Original Article

Human Chymase Expression in a Mice Induces Mild Hypertension with Left Ventricular Hypertrophy

Takafumi KOGA, Hidenori URATA^{*}, Yukiko INOUE^{*}, Takafumi HOSHINO, Takehiro OKAMOTO, Akira MATSUNAGA, Misao SUZUKI^{**}, Junichi MIYAZAKI^{***}, Munehito IDEISHI, Kikuo ARAKAWA, and Keijiro SAKU

A number of *in vitro* studies have suggested potential pathophysiological roles of human (h-) chymase. However, the lack of an appropriate animal model has left the *in vivo* roles of chymase unclear. To approach this problem, a transgenic mouse (TGM) model carrying the h-chymase gene was established. The h-chymase cDNA transgene was constructed with the chicken actin promoter and cytomegalovirus immediate early gene enhancer, and injected into mouse oocytes. Homozygous mice with a high copy number of the h-chymase gene suffered from intrauterine death. In three heterozygous TGM lines, h-chymase transgene expression was detected in entire organs, including the heart, vessels, skin, liver, lung, and brain. The h-chymase immunoreactivity was localized in the extracellular matrices of each organ, especially on the basement membranes of vessels. Aortic and hepatic chymase-dependent angiotensin II formations were significantly higher than those in the wild-type littermates. Three independent TGM lines showed the same phenotypic changes: elevation of blood pressure, left ventricular hypertrophy, emaciation with reduction in the lipid tissue, leukocytosis, and oligotrichia. The angiotensin II subtype 1 (AT₁) receptor antagonist valsartan suppressed the elevated blood pressure completely and left ventricular hypertrophy incompletely, but did not affect the other phenotypes. These data suggested that *in vivo* expression of h-chymase caused mild hypertension (AT₁ receptor-dependent) with left ventricular hypertrophy (partially AT₁ receptor-dependent), and also chronic inflammatory changes (AT₁ receptor-independent). (*Hypertens Res* 2003; 26: 759–768)

Key Words: chymase, transgenic mouse, cardiac hypertrophy, oligotrichia, leukocytosis

Introduction

Mast cell chymase is a potent chymotrypsin-like angiotensin (Ang) II-forming serine proteinase that is mainly produced by mast cells in vessels (1, 2) and in the heart (3) and that can convert big-endothelins to endothelins (1–31) (4). Chymase-dependent Ang II formation has been shown in the intact hamster, where Ang II converting enzyme (ACE) in-

hibitor could not prevent the pressor response of [Pro¹¹,D-Ala¹²]Ang I, which produces Ang II by incubation with purified chymase (5). In a study by Nishimura *et al.*, chymase-dependent Ang II formation was demonstrated in the intact hamster, and an ACE inhibitor could not prevent the pressor response of a modified Ang I ([Pro¹¹,D-Ala¹²]Ang I), which produces Ang II by incubation with purified chymase (5). In addition to its involvement in blood pressure regulation, Ang II plays a role in the stimulation of vascular smooth muscle

From the Department of Internal Medicine, Fukuoka University, School of Medicine, Fukuoka, Japan, ^{*} Department of Internal Medicine, Fukuoka University Chikushi Hospital, Chikushino, Japan, ^{**} Institute of Molecular Embryology and Genetics, Kumamoto University Medical School, Kumamoto, Japan, and ^{***} Department of Nutrition and Physiological Chemistry, Osaka University Medical School, Suita, Japan.

This study was supported by Grants-in-Aid for Scientific Research (B, 13470153; C, 10670690) from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

Address for Reprints: Hidenori Urata, M.D., Department of Internal Medicine, Fukuoka University Chikushi Hospital, 377-1 Zokumyoin, Chikushino 818-8502, Japan. E-mail: uratah@fukuoka-u.ac.jp

Received January 14, 2003; Accepted in revised form May 19, 2003.

Fig. 1. Schematic illustration of the human chymase transgene structure. The chymase transgene was constructed as described in Methods.

cell (SMC) growth and proliferation, vascular remodeling, and cardiac hypertrophy (6).

It has been suggested that chymase plays a significant role in cardiovascular diseases (7). For example, chymase mRNA and activity have been shown to be increased in the human heart after myocardial infarction and formation of atherosclerotic lesions (8, 9). In addition, chymase inhibitors have been shown to improve the survival rate after myocardial infarction in a hamster experimental model (10, 11), to significantly decrease atherosclerotic development in hamsters (12), and to reduce neointima formation in dog vein grafts (13).

In addition to the Ang II-forming activity (AIIFA) by h-chymase, the involvement of mammalian chymase in local inflammation has been suggested by several studies *in vitro*. Isolated chymase activates a potent proinflammatory cytokine, interleukin (IL)-1 β (14), degrades IL-4 (15), and cleaves a membrane-bound stem cell factor, provoking its release from cells (16). Isolated dog chymase is known to enhance histamine-induced vascular leakage in the skin of dogs (17). Human mast cell chymase also induces neutrophil and eosinophil recruitment in the skin of guinea-pigs as well as in the peritoneum of mice (18), suggesting a potential role of h-chymase in inflammatory cell migration. The participation of chymase in tissue matrix remodeling is also supported by a number of *in vitro* studies demonstrating that mammalian chymases activate procollagenase, degrade extracellular matrix (19), proteolytically liberate matrix-bound transforming growth factor (TGF)- β 1 (20), and process procollagen to form collagen fibrils (21). However, these results were obtained either by *in vitro* study or temporary treatment by recombinant or isolated, but not intrinsic, chymases.

Although the chymase selective substrate [Pro¹¹,D-Ala¹²]Ang I has been shown to cause a hypertensive reaction in hamsters (5) and baboons (22), the exact role of h-chymase in hypertension has not been clarified. In the present study, in order to test whether genetic introduction of the h-chymase gene causes hypertension or inflammation-related phenotypes, three independent transgenic mice (TGM) lines expressing the h-chymase gene were established and their phenotypes were analyzed.

Methods

Preparation of the h-Chymase Transgene

The isolated h-chymase cDNA (23) was subcloned into pCAGGS, a pKCR-3-based plasmid which contains a CMV-IE fragment and it lies upstream of the chicken β actin promoter (Fig. 1) (24). The h-chymase sequences were confirmed by the dideoxy sequencing method. The pCAGGS-h-chymase plasmid DNA was digested with SalI and KpnI, and the isolated h-chymase transgene fragment was gel-purified and used for microinjection (25).

Production of Transgenic Mice (TGM)

The C57BL/6 (B6) mouse strain was used to produce h-chymase TGM. Fertilized eggs were collected from the oviducts of superovulated B6 females that had been mated to males of the same genetic background. Several hundred molecules of the h-chymase transgene fragments were microinjected into the fertilized eggs of B6 mice according to the method previously described (26).

Newborn mice were screened for integration of the transgene by polymerase chain reaction (PCR) of tail DNA (1 ng) with the primers 5'-ACACTCCCCTTCCCATCACA-3' and 3'-GGGCTTTGCATCCGACCGTC-5', based on the h-chymase cDNA sequence. PCR was carried out in 1 $\times$ PCR buffer (50 mmol/l KCl, 1.5 mmol/l MgCl₂, 10 mmol/l Tris-HCl, pH 8.3), 200 μ mol/l dNTP, and 2.5 U of Taq polymerase. After the 30th cycling reaction of 96°C for 30 s (denaturing), 58°C for 30 s (annealing), and 72°C for 1 min (extension), the targeted sequence (297 bp) that is specific to the transgene was amplified. The presence of the transgene was also confirmed by Southern blotting of tail DNA using an h-chymase full-length cDNA probe. The inheritance pattern was compatible with a single autosomal integration site. Eight of 75 mice were positive for the h-chymase gene. Their offspring and the TGM and control mice were housed together under condition of controlled light (12 h light/12 h dark) and temperature (23 $\pm$ 2°C) with free access to food and water. The investigation conformed with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). All animal experiments were approved by our institutional committee and were performed according to its guidelines.

Measurement for Food Consumption

Adult female mice of TGM ($n = 6$) and control (wild type) mice ($n = 6$) were used in this experiment. Daily diet intake was determined by weighing the food remaining in each cage on the next day and replacing it with fresh preweighed diet. Diet intake was calculated by subtracting the weight of

the uneaten diet from the preweighed amount added to the cage. Spillage of diet was not measured.

Measurement of Blood Pressure and Rectal Temperature

Blood pressure was measured in both TGM and their control-group littermates at 12 weeks of age. The systolic blood pressure (SBP) was measured by a programmable sphygmomanometer (MK-2000; Muromachi, Tokyo, Japan) using the tail-cuff method. Unanaesthetized mice were introduced into a small holder during measurement at room temperature. The effect of the AT₁ receptor antagonist valsartan (Novartis Pharma, Basal, Switzerland) on blood pressure was also tested. The TGM group was separated into two groups, one that received, one that did not receive valsartan (14 mg/kg/day) in their drinking water. Measurement of the rectum temperature was performed using a Thermaleart TH-5 (Physitemp Instruments Inc., Clifton, USA).

Blood Cell Count

Complete blood cell counting was obtained by retro-orbital venous plexus sampling in polypropylene tubes containing EDTA. The number of blood cells was determined using an automatic cell counter (Celltac, MEK-6158; Nihon Kohden, Tokyo, Japan) and differential counts on zifiquick stain smears.

Preparation of Total RNA and Blot Hybridization with Chymase Probes

Total RNA was isolated from each tissue of TGM and their non-transgenic littermates by extraction with acid guanidinium thiocyanate, phenol, and chloroform. Total RNA from arterial walls and other tissues was subjected to electrophoresis in a formaldehyde (1%) gel and transferred to a nylon membrane. The filter was hybridized with a probe of the full-length h-chymase cDNA labeled by random primer methods with $\alpha^{32}\text{P}$ -dCTP.

Immunoblot Analysis

Each tissue (≈ 50 mg) was homogenized in 20 mmol/l Tris-HCl buffer at pH 7.4 and centrifuged at $40,000 \times g$ for 20 min. These homogenization and centrifugation steps were repeated twice. The pellet was resuspended in 20 mmol/l Tris-HCl buffer (pH 8.0) containing 2.0 mol/l potassium chloride and 1% Triton X-100 and incubated with gentle shaking at 4°C for 1 h. The mixture was centrifuged as described above, and the resultant supernatant was incubated with 50 μl of a soybean trypsin inhibitor immobilized-agarose suspension. After extensive washing with 20 mmol/l Tris-HCl buffer (pH 8.0), chymase bound to the gel was solubilized in 60 μl of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample loading buffer at 22°C

overnight. The solubilized protein in this loading buffer and pure h-chymase were electrophoresed on 14% sodium dodecyl sulfate-polyacrylamide gel, and the proteins were then transferred to a polyvinylidene difluoride microporous membrane using a semidry blotting system. After blocking and washing of the membrane, an anti-h-chymase monoclonal antibody solution (diluted 1:1,000) was added to the membrane and incubated overnight. After washing, a goat anti-mouse IgG solution (diluted 1:3,000) was added to the membrane, followed by incubation for 1 h at room temperature. The membrane was washed three times and then placed on a piece of Saran Wrap on a level surface. The substrate solution (10 ml of chemiluminescent substrate per 200 cm²) was added to the membrane and incubated for 5 min. The membrane was drained of excess liquid and exposed to X-ray or instant film at room temperature.

Preparation of Tissue Particle Fractions for the Assay of AIIFA

After being thawed and weighed, tissues were dissected into small pieces. They were placed in 50 mmol/l H₂PO₄ buffer (0.5 g/5 ml), pH 7.4, at 4°C and homogenized using a polytron homogenizer (Kinematica GMPH, Cincinnati, USA) at 9,000 rpm for 15 s at 4°C. The homogenate was centrifuged at $30,000 \times g$ for 20 min at 4°C. The supernatants were discarded, the pellets were resuspended in the same buffer, and the homogenization and centrifugation were repeated once. The final tissue pellets were resuspended in 50 mmol/l H₂PO₄ buffer, pH 7.4, containing 100 mmol/l NaCl and 10 mmol/l MgCl₂ using a hand-driven glass/glass homogenizer. The protein concentration of the particulate fraction was measured using Protein Assay Reagent (Pierce, Rockford, USA).

Assessment of Ang II Formation in Particulate Fraction

Assessment of Ang II formation in the tissue particulate fraction of the TGM and control mice was performed according to the method described previously (2). Briefly, a particulate fraction prepared as described above was incubated with synthetic Ang I (200 $\mu\text{mol/l}$) with or without inhibitors at 37°C for the appropriate time period. The formed Ang II was analyzed by reverse-phase high-performance liquid chromatography (HPLC; Shimadzu, Kyoto, Japan) using a C₁₈ reverse-phase HPLC column (2.2 $\times$ 25 cm; Vydac, Hesperia, USA) with a 15-min linear acetonitrile gradient (3% to 13%) in 25 mmol/l triethylamine-phosphate buffer, pH 3.0, at a flow rate of 2 ml/min. Ang II-forming activities were expressed as nmol of Ang II formed per milligram of protein. Chymostatin-inhibitable and aprotinin-insensitive Ang II formation were expressed as chymase-dependent Ang II-forming activity. All analyses for each sample were performed in duplicate, and the reproducibility and quality of all data were confirmed before the statistical analysis. The inter-assay and intra-assay coefficients of variation of this assay were 8.6%

Fig. 2. Representative Northern blot (a) and Western blot (b) analyses of several organs from h-chymase TGM. Total RNAs (a) and homogenates (b) prepared from various tissues of 10 week-old h-chymase TGM were analyzed as described in the Methods section. Lane numbers indicate different organs as follows: 1, muscle; 2, liver; 3, kidney; 4, lung; 5, brain; 6, stomach; 7, heart; 8, aorta in a and skin in b; respectively. T, TGM; C, control mice; Kd, kilodalton.

($n = 12$) and 5.1% ($n = 10$), respectively.

Measurement of Plasma Ang I, Ang II, Free T4, and Interleukin (IL)-1

Blood samples from TGM and control mice were collected into ice-cold microcentrifuge tubes containing 1 mmol/l EDTA, which were then immediately centrifuged in order to isolate the plasma fraction. Plasma Ang I and Ang II were measured by the method previously described (27). In brief, peptides in hamster plasma were extracted by ethanol precipitation and Sep-Pak purification. The residues containing Ang I or II were separated by a C_{18} reverse-phase HPLC column and the eluting peaks corresponding to authentic standard Ang I or II were collected to measure each peptide concentration by radioimmunoassay. Thyroid hormones in plasma were measured by a radioimmunoassay (28). IL-1 β in mouse plasma was measured by an immunoassay kit (Bio

Source International, Inc., Cmarillo, USA).

Immunohistochemical Studies

For pathological analysis, tissues were fixed with 10% formalin and embedded in paraffin wax, and then sections were stained with hematoxylin and eosin. Each tissue was immunohistochemically stained by the avidin-biotin alkaline phosphatase method. The paraffin-embedded sections were deparaffinized in xylene and dehydrated in graded series of ethanol solutions. Sections were then rinsed 3 times with a washing solution (50 mmol/l Tris-HCl buffer containing 145 mmol/l NaCl, pH 7.4) for 5 min at room temperature. After sections were incubated for 5 min in a blocking solution containing 10% normal nonimmune serum (horse or goat) from species from which the secondary antibody was obtained, sections were incubated for 1 h with the primary antibody (anti-h-chymase polyclonal antibody 50 μ g/ml) at room temperature. After being rinsed with the washing solution, sections were incubated with biotin-labeled secondary antibody (goat anti-rabbit immunoglobulin) for 30 min at room temperature and washed 3 times in the washing solution. Sections were applied to peroxidase-conjugated avidin for 30 min at room temperature. After being rinsed 3 times, sections were incubated in a freshly prepared 0.2 mol/l Tris buffer, pH 8.5, containing 0.5 mmol/l naphthol AS-BI-phosphoric acid, 0.27 mmol/l azotized new fuchsin, and 1 mmol/l levamisole. The staining was observed to be adequate (15 to 30 min). Sections were fixed in 10% buffered formalin for 10 min, counterstained with hematoxylin, and mounted. Negative controls were prepared by replacing the primary antibodies with preimmune rabbit IgG.

Inflammatory cell infiltration was quantitatively evaluated using antisera for leukocyte surface markers—LCA for lymphocytes and CD68 for macrophages (Dako; Dakocytomation A/S, Glostrup, Denmark)—as described above, and by direct cell counting for polymorphonuclear leukocytes. For each section, photomicrographs (100-fold magnification) of 20 randomly selected fields were obtained from the intermediate layers of the myocardium. LCA-positive cells, CD68-positive cells, and polymorphonuclear cells were counted and the number of inflammatory cells per unit area (1 mm²) was calculated.

Statistical Analysis

All values are expressed as the mean $\pm$ SD. Student's *t*-test was used for the statistical comparison of groups showing normal distribution, and one-way ANOVA was used for groups of unequal distribution. Values of $p < 0.05$ were considered statistically significant.

Table 1. Comparison of Various Parameters between Littermate Controls and h-Chymase Transgenic Mice (TGM)

Parameters	Control	(n)	TGM	(n)
Body weight (g)				
Male	26.6 ± 2.1	(6)	22.1 ± 2.1*	(7)
Female	20.1 ± 1.0	(7)	17.2 ± 1.6*	(7)
Food intake (g/mouse/day)	2.57 ± 0.23	(6)	3.77 ± 0.36*	(6)
Histological analysis for heart				
Myocyte size (μm)	33 ± 6	(6)	40 ± 5	(8)
Fibrotic area (% vs. total area)	3.2 ± 0.7	(6)	4.1 ± 0.5	(8)
Leukocyte number (/mm ²)	11.2 ± 2.1	(6)	24.5 ± 3.6*	(8)
Chymase dAIIFA (pmol/min/mg protein)				
Aorta	7.7 ± 3.1	(6)	25.0 ± 6.7*	(8)
Liver	22.2 ± 5.1	(6)	67.8 ± 32.6*	(6)
Plasma hormones				
Free T3 (pg/ml)	3.31 ± 0.58	(7)	3.20 ± 0.13	(5)
Angiotensin I (pg/ml)	3.03 ± 1.28	(8)	2.68 ± 1.30	(6)
Angiotensin II (pg/ml)	19.1 ± 15.5	(8)	18.5 ± 12.8	(6)
Interleukin-1β (pg/ml)	11.4 ± 2.9	(8)	47.1 ± 13.2*	(8)

SBP, systolic blood pressure; dAIIFA, dependent angiotensin II-forming activity; Free T3, active thyroid hormone; *n*, total number of mouse; **p* < 0.05 vs. control.

Results

Production and Establishment of the TGM Line

Eight lines of TGM were obtained. Two lines of the mice, lines 42 and 69, showed high copy numbers of the transgene, as estimated by Southern blot analysis using the tail DNA (data not shown). Line 69 had the highest copy number of the transgene. In the lines 42 and 69, the homozygous mice were suffered from intrauterine death. Although we established homozygous TGM in line 71, it was difficult to maintain homozygosity due to the low fertility. The heterozygous mice of lines 42 and 69 and the homozygous mice of line 71 showed similar phenotypic changes, as described below. Northern blot analysis using a h-chymase cDNA probe demonstrated that h-chymase mRNA was found in various tissues such as the heart, aorta, muscle, brain, liver, and kidney from TGM (Fig. 2a). In contrast, mRNA of the transgene was not detectable in any organs from the non-transgenic controls.

Western blot analyses of various tissues showed high levels of h-chymase immunoreactivity in the heart, muscle, stomach, and skin, and low levels in the liver (Fig. 2b). In addition, the 34-kDa immunoreactive bands corresponded to the molecular size of recombinant h-chymase.

AIIFA in Various Organs of TGM and Wild-Type Mice

Tissue AIIFA levels *in vitro* were determined in several organs. Aortic and hepatic chymase activities in TGM were significantly higher than those in the control mice (Table 1).

On the other hand, there was no significant difference in plasma Ang I or II levels between the TGM and control mice (Table 1).

Immunohistochemical Analyses of h-Chymase in TGM and Wild-Type Mice

Immunohistochemical analysis with anti-h-chymase antibody revealed that the majority of chymase immunoreactivity occurred in regions of the extracellular matrix such as the aortic adventitia and medial elastic fiber (arrows in Fig. 3d), and the basal membrane of both the bronchus and cerebral choroid plexus (data not shown). On the other hand, in control mice, there were no apparent immunohistochemical signals for h-chymase (Fig. 3a).

Phenotypic Changes

All phenotypic changes were examined in male and female TGM and there were no significant differences between the sexes in any of these changes except body weight (Table 1). Therefore, most of the following analyses were performed in female mice.

SBP and Left Ventricular Hypertrophy

SBP of TGM at week 12 was significantly higher than that of the littermate control mice (Fig. 4a, 102 ± 3 vs. 130 ± 3 mmHg, *n* = 8 each, *p* < 0.01), and a significant difference in blood pressure appeared from week 5. In addition, treatment with an Ang II type 1 (AT₁) receptor antagonist significantly reduced blood pressure to the level in the wild-type mice

Fig. 3. Representative aortic sections of wild-type (a, c) and h-chymase TGM (b, d) immunohistochemically stained with anti-h-chymase antisera. Photographs c and d are larger magnifications of a and b, respectively. Arrows in d indicate positive immunoreactivity (red color) in the adventitia, vs. a lack of positive immunoreactivity in wild-type mice (a, c). Bars represent 100 μ m in a and b, and 25 μ m in c and d.

Fig. 4. Comparison of systolic blood pressure (SBP; a) and the ratio of heart weight to body weight (HW/BW; b). The effects of the AT₁ receptor antagonist (ARB) on both parameters were examined in wild-type (Wild) and h-chymase transgenic mice (TGM). Both SBP and HW/BW were significantly elevated in TGM, and treatment with the AT₁ receptor antagonist significantly reduced both parameters.

(Fig. 4a, 97 ± 5 mmHg, $n = 8$, $p < 0.01$). At week 12, the ratio of the left ventricular weight to body weight of TGM was significantly greater than that of control mice (Fig. 4b,

5.83 ± 0.19 vs. 4.18 ± 0.13 , $n = 8$ each, $p < 0.05$), whereas the treatment with AT₁ receptor antagonist significantly reduced left ventricular hypertrophy without changing body weight

Fig. 5. Macroscopic (a) phenotypic comparison of wild-type (left) and h-chymase TGM (right) and a representative back skin section stained with hematoxylin and eosin of wild-type (b) and h-chymase TGM (c). h-chymase TGM mice at 10 weeks of age had a slightly smaller body size, as well as emaciation and oligotrichia, compared to the wild-type mice. The area set apart by arrows shows the thickness of skin fat. Note that the skin fat was reduced only in TGM (c). Bars represent 300 μm .

Fig. 6. Time-course changes in the body weight of wild-type (open circles) and h-chymase TGM (closed circles). There was a significant difference in body weight, but no growth retardation. * $p < 0.05$ vs. wild mice. $n = 6$ in each group.

(Fig. 4b, 5.19 ± 0.19 , $n = 6$, $p < 0.05$ vs. TGM and control). Both the inner diameters of the transverse section of myocytes and the cardiac fibrosis area were slightly increased, but these increases were not statistically significant (Table 1). Inflammatory cell infiltration into the interstitial area of the hearts of TGM was significantly greater than that of the control hearts (Table 1).

Other phenotypic changes in TGM were emaciation (Fig. 5a) due to a reduction in skin (Fig. 5b, c) and visceral fat, and oligotrichia (Fig. 5a). These were common phenotypes in the heterozygous mice with high copy number in either sex. This phenotypic change was therefore used as a positive marker to discriminate TGM from non-TGM.

A significant reduction of body weight was observed at week 4 and thereafter (Fig. 6). The mean body weight of

Fig. 7. Comparison of white blood cell density between wild-type mice (Wild; $n = 10$) and h-chymase TGM ($n = 8$). Closed column, granulocyte population; open column, non-granulocyte population. * $p < 0.01$ vs. control; ** $p < 0.05$ vs. control non-granulocytes.

TGM at week 12 was significantly lower than that of the littermate controls (Table 1). Daily diet intake in TGM was higher than that of the controls (Table 1). There were no significant differences in plasma free active thyroid hormone (T3) levels between TGM and control mice (Table 1).

Peripheral blood cells with leukocyte sub-populations were quantitated. An approximately 1.8-fold elevation of total leukocyte counts in TGM at week 10 was found compared with the littermate controls (Fig. 7, $13,275 \pm 1,575$ vs. $7,425 \pm 1,237/\mu\text{L}$, $n = 6$ each, $p < 0.01$). A similar leukocy-

toxis was observed in male TGM (data not shown). Similar results were confirmed in three independent TGM lines. Leukocytosis in TGM still existed at week 24 (data not shown).

The rectal temperature of wild-type mice at week 12 was $37.6 \pm 0.1^\circ\text{C}$ ($n = 5$) and that of TGM was $38.1 \pm 0.1^\circ\text{C}$ ($n = 5$, $p < 0.05$).

Discussion

The present study suggested that the transgene expression of h-chymase was associated with basal phenotypic changes such as mild hypertension, cardiac hypertrophy, leukocytosis, higher body temperature, oligotrichia, and emaciation due to reduction of fat tissue. Histological expression of the h-chymase transgene was observed in regions of the extracellular matrix, such as the aortic adventitia, medial elastic fiber, and basal membrane of both the bronchus and cerebral choroid plexus. These histological locations of h-chymase transgene expression were not markedly different from those of native h-chymase expression. In addition, the fact that three independent h-chymase TGM lines showed identical phenotypes suggested that these phenotypic changes were due to the transgene expression.

The hypertension observed in this TGM line was not severe, which is apparently different from Tsukuba Hypertensive Mice carrying the h-renin and angiotensinogen genes (29). It has been suggested that rat chymase transgenic expression in vascular smooth muscle cells is also associated with a moderate increase of blood pressure (30). These data potentially suggested that chymase gene expression causes a hypertensive response *in vivo* irrespective of whether the gene is α (human) or β (rat) chymase. It was important to determine whether this increase in blood pressure is mediated through the AT_1 receptor. Our data suggest that the increased blood pressure in TGM in the present study was AT_1 receptor-dependent because the increase was completely normalized by simultaneous treatment with an AT_1 receptor antagonist. Thus, chymase-dependent Ang II formation contributed to the slight increase in blood pressure in this TGM model.

h-Chymase TGM also showed cardiac hypertrophy. Histological examination of the cardiac sections suggested that both cardiomyocyte hyperplasia and an increase in the fibrotic area contributed to the cardiac hypertrophy. In addition, the increase in blood pressure in TGM was rather mild, but the degree of cardiac hypertrophy was relatively larger and was not proportional to the increase in blood pressure. Further, AT_1 receptor antagonist treatment significantly, but not completely, suppressed the cardiac hypertrophy. Therefore, we speculate that the cardiac hypertrophy was not due only to an increase in blood pressure. Since valsartan treatment completely normalized blood pressure, but not cardiac hypertrophy, it seemed that there was an AT_1 receptor-independent mechanism which caused cardiac hypertrophy in this

TGM line. One of the phenotypic changes of h-chymase TGM was chronic inflammation manifested by leukocytosis, increased body temperature, and emaciation. These inflammatory changes would seem to have been independent of the AT_1 receptor, since valsartan did not reverse them (data not shown). In addition, the fact that the inflammatory cell infiltrate in the interstitial area of the heart was significantly increased in the hearts of TGM suggests that inflammatory changes were directly involved in the cardiac hypertrophy. Since IL- β is known to be a chemoattractant for various inflammatory cells (31, 32) and to cause myocyte hypertrophy (33), it is possible that the increase in IL-1 β is related to the AT_1 receptor-independent hypertrophic change in the heart.

There was no significant difference in serum Ang II level between the TGM and control mice. The exact reason for this finding was not clear. Histologically, the transgene h-chymase expression was located mainly in the interstitial region of each organ, which is similar to the original location of native h-chymase (34). Therefore, it can be speculated that most of the tissue Ang II formation due to the transgene occurred in the interstitial region. In addition, the gene structure of h-chymase indicates that it is a proenzyme, and that liberation of both pre- and pro-sequences was essential to yield enzymatic activity. Such processing only occurs in cells containing a processing enzyme such as cathepsin C for h-chymase in their secretory granules (23). This could be a reason why tissue h-chymase activity was rather low compared to the level of the transgene expression, and why the plasma Ang II levels did not show a significant increase compared to that in wild-type mice.

In gross anatomical examination, we found a significant mass reduction in the dermal and visceral fat tissue of TGM. The amount of food intake in TGM did not decrease—and in fact increased—suggesting that appetite loss was not a cause of the emaciation. Since the thyroid function of TGM was within the normal limit, the weight reduction found in h-chymase TGM was not due to hyperthyroidism. Several phenotypic changes, such as leukocytosis, high body temperature, and increased IL-1 β suggested that TGM were complicated with chronic inflammation.

The leukocytosis observed in h-chymase TGM was due to the increase of both mononuclear and polymorphonuclear cell fractions. There was no significant increase in any fraction of peripheral leukocytes. In addition, the histological sections of the bone marrow showed hyperplastic changes in the myelocytic cell fraction, but not in the megakaryocyte or erythroblastic cell fractions (data not shown). Therefore, it was likely that the leukocytosis observed in h-chymase TGM was caused by direct stimulation of both the bone marrow and lymph nodes by a circulating humoral factor. Isolated chymase from mast cells induces neutrophilia and eosinophilia following injection into guinea-pig skin (18). The substrates upon which chymase may act *in vivo* to stimulate leukocytosis are not clear. One candidate is IL-1 β , the precursor of which is activated by chymase *in vitro* (14). IL-1 β

is able to induce the accumulation of eosinophils in rat skin (35) and of neutrophils in rabbit skin (31), and can prime human neutrophils *in vitro* (32, 36). IL-1 β is a potent proinflammatory cytokine expressed in a number of cell types, and leukocytosis and pyrexia are typical physical changes caused by increased IL-1 β . Therefore, chymase-dependent IL-1 β activation appears to have been involved in the leukocytosis and pyrexia observed in h-chymase TGM. Furthermore, since chymase is a potent Ang II-forming enzyme (3), the Ang II produced by chymase may induce expression of the potent eosinophil and monocyte chemoattractant factor (RANTES) (37), or of the monocyte chemoattractant protein-1 (38). These alternative proinflammatory pathways by Ang II could be additional stimuli for the inflammatory response observed in h-chymase TGM.

There has been no report describing the association between chymase and hair loss, although mast cells appear to be involved in the hair follicle cycle. Maurer *et al.* reported that mast cells are involved in regulating the hair follicle transformation from a resting state (telogen) to an active hair-growth state (anagen) and that skin mast cells may play a role in spontaneous hair follicle regression (catagen), which is typically characterized by massive epithelial cell apoptosis (39, 40). There are also clinical indications that mast cells are involved in the pathogenesis of several forms of hair loss and alopecia in humans. In some forms of scarring alopecias (41, 42) and in androgenetic alopecia (43), the number of skin mast cells is remarkably increased. These data support the notion that mast cells function as a hair cycle regulator and are involved in the control of hair follicle regression. Pathological examination in the present study revealed that the total number of hair follicles did not differ between h-chymase TGM and control mice (data not shown). Therefore, the oligotrichia appearing in this TGM line seemed to be related to the increased hair follicle turnover. The h-chymase TGM model presented in the present study should provide a new tool for examining the role of h-chymase on hair follicle cycling.

In conclusion, this study established the h-chymase TGM line and provided direct evidence of the pathological role of h-chymase *in vivo*. Our data also suggested that h-chymase might have been involved in the development of mild hypertension with left ventricular hypertrophy, chronic inflammation, and oligotrichia, and that the former phenotypic change appeared to be AT₁ receptor-dependent. However, the actions of h-chymase under a pathophysiological condition have been little investigated. In the future, we would like to address these issues using our transgenic model.

Acknowledgements

We thank Ms. Yuri Saito for her skillful technical assistance.

References

- Okunishi H, Oka Y, Shiota N, Kawamoto T, Song K, Miyazaki M: Marked species-difference in the vascular angiotensin II-forming pathways: humans *versus* rodents. *Jpn J Pharmacol* 1993; **62**: 207–210.
- Akasu M, Urata H, Kinoshita A, Sasaguri M, Ideishi M, Arakawa K: Differences in tissue angiotensin II-forming pathways by species and organs *in vitro*. *Hypertension* 1998; **32**: 514–520.
- Urata H, Kinoshita A, Misono KS, Bumpus FM, Husain A: Identification of a highly specific chymase as the major angiotensin II-forming enzyme in the human heart. *J Biol Chem* 1990; **265**: 22348–22357.
- Nakano A, Kishi F, Minami K, Wakabayashi H, Nakaya Y, Kido H: Selective conversion of big endothelins to tracheal smooth muscle-constricting 31-amino acid-length endothelins by chymase from human mast cells. *J Immunol* 1997; **159**: 1987–1992.
- Nishimura H, Buikema H, Baltatu O, Ganten D, Urata H: Functional evidence for alternative ANG II-forming pathways in hamster cardiovascular system. *Am J Physiol* 1998; **275**: H1307–H1312.
- Kim S, Iwao H: Molecular and cellular mechanisms of angiotensin II-mediated cardiovascular and renal diseases. *Pharmacol Rev* 2000; **52**: 11–34.
- Miyazaki M, Takai S: Local angiotensin II-generating system in vascular tissues: the roles of chymase. *Hypertens Res* 2001; **24**: 189–193.
- Ihara M, Urata H, Kinoshita A, *et al*: Increased chymase-dependent angiotensin II formation in human atherosclerotic aorta. *Hypertension* 1999; **33**: 1399–1405.
- Ihara M, Urata H, Shirai K, *et al*: High cardiac angiotensin II-forming activity in infarcted and non-infarcted human myocardium. *Cardiology* 2000; **94**: 247–253.
- Jin D, Takai S, Yamada M, Sakaguchi M, Miyazaki M: Beneficial effects of cardiac chymase inhibition during the acute phase of myocardial infarction. *Life Sci* 2002; **71**: 437–446.
- Hoshino F, Urata H, Inoue Y, *et al*: Chymase inhibition improves survival in hamsters with myocardial infarction. *J Cardiovasc Pharmacol* 2003; **41**: S11–S18.
- Uehara Y, Urata H, Ideishi M, Arakawa K, Saku K: Chymase inhibition suppresses high-cholesterol diet-induced lipid accumulation in the hamster aorta. *Cardiovasc Res* 2002; **55**: 870–876.
- Takai S, Jin D, Nishimoto M, *et al*: Oral administration of a specific chymase inhibitor, NK3201, inhibits vascular proliferation in grafted vein. *Life Sci* 2001; **69**: 1725–1732.
- Mizutani H, Schechter N, Lazarus G, Black RA, Kupper TS: Rapid and specific conversion of precursor interleukin 1 beta (IL-1 beta) to an active IL-1 species by human mast cell chymase. *J Exp Med* 1991; **174**: 821–825.
- Tunon de Lara JM, Okayama Y, McEuen AR, *et al*: Release and inactivation of interleukin-4 by mast cells. *Ann N Y Acad Sci* 1994; **725**: 50–58.
- Longley BJ, Tyrrell L, Ma Y, *et al*: Chymase cleavage of stem cell factor yields a bioactive, soluble product. *Proc Natl Acad Sci USA* 1997; **94**: 9017–9021.

17. Rubinstein I, Nadel JA, Graf PD, Caughey GH: Mast cell chymase potentiates histamine-induced wheal formation in the skin of ragweed-allergic dogs. *J Clin Invest* 1990; **86**: 555–559.
18. He S, Peng Q, Walls AF: Potent induction of a neutrophil and eosinophil-rich infiltrate *in vivo* by human mast cell tryptase: selective enhancement of eosinophil recruitment by histamine. *J Immunol* 1997; **159**: 6216–6225.
19. Vartio T, Seppa H, Vaheri A: Susceptibility of soluble and matrix fibronectins to degradation by tissue proteinases, mast cell chymase and cathepsin G. *J Biol Chem* 1981; **256**: 471–477.
20. Taipale J, Lohi J, Saarinen J, Kovanen PT, Keski-Oja J: Human mast cell chymase and leukocyte elastase release latent transforming growth factor-beta 1 from the extracellular matrix of cultured human epithelial and endothelial cells. *J Biol Chem* 1995; **270**: 4689–4696.
21. Kofford MW, Schwartz LB, Schechter NM, Yager DR, Diegelmann RF, Graham MF: Cleavage of type I procollagen by human mast cell chymase initiates collagen fibril formation and generates a unique carboxyl-terminal propeptide. *J Biol Chem* 1997; **272**: 7127–7131.
22. Hoit BD, Shao Y, Kinoshita A, Gabel M, Husain A, Walsh RA: Effects of angiotensin II generated by an angiotensin converting enzyme-independent pathway on left ventricular performance in the conscious baboon. *J Clin Invest* 1995; **95**: 1519–1527.
23. Urata H, Kinoshita A, Perez DM, Misono KS, Bumpus FM, Graham RM, Husain A: Cloning of the gene and cDNA for human heart chymase. *J Biol Chem* 1991; **266**: 17173–17179.
24. Miyazaki J, Takaki S, Araki K, *et al*: Expression vector system based on the chicken beta-actin promoter directs efficient production of interleukin-5. *Gene* 1989; **79**: 269–277.
25. Hoffmann S, Krause T, van Geel PP, *et al*: Overexpression of the human angiotensin II type 1 receptor in the rat heart augments load induced cardiac hypertrophy. *J Mol Med* 2001; **79**: 601–608.
26. Shimano H, Yamada N, Shimada M, *et al*: Hepatic and renal expression of rat apolipoprotein E under control of the metallothionein promoter in transgenic mice. *Biochim Biophys Acta* 1991; **1090**: 91–94.
27. Urata H, Khosla MC, Bumpus FM, Husain A: Evidence for extracellular, but not intracellular, generation of angiotensin II in the rat adrenal zona glomerulosa. *Proc Natl Acad Sci USA* 1988; **85**: 8251–8255.
28. Ekins R: One-step, labeled-antibody assay for measuring free thyroxine. 1: assay development and validation. *Clin Chem* 1992; **38**: 2355–2358.
29. Fukamizu A, Sugimura K, Takimoto E, *et al*: Chimeric renin-angiotensin system demonstrates sustained increase in blood pressure of transgenic mice carrying both human renin and human angiotensinogen genes. *J Biol Chem* 1993; **268**: 11617–11621.
30. Ju H, Gros R, You X, Tsang S, Husain M, Rabinovitch M: Conditional and targeted overexpression of vascular chymase causes hypertension in transgenic mice. *Proc Natl Acad Sci USA* 2001; **98**: 7469–7474.
31. Rampart M, Williams TJ: Evidence that neutrophil accumulation induced by interleukin-1 requires both local protein biosynthesis and neutrophil CD18 antigen expression *in vivo*. *Br J Pharmacol* 1988; **94**: 1143–1148.
32. Sullivan GW, Carper HT, Sullivan JA, Murata T, Mandell GL: Both recombinant interleukin-1 (beta) and purified human monocyte interleukin-1 prime human neutrophils for increased oxidative activity and promote neutrophil spreading. *J Leukoc Biol* 1989; **45**: 389–395.
33. Harada E, Nakagawa O, Yoshimura M, *et al*: Effect of interleukin-1 beta on cardiac hypertrophy and production of natriuretic peptides in rat cardiocyte culture. *J Mol Cell Cardiol* 1999; **31**: 1997–2006.
34. Urata H, Boehm KD, Philip A, *et al*: Cellular localization and regional distribution of an angiotensin II-forming chymase in the heart. *J Clin Invest* 1993; **91**: 1269–1281.
35. Sanz MJ, Weg VB, Bolanowski MA, Nourshargh S: IL-1 is a potent inducer of eosinophil accumulation in rat skin: inhibition of response by a platelet-activating factor antagonist and an anti-human IL-8 antibody. *J Immunol* 1995; **154**: 1364–1373.
36. Dularay B, Elson CJ, Clements-Jewery S, Damais C, Lando D: Recombinant human interleukin-1 beta primes human polymorphonuclear leukocytes for stimulus-induced myeloperoxidase release. *J Leukoc Biol* 1990; **47**: 158–163.
37. Wolf G, Ziyadeh FN, Thaiss F, *et al*: Angiotensin II stimulates expression of the chemokine RANTES in rat glomerular endothelial cells: role of the angiotensin type 2 receptor. *J Clin Invest* 1997; **100**: 1047–1058.
38. Chen XL, Tummala PE, Olbrych MT, Alexander RW, Medford RM: Angiotensin II induces monocyte chemoattractant protein-1 gene expression in rat vascular smooth muscle cells. *Circ Res* 1998; **83**: 952–959.
39. Maurer M, Paus R, Czarnetzki BM: Mast cells as modulators of hair follicle cycling. *Exp Dermatol* 1995; **4**: 266–271.
40. Maurer M, Fischer E, Handjiski B, *et al*: Activated skin mast cells are involved in murine hair follicle regression (catagen). *Lab Invest* 1997; **77**: 319–332.
41. Reed RJ, Clark WH, Mihm MC: The cutaneous mucinosis. *Hum Pathol* 1973; **4**: 201–205.
42. Rook AH, Prystowsky MB, Cassin M, Boufal M, Lessin SR: Combined therapy for Sezary syndrome with extracorporeal photochemotherapy and low-dose interferon alpha therapy: clinical, molecular, and immunologic observations. *Arch Dermatol* 1991; **127**: 1535–1540.
43. Lattanand A, Johnson WC: Male pattern alopecia a histopathologic and histochemical study. *J Cutan Pathol* 1975; **2**: 58–70.