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From Cell Biology to Tissue Engineering

Frontotemporal dementia-associated protein "phosphorylated TDP-43" localizes to atherosclerotic lesions of human carotid and main cerebral arteries

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Summary. The transactivation response DNA binding protein (TARDP) of 43 kDa (TDP-43) is a nuclear protein pivotal in RNA processing. Because phosphorylated (p) TDP-43 has been identified as a component of ubiquitin-positive and tau-negative inclusions in frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS), it is considered to play a major role in neurodegenerative processes. We investigated the immunolocalization of pTDP-43 in atherosclerotic lesions of human carotid and main cerebral arteries. Furthermore, we investigated the co-localization between pTDP-43 and 14-3-3 eta isoform or high mobility group box 1 (HMGB1).

pTDP-43 localized in the cytoplasm of many foamy macrophages located in the periphery of lipid-rich necrotic cores, and in the cytoplasm of infiltrated smooth muscle cell-like cells. pTDP-43 co-localized the 14-3-3 eta isoform in carotid plaques. pTDP-43 also co-localized HMGB1. This is the first demonstration of pTDP-43 immunolocalization in human carotid and main cerebral artery plaques. We believe that demonstration of the localization of pTDP-43 in atherosclerotic lesions is important as this may contribute to the establishment of the clinical diagnostic imaging of FTLD and ALS using the pTDP-43 epitope. Moreover, this finding may be useful for further understanding the role of TDP in cell death.

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Introduction

The TARDBP gene which encodes the transactivation response DNA binding protein of 43 kDa (TDP-43) is a nuclear protein possibly involved in RNA processing. TDP-43 has been identified as a component of ubiquitin-positive and tau-negative inclusions in frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS) (Arai et al., 2006; Neumann et al., 2006). This protein is usually located in the nucleus; however, in some pathological conditions, TDP-43 is fragmented, phosphorylated, and translocated in the cytoplasm (Arai et al., 2006; Neumann et al., 2006; Hasegawa et al., 2008). Phosphorylated (p) TDP-43-positive inclusions are a pathological hallmark of the majority of FTLD and ALS patients.

During the examination of a brain with dementia, we coincidentally found that pTDP-43 localizes to the atherosclerotic lesion of the human cerebral artery. Because close association between neurodegenerative dementia and vascular disturbance is attracting increasingly attention, it may be worth investingating immunolocalization of pTDP-43 located in these atherosclerotic lesions blood vesselsin the brain.

In the present study, we investigated the localization of pTDP-43 in atherosclerotic lesions of the human carotid and cerebral arteries.

Furthermore, we clarified the co-localization of pTDP-43, the 14-3-3 eta isoform, and high mobility group box 1 (HMGB1). 14-3-3 proteins are able to trap phosphorylated proteins and control the localization of some molecules (Aitken, 2006). We previously demonstrated the co-localization of pTDP-43 and the 14-3-3 eta isoform in human anterior horn cells of ALS patients (Umahara et al., 2016). HMGB1 is known to induce inflammatory cytokines (Yamada and Maruyama, 2007), and we previously demonstrated the localization of HMGB1 in human carotid plaques (Umahara et al., 2014).

Materials and methods

Specimens of carotid arteries were obtained from 18 patients (age range, 56-82 years, 11 men and 7 women) undergoing carotid endarterectomy (CEA). Specimens of the main cerebral arteries with atherosclerotic changes were obtained from 8 human patients at autopsy (49-93 years old at the time of death).

The 18 patients in whom CEA was performed did not include patients with FTLD or other types of dementia. Three out of the 18 patients had mild cognitive impairment. None of the 8 brains that were autopsied were positive for pTDP-43. Causes of death of the autopsied patients were pneumonia (4), ischemic heart disease (3), and abdominal trauma (1).

Tissue sections (5 μm thick) were prepared from formalin-fixed, paraffin-embedded blocks. We performed hematoxylin-eosin (HE), elastica-van Gieson (EVG), and Masson trichrome (MT) staining.

In this study, carotid plaques were classified into 3 types (Umahara et al., 2011, 2012) according to the American Heart Association (AHA) classification (Stary et al., 1995). AHA type IV indicates a thin fibrous cap plaque with a large lipid-rich necrotic core (carotid artery, n=4; cerebral artery, n=0). AHA type V indicates a thickened fibrous cap plaque with a lipid-rich necrotic core (carotid artery, n=6; cerebral artery, n=3). AHA type Vc indicates a thickened fibrous cap plaque without a lipid-rich necrotic core (carotid artery, n=8; cerebral artery, n=5).

Deparaffinized tissue sections placed in citrate buffer were heated in a pressure cooker for 20 min. Thereafter, the tissue sections were treated with 1% hydrogen peroxide for 30 min. They were then incubated with anti-phosphorylated TDP43 antibody (1:5000 mouse monoclonal, pS409/410, Cosmo Bio, Tokyo, Japan) at 4°C for 2 days. The tissue 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 min.

For immunofluorolabeling, after treatment with 0.5% normal goat and horse sera, we incubated deparaffinized tissue sections in a mixture of anti-phospho TDP-43 antibody (1:500), and either anti-CD68 antibody (rabbit polyclonal, 1:30, ab125047 abcam), anti-α-smooth muscle actin (SMA) l antibody (rabbit polyclonal ,1:30, ab5694 abcam), anti-14-3-3 eta isoform antibody (1:20, rabbit polyclonal, MGDREQLLQR, Immuno-Biological Laboratories, Gunma, Japan), or anti-HMGB1 protein antibody (1:200, rabbit polyclonal, KPDAAKKGVVKAEK; Shino-test, Kanagawa, Japan). These antibodies were then visualized with a mixture of anti-mouse IgG antibody made in goat serum conjugated with Alexa 488 (1:200; Molecular Probes, Eugene, OR, USA), and anti-rabbit IgG made in goat serum conjugated with Alexa 546 (1:200; Molecular Probes) for 2 hours. The fluorolabeled tissue sections were observed under a fluorescence microscope equipped with a laser confocal system (Leica SP5; Leica Microsystems GmbH, Heidelberg, Germany).

Results

Conventional immunohistochemistry

Carotid plaques

In the carotid artery lesions of the CEA specimens, pTDP-34-like immunoreactivity (IR) was observed in many areas of the cytoplasm of foam cells located in the periphery of lipid-rich necrotic cores (AHA types IV and V, Fig. 1B,C). These areas in the cytoplasm were diffusely immunopositive for pTDP-34. In 5 out of the 8 patients with AHA type Vc, pTDP-43-like IR was not observed, although a few foam cells were observed in the type Vc plaques. Cytoplasmic inclusions were not found.

pTDP-34-like IR was also observed in the cytoplasm of morphologically infiltrated smooth muscle cell-like spindle-shaped cells within the thickened fibrous area under the internal elastic lamina (mainly AHA types V and Vc; Fig. 1B,D). In 2 out of the 4 patients with AHA type IV, pTDP-43-like IR was not observed. Cytoplasmic inclusions were not found.

In some cases, pTDP-34-positive cells were only localized within a limited area near the eroded surface (Fig. 2C,D). In one carotid plaque with intramural hemorrhage (Fig. 3A), pTDP-34-positive cells were closely localized to the intramural hemorrhage (Fig. 3 B, C). pTDP-34-positive erythrocytes were not found.

Atherosclerotic lesions of main cerebral arteries

pTDP-34-like IR was observed in the cytoplasm of foam cells around the necrotic core (Fig. 4, square B) and in the cytoplasm of infiltrating cells (Fig. 4, square C) within the thickened intima similarly to the carotid

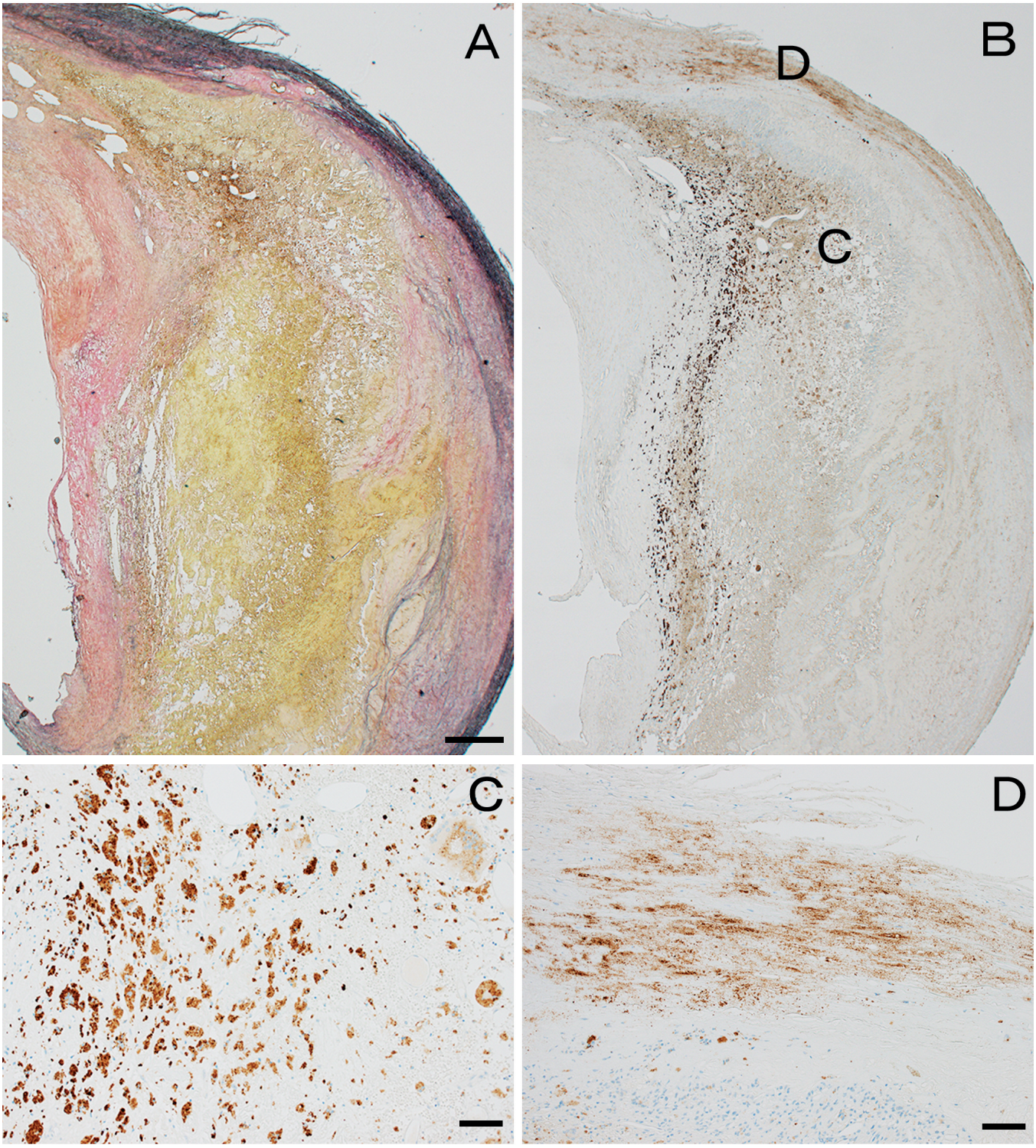

Fig. 1. A. Elastica-van Gieson (EVG) staining of carotid artery lesion specimen obtained by carotid endarterectomy (CEA). **B.** Immunohistochemical labeling of phosphorylated (p) TDP-43 in carotid artery lesion specimen obtained by CEA. pTDP-43-like IR in the CEA specimens was diffusely observed around the lipid core. **C.** pTDP-43-like IR in CEA specimens was observed in the cytoplasm of many foam cells and multinucleated giant cells. Fig. C represents the square marked as C in Fig. B. **D.** pTDP-43-like IR in CEA specimens was observed in spindle-shaped cells. Fig. D represents the square marked as D in Fig. B. Scale bars: A, 500 μm; C, D, 100 μm.

plaques. In 3 out of the 8 patients, pTDP-43-like IR was not observed. Cytoplasmic inclusions were not found.

Immunofluorolabeling

CD68-positive cells in carotid plaques

Many foam cells and multinucleated giant cells were positive for both pTDP-34 and CD68 (Fig. 5).

α-SMA–positive cells in carotid plaques

Many spindle-shaped cells were positive for both pTDP-34 and α-SMA (Fig. 6).

Co-localization between pTDP-34 and 14-3-3 eta isoform in carotid plaques

Many foam cells were positive for both pTDP-34 and 14-3-3 eta isoform (Fig. 7).

Co-localization between pTDP-34 and HMGB1 in carotid plaques

Many foam cells were positive for both pTDP-34 and HMGB1 (Fig. 8).

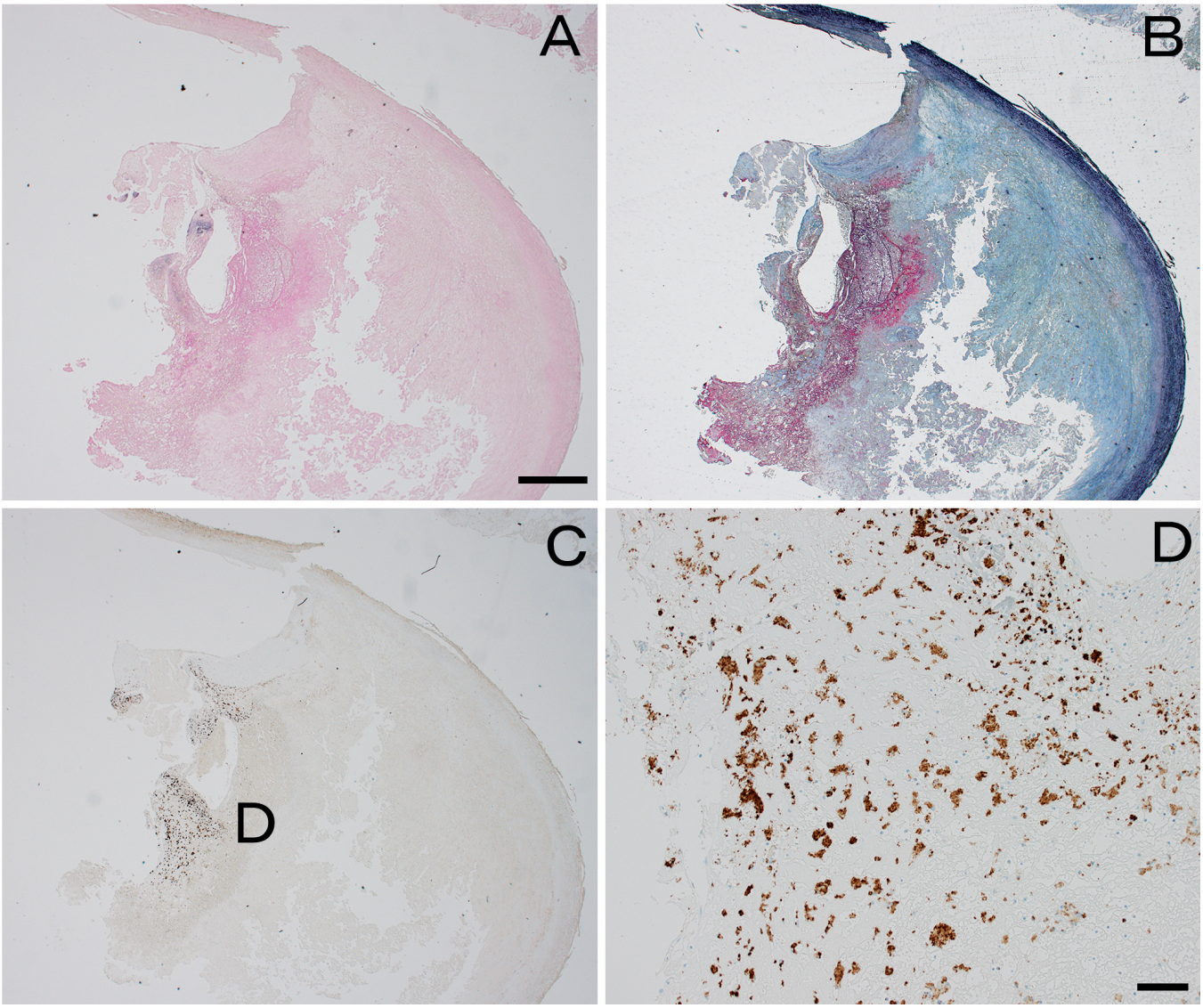

Fig. 2. A. Hematoxylin-Eosin (HE) staining of carotid artery lesion specimen obtained by CEA. **B.** Elastica-van Gieson (EVG) staining in carotid artery lesion specimen obtained by CEA. **C.** Immunohistochemical labeling of pTDP-34 in carotid artery lesion specimen obtained by CEA. pTDP-43-like IR was located in the eroded surface lesion. **D.** pTDP-43-like IR in CEA specimens was observed in the cytoplasm of many foam cells. D represents the square in Fig. C. Scale bars: A, 400 μm; D, 100 μm.

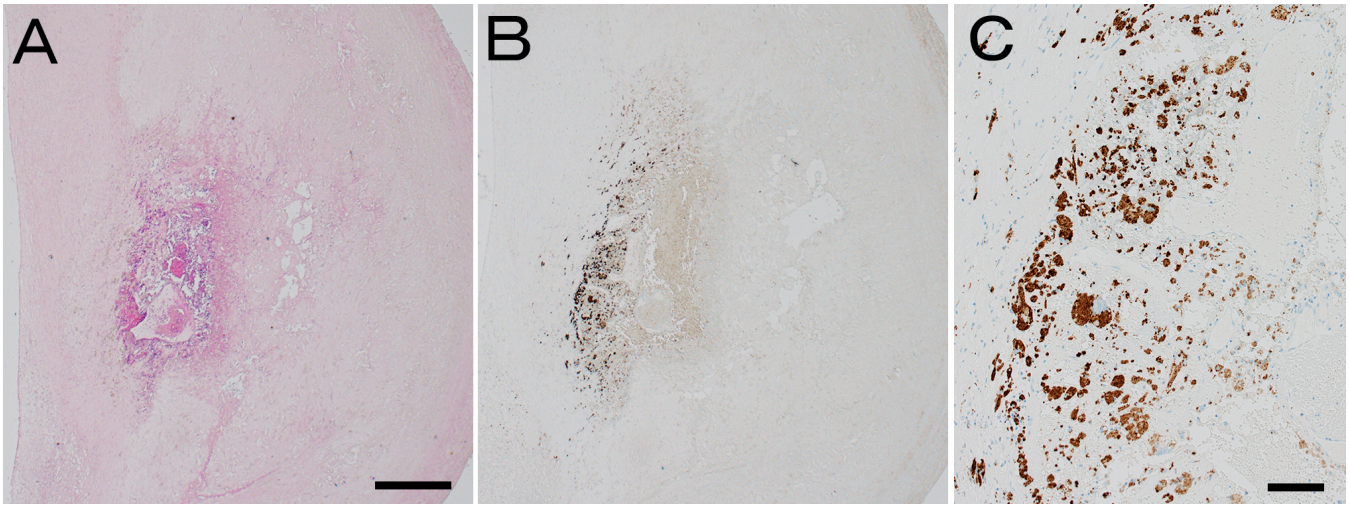

Fig. 3. A. Hematoxylin-Eosin (HE) staining of carotid artery lesion specimen obtained by CEA. Intramural hemorrhage is present. **B, C.** Immunohistochemical labeling of pTDP-43 in carotid artery lesion specimen obtained by CEA. pTDP-43-like IR in CEA specimens was observed in the cytoplasm of many foam cells around the intramural hemorrhage. Scale bars: A, B, 400 µm; C, 100 µm.

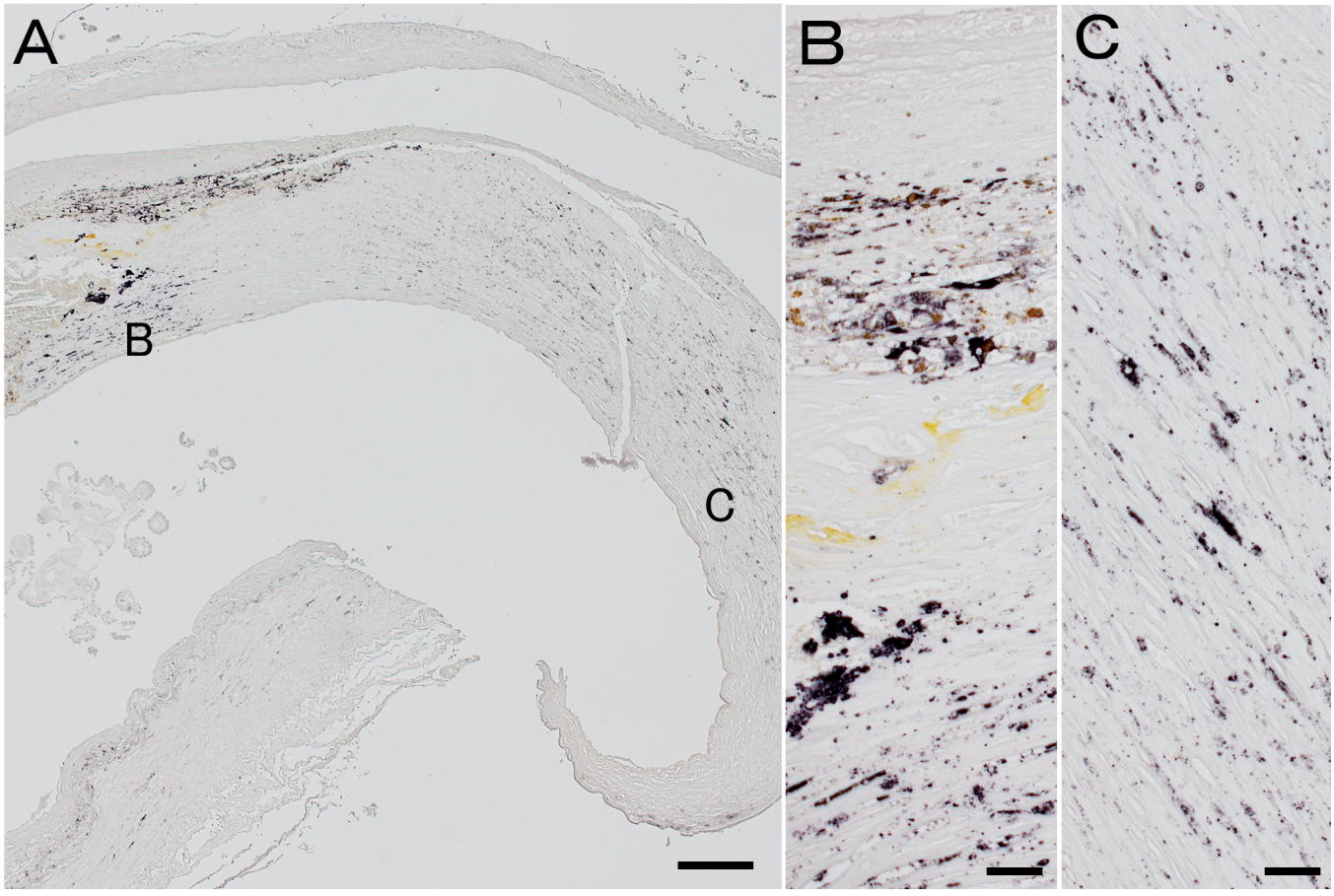

Fig. 4. Atherosclerotic lesion of the basilar artery. **A.** pTDP-34-like IR was observed around the necrotic core (square B) and in the infiltrating cells in the thickened intima (square C). **B, C.** Enlarged views of squares B and C in Fig. A. Scale bars: A, 400 µm; B, C, 100 µm.

Discussion

We believe that the demonstration of pTDP-43 localization to atherosclerotic lesions of the human carotid and main cerebral arteries is a very important finding. To the best of our knowledge, this is the first demonstration of the immunolocalization of pTDP-43 in human carotid and main cerebral artery plaques.

In atherosclerotic lesions, 2 types of cells, namely, macrophages, which are positive for CD68, and vascular smooth muscle cells (VSMCs), which are positive for α-SMA (Askari et al., 2002; Schwartz et al., 2000; Umahara et al. 2012), infiltrate the neo-intima and play important roles in atherogenesis. During atherogenesis, macrophages take up lipids (Umahara et al., 2011) and are then transformed into foam cells, whereas differentiated VSMCs in the contractile phase undergo phenotypic changes to an immature form in the synthetic phase. Both cell types (i.e., macrophages and VSMCs) were positive for pTDP-34 in the present study.

The pTDP-43 accumulation pattern in macrophages or infiltrated VSMCs of atherosclerotic plaques is different in neurons in FTLD or ALS. pTDP-43-positive cytoplasmic or intranuclear inclusion, which is observed in neurons in FTLD or ALS (Arai et al., 2006; Neumann et al., 2006), was never found in the macrophages or infiltrated VSMCs of the atherosclerotic plaques.

We believe that one possible role of TDP43 in atherosclerotic plaques is the activation of macrophages, as it has been reported that TDP43 activates microglia (Brettschneider et al., 2012). Similarly, pTDP-43 activates macrophages in atherosclerotic plaques.

pTDP-43-positive cells were closely localized to the intramural hemorrhage in the carotid plaque. As TDP43

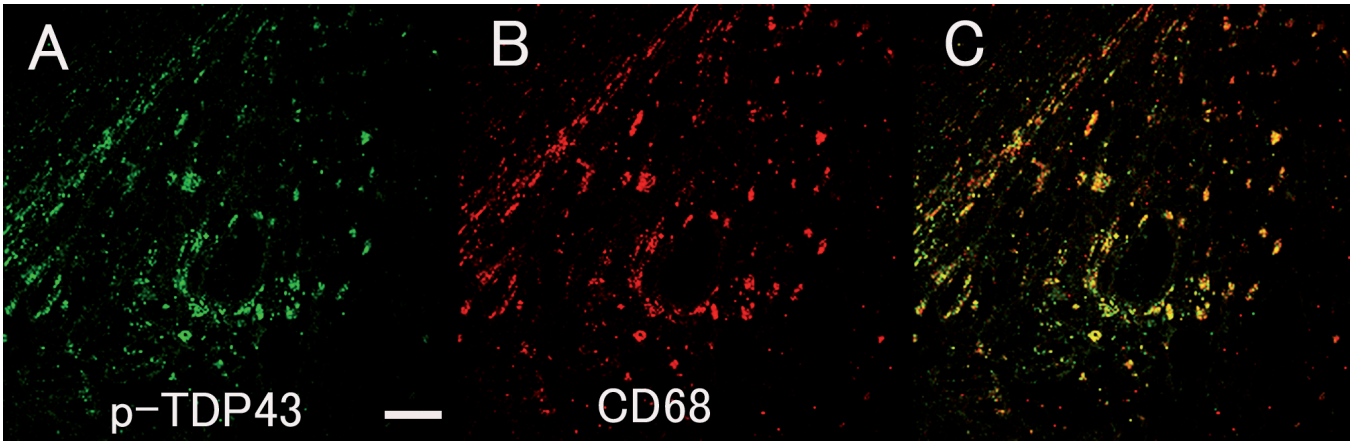

Fig. 5. Double immunofluorolabeling of pTDP-43 (**A**: green areas) and CD68 (**B**: red areas) in the carotid plaque. A merged image of pTDP-43 and CD68 is shown on image **C**. Scale bars: 100 μm.

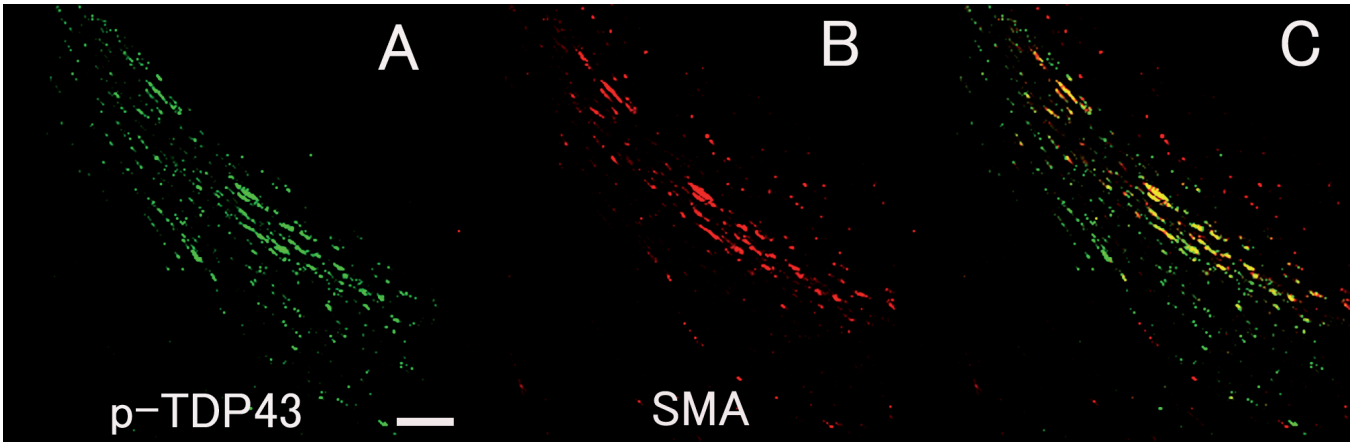

Fig. 6. Double immunofluorolabeling of pTDP-43 (**A**: green areas) and α-smooth muscle actin (α-SMA) (**B**: red areas) in a carotid plaque. A merged image of pTDP-43 and α-SMA is shown on image **C**. Scale bars: 500 μm.

has been reported to be associated with autophagy (Budini et al., 2017), these pTDP-43-positive cells might be associated with autophagy of a blood element.

Recently it was reported that pTDP-43 aggregates in the axial skeletal muscle of patients with sporadic and familial amyotrophic lateral sclerosis (Cykowski et al., 2018). Our result that VSMCs are positive for pTDP-43 might be some relevance to the results of this report.

The importance of pTDP-43 localization in atherosclerotic lesions of the human carotid and main cerebral arteries can also be considered from another viewpoint. pTDP-43 located in the carotid and cerebral arteries might affect future investigations in terms of pTDP-43 biomarker examination and pTDP-43 PET imaging for diagnosing FTD or ALS. The concentration of pTDP-43 in the blood is affected by the localization of pTDP-43 in the arterial wall. Furthermore, the concentration of pTDP-43 in the cerebrospinal fluid may be affected by the localization of pTDP-43 in the cerebral arterial wall. For the accurate detection of pTDP-43 accumulation in the cerebral parenchyma on PET, it may be necessary to eliminate the image of pTDP-43 accumulation in the cerebral arteries. Therefore, when considering the use of pTDP-43 for the diagnosis of central nervous system diseases, it is important to recognize the possibility that localization of pTDP-43 in arteriosclerotic lesions affects its accuracy.

Recently, it was reported that elimination of TDP-43 inclusions by using monoclonal antibody (Tamaki et al., 2018). Our result may contribute to grasping attention on future antibody therapy to TDP-43 related diseases.

14-3-3 proteins can trap and hold phosphorylated proteins and control the localization of some molecules (Aitken, 2006). An example of this is Forkhead box

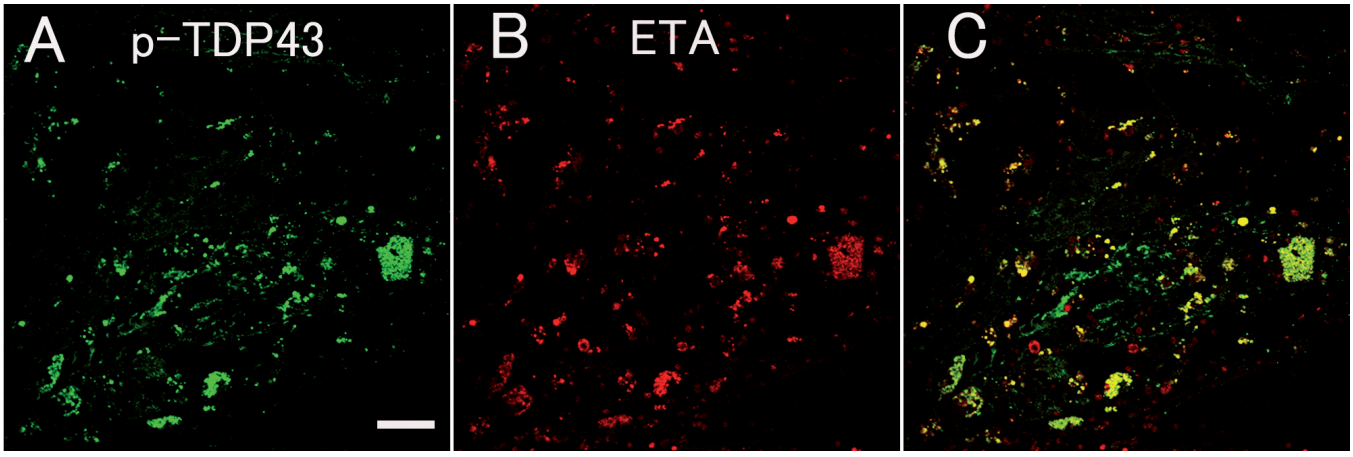

Fig. 7. Double immunofluorolabeling of pTDP-43 (**A**: green areas) and the 14-3-3 eta isoform (**B**: red areas) in the carotid plaque. A merged image of pTDP-43 and the eta isoform is shown on image **C**. Scale bars: 100 µm.

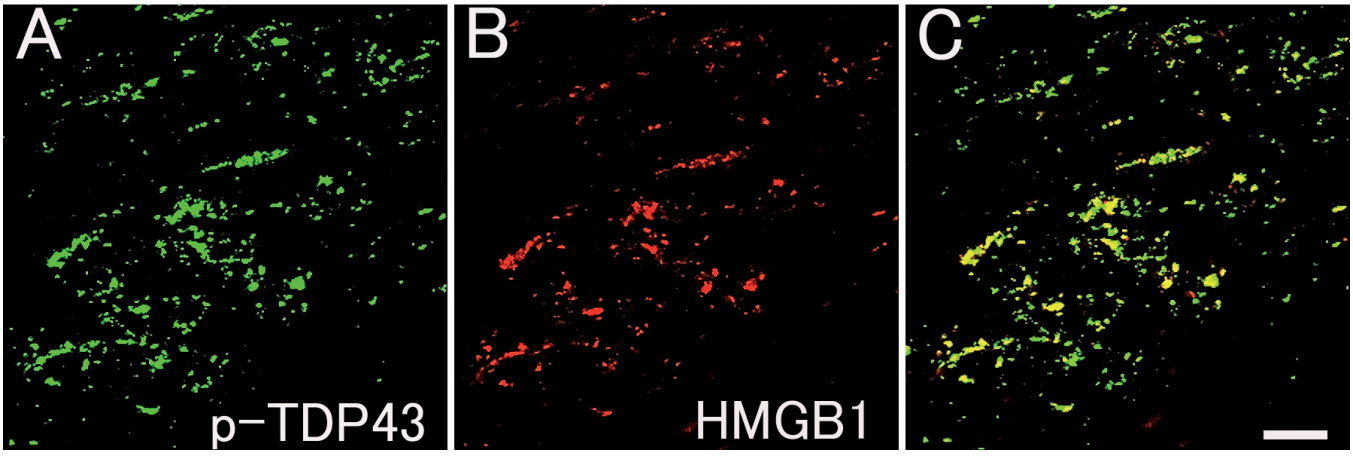

Fig. 8. Double immunofluorolabeling of pTDP-43 (**A**: green areas) and HMGB1 (**B**: red areas) in the carotid plaque. A merged image of pTDP-43 and HMGB1 is shown on image **C**. Scale bars: 100 µm.

protein O (FOXO), which is a regulatory factor of apoptosis. It was reported that TDP-43 competes against FOXO in binding to the 14-3-3 protein, and releases FOXO for nuclear translocation and activation (Zhang et al., 2014). We already demonstrated the co-localization between pTDP-43 and eta isoform in the granular structures of human anterior horn cells in ALS (Umahara et al., 2016). However, a pTDP-43-positive "inclusion" is not positive for the eta isoform. Moreover, we have already demonstrated that 14-3-3 proteins were located in human carotid plaques.

In this study, we demonstrated the co-localization between pTDP-43 and the eta isoform in foam cells in human carotid plaques. Because pTDP-43 and the eta isoform are co-localized in 2 completely different conditions, a universal association of pTDP-43 and the eta isoform was suggested. These findings may provide a basis for understanding the roles associated with TDP-43 and the eta isoform.

HMGB1 (Mosevitsky et al., 1989; Yamada and Maruyama, 2007) is a non-histone chromosomal protein found mainly in the nuclei of all cells in mammalian tissues. Essentially, HMGB1 located in the nucleus has multiple functions, including maintenance of the nucleosome structure, regulation of gene transcription, and involvement in DNA recombination. In some pathological conditions, HMGB1 translocates from the nucleus to the cytoplasm. Furthermore, HMGB1 is released into the extracellular space from necrotic cells and activated macrophages. HMGB1 binds to the receptors of advanced glycation end products (Stern et al., 2002) and the toll-like receptor (TLR) (Park et al., 2004), resulting in the induction of inflammatory cytokines. HMGB1 acts as a strong chemotactic agent for VSMCs (Degryse et al., 2001), triggering their migration from the tunica media to the tunica intima. We also demonstrated HMGB1 localization in human carotid plaques (Umahara et al., 2014).

In this study, we demonstrated the co-localization between pTDP-43 and HMGB1 in spindle and foam cells in human carotid plaques. To the best of our knowledge, this is the first report on pTDP-43 and HMGB1 co-localization.

It has been reported that lipopolysaccharide (LPS)-induced inflammation can promote TDP43 mislocalization and aggregation (Correia et al., 2015). LPS-induced inflammation is closely related to atherosclerosis via TLR4. HMGB1 also acts via TLR4. Thus, TDP43 and HMGB1 might be related via inflammation.

Conclusion

This is apparently the first demonstration of pTDP-43 immunolocalization in human carotid and main cerebral artery plaques. We believe that this demonstration of pTDP-43 localization in atherosclerotic lesions is important as it may contribute to the establishment of a clinical diagnosis of FTLD and ALS. Furthermore, we confirmed the co-localization between pTDP-43 and 14-3-3 eta isoform or HMGB1. These findings may prove useful for further understanding the roles of TDP-43 in cell death.

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