Molecular Therapy
Methods & Clinical Development
Original Article

Reversal of neuroinflammation in novel GS model mice by single i.c.v. administration of CHO-derived rhCTSA precursor protein

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Galactosialidosis (GS) is a lysosomal cathepsin A (CTSA) deficiency. It associates with a simultaneous decrease of neuraminidase 1 (NEU1) activity and sialylglycan storage. Central nervous system (CNS) symptoms reduce the quality of life of juvenile/adult-type GS patients, but there is no effective therapy. Here, we established a novel GS model mouse carrying homozygotic *Ctsa IVS6+1g→a* mutation causing partial exon 6 skipping with concomitant deficiency of Ctsa/Neu1. The GS mice developed juvenile/adult GS-like symptoms, such as gargoyle-like face, edema, proctoprosia due to sialylglycan accumulation, and neurovisceral inflammation, including activated microglia/macrophage appearance and increase of inflammatory chemokines. We produced human CTSA precursor proteins (proCTSA), a homodimer carrying terminal mannose 6-phosphate (M6P)-type N-glycans. The CHO-derived proCTSA was taken up by GS patient-derived fibroblasts via M6P receptors and delivered to lysosomes. Catalytically active mature CTSA showed a shorter half-life due to intralysosomal proteolytic degradation. Following single i.c.v. administration, proCTSA was widely distributed, restored the Neu1 activity, and reduced the sialylglycans accumulated in brain regions. Moreover, proCTSA suppressed neuroinflammation associated with reduction of activated microglia/macrophage and up-regulated Mip1α. The results show therapeutic effects of intracerebrospinal enzyme replacement utilizing CHO-derived proCTSA and suggest suppression of CNS symptoms.

INTRODUCTION

Galactosialidosis (GS) is an autosomal recessive genetic disorder caused by a primary defect of the protective protein cathepsin A (CTSA; EC3.4.16.1) gene (chromosomal locus, 20q13.1). CTSA forms a multienzyme complex with neuraminidase 1 (NEU1; EC 3.2.1.18) and acid β-galactosidase 1 (GLB1; EC3.2.1.23), activating the former enzyme and stabilizing the latter one.[1–3] Thus, a defect of CTSA results in secondary deficiencies of NEU1 and GLB1 and an intrinsic catalytic activity.[4,5] As a result, GS exhibits heterogeneous neurovisceral symptoms, including visual disturbance, myoclonus, cerebellar ataxia, cherry-red spots, coarse facies, and skeletal dysplasia.[6–8] GS's clinical phenotypes are classified into three subtypes: early infantile, late infantile, and juvenile/adult forms. The age of onset and severity are dependent on the kinds of gene mutation and the residual enzyme activities of mutant gene products.[9,10] Among them, the juvenile/adult type 2 patients, especially in the Japanese population, have the common single base substitution g to a at the 5′-splice donor site of the intron 7 of CTSA gene (CTSA IVS7+3a→g) in at least one allele, causing a splicing defect to decrease in the production of normal CTSA mRNA to less than 10% of normal level.[9,10] Most type 2 patients initially develop visual disturbance, action myoclonus, cerebellar ataxia, and gargoyle-like features at the age of 5 to 10 years, and relatively milder symptoms and slow progression due to the expression of a small amount of the normal CTSA.[6]

A GS model mouse (Ctsa-null, $Ctsa^{-/-}$) was generated by targeted disruption of the Ctsa gene allele and developed a clinical feature resembling the early onset and severe form of type 1 GS patients.[11] Symptoms include severe nephropathy, splenomegaly, progressive ataxia, and early death. In addition, this phenotype is associated with extensive lysosomal vacuolation and expansion in cells of most neurovisceral organs. $Ctsa^{-/-}$ mice have no cathepsin A activity and undetectable NEU1 activity. In contrast, Glb1 activity is partially reduced only in several cells of young mice but tends to increase in most tissues as the animals age. This model has been extensively

Received 12 January 2022; accepted 10 April 2022;
https://doi.org/10.1016/j.omtm.2022.04.001.

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utilized for the *in vivo* assessment of various therapeutic modalities, including bone marrow transplantation, bone marrow-mediated *ex vivo* gene therapy, AAV (adeno-associated virus)-mediated therapy, and enzyme replacement therapy (ERT).[12–15]

As for ERT for lysosomal storage diseases (LSDs), an intravenous (i.v.) or intracerebroventricular (i.c.v.) ERT has developed with recombinant enzyme drugs produced by mammalian cell lines, including the Chinese hamster ovary (CHO) and human fibrosarcoma (HT1080) cell lines stably overexpressing the human lysosomal enzyme genes. The i.v. ERT has been clinically applied for LSDs,[16–22] including Gaucher's disease, Fabry's disease, Pompe's disease, mucopolysaccharidoses (MPS) I, II, VI, and VII, and Wolman's disease. These multi-order disorders develop hepatosplenomegaly, vascular disease, cardiomegaly, and dysostosis multiplex. The i.v. ERT is based on the incorporation of a recombinant enzyme via cell surface glycan receptors, including the mannose receptor (MR)[16] and cation-independent mannose 6-phosphate receptor (CIM6PR)/insulin-like growth factor type II receptor,[17–22] into target organs and their subsequent delivery to intracellular lysosomes to degrade accumulated substrates. However, the i.v. ERT has little effect on the neurological symptoms of LSDs, because the enzyme drugs cannot go across the blood-brain barrier (BBB) into the brain parenchyma. On the other hand, the i.c.v. ERT as an alternative therapy has also been applied to treat neuronal ceroid lipofuscinosis type II (NCL2, tripeptidyl peptidase I deficiency) patients, in whom the recombinant enzyme drug is regularly administered via an Ommaya reservoir and catheter system to deliver the drugs into the cerebrospinal fluid and to a wide range of brain regions.[23] The i.v. or i.c.v. ERT utilizing recombinant human CTSA can be applicable for the treatment of GS patients,[24] although there is no fundamental therapy for GS.

In this study, we established a novel GS model mouse carrying the homozygotic Ctsa IVS6+1g→a mutation to analyze the pathogenic mechanism of relatively late-onset GS cases with central nervous system (CNS) symptoms and develop a novel treatment. The mouse developed relatively mild phenotypes similar to those in the juvenile/adult type 2 GS patients. Symptoms include myoclonic seizure, gargoyle-like features, edema, hernia, and renal failure. The phenotypes associated with sialylglycan accumulation and urinary secretion were due to the simultaneous Neu1 deficiency. Here, we demonstrated that a CHO-derived human CTSA precursor (proCTSA-6His) carrying the terminal M6P-type N-glycan suppressed the neuroinflammation in the brains of GS model mice on single i.c.v. administration.

RESULTS

Production of human CTSA precursor-6xHis by CHO cell line stably expressing *CTSA-6His*

We established a CHO-K1 cell line stably expressing human CTSA precursor protein fused to a 6× His tag at the C terminus (proCTSA-6His) by introducing fusion with pcDNA3.1 + expression vector and selected by drug-resistance with neomycin and hygromycin. As shown in Figure 1A, most of the gene products were secreted into the serum-free culture medium as an enzymatically inactive 52-kDa proCTSA-6His, although some parts of the products were transported to the lysosomes as the mature form with catalytic activity. The proCTSA-6His was purified by Ni-Sepharose chromatography (Figures 1B and 1C). The yield was about 1–2 mg/L medium. In addition, the proCTSA-6His was revealed to carry the terminal mannose 6-phosphate (M6P) carrying N-glycan located at the 32-kDa subunit region (Figure 1D) by lectin blotting with the recombinant CI-M6PR domain 9 (Dom9).[25]

Restoration and half-life of enzyme activities in GS fibroblasts by proCTSA-6His protein

The lysosomal enzyme activities of cathepsin A toward Z-Phe-Leu, neuraminidase toward 4-MU-NANA, and β-galactosidase toward 4-MU-β-Gal were restored in a human fibroblastic cell line ASVGS-1 (T1) derived from a Japanese GS patient 24 h after the addition of purified proCTSA-6His to the culture medium, as shown in Figures 2A–2D (CTSA, NEU1, GLB1, HEX). In contrast, the CTSA activity was not restored in the presence of 5 mM M6P (Figure 2A).

We next examined the intracellular half-life ($t_{1/2}$) of CTSA activity in T1 cells treated with proCTSA-6His for 24 h. After replacement with fresh medium, the intralysosomal mature CTSA activity exponentially decreased, and the $t_{1/2}$ was 18.6 h (Figure 3A). The $t_{1/2}$ of intracellular CTSA activity was very short compared with other lysosomal enzymes (e.g., HexA, a β-hexosaminidase isoform, 6.6 days). However, the $t_{1/2}$ of intralysosomal CTSA was found to be extended 59.0 h in the presence of 10 μM cysteine protease inhibitor leupeptin (Figure 3A), indicating that the very short intralysosomal half-life of CTSA should be due to the proteolytic degradation by other lysosomal proteases. On the other hand, the restored NEU1 activity was gradually reduced, and the effects of leupeptin were not observed (Figure 3B). The intracellular GLB1 activity was almost constant for 6 days and increased gradually in the presence of leupeptin because of inhibition of physiological degradation of GLB1 (Figure 3C), as previously reported.[26]

Intracellular distribution of expressed CTSA and accumulated sialylglycans

We performed indirect immunofluorescence analysis to visualize the proCTSA delivery to the lysosomes of T1 cells and accumulated sialylglycans as NEU1 substrate using anti-CTSA, anti-NEU1, and anti-terminal Sialα2-3 residue antibodies before and after the replacement of proCTSA-6His. As shown in Figure 4, the incorporated CTSA (Figure 4A) and restored NEU1 (Figure 4B) distributed in lysosomes colocalized with the lysosome-associated membrane protein LAMP1 as a lysosomal marker (Figure 4A). After treatment with proCTSA-6His, punctate fluorescence due to Sialα2-3 reduced (Figure 4C). From these results, the exogenously administered proCTSA-6His was incorporated via the M6P receptor and then associated with the intracellular NEU1 to activate the catalytic activity and reduce the sialylglycans accumulated in T1 cells.

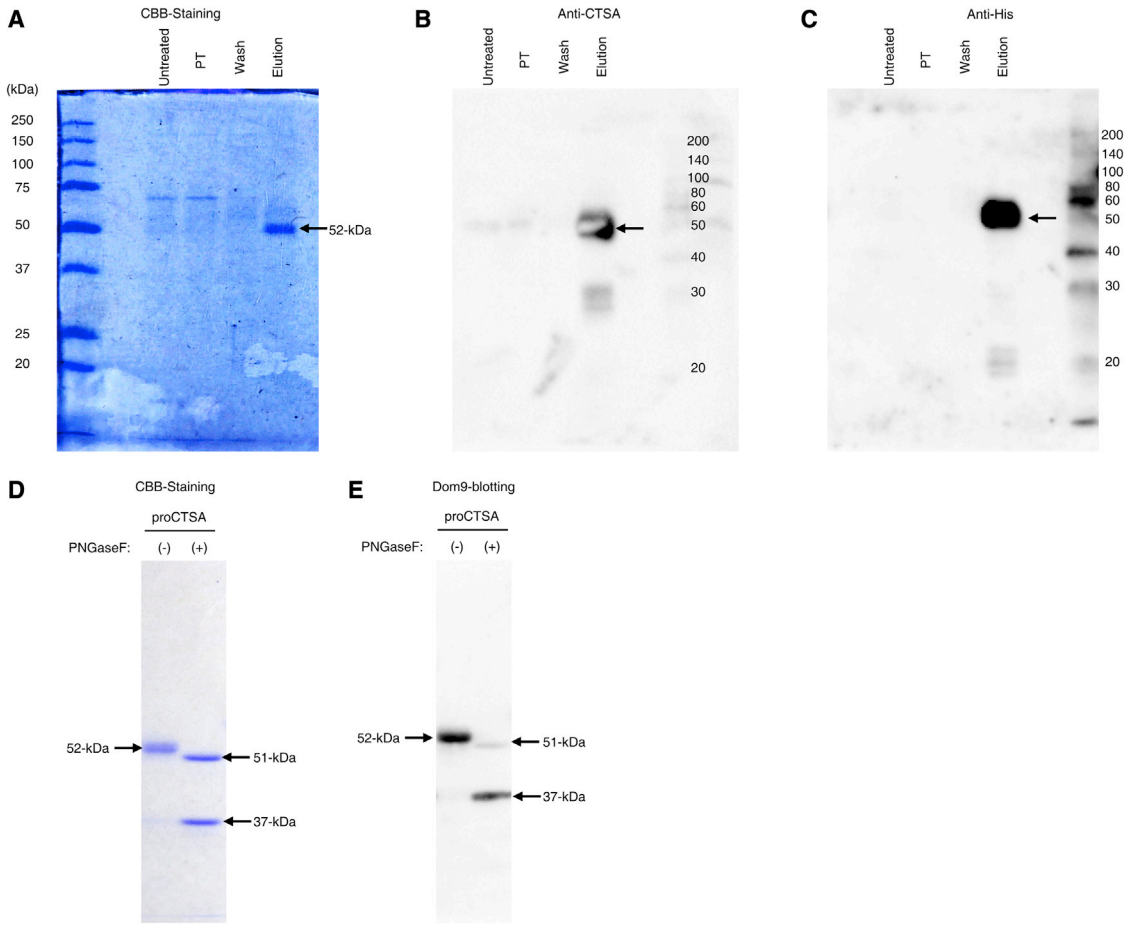

Figure 1. Biochemical characterization of proCTSA-6His purified from the conditioned medium of CHO/*CTSA-6His* cell line

(A–C) Proteins in each Ni-column fraction separated by SDS-PAGE. (A) Coomassie brilliant blue (CBB) staining, (B and C) immunoblotting with anti-CTSA and anti-His. Purified proCTSA-6His migrated to 52-kDa position. (D and E) Purified proCTSA-6His before and after treatment with PNGaseF. (D) CBB staining, (E) lectin blotting with CI-M6PR Dom9-FLAG-6His (PT, pass through, wash and eluate fractions contained 20 and 500 mM imidazole, respectively). The 37-kDa bands were PNGaseF-carrying FLAG tag reactive to anti-FLAG.

Establishment of a novel Ctsa/Neu1-deficient GS model mice

A targeting vector for homologous recombination at the murine *Ctsa* locus was constructed, as shown in Figure 5A. We generated a Ctsa gene-targeted mouse C57BL6 strain as GS model with homozygous single base substitution at the 5′ splice donor site of the intron 6(*IVS6,+1g→a*) in murine *Ctsa*, causing abnormal splicing of *Ctsa* mRNA (partial exon 6 skipping; Figure 5B). RT-PCR analysis (Figure 5C) of total RNA isolated from wild-type (WT, $Ctsa^{+/+}$) and Ctsa mutant tissues, including cerebrum, cerebellum, heart, liver, kidney, and muscle, confirmed the short Ctsa transcript due to partial exon 6 skipping in Ctsa mutant mice. In addition, the Ctsa mutant tissues are associated with simultaneous deficiencies of both Ctsa and Neu1 but not Glb1 rather than up-regulated activity.

Phenotype characterization of Ctsa mutant mice

The Ctsa mutant mice developed coarse faces (Figure 5D right and 5F) from around 4 weeks of age, with an apparent flattening, roundish nose and swelling forehead, eyelids, and forepaws (Figure 5H). Proctoptosia was also shown in the mutant mice (Figure 5I, right). The mutant mice showed a relatively long lifespan and died by 40 weeks (see Figure S2). The mutant mice showed progressively abnormal behavior with dull movement. However, as previously reported, no hepatic enlargement was observed in the dead Ctsa mutant mice compared with those in Ctsa full-KO mice.[11] The neurovisceral manifestations were similar to those of juvenile/adult forms of GS patients, including myoclonic seizure, coarse face, edema, and hernia.

Restoration of Ctsa and Neu1 activities in the brains of GS model mice after a single i.c.v. administration of proCTSA-6His

We evaluated the effects of single i.c.v. replacement of the proCTSA-6His on Ctsa/Neu1-deficient GS mice at the age of 10–12 weeks. One day after i.c.v. injection of the proCTSA-6His (3 mg/kg body weight [BW]), the CTSA (Z-Phe-Leu-degrading) activity was restored in

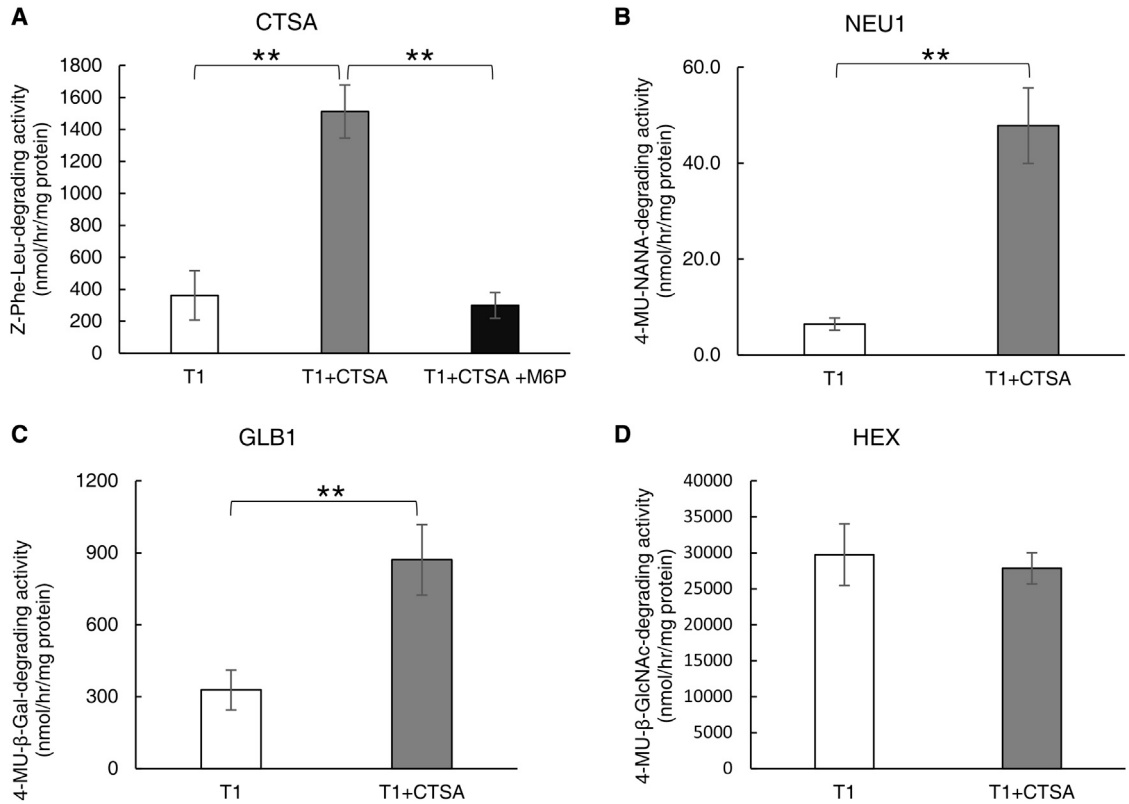

Figure 2. Recombinant enzyme replacement in cultured human fibroblasts derived from patients with GS

(A) Intracellular Z-Phe-Leu–degrading activity in GS fibroblasts (T1) after treatment with proCTSA-6His. Before enzyme treatment, fibroblasts were treated with M6P (+M6P). Error bars show mean ± SEM (n = 3). Student's t test; **p < 0.01. (B–D) Intracellular NEU1, GLB1, and HEX substrate-degrading activities in T1 cells after treatment with proCTSA-6His. Error bars show mean ± SEM (n = 3). Student's t test; **p < 0.01.

the brains to the same extent as that of WT mice (Figure 6A). Neu (4-MU-NANA-degrading) activity was restored to 67.3% of that of WT mouse brains 1 day after injection (Figure 6B). However, 1 week after i.c.v. injection of proCTSA-6His, the CTSA activity was turned back to the level of that of GS mouse (Figure 6A) without significant adverse and side effects, including anaphylactic shock after administration. This phenomenon was probably due to the short half-life of intracellular CTSA due to the proteolytic degradation within 1 week.

On the other hand, Neu activity maintained at 10.7% of that of WT mice 1 week after injection (Figure 6B). It was unknown why the intracellular neuraminidase activity remained under the circumstance of lack of CTSA after 1 week of injection. However, similar results were obtained for the NEU1 and CTSA activities in the replacement experiments using GS patient-derived fibroblastic T1 cells.

Reduction of sialylglycans accumulated in brains of GS model mice after a single administration of proCTSA-6His

To evaluate the effects of i.c.v. administration of proCTSA-6His on the accumulation of sialylglycans in the GS mouse brains, we analyzed them by the quantitative periodate-resorcinol method and qualitative immunohistochemistry (IHC).

By IHC, we first demonstrated the colocalization of LAMP1 and the accumulated sialylglycans-carrying terminal α-2,3-linked sialic acid residues detected by MAM lectin (Figure 7A), indicating lysosomal accumulation of sialylglycans. One week after i.c.v. injection of proCTSA-6His, the colocalization ratio of LAMP1 and MAM decreased in the GS cerebrum (Figure 7A). The MAM signals colocalized with F4/80 as a marker for microglia/macrophage were also decreased (Figure 7B). However, neither NeuN as a neuronal cell marker nor glial fibrillary acidic protein (GFAP) as an astrocyte marker colocalized with MAM signals. The GFAP-immunoreactivity increased in the GS cerebrum but was unchanged by proCTSA-6-His injection (Figure 7D). These results indicate that the murine Neu1 activated through binding with the proCTSA incorporated after a single i.c.v. injection could degrade the sialylglycans accumulated in microglial cells of the GS mouse brain regions (Figure S5).

In the quantitative periodate-resorcinol method, a significant increase in sialylglycoconjugates was observed in the extract derived from a part of the GS cerebrum compared with WT (Figure 7E). In contrast,

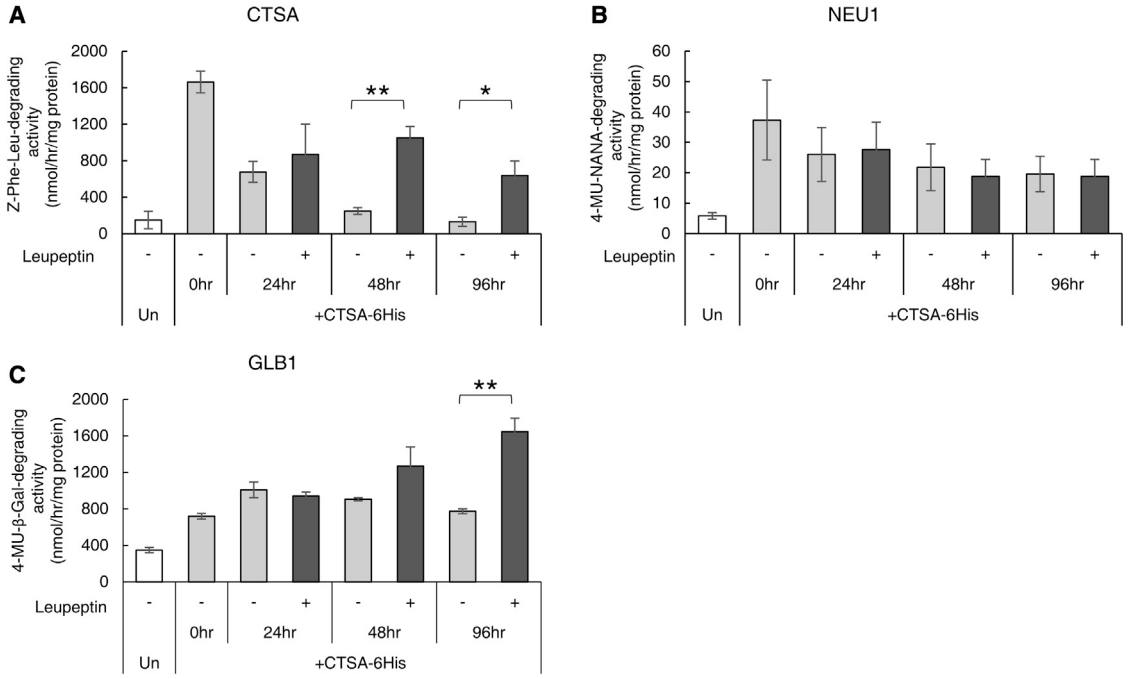

Figure 3. Changes over time of restored enzyme activities after replacement of proCTSA-6His

(A–C) Over time, intracellular substrate-degrading activities toward (A) Z-Phe-Leu, (B) 4-MU-NANA, and (C) 4-MU-β-Gal in T1 fibroblasts after treatment with proCTSA-6His. One day after replacement, the culture medium was exchanged to fresh medium, and then the T1 cells were cultured in the absence and presence of 10 μM leupeptin. "Un" means untreated T1 cells. Error bars show mean ± SEM (n = 3). Student's t test; *$p < 0.05$, **$p < 0.01$.

sialic acids derived from sialylglycoconjugates in the GS cerebrum were significantly reduced 1 week after by single i.c.v. injection of the proCTSA-6His.

Suppression of microglial activation and down-regulation of inflammatory chemokines in the brain after i.c.v. administration of proCTSA-6His

CD68-positive microglia were expanded in the cerebrum (Figure 8A), hippocampus (Figure 8B), and cerebellum (Figure 8C) of the GS model mouse at the age of 10–12 weeks, whereas little immunofluorescence was distributed in the brain of the WT mouse. In contrast, the CD68-positive microglia disappeared in the brain of the GS model mouse 7 days after a single i.c.v. administration of the proCTSA-6His (3 mg/kg BW).

The increase in murine macrophage inflammatory protein-1α (Mip-1α) was also observed in the GS brain extracts, consistent with microglial activation (Figure 8D). The result indicates that a neuroinflammatory response occurs in the GS brain. In Figure 8A, microglial activation seems completely reverted, but Mip-1α is almost three times that of the WT 7 days after injection of proCTSA-6His. It may have been due to the limited effects for astrocytes that may capture few enzymes. These findings suggest that the neuroinflammation in the GS mouse brain can be ameliorated by repeated i.c.v. administration, probably due to a reduction in sialylglycans accumulated in the activated microglia by the murine Neu1 resulting from the intracellular association with the human proCTSA.

DISCUSSION

Galactosialidosis (GS) is one of the lysosomal NEU1 deficiencies caused by autosomal recessive CTSA mutations, resulting in excessive accumulation of sialyloligosaccharides or sialylglycopeptides in neurovisceral organs of patients.[1,6,7,26] GS patients develop heterogeneous systemic manifestations similar to sialidosis, another NEU1 deficiency caused by autosomal recessive NEU1 gene mutations.[27] The latter lysosomal storage disease is also associated with sialylglycan storage in neurovisceral organs and tissues.[28–31] Hence, no curative therapies have been developed for these NEU1 deficiencies.

In this study, we produced the recombinant human proCTSA-6His carrying the M6P-type N-glycan by utilizing a CHO cell line stably expressing the CTSA-6xHis-fusion gene and GS models to evaluate its therapeutic potential for a novel i.c.v. ERT for GS. The M6P-type proCTSA-6His was incorporated by a GS patient-derived fibroblastic cell line T1 via cell surface cation-independent M6P receptor/insulin-like growth factor type II receptor. The CTSA, NEU1, and GLB1 activities deficient in the T1 cells were restored by incorporated proCTSA-6His. However, the catalytically active mature CTSA exhibited a very short half-life ($t_{1/2}$ = 18.6 h) due to proteolytic degradation by lysosomal cathepsins, because the $t_{1/2}$ was extended to 59.0 h in the presence of leupeptin in the culture medium. In contrast, the intralysosomal NEU1 activity did not decay rapidly and was maintained above 50% of the normal level 96 h after administration.

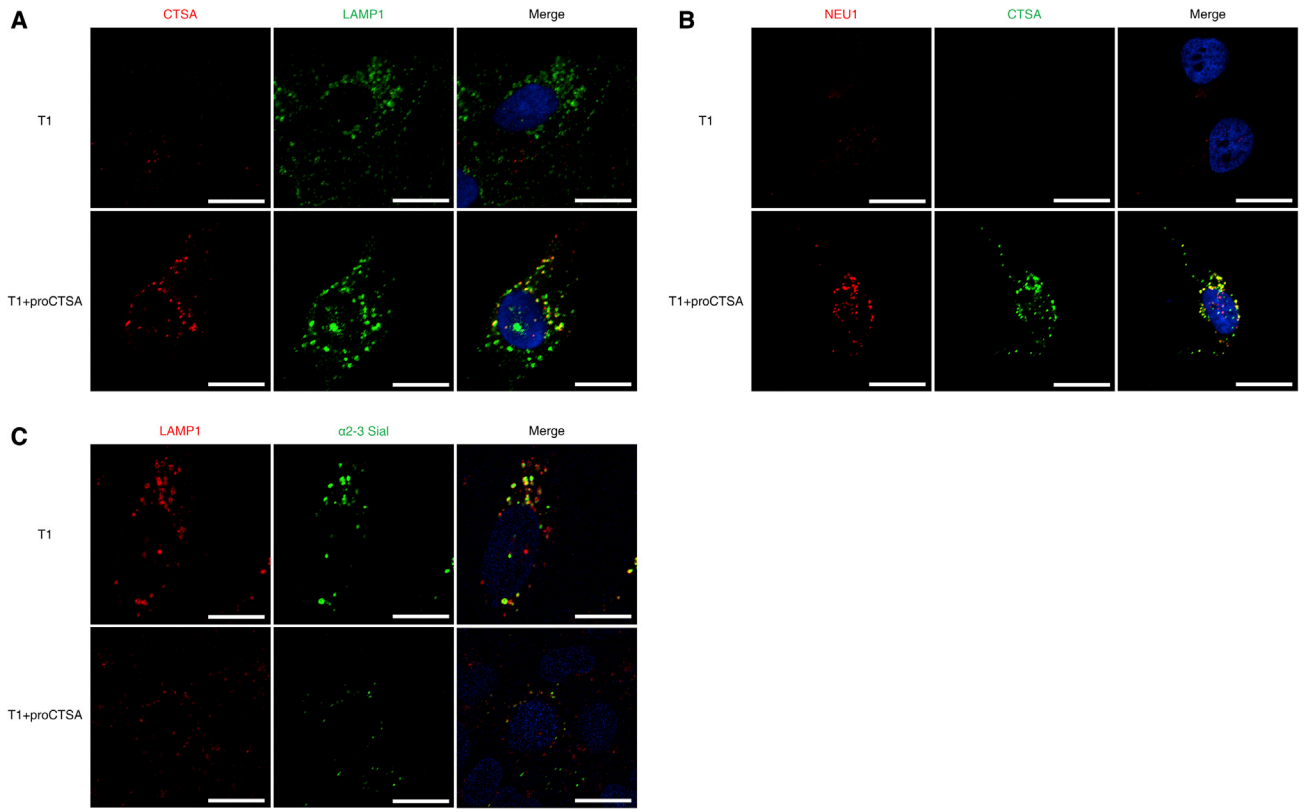

Figure 4. Lysosomal distribution of CTSA and NEU1 after replacement of proCTSA-6His

(A–C) Immunostained T1 fibroblasts treated with proCTSA-6His for 2 days (A and B) or 7 days (C). (A) CTSA (green) and LAMP1 (lysosomal marker, red), (B) NEU1 (red) and CTSA (green), and (C) Sialα2-3 (green) and LAMP1 (red). Scale bars, 20 μm.

It has been reported that the association with CTSA is essential for NEU1 activity expression.[1,32,33] The present results from our culture study suggest the NEU1 activity should be stabilized in lysosomes after association with exogenously incorporated proCTSA as the molecular chaperone and transportation, even after most of the mature CTSA was degraded. In parallel to the restoration of catalytic activity and lysosomal distribution of NEU1, the intracellular α2,3-sialylglycans were reduced, suggesting the efficacy to treat the NEU1 deficiency.

We also performed for the first time the i.c.v. administration of a CHO-derived proCTSA-6His to Ctsa/Neu1-deficient GS model mice. The Ctsa activity in the GS brains was restored to the WT level 24 h after the single i.c.v. injection, returning to the GS level in 7 days. The *in vivo* experiment results also indicate the short half-life of mature CTSA incorporated into the neural cells due to intralysosomal proteolysis. In contrast, the Neu1 activity was restored to about 30% of the WT level and maintained that for at least 7 days. Correspondingly, the amounts of sialylglycoconjugates in the brain extracts measured by periodate-resorcinol assay were significantly reduced 7 days after injection, suggesting that the accumulated substrates should be degraded by the activated NEU1 associating with the incorporated CTSA.

Here, we first demonstrated that the F4/80- and Cd68-positive activated microglia markedly increased in the brain regions of the GS model mice, including the cerebrum and cerebellum (see also Figure S5). As the co-localization of accumulated sialylglycans detected by MAM lectin specific for α2,3 sialic acid and F4/80 as a microglial marker was observed, the neural cells that contain the accumulated sialylglycans were suggested as the microglial cells. In parallel to the sialylglycans accumulation in the microglia, a remarkable increase and up-regulation of the Cd68-positive activated microglia and inflammatory chemokine, including Mip-1α, in the GS mouse brain were also observed. Microglial activation and neuroinflammation have been reported in other LSDs, including mucopolysaccharidosis[34] and GM1/GM2 gangliosidosis,[35] because of the natural substrate storage. The findings obtained in this study suggest that the storage of sialylglycans due to the Neu1 deficiency should cause the microglial activation and up-regulation of inflammatory chemokines.

Interestingly, not only the Cd68-positive activated microglia but also the up-regulated Mip-1α levels were decreased, but a single i.c.v. injection of the CHO-derived proCTSA-6His did not completely suppress the up-regulation at the 3 mg/kg BW dose to the GS model mice at 10–12 weeks. The results indicated that the M6P-proCTSA-6His was taken up by the activated microglia in the cerebrum and

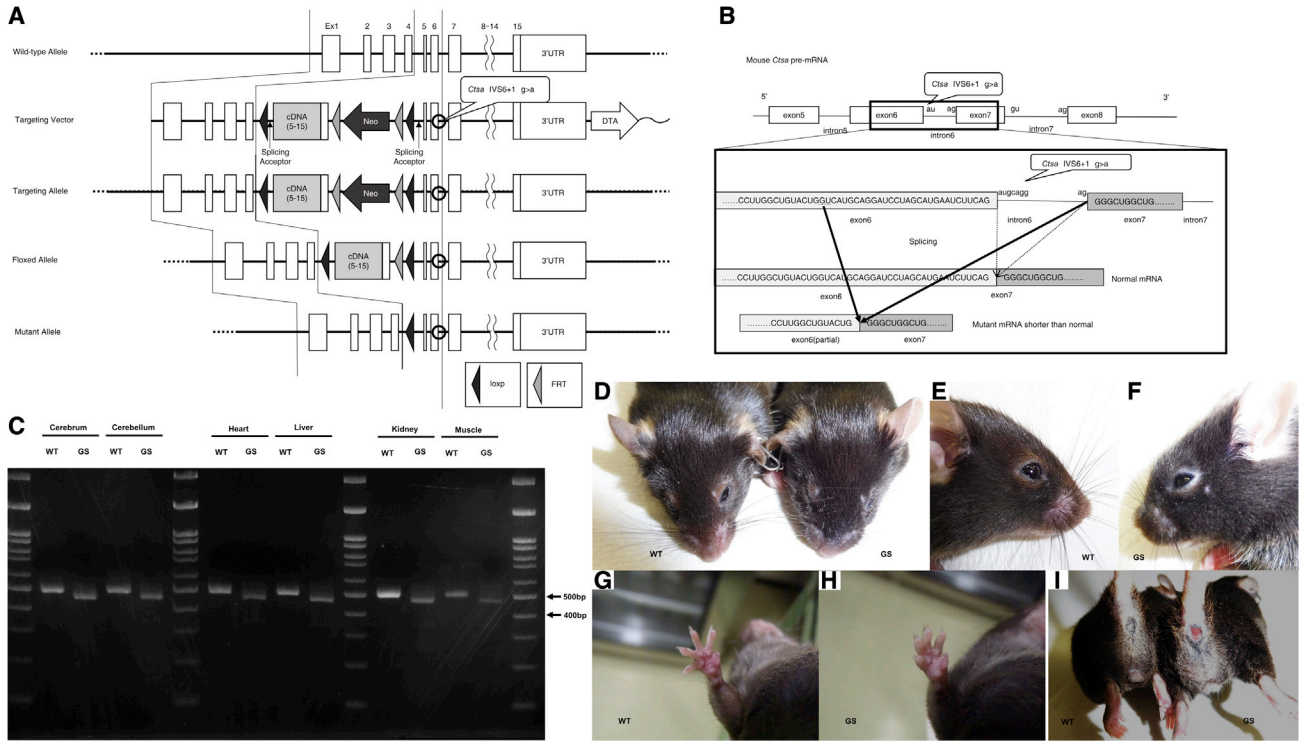

Figure 5. Generation and phenotype of Ctsa mutant mice

(A) Schematic representation of the mouse Ctsa gene (Wild-Type Allele), the targeting construct (Targeting Vector), and the mutant locus after the homologous recombination (Targeting Allele). White boxes show exons or 3′-UTR, and the black box shows cDNA containing exons 5 to 15. Triangles show *loxP* (black) or FRP (gray), arrows show PGK-neomycin (*Neo*) cassette (black) or DTA (white). In Floxed allele, the*Neo* cassette was removed by FLP. Mutant allele shows cDNA cassette after removal by Cre. (B) Schematic diagram of production of the defection of Ctsa. Intervening sequences 1, which work for 5′ splicing donor site base +1, was changed from guanine to adenine. The "gu" sequence located at 30 bases behind the base substitution was recognized as a new splicing doner region. As a result, mutant mRNAs were shorter than wild-type (WT). (C) Result of RT-PCR from cDNA derived from each organ. Primers were designed toward exons 4 to 7, Ctsa mutant mRNAs were shorter than WT. (D–I) The phenotype of Ctsa mutant mice. (D–F) The mutant mice have coarse faces, roundish nose tips (D, right) and swollen foreheads (F) compared with WT mice (E). (G and H) The forepaws of Ctsa mutant mice (H) were thicker than those of WT mice (G). (I) Proctoptosia was shown in the mutant mice (right).

cerebellum of the GS mice and then associated with the intracellular Neu1 to activate and degrade the accumulated sialylglycans in lysosomes. The incorporated proCTSA-6His can suppress the microglial activation and neuroinflammation in the GS mouse brain regions by i.c.v. injection. In contrast, the enhanced GFAP-immunoreactivity as an activated astrocyte marker in GS brain regions did not decrease by a single i.c.v. injection. The results indicate that the proCTSA-6His was not incorporated efficiently by the activated astrocytes to suppress the Mip-1α up-regulation. These findings strongly suggest that the repeated i.c.v. administration of the recombinant proCTSA produced by mammalian cell lines, including CHO and the human HT1080, should have therapeutic effects on neuroinflammation and neurological symptoms.

Existing studies[12–15,24] have focused on peripheral therapeutic effects with enzyme replacement and gene therapies, but they have no therapeutic effects on the brain and CNS symptoms. Recently, Cadaoas et al. have reported on the preclinical study of repeated intravenous ERT using CHO-derived human proCTSA and GS model mouse[11] with a null mutation at the Ctsa locus ($Ctsa^{-/-}$) at 0.2–20 mg/kg BW for 8 weeks.[24] They demonstrated a dose-dependent, systemic uptake by visceral organs and the restoration/normalization of the Ctsa, Neu1, and Glb1 activities, resolution of visceral histopathology, and reduction in sialyloligosaccharidurias. Although the efficacy to visceral symptoms and safety of i.v. ERT utilizing the CHO-derived M6P-proCTSA at doses ≧20 mg/kg over 8 weeks was shown, the i.v. ERT of recombinant proCTSA is not likely to be effective for correcting neurological symptoms, including myoclonus and cerebellar ataxia, commonly occurring in the juvenile/adult GS patients, because it scarcely goes across the BBB into the brain parenchyma.

Alternatively, the preclinical and clinical application of i.c.v. ERT utilizing the recombinant human lysosomal enzyme drugs produced by mammalian cells has been developed[23,36] for other LSDs. As mentioned above, the repeated i.c.v. ERT using the recombinant human tripeptidyl peptidase 1 (TPP1) drug (Cerliponase alfa, Brineura) at 300 mg/brain every other week is clinically applied to the patients with the late-infantile neuronal ceroid lipofuscinosis type 2 (CLN2) to treat the neurological manifestations.[23] Recently, Seo et al. performed an open-label, phase 1/2 clinical-trial study

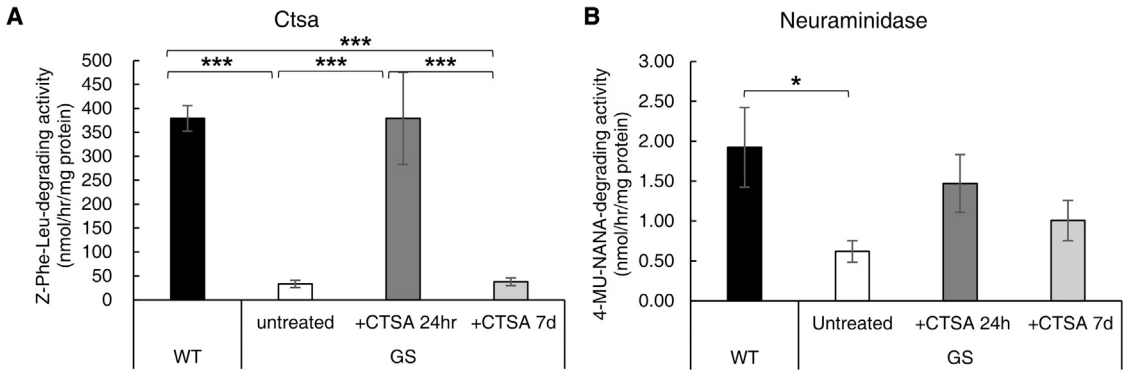

Figure 6. Restoration and decay of CTSA and NEU1 activities in GS mouse brains after single i.c.v. administration of proCTSA-6His

(A and B) The activity of neuraminidase (4-MU-NANA-degrading) (A) and Ctsa (Z-Phe-Leu-degrading) (B) in the brain of GS mouse 24 h and 7days after treatment with proCTSA-6His (3 mg/kg BW dose). Error bars show mean ± SEM (WT, n = 6; GS, n = 7; GS + CTSA 24 h, n = 3; GS + CTSA 7 days, n = 8). ANOVA with a Tukey-Kramer test; *$p < 0.05$, ***$p < 0.001$.

(JMACCT CTR JMA-IIA00350) and evaluated the efficacy and safety of i.c.v. ERT using Idursulfase beta for neuropathic mucopolysaccharidosis II (Hunter syndrome).[36] Chen et al. demonstrated suppression of astrogliosis and microgliosis in the GM1 gangliosidosis model *Glb1*$^{-/-}$ mice by i.c.v. replacement of the recombinant human GLB1 produced by a CHO cell line.[37,38] Stijn et al. have shown that i.c.v. injection of recombinant arylsulfatase A (ASA) improves walking function in ASA-deficient mice.[39] In those studies, continuous administration of the recombinant enzyme was performed using a cannula or pump, so we expect efficacy on CNS symptoms of GS mice by utilizing the same approach.

On the basis of our present findings, we propose that the continuous i.c.v ERT utilizing the recombinant human proCTSA will be clinically available to treat CNS symptoms of GS patients, including myoclonus and cerebellar ataxia. However, further study will be necessary to elucidate the underlying mechanism of replacing the proCTSA for the neural cells, microglia, neurons, astrocytes, and oligodendrocytes and correlate them with therapeutic effects on CNS symptoms. In addition, the efficacy of the continuous i.c.v. ERT may be expected to apply to visceral symptoms and chronic inflammation, because a part of the M6P-proCTSA-6His was delivered via cerebrospinal fluid into the circulation system after i.c.v. injection to the GS model mice (See Figure S4).

MATERIALS AND METHODS

Materials

The fluorogenic substrates, including the 4-methylumbelliferyl derivatives of *N*-acetyl neuraminic acids (4-MU-NANA), were purchased from Biosynth Carbosynth (Newbury, UK). *N*-acetyl-β-D-glucosaminide (4-MUG) and β-D-galactoside (4-MU-βGal), and 4-methylumbelliferone (4-MU), mannose 6-phosphate (M6P), nutrient mixture F-10 Ham, and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Geneticin (G418) was from InvivoGen (San Diego, CA, USA), and hygromycin B was from Wako Pure Chemical Industries (Osaka, Japan). The protease inhibitor leupeptin was purchased from the Peptide Institute (Osaka, Japan).

Antibodies and lectins

An anti-CTSA serum raised against human cathepsin A (CTSA) in rabbits was obtained from Sigma-Aldrich by outsourcing. Antiserum dilution was 1,000 times at western blotting. A rat hybridoma cell line producing the monoclonal anti-CTSA (anti-CTSA mAb) was obtained from Operon Biotechnologies Inc. (Huntsville, AL, USA) by outsourcing. The hybridoma supernatant dilution was 10 times at immunocytochemistry. Commercially available antibodies: anti-LAMP1 raised in rabbits (Abcam, ab24170; 1:400); anti-Siaα2-3 mAb (HYB4) raised in mouse (Wako, 011-25171; 1:50); anti-Neu1 mAb (F-8) raised in mouse (Santa Cruz Biotechnology, sc-166824; 1:100); anti-Iba1 antibody (1022-5) raised in mouse (Santa Cruz Biotechnology, sc-32725; 1:100), F4/80 (D2S9R) XP rabbit mAb (Cell Signaling Technology, 70076S; 1:200); anti-NeuN antibody raised in rabbits (Merck Millipore, ABN78; 1:400); anti-glial fibrillary acidic protein (GFAP) antibody produced in rabbit IgG fraction of antiserum, buffered aqueous solution (Sigma-Aldrich, G9269; 1:1,000) or anti-GFAP mAb produced in mouse (Sigma-Aldrich, G3893; 1:1000); anti-CD68 mAb (FA-11) raised in rat, eBioscience (Thermo Fisher Scientific, 14-0681-82; 1:400).

Biotin-conjugated lectins, including MAM, were purchased from Seikagaku (Tokyo, Japan). At lectin staining, biotinylated MAM was used at the concentration of 10 μg/mL. Recombinant cation-independent mannose 6-phosphate receptor (CI-M6PR) domain 9 (Dom9) was prepared according to the methods of Kiriyama &and Itoh.[25]

Cell culture

The CHO-K1 cell line was provided by RIKEN BioResource Center (Wako, Japan). Fibroblastic cell line ASVGS-1 (T1)[9] was cultured in nutrient mixture F-10 Ham (Sigma-Aldrich) medium containing 10% (v/v) FBS and antibiotics at 37°C in a humidified incubator continuously flushed with a mixture of 5% CO_2 and 95% air. The ethics committee of Tokushima University approved the use of patient-derived T1 cells for medical research.

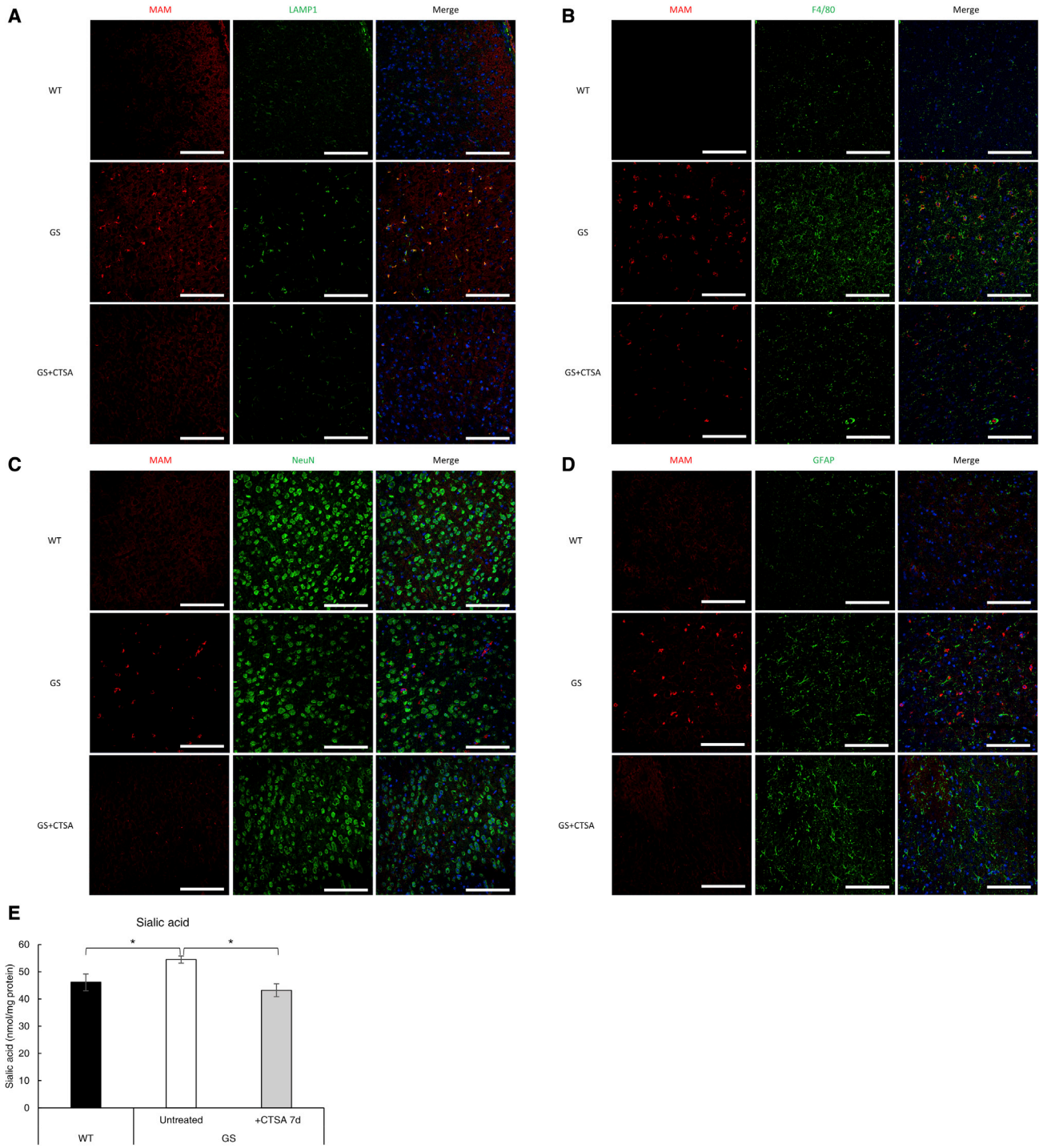

Figure 7. Sialylglycan accumulation in microglia in brain sections and reduction after single i.c.v. administration of proCTSA-6-His

(A–D) Immunohistochemical analysis of LAMP1 (a lysosomal marker), F4/80 (a microglial marker), NeuN (a neuronal marker), and GFAP (an astroglial marker), with each antibody and lectin staining with biotin-MAM and streptavidin-Cy5 and in the paraffin-embedded sections of the cerebrum of the GS mouse 7 days after treatment with proCTSA-6His (3 mg/kg BW dose). Blue, nuclei; red, MAM; green, (A) LAMP1, (B) F4/80, (C) NeuN, and (D) GFAP. Scale bar, 100 mm. Inset magnification ×3,200. (E) Sialic acids derived from sialylglycans in the mouse brain extracts were measured by the periodate-resorcinol method. Sialylglycans accumulated in the brain of GS mice at the age of 10–12 weeks were reduced 7 days after treatment with proCTSA-6His (3 mg/kg BW dose). Error bars show mean ± SEM (WT, n = 6; GS, n = 7; GS + CTSA 7 days, n = 8). ANOVA with a Tukey-Kramer test; *$p < 0.05$.

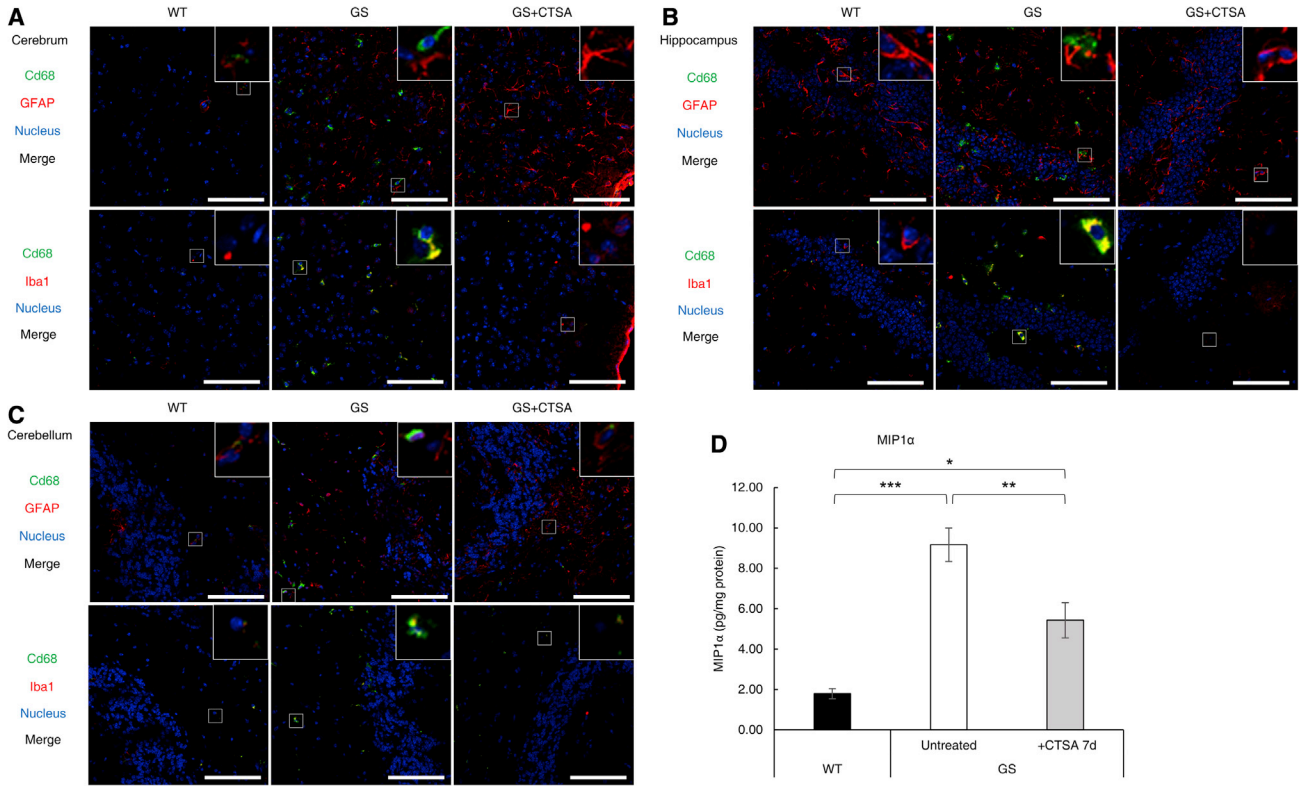

Figure 8. Microglial activation and up-regulation of inflammatory chemokine in GS mouse brain, and suppression and reversal after i.c.v. administration of proCTSA-6His

(A–C) Immunohistochemical analyses of Cd68 (activated glial marker) with anti-Cd68 in the cerebrum and cerebellum of GS mouse treated with proCTSA-6His (3 mg/kg BW dose). Blue, nuclei; green, Cd68; red, GFAP (top) or Iba1(bottom). Scale bar, 100 mm. Inset: magnification ×3,200. (D) Murine inflammatory protein 1α (Mip-1α) in the GS mouse brain extracts was quantified by ELISA. Mip-1α increased in the brain of GS mice at the age of 10–12 weeks and was reduced 7 days after treatment with proCTSA-6His (3 mg/kg BW dose). Error bars show mean ± SEM (WT, n = 6; GS, n = 7; GS + CTSA 7 days, n = 8). *p < 0.05, **p < 0.01, ***p < 0.001.

Mice

A transgenic mouse strain carrying a single-base substitution at the 5′-splice donor site of intron 6 of murine *Ctsa* gene causing partial exon 6 skipping has been established by outsourcing to the Unitech Co., Ltd. (Kashiwa, Chiba, Japan; see Figure 5A). Briefly, the DNA fragment of the mouse Ctsa gene was amplified and inserted upstream of the Diphtheria Toxin A gene in the targeting vector. The *IVS6,+1g→a* mutation was generated, and an array encoding *loxP Ctsa cDNA(exon5-15)_FRT_(PGK promoter)NeoR_FRT_loxp* was inserted into intron 4. The vector was electrophoresed into embryonic stem (ES) cells, and G418-resistant ES cells were screened for homologous recombination. Targeted ES clones were microinjected into C57BL/6J blastocysts and implanted into pseudo-pregnant female mice. Progeny male chimeras mated with C57BL/6J females, heterozygous (*WT/loxP_Ctsa cDNA(exon5-15)_FRT_(PGK promoter)NeoR_FRT_loxp*) mice were born. The mice mated with the other mice strain expressing Cre to remove Ctsa cDNA-NeoR cassette. The homozygotic mice (*Ctsa IVS6,+1g→a/IVS6,+1g→a*), designated Ctsa mutant in this paper, were produced by mating the heterozygous male and female mice. The mice were bred by crossing them with C57BL/6 mice (Japan SLC) and were maintained under specific pathogen-free conditions in the animal facilities at Tokushima University.

Establishment of CHO cell lines stably expressing CTSA-6His

DNA fragments, respectively encoding *CTSA*, were amplified using primer sets (forward: 5′-TTTTGGTACCCACCATGATCCGAGCCG CGCC-3′, reverse: 5′-TTTTGGGCCCGTATGGCTGCTTGTTCAG-3′). Both fragments for insertion and linearized pcDNA3.1+C-6His vector were digested with *Kpn*I and *Apa*I (TOYOBO) and purified. The digested DNA fragments were ligated with a DNA ligation kit (Takara Bio) according to the manufacturer's instructions. The resultant vector plasmid was designated pcDNA3.1+ CTSA-6His and used for transformation with MAX Efficiency DH5α Competent Cells (Invitrogen). Plasmid DNA-Lipofectamine 3000 (Invitrogen) complexes were transfected into CHO cells according to the manufacturer's instructions. Neomycin-resistant cell lines were established by G418 (InvivoGen) selection and the limiting dilution method.

DNA fragments encoding *CTSA-6His* were amplified using primer sets (forward: 5′-TTTTAGATCTCACCATGATCCGAGCCGCGCC-3′, reverse: 5′-AAAAGAATTCCTGATCAGCGGGTTTATC-3′) from

pcDNA3.1+ CTSA-6His as templates and ligated to produce an expression vector containing the sequence array of the CMV promoter CTSA-6His-IRES-HygroR. This DNA3.1+ CTSA-6His vector was transfected as described above. The hygromycin-resistant cell lines were selected in the presence of hygromycin B (Wako) by the limiting dilution method.

Purification of recombinant human cathepsin A precursor proteins from the conditioned medium

The cell line stably producing proCTSA-6His (1×10^7 cells/mL) was cultured in serum-free Ham's F-10 for 7 days, and then the supernatant was collected after being centrifuged at 1,500×*g* to remove floating cells as conditioned medium (CM). The pH of the supernatant was adjusted to below 7.5 and then was applied to the Ni-Sepharose column (6 Fast Flow, Cytiva). The column was washed with 20 mM sodium phosphate buffer (NaPB, pH7.5) containing 20 mM imidazole-HCl, and then the adsorbed proteins were eluted with NaPB (pH7.5) containing 500 mM imidazole-HCl. The eluate was concentrated, and the buffer composition was exchanged to PBS with Amicon Ultra 30k (30,000 MWCO; Millipore, Billerica, MA) and then stored at −30°C. The purity of the preparation was monitored by SDS-PAGE on a 12.5% polyacrylamide gel. Proteins were visualized with a PhastGel Blue R-350 (Cytiva). The purified proCTSA-6His was stored at −30°C before use.

Enzyme assays

Cells or tissues were sonicated with the extract buffer (50 mM sodium citrate buffer [pH 4.5]/150 mM NaCl/1% [v/v] Nonidet P-40 [NP-40] [Roche Diagnostics]/protease inhibitors). The lysosomal enzyme activities, including NEU1, HEX, and GLB1, were assayed fluorometrically with 4-methylumbelliferyl-N-acetyl-a-D-neuraminic acid or 4-methylumbelliferyl-β-D-galactopyranoside as substrates.[40] The activity of acid carboxypeptidase (CTSA) was measured at pH 5.6 with Z-Phe-Leu as a substrate.[27] Protein determination was performed with a DC assay kit (Bio-Rad, Richmond, CA, USA) with BSA as a standard (Sigma).

Immunocytochemical and immunohistochemical analyses

Cells (1×10^4 cells/well) were seeded onto eight-well Lab-Tek chamber slides. The proCTSA-6His (1 μg/well) were added, followed by incubation for 2 or 7 days. Cells were fixed with 4% paraformaldehyde. After the samples were removed by washing with PBS, the intracellular NEU1, CTSA, LAMP1, and Sialα2-3 were detected by each antibody.

For preparing the paraffin-embedded sections, mouse organs were soaked in 4% paraformaldehyde-PBS overnight. For paraffin-embedded sections, organs were soaked in 70% ethanol overnight, replaced by 70% to 100% ethanol and xylene, and paraffin embedded by Shandon Excelsior ES (Thermo Fisher Scientific). After being sliced by microtome (SM2010, Leica), intercepts dewaxed in 3× xylene, and from 100% to 70% ethanol, activated at 90°C for 40 min in HistoVTOne (Nacalai tesque). For frozen sections, organs were soaked in sucrose solution from 10% to 30% each overnight, embedded in OCT compound (Sakura Finetek) at −80°C. After being sliced by cryostat (CM3050S, Leica), intercepts were dried at room temperature and washed in PBS. Both frozen and paraffin-embedded intercepts were treated in blocking solution containing 5% goat serum (Cedarlane), 1% BSA, and PBS for 1 h at room temperature, and treated with first probe solution. In addition, each slice was treated with antibodies against Cd68, GFAP, and F4/80 diluted in Can Get Signal Immunoreaction Enhancer Solution (TOYOBO). The specimens were viewed with the LSM700. Image analyses were performed using the ZEN2012 program (Zeiss).

Enzyme replacement assay for T1 cells

Fibroblast cell line T1 was seeded onto a culture dish (Falcon). CTSA-6His was added, followed by incubation for 24 h. The cells were dissociated with a cell scraper (Sumitomo Bakelite) and sonicated with extract buffer (50 mM sodium citrate buffer([pH4.5]/150 mM NaCl/1% [v/v] NP-40/protease inhibitors). Then, intracellular 4-MU-NANA/Z-Phe-Leu/MU-βGal-degrading activities were measured. In the experiment to evaluate the intracellular half-life of CTSA, NEU1, and GLB1 activities, T1 cells were treated with the proCTSA-6His for 24 h, and then the culture medium was exchanged for a fresh medium following the monitoring of intracellular enzyme activities. Other experiments were performed in the presence of 10 μM leupeptin to inhibit the lysosomal proteases, including cathepsins B, L, and H.

Intracerebroventricular administration of human proCTSA to GS model mice

A 3 mg/kg BW dose of the recombinant human proCTSA, or the same volume of PBS, was singly injected into 10- to 12-week-old GS mice by i.c.v. administration.[41,42] Twenty-four hours or 1 week after the injection, the GS mice were euthanized, and their brains, livers, and spleens were dissected after PBS perfusion. Each tissue was frozen immediately. The sections were thawed and homogenized by sonication in extract buffer (50 mM sodium citrate buffer ([pH4.5]/150 mM NaCl/1% [v/v] NP-40/protease inhibitors). Before centrifugation at 13,200 × *g*, the NEU1 activity in brain homogenates was measured. After centrifugation, the supernatants were collected as tissue extracts. The lysosomal enzyme activities in the extracts were measured for CTSA, GLB1, and HEX, and protein concentration was determined.

Genotyping and RT-PCR

Genomic DNA was isolated from mouse tails by digesting with proteinase K (Merck) in 50 mM Tris-HCl (pH8.0)/20 mM EDTA/2% SDS. After heat treatment at 55°C overnight in the presence of NaCl at a final 1.3 M, the insoluble was removed by centrifuging at 13,200×*g* at 4°C for 10 min. Next, 2.5 vol of ethanol was added to the supernatants and centrifuged at 18,000×*g* at 4°C for 10 min. Finally, the pellets were dissolved in water, and the solutions were used as DNA samples. For genotype analysis, PCR was performed as follows: 1.25 U *Taq* DNA polymerase (Promega), 1×PCR buffer (Promega), 1.5 mM $MgCl_2$, 0.2 M dNTPs (Promega), 0.5 μM of

each primer set (forward: 5′-AGAGGGCAGGAGACTAGTTAGAG AC-3′, reverse: 5′-TTCAAGTACCCAGACATCCCTAAGA-3′), and DNA sample were mixed and amplified by thermal cycler (start at 94°C, 2 min, cycled 30 times at 98°C; 15 s, 60°C, 15 s, 72°C, 1 min; finish 4°C after 72°C, 1 min). Amplified DNAs were evaluated by agarose gel electrophoresis followed by ethidium bromide staining by 3% (w/v) agarose gel electrophoresis. WT allele appeared in 331 bp, and GS allele appeared in 365 bp, including the remaining *loxP* in intron 4 (See Figure S1).

Total RNA was isolated from brains (cerebrum and cerebellum), heart, kidney, liver, and muscle tissues of 18-week-old mice according to the protocol of ISOGEN (Nippon Gene). RNA (2 μg) was applied for RT-PCR by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to its protocol. The cDNAs prepared by RT-PCR were amplified in PCR by *Taq* DNA polymerase (Promega). Constitutions of the reaction mixture were the same as genotyping except for primer sets (forward 5′-AGATGGTGTCACCCTGGAGT-3′, reverse 5′-AGATTTGCCCACAATTCGAG-3′). The PCR conditions were as follows: start at 95°C for 2 min; cycled 35 times at 95°C for 30 s; 50°C for 30 s; 72°C for 30 s; and finish at 4°C. Amplified DNAs were evaluated by 3% (w/v) agarose gel electrophoresis followed by ethidium bromide staining. WT allele appeared in 496 bp, and GS allele appeared in 460 bp.

Western blotting

Each fraction, including pass-through (PT), washing, and eluate, obtained by Ni-column chromatography of the conditioned medium of CHO/*CTSA-6His* containing the proCTSA-6His was applied to SDS-PAGE under reducing conditions and then electroblotted onto polyvinylidene difluoride membranes (Immobilon; Nippon Millipore, Tokyo, Japan).[43] The blotted membranes were blocked with 50% Blocking One (Nacalai tesque, Kyoto, Japan) in Tris-buffered saline (TBS), followed by incubation with anti-CTSA or anti-His antibody and HRP-conjugated anti-rabbit IgG or HRP-conjugated anti-mouse IgG. The antibody-reacting bands were visualized with a chemiluminescence detection system (Western Lightning Chemiluminescence Reagent PLUS; PerkinElmer Life Sciences, Inc., Boston, MA) according to the manufacturer's protocol.

Deglycosylation of purified proteins by PNGaseF and CI-M6PR-Dom9 blotting

Reagents attached to the PNGaseF product were used. First, the purified proCTSA-6His was denatured in glycoprotein-denaturing buffer (0.5% SDS, 40 mM DTT) at 100°C for 10 min. Then, NP-40, GlycoBuffer, and PNGaseF were added and incubated for 1 h at 37°C. Next, the proCTSA-6His was separated by SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes. After blocking, membranes were treated with CI-PR-Dom9-FLAG-6His, anti-FLAG M2 antibody (Sigma-Aldrich, F3165) and HRP-conjugated Anti-mouse IgG. Finally, bands were visualized with a chemiluminescence detection system.[25]

Lectin staining

Lectin staining with a biotin-conjugated MAM lectin was performed according to Refs.[44,45]. The de-paraffinized sections were antigen activated at 90°C for 40 min by HistoVT One (Nacalai Tesque), washed with PBS, and then pretreated with 5% normal goat serum/1% BSA (Cedarlane Laboratory, Ontario, Canada) in PBS for 1 h at room temperature for blocking. Then, the sections were treated with biotin-conjugated MAM lectin (10 μg/mL) diluted in Can Get Signal Immunoreaction Enhancer Solution (TOYOBO) overnight at 4°C. After a washing with PBS, the cells were treated with streptavidin-CY5 (Southern Biotech, 7105-15, 1:1,000 diluted) for 1 h in the dark. The stained cells were examined with a confocal laser scanning imaging system (LSM510, Carl Zeiss).

Determination of sialic acids

Measurement of sialic acids derived from sialylglycoconjugates was performed according to the resorcinol method of Jourdian et al.[46]. N-acetylneuraminic acid was used as a standard. First, 50 μL of samples were added to 10 μL of 40 mM periodic acid solution. After the addition of the resorcinol reagent (125 μL; 0.6 g of resorcinol [Wako] in a solution containing 60 mL of 28% HCl, 40 mL of water, and 25 pmol of $CuSO_4$), the mixtures were mixed, placed in an ice bath for 5 min, and heated at 100°C for 15 min, and 125 μL of 95% *tert*-butyl alcohol was added. Samples were centrifuged at 13,200×*g* at 4°C for 3 min, the supernatant was collected, and the absorbances were determined at 630 μm.

Statistics

The statistical analyses were performed using the software EZR (Easy R).[47] We used the two-tailed unpaired t test. In comparisons of three or more groups, we used a one-way ANOVA with a Tukey-Kramer test. The data are presented as mean or average ±SEM. Values of $p < 0.05$ were considered statistically significant.

Study approval

All experiments were approved by the ethics committee on animal care at Tokushima University and were performed following institutional guidelines for animal care at Tokushima University. Cultured skin fibroblasts from patients were approved for use by the ethics committee at Tokushima University.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2022.04.001.

ACKNOWLEDGMENTS

We want to acknowledge the Ministry of Education, Culture, Sports, Science, and Technology (MEXT); the Japan Society for the Promotion of Science (JSPS); MEXT/JSPS KAKENHI (Grant Nos. 17H04102 to K.I. and D.T., 18K06658 to D.T., and 17H06157 to M.O.); the Japan Agency for Medical Research and Development (AMED) (TR-SPRINT Seeds A, A098 to K.I.); and the Ministry of Agriculture, Forestry and Fisheries of Japan (Research Project for Sericultural Revolution) to K.I. and S.N.

AUTHOR CONTRIBUTIONS

Y.H., D.T., and K.I. designed the research. Y.H., T.I., M.O., J.T., Y.T., H.I., Y.F., HA, Y.T., S.N., N.Y., and N.I. performed experiments. Y.H., D.T., M.I., Y.T., and K.I. analyzed data. Y.H. and K.I. wrote the manuscript.

DECLARATION OF INTERESTS

We confirm that there are no known conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome.

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