Alzheimer's Disease: X-ray Spectrometric Evidence of Aluminum Accumulation in Neurofibrillary Tangle-Bearing Neurons

Abstract. *The elemental content of neurons of the hippocampus was studied by a combination of scanning electron microscopy and x-ray spectrometry in autopsy-derived brain tissue from three cases of senile dementia (Alzheimer type) and three nondemented elderly controls. Foci of aluminum were detected within the nuclear region of a high percentage of neurons containing neurofibrillary tangles from the cases of senile dementia as well as the elderly controls. The adjacent normal-appearing neurons from both groups of patients were virtually free of detectable aluminum. These findings suggest that the association of aluminum to Alzheimer's disease extends to the neuronal level.*

The neurofibrillary tangle (NFT), since its original description by Alzheimer (*1*) in 1907, has been considered one of the principal miscroscopic features seen in the brains of individuals with senile dementia, Alzheimer's type (SDAT). The NFT is readily observed with the light microscope after silver impregnation staining and consists of parallel arrays of thickened, coarse, argyrophilic fibers within the neuronal cytoplasm. Studies of tissue obtained at autopsy show a striking correlation between the degree of clinical dementia and the extent of NFT formation in the hippocampus (*2*). Although small numbers of NFT's are encountered in the brains of nondemented elderly individuals, the density of NFT's in the hippocampus of patients with SDAT is 6 to 40 times greater than that of nondemented age-matched subjects (*3*). The pathogenesis of this cellular alteration, as well as that of SDAT itself, remains undetermined.

In 1973, Crapper and co-workers (*4*) reported the presence of increased amounts of aluminum in the brains of four individuals with SDAT. Their study was stimulated by the observations in rabbits (*5*) of the induction of neurofibrillary degeneration on direct exposure of central nervous system tissues to aluminum salts. The aluminum concentrations measured by Crapper *et al.* were extremely small, 7 to 9 μg of aluminum per gram (dry weight) of brain tissue in affected individuals compared to 2 μg/g in normal brain tissue. There was considerable regional variation in the aluminum concentration with increased amounts

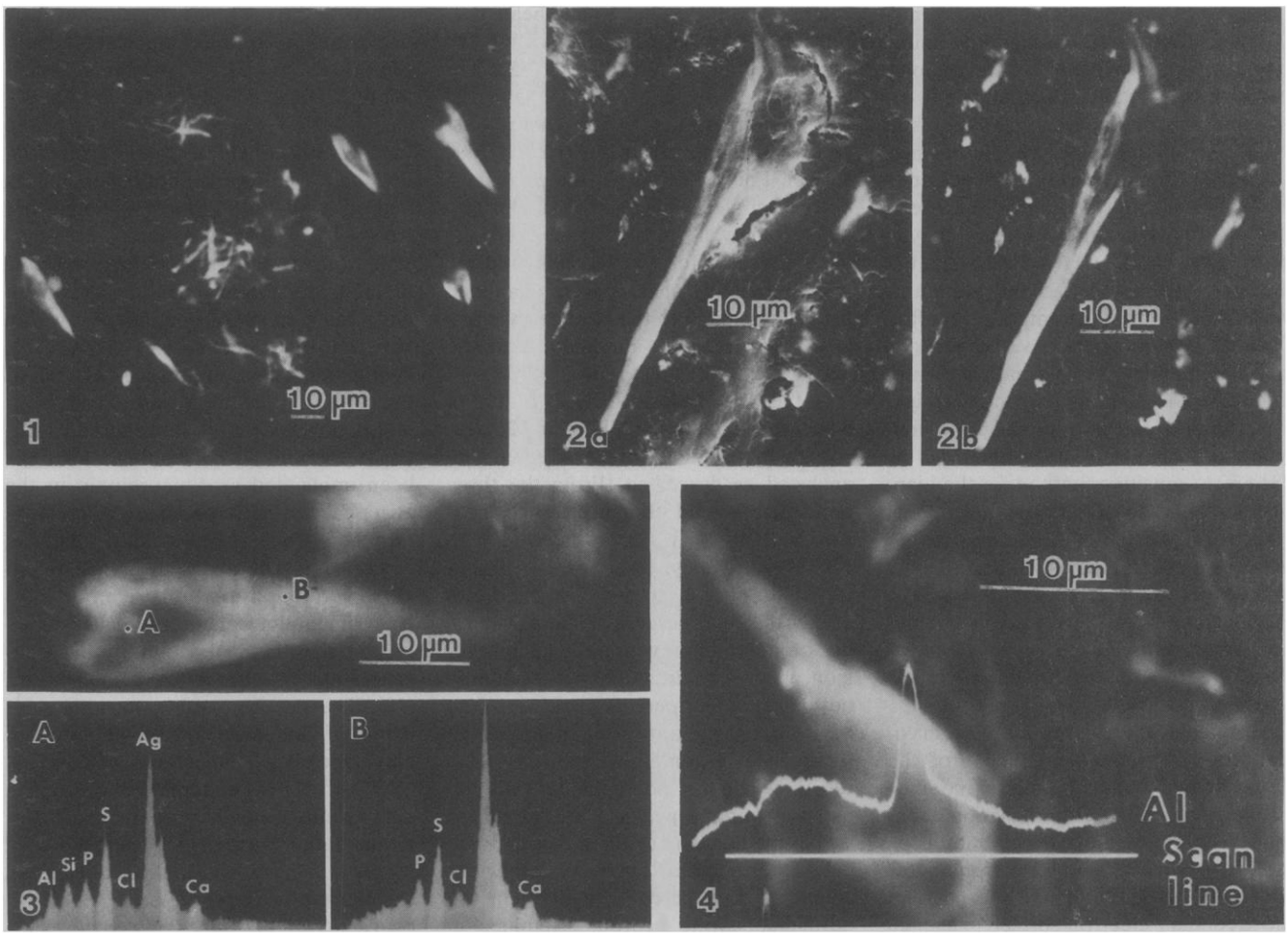

Fig. 1. Scanning electron micrograph–backscattered electron imaging of modified Bielschowski silver-stained section of the hippocampus, showing several NFT's and a senile plaque. Fig. 2. Scanning electron micrograph–secondary electron surface image (a) and backscattered electron image (b) of neuron containing a NFT (hippocampus-modified Bielschowski stain). Fig. 3. Scanning electron micrograph–backscattered electron image of a silver-stained neuron containing a NFT. Points A and B indicate the areas subjected to elemental analysis. Partial x-ray energy spectra are shown below corresponding to these two points. Note the prominent peaks for aluminum and silicon present in point A but not detected in point B. The counting time was 100 seconds. Fig. 4. Scanning electron micrograph–backscattered electron image of a silver-stained neuron with a NFT. A line scan is passed through the nuclear region. The above tracing demonstrates the distribution of energy detected along the line scan at the x-ray wavelength characteristic for aluminum. Note the focal intranuclear concentration of aluminum.

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Table 1. Percentage of neurofibrillary tangle-containing neurons and normal-appearing cells demonstrating the presence of magnesium, aluminum, or silicon. SDAT, 60 neurons from three cases (34 neurons with NFT's, 26 without); N-dE, nondemented elderly patients, 60 neurons from three cases (9 with NFT's, 51 without). A cell is considered positive if any of the four sites of analysis (four intranuclear, four cytoplasmic) show the presence of a peak corresponding to the Kα energy emission of either magnesium, aluminum, or silicon.

NFT	Cells positive (percent)					
	Magnesium		Aluminum		Silicon	
	SDAT	N-dE	SDAT	N-dE	SDAT	N-dE
			Nucleus			
Present	52.9 ± 8.6	100	91.2 ± 4.9	88.9 ± 10.5	50.0 ± 8.6	11.1 ± 10.5
Absent	3.8 ± 3.7	62.7 ± 6.8	3.8 ± 3.7	5.9 ± 3.3	30.8 ± 9.1	2.0 ± 2.0
			Cytoplasm			
Present	52.9 ± 8.6	100	29.4 ± 7.8	11.1 ± 10.5	29.4 ± 7.8	0
Absent	3.8 ± 3.7	49.0 ± 7.0	3.8 ± 3.7	2.0 ± 2.0	34.6 ± 9.3	7.8 ± 3.8

present predominantly in the areas containing the most prominent senile changes (frontal and temporal cortex and hippocampus). A subsequent report (*6*) provided aluminum concentrations from brains from ten additional patients with senile dementia as well as nine normal brains derived from nondemented individuals. The results refined the initial findings and provided further regional correlation of excess aluminum to areas of the brain containing large numbers of NFT's. Aluminum assays were performed by atomic absorption spectrometry with ashing of the tissues. Because of the destructive nature of the analytic procedure, Crapper *et al.* (*6*) were unable to further localize the aluminum within the tissues. Attempts to confirm Crapper's observations have been unsuccessful (*7*), and the association of aluminum to SDAT remains controversial.

Scanning electron microscopy in conjunction with x-ray spectrometry is an extremely sensitive method for the identification and localization of elemental constituents of biological tissues (*8, 9*). We now report on the use of this analytic procedure for the determination of the elemental content of neurons in the hippocampus of three cases of SDAT and of three elderly nondemented individuals (*10*).

Sections (20 μm) were cut on a cryostat from Formalin-fixed blocks of the hippocampus, stained with a modified Bielschowski silver impregnation technique, mounted onto pure carbon disks, and viewed in a scanning electron microscope (JEOL JSM-35). On the basis of the secondary electron surface image, 20 neurons were selected from the Sommer's sector of Ammon's horn. By means of back-scattered electron imaging (BEI) (*11*), the NFT's and the senile plaques within the tissues were identified by the bright appearance of the argyrophilic regions. The combination of the silver impregnation staining and BEI provides an image that is comparable to routine neurohistologic preparations (Figs. 1 and 2). Each preselected neuron was then evaluated by BEI for the presence or absence of a NFT (Fig. 2).

Under standardized conditions of accelerating voltage (25 kV) and beam current (1×10^{-9} A), four sites within the nuclear region, each measuring 0.5 μm in diameter, were chosen for elemental analysis by means of an energy dispersive x-ray spectrometer (Kevex 7000 series). Four sites within the perikaryal cytoplasm were selected for similar elemental analysis. X-rays from each site were collected for 100 seconds, and the resulting energy spectra were examined for peaks related to the Kα energy emission of magnesium, aluminum, and silicon (*12*) (Fig. 3).

In the three cases of SDAT, a total of 34 neurons containing NFT's and 26 neurons without NFT's were selected and analyzed as described. From the three nondemented elderly patients, 51 neurons without NFT's and 9 neurons with NFT's were analyzed (Table 1). Of neurons with NFT's examined in the cases of SDAT, 91.2 percent demonstrated a peak for aluminum within the nuclear region, whereas aluminum was essentially not detected (3.8 percent positive) in the adjacent normal-appearing neurons. In the few NFT-containing neurons from the three elderly nondemented individuals, aluminum peaks were identified within the nuclear region in eight of nine cells (88.9 percent) but were rarely encountered (5.9 percent positive) in the normal-appearing neurons examined in these cases. Although silicon was detected within the tangle-bearing neurons of the cases of senile dementia, this element was also encountered in the nontangled neurons. Silicon was rarely seen in the cells of the nondemented individuals. Magnesium was rather ubiquitous in all cell types except for the nontangled neurons of the SDAT cases. Data from the cytoplasmic regions closely paralleled that of the nuclei, except that the percentages of aluminum-positive cells were much lower (Table 1).

The accumulation of aluminum in the nuclear region of tangle-bearing cells appears to be focal. Of the determinations made within the nuclear region of tangle-bearing cells, only 38.4 percent demonstrated a peak for the presence of aluminum. The focal nature of the aluminum concentrations could also be demonstrated by evaluating many of the same tangle-bearing neurons by wavelength x-ray spectrometry, a technique which more closely resolves adjacent elemental peaks (*8*). Using a wavelength x-ray spectrometer, we confirmed the presence of focal aluminum concentrations in the nuclear region of NFT-bearing cells in all three senile cases (Fig. 4).

Our findings indicate an association of focal intranuclear accumulation of aluminum with the presence of neurofibrillary degeneration in the hippocampal neurons of cases of SDAT. A similar association is suggested by the data on the occasional NFT-containing neurons of the hippocampus of elderly nondemented individuals. Adjacent normal-appearing neurons from both groups of patients are free of detectable aluminum.

With the use of similar electron probe methods, focal concentrations of silicon and aluminum have been detected within the senile plaques of cases of SDAT (*13*). Aluminum, although present on much of the earth's surface, has no known biologic function and is not considered essential to the diet (*14*). Aluminum also has been implicated as a neurotoxin in the encephalopathy associated with long-term hemodialysis or so-called "dialysis dementia." Although the brains of patients with this condition are reported to have aluminum concentrations 12 times greater than normal, neurofibrillary degeneration has not been demonstrated (*15*). The precise nature, location, and extent of aluminum accumulation within cases of dialysis dementia as well as neurons with NFT's encountered in senility and various other neuropathologic conditions requires further investigation.

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Genetic Expression of Wilson's Disease in Cell Culture: A Diagnostic Marker

Abstract. *Wilson's disease fibroblasts have an elevated intracellular copper concentration as compared to cultured control cells. A decreased ratio of copper to protein was observed in cytoplasmic protein (or proteins) having a molecular weight ≥ 30,000 in Wilson's disease cells. The results of this culture study indicate its potential importance in the early unequivocal diagnosis of this disorder.*

Wilson's disease (hepatolenticular degeneration) is an autosomal recessively inherited metabolic dysfunction of copper metabolism (*1*). It is caused by excessive deposition of copper in various organs and, if untreated, the disease is invariably fatal. However, all the clinical manifestations can be prevented if the disease is diagnosed before functional impairment occurs (*2*). The onset of symptoms is usually in late childhood or early adulthood. The classical symptoms are Kayser-Fleischer rings, neurologic dysfunction, liver cirrhosis, and hypoceruloplasminemia. These symptoms may be absent in childhood. This, coupled with the absence of a simple noninvasive test, make early diagnosis of this disorder difficult (*3*).

The basic biochemical defect of Wilson's disease is still unclear. Abnormalities of the structure and metabolism of ceruloplasmin have been suggested (*4*). However, no confirmatory evidence has been found. Several investigators have suggested that metallothionein from the liver of patients with Wilson's disease has an abnormally high copper affinity (*5*); others have isolated a low-molecular-weight copper binding protein and found it to have a different quantity of bound copper as compared to normal liver (*6*). We now report that the genetic abnormality of Wilson's disease is expressed in cultured skin fibroblasts derived from these patients. Thus, cultured fibroblasts may serve (i) as an in vitro model for studying the pathogenesis of this disorder and (ii) as a noninvasive method for early diagnosis.

Skin fibroblast cultures were established from biopsies of typical Wilson's disease patients. Additional cultures were obtained from the Human Genetic Mutant Cell Repository (GM 32, 33). Cell cultures from individuals matched according to age and sex were used as controls. Both patients and controls included males and females ranging in age from 9 to 20 years. Cells were grown in 75-cm^2 plastic culture flasks containing 12 ml of Eagle's minimum essential medium with Hanks balanced salt solution, fetal calf serum (10 percent), and antibiotic-antimycotic (1 percent) solution and adjusted to *p*H 7.2 with 7.5 percent sodium bicarbonate solution. The cultures were incubated in a humidified environment of 5 percent carbon dioxide at 37°C. Only confluent cultures with a passage number between 6 to 13 were used. Confluent cultures were harvested with a rubber policeman, and the cells were suspended with 5 ml of deionized distilled water. Cells were lysed by rapidly freezing and thawing the suspension five times in Dry Ice and acetone. The lysate was centrifuged at 10,000*g* for 20 minutes. The supernatant was assayed for copper and cadmium with a Perkin-Elmer model 703 atomic absorption spectrophotometer equipped with a Perkin-Elmer model HGA-500 graphite furnace (*7*). Protein concentration of the lysates was determined by the method of Lowry *et al.* (*8*).

Table 1. Intracellular content of copper and cadmium in cultured fibroblasts. The results are expressed in nanograms (means ± standard deviation) of metal per milligram of soluble cell protein. The numbers in parentheses denote the number of different cultures (each from a different patient or control) tested.

Fibroblasts	Copper	Cadmium
Control (8)	101 ± 17	21.2 ± 5.0
Wilson's disease (7)	280 ± 19	20.5 ± 3.1

Wilson's disease cultured fibroblasts were morphologically normal and had a growth rate similar to control cells in basal medium. Completed media had a copper content of 25.4 ± 2.8 ng/ml and a cadmium content of 1.76 ± 0.14 ng/ml. The intracellular copper concentration of Wilson's disease cultured cells was significantly higher (approximately threefold) than that of normal cells (Table 1). The intracellular cadmium in both cell types was comparable. This observation indicates that the genetic abnormality of Wilson's disease, that is, elevated accumulation of copper, is expressed in cultured fibroblasts.

To examine the distribution of intracellular copper in Wilson's disease cultured cells, we fractionated cell lysates by gel filtration on a Sephadex G-50 column (2.5 by 60 cm) equilibrated with demineralized 0.02*M* tris-acetate (*p*H 8.6). Proteins were eluted from the column with the equilibrating buffer at a flow rate of 24 ml/hour. Proteins eluted were monitored by measuring the absorbance at 220 nm of the fractions (25 drops per fraction) with a Beckman ACTA III spectrophotometer. The copper concentration of the column fractions was determined by flameless atomic absorption spectroscopy at 324.7 nm. The fractionation procedures were carried out at 4°C (Fig. 1). A number of copper peaks are present in lysates from Wilson's disease cells but not in control lysates. These peaks had a very low absorbance at 220 nm. Only two copper peaks showed sig-

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