Distribution of Iron in a Single Neuron of Patients with Parkinson’s Disease

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Synchrotron radiation x-ray fluorescence (SRXRF) spectroscopy was applied to non-destructive elemental mapping in the melanized nigral neurons obtained from patients with Parkinson’s disease (PD) and a control subject. The cause of PD is unknown but many researchers consider that excessive accumulation of metallic elements (mainly iron) has a role in the generative process of PD. Microbeam imaging (mapping of the elements) with a beam size of 6 × 8 μm and an energy of 13.5 keV was carried out in single neurons. The distributions of trace elements in the neurons were obtained in an area of about 100 × 100 μm. It is demonstrated that iron was accumulated in the neuromelanin aggregates in and around the nigral neurons, coprecipitating with sulfur, calcium, zinc and copper to various extents in both the defined and control specimens. The average of the iron intensity measured inside of the melanin pigment granules of a PD case was about one order of magnitude higher than that of the control samples. Copyright © 1999 John Wiley & Sons, Ltd.

INTRODUCTION

It is highly probable that the function of a cell and in certain cases the cause of death of a cell are affected by the incorporation and/or accumulation of metallic elements in the cell. The elements that may be responsible for cell death are from a variety of sources with genetic or environmental origins. The kind of elements may be the heavy, medium- or light-mass elements. One of the well-studied elements related to excessive accumulation causing cell degeneration is iron. The study of the presence and distribution of iron was performed in the 1920s using histochemical and biochemical methods.¹,² The importance of the investigation of iron in the motor system is due to its distinct regional differences in predominant areas of the motor loop, its role in metabolic enzyme processes and its parallelism of distribution in the regions of the brain with the high concentration of neurotransmitter substances.¹ Iron has an oxidant role by donating an electron and enhancing the conversion reaction from the ferrous (Fe²⁺) to the ferric (Fe³⁺) state.³ A model for studying cell death in Parkinson’s disease (PD) is based on initiations of a PD-like syndrome by discovery of the selective nigral toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). This model considers the mechanism by which an exogenous toxic substance can initiate a neurodegenerative disorder.

Attempts have also been focused on investigating the causative factors in degenerative diseases such as PD such as environmental factors. However, there has not been a clear identification of the factors that may play a decisive role in the cases investigated.⁴,⁵

Based on the above considerations the basic questions in our study were as follows: (i) what is the elemental constituent of a single neuron obtained from patients with PD?; (ii) what are the relative concentrations of the elements and the absolute concentrations of the elements (quantification of elemental concentration)?; (iii) what is the distribution of a particular element (Fe, P, etc.) in the neuron?; (iv) what are the chemical states of iron and other elements? There is no single analytical method which can give answers to all these questions, especially when the contents of the elements are low. So far, conventional methods such as histochemical, biochemical, inductively coupled plasma spectroscopy,⁶ scanning electron microscopy with a wavelength-dispersive x-ray microanalyzer⁷ and nuclear microprobe (micro-PIXE)⁸ have been applied to investigate the trace elemental contents of the affected neurons.

In the past decade, synchrotron radiation x-ray fluorescence (SRXRF) spectroscopy⁹–¹¹ has been used for imaging trace element distributions¹²–¹⁵ in biopsy samples. SRXRF spectroscopy is a powerful method for biological samples, having the following advantages:¹⁶ (i) the detection limit is low (0.01 ppm level); (ii) the measuring time is relatively short (a few seconds for each point); (iii) heat damage is small compared with electron or iron microprobe methods; (iv) the measurements can be done in air.

PD is one of the major neurodegenerative diseases. Neuropathological studies have revealed that substantia nigra (SN), which is a part of midbrain, of patients with PD is damaged.¹⁷ The cause of neurodegeneration is unknown, but there are indications that iron in SN neurons is related to the neuronal degeneration. Many neuropathologists have pointed out that SN neurons of PD patients contain more iron than those of control subjects.¹⁸ The increased amount of iron in affected neurons may be responsible for the degenerative process in PD. Therefore, mapping of iron and other elements in a single neuron...
has great significance in understanding the role of metal elements in neurodegenerative processes.

EXPERIMENTAL

Beamline and main parameters
SRXRF analysis was carried out at the beamline 4A at the Photon Factory, KEK, Tsukuba, Japan. The x-ray beam was monochromatized with a synthetic multilayer film. The x-ray energy was 13.5 keV. Monochromatized x-rays were focused with Kirkpatrick–Baez optics. The cross-section of the beam was 6 × 8 µm on the sample. The sample stage was moved by x–y step pulse motors and the distributions (x-ray intensity maps) of Fe, Zn, Ca and S were obtained. The scanning area was 102.5 × 102.5 µm and was divided into 41 × 41 pixels. Each measurement point of the sample was irradiated for 5 s. The beam current of the storage ring was about 350 mA. Measurements were made in air. The beamline was equipped with a CCD camera in front of the sample holder. The image from this camera gave visual information on the measuring points.

An important problem to be solved during measurement was the handling of the specimen and observation of the measurement points for matching of the optical and XRF images. The specimens were monitored by a CCD camera during mapping. Using the CCD camera image, the scanning area was identified by distinguishable color of neuromelanin pigments. After XRF analysis, the same samples were deparaffinized and used for optical microscopic examination. The elemental maps were matched against the neuropathological findings obtained from the same tissue section stained with hematoxilin–eosin (HE) after analysis.

Clinical diagnosis and preparation of specimen
The specimens used in this study were obtained from patients who were clinically diagnosed to have PD Autopsy specimens of the midbrain, including the substantia nigra, were obtained from a 72-year-old male patient with PD and a 74-year-old male control subject. The specimens were fixed in 10% formalin and embedded in paraffin. Unstained sections with a thickness of 8 µm were cut from paraffin blocks and mounted on a Mylar film for elemental analysis. Neuromelanin is a central constituent of certain populations of dopaminergic neurons in the human substantia nigra, and is important for understanding neuron functioning and degeneration in PD. PD is characterized by reduction levels of the neurotransmitter dopamine, consequently leading to disturbance of the motor system with symptoms of tremor, rigidity and akinesia. In Parkinsonian substantia nigra, there is a marked accumulation of iron, especially in the melanized neurons of the pars compacta. The potential pathogenicity of iron in PD is related to its ability to generate free radicals and its selective binding to neuromelanin, producing Fe3+-melanin complexes. These complexes may promote the formation of cytotoxic free radicals and thereby increase lipid peroxidation and neuron death. There is relatively little information currently available on the cellular and subcellular distribution of iron in the nervous system in either normal or diseased cases.

RESULTS

In this section, experimental results are presented on the relative content and XRF imaging of iron and other trace elements in melanized nigral neurons. Emphasis is placed on a comparison between the neurons of diseased and normal cases. Using an SR microbeam, the constituent elements and their distributions in a single substantia nigra neuron were obtained. The same samples were studied histologically using an optical microscope after XRF spectroscopy.

Elemental content of melanin in normal and diseased neurons
XRF spectra were obtained at three different groups of points, namely (a) points inside of the melanin pigment granules released from a dead cell, (b) points outside the melanin pigment granules (control points) and (c) points at the boundary of the melanin pigment granules. One typical spectrum of each group is shown in Fig. 1(a)–(c), and the measurement points are indicated in Fig. 2 by (a), (b) and (c). Metallic elements (Fe, Zn and Cu) were detected inside the melanin pigment granules from the dead cells at high concentrations. The experiments were done in air, hence giving a constant value of the Ar peaks in all spectra. From each spectrum in groups (a) and (b), the peak areas of Fe, Cu, S, Ca and Ar were calculated. Averages, standard deviations and measurement errors of the peak areas are given in Table 1. The integrated values of the peak area of each element, after reduction of the background, were normalized by those of Ar. The average of the Fe intensity measured inside the melanin pigment granules was about 11 times higher than that of the control points. The average of the Cu intensity measured inside the melanin pigment granules was also about twice that of the control points. The measurement errors of the S and Ca peak areas in both groups were large.

Nevertheless, there was a substantial difference in the intensities of S in the three different regions.

<table>
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<th>Points</th>
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<th>S (a.u.)</th>
<th>Ca (a.u.)</th>
<th>Fe (a.u.)</th>
<th>Cu (a.u.)</th>
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</table>

* The measurement points were (A) inside the melanin pigment granules released from a dead cell and (B) outside the melanin granules.
Neuropathological investigations showed that the number of pigmented nigral neurons in a patient with PD is much lower than that of the control subject. In the tissue of patient with PD, various sizes of melanin pigment granules released from dying nigral neurons were scattered in a more condensed form (neuromelanin aggregates) than those contained in the nigral neurons of the control subject.

X-ray intensity maps of major elements in normal tissues were obtained and the results are shown in Fig. 3. In the normal nigral tissues, three normal pigmented neurons were observed. However, the elemental maps of Fe, S, Ca and Cu showed different patterns in each neuron [Fig. 3(A) and (B)]. The pigmented neurons (a) adjacent to a small vessel (v) showed strong x-ray intensities of all four elements, especially iron; neuron (c) showed a similar pattern but less marked in each element. In neuron (b), only the x-ray intensity of Fe was very strong, and the intensities of S, Ca and Cu were almost at the background intensity level.

**Imaging of nigral tissues from a PD patient**

X-ray intensity maps of major elements of the nigral tissues from a patient with PD were obtained and the results are shown in Fig. 4. In the PD nigral tissue [Fig. 4(A) and (B)], large aggregates (a) and small fragmented pieces (b) of neuromelanin pigments released from disappearing cell bodies were observed. There could also be seen an apparently normal nigral neuron (c) containing a small amount of neuromelanin pigments. A comparison between the x-ray intensities of the two cases mentioned above shows that the Fe content of the PD case was about one order of magnitude higher than that of the normal case.

The Fe (Kα) intensity from the large neuromelanin aggregates (a) was much stronger than that of the control subject, accompanying strong x-ray intensities of S, Ca and Zn. The small fragmented pieces of neuromelanin pigments (b) also showed a relatively strong x-ray intensity of Fe. However, neuron (c) which is apparently normal, showed no significant increase in the x-ray intensity of the four elements compared with the background level.

**Chemical state of iron**

An important consideration in investigating the influence of metallic ions on the functions of neurons is the understanding of the chemical state of the constituent elements. XRF spectroscopy and XAFS (EXAFS, XANES) are powerful techniques for understanding the physico-chemical
properties of the incorporated metals. However, two major problems exist in performing XAFS for ultra-low content metals in a single neuron. In the energy range 2.5–10 keV, which is needed to cover the energy range of XAFS for the elements of interest (P, Cl, Ca, Fe, Ni, Cu and Zn), neither the size nor intensity of the beam in the currently available SR facilities is sufficient. In a preliminary trial, we used beamline 4A at the Photon Factory with a beam radius of 500 µm and obtained fluorescence and transmission spectra in the energy range 7.10–7.161 keV. A typical spectrum is shown in Fig. 5. Because of the large size of the beam, we observed the possibility of presence of many states such as +2 and +3 and hydro compounds of Fe. The low intensity of the beam and the low content of iron in the neuron make it impossible to distinguish the positional variation of the charge state in this experiment. Using other available facilities, further experiments are being carried out in order to obtain a better understanding of the charge state of the elements in a single neuron.

Figure 3. (A) X-ray intensity maps of Fe, S, Ca and Cu and (B) photograph of the scanned area of the nigral tissue stained with HE for a normal case. The scanning area was about 75 × 75 µm and was divided into 30 × 30 pixels. The scale on the right of the x-ray intensity map shows the counts of the x-ray intensity. The range of mapping was 20–300 for Fe, 15–30 for S, 45–75 for Ca and 15–50 for Zn.

Figure 4. (A) X-ray intensity maps of Fe, S, Ca and Zn and (B) photograph of the nigral tissue stained with HE obtained from a patient with PD. The scanning area was about 102.5 × 102.5 µm and was divided into 41 × 31 pixels. The scale on the right of the x-ray intensity map shows the count of the x-ray intensity. The range of mapping was 20–450 for Fe, 15–40 for S, 15–40 for Ca and 5–15 for Zn.
DISCUSSION

In the present study we applied microbeam SRXRF to study single neurons from patients with PD and compared them with normal cases. The elemental content of the neuron, the relative concentration of the elements and the distribution of S, Ca and Fe in melanin pigment granules were measured. The average of the Fe peak area measured inside of the melanin pigment granules of the PD case was about one order of magnitude higher than that of the normal case. These results confirm previous results on the selective accumulation of iron in the neurons of patients with PD. The average of the Cu intensity measured inside the melanin pigment granules was also higher than that of the control points. There was also a difference in the intensity of S inside and outside the melanin pigment granules released from an apparently dead neuron.

Detailed investigation of the images of Fe (maps of Kα intensity of Fe) from the large neuromelanin aggregates showed that Fe had a much higher content in the neuromelanin released from a dead cell compared with that of the control subject. The same tendency was observed for the x-ray intensities of S, Ca and Zn. The neuromelanin from a surviving cell showed that the x-ray intensity of all four elements were almost at the background level.

Regarding the chemical state of iron and other elements that have a decisive role in the cytotoxicity of the elements that may result in neuron death, we demonstrated the possibility of the application of XAFS. The technical problems that are left to be solved are to have a beam size of a few µm (preferably 1 µm) in order to be able to make localized measurements and to have a higher photon flux in order to be able to measure ultra-low content elements. Using other available facilities with some improvement in beam optics, further experiments are being carried out in order to have a better understanding of the charge state of the elements in a single neuron.

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REFERENCES