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RBM3 AND CIRP MAY PLAY AN IMPORTANT ROLE AS AVASODILATOR. A PRELIMINARY STUDY

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ABSTRACT

It is now well accepted that e-NOs is one of target genes of HIF-1 and it plays an important role as a vasodilator or angiogenesis. We found in our series of immune his to chemical study that anti- RBM3 and CIRP antibodies were able to stain the nucleuses of neurons, myocardial cells and cells in the blood vessels existing in the newly infarction area of the brain and myocardium, although moderate or feeble reactivity with those antibodies were observed in sub-acute and/or chronic infarction area. The reactive manner of them were basically resemble to those by anti e-NOs antibody, and showed more intensive and clear compared with those by anti e-NOs antibody. It has been shown in our previous studies that expression of RBM3 and CIRP in the heart and/or brain occurred in response to cellular stressors, such as hypoxia and hypothermic conditions, where they may attenuate both apoptosis and necrosis. The results observed in this study additionally indicate that RBM3 and CIRP may also play an important role as a vasodilator mechanism in hypoxic-ischemic condition and show a priority role to detect the hypoxia status in the tissues obtained at forensic autopsies.

INTRODUCTION

RBM3 (RNA binding motif protein 3) is one of human RNA binding protein genes located in Xp11.23 (Derry *et al*., 1995) and CIRP (Cold-inducible RNA binding protein) is also the gene located 19p13.3 (Nishiyama *et al*., 1997). The genes produce RBM3 and CIRP proteins and the proteins play important roles in protection of cools stress (Ferry *et al*., 2011) and hypoxia (Furukawa *et al*., 2013). In our previous additional study we also showed that RBM3 and CIRP proteins were intensively expressed in the nucleuses of the brain and myocardium obtained from a victim with multiple brain infarctions (Furukawa *et al*., 2014), and individuals who died due to self-strangulation, hanging, manual strangulation, carbon monoxide poisoning and/or accidental hypothermia (Furukawa *et al*., 2013). During a series of our study we newly found that the reactivity of anti-RBM3 and CIRP antibodies showed resemble to those by anti e-NOs (endothelial nitric oxide synthase) antibody, especially in the endothelial cell nucleuses of blood vessels.

MATERIALS AND METHODS

Tissue sections from brains or hearts with different stages of infarction were collected during autopsy at Department of Legal Medicine, Shiga University of Medical Science during last three years and were process in routine his to-pathological techniques (10% formalin fixation, paraffin embedding, 3µm thick-section cutting), and stained by Hematoxylin-Eosin, and Azan stains. Cells involved in infarction areas were examined by an immune his to chemical technique using ABC technique (Nichirei Laboratories, Japan), and anti RBM3 antibody, anti e-NOs antibody and other antibodies, according to the manufacture protocol. In brief, paraffin-embedded sections were hydrated and washed in 10 m-mol of PBS (phosphate buffered saline). After blocking endogenous peroxidase by 3% H2O2 and incubation of suitable serum to prevent cross-reaction between antigens and antibodies, sections were incubated by antibodies against hypoxia related antigens for suitable hours. After rinsing with PBS, sections were incubated for one hour in suitable biotinylatedIgG solutions. Subsequently, sections were washed and ABC regent was added for 30 minutes. Finally, diaminobenzidine solution was added for several minutes, and slides was dehydrated and covered with a cover glass to prepared at microscope level observation. In the examination of myocardium Anti- RBM3, e-NOs and CCC9 antibodies were used to detection of existence of these antigens in the epithelial cells of the blood vessels since the expression manner in the nucleuses of myocardium had been described in a previous report (Furukawa *et al*., 2014; Furukawa *et al*., 2013).

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We used the additional antibodies listed in Table 1 to evaluate the changing of expression manner of the antigens detected by the antibodies during the process of brain infarction.

RESULTS

Myocardium

Anti RBM3 antibody showed intensive reactivity with the nucleuses of the myocardium obtained from individuals who died cardiac infarction, carbon monoxide poisoning, and hypothermia. Anti eNOs- and RBM3-antibodies stained endothelial cells of blood vessel contained thrombus, as shown in Figure 1, which showed acute myocardial infarction.

Brain infarction

In infarct areas with fresh bleeding, anti e-NOs- and RBM3-antibodies showed no reactivity with erythrocytes and clear reactivity with mononuclear cells in the bleeding and these antibodies stained cytoplasm and nucleuses in blood vessel around the bleeding, respectively, as shown in Figure 3. In sub-chronic infarct areas including acute and sub-acute infarction (Figure 4), both antibodies stained spheroids, mononuclear cells, macrophages and gemistocytes, although anti e-NOs antibody showed with cytoplasm and anti RBM3 antibody showed nucleuses. Both antibodies clearly stained cytoplasm or nucleuses in the epithelial cells of blood vessels that were newly produced vessels and covered with thin membrane just like as an envelopes (Figure 4).

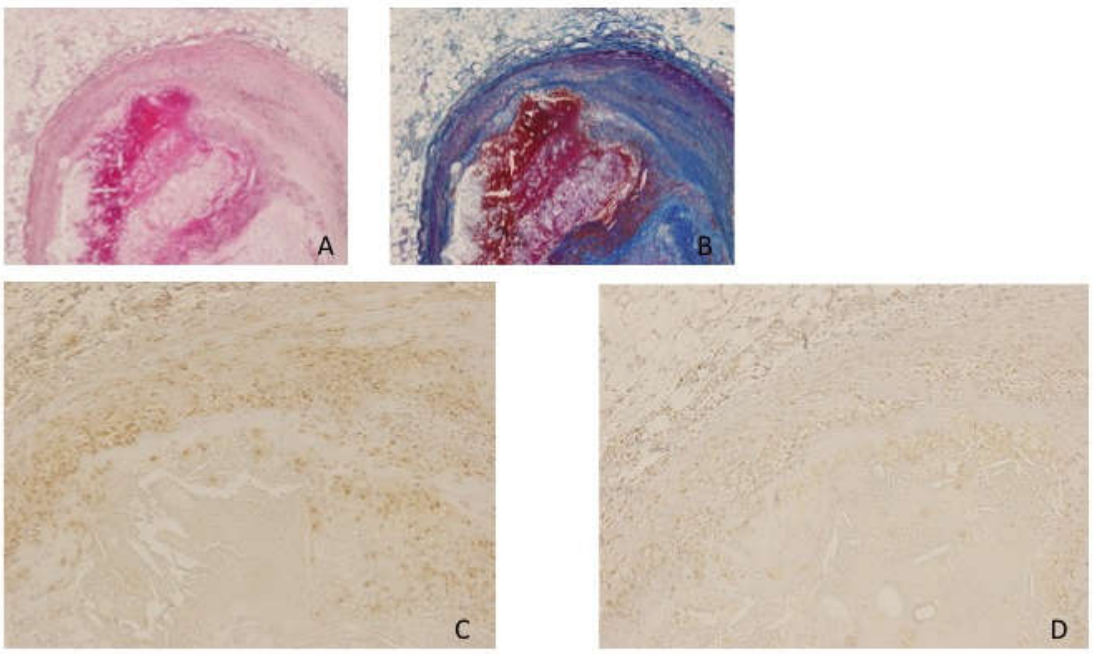

Figure 1. Staining of coronary artery containing thrombosis by HE (A), Azan (B), anti RBM3 antibody (C) and anti e- NOs antibody (D) The blood vessel was stained intensively by anti RBM3 antibody and weak by anti e- NOs antibody

These antibody also stained the nucleuses of endothelial cells of blood vessels in the infarct area where a large amount of neutrophils existed (Figure 2 which presented sub-acute infarction), however only anti RBM3 antibody showed good reactivity with nucleuses of blood vessels obtained from a patient with chronic hypoxia status caused by cardiac amyloidosis and no reactivity with both antibodies was observed in cells in blood vessels and fibroblast cells in the chronic fibrotic area. In the myocardium with contraction band necrosis there was no reactivity with this antibody, although anti e-NOs antibody showed weak reactivity with cytoplasm of cells with contraction band necrosis. Anti e-NOs antibody clearly stained the cytoplasm of the myocardium that was stained by anti RBM3 antibody, and showed no reactivity with blood vessels in chronic infarct area. Anti eNOs- and RBM3-antibodies stained endothelial cells of blood vessel contained thrombus in its lumen. The other antibodies examined showed no clear reactivity with cells in the blood vessels, except anti CCC9 (Complement component c 9) antibody, which showed clear reactivity with the nucleuses of endothelial cells from individuals who died due to hypothermia, but not cardiac infarction, however anti CCC9 antibody could stain the necrotic myocardium. Anti CIRP antibody showed good reactivity similar to those of anti RBM3 antibody.

In chronic infarct area with autolysis tissue and cavity with large amount of hemosiderin there was no reactivity with these antibodies (Figure 5). In the chronic infarct area, Anti CIRBP (cold inducible RNA binding protein)- and HSP70 (heat shock protein 70)-antibodies showed the similar stainability with those by Anti- RBM3 and e-NOs antibodies but relatively weak. Although anti HIF1(hypoxia inducible factor 1 α)- and VEGF (vascular endothelial growth factor)-antibodies showed weak reactivity with the several kinds of cells inside the infarction area.

Anti SIRT1 (sirtuin1) antibody showed clear reactivity with the nucleuses of the cell group containing giant granular corpuscle cells in the infarct area, while the antibody showed weak reactivity the cells outside infarction area. Anti AIF1 (apoptosis inducible factor 1)-, cFos (Fos-related antigen)- and Ngb (neuro globin)-antibodies showed weak reactivity with the cells inside the infarction area, anti p53(tumor protein 53)- and Wnt (wnt signaling) -antibodies showed feeble or no reactivity. Anti CCC9 antibody showed clear reactivity with the giant granular corpuscle cells in the infarction area, but not with endothelial cells of blood vessels. Anti CIRP antibody also showed good reactivity similar to those of anti RBM3 antibody.

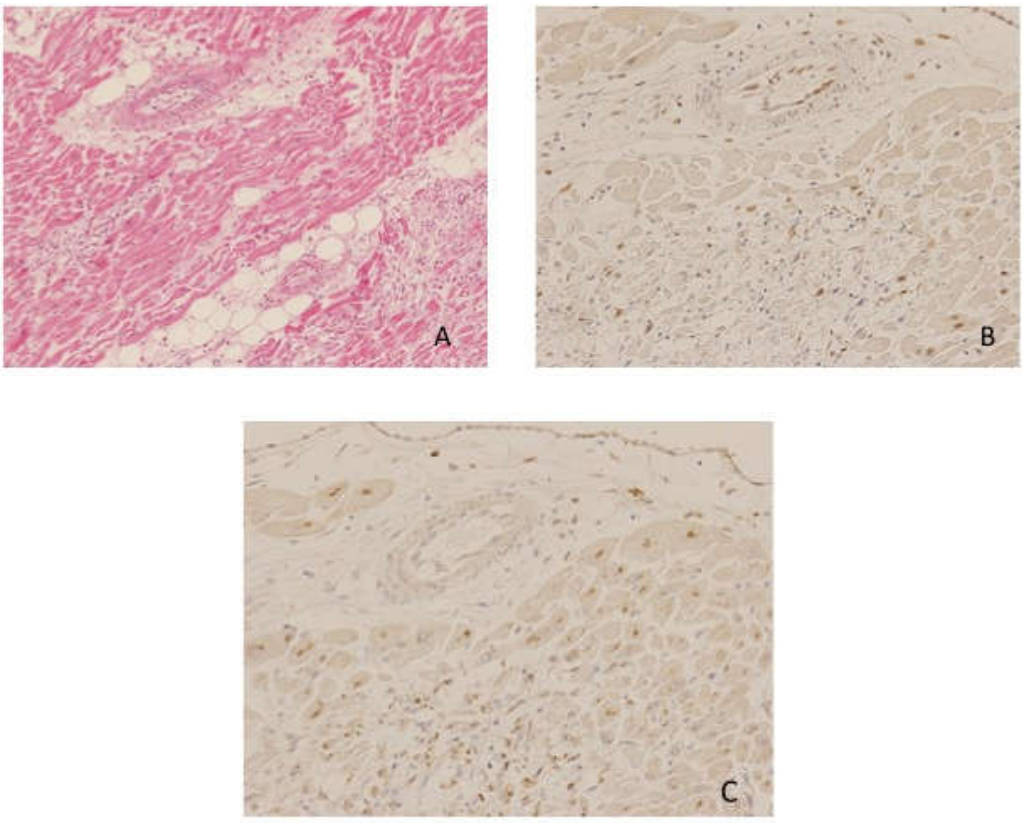

Figure 2. Staining cardiac infarct area by HE (A), anti RBM3 antibody (B) and anti e NOs antibody (C). Anti RBM3 antibody stained the nucleuses of epithelial cells of blood vessel and nucleuses of small amount of fibroblast or myocardial cells. Anti e-NOs antibody stained weakly blood vessel, and cytoplasm of the myocardial cells and fibroblast

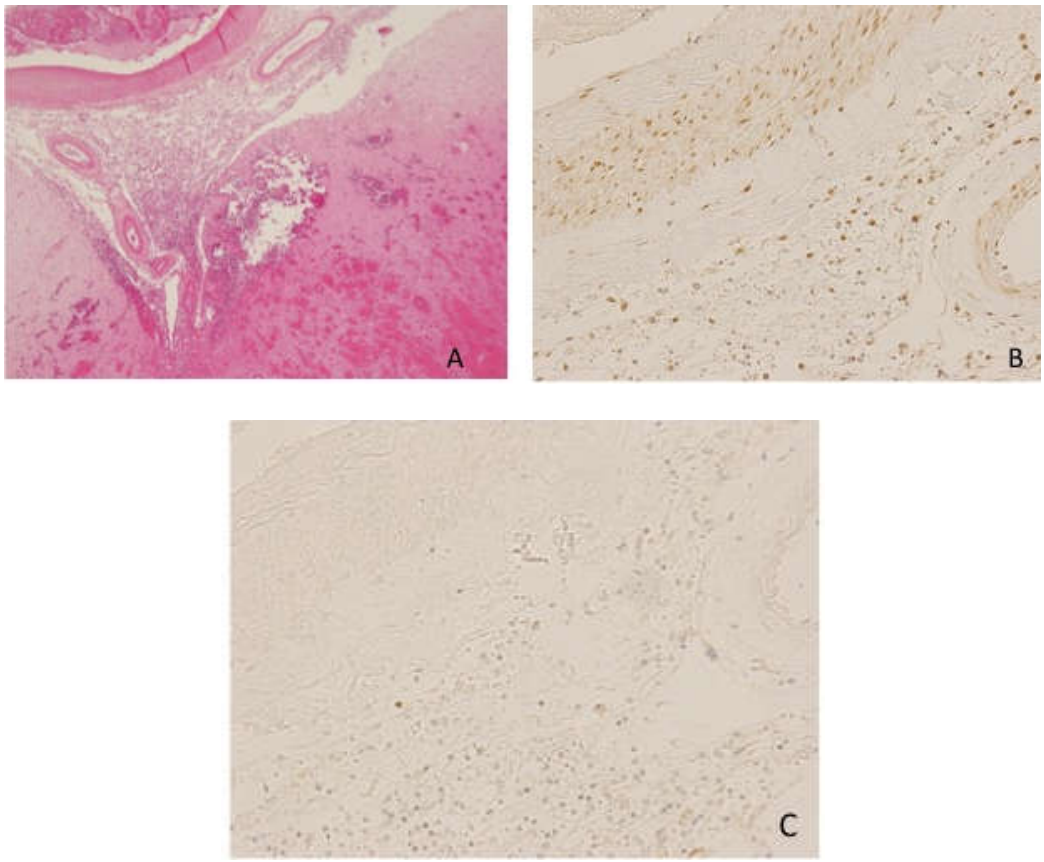

Figure 3. Staining of blood vessels aroundfresh brain infarct area by HE(A), anti RBM3 antibody (B), and anti e-NOs antibody (C). Anti RBM3 antibody stained cell nucleuses of blood vessels and large cells in the bleeding area. Antie-NOs antibody showed weak reactivity with the cells

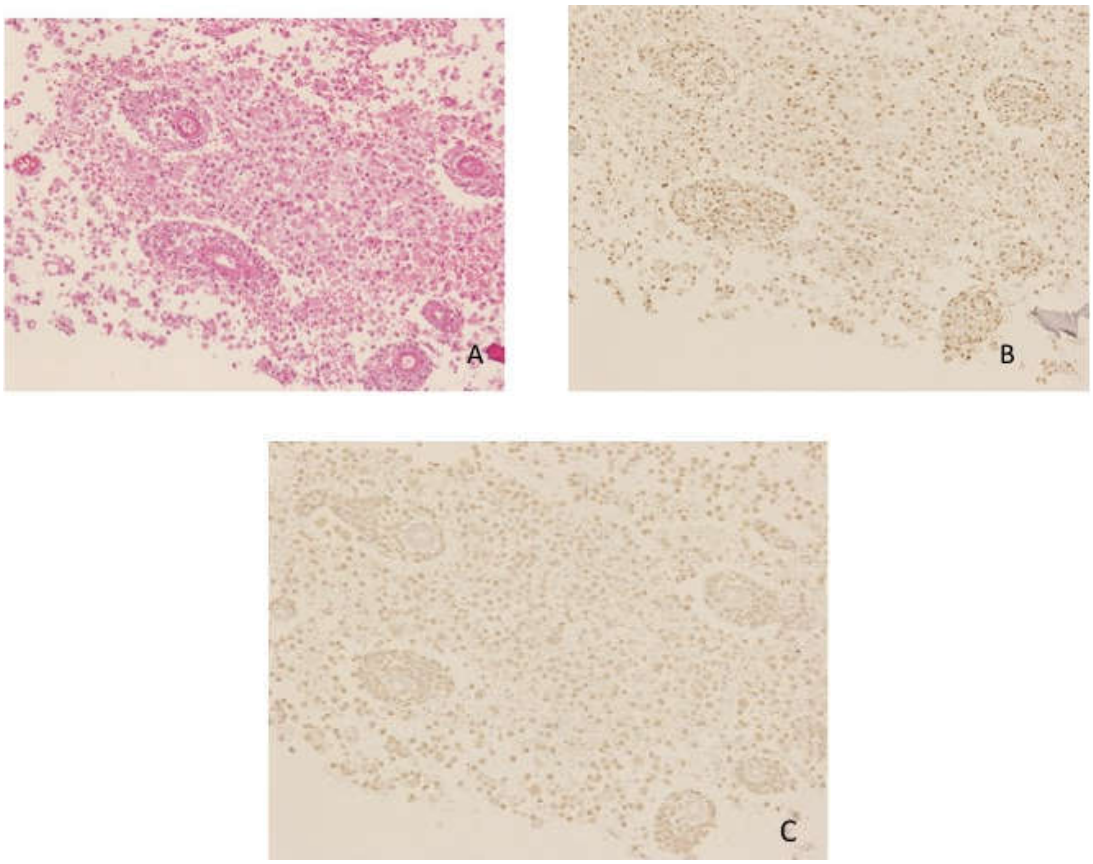

Figure 4. Staining of chronic brain infarct areaby HE (A), anti RBM3 antibody (B), and anti e-NOs antibody (C). Anti RBM3 antibody stained the nucleuses and anti e-NOS antibody stained cytoplasm of blood vessels covered by a thin membranous materials just like an envelope

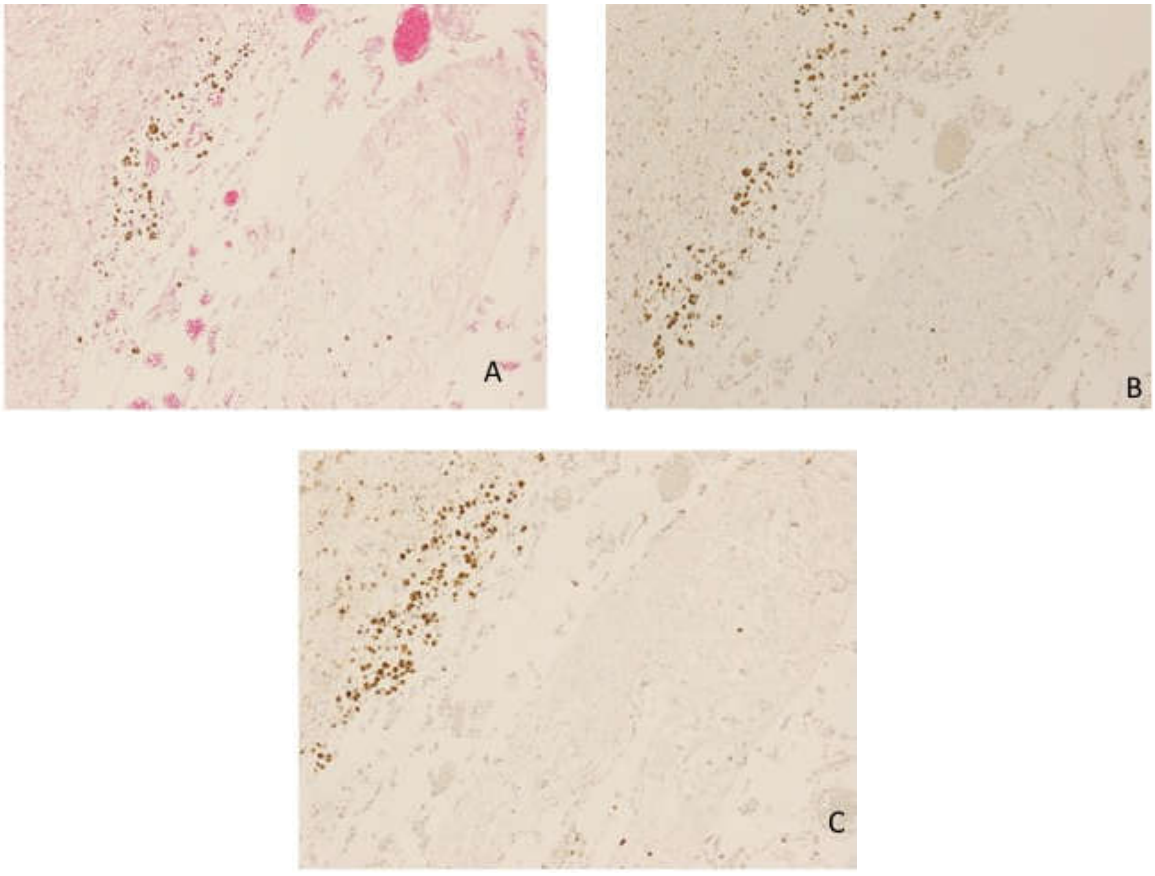

Figure 5. Staining of infarct cavity by HE (A), anti-RBM3 antibody and anti e-NOsantibody (C). A large amount of hemosiderin granules were existed in HE. Anti RBM3 antibody and anti e-NOs antibody showed no reactivity in thisarea

Table 1. Characteristics of the antibodies used in this study

Antibody	Maker	Clone	Species	AG-retrieval	Incubation	AB-dilution
CIRP	Protein Tech	10209-2-AP	R	autoclave	overnight	1: 400
RBM3	Protein Tech	14363-1-AP	R	autoclave	overnight	1: 400
HSP70	Santa cruz	Polyclonal	G	autoclave	overnight	1: 400
HIF-1α	Novus	NB 100-479	R	autoclave	overnight	1: 400
VEGF	Milipore	JH121	M	autoclave	overnight	1: 400
eNOS	Gene Tex	polyclonal	R	autoclave	overnight	pre-diluted
AIF- α	LSBio	aa-593-606	R	autoclave	overnight	1: 400
P53	Santa cruz	FL-393	G	autoclave	overnight	1: 400
cFOS	Gene Tex	polyclonal:	R	autoclave	overnight	1: 800
Ngb	SIGMA-ALDRICH	polyclonal:	R	autoclave	overnight	1: 400
wnt	Novus	6F2	M	autoclave	overnight	1: 400
SIRT1	Novus	E104:	R	autoclave	overnight	1: 400
CCC9	Leica	10A6	M	autoclave	overnight	1: 400

DISCUSSION

In our previous reports (Furukawa *et al*., 2013) we examined the expression of hypoxia related antigens in cerebral cortex and hypothalamus obtained from two individuals who died due to self-strangulation by ligature using antibodies against HIF1α-, HSP-70-, CIRP-, RBM3-, SIRT1-, p53-, e-NOs-, n-NOs-, COX-2-, and Wnt-antigens. Among these antibodies, anti CIRBP- and anti RBM3-antibodies showed good reactivity with pyramidal neurons in the cortex and hypothalamus, anti HSP-70- and anti e-NOs-antibodies showed moderate reactivity, and anti n-NOs-, anti COX-2- and anti Wnt-antbodies showed weak reactivity, however no reactivity was observed with anti HIF1α-, anti SIRT1- and anti p53-antibodies.

These results also indicate that CIRP and RBM3 protein consistently and early increased in the ischemic brain tissues compared with other antigens, although expression of the antigens might be influenced by the characteristics of the genes and/or timing of post-ischemic upregulation of the genes. In this study we could show that reactivity of anti RBM3and CIRP antibodies with nucleuses in the epithelial cells in blood vessels appeared in early infarct area and continued in the nucleuses in acute and sub-acute infarct area, however the reactivity was not detected chronic infarct area, such as cavity in the brain and the fibrosis in the myocardium. The reactivity of antie-NOs antibody with brain tissues and the myocardium was resemble to those with anti RBM3 anti body, although staining intensity was different and no reactivity with e-NOs was observed in the blood vessel from a patient with cardiac amyloidosis. RBM3 protein is a cold-induced member of the glycine-rich RNA-binding protein family.

Although a function for this protein has not yet been defined, it has been suggested that RBM3 may affect gene expression by facilitating translation at colder temperatures (Danno *et al*., 1997; Chappell *et al*., 2001). It is now well accept that RBM3 is ubiquitously expressed and it is the only transcript up-regulated in all tissues during torpor (Williams *et al*., 2005). Up-regulation of RBM3 and CIRP also occur in response to other cellular stressors, such as hypoxia and degenerative conditions, where it may attenuate both apoptosis and necrosis (Nishiyama *et al*., 1997; Wellmann *et al*., 2004; Kita *et al*., 2002). Although accumulating evidence suggests that RBM3 and CIRP play an important role in multiplecellular processes during development and during stress response to a variety of stressesincluding hypothermia (Nishiyama *et al*., 1997), the role of RBM3 and CIRP in cancer and infarct area remains equivocal.

Taken together, these observations suggest that RBM3 and CIRP may have a fundamental function in all cells that becomes of adaptive value under conditions of cellular stress, and of pathological significance in cell transformation. NO produced by e-NOs appears to protect the brain by enhancing brain blood flow in ischemic area and perhaps by its inhibitory effect on platelet and leukocyte adhesion. Enhanced NO production from e-NOs may also promote angiogenesis in damaged tissue (Veltkamp *et al*., 2002). Veltkamp *et al* (2002) reported that e-NOs was consistently increased in micro-vessels in the ischemic striatum after 24 to 168 hours of reperfusion after right middle cerebral artery of Wister rats for 75minutes. Their results indicate that the expression of e-NOs is delayed than our imagined times, since in our examination contained acute brain infarction death cases. Qing *et al* (2007) described that chronic hypoxemia is associated with the induction and stabilization of the transcription factor HIF-1 as well as its target genes, VEGF and eNOS, in the myocardium of infants with cyanotic cardiac defects. Althoughhypoxia is known to activate a number of regulatory responses within the cerebral cortex, including an increase in expression of HIF-1, nNOS, and VEGF to promote angiogenesis (Brune, 2000), .the functional interactions between HIF-1α, nNOS, and VEGF can differ depending on the level of tissue oxygen tension (Brune, 2000).

Under hypoxic conditions, increased NO can reduce cerebral HIF-1 α and VEGF expression (Huang *et al*., 1999). However, under normoxic conditions, increased NO may stabilize HIF-1 levels leading to transcription of HIF-1-responsive elements, including VEGF (18). These facts may affect the different reactivity among antibodies against hypoxic related antigens in the present examination. Concerning to RBM3 and CIRP, anti- RBM3 and CIRP antibodies were able to stain the nucleuses of neurons, myocardial cells and cells in the blood vessels existing in the newly infarction area of the brain and myocardium, although moderate or feeble reactivity with those antibodies were observed in sub-acute and/or chronic infarction area. These results may indicate that expression of them occur only in hypoxic or ischemic situation. The results obtained in this examination indicate that RBM3 and/or CIRBP induce vasodilation and angiogenesis mechanism in hypoxic tissues with the similarity of e-NOs protein. Additionally, the examination by means of immunostaining using anti- RBM3 and CIRP antibodies are able to have some superiority in the inference of existence of the hypoxic situations due to compression of the neck and/or asphyxia occurred before one's death, compared with other antibody against hypoxic related antigens.

As far as we are aware, this is the first report to show that RBM3 and/or CIRBP significantly induce vasodilator or angiogenesis in infarction of the brain and heart.

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