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From Cell Biology to Tissue Engineering

Phosphorylated TDP-43 localizes to chronic cerebral infarctions in human brains

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Summary. The transactivation response DNA-binding protein of 43 kDa (TDP-43) is a nuclear protein pivotal in RNA processing. Because phosphorylated TDP43 (pTDP-43) has been identified as a component of the ubiquitin-positive and tau-negative inclusions observed in the brains of frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS) patients, it is considered to play a major role in neurodegenerative processes. We previously reported that pTDP-43 is located in macrophages of atherosclerotic lesions of human carotid and major cerebral arteries. We hence hypothesized that pTDP-43 might be localized in the macrophages of other human brain lesions. Therefore, we investigated the immunolocalization of pTDP-43 in human brains with chronic cerebral infarction. Furthermore, we investigated the colocalization of pTDP-43 and the 14-3-3 eta isoform and found that pTDP-43 was localized in many macrophages located in chronic cerebral infarctions, in 6 out of the 15 human brains analyzed. pTDP-43 colocalized with the 14-3-3 eta isoform in these lesions. This is the first demonstration of pTDP-43 immunolocalization in chronic cerebral infarctions in human brains. We believe that our findings may be useful towards further understanding the pathophysiological roles of TDP-43 in various neurological disorders.

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Introduction

The transactivation response DNA-binding protein of 43 kDa (TDP-43) is a nuclear protein that is known to be involved in RNA processing. TDP-43 has been identified as a component of ubiquitin-positive and tau-negative inclusions in the brains of frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS) patients (Arai et al., 2006; Neumann et al., 2006). TDP-43 is usually located in the nucleus; however, in some pathological conditions, fragmented and phosphorylated TDP-43 is observed in the cytoplasm (Arai et al., 2006; Neumann et al., 2006; Hasegawa et al., 2008). Phosphorylated TDP-43 (pTDP-43)-positive inclusions are a pathological hallmark of the majority of FTLD and ALS patients. On the other hand, recently, the concept of limbic-predominant age-related TDP-43 encephalopathy (LATE) was proposed.

We previously reported that pTDP-43 is located in macrophages within atherosclerotic lesions of human carotid and main cerebral arteries (Umahara et al., 2020). We hence considered that pTDP-43 might be observed in macrophages within the lesion area of the brain parenchyma, as observed in atherosclerotic lesions. Therefore, we hypothesized that pTDP-43 is located in macrophages within other types of brain lesions. In the present study, we investigated the localization of pTDP-43 in human brains with chronic cerebral infarction,

because microglia/macrophages are known to be present in lesions of chronic cerebral infarction. Furthermore, we clarified the colocalization between pTDP-43 and the 14-3-3 eta isoform, because we previously demonstrated the colocalization between pTDP-43 and the 14-3-3 eta isoform in granular structures of human anterior horn cells in ALS patients (Umahara et al., 2016) and in macrophages of human carotid plaques (Umahara et al., 2020).

Material and methods

Specimens of brains from patients with chronic cerebral infarction were obtained at autopsy (49-74-years old at the time of death). Before autopsy consent was obtained from each patient's family regarding the use of the specimens for this study. There were 15 patients with chronic cerebral infarction (the disease durations of 10 of the patients were 3, 3, 4, 6, 6, 7, 7, 9, 10, and 12 months, and the disease durations of 5 patients were unknown); 3 patients had cardiogenic cerebral embolism (CE), 7 patients had atherothrombotic brain infarction (AT), and 5 patients had multilacunar (ML) infarction. Samples from 3 control patients were also analyzed (66, 71, and 77-years old); their causes of death were myocardial infarction, rectal cancer, and pneumonia. Tissue sections (5-μm thick) were prepared from formalin-fixed, paraffin-embedded blocks. Hematoxylin-eosin (HE) staining or immunohistochemistry was performed on the sections.

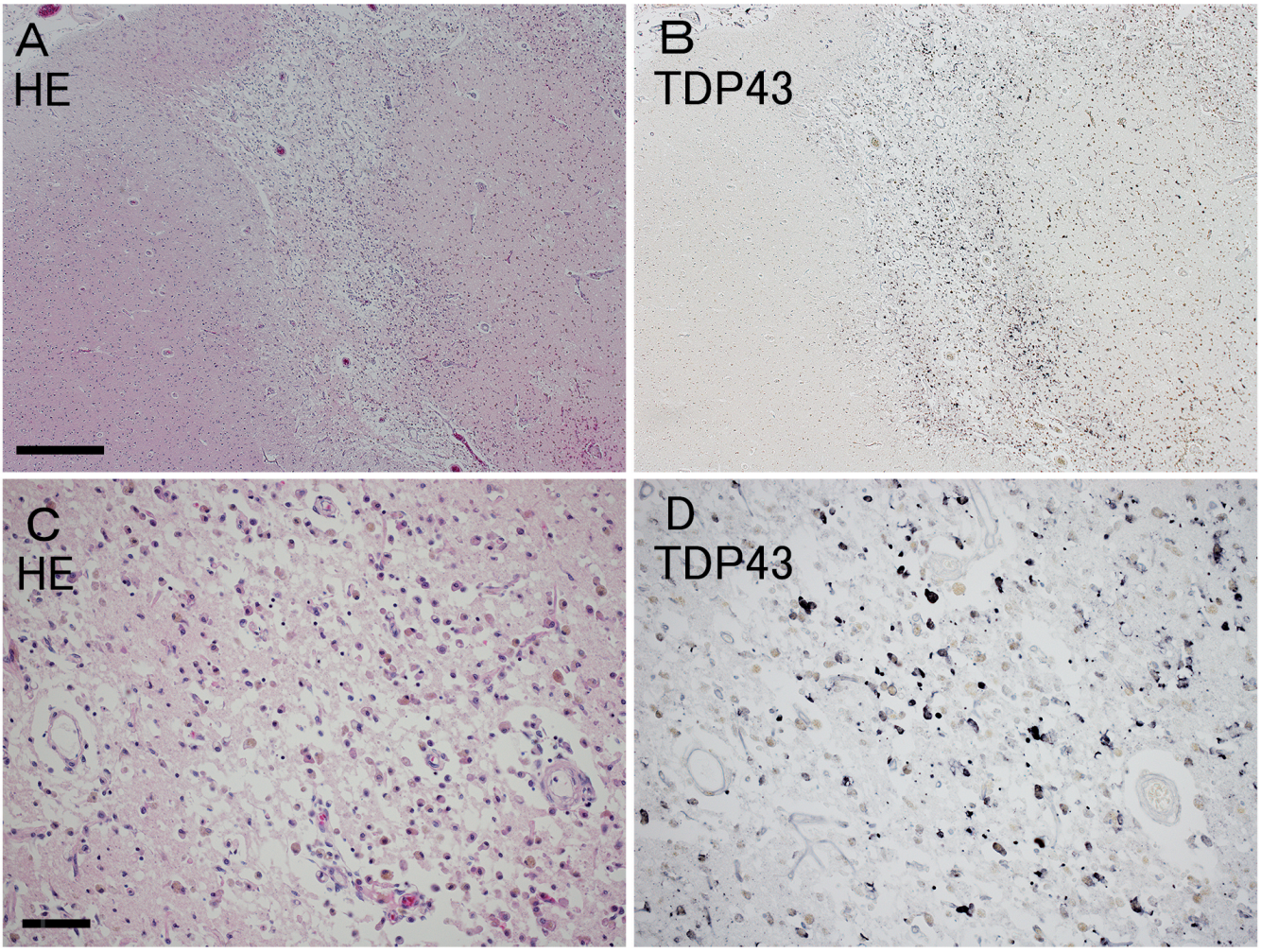

Fig. 1. Characteristics of TDP-43 in chronic cerebral infarctions. **A.** Low-magnification image of hematoxylin-eosin (HE) staining of a chronic cerebral infarction. **B.** Low-magnification image of immunohistochemical labeling of pTDP-43 in a chronic cerebral infarction. pTDP-43-positive cells are observed. **C.** High-magnification image of HE staining of a chronic cerebral infarction. Many macrophages are observed. **D.** High-magnification image of immunohistochemical labeling of pTDP-43 in a chronic cerebral infarction. pTDP-43-positive cells are observed. Scale bars: A, B, 1 mm; C, D, 100 µm.

For immunohistochemistry, 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-pTDP-43 antibody (1:5,000; 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 (1:1,000, 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 to 20 min.

For immunofluorolabeling, after treatment with 0.5% normal goat and horse sera, deparaffinized tissue sections were incubated in a mixture of anti-pTDP-43 antibody (1:500), and either ferritin (microglia/macrophage marker) (Kaneko et al., 1989), rabbit polyclonal, 1:500, DAKO) or an anti-14-3-3 eta isoform antibody (against MGDREQLLQR, 1:20, rabbit polyclonal, Immuno-Biological Laboratories, Gunma, Japan). These antibodies were then visualized with a mixture of anti-mouse IgG antibody in goat serum conjugated with Alexa 488 (1:200; Molecular Probes, Eugene, OR, USA), and anti-rabbit IgG in goat serum conjugated with Alexa 546 (1:200; Molecular Probes) for 2 hours. 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

In 6 out of the 15 samples from chronic cerebral infarction patients, in whom the durations of disease were 3 (CE), 4 (AT), 5 (AT), 12 (CE) months, and 2 (ML) unknown, macrophage-like cells positive for pTDP-43 were found mainly in the region surrounding the chronic ischemic cavity or ischemic loose lesions (Fig. 1). pTDP-43p staining was observed throughout the macrophage-like cells, including in their cytoplasm. TDP-43 staining was not observed in all other lesions.

Regarding the 3 control subjects, faint pTDP-43 immunoreactivity was observed in the hippocampus of the 77-year old subject, whereas immunoreactivity was not observed in the other 2 subjects.

Immunofluorolabeling

Almost all pTDP-43-positive cells in the chronic cerebral infarction lesions were also positive for ferritin (a microglia/macrophage marker) (Fig. 2). Furthermore, almost all pTDP-43-positive cells in the chronic cerebral infarction lesions were also positive for the 14-3-3 eta isoform (Fig. 3).

Discussion

To the best of our knowledge, this is the first demonstration of the immunolocalization of pTDP-43 in human brains of patients with chronic cerebral

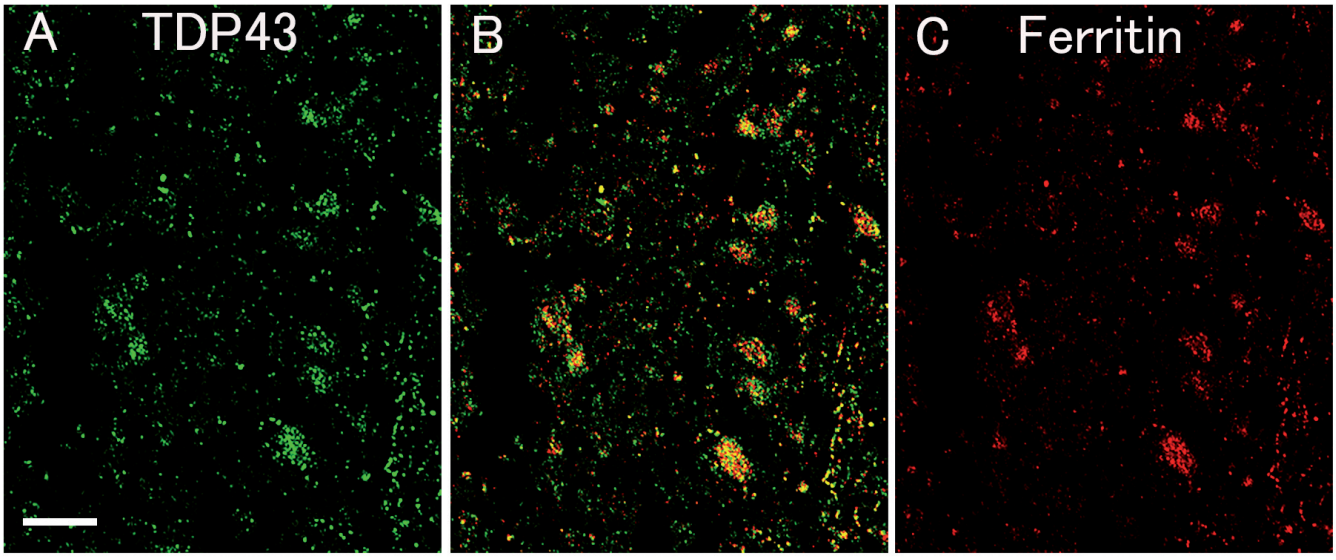

Fig. 2. Double immunofluorolabeling of pTDP-43 (**A**: green areas) and ferritin (**C**: red areas) in a chronic cerebral infarction. A merged image of pTDP-43 and ferritin is shown in **(B)**. Scale bar: 50 µm.

infarction. pTDP-43 was located in the ferritin, which is a well-established microglia/macrophage marker (Kaneko et al., 1989), positive cells, in chronic ischemic lesions.

It has been reported that TDP-43 immunoreactivity was negative in acute, subacute, and remote ischemic lesions of human brains (Lee et al., 2008). However, this previous study used an anti-TDP-43 antibody rather than an anti-pTDP-43 antibody as used in the present study. Furthermore, this previous study did not analyze pTDP-43 localization in chronic lesions of cerebral infarction. In another report, the cytoplasm of rat brain neurons in acute ischemic lesions were positive for TDP-43 but negative for pTDP-43 (Kanazawa et al., 2011). Unfortunately, this report also did not examine chronic cerebral infarctions. On the other hand, Thammistty et al. (2018) reported that pTDP-43 was observed in the cytoplasm of neurons in human post-mortem post-stroke brains autopsied at 1 to 5 days after the stroke.

Shindo et al. reported that in chronic ischemic lesions of mouse brains, pTDP-43 immunoreactivity was observed in the neuronal cytoplasm in the cerebral cortex and hippocampus (Shindo et al., 2013). However, in our present study, we did not observe immunolabeling of the neuronal cytoplasm in chronic cerebral infarctions of human brains using an anti-pTDP-43 antibody. Instead, we demonstrated the localization of pTDP-43 in macrophages of human brains with chronic cerebral infarction.

In our study, pTDP-43 positivity was only observed in the many foam cells (positive for a microglia/macrophage marker) in the lesions of only 6 out of the 15 samples of chronic cerebral infarction The durations of disease of these 6 patients were 3 to 12 months, and the infarction types varied (2 patients with CE, 2 with AT, and 2 with ML). Therefore, the results cannot be explained by the period after onset or the type of lesion. Further investigations are hence required to clarify the conditions that are associated with the localization of pTDP-43 in chronic ischemic lesions.

pTDP-43-positive cytoplasmic and intranuclear inclusions, which are observed in the neurons of FTLD and ALS patients (Arai et al., 2006; Neumann et al., 2006), were not observed in the macrophages of atherosclerotic lesions of human carotid and main cerebral arteries (Umahara et al., 2020). The pattern of pTDP-43 localization in macrophages within human chronic ischemic lesions is quite similar to the pattern in macrophages within atherosclerotic lesions of human carotid and main cerebral arteries.

One possible role of TDP-43 localization in human chronic ischemic lesions is the activation of macrophages, as it has been reported that TDP-43 activates microglia (Brettschneider et al., 2012). Another

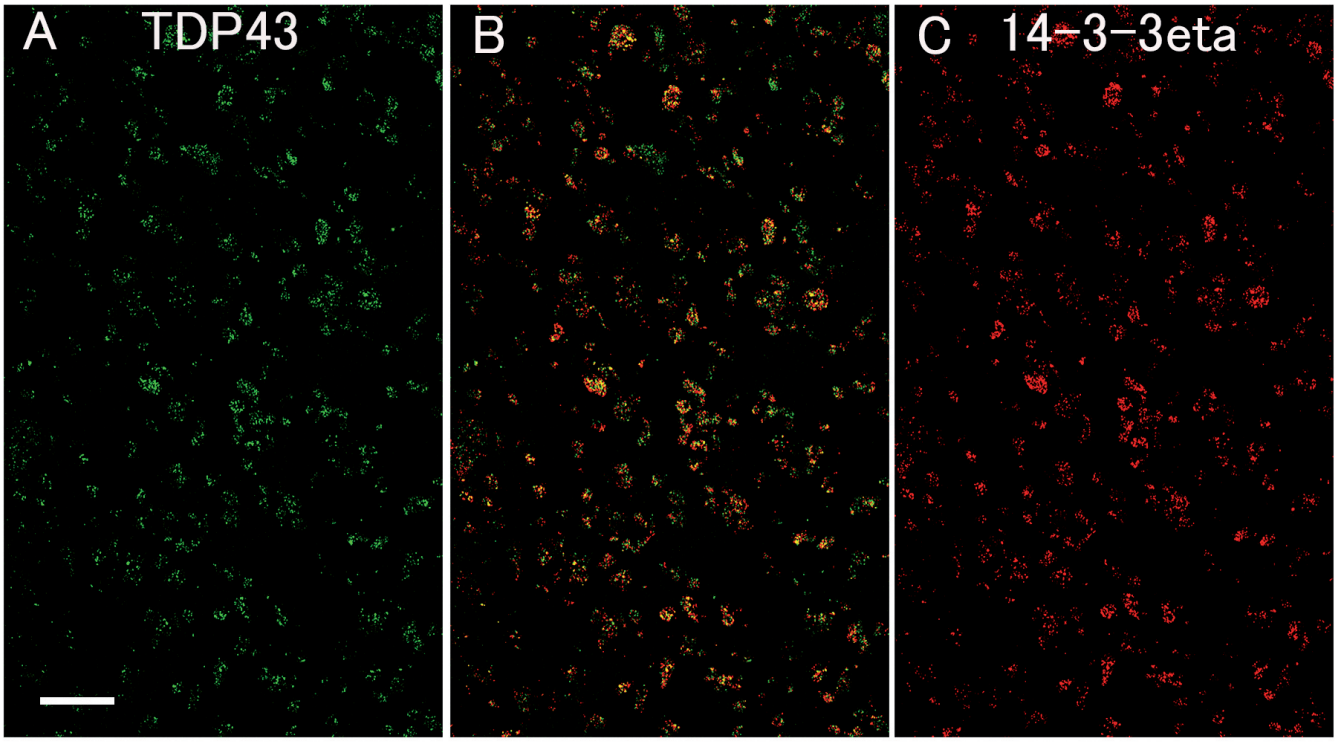

Fig. 3. Double immunofluorolabeling of pTDP-43 (**A**: green areas) and the 14-3-3 eta isoform (**C**: red areas) in a chronic cerebral infarction. A merged image of pTDP-43 and the 14-3-3 eta isoform is shown in **(B)**. Scale bar: 100 μm.

possible role might be associated with autophagy, as TDP-43 has been reported to be associated with autophagy (Budini et al., 2017). It was reported that subarachnoid hemorrhage enhances the expression of TDP-43 in the brains of experimental rats, including in their microglia (He et al., 2018), and inflammation was suggested to be an important factor in this enhancement of TDP-43 expression. Therefore, another possible explanation for the findings in our present study is the involvement of inflammation.

The importance of pTDP-43 localization in chronic ischemic lesions of human brains can be considered from other perspectives. The localization of pTDP-43 in chronic ischemic lesions should be clarified in the future when testing for the presence of pTDP-43 as a biomarker or during positron emission tomography with a pTDP-43 probe for the diagnosis of FTD or ALS. To improve the diagnostic accuracy of TDP proteinopathies, the localization of pTDP-43 in macrophages within chronic cerebral infarctions must be excluded. Recently, it was reported that TDP-43 inclusions can be eliminated using a monoclonal antibody (Tamaki et al., 2018). Our results may also contribute towards the establishment of antibody therapies for TDP-43-associated diseases, because pTDP-43 might be involved in the repair of chronic ischemic lesions, and its absence might inhibit lesion repair.

It has been reported that 14-3-3 proteins can trap and sequester phosphorylated proteins, and thereby control the localization of some molecules (Aitken, 2006). An example of this is Forkhead box protein O (FOXO), which is a regulatory factor of apoptosis. It was reported that TDP-43 competes with FOXO in binding to the 14-3-3 protein, and released FOXO molecules translocate to the nucleus and are activated (Zhang et al., 2014). We previously demonstrated the colocalization between pTDP-43 and the 14-3-3 eta isoform in granular structures of human anterior horn cells in ALS patients (Umahara et al., 2016). However, pTDP-43-positive inclusions were not positive for the 14-3-3 eta isoform. Moreover, we have demonstrated that 14-3-3 proteins are located in macrophages of human carotid plaques. In the present study, we demonstrated the colocalization between pTDP-43 and the 14-3-3 eta isoform in foam cells located in human chronic cerebral infarctions. Because pTDP-43 and the 14-3-3 eta isoform are colocalized in these 3 different conditions, a universal association of pTDP-43 and the 14-3-3 eta isoform was suggested. These findings may hence provide a basis for understanding the roles of TDP-43 in association with the 14-3-3 eta isoform.

In conclusion, to our knowledge, this is the first demonstration to date of pTDP-43 immunolocalization in human chronic cerebral infarctions. We believe that our results contribute towards the establishment of a clinical diagnosis method for TDP proteinopathies, such as FTLD, ALS, and LATE. Furthermore, we confirmed the colocalization between pTDP-43 and the 14-3-3 eta isoform in brain lesions. These findings are expected to contribute towards further understanding of the roles of TDP-43 and its phosphorylation in human central nervous system disorders.

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