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Systemic Amyloidosis in Transgenic Mice Carrying the Human Mutant Transthyretin (Met30) Gene

Pathologic Similarity to Human Familial Amyloidotic Polyneuropathy, Type I

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To analyze the pathologic processes of amyloid deposition in type I familial amyloidotic polyneuropathy (FAP), mice were made transgenic by introducing the human mutant transthyretin (TTR) gene. In these transgenic mice, amyloid deposition started in the gastrointestinal tract, cardiovascular system, and kidneys 6 months after birth and extended to various other organs and tissues with advancing age. At age 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. Amyloid deposition was shown to be composed of human mutant TTR and, in addition, mouse serum amyloid P component. These results clearly indicate that human variant TTR produced in transgenic mice deposits is a major component of amyloid fibrils in various organs and tissues. Thus this animal model is useful for analyzing how amyloid deposition initiates and proceeds in FAP. (Am J Pathol 1991, 138:403–412)

Familial amyloidotic polyneuropathy (FAP) is a heredofamilial amyloidosis transmitted by an autosomal dominant trait, occurring in middle-aged adults, and running a lethal clinical course.[1] Since the first description of Portuguese patients with FAP by Andrade in 1952,[2] many similar cases have been reported in various countries. It is classified by clinical workup into at least four types: type I (Andrade type), type II (Rukavina type), type III (Van Allen type), and type IV (Meretoja type).[3] Previous studies demonstrated that amyloid precursor protein in FAP type I and II is immunologically related to a serum protein, prealbumin, recently named transthyretin (TTR).[4] Furthermore biochemical analyses revealed that these amyloid deposits consist of variant TTR molecules.[5] So far six different types of variant TTR molecules have been identified and shown to form amyloid deposits in FAP.[6]

Type I FAP predominates over the other clinical types in Japan. Several families of FAP patients reported in Kumamoto and Nagano prefectures[7,8] are classified as type I, like FAP patients in Portugal[9] and Sweden.[10] Although the average age at onset differs significantly between Japanese, Portuguese, and Swedish patients, the pathologic distribution of amyloid deposition is similar. Amyloid deposition usually occurs in the peripheral and autonomic nerve tissues, cardiovascular system, kidneys, thyroid, or small and large intestines.[11,12] In all Japanese,[5] Portuguese,[9] or Swedish[10] patients with type I FAP, biochemical studies revealed that the major component of the amyloid deposits is a variant TTR with a single amino acid substitution of valine for methionine at position 30 (hMet30). Recent molecular biologic studies demonstrated one base change from G to A in the mutant TTR gene corresponding to the single amino acid substitution at position 30.[13,14] The presymptomatic diagnosis of FAP became possible by the detection of this variant TTR in the serum or the mutant TTR gene in the

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chromosome.[15] All FAP patients examined so far carried at least one mutant TTR gene, suggesting that this disease is mainly caused by the presence of the mutant TTR gene. However it remains unclear why the age at onset, clinical manifestation, and degree of amyloid deposition in various tissues vary so much from one case to another.

To elucidate the pathologic processes of amyloid deposition in FAP, we produced transgenic mice by the introduction of the human mutant TTR gene and found systemic amyloidosis in them.[16] This paper describes the patterns of amyloid deposition obtained from our histochemical, immunohistochemical, and ultrastructural studies and their similarity to human FAP.

Materials and Methods

Production of Transgenic Mice

An inbred strain of mouse, C57BL/6, was chosen for DNA microinjection to minimize the possible influence of genetic background on amyloid deposition. A chromosomal DNA segment covering the entire sequence for the mutant TTR gene associated with type I FAP was cloned as described previously.[13] The 7.8-kilobase pair *StuI-EcoRI* fragment was constructed by ligating the promoter region of the mouse methallothionein-I (MT-I) gene to the entire structural gene of the human mutant TTR gene (MT-hMet30).[16]

Approximately 200 copies of these constructs (MT-hMet30) were microinjected into fertilized eggs of C57BL/6 mice according to the method described elsewhere.[17] Four of twelve mice derived from eggs microinjected with the MT-hMet30 gene integrated it, as revealed by Southern blot analysis. These transgenic offspring were used in the following studies and were kept in plastic cages in our laboratory according to the guidelines of the Ministry of Education. They were bred by brother × sister mating.

DNA Isolation and Southern Blot Analysis

When the mice were 4 weeks old, genomic DNAs were extracted from a piece of tail and used for Southern blot analysis to examine whether the MT-hMet30 gene had integrated into the mouse chromosome.[18]

Western Blot Analysis

To analyze the production of human TTR in the mice, blood was taken from each transgenic mouse before they were killed and the sera were analyzed by Western blot assay.

Preparation and Fixation of Tissues

Transgenic mice were killed using ether anesthesia at 3-month intervals up to 24 months after birth. At each time point, we examined two to six transgenic mice and excised the 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. For light microscopy, tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Paraffin sections were used for histochemistry. For immunohistochemistry, part of the tissues were fixed in periodite-lysine-paraformaldehyde (PLP) fixative for 4 hours and cut by a cryostat (Bright; Huntingdon, UK). Small tissue blocks were obtained from the heart, kidneys, small intestine, and sciatic nerve, fixed in chilled 2.5% glutaraldehyde in 0.1 mol/l (molar) cacodylate buffer for 60 minutes, and submitted to electron microscopy.

Light Microscopy and Histochemistry

For light microscopy, paraffin sections were stained with hematoxylin and eosin. Semithin sections were cut from Epon-embedded blocks for electron microscopy and stained with 0.05% toluidine blue. For histochemical demonstration of amyloid, paraffin sections were stained by the Congo red method and some were treated with potassium permanganate ($KMnO_4$) before Congo red staining according to Wright's method.[19] To detect the emerald green birefringence emitted from amyloid deposits, the Congo red-stained paraffin sections were observed under a polarization microscope.

Immunohistochemistry

For immunohistochemical demonstration of the major components in amyloid deposits, formalin-fixed paraffin sections or PLP-fixed cryostat sections were immunostained by the avidin-biotin complex (ABC) method using polyclonal and monoclonal antibodies. The antibodies were anti-human TTR (Behringwerke; Marburg, FRG), anti-human serum amyloid A (SAA) (Dako; Santa Barbara, CA), anti-human serum amyloid P component (SAP) (Dako), anti-mouse SAA (supplied by Prof. S. Migita, Cancer Research Institute, Kanazawa University, Japan), and anti-mouse SAP (Behring Diagnostics; La Jolla, CA).

Electron Microscopy

After glutaraldehyde fixation, tissue blocks were post-fixed in 2% osmium tetroxide for 60 minutes, dehydrated in a graded series of ethanols, and embedded in Epok-812 (Oken; Tokyo, Japan). The blocks were cut by an ultrotome Nova (LKB, Upssala, Sweden), stained with uranyl acetate and lead citrate, and observed with an H-300 or 12-A electron microscope (Hitachi; Tokyo, Japan).

Results

Transgenic Mice

Transgenic mice showed normal appearance and good development in all lines until age 12 months. After age 15 months, hair became coarse in two lines, but no muscular atrophy or gait disturbance was found. Southern blot analysis indicated that the copy numbers of the integrated MT-hMet30 genes varied from 2 to 30 per diploid genome. The concentrations of the human mutant TTR in the blood varied among the mice and ranged from 1 to 5 mg/dl, one tenth to one half of that in FAP patients (Figure 1). When the mice were killed, no inflammatory lesions, such as pulmonary abscess or parasitic infestation, were found in any of these mice.

Amyloid Deposition

Table 1 shows the occurrence and degree of amyloid deposition in various tissues of the transgenic mice. Amyloid deposition was first found in the small intestine, stomach, and renal glomeruli at 6 months, and it occurred invariably in the gastrointestinal tract, kidneys, heart, and thyroid at 12 months and thereafter.

In the gastrointestinal tract, amyloid deposits occurred predominantly in the mucosa of the small intestine, particularly the terminal ileum. Amyloid deposition there was marked in the lamina propria of the intestinal villi and submucosal layer, and it occurred in or around the walls of small blood vessels later (Figure 2a and b). Electron microscopically, amyloid deposits were observed to consist of clusters of amyloid fibrils approximately 7 to 10 nm wide that varied in length, findings consistent with human FAP[11,12] or other types of amyloidosis (Figure 3).[20] In the initial stage, amyloid deposits were observed in the propria mucosa at the top of the intestinal villi, particularly beneath the epithelial cells. Also they were found in the basal area of the lamina propria, in the muscularis mucosae, and in the submucosa. In the submucosa, amyloid deposition occurred in the connective tissue and around small blood vessels. In the advanced stage, diffuse amyloid deposition was found in the mucosa, muscularis mucosae, submucosa, and subserosa.

In the wall of the stomach, amyloid deposits first occurred in the lamina propria of the ridge and were found in the submucosa, particularly the nonglandular part. In the large intestine, amyloid deposited in the submucosa of the cecum and anal ring.

In the kidneys, amyloid deposits occurred exclusively in the glomeruli at the initial stage, later involving the renal interstitium. Figure 4 shows the degree of amyloid deposition in the glomeruli. At age 6 months, slight amyloid deposits first were detected in the mesangial areas of about 10% of glomeruli. At 18 months, most of the glomeruli were affected by increased amyloid deposition. After 21 months, nearly 100% of the glomeruli were obliterated completely by massive amyloid deposits (Figure 2c and d). Ultrastructurally amyloid fibrils first were detected in

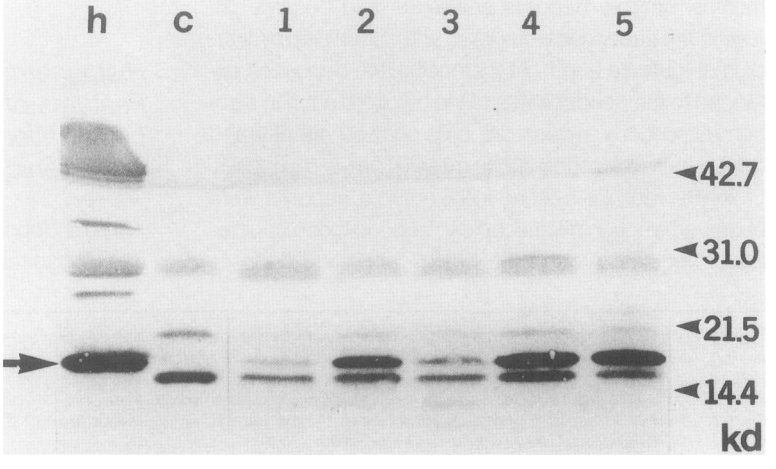


Figure 1. *Western blot analysis showing levels of expression of the MT-hMet30 gene in sera of transgenic mice. The size of the molecular weight in kilodaltons is indicated on the right of the panel. The arrow on the left indicates location of the human TTR monomer. Variable amounts of the human TTR were detected in the sera from all transgenic mice with amyloid deposits. Lane h, 0.5 μl of human serum; lane c, 1 μl of control mouse serum; lanes 1, 2, 3, 4, and 5, 1 μl of serum from each transgenic mouse at 12 months of age.*

Table 1. *Tissue Distribution of Amyloid Deposits in Transgenic Mice Carrying the MT-hMet30 Gene and Autopsy Cases of FAP[11] Determined Histochemically*

Organs	Transgenic mice: age examined (months)								Autopsy cases of FAP
	3	6	9	12	15	18	21	24	
Brain	−	−	−	−	−	−	−	−	−
Choroid plexus	−	−	−	−	−	−	−	−	+ +
Sciatic nerve	−	−	−	−	−	−	−	−	+ + +
Heart	−	−	−	+	+ +	+	+ +	+ + +	+ + +
Lung	−	−	−	−	−	−	±	±	±
Liver	−	−	−	−	−	−	±	±	±
Spleen	−	−	−	−	−	−	±	±	±
Kidney	−	+	−	+	+	+ +	+ + +	+ + +	+ + +
Pancreas	−	−	−	−	−	−	±	±	+
Thyroid gland	−	−	−	+	+	+ +	+ +	+ + +	+ + +
Stomach	−	+	−	+ +	+ +	+ +	+ +	+ + +	+
Intestine	−	+	−	+ +	+ +	+ + +	+ + +	+ + +	+
Lymph node	−	±	−	−	−	±	±	±	±

Amyloid deposits are absent, −; limited to the wall of small vessels, ±; observed in the wall of small vessels and their surrounding regions, +; moderate in the interstitium; + +; marked in the interstitium and parenchyma, + + +.
FAP, familial amyloidotic polyneuropathy.

the matrix around the mesangial cells or beneath the endothelial cells of glomerular capillaries. With age, the amount of amyloid fibrils increased and their clusters were observed in the lamina rara interna between the basal lamina and endothelial cells. At the advanced stage, clusters of amyloid fibrils were deposited massively in almost all parts of the mesangial matrix of glomeruli; the mesangial cells were swollen and the glomerular epithelial cells showed fusion of their foot processes (Figure 5). In the renal interstitium, amyloid deposition was found in the cortex after age 21 months but not in the medulla. Electron microscopically, amyloid deposition was observed to be particularly dense around the renal tubules.

In the cardiovascular system, amyloid deposits were marked. At age 12 months, slight, patchy amyloid deposition occurred in the subendocardial layer and in the superficial myocardium. Ultrastructurally clusters of amyloid fibrils in the superficial myocardium were observed initially around small blood vessels (Figure 6). After 18 months, amyloid deposits coalesced and became diffuse in the subendocardial layer and superficial myocardium (Figure 2e and f). At 24 months, the deposition extended to the deeper areas of the myocardium. On electron microscopy, myocardial cells showed atrophy and degenerative changes due to massive amyloid deposits.

In the vascular system, initial amyloid deposits were observed electron microscopically around blood capillaries or venules. Around the blood capillaries, deposits were observed beneath the basal lamina, extending to the perivascular region. Amyloid deposition in the arterial wall occurred mostly in the advanced stage, particularly in the adipose tissues and gastrointestinal tract. Also vascular amyloid deposition was found in the salivary glands, testes, lungs, and liver at age 15 months, and later in the pancreas, skeletal muscles, and splenic trabecles.

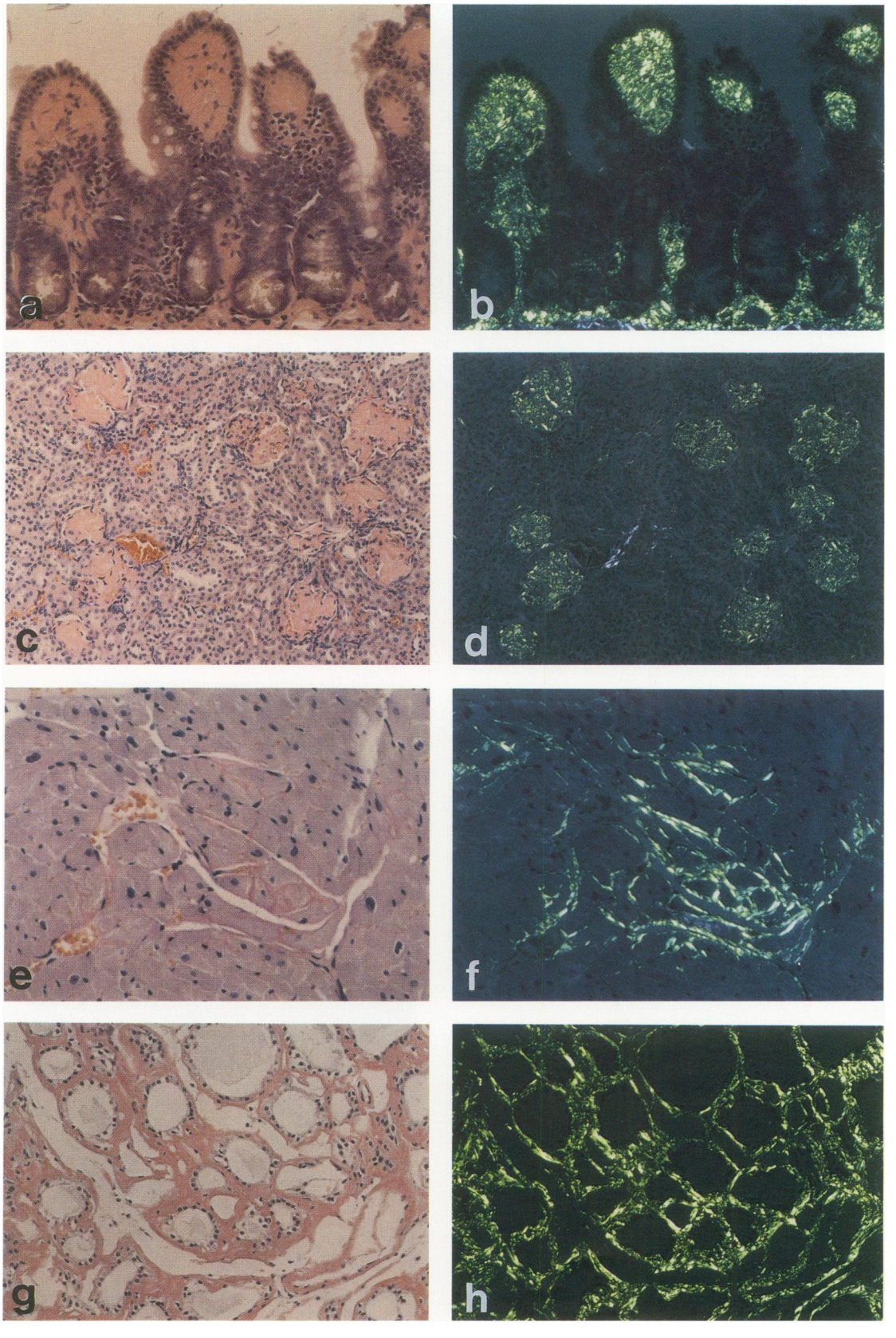
In the thyroid gland, amyloid deposits occurred around the interfollicular blood capillaries at 12 months, increased with age, and became prominent at 24 months. In the advanced stage, the thyroid follicles were compressed by marked interstitial amyloid deposition (Figure 2g and h).

In all the transgenic mice, no amyloid deposition was detected in the brain, choroid plexus, peripheral nerves, or in the hematopoietic tissues such as the bone marrow, spleen, liver, or lymph nodes. In the nontransgenic mice, no amyloid deposition was confirmed in any tissues examined up to age 24 months.

Histochemical and Immunohistochemical Features of Amyloid Deposits and Localization of Anti-human TTR in the Transgenic Mice

Amyloid deposits in the transgenic mice were stained with Congo red, showed resistance to treatment $KMnO_4$

Figure 2. *Light microscopic changes of principal organs in the transgenic mouse carrying the MT-hMet30 gene.* **a, b:** *Amyloid deposits of the terminal ileum at 12 months of age. Amyloid deposits were marked in the villous stroma and around the small vessels (×50).* **c, d:** *Amyloid deposits of the kidney at 24 months of age. All of the renal glomeruli were replaced by massive amyloid deposition (×25).* **e, f:** *Amyloid deposits of the heart at 18 months of age. Amyloid deposits were marked around the myocardial fibers (×60).* **g, h:** *Amyloid deposits of the thyroid gland at 24 months of age. Amyloid deposits were marked in the interstitium (×25). (***a, c, e,** *and* **g,** *Congo red and hematoxylin;* **b, d, f,** *and* **h,** *identical area viewed under polarizing light.)*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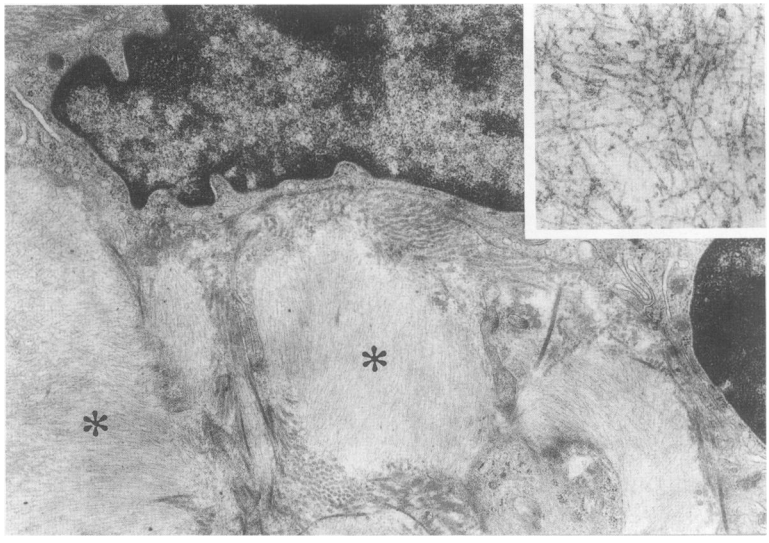

Figure 3. *Electron micrograph of submucosal tissue in the terminal ileum of the transgenic mouse at 12 months of age. Clusters of amyloid fibrils were found among collagen fibers (asterisk) in the extracellular space, but not in the cytoplasm of fibroblasts (×15,000). Inset: Higher magnification of amyloid fibrils. Amyloid fibrils measured approximately 7 to 10 nm in width (×40,000).*

by Wright's method,[18] and emitted an emerald-green birefringence under polarized light. By the ABC method, the amyloid deposits reacted with anti-human TTR (Figure 7a) and anti-mouse SAP (Figure 7b) antisera, but not with anti-mouse SAA, anti-human AA, or anti-human SAP antisera.

In the transgenic mice, liver cells, ductal epithelia of the salivary glands, pancreatic exocrine cells, epithelial cells of renal tubules at the proximal convulsion, myocardial cells, and part of the skeletal muscle cells showed immunoreactivity with anti-human TTR, but the epithelial cells of the choroid plexus were nonimmunoreactive.

The nontransgenic C57BL/6 mice demonstrated no immunoreactivity with anti-human TTR in any kind of cell.

Discussion

In this paper we demonstrated clearly that hMet30 could be deposited as amyloid fibrils in transgenic mice carrying the human mutant TTR gene (MT-hMet30). Amyloid deposition occurred predominantly in the intestinal mucosa, renal glomeruli, myocardium, small vascular walls, and thyroid. With age, amyloid deposition became marked and was found in various other organs and tissues, except for the nervous tissues. The possibility of the development of age-associated amyloid deposition in transgenic mice can be ruled out for the reasons described below.

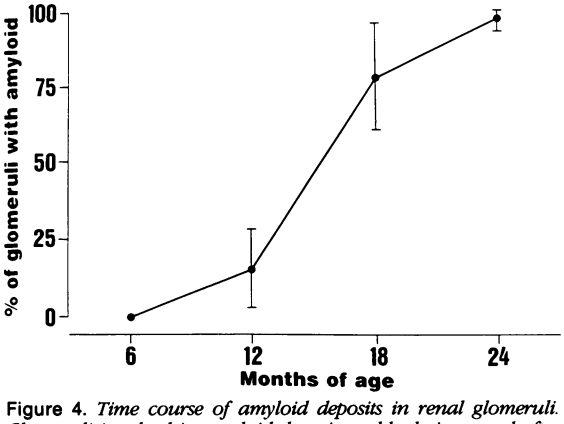

Figure 4. *Time course of amyloid deposits in renal glomeruli. Glomeruli involved in amyloid deposits suddenly increased after age 12 months. Renal amyloid deposition was expressed as the rate of affected glomeruli to total glomeruli on a section. Each point represents mean ± SD.*

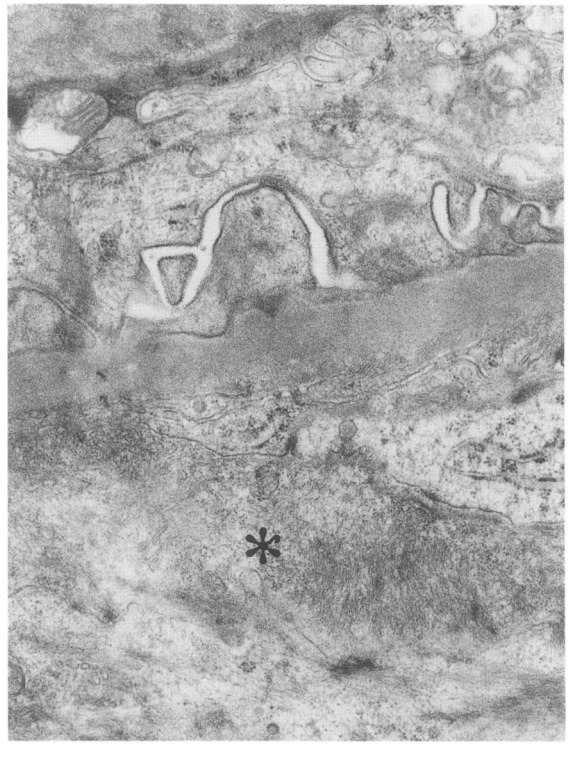

Figure 5. *Electron micrograph of a renal glomerulus in the transgenic mouse at age 21 months. Amyloid fibrils (asterisk) were found in the mesangial matrix (×17,000).*

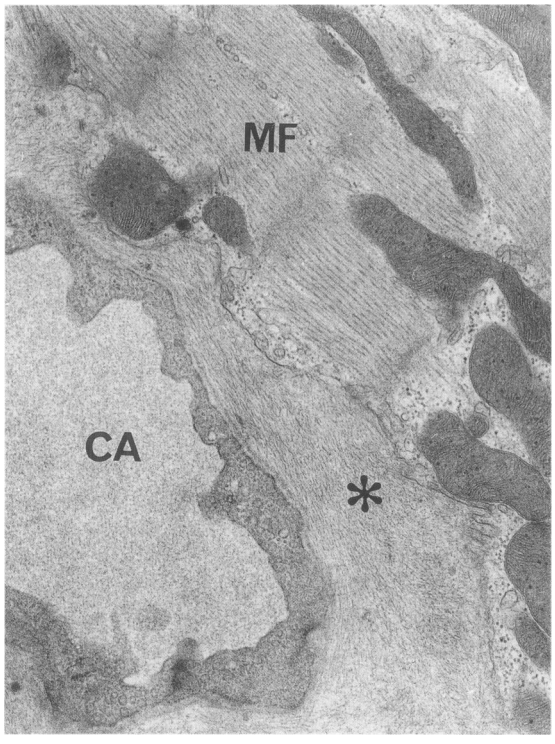

Figure 6. *Electron micrograph of the myocardium in the transgenic mouse at age 12 months. Amyloid fibrils (asterisk) were observed around small blood vessels (×17,000). CA, capillary lumen; MF, myocardial fibers.*

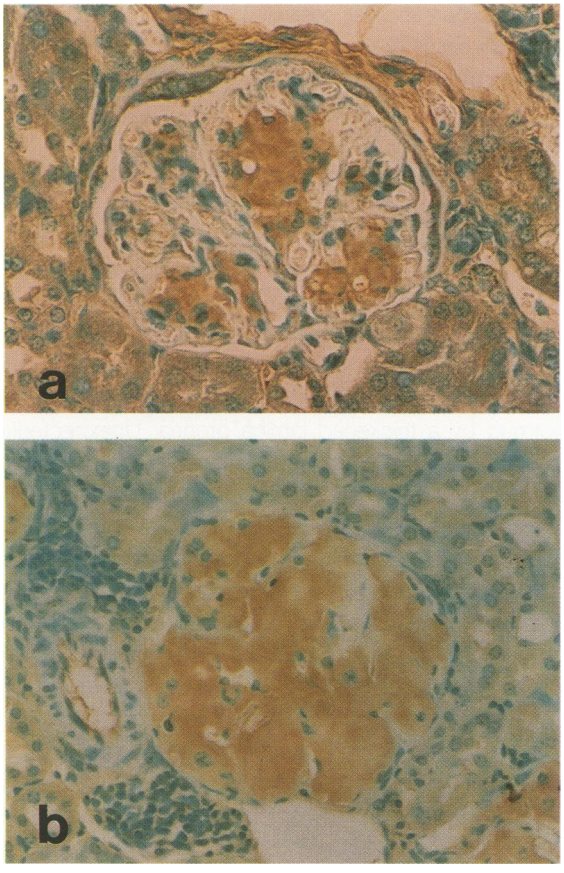

Figure 7. *Amyloid deposits in renal glomeruli showing positive staining with anti-human TTR* **(a)** *and anti-mouse SAP* **(b)** *antisera (ABC method, ×100).*

Senile amyloidosis is known to occur spontaneously in various strains of mice, including C57BL mice, and to involve the spleen, liver, heart, kidneys, or gastrointestinal tract.[21–26] In senile amyloidosis, renal amyloid deposition occurs mainly in the renal papilla or interstitium, and glomerular involvement is slight or absent.[21–25] In senile amyloidosis of C57BL mice, amyloid deposition was reported to occur in the renal papilla in 50% of the animals at age 18 months and to involve the spleen, particularly around the white splenic pulp; however glomerular amyloid involvement is slight.[21] By contrast, in transgenic mice amyloid deposition is prominent in the renal glomeruli but slight or absent in the spleen or liver. Compared to the development of spontaneous senile amyloidosis in mice,[22] in transgenic mice amyloid deposition occurs at least 6 months earlier. Another definitive difference involves amyloid precursor protein. Although the amyloid precursor protein in human senile amyloidosis is also TTR,[27,28] it has not been determined yet in mice. Non-AA[24] or apoprotein A II (Pro^5-Gln)[29] have been mentioned. In transgenic mice, however, we demonstrated clearly that the amyloid precursor protein is human variant TTR. In the following section, four subjects will be discussed in relation to the pathologic similarity and dissimilarity between the transgenic mice and human FAP autopsy cases.

First the pathologic changes of the transgenic mice correlate well with those reported in previous pathologic studies of FAP.[30–32] Some of the present authors recently reported in nine autopsy cases of type I FAP that amyloid deposition occurred predominantly in the cardiovascular system, peripheral and autonomic nervous system, choroid plexus, kidneys, thyroid, and gastrointestinal tract.[11,12] However it was slight or minimal in the pancreas, bone marrow, spleen, lymph nodes, or liver, and absent from the brain parenchyma. The major sites and pattern of amyloid deposition in the transgenic mice are similar to these human autopsy cases, except for the peripheral and autonomic nervous tissues. In the mice, cardiac amyloid deposition initially occurs in the subendocardial layer and in the superficial areas of myocardium, and then it extends deeper into the cardiac wall. This pattern is similar to that in the heart of human cases confirmed by our pathologic study.[11,12] In the kidneys of transgenic mice, amyloid deposition first occurred in the mesangial areas of the glomeruli. The number of involved glomeruli and the grade of glomerular involvement increased with age and amyloid deposition was found around the renal tubules in advanced age. These find-

ings also are almost consistent with those reported in autopsy cases of type 1 FAP.[11,12,31] Amyloid deposition in the thyroid gland of transgenic mice is prominent and its pattern closely resembles that of human cases of FAP.[11,12]

On the other hand, in the gastrointestinal tract of transgenic mice, amyloid deposition is more prominent than in the human autopsy cases of FAP and occurs predominantly in the mucosa of the terminal ileum, in the gastric submucosa at the ridge of the nonglandular part, and around the anal ring. In the mouse, the stomach is divided into two parts, the left nonglandular side and the right glandular side, by the U-shaped ridge. This ridge is formed by a thickened lamina propria. Unexpectedly amyloid deposition is more prominent in the nonglandular side, including the ridge, than in the glandular side. A similar pattern of amyloid deposition was observed around the anal ring: it was more prominent under the squamous epithelium than the glandular epithelium. It is interesting to note that such a peculiar pattern of amyloid involvement in the stomach of transgenic mice is also found in spontaneous senile amyloidosis of mice.[22,23] These results suggest that the difference of amyloid deposition in the alimentary tract between transgenic mice and FAP patients is due to the difference in anatomic structure and that such microenvironments, including the fine anatomic structure or local blood flow, are involved in the amyloid deposition.

One important question is how the amyloid deposition initiates and proceeds in the early stage of FAP. So far there has been no clear data on this subject, partly because detailed pathologic tissue analysis is only possible at autopsy. By that time a large amount of amyloid usually has accumulated in many tissues, as discussed earlier. The main target tissues for amyloid deposition in FAP patients are not the liver and the choroid plexus where the hMet30 gene is expressed. This suggests that hMet30 is transported *via* blood or lymphatic circulation into tissues to deposit as a major amyloid component *in loco*. Transgenic mice presented here made it possible to analyze the initial stage of amyloid deposition. As expected, amyloid deposition initiates around blood capillaries and extends to the perivascular region. From this point of view, it is reasonable that amyloid deposition is most prominent in tissues with a rich blood supply, such as kidneys, heart, and thyroid glands. Although the MT-hMet30 gene is expressed in a variety of tissues, the distribution pattern of amyloid deposition is not related to the tissue specificity of the MT-hMet30 gene expression.[33] This finding is consistent with the notion that amyloid fibrils are derived from the blood.

It is a well-known fact that SAP is a minor component of all types of amyloidosis.[34–36] Although its role in amyloid deposition has not been elucidated clearly yet, SAP is known to exist in the sera of various mammalian species and to have a binding capacity to specific ligands in the presence of calcium.[37] In our transgenic mice, a concomitant deposition of mouse SAP was demonstrated immunohistochemically in almost all amyloid deposits. These results suggest that the pathologic role of mouse SAP is the same as that of human SAP, although SAP is a typical acute-phase reactant in mice but not in humans. Further investigation is needed to determine whether SAP is involved in the initiation or acceleration of amyloid deposition.

Although the most striking pathologic feature of FAP is amyloid deposition in the peripheral and autonomic nervous tissues, no amyloid deposition was observed in the nervous tissues of the transgenic mice examined up to age 24 months. In all the autopsy cases of FAP, we also observed marked amyloid deposition in the choroid plexus and substantiated the histochemical localization of human TTR in the choroid plexus epithelial cells.[11,12] The choroid plexus and liver are considered the major sites of human TTR production.[38] If we consider the fact that the peripheral nerve is open-ended with respect to the subarachnoid space,[39] we can speculate that the production of human variant TTR by the choroid plexus epithelia is related to amyloid deposition in the peripheral nerve tissues. In the transgenic mice, however, we demonstrated immunohistochemically little or no production of human TTR in the epithelial cells of the choroid plexus. This could be the reason for the absence of amyloid deposition there and in the peripheral and autonomic nervous system in transgenic mice. A second possibility is the low-level expression of the hMet30 gene in these transgenic mice. To provide more definite evidence for this speculation, we are making mice transgenic that produce a high level of hMet30 in the epithelial cells of the choroid plexus. A third possibility lies in the characteristic metabolic features of the mouse itself. For example, a mutant mouse deficient for the enzyme in purine metabolism, hypoxanthine-guanine phosphoribosyl transferase (HPRT), was produced;[40,41] however no symptoms have developed yet in this mouse. In humans HPRT deficiency causes severe neurologic disorder and hyperuricemia (Lesch-Nyhan syndrome). Interestingly degenerative changes in the peripheral nerve tissues before amyloid deposition were reported in FAP patients.[11,12] Thus another intrinsic factor(s) may be involved in amyloid deposition in the nervous tissues of FAP.

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