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ORIGINAL ARTICLE

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Immunohistochemical localization of c-fos in the nuclei of the medulla oblongata in relation to asphyxia

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Abstract The immediately early gene product c-fos is known to be induced in neurons under noxious stimuli. Therefore, the immunohistochemistry of c-fos expression in human brains might offer information on the localization of stimulated neurons. In this study, the immunohistochemical localization of c-fos was studied in the neurons of the hypoglossal nucleus (XII), the dorsal motor nucleus of the vagal nerve (X), the nucleus solitarius (Sol), the accessory cuneate nucleus (Cun), the spinal trigeminal nucleus (V) and the inferior olive (Oli) of the human medulla oblongata from forensic autopsy cases. The neurons in the X nucleus showed the highest percentage of positive reactions for c-fos, followed in descending order by the Cun, V, Oli, XII and Sol. The c-fos immunoreactivity in the Cun and X was statistically significantly higher than in the Sol, XII and Oli. Although neurons in the Sol are known to be involved in respiration, there was no statistically significant difference in the c-fos immunoreactivity in the neurons in the Sol between asphyxia and non-asphyxia cases. On the other hand, the percentage of neurons positive for the c-fos immunoreactivity was statistically significantly higher in the Oli of asphyxia cases than of non-asphyxia cases. Our results indicate the difference in the immunoreactivity of c-fos among the nuclei of the human medulla oblongata and that the c-fos immunoreactivity in the Oli might assist the diagnosis of asphyxia.

Key words Immunohistochemistry · C-fos · Medulla oblongata

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Introduction

Several markers have been studied regarding cell injury, including tumor necrosis factor-alpha and nuclear DNA fragments (Betz et al. 1997; Kita et al. 1997). The immediately early gene product c-fos is known to increase in neurons under noxious stimulation in experimental animals (Bullitt 1990). However, only limited studies have been done on the c-fos protein in human brains (Zhang et al. 1992; Leifer and Kowall 1993; Anderson et al. 1994; MacGibbon et al. 1997). The rational of this study was to examine if the immunohistochemistry of c-fos in human brains might offer information on the localization of stimulated neurons after human autopsies. In this study, c-fos immunoreactivity was studied in the nuclei of the human medulla oblongata with special reference to asphyxia.

Materials and methods

Brains from 42 autopsy cases were studied, including 13 cases of acute asphyxia (mechanical obstruction of the airway) by strangulation, hanging, smothering, or drowning (Table 1). Brains were fixed in 10% formalin for about 1 week, 5 μm sections were cut from the medulla oblongata and immunostained with anti-c-fos rabbit polyclonal IgG antibody (sc-52, Santa Cruz Biotechnology, Calif.) by the avidin biotin complex (ABC) method using the HistoMark Streptavidin-HRP Systems kit (Kirkegaard and Perry Laboratories, Md.). Briefly, the sections were deparaffinized in xylene and rehydrated. Endogenous peroxidase was blocked by incubating with 3% hydrogen peroxide for 5 min. After washing in phosphate-buffered saline (PBS), the sections were incubated with 10% normal goat serum. The sections were incubated with the 1:100 diluted anti-c-fos antibody at room temperature for 1 h. After washing in PBS for 5 min, the sections were incubated with the biotinylated goat anti-rabbit IgG antibody supplied in the kit for 30 min. The sections were washed in PBS and incubated with strepavidin-peroxidase for 30 min. After washing in PBS, the sections were stained with 3–3′-diaminobenzidine tetrahydrochloride (DAB, Sigma, Mo.) solution (30 mg of DAB and 0.5 ml of 0.3% hydrogen peroxide in 100 ml of 0.05 M Tris-HCl, pH 7.6) and counterstained with Mayer's hematoxylin. In order to confirm the specificity of c-fos immunostaining, the blocking peptide (final concentration 10 μg/ml, sc-52P, Santa Cruz Biotechnology, Calif.) was coincubated with the anti-c-fos antibody and treated in the same way.

Table 1 Case profiles of this study

Age	M/F	Cause of death	AMI	PMI
Asphyxia cases				
0	M	Strangulation	0	5
29	M	Strangulation	0	7
74	F	Strangulation	0	9
76	F	Strangulation	0	18
2	M	Strangulation	0	24
0	M	Strangulation	0	35
19	F	Strangulation	0	50
64	F	Strangulation	0	uk
51	M	Strangulation, Hemorrhage	0	uk
47	F	Hanging	0	24
56	M	Hanging	0	24
0	M	Smothering	0	uk
8	F	Drowning	0	23
Non-asphyxia cases				
41	M	Hemorrhage	0	48
61	F	Hemorrhage	0	72
23	F	Hemorrhage	0.33	9
35	M	Hemorrhage	0.67	12
53	M	Hemorrhagic shock	2.5	20
58	M	Hemorrhagic shock	3.5	32.5
39	M	Hemorrhagic shock	11	uk
18	M	Hemorrhagic shock	24	18
53	M	Traumatic shock	7	6
81	M	Traumatic shock	13	7
44	M	Traumatic shock	uk	uk
93	M	Pulmonary embolism	6	21
70	F	Heart failure	4.5	17
65	M	Heart failure	22	18
32	F	Multiple organ failure	864	14
47	M	Head injury	6	12
53	M	Brain stem contusion	0	7
55	M	Brain stem contusion	0	16
56	M	Brain injury	115	14
36	F	Cerebral hemorrhage	204	19
49	M	Head and chest injury	1	11
uk	M	Chest and abdominal injury	0	14
76	M	Chest and abdominal injury	1.5	15
28	M	Abdominal injury	0.67	3.5
56	F	Peritonitis	30	19
48	M	Starvation	uk	56
35	M	Acetaldehyde intoxication	6	uk
26	F	Unknown	72	8
36	M	Unknown	uk	8

M male, *F* female, *AMI* antemortem interval (hr) between the onset of the injury or abnormality and the death, *PMI* postmortem interval (hr) between the death and the autopsy, 0 in *AMI*: immediate death, uk: unknown

The neurons in the hypoglossal nucleus (XII), the dorsal motor nucleus of the vagal nerve (X), the nucleus solitarius (Sol), the accessory cuneate nucleus (Cun), the spinal trigeminal nucleus (V) and the inferior olive (Oli) of the medulla oblongata which were positively stained by the anti-c-fos antibody were counted and estimated as a percentage of the total neurons. The positive neurons were defined as those with a clearly stronger staining than in the surrounding interstitium.

The data were expressed as the mean ± the standard errors of the mean (SEM). Friedman's test was used to confirm the statistically significant differences between the nuclei paired in each case. Dunn's test was used for the comparison among these nucleis and the Mann-Whitney test was used to compare two groups of data. Linear regression was used for the correlation between the AMI (antemortem interval between the onset of injury or abnormality and death) or PMI (postmortem interval between death and the autopsy) and the percentage of positive c-fos immunoreactivity. A P value less than 0.05 was considered statistically significant.

Results

The mean AMI was 35.8 ± 22.6 h (n = 39), whereas the mean PMI was 19.9 ± 2.6 h (n = 36).

The c-fos immunoreactivity was present in the cytoplasm of some neurons in the XII, X, Sol, Cun, V and Oli (Fig. 1). The c-fos immunoreactivity was completely blocked by co-incubation with the blocking peptide with the anti-c-fos antibody, confirming the specificity of the antibody. The percentage of neurons positive for c-fos immunoreactivity was the highest in the X, followed by the Cun, V, Oli, XII, and Sol in descending order (Fig. 2). The Cun and X nuclei showed statistically significantly higher c-fos immunoreactivity than the Sol, XII, and Oli (Dunn's test $P < 0.01$, Fig. 2).

There was no statistically significant correlation between the AMI or PMI and the percentages of c-fos positive neurons in any of the nuclei studied.

The percentage of neurons positive for c-fos immunoreactivity in the Oli was statistically significantly higher in the asphyxia cases than in the non-asphyxia cases (Mann-Whitney test $P < 0.01$). However, there was no statistically significant difference in the c-fos immunoreactivity of the neurons in the XII, X, Sol, V, or Cun between asphyxia and non-asphyxia cases (Mann-Whitney test, data for the Sol, XII, and Oli are shown in Fig. 3).

Discussion

The immunolocalization of c-fos protein has been reported to be mainly in the cell nucleus, but also in the cytoplasm in some human studies (Zhang et al. 1992; Leifer and Kowall 1993; Anderson et al. 1994; MacGibbon et al. 1997). Our results showed the presence of c-fos immunoreactivity in the cytoplasm of neurons in the human specimens taken from the medulla oblongata. The specificity of the c-fos immunoreactivity in this study has been confirmed by co-incubation with the blocking peptide.

Our results indicate that there is a statistically significant high incidence of positive c-fos immunoreactivity in the cytoplasm of neurons in the X and Cun compared with the Sol, XII, and Oli. We have previously shown that the neuron-specific enolase immunoreactivity is higher in the XII neurons than in the X (Nogami et al. 1998). Interestingly, our study showed the localization of the c-fos immunoreactivity to be higher in the X than in the XII, which

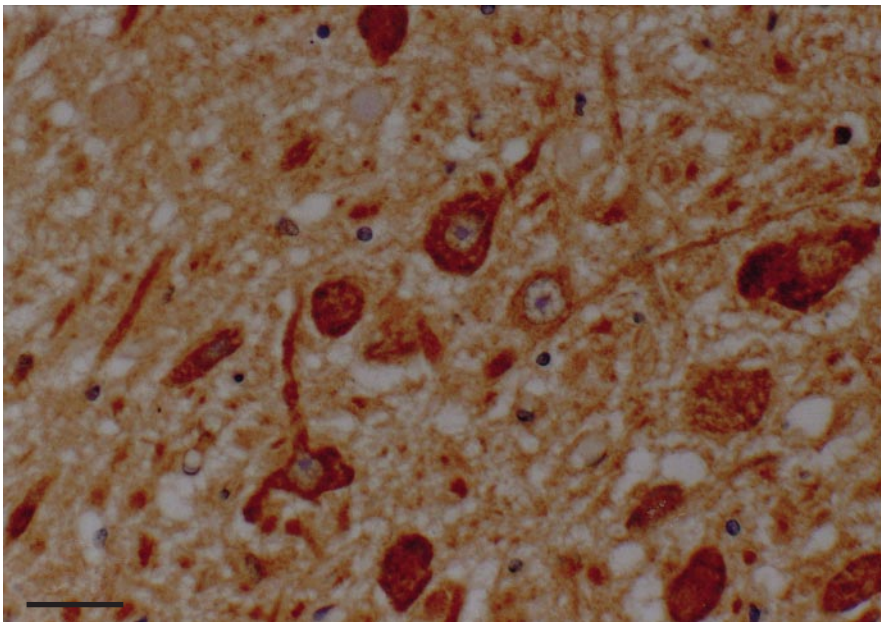

Fig. 1 Immunohistochemical staining of c-fos in the dorsal motor nucleus of the vagal nerve of the human medulla oblongata from a 56-year-old male who died from hanging (× 400, bar = 20 μm). Note the positive staining in the neuron cytoplasm

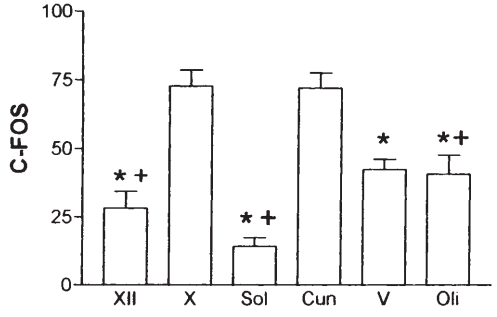

Fig. 2 Percentages of neurons with positive c-fos immunoreactivity in the medulla oblongata nuclei

XII hypoglossal nucleus, *X* dorsal motor nucleus of the vagal nerve, *Sol* nucleus solitarius, *Cun* accessory cuneate nucleus, *V* spinal trigeminal nucleus, *Oli* inferior olive, *C-FOS* percentage of neurons with positive c-fos immunoreactivity
The bars show the means ± SEM.
* Statistically significant compared with the X ($P < 0.01$ for the XII, Sol, and Oli, and $P < 0.05$ for the V)
+ Statistically significant compared with the Cun ($P < 0.01$)

indicates an immunohistochemical difference in the characteristics of the nuclei studied.

The c-fos immunoreactivity in neurons has been reported to increase in experimental animals given noxious stimuli (Bullitt 1990). The c-fos immunoreactivity in the neurons has been regarded as a useful tool to demonstrate the stimulus-related local neuronal activation on a single cell level and may be useful to complement other mapping techniques such as electrophysiological recording or 2-deoxyglucose autoradiography (Sagar et al. 1988; Ehret and Fischer 1991). Therefore, the higher incidence of c-fos immunoreactivity in the X and Cun neurons might reflect the higher basic activity of these neurons.

Some studies have indicated an increase in c-fos immunoreactivity in the neurons of the Sol by hypotension or noxious stimulation of the stomach (Chen et al. 1995; Ruggiero et al. 1996). The neurons in the Sol have been regarded to be involved in respiratory activity (Carpenter 1991), whereas the neuronal activity in the XII has been reported to decrease in hypercapnia (Bartlett et al. 1987). In our study, neurons in the Sol and XII showed the lowest c-fos immunoreactivity among the nuclei studied and there was no statistically significant difference in the c-fos immunoreactivity in the neurons of the Sol or XII between the asphyxia and non-asphyxia cases. Therefore, c-fos immunoreactivity in the Sol or XII may not clearly reflect the asphyxic state in humans.

On the other hand, the percentage of neurons positive for c-fos immunoreactivity in the Oli was statistically significantly higher in asphyxia than in non-asphyxia cases. The reason for the selectively higher c-fos immunoreactivity in the Oli from the asphyxia cases is unknown. The Oli is known to send fibers to the cerebellum (Carpenter 1991). Since asphyxia caused death in a few minutes in the cases studied, this increase is unlikely to be due to new protein induction. C-fos is known to accumulate in the nucleus (Morgan and Curran 1989) and therefore the translocation of the c-fos protein from the peripheral neuropil to the perikaryon in the short time of asphyxia until death, might take place due to an increase in the neuronal activity. Another possibility is that the postmortem degradation of c-fos in the Oli might be different between asphyxia and non-asphyxia. There was no statistically significant difference in the postmortem time until the autopsy between the asphyxia and non-asphyxia cases.

In conclusion, the c-fos immunoreactivity showed a difference among the nuclei of the human medulla oblongata with higher levels in the X and Cun than in the Oli, XII and Sol. The higher percentage of neurons positive for c-fos in the Oli might assist the diagnosis of asphyxia in the postmortem examination.

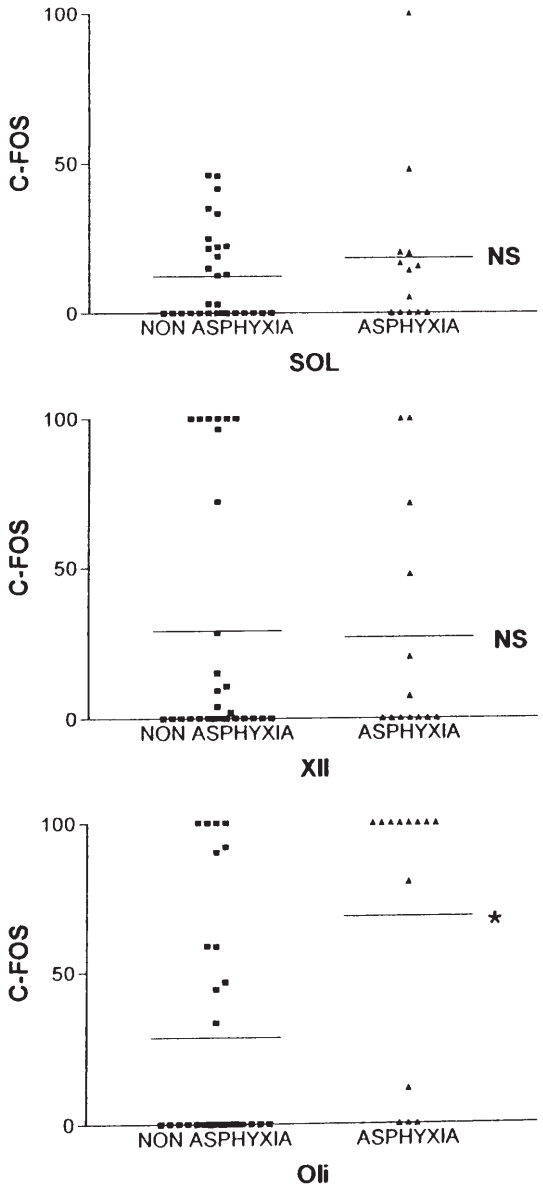

Fig. 3 Percentages of neurons positive for c-fos immunoreactivity in asphyxia and non-asphyxia cases

Sol nucleus solitarius, *XII* hypoglossal nucleus, *Oli* inferior olive, *C-FOS* percentage of neurons with positive c-fos immunoreactivity The bars show means
* Statistically significant compared with the non-asphyxia cases ($P < 0.05$) *NS* not statistically significant

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