Fibroblasts endocytose and degrade transthyretin aggregates in transthyretin-related amyloidosis

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Transthyretin (TTR)-related amyloidosis is a fatal disorder characterized by systemic extracellular deposition of TTR amyloid fibrils. Mutations in the TTR gene cause an autosomal dominant form of the disease—familial amyloidotic polyneuropathy (FAP). Wild-type (WT) TTR can also form amyloid fibrils in elderly patients with senile systemic amyloidosis. Regression of amyloid deposits in FAP patients who undergo liver transplantation to remove the main source of mutant TTR suggests the existence of mechanisms for the clearance of TTR deposits from the extracellular matrix (ECM), but the precise mechanisms are largely unknown. Because fibroblasts are abundant, playing a central role in the maintenance of the ECM and because the skin is one of the major sites of soluble TTR catabolism, in the present study, we analyzed their role in clearance of TTR aggregates. *In vitro* studies with a fibroblast cell line revealed that fibroblasts endocytosed and degraded aggregated TTR. Subcutaneous injection of soluble and aggregated TTR into WT mice showed internalization and clearance over time by both fibroblasts and macrophages. Immunohistochemical studies of skin biopsies from V30M patients, asymptomatic carriers, recipients of domino FAP livers as well as transgenic mice for human V30M showed intracellular TTR immunoreactivity in fibroblasts and macrophages that increased with clinical status and with age in transgenic mice. Overall, the present *in vitro* and *in vivo* data show that fibroblasts endocytose and degrade TTR aggregates. The function or dysfunction of TTR clearance by fibroblasts may have important implications for the development, progression, and regression of TTR deposition in the ECM.

Laboratory Investigation (2013) **93**, 911–920; doi:10.1038/labinvest.2013.83; published online 1 July 2013

KEYWORDS: amyloidosis; endocytosis; extracellular matrix; familial amyloidotic polyneuropathy; fibroblasts; transthyretin; TTR degradation

Transthyretin (TTR)-related amyloidoses are fatal systemic amyloidoses associated with systemic deposition of TTR extracellular amyloid fibrils. TTR-related familial amyloidotic polyneuropathy (FAP) is an autosomal dominant disease related to mutant TTR deposition in various organs and systems, including the peripheral nervous system, eyes, heart, gastrointestinal tract, kidneys, and skin. As of 2012, more than 120 TTR mutations have been discovered, most of which lead to FAP development (http://www.fapwtr.org/ram_fap.htm); TTR V30M is the most common disease-causing mutation and occurs in large foci of patients throughout the world.¹ Senile systemic amyloidosis (SSA) is an age-related nonhereditary systemic amyloidosis and mainly affects cardiac functions in elderly people where wild-type (WT) TTR forms amyloid in different tissues.²

TTR is primarily synthesized by the liver, retina, and choroid plexus of brain, circulates as a soluble tetramer form in blood,

cerebrospinal fluid, and aqueous humor, and transports thyroxine and retinol-binding protein. TTR dissociation into monomers is a determining step for partial misfolding, oligomer formation, and fibrillogenesis in tissues. The rate of TTR tetramer dissociation is increased by amyloidogenic mutations.³ The pathogenesis of TTR-related amyloidosis, however, cannot be fully explained by instability of the tetrameric structure that results from mutations because of the following: (1) the penetrance, age at onset, clinical symptoms, and prognosis differ depending on race and pedigree, even for the same mutant TTR;⁴ (2) WT TTR also forms amyloid fibrils and causes SSA in elderly people, especially men;² and (3) pathological studies reveal organ and tissue tropism of TTR amyloid.⁵ These data indicate the presence of complex mechanisms of amyloid fibril formation and clearance *in vivo*, mechanisms that may be affected by unknown genetic and environmental factors, aging, and sex differences.

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Received 15 February 2013; revised 24 May 2013; accepted 3 June 2013

Previous histopathological observations on V30M individuals showed extracellular oligomeric non-fibrillar Congo red-negative TTR forms in tissues of FAP patients, asymptomatic carriers, recipients of livers transplanted from FAP patients and transgenic mouse models expressing amyloidogenic TTR V30M.^{6–8} Interestingly, pathological and biochemical findings of FAP patients who underwent liver transplantation to remove the main source of mutant TTR showed regression of amyloid deposits in aspirated abdominal fat tissues and gastrointestinal mucosa.⁹ Although cardiac amyloid deposition reportedly sometimes progressed after liver transplantation, proteomics analyses revealed that amyloid fibrils in these patients were derived primarily from WT TTR, which the normal liver graft must have produced.¹⁰ These findings suggest the presence of a dynamic balance between the formation and degradation of TTR amyloid in the extracellular matrix (ECM).

A kinetic study of serum WT TTR showed that TTR is degraded in the liver, muscle, skin, and kidney.¹¹ Different tetrameric stabilities of WT and mutant TTRs cause different clearance patterns from plasma.¹² Previous *in vitro* studies showed that WT and mutant TTR are internalized by different types of cells, including hepatocytes, mouse embryonic fibroblasts, yolk sac cells, and sensory neurons.^{13–15} Whether these cells could endocytose and degrade TTR aggregates *in vivo* needs investigation.

The present report addresses this question in a fibroblast cell line and an experimental mouse model after subcutaneous injection of TTR aggregates. Furthermore, we inspected the presence of TTR in fibroblasts in skin biopsies of V30M FAP patients, asymptomatic carriers, recipients of domino FAP transplants, and also in V30M transgenic mice at various ages.

MATERIALS AND METHODS

Tissue Samples

Archival skin biopsy samples obtained from FAP V30M patients ($n = 4$), asymptomatic carriers ($n = 4$), domino liver transplant recipients ($n = 15$), and normal control subjects ($n = 4$) were previously characterized for TTR deposition after informed consent.⁷ Autopsy heart tissues from six patients with FAP V30M and six non-FAP patients who had no heart disease were obtained at Kumamoto University Hospital. The Ethical Committee of Kumamoto University gave approval for the study.

Skin specimens from transgenic mice expressing human TTR V30M in a TTR null background at the ages of 3 ($n = 5$), 6 ($n = 5$), and 15 ($n = 5$) months were available at the laboratory.¹⁶

Subcutaneous (s.c.) Injection of TTR into Mice

Mice were housed in pathogen-free conditions; all animal experiments were performed according to the European Communities Council Directive. To investigate the function of fibroblasts in the degradation of TTR *in vivo*, we injected

6-month-old SV129 WT mice s.c. in three different spots in the back using 29 G syringes with preparations containing 0.05 mg (in 200 μ l volume) of either soluble or aggregated WT TTR and V30M TTR ($n = 3$ /group). Skin samples were collected (including approximately 2 mm of surrounding tissue from the center of injected site) 3, 8, and 24 h after injection, subsequently fixed in formalin, and embedded in paraffin. Three-micrometer slices were cut and evaluated by immunohistochemistry.

Preparation of Soluble TTR and Aggregated TTR

Recombinant WT and V30M TTRs were produced in an *Escherichia coli* expression system. Protein isolation and purification were performed as previously described.¹⁷ Aggregated TTR forms were generated by incubating the protein (0.25 mg/ml), with stirring, at room temperature for 7 days.¹⁸ Aggregated TTR was diluted 1:9 (v/v) with 30 μ M thioflavin T in Glycine Buffer (pH 9.0), and the oligomeric preparations were tested by thioflavin T spectrofluorometric assays (excitation at 440 nm). Electron microscopy was also performed to check the aggregates formation. For fluorescent TTR, the protein was labelled with the fluorescent dye Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Cell Culture

The human fibroblast cell line NIH3T3 was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (0.1 mg/ml) at 37 °C in a 5% CO₂ humidified atmosphere. Multi-spot glass slides (Greiner Bio-One, Frickenhausen, Germany) and 12-well plates (Orange Scientific, Braine-l'Alleud, Belgium) were coated with 250 ng of aggregated TTR prepared as described above, air-dried, and incubated with NIH3T3 cells (1×10^4 cells/ml) in DMEM for up to 48 h. For immunocytochemistry, cells on glass slides were washed twice with phosphate-buffered saline (PBS) and fixed for 30 min with 2% paraformaldehyde before incubation with selected antibodies; for immunoblotting assays, the 12-well coated plates were treated with 20 mM HEPES-NaOH (pH 7.4) containing 1% Triton X-100 before application into filters. In another experiments, fibroblasts were incubated with unlabelled TTR aggregates (1 μ g/ml) in 24-well plates (Orange Scientific), and supernatants were collected at 0, 3, 8, 24, and 48 h (three wells per time point) and measured for total and aggregated TTR content (see below). For live cell culture studies, cells were incubated in chamber slides (Ibidi, Munich, Germany) with either synthetic soluble Alexa-TTR or Alexa TTR aggregates for 24 h and then incubated with red fluorescent LysoTracker (Invitrogen Molecular Probes, Eugene, OR, USA) for 60 min, after which images of the fluorescence were acquired with a confocal microscope (Leica TCS SP2 AOBS; Leica Microsystems, Heidelberg, Germany). Experiments were performed in duplicate and repeated three times.

Dot-Blot and Filter Assays

Amounts of total TTR and TTR aggregates were analyzed by dot-blot and filter assays, respectively, as previously described.¹⁹ For the dot-blot assay, cell culture medium or lysate was applied to a nitrocellulose membrane and dried. For the filter assay, to separate soluble proteins from aggregates, samples were applied to a 0.2- μ m-pore cellulose acetate membrane filter (Schleicher and Schuell, Dassel, Germany), by using a filtration manifold system (Gibco BRL, Gaithersburg, MD, USA). The membrane/filter were blocked with 5% (w/v) skim milk in PBS for 1 h, incubated with a rabbit anti-human TTR antibody (1:500; Dako, Glostrup, Denmark) for 1 h, and then incubated with a horseradish peroxidase-conjugated anti-rabbit IgG (1:500). TTR signals were detected with the ECL Kit (Enhanced Chemiluminescence; GE Healthcare, Buckinghamshire, UK) and were quantified by using the public domain NIH ImageJ program.

Immunohistochemistry and Immunofluorescence

For immunohistochemistry, tissue sections were treated with 3% hydrogen peroxide in methanol for 30 min. Slides were incubated in 4% FBS and 1% bovine serum albumin in PBS at room temperature. After overnight incubation with anti-human TTR antibody (1:1000; Dako) at 4 °C, reactivity was visualized via the biotin-extravidin-peroxidase kit (Sigma-Aldrich, St Louis, MO, USA). Images were acquired from 10 visual fields per sample at an original magnification of $\times 40$. Calculation of the percentage of TTR-immunopositive area was performed by using NIH ImageJ, as previously described.²⁰

For immunofluorescence, primary antibodies were: sheep anti-human TTR (1:500; Abcam, Cambridge, UK), goat anti-S100A4 (1:50; Acris Antibodies GmbH, Herford, Germany), goat anti-Iba1 (1:50; Abcam), and rabbit anti-Lamp2 (1:100; Sigma-Aldrich), which were incubated overnight at 4 °C in blocking buffer. Secondary antibodies were Alexa Fluor 568 anti-rabbit, Alexa Fluor 568 anti-goat, and Alexa Fluor 488 anti-sheep (1:1000; Invitrogen Molecular Probes), with incubation for 1 h at room temperature. Slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) and visualized by confocal microscopy (Leica TCS SP2 AOBs). TTR-immunopositive fibroblasts were detected by merged images with anti-TTR antibody (green) and fibroblast marker (S100A4; red). The number of TTR-immunopositive cells in each group was calculated as an average of 10 visual fields (200 μ m $\times$ 200 μ m) per sample (1 specimen/sample) at an original magnification of $\times 40$.

Congo Red Staining

Paraffin-embedded sections and paraformaldehyde-fixed cells were stained with phenol Congo red solution and were viewed by bright field or polarized light microscope.

Statistics

All data are expressed as means $\pm$ SD. Controls and treated groups were compared by ANOVA followed by the Tukey *post hoc* test. Accepted level of significance was $P < 0.05$.

RESULTS

Fibroblasts Endocytose and Degrade TTR Aggregates *In Vitro*

We performed different types of '*in vitro*' studies in NIH3T3 fibroblasts incubated with aggregates preparations formed from WT-TTR. This preparation contains both soluble and oligomeric TTR, with amyloid-like properties as assessed by thioflavin T (Figure 1a) filter immunoassay (Figure 1b), and electron microscopy (Figure 1c); immunofluorescence analysis with anti-TTR on aggregate-coated glass surfaces (coverslips) showed an heterogeneous preparation of different size aggregates, which is drastically reduced upon addition and incubation with fibroblasts (Figures 1d and e); in some instances, aggregates are observed in close contact with the plasma membrane.

The proportion of soluble and aggregated forms was quantified in coated wells in the absence/presence of fibroblasts; when fibroblasts were added, the intra and extracellular total levels of TTR (soluble and aggregated) were significantly reduced (Figure 1f); similarly, levels of aggregated TTR, as assessed by filter assays were also reduced, as compared with the control situation without cells. All together, the data suggested that not only soluble but also aggregated TTR had been degraded. Finally, we found that the levels of total and aggregated TTR decreased in conditioned media over time upon incubation with fibroblasts (Figure 1h).

To investigate internalization of TTR aggregates, we incubated fluorescently labeled soluble and aggregated TTR with fibroblasts and used live cell imaging by confocal microscopy. Consecutive cross-sectional images revealed that indeed fibroblasts internalized small TTR aggregates (Figures 2a and c) observed intracellularly as punctuate material. By contrast, cells cultured with soluble TTR did not display TTR signal (Figure 2b), probably because of inefficient internalization and/or insufficient weak signal. Further incubation with LysoTracker demonstrated co-localization of TTR aggregated material within lysosomes (Figure 2d). Intracellular amyloid formation was not observed in Congo red staining (Supplementary Figure S1a).

Fibroblasts Endocytose and Degrade Soluble and Aggregated TTR in Mouse Skin

To investigate internalization and degradation '*in vivo*', we injected mouse skin with WT and V30M with both soluble and aggregated preparations, killed animals 8 h later, and searched for TTR immunoreactivity in skin sections. TTR immunoreactivity was observed within a perimeter of approximately 14 mm² and localized in the dermis and subcutaneous layers but not in the epidermis and muscle

Figure 1 NIH3T3 fibroblasts removed TTR aggregates *in vitro*. (a) Thioflavin T (ThT) fluorescence spectra of TTR preparations before (–) and after (+) oligomer formation and respective immunoblotting image obtained via (b) the filter assay and (c) electron microscopy. Bar = 100 nm. (d, e) Immunofluorescence images of TTR aggregates (green) on the glass surface after incubation without cells (d) and with NIH3T3 cells (e). Nuclei stained blue with DAPI. Magnification $\times 400$. (f) Quantification of total (soluble and aggregated) TTR on the glass surface as obtained by a dot-blot assay after incubation with and without NIH3T3 cells for 48 h. (g) Quantification of aggregated TTR on the glass surface as obtained by a filter assay after incubation with and without NIH3T3 cells for 48 h. (f, g) $*P < 0.01$. (h) Quantification over time of total and aggregated TTR in cell culture media as obtained by dot-blot and filter assays, respectively.

(Figure 3a), in the interstitium, specially around blood vessels and sebaceous glands, and intracellularly in fibroblasts and macrophages (Figure 3a) as confirmed with consecutive cross-sectional confocal microscopic images with fibroblast and macrophage markers, respectively (Figures 3b and c). Furthermore confocal microscopic images with a lysosomal marker showed that intracellular TTR signals partially co-localized with lysosome (Figure 3d). Other types of cells, including myocytes, epithelial cells, and endothelial cells, showed no intracellular TTR immunoreactivity. No differences in TTR tissue distribution were noticed among tissues from animals injected with WT or V30M either in soluble or aggregated form.

To determine changes in levels of intracellular and extracellular TTR over time, we collected skin samples at 3, 8, and 24 h after injection and semiquantified total TTR levels and

number of immunoreactive fibroblasts. Total TTR, particularly extracellular TTR, constituted most of the TTR found decreased to residual levels at 8 h and was not detected at 24 h (Figures 3f–i). The number of TTR-immunopositive fibroblasts, however, increased between 3 and 8 h and decreased thereafter (Figures 3f–h and j). Similar decay and/or number of immunoreactive fibroblast patterns were found in the four groups of injected mice (with soluble and aggregated WT and V30M TTR). Intracellular amyloid formation was not detected with Congo red staining (Supplementary Figure S1b).

TTR Co-Localized with Fibroblasts in Transgenic Mice Expressing Human V30M TTR

In another study, we analyzed skin from 15-month-old transgenic mice expressing human TTR V30M in a TTR null

Figure 2 Internalization of TTR aggregates *in vitro* by NIH3T3 fibroblasts. Fluorescence images of NIH3T3 cells incubated with (a) TTR aggregates (green) or (b) soluble TTR in cell culture media at 0, 3, and 24 h. Arrow heads indicate TTR aggregates close contact with cell membranes. Nuclei stained blue with DAPI. Magnification $\times 400$. (c) Consecutive cross-sectional (A–D) images of NIH3T3 cells after incubation with aggregated fluorescent TTR for 24 h. Magnification $\times 400$. (d) Image of live NIH3T3 cells incubated with fluorescent TTR aggregates (green) and LysoTracker (red). Arrows indicate co-localization of TTR aggregates in lysosomes. Magnification $\times 400$.

background; this strain shows deposition of non-fibrillar TTR after 3 months of age, especially in the gastrointestinal tract and skin; small amounts of TTR amyloid fibrils are detected in the gastrointestinal tract after 9 months of age.¹⁶ Immunohistochemistry and immunofluorescence with anti-TTR antibody and a fibroblast and/or macrophage marker demonstrated TTR in some fibroblasts and macrophages in the skin, besides the typical extracellular TTR deposits (Figures 4a–c), and shared a similar histology with specimens from WT mice that received s.c. TTR injections as described above; immunopositive tissues from the gastrointestinal tract also disclosed TTR-positive fibroblasts and macrophages (data not shown).

We further investigated TTR deposition at different ages in this mouse model. Extracellular TTR deposition increased in skin with advancing age, as in previous reports.²¹ Concerning intracellular TTR, the number of TTR-immunopositive fibroblasts significantly increased between 3 and 6 months and remained unchanged at 15 months (Figures 4d–g).

TTR Co-Localized with a Fibroblast Marker in Skin and Heart Specimens from FAP Patients

Finally, to determine whether fibroblasts in human tissues internalize TTR, we performed TTR immunohistochemistry

Figure 3 Endocytosis by fibroblasts of TTR injected into mouse skin. (a) TTR immunostaining of a skin sample taken at 8 h after V30M TTR aggregates injection. Different skin areas are indicated in the left. Magnification $\times 200$. (b) Confocal microscopy image with anti-TTR antibody (green), fibroblast marker (S100A4; red), (c) macrophage marker (Iba1; red), and (d) lysosome marker (Lamp2; red). Nuclei stained blue with DAPI. Arrows indicate co-localization of TTR aggregates in lysosomes. (e–h) TTR immunostaining of skin samples taken at (e) 3 h after PBS injection and 3 h (f), 8 h (g), and 24 h (h) after injection of V30M TTR aggregates. Magnification $\times 400$. (i, j) Semiquantification of (i) extracellular TTR-immunopositive areas and (j) the number of TTR-immunopositive fibroblasts after injection of different TTR preparation, both soluble and aggregated TTR derived from WT and V30M mutant. Insets show boxed areas at greater magnification.

and confocal microscopy with the fibroblast marker in skin biopsy samples and autopsy heart tissues obtained from FAP V30M patients containing amyloid deposits, asymptomatic carriers with non fibrillar TTR deposition, domino liver transplant recipients (with non-fibrillar and amyloid deposition), and controls (Figure 5). Except for control skin, all the groups analyzed had TTR-immunoreactive fibroblasts (Figures 5a–c), as confirmed by confocal microscopy with the fibroblast marker (Figure 5d). The proportion of intracellular TTR immunoreactivity was not much, because TTR aggregates were mainly deposited in the extracellular space in tissues from patients. The proportion of fibroblasts with intracellular TTR varied among the analyzed groups as summarized in Table 1; thus, three out of the four skin

biopsies from FAP patients presented TTR in fibroblasts, whereas only one out of four asymptomatic carrier samples were positive. As for FAP domino liver transplant recipients, we analyzed skin biopsies post transplant collected after 1–4 years, which were both Congo red and TTR negative and collected after 4 years of transplant; the latter group was positive for TTR and included samples with and without amyloid deposition. Two out five samples that were positive for TTR but negative for Congo red birefringence (CR–TTR+) showed TTR-immunoreactive fibroblasts, whereas this proportion increased to four out of five in skin biopsies positive for amyloid (CR+TTR+). The levels of TTR-immunoreactive fibroblasts increased significantly in FAP patients as presented in Figure 5e.

Figure 4 TTR-immunopositive fibroblasts in transgenic mice. (a) TTR immunostaining of a skin sample from transgenic mouse expressing the human amyloidogenic V30M TTR. Insets show boxed areas at greater magnification representing macrophages (upper image) and fibroblasts (lower image). Magnification $\times 200$. (b) Confocal microscopy image with anti-TTR antibody (green), fibroblast marker (S100A4; red), and DAPI (blue). (c) Confocal microscopic image with anti-TTR antibody (green), macrophage marker (Iba1; red), and DAPI (blue). (d) Semiquantification of the number of TTR-immunopositive fibroblasts in mouse skin of different ages. (e–g) Representative images of TTR immunostaining of skin samples from transgenic mice at the ages of 3 (e), 6 (f), and 15 (g) months. Magnification $\times 400$. * $P < 0.01$.

Also, in the heart, certain fibroblasts from FAP patients manifested TTR immunoreactivity in their cytoplasm, especially fibroblasts in the epicardium and endocardium (Figures 5f–h).

DISCUSSION

Amyloid deposits preferentially in the ECM, especially at basement membranes, in most types of amyloidosis, including Alzheimer's disease, AL amyloidosis, AA amyloidosis, and TTR-related amyloidosis.^{22,23} The ECM microenvironment is thought to have a key role in amyloid fibril formation in TTR-related amyloidosis, because prefibrillar aggregate

and fibrillar forms of TTR exist in the ECM generated from blood-soluble TTR.

Studies on long-term course of FAP patients after liver transplantation showed that the total amount of amyloid deposits in abdominal fat tissues decreased, and contribution of WT TTR to mutant TTR increased, which may indicate the presence of a dynamic process of amyloid fibril formation and metabolism in the ECM.^{9,10}

The ECM maintains tissue architecture and homeostasis and contains numerous components, including collagens, proteoglycans, glycosaminoglycans, growth factors, and proteases, that are produced and secreted by various stromal

Figure 5 TTR-immunopositive fibroblasts in human tissues. (a–c) Representative images of TTR immunostaining of skin specimens from a (a) control subject, (b) asymptomatic carrier, and (c) FAP patient. Magnification $\times 200$. (d) Confocal microscopy image of a skin specimen from an FAP patient with anti-TTR antibody (green) and fibroblast marker (S100A4; red). Magnification $\times 400$. (e) Semiquantification of the number of TTR-immunopositive fibroblasts in the skin from asymptomatic carriers, FAP patients, and FAP domino liver transplant recipients. $*P < 0.05$. (f, g) Representative images of TTR immunostaining of heart specimens from (f) control and (g) FAP patients. Magnification $\times 200$. (h) Confocal microscopy image of a heart specimen from an FAP patient with anti-TTR antibody (green) and fibroblast marker (S100A4; red). Magnification $\times 400$. Insets show boxed areas at greater magnification. Arrows point to TTR-immunopositive fibroblasts.

cells, predominantly fibroblasts.²⁴ Fibroblasts, together with macrophages, also have a central role in degrading and remodelling the ECM. Although, different types of cells reportedly exhibit varying degrees of TTR internalization activity *in vitro*,^{13–15} fibroblasts and macrophages have higher migratory potential and more accessibility to TTR deposits in ECM. Previous ultrastructural analyses revealed amyloid deposits at the outer layer of the basement membrane, which occurs at the base of all epithelial linings, underlying endothelia of capillaries and venules, and around Schwann cells, adipocytes, and muscle cells.^{22,25} Therefore, these cells are separated from amyloid deposits by their own basement

membranes, and they barely make contact and degrade amyloid fibrils. Fibroblasts and macrophages, however, are in close proximity to amyloid deposits but their interactions are still unclear.

Macrophages perform a significant phagocytic function: removing dead cell materials, necrotic tissue components, and foreign materials such as pathogens, and they also reportedly phagocytosed and degraded amyloid β ($A\beta$), β_2 -microglobulin (β_2m), and AA amyloid fibrils.^{26–29} Conversely, macrophages also reportedly promoted amyloid formation by phagocytosis of amyloid precursor proteins, including $A\beta$ and SAA.^{29,30}

Table 1 Presence of TTR-immunopositive fibroblasts in skin tissues obtained from patients and controls

Controls	Carriers	FAP patients	Domino LT recipients			
			CR –	TTR –	CR –	TTR +
0/4	1/4	3/4	0/5	2/5	CR +	TTR +
					4/5	

Abbreviations: LT, liver transplant; CR, Congo red staining; TTR, anti-TTR immunostaining.

Fibroblasts produce ECM remodelling enzymes, including matrix metalloproteinases (MMPs) and their specific inhibitors (tissue inhibitor of metalloproteinases).³¹ MMPs work cooperatively to degrade most ECM components. MMPs 2, 3, and 9 were shown to degrade A β fibrils *in vitro*,³² and are upregulated in brain tissues obtained from Alzheimer's disease patients and transgenic model mice.³³ Similarly, MMPs degraded TTR fibrils and prefibrillar aggregates *in vitro* and were upregulated in FAP patients,³⁴ transgenic animals,³⁵ and a cultured cell line.³⁶

In this report, we show the capacity of fibroblasts to endocytose and degrade small TTR aggregates in lysosomes, both '*in vitro*' and in an '*in vivo*' model, after s.c. injection of TTR aggregates into WT mice, whereby soluble and aggregated TTR were endocytosed by fibroblasts and macrophages that efficiently cleared both types of TTRs with time. Recently, intracellular localization of TTR V30M in glomerular podocytes as well as in kidney ECM was shown in transgenic mice. Increased deposition was demonstrated in mice housed in non-specific pathogen-free conditions, suggesting environmental conditions influence both glomerular deposition and podocytic localization of TTRV30M.³⁷ Similarly, a recent study with a mutant TTR transgenic fly model revealed that TTR aggregates were internalized by body fat cells *in vivo*.³⁸ The authors hypothesized that the prefibrillar TTR aggregates matured inside the cells and amyloid fibrils released by cell death. In our cultured cell line and TTR-injected mouse skin, however, formation of amyloid fibrils inside the cytoplasm was not observed.

Intracellular accumulation of aggregated material derived from the ECM was previously reported, not only in fibroblasts but also from other cell types. In one research study of β_2 m amyloidosis, in which amyloid fibrils were deposited mainly in the synovium and cartilage, human synovial fibroblasts endocytosed β_2 m that had been modified with advanced glycation end products.³⁹ Another cell type, astrocytes, which are the most abundant cells in the central nervous system, have functions similar to those of fibroblasts in the maintenance of homeostasis, in which these cells produce ECM components and MMPs.^{40,41} Previous studies provided evidence from brain specimens from Alzheimer's disease patients that astrocytes degraded A β oligomers and amyloid fibrils *in vitro* and *in vivo* and manifested intracellular A β .^{42,43} Similarly, in primary cutaneous amyloidosis, intracellular

oligomers were observed in the basal cell layer of the epidermis and in surrounding cells, including fibroblasts.⁴⁴

As Figures 4 and 5 illustrate, some fibroblasts in tissues obtained from FAP patients, asymptomatic carriers, domino liver transplant recipients, and transgenic mice contained TTR. It is known that biosynthesis and serum concentrations of TTR decreased with disease progression, but extracellular TTR aggregates accumulated at an accelerated rate.⁴⁵ In our study, abundance of TTR-immunoreactive fibroblasts showed a tendency to increase with progression of amyloid deposition. Most likely, dysfunctional clearance of TTR aggregates by fibroblasts resulting from inability of lysosomes to deal with the intracellular load of aggregated TTR material may cause development and progression of TTR-related amyloidosis. In addition to the intracellular uptake of TTR, as shown here, fibroblasts may have the ability to degrade TTR aggregates and fibrils extracellularly by secreting MMPs. Further studies are needed to clarify these multiple cellular and molecular mechanisms of fibroblasts in the clearance of TTR aggregates, which may provide etiological and therapeutic clues in TTR-related amyloidosis.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

ACKNOWLEDGEMENTS

We would like to thank Paula Gonçalves from IBMC for tissue specimen preparation; Paula Sampaio and Joana Magalhães for support with confocal microscopy and oligomer experiments, respectively. This work is supported by grants from the Amyloidosis Research Committee, the Pathogenesis, Therapy of Hereditary Neuropathy Research Committee, the Surveys and Research on Specific Disease, the Ministry of Health and Welfare of Japan, and Grants-in-Aid for Scientific Research: Strategic Young Researcher Overseas Visits Program for Accelerating Brain Circulation (S2210) from the Ministry of Education, Science, Sports and Culture of Japan to YA and YM and FEDER funds through the Operational Competitiveness Programme—COMPETE and by national funding from Portuguese Foundation for Science and Technology (FCT) under the projects FCOMP-01-0124-FEDER-022718 (PEST-c/SAU/LA0002/2011 and PTDC/SAU-ORG/111313/2009) to MJS, and fellowship to NPG (SFRH/BD/74304/2010).

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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