Int J Legal Med (2001) 114 : 310–315

ORIGINAL ARTICLE

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Intranuclear ubiquitin immunoreactivity in the pigmented neurons of the substantia nigra in fire fatalities

Received: 30 June 2000 / Accepted: 11 October 2000

Abstract To evaluate the significance of immunohistochemical staining of ubiquitin (heat shock protein) in the midbrain for medico-legal investigation of death in fires, we examined forensic autopsy cases of fire fatalities (n = 35) in comparison with controls (n = 27; brain stem injury, acute myocardial infarction and carbon monoxide poisoning other than fire fatality). There were two intranuclear staining patterns in the nuclei of pigmented substantia nigra neurons: a type of inclusion (possible Marinesco bodies) and a diffuse staining. Percentage of nuclear ubiquitin positivity (Ub-positive %) in fire fatalities (2.7–44.7%; mean, 18.5%) was significantly higher than in brain stem injury (n = 9; 0–10.4%; mean, 4.5%) and myocardial infarction (n = 14; 1.5–14.6%; mean, 6.9%), independently of blood carboxyhemoglobin (COHb) levels. Age-dependent increase in Ub-positive % was observed in lower COHb (< 60%) cases. The intranuclear diffuse ubiquitin staining was not observed in cases of high blood cyanide level (> 1.0 μg/ml). These observations showed that intranuclear ubiquitin immunoreactivity of the pigmented substantia nigra neurons in the midbrain was induced by severe stress in fires.

Key words Ubiquitin · Pigmented substantia nigra neurons · Immunohistochemistry · Fire fatality · Physical activity

Introduction

Heat shock proteins, including ubiquitin, rapidly respond to various types of stress [1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14]. Changes in these proteins and related acute reactants have been studied by the immunohistochemical and biochemical methods in fire fatalities [15, 16, 17], brain injury [18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28], asphyxia [9] and sudden infant death [29, 30]. No studies have been undertaken of the central nervous system (CNS) in fire deaths. Pigmented neurons of the substantia nigra (dopamine-neurons) of the midbrain have a function related to skeletal muscle movement under the control of the cerebral cortex [31]. The pigmented neurons in the eldery deceased often contain non-viral eosinophilic intranuclear inclusions (Marinesco bodies) [32], which are considered to be degenerative products of neuromelanin or other dopamine-neuron specific intranuclear components [33, 34, 35, 36, 37, 38], although the pathological significance still remains obscure. Ubiquitin immunoreactivity in these pigmented neurons, in relation to the neuronal degeneration including the appearence of Marinesco bodies, may be a useful marker for the stress on CNS resulting from the physical activity before death in fires.

In the present study, on forensic autopsy materials, the significance of immunohistochemical staining of ubiquitin in the midbrain was evaluated in deaths in fires.

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Materials and methods

Formalin-fixed paraffin-embedded midbrain tissue specimens (horizontal section, sampled at the under-mentioned post-mortem time) of 35 forensic autopsy cases of fire fatality, without any significant preexisting disease, concomitant trauma or hypnotic-sedative drug-influence, at our institute were examined. The fatal fire cases (survival time, < 0.5 h; without post-mortem thermal damage to the midbrain) were subdivided into two groups by blood carboxyhemoglobin (COHb) levels: COHb < 60% (n = 25, 17 males and 8 females, 47–86 years of age, 6–37 h post-mortem) and COHb > 60% (n = 10, 3 males and 7 females, 42–78 years of age, 6–32 h post-mortem). Control groups consisted of fatalities from brain stem injury (n = 9, males; 47–85 years of age; survival time, minutes; 8–38 h post-mortem), acute myocardial infarction (n = 14; 11 males and 3 females; 39–84 years of age; survival time, < 0.5–15 h; 6–34 h post-mortem) and fatalities from acute carbon monoxide (CO) poisoning due to incomplete combustion of coal/gas (n = 4, 3 males and 1 female; 18–76 years of age; survival time, < 0.5–1 h; 16–36 h post-mortem).

Tissue sections

Serial sections (4 μm thick) were prepared from the tissue specimens of the midbrain. The tissue sections were stained with haematoxylin-eosin (H&E) and immunohistochemistry.

Immunohistochemistry

Polyclonal rabbit anti-ubiquitin (Dako A/S, Denmark) was used at a 100-fold dilution, with a 3 h incubation at 37°C, on a Vectastain Universal Elite ABC Kit (DAB) (Vector Laboratories, Burlingame, Calif.) according to the manufacturer's instructions (counterstaining with hematoxylin). Endogenous peroxidase was inactivated by incubation with 3% hydrogen peroxide for 10 min. As the control to confirm the specificity of immunostaining, phosphate buffered saline or normal rabbit serum was substituted for the primary antibody.

Quantitative analysis of Marinesco bodies and ubiquitin-staining in the nuclei of pigmented substantia nigra neurons

Marinesco bodies were identified as eosinophilic nuclear inclusions in addition to the nucleolus in the pigmented neurons visible on the H&E sections. The neurons with nuclei in which Marinesco bodies were detected were counted: the number of total pigmented neurons and that of Marinesco body-containing neurons were counted in 10 fields under a 200 × magnification, and the percentage (%) was estimated.

Ubiquitin-positive pigmented neurons were quantitatively analysed in a similar manner: the number of neurons with nuclei in which ubiquitin immunoreactivity was detected were counted and the percentage of nuclear ubiquitin positivity (Ub-positive %) was estimated as described above.

Chemical analyses

Blood COHb % saturation was analysed on a CO-oximeter system (Ciba-Corning 270, New York). Cyanide and alcohol were determined by head-space gas chromatography/mass spectrometry [39]. Drug analyses were performed by gas chromatography/mass spectrometry.

Statistical analyses

A regression equation analysis was used to examine the relationship of Marinesco bodies and nuclear ubiquitin-positivity with the age of victims, COHb and cyanide levels. Comparison between groups were performed by Students *t*-test. Logistic regression and stepwise regression tests were used in the multivariate analyses. A P value less than 0.05 was considered statistically significant.

Results

Immunohistochemical distribution of ubiquitin

Intense immunostaining for ubiquitin was frequently observed in the cerebral peduncles and the ependymal cells of the cerebral aqueduct irrespective of the cause of death. The microglial cells in the central gray matter showed scattered positive staining in some cases. A characteristic staining was found in the nuclei of some pigmented substantia nigra neurons. The staining was clearly identifiable and showed two patterns: a type of inclusion body and a diffuse staining (Fig. 1a and c). The diffuse staining usually involved ubiquitinated inclusions. Typical intranuclear inclusions were eosinophilic in H&E staining of the adjacent sections, and were identified as Marinesco bodies (Fig. 1b and d). The ubiquitinated nuclei were markedly larger and less stained than the others in H&E sections.

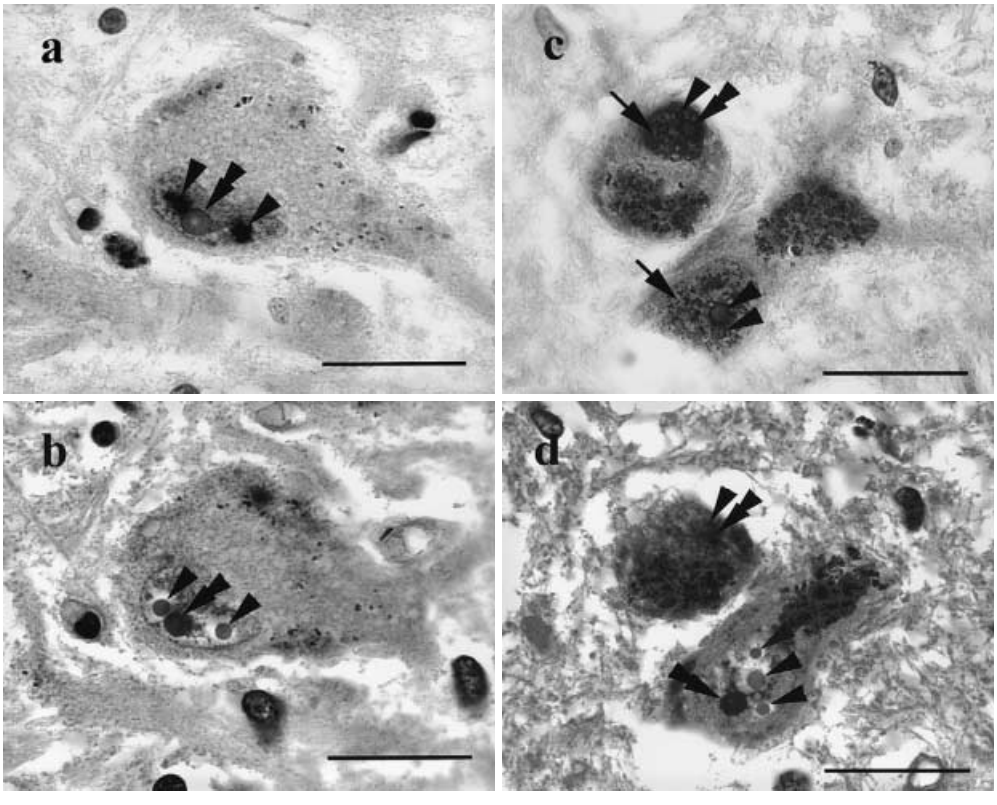

Fig. 1 a–d Intranuclear inclusions and ubiquitin immunoreactivity of the pigmented substantia nigra neurons in the midbrain (Original magnification × 1000, bar = 10 μm). **a** Intranuclear ubiquitin immunoreactivity (*arrowheads*) and **b** intranuclear inclusions (Marinesco bodies) by H & E staining (*arrowheads*) on serial sections, **c** intranuclear diffuse immunostaining of ubiquitin (*arrows*) involving inclusions (*arrowheads*) and **d** the H & E finding of intranuclear inclusions (*arrowheads* Marinesco bodies, *double arrowheads* nucleoli)

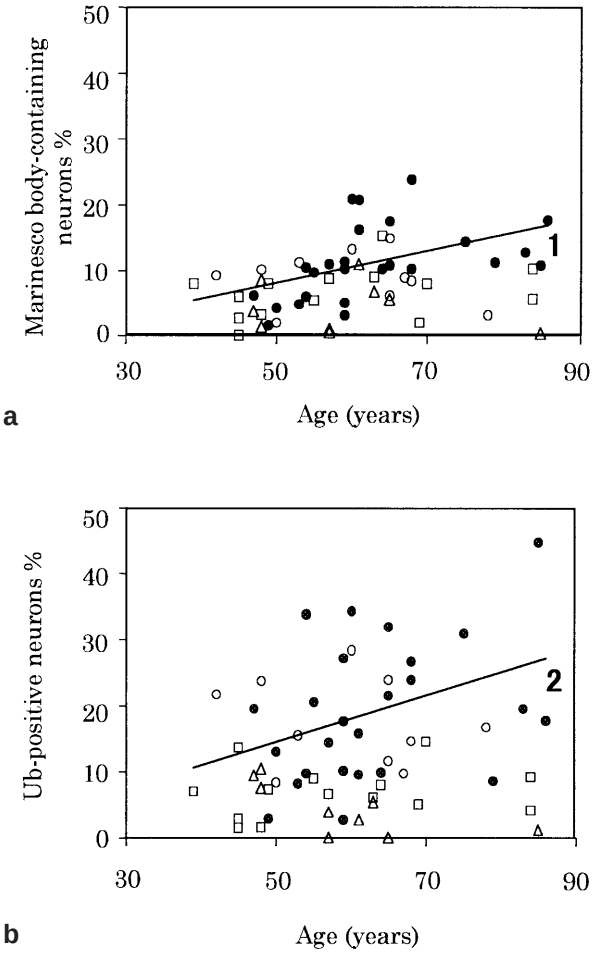

Fig. 2 Relationship of **a** Marinesco body and **b** nuclear ubiquitin positivity frequency in the pigmented substantia nigra neurons with the age of victims (● fire death, COHb < 60%; ○ fire death, COHb > 60%; △ brain stem injury; □, acute myocardial infarction. Correlation equations for fire death with lower COHb (< 60%): 1, $y = 0.25x–4.27$, $n = 25$, $r = 0.47$; 2, $y = 0.35x–3.08$, $n = 25$, $r = 0.36$. There was no significant correlation for the other groups; fire death with COHb higher then 60% and the control groups)

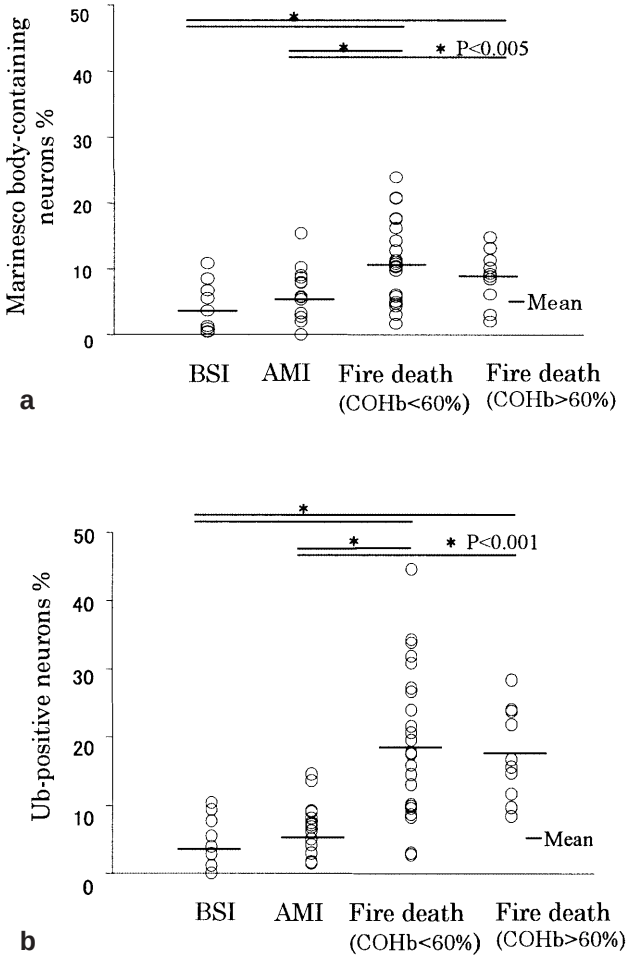

Fig. 3 Comparison of **a** Marinesco body and **b** nuclear ubiquitin-positivity frequencies in the pigmented substantia nigra neurons between fire victims and control cases (*BSI* brain stem injury, *AMI* acute myocardial infarction)

Quantitative analysis of Marinesco bodies

The ratio (%) of the pigmented substantia nigra neurons containing Marinesco bodies gradually increased depending on the age of victims in fire death cases with lower COHb (< 60%), showing a correlation, whereas age-dependency was not significant in higher COHb (> 60%) cases and the control groups (Fig. 2 a). The mean percentages of Marinesco body-containing neurons in fire death with lower COHb (11.2%; mean age, 63.0 years) and higher COHb (8.7%; mean age, 59.6 years) were significantly higher than those in brain stem injury (4.3%; mean age, 59.0 years) and in acute myocardial infarction (6.5%; mean age, 58.4 years) (Fig. 3 a). In CO poisoning other than fire fatality (COHb, 71.7–89.1%; mean age, 41 years), the frequency of Marinesco body-containing neurons was low (0–7.7%; mean, 2.0%).

In fire victims, there was no significant difference in the frequency of Marinesco body-containing neurons between the lower and higher COHb groups. In a multivariate analysis of all cases using logistic regression test (odds ratios and P values), the frequency of Marinesco body-containing neurons showed a correlation with the age (0.92, $P = 0.01$) and was significantly high in fire death cases (0.09, $P = 0.006$), without relationship to gender, postmortem time and survival time.

Quantitative analysis of nuclear ubiquitination in the pigmented substantia nigra neurons

The total Ub-positive % showed a correlation with the age of victims in fire death cases with lower COHb, whereas age-dependency was not significant in higher COHb cases and the control groups (Fig. 2 b). The Ub-positive % in fire victims ranged from 2.7% to 44.7% (mean, 18.5%) and was significantly higher than that of the fatalities from brain stem injury (mean, 4.5%) and acute myocardial in-

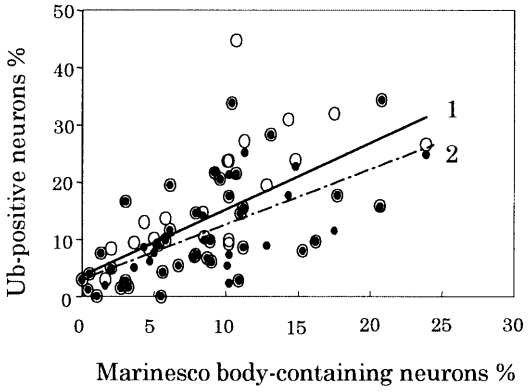

Fig. 4 Relationship between Marinesco body and nuclear ubiquitin-positivity frequencies in the pigmented substantia nigra neurons (○, Total ubiquitin (Ub)-positive cells; 1, $y = 1.17x+3.56$, $n = 58$, $r = 0.63$, $P < 0.0001$; ● inclusion-type ubiquitin (Ub)-positive cells; 2, $y = 0.96x+3.15$, $n = 58$, $r = 0.62$, $P < 0.0001$)

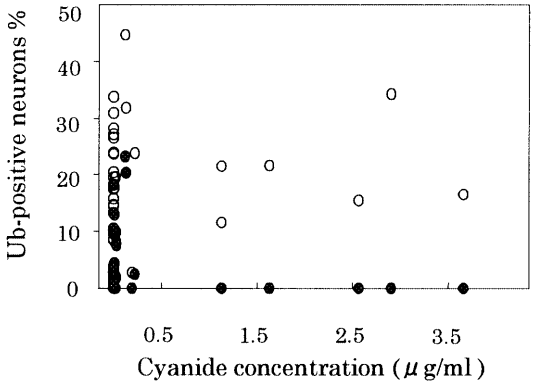

Fig. 5 Relationship between nuclear ubiquitin-positivity frequencies (○ total, ● diffuse type) in the pigmented substantia nigra neurons and blood cyanide level in fire victims

farction (mean, 6.9%). In CO poisoning other than fire fatality, the Ub-positive % was low (0–15.0%; mean, 4.6%). In a multivariate analysis of all cases using logistic regression test, the Ub-positive % was significantly high in fire death cases (0.07, $P = 0.0008$), without relationship to the age, gender, post-mortem time and survival time.

The Ub-positive % in the total and that of the inclusion-type correlated with the frequency of Marinesco body-containing neurons, showing a somewhat higher value (Fig. 4). In fire death, there was no correlation between the Ub-positive % and postmortem COHb or cyanide level in the right heart blood in a regression equation analysis (correlation coefficient $R = 0.28$ and $R = 0.11$, respectively) and in a multivariate analysis using stepwise regression test. However, total Ub-positive % in cases of lower COHb showed a wider distribution than in cases of high COHb (Fig. 3b). The Ub-positive % of the diffuse staining pattern showed a tendency to decrease, as cyanide level elevated (Fig. 5). This staining pattern was not found in cases with a blood cyanide level over 1.0 μg/ml. The relationship with blood alcohol level (0–2.68 mg/ml) was not clear.

Discussion

In the present study, immunohistochemical analysis of ubiquitin focused on staining the pigmented substantia nigra neurons, since they were very easy to identify and showed a marked case-to-case difference. Intranuclear ubiquitinated inclusions of the neurons have been investigated in relation to some neurodegenerative disorders [40, 41, 42, 43]. It is also known that intranuclear inclusions including Marinesco bodies increased with aging [33, 34, 35, 36, 37], and an increase in such inclusions by electric stimulation has been suggested using animal experimental models [38, 44, 45].

The present study showed that the frequencies of Marinesco body-containing neurons and nuclear ubiquitin-positivity were not dependent on age in the control groups and fire victims with higher COHb, whereas an age-dependency was observed in fire victims with lower COHb (death due to burns). A higher nuclear ubiquitin-positivity than the Marinesco body positive value, showing a correlation, was considered to be due to the higher detectability by immunostaining. In this analytical condition, fire fatalities showed significantly higher frequencies of Marinesco bodies and nuclear ubiquitin-positivity than controls. Although the total ubiquitin-positivity did not correlate with blood COHb (hypoxia) or cyanide level, an inverse relationship was observed between the diffuse staining pattern and the blood cyanide level. Thus, it was suggested that intranuclear ubiquitin immunoreactivity of the pigmented substantia nigra neurons was induced by fatally severe stress in fires. Additionally, the nuclear ubiquitin-positivity may be partially suppressed by cyanide poisoning, which can be a factor to reduce the survival time and physical activity, although further investigations are required. Hypoxia did not appear to have an essential role in nuclear ubiquitin-positivity of the nerve cells, since it did not correlate with elevation of COHb level.

Biochemical investigations have led to the hypothesis that ubiquitin can move rapidly from the cytoplasm into the nucleus, combine with denatured proteins to form macromolecular polymeric aggregates and finally degrade into the mononers [46, 47, 48]. On the basis of this biochemical hypothesis, it has been suggested that immunostaining of ubiquitin may indicate the possible activation and redistribution to form larger aggregates rather than the de novo synthesis. It can also explain very prompt ubiquitination. It has been proposed that selected damage of pigmented substantia nigra neurons in the midbrain may be caused by loss of neurotransmitters, energy crisis, oxidative stress, neurotoxins, excitotoxicity (calcium hypothesis), loss of neurotropic factors and apoptosis [49, 50, 51, 52, 53, 54, 55, 56, 57]. A likely explanation for substantial increase of nuclear ubiquitin-positivity in fire death may be neurodegeneration due to energy crisis or excitotoxicity (over excitation), since it was apparently dependent on the acute stress in fires to the victims without any preexisting CNS complications, and was independent of hypoxia or toxic gas levels. The above-described age-dependency of

Marinesco bodies and nuclear ubiquitin-positivity in death due to burns suggests that such neurodegeneration may be magnified in older victims.

The above-described observations suggest an acute degeneration due to hyperactivity of the neurons, which have a function related to skeletal muscle movement [31]. A similar response in c-fos immunoreactivity in the nuclei of the medulla oblongata was reported in asphyxia, suggesting the stimuli-related local neuronal activation [9, 10, 11, 12, 13, 14]. A tentative speculation, in consideration of the function of the neurons, cortical regulation of the neuronal activity and also animal experimental findings [38, 44, 45], may be a contribution of cortical stimulation to the neurodegeneration, during alert consciousness, which are related to physical activity. With respect to this hypothesis, further investigations are required to clarify the factors involved in the nuclear ubiquitin-positivity of pigmented substantia nigra neurons in fire death, including the effect of toxic gases and CNS depressants.

In conclusion, the present study showed that intranuclear ubiquitin immunoreactivity of pigmented substantia nigra neurons in the midbrain was induced by fatally severe stress in fires, suggesting a possibility for elavuation of the stress on CNS resulting from the physical activity before death in fires.

Acknowledgements This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan (Grants No. 11670425 and No. 12470109).

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