Antioxidants in Neoplastic Cells: II. Isolation and Partial Characterization of a Phenolic Antioxidant from Differentiated Mouse Neuroblastoma Cells

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ABSTRACT

The generation of an antioxidant has been shown to be associated with the dramatic increase in resistance to lipid peroxidation which occurs during the differentiation of mouse neuroblastoma cells in culture. The antioxidant has been isolated from the neuroblastoma neutral lipid fraction and partially characterized by means of low-resolution and high-resolution mass spectrometry and other lines of evidence. All presently available information suggests that this antioxidant is a highly aromatic, monosubstituted phenol having the molecular formula $C_{19}H_{14}O_2$.

INTRODUCTION

A variety of treatments has been shown to cause mouse neuroblastoma cells growing in culture to undergo a process of morphological and biochemical specialization which strongly resembles neuronal differentiation (1). During this process, the round, logarithmically growing cells flatten and extend long, axon-like processes termed neurites, while cell division is generally diminished or arrested (1).

During differentiation, neuroblastoma membranes exhibited a dramatic increase in resistance to lipid peroxidation (2). This observation suggested that the molecular mechanism underlying resistance to peroxidation might be identified by comparing the compositions of low-resistance and high-resistance cells. Following this approach, we have demonstrated a threefold enhancement in the antioxidative capacity of the neutral lipid fraction of neuroblastoma cells during differentiation (2).

In this paper, we report the isolation and partial characterization by mass spectrometry of a highly aromatic, apparently phenolic antioxidant which is generated during the differentiation of neuroblastoma cells. For convenience, we have designated this antioxidant A274.

METHODS

Mouse neuroblastoma cells (C 1300, clone N-18) were cultured in Eagle's medium as modified by Dulbecco (Gibco, Grand Island, NY) in 10% CO$_2$-90% air with 10% fetal bovine serum (Microbiological Associates, Bethesda, MD). Harvesting of cells and the preparation of neutral lipid extracts were carried out as previously described (2). Preparations of differentiated or undifferentiated cells were produced by two protocols reported in the preceding paper (2). In the first protocol, cells were cultured in 10% fetal bovine serum and harvested on the third day of subculture (3-day cells). In the second protocol, differentiation was induced by withdrawing fetal bovine serum from the medium on the fourth day of subculture; fresh serum-free medium was introduced on the fifth day, and the cells were harvested on the sixth day (6-day cells). When cultured in this manner, 3-day cells were undifferentiated and readily underwent peroxidation, while 6-day cells had formed neurites and were highly resistant to peroxidation (2). The test system for assessing lipid peroxide production by neuroblastoma membranes has been described (2). Protein was estimated by the method of Lowry and co-workers (3).

The assay developed in this laboratory (2) for estimating the antioxidative activity of lipid extracts was modified as follows in order to increase its sensitivity: an extract sample was tested in a final volume of 250 $\mu$L benzene containing 1.2 M isopropylbenzene (cumene, Eastman Organic Chemicals, Rochester, NY) and 0.1 mM azobis(isobutyronitrile) (AIBN, Aldrich Chemical Co., Milwaukee, WI). This mixture was incubated for 15 min in a sealed tube at 60 C in a shaking water bath. Cumyl hydroperoxide formation was determined by the ferrous thiocyanate method (4).

Extracts of neutral lipid from neuroblastoma cells were fractionated on an LH-20 Sephadex column using freshly distilled ethanol as the eluting solvent (5). A column 19 cm in height and having a bed volume of 17 ml was generally employed, but smaller columns were also used, including some made from Pasteur pipets. The Pasteur pipet columns were 7.0 cm in height and had a bed volume of 1.4 ml. Neutral lipid
samples derived from neuroblastoma (100 mg to 300 mg protein) were dissolved in 0.3 ml ethanol, placed on the large column, and eluted with ethanol; 1.0 ml fractions were collected. Since ethanol interferes with the cumene-AIBN assay, column fractions were dried in a vacuum oven at 60 C and redissolved in an appropriate volume of benzene. A water aspirator provided the vacuum. Experiments have shown that the antioxidant activity of column fractions is stable to incubation in a vacuum oven up to at least 135 C. Aliquots (10 ~μl-20 μl) from column fractions were assayed by the cumene-AIBN procedure and the results expressed as percent inhibition. The mid-peak tubes from the inhibitory peak (containing about three-fourths of the total activity of A274) were pooled and stored at -85 C.

Silica gel TLC medium (ITLC-SA) was purchased from Gelman Instrument Co., Ann Arbor, MI. Silicic acid, type CC-7 (Mallinckrodt Chemical Works, St. Louis, MO) was activated for 1 hr at 110 C. Silicic acid columns ca. 1 cm in height were prepared using Pasteur pipets and washed with several column volumes of ethanol prior to use.

The monoacetate of A274 was prepared by a modification of Gill’s method (6). The acetic anhydride used was twice-distilled. Sylon BTZ (Supelco, Inc., Bellefonte, PA) was employed to prepare the monois(trimethylsilyl) ether of A274; the sample of A274 was evaporated to dryness with nitrogen in a 0.3 ml reaction vial (Pierce Chemical Co., Rockford, IL), whereupon the vial was sealed, and 20 μl Sylon BTZ was injected. The contents were mixed, allowed to stand for 10 min at room temperature, and then incubated at 60 C for 30 min. The monomethyl derivative of A274 was prepared using diazomethane generated from N-methyl-N-nitroso-p-toluensulfonamide (Alrich Chemical Co., Milwaukee, WI) by the micromethod of Schlenk and Gellerman (7).

Low-resolution mass spectrometry was carried out using the following Finnigan components: a 1015D electron impact mass spectrometer and a 3200 chemical ionization mass spectrometer were separately interfaced to 9500 gas chromatographs and collectively interfaced to a 6000 minicomputer data system. Gas chromatography-mass spectrometry (GC-MS) was carried out using a 3% SP-400 column (80/100 Supelcoport, 2 mm ID, length, 45 cm) (Supelco, Inc., Bellefonte, PA). The temperature program generally employed was 150-240 C at 10 C/min. Methane (18 ml/min) served as both the carrier gas and the reagent gas for chemical ionization. A direct insertion probe was also used to introduce samples into the source. These studies employed 20-μl samples in benzene, which were derived from ca. 1 mg protein. About 5 μl of a sample was used for each test.

High-resolution GC-MS was carried out with an A.E.I. MS50/DSS50 system, using a 2% SE-30 column with helium (35 ml/min) as the carrier gas. Data were obtained during the course of each run by rapid, repetitive scanning at high resolution (>10,000 M/ΔM).

RESULTS

A 120-fold increase in the cumene/AIBN ratio afforded a 16-fold increase in the sensitivity of the assay. The kinetics of the high-sensitivity assay were similar to those reported for the original assay (2), except that the inhibitory potency of a lipid extract was no longer approximately constant with time but declined measurably during the course of the incubation. This result is reasonable since lower extract concentrations were employed, and essentially all chain-breaking antioxidants are degraded in the course of their action (8).

Chromatography of the neutral lipid fraction from differentiated, 6-day neuroblastoma cells on LH-20 Sephadex resulted in two major peaks whose antioxidant activity could be demonstrated by the cumene-AIBN assay (Fig. 1). Using the large column, we found these peaks at 14 ml (peak I, 0.82 Vbed) and 24 ml (peak II, 1.4 Vbed). An identical elution pattern was observed on the smaller columns. We believe these two peaks are due to different chemical substances. When peak I was rechromatographed, the elution volume of the activity was still 0.82 Vbed and no activity appeared in the region of peak II. Similarly, peak II rechromatographed at 1.4 Vbed and no activity appeared in the region of peak I.

In contrast to neutral lipid extracts from 6-day cells, extracts from undifferentiated, 3-day cells clearly exhibited peak I but showed only a trace of peak II when chromatographed on LH-20 Sephadex (Fig. 2). Comparison of Figures 1 and 2 indicated that peak II activity was associated with differentiation in neuroblastoma cells, whereas differentiated and undifferentiated cells yielded comparable peak I activity.

Trace contaminants found in a variety of polar solvents also inhibit the peroxidation of cumene, and these contaminants have proved difficult to remove. For example, a trace of antioxidant activity derived from the solvent remained in the column fractions after the 60 C vacuum evaporation step. One-milliliter portions of ethanol were concentrated