metabolizing enzymes is lecithin retinol acyltransferase (LRAT), which catalyzes the transformation of ATRol into inactive retinyl esters. LRAT is highly expressed in human melanoma cells. The aim of this study was to identify the mechanisms in retinol metabolism that are responsible for cellular retinoid sensitivity in the murine melanoma cell line B16F10. **Methods:** mRNA expression analysis, cell viability assessment and determination of intracellular retinoid levels using HPLC analysis of a generated LRAT-overexpressing B16F10 cell line compared to the control B16F10 cell line. **Results:** We found that the murine retinoid-sensitive B16F10 cell line does not express the enzyme LRAT. LRAT overexpression decreased the antiproliferative effects of retinoid treatment in these melanoma cells. The RAR-regulated enzyme Cyp26a1 showed a significantly lower expression in LRAT-overexpressing B16F10 cells. Cyp26a1 expression was restored after ATRA incubation. HPLC analysis revealed that the level of inactive retinyl ester increased after ATRol treatment, and levels of the substrate ATRol and biologically active ATRA significantly decreased in LRAT-overexpressing murine melanoma. Consistently with this, levels of 4-oxo-retinoic acid, an ATRA metabolite and Cyp26a1 product, were also decreased in LRAT-overexpressing cells. **Conclusion:** Our results revealed a direct link between LRAT expression and regulation of ATRA levels indicating that the absence of LRAT-catalyzed retinol esterification is important for mediating retinoid sensitivity in murine melanoma cells. Thus, our data suggest that LRAT overexpression represents a novel mechanism by which tumor cells can escape high supplementary ATRA levels that mediate tumor-suppressive RAR signaling.

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Introduction

Vitamin A derivatives (retinoids), like all-*trans*-retinoic acid (ATRA), are essential for many physiologically relevant processes like cell growth and differentiation during embryogenesis and in the adult organism, and are also important for skin physiology [1–4]. At the molecular level, ATRA binds and activates the retinoic acid receptors (RARs), which directly regulate the transcription of various target genes [1]. Intracellular retinoid levels are regulated by mechanisms that are not completely understood [5, 6]. For ATRA synthesis, vitamin A (all-*trans*-retinol, ATRol) can be oxidized via all-*trans*-retinal (ATRal) as a metabolic intermediate [7, 8]. Due to the antiproliferative and prodifferentiation effects of ATRA [9], it is successfully used to treat several types of cancers [10, 11]. However, many human melanoma cell lines are highly resistant to ATRA treatments [12, 13], and ATRA and its isomer 13-*cis*-retinoic acid are poor therapeutics for human melanoma [14–17].

The lack of information about the mechanism of ATRA resistance in human melanoma cells greatly hampers efforts to improve clinical protocols that use this drug. Recently, efforts have been made to identify these tumor-specific mechanisms of retinoid resistance and metabolic response in melanoma cells [18, 19]. Aberrant vitamin A metabolism is an attribute of cancer cells presenting such a potential retinoid resistance mechanism. It was shown that cancer-specific modulations in retinoid metabolism reduce ATRA levels in cancer cells and that both the synthesis and metabolism of the bioactive metabolites of retinol are impaired in cancer cells relative to normal cells [10, 11]. Recently, we identified alterations in retinoid metabolism in human melanoma cells [6, 20]. ATRol can be oxidized to ATRA or alternatively be esterified into biologically inactive retinyl esters. This key metabolic step is catalyzed by the enzyme lecithin retinol acyltransferase (LRAT) [21]. We showed that LRAT expression is highly upregulated in human melanoma cells as compared to benign melanocytes [20]. We believe that LRAT may be important for the removal of ATRol which is the precursor for ATRal and ATRA production.

Importantly, in contrast to human melanoma, many studies showed that murine melanoma cell lines, such as B16F10, are highly sensitive to retinoids both in vitro and in vivo, and that retinoids exhibit significant effects on cell differentiation, proliferation, invasion and metastasis formation [22–31]. The purpose of this study was to identify the mechanisms in retinol metabolism that can be responsible for cellular retinoid sensitivity in the murine melanoma cell line B16F10.

Materials and Methods

Cell Culture and Reagents

The murine melanoma B16F10 cell line was cultivated at 37°C in an atmosphere of 5% CO_2 in RPMI 1640 with 2 mM L-glutamine (Gibco/Invitrogen, Darmstadt, Germany), supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany). ATRol, ATRal and ATRA were purchased from Sigma (Taufkirchen, Germany).

LRAT Overexpression

Plasmid amplification was performed in *Escherichia coli* using mouse LRAT cDNA plasmid (MR225686, Origene, Rockville, Md., USA) or pEntry (PS100001, Origene) as the control according to the manufacturer's protocol.

Plasmid DNA from bacterial lysates was purified using the HiSpeed Plasmid Maxi Kit (Qiagen, Hilden, Germany).

B16F10 melanoma cells were plated 8 h before transfection at 70% confluency. DNA transfections were performed using the X-tremeGENE 9 DNA Transfection Reagent (Roche, Penzberg, Germany) according to the manufacturer's recommendations. The chosen ratio of transfection reagent:DNA was 6:2. After transfection, selection was made by adding 2 μg/ml G418 (Roth, Karlsruhe, Germany) to the medium overnight. Subsequently, the cells were washed and treated with 1 μM ATRol, ATRal or ATRA for 24 h, or left untreated.

RNA Isolation

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions, including the on-column digestion of DNA with RNase-free DNase I. The RNA was quantified by photometric measurement (NanoDrop Technologies, Wilmington, Del., USA), and its integrity was analyzed on a 2100 bioanalyzer (Agilent Technologies, Palo Alto, Calif., USA).

Quantitative Reverse Transcription Polymerase Chain Reaction

Quantitative reverse transcription polymerase chain reaction was performed as previously described [32]. Purified RNA was reverse transcribed with the SS VILO Mastermix (Life Technologies, Carlsbad, Calif., USA) according to the manufacturer's instructions. TaqMan experiments were carried out on an ABI Prism 7300 sequence detection system (Applied Biosystems, Weiterstadt, Germany) using Assays-on-Demand gene expression products for murine LRAT (Mm00469972_m1) and murine Cyp26a1 (Mm00514486_m1) according to the manufacturer's recommendations. An Assay-on-Demand product for murine GAPDH (Mm99999915_g1) was used as an internal reference to normalize the target transcripts. All measurements were performed in triplicate in separate reaction wells. Experiments were repeated twice.

Cell Viability

To analyze the influence of retinoids on cellular viability, 1×10^5 B16F10 cells were plated in 6-well plates and were treated after 24 h with 1 μM ATRol, ATRal or ATRA, or left untreated. Proliferation and cytotoxicity were assayed using alamarBlue (AbD Serotec, Düsseldorf, Germany) according to the manufacturer's protocol. After 48 h, B16F10 cells were incubated with 10% (v/v) alamarBlue reagent for 2 h at 37°C, then the absorbance of samples was measured at 560 and 600 nm with a SpectraMax 250 reader

Fig. 1. Endogenous expression, overexpression and regulation of LRAT and its induced metabolic transformation of retinol to retinyl esters in the murine melanoma B16F10 cell line. **a** Taqman analysis of LRAT mRNA expression 24 h after treatment with 1 μM ATRol, ATRal or ATRA in B16F10 cells transfected with LRAT (+LRAT) or control plasmid. Differences between control cells and LRAT+ cells were significant (p < 0.001). **b** ATRP levels in control and LRAT+ cells after 24 h incubation with the substrate ATRol (1 μM) as determined by LC-MS analysis. All experiments were performed in triplicate and were repeated twice.
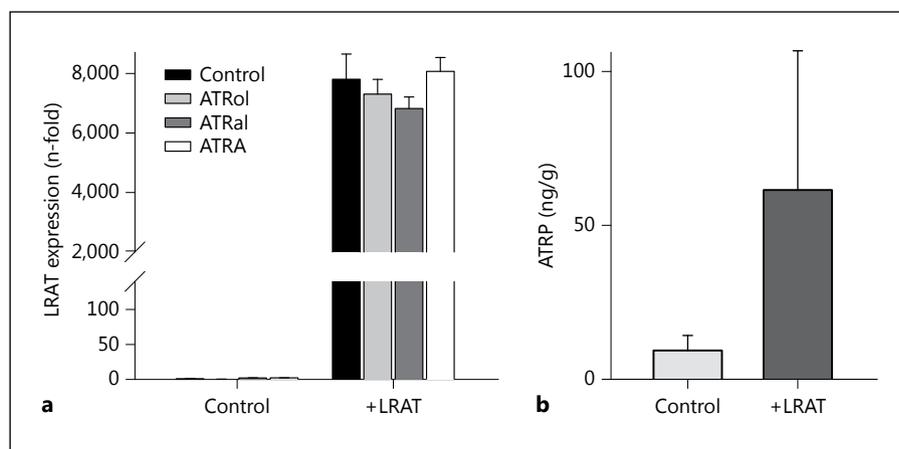
(Molecular Devices, Ismaning, Germany). Data analysis was performed according to the manufacturer's recommendations. Experiments were repeated twice.

Retinoid Analysis
High-performance liquid chromatography mass spectrometry-mass spectrometry (LC-MS) analyses were performed using a standard protocol of a previously published methodology under dark yellow/amber light [33]. The concentrations of 4-oxo-retinoic acid (4-oxo-RA), ATRol, all-*trans*-retinyl palmitate (ATRP) and ATRA were determined in the collected cells originating from cell culture in triplicate. Quantification was performed as previously described [33]. In a draft, sample preparation was performed using 100 mg of the sample (if samples were under 100 mg, water was added up to the used standard weight: 100 mg), vortexed for 10 s, put in an ultrasonic bath for 5 min, shaken for 6 min and centrifuged at 13,000 rpm in a Heraeus Biofuge Fresco at 4°C. After centrifugation, the supernatants were dried in an Eppendorf concentrator 5301 (Eppendorf, Germany) at 30°C. The dried extracts were resuspended with 60 μl of methanol, diluted with 40 μl of a 60-mM aqueous ammonium acetate solution, transferred to an autosampler and subsequently analyzed.

Statistical Analysis
Statistical analysis was performed with Sigmaplot version 11 using Student's t test. Data are given as arithmetical means ± standard error of the mean. Values of p < 0.05, p < 0.01 and p < 0.001 were considered significant and are indicated in the figures.

Results

Endogenous Expression, Overexpression and Regulation of LRAT in the Murine Melanoma Cell Line B16F10

In previous work, we had hypothesized that LRAT expression may be important for reduced ATRol levels in retinoid-resistant human melanoma cell lines [20]. Therefore, we wanted to evaluate the role of LRAT in the retinoid metabolism of the murine retinoid-sensitive cell line B16F10. Interestingly, in contrast to human melanoma cell lines [20], we found that B16F10 cells do not express LRAT mRNA (C_t value 34.4; fig. 1a). Next, we generated an LRAT-overexpressing B16F10 cell line by transient mouse LRAT transfection (B16F10 + LRAT). LRAT expression was significantly increased (C_t value 21.2; p < 0.001) in B16F10 + LRAT cells as compared to B16F10 transfected with the control vector (control cells; fig. 1a). Expression of LRAT did not change after treatment with its substrate ATRol (1 μM), ATRal (1 μM) or ATRA (1 μM; fig. 1a).

LRAT esterifies ATRol to all-*trans*-retinyl ester, mainly ATRP [21]. LRAT overexpression was confirmed by quantification of ATRP by sensitive LC-MS measurements. After ATRol incubation (1 μM for 24 h), higher ATRP mean levels were detected in B16F10 + LRAT cells when compared to control cells according to the higher LRAT expression and higher esterification reaction in the B16F10 + LRAT cells (fig. 1b).

LRAT Overexpression Diminished Responsiveness to Retinoid Treatment

The antiproliferative effects of retinoids are well established in the murine melanoma cell line B16F10 [23, 26, 27, 30, 31]. To determine the effects of LRAT overexpression on retinoid sensitivity, we treated B16F10 control and +LRAT cells with 1 μM ATRol, ATRal or ATRA for 48 h and assessed cellular viability by the alamarBlue assay. Importantly, LRAT overexpression significantly diminished responsiveness to retinoid treatment (p = 0.026 for ATRal and p < 0.001 for ATRA; fig. 2).

LRAT Influences Retinoid Levels and
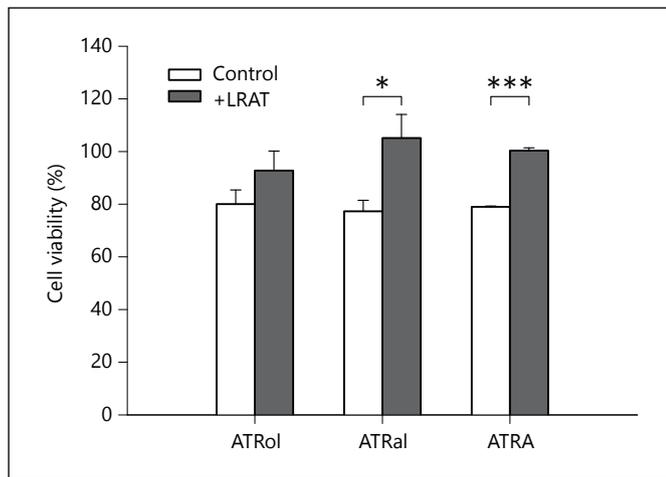Retinoid Resistance in Murine Melanoma

Fig. 2. LRAT overexpression reduces retinoid antitumor efficacy in the murine melanoma B16F10 cell line. Influence of retinoids on cell viability. B16F10 control cells and B16F10 + LRAT cells were treated with 1 μM ATRol, ATRal, ATRA or were left untreated for 48 h. Measurements were performed by the alamarBlue assay, and the results were related to the untreated control. * p < 0.05, *** p < 0.001.

Fig. 3. Overexpression of LRAT reduces expression of Cyp26a1, a retinoid-regulated marker gene used to monitor retinoid signaling. Taqman analysis was performed to measure Cyp26a1 mRNA expression in B16F10 control and B16F10 + LRAT cells and after 24 h of treatment with 1 μM ATRA. * p < 0.05.

Role of LRAT in Retinol Metabolism of Murine Melanoma

Decreased levels of biologically active retinoids could be the reason for reduced retinoid responsiveness in B16F10 + LRAT cells. Therefore, we determined the ATRA-metabolizing enzyme Cyp26A1 in this study functioning as a molecular marker to monitor the intracellular retinoid status [34–37]. Cyp26a1 mRNA expression was significantly reduced in B16F10 + LRAT cells as compared to control cells (fig. 3). If the observed effect on Cyp26a1 expression is mediated by an LRAT-induced deficiency of biologically active retinoids, the addition of ATRA should rescue the cells. Indeed, 24-hour incubation with ATRA partly re-elevated ('rescued') the expression of Cyp26a1 in B16F10 + LRAT cells (fig. 3). The difference between B16F10 control cells and B16F10 + LRAT cells treated with ATRA was not significant. These results indicate that B16F10 + LRAT cells possess decreased levels of biologically active retinoids.

LRAT Overexpression Diminishes Intracellular Levels of Biologically Active Retinoids

To gain further insight into the role of LRAT in retinol metabolism, its substrate ATRol and its biologically active metabolite ATRA were determined using LC-MS in B16F10 + LRAT and control cells. The Cyp26a1 product, 4-oxo-RA, which represents a metabolite of A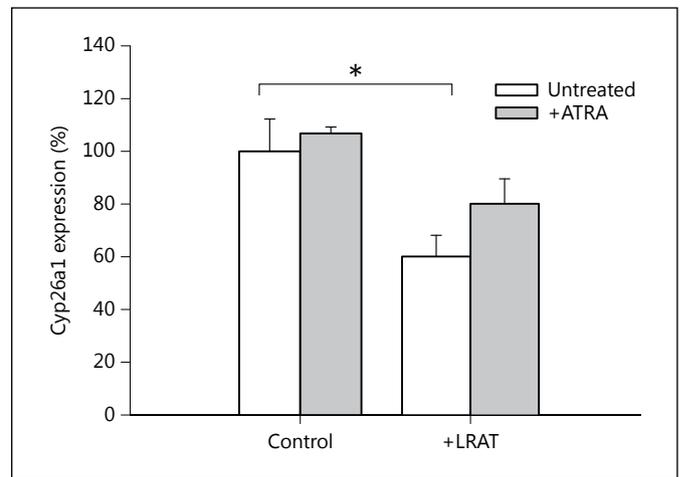TRA, was also analyzed. Endogenous ATRol remained unchanged (p = 0.636), while endogenous levels of ATRA and 4-oxo-RA were under the detection limit in both clones (fig. 4a). Importantly, after ATRol incubation (1 μM for 24 h), B16F10 + LRAT cells showed significantly reduced ATRol levels (p = 0.005), reduced ATRA levels (p = 0.032) and reduced 4-oxo-RA levels (p = 0.026) as compared to the control cells (fig. 4b).

Discussion

There is an obvious association between vitamin A status, retinoid signaling and cancer development that led to the examination of retinoids as preventive and therapeutic agents for a variety of cancers [10, 11, 38]. Accordingly, the retinol-esterifying enzyme LRAT has been the subject of retinoid and tumor research for many years. Recently, we found that human melanoma possesses high LRAT expression and high esterification potential when compared to human melanocytes [20]. By studying retinoid metabolism in retinoid-responsive murine B16F10 cells, we hoped to discover novel successful strategies to overcome resistance of human melanoma to retinoid treatment.

Studies in LRAT knockout mice revealed that LRAT is the predominant retinol acyltransferase regulating retinol levels [37, 39–41]. To our knowledge, this is the first study presenting evidence that LRAT expression is near-
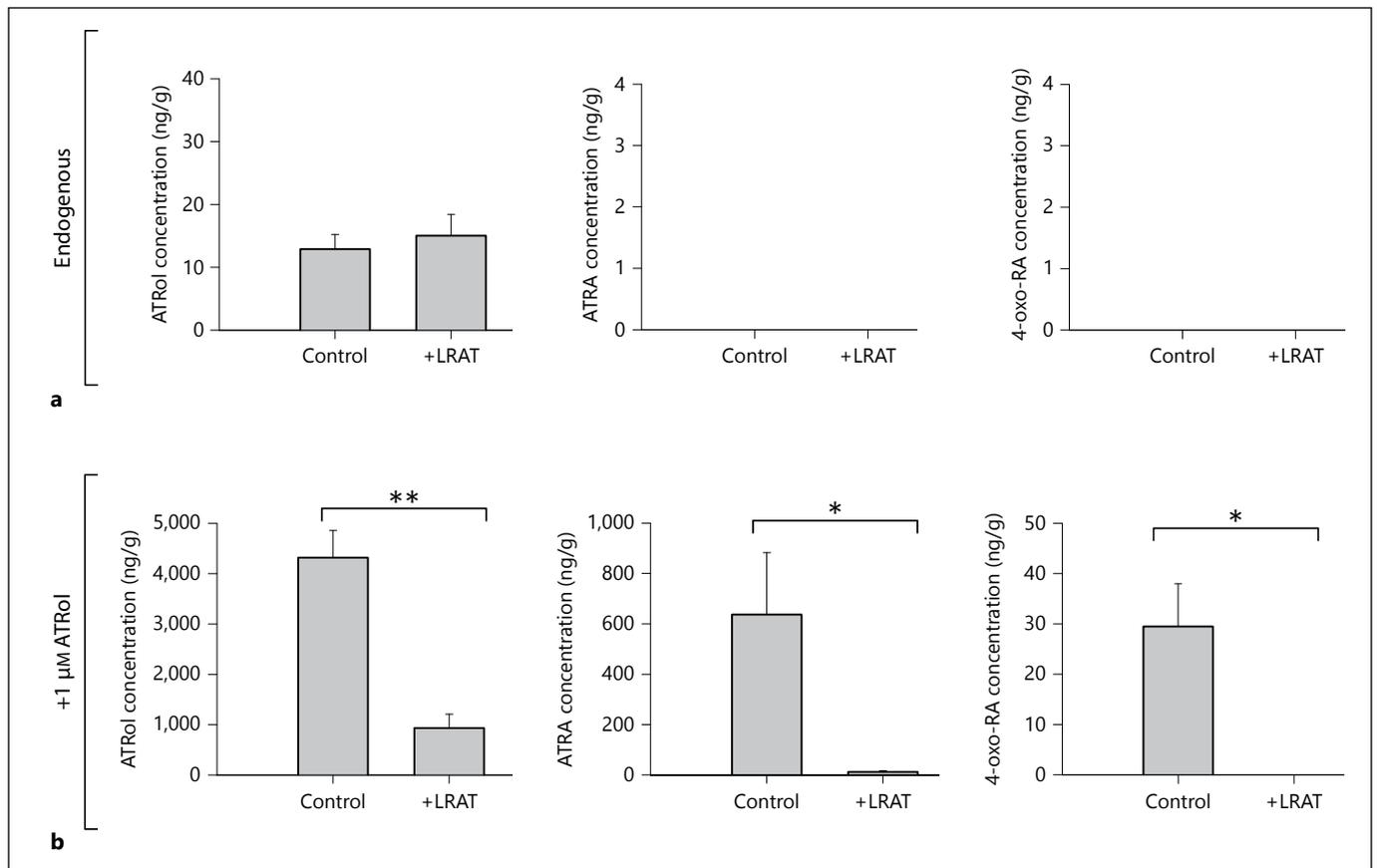
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Fig. 4. LRAT overexpression reduces levels of biologically active retinoids in the murine melanoma B16F10 cell line. **a** Endogenous levels of ATRol, ATRA and 4-oxo-RA in B16F10 melanoma cells were determined by LC-MS analysis. Levels of ATRA and 4-oxo-RA were below the detection limit. **b** Levels of ATRol, ATRA and 4-oxo-RA 24 h after incubation with 1 μM ATRol. Experiments were performed in triplicate and were repeated twice. * p < 0.05, ** p < 0.01: considered as significant.

ly absent in the retinoid-sensitive murine melanoma cell line B16F10. Additionally, retinoids did not influence LRAT expression in these murine cells as was previously described in human melanoma cell lines [20].

The growth-inhibitory and prodifferentiating effects of various endogenous and synthetic retinoids are well characterized in murine melanoma cell lines like B16F10 [23, 26, 27, 30, 31]. For this reason, this cell line was chosen for our experiments. A hallmark of cancer cells is the alteration of metabolic pathways [42]. However, the molecular changes responsible for the biological activity of retinoids in murine melanoma are not well understood. It was shown in B16 melanoma cells that ATRA treatments induce an increase in protein kinase C RNA and protein levels, thus resulting in altered gene transcription via phosphorylation of the transcription factor complex activator protein-1 [30], the activity of which is itself also increased by ATRA in B16 cells [43], and activator pro-

tein-1 was assumed to contribute to the final phenotype of growth arrest and differentiation by ATRA [26]. Estler et al. [44] performed microarray analysis and showed that ATRA 'normalized' the expression of genes involved in energy metabolism, DNA replication, DNA repair and differentiation in B16 melanoma cells. Pathway analysis suggested that CDC2, CHEK1, CDC45L and MCM6 are key players in mediating the biological activity of ATRA in B16 melanoma cells [44]. Importantly, we showed that LRAT overexpression decreased these retinoid-induced antiproliferative effects in B16F10 cells in vitro, thus indicating that retinol metabolism is important for the mediation of tumor resistance towards retinoid treatment.

Regulation of retinoid metabolism is complex [1, 5, 6], and aberrant vitamin A metabolism in cancer cells could represent a potential retinoid resistance mechanism in tumor cells. Accordingly, both the synthesis and metabolism of the bioactive metabolites of ATRol are sometimes
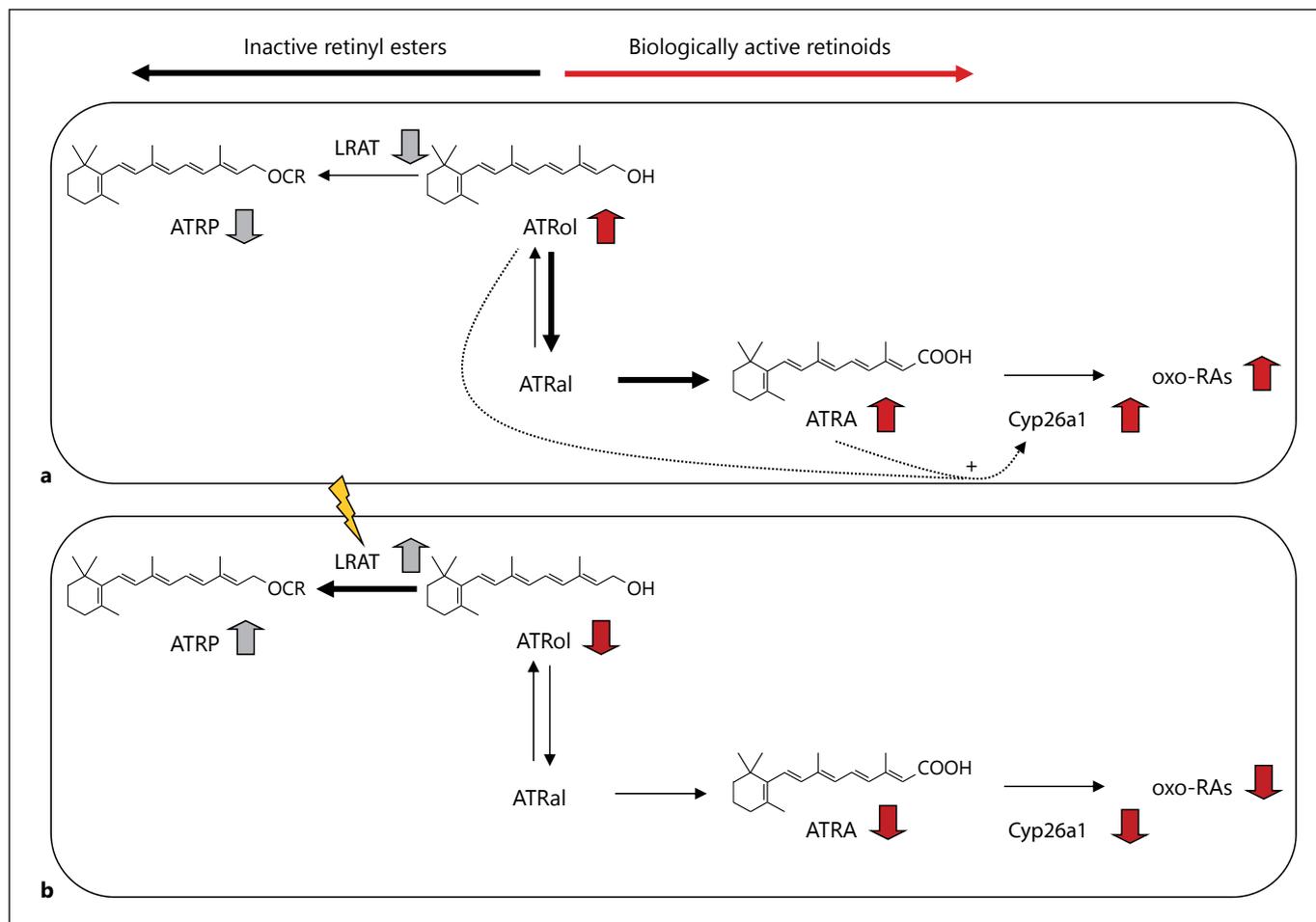
Fig. 5. Direct link between LRAT expression and regulation of biologically active ATRA levels in B16F10 cells (**a**) and LRAT-overexpressing B16F10 cells (**b**). Various enzymes, binding proteins and further retinoid derivatives are not illustrated in this schematic. Dotted arrows (+) represent gene upregulation pathways induced by retinoids.

impaired in cancer cells [10, 11]. The major catabolic enzyme known to regulate ATRA levels is Cyp26a1 [34, 45]. It can decrease intracellular levels of ATRA by metabolism to its oxo- and hydroxy-derivatives [46]. For example, in squamous cell carcinoma, Cyp26a1 expression, activity, turnover rate and catabolism of ATRA are increased when compared to normal keratinocytes [47]. Cyp26a1 was also previously described as an autoregulated retinoid target gene [34–37]. Its expression correlates with the tissue retinol level [36], and its expression levels were used to monitor intracellular retinoid levels and retinoid signaling [37]. Previous reports described Cyp26a1 regulation in LRAT knockout mice [37, 48]. Cyp26a1 expression in different tissues increased in LRAT knockout animals when compared to wild-type animals [48] and correlated with the tissue retinol level in

the LRAT knockdown mice [37]. Consistently with low ATRol and ATRA levels in transfected B16F10 + LRAT cells, Cyp26a1 expression was reduced when compared to B16F10 control cells lacking LRAT (fig. 3). If the observed effect on Cyp26a1 expression is mediated by an LRAT-induced deficiency of biological active retinoids, the addition of ATRA should diminish this effect. Indeed, in this rescue experiment, we showed that ATRA treatment leads to the upregulation of Cyp26a1 expression in B16F10 + LRAT cells. These results provide evidence that the effects of LRAT overexpression on Cyp26a1 expression can be decreased after ATRA treatment. The regulation of Cyp26a1 expression in the LRAT-overexpressing B16F10 cells suggests that LRAT also functions as an important regulator of intracellular retinoid concentrations and signaling.

Amann/Czaja/Bazhin/Rühl/Eichmüller/
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Another enzyme, the acyl-CoA:diacylglycerol acyltransferase 1, functions as an acyl-CoA:retinol acyltransferase in murine skin [49]. Acyl-CoA:diacylglycerol acyltransferase 1 deficiency resulted in elevated levels of ATRA in the skin and caused enhanced sensitivity to topically administered retinol [49]. This is consistent with our results indicating retinol esterification as an important regulatory mechanism influencing the synthesis of ATRA from its precursor ATRol.

To verify the retinoid signaling effects induced by LRAT overexpression, endogenous retinoids were determined using LC-MS measurements. Endogenous levels of retinoids were comparable in the B16F10 + LRAT cell and its control. However, inactive retinyl ester levels increased, and levels of the substrate ATRol and biologically active ATRA significantly decreased in LRAT-overexpressing B16F10 cells incubated with ATRol.

These results revealed a direct link between LRAT expression and the regulation of ATRA levels (fig. 5). We assume that in the presence of LRAT, ATRol can be efficiently converted to retinyl esters in B16F10 + LRAT cells. Therefore, less ATRol is available for ATRA synthesis and retinoid signaling monitored by the expression of the ATRA-induced Cyp26a1 protein is reduced. Interestingly, the ATRA metabolite and product of Cyp26a1, 4-oxo-RA, which is normally quickly further metabolized to water-soluble 4-oxo-retinoids, was also shown to possess biological activity in the skin [50]. We found that its levels are also decreased in LRAT-overexpressing melanoma cells.

In conclusion, our results indicate that the absence of retinol esterification led to higher ATRol levels remaining for further metabolism to active ATRA. Therefore, LRAT deficiency is important for mediating retinoid responsiveness in the murine melanoma cells. Our data suggest that LRAT overexpression represents a novel mechanism by which tumor cells can escape ATRA-induced tumor-suppressive RAR signaling.

Disclosure Statement

The authors have no conflict of interest to declare.

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