学位論文

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Original Article

Immunoreactivities for hepcidin, ferroportin, and hephaestin in astrocytes and choroid plexus epithelium of human brains

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Iron plays essential roles in the central nervous system. However, how the iron level is regulated in brain cells including glia and neurons remains to be fully clarified. In this study, the localizations of hepcidin, ferroportin, and hephaestin, which are known to be involved in iron efflux, were immunohistochemically examined in autopsied human brains. Immunoreactivities for hepcidin and ferroportin were observed in granular structures within the cytoplasm of reactive astrocytes and epithelial cells of the choroid plexus. Granular structures showing immunoreactivities for hepcidin and ferroportin were also stained with antibodies for early endosome antigen 1 (EEA1). In addition, immunoreactivity for hephaestin was observed in the cytoplasm of epithelial cells of the choroid plexus as well as reactive astrocytes. Immunoreactivity for hephaestin in the cytoplasm of reactive astrocytes was occasionally colocalized with immunoreactivity for EEA1, while that of hephaestin was frequently observed in the cytoplasm showing no immunoreactivity for EEA1. These findings suggest that immunoreactivities for hepcidin and ferroportin are localized in close proximity to granular structures showing immunoreactivity for EEA1 in the cytoplasm of human brain astrocytes. They also suggest that immunoreactivity of hephaestin is localized in the cytoplasm of the choroid plexus epithelium as well as reactive astrocytes of human brains.

Key words: astrocyte, choroid plexus, ferroportin, hepcidin, hephaestin.

INTRODUCTION

Iron homeostasis is essential for brain functioning. It is considered that iron moves from the systemic circulation into the central nervous system and is taken up into several kinds of cells in the brain.[1,2] Iron plays important roles in several kinds of brain functions, such as the biosynthesis of neurotransmitters, myelin formation, and energy metabolism. On the other hand, excess intracellular iron is considered to increase the oxidative load on cells. Actually, brain iron accumulation due to a lack of ceruloplasmin ferroxidase activity caused by aceruloplasminemia induces progressive neurodegeneration.[3] Neuronal iron accumulation has also been observed in the cortex of Alzheimer's disease patients,[4,5] the substantia nigra in Parkinson's disease patients,[6–9] and in various brain regions of patients with tauopathies.[9,10] Increasing evidence highlights the contribution of key proteins associated with neuronal degeneration to iron accumulation. The loss of soluble tau contributes to toxic neuronal iron accumulation in patients with Alzheimer' disease, Parkinson's disease, and tauopathies.[11] Amyloid-β (Aβ) precursor protein (APP) stabilizes the cell surface ferrous iron exporter ferroportin, and loss of tau causes iron retention by decreasing the surface trafficking of APP.[11,12] In addition, excess iron accumulation and deposition in astrocytes could result in oxidative damage to cells and induce glial degeneration, followed by neuronal degeneration.[13] These results suggest that abnormalities of iron metabolism and transport are associated with the pathogenesis of common neuronal degenerative disorders.

There is increasing evidence indicating that astrocytes are key regulators of iron metabolism in the brain.[14] Intracellular iron in astrocytes is considered to be stored in ferritin or exported from astrocytes via the only known iron export protein, ferroportin.[15] Hepcidin is known to induce the internalization of ferroportin into endosomes for

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degradation in lysosomes.[16–18] In this way, the ferroportin-hepcidin system likely represents the main pathway for cellular iron egress and also regulates cellular iron levels in astrocytes. Hephaestin is known to be a large membrane-anchored multicopper ferroxidase involved in iron metabolism.[19] Dietary iron is exported across the basolateral membrane of enterocytes by the ferrous iron transporter ferroportin, and hephaestin increases the efficiency of this process by oxidizing the transported iron to its ferric form and promoting its release from ferroportin. Hephaestin was also reported to be expressed in astrocytes of human brains.[20] In addition, the expression of several iron-related proteins including hepcidin, ferroportin, and hephaestin has been extensively investigated in rats using primary astrocytes as well as endothelial cells.[21]

Several key proteins for iron efflux have also been reported to be expressed in the choroid plexus of rodents. Experimental findings using an *in situ* hybridization technique show that several iron-related proteins, such as ferroportin, hephaestin, and the ferritin heavy chain as well as the transferrin receptor, are also expressed in the choroid plexus of the mouse brain,[22] indicating that the choroid plexus may have a much greater role than previously considered in brain iron transport. Other experimental findings indicate the expression of messenger RNA (mRNA) and protein of hepcidin in the normal rat brain and mRNA expression of hepcidin in the choroid plexus.[23] However, it is unclear whether immunoreactivity of hepcidin exists in the choroid plexus of human brains. It was reported that immunoreactivity of ferroportin was present in the choroid plexus[24–26] as well as astrocytes[27] of human brains, while that of hephaestin was seen in astrocytes, but not in the choroid plexus, of human brains.[20] From the above findings, it remains unclear whether these iron-related proteins are expressed in both astrocytes and epithelial cells of the choroid plexus in human brains, and where these proteins are localized within the cells.

Accordingly, in this study, we immunohistochemically examined the localizations of hepcidin, ferroportin, and hephaestin, which are involved in iron efflux, in astrocytes and epithelial cells of the choroid plexus using autopsied human brains.

MATERIALS AND METHODS

Human brain samples were obtained at autopsy from five patients with or without neurological abnormalities in Kagawa University Hospital, as previously reported.[28] Brief clinical information and the postmortem delay time of materials resected at autopsy in five patients are shown in Table 1. The main diagnosis of each patient was established according to clinical and autopsy findings.[28] This study using human brains was approved by the institutional ethics committee of the Faculty of Medicine, Kagawa University. The brains were fixed in 10% buffered formalin and processed for immunohistochemical examination. The brain samples were embedded in paraffin and sectioned at a thickness of 4 μm. The primary rabbit antibodies used in this study were antibodies against hepcidin (ab30760; Abcam, Cambridge, UK),[27] ferroportin (SLC40A1) (ab78066; Abcam),[29] hephaestin (NBP1-85483; Novus Biologicals, Centennial, CO, USA), ferritin heavy chain (ab75972, Abcam),[30] and early endosome antigen 1 (EEA1) (NBP1-30914); Novus),[31] while the primary mouse antibodies were those against ferroportin (ab239583; Abcam), glial fibrillary acidic protein (GFAP) (ab10062; Abcam),[28] and EEA1 (NBP2-36568; Novus). Information on the antibodies is summarized in Table 2. Before incubation with the antibodies for hepcidin, ferroportin, and hephaestin, antigen retrieval was performed by heating sections in 10 mmol/L sodium citrate buffer (pH 6) at 95°C for 20 min. Before incubation with antibodies for ferritin, antigen retrieval was performed by heating sections in 1 mmol/L Tris-ethylenediaminetetraacetic acid (EDTA) (pH 9.0) at 95°C for 20 min. After treatment with hydrogen peroxide and blocking with 2% bovine serum albumin in phosphate-buffered saline (PBS) for 30 min, the sections were incubated with primary antibodies at room temperature (RT) for 90 min. Staining was achieved with a Simple Stain kit (Nichirei, Tokyo, Japan) and developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB) and

Table 1 Clinical information and the postmortem delay time of materials resected at autopsy in five patients

Case	Age/sex	Main diagnosis	Postmortem delay (h)
1	30–40/F	Brainstem hemorrhage	2.5
2	70–80/M	Cerebellar tuberculosis	1
3	60–70/F	Thalamic hemorrhage	5
4	70–80/F	Pneumonia	4
5	70–80/M	Unstable angina	3

Table 2 Summary of antibodies used in this study

Antibody	Source	Host species and dilution
Hepcidin	Abcam, ab30760 [27]	Rabbit, 1:200†
Ferroportin	Abcam, ab78066 [29]	Rabbit, 1:25†
Ferroportin	Abcam, ab239583	Mouse, 1:50
Hephaestin	Novus, NBP1-85483	Rabbit, 1:50†
Ferritin HC	Abcam, ab75972 [30]	Rabbit, 1:100‡
GFAP	Abcam, ab10062 [28]	Mouse, 1:200†
EEA1	Novus, NBP2-36568	Mouse, 1:100‡
EEA1	Novus, NBP1-30914 [31]	Rabbit, 1:100

†Antigen retrieval with citrate buffer (pH 6.0) is needed prior to the application of the primary antibody. ‡Antigen retrieval with Tris-EDTA buffer (pH 9.0) is needed prior to the application of the primary antibody.

hydrogen peroxide (Nichirei) at RT for 5–7 min. The sections were counterstained with hematoxylin.

For double immunofluorescence staining of iron-associated proteins and a marker protein for astrocytes or endosomes, the sections were incubated at RT for 90 min with the rabbit antibody against hepcidin, ferroportin, or hephaestin and the mouse antibody against GFAP (ab10062) or EEA1 (NBP2-36568), followed by incubation at RT for 60 min in Alexa Fluor-conjugated secondary antibodies (1:200, Molecular Probes, Eugene, OR, USA). Before incubation with primary antibodies, antigen retrieval was performed by heating sections in 10 mmol/L sodium citrate buffer (pH 6.0) or in 1 mmol/L Tris-EDTA (pH 9.0) at 95°C for 20 min. In addition, double immunofluorescence staining using the mouse antibody against ferroportin (ab239583) and the rabbit antibody against hepcidin (ab30760) was performed according to the procedures described above, while staining used for the mouse antibody against ferroportin (ab239583) and the rabbit antibody against EEA1 (NBP1-30914) was performed according to modified procedures without antigen retrieval. The sections were then incubated for 60 min at RT in monomeric cyanine nucleic acid stain (TO-PRO-3, Molecular Probes), which was diluted to 2.5 μmol/L in PBS. The fluorescent signals were viewed under a confocal microscope (Carl Zeiss LSM700, Oberkochen, Germany). As a control experiment, we performed an identical immunohistochemical procedure with omission of the primary antibodies.

RESULTS

Immunohistochemical examination using an antibody for hepcidin showed the immunoreactivity of granular structures in the cytoplasm of glial cells and epithelial cells of the choroid plexus in all human brains examined (Fig. 1A, B). Immunohistochemical examination using an antibody against ferroportin showed immunoreactivity in granular structures within the cytoplasm of some glial cells and a few epithelial cells of the choroid plexus (Fig. 1C, D). Immunohistochemical examination using an antibody against hephaestin showed immunoreactivity in the cytoplasm, occasionally in granular structures, of glial cells and cerebral vessels, and in the cytoplasm of epithelial cells in the choroid plexus (Fig. 1E, F). Immunohistochemical examination using an antibody against the ferritin heavy chain also showed strong immunoreactivity throughout hypertrophic cell bodies and processes of glial cells and a few epithelial cells of the choroid plexus (Fig. 1G, H). These glial cells shown in Figure 1A, C, E, and G were considered to be reactive astrocytes as they had hypertrophic cell bodies and processes. In addition, there were differences in the proportion of immunopositive epithelial cells of the choroid plexus among five brains examined in this study (Fig. 1B, D, F, H).

Double immunofluorescence examination showed that immunoreactivities for hepcidin (Fig. 2A–C), ferroportin (Fig. 2D–F), and hephaestin (Fig. 2G–I) were locally or entirely observed in GFAP-positive astrocytes. Granular immunoreactivity for hepcidin was adjacent to that of GFAP-positive fibrillary structures or partially colocalized (Fig. 2A–C). Granular immunoreactivity for ferroportin was mostly colocalized with that of GFAP (Fig. 2D–F). Immunoreactivity for hephaestin was mostly colocalized with that for GFAP (Fig. 2G–I). Immunoreactivities for hepcidin, ferroportin, and hephaestin were commonly observed in GFAP-positive glial cells with hypertrophic cell bodies and processes, indicative of reactive astrocytes.

Double immunofluorescence examination using the mouse antibodies against EEA1, an early endosome marker, and the rabbit antibodies against hepcidin, ferroportin, or hephaestin was performed. Immunoreactivity for EEA1 was partially colocalized with or closely adjacent to that for hepcidin (Fig. 3A–C). Immunoreactivity for EEA1 was partially colocalized with or closely adjacent to that for ferroportin (Fig. 3D–F). Immunoreactivity for hephaestin was occasionally colocalized with that for EEA1, while that for hephaestin was frequently observed in the cytoplasm showing no EEA1 immunoreactivity (Fig. 3G–I).

Double immunofluorescence examination using the mouse antibody against ferroportin and the rabbit antibody against hepcidin or EEA1 was also performed. Immunoreactivity for ferroportin was mostly colocalized with that of hepcidin (Fig. 4A–C) and partially colocalized with that for EEA1 (Fig. 4D–F).

DISCUSSION

In this study, some antibodies against iron-related proteins (shown in Table 2), which have been employed in previous immunohistochemical experiments, were used. The findings presented here were mostly consistent with previously reported ones, supporting the validity of the present results. In addition to the already-known findings, immunoreactivities for hepcidin and ferroportin was observed, at least partially, in granular structures of reactive astrocytes, showing EEA1 immunoreactivity, in human brains. Accordingly, it was suggested that hepcidin-bound ferroportin may be internalized in some endosomal structures of human brain astrocytes. The data reported by Hamasaki et al. suggest that EEA1 functioned in macropinosome fusion in epidermal growth factor-stimulated A431 cells.[32] Based on the data, it is likely that the iron transport regulated by hepcidin and ferroportin may be

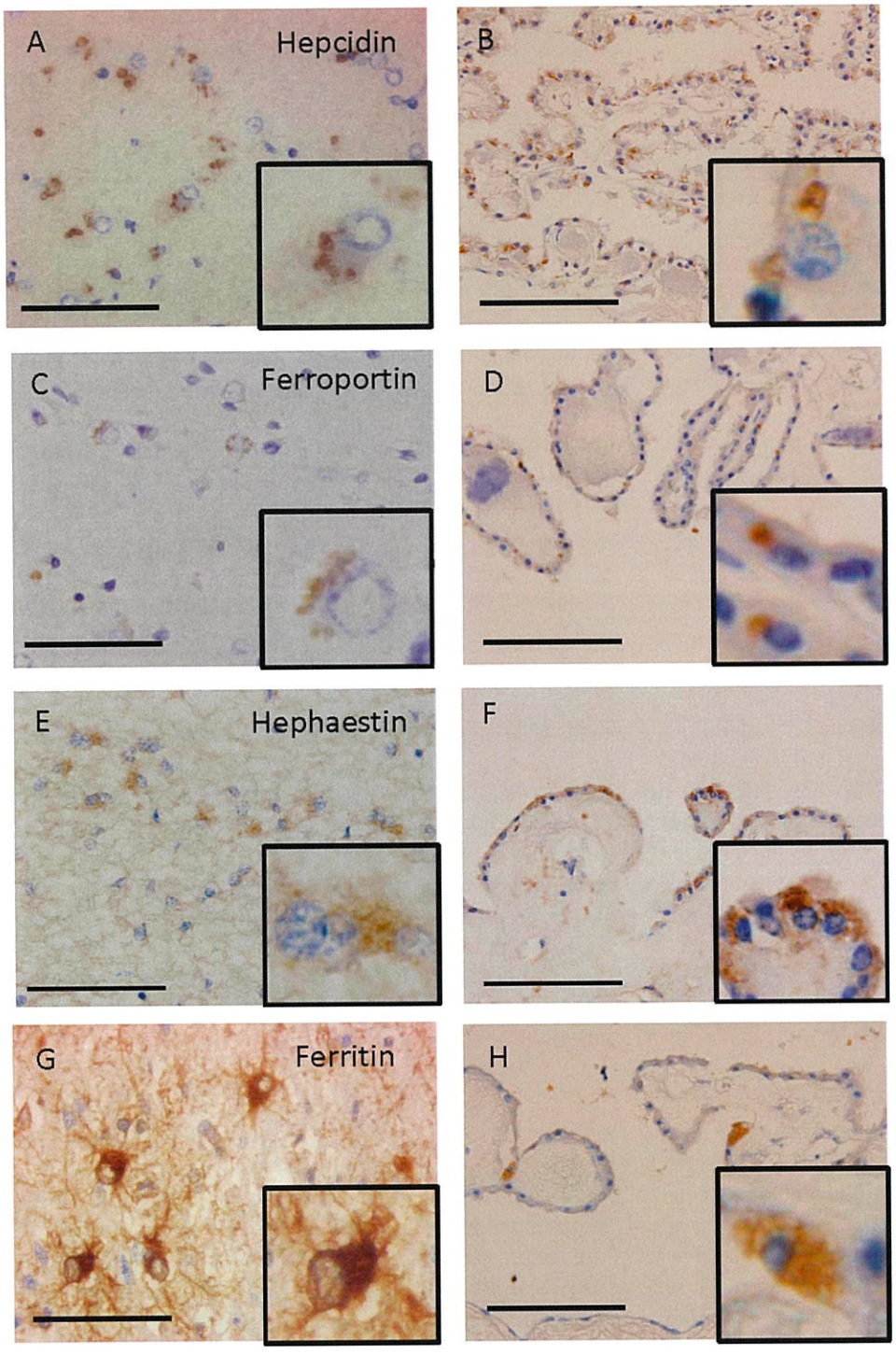

Fig. 1 Representative microphotographs of immunohistochemical staining using antibodies against hepcidin (A, B), ferroportin (C, D), hephaestin (E, F), and ferritin (G, H) in brain parenchyma (A, C, E, G) and the choroid plexus (B, D, F, H). Immunoreactivities for these proteins are observed in the cytoplasm of glial cells (A, C, E, G) of human brains. The inset of each image shows an enlarged image (A, C, E, G). Immunoreactivities for hepcidin (A) and ferroportin (C) are observed in granular structures within the cytoplasm, while immunoreactivity for hephaestin (E) is observed in the cytoplasm, occasionally in granular structures, of glial cells. Immunoreactivity for ferritin (G) is seen throughout the cytoplasm with fine processes of glial cells. Immunoreactivities for hepcidin (B) and ferroportin (D) are observed in granular structures in the cytoplasm of epithelial cells of the choroid plexus, while immunoreactivity for hephaestin (F) is observed in the supranuclear area in the cytoplasm of epithelial cells. Immunoreactivity for ferritin (H) is observed in the cytoplasm of a few epithelial cells. The inset of each image shows enlarged images (B, D, F, H). Scale bars: 100 μm (A-H).

associated with the macropinocytic pathway. In addition, immunoreactivity for hepcidin was confirmed to be immunohistochemically expressed in epithelial cells of the choroid plexus of human brains. In this study, we also observed the immunoreactivity for ferritin in astrocytes with hypertrophic cell bodies and processes, as previously reported.[33]

Hephaestin has been reported to be expressed in astrocytes of white matter in the human brains of multiple sclerosis patients and controls using an anti-hephaestin antibody (sc-49969; Santa Cruz Biotechnology, Santa Cruz, CA, USA).[20] We confirmed that immunoreactivity for hephaestin was observed in the cytoplasm of human brain astrocytes. Hephaestin, also known as a ferroxidase,

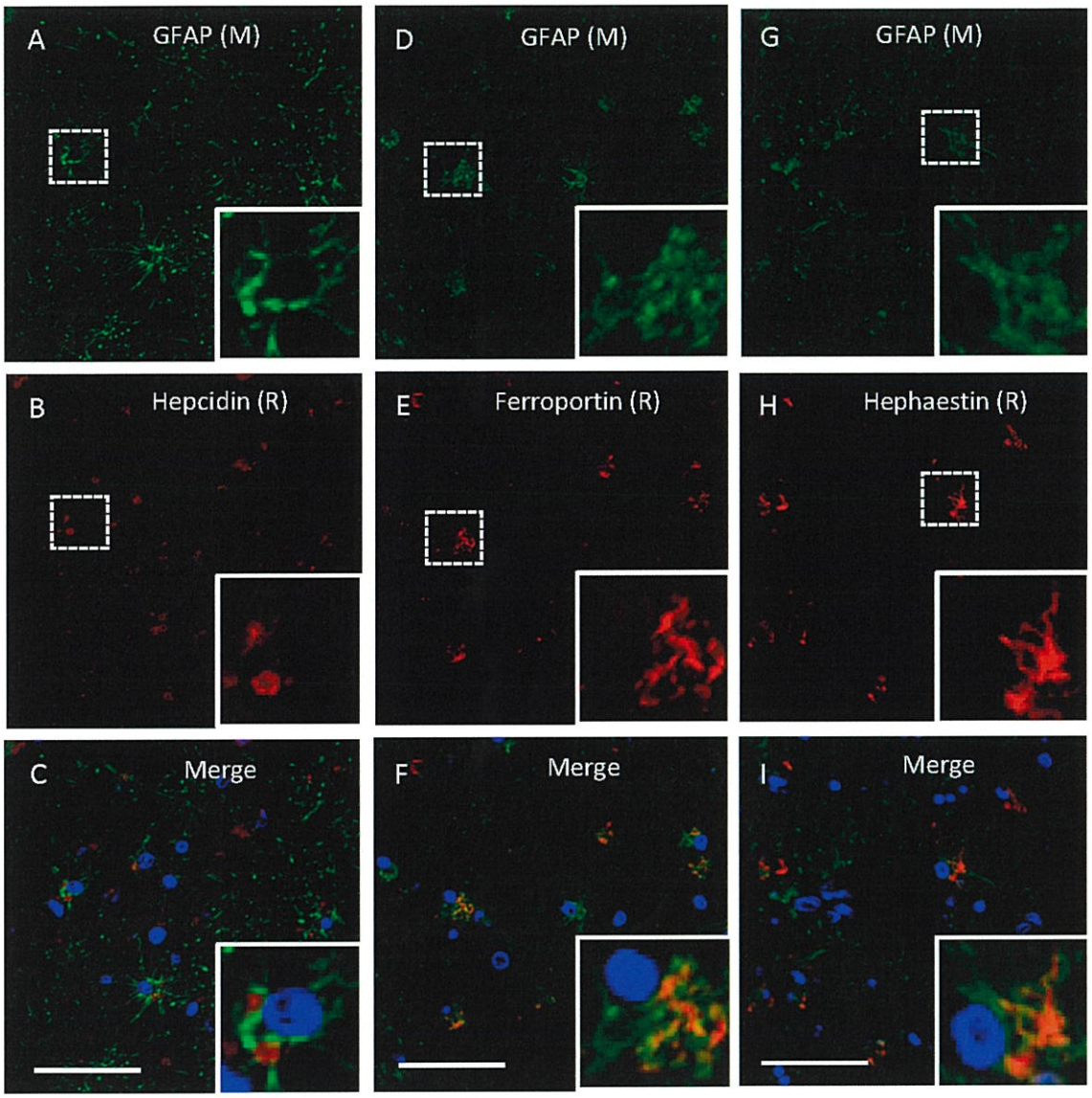

Fig. 2 Representative confocal microscopic images. Glial cells immunostained with the mouse (M) anti-GFAP antibody (visualized as green in A, D, or G) and the rabbit (R) anti-hepcidin, ferroportin, or hephaestin antibody (visualized as red in B, E, or H, respectively), nuclear staining with TOPRO-3 stain (visualized as blue), and merged images (C, F, or I, respectively) are shown. The inset of each image (A–I) shows enlarged images (indicated by a square in A, B, D, E, G, or H). Scale bars: 50 μm (A-I).

was reported to be expressed in a secreted form.[19] Burkhart et al. reported that immunoreactivity for hephaestin was localized at both the cellular membrane and cytoplasm using primary astrocytes.[21] In addition, immunoreactive hephaestin and ferroxidase activity were present in the cytosolic fraction of rat enterocytes.[34] Accordingly, it is plausible that immunoreactivity of hephaestin is localized in the cytoplasm of astrocytes. In addition, we newly found that immunoreactivity for hephaestin was also observed in the cytoplasm of epithelial cells of the choroid plexus in human brains. Kuo et al. reported that hephaestin is primarily located in a supranuclear compartment of both intestinal enterocytes and cultured cells.[35] The results are consistent with our findings showing localization of hephaestin immunoreactivity in supranuclear areas of the cytoplasm in epithelial cells of the choroid plexus. However, the use of paraffin-embedded sections taken from autopsied human brains makes it difficult to identify the exact location of immunoreactive substances.

From this study's findings, immunohistochemical localizations of hepcidin, ferroportin, and hephaestin were immunohistochemically confirmed in the cytoplasm of reactive astrocytes and epithelial cells of the choroid plexus in human brains. In addition, it was suggested that immunoreactivity for ferroportin was located in association with endosomal structures, possibly macropinosomes,

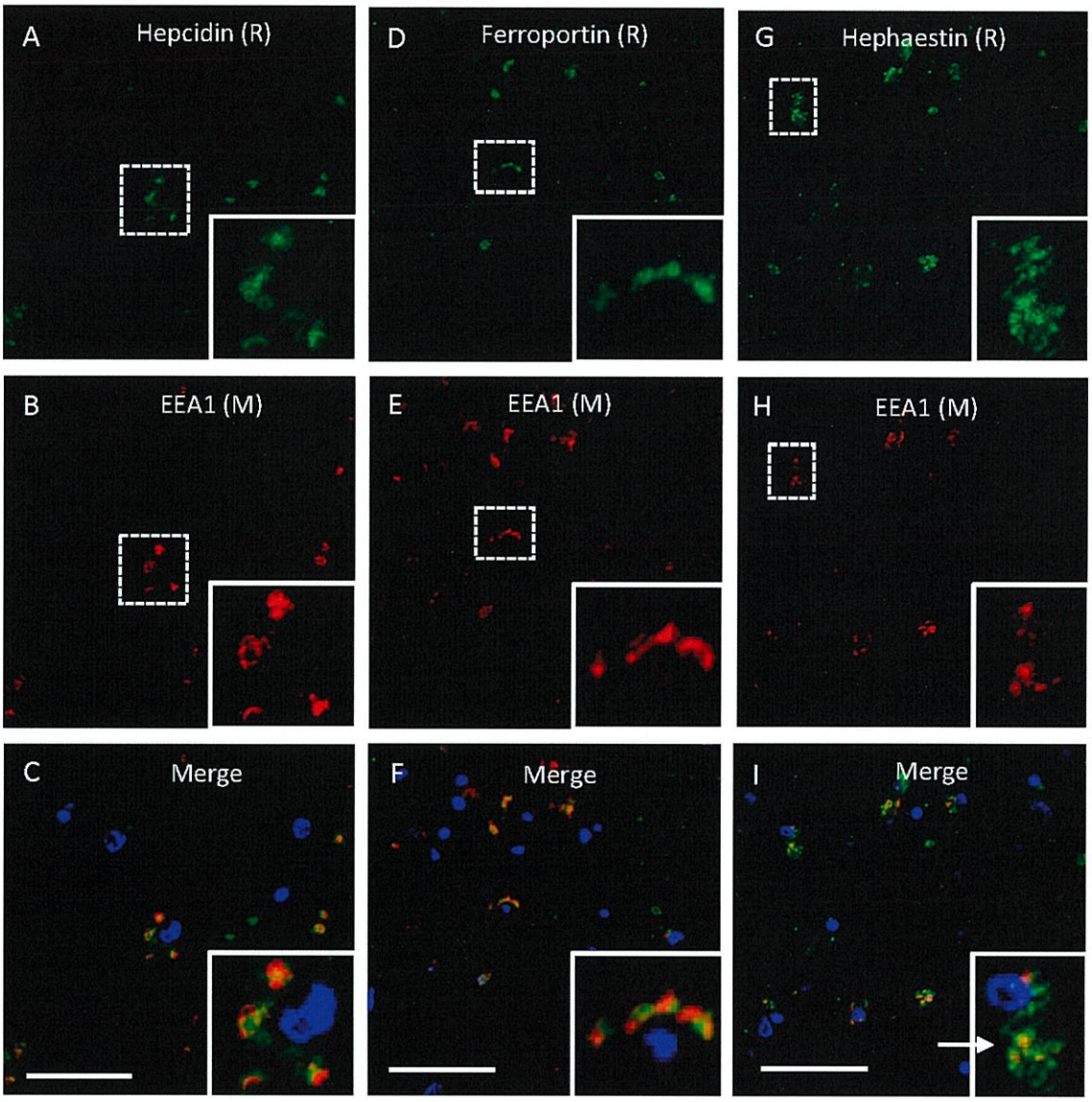

Fig. 3 Representative confocal microscopic images. Immunohistochemical observations with the rabbit (R) anti-hepcidin, ferroportin, or hephaestin antibody (visualized as green in A, D, or G, respectively) and the mouse (M) anti-early endosome antigen 1 (anti-EEA1) antibody (visualized as red in B, E, or H), nuclear staining with TOPRO-3 stain (visualized as blue), and merged images (C, F, or I, respectively) are shown. The inset of each image (A–I) shows enlarged images (indicated by a square in A, B, D, E, G, or H). An arrow in image (I) shows double immunoreactivity for hephaestin and EEA1, while other green signals seen in (G) show only immunoreactivity for hephaestin. Scale bars: 50 μm (A-I).

of reactive astrocytes. Recent experimental findings on the clearance of intracerebral substances suggest that proteolytic degradation of soluble amyloid-beta (Aβ) in macropinosomes of astrocytic cells is a significant mechanism underlying Aβ clearance.[36,37] Accordingly, it is likely that these proteins may also regulate intracellular iron levels in astrocytes. However, in this study, we only observed immunohistochemical findings. Further studies are needed to confirm whether these proteins are functioning in these cells.

In this study, some cells of the choroid plexus epithelium were stained with these antibodies, while others were not. Previous studies[28] using antibodies for transporters on clearance of Aβ also showed differences in the proportion of immunoreactive cells among brains. It remains to be clarified why there were differences in the proportion of immunoreactive epithelial cells of the choroid plexus among brains. In this study, the proportion of cells immunoreactive for iron-related proteins tended to be relatively high in two brains with ischemic vascular changes from cases 2 and 5 as shown in Table 1. It may depend on the presence or absence of neurological abnormality including ischemic changes or heterogeneity of epithelial cells in the choroid plexus.[38,39] Further studies using autopsied human

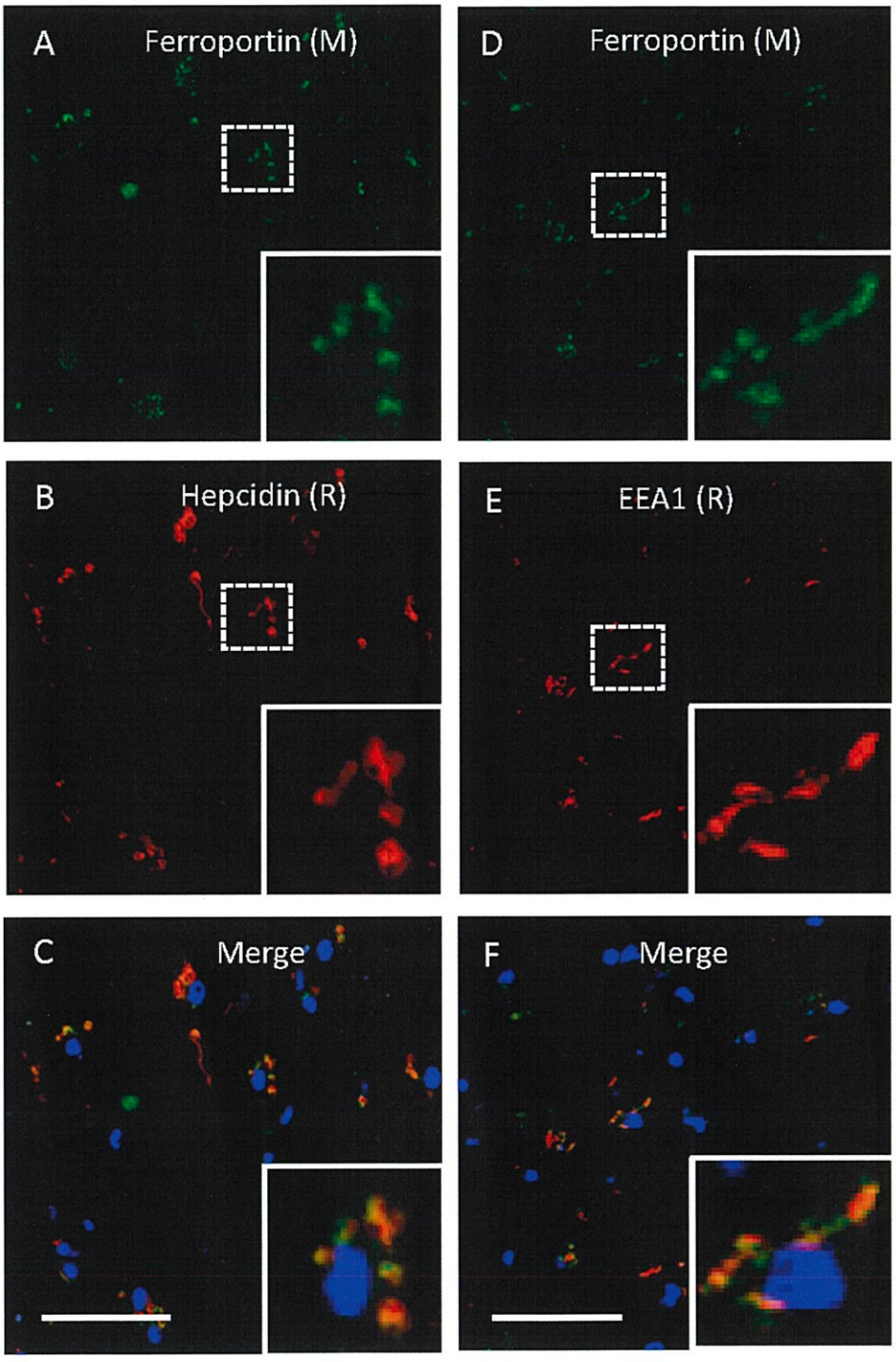

Fig. 4 Representative confocal microscopic images. Immunohistochemical observations with the mouse (M) anti-ferroportin antibody (visualized as green in A or D) and the rabbit (R) anti-hepcidin or anti-EEA1 antibody (visualized as red in B or E, respectively), nuclear staining with TOPRO-3 stain (visualized as blue), and merged images (C or F, respectively) are shown. The inset of each image (A–F) shows enlarged images (indicates by a square in A, B, D, or E). Scale bars: 50 μm (A-I).

brains of patients with diseases such as dementia and cerebral infarction are also needed.

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KY and NU performed immunostaining and analyzed stained cells, YC, RM, KM, and MU conducted pathological dissection, RM, RF, and NA created figures, KY and NA created microphotographs with a confocal microscope and examined the relationship between immunopositive substances and endosomes, GS and MU interpreted the data, and KM, RF, and GS revised it critically for content. All authors gave approval for the final version to be published and agreed to be accountable for all aspects of the work.

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DISCLOSURE

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