Amyloid deposition in a mouse model humanized at the transthyretin and retinol-binding protein 4 loci

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Received: 29 August 2017 / Revised: 29 November 2017 / Accepted: 2 December 2017
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Abstract

Familial amyloidotic polyneuropathy is an autosomal dominant disorder caused by a point mutation in the transthyretin (*TTR*) gene. The process of *TTR* amyloidogenesis begins with rate-limiting dissociation of the *TTR* tetramer. Thus, the *TTR* stabilizers, such as Tafamidis and Diflunisal, are now in clinical trials. Mouse models will be useful to testing the efficacy of these drugs. Although several mouse models have been generated, they all express mouse *Rbp4*. Thus, human *TTR* associates with mouse RBP4, resulting in different kinetic and thermodynamic stability profiles of *TTR* tetramers. To overcome this problem, we previously produced humanized mouse strains at both the *TTR* and *Rbp4* loci (*Ttr*^{*hTTRVal30*}, *Ttr*^{*hTTRMet30*}, and *Rbp4*^{*hRBP4*}). By mating these mice, we produced double-humanized mouse strains, *Ttr*^{*hTTRVal30/hTTRVal30*}·*Rbp4*^{*hRBP4/hRBP4*} and *Ttr*^{*hTTRVal30/Met30*}·*Rbp4*^{*hRBP4/hRBP4*}. We used conventional transgenic mouse strains on a wild-type (*Ttr*^{*+/+*};*Tg*[6.0*hTTRMet30*]) or knockout *Ttr* background (*Ttr*^{*-/-*};*Tg*[6.0*hTTRMet30*]) as reference strains. The double-humanized mouse showed 1/25 of serum hTTR and 1/40 of serum hRBP4 levels. However, amyloid deposition was more pronounced in *Ttr*^{*hTTRVal30/Met30*}·*Rbp4*^{*hRBP4/hRBP4*} than in conventional transgenic mouse strains. In addition, a similar amount of amyloid deposition was also observed in *Ttr*^{*hTTRVal30/hTTRVal30*}·*Rbp4*^{*hRBP4/hRBP4*} mice that carried the wild-type human *TTR* gene. Furthermore, amyloid deposition was first observed in the sciatic nerve without any additional genetic change. In all strains, anti-*TTR* antibody-positive deposits were found in earlier age and at higher percentage than amyloid fibril deposition. In double-humanized mice, gel filtration analysis of serum revealed that most hTTR was free of hRBP4, suggesting importance of free *TTR* for amyloid deposition.

Introduction

Transthyretin (*TTR*)-associated amyloidosis (ATTR) is an autosomal dominant disorder caused by a point mutation in the *TTR* gene. According to the online registry for hereditary amyloidosis mutations (<http://www.amyloidosismutations.com>), 138 *TTR* mutations are associated to human amyloidosis. *TTR* is a 127-amino acid, 55-kDa protein composed of four identical, non-covalently associated subunits [1, 2]. *TTR* serves as a transport molecule for thyroxine (T4) and retinol-binding protein 4 (RBP4). The process of *TTR* amyloidogenesis involves rate-limiting dissociation of the *TTR* tetramer, followed by partial unfolding of monomers to yield non-fibrillar aggregates, protofibrils, and mature amyloid fibrils [3–5]. Structural studies of the *TTR* tetramer yielded several compounds that bind to the T4 hormone pocket of *TTR*, consequently stabilizing *TTR* and inhibiting fibril formation [6–11].

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To gain insight into the pathogenesis of ATTR, several groups have generated transgenic mice that carry *TTR* genes with various mutations, such as Met30, Ser10/Met30, Pro55, or Ser84 [12–18]. Using the mouse metallothionein promoter or the human homologous *TTR* promoter, we produced transgenic mice in which amyloid deposition was observed in similar tissues as in human autopsy cases, except for its absence in the peripheral and autonomic nervous systems [15, 18]. Teng et al. [16] have also reported amyloid deposition in transgenic mice carrying the wild-type human *TTR* gene. By contrast, transgenic mouse strains with amyloidogenic Pro55 develop non-fibrillar *TTR* deposition, but fail to develop amyloid deposits in a 129S1/Sv background [12]. Later, Reixach et al. [19] demonstrated that heterotetramers comprising mouse and human subunits are kinetically more stable than those of human homotetramers and are considered to be inhibitory to dissociation and subsequent amyloid formation. Sousa et al. [12] reported the presence of amyloid fibrils in Pro55 transgenic mice on a *TTR*-null background.

Although liver transplantation is a promising approach for preventing disease progression, its utility of liver transplantation is limited by restricted donor availability, patient eligibility, and the risk of adverse events [20–26]. Several compounds that bind to the T4 hormone pocket were shown to stabilize the *TTR* tetramer thereby inhibiting fibril formation. Two *TTR* stabilizers, such as Tafamidis and Diflunisal, are now in clinical trials, and Tafamidis was approved in the European Union in 2011. Although both drugs seem to slow disease progression, no therapy can reverse the disease course [27–30]. Thus, there is an urgent medical need for new treatments that can halt or reverse the progression of amyloidosis and that are effective across all genotypes and disease stages.

Animal models that reproduce the pathology of human *TTR*-mediated familial amyloidosis are required to assess the *in vivo* efficacy of drug candidates. We previously produced humanized mice (*Ttr*^{hTTRVal30} and *Ttr*^{hTTRMet30}) carrying either human normal (*hV*) or mutant (*hM*) *TTR* cDNA at the mouse *Ttr* locus using a Cre-mediated recombination system [31]. To test the stabilizing activity of drugs *in vivo*, we think it is essential to mimic the physiologic condition of human serum, where human *TTR* binds to human RBP4. Thus, we also created a humanized mouse (*Rbp4*^{hRBP4}) carrying the human *RBP4* (*hR*) cDNA at the mouse *Rbp4* locus using the same strategy [32–34]. We then crossed these lines to generate mice humanized mice with a normal (*Ttr*^{hTTRV30/hTTRV30};*Rbp4*^{hRBP4/hRB4}, abbreviated as *hV/hV:hR/hR*) or patient genotype (*Ttr*^{hTTRV30/hTTRM30};*Rbp4*^{hRBP4/hRB4}, abbreviated as *hV/hM:hR/hR*). Here we examined amyloid deposition in these double-humanized mice compared with conventional transgenic mouse strains on a wild-type (*Ttr*^{+/+;Tg}[6.0h*TTRMet30*],

abbreviated as *+/+;Tg*) or knockout *Ttr* background (*Ttr*^{-/-;Tg}[6.0h*TTRMet30*], abbreviated as *-/-;Tg*).

Materials and methods

Conventional Transgenic Mouse Strains

Wild-type (*+/+;Tg*) or knockout *Ttr* background (*-/-;Tg*) hemizygous mice for the 6.0h*TTRMet30* gene were used as control. These mice contained ~30 copies of the 6.0h*TTRMet30* gene [35].

Construction of the Targeting/Replacement Vectors and the Isolation of Targeted/Replaced ES Cells

The construction of the targeting vector and the isolation of targeted ES cells for the mouse *Ttr* locus were achieved using a previously described method [31]. Briefly, a 3-kb 5' homologous region upstream of the ATG codon and a 6.7-kb 3' homologous region downstream of the ATG codon were ligated into a p71neoP cassette containing lox71-PGK-neo-loxP-polyA, to produce the *TTR71neoPTTR* construct that comprised 5' *Ttr* homologous region-lox71-PGK-neo-loxP-polyA-3' *Ttr* homologous region. Finally, a diphtheria toxin A (DT-A) fragment with an MC1 promoter was ligated to the 5'-end of *TTR71neoPTTR* to produce the targeting vector (Fig. 1a). The targeting vectors were introduced into TT2 ES cells [36] derived from an F1 embryo obtained from a mating between C57BL/6 and CBA mice (Charles River, Inc., Yokohama, Japan). To generate the replacement vector, we first made a cassette that contained a rabbit β -globin intron II, a *hV* cDNA or a *hM* cDNA, and a puromycin resistance gene with the phosphoglycerate kinase promoter (*PGKpuro*) flanked by two FLP recognition target (FRT) sequences. Then, the cassette flanked by lox66 and loxP sites was inserted into pSP73 (Promega, Tokyo, Japan) (Fig. 1a). The ES cell lines with the *Ttr*-targeted null allele were coelectroporated (Bio-Rad Gene Pulser at 400 V and 125 μ F) with 20 μ g of replacement vector plasmid and 20 μ g of pCAGGS-*Cre* plasmid [37] to produce ES clones with the replaced allele.

The construction of the targeting vector and the isolation of targeted ES cells for the mouse *Rbp4* locus were achieved using a previously described method (Fig. 1b) [32, 34]. Briefly, a 2.8-kb 5' homologous region upstream of the ATG codon and a 6.7-kb 3' homologous region downstream of the ATG codon were ligated into a p71neoP cassette containing lox71-PGK-neo-loxP-polyA, to produce the *RBP471neoPRBP4* construct (5' *Rbp4* homologous region-lox71-PGK-neo-loxP-polyA-3' *Rbp4* homologous region). Finally, a diphtheria toxin A (DT-A) fragment with an MC1 promoter was ligated to the 3'-end of 3' *Rbp4* homologous

Fig. 1 Creation of targeted and replaced alleles. **a** *Ttr* locus. Homologous recombination between the wild-type allele and the targeting vector resulted in the creation of a targeted null allele carrying the PGK-*neo* gene flanked by *lox71* and *loxP*. In the second step, the targeted clones were electroporated with the replacement vector containing *hV* or *hM* cDNA flanked by *lox66* and *loxP*. Site-directed recombination occurred between *lox71/lox66* and *loxP/loxP*, resulting in the creation of the replaced allele, *Ttr*^{hV} or *Ttr*^{hM}. **b** *Rbp4* locus. Homologous recombination between the wild-type allele and the targeting vector yielded a targeted null allele carrying the PGK-*neo* gene flanked by *lox71* and *loxP*. In the second step, targeted clones were electroporated with the replacement vector containing *hRBP4* ORF flanked by *loxKMR3* and *loxP*. Site-directed recombination between *lox71/loxKMR3* and *loxP/loxP* resulted in the creation of the replaced allele, *Rbp4*^{hR}.

region to produce the targeting vector. The targeting vectors were introduced into TT2 ES cells [36]. To construct the replacement vector, we first made a cassette that contained a human *RBP4* cDNA and a puromycin resistance gene with the PGK promoter (PGK_{puro}) flanked by two FRT sequences. Then, the cassette flanked by *loxKMR3* and *loxP* sites was inserted into pSP73 (Promega, Tokyo, Japan). The ES cell lines with the *Rbp4*-targeted null allele were co-electroporated (Bio-Rad Gene Pulser at 400 V, 125 μ F) with 20 μ g of replacement vector plasmid and 20 μ g of pCAGGS-*Cre* plasmid [37] to produce ES clones with the replaced allele.

PCR and Southern Blot Analyses of Isolated Targeted ES Clones

The *Ttr* targeted ES clones were screened and confirmed by long PCR and Southern blot analyses using genomic DNA

purified by phenol, chloroform and ethanol precipitation from ES cells. In the long PCR analysis, the primers, 2-20-1S (5'-GTAAGCAATCTTAGCCAGGCTCTCC-3') and *lox71*-PR (5'-TATACGAACGGTATAGGTCCCTCGAC-3'), were used to detect the 5' end of the targeted allele (3.0 kb). The primers mTtr6 (5'-TGGGCTGAGTCTCTCAATTCTG-3') and neo-F (5'-AGAGGCTATTCGGC-TATGAC-3') were used to detect the 3'-end of the targeted allele (9.0 kb). For Southern blot analysis, DNAs from ES cells was digested with BamHI or XbaI and were analyzed for the presence of the targeted allele using a neo probe. Digested DNAs was electrophoresed on a 1.2% agarose gel and blotted onto nylon membranes (Hybond-N+; Amersham, Tokyo, Japan). After the membranes were cross-linked by exposure to ultraviolet light (UV Stratalinker 1800; Stratagene, La Jolla, CA, USA), hybridization was performed using a neomycin-specific probe prepared using a DIG DNA labeling and detection kit (Roche, Tokyo, Japan).

The *Rbp4*-targeted ES cell clones were confirmed by Southern blot analyses as described previously [34]. For detection of homologous recombination in the 5' region, DNA from ES cells was digested with *XhoI/SalI* or *MunI* and the targeted allele was detected with a neo probe and a 5' probe; for detection of homologous recombination in the 3' region, *AgeI/HpaI* or *SacII/HpaI* digestion and a neo probe and 3' probe were used, respectively.

PCR and Southern Blot Analyses for Genotyping of ES Clones or Mice

Genotypes of ES cells and mice at the *Ttr* locus were determined by PCR analyses and confirmed by Southern blot analysis using genomic DNA from ES cells or tails according to the previously described methods [31]. Briefly, in the PCR analysis, the primers mTtr A9-s (5'-CGTAGAGCGAGTGTTCG-3') and mTtr19 (5'-CAGCTGTTGCTATAGTAATTCCC-3') were used to detect the wild-type mouse *Ttr* allele (864 bp); the primers neo-R (5'-CACCATGATATTCGGCAAGC-3') and neo-F (5'-AGAGGCTATTCGGCTATGAC-3') were used to detect the targeted allele (545 bp); and the primers A9-s and SP-A (5'-CAGTGTATATCATTTGTAACC-3') were used to detect the replaced allele (783 bp). For Southern blot analysis, 10 µg of DNA from mice with the targeted allele and replaced allele was digested with *BamHI* or *XbaI* and *BamHI* or *BglII*, and was analyzed using a neo-specific or puromycin-specific probe, respectively.

Genotypes of ES cells and mice at the *Rbp4* locus were determined by PCR analyses and confirmed by Southern blot analysis using genomic DNA from ES cells or tails according to previously described methods [32, 34]. Briefly, in the PCR analysis, the primers neo-R (5'-CACCATGATATTCGGCAAGC-3') and neo-F (5'-AGAGGCTATTCGGCTATGAC-3') were used to detect the targeted allele (545 bp); the primers mRbp4 GSPF1 (5'-CTCGGCTCCGTCGCTCCACG-3') and mRbp4 GSPR1 (5'-CCAGAGCCCAGAGAACTGAG-3') were used to detect the wild-type mouse *Rbp4* allele (403 bp) and the replaced allele (3421 bp). To detect homologous recombination in the 5' region, DNA from ES cells was digested with *XhoI/SalI* or *MunI* and analyzed for the presence of the targeted allele using a neo and 5' probes. To detect homologous recombination in the 3' region, DNA from ES cells was digested with *AgeI/HpaI* or *SacII/HpaI* and analyzed for the presence of the targeted allele using neo and 3' probes. To detect the replaced allele genomic DNA from ES cells was digested with *BamHI* or *BglII* and was used for Southern blot analysis with a puromycin-specific probe.

Generation of Mouse *Ttr* or *Rbp4* Knockout Mice and Human *TTR* or *RBP4* Knock-in Mice

Chimeric mice were produced by aggregation of ES cells with eight-cell embryos from ICR mice according to previously described methods [31]. Chimeric male mice were backcrossed to C57BL/6 females (Nippon Clea, Kanagawa, Japan) and mice from after the tenth generation were used in the following experiments. The experimental protocols that involved animals were approved by the Kumamoto University Ethics Committee for Animal Experiments (F25-329, F27-122), and all experiments were performed in accordance with the institute's guidelines.

Serum hTTR, mRBP4 and hRBP4 levels

Commercial ELISA kits were used according to the manufacturers' instructions to determine the serum concentrations of hTTR (KA0495, Abnova, Taipei, China), mRBP4 (Mouse Retinol-Binding protein 4 ELISA Kit SimpleStep, ab202404, Abcam, Tokyo, Japan) and hRBP4 (AG-45A-0035YEK-KI01, AdipoGen, San Diego, CA, USA). Elisa assay was performed when the male mice were at 3, 12, and 24 months of age.

Histochemical and Immunohistochemical Analysis

Male mice were used for histochemical and immunohistochemical analyses. Mice were killed by cervical dislocation at 12, 18, and 24 months of age. Tissues—brain, heart, kidneys, spleen, skeletal muscle, stomach, small and large intestines, and sciatic nerves—were excised, fixed in 10% neutral-buffered formalin, and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin. For histochemical demonstration of amyloid, the serial sections were stained with Congo Red according to Wright's method. To detect the emerald green birefringence emitted from the amyloid deposits, a polarized microscope was used to examine the Congo Red stained sections.

For immunohistochemical demonstration of the major components of amyloid deposits, the serial sections were immunostained by the indirect immunoperoxidase method. The primary antibodies were rabbit anti-human TTR (diluted 1:500; Sigma-Aldrich, Tokyo, Japan) or goat anti-mouse serum amyloid A (SAA) (15 µg/ml, R&D Systems, Tokyo, Japan) polyclonal antibodies. The primary antibodies were detected with biotinylated anti-rabbit or anti-goat secondary antibody (diluted 1:200; Vectastain ABC Kit; Vector Laboratories, Burlingame, CA) and DAB detection kit (Ventana Medical Systems, Tucson, AZ) according to the manufacturer's instructions. To obtain negative controls, the same procedure was done without the primary antibodies.

Fig. 2 Serum levels of hTTR and hRBP4. **a** Serum hTTR levels in $+/+;Tg$ and $-/-;Tg$ mice. **b** Serum hTTR levels in $hV/hV:hR/hR$ and $hV/hM:hR/hR$ mice. **c** Serum mRBP4 levels in $+/+;Tg$ and $-/-;Tg$ mice. **d** Serum hRBP4 levels in $hV/hV:hR/hR$ and $hV/hM:hR/hR$ mice. There was no significant difference in mean hTTR, mRBP4, and hRBP4 serum levels among age groups

Gel Filtration Chromatography to Separate RBP4–TTR Complexes

Plasma (0.1 ml) diluted with same volume of buffer (100nmol/L $\text{Na}_2\text{PO}_4\text{-NaH}_2\text{PO}_4$, pH7.4) was loaded onto a Superdex 200 Increase 10/300 GL column (GE Healthcare, Tokyo, Japan) column connected to an LC-8020 HPLC system (Toso, Tokyo, Japan). Protein complexes were separated by passing phosphate-buffered saline through at 0.5 ml/min for 50 min at room temperature. Eluted proteins were detected by their absorbance at 280 nm and collected in 0.5-ml fractions for analysis by SDS–PAGE.

Statistical Analysis

Amyloid deposition was widely distributed in tissues. Thus, it is difficult to use ImageJ to quantify tissue deposition. Instead, we used simply pathological criteria to quantify tissue deposition. For example, no amyloid deposition was scored as 0; $\pm$ (deposition limited to the walls of small vessels) as 1; +(deposition in walls of small vessels and surrounding areas) as 2; ++ (moderate deposition in interstitium) as 3; and +++ (marked deposition in interstitium and parenchyma) as 4. Mann–Whitney *U*-test was used for the statistical analysis. $P < 0.05$ was considered to indicate a significant difference.

Results

Establishment of ES Clones and Mouse Strains with the Targeted Null Allele or Replaced Allele

The mouse lines carrying either *hV* or *hM* at the mouse *Ttr* locus have been described previously [31]. Mouse lines with *hR* at the mouse *Rbp4* locus (humanized *Rbp4* mice) were produced in a similar way as humanized TTR mice [32]. Briefly, the replaced allele was created in two steps. First, the targeted null allele was generated by disrupting the ATG exon, which is in exon 1 in the *Ttr* gene (Fig. 1a) and exon 2 in the *Rbp4* gene (Fig. 1b), using a targeting vector containing a neomycin (neo) resistance gene flanked by *lox71* and *loxP* sites. Second, the *Cre*-mediated site-specific introduction of *hV*, *hM* or *hR* cDNA was performed to produce the replaced allele (Fig. 1a, b).

These humanized mice were bred to produce two mouse strains: $hV/hV:hR/hR$ and $hV/hM:hR/hR$. Mice carrying the humanized allele were bred to homozygosity [33]; heterozygous and homozygous mice were born at the expected from Mendelian ratio.

In addition, we used two other transgenic strains, $+/+;Tg$ [38] and $-/-;Tg$ that were established by mating $+/+;Tg$ with *Ttr* knockout mice. These mice carry the *hM* gene harboring a 6.0-kb upstream region on the C57BL/6 background or the *Ttr*-null background, respectively.

Serum hTTR, mRBP4, and hRBP4 Levels

Four mice in each strain were used for the examination of serum hTTR, mRBP4, and hRBP4 levels. In $+/+;Tg$ mice, mean ($\pm$ SEM) hTTR serum levels at 3, 12, and 24 months of age were 149 ± 7.53 , 148 ± 12.17 , and 142 ± 16.19 μ g/ml, respectively (Fig. 2a, left panel). In $-/-;Tg$ mice, mean ($\pm$ SEM) hTTR serum levels at 3, 12, and 24 months of age were 143 ± 5.12 , 141 ± 9.39 , and 131 ± 11.62 μ g/ml, respectively (Fig. 2a, right panel). The corresponding values for $hV/hV:hR/hR$ mice were 6.62 ± 0.37 , 6.40 ± 0.28 , and 6.06 ± 0.49 μ g/ml, respectively (Fig. 2b, left panel), while in $hV/hM:hR/hR$ mice they were 5.77 ± 0.16 , 5.94 ± 0.50 , and 5.53 ± 0.64 μ g/ml, respectively (Fig. 2b, right panel). In all mouse strains, there was no significant difference in mean hTTR serum levels among age groups. Although there was a trend of lower TTR levels in $hV/hM:hR/hR$ mice than in $hV/hV:hR/hR$ mice, the difference was not significant. Thus, the serum hTTR level in $hV/hV:hR/hR$ mice or $hV/hM:hR/hR$ mice was $\sim 1/25$ of that in $+/+;Tg$ or $-/-;Tg$ mice.

In $+/+;Tg$ mice, mean serum levels of mRBP4 at 3, 12, and 24 months of age were 25.2 ± 1.77 , 25.1 ± 1.28 , and 24.7 ± 1.06 μ g/ml, respectively (Fig. 2c, left panel). The corresponding serum mRBP4 levels were 26.5 ± 0.69 , 25.4 ± 0.89 , and 24.9 ± 0.87 μ g/ml, respectively, in $-/-;Tg$ mice (Fig. 2c, right panel). In $hV/hV:hR/hR$ mice, mean serum levels of hRBP4 at 3 months, 12 months and 24 months of age were 0.60 ± 0.05 , 0.56 ± 0.04 , and 0.55 ± 0.03 μ g/ml, respectively (Fig. 2d, left panel). The corresponding mean serum hRBP4 in $hV/hM:hR/hR$ mice were 0.46 ± 0.02 , 0.47 ± 0.03 , and 0.44 ± 0.03 μ g/ml, respectively (Fig. 2d, right panel). In all mouse strains, there were no significant differences in serum mouse or human RBP4 levels among age groups. As we reported previously [32], the serum level of hRBP4 in hR/hR ($Rbp4^{hRBP4/hRBP4}$) mice was 7.8 ± 0.53 μ g/ml. Thus, these data suggested that low serum hTTR levels resulted in low RBP4 levels, probably due to a low percentage of TTR-bound hRBP4 and glomerular filtration of non-TTR-bound RBP4 into urine. Again, there was a trend of lower RBP4 in $hV/hM:hR/hR$ mice than in $hV/hV:hR/hR$ mice, but this difference was not significant.

Physical Features

As the serum TTR and RBP4 levels were considerably low, we examined the mortality rate, body weight, food consumption, and clinical chemistry to verify the effects of gene modification. Among 15 mice in each group, 4, 3, 5, and 6 mice were found dead in the $+/+;Tg$, $-/-;Tg$, $hV/hV:hR/hR$, and $hV/hM:hR/hR$ groups, respectively, before completion of the 24-month observation period. Thus, the

Fig. 3 Time-courses of the mean body weight gain. Although the mean body weight of $hV/hV:hR/hR$ and $hV/hM:hR/hR$ mice was slightly lower at some points, there was no difference at 24 months of age among the four strains

number of dead animals was similar among the four strains. The cause of death was not investigated. The time course of the mean body weight gain is shown in Fig. 3. Although the mean body weight of $hV/hV:hR/hR$ and $hV/hM:hR/hR$ mice was slightly lower at some points, there was no difference at 24 months of age. Food consumption was measured for one week when mice were 6 months of age and was similar among the four mouse strains. In addition, the results of the clinical chemistry and hematology analyses of blood samples were similar among the four strains.

Onset and Degree of Amyloid Fibril Deposition

Amyloid deposition was not observed in the brain, liver, or spleen of all mouse strains. Amyloid fibrils were stained with the anti-TTR antibody, but not the anti-SAA antibody, suggesting that secondary amyloidosis did not occur under the conventional conditions of the mouse facility.

Amyloid deposition was observed only in the intestinal tract in $+/+;Tg$ (Fig. 5a, e, i) and $-/-;Tg$ mice (Fig. 5b, f, j). Amyloid deposition was first found in the alimentary tract at 12 months of age (Fig. 4, Table 1). The amount of amyloid deposits in the alimentary tract was increased with age (Fig. 4, Table 1). However, amyloid deposition in the heart and kidney was not found, even at 24 months of age, in these lines under the conventional conditions used in this study (Table 1). In general, there was less amyloid deposition in this experiment than that previously reported [15, 39].

Amyloid deposition was first found in the alimentary tract at 12 months of age in the $hV/hV:hR/hR$ line but at 18 months of age in the $hV/hM:hR/hR$ line (Table 1). Amyloid deposition in the $hV/hV:hR/hR$ and the $hV/hM:hR/hR$ lines at 24 months of age was shown in Fig. 5c, g, and k and Fig. 5d, h, and l, respectively. In these lines, the amount

Fig. 4 Amounts of amyloid deposition. **a** At 12 months of age. Amyloid deposition in the alimentary tract was observed in $+/+$:Tg, $-/-$:Tg and $hV/hV:hR/hR$ lines. **b** At 18 months of age. Amyloid deposition in the alimentary tract was observed in all lines. **c** At 24 months of age. The amounts of amyloid deposition in the alimentary tract was higher in the $hV/hV:hR/hR$ and $hV/hM:hR/hR$ lines than those in the $+/+$:Tg and $-/-$:Tg lines. Amyloid deposition in the heart was observed only in the $hV/hV:hR/hR$ and $hV/hM:hR/hR$ lines. Amyloid deposition in the sciatic nerve was observed only in the $hV/hM:hR/hR$ line

of amyloid deposits in the alimentary tract increased with age and was higher than that in the $+/+$:Tg and $-/-$:Tg lines (Fig. 4, Table 1). Amyloid deposition was first found in the stomach or small intestine and then in the large intestine. The major sites of amyloid deposition in the alimentary tract were similar to those previously described [15, 18]. In addition, amyloid deposition was observed in the heart in both $hV/hV:hR/hR$ (Fig. 5m, n) and $hV/hM:hR/hR$ (Fig. 5o, p) strains at 24 months of age, but there were more amyloid deposits in the $hV/hM:hR/hR$ line than in the $hV/hV:hR/hR$ line (Fig. 4). Surprisingly, amyloid deposition was found in the perineurium of the sciatic nerve of the $hV/hM:hR/hR$ mice at 24 months of age (Fig. 5q–s; Table 1). We believe that this is the first case of amyloid deposition in the peripheral nerve in the model mouse, although non-fibrillar deposits were observed in Tg(6.0hTTRV30M):*Hsf1*^{−/−} (designated as TTR/HSF1) mice [40]. Amyloid deposition in the kidney was observed in only the wall of a small vessel in one $hV/hV:hR/hR$ mouse. Taken together,

there was more amyloid deposition in the $hV/hV:hR/hR$ and $hV/hM:hR/hR$ lines than in the $+/+$:Tg and $-/-$:Tg lines. These results were surprising because serum hTTR levels in the $hV/hV:hR/hR$ and $hV/hM:hR/hR$ lines were $\sim 1/25$ compared with those in the $+/+$:Tg and $-/-$:Tg lines. These results suggest that the kinetic and thermodynamic stability of the TTR:RBP4 complex is important for amyloid deposition that replicating the human disease.

Assessment of TTR deposition by immunohistochemistry

We analyzed anti-human TTR-positive deposits by immunohistochemistry at 12, 18, and 24 months of age (Table 2). At 12 months of age, anti-human TTR-positive deposits was observed in the small intestine of all mice except $hV/hM:hR/hR$ mice in which 30% of them showed TTR-positive deposits. In the heart and kidneys, 10–30 % of mice showed TTR-positive deposits, while no amyloid fibril

Table 1 Tissue distribution of TTR amyloid deposits

	<i>Ttr</i> ^{+/+} : <i>Tg</i> (6.0- <i>hMet30</i>)	<i>Ttr</i> ^{-/-} : <i>Tg</i> (6.0- <i>hMet30</i>)	<i>Ttr</i> ^{<i>hVal30/hVal30</i>} : <i>Rbp4</i> ^{<i>hRBP4/hRBP4</i>}	<i>Ttr</i> ^{<i>hVal30/hMet30</i>} : <i>Rbp4</i> ^{<i>hRBP4/hRBP4</i>}
12 m				
Heart	0/10	0/10	0/10	0/10
Kidney	0/10	0/10	0/10	0/10
Skeletal m	0/10	0/10	0/10	0/10
Stomach	2/10 (±, ±)	0/10	3/10 (1+, 1+, 1+)	0/10
Small intestine	8/10 (±, ±, ±, 1+, 1+, 1+, 1+, 2+)	5/10 (2+, 2+, 2+, 2+, 2+)	10/10 (1+, 1+, 1+, 1+, 1+, 2+, 2+, 2+, 2+, 3+)	0/10
Large intestine	0/10	0/10	0/10	0/10
Sciatic nerve	0/8	0/10	0/10	0/10
18 m				
Heart	0/7	0/7	0/8	0/6
Kidney	0/7	0/7	0/8	0/6
Skeletal m	0/7	0/7	0/8	0/6
Stomach	1/7 (2+)	0/7	3/8 (2+, 3+, 3+)	2/6 (2+, 3+)
Small intestine	6/7 (2+, 2+, 2+, 2+, 2+, 3+)	5/7 (2+, 2+, 2+, 2+, 2+)	7/8 (2+, 2+, 2+, 3+, 3+, 3+, 3+)	6/6 (2+, 2+, 2+, 2+, 3+, 3+)
Large intestine	0/7	0/7	0/7	0/6
Sciatic nerve	0/7	0/7	0/7	0/6
24 m				
Heart	0/12	0/12	6/12 (1+, 1+, 2+, 2+, 2+, 2+)	8/8 (±, 1+, 1+, 2+, 2+, 2+, 2+, 2+)
Kidney	0/12	0/12	1/12 (±)	0/8
Skeletal m	0/12	0/11	0/12	0/8
Stomach	8/12 (2+, 2+, 2+, 2+, 2+, 2+, 2+, 2+, 2+, 2+, 2+)	8/11 (2+, 2+, 2+, 2+, 2+, 2+, 2+, 2+, 2+, 2+)	12/12 (2+, 2+, 2+, 2+, 2+, 2+, 2+, 2+, 2+, 2+, 2+)	8/8 (2+, 2+, 2+, 2+, 3+, 3+, 3+, 3+)
Small intestine	12/12 (2+, 2+, 2+, 3+, 3+, 3+, 3+, 3+, 3+, 3+, 3+)	12/12 (2+, 2+, 2+, 3+, 3+, 3+, 3+, 3+, 3+, 3+, 3+)	12/12 (3+, 3+, 3+, 3+, 3+, 3+, 3+, 3+, 3+, 3+, 3+)	8/8 (3+, 3+, 3+, 3+, 3+, 3+, 3+, 3+)
Large intestine	6/12 (1+, 2+, 2+, 2+, 2+, 2+, 2+)	0/12	11/12 (2+, 2+, 2+, 2+, 2+, 2+, 2+, 2+, 2+, 2+, 2+)	8/8 (2+, 2+, 2+, 2+, 2+, 2+, 2+, 2+)
Sciatic nerve	0/12	0/9	0/12	4/7 (1+, 1+, 1+, 1+)*

– no amyloid deposits, ± deposition limited to the walls of small vessels, + deposition in walls of small vessels and surrounding areas, ++ moderate deposition in interstitium, +++ marked deposition in interstitium and parenchyma.

*Positive in epineurium

deposits were observed. At 18 months of age, anti-human TTR-positive deposits was observed in the small intestine of all mice. In the heart and kidneys, 30–50 % of mice showed TTR-positive deposits, while no amyloid fibril deposits were observed. At 24 months of age, anti-human TTR-positive deposits was observed in the small intestine of all mice. In the heart and kidneys, 30–100 % of mice showed TTR-positive deposits. Thus, anti-human TTR-positive deposits were found in earlier age and higher percentage in all strains than amyloid fibril deposits.

Gel filtration chromatography

To analyze the kinetic and thermodynamic stability of the TTR:RBP4 complex, plasma samples from +/+, *hV/hV:hR/hR*/

hR and *hV/hM:hR/hR* mice were subjected to gel filtration chromatography, and column fractions were analyzed by western blotting. The majority of immunoreactive RBP4 and TTR eluted together in fractions 28–31 in the control mouse (Fig. 6). A small amount of immunoreactive RBP4 eluted after this primary peak in subsequent fractions 32–39 (Fig. 6). These secondary fractions represent non-TTR-bound RBP4 because no associated TTR immunoreactivity was observed (Fig. 6). The percentage of the TTR-RBP4 bound form in +/+ mice was 82.0 ± 2.2 (Fig. 6). However, most of the RBP4 in the *hV/hV:hR/hR* and *hV/hM:hR/hR* mice were not bound to TTR, indicating that the hTTR was also free. These results suggest that the increase in free hTTR accelerates amyloid deposition, even under conditions of low serum hTTR levels.

Fig. 5 Histochemical and immunohistochemical analyses. Histochemical and immunohistochemical findings at 24 months of age in the small intestine (**a–l**), the heart (**m–p**), and the sciatic nerve (**q–s**). The sections stained with Congo Red (**a–d**, **m**, **o**, and **q**) showed apple green birefringence under polarizing light (**e–h**, **n**, **p**, and **r**). Tissue sections were also stained with anti-human TTR (**i–l**, and **s**). Amyloid deposition was observed only in the intestinal tract in $+/+;Tg$ (**a**, **e**,

and **i**) and $-/-;Tg$ mice (**b**, **f**, and **j**). By contrast, amyloid deposition was observed in the intestinal tract (**c**, **g**, and **k**) and heart (**m** and **n**) in $hV/hV:hR/hR$ mice and in the intestinal tract (**d**, **h**, and **l**), heart, (**o** and **p**) and sciatic nerve (**q–s**) in $hV/hM:hR/hR$ mice. Scale bar: 100 μ m in **a–p** and 50 μ m in **q–s**

Table 2 Anti-TTR antibody-positive and amyloid fibril tissue deposition

Age	Strain	+/+:Tg		-/-:Tg		hV/hV:hR/hR		hV/hM:hR/hR	
		TTR+	CR+	TTR+	CR+	TTR+	CR+	TTR+	CR+
12 months	Small intestine	10/10	8/10	8/10	5/10	10/10	10/10	3/10	0/10
	Heart	3/10	0/10	3/10	0/10	2/10	0/10	1/10	0/10
	Kidney	2/10	0/10	2/10	0/10	1/10	0/10	1/10	0/10
	Sciaic nerve	0/8	0/8	0/10	0/10	0/10	0/10	0/10	0/10
18 months	Small intestine	7/7	6/7	6/7	5/7	8/8	7/8	6/6	6/6
	Heart	2/7	0/7	2/7	0/7	3/8	0/8	4/6	0/6
	Kidney	2/7	0/7	3/7	0/7	3/8	0/8	3/6	0/6
	Sciaic nerve	2/7	0/7	1/7	0/7	0/7	0/7	0/6	0/6
24 months	Small intestine	12/12	12/12	12/12	12/12	12/12	12/12	8/8	8/8
	Heart	5/12	0/12	4/12	0/12	8/12	6/12	8/8	8/8
	Kidney	4/12	0/12	3/12	0/12	3/12	1/12	4/8	0/8
	Sciaic nerve	2/12	0/12	1/9	0/9	2/12	0/12	5/7	4/7

TTR+ anti-TTR antibody-positive, CR+ congo red positive

Fig. 6 Gel filtration chromatography. Each fraction was analyzed by western blotting using anti-RBP4 and anti-TTR antibodies. The bound form of RBP4 and TTR was found in fractions 28–31, whereas the free form of RBP4 was found in fractions 32–39. The TTR–RBP4 complex was barely detected in the *hV/hV:hR/hR* and *hV/hM:hR/hR* lines

Discussion

In this study, we analyzed amyloid deposition in two double-humanized mouse strains, *hV/hV:hR/hR* and *hV/hM:hR/hR*. The serum levels of hTTR and hRBP4 were much lower in the double-humanized mice than in conventional transgenic mice, Tg(6.0-hMet30), on the wild-type *Ttr* or the *Ttr*-null background. Nevertheless, amyloid deposition was more prominent in *hV/hV:hR/hR* and *hV/hM:hR/hR* mice than in conventional transgenic mice. In addition, amyloid deposition was also observed in *hV/hV:hR/hR* mice, carrying the normal human *TTR* gene. Amyloid deposition started later in *hV/hM:hR/hR* mice than in *hV/hV:hR/hR* mice, but *hV/hM:hR/hR* showed more amyloid deposition at 24 months of age. Furthermore, amyloid deposition was first observed in the sciatic nerve without any additional genetic change.

Previously, we reported no amyloid deposition in transgenic mice carrying the wild-type *hTTR* gene (designated as C57BL/6-Tg(7.2TTRVal30)) [14]. By contrast, non-fibrillar deposits were reported in mice transgenic for the wild-type *hTTR* gene (designated as B6D2F2-Tg(19.2TTRVal30)) by other investigators [16, 41]. Serum concentrations of hVal30 were 26–80 µg/ml in C57BL/6-Tg(7.2TTRVal30) mice and 1–3.5 mg/ml in B6D2F2-Tg(19.2TTRVal30) mice. As the serum levels of hVal30 in B6D2F2-Tg(19.2TTRVal30) mice were 10–100 times higher than those in C57BL/6-Tg(7.2TTRVal30) mice, the high Val30 expression was thought to be required for amyloid deposition of wild-type hTTR. However, the serum concentration of hTTR in our *hV/hV:hR/hR* mice was 6.62 ± 0.37 µg/ml, 10-fold lower than that in C57BL/6-Tg(7.2TTRVal30) mice and 100-fold lower than that in B6D2F2-Tg(19.2TTRVal30) mice. We also reported that the serum levels of human mutant TTR are correlated, to some extent, with the occurrence and degree of amyloid deposition in C57BL/6-Tg(7.2TTRVal30) mice [15]. However, amyloid deposition was more prominent in *hV/hV:hR/hR* and *hV/hM:hR/hR* mice than in *+/+:Tg* and *-/-:Tg* mice. Furthermore, we are the first to observe amyloid deposition in the sciatic nerve without additional genetic change, such as *Hsf1* deficiency, in the *hV/hM:hR/hR* line.

As reported by other investigators [12, 13, 16], anti-TTR antibody-positive deposits were found in earlier age and higher percentage in all our mice than amyloid fibrillar deposits. Teng et al. demonstrated that both amyloid and nonamyloid deposits were intact human TTR monomers with no evidence of proteolysis by amino terminal amino acid sequence analysis and mass spectrometry [16]. Thus, the anti-TTR antibody-positive deposits may represent a preamyloid intermediate state.

In our models, the mouse *Ttr* and *Rbp4* genes were replaced with the human *TTR* and *RBP4* genes, respectively. Thus, there is no expression of mouse *TTR*. It is well known that heterotetramers of human and mouse TTR are present in the serum of these transgenic mice and that these heterotetramers are more stable than hTTR homotetramers [19]. In fact, amyloid deposition was accelerated by TTR-Leu55Pro on a *Ttr*-null background (designated as *Ttr*^{-/-}:129S1/Sv-Tg(*TTRPro55*)) [12] or *Ttr*^{-/-}:B6D2F2-Tg(*TTRPro55*) [13]. By contrast, Kohno et al. [35] demonstrated no differences in the onset, progression, or tissue distribution of amyloid deposition in Tg(6.0h*TTRMet30*) mice with or without the murine *Ttr* gene (designated as *Ttr*^{+/+}:C57BL/6-Tg(6.0h*TTRMet30*) or *Ttr*^{-/-}:Tg(C57BL/6–6.0h*TTRMet30*)). The serum levels of TTRPro55 in *Ttr*^{-/-}:129S1/Sv-Tg(*TTRPro55*), *Ttr*^{-/-}:B6D2F2-Tg(*TTRPro55*), and *Ttr*^{-/-}:Tg(C57BL/6–6.0h*TTRMet30*) mice were 10–200, 20–35, and 240–340 µg/ml, respectively. These data suggest that amyloid deposition was affected by the presence or absence of mouse *Ttr* under conditions of low serum levels of human mutant TTR. Amyloid deposition may be facilitated by the absence of mouse TTR, probably because of the instability of human TTR tetramers. Interestingly, hTTRVal30 and hTTRMet30 tetramers are more unstable in mouse serum, when the serum human TTR levels were low [31]. Human *TTR* mRNA and TTR protein expression levels in the liver of homozygous *hV/hV* mice were approximately twice those in heterozygous *+/hV* mice. However, the serum human TTR levels were much lower in *hV/hV* mice than in *+/hV* mice. The low mean serum hTTR levels could be due to shunting to the ERAD pathway. In a previous study [31], we examined morphological abnormality by transmission electron microscopy and the localization of hTTR using immunofluorescent detection with anti-calnexin and anti-GM130 antibodies. We did not find any abnormality, suggesting that hTTR was secreted by normal pathway.

RBP4 may also influence the amyloid deposition. As we reported previously, the serum level of hRBP4 in *Rbp4*^{hR/hR} (*hR/hR*) mice was 7.4 ± 1.50 µg/ml [32], whereas the serum levels of hRBP4 levels in *hV/hV:hR/hR* and *hV/hM:hR/hR* mice were 0.60 ± 0.05 and 0.46 ± 0.02 µg/ml, respectively, indicating a considerable reduction in these mice with serum hTTR levels ~1/25 of normal. This decrease in RBP4 may be due to glomerular filtration of non-TTR-bound RBP4. As shown in Fig. 6, the hTTR-bound form of hRBP4 was barely detected, although ~80–90% of RBP4 is TTR bound under normal conditions [32, 42]. Thus, an increase in non-RBP4-bound TTR potentially accelerated amyloid deposition in our double-humanized mice. In fact, RBP4 can prevent TTR amyloid formation at acidic pH [43].

To the best of our knowledge, neither non-fibrillar nor fibrillary deposits have been observed in the peripheral and autonomic nervous systems of conventional TTR transgenic

mouse models. Non-fibrillar deposits accompanied by the induction of pro-inflammatory cytokines, RAGE up-regulation, and NF-κB activation were first reported in the sciatic nerve, dorsal root ganglia (DRG), and autonomic ganglia of Tg(6.0h*TTRV30M*):*Hsf1*^{-/-} (designated as *TTR/HSF1*) mice [40]. This result suggested that disrupting the heat shock response would aggravate TTR deposition and that HSF1 is involved in familial amyloidotic polyneuropathy (FAP) pathogenesis as a defense mechanism against extracellular TTR deposits. In our *hV/hM:hR/hR* mice, we observed amyloid deposition in the perineurium of the sciatic nerve. However, no such inflammatory response was observed in our *hV/hM:hR/hR* mice. The inflammatory response might be important for neurodegeneration, but the physico-chemical properties of the TTR–RBP4 complex in the microenvironment surrounding the peripheral nerve or autonomic nerve may be important for non-fibrillar and fibrillary deposits.

We previously reported that amyloid deposition was observed in the heart and kidney of the *+/+:Tg* and *-/-:Tg* lines [15, 35]. However, this result was not reproduced in this experiment, perhaps because of different housing conditions, as amyloid deposition is greatly affected by environmental conditions [39]. The amount of amyloid deposits differs under various conventional conditions [44].

In human, serum levels of TTR decreased with aging [45–47]. Kohno et al. [35] examined the serum hTTR levels in *+/+:Tg* and *-/-:Tg* strains at 11, 14, and 18 months of age, but there was no significant difference among age groups. We also examined the serum levels of hTTR, mRBP4, and hRBP4 at 12 and 24 months of age. However, there was no significant difference among age groups in all mouse strains. It is known that the serum TTR level is a good biomarker for nutritional state [48, 49]. As experimental mice are kept in good housing conditions, the serum TTR levels may not change under such conditions.

Gel filtration assay revealed that most hRBP4 was free of hTTR. Free hRBP4 was easily excreted to urine due to its small protein size. However, serum RBP4 levels were 0.5–0.6 µg/ml and the ratio of RBP4 to TTR (5.7–7.5 µg/ml) was about 1:10 in our double-humanized mice. This ratio is similar to human status which is 1/5 to 1/10. Thus, we do not know why the hTTR/hRBP4 ratio was kept within normal range despite most hRBP4 was free of hTTR. Before gel filtration assay, the serum sample was diluted two times with phosphate buffer. This treatment may cause dissociation of TTR/RBP4 complex, especially when TTR and RBP4 concentration is low.

Taken together, the presence of human RBP4 instead of mouse RBP4 and the absence of mouse TTR might be responsible for the accelerated amyloid deposition in our *hV/hV:hR/hR* mice despite the low serum hTTR level. These data suggest that our models recapitulated the pathological

findings in FAP more precisely. In addition, this model will be necessary for the appropriate evaluation of effective therapeutics. However, our mice does not represent human TTR status as far as the low serum hTTR level and high percentage of unbound TTR with RBP4 are concerned. In our double-humanized mice, mean serum RBP4 levels were 0.5–0.6 µg/ml and the ratio of RBP4 to TTR was ~1:10. This ratio is similar to that of the human situation (1/5–1/10). We wondered whether amyloid deposition occurred under such a low serum level of hTTR. RNA interference (RNAi) therapy is now under investigation. In this case, we expect very low level of serum hTTR and RBP4. So, the question is that can we stop amyloid deposition by lowering the hTTR expression. Serum level of hTTR in our mice is 1/25 of that in human. Thus, our data might be useful to consider the effect of RNAi therapy. The double-humanized mouse at the *Ttr* and *Rbp4* loci, in which serum TTR and RBP4 levels are equivalent to those in humans, represents a promising approach to replicating more precisely the disease process.

Acknowledgements The manuscript has been carefully edited by Nature Research Editing Service (<http://authorservices.springernature.com>). This study was partly funded by Chiesi Farmaceutici, Parma, Italy. This work was supported by Grants-in-Aid for Scientific Research (24590404 to Z.L.) and (S) (21220010 to K.Y.) from the Japan Society for the Promotion of Science, the National Natural Science Foundation of China (grant number 81670028 to S.J.), the Major Program of Natural Science Foundation of Heilongjiang Province of China (ZD2016014 to S.J.); the Dean Foundation and Foster Foundation of the Fourth Hospital of Harbin Medical University (to S.J.); Scientific Support Programs for Cancer Research, a Grant-in-Aid for Scientific Research on Innovative Areas, the Ministry of Education, Culture, Sports, Science and Technology (221S0001 to K.Y.); and CREST (Japan Science and Technology Agency) (to K.Y.).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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