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Animal Model

Cysteine 10 Is a Key Residue in Amyloidogenesis of Human Transthyretin Val30Met

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Type I familial amyloidotic polyneuropathy (FAP), a systemic amyloidosis, is characterized by aggregation of variant transthyretin (TTR Val30Met) into stable, insoluble fibrils. This aggregation is caused by genetic and environmental factors. Genetic factors have been studied extensively. However, little is known about environmental or physiological factors involved in the disease process, and their identification may be important for development of effective treatment. X-ray crystallography of normal and amyloidogenic human TTR Val30Met in type I FAP showed that the −SH side chain of cysteine at position 10 (Cys10) forms a hydrogen bond with Gly57 in normal TTR but not in TTR Val30Met. This result suggests a crucial role for the free Cys10 residue and possible involvement of physiological factors affecting Cys residue reactivity in TTR amyloidogenesis. To analyze amyloidogenesis *in vivo*, our group generated murine FAP models by transgenic technology, with human TTR Val30Met. The three lines of transgenic mice expressed amyloidogenic mutant TTR (Cys10/Met30), wild-type TTR (Cys10/Val30), and artificial Cys-free mutant TTR (Ser10/Met30). Histochemical investigation showed deposition of amyloid derived from human TTR only in amyloidogenic mutant TTR (Cys10/Met30) mice. Thus, the −SH residue in Cys10 plays a crucial role in TTR Val30Met amyloidogenesis *in vivo*. These data suggest the possibility of innovative treatment via physiological factors modulating Cys10 residue reactivity. *(Am J Pathol 2004, 164:337–345)*

Type I familial amyloidotic polyneuropathy (FAP) is caused by deposition of stable and insoluble protein fibrils called amyloid.[1] The formation of amyloid fibrils is initiated by overproduction, mutation, or abnormal processing of amyloidogenic proteins.[2,3] In the case of FAP, mutant transthyretin (TTR) is involved in amyloid fibril formation.[4–6] Type I FAP, caused by the substitution Val to Met at position 30 of TTR (TTR Val30Met), predominates over other types of FAP in Japan, Portugal, Sweden, and the United States.[7–13] This disorder has an autosomal dominant inheritance and eventually leads to death.

TTR is a homotetrameric plasma protein that under normal conditions carries thyroxine (T4) and retinol with retinol-binding protein.[14] It is mainly synthesized by the liver and choroid plexuses; trace amounts are also found in the retina of the eye, the pancreas, and other tissues.[15]

Amyloid formation is a complicated process involving not only genetic but also various physiological (and environmental) factors. The process leading from the amyloidogenic protein to the resultant amyloid fibrils is a primary focus of amyloid research; genes and genetic factors responsible for the disease have also been extensively studied. Important approaches have been used in an attempt to understand this process such as structural biology, *in vitro* study, and animal model.

We previously used a structural biology approach in our previous investigations. Our fine three-dimensional structure analysis of human mutant TTR by X-ray crystallography showed that the Cys10 residue in TTR Val30Met is free from an internal hydrogen bond and suggested that Cys10 may play a crucial role in TTR amyloidogenesis.[16] This result implies that physiological factors affecting reactivity of the cysteine residue may be involved

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in the amyloidogenesis of TTR. Also, Lai and colleagues,[17] Liu and colleagues,[18] Colon and Kelly,[19] and Lashuel and colleagues[20] reported that TTR can be induced to be partially separated into monomers *in vitro* under low pH and can self-assemble into amyloid fibrils in acidic environment. These results indicate that the misfolded monomer may be a cause of amyloidogenesis and an environmental factor (or factors) may be important in amyloidogenesis.

We previously developed transgenic mouse lines expressing variant TTR that may allow us to analyze the physiology of amyloid formation *in vivo*.[21–24] In this report, we describe experiments designed to study the effect of Cys10 on deposition of human variant TTR-derived amyloid. We compared amyloid formation in three new lines of mice that we developed: amyloidogenic transgenic mice (7.2-hMet30) carrying the human mutant TTR (Cys10/Met30) gene, control transgenic mice (7.2-hTTR) carrying the human wild-type TTR (Cys10/Val30) gene, and Cys-free variant transgenic mice (7.2-hSerMet) carrying the human artificial TTR (Ser10/Met30) gene. By using this *in vivo* experimental system, we found amyloid deposits in only 7.2-hMet30 mice, which means that Cys10 is indeed a crucial factor in TTR amyloidogenesis. Our findings here imply possible control of TTR amyloid formation through physiological or environmental factors that modulate reactivities of the thiol residue of cysteine—findings that may thus provide a new pharmacological target in the development of drugs for this intractable disorder.

Materials and Methods

Construction of Transgenes and Production of Transgenic Mice

A 7.2-kb fragment of the human TTR promoter region (a kind gift from Professor S. Maeda, University of Yamanashi School of Medicine, Yamanashi, Japan), which was derived from LmPAE-7,[25] was digested with *Eco*RI and *Bam*HI. The 7.2-kb promoter fragment replaced an MHC class I promoter fragment of a cDNA expression vector pLG1,[26] and the resultant vector was designated pTG.

Three recombinant cDNAs[10] encoding normal TTR, TTR Val30Met, and Ser10-TTR Val30Met were introduced into the cDNA cloning site of the plasmid pTG,[27] and we then checked the DNA sequence.[28] The resultant plasmids were designated 7.2-hTTR, 7.2-hMet30, and 7.2-hSerMet, respectively. Before microinjection of each plasmid into mouse eggs, the plasmids were digested with *Kpn*I and *Sac*II. A 8.95-kb fragment that was obtained was isolated as a transgene. Approximately 200 copies of the transgene were microinjected into the pronucleus of fertilized eggs of C57BL/6J mice (Clea Japan, Tokyo, Japan), as previously described.[29]

The presence of the transgene in mice was checked when the mice were 4 weeks old by use of Southern blot analysis,[30] with the *Sca*I-*Xho*I fragment as a probe. The serum level of human TTR in each transgenic mouse was determined by Western blotting to allow selection of two or three producers of high levels of human TTR (strains) for each construct. The resultant transgenic mice were kept under the same specific pathogen-free conditions until the age of 8 months, when they were transferred to conventional housing conditions in Inoue Experimental Animal Center (Kumamoto, Japan).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

For each transgenic mouse strain, the tissue specificity of transgene expression was determined by RT-PCR.[23] Total RNAs extracted from various tissues via the guanidine thiocyanate procedure were reverse-transcribed into cDNA by SuperScript RT (Gibco/BRL SuperScript Preamplification System; Life Technologies, Inc., Gaithersburg, MD). To avoid co-amplification of endogenous mouse TTR, two transgene-specific primers were synthesized for PCR. One primer (5′-CCTACAGCTCCTGGGCAACGTGCT-3′) was derived from the sequence flanking the 5′ end of the inserted human TTR cDNA and the other primer (5′-GGCATTGGCCACACCAGCCACCACCAC-3′) was from the sequence flanking the 3′ end of the cDNA. PCR was performed with a reaction mixture (50 μl) containing 5 pmol of primers, 1 U of AmpliTaq DNA polymerase (Perkin-Elmer/Cetus, Tokyo, Japan), 1× PCR buffer, and 200 μmol/L dNTPs on a heating block (PC-700; ASTEC, Fukuoka, Japan). After 30 cycles of amplification (1 cycle: denaturation at 94°C for 60 seconds, annealing at 58°C for 60 seconds, and extension at 72°C for 120 seconds), PCR products were processed by electrophoresis on an agarose gel. A 630-bp product was detected by use of ethidium bromide.

Immunoblot Analysis and Quantification of Human TTR in Serum

To select the transgenic mice for this research, human TTR levels in mouse sera were determined by immunoblotting according to a previously reported method,[23] but without the use of radioisotopic detection. This immunoblot-based method, free from the effect of endogenous mouse TTR, allows use of our human TTR antibody that reacts with the mouse TTR and the human TTR concentrations in mouse sera were comparable to those found in our previous research.[23,31]

Mouse serum samples were subjected to 17% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were electroblotted onto methyl alcohol-treated nylon membranes (Hybond N^+; Amersham, Buckinghamshire, UK). The membranes were incubated with rabbit anti-human prealbumin antibody (MBL, Nagoya, Japan) and were then treated with horseradish peroxidase-conjugated goat anti-rabbit IgG. The membranes were developed according to Amersham's enhanced chemiluminescence protocol. After Western blotting, the serum concentration of human TTR in each transgenic mouse strain was determined by a gel plotting macro of the NIH Image program (Wayne Rasband, National Institutes of

Health, Bethesda, MD). There were approximately linear relationships between the human TTR control and the imaging program intensity of the bands until 20 mg/dl. We chose two or three transgenics of nearly the same human TTR level for each line.

Histochemical Analysis

Transgenic mice were killed under ether anesthesia at 9, 15 to 19, and 21 to 24 months of age. Tissues—heart, kidneys, spleen, liver, lungs, pancreas, stomach, small and large intestines, urinary bladder, thyroid gland, lymph nodes, bone marrow, sciatic nerves, autonomic nerves, and brain—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 same sections were stained with Congo Red according to Wright's method, ie, with potassium permanganate ($KMnO_4$) before Congo Red staining.[32] To detect the emerald green birefringence emitted from the amyloid deposits, a polarized microscope was used to examine the Congo Red-stained sections.

Immunohistochemical Analysis

For immunohistochemical demonstration of the major components of amyloid deposits, the tissues were embedded in OCT compound (Miles, Elkhart, IN), frozen in liquid nitrogen on a floating plastic vessel, and sectioned by use of a cryostat (Bright, Huntington, UK). After inhibition of endogenous peroxidase activity,[33] the sections were immunostained by the indirect immunoperoxidase method.[23] The first antibodies were rabbit anti-human prealbumin (transthyretin) (DAKO, Glostrup, Denmark) and anti-mouse SAP (Behring Diagnostics, La Jolla, CA), and the second antibody was the anti-rabbit immunoglobulin horseradish peroxidase-linked $F(ab')_2$ fragment (Amersham, Poole, UK). After visualization with 3,3′-diaminobenzidine (Sigma Chemical Co., St. Louis, MO), sections were stained with hematoxylin and mounted with malinol. For detection of AA amyloid, sections were stained by using the mouse anti-human amyloid A monoclonal antibody (DAKO) and the MOM kit (Vector, Burlingame, CA) according to the manufacturers' instructions, as described previously.[34] To obtain negative controls, the same procedure was done without the primary antibodies.

Results

Construction of Transgenes and Generation of Transgenic Mice

Our previous X-ray study showed that the Cys10 of TTR Val30Met is free from the intermolecular hydrogen bond found in normal TTR (Figure 1)[16] and led us to the hypothesis that the free cysteine residue may play a critical role in amyloidogenesis. To investigate the relationship

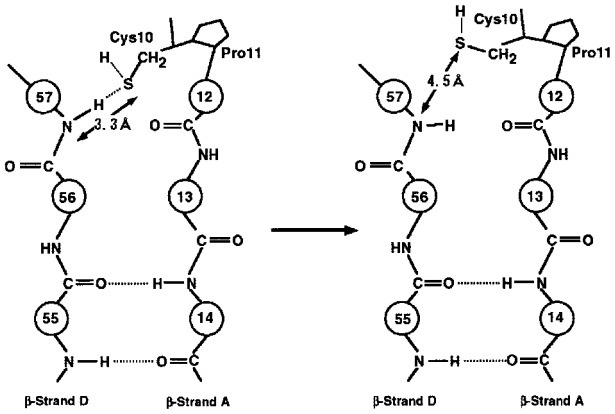


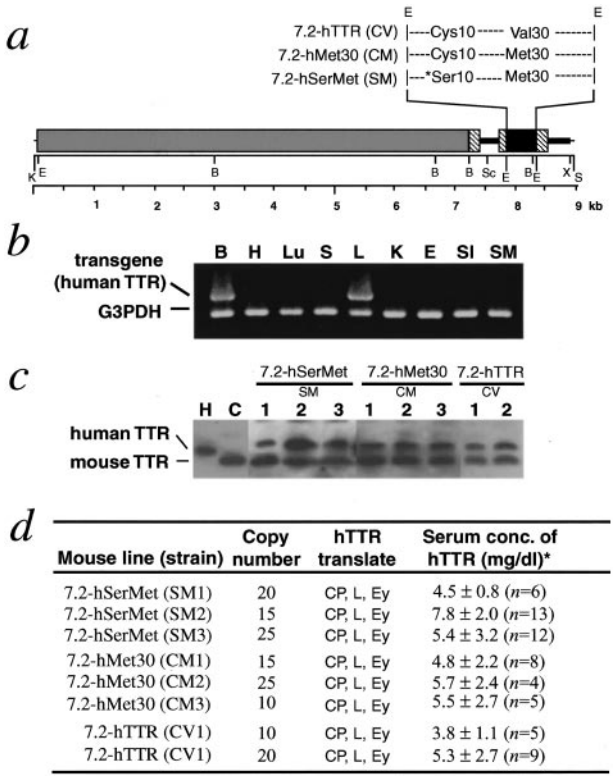
Figure 1. Schematic drawing of changes in interaction of Cys10 (from Terry et al[17]). **Left**, normal TTR: the −SH group of Cys10 interacted with the NH group of residue 57 (NH57) by means of a hydrogen bond (3.3 ± 0.2 Å). **Right**, TTR Val30Met: the structural effect of the Met30 substitution is breakage of the hydrogen bond between Cys10 and NH57.

between Cys10 and amyloid deposits, we developed transgenic mice expressing human TTRs with appropriate amino acid substitutions. First, we constructed three transgenes with a 7.2-kb fragment of human TTR promoter, as described in Materials and Methods. Genetic types of those transgenes are Cys10/Val30 for the wild-type TTR, Cys10/Met30 (TTR Val30Met) for the amyloidogenic mutant TTR, and Ser10/Met30 (artificial TTR: Val30Met with Cys10Ser) for an artificial Met30 TTR without the cysteine residue (Figure 2a). Serine was chosen as the replacement because it is structurally similar to cysteine but is chemically inert, that is, the only −SH residue in cysteine is replaced by −OH in serine.

Second, these transgenes were injected into fertilized eggs. The mice thereby produced were used for Southern and Western blot analysis (Figure 2c), and two or three strains were chosen for each mouse line: 7.2-hTTR, strains CV1 and CV2; 7.2-hMet30, strains CM1, CM2, and CM3; and 7.2-hSerMet, strains SM1, SM2, and SM3. The copy number of the transgene in each strain was 10 to 25 (Figure 2d). At 8 weeks of age, these mice were mated with wild-type C57BL/6J mice. All lines of transgenic mice transmitted the transgene to their offspring, and these offspring were used for analysis. All of the mice were housed under specific pathogen-free conditions for 8 months and were then transferred to conventional housing conditions, in which amyloidosis is expected to be enhanced.

Transgene Expression

The transgenic mice were further characterized. To examine the tissue specificity of transgene expression in all transgenic mouse strains, total RNAs were extracted from various tissues (brain, heart, lung, spleen, liver, kidney, esophagus, small intestine, and skeletal muscle) and were subjected to RT-PCR analysis. PCR products were detected only in liver and brain (Figure 2b). Immunohistochemical analysis showed that the transgenes were translated at the choroid plexus in the brain, liver, and retina of the eye, as is the case in humans (data not



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Mouse line (strain)	Copy number	hTTR translate	Serum conc. of hTTR (mg/dl)*
7.2-hSerMet (SM1)	20	CP, L, Ey	4.5 ± 0.8 (*n*=6)
7.2-hSerMet (SM2)	15	CP, L, Ey	7.8 ± 2.0 (*n*=13)
7.2-hSerMet (SM3)	25	CP, L, Ey	5.4 ± 3.2 (*n*=12)
7.2-hMet30 (CM1)	15	CP, L, Ey	4.8 ± 2.2 (*n*=8)
7.2-hMet30 (CM2)	25	CP, L, Ey	5.7 ± 2.4 (*n*=4)
7.2-hMet30 (CM3)	10	CP, L, Ey	5.5 ± 2.7 (*n*=5)
7.2-hTTR (CV1)	10	CP, L, Ey	3.8 ± 1.1 (*n*=5)
7.2-hTTR (CV1)	20	CP, L, Ey	5.3 ± 2.7 (*n*=9)

Figure 2. Characterization of transgenic mice. **a:** Structures of three transgenes used in human TTR transgenic mice. Each transgene contained 7.2 kb of the human TTR promoter region (indicated by the **gray box**); rabbit β-globin gene and human TTR cDNA encoding Val30Met as an amyloidogenic mutant TTR; and Cys10Ser Val30Met as an artificial mutant TTR without the −SH residue of Cys10 or normal TTR. The **black box** indicates TTR cDNA that was inserted into the rabbit β-globin gene exon (indicated by the **striped box**) and intron (**bold line**) to express the cDNA gene *in vivo*. After construction, the plasmid was digested by *Kpn*I and *Sac*II to exclude the vector sequence from the transgene. Symbols for restriction endonucleases are as follows: B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; S, *Sac*II; Sc, *Sca*I; X, *Xho*I. **b:** Expression of the transgene in transgenic mice as detected by RT-PCR. Expression of the transgene was detected in the brain and liver of all transgenic mice. G3PDH was used as a loading control. Expression patterns in all transgenic mice were the same. Symbols for tissues are as follows: B, brain; H, heart; Lu, lung; S, spleen; L, liver; K, kidney; E, esophagus; SI, small intestine; SM, skeletal muscle. **c:** Detection of human TTR in mouse serum. Serum from transgenic mice was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with anti-human TTR antibody. All strains—CM1, CM2, and CM3 (7.2-hMet30 mice); SM1, SM2, and SM3 (7.2-hSerMet mice); CV1 and CV2 (7.2-hTTR mice)—contained both mouse and human TTR. **Lane H**, human serum; **lane C**, mouse serum. **d:** Characterization of transgenic mice. The patterns of human TTR (hTTR)-translated tissues in all transgenics were demonstrated by use of immunohistochemistry. No significant difference in the serum concentration of human TTR in each strain was found, except for the concentration in the SM2 line, which was higher than that in the CV1 line ($P < 0.05$). Symbols for tissues are as follows: CP, choroid plexus; L, liver; Ey, retina of eye. *, Data are means ± SD.

shown). Concentrations of human TTR in the two 7.2-hTTR mouse strains CV1 and CV2, the three 7.2-hMet30 transgenic mouse strains CM1, CM2, and CM3, and the three 7.2-hSerMet mouse strains SM1, SM2, and SM3 are given in Figure 2d.

A previous study suggested that amyloid deposition depends on the serum levels of human TTR in the case of 0.6-hMet30 mice (2.0 mg/dl) and 6.0-hMet30 mice (14.5 mg/dl).[24] It is important to compare similar levels of human TTR concentration in the different mouse lines. In our three lines, there was no statistically significant difference in the human TTR concentrations. Only the SM2 strain had a higher serum concentration of human TTR than did the CV1 strain ($P < 0.05$). Nontransgenic littermates showed no detectable level of human TTR in serum samples (Figure 2c).

Table 1. Deposition of Amyloid Derived from Human TTR

Mouse line (strain)	No. of mice with amyloid/total no. of mice		
	Age 9 months	Age 15 to 19 months	Age 21 to 24 months
7.2-hMet30 (CM1)	0/3	0/5	5/10*
7.2-hMet30 (CM2)	0/1	0/7	7/12*
7.2-hMet30 (CM3)	—	0/7	2/5*
7.2-hSerMet (SM1)	—	0/2	0/6
7.2-hSerMet (SM2)	0/1	0/4	0/25†
7.2-hSerMet (SM3)	0/3	0/5	0/9
7.2-hTTR (CV1)	—	0/2	0/4
7.2-hTTR (CV2)	0/1	0/6	0/23
Control (littermate)	0/1	0/6	0/18

*Statistical difference noted between 7.2-hMet30 line and the other two lines.
†Only one mouse with AA amyloid deposition (TTR negative).

Evaluation of TTR Amyloid Deposition by Congo Red Staining and Immunohistochemistry

We examined each transgenic line at 9, 15 to 19, and 21 to 24 months after birth in an effort to detect amyloid deposits. We used tissue samples from transgenic and nontransgenic mice, both male and female, for each time point. Paraffin sections were stained by the Congo Red method as noted in Materials and Methods and were then subjected to examination with a polarized microscope. Amyloid deposits were found only in the 7.2-hMet30 line, in 5 of 10 CM1 mice, 7 of 12 CM2 mice, and 2 of 5 CM3 mice at 21 to 24 months of age (Table 1). Table 2 shows the location and relative amount of amyloid deposits in the various tissues examined: heart, thyroid, lung, liver, kidney, esophagus, upper stomach, urinary bladder, testis, and large and small intestines—the same pattern of deposition that was observed in other TTR transgenic mice and reported previously.[23,24] No significant sex difference was observed in the pattern of deposits. Marked deposition was found in the kidney (Figure 3a), the interstitium of the thyroid (Figure 3d), and the small intestine (Figure 3f). These tissues demonstrated an apple green birefringence under polarized light (Figure 3; b, e, and g). No pathological change was observed in the sciatic nerve (data not shown).

As an example of immunohistochemical analysis, Figure 3c shows that amyloid deposits reacted to anti-human TTR antibody. The result demonstrates that these amyloid deposits were of human variant TTR origin. The amyloid deposits also reacted to anti-mouse serum amyloid P component (SAP) antibody (data not shown), which indicated that murine SAP binds to human TTR Val30Met-derived amyloid.

No amyloid deposition was detected in 7.2-hTTR mice (strains CV1 and CV2) carrying the wild-type human TTR gene, however. Also, nontransgenic littermates showed

Table 2. Tissue Distribution of TTR Amyloid Deposits in 7.2-hMet30 Line at the Age of 21 to 24 Months

	CM1	CM2	CM3
Total no. of mice with amyloid/total no. of mice without amyloid	5/5	7/5	2/3
Number of mice with amyloid (total examined), ♂/♀	4 (7)/1 (3)	6 (10)/1 (2)	0 (2)/2 (3)
Brain	−	−	NT
Sciatic nerve	−	−	NT
Heart	± to ++	± to +	±
Thyroid	+ to ++	+	NT
Lung	− to ±	− to ±	NT
Liver	− to ±	− to ±	NT
Kidney	++ to +++	++ to +++	++
Spleen	−	−	NT
Esophagus	− to ++	− to +	NT
Upper stomach	± to ++	± to +	NT
Large intestine	− to ++	± to ++	NT
Small intestine	++ to +++	++ to +++	NT
Urinary bladder	− to +	−	NT
Testis	− to ±	−	NT

−, 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; NT, not tested.

no amyloid deposition at 24 months of age (Table 1). These results clearly indicate that amyloid deposition in the 7.2-hMet30 mice was mutation-dependent, as in the case of FAP patients (Table 3). In mice with Congo Red-negative results, nonfibrillar aggregates of human TTR were detected by immunohistochemical staining in one line of each strain. Nonfibrillar deposits were found in both males and females at 21 to 24 months of age, as in other previous reports (Figure 4).[34,35]

Role of Cys10 in TTR Amyloidogenesis

To analyze the role of Cys10 in amyloidogenesis of TTR V30M, 7.2-hSerMet mouse strains were prepared. Samples of tissues, including different organs, skeletal muscle, and various neural tissues, were taken from all three strains of 7.2-hSerMet mice at 9, 15 to 19, and 21 to 24 months age and were subjected to Congo Red staining. No TTR-positive amyloid deposition was observed in any strain. Only one mouse of the 7.2-hSerMet line (SM2 strain) showed a very weak congophilic anti-human TTR-negative deposit in the kidney after $KMnO_4$ treatment. This deposit reacted with AA amyloid antibody but not with human TTR antibody (data not shown). Table 3 indi-

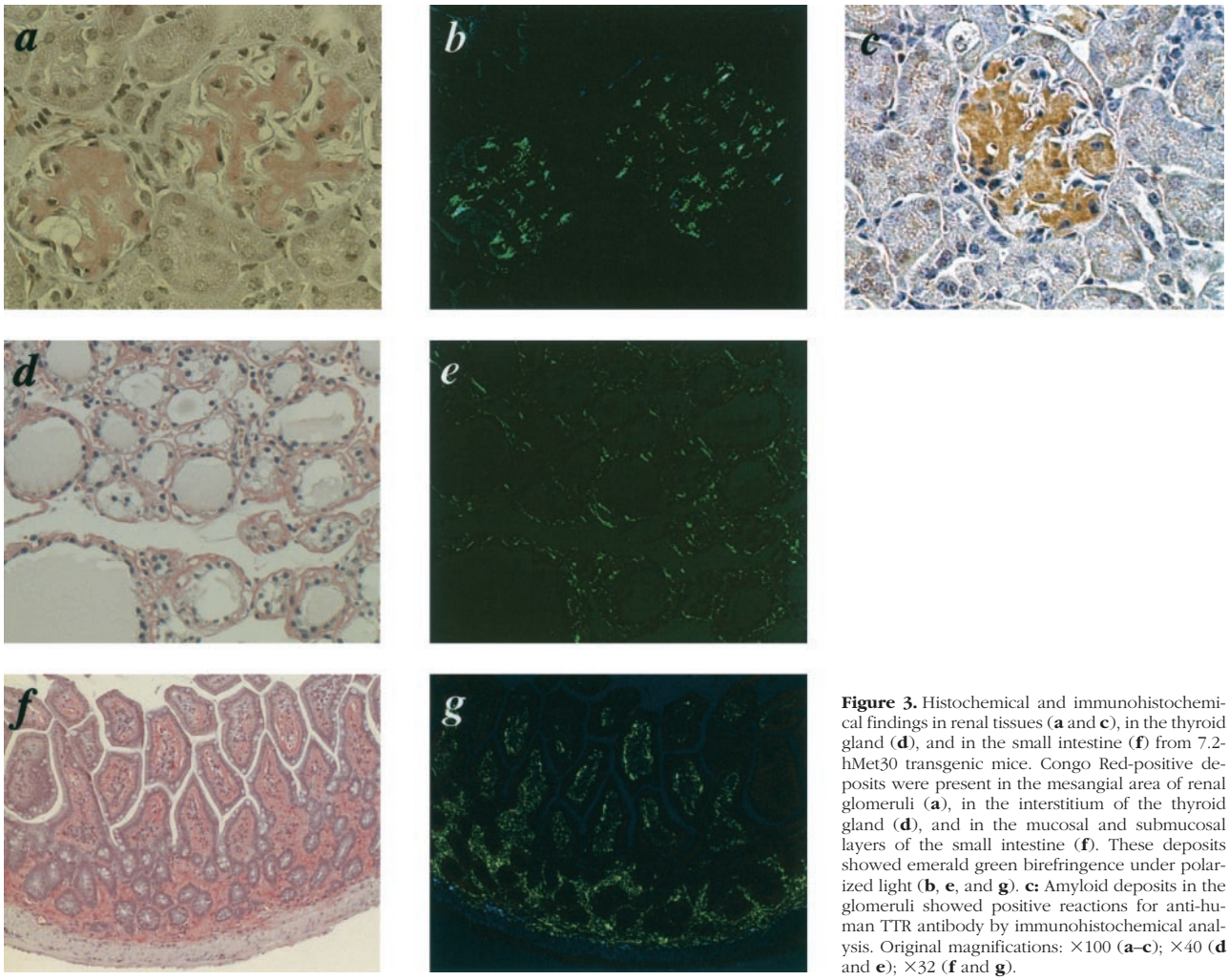


Figure 3. Histochemical and immunohistochemical findings in renal tissues (**a** and **c**), in the thyroid gland (**d**), and in the small intestine (**f**) from 7.2-hMet30 transgenic mice. Congo Red-positive deposits were present in the mesangial area of renal glomeruli (**a**), in the interstitium of the thyroid gland (**d**), and in the mucosal and submucosal layers of the small intestine (**f**). These deposits showed emerald green birefringence under polarized light (**b**, **e**, and **g**). **c:** Amyloid deposits in the glomeruli showed positive reactions for anti-human TTR antibody by immunohistochemical analysis. Original magnifications: ×100 (**a**–**c**); ×40 (**d** and **e**); ×32 (**f** and **g**).

Table 3. Relation Between Amino Acid Content of Human TTR and Amyloid Deposition

Mouse line	Genotype of human TTR	Amino acid characterization				TTR amyloid deposition
		Cys10	Ser10	Val30	Met30	
7.2-hTTR	Normal	+	−	+	−	−
7.2-hMet30	Amyloidogenic	+	−	−	+	+
7.2-hSerMet	Artificial-SH free	−	+	−	+	−

+, present; −, absent.

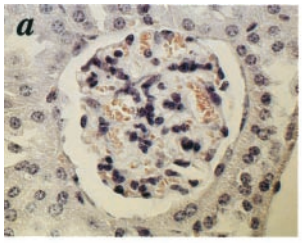
cates that both 7.2-hSerMet and 7.2-hMet30 mice had the same Val30Met mutation in the human TTR, but the former transgene of artificial human TTR had Ser10 and not Cys10 so that there was no thiol residue. Ser10 completely suppressed the amyloid deposition of TTR Val30Met (0 of 6, 0 of 25, and 0 of 9 in SM1, SM2 and SM3, respectively), compared with amyloid deposition in all 7.2-hMet30 mouse strains (5 of 10, 7 of 12, and 2 of 5 in CM1, CM2, and CM3, respectively) at 21 to 24 months (Table 1). These results indicate that the thiol residue of Cys10 is a key factor in amyloidogenesis of TTR Val30Met. In addition, the finding of Congo Red-negative nonfibrillar deposits in all strains suggests that Cys10 may play a role in fibrillogenesis.

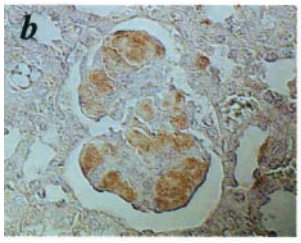
Discussion

In intricate diseases such as amyloidosis, both genetic and environmental factors are involved in the disease process. Recent advances in genomics have accelerated the identification of genetic factors, but the way in which environmental factors affect that process is not completely understood. Although no solid approach exists for identification of such environmental factors, the purpose of the present work is to report that careful analysis of genetic factors may provide clues to the environmental influences. X-ray crystallography of TTR Val30Met, which is responsible for type I FAP,[7] showed that the Met30 substitution induces a very small change in the structure of TTR,[16] and a significant change caused by Met30 clearly breaks the apparent hydrogen bond between Cys10 and Gly57 and moves the cysteine side chain slightly out of its pocket (Figure 1). This structural alteration led us to the hypothesis that a cysteine residue is involved in the Val30Met type of FAP amyloidogenesis. In this study, we show that Cys10 of TTR Val30Met is indeed a prerequisite for amyloidogenesis. This result suggests the importance of environmental and physiological factors affecting the reactivity of the thiol residue of Cys10 in FAP amyloidogenesis.

There have been two previous reports of FAP research on Cys10 (Cys-free studies): a study of a TTR Cys10Arg kindred[36] and an *in vitro* TTR Cys10Ala study.[37] The first study reported the late onset (older than 60 years of age) of FAP, which is different from the early onset (~25 to 35 years of age) of TTR Val30Met FAP. This finding suggested that amyloidogenesis of Cys10Arg is not the same as that of Val30Met. In the case of TTR Cys10Arg, the van der Waals volume of arginine (148 Å^3) is higher than that of cysteine (86 Å^3); this difference leads to a conformational modification of the TTR protein that may result in the late onset of TTR Cys10Arg amyloidosis. The second study, comparing the role of Cys10 TTR (Cys10Ala) with wild-type TTR, reported equal structural stability and capability of fibril formation *in vitro*. However, this study examined only the Val30 type, not the Val30Met mutant.

Both studies, therefore, do not contradict our hypothesis that Cys10 plays a very important role in amyloido-





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Mouse line (strain)	No. of mice with TTR staining / total no. of mice with Congo red-negative staining			Tissue distribution of nonfibrillar deposition
	Age 15-19 months	Age 21-24 months		
7.2-hMet30 (CM1)	0 / 1	0 / 5	2 /12 (16.7%)	male:1, lung female:1, tyroid, vascular endothelium
7.2-hMet30 (CM2)	—	0 / 4		
7.2-hMet30 (CM3)	0 / 2	2 / 3		
7.2-hSerMet (SM1)	0 / 1	0 / 4	3 /28 (10.7%)	male:2, glomeruli female:1, glomeruli
7.2-hSerMet (SM2)	0 / 5	3 / 17		
7.2-hSerMet (SM3)	0 / 2	0 / 7		
7.2-hTTR (CV1)	0 / 2	2 / 4	3 /18 (16.7%)	male:2, glomeruli, small intestine female:1, lung
7.2-hTTR (CV2)	—	1 / 14		

Figure 4. Analysis of nonfibrillar TTR deposition in Congo Red-negative transgenics. **a:** The glomerulus did not show any staining, but erythrocytes in the capillary lumen of glomerular tufts had slight Congo Red staining. **b:** Immunohistochemical staining in the same transgenic mouse demonstrated TTR deposition in the mesangial area of the glomerulus. **a** and **b** were not adjacent serial sections but were from the same 7.2-hTTR mouse. **c:** Congo Red-negative TTR deposition was found in one strain of each line (CM3 and SM2) and in CV1 and CV2 strains at 21 to 24 months of age. Original magnifications, ×200 (**a** and **b**).

genesis of TTR Val30Met. In addition, wild-type TTR causes senile amyloidosis, as does Cys10Arg in that kindred. Although we could not identify the pathogenesis with certainty, both studies suggested that a Cys10-unrelated process may exist for formation of amyloid in types of FAP amyloidosis other than Val30Met. To best study such a process, *in vitro* physiological conditions should be similar to physiological conditions found in patients, but *in vitro* reproduction of these *in vitro* physiological conditions is difficult. We used our transgenic mouse model of TTR Val30Met amyloidosis, because this model provides a suitable experimental *in vivo* system.

The human artificial TTR (Ser10/Met30) gene was used to study the role of Cys10 in amyloidogenesis of TTR Val30Met. Although we do not have a crystal structure of the artificial TTR molecule (Ser10/Met30), no dynamic conformational change would occur between Val30Met TTR and artificial TTR because Cys10 is not one of the backbone (β-strand) amides in which the conformational instability of TTR is located.[18] In addition, many researchers use cysteine-to-serine substitution, which results in a similar protein folding,[38] because cysteine resembles serine in molecular structure and van der Waals volume (86 $Å^3$ for cysteine *versus* 73 $Å^3$ for serine).

TTR amyloid deposits were observed in all 7.2-hMet30 strains in which Cys10 was included in the human TTR, but not in the 7.2-hSerMet line (Table 1). This result indicates that amyloid deposition was suppressed by substituting the chemically inert serine for cysteine, which suggests that Cys10 is an essential residue in TTR amyloidogenesis *in vivo*.

Only one 7.2-hSerMet (SM2) mouse (24 months old) demonstrated a very weak Congo Red-positive and TTR-negative deposit. The paraffin sections showed very slight staining with $KMnO_4$ treatment before use of the Congo Red method, which indicates weak $KMnO_4$ reactivity.[32] This deposit reacted with AA amyloid (data not shown). Indeed, another research group has reported that TTR-free AA amyloid deposits were found in the negative littermates of TTR Leu55Pro transgenic mice.[35] In addition, seven of the 7.2-hSerMet mice had no amyloid deposition at 26 to 31 months of age (data not shown).

The proportion of amyloid deposition in 7.2-hMet30 mice was lower (50 to 60%) than that in 6.0-hMet30 mice.[22] One possible reason for this is the serum level of human variant TTR: the serum concentration in 7.2-hMet30 mice was half that in 6.0-hMet30 mice and is double that in 0.6-hMet30 mice.[31] However, a previous study found no correlation between the serum levels of human variant TTR[23] in the amyloid mouse model. Another possibility was an environmental factor: housing under specific pathogen-free conditions for 8 months could have resulted in the difference between 7.2-hMet30 and 6.0-hMet30 mice. In the 6.0-hMet30 model, no amyloid deposition was found under specific pathogen-free conditions, but the conventional condition was an amyloidogenic environment.[39] The deposition may arise from factors other than the genetic background. Indeed, all six 7.2-hMet30 mice showed amyloid deposition at 24 months when mice were kept only under conventional conditions (data not shown), as in our results for 6.0-hMet30 mice kept under conventional conditions.[24] Further analysis is needed to identify the relation between environmental factor(s) and amyloidogenesis.

Because cysteine is reactive, it is likely that the thiol residue of Cys10 is involved in amyloid formation through its oxidation. Indeed, severe oxidative stress was observed in FAP patients,[40] and in one FAP patient who possessed a mutant extracellular superoxide dismutase gene, massive amyloid depositions were much more severe than those in other FAP patients.[41] In addition, it is notable that the amyloid of TTR Val30Met includes disulfide bridges, which may have been caused by oxidation of the thiol residue of cysteine.[42,43] These findings thus also suggest the importance of the oxidation of Cys10, the sole cysteine residue of TTR,[44] in TTR amyloidogenesis.

Oxidation plays an important role in various degenerative diseases including amyloidosis.[45] For example, oxidative stress[46] and modification of the cysteine residue of β amyloid precursor protein[47,48] have been observed in Alzheimer's disease. These observations provide additional evidence supporting oxidative stress and cysteine modification as key factors in amyloid research.

In 7.2-hTTR strains overexpressing human wild-type TTR, nonfibrillar TTR deposits were found in mice at 21 to 24 months of age but amyloid deposits were not seen up to 30 months of age. Teng and colleagues[35] reported that transgenics overexpressing wild-type human TTR can produce both fibrillar and nonfibrillar deposits. This result is not consistent with our findings for amyloid deposits. The reason may be a difference in genetic background: Teng and colleagues[35] used C57BL/6 $\times$ DBA/2 mice and we used C57BL/6 mice. Our previous study suggests this possibility because, in the case of Val30Met TTR overexpressed by a metallothionein promoter, C57BL/6 mice showed amyloid deposits[49] but (C57BL/6 $\times$ C3H)F1 $\times$ BALB/c mice[50] did not. This means that a different genetic background leads to the presence or absence of amyloid deposition. That is, TTR amyloid deposition in our mouse models with the same genetic background are Met30-dependent, as in the case of amyloid formation in FAP patients.

We are also now using our mouse models to observe the effect of antioxidants such as glutathione and tocopherol on TTR amyloid formation. We believe that our mouse system is useful for monitoring the effects on amyloid formation of antioxidants and other environmental and physiological factors that modulate oxidation of the cysteine. Our research team is currently conducting these investigations.

DNA technology has enabled presymptomatic and prenatal diagnoses of FAP, and *in vitro* research has suggested the possibility of treatment of TTR amyloidosis.[51] However, no effective therapy has yet been developed except for liver transplantation, which is applicable to a very limited number of patients.[52,53] Therefore, there has been keen interest in development of suitable treatment of the disease. The present study may provide a rationale for developing an innovative treatment of amy-

loidosis in type I FAP by suppressing reactivity of the thiol residue of Cys10.

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