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Growth factor-stimulated trans plasma membrane electron transport in HL-60 cells

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Electron flow across the plasma membrane of living cells and its rapid modulation by growth factors has been measured continuously through a simple assay procedure whereby the transported electrons are captured by ascorbate free radical to slow the rate of chemical oxidation of ascorbate. The assay provides a direct demonstration of electron transport to an external electron acceptor that is both physiological and impermeant. The reduction of external ascorbate free radical is stimulated by the growth factors, EGF and transferrin, and is inhibited by wheat germ agglutinin. The results demonstrate, under physiological conditions, the operation of a growth factor- and lectin-responsive electron transport system at the cell surface using a cultured human cell line.

Plasma membrane; Electron transport; Ascorbate

1. INTRODUCTION

An electron transport system in the plasma membrane is apparently ubiquitous in eukaryotic cells [1]. Agents that stimulate growth frequently stimulate this electron transport whereas agents that inhibit growth may inhibit electron transport. Experiments studying these effects have been criticized because reduction of external ferricyanide, a non-physiological low potential electron acceptor [2], has been employed to demonstrate transmembrane electron flow. Use of ferricyanide has been defended on the basis that ferricyanide will, of itself, reduce the serum requirements for growth of cultured mammalian cells [3]. The utilization of the putative natural electron acceptor, oxygen [4], has been difficult to interpret with living cells due to the ready entry of oxygen into cells and its many internal roles.

As an alternative, we have developed an assay based on the ability of transported electrons to prevent oxidation of external ascorbate to monitor transmembrane electron transport by mammalian cells under physiological conditions.

Abbreviations: A·, ascorbate radical; EGF, epidermal growth factor; Fe_2TF, diferric transferrin; WGA, wheat germ agglutinin.

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2. MATERIALS AND METHODS

HL-60 cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B. Cells were concentrated from stock cultures by centrifugation at 1,000 × *g* and washed twice with serum-free RPMI-1640 medium and resuspended for assay in 100 mM Tris–HCl, pH 7.4. All chemicals were from Sigma unless otherwise specified. Diferric transferrin was human holo (iron saturated) from Boehringer-Mannheim (Lot #Fe_2TF BLA 108). The epidermal growth factor was from mouse, culture grade from Upstate Biotechnology Inc. (Lake Placid, NY. Lot #EGF 10166).

Ascorbate oxidation was determined by a direct reading at 265 nm [5] using 5×10^6 cells in a 2 or 3 ml final volume. Ascorbate was monitored continuously with time using a Beckman Model DU-7 or a Shimadzu UV-160 spectrophotometer over 10 min. An extinction coefficient for ascorbate determined for Tris–HCl buffer at pH 7.4 of 11.2 $mM^{-1} \cdot cm^{-1}$ [6] was used.

3. RESULTS

Under the assay conditions described, a linear decrease in absorbance at 265 nm was observed in the absence of cells (Fig. 1). This decrease was slowed with cells present and the rate of electron flow across the plasma membrane slowed in proportion to the number of cells present (Figs. 1 and 2).

Within the linear range of the assay (0.2 mM ascorbate, 2–5 × 10^5 cells per ml), the rate of ascorbate oxidation was slowed by the addition of the growth factors, differic transferrin (Fe_2TF) and epidermal growth factor (EGF) (Table I). With EGF (Fig. 3) and Fe_2TF (Fig. 4) the response was proportional to growth factor concentration and was saturating within a physiological range. Surfaces of HL-60 cells do contain re-

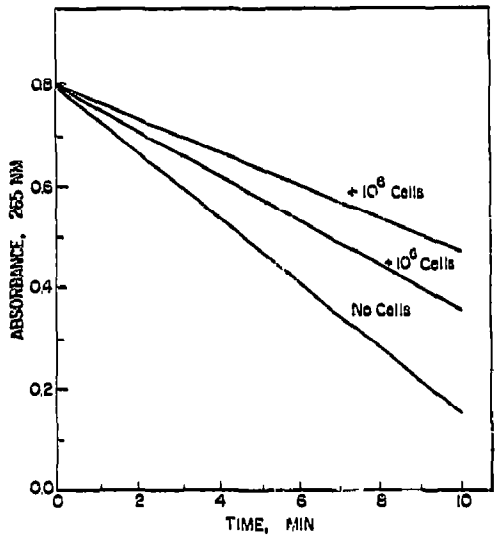

Fig. 1. Time-course of ascorbate oxidation in the presence or absence of 10^6 or 5×10^5 HL-60 cells.

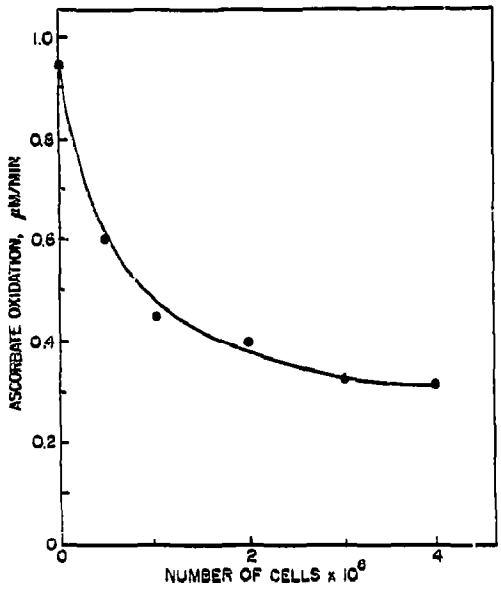

Fig. 2. Ascorbate autooxidation ($\mu M \cdot min^{-1}$) in the presence of varying numbers of HL-60 cells.

ceptors for both Fe_2TF [7] and EGF [8]. At saturating EGF and Fe_2TF, the responses were not additive (Table I). The order of growth factor addition was unimportant and both growth factors could be added simultaneously with similar effects.

Lectins such as wheat germ agglutinin (WGA) (Table II) or concanavalin A (Con A) (not illustrated) that bind to HL-60 cells or to right-side-out plasma membrane vesicles from HL-60 cells reduce the cells' ability to slow ascorbate oxidation as well as to inhibit the reduction of ascorbate free radical by rat liver plasma membranes in the assay. Lectins were reported previously to inhibit NADH-ascorbate free radical oxido-reductase of rat liver plasma membranes [9].

Evidence for the specificity of the assay is summarized as well in Table II. Boiled cells were without activity. Plasma membranes prepared by aqueous two-phase partition [10] from HL-60 cells, in contrast, were active. However, with the isolated plasma membrane, it was necessary to add exogenous NADH as a source of electrons. Boiled plasma membranes and plasma membranes without NADH were without effect.

4. DISCUSSION

That the altered rates of ascorbate oxidation were dependent on electron flow across the plasma membrane of the suspended cells was supported by independent measurements of internal cellular levels of pyridine nucleotides. NADH, rather than NADPH, appeared to be the natural donor for the trans plasma membrane redox system [11]. Accordingly, addition of a mixture of ascorbate and dehydroascorbate to HL-60 cells to generate ascorbate free radicals induced a rapid

Table I

Stimulation by growth factors of ascorbate autooxidation by induced HL-60 cells

Addition	Ascorbate autooxidation ($\mu M \cdot min^{-1}$)	Change due to additions
NONE	1.0	–
EGF (5 ng/ml)	1.0	0[a]
Fe_2TF (20 μM)	0.95	0.05[a]
Cells	0.6	–
Cells + EGF (5 ng/ml)	0.4	0.2[b]
Cells + FE_2TF (20 μM)	0.3	0.3[c]
Cells + EGF followed by Fe_2TF	0.2	0.4[d]
Cells + FE_2TF followed by EGF	0.2	0.4[d]

Each assay contained 5×10^5 cells = 65 μg protein in a final volume of 3 ml. Values not followed by the same letter are significantly different ($P < 0.01$).

Table II

Specificity of inhibiton of ascorbate autooxidation as a measure of plasma membrane electron transport

Addition	Ascorbate autooxidation
NONE	1.00
Cells	0.30
Boiled cells	0.98
Plasma membranes (w/NADH)	0.50
Boiled plasma membranes	0.90
WGA	1.00
Cells + WGA	0.70
Plasma membranes + WGA	0.80

Each assay contained 1×10^6 cells = 125 μg protein or 40 μg/ml of plasma membrane in a final volume of 2 ml. Wheat germ agglutinin (WGA) was at a final concentration of 300 ng/ml. Ascorbate autooxidation rates are $\mu M \cdot min^{-1}$.

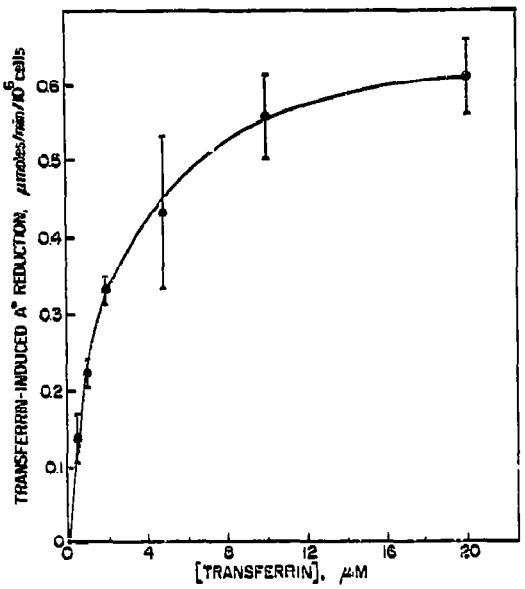

Fig. 3. Diferric transferrin-induced slowing of ascorbate autooxidation (reduction of ascorbate radical, A•) in the presence of 10^6 induced HL-60 cells. Values are means of three experiments with duplicate or triplicate determinations in each experiment ± standard deviations.

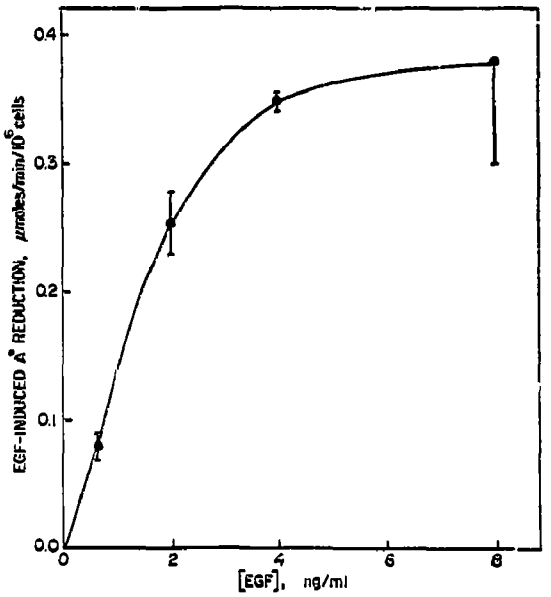

Fig. 4. Epidermal growth factor (EGF)-induced slowing of ascorbate autooxidation (reduction of ascorbate radical, A•) in the presence of 10^6 induced HL-60 cells. Conditions are as in Fig. 3.

oxidation of intracellular NADH with a corresponding appearance of NAD^+ [12]. The total amount of pyridine nucleotide remained unchanged in these experiments.

The nature of the electron transport chain involved in plasma membrane redox reactions is not known although a number of potential components have been identified as redox constituents of the mammalian plasma membrane [2]. However, the ability of cells to regenerate ascorbate parallels NADH oxidation [6] and oxygen reduction [4]. Both are inhibited by lectins. Additionally, treatment of cells 2 h at 37°C with 1–3 μM tunicamycin to inhibit glycoprotein glycosylation inhibited the prevention of ascorbate free radical reduction by the cells by nearly 50%. These findings, taken together with the stimulation of an NADH oxidase of the mammalian plasma membrane by EGF and Fe_2TF [13], indicate that measurement of ascorbate free radical reduction by whole cells may provide a convenient measure of the activity of the hormone- and growth factor-responsive electron transport chain of the mammalian cell surface.

Transmembrane transport of electrons from cytoplasmic NADH to impermeant artificial electron acceptors, such as ferricyanide, is a ubiquitous property of erykaryote plasma membranes [1,2]. However, if trans plasma membrane electron transport is to serve a physiological function, then a natural electron acceptor for NADH oxidation at the plasma membrane, most likely oxygen, would be required.

An enzymatic transfer of electrons from reduced pyridine nucleotide (NADH) to molecular oxygen in the absence of added electron acceptors defines an NADH oxidase activity recently identified through analyses of highly purified isolated plasma membrane vesicles from both animals [14] and plants [15]. The activity appears to exhibit some relationship to growth control and to fulfill a regulatory function as a terminal oxidase of plasma membrane electron transport [4,14]. Comparing purified plasma membranes from different sources, the oxidase is stimulated by growth factors and hormones in liver [14] and in plants [15] and is inhibited by growth inhibitory concentrations of retinoic acid and calcitriol (1-alpha-1,25-dihydroxy vitamin D_3) with human keratinocytes [16].

The plasma membrane NADH oxidase differs from mitochondrial oxidases and cellular peroxidases in being insensitive to cyanide. This may account for the stimulation of NADH oxidation by transferrin previously attributed to NADHidiferric transferrin oxidoreductase [7] and to earlier reported NADH oxidations by artificial electron acceptors stimulated by growth factors and hormones [18].

It is the oxidase which appears to be rate limiting to trans plasma membrane electron transport. Both the trans plasma membrane electron transport as measured by the reduction of external ascorbate free radical (slowing of the rate of chemical oxidation of ascorbate), and the plasma membrane oxidase as measured by the oxidation of NADH by isolated plasma membranes appear to be growth factor- and lectin-responsive. Thus it may be that the assay procedure described here and applied to living cells with internal NADH as the elec-

tron donor represents an in situ equivalent to a direct assay of the NADH oxidase of plasma membranes with external NADH as electron donor.

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