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INHIBITION OF 3T3 ADIPOGENESIS BY RETINOIC ACID IS NOT MEDIATED BY CYTOPLASMIC RETINOIC ACID-BINDING PROTEIN

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SUMMARY: Retinoic acid (RA) inhibits 3T3 adipogenesis in a dose-dependent and reversible manner, but its mechanism of action remains unknown. 3T3-F442A cell variants obtained by mutagenesis with nitrosoguanidine and/or selection with high RA concentrations showed different resistance to RA cytotoxicity and underwent adipose conversion of various extents when they were cultured in adipogenic conditions. Commitment to adipose differentiation was also inhibited by RA in these clones. Gel filtration chromatography showed the presence of a cytosolic RA-binding activity in the parental cells but not in three of the variant clones isolated. We demonstrate that cytoplasmic RA-binding activity is not essential for the inhibitory effects of the retinoid on 3T3 adipogenesis, or for resistance to RA cytotoxicity. Other mechanisms should be involved to explain the inhibition of adipose differentiation by RA.

Retinoic acid (RA) modulates proliferation and differentiation in many cell types (1, 2), but its mechanism of action remains unclear. Two types of retinoid nuclear receptors mediate the effects of these compounds (3, 4). Various cytoplasmic retinoid binding proteins have also been described (5, 6), although their function is still controversial. Some studies have shown a direct relationship of RA biological activity with its binding affinity by cytoplasmic retinoic acid-binding protein (CRABP) (7, 8). Embryonic carcinoma F9 cell mutants lacking CRABP activity are not induced to differentiate by RA (9, 10), and F9 mutants that overexpress CRABP mRNA are more sensitive to induction of differentiation by RA (11). However, other experiments suggested that CRABP activity might not be essential for RA modulation of gene expression (8, 12-14), or that CRABP role might depend on cell type.

Retinoic acid reversibly inhibits adipogenesis of 3T3-F442A cells by blocking some events prior to cell commitment (15, 16), but the mechanism by which RA exerts its action remains unknown. To study the role of CRABP in RA inhibition of adipose differentiation, 3T3-F442A cell variants were obtained and analyzed for their response

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to RA and CRABP activity. Our results show that 3T3-F442A preadipocytes have CRABP activity, and that three variant clones lacking CRABP activity were similarly inhibited by RA. Thus, CRABP activity is not essential for inhibition of adipose differentiation by RA; other mechanisms should be involved.

MATERIALS AND METHODS

Materials: Calf serum was obtained from HyClone Labs. (Logan, UT) and cat serum from Colorado Serum Co. (Denver, CO). N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG) was from K & K Labs. (Plainview, NY). [^{3}H]All-trans-retinoic acid was a gift from Hoffman-LaRoche (Nutley, NJ). Insulin, d-biotin, bovine serum albumin (BSA), all-trans-retinoic acid, and Oil red O were from Sigma Chemical Co. (St. Louis, MO). Sephadex G75 was obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden). All other reagents were analytical grade.

Cell culture conditions: 3T3-F442A cells or the variant clones were inoculated in Corning tissue culture dishes at 7 x 10^2 cells/cm^2 in Eagle's medium modified by Dulbecco and Vögt (DMEM), supplemented with 5% (v/v) calf serum. Three days after, the cells were changed to adipogenic medium: DMEM containing 5% calf serum, 5 µg/ml insulin and 1µM d-biotin (17), containing or not 10 µM RA or non-adipogenic medium: DMEM containing 5% cat serum, 5 µg/ml insulin and 1µM d-biotin(18). Cells were maintained as previously described (15) and adipose conversion was quantitated (19).

Isolation of RA-resistant cell variants: Exponentially growing cultures of 3T3-F442A cells were incubated for 16 hrs. in medium containing 1.0 or 2.0 µg/ml of MNNG. Then, cultures were washed exhaustively with serum-free DMEM and refed with medium containing 5% calf serum. After 3 days, cultures were trypsinized and cells were inoculated at 100 cells per 60 mm dish in medium supplemented with 10% calf serum. Three days after, cultures were refed with medium containing 5% calf serum and 0.1 mM or 0.2 mM RA. After ten days, individual colonies resistant to the high RA concentrations were isolated using stainless-steel cloning cylinders. Some clones were not treated with MNNG and they were isolated by their resistance to 0.1 mM RA.

Determination of CRABP activity: CRABP was determined by gel filtration chromatography of cytosolic extracts incubated with [^{3}H] all-trans-retinoic acid ([^{3}H]RA) (14, 20). Confluent cultures of preadipocytes were rinsed three times with PBS, and the cytosolic fraction was obtained (20). Five mg of protein were mixed with [^{3}H]RA (15.8 Ci/mmol) to a final RA concentration of 100 nM in the presence or absence of a molar excess of 1000-fold non-labeled RA to determine specific binding of [^{3}H]RA. Assay mixtures were incubated in the dark for 16 hrs. at 4° C, and chromatographed through a Sephadex-G75 column (0.7 x 30 cm) previously equilibrated with extraction buffer (20). Elution volume for CRABP was determined using ribonuclease A (13,700 Da) as molecular weight marker. The column was eluted with the same buffer solution; 0.3 ml fractions were collected, and radioactivity was quantitated by liquid scintillation counting.

RESULTS

We obtained 30 variant clones from 3T3-F442A cells by mutagenesis and/or selection with high concentration of RA as described in Materials and Methods. The variant

clones were cultured in adipogenic medium with or without RA to evaluate their ability to differentiate into adipocytes, and whether they were inhibited by the retinoid. Twenty days after inoculation, some clones (e.g., 3T3-F442A/C54 and 3T3-F442A/C4) exhibited a high level of adipose differentiation similar to the parental cells, while other clones (e.g., 3T3-F442A/C5 and 3T3-F442A/C60) showed very low or null differentiation (Table 1). All adipogenic clones were inhibited by 10 μM RA in a similar way as the parental cells had been (Table 1). To test whether RA inhibits commitment to differentiation of the variant clones as in the parental cells (15), some of the cultures of each clone treated with RA were refed with adipogenic or non-adipogenic medium without the retinoid and maintained in these conditions from day 11 to day 20 of culture (15, 16). As is shown in Table 2, both the parental 3T3-F442A cells and the variant clone 3T3-F442A/C6 reversed RA inhibition when they were cultured under adipogenic medium but not under non-adipogenic medium. Similar results were obtained with other variant clones, suggesting that RA inhibits commitment to differentiation in the variant clones tested, as reported for the parental cells (15, 16).

The binding of [^{3}H]RA to cytosolic fractions was assayed to determine the activity of CRABP in the variant clones. Figure 1 shows the elution profiles obtained by gel filtration chromatography; a specific radioactive peak of [^{3}H]RA binding fraction that

Table 1. Adipose Differentiation of 3T3-F442A Cells and Variant Clones

Clone		Adipose Differentiation (Normalized)[a]	
		Adipogenic medium	Adipogenic medium +10 μM RA
3T3-F442A		1.00	0.00
3T3-F442A	/C4 [b], /C6 [b], /C23, /C36, /C42, /C44, /C45, /C46, /C51, /C54	1.00-0.75	0.00
3T3-F442A	/C15, /C20, /C21, /C37, /C48, /C57, /C59	0.75-0.50	0.00
3T3-F442A	/C1 [b], /C2 [b], /C 3, /C5 [b] /C13, /C43	0.50-0.01	0.00
3T3-F442A	/C12, /C16, /C28, /C29, /C52, /C60	0.00	0.00

[a] 3T3-F442A or variant preadipocytes were cultured as indicated. After 20 days, adipose differentation was quantitated (19). Data were normalized using fully differentiated 3T3-F442A cells as reference.

[b] Spontaneous variants obtained only by selection with 0.1 mM RA.

Table 2. Recovery of RA Inhibition of Adipose Differentiation

Initial culture conditions	Final culture conditions	Adipose conversion (%)	
		3T3-F442A	3T3-F442A/C6
Adipogenic	Adipogenic	100.0	100.0
Non-adipogenic	Non-adipogenic	13.0	9.5
Adipogenic + RA	Adipogenic + RA	5.8	7.3
Adipogenic + RA	Adipogenic	33.0	28.1
Adipogenic + RA	Non-adipogenic	5.8	4.4

3T3-F442A or variant preadipocytes were cultured during eleven days under the initial culture conditions and then changed to the final culture conditions. Adipose differentiation was quantitated (19) after 20 days of culture.

coeluted with ribonuclease A was observed in the parental 3T3-F442A cells (Fig. 1), indicating that 3T3-F442A preadipocytes have a specific CRABP activity. However, a similar binding activity was not observed in the variant clones 3T3-F442A/C4,

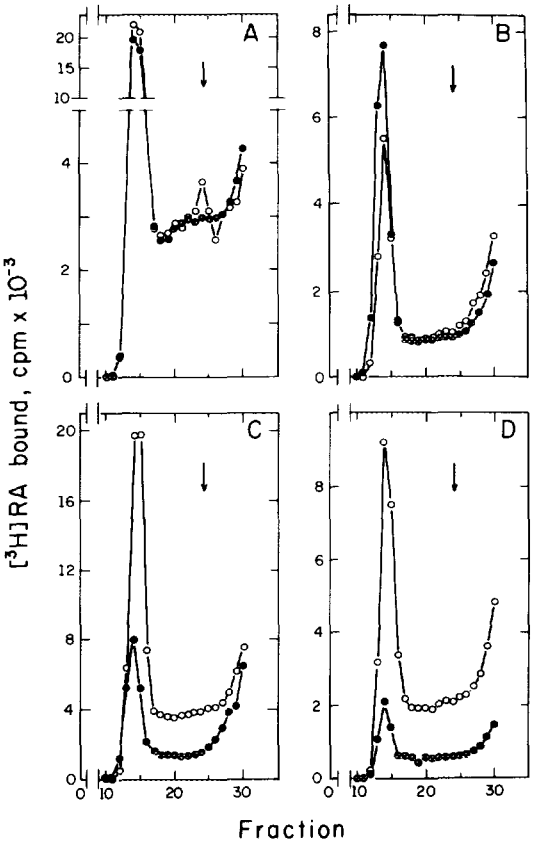

Figure 1. Determination of CRABP activity. Gel filtration analyses of CRABP were carried out as described (see Materials and Methods) incubating cytosolic cell extracts with [^{3}H]RA (15.8 Ci/mmol) in the presence (●) or absence (○) of a molar excess of 1000-fold of non-labelled RA. Fractions 12 to 16 correspond to void volume. Arrow indicates the elution volume of CRABP. A) 3T3-F442A; B) 3T3-F442A/C4; C) 3T3-F442A/C6; D) 3T3-F442A/C44.

3T3-F442A/C6, and 3T3-F442A/C44 (Fig.1B-D). These results indicate that CRABP activity is not detectable in the variant clones and, since these variants were inhibited by RA similarly as the parental cells (Table 1), this activity did not mediate the inhibitory action of RA on 3T3 adipogenesis.

Chemically induced mutants may become genetically unstable after withdrawal of the selecting agent. Thus, the variant clones resistant to high RA concentrations were subcultured several times without RA, and their colony forming efficiency (CFE) was determined in medium containing high RA concentrations. The clones 3T3-F442/C4 and 3T3-F442/C6 exhibited higher CFE than parental cells, both in the absence and in the presence of all retinoid concentrations tested, showing that these variants did not undergo reversion into a less resistant phenotype. The other clone, 3T3-F442A/C44, became more susceptible to the cytotoxic effects of RA (Fig. 2) suggesting that the resistant phenotype of this clone probably was undergoing reversion into a phenotype sensitive to RA cytotoxicity.

DISCUSSION

The role of CRABP in the signaling pathway of RA remains controversial. Initially, CRABP was viewed as a "shuttle" that transports RA to the cell nucleus (7, 21), but recent studies suggested that this protein has a role in controlling the actual concentration of free RA (22, 23), as well as RA metabolism and its intracellular half-life (24, 25). Our experiments by gel filtration chromatography show that CRABP

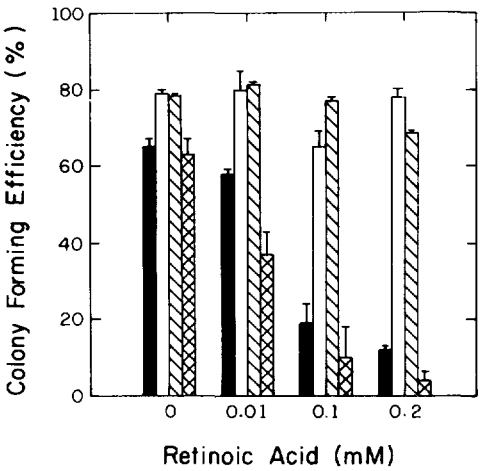

Figure 2. Resistance to RA cytotoxicity. 3T3-F442A preadipocytes (■) or those of the variant clones 3T3-F442A/C4 (□), 3T3-F442A/C6 (▨), or 3T3-F442A/C44 (⊠) were inoculated at 100 cells per 60 mm dish in DMEM supplemented with 10% calf serum. Three days after, cultures were refed with DMEM containing 5% calf serum and the indicated RA concentrations. After 10 days, the cultures were fixed and stained with hematoxylin and colony forming efficency was determined.

activity was present in 3T3-F442A parental preadipocytes but not in the three variant clones tested. In the parental cells, as well as in the variant clones tested, RA inhibited commitment to adipose differentiation. If RA exerts its action at the nuclear level, the transport of RA by CRABP to the cell nucleus seems not to be the mechanism that mediates RA inhibition of adipogenesis in these cells. Thus, other pathways, however unlikely, could be in place for RA to reach and interact with the nuclear receptors present in 3T3 preadipocytes (26, 27).

An alternative hypothesis on the mechanism of action of the retinoids suggests that these compounds can change the pattern of cell surface glycosylation (28). Recent works have shown that RA modulates the adhesion of mouse fibroblasts to different components of extracellular matrix (29), alters the expression of glycoconjugates on the surface of squamous carcinoma cells (30), and induces changes in the properties of glycosaminoglycans of melanoma B16 cells (31). Also, RA modulates the function of some membrane receptors modifying the structure of their glycosidic portion (32), or altering the receptor number on the plasma membrane (33, 34). moreover, RA inhibits the expression of lipogenic enzyme activities in 3T3-F442A preadipocytes by changing cytoskeleton assembly (15), which could be explained by changes in fibronectin levels (35, 36). These data, and our results showing a RA effect independent of CRABP in the 3T3 preadipocytes, make attractive the idea to explore other mechanisms of action. It is possible that in 3T3 preadipocytes RA may alter the interaction of the adipogenic factors with their cell surface receptors and modulates cell cytoskeleton assembly in order to inhibit commitment to differentiation and the expression of lipogenic enzymes.

The role of CRABP on RA cytotoxicity, as its role on RA modulation of gene expression, remains unclear. It has been proposed that CRABP could control the availability of free RA (22, 23), or that the resistance to RA cytotoxicity might not depend on the absence of CRABP activity (37, 38). Our experiments showing that 3T3 preadipocyte variants lacking detectable CRABP activity exhibit distinct susceptibility to cytotoxic effects of RA (Fig. 1), seem to reinforce the view that resistance to RA cytotoxicity does not depend on the absence of CRABP activity (37, 38).

In conclusion, our results indicate that CRABP does not mediate either the RA inhibition of 3T3 adipogenesis or the resistance to RA cytotoxicity, since cell variants lacking CRABP activity are similarly inhibited in their differentiation by the retinoid and since the absence of CRABP activity does not confer resistance to RA cytotoxicity. Our results suggest that other mechanisms could mediate RA effects on adipose differentiation. Changes in glycosylation induced by RA at the level of cell membrane, specially on adipogenic factor receptors, should be studied in some of the clones lacking CRABP activity that we have isolated.

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