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RESEARCH ARTICLE

# Interaction between mevalonate pathway and retinoic acid-induced differentiation

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All *trans* retinoic acid (ATRA) is a potent inducer of differentiation of HL-60 cell line. The pretreatment of the cells by compactin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl (HMG) CoA reductase, during 24 hours, enhances the ATRA-induced cell differentiation. At 50 nM, the percentage of cell differentiation is 34.9% ± 2 and 73% ± 2.96 in the control and compactin-treated cells, respectively. The removal of compactin boosts the level of HMG-CoA reductase and therefore the biosynthesis of sterol and nonsterol isoprenoid compounds. The participation of sterol and nonsterol pathway was then investigated. The supply of an excess of cholesterol (up to 80 $\mu$g/ml of LDL) leads to a significant decrease of cell differentiation by ATRA from 78% ± 0.1 to 54% ± 2.8. A concomitant decrease of cell growth (51% ± 6.4) was observed. The pretreatment of cells by the geranylgeranyltransferase inhibitor (GGTI-298) has no effect on the cell differentiation process. By contrast, the farnesyltransferase inhibitors (FTI-II and FTI-277) completely abolish the ATRA-induced differentiation, thus confirming the involvement of farnesylated proteins in the differentiation mechanism.

## INTRODUCTION

The retinoids play an important role in regulating a broad range of biological processes such as growth differentiation and development in a variety of cell types and tissues [1].

All *trans* retinoic acid (ATRA) exerts a potent differentiating action on human myelogenous leukemia HL-60 cell line and primary bone marrow cultures from patients with acute myelogenous leukemia (AML) [2, 3]. One mechanism for the differentiating activity of ATRA in cells involves the RA nuclear receptors (RARs and RXR) [4], which have specific high-affinity binding sites for ATRA and some of its metabolites [5–7]. Other mechanisms may also be involved in RA-induced differentiation including retinoic acid acylation [8, 9].

A potent differentiating action of ATRA on leukemic cell line is accompanied by a marked cell growth inhibition, as evidenced by an increase of cells in $G_0$ and a reduction of cells in S phase [10]. However, until now, no clear evidence has been given to prove if both processes are independent or closely related. Our work focused on a possible common pathway based on mevalonate (MVA) metabolism.

Proliferation of cells is known to require at least 2 products synthesized from mevalonate: cholesterol and nonsterol isoprenoid derivatives, including farnesylated proteins especially those of *ras* family [11].

HMG-CoA reductase functions as the rate-limiting enzyme of the MVA pathway. It is highly regulated by a negative feedback mechanism. Indeed, the sterols repress transcription of the HMG-CoA reductase gene through a short sequence in the 5′ flanking region of the gene designated SRE-1 [12–14]. Nonsterol and sterol compounds control HMG-CoA reductase translation [15] and degradation rate [16, 17].

Mevalonate homeostasis is achieved through (i) sterol-mediated feedback repression of the genes for HMG-CoA synthetase, HMG-CoA reductase, and the LDL receptor and (ii) posttranscriptional regulation of HMG-CoA reductase [11].

The studies have identified a number of proteins with mevalonate-derived prenyl groups attached post-translationally [18, 19]. Growth-regulating p21ras proteins encoded by *ras* protooncogenes and oncogenes are covalently attached to farnesyl residues which anchor them to the cell membrane.

In the search for MVA-derived compounds involved in growth control, much attention has been aimed at prenylated proteins [20]. Among them, Ras proteins have attracted special interest [21]. Indeed, p21ras proteins are involved in cell growth, and oncologically mutated forms of *ras* are found in a wide variety of human tumors [18, 22, 23]. Interestingly, Prendergast *et al.* [24] reported that inhibition of *ras* farnesylation with farnesyltransferase inhibitors leads to transformed phenotype reversion. However, these authors suggested that other prenylated proteins than Ras might be implicated in this mechanism.

Ras proteins are processed through a series of reactions that result in either farnesylation or geranylgeranylation at a cysteine residue at the fourth amino acid position from the carboxyl-terminal end [25].

The maturation of Ras proteins, heterodemic G proteins

($\gamma$ subunit), nuclear lamins (A and B, and rhodopsin kinase, among others, requires their covalent attachment to $C_{15}$ (farnesyl) or $C_{20}$ (geranylgeranyl) isoprenoids derived from mevalonate [11, 26, 27]. Isoprenylation-dependent membrane anchorage and subcellular localization of Ras protein is often required for their maturation and function[26–29].

The farnesylated proteins are necessary for cell growth [18, 19]. On the other hand, induction of differentiation is well known to parallel a decrease of cell growth [10]. Here, we put in evidence that nonsterol compounds, derived from mevalonate pathway, could be a common key element involved in these two processes.

## MATERIALS AND METHODS

### *Cell culture and reagents*

HL-60 promyelocytic leukemic cells (American Type Culture collection, Rockville, MD) were grown in RPMI 1640 (GIBCO, France) supplemented with 15% heat inactivated fetal calf serum (GIBCO, France) and 2 mM L-glutamine in a humidified atmosphere of 95% air, 5% $CO_2$. ATRA, from Hoffman-La Roche, France, was dissolved in dimethylsulfoxide (DMSO) at an initial stock concentration of 0.01 M and stored at −20°C. At the time of analysis all samples were allowed to thaw in the dark at room temperature and diluted at the appropriate concentration in RPMI 1640 medium. In all cell cultures, the concentration of DMSO never exceeded 0.01%. Compactin was obtained from Sigma and Farnesyltransferase inhibitor II from Calbiochem. Farnesyltransferase inhibitor 277 (FTI-277) and geranylgeranyltransferase inhibitor 298 (GGTI-298) were kind gifts from Said M. Sebti and Michelle A. Blaskovich (University of South Florida, H. Lee Moffitt Cancer Center & Research Institute).

### *Low-density lipoprotein (LDL) preparation*

Human LDL was prepared by ultracentrifugation using a Beckman TL 100 ultracentrifuge and a Beckman TL 100.2 fixed angle rotor [30].

### *Induction of cell differentiation*

HL-60 cells were suspended in growth medium at $10 \times 10^4$ cells/ml in the presence or absence of the indicated agent. The cell viability was assessed by trypan blue exclusion test. Differentiation was estimated by NBT reduction as previously described [31]. A minimum of 300 cells was counted and the percentage of differentiation (*i.e.*, percentage of NBT positive cells) was calculated.

### *Western blotting*

Cells were lysed with 2× Laemmli sample buffer. Samples ($10^6$ cells/100 $\mu$l) were boiled, sheared, and clarified by centrifugation in a microcentrifuge prior to storage at −20°C. Samples were separated on a 12.5% SDS polyacrylamide gel and electrophoretically transferred to nitrocellulose membrane. Membrane was blocked for 2 hours in TBS (50 mM Tris, 150 mM NaCl, pH 7.5) containing 0.1% Tween 20 (TBST) and 5% powdered milk before antibody addition. Membrane was probed with monoclonal antibody directed against *H-ras*, or with monoclonal antibody directed against *K-ras* or with polyclonal antibody directed against *N-ras*, all from Tebu-Santa Cruz, France. Detection was performed with ECL chemiluminescence system (Amersham).

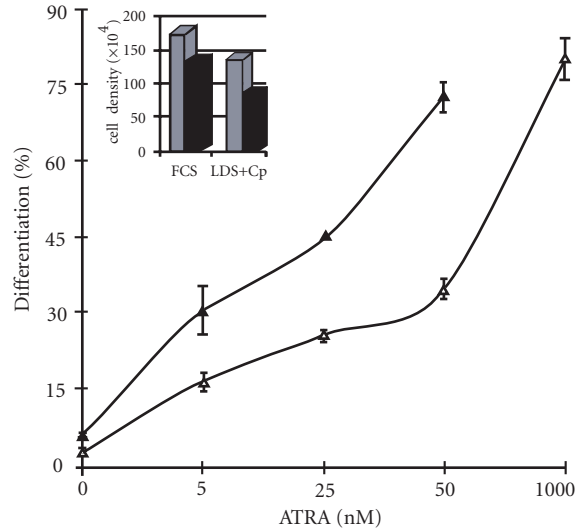


Figure 1: Effect of ATRA on HL-60 cell differentiation. HL-60 cells were incubated for 24 hours in foetal calf lipoprotein deprived serum (LDS medium) supplemented with compactin (2 $\mu$M). Then, they were washed in phosphate buffered saline and cultured during 5 days in LDS medium containing increasing concentrations of ATRA (▲). The control cells were cultured during 5 days in FCS medium containing increasing concentrations of ATRA (△). At day 5, cell differentiation was determined by NBT reduction and viable cell number as determined by Trypan blue exclusion. Insert: Effect on cell growth. ATRA-treated cells 50 nM (■) and control cells (■).

## RESULTS

### *Effect of ATRA on differentiation of compactin-HL-60 treated cells*

The presence of compactin and cholesterol-deprived medium (LDS) had no effect on the basal differentiation activity of HL-60 cells. The treatment of HL-60 cells with ATRA induced a gradual increase of differentiated cells. When endogenous cholesterol synthesis was inhibited, in a first step for 24 hours by incubation in LDS containing compactin (2 $\mu$M), then the removal of compactin induced an increase of the sterol and nonsterol pathways, a possible consequence of the overexpression of HMG-CoA reductase, ATRA induced cell differentiation but the effect was more pronounced in the cells temporarily treated with compactin. At 50 nM, ATRA induced, respectively, 34.9% ± 2 and 73% ± 2.96 of cell differentiation (Figure 1), in the control and compactin-treated cells, whereas 1000 nM was required to obtain 80% ± 4 in control cells.

Furthermore, at 50 nM of ATRA, a strong increase of growth inhibition was observed in the differentiated compactin-treated cells compared with control cells (80.5 ± 5 *versus* 132.5 ± 30) (Figure 1).

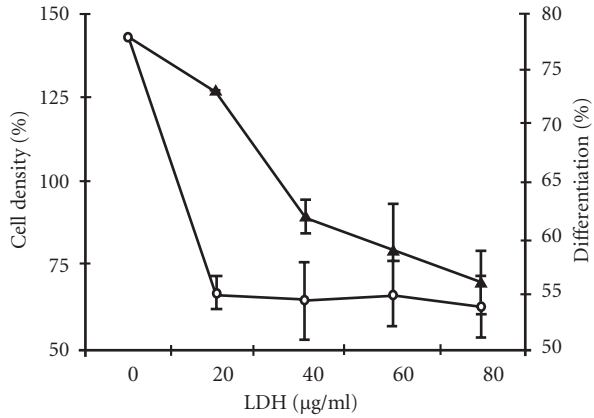


FIGURE 2: Effect of higher low-density lipoprotein concentrations on growth and differentiation of HL-60 cells by ATRA. HL-60 cells were incubated during 5 days with 1 $\mu$M ATRA and increasing concentrations of LDL. Differentiation as determined by NBT reduction (◦) and viable cell number as determined by Trypan blue exclusion (▲).

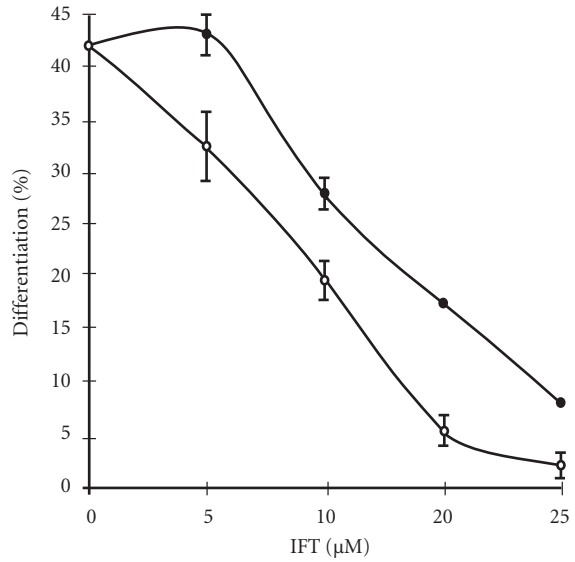


FIGURE 3: Effect of farnesyltransferase inhibitors on differentiation of HL-60 cells by ATRA. HL-60 cells were pretreated with increasing concentrations of FTI-II (•) or FTI-277 (◦). After 24 hours, The ATRA (1 $\mu$M) was added to culture medium. Cell differentiation was determined at day 4.

### *Effect of increasing concentrations of low-density lipoprotein on differentiation of HL-60 cells by ATRA*

In order to test the cholesterol effect on ATRA-induced cell differentiation, the cells were incubated in the presence of ATRA (1 $\mu$M) and increasing concentrations of LDL, up to 80 $\mu$g/ml. Under such conditions, the differentiation of ATRA-treated cells was decreased from 78% to 54% ± 2.8. Interestingly, a concomitant decrease of cell growth (48%) was evidenced in the presence of high concentrations of LDL (Figure 2).

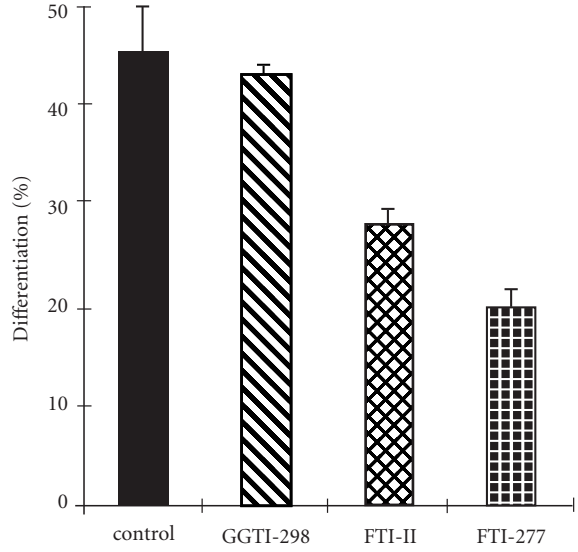


FIGURE 4: Effect of prenylation protein inhibitor on differentiation of HL-60 cells by ATRA. HL-60 cells were pretreated with 10 µM of GGTI-298 or FTI-II or FTI-277. After 24 hours, The ATRA (1 µM) was added to culture medium. Cell differentiation was determined at day 4.

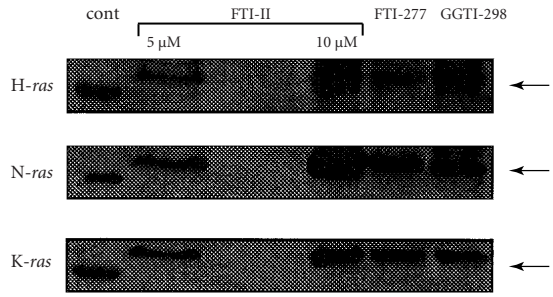


FIGURE 5: Inhibition of *H-ras*, *N-ras*, and *K-ras* prenylation by FTI-II, FTI-277, and GGTI-298 HL-60 cells were treated with 5 or 10 $\mu$M of FTI-II or 10 $\mu$M of FTI-277 or GGTI-298 for 24 hours. Cell lysates were analyzed by Western blotting with either a *H-ras* monoclonal antibody or a *N-ras* polyclonal antibody or a *K-ras* monoclonal antibody. Arrows, unprenylated *ras* bands.

### *Evidence for the involvement of prenylated proteins in HL-60 cell differentiation*

The cells were preincubated in the presence of the farsnesyltransferase inhibitor II or farnesyltransferase inhibitor FTI-277 during 24 hours, then treated by ATRA during 3 days (Figure 3). A strong decrease of ATRA-induced differentiation was observed with increasing concentrations of both inhibitors. FTI-277 was a more potent inhibitor compared with FTI-II (Figure 3). Indeed, the differentiating activity by ATRA

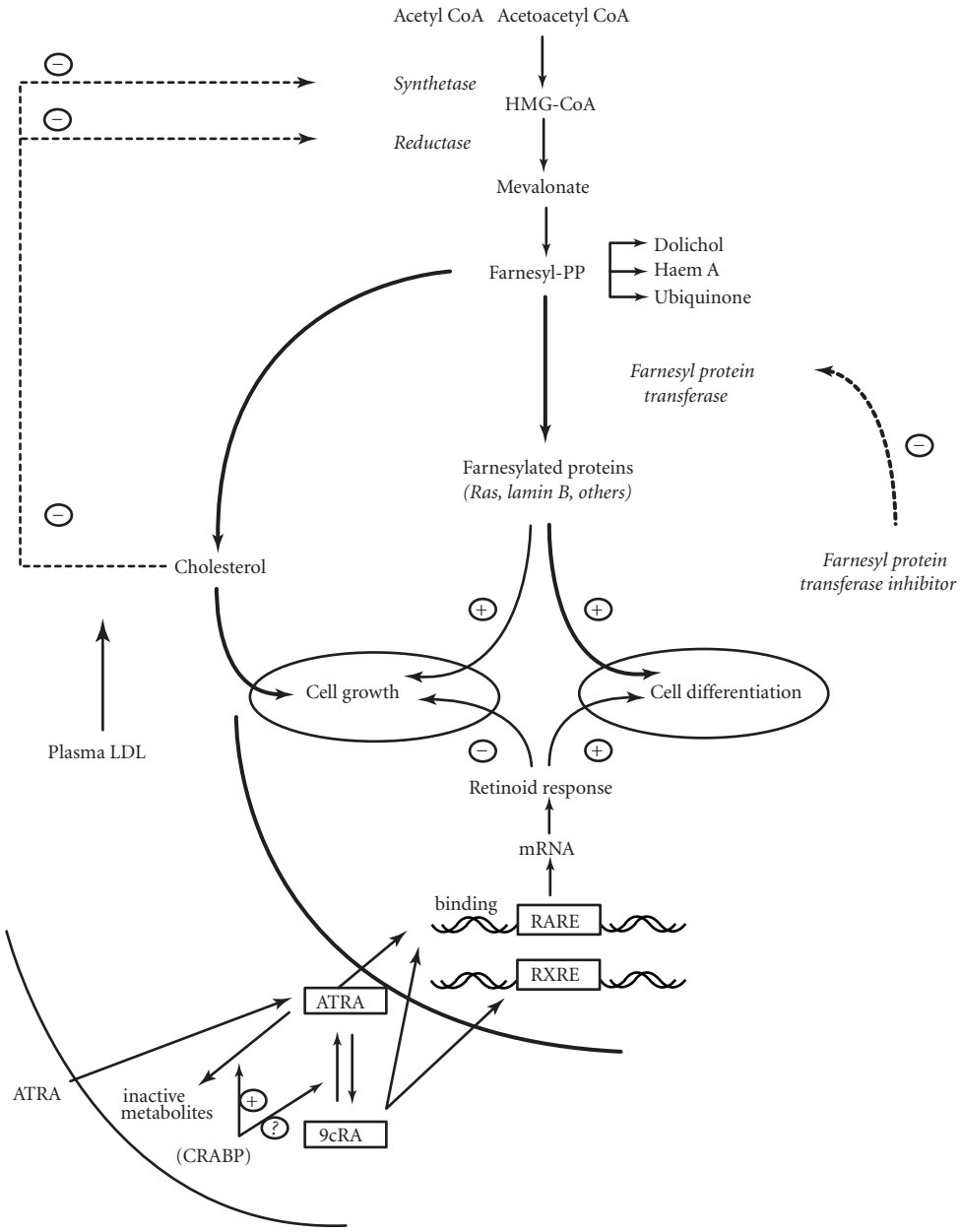


Figure 6: Interaction between mevalonate pathway and retinoic acid-induced differentiation.

was totally abolished at 25 $\mu$M of FTI-277 (Figure 3) and 40 $\mu$M of FTI-II (data not shown). These results confirmed the involvement of the nonsterol mevalonate pathway in cell differentiation.

When the cells were preincubated in the presence of 10 $\mu$M of GGTI-298 during 24 hours, then treated by ATRA during 3 days, no significant result was observed compared with control cells whereas, at the same concentration (10 $\mu$M), FTI-II and FTI-277 decrease ATRA-induced differentiation of the cells (Figure 4). The prenylation of *H-ras*, *K-ras*, and *N-ras* in the cells is effectively inhibited under the conditions tested (Figure 5). This result suggested that farnesylated proteins, such as *H-ras*, *K-ras*, and *N-ras*, could be implied in the mechanism of cell differentiation by ATRA.

## DISCUSSION

At 50 nM, ATRA exhibited 34.9% and 73% of cell differentiation in control and compactin-treated cells, respectively. A 24 hours treatment of HL-60 cells by compactin, a potent HMG-CoA reductase inhibitor, increased their sensitivity to ATRA. In fact, HMG-CoA reductase is one of the most highly regulated enzymes. Compactin is well known as inducing an overexpression of reductase protein. In cultured cells, com-

pactin blocks the synthesis of MVA and triggers adaptive reactions that yield a 200-fold increase in reductase protein within a few hours [11]. After removal of compactin, this enzyme becomes active, boosts the mevalonate pathway with concomitant increase of the sterol and nonsterol products [11]. The increase of ATRA differentiating activity in compactin-treated cells could be therefore a consequence of an increase in the sterol or nonsterol compounds or both.

A first attempt was made to check the effect of an excess of cholesterol supply. Increasing concentrations of LDL (up to 80 $\mu$g/ml) enhanced growth inhibition and decreased cell differentiation induced by 1 $\mu$M of ATRA (Figure 3). In the two cases, a limit was reached and this could be a consequence of the remaining activity of endogenous mevalonate pathway [11]. HL-60 cells have numerous receptors for LDL which can therefore deliver cholesterol to the cells [32]. The inhibitory effects could be attributed to the decrease of the nonsterol products.

To confirm the effect of nonsterol products, on cell differentiation, HL-60 cells were preincubated with the prenylation inhibitors. In the presence of FTI-II, FTI-277 specific inhibitors for farnesyltransferase, ATRA differentiating activity can be totally abolished. By contrast, GGTI-298 has no significant effect. Comparison of the results confirm a predominant role for farnesylation (inhibited by FT-277) compared with geranygeranylation (inhibited by GGTI-298). A less specific inhibitor FTI-II, acting on the two types of isoprenoid compounds, has less pronounced effects. Western blots electrophoresis confirms that, at the concentration used, farnesylation and geranygeranylation reactions were actually inhibited.

Farnesyl proteins include Ras proteins that are involved in the transduction of mitogenic signals. In addition, our results show that compounds of the farnesyl proteins family including *H-ras*, *N-ras*, and *K-ras* could be involved in the retinoic acid-induced differentiation of HL-60 cells. Figure 6 shows the regulation of HMG-CoA reductase activity and the role of farnesylated proteins on growth and cell differentiation. ATRA is well known to act up on these processes but, in addition, our results show that farnesyl proteins play a key role in the ATRA-induced differentiation of HL-60 cells. A mechanism, involving RAR and/or RXR retinoic acid receptors, cannot be excluded but requires further investigations.

Taken together, these results (i) suggested the involvement of mevalonate pathway in ATRA differentiating effect on HL-60 cells, and (ii) support the use of HMG-CoA reductase inhibitors as potential adjuvant therapeutic agents in differentiating chemotherapy.

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