Cerebral small-vessel disease protein HTRA1 controls the amount of TGF-β1 via cleavage of proTGF-β1

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Received December 14, 2010; Revised January 27, 2011; Accepted February 8, 2011

Cerebral small-vessel disease is a common disorder in elderly populations; however, its molecular basis is not well understood. We recently demonstrated that mutations in the high-temperature requirement A (HTRA) serine peptidase 1 (HTRA1) gene cause a hereditary cerebral small-vessel disease, cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL). HTRA1 belongs to the HTRA protein family, whose members have dual activities as chaperones and serine proteases and also repress transforming growth factor-β (TGF-β) family signaling. We demonstrated that CARASIL-associated mutant HTRA1s decrease protease activity and fail to decrease TGF-β family signaling. However, the precise molecular mechanism for decreasing the signaling remains unknown. Here we show that increased expression of ED-A fibronectin is limited to cerebral small arteries and is not observed in coronary, renal arterial or aortic walls in patients with CARASIL. Using a cell-mixing assay, we found that HTRA1 decreases TGF-β1 signaling triggered by proTGF-β1 in the intracellular space. HTRA1 binds and cleaves the pro-domain of proTGF-β1 in the endoplasmic reticulum (ER), and cleaved proTGF-β1 is degraded by ER-associated degradation. Consequently, the amount of mature TGF-β1 is reduced. These results establish a novel mechanism for regulating the amount of TGF-β1, specifically, the intracellular cleavage of proTGF-β1 in the ER.

INTRODUCTION

Ischemic cerebral small-vessel disease is a common disorder in elderly populations and contributes to cognitive decline and stroke (1). However, little is known about the molecular basis of the disease (1). We recently determined that mutations in the high-temperature requirement A (HTRA) serine peptidase 1 (HTRA1) gene cause a hereditary cerebral small-vessel disease, cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL). HTRA1 belongs to the HTRA protein family, whose members have dual activities as chaperones and serine proteases and also repress transforming growth factor-β (TGF-β) family signaling. We demonstrated that CARASIL-associated mutant HTRA1s decrease protease activity and fail to decrease TGF-β family signaling. However, the precise molecular mechanism for decreasing the signaling remains unknown. Here we show that increased expression of ED-A fibronectin is limited to cerebral small arteries and is not observed in coronary, renal arterial or aortic walls in patients with CARASIL. Using a cell-mixing assay, we found that HTRA1 decreases TGF-β1 signaling triggered by proTGF-β1 in the intracellular space. HTRA1 binds and cleaves the pro-domain of proTGF-β1 in the endoplasmic reticulum (ER), and cleaved proTGF-β1 is degraded by ER-associated degradation. Consequently, the amount of mature TGF-β1 is reduced. These results establish a novel mechanism for regulating the amount of TGF-β1, specifically, the intracellular cleavage of proTGF-β1 in the ER.

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proteases (9). Studies have shown that members of the HTRA family decrease transforming growth factor-β (TGF-β) family signaling (10, 11). We have previously shown increased expression of TGF-β1 associated with CARASIL (2). We have also shown that CARASIL-associated mutant HTRA1s exhibit decreased protease activity and fail to decrease TGF-β family signaling (2). Moreover, the ED-A fibronectin and versican, which are induced by increased TGF-β signaling, accumulate in the hypertrophic intima of cerebral small arteries in patients with CARASIL (2). Finally, TGF-β1 is increased in the cerebral small arteries of patients with CARASIL (2). These findings indicate that increased TGF-β signaling plays a pivotal role in the pathogenesis of cerebral small-vessel disease in CARASIL.

TGF-β signaling is temporally and spatially regulated by a balance among maturation, sequestration and presentation (12–14). TGF-β is synthesized as a homodimeric proprotein (proTGF-β) and is subsequently cleaved into an N-terminal dimeric propetide, latency-associated peptide (LAP) and a C-terminal mature TGF-β by a proprotein convertase, such as furin, in the trans-Golgi network. LAP forms a non-covalent complex with a dimer of mature TGF-β. This complex binds to a latent TGF-β-binding protein (LTBP), and the bound complex is then secreted and anchored to the extracellular matrix, resulting in sequestration of the mature TGF-β in the extracellular space. The sequestered mature TGF-β is activated by serine protease, matrix metalloproteinase or acidic microenvironments in the extracellular space (13). The extracellular matrix, which stores TGF-β in a complex with LAP and LTBP, also regulates the bioavailability of TGF-β (14). The activation of mature TGF-β is the rate-limiting step for TGF-β signaling. The tight regulation of bioavailability of TGF-β in intracellular and extracellular spaces is important to regulate its signaling.

HTRA1 decreases TGF-β signaling through its protease activity and might play a pivotal role in human cerebral small-vessel disease; however, the precise molecular mechanism by which HTRA1 decreases TGF-β signaling remains unknown (11). Previous studies suggested the possibility of cleavage of mature TGF-β by HTRA1 in the extracellular space, but the issue remains controversial (15, 16). Here, we show that the increased endogenous and exogenous HTRA1 effectively decreases the TGF-β1 signal introduced by co-transfected proTGF-β1 but not mature TGF-β1 in culture medium. Furthermore, we show that HTRA1 cleaves the pro-domain of proTGF-β1 in the endoplasmic reticulum (ER), before furin processes proTGF-β1 in the trans-Golgi network. The aberrant cleaved products of proTGF-β1 are degraded by the ER-associated degradation (ERAD) system, leading to a reduced amount of mature TGF-β1. The intracellular cleavage of proTGF-β1 is a novel mechanism to regulate the amount of TGF-β1 in human cerebral small vessels.

RESULTS
Increased TGF-β1 signaling plays a pivotal role in pathogenesis of cerebral small-vessel arteriopathy in CARASIL

We have previously shown increased expression of TGF-β and ED-A fibronectin, induced by TGF-β signaling, in small arterial walls of patients with CARASIL (2) (Fig. 1A–D). To elucidate whether the increased TGF-β1 signaling was restricted to cerebral small arteries, we investigated the expression of ED-A fibronectin in other arteries that did not show any pathologic change (4–6). We found no expression of ED-A fibronectin in arterial walls of coronal tissue, renal arteries or the aorta from a patient with CARASIL (Fig. 1E–J). In addition, we found expression of hyaluronan in the small cerebral arterial walls in the patient with CARASIL (Fig. 1K and L). Hyaluronan is an extracellular matrix protein that is induced by TGF-β1 signaling (17). In endothelial cells of small cerebral arteries from the patient, the expression of phosphorylated Smad2, which is induced by TGF-β1 signaling, increased (Fig. 1M and N). In addition, the small cerebral arterial wall of the patient exhibited increased immunostaining for LAP, suggesting that synthesis of TGF-β increases in small cerebral arteries in CARASIL (Fig. 1O and P).

HTRA1 decreases the TGF-β1 signaling pathway via its interaction with proTGF-β1

To elucidate the molecular mechanism by which HTRA1 decreases TGF-β signaling, we first addressed whether HTRA1 affects TGF-β signaling via its interaction with mature TGF-β1 or proTGF-β1. We investigated the effect of HTRA1 by two different methods: by stimulation of TGF-β1 signaling via transfection with a constitutively active proTGF-β1 (C223S/C225S) expression vector or by addition of a recombinant mature TGF-β1 in culture medium. Both methods activated TGF-β1 signaling, and HTRA1 decreased TGF-β1 signaling induced by a transfected constitutively active proTGF-β1 (C223S/C225S) expression plasmid in C2C12 cells (Fig. 2A, lanes 1, 5–7) (2, 18). In contrast, HTRA1 failed to decrease TGF-β1 signaling induced by the recombinant mature TGF-β1 in the culture medium (Fig. 2A, lanes 1–4). Similar results were obtained in HEK293T and HepG2 cells (data not shown). Co-immunoprecipitation experiments demonstrated an association between wild-type proTGF-β1 and wild-type or the S328A mutant of HTRA1 in HEK293T cells (Fig. 2B and C). The S328A mutant of HTRA1 is an artificial protein that abolishes protease activity in HTRA1 (19). These results suggest that HTRA1 decreases TGF-β1 signaling via interaction with proTGF-β1 but not mature TGF-β1.

Next, to determine whether HTRA1 decreases TGF-β1 signaling in the intracellular or extracellular space, we carried out cell-mixing experiments (Fig. 2D and E) (20). We prepared two populations of HEK293T cells: responding and stimulator cells. The responding cells contained the TGF-β reporter (SβE)a-Luc vector (R in Fig. 2D); the stimulator cells contained the constitutively active proTGF-β1 (C223S/C225S) expression plasmid (S in Fig. 2D). As expected, stimulator cells induced TGF-β1 signaling in responding cells (Fig. 2E, lanes 1 and 2). Next, we transfected a wild-type or S328A HTRA1 expression vector into stimulator or responding cells, followed by a TGF-β reporter assay. The wild-type HTRA1 efficiently decreased TGF-β1 signaling when it was expressed in the stimulator cells but not in the responding cells (Fig. 2E, lanes 3 and 5). In addition, the culture media from cells expressing wild-type HTRA1 in stimulator cells,
but not in responding cells, decreased the phosphorylation of Smad2, a downstream effector of the TGF-β family signaling pathway (Fig. 2F). In contrast, the cells transfected with S328A HTRA1 showed no effect on TGF-β1 signaling or phosphorylation of Smad2. These results indicate that HTRA1 decreases TGF-β1 signaling in the intracellular space in a protease activity-dependent manner.

Endogenous HTRA1 decreases TGF-β1 signaling in fibroblasts from CARASIL with R370X mutation

To avoid overexpression-related artifacts, we investigated the effect of endogenous HTRA1 on TGF-β signaling. We had previously shown that R370X HTRA1, which is a nonsense mutation observed in patients with CARASIL, retained its protease activity (2). However, the R370X mutation resulted in marked depletion of HTRA1 mRNA by nonsense-mediated mRNA decay (2). It has been shown that the aminoglycoside antibiotics can enable read-through of a premature stop codon, resulting in stabilization of nonsense-containing mRNA and subsequently translating full-length proteins (21,22). Thus, the expression of endogenous HTRA1 could be induced by gentamicin treatment in fibroblasts derived from a CARASIL patient with the R370X mutation. We found that the R370X HTRA1 protein co-immunoprecipitated with wild-type proTGF-β1 (Fig. 3A). Under gentamicin treatment, the HTRA1 mRNA level in R370X fibroblasts increased from ~5 to ~40% of that in control fibroblasts (Fig. 3B). In addition, gentamicin increased the amount of R370X HTRA1 protein and a small amount of full-length HTRA1 protein (Fig. 3C and D). The TGF-β reporter assay demonstrated that gentamicin treatment restored TGF-β1 signaling in the R370X fibroblasts to a level comparable with that of control fibroblasts (Fig. 3E). In contrast, gentamicin treatment had no effect on TGF-β1 signaling triggered by recombinant mature TGF-β1 in the cultured medium (Fig. 3E). Gentamicin did not affect the level of TGF-β1 signaling-related gene mRNA expression, including Smad2, Smad3, Smad4, TGF-β receptor-1 (TGFBR1), TGF-β receptor-2, (TGFBR2) and TGF-β1 (Supplementary Material, Fig. S1). These results indicate that intrinsic HTRA1 decreases TGF-β1 signaling via interaction with proTGF-β1 under physiological conditions.

HTRA1 cleaves LAP and reduces the amount of mature TGF-β1

Because HTRA1 is a serine protease, we investigated whether HTRA1 cleaves proTGF-β1. HEK293T cells were
HTRA1 decreases TGF-β1 signaling via interaction with proTGF-β1 in the intracellular space. (A) HTRA1 decreased TGF-β1 signaling induced by proTGF-β1. Amino acid substitution of the serine protease motif S328A, which abolishes the protease activity in HTRA1, was used as a negative control (19). C2C12 cells were co-transfected with wild-type (WT) or mutated HTRA1 (S328A) expression plasmid and the following constructs: (SBE)₄-firefly luciferase expression plasmid (internal control reporter vector) and vectors containing Smad2 and Smad4. In lanes 5–7, the cells were also co-transfected with proTGF-β1 with two point mutation (C223S/C225S) expression vectors (2,18). In lanes 2–4, the cells were treated with 10 ng/ml mature TGF-β1 for 24 h in culture medium. Note that HTRA1 decreases TGF-β1 signaling induced by proTGF-β1 (lane 6) but not mature TGF-β1 in the culture medium (lane 3). Data represent the mean with standard error of normalized firefly luciferase/renilla luciferase activities from three independent experiments. Asterisk indicates significant difference (*P < 0.05, Tukey multiple-comparison test). (B and C) HTRA1 binds to proTGF-β1. HEK293T cells were transfected with a wild-type proTGF-β1 expression vector either alone or in combination with a vector encoding V5-tagged HTRA1 wild-type or V5-tagged HTRA1 S328A. Cell lysates were subjected to immunoprecipitation (IP) with the use of the anti-V5 (B) or anti-LAP antibody (C). ProTGF-β1 and HTRA1 were shown by immunoblotting (IB) with the use of the anti-LAP antibody for proTGF-β1 or the anti-V5 antibody for HTRA1. An aliquot of each cell lysate before immunoprecipitation (input) was used as a loading control. (D–F) Cell-mixing experiments were carried out with the use of HTRA1. (D) Schematic diagram of cell-mixing experiments. We prepared two populations of HEK293T cells: responding and stimulator cells (20). The responding cells (white box: R) were co-transfected with pRL-TK renilla luciferase expression plasmid, (SBE)₄-Luc, and vectors containing Smad2 and Smad4. The stimulator cells contained the proTGF-β1 C223S/C225S expression plasmid (gray box: S) (18). +, HTRA1 indicates transfection with HTRA1 expression plasmid. (E) HTRA1 decreased TGF-β1 signaling in the intracellular space. Note that in lane 3, HTRA1 efficiently blocked signaling only when expressed in cells producing proTGF-β1 C223S/C225S. Data represent the mean with standard error of normalized firefly luciferase/renilla luciferase activities from three independent experiments. Asterisk indicates significant difference (*P < 0.05, Tukey multiple-comparison test). (F) HTRA1 decreased the subsequent phosphorylation of Smad proteins in the intracellular space. HepG2 cells were incubated for 1 h with conditioned media collected from cells shown in (E). Cells were harvested for immunoblotting with anti-phospho-Smad2, as a read-out of mature TGF-β1 available in conditioned media. The phosphorylated Smad proteins were shown by immunoblotting with anti-Smad2/3 or anti-phospho-Smad2/pSmad2. Anti-Smad2/3 immunoblotting served as a loading control.

Simultaneously transfected with HTRA1 and wild-type proTGF-β1 constructs, followed by immunoblotting with an anti-LAP antibody that recognizes a pro-domain of proTGF-β1. We observed ~20 to ~30 kDa products that increased according to the amount of transfected HTRA1 constructs (Fig. 4A, upper panel, lanes 1–4). Moreover, the amount of mature TGF-β1 in culture media decreased as the amount of transfected HTRA1 increased (Fig. 4A, middle panel, lanes 1–4 and Fig. 4B). In contrast, we did not observe these cleaved products and decrement of the mature TGF-β1 in S328A HTRA1 (Fig. 4A upper and middle panels, lanes 5 and 4B). The possibility that HTRA1 influences the level of exogenous proTGF-β1 mRNAs was excluded by quantitative real-time reverse transcription–polymerase chain reaction (RT–PCR) (Fig. 4C).

Next, we investigated whether HTRA1 cleaves LAP, which is the N-terminal of proTGF-β1, or mature TGF-β1, which is the C-terminal of proTGF-β1. We co-incubated recombinant HTRA1 (500 nm) with either recombinant LAP (100 nm) or recombinant mature TGF-β1 (100 nm) at two time points and analyzed the materials by western blotting (Fig. 4D) (23). HTRA1 cleaved LAP in a time-dependent manner (Fig. 4D, upper panel, lanes 2 and 5), whereas HTRA1 failed to cleave mature TGF-β1 (Fig. 4D, bottom panel, lanes 2 and 5).

Because the bacterial HtrA homolog DegP has chaperone activity at low temperatures, it has been thought that human HTRA1 also has this activity (24). However, the chaperone
Figure 3. Restoration of intrinsic HTRA1 decreases the increasing TGF-β1 signaling in fibroblasts from a patient with CARASIL. (A) R370X HTRA1 binds to proTGF-β1. HEK293T cells were transfected with a wild-type proTGF-β1 expression vector either alone or in combination with a vector encoding V5-tagged HTRA1 wild-type or R370X. Cell lysates were subjected to immunoprecipitation (IP) with the use of the anti-LAP antibody. ProTGF-β1 and HTRA1 were shown by immunoblotting (IB) with the use of the anti-LAP antibody for proTGF-β1 or the anti-V5 antibody for HTRA1. An aliquot of each cell lysate before immunoprecipitation (input) was used as a loading control. (B–D) Gentamicin treatment increased the abundance of HTRA1 mRNA and protein in fibroblasts from a patient with CARASIL carrying the R370X mutation. (B) HTRA1 mRNA levels in cultured skin fibroblasts from the subject carrying R370X HTRA1 as a percentage of their levels in cells from control subjects (n = 2) with or without gentamicin for 48 h. The bars represent the standard errors. (C and D) Western blot analysis of HTRA1 using the cultured skin fibroblasts of the subject carrying R370X and the control subject. Full-length HTRA1 is shown in a long exposure (D). HTRA1 mRNA (B) and truncated protein (C) expression were increased by increasing the concentrations of gentamicin. (E) Fibroblasts from two control subjects and a subject carrying R370X HTRA1 were co-transfected with (SBE)₄-Luc vector, pRL-TK renilla luciferase expression vectors (18) or treated with 10 ng/ml mature TGF-β1, LAP and LTBP, we investigated whether the presence of LTBP influences cleavage of proTGF-β1 or reduction of secreted mature TGF-β1 by HTRA1. Specifically, we downregulated the expression of LTBP-1 using siRNA. We found that downregulation of LTBP1 showed no effect on the cleavage activity of HTRA1 by regard to wild-type proTGF-β1 or decreased the amount of secreted mature TGF-β1 (Supplementary Material, Fig. S3).

ProTGF-β1 products cleaved by HTRA1 are degraded by ERAD

Previous studies have revealed that furin cleaves proTGF-β in the trans-Golgi network (12,26,27). To investigate the cell organelle in which HTRA1 cleaves proTGF-β, we transfected HTRA1 or furin and wild-type proTGF-β1 in HEK293T cells and treated the cells with brefeldin A, which disassembles the Golgi apparatus, or monensin, which prevents transfer of proteins from the Golgi apparatus to the plasma membrane. As expected, the cleavage of wild-type proTGF-β1 by furin was inhibited by brefeldin A (Fig. 5A, lanes 1 and 2) but not by monensin (Fig. 5A, lane 1 and 3). In contrast, the cleavage of wild-type proTGF-β1 by HTRA1 was not inhibited by either brefeldin A or monensin (Fig. 5A, lanes 5 and 6), indicating that HTRA1 cleaves proTGF-β1 in the ER. Confocal microscopy demonstrated that HTRA1-GFP and wild-type proTGF-β1 consistently overlapped with the ER (Fig. 5B).

We then investigated whether these HTRA1-cleaved products are processed by furin, which is a proprotein convertase enzyme important in TGF-β1 maturation. HTRA1 and wild-type proTGF-β1 were co-transfected with or without furin in HEK293T cells. As expected, when wild-type proTGF-β1 and furin were co-transfected, LAP and secreted mature TGF-β1 were increased (Fig. 5C, lane 2). However, in the presence of HTRA1, furin had no apparent effect on the production of cleaved products and the amount of secreted mature TGF-β1. These results indicate that these cleaved products are not processed by furin (Fig. 5C, lanes 3 and 4).

If proper maturation fails, the aberrant products are degraded. Therefore, we speculated that the aberrant cleaved products produced by HTRA1 can be degraded by proteasome-dependent machinery. Treatment with proteasome inhibitors, MG-132 and epoxomicin, increased the amount of...
HTRA1-cleaved products (Fig. 5D, lanes 3 and 6). Furthermore, these cleaved products were increased in the presence of eeyarestatin I, which is the ERAD-specific inhibitor (Fig. 5E, lanes 2 and 3) (28). Taken together, these data indicate that HTRA1 cleaves proTGF-β1 in the ER and these cleaved products are degraded by ERAD, resulting in reduction of the amount of secreted mature TGF-β1.

To investigate whether HTRA1 cleaves proTGF-β1 under physiologic conditions, we performed immunoprecipitation and western blot experiments on fibroblasts from a control and the patient carrying the R370X mutation. We found endogenous cleaved proTGF-β1 products in both control and R370X fibroblasts (Fig. 5F, lane 1 and 2). Since we had shown that HTRA1-cleaved products are degraded by ERAD, we investigated whether these products were increased by MG-132. Treatment with MG-132 increased the amount of cleaved products in a control, but not in the R370X fibroblast (Fig. 5F, lane 3 and 4).

CARASIL-associated mutated HTRA1s bind proTGF-β1 but are unable to cleave it

Next we investigated whether CARASIL-associated mutated HTRA1s bind proTGF-β1 and cleave it. Co-immunoprecipitation assays revealed that CARASIL-associated mutated HTRA1s bind to wild-type proTGF-β1 (Fig. 6A and B); however, we did not detect the cleaved products of wild-type proTGF-β1 in the CARASIL-associated mutated HTRA1-transfected cells (Fig. 6C, upper panel). Moreover, CARASIL-associated mutated HTRA1s did not alter the amount of mature TGF-β1 in conditioned media (Fig. 6C, middle panel, and D).

DISCUSSION

We demonstrated that HTRA1 decreases TGF-β1 signaling triggered by proTGF-β1 in the intracellular space but not mature TGF-β1 in the extracellular space. We further showed that HTRA1 cleaved LAP, the N-terminal of proTGF-β1, in the ER, and the cleaved products were degraded by ERAD (Fig. 7). The intracellular aberrant cleavage of proTGF-β1 is a novel mechanism to regulate the amount of mature TGF-β1. The result that endogenous R370X HTRA1 decreases TGF-β1 signaling triggered by proTGF-β1, but not mature TGF-β1, further confirms our conclusion that HTRA1 interacts with proTGF-β1 under physiologic conditions.
One might suggest that TGF-β1 or LAP is cleaved by HTRA1 in the extracellular space; however, this is not likely. HTRA1 decreases TGF-β1 signaling; however, cleavage of LAP in the extracellular space may release mature TGF-β, resulting in increased TGF-β1 signaling. With regard to TGF-β1, the mature TGF-β is rapidly degraded under physiological conditions (29). Moreover, we and others have demonstrated that HTRA1 is unable to decrease TGF-β1 signaling triggered by recombinant mature TGF-β1 in culture medium (15).

**Figure 5. HTRA1 cleaves proTGF-β1 in the ER and cleaved proTGF-β1 is degraded by ERAD.**

(A) HTRA1 cleaves proTGF-β1 in the ER. HEK293T cells were co-transfected with the indicated expression vectors in the presence of 2 μg/ml breafeldin A (BFA) or 5 μM monensin (MON) for 16 h and then lysed. Cell lysates were subjected to immunoblotting with the anti-LAP antibody. The 30 kDa cleaved products representing LAP are shown in lanes 1 and 3. Cleaved proTGF-β1 products by HTRA1 were increased in cell lysates treated with either breafeldin A or monensin (lanes 5 and 6). (B) Localization of HTRA1 and proTGF-β1 in the ER. HEK293T cells were transfected with GFP-tagged HTRA1 and stained with ER-tracker. A confocal image of HEK293 cells shows HTRA1 (green), ER (red) and the overlay. The merged image indicates that GFP-tagged HTRA1 was localized in the ER. HEK293T cells were co-transfected with wild-type proTGF-β1 expression plasmid and ER-targeted plasmid, followed by immunostaining with an anti-LAP antibody. A confocal image shows proTGF-β1 (green), ER (red) and the overlay. The merged image indicates that proTGF-β1 was localized in the ER. Scale bar represents 10 μm. (C) Furin does not process cleaved TGF-β1. There was no difference in the pattern of cleaved products and the amount of secreted mature TGF-β1 regardless of furin (lanes 3 and 4), indicating that furin does not influence these cleaved products derived from wild-type proTGF-β1. (D) Treatment with proteasome inhibitor accumulates the fragments of proTGF-β1 generated by HTRA1. Both MG-132 and epoxomicin increased the amount of cleaved products, indicating that these products may be degraded by the ubiquitin-proteasome pathway. (E) ERAD inhibitor leads to an accumulation of the fragments of proTGF-β1 generated by HTRA1. HEK293T cells co-transfected with HTRA1 and wild-type proTGF-β1 were treated with 20 nM eeyarestatin I, a chemical inhibitor that can block ERAD, for 12 h. (F) ProTGF-β1 cleaved products in fibroblast from a control and a patient with CARASIL carrying the R370X mutation. Cell lysates from fibroblasts were subjected to immunoprecipitation (IP) with the use of the anti-LAP antibody. ProTGF-β1 was shown by immunoblotting (IB) with the use of the anti-LAP antibody. In addition to a 45 kDa product corresponding to endogenous proTGF-β1, additional cleaved products were observed around ~20 kDa to ~30 kDa. MG-132 increased the amount of cleaved products in control fibroblasts.
It has been suggested that TGF-β synthesis is in excess and its activation is a rate-limiting step in its bioavailability (30). However, it has recently been reported that Emilin1 decreases TGF-β1 expression by binding to proTGF-β1 and preventing its processing by furin (20). This mechanism is similar to that of HTRA1, as reported in this paper. Indeed, wild-type and disease-associated mutants of HTRA1 bind to the proTGF-β1; however, only the wild-type HTRA1 is able to cleave the proTGF-β1 and decrease the amount of mature TGF-β1. Thus, the cleavage of proTGF-β1 by HTRA1 is crucial to the molecular mechanism that decreases TGF-β1 signaling by HTRA1.

We are unable to exclude the possibility that HTRA1 also interacts with proTGF-β1 in the extracellular space where it interferes with proper processing. In some tissues, proTGF-β1 is directly secreted into the extracellular space where it is processed into mature TGF-β1 by proprotein convertase (20,31). However, the processing of proTGF-β1 into mature TGF-β1 mainly occurs in the intracellular space in many tissues and cells (14,30). Furthermore, the results of the cell-mixing assay clearly show that HTRA1 decreases TGF-β1 signaling in the intracellular space.

It remains unclear why an increase in the synthesis of mature TGF-β1 results in the narrowly restricted clinical phenotype associated with CARASIL. The increased TGF-β1 signaling was not observed in the aorta and renal arteries, which are intact in patients with CARASIL. In addition, the amount of TGF-β1 increased in the cerebral small arterial wall from CARASIL tissues. Furthermore, we have previously shown that the endothelial cells in the cerebral small arteries respond to TGF-β signaling (2). Thus, the regulation of TGF-β signaling by HTRA1 plays an important role in the cerebral small vessels. Indeed, HTRA1 is expressed in cerebral small vessels (32). In human cerebral small vessels, HTRA1 might function as a tissue-specific regulator of TGF-β signaling.

We have shown endogenous cleaved products of proTGF-β1 in human fibroblasts. Although we also detected cleaved products of proTGF-β1 in fibroblasts from the patient carrying the R370X mutation, treatment with MG-132 increased the amount of endogenous cleaved products of proTGF-β1 in control, but not in R370X, fibroblasts. These results indicate that the cleaved products in R370X fibroblasts did not undergo ERAD. We have shown in this paper that HTRA1-cleaved products of proTGF-β1 undergo ERAD. In addition, we have shown that TGF-β signaling triggered by transfected proTGF-β1 increased in the fibroblasts from the patient carrying the R370X mutation compared to the cell-mixing assay.
with that in control fibroblasts. Thus, we can speculate that these products in R370X fibroblasts may be cleaved by other proteases, but not by HTRA1.

To increase endogenous HTRA1, we treated fibroblasts from R370X mutations with gentamycin, which can suppress the accurate identification of translation termination codons. Gentamicin increased the amount of read-through full-length HTRA1, as well as HTRA1 R370X protein. R370X HTRA1 protein retained serine protease activity comparable to that of HTRA1 wild-type in vitro (2). Thus, endogenous R370X HTRA1 protein, as well as small amounts of full-length HTRA1, affects proTGF-β1. The increased amount of R370X HTRA1 protein generated by gentamycin might result from increasing the stability of the R370X mRNA. The gentamicin cloaks the premature termination codon of R370X mRNA in a pioneer round of translation and results in inhibition of nonsense-mediated mRNA decay and stabilization of R370X mRNA (21,22,33). This strategy could become a remedy for patients with CARASIL carrying the R370X mutation (34,35).

In conclusion, we have shown that HTRA1 binds to and cleaves the pro-domain of proTGF-β1 in the ER, resulting in a decreased amount of secreted mature TGF-β1. Our findings provide a new regulatory mechanism for TGF-β1 signaling in human cerebral small vessels and contribute to our understanding of the molecular basis for the pathogenesis of CARASIL.

MATERIALS AND METHODS

Plasmid constructs

Full-length human wild-type proTGF-β1 expression plasmid and human HTRA1 complementary DNA (cDNA) were obtained from ORIGENE (Rockville, MD, USA) and Open Biosystems (Huntsville, AL, USA), respectively. Constitutively active proTGF-β1 (C223S/C225S) and mutant HTRA1 (V297M, A252T, S328A, R370X) expression plasmids were generated with the use of the GeneTailor site-directed mutagenesis system (Invitrogen, Carlsbad, CA, USA) (2,18). We are grateful to Dr Kazuhiwa Nakayama (Kyoto University) for the human furin expression vector (36). For the luciferase assay, we are grateful to Dr Bert Vogelstein (Howard Hughes Medical Institute and Sidney Kimmel Comprehensive Cancer Center) for the (SBE)₅-Luc reporter vector and to Dr T. Katagiri (Saitama Medical School Research Center for Genomic Medicine) for the mouse full-length Smad4. The pRL-TK plasmid was obtained from Promega (Madison, WI, USA).

Cell culture and transfection

We cultured HEK293T, C2C12 cells (American Type Culture Collection, Manassas, VA, USA) and fibroblasts from the patient with CARASIL carrying the R370X mutation in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO₂. To inhibit the proteasome-dependent degradation pathway, we treated the cells with MG-132 (50 μM) or epoxomicin (100 μM) for 12 h. To inhibit ERAD, we treated cells with eeyarestatin I (20 μM, purchased from Tocris Bioscience, Bristol, UK) for 12 h. Plasmid DNAs were transfected into these cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The transfected cells were cultured in the medium containing 10% FBS serum for 24 h, followed by culture in serum-free medium for an additional 24 h prior to analysis. Each experiment was repeated at least three times.

TGF-β1 digestion in vitro

We generated His-tagged recombinant HTRA1 protein in the FreeStyle 293 expression system according to the manufacturer’s instructions (Invitrogen). The 293-F cells were transfected with the expression vectors using 293fectin (Invitrogen) and grown in serum-free FreeStyle™ 293 Expression medium for 72 h. The conditioned media were cleared by centrifugation, and recombinant proteins were purified with the HisTrap™ FF crude kit (GE Healthcare, Waukesha, WI, USA) and dialyzed against Tris-buffered saline buffer (50 mM Tris–HCl, pH 8.5, 150 mM NaCl). Recombinant LAP and recombinant mature TGF-β1 were purchased from R&D systems (Minneapolis, MN, USA) and Sigma (St Louis, MO, USA), respectively. They were co-incubated at 37°C for 30 min or 3 h in 50 mM Tris–HCl, pH 8.5, 150 mM NaCl.

Immunoblotting and co-immunoprecipitation analysis

Equal amounts of protein from cell lysates or cultured medium were analyzed by immunoblotting. V5-tagged HTRA1 was detected with the anti-V5 antibody (Invitrogen). ProTGF-β1 was detected with the anti-LAP antibody (R&D Systems), and mature TGF-β1 was detected with the anti-TGF-β1 antibody (Promega). We detected Smad2 and phosphorylated Smad2 proteins using anti-Smad2/3 and anti-phospho-Smad2 (Cell Signaling, Beverly, MA, USA) antibodies, respectively. Reactive bands were quantified with ImageQuant TL software (Amersham Biosciences, Piscataway, NJ, USA). For co-immunoprecipitation experiments, cells were homogenized in lysis buffer [25 mM Tris, pH 7.4, 150 mM NaCl, 1% CHAPS (w/v)] and mammalian protease inhibitor cocktail (Sigma; 1:200 dilution) at 4°C. Lysates were centrifuged for 10 min at 10 000 g, and the resulting supernatant fractions were pre-cleared with the use of a mixture of protein G or A agarose beads (Pierce, Rockford, IL, USA) for 2 h at 4°C. We brought 4 mg of each pre-cleared cell lysate to 1 ml with lysis buffer and incubated the lysates with 1 μg of the appropriate antibody plus 50 μl of protein G or A agarose beads at 4°C for 1 h. Immunocomplexes were recovered by centrifugation (3000 g for 3 min), washed four times with 1 ml of lysis buffer and once with 50 mM HEPES (pH 7.4) and resuspended in Laemmli buffer followed by immunoblot analysis. For the immunoprecipitation analysis in human fibroblasts, cells were homogenized in lysis buffer [25 mM Tris, pH 7.4, 150 mM NaCl, 1% CHAPS (w/v)] at 4°C. Lysates were centrifuged for 20 min at 10 000 g, and the resulting supernatant fractions were pre-cleared with the use of a mixture of protein G agarose beads for 2 h at 4°C. The lysates were incubated with 1 μg of the anti-LAP antibody for 2 h, followed by incubation with 25 μl of protein G beads at 4°C for 1 h.
Real-time quantitative RT–PCR analysis

Total RNA was isolated from transfected HEK293T cells or cultured skin fibroblasts with the RNasy plus mini kit (Qiagen, Valencia, CA, USA). cDNA was synthesized with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Real-time quantitative RT–PCR was conducted with the SYBR green PCR master mix and the ABI Prism 7700 Sequence Detection System (Applied Biosystems). To assay HTRA1, TGF-β1, Smad2, Smad3, Smad4, TGFBR1 and TGFBR2 mRNA, we performed real-time quantitative RT–PCR in relation to the expression of glyceraldehyde-3-phosphate dehydrogenase. We designed primer pairs for HTRA1, TGF-β1, Smad2, Smad3, Smad4, TGFBR1 and TGFBR2 as follows: HTRA1 forward: 5′-ggaagatcccaacagtttg-3′, HTRA1 reverse: 5′-ggaagatcccaacagtttg-3′, TGF-β1 forward: 5′-ggaagatcccaacagtttg-3′, TGF-β1 reverse: 5′-ggaagatcccaacagtttg-3′, Smad2 forward: 5′-ggaagatcccaacagtttg-3′, Smad2 reverse: 5′-ggaagatcccaacagtttg-3′, Smad3 forward: 5′-ggaagatcccaacagtttg-3′, Smad3 reverse: 5′-ggaagatcccaacagtttg-3′, Smad4 forward: 5′-ggaagatcccaacagtttg-3′, Smad4 reverse: 5′-ggaagatcccaacagtttg-3′, TGFBR1 forward: 5′-ggaagatcccaacagtttg-3′, TGFBR1 reverse: 5′-ggaagatcccaacagtttg-3′, TGFBR2 forward: 5′-ggaagatcccaacagtttg-3′, TGFBR2 reverse: 5′-ggaagatcccaacagtttg-3′.

Immunohistochemical analysis

We carried out immunoperoxidase staining on formalin-fixed, paraffin-embedded brains, obtained from two autopsied patients with CARASIL and autopsied control subjects (an 84-year-old woman with stroke, a 36-year-old woman with amyotrophic lateral sclerosis). The primary antibodies were against LAP (R&D Systems), phosphorylated Smad2 (Ser456/467) (Cell Signaling) and fibronectin ED-A (Abcam, Cambridge, UK). For detection of hyaluronan, sections were reacted with biotin-labeled HABP (Seikagaku Biobusiness, Tokyo, Japan) for 2 h at room temperature, followed by avidin-conjugated horseradish peroxidase (Vector ABC kit, Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. Diaminobenzidine (Vector Laboratories, Burlingame, CA, USA) was used as a chromogen. Images were obtained with an inverted microscope (TE-300NT; Nikon, Tokyo, Japan) and a confocal microscope (CSU-10; Yokogawa Electric Corp, Tokyo, Japan) equipped with a ×40 objective (NA 0.80; Olympus, Tokyo, Japan).

Luciferase assay

Luciferase assays were performed as described previously (2,11,37). Mouse C2C12 myoblasts were co-transfected with HTRA1 expression vectors and the following constructs: (SBE)4-firefly luciferase vector (TGF-β responsive reporter vector), pRL-TK renilla luciferase expression plasmid (internal control reporter vector) and vectors containing Smad2, Smad4 and TGF-β1 [encoding proTGF-β1 with two point mutations (C223S, C225S)] (18). Cell extracts were assayed for luciferase activity with the use of the Dual-Luciferase Reporter Assay System (Promega). For cell-mixing experiments, HEK293T cells were transfected with plasmids as described in the figure legends. Twenty-four hours after transfection, responding and stimulator cell populations were trypsinized, mixed together and replated in 24-well plates. Twenty-four hours after starvation in DMEM supplemented with 0.1% FBS, cells were harvested for a luciferase assay. For a luciferase assay using skin fibroblasts, fibroblasts derived from controls or patients with CARASIL were treated with or without gentamicin at a concentration of 500 μg/ml for 48 h. After gentamicin treatment, cells were trypsinized and replated in 24-well plates. After 24 h, transfection was performed. All (SBE)4-firefly luciferase activity was corrected by using pRL-TK renilla luciferase activity for transfection efficiency. Every sample was transfected in triplicate, and every experiment was repeated three times.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

We thank the patients and family members for their participation and M. Tsuchiya for technical assistance. We are extremely grateful to Drs B. Vogelstein (Howard Hughes Medical Institute and Sidney Kimmel Comprehensive Cancer Center), T. Katagiri (Saitama Medical School Research Center for Genomic Medicine) and K. Nakayama (Kyoto University) for their generous gifts of plasmids.

Conflict of Interest statement. None declared.

FUNDING

Grant-in-Aid for Scientific Research on Priority Areas ‘Advanced Brain Science Project’ and ‘Applied Genomics’ and Grant-in-Aid for Scientific Research (B) from the Japan Society for the Promotion of Science, and Grant-in-Aid for the Research Committee for Hereditary Cerebral Small Vessel Disease from the Ministry of Health, Labor and Welfare, Japan.

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