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Taisuke Kato, …, Shoji Tsuji, Osamu Onodera

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Candesartan prevents arteriopathy progression in cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy model

Taisuke Kato,1 Ri-ichiroh Manabe,2 Hironaka Igarashi,3 Fuyuki Kametani,4 Sachiko Hirokawa,5 Yumi Sekine,5 Natsumi Fujita,5 Satoshi Saito,1 Yusuke Kawashima,7 Yuya Hatano,6 Shoichiro Ando,6 Hiroaki Nozaki,4 Akihito Sugai,5 Masahiro Uemura,5 Masaki Fukunaga,5 Toshiya Sato,10 Akihide Koyama,15 Rie Saito,17 Atsushi Sugie,13 Yasuko Toyoshima,12 Hirotoshi Kawata,14 Shigeo Murayama,15,16 Masaki Matsumoto,17 Akiyoshi Kakita,12 Masato Hasegawa,4 Masafumi Ihara,6 Masato Kanazawa,5 Masatoyo Nishizawa,18 Shoji Tsuji,19 and Osamu Onodera5

1Department of System Pathology for Neurological Disorders, Brain Science Branch, Brain Research Institute, Niigata University, Niigata, Japan. 2Laboratory for Comprehensive Genomic Analysis, Center for Integrative Medical Sciences, RIKEN, Kanagawa, Japan. 3Center for Integrated Human Brain Science, Brain Research Institute, Niigata University, Niigata, Japan. 4Department of Brain and Neuroscience, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan. 5Department of Neurology, Clinical Neuroscience Branch, Brain Research Institute, Niigata University, Niigata, Japan. 6Department of Neurology, National Cerebral and Cardiovascular Center, Suita, Japan. 7Department of Applied Genomics, Kazusa DNA Research Institute, Chiba, Japan. 8Department of Medical Technology, Graduate School of Health Sciences, Niigata University, Niigata, Japan. 9Division of Cerebral Integration, Department of System Neuroscience, National Institute for Physiological Sciences, Aichi, Japan. 10Department of Laboratory Animal Science, Katsato University School of Medicine, Kanagawa, Japan. 11Department of Legal Medicine, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan. 12Department of Pathology, Clinical Neuroscience Branch and 13Department of Neuroscience of Disease, Brain Research Institute, Niigata University, Niigata, Japan. 14Department of Pathology, Jichi Medical University, Tochigi, Japan. 15Brain Bank for Aging Research, Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology, Tokyo, Japan. 16Brain Bank for Neurodevelopmental, Neurological and Psychiatric Disorders, United Graduate School of Child Development, University of Osaka, Osaka, Japan. 17Department of Omics and Systems Biology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan. 18Brain Research Institute, Niigata University, Niigata, Japan. 19Department of Neurology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan.

Introduction

Cerebral small vessel disease (CSVD) causes dementia and gait disturbance due to arteriopathy. Cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL) is a hereditary form of CSVD caused by loss of high-temperature requirement A1 (HTRA1) serine protease activity. In CARASIL, arteriopathy causes intimal thickening, smooth muscle cell (SMC) degeneration, elastic lamina splitting, and vasodilation. The molecular mechanisms were proposed to involve the accumulation of matrisome proteins as substrates or abnormalities in transforming growth factor β (TGF-β) signaling. Here, we show that HTRA1−/− mice exhibited features of CARASIL-associated arteriopathy: intimal thickening, abnormal elastic lamina, and vasodilation. In addition, the mice exhibited reduced distensibility of the cerebral arteries and blood flow in the cerebral cortex. In the thickened intima, matrisome proteins, including the hub protein fibronectin (FN) and latent TGF-β binding protein 4 (LTBP-4), which are substrates of HTRA1, accumulated. Candesartan treatment alleviated matrisome protein accumulation and normalized the vascular distensibility and cerebral blood flow. Furthermore, candesartan reduced the mRNA expression of Fn1, Ltbp-4, and Adamts12, which are involved in forming the extracellular matrix network. Our results indicate that these accumulated matrisome proteins may be potential therapeutic targets for arteriopathy in CARASIL.

Cerebral small vessel disease (CSVD) is a sporadic and hereditary arteriopathy of the cerebral small vessels that results in dementia and gait disturbance (1). The molecular mechanism of CSVD differs from that of large vessel disease, but the precise mechanism is still unknown (2). Therefore, therapeutic strategies based on the molecular mechanism have not been developed for CSVD.

Cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL) is a hereditary form of CSVD caused by a loss of function of high-temperature requirement A1 (HTRA1), a serine protease (3, 4). CARASIL is characterized by leukoencephalopathy, multiple lacunas, and reduced cerebral blood flow without hypertension (5). The arteriopathy of CARASIL shows intimal thickening, degeneration of smooth muscle cells (SMCs), splitting of the internal elastic lamina, and dilatation of vessels, and thus resembles sporadic CSVD (6–8). Intimal thickening is widespread in intracranial cerebral arteries, including large arteries as well as pial arteries and arterioles (9, 10). In contrast, degeneration of SMCs is most prominent in arterioles (9). It is unclear which morphological changes play a pivotal role in the pathogenesis of CARASIL.

Arteriopathy in CARASIL has been suggested to be caused by the accumulation of matrisome proteins, which are components of the extracellular matrix (ECM) (11–13). Matrisome proteins
are composed of ECM proteins (core-matrisome proteins) and ECM-modifying proteins (matrisome-associated proteins; refs. 14, 15). Indeed, matrisome proteins accumulate in the thickened intima in individuals with CARASIL (3, 13, 16). Some of these accumulated matrisome proteins, including fibronectin (FN), are substrates for HTRA1 (16, 17).

Another hypothesis has been proposed regarding changes in transforming growth factor β (TGF-β) signaling. HTRA1 overexpression suppresses TGF-β signaling in cultured cells (3, 18, 19). HTRA1 cleaves pro-TGF-β (16). TGF-β and proteins induced by TGF-β signaling accumulate in the cerebral small vessels of patients with CARASIL, suggesting that TGF-β signaling is enhanced in CARASIL (3, 16). On the other hand, decreased TGF-β signaling has also been proposed (20). HTRA1 digests latent TGF-β-binding protein 1 (LTBP-1), which anchors inactive TGF-β to the ECM (20). Decreased TGF-β signaling has been demonstrated in cultured fibroblasts from HTRA1−/− mice (20). Decreased TGF-β signaling may induce degeneration of SMCs (21). Therefore, it remains unclear how TGF-β signaling contributes to arteriopathy in CARASIL (20, 22–25).

If perturbation of the matrisome in cerebral small vessels or an increase in TGF-β signaling plays an important role in developing arteriopathy in CARASIL, we hypothesized that angiotensin II type 1 receptor antagonists (AT1RBs) may be a candidate for treatment of arteriopathy. AT1RBs, including candesartan cilexetil, attenuate arteriopathy in several other organs by normalizing the perturbed matrisome (26). Candesartan suppresses FN expression (27) and inhibits TGF-β signaling in the brain (28–30). In the present study, we aimed to elucidate the molecular mechanism of arteriopathy in CARASIL by examining whether candesartan cilexetil improves arteriopathy in HTRA1−/− mice.

Results
HTRA1 is expressed in the endothelial cells of the pial arteries. The loss of HTRA1 expression in HTRA1−/− mice was confirmed by RT-PCR (Supplemental Figure 1, A–C; supplemental material available online with this article; https://doi.org/10.1172/JCI140555DS1). HTRA1 is a secretory protein. Thus, we investigated the distribution of Htra1 mRNA by performing in situ hybridization to detect cells expressing HTRA1 (Supplemental Figure 1D). In HTRA1−/− mice, Htra1 mRNA was expressed in astrocytes but not in neurons or microglia (Supplemental Figure 2A) (24). In pial arteries, Htra1 mRNA was expressed in endothelial cells (Supplemental Figure 2B), while in arterioles and capillaries, few endothelial cells expressed Htra1 mRNA (Supplemental Figure 2, C and D). Htra1 mRNA was not expressed in SMCs or pericytes (Supplemental Figure 2, B and E). Htra1 mRNA expression in astrocytes and endothelial cells was confirmed in cultured primary cells from HTRA1−/− mice (Supplemental Figure 2, F and G).

Accumulation of matrisome proteins in the pial arteries and arterioles of HTRA1−/− mice and patients with CARASIL. HTRA1−/− mice showed normal blood pressure and normal blood glucose levels (Supplemental Figure 3, A and B). By 24 months of age, HTRA1−/− mice displayed no motor deficits, white matter lesions, or ischemic lesions compared with HTRA1+/+ mice (Supplemental Figure 6, F and G). Accumulation was also occasionally observed in the subendothelial layer of the thickened intima of pial arteries (Supplemental Figure 4, Figure 2A), but not LTBP-1 (P = 0.064; Supplemental Figure 5A), in HTRA1−/− mice were confirmed. The cellular type of FN (EDA+ FN) was increased (Figure 2A), while the plasma type of FN was not increased in blood plasma (Supplemental Figure 5B). See complete unedited blots in the supplemental material.

Next, we assessed FN, LTBP-1, fibulin-5, and elastin by immunohistochemical analysis. As reported by Monet-Lepretre et al., we could not find a suitable antibody for detecting mouse TIMP3 by immunohistochemical analysis (33). FN, LTBP-4, and fibulin-5 markedly accumulated in the intima of large cerebral arteries, pial arteries, and arterioles but not in capillaries of HTRA1−/− mice (Figure 2, B–D and Supplemental Figure 5, C–H). Immunohistochemical analysis and immunoelectron microscopy revealed that FN, LTBP-4, and fibulin-5 accumulated in the subendothelial layer of the thickened intima of pial arteries (Supplemental Figure 6, A–E) and arterioles (Supplemental Figure 6, F and G). Accumulation was also occasionally observed in the tunica media (Supplemental Figure 6H). We also found double-layered elastic lamina formation in the intima of pial arteries of HTRA1−/− mice (Figure 2E). The elastic lamina on the luminal side was not uniform in thickness and was partially fragmented and colocalized with LTBP-4 (Figure 2E).

In patients with CARASIL, the accumulation of FN, LTBP-4, TIMP3, and fibulin-5 was confirmed in the pial arteries and the
sis, immunoblotting analysis revealed increased levels of TGF-β1 (latency-associated peptide for TGF-β1 [LAP-β1]) (Supplemental Figure 8B). A significant increase in TGF-β2 levels, though lower than that in TGF-β1 levels, was also observed (Supplemental Figure 8B). TGF-β1 was detected in the intima of the pial arteries and the arterioles but not in the capillaries at 24 months of age (Supplemental Figure 8, C–E). However, immunohistochemical and immunoblotting analysis of the cerebral arteries and the cortices of HTRA1−/− mice did not reveal any changes in phosphorylated SMAD (pSMAD) levels (Supplemental Figure 8, F–J). We also did not find any changes in noncanonical SMAD-independent TGF-β signaling (Supplemental Figure 9, A–C) or in the expression levels of TGF-β–regulated genes (Supplemental Figure 9D).

Candesartan ameliorates the accumulation of matrisome proteins and prevents vascular remodeling. AT1RBs, including candesartan cilexetil, have been reported to reduce matrisome protein accumulation (Supplemental Figure 7, A–D). FN, LTBP-4, and fibulin-5 were found in the thickened intima, while TIMP3 was found in the intima but was preferentially detected in the tunica media. In some vessels, LTBP-4 and fibulin-5 displayed multilayered deposits (Figure 3, H and W). Notably, LTBP-4 and fibulin-5 were frequently observed on the luminal side of multilayered elastin (Figure 3, Y and Z).

We investigated whether HTRA1 digests LTBP-4 and elastin since FN, TIMP3, and fibulin-5 have been reported to be substrates of HTRA1 (13, 17, 34). HTRA1 digested LTBP-4 and fibulin-5 at the same concentration at which it digested FN (Supplemental Figure 8A). However, elastin was not digested by HTRA1 even at a dose that was more than enough to degrade FN and LTBP-4 (Supplemental Figure 8A).

Because of the potential variability of TGF-β signals in CARASIL, we analyzed the accumulation of TGF-β and alterations in TGF-β signaling in HTRA1−/− mice. Consistent with the MS analysis, immunoblotting analysis revealed increased levels of TGF-β1 (latency-associated peptide for TGF-β1 [LAP-β1]) (Supplemental Figure 8B). A significant increase in TGF-β2 levels, though lower than that in TGF-β1 levels, was also observed (Supplemental Figure 8B). TGF-β1 was detected in the intima of the pial arteries and the arterioles but not in the capillaries at 24 months of age (Supplemental Figure 8, C–E). However, immunohistochemical and immunoblotting analysis of the cerebral arteries and the cortices of HTRA1−/− mice did not reveal any changes in phosphorylated SMAD (pSMAD) levels (Supplemental Figure 8, F–J). We also did not find any changes in noncanonical SMAD-independent TGF-β signaling (Supplemental Figure 9, A–C) or in the expression levels of TGF-β–regulated genes (Supplemental Figure 9D).
LTBP-4 and fibulin-5 were almost halved in candesartan-treated HTRA1−/− mice (Figure 4, A and B and Supplemental Figure 11, C–F). In contrast, amlodipine slightly reduced the FN levels but did not reduce the levels of LTBP-4 and fibulin-5 (Figure 4, A and B and Supplemental Figure 11, C–F). Immunoblotting analysis of the cerebral arteries of HTRA1−/− mice confirmed that FN, LTBP-4, EDA+ FN, and elastin levels were decreased in candesartan-treated HTRA1−/− mice (Figure 4C). In addition, the accumulation of TIMP3, fibulin-5, and TGF-β1 was attenuated by candesartan treatment (Supplemental Figure 11G). Concomitantly, the levels of pSMAD3 but not pSMAD5 or pErk1/2 were downregulated by candesartan treatment (Supplemental Figure 11G and ref. 40).

In HTRA1−/− mice, the accumulation of FN and LTBP-4 began at 4 months of age and increased with age (Supplemental Figure 10, A and B). Therefore, we began administration of candesartan and the calcium-channel blocker amlodipine, a control with comparable antihypertensive effects, to HTRA1−/− mice at 4 months of age and examined the effects of these drugs at 24 months of age (Supplemental Figure 11, A and B). Immunohistochemical analysis revealed that FN levels were substantially reduced and that the levels of LTBP-4 and fibulin-5 were almost halved in candesartan-treated HTRA1−/− mice (Figure 4, A and B and Supplemental Figure 11, C–F). In contrast, amlodipine slightly reduced the FN levels but did not reduce the levels of LTBP-4 and fibulin-5 (Figure 4, A and B and Supplemental Figure 11, C–F). Immunoblotting analysis of the cerebral arteries of HTRA1−/− mice confirmed that FN, LTBP-4, EDA+ FN, and elastin levels were decreased in candesartan-treated HTRA1−/− mice (Figure 4C). In addition, the accumulation of TIMP3, fibulin-5, and TGF-β1 was attenuated by candesartan treatment (Supplemental Figure 11G). Concomitantly, the levels of pSMAD3 but not pSMAD5 or pErk1/2 were downregulated by candesartan treatment (Supplemental Figure 11G and ref. 40).
We further conducted MS analysis of the cerebral vessels of candesartan-treated and untreated 24-month-old HTRA1−/− mice. We identified 6123 proteins, and 1107 showed significant changes in abundance in candesartan-treated HTRA1−/− mice (increased proteins: 804, decreased proteins: 303; Supplemental Table 6). Among the 360 proteins that were increased in untreated HTRA1−/− mice compared with untreated HTRA1+/+ mice and identified by 2 or more unique peptides in the second MS analysis, 48.9% of the proteins were decreased by candesartan treatment (group 1; Supplemental Tables 2, 6, and 7). GO enrichment analysis of group 1 revealed notable enrichment into ECM components (FDR = 1.03 × 10−52; Figure 4D, Supplemental Figure 12A, and Supplemental Table 9). In contrast, among the proteins that were increased in untreated HTRA1−/− mice compared with untreated HTRA1+/+ mice but not decreased by candesartan treatment (group 2), no such characteristic enrichment

Figure 3. Accumulation of matrisome proteins and colocalization of elastic lamina and LTBP-4 or fibulin-5 in the cerebral vessels of patients with CARASIL. (A–X) FN (A–F), LTBP-4 (G–I), TIMP3 (M–R), and fibulin-5 (S–X) immunoreactivity in the cerebral arteries of controls (A, D, G, J, M, P, S, and V) and patients with CARASIL (B, C, E, F, H, I, K, L, N, O, Q, R, T, U, W, and X). Panels B, C, E, H, K, O, Q, and W were obtained from patient 1 (a 46-year-old woman), and panels F, I, L, N, R, T, U, and X were obtained from patient 2 (a 54-year-old man); both patient 1 and patient 2 had a homozygous nonsense mutation in the HTRA1 gene (p.R302ter). Apparent accumulation of FN, LTBP-4, TIMP3, and fibulin-5 was detected in the cerebral vessels of patients with CARASIL. For comparison, autopsy samples from 4 control subjects (a 49-year-old woman, a 51-year-old woman, an 89-year-old man, and a 51-year-old man) were subjected to the same immunohistochemical analysis. No evident accumulation of these molecules was found in the cerebral arteries of the controls. Representative micrographs of pial (A–C, F–I, M–O, S–U, and W) and parenchymal (D, E, J, K, L, P, Q, R, V, and X) arteries are shown. Scale bars = 100 μm. (Y and Z) Simultaneous detection of elastin and LTBP-4 (Y) or fibulin-5 (Z) in cerebral arteries of a CARASIL patient (a 46-year-old woman). In the LTBP-4 or fibulin-5 accumulation sites, colocalization with elastin was frequently found. Details of the patients with CARASIL and controls are shown in Supplemental Table 18. L: vascular lumen. Scale bars = 50 μm.
into ECM components was observed (Figure 4D, Supplemental Tables 8 and 10). Indeed, of the 145 matrisome proteins that were increased in untreated HTRA1−/− mice and identified by 2 or more unique peptides in the second MS analysis, 88 proteins (60.7%) were decreased by candesartan treatment (Figure 4E and Supplemental Table 11).

Immunohistochemical analysis of the pial arteries of 24-month-old HTRA1−/− mice showed intimal thickening and lumi-
nal dilatation resembling those observed in CARASIL patients (Figure 5, A–C). These alterations were improved by candesartan treatment but not amlodipine treatment (Figure 5, A–C). On the other hand, the cross-sectional areas (CSAs) of the tunica media and the number of SMCs were not different (Figure 5, A and D and Supplemental Figure 12B). Even in the arterioles, where CARASIL induced the most prominent degeneration of the tunica media, there was no change in staining for α smooth muscle actin (αSMA; Supplemental Figure 12C and ref. 9). At 16 months of age, there was no increase in lumen diameter (Supplemental Figure 12D).

We further analyzed the lumen diameter under physiological pressure loading using the middle cerebral artery. An increase in vessel diameter in HTRA1−/− mice was also demonstrated under physiological pressure loading (Figure 5E).
Candesartan prevents a decrease in cerebral blood flow in HTRA1−/− mice. We examined cerebral blood flow in HTRA1−/− mice from 16–20 months of age using magnetic resonance imaging (MRI). The resting cerebral blood flow was significantly reduced in the cerebral cortex but not in the striatum of HTRA1−/− mice (Figure 6, A and B, Supplemental Figure 12E, refs. 41–43). Notably, candesartan administration normalized cerebral blood flow in HTRA1−/− mice (Figure 6, A and B). In the cerebral cortex, the vascular response to hypercapnia (10% CO₂ inhalation), a known cerebral vasodilator, did not differ between HTRA1−/− mice and HTRA1+/+ mice (Figure 6, A and C). Candesartan prevents a decrease in cerebral blood flow in HTRA1−/− mice. Candesartan was administered for a short (1 week at 4 months of age) or long (from 4 to 24 months of age) period. First, we identified the genes with expression levels that were altered by HTRA1 deficiency. We identified 12 and 11 differentially expressed genes at 4 and 24 months of age, respectively. There were, however, no overlapping genes between the groups (Supplementary Figure 13, A and B, and Supplemental Tables 12 and 13). The changes in the expression of only 4 and 6 of these genes were reproduced by droplet digital PCR (ddPCR) analysis (Supplementary Figure 13, A and B, and Supplemental Tables 14 and 15).

Regarding pericytes, electron microscopy revealed that pericytes were significantly detached from endothelial cells in HTRA1−/− mice (Figure 7C). The pericyte coverage rate of the capillary wall was also significantly reduced in HTRA1−/− mice (Figure 7D). However, treatment with candesartan did not improve the pericyte coverage rate (Figure 7D). Blood-brain barrier function was preserved in HTRA1−/− mice (Supplemental Figure 12, F and G and ref. 44).

Candesartan decreases the expression of Adamtsl2, Fn1, Ltbp-4, and Fbln5. To explore the molecular pathways affected by candesartan, we performed RNA sequencing (RNA-seq) analysis of the anterior and middle cerebral arteries of HTRA1+/+ mice and HTRA1−/− mice with or without candesartan treatment at 4 and 24 months of age. Candesartan was administered for a short (1 week at 4 months of age) or long (from 4 to 24 months of age) period. First, we identified the genes with expression levels that were altered by HTRA1 deficiency. We identified 12 and 11 differentially expressed genes at 4 and 24 months of age, respectively. There were, however, no overlapping genes between the groups (Supplementary Figure 13, A and B, and Supplemental Tables 12 and 13). As compared with gene expression levels of candesartan-treated and untreated HTRA1−/− mice, we identified 10 and 84 genes that were differentially expressed in short-term and long-term candesartan-treated HTRA1−/− mice, respectively (Figure 8, A and B and Supplemental Tables 14 and 15). Adamtsl2, which encodes a matrisome-associated protein involved in FN network formation, was the only gene that overlapped between the groups (Figure 8, A-C and refs. 46, 47). ddPCR analysis showed that short-term treatment with amlopidine also slightly reduced Adamtsl2 expression, whereas candesartan treatment more highly reduced Adamtsl2 expression (Figure 8, D and E). Adamtsl2 mRNA expression was decreased by 69.5% and 49.0% in short-term and long-term candesartan-treated HTRA1−/− mice, respectively, compared with untreated HTRA1−/− mice. In addition, ddPCR analysis revealed that candesartan but not amlopidine exerted inhibitory effects on Fn1, EDα, Fn1, Ltbp-4, and Fbln5 mRNA expression (Figure 8F) and that Timp3 mRNA expression was downregulat-
did not show degeneration of SMCs, they exhibited intimal thickening, alteration of the elastic lamina, accumulation of matrisome proteins in the intima, and vascular enlargement. In addition, HTRA1−/− mice showed decreased vascular distensibility and cerebral blood flow. Most importantly, we showed that the candesartan administration substantially improved these changes.

We first discuss which changes primarily contribute to arterial dysfunction. Thickening of the intima and degeneration of the tunica media are pathological hallmarks of CARASIL (7, 9, 48). In HTRA1−/− mice, intimal thickening developed, and matrisome

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Discussion

Here, we showed that the cerebral arteries of HTRA1−/− mice exhibited CARASIL-like pathological features. Although HTRA1−/− mice

Figure 7. Candesartan fails to prevent mural cell alterations in HTRA1−/− mice. (A) Quantification of the expression of contractile SMC markers in the anterior and middle cerebral arteries at 4, 12, 16, and 24 months of age. Only HTRA1−/− mouse data are shown as a ratio relative to the HTRA1+/+ mice (n = 5–8 animals per group). (B) The effect of candesartan treatment on the reduction in contractile SMC gene expression in HTRA1−/− mice. Expression levels relative to those in HTRA1+/+ mice are presented (n = 5 animals per group). (C) The pericyte-capillary adhesion ratio was estimated by analysis of electron micrographs. The upper panels show the formula used to determine the pericyte adhesion ratio. The pericyte adhesion ratio is the ratio of the length of the adhesion region between a pericyte and a capillary to the perimeter of the pericyte. The lower images are representative electron microscopic images of capillaries in 24-month-old HTRA1−/− mice with or without candesartan treatment. CD13-immunopositive pericytes (green) covering brain capillaries visualized by lectin staining (red). Scale bars = 50 μm. The bar graph shows pericyte coverage of brain capillaries in the cerebral cortex (n = 4 mice per group; 2–4 images per animal were analyzed). The data represent the mean ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001 with Bonferroni’s post hoc test (A, B, and D). *P < 0.05 with 2-tailed unpaired t test (C).
and refs. 49, 50). These results indicate that the matrisome protein accumulation contributes to arterial dysfunction in HTRA1−/− mice. In other organs, the accumulation of matrisome proteins was shown to increase arterial stiffness and reduce vascular distensibility, resulting in reduced blood flow (51–53). Therefore, even in HTRA1−/− mice, we speculate that the accumulated matrisome proteins promote stiffening of the cerebral arteries and reduce vascular distensibility, which may lead to a decrease in cerebral blood flow.

We considered which of the accumulated matrisome proteins are important in the development of arteriopathy. Decreased proteins accumulated in the intima. While no degeneration of the tunica media was observed, expression of contractile genes in SMCs and pericyte coverage were decreased. Importantly, candesartan treatment alleviated the accumulation of matrisome proteins in the intima and improved vascular distensibility and cerebral blood flow. On the other hand, the decreased expression of contractile genes in SMCs and reduced pericyte coverage were not improved. In addition, the functional impact of the reduced mRNA levels of contractile genes in SMCs may be weak because these changes were not reflected in the protein levels (Supplemental Table 1 and refs. 49, 50).
activity of HTRA1 resulted in the accumulation of its substrates FN, LTBP-4, TIMP3, fibrulin-5, and TGF-β1. Topological analysis of protein-protein interactions showed that FN is an important hub protein of the network of matrisome proteins accumulated in the arteries of HTRA1−/− mice. LTBP4 binds to the TGF-β1 signal protein of the network of matrisome proteins accumulated in the expression of the causative gene in each vessel. HTRA1 activity may be novel therapeutic candidates for CARASIL as well as sporadic CSVD. These approaches may open new avenues for preventing the progression of CSVD.

Methods
See Supplemental Data for additional methods.

Animals and study design. HTRA1−/− mice were obtained from Lexicon Pharmaceuticals. HTRA1−/− mice were backcrossed with C57BL/6Jc mice more than 10 times. Mouse genotypes were determined by PCR using tail DNA (Supplemental Figure 1, A and B and Supplemental Table 17). HTRA1−/− littermates were used as controls. For evaluation of cerebral blood flow, male HTRA1+/+ and HTRA1−/−
mice were used. In the other experiments, both female and male HTRA1+/− and HTRA1−/− mice were used to ensure an equal sex ratio.

**Sample preparation for data-independent acquisition (DIA) MS analysis.** For this analysis, we used 3 animals per group. Independent sample sets from different mice were used in the first and second MS analyses. Anterior and middle cerebral artery samples were precipitated in acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA) by using a water bath-type sonicator and then centrifuged at 15,000 g for 15 minutes at 4°C to remove the supernatant. The precipitate was extracted in 0.5% sodium dodecanoate and 100 mM Tris-HCl (pH 8.5) by using a water bath-type sonicator. The concentrations of the extracted proteins were measured by using a BCA protein assay kit (Thermo Fisher Scientific) and adjusted to 1 mg/mL with 0.5% sodium dodecanoate and 100 mM Tris-HCl (pH 8.5). A total of 20 μg protein extract was treated with 10 mM dithiothreitol at 50°C for 30 minutes and then subjected to alkylation with 30 mM iodoacetamide in the dark at room temperature for 30 minutes. The reaction with iodoacetamide was stopped with 60 mM cysteine for 10 minutes. The mixture was diluted with 150 μL of 50 mM ammonium bicarbonate and digested by adding 1 μg trypsin/Lys-C mix (Promega) overnight at 37°C. The digested sample was acidified with 30 μL of 5% TFA and then sonicated. The mixture was shaken for 5 minutes and centrifuged at 15,000 g for 5 minutes. The supernatant was desalted by using C18-StageTips and then dried with a centrifugal evaporator. The dried peptides were redissolved in 5% ACN and 0.1% formic acid (FA). The redissolved peptides were measured by using a colorimetric peptide assay kit (Thermo Fisher Scientific) and transferred to a hydrophilic-coated low-adsorption vial (ProteoSave vial; AMR Inc.).

**DIA-MS analysis.** Pretreatment for shotgun proteome analysis was performed as previously reported (78). Peptides were directly injected onto a 75 μm x 12 cm nanoLC capillary column (Nikkyo Technos Co.) at 40°C and then separated over an 80-min gradient at a flow rate of 100 nL/min using an Ultimate 3000 RSLCnano LC system (Thermo Fisher Scientific). Peptides eluted from the column were analyzed on a Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific) for overlapping window DIA-MS (78, 79). MS1 spectra were collected in the range of 495 to 785 m/z at 30,000 resolution, the automatic gain control target was set to 3 × 106, and the maximum injection time was set to auto, and the stepped normalized collision energies were 22, 26, and 30. The isolation width for MS2 was set to 4 m/z, and overlapping window patterns in 500 to 7780 m/z were used as window placements optimized by Skyline (80). Raw data files of the LC-MS/MS analyses have been deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the JPOST partner repository (http://jpostdb.org) with the following identifiers: JPOST Repository Database IDs, JPST000994 and JPST001052; ProteomeXchange IDs, PXD022298 and PXD023477. RS proteome samples were searched against a mouse spectral library using Scaffold DIA (Proteome Software, Inc.). The mouse spectral library was generated from the mouse protein sequence database (UniProt id UP000000589, reviewed, canonical, 17,042 entries) by Prosite (81). The Scaffold DIA search parameters were as follows: experimental data search enzyme, trypsin; maximum missed cleavage sites, 1; precursor mass tolerance, 10 ppm; fragment mass tolerance, 10 ppm; and static modification, cysteine carbamidomethylation. The protein identification threshold for both peptides and proteins was set to FDR of less than 1%. Peptide quantification was performed with the EncyclopeDIA algorithm (82) in Scaffold DIA. For each peptide, the 4 highest quality fragment ions were selected for quantification. Protein quantification was estimated from the summed peptide quantification. Proteins identified by 2 or more unique peptides were compared between groups. Matrisome proteins were extracted according to MatrisomeDB (http://matrisomedb.pepchem.org/) (83). GO enrichment analysis was performed with PANTHER (http://geneontology.org/). An adjusted P less than 0.05 and a fold change in protein abundance greater than 1.5 or less than 0.5 were set as significance thresholds. To assess the relative protein abundance for different proteins, we normalized DIA protein intensity to molecular weight to correct for the higher probability of peptide identification in larger proteins (84, 85).

**Protein-protein interaction network analysis.** The protein-protein network was constructed, visualized, and topologically analyzed with Cytoscape 3.8.0 (86, 87). Specifically, the Cc, Cp, and D were calculated using the Cytoscape Network Analysis module.

**Vascular and brain fresh tissue collection.** Blood was removed by transcardial perfusion with HBSS. Segments of the anterior and middle cerebral arteries with medium-sized branches were isolated from excised mouse brains in ice-cold MEM under a dissecting microscope, immediately frozen on dry ice, and stored at −80°C (88). The cerebral cortex was dissected, and the pia matter was removed by rolling on dry filter paper.

**ECM preparation.** ECM was prepared according to a previously described method (89). Briefly, anterior and middle cerebral arteries were homogenized in sodium deoxycholate (DOC) lysis buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium DOC, and 50 mM Tris-HCl [pH 7.5]) and then cleared by centrifugation. After the soluble tissue materials were collected, the insoluble fraction (ECM fraction) was homogenized in SDS lysis buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, and 0.5% NP-40). After homogenization, 2-mercaptoethanol was added to the lysates at a concentration of 5%, and then the lysate was incubated for 30 minutes at room temperature. After centrifugation, SDS-solubilized ECM fractions were subjected to immunoblotting as described below.

**Immunoblotting.** Protein samples were separated by SDS polyacrylamide gel electrophoresis and transferred onto PVDF membranes. The required volume of each ECM fraction sample was determined according to the protein content in the sodium DOC-soluble fraction. The membranes were probed with the following antibodies: mouse anti-LTBP-4 antibody (AF2885; 1:2000; R&D Systems), rabbit anti-LTBP-1 antibody (ab78294; 1:1000; Abcam), rabbit anti-FN antibody (ab2413; 1:1000; Abcam), mouse anti-EDA’ FN antibody (F6140; 1:3000; Sigma), rabbit anti- elastin antibody (PR385; 1:2000; Elastin Products), goat anti-LAP-β1 (AF-246-NA; 1:100; R&D Systems), rabbit anti-TIMP3 antibody (ab58804; 1:500; Abcam), rabbit anti-fibulin-5 (ab109428; 1:1000; Abcam), rabbit anti-TGF-β2 antibody (ab36495; 1:1000; Abcam), mouse anti-β-actin antibody (M177-3; 1:5000; Medical and Biological Laboratories Co.), rabbit anti-phospho-SMAD2 antibody (3108; 1:500; Cell Signalling Technologies), rabbit anti-phospho-SMAD3 antibody (ab52903; 1:1000; Abcam), rabbit anti-phospho-SMAD1 antibody (ab214423; 1:1000; Abcam), rabbit anti-phospho-SMAD5 antibody (ab92698; 1:5000; Abcam), rabbit anti-phospho-Erk1/2 (p44/42 MAPK) antibody (4370S; 1:1000; Cell Signalling Technologies), rabbit anti-phospho-Erk5 antibody (3371S; 1:1000; Cell Signalling Technologies), rabbit anti-phospho-p38
The animals were anesthetized with 3% to 5% isoflurane for induction of anesthesia described by Ewing et al. (96). The maps were generated from cerebral perfusion image, the T1 map, and the MTR map according to the delay. Quantitative cerebral blood flow maps were obtained from the ic secant inversion pulse (95) and a 32-point (20–5000 ms) inversion recovery sequence performed using a centric ordered snapshot-FLASH with a hyperbolic sinc function for adiabatic inversion. T1 measurements were acquired under the same conditions used for cerebral perfusion measurements but without an axial gradient for adiabatic inversion. The first branch-free segment of the middle cerebral artery was carefully isolated and mounted on glass cannulas in a pressure myograph chamber (Danish Myo Technology [DMT]). Vessels were bathed with warm oxygenated PSS at an intraluminal pressure of 80 mmHg, and the vessels were allowed to equilibrate for 30 minutes. The passive structure of vessels was assessed with increasing intraluminal pressure from 5 to 140 mmHg in 20-mmHg increments. Vessel lumen inner diameters (IDs) were tracked and recorded after 5 minutes at each pressure step by video microscopy using Myoview software (DMT). Passive distensibility was calculated as the percent change in ID from the original diameter (IDO, at 5 mmHg) at a given pressure (IDP): [(IDP - ID0) / ID0] × 100 (%). (91–93).

MRI measurements. MRI experiments were performed with an 18-cm bore 7 T horizontal magnet (Magnex Scientific) and a Varian Unity-INOVA-300 system (Varian Inc.) equipped with an actively shielded gradient. A birdcage transmission coil with a 6-cm internal diameter and quadrature receiving coils were used in the study. Cerebral perfusion was assessed by continuous arterial spin labeling (CASL; ref. 94) with a centric ordered variable tip angle gradient-echo (VTE-GRE). Three seconds of radiofrequency (RF) at ± 10 mm from the imaging slice were irradiated with a 1 g/cm axial gradient alternately in 0.8 seconds before the VTE-GRE sequence. Sixty-four pairs of images were summed to enhance the signal-to-noise ratio. The magnetization transfer ratio (MTR) was evaluated under the same conditions used for cerebral perfusion measurements but without an axial gradient for adiabatic inversion. TI measurements were performed using a centric ordered snapshot-FLASH with a hyperbolic secant inversion pulse (95) and a 32-point (20–5000 ms) inversion delay. Quantitative cerebral blood flow maps were obtained from the cerebral perfusion image, the T1 map, and the MTR map according to the theory described by Ewing et al. (96). The maps were generated using MRI image calculation software (MR Vision, MR Vision Co.). The animals were anesthetized with 3% to 5% isoflurane for induction followed by 1.2% isoflurane for maintenance. Cerebral blood flow in normocapnia was measured under 1 L/min of 30% O2/70% N2O under spontaneous breathing through a face mask. Hypercapnia was induced with 1 L/min of 30% O2/60% N2O/10% CO2. Cerebral blood flow in hypercapnia was measured after 10 minutes of prolonged 10% CO2 exposure following induction of hypercapnia under spontaneous breathing. The rectal temperature was maintained at 37°C ± 0.5°C throughout the measurements using a homemade air conditioning system. The cerebral blood flow was assessed in the cerebral cortex and striatum. Data for the mouse white matter, which is small in size, could not be obtained by CASL due to the partial volume effect (41).

RNA-seq and analysis. For this analysis, we used 3 animals per group. Polyadenylated mRNA was enriched by an oligo-dT probe. The RNA-seq library was prepared using an Illumina TruSeq Stranded mRNA Sample Prep Kit according to the manufacturer’s instructions. Quality control and quantification of the library were performed using an Agilent Bioanalyzer and a KAPA Library Quantification Kit (KAPA Biosystems). All 9 libraries were pooled and sequenced on the Illumina HiSeq 2500平台 (high output mode, 50-base single read) on a single flow cell lane. Raw reads were adapter trimmed and quality filtered using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html), and resulting reads with N and those derived from ribosomal RNA were excluded. The clean reads were mapped to the mouse reference genome sequence (Grcm38/mm10) using TopHat (97). Mapped reads were counted with HTseq (98). We used DEseq2 to identify differentially expressed genes with an adjusted p value less than 0.05 (99). Genes containing samples with a read count of 0 were excluded from subsequent analyses. GO enrichment analysis was performed with PANTHER (http://geneontology.org/). KEGG pathway enrichment analyses were performed with the clusterProfiler R package (100). These data are available from the DDBJ Sequence Read Archive (https://ddbj.nig.ac.jp/DRASearch/) (accession numbers DRA011075 and DRA011076).

Statistics. Statistical computation was performed using IBM SPSS 22. The data were first subjected to the Shapiro-Wilk test (for fitting to the Gaussian distribution) and Levene’s test (for equal variance). One-way ANOVA, 2-way repeated ANOVA, or 2-tailed unpaired t test was used for data with a Gaussian distribution and equal variance. Subsequently, the data were analyzed by the Bonferroni’s post hoc test. Alternatively, data with unequal variance were analyzed by the Steel-Dwass test or Mann-Whitney U test. P less than 0.05 was regarded as statistically significant. The number of mice used for each analysis is indicated in the figure legends. All data are presented as the mean ± SD. DEseq2 was used to identify differentially expressed genes in the RNA-seq data with an adjusted P value less than 0.05 (99, 101). Differential protein levels from DIA-MS proteomics were analyzed using ROPECA with FDR cutoff set to 0.05 (102, 103).

Study approval. All animal experiments described were approved by the Animal Use and Care Committee of Niigata University and followed the guidelines of the National Institutes of Health. The human study was approved by the Institutional Ethical Review Board of Niigata University. Written informed consent was obtained from the families, and all clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki.

Author contributions. TK designed the research study, conducted the experiments, acquired the data, analyzed the data and wrote the manuscript.
RM, YS, NF, SH, TS, AK, YK, and MU conducted the experiments and acquired and analyzed the data. SS, FK, and HI conducted the experiments, acquired and analyzed the data and wrote the manuscript. MF, MM, and YH analyzed the data and provided expertise related to the experiments. AS, RS, SA, MK, and AS designed the research study and wrote the manuscript. MH, MI, and MN designed the research study and provided expertise related to experiments. HK and SM provided human materials. HN and ST designed the research study. OO designed the research study, analyzed the data and wrote the manuscript. All authors participated in interpreting the results and revising the manuscript.

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Address correspondence to: Osamu Onodera, 1-757 Asahimachi-dori, Chuo-Ku, Niigata City, Niigata 951-8585, Japan. Phone: 81.25.227.0684; E-mail: onodera@bri.niigata-u.ac.jp.


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