

THE CONTROL OF CELL DIFFERENTIATION AND GROWTH BY RETINOIC ACID MAY BE RELATED TO ITS EFFECT ON TRANSPLASMA-MEMBRANE DIFERRIC TRANSFERRIN REDUCTASE

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INTRODUCTION

The induction of cell differentiation or maturation by retinoic acid is well documented (Amatruda and Koeffler, 1986; Chou, 1982; Chou and Ito, 1984; Lotan and Lotan, 1980; Sherman, 1986; Strickland and Mahadavi, 1978; Strickland, *et al.*, 1980; Yen, *et al.*, 1987). Retinoic acid also inhibits the growth of several types of cells (Lotan, 1980; Roberts and Sporn, 1984). Relatively little is known about the mechanism of retinoid-induced cell differentiation. It is generally believed that a cellular retinoid-binding protein mediates the control of cell differentiation (Chytil and Ong, 1984; Sherman, 1986). Additionally, a retinoic acid-binding protein (site of action) in plasma membrane has also been described (Cope and Boutwell, 1983; Sani, 1979).

Reduction of impermeable oxidants by a transplasma membrane electron transport system stimulates proliferation of HeLa (Sun, *et al.*, 1985) and melanoma cells (Ellen and Kay, 1983). This electron transport system acts as a diferric transferrin reductase (Navas, *et al.*, 1986), because the external diferric transferrin is reduced, while cytosolic NADH is oxidized (Löw, *et al.*, 1987). Transferrin is a component of serum, and it has been shown to replace serum in the growth of many types of cells (Hutchings and Soto, 1978). Furthermore, cell proliferation has been related to expression of transferrin receptor (Miskimins, *et al.*, 1986). Therefore, diferric transferrin (Fe_2Tf) can be the natural oxidant for growth stimulation by transplasma membrane electron transport (Sun, *et al.*, 1984). In this study, retinoic acid is shown to inhibit the transplasma membrane NADH diferric transferrin reductase activity. The inhibition of this enzyme may explain the growth inhibition by retinoic acid. The study with BALB/3T3 and SV/T2 cells also indicates that a correlation exists between morphology, proliferation index, and this enzyme activity. Recently, an association with growth arrest has been observed during retinoic acid-induced differentiation of F9 cells (Dean, *et al.*, 1986). The inhibition of this diferric transferrin reductase may provide a basis for cell differentiation and growth control by retinoic acid.

MATERIALS AND METHODS

3T3 cells (SV/T2, SV40 transformed, and BALB/3T3 non-transformed) were grown under an atmosphere of 5% CO_2 and 95% air at 37°C. The growth medium was a minimal essential medium containing 10% serum, 100 U of penicillin, and 100 μml of streptomycin at pH 7.4. Cells were maintained in a similar medium

containing 2% serum (Sun, *et al.*, 1983). Confluent monolayer cultures were then prepared for study by pelleting the trypsinized, suspended cells at 15,000 g. The pellet was diluted with TD-Tris buffer (NaCl 8 g/l, KCl 0.38 g/l, Na₂HPO₄ 0.1 g/l, and Trizma base 3 g/l, pH 7.4) to a final concentration of 0.1 g cell wet weight/ml (gww/ml). The harvested cells were in exponential growth phase.

The effect of all trans retinoic acid on cell growth was examined by growing cells in various concentrations of serum with and without the presence of retinoic acid. After two days of incubation at 37°C, cell morphology was examined under the microscope, cells were then harvested, and a cell survival count was immediately taken. Survival was determined by an eosin Y exclusion method as described by Mitchell and Shrigi (1980).

Transmembrane electron transport activity was assayed by measuring the reduction of iron in diferric transferrin by whole cells. The formation of ferrous bathophenanthroline disulfonate was determined according to Avron and Shavit (1963). Absorbance changes between 535 nm and 600 nm were determined in the dual beam mode with the DW2a Aminco spectrophotometer. An extinction coefficient of 17.1 mM⁻¹ cm⁻¹ was used. The assay mixture in 2.8 ml TD buffer contained 3.4 μM diferric transferrin, 3.3 μM bathophenanthroline disulfonate, and 0.015 gww of cells. Diferric transferrin was obtained from Miles Laboratories. Assay temperature was 37°C. Proton release was performed as described previously (Sun and Crane, 1985).

Transmembrane electron transport activities could also be determined by measuring ferricyanide reduction with intact cells. Ferricyanide reduction by cells were performed in an Aminco DW2a dual beam spectrophotometer equipped with a 37°C temperature-controlled chamber and a magnetic stirrer (Crane, *et al.*, 1982). TD-Tris buffer was used. Absorbance changes were measured in the dual wavelength mode using 420-500 nm. The rates were recorded with a Linear recorder. A millimolar extinction coefficient of 1.0 mM⁻¹ cm⁻¹ was used for potassium ferricyanide. There is an initial fast rate of ferricyanide reduction by cells for 1-2 min, followed by a steady slower rate which continues for 10 min or longer (Clark, *et al.*, 1981).

The application of flow cytometer to study the cell cycle distribution has been described previously (Krishan, 1975; Stern and Lindmo, 1979). The cell grew for 72 hr. with or without the presence of retinoic acid. The total DNA content of the cells was measured with a fluorescent dye, propidium iodide, which bound to DNA. After treatment of the cells (10⁶/ml) with propidium iodide (50 μg/ml in 0.1% sodium citrate) for 2 hr., the treated cells were then injected into the nozzle by a motor-driven syringe. The fluorescence resulting from the dye-DNA complex in each cell was quantitated and displayed as a histogram by a computer.

RESULTS

To reduce impermeable oxidants by cellular internal NADH, a redox system is necessary to carry electrons across the plasma membrane. The use of intact cells, therefore, provides the best assay of transmembrane redox enzyme activity. The reduction of Fe₂Tf by whole cells is measured by the formation of a chelate, ferrous bathophenanthroline disulfonate in the external media (Avron and Shavit, 1963). The effect of retinoic acid on Fe₂Tf reductase of BALB/T2 and SV/T2 cells was examined. The results are seen in Table 1. Very little effect of retinoic

TABLE 1. Effect of retinoic acid on the reduction of diferric transferrin by 3T3 cells.

Concentration of Retinoic Acid	Fe ² Tf Reduction (nmoles min ⁻¹ gww ⁻¹)	
	SV/T2 cells	BALB/3T3 cells
None	5.4	12.5
10 ⁻⁸ M	5.1	11.2
10 ⁻⁷ M	4.9	8.4
10 ⁻⁶ M	4.7	6.2
10 ⁻⁵ M	4.3	4.1

TABLE 2. Effect of retinoic acid on redox activities of 3T3 cells.

Redox Activities	Addition	Reduction Rate (n moles/min/gww)	
		SVT2 (Transformed)	BALB/3T3 (Non-Transformed)
Diferric Transferrin Reduction	None	2.1	15.5
	Retinoic Acid (1 x 10 ⁻⁵ M)	1.8	4.93
Ferricyanide Reduction (Fast Rate)	None	93	168
	Retinoic Acid (1 x 10 ⁻⁵ M)	81	24
Ferricyanide Reduction (Slow Rate)	None	15	108
	Retinoic Acid (1 x 10 ⁻⁵ M)	18	0

acid is seen with SV/T2 cells. However, retinoic acid gives up to 70% inhibition of reductase activity with BALB/3T3 cells. A similar effect is also observed for ferricyanide reduction by the non-transformed cells (Table 2).

Redox coupled proton release by BALB/3T3 cells is also inhibited by retinoic acid to an extent similar to electron transport (Table 3), whereas proton release induced by Fe₂Tf with SV/T2 cells gives only a ~10% effect of retinoic acid. Instead of inducing proton release, Fe₂Tf induces proton up-take by SV/T2 cells.

The effect of retinoic acid on cell growth was also examined. Significant growth inhibition caused by retinoic acid is shown by BALB/3T3 cultures only. An insignificant effect is seen with SV/T2 cultures (Table 4).

The morphological study shows that retinoic acid induces several interesting features in BALB/3T3 cultures but causes no significant morphological changes in ST/T2 cultures. These interesting features include: 1) induction of gaps among cells; 2) formation of slim shape with tails; and 3) enlargement of cell size in some cells (Figure 1). There are no morphological differences between the untreated cultures of BALB/3T3 and SV/T2 cells.

The study with flow cytometry provides a study of cell cycle distribution through the fluorescence measurement (DNA content). The various phases of the

TABLE 3. Effect of retinoic acid on redox induced proton release by 3T3 cells.

Redox Agents	Addition	Rate of H ₊ Release (n moles/min/gww)	
		SVT2 (Transformed)	BALB/3T3 (Non-Transformed)
Diferric Transferrin	None	-166	617
	Retinoic Acid (1 x 10 ⁻⁵ M)	-154	221
Potassium Ferricyanide	None	551	904
	Retinoic Acid (1 x 10 ⁻⁵ M)	448	147

(-) indicates proton uptake.

TABLE 4. Effect of retinoic acid on the growth of 3T3 cells.

Concentration of Retinoic Acid	No. Cells/25 cm ₂ flasks (x 10 ⁻⁶)	
	SV/T2 (Transformed)	BALB/3T3 (Non-Transformed)
Control	4.36	5.67
10 ⁻⁹	4.25	5.42
10 ⁻⁸	4.13	4.77
10 ⁻⁷	4.12	4.34
10 ⁻⁶	4.0	4.30
10 ⁻⁵	4.16	4.32

cell cycle provided from the fluorescence measurement give an index of the proliferation pattern. It is interesting to compare the cell cycle parameters of BALB/3T3 and SV/T2 cells before and after treatment with retinoic acid (Table 5). SV/T2 cells treated with retinoic acid show a decrease in the percentage of S phase and a significant increase in the percentage of G2/M phase. In contrast, BALB/3T3 cells show a large increase in the percentage of S phase, while the percentage of G2/M phases drops proportionally after retinoic acid treatment. These opposing effects may explain the rapid growth of SV/T2 cells and the slow growth of BALB/3T3 cells, while retinoic acid (1 x 10⁻⁵ M) is present in cultures.

DISCUSSION

The basis for growth control by transmembrane electron transport is unclear. However, the known effect of this redox activity is the oxidation of cytosolic NADH to increase NAD concentration (Navas, *et al.*, 1986) which can act to regulate inosine monophosphate dehydrogenase, a key enzyme in purine (DNA building block) biosynthesis. Evidence has also shown that this transmembrane redox system is responsive to agents which stimulate cell growth, such as insulin, (Crane, *et al.*, 1982) at the concentration which promotes cell proliferation and is inhibited by antitumor drugs at a concentration which shows killing effects (Crane, *et al.*, 1985; Sun and Crane, 1985). Therefore, it is reasonable to propose

TABLE 5. Effect of retinoic acid on cell cycle parameters.

Cells/Treatment	Cell cycle		
	S (%)	G ² /M (%)	G ⁰ /G (%)
BALB/3T3 (control)	27.55	41.47	30.97
BALB/3T3 (plus RA)	62.45	5.60	31.93
SV/T2 (control)	35.61	16.11	48.27
SV/T2 (plus RA)	17.15	36.38	56.45

RA = Retinoic acid (1×10^{-5} M).

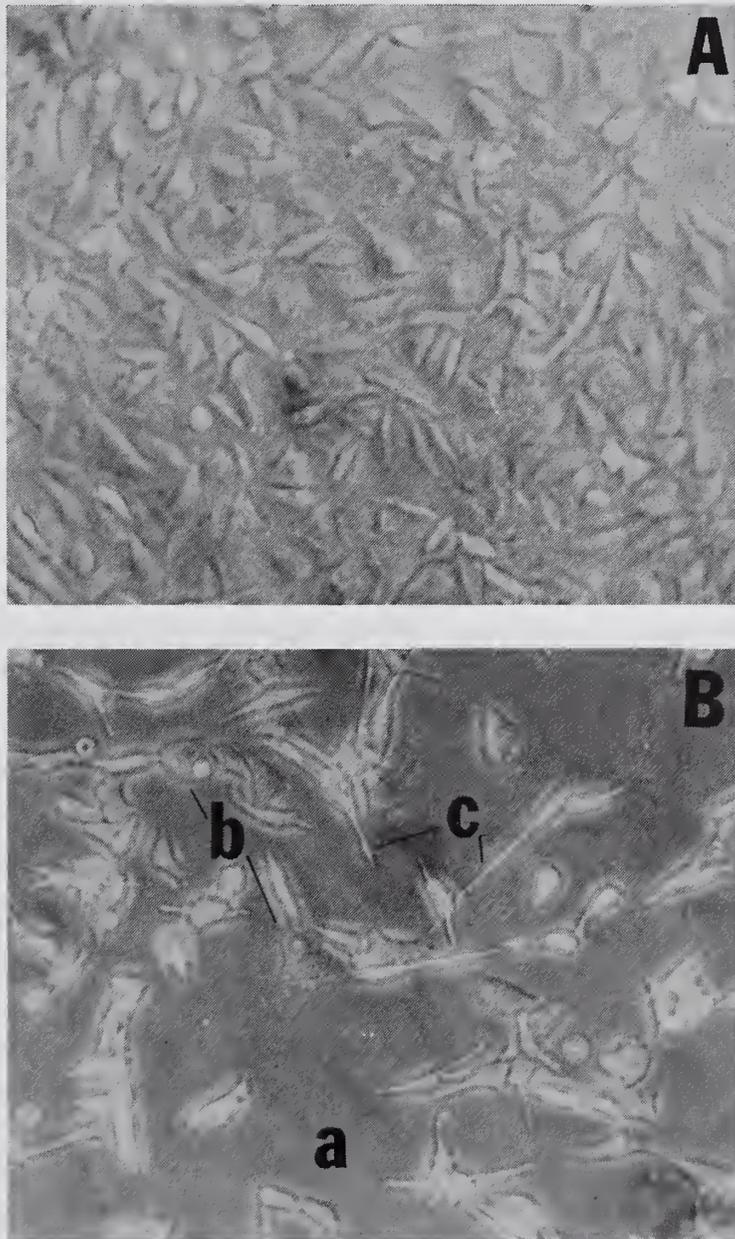


FIGURE 1. Effect of retinoic acid on the morphology of BALB/3T3 cultures: (A) BALB/3T3 controlled cultures; (B) BALB/3T3 treated cultures (a) gapping, (b) enlargement, and (c) tail formation. Treated cells were grown in media containing retinoic acid (10^{-5} M) for 72 hours.

that the control of cell differentiation and growth by retinoic acid may be related to its effect on transplasma membrane redox activities. Data presented in this study, which shows a correlation between morphology, proliferation index, and transplasma-membrane enzyme activity after treatment with retinoic acid, strongly supports the idea.

The difference in sensitivity to retinoic acid between BALB/3T3 cells and SV/T2 cells is similar to that observed by Jetten, *et al.* (1979) in which the inhibition of exponential cell growth and a reduction in saturation density by retinoic acid between transformed and non-transformed mouse fibroblast cells were compared. The *RLA209-15* cell line was established by transforming primary fetal rat hepatocytes with a simian virus 40 (tsA209 virus) that is temperature sensitive for maintenance of transformation. At the nonpermissive temperature (40°C), these cells did not show characteristics of malignant transformation, as they did at the permissive temperature (33°C) but regained the normal differentiated phenotype characterized by an induction of albumin and α -fetoprotein. Under the treatment of retinoic acid, an alteration of the ratio of the functional liver proteins in these cells has been described with a decrease of α -fetoprotein and an increase of albumin production (Chou and Ito, 1984). However, insignificant difference has been observed in cells with transformed phenotype (cell grown at 33°C), for they have very low level of functional liver proteins either with or without the presence of retinoic acid. All these evidences indicate a retinoic acid resistance shown by SV40 viral transformed cells, whereas a strong sensitivity was observed on non-transformed cultures.

A study of retinoic acid on cell cycle parameters indicates that SV/T2 cells show a quick shift of G2/M phases from S phase (Table 5). This may explain why transformed SV/T2 cells are a fast grower and lack cell regulation. In contrast, BALB/3T3 cells show an increase of DNA during the replication phase and a dramatic decline of G2/M phase. The S phase arrest without proceeding to the post-DNA replication phase or mitotic division may explain why the cell size of BALB/3T3 is enlarged, and this is how the alteration of cell morphology (differentiation) is observed on BALB/3T3 cultures. An increase of the relative frequency of myotube-like giant cell formation in rat rhabdomyosarcoma (Gabbert, *et al.*, 1988) and induction of cell flatterring and gapping in B16-F10 (lung tumor) cells (Lotan and Lotan, 1980) by retinoic acid have also been described.

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