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ORIGINAL ARTICLE

EXPRESSION OF SYNPHILIN-1 IN HUMAN VASCULAR ENDOTHELIAL CELLS

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Abstract: Synphilin-1 was originally identified as a binding protein of α-synuclein, a major component of Lewy bodies in Parkinson's disease. Synphilin-1 localizes mainly in the synaptic terminals and is also found in Lewy bodies. We previously demonstrated the expression of α-synuclein in vascular endothelial cells. The present study was undertaken to address the expression of synphilin-1 in endothelial cells. Synphilin-1 immunoreactivity was detected in the vessel walls, particularly in endothelial cells, in postmortem human brain. Expressions of mRNA and protein for synphilin-1 in human umbilical vein endothelial cells in culture were demonstrated by reverse-transcription polymerase-chain reaction and western blotting. Synphilin-1 is constitutively expressed in endothelial cells and may have some physiological function in the vascular wall.

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Key words: synphilin-1; vascular endothelial cells; α-synuclein.

原 著

ヒト血管内皮細胞におけるシンフィリン-1の発現

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抄録 シンフィリン-1は，パーキンソン病におけるレヴィー小体の主要成分であるα-シヌクレインの結合タンパク質として最初に発見され，シナプス終末やレヴィー小体中に存在する．われわれは血管内皮細胞においてα-シヌクレインが発現していることを明らかにしたが，本研究では，内皮細胞におけるシンフィリン-1の発現について検討を行った．その結果ヒト剖検脳の血管壁，特に内皮細胞においてシンフィリン-1が免疫組織学的に検出された．また，培養ヒト臍帯静脈内皮細胞においてシンフィリン-1のmRNAとタンパク質の発現が，それぞれreverse-transcription polymerase-chain reactionとウェスタン・ブロッティングにより確認された．シンフィリン-1は血管内皮細胞において恒常的に発現しており，血管においてなんらかの生理機能を有するものと考えられた．

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Introduction

Synphilin-1 is a component of Lewy bodies in Parkinson's disease[1-4]. It was originally identified as a binding protein of α-synuclein[5], which is the precursor of the non-amyloid β (Aβ) component of Alzheimer's disease amyloid and a major component of Lewy bodies in Parkinson's disease[6]. Two types of mutation in α-synuclein gene are associated with familial Parkinson's disease[7,8], and α-synuclein-positive filamentous inclusions have been reported in diseases as Lewy body disorders and multiple system atrophy[9]. α-Synuclein is expressed mainly in presynaptic nerve terminals[10] and considered to play roles in synaptic function and neuronal plasticity[11].

Synphilin-1 is also detected in presynaptic membrane and neuronal cytoplasm.[12] There are several lines of evidence for the important role of synphilin-1 and its interaction with α-synuclein in the formation of intracellular inclusions in neurodegenerative diseases. Synphilin-1 has protein binding motifs as ankyrin-like domain and coiled-coil domain[13], and a type of mutation in α-synuclein, found in familial Parkinson's disease, is demonstrated to have a stronger interaction with synphilin-1 as compared to the wild type[14]. Transfection of HEK293 cells with synphilin-1 gene resulted in the formation of cellular inclusions similar to Lewy bodies[15]. Synphilin-1 is known to be a substrate of ubiquitin ligase parkin[16], of which mutation is demonstrated in familial Parkinson's disease[17]; and impairment of the ubiquitin-proteasome pathway causes the cell death with synphilin-1 accumulation[18].

We previously reported the expression of α-synuclein in human umbilical vein endothelial cells (HUVECs)[19]. In regard to the pathogenetic significance of the interaction between α-synuclein and synphilin-1, the present study was undertaken to address synphilin-1 expression in vascular endothelial cells; and we herein report the expression of synphilin-1 in human cerebral blood vessels and vascular endothelial cells in culture.

Methods

We examined brain tissues from five (2 male and 3 female) patients aged 62-86 yeas. All of them were autopsied in the Hirosaki University Hospital and free of any neurological diseases. Tissues were fixed with 4% paraformaldehyde in 20 mM phosphate-buffered saline, pH 7.4 (PBS), for three weeks and embedded in paraffin. Sections of 4 μm thick were subjected to immunohistochemical procedure by avidin-biotin-peroxidase complex method. The primary antibody was a polyclonal anti-synphilin-1 antibody (1:500; Chemicon, Temecula, CA, USA).

Synphilin-1 mRNA and protein expression was analyzed in HUVECs in culture. HUVECs were isolated with collagenase and cultured in a gelatin-coated culture dish as described[20]. The culture medium was Humedia EB-2 (Kurabo, Osaka, Japan) supplemented with 2% fetal bovine serum, 10 ng/ml recombinant human (r(h)) epidermal growth factor, 1 μg/ml hydrocortisone, 5 ng/ml r(h) basic fibroblast growth factor and 10 μg/ml heparin. When the cultures reached about 80% confluence, the medium was changed to Humedia EB-2 containing 20% human serum, and tightly confluent monolayers were used for the experiments.

Levels of synphilin-1 mRNA were analyzed by reverse-transcription polymerase-chain reaction (RT-PCR). Total RNA was extracted from cells using a total RNA isolation kit (Qiagen, Hilden, Germany). Single strand cDNA for a PCR template was

synthesized from 1 μg of total RNA using primer oligo(dT)$_{12-18}$ and M-Mulv reverse transcriptase (Gibco-BRL, Gainthersburg, MD, USA). Each cDNA was amplified by PCR using *Taq* DNA polymerase (Qiagen). The sequences of the primers were as follows:

synphilin-1-F (5'-CATCAGGGGGACGCAG GTTT-3'),
synphilin-1-R (5'-TGTGGAGGTCCGCTGG AGAG-3'),
GAPDH-F (5'-CCACCCATGGCAAATTCC ATGGCA-3'),
and GAPDH-R (5'-AGACCACCTGGTGCT CAGTGTAGC-3').

Condition for reaction was 1×(94℃, 1 min), 30×(94℃, 1 min; 57℃, for synphilin-1 or 55℃ for GAPDH, 1 min; 72℃, 1 min), and 1×(72℃, 10 min). PCR products were analyzed by electrophoresis on a 1.2% agarose gel containing 500 ng/ml ethidium bromide. Expected size for the products for synphilin-1 and GAPDH was 340 and 598 bp, respectively.

Cellular levels of synphilin-1 protein were analyzed by western blotting. HUVECs were washed with 20 mM phosphate-buffered saline (PBS) and lysed using cell lysis buffer (PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and 0.01% protease inhibitor cocktail). Lysates were passed through a 23-gage needle and centrifuged. The supernatants were subjected for SDS-polyacrylamide gel (4-20%) electrophoresis. Proteins were blotted to a PVDF membrane (Millipore Japan, Tokyo, Japan), and the membrane was incubated with a Superblock® blocking agent (Pierce, Rockford, IL, USA). Then the membrane was incubated with an anti-synphilin-1 antibody (1:2,000) and HRP-conjugated goat anti-rabbit IgG (1:10,000; Kirkegaard Perry Laboratories, Gaithersburg, MD, USA). HRP reaction was preformed using a chemiluminescence substrate (SuperSignal West Pico®; Pierce), and the membrane was exposed to a Kodak BioMax® film (Kodak, Rochester, NY, USA). The protein extract from a rat brain homogenate and the extract from BEAS-2B bronchial epithelial cells were also examined as positive and negative controls. Specificity of the anti-synphilin-1 antibody was also examined by western blot analysis on the extract from the BEAS-2B cells rendered to overexpress synphilin-1. The cells were transfected with cDNA for FLAG-synphilin-1 fusion protein using an Effectene transfection reagent (Qiagen). Expression of α-synuclein was also examined in a similar manner using an anti-α-synuclein antibody instead of anti-synphilin-1.

HUVECs were also subjected to immunofluorescent staining for synphilin-1. The cells were fixed with 4% paraformaldehyde in PBS and incubated sequentially with a Superblock® blocking solution, an anti-synphiln-1 antibody (1:500), biotin-labeled anti-rabbit IgG (1:200) and streptavidin-FITC. Cells were examined using a laser confocal microscope (Carl Zeiss, Jena, Germany).

Results

Immunohistochemical identification of synphilin-1 in human brain tissue is shown in Figure 1. The cerebellar cortex was positively stained for synphilin-1. As already reported[3,4], nerve terminals were positively stained; and cell bodies and nerve processes were also weakly positive (Figure 1a). The vascular walls of cerebral leptomeningeal arteries (Figure 1b) and artery and vein in the subarachnoid space of the cerebrum or around the brainstem (Figure 1c) showed high levels of synphilin-1 immunoreactivity (Figure 1d). Higher magnification revealed the presence of the immunoreactivity in

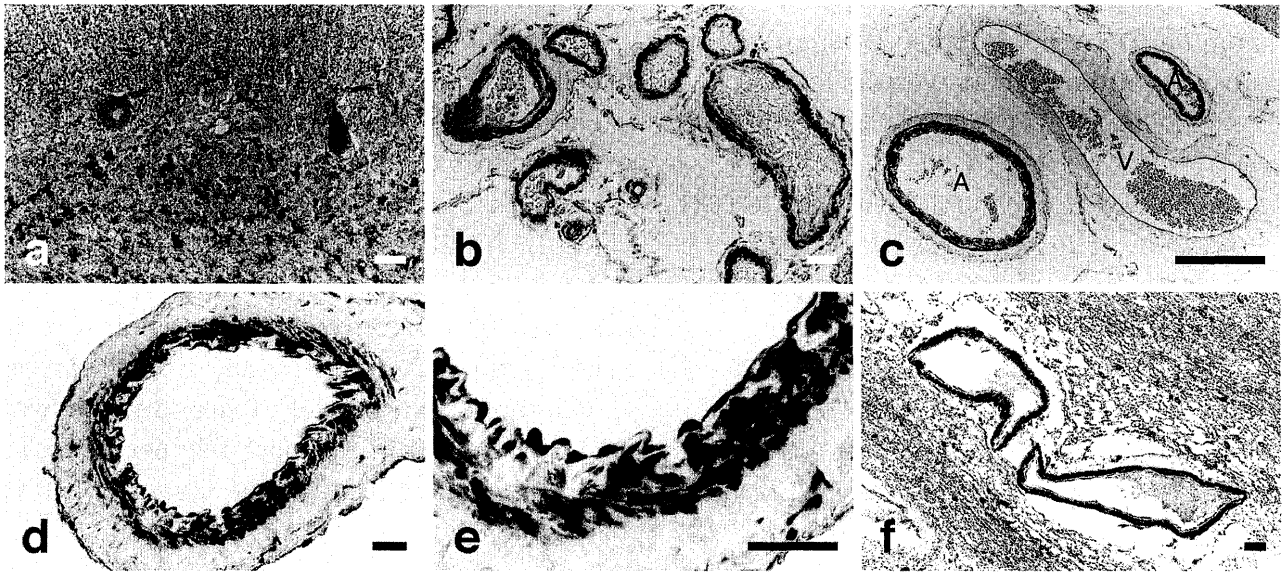

Figure 1 Immunohistochemical identification of synphilin-1 in human brain. (a) The cerebellar cortex with synphilin-1 immunoreactivity in nerve terminals. Nerve cell bodies and processes are also weakly immunoreactive. (b) The cerebral leptomeningeal arteries showing synphilin-1 immunoreactivity. (c) Arterial (A) and venous (V) blood vessels in the subarachnoid space of the cerebrum. (d) The artery in the subarachnoid space around the brainstem with synphilin-1 immunoreactivity. (e) Higher magnification of the arterial wall in (d) demonstrates intense immunoreactivity in the endothelium and smooth muscle layers. (f) Two intraparenchymal vessels in the midbrain. Scale bars, 25 μm in a, b, d-f; 250 μm in c.

both endothelial and smooth muscle cells (Figure 1e). The vessel walls in the brain parenchyma were also positive for synphilin-1 (Figure 1f).

RT-PCR analysis on the expression of synphilin-1 in HUVECs in culture is shown in Figure 2. HUVECs were found to express synphilin-1 mRNA under basal condition, and a single band of 340 bp was amplified by RT-PCR specific for synphilin-1 (Figure 2A). Expression of synphilin-1 protein in HUVEC lysates was detected in western blot analysis as shown in Figure 2B. Synphilin-1 protein was observed as a band of 90 kDa. A similar band was also detected, along with a few unidentified cross-reacting bands, in rat brain homogenate in a parallel experiment. Also, BEAS-2B bronchial epithelial cells were negative for synphilin-1 protein, and the lysate of BEAS-2B cells transfected with cDNA for FLAG-synphilin-1 was positive for immunoreactivity with an expected

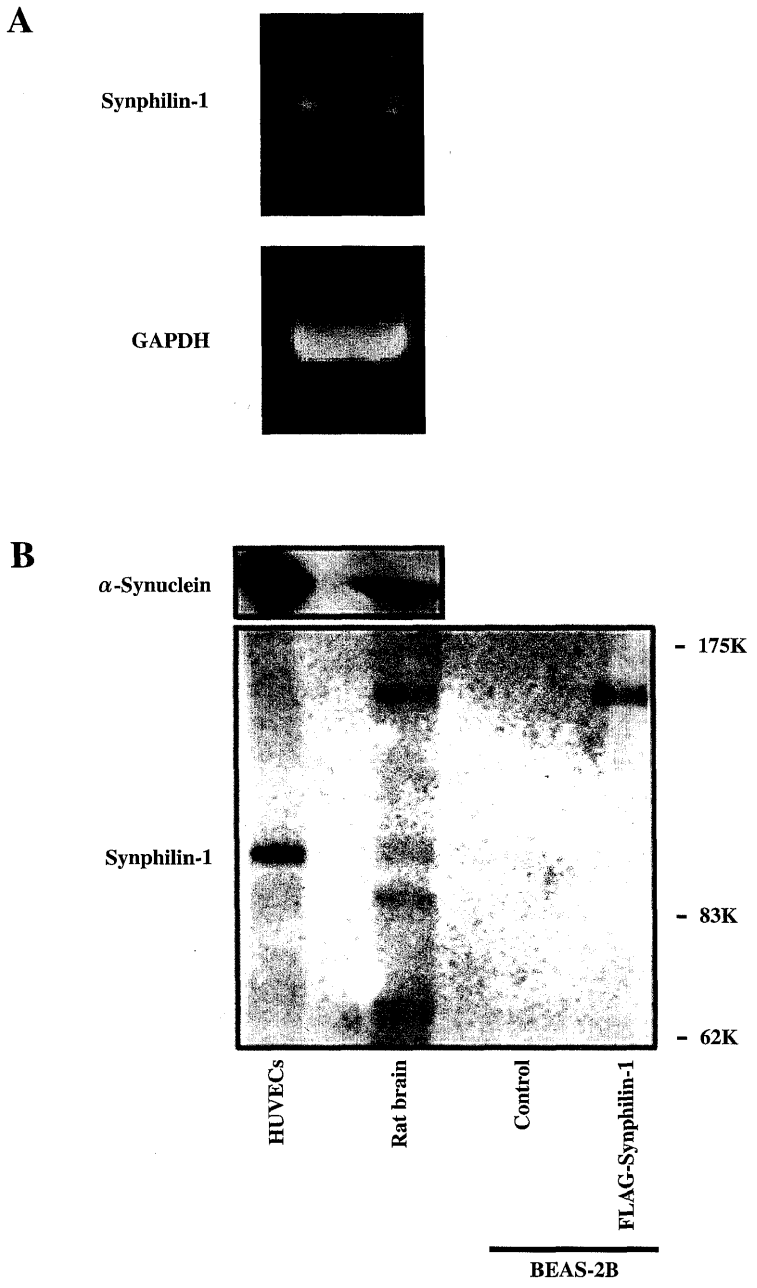

Figure 2 Expression of mRNA and protein of synphilin-1 in HUVECs. (A) Expression of mRNA for synphilin-1 and GAPDH was analyzed by RT-PCR. (B) Expressions of synphilin-1 and α-synuclein proteins were analyzed by western blotting. Synphilin-1 from HUVECs was detected as a 90 kDa protein, and a similar band was found in rat brain homogenate analyzed as a positive control. BEAS-2B bronchial epithelial cells were negative for synphilin-1 expression, and the cells rendered to express FLAG-synphilin-1 fusion protein were found to positive for the protein with an expected molecular size.

molecular size of about 120 kDa[21]. The size of this band exceeds that deduced from amino acid composition of the protein, but a previous study[21] suggested the presence of the modification process that may lead to the expression of a higher molecular mass. Expression of α-synuclein was confirmed both in HUVECs and rat brain.

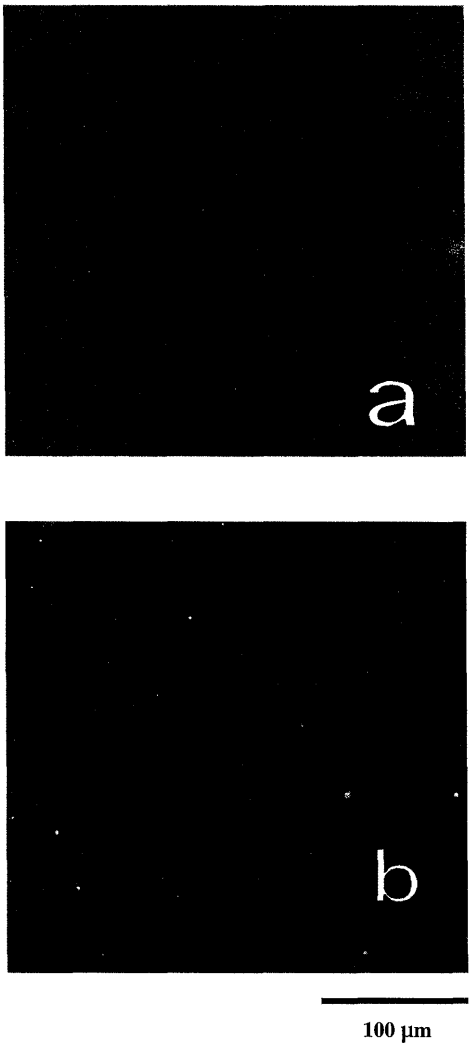

Figure 3 Immunofluorescent staining for synphilin-1 in HUVECs. (a) HUVECs were fixed with 4% paraformaldehyde, and incubated with non-immune IgG, biotin-labeled anti-rabbit IgG and streptavidin-FITC. (b) HUVECs were treated as in (a) except for the use of anti-synphilin-1 IgG instead of non-immune IgG. Scale bar, 100 μm.

Immunofluorescent staining revealed the presence of synphilin-1 in HUVECs, under basal culture condition, as shown in Figure 3. Synphilin-1 immunoreactivity was observed as cytoplasmic fluorescence (Figure 3b), and the cells stained using a control antibody were virtually negative for immunofluorescence (Figure 3a).

Discussion

Recent studies have shown that synphilin-1 immunoreactivity is present in Lewy bodies in Parkinson's disease and dementia with Lewy bodies as well as in glial cytoplasmic inclusions in multiple system atrophy. Various neuronal and glial

inclusions in neurodegenerative disorders other than Lewy body disease and multiple system atrophy are synphilin-1-negative. We previously reported the expression of α-synuclein in human vascular endothelial cells and smooth muscle cells[19]. The present study also demonstrated the expression of synphilin-1 in the vascular wall. Synphilin-1 was detected in the vascular endothelial cells and smooth muscle cells in normal human brain tissue. Synphilin-1 was also detected in tissues from cerebellar cortex examined as a positive control. RT-PCR and western blotting demonstrated that synphilin-1 mRNA and protein were expressed in HUVECs under basal condition; and immunofluorescent staining demonstrated the presence of synphilin-1 immunoreactivity in HUVECs under basal culture condition. Human synphilin-1 and rat homologue consist of 919 and 953 amino acids, respectively; and overall identity between the two proteins at amino acid level is 85%. In the present study, western blot analysis of rat brain extract detected a single band with a molecular size similar to human synphilin-1 (90 kDa).

Although oxidative stress is known to induce the intracellular accumulation of α-synuclein[22], there is no clear evidence for the regulatory mechanism of synphilin-1 expression. Vascular endothelial cells may serve as one of the effective experimental models for the studies of the role of synphilin-1 in the interaction with α-synuclein and formation of intracellular inclusions.

In conclusion, vascular endothelial cells express synphilin-1, which may bear some insight into the function of this protein.

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