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EVIDENCE FOR A PLASMA MEMBRANE REDOX SYSTEM ON INTACT ASCITES TUMOR CELLS WITH DIFFERENT METASTATIC CAPACITY

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Summary

A NADH-ferricyanide reductase of the external surface of intact mouse ascites tumor cells grown in culture was shown. The oxidation/reduction reaction was due to enzymatic rather than inorganic iron catalysis as demonstrated by the kinetics and specificity of the reaction. Activities of three markers for cytoplasmic contents were lacking with the intact tumor cells. The dehydrogenase activity was inhibited by *p*-chloromercuribenzoate, bathophenanthroline sulfonate, and the anticancer drug adriamycin. Sodium azide and potassium cyanide inhibited partially. The response to inhibitors resembled that of isolated plasma membranes rather than that of mitochondria. Concurrent with these findings, neither superoxide dismutase nor rotenone affected the redox activity. The findings provide evidence for the operation of a plasma membrane redox system at the surface of intact, living cells.

Introduction

The primary locations of enzymes that catalyse oxidation/reduction reactions are mitochondria, endoplasmic reticulum and the cytosol. More recently, evidence has been presented for the existance of a redox enzyme on the plasma membrane (Refs. 1—3 and literature cited). With the aid of electron micro-

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scopy and enzymatic markers to monitor relative purity [1], plasma membrane preparations have shown redox activities well in excess of the 2% of contaminating membranes and exhibit differences with respect to substrate and inhibitor specificities, when compared to redox enzymes of mitochondria and various endomembranes. However, additional lines of evidence for a plasma membrane redox system have been sought.

In this report we provide enzymatic evidence for an NADH-ferricyanide reductase present on the external surface of ascites tumor cells grown in suspension culture.

Materials and Methods

Tumor cell lines and maintenance. The tumor line ESb (previously L5178Y-ES) [4,5] is a spontaneous variant of the chemically induced DBA/2 lymphoma Eb (previously L5178Y-E). It arose in 1968 during routine intraperitoneal transplantation in Professor Alexander's laboratory (Chester Beatty Research Laboratories, U.K.) as a variant with greatly increased metastatic capacity. The ascites tumor lines were obtained in 1977 and maintained by intraperitoneal transplantation in syngeneic DBA/2 male mice and stored in liquid nitrogen. Some of the characteristic differences between the two lines Eb and ESb have been described recently [5—9].

The ascites tumor cells were adapted to tissue culture in vitro using RPMI-1640 culture medium (Gibco) with sodium bicarbonate (1 g/500 ml) and 10% fetal calf serum. As described previously [9], the culture of ESb tumor cells required the addition of 2-mercaptoethanol at a concentration of about 10^{-5} M. Cells were incubated at an initial density of 10^5/ml for 3—4 days at 37°C in a 5% CO_2 atmosphere. Cell density at the time of harvest was between 1.5 and $2.0 \cdot 10^6$ viable cells per ml as determined by trypan blue exclusion. Non viable or broken cells never exceeded 1% of the total population by this criterion. Cells were prepared for study by pelleting suspension cultures at 1500 rev./min in an HB-4 Spinco swinging bucket rotor at 4°C for 5 min and washing three times in phosphate buffered saline (0.137 M NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, 8.4 mM NaH_2PO_4, and 8.8 mM K_2HPO_4) plus sucrose (2.5 g/l), pH 6.8. The final pellet was diluted with phosphate buffered saline plus sucrose to a final protein concentration of 5 mg/ml as determined via the Biuret method and maintained on ice prior to incubation.

NADH dehydrogenase activities. All enzyme assays were with an Aminco DW-2a dual beam spectrophotometer with linear recorder, cuvette stirrer, and 37°C temperature controlled cuvette chamber. Glass distilled water was used in preparation of solutions and in washing cuvettes.

The rate of reaction was determined by recording oxidation of NADH or NADPH at 340 nm with reference at 600 nm for NADPH- and 581 nm for NADH-cytochrome *c*. Reduction of ferricyanide was at 417 nm with reference at 620 nm and reduction of cytochrome *c* was at 550 nm with reference at 400 nm. Reactions were linear for at least 5 min under the conditions described and steady state rates were monitored routinely for 2 min and corrected for endogenous activity (—acceptor). Endogenous activity (—acceptor) for NADH oxidation in the absence of ferricyanide, for detergent-lysed cells, for example,

stabilized quickly after 1 min to a rate about 10% that with acceptor added.

Reaction mixtures contained either 0.25 mM ferricyanide or 0.5 mg/ml cytochrome *c*, $3.5 \cdot 10^{-4}$ M NADH, $3.3 \cdot 10^{-4}$ M NADPH, inhibitors, and/or detergents as indicated, 0.1 ml of cell suspension, and 2.5 ml of phosphate buffered saline plus sucrose, pH 6.8. Extinction coefficients used in calculation of specific activities were 6.23 $cm^{-1} \cdot mmol^{-1}$ for NADH and NADPH oxidation, 1.0 $cm^{-1} \cdot mmol^{-1}$ for ferricyanide reduction, and 18.5 $cm^{-1} \cdot mmol^{-1}$ for cytochrome *c* reduction.

Succinate dehydrogenase activity. The reaction mixture contained 40 mM sodium succinate, 30 mM indophenol, and 0.3 mg/ml phenazine methosulfate in phosphate buffered saline plus sucrose. After addition of 50 µl of washed cell suspension diluted to 5.0 mg/ml protein, the reduction of indophenol was recorded at 600 nm with reference at 505 nm. The extinction coefficient was 11.0 $cm^{-1} \cdot mmol^{-1}$.

Lactate dehydrogenase activity. The reaction mixture contained 0.14 mM NADH, 0.20 mM pyruvate, and 50 µl of the washed cell suspension adjusted to 5.0 mg/ml protein. The oxidation of NADH was recorded at 340 nm with reference at 600 nm.

Results

NADH-ferricyanide reductase activity was first order with respect to protein concentration for either NADH oxidation or ferricyanide reduction (Fig. 1). The K_m values for NADH to ferricyanide (range $3 \cdot 10^{-4}$—$3 \cdot 10^{-3}$ M NADH) were $1.5 \cdot 10^{-4}$ M for Eb cells and $5.0 \cdot 10^{-4}$ M for ESb cells at pH 6.8. The *V* of the oxidation reaction was similar for both cell lines, 10 nmol $\cdot$ $min^{-1} \cdot mg^{-1}$

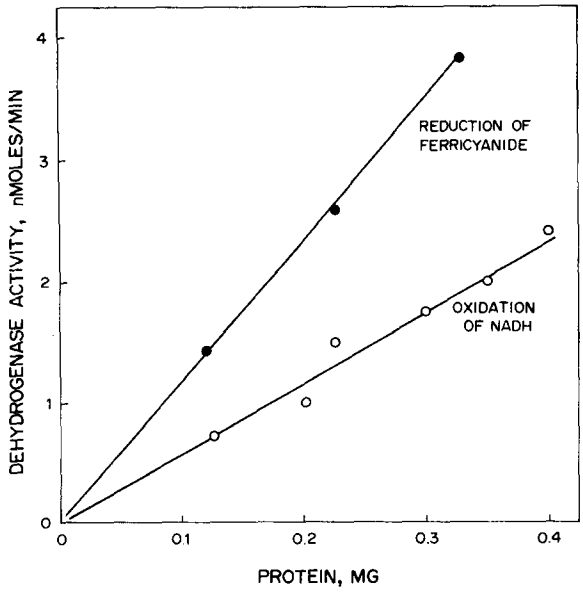

Fig. 1. Relationship between NADH-ferricyanide reductase recording NADH oxidation or ferricyanide reduction and protein concentration with intact ascites tumor cells (ESb).

TABLE I

NADH DEHYDROGENASE ACTIVITIES OF INTACT ASCITES CELLS BEFORE AND AFTER DETERGENT TREATMENT

Specific activities were determined either by observing NADH oxidation or ferricyanide reduction as indicated at pH 6.8 with and without addition of detergents. Results are from three different cell preparations ± standard deviation (S.D.)

Substrate		Detergent	Dehydrogenase activity ± S.D. (nmol · min^{-1} · mg^{-1})			
			Oxidation		Reduction	
			Eb	ESb	Eb	ESb
NADH	Ferricyanide	None	5.1 ± 1.1	7.8 ± 2.7	13.2 ± 2.1	18.2 ± 4.0
		0.012% Triton	117.3 ± 5.7	79.5 ± 11.7	168.5 ± 50.6	143.0 ± 29.8
		0.4% Triton	—	—	128.0 ± 11.9	131.3 ± 26.3
		0.02% Saponin	120.3	120.3	161.4	162.9
NADH	Cytochrome c	None	0.00	0.00	1.6 ± 0.3	1.2 ± 1.4
		0.012% Triton	1.0 ± 0.1	0.9 ± 0.1	10.1 ± 2.6	9.8 ± 4.5

for both lines. Observing the reduction reaction, the V was 17 nmol · min^{-1} · mg^{-1}. The oxidation reaction involved donation of two electrons per NADH molecule and the reduction reaction involved the acceptance of one electron per molecule of ferricyanide. Thus the reductase activities determined by measuring acceptor reduction were comparable to those determined from NADH oxidation and produced the expected 2 : 1 ratio. In contrast to intact cells, oxidation was disproportionately greater than reduction for broken cells (Table I) suggesting that here some NADH oxidation was due to endogenous acceptors and not all due to ferricyanide. The cells showed increased activity with increasing pH to an optimum between pH 8.0 and 8.5 (Fig. 2A; see also Strittmatter and Velick [10] for microsomal NADH-ferricyanide reductase). Differences in rates between the two cell lines were neither reproducible among different isolates nor statistically significant.

The NADH dehydrogenase activities were greatly augmented when cells were ruptured by the addition of either Triton X-100 or saponin (Table I). No inhibition of activity was observed with Triton X-100 even at concentrations in excess of that required to break the cells (Table I).

Three marker enzymes were assayed, succinate dehydrogenase for mitochondria, lactate dehydrogenase for the soluble cytoplasm and NADPH oxidoreductase for endoplasmic reticulum (microsomes). No succinate dehydrogenase was detected until detergent was added (Table II). A small lactate dehydrogenase activity, which was first order with respect to protein concentration (not shown) was detected (Table II). The specific activity of the lactate dehydrogenase was reduced to 25 nmol · min^{-1} · mg^{-1} with repeated washings of the cells with phosphate buffered saline plus sucrose. Moreover, lactate dehydrogenase activity was increased 36-fold or more with the addition of detergents (Table II). No activity was detected when NADPH was substituted for NADH in the dehydrogenase assays (Table II). Also no reductase activity was detected for NADH and cytochrome c when NADH oxidation was recorded. A slow rate was obtained when cytochrome c reduction by NADH was followed (Table I).

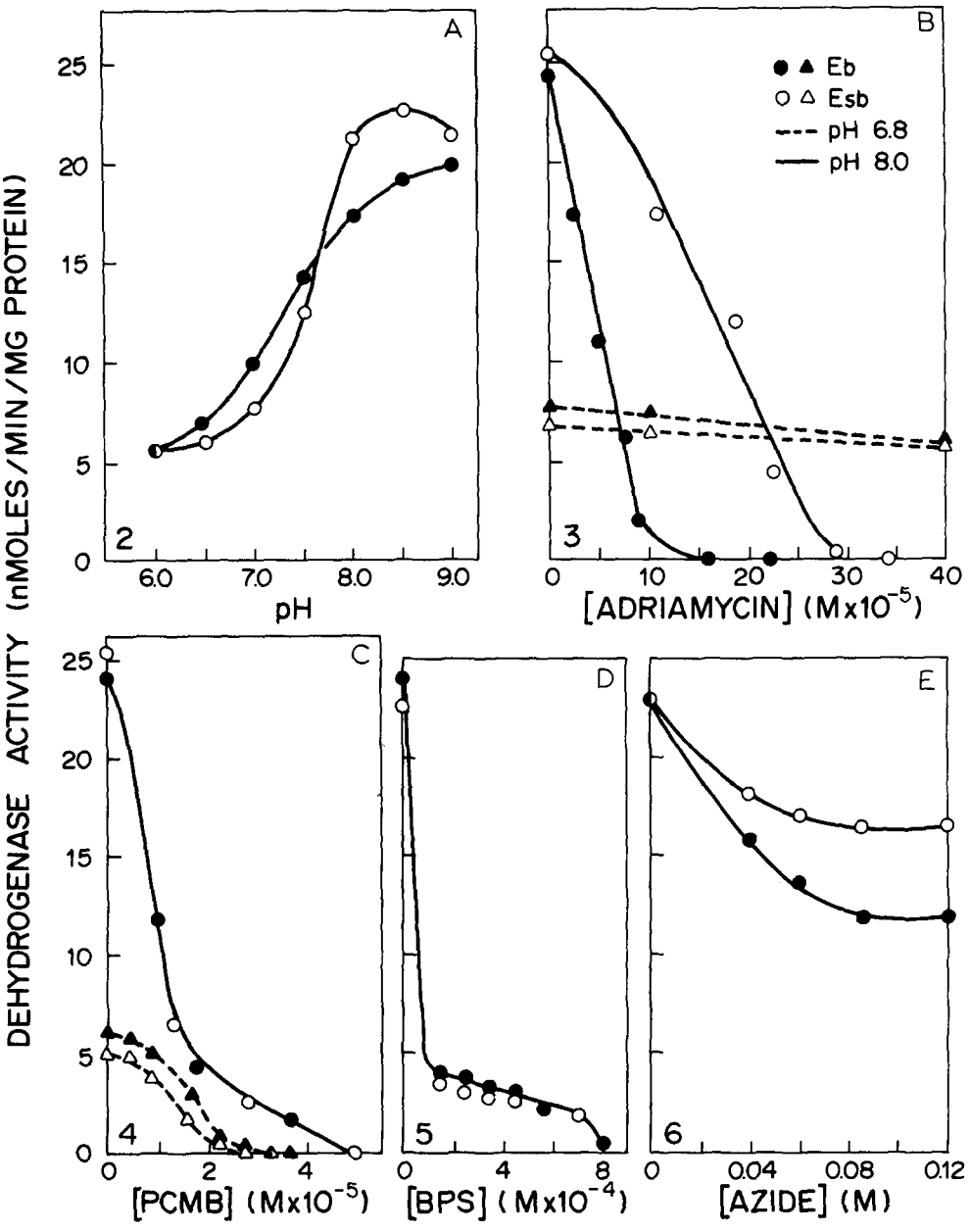

Fig. 2. pH dependency (A) and inhibition of NADH-ferricyanide reductase in ascites cells recording NADH oxidation with adriamycin (B), *p*-chloromercuribenzoate (PCMB) (C), bathophenanthroline sulfonate (BPS) (D), and azide (E). Phosphate buffered saline was adjusted to the pH indicated.

The dehydrogenase activity of the cell surface appeared not to have a major requirement for ions. The activity was unaffected by the substitution of phosphate buffer for phosphate buffered saline in the reaction mixture but the lactate dehydrogenase activity was reduced.

The anticancer drug adriamycin inhibited the non-metastatic Eb line at 15

TABLE II

MARKER ENZYME ACTIVITIES OF INTACT ASCITES CELLS BEFORE AND AFTER DETERGENT TREATMENT

Succinate dehydrogenase and lactate dehydrogenase activities were low or lacking in washed intact cells. Activity increased greatly with the addition of detergents. Results are from three different cell preparations ± standard deviation (S.D.)

Enzyme	Acceptor	Detergent	Dehydrogenase activity ± S.D. (nmol · min^{-1} · mg^{-1})	
			Eb	ESb
Succinate dehydrogenase		None	0.0 ± 0.1	0.0 ± 0.1
		0.012% Triton	2.3 ± 1.0	4.6 ± 2.7
Lactate dehydrogenase		None	23.6 ± 8.7	25.6 ± 7.2
		0.012% Triton	1103 ± 171	1047 ± 110
		0.40% Triton	1388 ± 146	—
NADPH dehydrogenase				
	Ferricyanide	None	0.0 ± 0.0	0.0 ± 0.0
		0.012% Triton	9.1 ± 1.9	14.9 ± 9.9
	Cytochrome *c*	None	0.0 ± 0.0	0.0 ± 0.0

mM at pH 8.0 while ESb was not completely inhibited until 30 mM (Fig. 2B). Adriamycin was a less effective inhibitor when tested at pH 6.8. Low concentrations of *p*-chloromercuribenzoate completely inhibited the NADH-ferricyanide activity of the cells at both pH 6.8 and 8.0 (Fig. 2C). Bathophenanthroline sulfonate also severely inhibited the dehydrogenase activity (Fig. 2D). The two cell lines were identical in their response to these inhibitors. Sodium azide (Fig. 2E) and potassium cyanide (not shown) partly inhibited (maximum 50%) with Eb being more sensitive than ESb to azide. Superoxide dismutase and rotenone were without effect (not shown).

Discussion

Previous studies have indicated NADH ferricyanide reductase activity of plasma membrane located on the inside of the erythrocyte membrane [10] and in a transmembrane position [11], but this is the first indication of a NADH dehydrogenase located on the outside.

Three lines of evidence are presented from mouse lung carcinoma ascites cells for the existance of a NADH-ferricyanide reductase on the exterior of the plasma membrane: (1) The oxidation/reduction reaction results from enzymatic catalysis and not from catalysis by a metal ion, such as iron, bound to the cell surface. (2) The activity is due to an enzymatic catalyst located on the outsides of intact cells and not the result of mitochondrial, endoplasmic reticulum, or cytoplasmic enzymes released from broken cells. Also excluded was a transport system induced by NADH whereby the redox reaction occurred via reducing agents exported out of the cell [12]. (3) The redox activity responded to inhibitors in a manner similar to purified preparations of plasma membranes of other cell types [1—3].

The reaction between NADH and ferricyanide was recorded by observing either oxidation or reduction. No reaction, oxidation or reduction, was ob-

served between NADPH and ferricyanide, or between NADPH and cytochrome *c*. Also, no oxidation was observed between NADH and cytochrome *c*. By following the reduction of cytochrome *c*, a slow reaction was detected. This may indicate a cytochrome site partly exposed at the external surface of the plasma membrane or the export of a reducing agent from the cell which can reduce cytochrome *c*.

A possibility in considering the mechanism of the observed oxidation/reduction reaction was the production of superoxide by an enzyme located on the plasma membrane as proposed for leucocytes [13—15]. Superoxide produced by oxidation of NADH would reduce ferricyanide or cytochrome *c* (and also the reaction from NADPH to ferricyanide or cytochrome *c*). Another possible catalyst was membrane bound iron. Here too the reaction would show no specificity. The activity observed was specific for NADH with preference of ferricyanide over cytochrome *c*. As is common for plasma membrane dehydrogenases, NADH was preferred over NADPH to the point of excluding microsomal dehydrogenases as the source of activity. The NADPH dehydrogenases present in plasma membranes are normally between 1/25 and 1/60 the activity of the endoplasmic reticulum [16—19]. In erythrocyte ghosts the total absence of NADPH dehydrogenase from plasma membrane has been shown concurrent with the absence of endoplasmic reticulum from these cells.

Manganese dependent aerobic oxidation of NADH with the formation of free radical products was reported by Klebanoff [20] and Halliwell [21]. If such enzymes produced the activity, the addition of manganese should stimulate the reactions. When manganese (0.6 mM) was added, no change in activity was observed. The possibility that a superoxide generating system was producing this dehydrogenase activity was eliminated by the specificity of the reaction for ferricyanide and not cytochrome *c*, the lack of inhibition by superoxide dismutase and the fact that ferricyanide reduction occurred under an argon atmosphere (not shown).

One aspect of this research was to compare the relative enzymatic activities of metastatic and nonmetastatic ascites tumor lines which originated from the same original lymphoma line. The previously observed dependency of ESb but not Eb tumor cells on the presence of low concentrations of 2-mercaptoethanol in the culture medium [8,9] prompted us to look for possible differences between these lines in membrane redox enzyme activities. The affinity of the enzyme differed between the two tumor lines with the metastatic cells (ESb) having the greater affinity for NADH compared to the nonmetastatic cells. Both lines exhibited approximately the same V. The two lines responded similarly to inhibitors except for adriamycin and azide where the metastatic line was significantly less sensitive. This adriamycin inhibition of activity may have some relevance to its anticancer action. It has been proposed that adriamycin intercalates with DNA [22]. However, recent work has drawn attention to derivatives of adriamycin which are unable to intercalate with DNA but still retain the antitumor activity [23]. Thus a membrane interaction of adriamycin may be important to its mode of action.

In summary, we present evidence for the existance of a NADH-ferricyanide reductase acting as an ecto-redox enzyme on intact mouse ascites tumor cells grown in culture. The function and exact nature of the redox system are not known.

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