Time-dependent and lesion-dependent HMGB1-selective localization in brains of patients with cerebrovascular diseases

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Summary. High mobility group box 1 protein (HMGB1) has multiple functions, including the maintenance of nucleosomes and the regulation of gene transcription. HMGB1 is released from activated macrophages, resulting in the induction of inflammatory cytokines. Recently, much research about the role of HMGB1 in cerebrovascular disease (CVD) has been reported. In an animal model, HMGB1 neutralization ameliorates brain infarction, there is an early release of HMGB1 from neurons, and HMGB1 antibody attenuates delayed cerebral vasospasm in experimental subarachnoid hemorrhage. It was also reported that elevation of HMGB1 in serum correlates with severity of acute intracerebral hemorrhage.

However, the evidence of HMGB1 localization in brains of patients with CVD is very limited. Therefore, we investigated at autopsy the immunolocalization of HMGB1 in brains of patients with CVD (acute and chronic cerebral infarction, acute cerebral hemorrhage, subarachnoid hemorrhage). In 3 out of 10 acute cerebral infarction cases, the cytoplasm of neurons located around the ischemic core (i.e., penumbra) was positive for HMGB1. In the chronic stage of cerebral infarction, macrophages located in some ischemic regions were positive for HMGB1. Around the hematoma in the basal ganglia, HMGB1-like immunoreactivity (IR) was intense in macrophages. However, around the subarachnoid hematoma, HMGB1-like IR was not seen in the cortex. In arteries surrounded by subarachnoid hematoma, HMGB1-like IR was located in the cytoplasm of vascular smooth muscle cells. These findings, which partially differ from animal model results, may provide translational research and a basis for understanding the role of HMGB1 in brains of patients with CVD.

Key words: HMGB1, Cerebral infarction, Cerebral hemorrhage, Subarachnoid hemorrhage, Penumbra

Introduction

High mobility group box 1 protein (HMGB1) (Mosevitsky et al., 1989; Bustin, 1999; Yamada and Maruyama, 2007) is a non-histone chromosomal protein that is highly conserved, ubiquitous, and widely distributed, mainly in the nuclei of all cells in mammalian tissues. HMGB1 essentially has multiple functions located in the nucleus, including the maintenance of nucleosome structure, the regulation of gene transcription, and involvement in DNA recombination. As currently recognized, HMGB1 has a wide range of potential functions and pathological relevance. In some pathological conditions, HMGB1 was translocated to the cytoplasm from the nuclei. Furthermore, HMGB1 is released into the extracellular space from necrotic cells and from activated macrophages. HMGB1 binds to the receptors for advanced glycation end products (Stern et al., 2002) and the toll-like receptor (Park et al., 2004), resulting in the induction of inflammatory cytokines.
Increasing attention is being paid to HMGB1 and the number of publications on HMGB1 is steadily increasing (Kim et al., 2006; Sumiyoshi et al., 2015). For example, in an animal model, treatment with neutralizing anti-HMGB1 monoclonal antibody ameliorated brain infarction (Liu et al., 2007; Zhang et al., 2011) and HMGB1 was increased in the brain after acute subarachnoid hemorrhage (SAH) (Murakami et al., 2011). It was also reported that an elevation of HMGB1 in serum correlates with severity of acute intracerebral hemorrhage (Zhou et al., 2010). A clinical trial “The Role of HMGB-1 in Chronic Stroke” by a service of the U.S. National Institutes of Health has been implemented to measure the presence of HMGB1 in the blood after stroke (https://clinicaltrials.gov/show/NCT01705353). Most recently, startling findings (Haruma et al., 2016) were reported that HMGB1 antibody attenuates delayed cerebral vasospasm after SAH in rats, and that the administration of anti-HMGB1 antibody restored most of the intranuclear localization of HMGB1 in vascular smooth muscle cells (VSMCs) of the basilar artery in rats after SAH.

However, the evidence of HMGB1 localization in the brain with CVD is only from animal models. Rigorous immunohistopathological demonstration of HMGB1 expression in human brains with CVD is very limited, although HMGB1 localization in main cerebral arteries with atherosclerotic lesions was already reported by us (Umahara et al., 2014). We are concerned that the lack of studies using human brain tissue might misguide the HMGB1 therapy for CVD.

We investigated the immunolocalization of HMGB1 at autopsy in brains of patients with CVD using a specific antibody, and confirmed the detailed localization and specific cell types. It is imperative in translational research to confirm the immunolocalization of HMGB1 in brains of patients with CVD. This examination must facilitate translation from the scientific discoveries from animal stroke model research into the development of new strategies for prevention and treatment of human stroke.

Materials and methods

Specimens of brains from patients with CVD were obtained at autopsy (49–93 years old at the time of death). There were 10 cases of acute and subacute cerebral infarction (case durations were 1, 2, 3, 4, 4, 9, 11, 17, 23, and 29 days). Six out of these 10 cases were acute cardioembolic stroke; 4 cases were hemorrhagic infarction; 3 cases were acute atherothrombotic brain infarction; 1 case was artery-to-artery embolism. There were 6 cases of chronic stage cerebral infarction (all greater than 2 months). There were 4 cases of acute cerebral hemorrhage (3–28 days). There were 3 cases of acute subarachnoid hemorrhage (2, 4, and 4 days). There were 3 control cases (68, 72, and 79 years old); the causes of death were myocardial infarction, rectal cancer, and pneumonia. Tissue sections (5 µm thick) were prepared from formalin-fixed, paraffin-embedded blocks. We performed hematoxylin-eosin (HE) staining.

Deparaffinized sections were placed in citrate buffer and heated in a pressure cooker for 20 minutes. They were treated with 1% hydrogen peroxide for 30 minutes. They were then incubated with the primary antibody produced in rabbits against human HMGB1 (1:2000, KPDAAKKGVVKAEK; Shino-test, Kanagawa, Japan) at 4°C for 2 days. The sections were then incubated with the appropriate biotinylated secondary antibody for 2 hours. After incubation with the avidin-biotin-peroxidase complex (1:1000, ABC Elite; Vector, Burlingame, CA, USA) for 1 hour, peroxidase labeling was visualized with a mixture of 0.03% 3,3-diaminobenzidine, 0.6% nickel ammonium sulfate, 0.05 M imidazole and 0.00015% hydrogen peroxide. A brown (3,3-diaminobenzidine only) or a deep purple (a mixture of 3,3-diaminobenzidine and nickel ammonium sulfate) immunoreaction product appeared after 15-20 minutes.

For immunofluorolabeling, after treatment with 0.5% normal goat serum, we incubated deparaffinized sections with a mixture of the anti-HMGB1 antibody (1:200), and anti-human CD68 (1:100, mouse monoclonal antibody clone KP1; Dako), for 48 hours. These antibodies were then visualized with a mixture of anti-mouse IgG antibody made in goat serum conjugated with Alexa 488 (1:100; Molecular Probes, Eugene, OR, USA), and anti-rabbit IgG made in goat serum conjugated with Alexa 546 (1:100; Molecular Probes) for 2 hours. They were then incubated with 4',6-diamidino-2-phenylindole (DAPI). The fluorolabeled sections were observed under a fluorescence microscope equipped with a laser confocal system (Leica SP5; Leica).
Microsystems GmbH, Heidelberg, Germany).

Results

Normal controls

In all normal control subjects, a small number of HMGB1-positive nuclei of glial cells were observed in cerebral tissues; however, a small number of HMGB1-positive nuclei or cytoplasmic regions of neurons were seen (data not shown, please see normal area of Fig. 1).

Acute and subacute cerebral infarction (Fig. 1)

In 3 out of 10 cases, the cytoplasm of neurons located around an ischemic core (i.e., penumbra) was

Fig. 2. Chronic stage of cerebral infarction. a. Low magnification of chronic cerebral infarction; hematoxylin-eosin (HE) staining. Chronic cerebral infarction regions are indicated by “*” and “#” in the figure. b. High magnification of chronic cerebral infarction region (“*” in Fig. 2a); HE staining. Many macrophages are seen. c. High magnification of another chronic cerebral infarction region (“#” in Fig. 2a); HE staining. Many macrophages are seen. This finding (c) has almost the same appearance as finding (b). d. Immunohistochemical labeling of HMGB1: chronic cerebral infarction region “*”. HMGB1-positive macrophages are not seen. e. Immunohistochemical labeling of HMGB1: chronic cerebral infarction region “#”. HMGB1-positive macrophages are seen. Scale bars: a, 1 mm; b, c, 100 μm; d, e, 50 μm.
positive for HMGB1, although nuclei of neurons located around the ischemic core were negative or faintly positive for HMGB1. Of these 3 cases, 2 cases were atherothrombotic brain infarction and 1 case was artery-to-artery embolism.

Both cytoplasm and nuclei of neurons located in the ischemic core and the area far from the ischemic core (normal area) were negative for HMGB1. The duration of these 3 cytoplasm-HMGB1-positive cases was 4, 11, and 29 days. These 3 cases did not include hemorrhagic infarction. In the other 7 cases, HMGB1-positive neurons were not seen.

Chronic stage of cerebral infarction (Fig. 2a)

Neurons were negative for HMGB1. Macrophages located in some ischemic regions (Fig. 2b) were positive for HMGB1 (Fig. 2d). However, in the same section, macrophages located in other ischemic regions (Fig. 2c) were negative for HMGB1 (Fig. 2e). In all cases, to varying degrees, HMGB1-positive macrophages were seen in chronic ischemic regions. Reactive astrocytes around the ischemic regions, in which HMGB1-positive macrophages were seen, were negative for HMGB1 (Fig. 3).

Fig. 3. Chronic stage of cerebral infarction. a. Many reactive astrocytes around the ischemic regions are seen (arrows); HE staining. b. Reactive astrocytes around the ischemic regions are negative for HMGB1, although HMGB1-positive macrophages are seen in these regions. Scale bars: 100 μm.

Fig. 4. Acute cerebral hemorrhage. a. Around a hematoma in the basal ganglia, HMGB1-like immunoreactivity (IR) is intense (arrow). b. High magnification of arrow point region indicated in Fig. 4a. Scale bars: a, 200 μm; b, 20 μm.
Acute intracerebral hemorrhage (ICH)

Around hematomas in all cases, HMGB1-like immunoreactivity (IR) was intense in localized cells (Fig. 4). In double immunofluorolabeling, some of these cells were positive for both HMGB1 and CD68 (Fig. 5).

Acute subarachnoid hemorrhage

In the cortex around subarachnoid hematomas in all cases, HMGB1-like IR was not seen (Fig. 6) except for a small number of HMGB1-positive nuclei of glial cells; this finding is similar to normal control cases.

In arteries (Fig. 7a, arrows) surrounded by subarachnoid hematomas, HMGB1-like IR was located in the cytoplasm of VSMCs (Fig. 7b, arrows) in all SAH cases. On the other hand, in arteries separated by subarachnoid hematomas, HMGB1-like IR was located in only a small number of nuclei in VSMCs (Fig. 7d, arrows) in all SAH cases, although HMGB1-like IR was seen in arteries with atherosclerotic lesions, independent of location of the subarachnoid hematomas (data not shown).

Discussion

Overall, our results of HMGB1 localization patterns in the brain of patients with CVD were partly similar to those of animal models. However, under some conditions we failed to obtain the results from animal
models. The differential findings of HMGB1 localization between our results in human brains and those of animal models are important in understanding the role for future therapy of human patients with CVD.

**Acute and subacute cerebral infarction**

In an animal model of ischemic brain, HMGB1 translocation to the cytoplasm from nuclei in neurons located in the penumbra occurred consistently, and two hours after the onset of ischemia, HMGB1 was stained both in the cytoplasm and nuclei, suggesting the translocation of HMGB1 in ischemic rat brain (Zhang et al., 2011). This animal model result has indicated that ischemic stress may lead to such HMGB1 translocation and that HMGB1 is a key mediator of the immune mechanism in ischemic stroke.

In 3 out of 10 cases, cytoplasm of neurons located around an ischemic core (penumbra) was positive for HMGB1 and we failed to detect HMGB1 immunoreactivity in ischemic neurons at the earliest phase (case durations of 1, 2, and 3 days). This result

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**Fig. 7.** Cerebral arteries in and out of the subarachnoid hematoma. HMGB1-like IR is not seen. Cerebral artery in the subarachnoid hematoma. a. HE staining. b. HMGB1. Weak HMGB1-like IR is seen. In arteries surrounded by subarachnoid hematoma, HMGB1-like IR is located in the cytoplasm of VSMCs in all cases (c). On the other hand, in arteries separated by subarachnoid hematoma, HMGB1-like IR is located in a small number of nuclei in VSMCs in all SAH cases (d). Scale bar: 50 μm.
partly agrees with the results from animal models. However, this result may reflect the difference in the role of HMGB1 expression between human and animal models. In our results, only 3 cases showed HMGB1-positive cytoplasm in neurons. In all 10 cases, neuronal nuclei were commonly negative for HMGB1. We speculated that, in the human brain, HMGB1 was expressed in neuronal cytoplasm within a limited time from ischemia onset and with a limited degree of ischemic stress.

The only thing we can say is that if treatment with neutralizing anti-HMGB1 monoclonal antibody is attempted as a new treatment for human cerebral infarction, the time of administration and degree of ischemic stress should be considered; this is because in cerebral infarction cases with unexpressed HMGB1, HMGB1 neutralization is thought to be ineffective. However, in limited autopsy samples it is difficult to determine the target time and degree of ischemic stress, and to consider the differences in time axis between humans and other animals.

### Chronic stage of cerebral infarction (Fig. 2a)

The pathogenesis explaining the differences in ischemic regions between those containing HMGB1-positive macrophages and HMGB1-negative macrophages in our results is unclear. In HE staining, the appearance of those regions is almost the same in the same section. Although we demonstrated the fact of HMGB1 localization, we cannot present the reason why both HMGB1-positive and HMGB1-negative macrophages were seen in the chronic stage of cerebral infarction. Unfortunately, there are limitations to using human autopsy sections.

We considered two hypotheses explaining the existence of HMGB1-positive macrophages in chronic ischemic regions. One is that HMGB1-associated inflammation partially continues to the chronic stage from acute infarction. Another is that HMGB1-positive macrophages might serve a role in autophagy in chronic ischemic lesions because HMGB1 might sustain autophagy (Kang et al., 2010). Our results might contribute to explaining the results of the clinical trial “The Role of HMGB-1 in Chronic Stroke” by a service of the U.S. National Institutes of Health (https://clinicaltrials.gov/show/NCT01705353).

It was reported in the animal model that reactive astrocytes in ischemia are positive for HMGB1 (Hayakawa et al., 2012, 2013). HMGB1 can play a surprisingly beneficial role during stroke recovery by promoting endothelial progenitor cell function and vascular remodeling in cortical gray (Hayakawa et al., 2012) and white (Hayakawa et al., 2013) matter in animals. We failed to demonstrate the localization of HMGB1 in human reactive astrocytes around chronic ischemic lesions. Of course our results cannot disavow the hypothesis that HMGB1 is expressed at a limited time after ischemic stress in human astrocytes because our finding is only for reactive astrocytes at the chronic stage. We might only suggest that further examination of the HMGB1 expression pattern in astrocytes in the human brain is needed.

### Acute ICH

Around hematomas in all cases, HMGB1-like IR was intense in localized cells (Fig. 4). In double immunofluorolabeling, some of these cells were positive for both HMGB1 and CD68 (Fig. 5). Our results may support the report (Zhou et al., 2010) that elevation of HMGB1 in serum correlates with severity of acute intracerebral hemorrhage. Furthermore it was reported that Anti-HMGB1 antibody inhibits hemorrhage-induced brain injury and improved neurological deficits in rats (Wang et al., 2017). HMGB1 might be a new target for prevention and treatment, not only for cerebral infarction, but also for ICH.

### Acute subarachnoid hemorrhage

From the results of the animal model (Murakami et al., 2011) revealing that HMGB1 was located or expressed in microglia, astrocytes, and neurons around SAH, HMGB1 is expected to be a treatment target for SAH. Therefore, the result that we failed to demonstrate HMGB1 localization in the human brain with SAH was unexpected for us, although a small number of HMGB1-positive nuclei of glial cells were seen (this finding is commonly observed in unaffected human brain).

A recent study using an animal model (Sun et al., 2014) reported that the peak of HMGB1 expression is day 1. Although we could not obtain any human section with day 1 SAH, in day 2 and day 4 human SAH we failed to demonstrate HMGB1-positive cytoplasm of any cells in the cortex. Of course this time lag cannot be ignored and is one of the limitations of examination using human material. The difference between the animal model cortical findings and human cortical findings with SAH is unexplainable for us.

On the other hand, HMGB1 localization in human cerebral arteries with SAH is similar to its localization in basilar arteries in rats after SAH (Haruma et al., 2016).

We already reported (Umahara et al., 2014) that in human basilar arteries in normal control cases, a small number of nuclei in VSMCs were positive for HMGB1. In arteries separated by subarachnoid hematoma in all our SAH cases, HMGB1-like IR was similar to this finding and to a normal rat artery finding (Haruma et al., 2016). We also already reported (Umahara et al., 2014) that in human main cerebral arteries with atherosclerotic lesions, HMGB1-like IR was positive for cytoplasm of macrophages and infiltrated VSMCs. In arteries separated by subarachnoid hematomas in all our SAH cases, coincidental atherosclerotic lesions were positive for HMGB1.

In arteries surrounded by subarachnoid hematomas in our cases, HMGB1-like IR was located in the...
cytoplasm of VSMCs. This cytoplasmic localization of HMGB1 in VSMCs might be the result of transfer from nuclei to cytoplasm, as it was demonstrated in rat basilar arteries (Haruma et al., 2016). Our finding may support the potential of HMGB1 antibody therapy to delay cerebral vasospasm after SAH in humans as in rats.

**Conclusions**

We demonstrated time-dependent and lesion-dependent HMGB1-selective localization in human brains with CVD.

In 3 out of 10 acute cerebral infarction cases, the cytoplasm of neurons located around the ischemic core (i.e., penumbra) was positive for HMGB1. In the chronic stage of cerebral infarction, macrophages located in some ischemic regions were positive for HMGB1. Around the hematomas in the basal ganglia, HMGB1-like IR was intense in macrophages. However, around the subarachnoid hematomas, HMGB1-like IR was not seen in the cortex. In arteries surrounded by subarachnoid hematomas, HMGB1-like IR was located in the cytoplasm of vascular smooth muscle cells.

These findings, which partially differ from animal model results, may provide a basis for understanding the role of HMGB1 in the brains of patients with CVD and contribute to further drug development for treating CVD.

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