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The damaging in the granular cells and expression patterns of CIRBP, RBM3, HSP70, HIF-1, AIF1, SIRT1,Ngb, cFos, p53 and CCC9 in the postmortem human cerebellums obtained from individuals who died due to hanging, strangulation, drowning, or asphyxia by anaphylaxis or food aspiration may be closely linking with agonal duration.

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ABSTRACT

We encountered several cases concerning to ischemic and hypoxic deaths at routine autopsy cases. These victims were individuals who died due to suicidal hanging, autoerotic accidental hanging, self-ligature strangulation, manual strangulation, strangulation by a rope, accidental and suicidal drowning, anaphylaxis shock, and asphyxia by food aspiration. Theiragonal duration and postmortem interval were different, that is, agonal duration distributed from direct death to 4days and postmortem interval was from

12hours to 40 days, respectively. We examined the damaging degree of the granular cell layer in the each cerebellum using an application of Photshop andexpression pattern of antigens in the cerebellums by immunohistochemstry using anti CIRBP, RBM3, HIF-1, AIF1, SIRT1, Ngb, HSP 70, cFos, p53 and CCC9 antibodies. The results indicate the co-relationship between damaging of the granular cells and agonal duration, andshowed that expression pattern of antigens may be relate

with agonal duration, although it was not significant. The antigens expressed during agonal duration might be kept even in postmortem interval. In a special case in which an individual was killed and buried for 40 days under ground after being strangulated, the antigens related to stress and ischemic signaling pathway such as HSP70, CCC9, CIRBP, RBM3 and SIRT 1 were detectable in the molecular layer and dentate nucleus of the cerebellum. The co-relationship between the damaging of granular cells and agonal duration may be more reliable compared with those of Purkinje cells and agonal duration. The results obtained in this study indicate that the damaging of granular cells is useful tool to estimate agonal duration, and postmortem humanbrain tissue may be utilizable for diagnosis and examination of brain diseases. The results obtained in this study indicated that CIRBP and RBM3 were hypoxic or ischemic related antigens and not the cold inducible antigens.

Keywords: Cerebellum, Purkinje cells, Granular cell layer, Dentate

nucleus, Hypoxia, Ischemia, CIRBP, RBM3, SIRT1, Ngb, CCC9 and HSP 70

Introduction

The cells could bring their action to protect harmful effects assigned by the environmental items. Although cerebral ischemia occurs in the context either of focal reduction in blood flowing to the brain regions and/or cerebral infarction, a global deficit status occurs only in animal study. However the global deficit actually occurs in forensic practical cases such as hanging, strangulation, food aspiration, drowning death and/or anaphylactic reaction.

The brain is vulnerability to ischemic and hypoxic status. It is widely accepted that in hypoxia and ischemic cases an increase in the transcription level of specific genes, including those encoding for hypoxia-inducing factor

1(HIF1)(1,2), apoptotic inducing factors (AIF-1) (3), and stress related factors containingCold inducing RNA binding protein (CIRBP)(4)and RNA binding motif 3 (RBM3)(5) which are recognized as a cold stress inducing protein and were intensively expressed in the postmortem human heart muscle obtained from accidental hypothermic death cases (6). Neuroglobin (Ngb)(7), Complement component C 9(8,9), c-Fos(10), Protein 53(p53)(11), Sirtuin 1 (SIRT1)(12) andHeat shock protein 70(HSP70)(13) were also expect to express in the hypoxia or ischemic cerebellum.

The morphological and histological changes recognized in the cerebellums obtained from brain death cases have been investigated around 1990 (14), howeverimmunohistochemical studies using antibodies against apoptotic-, stress-, necrotic- and/or hypoxic-related antigens are rare until recent. We have been performing the forensic autopsy as a routine working and encountered several suicidal hanging and self-ligature strangulation cases in which there are directly, acute, sub-acute and prolong death individuals (15), and other deaths due to different types of asphyxia and with different durations.

Although various studies have been examined the consequences of hypoxia on cerebellar neurons, a controversy still exists concerning the mode of toxicity expressed in Purkinje cells following hypoxic insult (16). In the present study we examined the degree of damaging of granular cellsand

Purkinje cells during agonal time in the first step and the differences among the expression of stress, hypoxic and apoptotic related antigens described above were studied in the next step.

Concerning hypoxic changes in Purkinje cell, Hausmann et al(17) studied using 52 individuals with a history of acute or prolonged cerebellar hypoxia/ischemia before death. Various and different kind of cause of death were contained in their study.In this study we examined the relationship between degree of damaging granular cells and expression pattern of hypoxia, apoptotic and/or necrotic antigens in the cerebellum obtained from individuals who died mainly due to compression of cervical arteries.

Concerning to the using of postmortem human brain to investigate the brain disorder, Sheedy et al (18) proposed that histological situation of cerebellar granule cell layer is a useful marker of tissue integrity. We also like to describe some comments on the autolysis of the brain tissue during the postmortem interval.

Materials and Methods

In this study we selected and examined the cerebellum obtained from suicidal hanging cases containing direct, sub-acute and delayed death cases， self strangulation cases, manual strangulation cases, drowning cases, anaphylaxis case, and asphyxia case due to food aspiration to the larynx and/or the trachea. We did not use the cerebellum obtained from patients who died due to illness such as cardiac infarction, pulmonary diseases, and or cerebral infarction, since it is difficult to determine the onset of ischemia or hypoxic state to the cerebellum in these illness. For example, we have one autopsy case where the granular cells were obviously damaged in 54 year-old-man who died due to cardiac infarction with coronary artery thrombosis during right in driving his car. The autopsy revealed that he had been have several times heart attach. Characterization of antibodies used or examined in this study was summarized in Table 1.

Table 1. Characteristics of the antibodies used in this study

Antibody	Maker	Clone	Species	AG-retrieval	incubation	AB-dilution
CIRBP	Protein Tech	10209-2-AP	R	autoclave	overnight	1:400
RBM3	Protein Tech	14363-1-AP	R	autoclave	overnight	1:200
SIRT1	Novus	E104:	R	autoclave	overnight	1:400
HSP70	santa cruz	polyclonal:	G	autoclave	overnight	1:400
HIF-1α	NOVUS	NB100-479	R	autoclave	overnight	1:400
P53	santa cruz	FL-393 :	G	autoclave	overnight	1:400
C-Fos	BioLegend	Poly6414	R	autoclave	overnight	1:400
Ngb	Sigma-Aldrich	Poly-clone	R	autoclave	overnight	1:400
AIF1	Lifespan	Poly-clone	R	autoclave	overnight	1:400
CCC9	Leica	10A6	M	autoclave	overnight	1:400

[1] Tissue preparation and analysis of Granular cell Damaging

The tissue sections with about 0.5cm thickness were obtained from the cerebellum at autopsy, Department of Legal Medicine, Shiga University of Medical Science. Tissues containing the dentate nucleus, and cerebellar cortex were fixed in 10% of formaldehyde for 24 hours and embedded in paraffin. Threeμm of tissue sections were prepared for conventional and immunihistochemical staining using antibodies described in Table 1. The staining procedure for immunohistochemistry was followed our previous report (4).

Damage of granular cell layer was evaluated by an observer blinded to the group assignment of the section according to the report by our previous report(19). In shortly the evaluation of alteration of the granular cells was performed using a Photoshop application. After taking each photograph at microscopy level the area with blue color in the granular cell layer, that is, nucleuses of granular cells stained by hematoxylin, was measured by an application and with same magnification, that is, blue color area indicates existence of live nuclear of the granular cells. The frequency of remaining granular cells was indicated as percentage of the total number of pixel and was grouped. The numerical value was the mean value of five different areas.

The damage and decreasing of the Purkinje cells was also evaluated by image analytic technique. The images of cerebellar sections stained by HE were captured using an objective lens with a magnification of 4. The total length of the Purkinje cell layer from 8 images from each individual was calculated using BZ-II Image Analysis Application. And then the number of the Purkinje cells

with nucleus was counted. The existence of the Purkinje cells was showed a number per 1mm of the Purkinje cell layer.

[2] Cases examined.

Case 1. (Autopsy No.1222)

A 39 year-old woman with depression died due to hanging using a vinyl rope.

Small number of petechea was detected in her conjunctiva, and single ligature mark was clearly observed. In this case re-perfusion of blood to the brain was not presumed.

Case 2. (Autopsy No. 1225)

A 38-year-old woman died due to hanging by a rope after she killed her own

son. At autopsy clear ligature mark was found around her neck and ligature mark indicated typical hanging manner. In this case re-perfusion of blood to the brain was not presumed, however her postmortem interval was presumed around two days.

Case 3. (Autopsy No. 58749)

A 74 year-old housewife was found to be hanging by her husband. When the husband immediately cut the ligature, her body full down and she got a bruise on the posterior of head. The autopsy revealed the no facial congestion, multiple costal fractures, bleeding in the fatty tissue in the heart and left temporal of the head and mild cerebral contusion on the lower part of both side in frontal lobes. In this case, occlusion of cervical arteries occurred in a short time, and re-perfusion of blood to the brain might be performed in a few minutes.

Case 4. (Autopsy No. 1257)

A 45 year-old woman was found her death due to hanging. The body without clothing except a scanty was standing by a stairs in her house. Her face showed severe congestion and number of petechea in the conjunctivae was detected. Her death seemed to be accident due to autoerotic performance.

The autopsy revealed a small amount of bleeding in the right sternocleidomastoid muscle and no fracture of hyoid bone and thyroid cartilage. The brain showed congestion and no bleeding. In this case intermittent occlusion may be occurred during her autoerotic performance.

Case 5. (Autopsy No. 1320)

A 43 year-old man was found in his working car. The body lied on the back seat of a low displacement car and covered with a blanket. There was a ligature mark on his neck but no ligature in the car. The result of autopsy suspected that he attempted suicidal hanging but he could not die because of snapping a ligature. His death was presumed relayed hanging death, and agonal duration was presumed two or three hours. Re-perfusion of blood to the brain obviously was presumed.

Case 6. (Autopsy No. 1255)

A 67-year-old woman performed hanging with sitting position. Although she carried to a hospital by an emergency car, she died 20 hours after finding of her hanging. Re-perfusion of blood to the brain obviously was presumed.

Case 7. (Autopsy No. 1117)

A 51 year-old man was found death due to self-strangulation with a cotton rope in his room. The severe congestion over the ligature mark, and small blisters were observed between two ligature marks. A large number of petechiea in the conjunctiva and mouth mucosa was also recognized.Agonal duration presumed to be longer than hanging death.

Case 8. (Autopsy No. 1239)

A 31 year-old man was found death in a prison room. He wound a sleeve of his T-shirt around his neck, and severe congestion and petechia on his face, conjunctiva and mouth mucosa. Agonal duration presumed to be longer than hanging death.

Case 9. (Autopsy No.1254)

A 26 year-old man drowned in a public poor during his diving training and he was rescued and transformed to a hospital, however he died after 20 hours.

Case 10. (Autopsy No.59458)

A 71 year-old woman japed into a river. After being transformed to a hospital for rescue and medical treatment, she died two days later.

Case 11. (Autopsy No.1142)

A 54-year-old woman was killed by her acquaintance with manual strangulation. In one hour after hear death the body was found by her daughter in her living room. The agonal duration was presumed to be a bit longer than self-strangulation cases, since it may be occur the defense to murderer.

Case 12. (Autopsy No.1272)

A 47-yaer-old woman was found under the ground in front of her house.

Police research revealed that her husband killed her with manual strangulation and after keeping her body in a forest for several days, she had been burying for about 40 days. At discovering her body, the body was rapped with thin plastic film and covered with plastic seat.The agonal duration was presumed to be a bit longer than self-strangulation cases, however the postmortem interval was longest in this examination.

Case 13. (Autopsy No.1242)

A-51-year old woman fell into anaphylaxis shock by intake of barium salt for stomach examination, and she died after 4 days in an emergency hospital.

Case 14. (Autopsy No.1359)

A 53-year old man was found death. Autopsy revealed that food mass entered to larynx and he died due to accidental ingestion. His agonal duration was presumed to be several minutes.

The postmortem interval of each case was within4 days except one individual from case 12.

Results

The damaging in the granular cells and Purkinje cells of each individual was described in Table 2, and results obtained by immunohistchemistry were listed in Tables3-1 and 3-2.

Table 2. Remaining of the granular cells and Purkinje cells in the cerebellum.

Case	NO.	Sex	Age	Remain GC	Remain PC	Esti-PI	Esti-AG	Cause of Death
1	1222	F	39	52.4%	6.1cells	22h	3m	Hanging
2	1225	F	38	47.1%	5.8cells	36h	3m	Hanging
3	58749	F	74	52.5%	6.5cells	24h	3m	Hanging with brain contusion
4	1257	F	45	38.3%	3.7cells	50h	3m*	Accidental hanging
5	1320	M	49	19.1%	3.6cells	4d	3h	Delayed hanging death
6	1255	F	62	8.4%	3.4cells	25h	20h	Brain death due to hanging
7	1117	M	51	46.1%	4.1cells	8h	15m	Self-ligature strangulation
8	1239	M	31	46.8%	3.1cells	36h	15m	Self-ligature strangulation
9	1254	M	26	25.4%	3.5cells	30h	20h	Drowning
10	59458	F	71	18.5%	0.8cells	25h	2d	Drowning
11	1142	F	54	36.5%	2.8cells	12h	20m	Manual strangulation
12	1272	F	36	20.3%	3.1cells	43d	20m	Manual strangulation
13	1242	F	51	12.2%	1.7cells	24h	4d	Anaphylaxis
14	1359	M	53	36.2%	1.5cells	34h	10m	Aspiration

Remain GC; Frequency of remaining granular cells, Remain PC; Remaining Purkinje cells, Esti-PI; Estimated postmortem interval (hours or days), Esti-AG; Estimated agonal durati (minutes or days), : *; autoerotic case, repeated performance was estimated. Theevaluation procedures for the granular cells and Purkinje cells were described in the text.

Table 3-1. Results obtained from immunohistochemistry

Case No.	Cells	Antibodies									
		CI	RB	HS	SIR	HIF	Ngb	c-Fos	p-53	AIF	CCC9
1.1222	Gc*	+	+	+	-	-	-	-	-	-	-
	PcN	+	+	-	-	-	-	-	-	-	-
	PcC	+	+	-	-	-	+	+	-	-	-
	Mc*	+	+	+	-	-	+	+	-	-	-
	DN	+	+	-	-	-	-	-	-	-	+
	DC	+	+	+	+	+	+	+	+	+	+
	W*	+	+	+	-	-	+	+	-	+	-
2.1225	Gc*	+	+	-	-	-	-	-	-	-	+
	PcN	+	+	-	-	-	-	-	-	-	-
	PcC	+	+	-	-	-	+	+	+	+	+
	Mc*	+	+	+	-	-	-	-	-	+	+
	DN	+	+	-	-	-	-	-	+	+	-
	DC	+	+	+	-	-	+	+	+	+	+
	W*	+	+	+	-	+	+	-	+	+	+
3.58749	Gc*	+	+	+	-	-	-	+	+	+	+
	PcN	+	+	-	-	-	-	-	-	-	-
	PcC	+	-	-	-	+	+	+	+	+	-
	Mc*	+	+	+	-	+	+	+	+	+	+
	DN	+	+	-	-	-	-	-	-	-	+
	DC	+	+	+	-	+	+	+	+	+	+
	W*	+	+	+	-	-	+	+	-	+	-
4.1257	Gc*	+	+	+	-	-	+	+	+	+	+
	PcN	+	+	-	-	-	-	-	-	-	-
	PcC	+	-	-	-	-	-	+	+	+	+
	Mc*	+	+	+	-	-	-	-	-	+	+
	DN	+	+	-	-	-	-	-	-	-	+
	DC	+	+	+	-	-	-	-	-	-	-
	W*	+	+	+	-	+	-	-	-	-	+
5.132	Gc*	+	+	-	-	+	-	+	-	+	+
	PcN	+	+	-	-	-	-	-	-	-	+
	PcC	+	+	-	-	-	+	+	+	+	+
	Mc*	+	+	+	-	+	-	-	-	+	+
	DN	+	+	-	-	-	-	-	-	+	-
	DC	+	+	+	+	+	+	+	+	-	+
	W*	+	+	+	+	+	-	+	+	+	+
6.1255	Gc*	-	-	+	-	-	+	+	+	-	-
	PcN	+	+	-	-	-	-	-	-	-	-
	PcC	-	+	-	-	-	+	+	+	-	-
	Mc*	+	+	+	-	+	-	+	+	-	-
	DN	-	-	-	-	-	-	-	-	-	-
	DC	+	+	+	-	-	-	-	-	-	+
	W*	+	+	+	+	-	+	+	-	+	-
7.1117	Gc*	+	+	+	-	-	-	-	-	-	+
	PcN	+	+	-	-	-	-	-	-	-	-
	PcC	+	+	-	-	-	+	+	+	+	+
	Mc*	+	+	+	-	-	-	-	-	-	+
	DN	+	+	-	-	-	-	-	-	-	+
	DC	+	+	+	-	-	-	+	+	-	+
	W*	+	+	-	-	-	+	+	+	+	+

CI;CIRBP, RB;RBM3, HS;HSP-70, SIR;SIRT 1, HIF;HIF-1, Nbg; Neuro-globin, GC; Granular cells, PcN; Purkinje cell nucleus, PcC; Purkinje cell cytoplasm, Mc; Cells in molecular cell layer , Dn; Dentate cell nucleus, Dc;Dentate cell cytoplasm, Reactivity; +; positive, -;negative, *; result of reactivity detected in cytoplasm or nucleus was described.

Table 3-2. Results obtained from immunohistochemistry

Case No.	Cells	Antibodies									
		CI	RB	HS	SIR	HIF	Ngb	c-Fos	p-53	AIF	CCC9
8.1239	Gc *	+	+	+	-	-	-	-	-	-	+
	PcN	+	+	-	-	-	-	-	-	-	-
	PcC	+	+	-	-	-	+	+	+	+	+
	Mc*	+	+	+	-	-	-	-	-	-	+
	DN	+	+	-	-	-	-	-	-	-	-
	DC	+	+	+	-	-	-	+	+	+	+
	W*	+	+	-	-	-	+	+	+	+	+
9.1254	Gc*	+	+	+	-	-	-	-	-	-	-
	PcN	-	-	-	-	-	-	-	-	-	-
	PcC	-	-	-	-	-	-	+	-	-	-
	Mc*	+	+	-	-	+	-	-	-	-	+
	DN	-	-	-	-	-	-	-	-	-	+
	DC	-	-	-	-	-	-	-	-	-	-
	W*	+	+	+	-	-	-	+	-	-	+
10.59458	Gc*	+	+	-	-	+	-	+	-	+	+
	PcN	+	+	-	-	-	-	-	-	-	+
	PcC	+	+	-	-	-	+	+	+	+	+
	Mc*	+	+	+	-	+	-	-	-	+	+
	DN	+	+	-	-	-	-	-	-	+	-
	DC	+	+	+	+	+	+	+	+	-	+
	W*	+	+	+	+	+	-	+	+	+	+
11.1142	Gc*	+	+	+	-	-	+	+	+	+	+
	PcN	+	+	-	-	-	-	-	-	-	-
	PcC	+	-	-	-	-	-	-	-	-	-
	Mc*	+	+	+	-	-	-	-	-	-	-
	DN	+	+	-	-	-	-	-	-	-	-
	DC	+	+	+	-	+	-	-	-	-	-
	W*	+	+	+	-	-	-	-	-	-	-
12.1272	Gc*	-	-	-	-	-	-	-	-	-	-
	PcN	-	-	-	-	-	-	-	-	-	-
	PcC	+	-	-	-	-	-	-	-	-	-
	Mc*	+	+	+	+	-	-	-	-	-	+
	DN	+	+	+	-	-	-	-	-	+	-
	DC	+	+	-	-	-	-	-	-	-	+
	W*	+	+	-	+	-	-	-	+	-	-
13.1242	Gc*	-	-	-	-	-	-	-	-	-	-
	PcN	-	-	-	-	-	-	-	-	-	-
	PcC	-	-	-	-	-	-	-	-	-	-
	Mc*	-	-	-	-	-	-	-	-	-	-
	DN	-	-	-	-	-	-	-	-	-	-
	DC	+	+	+	+	+	+	+	+	+	+
	W*	-	-	-	-	-	+	+	-	-	-
14.1359	Gc*	+	+	+	-	-	+	+	+	+	+
	PcN	+	+	-	-	-	-	-	-	-	-
	PcC	+	-	-	-	-	-	-	-	-	-
	Mc*	+	+	+	-	-	-	-	-	-	-
	DN	+	+	-	-	-	-	-	-	-	-
	DC	+	+	+	-	+	-	-	-	-	-
	W*	+	+	+	-	-	-	-	-	-	-

CI;CIRBP, RB;RBM3, HS;HSP-70, SIR;SIRT 1, HIF;HIF-1, Nbg; Neuro-globin, GC; Granular cells, PcN; Purkinje cell nucleus, PcC; Purkinje cell cytoplasm, Mc; Cells in molecular cell layer , Dn; Dentate cell nucleus, Dc;Dentate cell cytoplasm, Reactivity; +; positive, -;negative, *; result of reactivity detected in cytoplasm or nucleus was described.

1. Granular cells

[1]Hematoxylin-Eosin staining

In the granular cell layer the morphology was different, that is, complete disappear of cells with cell debris or fibrous material in some individuals, remarkable decreasing of cells in other individuals and numerous remaining cells in another individuals. For example, the granular cell layer from severe level degeneration individuals showed a diffuse decrease in number the nuclei stained by hematoxylin and diffuse pallor of the background without glial reaction and with cell debris as shown in Figure 1, and the changes in high remaining individuals were not obvious and showed no remarkable difference compared with those from brain injure deaths due to traffic

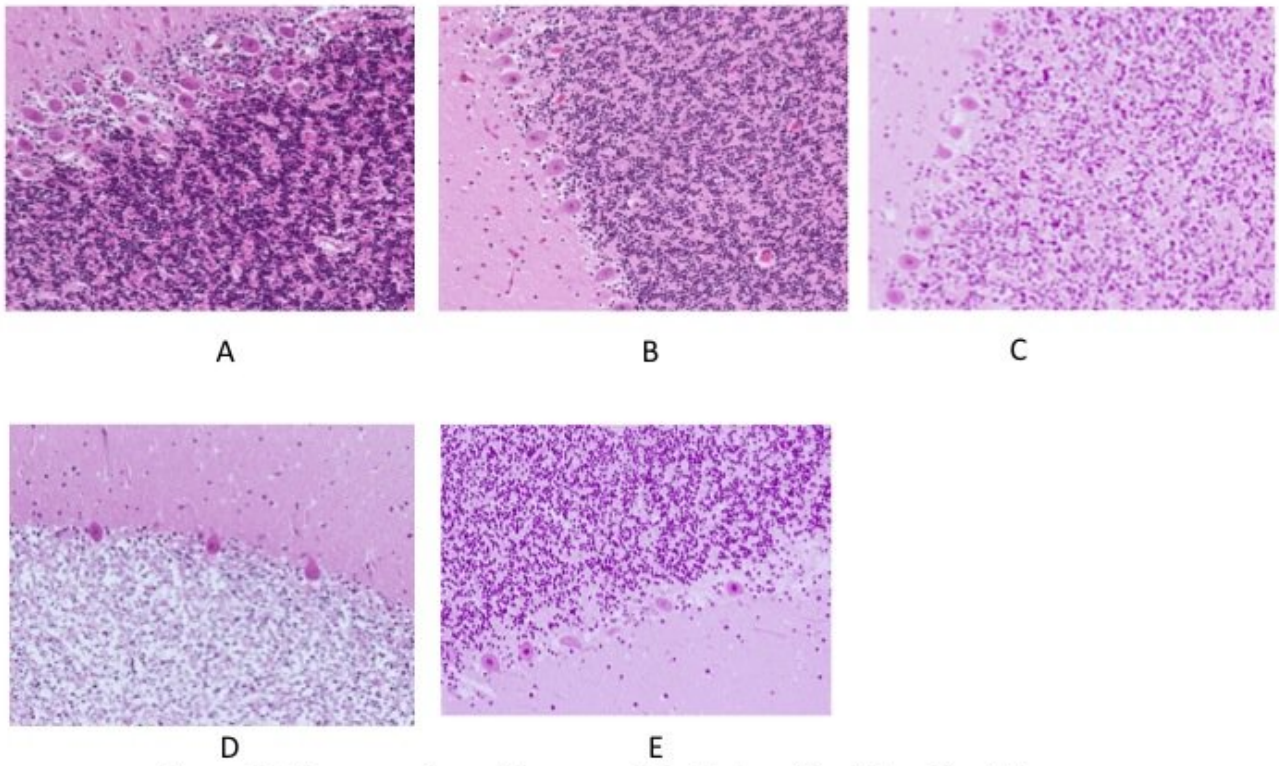


Figure 1. The granular cell layer and Purkinje cells stained by HE
A; High remaining level from Case 3. B; middle remaining level from case 4.
C; low remaining level from case 9, D; severe level from case 6. E;
low remaining from case 12. The granular cells were damaged with agonal
duration but Purkinje cells kept the morphology.

accidents (date not shown).

The results obtained from evaluation of the damaging of the granular cell layer using an application of Photo-shop were listed in Table 2. The frequency of remaining granular cells was indicated as percentage of the total number of pixel and classed into four degrees, that is, high remaining level; over or around 50% of cell was stained by hematoxylin, middle remaining level; from 30 to 40% of the cells were stained, low remaining level; around 20% of cells were stained, and severe degeneration level; around 10% of cells were remained with large amount of cell debris (synaptic glomeruli). Case 1,2,3,7,and 8 were grouped into high remaining level, case 4,11 and 14 were in moderate group, case 5, 9, 10 and 12 were in low group and case 6 and 13 were grouped into severe level. As an interesting case, the granular cell layer of the cerebellum obtained from case

12 individual whose postmortem interval was presumed over 40 days, decreased the stain-ability, however the morphology of the granular cells have been well keeping compared with the longer agonal duration cases that was classed into severe level.

Changing of granular cell layer was remarkable compared with that of Purkinje cells in lower and severe level groups where the morphology of Purkinje cells was still remained.

[2] Immunohistochemical staining

In the granular cell layer at high level remaining, a large amount of granular cell nucleus showed good and intensive reactivity with anti CIRBP and RBM3 antibodies. HSP70 showed weak or no

reactivity with the nucleus of the granular cells, however it showed good reactivity with cytoplasm and synaptic glomeruli of some cells, presumed Golgi cells and Lugar cells in the granular cell layer and the Purkinje cells layer, respectively. Antibodies againstcFos, AIF1, p53, Ngb and CCC9 showed weak reactivity with cytoplasm of the granular cells and in the synaptic glomeruli of the granular cell layer. The synaptic glomeruli showed just like cell debris.

The granular cells at middle remaining level showed moderate reactivity with anti RBM3 antibody and weak reactivity with CIRBP antibody in the nucleus, and anti HIF1, SIRT1 and Ngb showed no reactivity with the granular cells at middle remaining level. There is slight reactivity between cell debris, synaptic glomeruli, with anti CIRBP, RBM3, HIF1, SIRT1 and Ngb in the granular cell layer at low level remaining.

2. Purkinje cells

[1]Hematoxylin-Eosin staining

The Purkinje cells showed various changes such as disappear, shrunken eosionophilic cytoplasm with smudgy dark nuclei, or vacuolated, homogenous, eosinophilic or pale cytoplasm with pale or piknotic nuclei.

The Purkinje cells exhibited homogeneity in color and shape at high remaining level. With decreasing of the cells in the granular cell layer, the degeneration of Purkinje cells was also progressed. Purkinje cells from case

12 remained the morphology with normal nucleus in many cells, although staining ability was decreased.

[2]Immunohistochemical staining

Purkinje cells in the cerebellum at high remaining level showed clear reactivity with anti CIRBP both in the nucleus and cytoplasm, and intensive reactivity with RBM3 only in the nucleus (Figure 2).

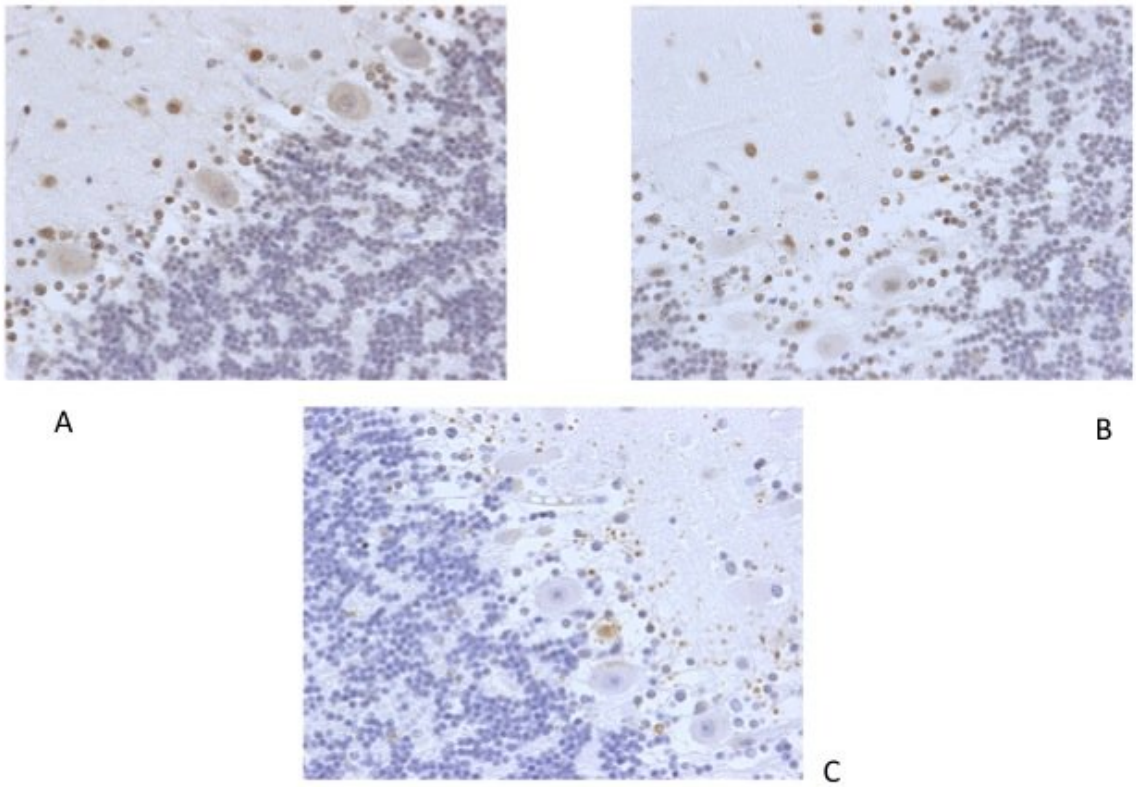


Figure 2. Results of immunohistochemical staining of Purkinje cells from case 3. A; Anti CIRBP showed good reactivity with cytoplasm of the cells, B; Anti RBM3 stained the nucleuses of Purkinje cells, C; Anti HSP70 showed clear reactivity with cytoplasm in cells around Purkinje cells but no with Purkinje cells. (×400)

Anti cFos, Ngb and

CCC9 showed weak reactivity with cytoplasm and no with nucleus. Anti

HSP70 showed good reactivity cytoplasm in the cells around Purkinje cells (Figure 2), however, reactivity of the antibody with cytoplasm in the Purkinje cells decreased with progression of damaging of granular cells.

Purkinje cells at middle remaining level showed slight reactivity with CIRBP in cytoplasm and clear reactivity with anti RBM3 in the nucleus.

Although no reactivity with anti HIF1, SIRT1 and CCC9 was recognized, antiNgb, cFos and HSP antibodies showed weak reactivity in the cytoplasm of some Purkinje cells.

Purkinje cells at low remaining level showed no reactivity with anti CIRBP, RBM3, HIF1, and SIRT1, however anti CCC9 (Figure 3), Ngb, cFps and HSP, showed weak reactivity with cytoplasm of some Purkinje cells which were keeping the morphology.

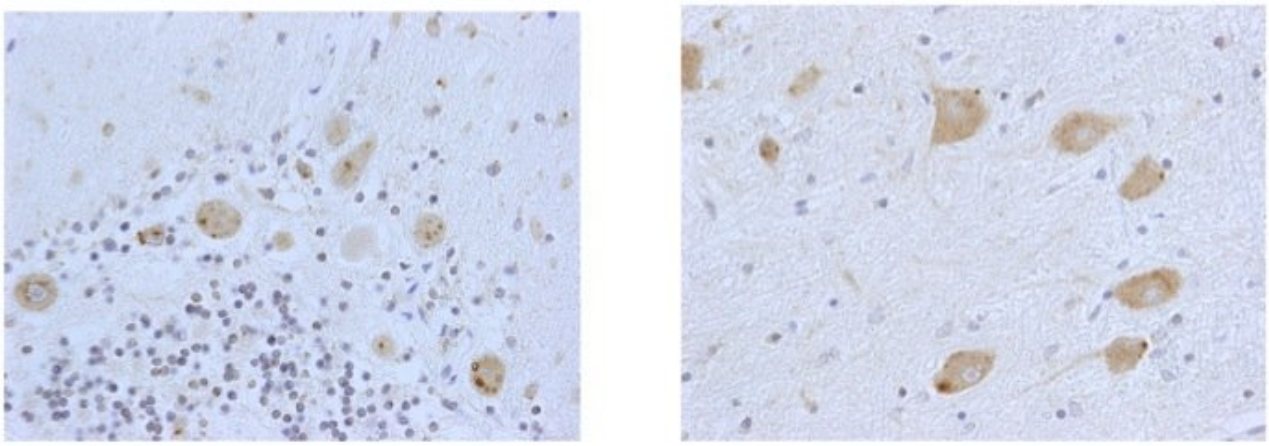


Figure 3. Anti CCC9 showed weak reactivity with Purkinje cells (A) and clear with the dantate nuclear cells (B) from Case 5.

3. Molecular layer

[1]Hematoxylin-Eosin staining

The cells in the molecular layer were detected even in individuals with severe decreasing level of granular cell layer, although the number of remaining cells was decreased, however it was difficult to identify the basket or satellite cells.

[2] Immunohistochemical staining

At high remaining level the nucleuses of many cells in the molecular layer, but not all cell, showed intensive reactivity with anti CIRBP and RBM3, and moderate reactivity with anti HSP. Although weak reactivity with cells and anti CIRBP, RBM3, HSP and cFos antibodies was recognized at low and middleremaining level, anti HIF1 and Ngb showed no reactivity with the cells. At moderate and low remaining level anti SIRT 1and CCC9 stained weakly with nucleus of some cells in the molecular layer but not all cell. At severe level weak reactivity of CIRB, RBM3, cFos and HSP with the nucleus of the some cells was detected and no or feeble with other antibodies.

4. Cells in the Dentate Nucleus

[1]Hematoxylin-Eosin staining

With the progressing of the agonal duration, the degeneration of the dentate nuclear cells increased. However cells were able to keep the morphology even in severe level cases where it was difficult to detect the cell nucleus.

[2]Immunohistochemical staining

Anti CIRBP, RBM3 and HSP showed intensive reactivity with cytoplasm of the dentate nucleus cells at high remaining level, the reactivity with these antibodies decreased with increasing of damaging of the granular cells, however weak reactivity with all antibody (Figure4), except anti HIF1, examined in this study was kept even in severe damage level of the cerebellum obtained from Case 6 whose evaluation numerical value was calculated at 8.4%.

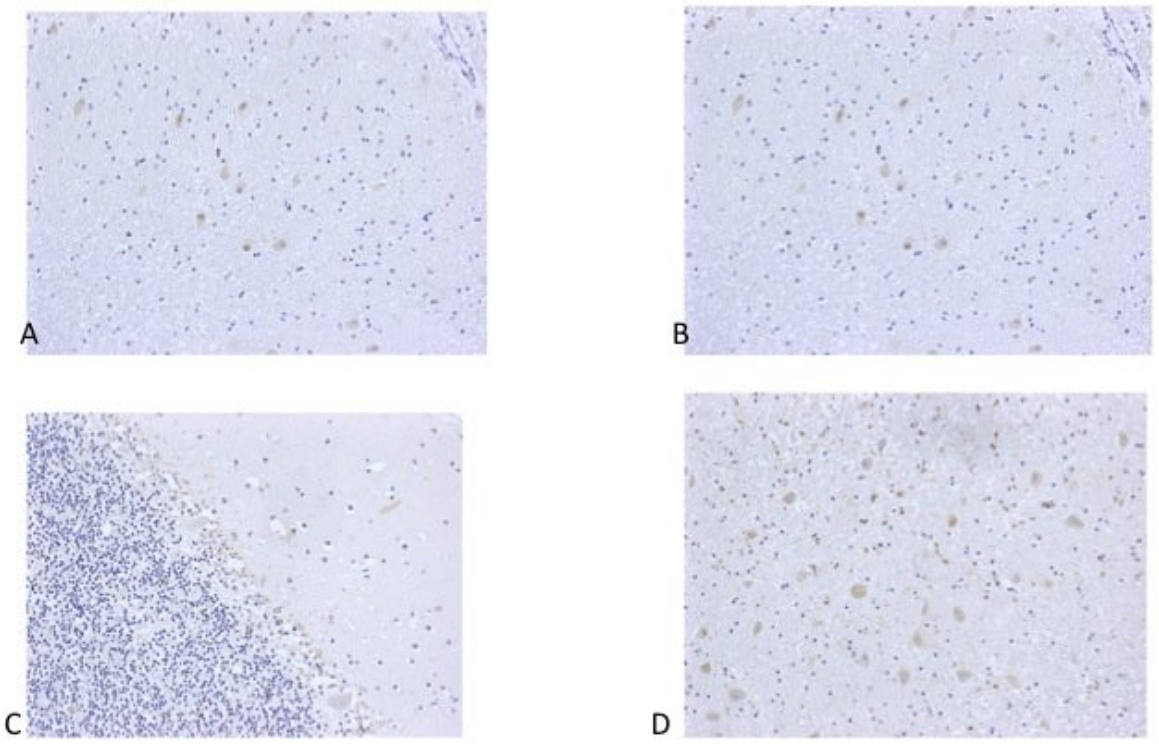


Figure 4. Cells in the molecular layer (A) and dentate nuclear cells(B) remained reactivity with anti CIRBP, and cells around Purkinje cells (C) and dentate nuclear cells(D) remained reactivity with anti HSP70. (×100) The cerebellum was obtained from case 12.

Anti SIRT 1 showed moderate reactivity with the cell nucleus around the dentate nucleus and cytoplasm of the dentate nucleus cells at moderate remaining level.

5. The cells in the white matter

[1]Hematoxylin-Eosin staining

The cells in the white matter of the cerebellum were remained even in severe level individuals, although their number was remarkably decreased.

[2]Immunohistochemical staining

Anti CIRBP, RBM3, HSP70 and cFos antibodies showed weak reactivity with nucleuses and cytoplasm of the remaining cells even in the severe level individuals. Anti AIF1, SIRT1 and Ngbalso showed weak reactivity with the cytoplasm of the remaining cells in severe damaged level individuals, however, reactivity with HIF1 was not detected.

Some cells such as cells in the dentate nucleus cells and molecular layer in the cerebellum from case 12 remained weak reactivity with anti CIRBP, HSP70 and CCC9 as shown in Figure 4.

Discussion

In this study could indicate that many antigens are expressed during agonal duration as the vital reaction or biological reaction of the bodies. In forensic field it is known as the obvious evidence that the pressure ulcer could occur during alive and not occur or not become more seriously after one's death.

Concerning to agonal duration in hanging cases, Sauvageau et al (20) reported that the death occurred at 4mintes 12 seconds± 2 minutes 29 seconds, and in our experience the death occurred at 2mints 38seconds (21).

Since in typical hanging case both side of carotid and vertebral arteries are obstructed, the death comes earlier than other death due to self-strangulation and/or manual strangulation.

The brain is critically dependent on its blood flow for a continuous supply of oxygen and glucose. Only a few minutes of severe ischemia induce the selective degeneration of certain neuronal populations, including pyramidal neurons in the CA1 region of the hippocampus, neocortical neurons in layers

3,5, and 6, and cerebellar Purkinje cells (22). As describing in a review article bySarna and Hawkes(23), it is also well known the vulnerability of the Purkinje cells to severe and transient global brain ischemia, chronic hypoxia experiments, intermittent hypoxia(24,25), toxic situation (26)and many neuronal degeneration diseases. This study indicates that the vulnerability of the granular cells may be more obvious and convenience for analysisthan that of Purkinje cells, since the changing of granular cell layer was remarkable and homogeneous compared with that of Purkinje cells in lower and severe level groups where Purkinje cells was still remained and showed various changes such as disappear, shrunken eosinophilic cytoplasm with smudgy dark nuclei, or vacuolated, homogenous, eosinophilic or pale cytoplasm with pale or pyknotic nuclei, showing difficulty in the evaluation and analysis.

There was the difference of the degree of damaging in the granular cell layer between case 11 and 12, although they were killed by manual strangulation andagonal durations of them were presumed almost the same. The differencein partly seemed to relate their postmortem intervals. There was also difference of damaging level among case 6, 10 and 13. This difference may arise from obstruction of cervical vessels in case 6 and global anoxia in cases1 10 and 13.

In our previous reports (6), antigens such as CIRBP, RBM3 and SIRT1 were expressed in the nucleus of the cardiac calls obtained from hypothermic and/or carbon monoxide intoxication death. In this study, reactivity with anti-CIRBP and RBM3 antibodies was detected in nucleuses in the cells of the cerebellum, however that of anti SIRT1 antibody was not detected in the granular cells and Purkinje cells, although the reactivity was recognized in the cytoplasm of the dentate nucleus cells of the cerebellum. The same result was observed in the cerebral cortex (15), indicating that expression of SIRT1 which plays important role to protect the brain function from many stress (27)may be most delayed one in the brain.

It is well known that CIRP and RBM3 were discovered after exposure to a moderate cold-schock and other cellular stress such as UV radiation and hypoxia(28). Although at normal temperature, CIRBP

mRNA expression was detected in the rat brain(29), Wellmann et al (30) reported that hypoxia significantly induced CIRBP and RBM3 expression in vitro. We also reported that intensive reactivity of anti CIRBP and RBM3, weak reactivity of HIF1 and HSP-70 and no reactivity with SIRT1 in the nucleus in the neuron of the frontal cortical region obtained from two male who died suicidal strangulation using a rope or T-shirt were detectable (15). These results indicate that CIRBP and RBM3 may be ischemic and/or hypoxic related antigens, although these antigens were discovered as the cold inducible antigens.

HIF-1 is a hypoxic inducing factor and detected in hypoxic tissues (1), and oxygen-regulated expression of CIRBP and RBM3 by a HIF-1-independent mechanism was reported usingthe human leukemic cell line (30), however in this study HIF1 could show weakor feeble reactivity with Purkinje cells and confirm the repot from Wellmann et al (30). Chen et al (12) reported a bidirectional link of HIF and Sirt1 signaling during hypoxia, and then weak reactivity of HIF and Sirt 1 in the celebbellum may be caused by the linkage of the genes encoded expression of HIF and Sirt 1 in the this organ.

Concerning the expression of HSP70 many studies were reported, and the expression of HSP70 in Purkinje cells reduced in patients with schizophrenia compared with individual without disease (31). In this study the expression of HSP70 was not detected in Purkinje cells, although the clear expression of the antigen in the cells was recognized in Purkinje cell layer, granular cell layer, molecular layer, dentate nucleus, and cerebellar white matter. In our previous experience (15), reactivity with HSP70 and neurons in the cerebral cortex was remarkably detected in the case 7 and 8 of this study.These results may cause by the difference of stability against the stress among the cells, indicating that the HSP70 expression system in Purkinje cell is most vulnerability, different among the brain lesions or early disappear in the cells.

Schaefer et al (32) described deposition of C5b-9 complexes can already occur at the cytoplasmic membrane surface of ischemic cells, and immnocytochemical detection of C5b-9 provides a powerful tool for detecting even single cell necrosis in the cardiac infarction cases. In this study the reactivity of CCC9 was detected mainly in the cytoplasm of the dentate nucleus cells. The cells stained by anti CCC9 may be in necrotic stage and shows the latest stage in the cell biological reaction against ischemic, because some heart cells were stained by CCC9 and other cells with normal morphology did not show the reactivity in acute cardiac infarction cases.

In this study the Purkinje cells seemed to be destroyed with increasing of disappearing of granular cells and showed clear difference in the expression pattern of CIRBP and RBM3, that is, CIRBP showed its reactivity in the cytoplasm and RBM3 was detected in the nucleus of the Purkinje cells at the level of normal stainability of granular cells. This phenomenadiffer from those in the heart muscle in which these antigens were expressed mainly in the nucleus of the cells affected in hypoxic situation (6).This result indicates that the difference in the expression of these antigens exists among the organs.

Conclusion

The results obtained in this study indicate that the intracellular distribution of each antigen confirmed the previous reports. The intercellular distribution of each antigen was different in the cerebellum. The expression of hypoxia, ischemia, apoptotic and necrotic related antigens may be expressed according to each encoded gene during agonal duration and the activity of its expression,

cascade of antigen expression pathway, may be stopped at cellular death and situation of expressed antigen and cell morphology would be kept even in postmortem interval when there was no putrefy of the body.

The cells in the dentate nucleus and white matter may be most stable to the changing during agonal duration and postmortem interval. The evaluation of granular cell layer might be useful to presume the agonal duration, confirming to the report described by Sheedy et al (18), however, we should know that the granular cells were kept their morphology for a long time in special situation such as under ground.

The present study indicates that once vessel occlusion has occurred, the concerned tissue depleted of blood supply undergoes causing neuronal injury and many signal pathways are activated leading to cell death, immune, and other responses, and also indicates that the results by cascade of signal pathways may be kept the situation that the each cascade was stopped. This phenomenonresembles to pressure ulcer (decubitus), that is, the pressure ulcer never newly becomes in postmortem interval.

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