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Transformed Liver Cells have Modified Transplasma Membrane Redox Activity which is Sensitive to Adriamycin

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Electron transport across the plasma membrane is found in all cells which have been tested. This activity has been implicated in control of cellular growth, transport and hormone response. In virus transformed cells and tumor cells we find the activity is decreased and becomes sensitive to the antitumor drug adriamycin. Inhibition of transmembrane redox by adriamycin parallels cytoxicity to transformed cells.

Adriamycin is an antitumor drug which has extensive use in cancer therapy (1). It has been shown to interfere with DNA and RNA synthesis by intercalation with the DNA molecule (2,3,4). Recent evidence has shown that adriamycin acts at sites on the plasma membrane (5,6,7), because this drug retains cytotoxicity when bound to impermeable agarose beads. Certain membrane functions are affected by adriamycin (8,9) and the NADH ferricyanide reductase of isolated plasma membranes is inhibited by this drug (10,11,12,13). We examined the effect of adriamycin on the ferricyanide reductase activity of intact cells to see if inhibition at an external site could be related to its cytotoxic effect and to determine if adriamycin-induced inhibition is correlated with its antitumor activity. We conclude that the transmembrane redox system in virus transformed liver cells and in hepatoma cells is modified to

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decrease its activity and increase its susceptibility to inhibition by anthracyclines.

Materials and Methods

The cell model system used in this study is a simian virus 40 (SV40) temperature sensitive (ts) A209 virus-transformed fetal liver RLA209-15 cell line that is temperature sensitive for growth and differentration (17). They exhibit the transformed phenotype at the permissive temperature (33°C) but mimic the normal, nontransformed hepatocytes at the nonpermissive temperature (40°C). As controls, we used the McA-RH7777 hepatoma and primary rat fetal hepatocyte cells.

All cells that have been tested have a plasma membrane redox system which reduces external ferricyanide (14,15,16). Ferricyanide reduction was measured at 37° in an Aminco DW2a spectrophotometer using a dual beam to subtract adsorption at 500 nm from adsorption at 420 nm. Two to 20 mg wet weight of cells are placed in a cuvette with Tris-EDTA buffer (140 mM NaCl, 2.5 mM KCl, 0.6 mM Na_2HPO_4, 25 mM Trizma base and 0.05 mM EDTA, pH 7.4). Drugs are incubated with the cells for various times before running the assay. The reaction may be started by adding ferricyanide at 0.2 mM final concentration or by adding cells. The cuvette is equipped with a magnetic stirrer to keep the cells in suspension. Blanks were run with ferricyanide plus adriamycin and no cells. Ferricyanide extinction coefficient is taken as $1cm^{-1}mM^{-1}$. As with most cells which we have tested, ferricyanide reduction shows a fast rate for about two minutes followed by a slower phase which can maintain a steady rate for 10-30 min (14,16). To check for ferricyanide reduction by mitochondria released from broken cells, the activity is measured in the presence of rotenone or antimycin (14). We find these agents do not inhibit either phase of ferricyanide reduction with these cells. RLA209-15 cells were cultured as described (17) and released by trypsinization with 0.05% trypsin and 0.02% EDTA for about 4 min. Cell survival was tested by eosin exclusion (18). External NADH ferricyanide reductase was assayed under the same conditions described for transmembrane ferricyanide reduction except for the addition of 50 μM NADH as substrate (11) and following the decrease in absorbance at 340 nm.

Results and Discussion

RLA209-15 liver cells grown at 33°C (transformed phenotype) show a much slower rate of ferricyanide reduction in both the fast and slow phase than do the cells which have been grown at 40°C (nontransformed phenotype) (Table 1). A similar difference has been observed with SV40 transformed 3T3 and nontransformed 3T3 cells (Löw, Grebing, Crane, unpublished). Hepatoma cells have a rate of ferricyanide reduction thirty percent less in the fast phase and 60 percent less in the slow phase than isolated fetal liver cells. High rates of ferricyanide reduction have also been observed with adult liver cells (14).

Adriamycin inhibits the ferricyanide reduction by transformed RLA209-15 liver cells in both the fast and slow phase. However, the same concentration of drug gives much less inhibition of ferricyanide reduction by those cells

TABLE I

Transmembrane ferricyanide reductase activities of RLA209-15 fetal liver cells grown at 33°C and 40°C, primary fetal liver cells, and hepatoma cells.

Cell lines	culture conditions	Ferricyanide reduction rate nmole/min/gww	
		fast phase	slow phase
RLA209-15	33° (transformed phenotype)	260±53(13)	99±42(13)
RLA209-15	40° (nontransformed phenotype)	709±114(9)	320±105(9)
McA-RH7777 (hepatoma)	33°C	836 (2)	211±22(3)
primary fetal liver cells	33°C	1210±37(3)	605±109(3)

The culture conditions for RLA209-15 cells were as described in the legend to Fig. 1. Primary rat fetal liver cells from 18-day old rat embryonic liver were prepared as previously described (17). Ferricyanide reductase activity in these primary hepatocytes was measured 3 days after they attached to the plastic flasks. The primary fetal hepatocytes, cultured under these conditions, produced high levels of α-fetoprotein, albumin, and transferrin in culture (data not shown). McA-RH777 cells in mid-logarithmic phase of growth were used in this study.

exhibiting a nontransformed phenotype (Table II). Cultured hepatoma cells, like transformed RLA209-15 cells, also show inhibition by adriamycin. On the other hand, primary fetal liver cells, like nontransformed RLA209-15 cells, show very little response to adriamycin. These effects are seen with relatively short term exposure of the cells to adriamycin. The sensitivity is increased by longer preincubation of cells with adriamycin before measuring the ferricyanide reduction rate. With transformed RLA209-15 fetal liver cells the fast phase shows 63% inhibition and the slow phase shows 25% inhibition after 40 min preincubation with 10^{-7}M adriamycin at room temperature (Fig. 1). For RLA209-15 cells, the adriamycin-induced cytotoxicity parallels inhibition of the slow phase ferricyanide reduction (Fig. 1). Primary fetal hepatocytes, like RLA209-15 cells grown at 40°C, are not sensitive to killing by low concentrations of adriamycin. A 7% decrease in survival rate is observed for these primary hepatocytes after prolonged incubation (80 min) with this drug at 10^{-7}M. Although both external and transmembrane NADH dehydrogenases can be detected with intact cells (11,19), the adriamycin sensitive enzyme is the trans-

TABLE II

Effect of adriamycin and AD32 on transmembrane ferricyanide reductase activities of RLA209-15 fetal liver, McA-RH7777 hepatoma, and primary fetal liver cells.

Cells, culture temperature	percent inhibition by:		
	adriamycin		AD32
	10^{-6}M	5×10^{-6}M	10^{-6}M
Fast phase:			
RLA209-15 (33°C) (transformed)	49±12	72±7	40
RLA209-15 (40°C) (nontransformed)	-2±4	20±9	-6
McA-RH7777 (33°C)	35	57	-
primary fetal liver cell (33°C)	-	11	12
Slow phase:			
RLA209-15 (33°C) (transformed)	55±16	56±16	36
RLA209-15 (40°C) (nontransformed)	3±4	12±7	-4
McA-RH-7777 (33°C)	20	48	-
primary fetal liver cells (33°C)	-	8	6

[a](-) indicates stimulation instead of inhibition.

membrane dehydrogenase since the external NADH dehydrogenase in RLA209-15 cells grown both at 33°C and 40°C is not sensitive to this drug at concentrations below 5×10^{-6}M.

AD32 (N-trifluoroacetyl adriamycin-14-valerate), is an adriamycin analog which does not intercalate with DNA (20). AD32 also inhibits the transmembrane ferricyanide reductase. It appears that the effect of adriamycin on the membrane redox system does not involve prior action at the level of DNA but is a direct action of this drug on plasma membrane function.

Since both transformed and nontransformed fetal liver cells are derived from the same clone of RLA209-15 cells, our data unequivocally demonstrate that transformed cells are more sensitive than nontransformed cells to adriamycin-

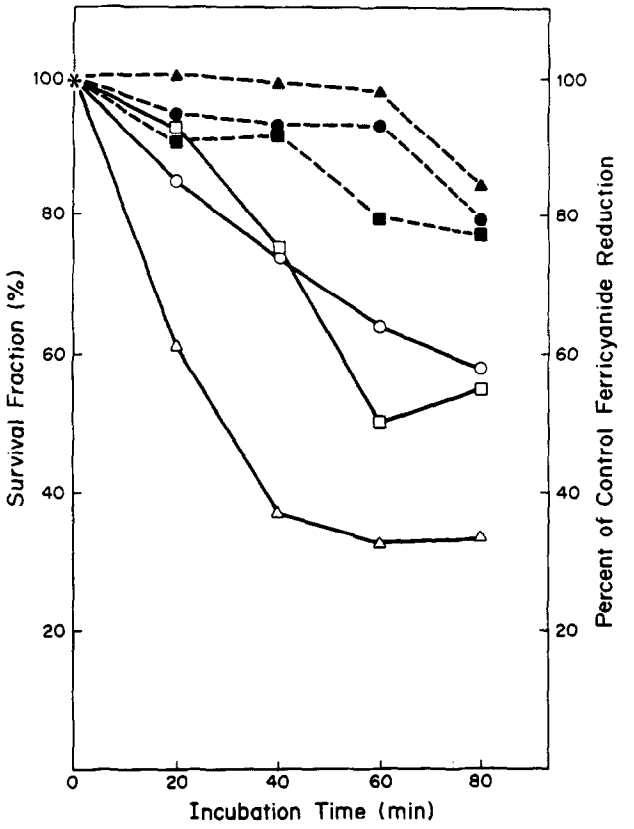

Fig. 1. Effect of adriamycin on the survival fraction and ferricyanide reduction of RLA209-15 cells. Two parallel sets of RLA209-15 cultures were plated initially at 33°C. The first set of cultures was maintained at 33°C throughout growth. The second set of cultures were shifted from 33°C to 40°C after 3 days growth at 33°C. After an additional 3 days incubation at 33°C and 40°C, cells were trypsinized and resuspended in tris-EDTA buffer pH 7.4 plus 2% of fetal calf serum in a concentration of 0.1 gw.w./ml, and incubated with adriamycin (10^{-7}M) at 22°C water bath with shaking. Samples were taken periodically, chilled in an ice bath, and diluted 10 fold with ice-cold tris-EDTA buffer plus 2% fetal calf serum. Survival fraction was measured immediately after spinning the cells and resuspending in tris-EDTA buffer plus 2% fetal calf serum by using eosin Y exclusion method. Survival fraction (40°C) (●), slow rate ferricyanide reduction (40°C), (■) fast rate ferricyanide reduction (40°C) (▲), survival fraction (33°C) (○), slow rate ferricyanide reduction (33°C) (□) and fast rate ferricyanide reduction (33°C) (△).

induced cytotoxicity. Furthermore, such cytotoxicity may be linked to the inhibition of transmembrane ferricyanide reductase by this antitumor drug. Recently addition of ferricyanide has been shown to stimulate growth of melanoma cells and the effect has been attributed to the transmembrane electron transport system (21). There are several other functions or properties of the plasma membrane which have been shown to be affected by adriamycin. Among

these are ion transport (22,23), membrane fluidity (8), membrane fusion and response to hormones (5). Each of these responses to adriamycin can be based on adriamycin inhibition of a protonophoric transmembrane dehydrogenase (15,16,24,25) which can modify membrane potential (26), drive amino acid transport (27) and control the activity of adenylate cyclase (28) or reduce external iron for uptake (29). The redox system in the plasma membrane, which can be assayed by external ferricyanide reduction provides a system to measure direct effects of adriamycin on the plasma membrane. The redox system may also provide an approach to study the mechanism of adriamycin action at the plasma membrane.

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