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Transformation with SV40 Virus Prevents Retinoic Acid Inhibition of Plasma Membrane NADH Diferric Transferrin Reductase in Rat Liver Cells

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Abstract

Retinoic acid inhibits the reduction of diferric transferrin through the transplasma membrane electron transport system on fetal rat liver cells infected with a temperature-sensitive SV40 virus when the cells are in the nontransformed state cultured at 40°C. When the cells are in the transformed state (grown at the permissive 33° C temperature), retinoic acid does not inhibit the diferric transferrin reduction. Inhibition of activity of nontransformed cells is specific for retinoic acid with only slight inhibition by retinol and retinyl acetate at higher concentrations. Isolated rat liver plasma membrane NADH diferric transferrin reductase is also inhibited by retinoic acid. The effect of transformation with SV40 virus to decrease susceptibility to retinoic acid inhibition stands in contrast to much greater adriamycin inhibition of diferric transferrin reduction in the transformed cells than in nontransformed cells.

Key Words: Transplasma membrane electron transport; plasma membranes; retinoic acid; cell differentiation; SV40 transformation; adriamycin.

Introduction

Retinoic acid has strong inhibitory effects on the proliferation of some melanoma and other tumor cells (Lotan, 1980; Roberts and Sporn, 1984; Lotan and Nicholson, 1977). Retinoic acid is also effective in causing the differentiation and maturation of myeloid and other cells (Amatruda and

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Koeffler, 1986; Breitman *et al.*, 1980; Strickland and Mahdavi, 1978; Yen *et al.*, 1987; Sherman, 1986), including fetal rat liver cells (Chou and Ito, 1984). The basis of the retinoic acid effects is unknown but its site of action is presumed to be in the nucleus mediated by specific cytosolic retinoic acid or retinol-binding proteins (Sherman, 1986; Chytil and Ong, 1984; Liau *et al.*, 1985). Some evidence also has been presented for a site of action at the plasma membrane (Amatruda and Koeffler, 1986; Yen *et al.*, 1984; Lipkin *et al.*, 1978; Manino *et al.*, 1978).

The plasma membranes of all cells which have been studied contain a transmembrane electron transport system which reduces external impermeable oxidants (Crane *et al.*, 1985; Löw *et al.*, 1986) while cytosolic NADH is oxidized (Navas *et al.*, 1986). The oxidants which can act as electron acceptors for this transmembrane electron transport stimulate the growth of several types of cells in serum-deficient media (Sun *et al.*, 1986a; Kay and Ellem, 1986; Sun *et al.*, 1984). Diferric transferrin can act as a natural electron acceptor for the transplasma membrane electron transport from cytosolic NADH (Löw *et al.*, 1986). Further evidence for this transmembrane diferric transferrin reductase derives from the demonstration that isolated liver plasma membranes have NADH diferric transferrin reductase activity.

Diferric transferrin is necessary for the serum-free growth of many cell types (Barnes and Sato, 1980; Kan and Yamane, 1984). Part of this stimulation of growth can be based on the action of diferric transferrin as an external electron acceptor in addition to its role in iron supply, since nonferric oxidants also cause growth stimulation (Sun *et al.*, 1984; Crane *et al.*, 1987; Laliberte *et al.*, 1987). The basis for growth stimulation by transplasma membrane electron transport and consequent oxidation of cytosolic NADH is not known. The reduction of external oxidants including diferric transferrin is accompanied by activation of the Na^+/H^+ antiport (Garcia-Cañero *et al.*, 1987; Sun *et al.*, 1987a) which has been shown to activate cell growth (Moolenaar *et al.*, 1986; Chambard and Pouyssegur, 1986).

The use of cells infected with a temperature-sensitive strain of Simian virus 40 (SV40) permits the study of cellular function in isogenic transformed and nontransformed cells. We have shown that the transplasma membrane electron transport is changed in the transformed fetal rat liver cells infected with the temperature-sensitive SV40 virus and grown at the permissive temperature of 33°C in comparison to the activity of cells held at 40°C which have the nontransformed phenotype (Sun *et al.*, 1983, 1987a). The transmembrane electron transport in the transformed cells, grown at 33°C, exhibits much greater inhibition by the antitumor drug, adriamycin, than does activity in nontransformed cells (Sun *et al.*, 1983). In this paper we present evidence that the transplasma membrane diferric transferrin reduction by fetal rat liver cells is inhibited by retinoic acid in the nontransformed

cells whereas the SV40 transformed cells lose the sensitivity to retinoic acid. NADH diferric transferrin reductase activity of isolated rat liver plasma membrane also is inhibited by retinoic acid.

Methods

Growth of the temperature-sensitive SV40 fetal rat liver cells has been described (Chou and Ito, 1984). Cells are cultured as transformed cells at 33°C for three days. Incubation at 40°C for three days converts the cells to the nontransformed type (Sun *et al.*, 1986b; Chou and Schlegel-Haueter, 1981).

Diferric Transferrin Reduction by Cells

Diferric transferrin reduction by cells was measured with an Aminco DW2a spectrophotometer in the dual-beam mode to follow formation of ferrous bathophenanthroline disulfonate (BPS) at 535 nm. The reference wavelength was 600 nm. At these wavelengths the extinction coefficient difference for the complex is 17.6 $mM^{-1}cm^{-1}$. Reduction was carried out in 2.8 ml of reaction mixture containing salts-Tris (TD) buffer (140 mM NaCl, 25 mM KCl, 0.6 mM Na_2PO_4, 25 mM Trizma base, pH 7.4), 10 μM bathophenanthroline disulfonate, 10 μM diferric transferrin (absorbance ratio at 465 : 280 greater than 0.04), and 0.01–0.03 g wet weight of cells (Löw *et al.*, 1986). All assays were at 37°C.

Ferricyanide Reduction by Cells

Ferricyanide reduction was measured as described previously by measuring Δ absorbance 420 minus 500 nm (Sun *et al.*, 1986b). Proton release was measured in 150 mM NaCl with 1.5 μm Tris chloride buffer by following change in pH of the media with a Corning electrode as described (Sun *et al.*, 1987a).

NADH Diferric Transferrin Reductase in Membranes

NADH diferric transferrin reductase was measured by following decrease of NADH in the presence of diferric transferrin and plasma membranes at 340 nm with reference at 430 nm using the dual beam. The extinction coefficient used was 6.22 $mM^{-1}cm^{-1}$. The reaction mixture at 37°C contained sodium phosphate, pH 7.4, at 0.05 M, 25 μm NADH, 10 μM diferric transferrin, 1 mM KCN, and 0.1–0.02 mg protein in a total volume of 2.8 ml. NADH oxidation without transferrin was subtracted (Löw *et al.*,

1986). Diferric transferrin was prepared according to Karin and Mintz (1981) or obtained from Boehringer-Mannheim (Indianapolis).

Plasma Membrane Isolation

Rat liver plasma membranes were isolated and characterized according to Yunghans and Morré (1973).

Results

Both transformed (33°C) and nontransformed (40°C) fetal liver cells reduce diferric transferrin with release of ferrous ions for ferrous bathophenanthroline sulfonate formation. With the nontransformed cells, addition of retinoic acid to cells 3 min before addition of the diferric transferrin inhibits the diferric transferrin reduction. Inhibition is apparent at 10^{-7} M and increases with concentration of retinoic acid up to 10^{-5} M. When retinoic acid is added to transformed cells (33°C), there is no inhibition of the diferric transferrin reductase (Fig. 1).

The rate of NADH diferric transferrin reductase in isolated rat liver plasma membrane also is inhibited by retinoic acid. Inhibition is apparent at

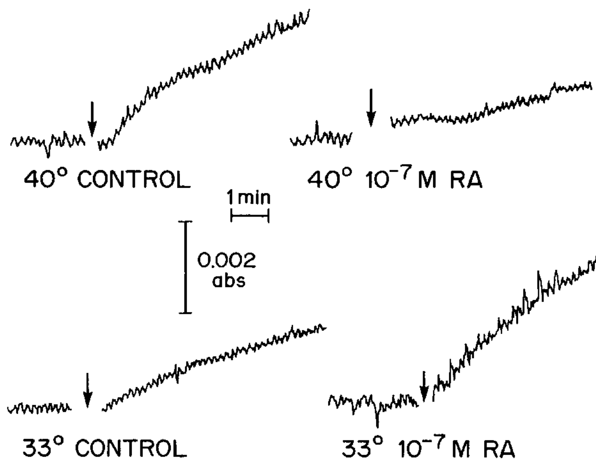

Fig. 1. Effect of retinoic acid on formation of ferrous bathophenanthroline disulfonate measured at 535 nm with rat liver cells and diferric transferrin. Cells held at indicated temperature for 3 days. 40°C exposure produces the nontransformed phenotype, whereas 33°C cells express the SV40 transformation. Assay as in Methods. 0.015 g wet weight cells used for assay of 40°C cells, 0.02 g wet weight for 33°C control, and 0.03 g wet weight for 33°C with 10^{-7} M retinoic acid (RA). Diferric transferrin: 10 mM added at the arrow. All assays at 37°C. Note that the apparent difference in activity for 33°C control and 33°C retinoic acid-treated cells is caused by the difference in amount of cells used.

10^{-7} M and increases up to 10^{-5} M as is observed with the nontransformed cells (Table I).

The effect of retinoic acid on the diferric transferrin reduction by the nontransformed cells is specific for retinoic acid. Retinol or retinyl acetate do not show significant inhibition below 10^{-5} M (Table II).

Reduction of ferricyanide by cells is not strongly inhibited by apotransferrin or antitransferrin receptor antibodies, so the reduction primarily involves the electron transport system but not the transferrin receptor (Löw *et al.*, 1987). Ferricyanide reduction by nontransformed cells is inhibited by retinoic acid at concentrations similar to those which inhibit diferric transferrin reduction. There is very little inhibition of ferricyanide reduction with transformed (33°C) cells (Table III).

When diferric transferrin is added to cells, the Na^+/H^+ antiport is activated to release H^+ from the cells so long as the external Na^+ concentration

Table I. Inhibition of NADH Diferric Transferrin Reductase Activity of Rat Liver Plasma Membrane[a]

Retinoic acid concentration (M)	NADH diferric transferrin reductase activity (nmol min^{-1} mg $protein^{-1}$)
None	7.1 ± 0.3 (3)
10^{-7}	6.8
10^{-6}	4.4
5×10^{-6}	3.8
10^{-5}	3.3
5×10^{-5}	2.8

[a]Control rate with standard deviation and number of assays.

Table II. Effect of Retinoid Compounds on the Rate of Diferric Transferrin Reduction by SV40 Rat Liver Cells under Transformed and Nontransformed Conditions

Cell culture conditions (°C)	Addition (M)	Diferric transferrin reduction rate (nmol min^{-1} g wet $weight^{-1}$)
40	None	5.7 ± 0.5 (3)
40	Retinoic acid 10^{-8}	5.1
40	Retinoic acid 10^{-7}	3.2
40	Retinoic acid 10^{-6}	2.8
40	Retinoic acid 10^{-5}	0.2
40	Retinol 10^{-7}	5.6
40	Retinol 10^{-5}	4.1
40	Retinyl acetate 10^{-7}	5.5
40	Retinyl acetate 10^{-5}	3.0
33	None	1.2
33	Retinoic acid 10^{-5}	1.4
33	Retinol 10^{-5}	1.9
33	Retinyl acetate 10^{-5}	1.4

Table III. Effect of Retinoic Acid on the Ferricyanide Reduction by SV40-Infected Rat Liver Cells in Transformed (33°C) and Nontransformed (40°C) Condition[a]

Concentration of *all trans*-retinoic acid[a] (M)	Ferricyanide Reduction rate for cells under different culture conditions ($nmol\ min^{-1} g$ wet weight^{-1})	
	40° cells	33°C cells
None	644 ± 9 (3)	182 (2)
10^{-9}	567	182
10^{-8}	364	189
10^{-7}	196	196
10^{-6}	70	154

[a]Note that retinoic acid at concentrations above 10^{-6} M interferes with the ferricyanide reduction assay.

can support the exchange (Sun *et al.*, 1987a). Retinoic acid also stimulates this Na^+/H^+ exchange (Ladoux *et al.*, 1987). The stimulation of H^+ release by diferric transferrin and retinoic acid are not additive. Both transformed and untransformed cells are stimulated by retinoic acid to release H^+. Retinoic acid does not stop H^+ release in nontransformed cells even when diferric transferrin is present.

In contrast to the lack of retinoic acid effect on transferrin reductase activity of the SV40-transformed liver cells, adriamycin strongly inhibits the diferric transferrin reductase activity of the transformed cells but has much less effect on the untransformed cells (Table IV) (Sun *et al.*, 1983).

Discussion

Control of the transplasma membrane electron transport has been related to control of growth in several transformed cell lines including

Table IV. Effect of Adriamycin on the Diferric Transferrin Reduction by SV40-Infected Rat Liver Cells in the Transformed (33°C) and Nontransformed (40°C) Condition

Cell culture condition (°C)	Concentration of adriamycin added (M)	Diferric transferrin reduction ($nmol\ min^{-1} g$ wet weight^{-1})
40	None	8.8 (2)
40	10^{-8}	9.2
40	10^{-7}	11.2
40	10^{-6}	9.0
40	10^{-5}	5.7
33	None	1.8 ± 0.3 (3)
33	10^{-8}	0.9
33	10^{-7}	0.4
33	10^{-6}	0.2

HeLa (Sun *et al.*, 1984, 1985), HepG2 (Crane *et al.*, 1987), Ehrlich ascites (Waranimman *et al.*, 1986), Swiss 3T3 and SV40-transformed 3T3 (Löw and Crane, unpublished), SV40-transformed fetal liver (RLA209-15) (Sun *et al.*, 1986a), L1210 (Sun *et al.*, 1986a), and melanoma (Kay and Ellem, 1986). Oxidants that accept electrons from the enzyme at the cell surface stimulate growth in serum-free media, whereas oxidants that cannot act as electron acceptors for the enzyme do not stimulate growth (Sun *et al.*, 1984). Insulin which acts as a growth factor for HeLa cells stimulates transmembrane electron transport at the same concentrations which stimulate growth and greatly increases the growth stimulation seen with ferricyanide as an external oxidant (Sun *et al.*, 1985).

Several antitumor drugs have been shown to inhibit the transmembrane electron transport to ferricyanide at concentrations which inhibit growth. The effective drugs include adriamycin (Sun and Crane, 1984a; Sun *et al.*, 1987b), *cis*-diammine dichloroplatinum II (Sun and Crane, 1984b), actinomycin D (Sun and Crane, 1981), and bleomycin (Sun and Crane, 1984c). As shown here for HeLa cells (Sun *et al.*, 1986c), the diferric transferrin reduction can also be inhibited by adriamycin. The inhibition of ferricyanide reduction by these drugs and retinoic acid indicates that their action is primarily on the electron transport system rather than on the transferrin receptor. If the inhibition site was on the transferrin receptor, we would expect the drugs to inhibit diferric transferrin reduction selectively. For example, apotransferrin or the B3/25 monoclonal transferrin receptor antibody strongly inhibits diferric transferrin reduction but has only a small effect of ferricyanide reduction (Löw *et al.*, 1986). Inhibition of the NADH diferric transferrin reductase activity in isolated liver plasma membranes is further evidence that a site of adriamycin inhibition is at a component in the membrane as proposed by others (Tritton *et al.*, 1983; Rogers *et al.*, 1983). Inhibition of electron transport in isolated plasma membrane by retinoic acid is evidence for a site of retinoic acid action in the plasma membrane.

With fetal liver cells and pineal cells the SV40-transformed cells are much more sensitive to adriamycin inhibition of electron transport and growth than the nontransformed cells (Sun *et al.*, 1983, 1986c). The effects of retinoic acid are in marked contrast since retinoic acid inhibits in nontransformed cells and is relatively ineffective with the SV40-transformed cells. Although retinoic acid has been shown to inhibit HeLa cell growth (Dion and Gifford, 1980) and diferric transferrin reduction (Sun *et al.*, 1987c), it does not inhibit growth of SV40-infected 3T3 cells (Lotan and Nicholson, 1977; Jetten *et al.*, 1979; Dion *et al.*, 1978). On the other hand, retinoic acid has been shown to increase maturation of untransformed fetal liver cells (Chou and Ito, 1984). The evidence indicates that SV40 transformation modifies the transplasma membrane electron transport system to increase its inhibition by adriamycin and decrease its response to retinoic acid.

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