Animal Model

Evidence for Early Cytotoxic Aggregates in Transgenic Mice for Human Transthyretin Leu55Pro

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Familial amyloidotic polyneuropathy (FAP) is a lethal autosomal dominant disorder characterized by systemic extracellular deposition of transthyretin (TTR) amyloid fibrils. Several groups have generated transgenic mice carrying human TTR Val30Met, the most common mutation in FAP. To study amyloidogenicity and cytotoxicity of different TTRs, we produced transgenic mice expressing human TTR Leu55Pro, one of the most aggressive FAP-related mutations. TTR deposition and presence of amyloid fibrils was investigated and compared to animals carrying the human TTR Val30Met gene kept under the same conditions. Deposition in a C57BL/6J background (TTR-Leu55Pro mice) and in a TTR-null background [TTR-Leu55Pro X TTR-knockout (KO) mice] was compared. Animals in a C57BL/6J background presented early (1 to 3 months) nonfibrillar TTR deposition but amyloid was absent. In a TTR-null background, presence of amyloid fibrils was detected starting at 4 to 8 months with a particular involvement of the gastrointestinal tract and skin. This data suggested that TTR homotetramers are more prone to fibril formation than TTR murine wild-type/human mutant heterotetramers. The nature of the deposited material was further investigated by immunocytochemistry. Both amorphous aggregates and small TTR fibrils were present in TTR-Leu55Pro X TTR-KO transgenics. We observed that these TTR deposits mimic the toxic effect of TTR deposits in FAP: animals with TTR deposition, present approximately twofold increased levels of nitrotyrosine in sites related to deposition. The TTR-Leu55Pro X TTR-KO mice here described are an important tool for the dual purpose of investigating factors involved in amyloidogenesis and in cytotoxicity of deposited TTR. (*Am J Pathol* 2002, 161:1935–1948)

Familial amyloidotic polyneuropathy (FAP) is an autosomal-dominant disease characterized by the extracellular deposition of amyloid fibrils in several tissues namely in the peripheral and autonomic nervous systems, cardiovascular system, kidney, thyroid, and gastrointestinal tract.¹ The first symptoms of the disease usually appear in individuals between 20 and 45 years of age and are generally progressive and fatal in ~10 to 20 years. The main component of the fibrils in FAP patients is mutated transthyretin (TTR).² Several mutations have been described in this protein, the most common being a Val for Met substitution at position 30 (Val30Met).³ Leu55Pro represents one of the most aggressive FAP-related mutations because the patients present a more severe and early onset.^{4,5} Under normal physiological conditions, TTR, a homotetrameric plasma protein,⁶ functions as the carrier for both thyroxine (T4) and retinol (vitamin A); in the latter case through binding to retinol-binding protein. TTR is mainly synthesized by the liver and the choroid plexuses of the brain and in lesser amounts by the retina of the eye, pancreas, and other tissues.⁷

We have previously assessed nerve biopsies from asymptomatic carriers of Val30Met TTR and FAP patients in different stages of disease progression for TTR deposition and presence of amyloid fibrils. Early in FAP, TTR is already deposited in an aggregated nonfibrillar form, negative for Congo Red birefringence.⁸ This suggested that preamyloidogenic forms of TTR exist in the nerve of FAP patients, in a stage before fibril formation. Nonamyloid TTR deposits have also been demonstrated in transgenic mice for human wild-type (wt) TTR.⁹ In human FAP nerves, when cytotoxicity of nonfibrillar TTR was assessed by immunohistochemistry for inflammation and oxidative stress-associated molecules, we observed in-

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Figure 1. Transgenesis analysis. **A:** Schematic representation of the final expression vector used to generate TTR-Leu55Pro transgenic mice. p169ZT-hTTR55 contained the sheep metallothionein 1 promoter (SMT-1), a β -globin splice site (bSS) preceding human TTR Leu55Pro cDNA (TTR), followed by the SV40 polyA signal (pA). The construct was linearized with *Bss*III, isolated from the bacterial sequences and used for microinjection. **B:** Southern analysis of tail genomic DNA from TTR-Leu55Pro mice, lines 1, 7, and 9 (lanes 1, 2, and 3, respectively), cut with *Eco*RI and probed for hTTR cDNA. The resulting fragment containing human TTR (~500 bp) was seen in all of the transgenic founders generated. **C:** PCR of genomic DNA from tail biopsies of TTR-Leu55Pro mice lines 1, 7, and 9 (lanes 4, 5, and 6, respectively) using primers that amplify hTTR cDNA; the construct used for microinjection was the positive control (lane 2) and DNA extracted from a TTR-KO mouse was the negative control (lane 3); 1-kb DNA ladder (lane 1).

creased axonal expression of these markers, showing that early aggregates, in a presymptomatic phase, are toxic to cells.^{8,10} This toxicity is most probably related to activation of nuclear factor (NF)- κ B by TTR aggregates,¹¹ as some inflammation- and oxidative stress-related molecules are targets of the NF- κ B transcription factor,¹² and activation of NF- κ B is found at sites of TTR deposition in FAP nerves.¹¹

To understand the mechanisms underlying fibril formation, deposition, and cytotoxic effects caused by aggregates, many questions remain to be investigated. In an attempt to gain insights in the pathogenesis of FAP, several groups have generated transgenic mice carrying the human TTR Val30Met gene. Breeding of transgenic mice carrying human TTR Val30Met fused to the mouse metallothionein promoter in the C57BL/6J background showed that amyloid deposition in the mucosa and small intestine starts at age 6 months.¹³ Using the human homologous TTR promoter sequences to generate TTR Val30Met transgenics, amyloid was observed starting at 6 months and at the age of 24 months the pattern of amyloid deposition was similar to that observed in human autopsy cases of FAP, except for its absence in the choroid plexus and in the peripheral and autonomic nervous systems.¹⁴ The same pattern of amyloid deposition was reported when these transgenics were backcrossed to a TTR-null background.¹⁵ Several laboratories have produced transgenic animals with constructs bearing different amyloidogenic TTR mutations (TTR Leu55Pro, TTR Ile84Ser) but human TTR amyloid deposits and/or TTR deposition have not been detected,^{9,16} probably because of low levels of protein synthesis and/or the effect

of mouse endogenous TTR and formation of murine wt/human mutant TTR heterotetramers. Amyloid and nonfibrillar deposits in mice overexpressing wt human TTR have been reported.⁹ In these mice TTR deposits occurred primarily in the heart and kidney in the majority of the animals older than 18 months. In none of these animal models was the possible cytotoxic effect of TTR ever evaluated.

Here we assess TTR deposition in mice bearing human TTR Leu55Pro both in a C57BL/6J and TTR-null backgrounds, in comparison with transgenic mice for TTR Val30Met, and study the possible cytotoxicity of TTR deposits in these animal models.

Materials and Methods

Vector Constructions (Figure 1)

The sheep metallothionein promoter-1 (SMT-1) was excised from pBluescript II KS with *Sac*I and *Bam*HI (this vector, p169, was provided by Dr. Kevin Ward, Commonwealth Scientific and Industrial Research Organization, Blacktown, New South Wales, Australia). The β -globin splice site and the SV40 polyA tail were excised from pZT (a kind gift from Dr. José Belo, Gulbenkian Institute for Science, Portugal) with *Bam*HI and *Kpn*I. The *Kpn*I-blunted-*Bam*HI β -globin splice site-polyA insert was subcloned in *Sac*I-blunted *Bam*HI sites of p169. The vector so generated, carrying SMT-1, β -globin splice site, and polyA was termed p169ZT and was subsequently used to subclone human TTR cDNA (hTTR) from the start codon

to the stop codon,¹⁷ in the *NheI*-blunted site. The Leu55Pro mutation was inserted by site-directed mutagenesis using the Quickchange mutagenesis kit (Stratagene, Lisboa, Portugal) and following the manufacturer's instructions and the appropriate primers: the sense primer was 5'-TCTGGAGAGCCGCATGGGCT-3' and the anti-sense was 5'-AGCCCATGCGGCTCTCCAGA-3'. The construct obtained was termed p169ZT-hTTR55. Constructs were subjected to dideoxy sequencing using the T7 sequencing kit (Pharmacia, Freiburg, Germany).

Transfection and TTR Expression in Cell Culture

The ability of p169ZT-hTTR55 to express TTR was assessed by transient transfection of rat hepatomas (FAO) (American Type Cell Collection, Rockville, MD) following the CaPO₄-DNA precipitate method.¹⁸ The presence of TTR in cell culture media was tested by quantitative enzyme-linked immunosorbent assay (ELISA)¹⁹ 48 hours after transient transfection. Experiments were performed both in the absence and in the presence of 10 mmol/L of ZnSO₄ in the cell culture media to test activation of the SMT-1 promoter.

Microinjection, Generation of Transgenic Mouse Lines, and Transgenesis Analysis

The C57BL/6J inbred mouse strain was chosen for DNA microinjection to minimize the possible influence of genetic background on amyloid deposition.²⁰ p169ZT-hTTR55 linearized with *Bss*HI was injected into fertilized C57BL/6J eggs. The manipulated eggs were subsequently implanted in foster mothers as described.²¹ All animals were fed standard rodent chow with water supplemented with 50 mmol/L of ZnSO₄ (to ensure induction of TTR via the metal-sensitive SMT-1 promoter) provided *ad libitum* and were maintained in a conventional animal facility with no more than five animals per cage. Identification of the transgene in founders and their progeny was performed by Southern blot and polymerase chain reaction (PCR). Southern blot analysis was performed from *Eco*RI-digested genomic DNA isolated from tail biopsies by standard procedures.¹⁸ Hybridization was performed overnight at 60°C with a probe for human TTR cDNA obtained by PCR and washes were done at 50°C. PCR was performed with primers for hTTR (sense: 5'-ATG-GCTTCTCATGGTCTG-3'; anti-sense: 5'-GAAGTCCCT-CATTCTTG-3') using 30 cycles of denaturation at 95°C for 1 minute; annealing at 65°C for 1 minute; and extension at 72°C for 1 minute. The copy number of the SMT1-TTR Leu55Pro transgene was estimated from the relative intensity of the hybridizing band for human and endogenous mouse TTR.¹⁵

Northern Blot Analysis

RNA was isolated from the intestine, stomach, kidney, heart, lung, liver, spleen, eye, and brain by homogenization in Trizol solution (Gibco, Barcelona, Spain) following

the manufacturer's instructions. RNA samples (20 µg) were electrophoresed and blotted following standard procedures.¹⁸ Hybridization was performed at 42°C with a probe for human TTR cDNA and washes were at 37°C. All membranes were normalized with a mouse probe for hypoxanthine phosphoribosyltransferase. Tissues in which TTR expression was absent by Northern blot were further assessed by reverse transcriptase-PCR. Briefly, total RNA was extracted as already described and cDNA was produced using the reverse transcription kit from Promega and following the manufacturer's instructions. The produced cDNA was subjected to PCR for human TTR as previously described in this section. Standardization was done by PCR for mouse β-actin.

Protein Expression

Plasma was collected from transgenic mice after 1 week of drinking water supplemented with 50 mmol/L of ZnSO₄, and the concentration of human TTR in the plasma was measured by ELISA and calculated from a standard curve ranging from 5 to 250 ng wt human TTR/ml. To check if the produced TTR had the expected molecular weight, immunoblot analysis of 3 µl of plasma was performed. TTR was visualized using polyclonal rabbit anti-human TTR (DAKO, Glostrup, Denmark) as previously described.¹⁹ The formation of heterotetramers between mouse and human monomers was assessed by TTR immunoprecipitation experiments: 100 µl of plasma were incubated overnight with 30 µl of rabbit anti-human TTR (DAKO). After centrifugation at 14,000 rpm for 30 minutes, antigen-antibody complexes were obtained, washed with phosphate-buffered saline (PBS), and re-suspended in 30 µl of standard sodium dodecyl sulfate (SDS) sample buffer with β-mercaptoethanol. The solubilized pellet was separated in 15% SDS-polyacrylamide gel electrophoresis (PAGE) followed by silver staining to check for the presence of human and mouse TTRs. Human and mouse TTR standards were obtained by affinity chromatography to a retinol-binding protein column as described elsewhere.¹⁵

Backcrossing TTR-Leu55Pro Transgenics to a TTR Knockout (KO) Background: Generation of TTR-Leu55Pro X TTR-KO Lines

TTR KO mice have been generated and characterized.²² These animals were obtained from the Jackson Laboratory (Bar Harbor, ME) and were crossed to the 129S1/Sv background for more than 10 generations. Transgenic mice carrying the human TTR Val30Met gene (TTR-Val30Met) had previously been backcrossed to the TTR KO background (TTR-Val30Met X TTR-KO).¹⁵ More than 10 backcrosses of transgenic mice carrying human TTR Leu55Pro to the TTR KO background were performed before starting the experiments, therefore at least 99.5% of the genetic background of the TTR-Val30Met X TTR-KO and TTR-Leu55Pro X TTR-KO strains is 129S1/Sv. Mice were tested by ELISA for the presence of human

TTR in plasma and for the absence of mouse endogenous TTR by PCR using genomic DNA with primers for the disrupted exon 2 of mouse TTR.¹⁵

Light Microscopy and Immunohistochemistry

Transgenic mice with ages ranging from 1 to 24 months, were killed after anesthesia with ketamine/xylazine. Heart, kidneys, spleen, liver, lungs, pancreas, esophagus, stomach, small and large intestines, muscle, tongue, and eye were immediately excised and processed. For light microscopy, tissues were fixed in 4% neutral buffered formalin and embedded in paraffin. For immunohistochemistry, 5- μ m-thick sections were deparafinized in xylol and dehydrated in a descendent alcohol series. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide/100% methanol and sections were blocked in blocking solution (4% bovine serum and 1% bovine serum albumin in PBS). Primary antibodies used were mouse monoclonal anti-TTR mAb 39-44,²³ diluted 1:3 and anti-nitrotyrosine (Chemicon, Hofheim, Germany) diluted 1:1000 in blocking solution and incubated overnight at 4°C.

Immunohistochemistry using the TTR monoclonal antibody was performed using the MOM kit (Vector, Burlingame, CA) according to the manufacturer's instructions. Antigen visualization was performed with the biotin-extravidin-peroxidase kit (Sigma), using 3-amino-9-ethyl carbazole (Sigma) or diaminobenzidine as substrates. On parallel control sections, no primary antibody and/or anti-TTR preabsorbed with excess TTR (300 μ g of recombinant TTR²⁴/ μ L antibody, in a final volume of 100 μ l) were used. Semiquantitative analysis of immunohistochemical images was performed with the Universal Imaging system (NIH) that performs automated particle analysis in a measured area, ie, the area occupied by pixels corresponding to the immunohistochemical substrate's color is counted and normalized relative to the total area. Each slide used in semiquantitative immunohistochemistry was analyzed in five different selected areas. Results shown represent percent occupied area $\pm$ SD.

Congo Red and I-DOX Binding to Tissue Sections

The presence of amyloid in tissue sections was investigated after staining with Congo Red and observation under polarized light.²⁵

The anthracycline 4'-iodo-4'-deoxydoxorubicin (I-DOX), reported to co-localize with amyloid deposits in tissue sections of patients with FAP,²⁶ was used to stain tissue sections from transgenic animals. Briefly, deparafinized sections were incubated for 20 minutes with 10^{-5} mol/L I-DOX in 80% ethanol saturated with NaCl. Anthracycline fluorescence was determined with a Bio-Rad MRC confocal microscope (Bio-Rad, Hercules, CA) using a 568-nm excitation filter.

Electron Microscopy and Immunogold TTR Labeling

Small skin pieces were fixed in 2.5% glutaraldehyde in PBS for 2 hours and subsequently washed three times in PBS. Semithin sections (1 μ m) were cut from epon-embedded blocks and stained with toluidine blue. Ultrathin sections (500 Å) were cut in an ultratome (LK Brumma Nova), stained with uranyl acetate (2 minutes) and lead citrate (3 minutes), and observed on a Zeiss electron microscope. Ultrathin sections were mounted on nickel grids. After washing with distilled water, grids were blocked on the surface of a drop of 1% bovine serum albumin in PBS for 30 minutes and subsequently washed in PBS. Rabbit anti-human TTR (DAKO) diluted 1:100 was added for 1 hour at room temperature and subsequently grids were immersed in PBS. Control grids were incubated with preabsorbed anti-human TTR. Incubation with anti-rabbit immunoglobulins coupled to 10-nm gold particles (Amersham, Freiburg, Germany) diluted 1:15 in 1% bovine serum albumin was performed for 30 minutes at room temperature. The grids were then placed on the surface of Tris-HCl buffer, pH 7.6, and stained with uranyl acetate (3 minutes) and lead citrate (2 minutes).

Aggregate and Fibril Extraction

A modification of the protocol of Kaplan and colleagues²⁷ was followed. Briefly, each tissue specimen (~100 mg) was homogenized manually with a glass rod with 1 ml of ice-cold PBS and centrifuged for 10 minutes at $14,000 \times g$. This procedure was repeated three times to remove soluble blood components. A final wash with 1% SDS in PBS was performed. The resulting pellet was resuspended in 1 ml of 20% acetonitrile containing 0.1% trifluoroacetic acid. The mixture was incubated at room temperature for 1 hour with moderate mixing and centrifuged again. The incubation and centrifugation steps were repeated twice and the supernatants were pooled, lyophilized, and resuspended in 20 μ l of water for immunochemical characterization of amyloid proteins. Coomassie staining of 15% SDS-PAGE and anti-TTR immunoblotting, as already described, were performed in the obtained extracts.

Results

Vector Construction, Generation of Transgenic Mouse Lines, and Transgenesis Analysis

The final construct bearing human TTR Leu55Pro under the control of the SMT-1 promoter, p169ZT-hTTR55, was produced as described in Materials and Methods (Figure 1A). The SMT-1 promoter, was chosen to generate Leu55Pro transgenics for two main reasons: it is active in virtually all cells, a feature that may be advantageous in achieving high serum concentrations of amyloid precursors; and the expression of metallothionein promoter fusion genes is high in the liver,²⁸ the major physiological

6site of TTR production. Demonstration of TTR production was performed by quantitative ELISA of cell media 48 hours after transfection of FAO cells in the presence of 10 mmol/L of ZnSO₄. TTR Leu55Pro production was ~300 ng/10⁶ cells/48 hours; in the absence of ZnSO₄, constitutive levels were undetectable (data not shown). As expression was proven the construct was linearized by digestion with *Bss*HII and microinjected.

Three founders showed integration of the transgene: lines number 1, 7, and 9. Founders and their positive progeny were identified by Southern analysis of genomic DNA extracted from a tail biopsy and probed with TTR cDNA (Figure 1B). Alternatively, PCR using primers that amplify human TTR cDNA was used (Figure 1C). All founders transmitted the transgene to their offspring. The estimated copy number of the human TTR Leu55Pro cDNA per diploid genome was four copies in transgenics from line 1 (data not shown).

Transgene Expression

In TTR-Leu55Pro mice, average plasma TTR levels for line 1 were 50 to 150 µg/ml; for line 7, 50 to 200 µg/ml; and for line 9, 10 to 50 µg/ml. Six animals from line 1 were followed for TTR-circulating levels for a period of 12 months. A peak of expression generally occurred between days 3 and 6 after ZnSO₄ induction and the levels were maintained throughout time presenting a slight decrease (~10%) 1 month after induction.

The sites of TTR expression in TTR-Leu55Pro mice were studied by Northern analysis of several tissues of 3-month-old animals that had been under ZnSO₄ induction in the previous 2 months. All lines had the liver as the major site of TTR expression (Figure 2A). In line 7, animals presented additional high expression levels of TTR in the intestine (Figure 2A, middle) that was not observed in the other two lines. Membranes were normalized with a HPRT probe and all samples had the same amount of loaded RNA (data not shown). To assess sites with low TTR expression levels, we performed reverse transcriptase-PCR on organs that appeared negative on Northern analysis and found that the kidney, stomach, spleen, and eye expressed human TTR Leu55Pro (data not shown).

The electrophoretic mobility and apparent molecular weight of human TTR Leu55Pro circulating in plasma of transgenic mice was the same as the one presented by a TTR standard as assessed by anti-TTR immunoblot on SDS-PAGE (Figure 2B, left). In the SMT-1-driven transgenics, as one of the major sites of TTR expression is the liver, human TTR synthesis is occurring at the same time as the synthesis of endogenous mouse TTR. Thus, it is possible that heterotetramers composed of both mouse and human monomers are formed. We performed immunoprecipitation of plasma TTR from TTR-Leu55Pro mice with a polyclonal anti-human TTR antibody and both the mouse endogenous and human TTR were observed (Figure 2B, right) thus suggesting the existence of heterotetrameric TTR molecules. Previous immunoprecipitation experiments using this antibody with plasma from non-transgenic mice did not immunoprecipitate mouse TTR

A.

B.

Figure 2. TTR expression in the TTR-Leu55Pro transgenic lines. **A:** Northern analysis of RNA extracted from several organs (K, kidney; I, intestine; E, eye; B, brain; H, heart; S, stomach; L, lung; Li, liver; Sp, spleen) of TTR-Leu55Pro transgenics from lines 1, 7, and 9. **B:** Circulation of human TTR in TTR-Leu55Pro transgenic lines. **Left:** Anti-TTR immunoblot of isolated recombinant human TTR standard (lane 1) and plasma from TTR-Leu55Pro line 1 transgenic mice (lane 2). **Right:** Analysis of mouse endogenous/human mutant TTR heterotetramer formation. Silver staining of immunoprecipitated plasma from TTR-Leu55Pro line 1 (lane 3). As a control, a mix of isolated human and mouse TTR were run alongside (lane 4).

homotetramers. This further suggests that mouse TTR observed by immunoprecipitation of plasma from TTR-Leu55Pro transgenic mice originates from circulating mouse endogenous/human TTR heterotetramers.

Table 1. TTR Deposition in TTR-Leu55Pro and TTR-Val30Met Mice Ranging from 1 to 24 Months of Age as Evaluated by TTR Immunohistochemistry (TTR) and By the Presence of Amyloid Fibrils Positive by Congo Red Staining

		1–3 months			4–8 months			9–12 months				13–18 months				19–24 months					
TTR-Leu55Pro		<i>n</i> = 15			<i>n</i> = 13			<i>n</i> = 5				<i>n</i> = 11				<i>n</i> = 11					
	TTR	Total	I	Sk	Total	I	Sk	Total	I	Sk	Total	I	Sk	Total	I	Sk	Total	I	Sk		
		14	10	0	12	10	1	5	2	1	11	4	9	11	7	2					
	Congo Red	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0					
TTR-Val30Met		<i>n</i> = 16				<i>n</i> = 16				<i>n</i> = 25				<i>n</i> = 25				<i>n</i> = 25			
	TTR	Total	I	Sk	St	Total	I	Sk	St	Total	I	Sk	St	Total	I	Sk	St	Total	I	Sk	St
		14	9	0	12	16	13	0	14	25	13	0	25	20	17	1	15	20	17	1	15
	Congo Red	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0

Total refers to the total number of animals either TTR- or Congo Red-positive in each age range. Major sites with TTR deposition (I, intestine; Sk, skin; and St, stomach) are discriminated.

Assessment of TTR Deposition by Immunohistochemistry and Congo Red Staining

Transgenic Mice in a C57BL/6J Background (TTR-Leu55Pro)

We started by evaluating TTR deposition by immunohistochemistry in TTR-Leu55Pro mice from line 1 ranging from 1 to 24 months of age (Table 1). Animals from line 1 presented the most widespread TTR staining with particular involvement of the gastrointestinal tract, starting as early as 1 to 3 months in ~90% of the animals (*n* = 15); older than 4 months of age, TTR deposition was observed in nearly all animals. TTR deposits were first found in the small intestine, especially in the terminal ileum and duodenum. Deposits were found at the top of the intestinal villi, becoming more widespread with advancing age. Other major sites of TTR deposition included the skin and kidney starting at 4 to 8 months of age. In animals from line 7, TTR staining was found predominantly in the intestine overlapping with secretory vesicles, starting at 3 months. The origin of TTR staining, ie, deposition and/or synthesis was not possible to distinguish because these animals produce TTR in the intestine visible by immunohistochemistry. In line 9, TTR immunoreactivity was very low and started only at 9 months. It is possible that the differences observed both in the pattern and penetrance of TTR deposition in the three TTR-Leu55Pro lines were related to differences in the site of integration of the

transgene. However, it should be noted that in all these animal lines, the major site of TTR deposition was the intestine. The deposited material in the TTR-Leu55Pro transgenics was not Congo Red-positive in any of the analyzed animals until 24 months (Table 1).

In TTR-Val30Met, the observed phenotype was very similar to the one seen in TTR-Leu55Pro although the preferential site of TTR deposition was the stomach, affecting ~90% of the animals 1 to 3 months old (*n* = 16) (Table 1). It is noteworthy that with advancing age, in the TTR-Val30Met the pattern of deposition was more widespread than in the TTR-Leu55Pro with involvement of the intestine, kidney, skin, pancreas, spleen, and muscle. Congo Red-positive deposition was only observed in one animal from the 19- to 24-month-old group (Table 1). In TTR-Val30Met examined in other laboratories,¹³ amyloid deposition was observed in all of the mice after 21 months of age and starting at 9 months. The delay in amyloid deposition when comparing to the same transgenics kept in other facilities might be related to environmental factors.

TTR Transgenic Mice in a KO Background (TTR-Leu55Pro X TTR-KO)

After backcrossing TTR Leu55Pro into a TTR KO background, we observed that amyloid fibril deposition was now detected earlier albeit with low penetrance: at 4 to 8 months Congo Red-positive material was found in ~20%

Table 2. TTR Deposition in TTR-Leu55Pro X TTR-KO and TTR-Val30Met X TTR-KO Mice Ranging from 1 to 24 Months of Age as Evaluated by TTR Immunohistochemistry (TTR) and by the Presence of Amyloid Fibrils Positive for Congo Red Staining

		1-3 months				4-8 months				9-12 months											
TTR- Leu55Pro X TTR-KO		<i>n</i> = 30				<i>n</i> = 16				<i>n</i> = 17											
		Total	I	Sk		Total	I	Sk		Total	I	Sk									
	TTR	30	24	26		14	11	14		17	12	14									
	Congo	0	0	0		3	0	3		3	0	3		13-18 mo				19-24 mo			
	Red																				
TTR- Val30Met X TTR-KO		<i>n</i> = 18				<i>n</i> = 19				<i>n</i> = 21				<i>n</i> = 27				<i>n</i> = 28			
		Total	I	Sk	St	Total	I	Sk	St	Total	I	Sk	St	Total	I	Sk	St	Total	I	Sk	St
	TTR	15	10	0	14	19	5	16	8	20	8	11	11	26	14	11	21	23	27	5	28
	Congo	0	0	0	0	1	0	1	0	2	0	0	0	13	0	0	13	16	4	0	16
	Red																				

Total refers to the total number of animals either TTR- or Congo Red-positive in each age range. Major sites with TTR deposition (I, intestine; Sk, skin; and St, stomach) are discriminated.

of the animals ($n = 16$) (Table 2). TTR deposition occurred mainly in the intestine and skin. This observation suggested that the presence of murine wt TTR/human Leu55Pro TTR heterotetramers causes delayed amyloid formation when compared to TTR Leu55Pro homotetrameric molecules. A similar penetrance and organ involvement of TTR amyloid fibril deposition was observed in older animals (9 to 12 months, Table 2).

Figure 3 shows adjacent sections of Congo Red-positive/TTR-positive (+/+) (Figure 3, top), Congo Red-negative/TTR-positive (-/+) (Figure 3, middle panels, and G) and Congo Red-negative/TTR-negative (-/-) (Figure 3H) TTR-Leu55Pro X TTR-KO skins, stained for TTR (Figure 3, left panels, and C and H) and Congo Red (Figure 3, B and E). It should be noted that the pattern of TTR deposition in the skin of TTR-Leu55Pro X TTR-KO is distinct from the one observed in FAP patients: the prominent glandular involvement in patients is replaced by a more widespread TTR staining in these animals, in which collagen fibrils of the reticular layer and epidermis appear decorated with TTR deposits both in +/+ and in -/+ skins (Figure 3, A and D, respectively). Under a higher magnification, TTR deposits were found surrounding blood vessels (arrow in Figure 3G), muscular and fat tissue (arrowhead in Figure 3G), and hair follicles and sebaceous glands (asterisk in Figure 3G). As referred to, some skins were negative both for Congo Red and TTR staining (Figure 3H). The specificity of TTR staining was assessed by immunohistochemistry in contiguous sections of +/+ skins using the anti-human TTR antibody preabsorbed with excess TTR (Figure 3C). As shown, after preabsorption, staining for TTR was absent.

As already mentioned, some of these TTR deposits showed the characteristic green birefringence after staining with Congo Red (Figure 3B), whereas in others no Congo Red staining was observed despite the presence of TTR deposits (Figure 3E). The anthracycline I-DOX is known to bind amyloid fibrils in tissue sections from FAP patients.²⁶ When I-DOX binding was studied in TTR-Leu55Pro X TTR-KO sections, we observed that I-DOX was able to bind not only +/+ sections, but also micro deposits in -/+ sections (arrows in Figure 3F). This suggested that I-DOX is more sensitive to detect TTR deposits negative for Congo Red. As expected, mice in which the TTR deposition had not been found did not bind I-DOX (Figure 3I).

In TTR-Val30Met X TTR-KO, amyloid deposits were occasionally observed before 12 months (3 of 58 animals from 1 to 12 months), but at 13 to 18 months ~50% of the animals ($n = 27$) had Congo Red-positive material (Table 2). The major affected organs were the esophagus and stomach. With advancing age, the pattern of deposition became widespread with involvement of the intestine, kidney, skin, pancreas, spleen, and muscle. When compared to TTR-Val30Met in which only one animal of the 19 to 24 months group had amyloid fibrils (Table 1), the TTR-Val30Met X TTR-KO of the same age presented ~60% of animals with amyloid deposits (Table 2). This earlier amyloid deposition and higher penetrance reinforced the hypothesis we raised by comparison of TTR-Leu55Pro and TTR-Leu55Pro X TTR-KO, ie, that human homotetrameric mutant TTR molecules are more prone to

fibril formation than heterotetrameric wt mouse/human mutant TTR molecules.

There was no significant difference in the onset of TTR deposition and fibril formation between TTR-Val30Met X TTR-KO and TTR-Leu55Pro X TTR-KO (Table 2). The main distinctions between both strains were the sites where amyloid deposits were found: whereas TTR-Val30Met X TTR-KO have a widespread deposition with particular involvement of the stomach and skin, the TTR-Leu55Pro X TTR-KO showed deposition mainly in the intestine and skin.

Assessment of TTR Deposition by Electron Microscopy and Fibril Extraction

To investigate the nature of the TTR material found on the Congo Red-positive deposits and on the Congo Red-negative/TTR-immunoreactive deposits, we performed immunogold labeling on epon-embedded skin of Congo Red-positive/TTR-immunoreactive (+/+), Congo Red-negative/TTR-immunoreactive (-/+), and Congo Red-negative/TTR-negative (-/-) tissues. In tissues in which Congo Red-positive material had been detected (+/+), TTR fibrils and small aggregates were clearly visible by immunoelectron microscopy using a polyclonal anti-human TTR antibody (arrows and arrowheads, respectively, in Figure 4A, top left). TTR fibrils were closely apposed to collagen fibrils (labeled as C) (Figure 4A, top left) as is the case in FAP tissues. The length and diameter of the microfibrils was assessed from amplified photographs and the obtained diameter (8.6 nm) was within the range described for the human TTR fibrils found in FAP tissues (7 to 12 nm). The specificity of TTR staining was demonstrated by performing immunoelectron microscopy of +/+ skin sections using the anti-TTR antibody preabsorbed with TTR. As shown (Figure 4A, top middle) no labeling is observed both in fibrillar and aggregated material thus proving specificity. In skins that had been scored -/-, no fibrils and no TTR labeling was found (Figure 4A, top right). In tissues that had been scored Congo Red-negative/TTR-immunoreactive (-/+), the presence of small amorphous aggregates of TTR was detected (arrowheads in Figure 4A, bottom left). This amorphous material was also in close contact with collagen fibrils (Figure 4A, bottom) and seemed to physically displace them (Figure 4A, bottom right and left). The less organized, nonfibrillar nature of the amorphous deposits was evident in some areas of the sections analyzed (Figure 4A, bottom middle). It is interesting to note that the aggregates observed in TTR-Leu55Pro X TTR-KO mice here described are similar to the ones present in the wt TTR transgenics previously reported.⁹ Using the antibody preabsorbed with an excess of TTR no labeling was seen (Figure 4A, bottom right) thus showing that the nonfibrillar deposits are composed of TTR. These aggregates probably represent early deposition events that precede fibril formation.

To further determine that TTR was present in the deposited material, we performed extraction of fibrils from the skin of TTR-Leu55Pro X TTR-KO animals Congo Red-

Figure 3. Analysis of TTR deposition in the skin of TTR-Leu55Pro X TTR-KO transgenics. Immunohistochemical analysis of TTR deposition in Congo Red-positive/TTR-positive (**A**) (+/+), Congo Red-negative/TTR-positive (-/+) (**D** and **G**), and Congo Red-negative/TTR-negative (-/-) (**H**) skins. Specificity of TTR labeling was demonstrated by the absence of immunoreactivity in a +/+ skin when the antibody was preabsorbed with excess of TTR (**C**). Presence of amyloid deposits was assessed by Congo Red staining in adjacent sections (**B** and **E**) and allowed the scoring of TTR deposits as Congo Red-positive (**B**) or Congo Red-negative (**E**). Binding of I-DOX was assessed in -/+ (**F**) and -/- (**I**) sections. Original magnifications: $\times 25$ (**A–F**, **H**, and **I**); $\times 50$ (**G**).

A

B

Figure 4. Analysis of TTR deposition in TTR-Leu55Pro X TTR-KO by immunoelectron microscopy and fibril extraction. **A:** Immunoelectron microscopy of +/+ skin using polyclonal anti-TTR (**top left**) or anti-TTR preabsorbed with excess TTR (**top middle**). A negative control skin (-/-) was included for TTR immunocytochemistry (**top right**). Immunoelectron microscopy of -/+ skin using polyclonal anti-TTR (**bottom left** and **bottom middle**) or anti-TTR preabsorbed with excess TTR (**bottom right**). C, collagen; **arrows**, TTR fibrils; and **arrowheads**, TTR amorphous aggregates. Scale bar, 100 nm. **B:** Anti-TTR immunoblot analysis of fibrils extracted from the skin of TTR-Leu55Pro X TTR-KO and TTR KO mice. Recombinant TTR standard (**lane 1**), TTR KO mice (**lanes 2 and 3**), -/- (**lanes 4 and 5**), -/+ (**lanes 6 and 7**), and +/+ (**lanes 8 and 9**) TTR-Leu55Pro X TTR-KO.

positive/TTR-immunoreactive (+/+), Congo Red-negative/TTR-immunoreactive (-/+), and Congo Red-negative/TTR-negative (-/-). The fibrillar material extracted from +/+ skins of TTR-Leu55Pro X TTR-KO mice ($n = 6$) contained TTR (Figure 4B). This result further confirmed the presence of TTR fibrils in TTR-Leu55Pro X TTR-KO mice. In some cases, animals with TTR deposition but Congo Red-negative ($n = 6$) also showed TTR in the extracts. This can be explained by the fact that small aggregates might also be extracted by the experimental protocol used. The sensitivity of the method is probably related to the amount of deposited nonfibrillar material. As a negative control, TTR-Leu55Pro X TTR-KO transgenics with no deposition (-/-, $n = 6$) and skins from TTR KO mice were used. These never showed TTR in the extracts (Figure 4B).

Cytotoxicity of Deposited TTR

We have previously determined that in FAP, TTR aggregates are cytotoxic for cells in the vicinity of the deposits.^{8,10} Signs of increased oxidative stress, namely the presence of nitrotyrosine (NT) epitopes are observed early in FAP.¹⁰ To investigate whether this phenomenon is also present in TTR transgenics, we performed semiquantitative immunohistology of NT staining of skins of TTR-Leu55Pro X TTR-KO mice. We observed that NT epitopes were increased in sites related to TTR deposition in the skin (Figure 5A) and intestine (data not shown). There was a ~1.5-fold increase ($P < 0.0001$) in NT epitopes in the Congo Red-negative/TTR-immunoreactive (-/+) TTR-Leu55Pro X TTR-KO skins ($n = 11$) when compared to Congo Red-negative/TTR-negative (-/-) skins ($n = 7$) (Figure 5B, left). These results suggested that the amorphous deposited TTR material found in the TTR-Leu55Pro X TTR-KO, in a stage before fibril formation might already be responsible for the cytotoxic effects in surrounding cells. Therefore these animals represent suitable animal models for studying molecular cascades involved in degeneration caused by extracellular protein deposition.

To compare the toxicity caused by TTR Leu55Pro and Val30Met, we performed similar semiquantitative analysis of NT staining in the intestine of both TTR-Val30Met X TTR-KO and TTR-Leu55Pro X TTR-KO animals. As shown (Figure 5B, right), there was an approximately twofold increase ($P < 0.0001$) in NT epitopes in the Congo Red-negative/TTR-immunoreactive (-/+) intestines when compared to Congo Red-negative/TTR-negative (-/-) ones, both in TTR-Val30Met X TTR-KO and TTR-Leu55Pro X TTR-KO (Figure 5B). No significant difference was observed in NT quantitation in the intestine of -/+ TTR-Val30Met X TTR-KO and TTR-Leu55Pro X TTR-KO. This data suggests that Val30Met and Leu55Pro TTR aggregates exert similar effects when deposited in tissues (Figure 5B).

Discussion

In this report we present evidence that transgenic mice carrying human TTR Leu55Pro in a TTR-null background (TTR-Leu55Pro X TTR-KO) have early TTR deposition and show increased cytotoxic stress in sites related to TTR deposition.

Leu55Pro TTR is associated with clinically aggressive FAP and has a high amyloidogenic potential.^{4,5} TTR Leu55Pro forms fibrils *in vitro* enabling detailed analyses of TTR fibrillogenesis; at physiological pH we recently proposed a model in which fibrillar TTR structures are composed of several elementary protofilaments that appear in the initial phases of fibrillogenesis, with each protofilament being a vertical stack of structurally modified TTR monomers.²⁹ The model presented for the molecular arrangement of TTR monomers in protofilaments within fibrils may resemble early deposition events *in vivo* as the ones here described for the TTR Leu55Pro transgenics.

TTR Leu55Pro transgenic mice previously generated by other groups showed no TTR deposition.⁹ The authors speculated that this might be related to the low circulating levels of human mutant protein (10 to 30 $\mu\text{g/ml}$). In this respect we would stress that we did not find any correlation between TTR deposition and TTR levels in the plasma of transgenic mice. The TTR-Leu55Pro mice here described present relatively low levels of plasma TTR, having however a similar TTR deposition as TTR Val30Met transgenics that present high levels of circulating human protein.¹⁵ It is also noteworthy that TTR-Leu55Pro transgenics previously reported were not in a TTR-null background. It is therefore possible that some of the differences observed between previous reports and ours are in part related to the presence of mouse wt/human mutant heterotetramers.

It has been previously described that the pattern of amyloid deposition of heterozygous (TTR-Val30Met) versus homozygous (TTR-Val30Met X TTR-KO) Val30Met transgenics did not differ,¹⁵ ie, that the presence of heterotetrameric molecules would not delay amyloid formation. However, in our animal facility, when TTR-Val30Met mice were compared to TTR-Val30Met X TTR-KO mice, a difference in amyloid deposition was found, suggesting that TTR homotetramers are more amyloidogenic than the wt murine/mutant human TTR heterotetrameric forms present in TTR-Leu55Pro and TTR-Val30Met strains. In humans no correlation has been found between amyloidogenicity and the presence of homotetrameric or heterotetrameric TTR Val30Met: homozygous patients do not show a more severe clinical presentation or an earlier onset of the disorder.³⁰ It is also interesting to note that monozygotic twins with FAP were discordant for age of onset and clinical manifestations.³¹ Therefore, there must be a significant contribution from nongenetic factors to the phenotypic variability of FAP, namely environmental factors. Such factors may also account for the different deposition observed in TTR-Val30Met and TTR-Val30Met X TTR-KO transgenic mice bred in different animal facilities.¹⁵ In regard to this, the importance of diet on amyloid formation has been given special relevance. In Alzhei-

Figure 5. Oxidative stress in TTR-Val30Met X TTR-KO and TTR-Leu55Pro X TTR-KO transgenics. **A:** NT staining (**left**) of -/- (**top**) and -/+ (**bottom**) TTR-Leu55Pro X TTR-KO skins (original magnifications, $\times 25$). **Right:** TTR deposition assessed by immunohistochemistry in adjacent sections. **B:** Quantitation of NT staining in: **left:** -/- ($n = 7$) and -/+ ($n = 11$) TTR-Leu55Pro X TTR-KO skins; **right:** intestine of TTR-Leu55Pro X TTR-KO (HM55) and TTR-Val30Met X TTR-KO (HM30) transgenics -/- ($n = 7$ and $n = 11$, respectively) and +/- ($n = 14$ and $n = 15$, respectively). *, $P < 0.0001$.

mer's disease, high dietary cholesterol increases Abeta accumulation and accelerates the Alzheimer's disease-related pathology observed in animal models.³² It has also been shown that hyperglycemia and amyloid formation are correlated in transgenic mice for human islet amyloid polypeptide.³³ The influence of environmental factors such as the diet should be addressed in FAP. The relative abundance of other factors such as the serum amyloid P component (SAP) might influence phenotypic

variation. SAP is universally present in amyloid deposits³⁴ and it has previously been reported that human TTR and mouse SAP are deposited as amyloid in tissues of transgenic mice for human TTR.³⁵ Furthermore, in mice and hamsters, plasma concentrations of SAP were shown to be closely related with amyloidogenesis.³⁴ However, in TTR transgenic mice, the induction of SAP synthesis by acute inflammation did not affect the onset and extent of TTR deposition;³⁶ in double-transgenic mice carrying

both the human mutant TTR gene and the human SAP gene onset, progression and tissue distribution of amyloid deposition were the same as those in single transgenic mouse despite higher levels of plasma SAP.³⁷ These results suggested that SAP is not important for the initiation and progression of amyloid deposition in FAP animal models. In view of the recent report on the possible therapeutic potential of depleting SAP for the treatment of amyloidosis by inhibitors of SAP binding,³⁴ the animal models here described become important in the future for testing of the effect of such types of drugs in amyloid deposition in FAP.

Transgenic mice for human mutant TTR so far generated present deposited material especially in the gastrointestinal tract.^{13–15} One can speculate that in mice, this tissue might contain specific factors that make it more prone to aggregation and/or fibril formation. It is possible that TTR deposition in the skin of the SMT-1 transgenics might be related to the SMT-1 promoter-driven expression present in our constructs, although no human TTR mRNA has been detected in the skin of these animals (data not shown). We can also not exclude an effect of ZnSO₄ because it has been previously shown that sulfate seems to promote aggregation of pre-existing TTR amyloid fibrils.³⁸

In transgenic mice overexpressing human wt TTR, nonfibrillar TTR aggregates have been previously reported.⁹ It is interesting to note that the aggregates observed by Teng and colleagues⁹ by immunoelectron microscopy of tissues from wt TTR transgenic mice look very similar to the ones described in this report for the TTR-Leu55Pro transgenics. However, whereas half of the wt TTR transgenics presented non-Congophilic TTR deposits between 12 and 17 months of age, the majority of the TTR-Leu55Pro transgenics between 1 to 3 months already showed nonfibrillar TTR deposition. Another major difference between these two strains of animals is the preferential deposition site of TTR: wt TTR transgenics⁹ show a particular involvement of the kidneys and heart whereas the TTR-Leu55Pro present preferential deposition in the intestine and skin. We had also already reported the existence of Congo Red-negative TTR deposition in human nerves in early stages of disease progression.⁸ In humans, less structured, nonfibrillar deposits that are able to produce significant clinical pathology have also been described in light chain deposition disease and light and heavy chain deposition disease.³⁹ The presence of such aggregates in the different TTR transgenics here described, further confirms their existence *in vivo* and suggests that these may precede protofibril and fibril formation.

In FAP, the observation that tissue dysfunction may precede amyloid fibril deposition suggested a possible role to other intrinsic factors such as the cytotoxicity of these early nonfibrillar TTR deposits. We have previously shown that deposition of TTR in the form of small nonfibrillar aggregates is toxic to surrounding cells, leading to a sustained inflammatory response and oxidative stress.^{8,10} Furthermore, small TTR aggregates rather than mature fibrils were shown to be toxic in cell culture.⁸ There is, however, a considerable debate as to whether

fully formed mature amyloid fibrils or nonfibrillar aggregates are the primary pathogenic species responsible for the onset of disease and cellular damage. Although there is evidence for the toxicity of mature fibrils in some amyloid diseases,⁴⁰ there is an increasing body of evidence that supports the suggestion that at least in some cases the nonfibrillar aggregates may be the primary toxic species. *In vitro* studies with prion protein fragment, α -synuclein, amyloid β protein ($A\beta$), TTR, and proteins that are not disease-related^{8,41–43} showed that mature fibrils were essentially harmless to cells, supporting that amyloid fibrils are not cytotoxic and reinforcing the findings on cytotoxicity of prefibrillar assemblies. In the case of Alzheimer's disease, this hypothesis was further demonstrated in rats *in vivo* by performing cerebral microinjection of $A\beta$ oligomers in the absence of amyloid fibrils and showing a marked inhibition of hippocampal long-term potentiation and disruption of synaptic plasticity.⁴⁴

The possibility that animal models of TTR amyloidosis might mimic this stress response had never been evaluated. In this respect, transgenic mice expressing mutant amyloid precursor protein have provided important information about the pathogenesis of Alzheimer's disease. In these animals small assemblies of $A\beta$ that occur before $A\beta$ fibril formation are neurotoxic leading to neurodegeneration and to defects in cognition and memory.⁴⁵ In amyloid precursor protein transgenic mice, microglia and astrocytes were activated by aggregated nonfibrillar forms of $A\beta$.⁴⁶ Therefore, as the formation of amyloid plaques is dissociated in time from the early phenotype of these animals, the neurotoxic and inflammatory potential has been attributed to nonfibrillar $A\beta$ aggregates. We now show that in the case of animal models for TTR amyloidosis, both TTR-Val30Met X TTR-KO and TTR-Leu55Pro X TTR-KO present an increase in the oxidative stress marker NT in sites related to deposition of TTR, even before the appearance of detectable amyloid fibrils. Nitration of protein tyrosine residues has been widely used as a footprint for the *in vivo* formation of peroxynitrite and other reactive nitrogen species that result in oxidative damage to proteins.⁴⁷ This results from the fact that when superoxide reacts with nitric oxide to generate peroxynitrite, it forms biologically active nitrating agents that can convert native tyrosine residues in proteins into 3-NT. There is a large body of evidence implicating oxidative damage in the pathogenesis of both normal aging and neurodegenerative diseases and nitration correlates well with many disease states including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, dementia with Lewy bodies, Huntington's disease, among others^{47–49} including FAP. In FAP we showed increased nitration in tissues of asymptomatic carriers of Val30Met with nonfibrillar TTR deposition.¹⁰ However, the role of nitration in disease pathogenesis is still not clear and deserves further clarification concerning the potential consequences to protein function, pathogenesis, effects on signal transducing pathways, and metabolism. Transgenic animal models for mutant TTR represent therefore important tools in unraveling the cytotoxicity of small aggregates in tissue dysfunction and in the involvement of environmental factors in TTR deposition. In addi-

tion they are invaluable for testing the effect of drugs in preventing or reversing TTR deposition.

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