Impaired Autophagic Degradation of Transforming Growth Factor-β-Induced Protein by Macrophages in Lattice Corneal Dystrophy

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PURPOSE. Lattice corneal dystrophy (LCD) is related to the denaturation of transforming growth factor-β-induced protein (TGFBIp). Autophagic degradation of the denatured proteins by macrophages is one pathway to remove the denatured proteins. Thus, we investigated the role of autophagy in the degradation of mutant (MU) TGFBIp in macrophages.

METHODS. Corneas from participants were observed by slit-lamp photography and subjected to histopathologic and genetic analysis. Wild-type (WT) and MU TGFBIp were recombined and expressed. Macrophages from MU participants were isolated and cocultured with the recombinant TGFBIp. Colocalization of the two molecules was observed by immunofluorescence microscopy. Enzyme-linked immunosorbent assay, Western blotting, and flow cytometry were used to detect changes in molecule expression related to the phenotype and autophagy process.

RESULTS. Fourteen members from a family of 25 were identified as LCD sufferers. Significant TGFBIp aggregates and macrophage infiltration were found only in the corneas of LCD sufferers. Marker accumulation of TGFBIp was found in macrophages exposed to WT TGFBIp, but not to MU TGFBIp. Impaired autophagic flux due to defective autophagosome fusion to lysosomes was found in macrophages exposed to MU TGFBIp. Blockage of the autophagic process suppressed the expression of CD68 and CD36 in macrophages exposed to MU TGFBIp. However, the exact molecular mechanisms, including the clearance mechanism, are not fully understood.

CONCLUSIONS. Our results suggested that reversion of the defective autophagic process in macrophages may be a therapeutic strategy for patients with LCD.

Keywords: autophagy, transforming growth factor-β-induced protein, lattice corneal dystrophy, macrophages, phagocytosis, protein degradation
characterized by accumulation of amyloid-β plaques that are directly toxic to neurons, resulting from inadequate amyloid-β clearance in relation to amyloid-β production. Although the role of macrophages (microglia) in the clearance of amyloid-β is controversial, a few lines of evidence have indicated that microglia are responsible for the clearance of amyloid-β plaques by phagocytosis in AD brains. Therefore, recent studies have sought new approaches to increase the macrophage-mediated clearance of amyloid-β plaques, indicating a possible alternative strategy for AD treatment in the future.

To remove amyloid aggregates in extracellular stroma, macrophages must infiltrate into the stroma and clear the amyloid aggregates by phagocytosis. The phagocytized amyloid aggregates then are mainly degraded by the ubiquitin proteasome or autophagy pathways. The ubiquitin proteasome pathway selectively degrades phagocytized (substrate) proteins through ubiquitin modifiers in 26S proteasomes, whereas autophagy degrades denatured proteins or organelles via lysosomes. Any alterations in the processes of these proteolytic pathways may result in the extracellular or intracellular accumulation of denatured proteins, causing pathologic processes and disease. Recently, a study found alterations of autophagic clearance of TGFBIp in corneal fibroblasts from patients with LCD type II.

Since amyloid deposits have a major role in LCD pathology, just like the role of macrophages in the clearance of amyloid-β aggregates in AD, it is conceivable that the immune clearance of amyloid aggregates by macrophages may be important in the LCD mechanism. We reported on a family of 25 members with 14 LCD sufferers associated with the p.(Leu558Pro) mutation in the TGFBI gene, the same mutation in the TGFBI gene that was described previously. We thoroughly investigated whether macrophages have a role in the clearance of amyloid aggregates in this family. There was impaired autophagic clearance of MU TGFBIp by macrophages in the LCD sufferers compared to the normal family members. Our results provided a better understanding of the disease mechanism and may pave the way to therapeutic development.

**MATERIALS AND METHODS**

**Subjects and Clinical Evaluation**

We strictly followed the Declaration of Helsinki in the treatment of the participants. This study was approved by the ethics committee of Hainan Medical College, China. A three-generation LCD pedigree was collected in Hainan Province, China, from June 2012 to July 2013 (Fig. 1A). Written informed consent was obtained from all 25 participants (14 affected and 11 unaffected). The family members ranged from 10 to 89 years old. After informed consent was obtained, a detailed ophthalmologic examination was performed to determine the status of the corneas of all participants. Corneal phenotypes were detected by slit-lamp photography. The clinical history, including age at onset, initial presenting signs, clinical symptoms, and treatment history, also were obtained in detail.

**Histopathologic and Genetic Analyses**

Corneal tissue from the left eye of the proband excised at penetrating keratoplasty was fixed and analyzed using light microscopy following staining with hematoxylin and eosin (H&E), Congo red, and periodic acid-Schiff (PAS). In addition, peripheral blood was collected from all study subjects.
Impaired Autophagy to LCD TGFBIp in Macrophages

Genomic DNA was extracted from the peripheral blood using the QiAamp DNA blood Mini Kit (Qiagen, Hilden, Germany). All 17 coding exons of the TGFBI gene were amplified by polymerase chain reaction (PCR) using the previously reported primers and conditions. Gene sequencing for each PCR product was entrusted to a commercial company (Takara, Dalian, China). The sequencing results were compared to the TGFBI gene in GenBank (MIM 601692).

Corneal Immunohistochemistry

Immunohistochemical detection was performed using the ABC kit (Vector Labs, Inc., Burlingame, CA, USA). In brief, 5-μm sections of cornea from the proband and an age-matched victim of an eye accident were dewaxed with xylene and hydrated with graded ethanol. Thereafter, the sections were blocked in 2% BSA in PBS and stained overnight with unconjugated anti-TGFBIp (Abcam, Cambridge, UK) or anti-CD11b (Abcam). On the next day, a Cy2 or Cy3-conjugated secondary antibody (Abcam) was used to detect anti-TGFBIp and anti-CD11b, respectively. 4'-6-Diamidino-2-phenylindole (DAPI) was used for nuclear counterstaining. Images were taken with a fluorescence microscope (80i; Nikon, Tokyo, Japan).

Preparation of Recombinant TGFBIp

Recombinant wild type (WT) and MU TGFBIp proteins were prepared as previously reported. Briefly, Spodoptera frugiperda (Spf) insect cells were propagated in 30 mL 28°C BacVector insect cell medium (Darmstadt, Germany) in 250 mL flasks with 1,040-rev/minutes shaking. Spf cells were transfected with a plasmid encoding a 6-His-tagged TGFBI cDNA amplified from WT and MU participants, respectively. Two days after transfection, the conditioned medium was exchanged to 10 mM imidazole in 0.3 M NaCl, 50 mM sodium phosphate buffer, pH 8 (Buffer B) using a 30-kDa cutoff membrane in a flow-cell apparatus. The retentate was applied over a 0.3 mL bed volume of Ni-NTA affinity resin that subsequently was washed with Buffer B. Increasing imidazole from 10 to 250 mM in Buffer B eluted TGFBIp. Western blots (WB) identified the fractions containing TGFBIp, which were subsequently was washed with 10% autologous serum and penicillin/streptomycin/fungizone and were incubated for 7 to 10 days until adherent macrophages were differentiated. Macrophages were treated with recombinant TGFBIp (2 µg/mL dissolved in dimethyl sulfoxide [DMSO]) at appropriate dilutions. After overnight incubation, the cells were washed, fixed using 4% paraformaldehyde, stained with first antibodies (anti-TGFBIp or anti-CD11b), and subsequently with secondary immunofluorescent-conjugated antibody (all antibodies were from Abcam). At the same time, DAPI was used to stain the cell nuclei. Thereafter, the fluorescent images were captured by confocal laser scanning microscopy (Olympus, FV3000).

TGFBIp Phagocytosis by Macrophages

Isolation of mononuclear cells and preparation of mature monocyte-derived macrophages from the WT and MU participants were performed as described previously. In brief, peripheral blood mononuclear cells (PBMCs) were isolated using the Ficoll-Hypaque technique; 50,000 PBMCs were placed into each well of an 8-well plate in Iscove’s medium with 10% autologous serum and penicillin/streptomycin/fungizone and were incubated for 7 to 10 days until adherent macrophages were differentiated. Macrophages were treated with recombinant TGFBIp (2 µg/mL dissolved in dimethyl sulfoxide [DMSO]) at appropriate dilutions. After overnight incubation, the cells were washed, fixed using 4% paraformaldehyde, stained with first antibodies (anti-TGFBIp or anti-CD11b), and subsequently with secondary immunofluorescent-conjugated antibody (all antibodies were from Abcam). At the same time, DAPI was used to stain the cell nuclei. Thereafter, the fluorescent images were captured by confocal laser scanning microscopy (Olympus, FV3000).

Immunofluorescent Detection of Colocalization

Cells were fixed with 4% paraformaldehyde for 30 minutes, washed with PBS, and then incubated with 0.1% Triton X-100 for permeabilization. Cells were stained with anti-LC3B polyclonal antibody, TGFBIp, or anti-LAMP1 at 4°C overnight, and subsequently stained with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Abcam, ab150077) or Cy3-conjugated second antibody (Abcam) at 37°C for 1 hour. Nuclei were stained with DAPI for 5 minutes. Images were captured using confocal laser scanning microscopy (Olympus, FV3000).

Enzyme-Linked Immunosorbent Assay (ELISA)

The concentrations of TGFBIp in cell culture medium at different time points were measured by ELISA using kits (BD Biosciences, San Jose, CA, USA) and antibody against TGFBIp (Abcam) according to the manufacturer’s protocol. Enzyme activity was measured with an ELISA reader (Bio-Tek, Winooski, VT, USA) as described previously.

WB Analysis

Cells were treated with or without chemical agents, such as 3-methyladenine (3-MA), MG132, and leupeptin, as reported previously. Cells were lysed with RIPA buffer (50 mM Tris, 1.0 mM EDTA, 150 mM NaCl, 0.1% Triton X-100, 1% sodium deoxycholate, and 1 mM PMSF). Protein was measured using a BCA protein assay kit (Pierce, 23225, Rockford, IL, USA) and resolved by 12% SDS-PAGE, and then transferred to polyvinylidene fluoride (PVDF; BioRad Laboratories, Hercules, CA, USA) membranes. After blocking, the membranes were incubated with primary antibodies at 4°C overnight, and then incubated with secondary antibodies at room temperature for 1 hour. Target proteins were examined using ECL reagents (WBKLS0100; Millipore, Billerica, MA, USA). Semiquantitative analysis was performed by measuring band density using ImageJ software (V6, NIH).

Flow Cytometry

Cell surface proteins CD68 and CD36 were detected with a BD FACSCalibur Flow cytometer, and the resultant data were analyzed by FlowJo software (BD Biosciences). Briefly, anti-CD68 or anti-CD36 antibodies (Abcam) were used to stain the cells followed by a secondary antibody conjugated with either FITC- or PE. For intracellular staining, the cells first were fixed, permeabilized, and stained with corresponding first antibodies and FITC- or PE-conjugated secondary antibodies. Thereafter, the stained cells were analyzed by cytometric analysis.

Statistical Analysis

Statistical analysis was performed using Prism 6 (GraphPad Software, Inc., San Diego, CA, USA). Statistical differences were determined by a nonparametric 2-tailed Student’s t-test in experiments comparing two samples and 1- or 2-way ANOVA with Bonferroni’s test for multiple comparisons when more than two samples were analyzed. Data are expressed as means ± SD and P < 0.05 was deemed statistically significant. Error bars indicate SD unless otherwise indicated.

RESULTS

Clinical Characteristics

The pedigree of the participant family is shown in Figure 1A. Age at onset was 25 to 43 years in the affected family members.
The main complaints and initial symptoms were photophobia, visual acuity decrease, and foreign body sensation. The progress of corneal opacities in all affected members was bilateral symmetry. The proband (Fig. 1A, II-3) was a 51-year-old man with a 7-year history of photophobia and loss of visual acuity in each eye, but without any history of recurrent corneal erosions. Ultimately, penetrating keratoplasty was performed on his right eye. Slit-lamp biomicroscopic examination of a 27-year-old daughter of the proband (Fig. 1A, III-5) showed linear mid and deep stromal opacities that also were found in most affected members’ corneas (Fig. 1B).

Histopathologic analysis was performed on the proband’s corneal button that underwent penetrating keratoplasty. Variably sized, irregularly shaped deposits were situated mainly in the anterior and middle corneal stroma. The deposits were stained positively with Congo red, indicating that they were amyloid in nature (Fig. 1C). In addition, direct sequencing of TGFBI exons 1–17 in all affected members demonstrated a previously described heterozygous missense mutation. Exon 12 exhibited a T>C heterozygous substitution at nucleotide position 1673 that causes a leucine-to-proline amino acid substitution in the protein (Leu558Pro; Fig. 1D).

Macrophage Infiltration in LCD Corneal Stroma

To confirm the presence of resident macrophages in LCD corneas and the relationship between infiltrated macrophages and amyloid aggregates, corneal sections from the proband and control patient were double-stained with anti-TGFBIp and anti-CD11b (a monocyte/macrophage marker), which show as green colors, respectively, and nuclei were stained by DAPI. Images are representative of three independent experiments.

Normal Initial Phagocytosis but Impaired Degradation of TGFBIp in Macrophages

Macrophages were isolated from the TGFBI MU sufferers. These macrophages had the obvious expression of CD11b and CD14 (Supplementary Fig. S1A), and did not markedly express endogenous TGFBIp (Supplementary Fig. S1B) by RT-PCR and WB. In addition, we also observed the morphology of recombinant MU and WT TGFBIp by transmission electron microscope, respectively. We found their morphology to be different, with the recombinant MU-TGFBIp in a form of long and straight fibrils (Supplementary Fig. S1C). To study whether macrophages normally phagocytose and degrade MU TGFBIp, we treated the macrophages from LCD sufferers with recombinant MU TGFBIp or its normal counterpart (WT) first for 5 hours and then removed the TGFBIp for the second 5 hours according to the protocol shown in Figure 3A. Representative images showed that uptake of MU and WT TGFBIp was almost the same at 2.5 and 5 hours (Fig. 3B). In contrast, intracellular MU TGFBIp was unchanged at 7.5 and 10 hours, but the intracellular WT TGFBIp significantly decreased at 7.5 hours and almost disappeared at 10 hours (Fig. 3B), indicating the accumulation of MU TGFBIp in macrophages exposed to MU TGFBIp. Quantification of the green signals in the cells also showed similar results in MU and WT TGFBIp by macrophages as shown in representative images (Fig. 3C). These phenomena were confirmed further by WB analysis. The WB results showed that the level of the intracellular MU TGFBIp was unchanged at each time point, but the level of WT TGFBIp significantly decreased at 7.5 hours and almost disappeared at 10 hours (Figs. 3D, E). We also found the same results in the macrophages from the non-LCD–affected family members (Supplementary Fig. S1D). To observe whether the intracellular TGFBIp was colocalized with LAMP1, we double stained the macrophages with antibodies against TGFBIp and LAMP1 (a lysosome marker), and found that there was obvious colocalization of GFBIp and LAMP1 in the macrophages exposed to WT TGFBIp, but not in those exposed to WU TGFBIp (Supplementary Fig. S1E). In addition, we also detected the concentration of TGFBIp in the culture medium by ELISA and WB using antibody against TGFBIp and 6-His tag, respectively. The results showed that the concentration of MU TGFBIp in the culture medium keep almost no change at 2.5 and 5 hours compared to the concentration of WT TGFBIp (Fig. 3F, Supplementary Fig. S1F). Taken together, these results suggested that MU TGFBIp, and not MU types of macrophages, cause initial normal phagocytosis but impaired degradation or accumulation of TGFBIp in macrophages.

Implied Phagocytic Activation of Macrophages With MU TGFBIp

The expressions of phagocytic macrophage marker CD68 and surface scavenger receptor CD36 were detected to evaluate whether the macrophages had activated phagocytosis. The macrophages from the TGFBI MU sufferers were exposed to MU and WT TGFBIp as above. CD68 or CD36 expression was first detected by WB analysis. Compared to the unexposed
cells (time 0 hours), macrophages exposed to MU and WT TGFBIp had high expression of CD68 and CD36, with more significant expression in cells exposed to WT TGFBIp (Figs. 4A–C). In addition, the expressions of CD68 and CD36 were decreased in a time-dependent manner, especially in the macrophages after the WT TGFBIp was withdrawn, but almost unchanged in macrophages after MU TGFBIp was withdrawn (Figs. 4A–C). These results were strongly confirmed by flow cytometry analysis, which was similar to WB analysis (Figs. 4D–F). Together, these results indicated that phagocytic activation in macrophages exposed to MU TGFBIp was impaired compared to that in macrophages exposed to WT TGFBIp.
Incomplete Autophagic Flux in Macrophages Exposed to MU TGFBIp

As autophagy is a common pathway to degrade unnatural or aggregate proteins, we investigated whether complete autophagic flux may be induced in macrophages after exposure to MU TGFBIp. We first detected the conversion of LC3-I to lipidated LC3-II, a classical marker of autophagosome formation. After macrophages were exposed to MU or WT TGFBIp, increased LC3-II conversion was found at 2.5 and 5 hours (Figs. 5A, 5B). However, the high LC3-II conversion persisted in macrophages exposed to MU TGFBIp, but not in those exposed to WT TGFBIp (Figs. 5A, 5B).

To verify the formation of autophagosomes in macrophages exposed to MU and WT TGFBIp, we analyzed the distribution of endogenous LC3 puncta, another classical marker of autophagosome formation. Markedly increased endogenous LC3 puncta were found in macrophages exposed to MU and WT TGFBIp at 2.5 and 5 hours, which persisted in the macrophages exposed to MU TGFBIp, but not in those exposed to WT TGFBIp at 7.5 and 10 hours (Figs. 5D, 5E).

Since the inhibition of autophagosome turnover at the late stage also leads to LC3-II conversion and the accumulation of LC3 puncta, the above phenomena do not mean that the autophagy progress was complete. Sequestosome-1 (SQSTM1) protein is involved in the targeting of polyubiquitinated proteins to autophagosomes and is selectively degraded via autophagy. Thus, we observed SQSTM1 expression in macrophages exposed to MU and WT TGFBIp as above. As shown in Figures 5A and 5E, the expression of SQSTM1 was unchanged in macrophages not exposed to any TGFBIp (0 hours) and in those after MU and WT TGFBIp were withdrawn (7.5 and 10 hours). However, the expression of SQSTM1 significantly decreased in macrophages exposed to WT TGFBIp at 2.5 and 5 hours (Figs. 5A, 5C). These results strongly indicated that the autophagic flux (progress) in macrophages exposed to MU TGFBIp was not completed.

TGFBIp Accumulation in Macrophages due to Impaired Autophagosome Fusion to Lysosomes

To further verify whether the incomplete autophagic flux was related to impaired autophagosome fusion with lysosomes, we first blocked the autophagic process with 3-MA (an autophagy inhibitor) and the ubiquitin proteasome pathway by MG132, respectively. Figure 6A shows that neither 3-MA nor MG132 treatment influenced MU TGFBIp uptake by macrophages, and
MU TGFβp accumulated in the macrophages at 7.5 and 10 hours after MU TGFβp was withdrawn. However, WT TGFβp had markedly accumulated in macrophages after WT TGFβp was removed when the autophagic process was blocked by 3-MA, a result similar to MU TGFβp exposure (Fig. 6A). Blockage of the ubiquitin proteasome pathway by MG132 did not influence WT TGFβp uptake and caused WT TGFβp accumulation in macrophages (Fig. 6A). Next, leupeptin, a moderate cell-permeable inhibitor of lysosomal cysteine proteases, was used to treat macrophages to block lysosome fusion with autophagosomes. Figure 6B shows that leupeptin treatment did not affect MU TGFβp uptake by macrophages, and MU TGFβp accumulated in macrophages at 5 and 10 hours after MU TGFβp was removed. In macrophages exposed to WT TGFβp, leupeptin treatment did not affect WT TGFβp uptake, but remarkably influenced the degradation of WT TGFβp after WT TGFβp was removed (Fig. 6B). Moreover, obvious colocalization of endogenous LC3 puncta with LAMP1 (a lysosome marker) was found in macrophages exposed to WT TGFβp (Figs. 6C, 6D). Taken together, these results strongly indicated that autophagy is the major pathway by which uptake of TGFβp in macrophages is degraded, and that the impaired autophagosome fusion to lysosomes causes the accumulation of MU TGFβp in macrophages.

Suppression of Phagocytic Activation in Macrophages Exposed to WT TGFβp by Blocking Autophagy

To investigate whether blockage of autophagy suppresses the phagocytic activation of macrophages exposed to WT TGFβp,
we blocked the autophagic process with 3-MA and detected the expressions of CD68 and CD36 in the macrophages. Blockage of autophagy by 3-MA suppressed the expressions of CD68 and CD36 in macrophages exposed to WT TGFBIp, which were active in the macrophages exposed to WT TGFBIp only (Fig. 7, Supplementary Fig. S2). This was evidenced by almost the same expression levels of CD68 and CD36 in macrophages exposed to WT TGFBIp only (Fig. 7, Supplementary Fig. S2). This was evidenced by almost the same expression levels of CD68 and CD36 in macrophages exposed to WT TGFBIp only and WT TGFBIp with 3-MA by WB analysis (Figs. 7A–C). Similar results were found by flow cytometry analysis (Figs. 7D–F). In addition, we also blocked the autophagic process with RNA interfering (siRNA) against Beclin 1 (siBeclin 1) and then detected the expression of CD68 and CD36 by WB. Similar results of CD68 and CD36 expression as blockage of the autophagic process by 3-MA were found (Supplementary Fig. S2A). Moreover, the expression of CD68 and CD36 in the macrophages not exposed to any MU or WT TGFBIp was not affected by 3-MA or siBeclin 1 (Supplementary Fig. S2B). These results are completely different from those shown in Figure 4. They indicated that phagocytic activation in macrophages exposed to WT TGFBIp is reversed to a similar level as that in macrophages exposed to MU TGFBIp only.

**DISCUSSION**

We reported the clinical, histopathologic, and molecular genetic features and possible molecular mechanism of a variant of LCD (p.[Leu558Pro] mutation) in a Chinese family, which has been previously reported in a Spanish family.\(^\text{15}\) TGFBI-associated corneal dystrophies are caused by extracellular depositions of insoluble protein aggregates, which can be amyloid and/or granular.\(^\text{47}\) The protein encoded by the TGFBI gene is 683 amino acids long and contains four tandemly repeated FAS-1 domains.\(^\text{48}\) Previous reports have indicated that most TGFBI mutations associated with corneal dystrophies are located in the fourth FAS-1 domain, except for those affecting amino acid residues 113 and 123–126 in the first FAS-1 domain and amino acid 501 in the third FAS-1 domain.\(^\text{10,47–50}\) Several lines of evidence show that mutations in the fourth FAS-1 domain cause MU protein deposition by altering TGFBIp
structure, stability, and subsequent protein processing, or even by affecting TGFBIp fibrillation rates and protein turnover.51 A proteolytic cleavage site between the wild-type Arg557 and Leu558 residues in the fourth FAS-1 domain has been found by Underhaug et al.52 Therefore, the p.(Leu558Pro) mutation in our current study may encode a mutant TGFBIp that is significantly less susceptible to proteolysis. The resultant mutant TGFBIp may further disrupt the normal degradation and turnover of corneal TGFBIp, as demonstrated for the p.(Arg555Trp) mutation, leading to dystrophic corneal deposition.52 In addition, the phenotypic characteristics in our study do not present with those in the missense mutation involving Val113,52 suggesting that the disruption of the proteolytic cleavage site is of primary importance in terms of its effect on TGFBI function.

LCD is a degenerative disorder that causes loss of corneal transparency and eventually leads to loss of vision.4–6 Degenerative disorders are characterized mainly by the accumulation of extracellular or intracellular denatured protein aggregates that cause disease states, such as LCD in the eye or AD in the brain. At present, macrophages are thought to be the major cell components that clear extracellular denatured protein aggregates (such as amyloid deposits) so as to avoid disease states.53,54 Two important proteolytic systems, the ubiquitin/proteasome and the autophagy pathways, are involved in the degradation of denatured protein aggregates after they have been taken up into macrophages.55 Several studies have reported that autophagy is involved in the clearance of TGFBIp, and impaired autophagic degradation of TGFBIp is found in the fibroblasts of granular corneal dystrophy type 2 (GCD2).56–58 We reported on a family suffering from LCD due to a Leu558Pro mutation in exon 12 with a T > C heterozygous substitution. We then used the corneas from the proband and a normal subject to investigate the role of macrophages in the pathogenesis of LCD by directly observing macrophage infiltration in the proband’s cornea. Our results showed that there was obvious TGFBIp accumulation surrounded by CD11b+ macrophages in the TGFBI mutant proband’s cornea, but not in the control normal subject. Although normal phagocytosis of mutant and wild-type TGFBIp was found in macrophages from mutant patients and normal family members, the degradation of mutant TGFBIp, but not wild-type TGFBIp, was impaired in macrophages from mutant patients, indicating that the mutant TGFBIp itself, and not the mutant macrophages, causes the accumulation and impaired degradation of mutant TGFBIp. Macrophages have an important role in phagocytosis and the removal of amyloid aggregates in the extracellular stroma. As autophagy is a pathway that degrades the phagocytic components in macrophages,59 we, thus, investigated the role of autophagy in the degradation of mutant compared to wild-type TGFBIp by measuring the extent of autophagosome formation and the status of autophagy in macrophages from LCD sufferers. Our data demonstrated that autophagy was the main intracellular degradation mechanism for wild-type TGFBIp, but impaired autophagy was found for mutant TGFBIp because mutant TGFBIp, but not wild-type TGFBIp, accumulated in macrophages from mutant and normal subjects. The impaired intracellular degradation in macrophages was due to incomplete autophagic flux that resulted from impaired autophagosome fusion to
Impaired Autophagy to LCD TGFBIp in Macrophages

In summary, we found impaired autophagic degradation of TGFBIp in macrophages after exposure to MU or WT TGFBIp. However, when MU or WT TGFBIp exposure was removed, significant accumulation of TGFBIp was found only in macrophages exposed to MU TGFBIp and not to WT TGFBIp. These results indicate that WT and MU TGFBIp can be taken up by macrophages after initial exposure to WT and MU TGFBIp, and that the uptake and degradation of WT TGFBIp is a continuous process, but the process almost stops in the macrophages exposed to MU TGFBIp, meaning that there is impaired degradation of MU TGFBIp in macrophages. To investigate the possible mechanism, we analyzed the phenotypes of macrophages exposed to MU or WT TGFBIp by detecting the expression of CD68 (a phagocytic marker) and CD36 (a surface scavenger receptor), respectively. WB and flow cytometry analysis showed that high expression of CD68 and CD36 was found mainly in macrophages exposed to WT TGFBIp, and the levels of CD68 and CD36 in these macrophages were similar to those in normal macrophages not exposed to any TGFBIp. In addition, the expressions of CD68 and CD36 were decreased in a time-dependent manner especially in macrophages after WT TGFBIp was withdrawn, but almost unchanged in macrophages after the MU TGFBIp was withdrawn. Moreover, the high expressions of CD68 and CD36 in macrophages exposed to WT TGFBIp ceased when the autophagic process was blocked by 3-MA. Taken together, these results indicated that the defective autophagic activation by MU TGFBIp results in impaired phagocytic activation. Although the relationship between impaired degradation of MU TGFBIp, impaired phagocytic activation of macrophages, and incomplete autophagic flux have been illuminated, the detailed molecular mechanisms still need further investigation.

In summary, we found impaired autophagic degradation of MU TGFBIp due to incomplete autophagy flux in macrophages. Moreover, the incomplete autophagy flux prevents the further phagocytic activation of macrophages, which results in defective further uptake of MU TGFBIp. Our results revealed the relationship among impaired degradation of MU TGFBIp, impaired phagocytic activation of macrophages, and incomplete autophagic flux in macrophages exposed to MU TGFBIp. Although the detailed molecular mechanisms leading to the defective autophagy remain unknown, our results suggested that reversion of the defective autophagic process in macrophages may be a therapeutic strategy for patients with LCD.

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Impaired Autophagy to LCD TGFBIp in Macrophages


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