Cloning of the cDNA encoding mast cell tryptase of Mongolian gerbil, *Meriones unguiculatus*, and its preferential expression in the intestinal mucosa

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By using the combination of reverse-transcription PCR and rapid amplification of cDNA ends methods, a cDNA encoding mast cell tryptase was successfully cloned from the small intestine of Mongolian gerbil, *Meriones unguiculatus*, infected with *Nippostrongylus brasiliensis*. The cDNA was 1219 bp long including 810 bp of an open reading frame. Based on the deduced amino acid sequences of known mast cell tryptases of other species, the gerbil mast cell tryptase (gMCT) was highly similar to mouse mast cell protease (mMCP)-7, and seems to be translated as a prepro-enzyme with 25 amino acids of signal and activation peptides and 245 amino acids of mature enzyme. The gMCT

mRNA was preferentially transcribed in the intestinal mucosa and to a far lesser extent in the connective tissue such as skin and tongue. Moreover, kinetic study after infection revealed that the amount of gMCT mRNA in the small intestine correlated well with the degree of intestinal mastocytosis. Throughout the course of infection, enzyme-histochemically detectable tryptase activity was limited to mucosal mast cells. Since mucosal mast cells of other rodents, including mice and rats, do not express tryptases, this is the first report of rodent mast cell tryptase expressed in the intestinal mucosa.

INTRODUCTION

Mast cells are usually classified as two subsets, namely mucosal mast cells (MMC) and connective tissue mast cells (CTMC), depending on their tissue distribution, histochemical properties and cytokine dependency [1]. Mast cells contain chymotrypsin-like (chymase) and trypsin-like (tryptase) neutral serine proteases (mast cell proteases; MCPs) in their granules [2–4]. MCPs can also be used as the phenotypic markers of MMC and CTMC [5,6] because of the markedly different tissue distributions. Mouse MCP (mMCP)-1 to mMCP-5 are chymases and mMCP-6 and -7 are tryptases [6–12], and their expressions are restricted to particular subtypes and/or maturation stages of mast cells [6,13]. Similarly, rat MCP (rMCP)-1 and -2, are defined as specific chymases for CTMC and MMC respectively [14–16]. Rat tryptase was purified from peritoneal mast cells, a representative of CTMC [17]. In mice and rats, tryptases were not detected in MMC by RNA blot analyses nor by enzyme histochemistry, whereas tryptases and certain chymases were expressed in CTMC [18–20]. Recently we found that mast cell subsets of Mongolian gerbil, *Meriones unguiculatus*, were quite distinct from those of mice and rats in terms of histochemical properties of granular proteoglycan [21] and protease activities [22]. Of most interest was that MMC in the jejunum of gerbils had tryptase activity [22]. We also found that Mongolian gerbils were unable to expel *Strongyloides ratti* [23] or *Strongyloides venezuelensis* [24,25], expulsion of which from the intestine was known to be entirely dependent on MMC in mice and rats [26–30]. Therefore, incapability of gerbils to expel particular intestinal nematodes might be related to the unusual phenotypic expressions, including proteases, of mast cell subsets of this animal. Here we report that

cDNA of gerbil mast cell tryptase (gMCT) was successfully cloned and sequenced from the jejunum of Mongolian gerbils infected with *Nippostrongylus brasiliensis*, a potent inducer of MMC in rodents including gerbils. The mRNA was preferentially detected in the intestinal mucosa and to a far lesser extent in the skin and tongue.

MATERIALS AND METHODS

DNA and RNA extraction

Genomic DNA was purified from liver of an 8-week-old gerbil by SepaGene (Sanko Junyaku Co., Ltd., Tokyo, Japan) according to the manufacturer's protocol. Total RNAs were extracted by ISOGEN (Nippongene, Tokyo, Japan) from small intestine, skin and tongue of 8–10-week-old gerbils before and 7, 10, 14 and 21 days after infection with *N. brasiliensis*. Poly(A)⁺ RNA selection was performed by oligo(dT) latex (Oligotex-dT30; Nippon Roche, Tokyo, Japan).

Reverse transcription PCR (RT-PCR) cloning and sequencing

A 1 µg amount of poly(A)⁺ RNA extracted from gerbil small intestine infected with *N. brasiliensis* was reverse-transcribed by random hexamer [pd(N)₆] and SuperScript reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD, U.S.A.). The resulting cDNA was subjected to PCR for 30 cycles with the primers (Figure 1a, P1 and P2, sequences of which are shown in the Primers and anchor section) designed in the well-conserved regions of known mast cell tryptase cDNA sequences [9,12,31–34]. The P1 primer sequence is absolutely conserved

Abbreviations used: MMC, mucosal mast cells; CTMC, connective tissue mast cells; MCP, mast cell protease; mMCP, mouse MCP; gMCT, gerbil mast cell tryptase; RT-PCR, reverse transcription PCR; RACE, rapid amplification of cDNA ends; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; VCU, villous crypt unit.

The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the accession number D31789.

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Figure 1 Overview of the gMCT mRNA and its product and the scheme of RT-PCR (a), and 3'- (b) and 5'- (c) RACEs for cDNA sequencing

The locations and directions of the primers used are also indicated. aa, Amino acids.

among known tryptases. The P2 primer sequence differs from mMCP-6 and dog tryptase by only one nucleotide near the 5'-end of the primer. The thermal cycle profile was 1 min at 94 °C, 2 min at 50 °C, 3 min at 72 °C. After electrophoresis, the PCR product of the expected size was cut from 2% agarose gel, purified by GeneClean II (Bio 101 Inc., La Jolla, CA, U.S.A.) and cloned into a TA cloning vector, pCR II (Invitrogen, San Diego, CA, U.S.A.). The nucleotide sequence was determined by the dideoxy chain-termination method as described previously [35].

Rapid amplification of cDNA ends (RACE)

A 1 µg amount of poly(A)⁺ RNA and 5 µg of total RNA extracted from small intestine of infected gerbil were reverse-transcribed with DT7 primer and P5 primer, for 3' and 5' RACE respectively [36,37]. The DT7 primer contains oligo(dT)₁₇ at the 3'-end followed by PT7 primer sequence, while the P5 primer is specific for gMCT. For 3' RACE, the first-strand cDNA was amplified by PCR with the primers P3 and PT7 and the resulting first-round RACE products were amplified again with the primers P4 and PT7 (Figure 1b, sequences of which are shown in the Primers and anchor section). For 5' RACE, we used 5'-AmpliFINDER RACE Kit (Clontech, Inc., Palo Alto, CA, U.S.A.). Briefly, the single-stranded oligonucleotide anchor (5' anchor) was directly ligated to the 3'-end of the first-strand cDNA with T4 RNA ligase and the resulting anchor-ligated cDNA was amplified by PCR with the primer P6 and the anchor primer P7 (Figure 1c, sequences of which are shown in the Primers and anchor section). These RACE products were also purified, cloned and sequenced as described above.

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1      gagcaggttcaaaagaca
18     accaaggtggccccctcccagagcctctgacgtctgcatgctggccaagctgactcgaa
78     ctcccgaattggacactaccttctgctccctggttctctggcctgttctgcaccgacaa
138    tctgttgacactagccagcctttacctccaactaggttcacactactcactgtttccaa

198    atgctgaagctgctgctgctggcactgccctgttcagcctgatgcatcggtcccccgtg
(-25) M L K L L L L A L P L F S L M H R S P L

258    tggcaagagtggggcattgttgggggacaggaggacactgggaacaagtggccctggcag
(-5) C Q E W G I V G G Q E A P G N K W P W Q

318    gtgagcctctgctgccaatgaaacactactggagcatttctcgccgggctccctcatccac
(16) V S L R A [N] E T Y W R H F C G G S L I H

378    ccacagtgggtgctcaccgcggcactgtgtgggacgactattgctgatcccaacaag
(36) P Q W V L T A A H C V G P T I A D P N K

438    gtcagagtacagcttcgaaagcagtcacttattaccacgaccacactgtgctgtgagc
(56) V R V Q L R K Q Y L Y L Y H L D H L A V S

498    cggatcatcacacaccgacattctatgccaccagaatggggcgacatgcctactt
(76) R I I T H P T F Y A T Q N G A D I A L L

558    gagctcaagaacctgtgaaacatttcagcattgtccacccgctctccctgctcctgcc
(96) E L K N P V [N] I S S H V H F V S L P P A

618    tcagagaccttccctcaggaacattgtgctgggtgacaggtggggaacatcgacaat
(116) S E T F P S G T L C W V T G W H N I D N

678    gatgtgagcctgccaccgccatttccctgaaggaggtgcaagttcccgctgtggaacac
(136) D V S L P P P F P L K E V Q V P V V E N

738    cagctttgtgacctaagtatcacaagggtgtctacacaggggacaacatccacattgtc
(156) Q L C D L K Y H K G V Y T G D N I H I V

798    cgagacgacatgctgtgctgggaacgaaggacagcactcctgccagggtgactccgga
(176) R D D M L C A G N E G H D S C Q G G D S G

858    ggacctctggtctgcaaggtaaacggtacgtgctgacggcaggtgtggtcagctgggtg
(196) G P L V C K V [N] G T W L Q A G V V S W G

918    gagggctgtgctctgccaacaggcctggcatctacactcgggtcacctattacttgagc
(216) E G C A L P N R P G I Y T R V T Y Y L D

978    tggatccacgctatgtccccaaggacttctgaatcacctccagagtcaggaggagaacca
(236) W I H R Y V P K D F *

1038   gatctctgctgtccctacacgctgcttctgcccgggggagatcctgtgctctccta
1098   ccacctcccatcctgtggtgctcctcctgagccctggccactcctgtccctccctc
1158   ccaggcagccccactatgtagccagccactcttctgtatggctc[cccaaa]atgcacgaa
1218   gc

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Figure 2 Nucleotide and deduced amino acid sequences of gMCT

The symbols used indicate as follows: signal and activation peptides, —; stop codon, *; polyadenylation signal, [N]; serine protease catalytic triad residues, ●; potential N-linked glycosylation sites, □. The double underlining indicates the 'ccaa' sequence, which may be a consequence of a mutation in the 3' acceptor site of the first intron in mMCP-7 [12]. The amino acids are numbered, with position one set to the start of the mature enzyme, in brackets on the left-hand side.

Primers and anchor

Primers and anchor sequences used were as follows:

Primer P1 (sense), 5'-AAGTGGCCCTGGCAGGTGAGCCT-3';
 primer P2 (antisense), 5'-GGGGACATAGTGGTGGATCCA-3';
 primer P3 (sense), 5'-ACCTACTGGAGGCATTCTG-3';
 primer P4 (sense), 5'-ATCCACCCACAGTGGGTGCT-3';
 primer P5 (antisense), 5'-CGGTCCCACACAGTGTGCCG-3';
 primer P6 (antisense), 5'-GGTGAACACCCACTGGGGGT-3';
 primer P7, 5'-CTGGTTCGGCCACCTCTGAAGGTTCCA-GAATCATAG-3';
 primer PT7, 5'-AATACGACTCACTATAG-3';
 primer DT7, 5'-TAATACGACTCACTATAGGGATTTTTT-3';
 5' anchor, 3' NH₂-GGAGACTTCCAAGGTCTTAGCTATC-ACTTAAGCAC-p 5'.

The directions and locations of these primers and anchor ligation site are shown in Figure 1a–c. After sequencing of the RT-PCR products using the primers P1 and P2, which were designed in the well-conserved regions of known mast cells tryptase cDNA

Figure 3 Comparison of the deduced amino acid sequence of gMCT with those of other known mast cell tryptases

The mouse (mMCP-6 and -7), human and dog sequences were taken from [9,12,31 and 34], respectively. In this comparison, the human tryptase sequence reported by Miller et al. [31] was used as the representative of five human tryptases reported to date [31–33]. Identical amino acid residues among the five sequences are indicated by *. The other symbols used indicate as follows: predicted cleavage sites of signal and activation peptides, †; potential N-linked glycosylation sites, □; cysteine residues building the predicted intrachain disulphide bonds, ●; serine protease catalytic triad residues, ■.

sequences, the primers P3–P6 were designed in gerbil specific inner regions.

Southern and RNA blot analysis

Three restriction enzymes, *Eco*RI, *Hind*III and *Xho*I were purchased from TOYOBO (Osaka, Japan). A 10 µg amount of gerbil genomic DNA digested by one of these endonucleases was electrophoresed on 1% agarose gel and transblotted onto Hybond-N nylon membrane (Amersham, Buckinghamshire, UK). Total RNAs (30 µg each) from the small intestine, skin and tongue of uninfected and infected gerbils were also electrophoresed on 1% formaldehyde agarose gel and transblotted onto nylon membrane. Hybridization was performed under high stringency conditions using ³²P-labelled probes as described [35]. The probes used were 3'-RACE products (corresponding to nucleotide positions 372–1219) for RNA blot analysis and PCR product (corresponding to 798–998 and having no internal restriction sites by the endonucleases used in this study) for Southern blot analysis. The control human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) probe, which strongly cross-hybridizes to rodent G3PDH mRNA, was purchased from Clontech.

Enzyme histochemistry

Small pieces of jejunum, resected at about 10 cm distal to pylorus, were obtained from before and 7 and 14 days after *N.*

brasilensis infection as described previously [21,22]. The tissues fixed in Carnoy's fluid for 3 h were dehydrated, cleared in *n*-limonene, and embedded in paraffin wax. Tryptase activity in mast cells was detected by the methods of Lojda et al. [38] and Osman et al [39] using Z-Ala-Ala-Lys-4-methoxy-2-naphthylamide (Enzyme Systems Products, Livermore, CA, U.S.A.) as a substrate. The reaction medium was prepared as follows: 4 mg of substrate dissolved in 0.5 ml of dimethylformamide was added to 10 ml of 0.1 M Tris-HCl buffer (pH 7.1) containing 10 mg of Fast Blue B double zinc salt. The mixture was filtered immediately before use. Sections were incubated in the reaction medium for 30 min at 37°C, rinsed in water and mounted in glycerinated gelatin. An adjacent section was stained with Alcian Blue (pH 0.3) and Safranin O (pH 0.1) for counting the number of mast cells per 10 villous crypt units (VCU) and to confirm enzyme activity in mast cells.

RESULTS

Sequencing of gMCT cDNA

To obtain the full-length gMCT cDNA, we first performed RT-PCR with primers P1 and P2, which were designed in the well-conserved region of known tryptase cDNAs of other species (Figure 1a). The expected size (approximately 600 bp) of RT-PCR product was obtained and sequenced. Based on this partial cDNA sequence, both 5'- and 3'-RACE were performed using the primers P3–P6 (Figures 1b and 1c), which were specific for gerbil. By using these steps, the full-length cDNA sequence was

Figure 4 Southern-blot analysis of gerbil genomic DNA digested with *EcoRI* (lane 1), *HindIII* (lane 2), and *XhoI* (lane 3)

The blot was performed under high-stringency conditions and was probed with PCR products corresponding to nucleotide positions 798–998 and having no internal restriction sites for the endonucleases used. Note that two apparent DNA fragments were detected regardless of the restriction enzymes used.

obtained. At least six individual clones each from the RT-PCR and RACE products were sequenced and all sequencing was done once or more in both directions to obtain maximum accuracy for the sequence given. The total 1219 bp of gMCT nucleotide sequence and its deduced amino acid sequence are shown in Figure 2. An ATG codon was found 198 nucleotide residues downstream of the 5'-end. A stop codon (numbered 1008) and the following 209 bp non-coding region including a polyadenylation signal, ATTAAA (numbered 1202–1207), were found at the 3'-end. Thus, a peptide with 270 amino acids was encoded by the single open reading frame. The first 25 amino acids probably represent the signal peptide and the activation peptide, based on a comparison with known tryptases of other species. Consequently the initial Met was numbered –25 and the following Ile, considered to be the N-terminal of the mature protein, was numbered 1.

Comparison of the deduced amino acid sequence with that of other species

When the deduced amino acid sequence of gMCT was compared with those of other species' mast cell tryptases (Figure 3), serine protease catalytic triad residues (His⁴⁴, Asp⁹¹ and Ser¹⁹⁴ and eight cysteine residues building the predicted intrachain disulphide bonds were well-conserved. However, potential N-linked glycosylation sites were variable, except for Asn¹⁰² which was conserved in all species. The N-linked glycosylation site at Asn²¹ was seen in gMCT and mMCP-7, whereas Asn²⁰³ was observed in gMCT and human tryptase. The sequence identity of gMCT with mMCP-7 was 88.2% in amino acids for mature enzyme, whereas that with mMCP-6, human tryptase and dog tryptase were 74.3%, 73.9% and 72.2% respectively.

Southern blot analysis

In Southern blot analysis under high-stringency conditions (Figure 4), two apparent DNA fragments were detected regardless of the restriction enzymes used. Since the probe used has

Figure 5 RNA blot analysis of gMCT before and after infection with *N. brasiliensis*

A 30 µg amount of the RNAs derived from various tissues at different times of infection were loaded. (a) Gerbil skin (lanes 1 and 2) and tongue (lanes 3 and 4) before (lanes 1 and 3) and 14 days after (lanes 2 and 4) infection; (b) gerbil jejunum before (lane 1) and 7 (lane 2), 10 (lane 3), 14 (lane 4) and 21 (lane 5) days after infection with *N. brasiliensis*. The numbers of mast cells per 10 VCU in the jejunum of gerbil were counted and are indicated in (b). The blot was performed under high-stringency conditions and was probed with 3'-RACE products corresponding to nucleotide positions 372–1219. The films were exposed for 10 days (a) and 3 days (b) at –70°C.

no internal restriction sites and since the corresponding region is thought not to be divided by insertion of an intron [9,12], this result indicates the probable presence of at least one additional gMCT-like gene, like mouse tryptases (mMCP-6 and -7) and human tryptases. However, all 16 individual clones of RT-PCR products from gerbil small intestine had the same gMCT sequence and no distinct additional gMCT-like cDNA was detected. Therefore, the major tryptase of jejunal mucosal mast cells of gerbil is probably the gMCT reported here.

RNA blot analysis

To examine the expression of gMCT gene in different tissues before and after infection, RNA blot analysis was performed. Similar to our previous results of enzyme histochemistry [22], the gMCT had a unique tissue distribution, being preferentially transcribed in the intestinal mucosa and to a far lesser extent in connective tissue such as skin or tongue (Figures 5a and 5b). The gMCT mRNA levels in the skin and tongue remained unchanged during infection (results not shown) whereas those in the small

Figure 6 Enzyme-histochemical staining of tryptase in the jejunum of Mongolian gerbils before (day 0) and 14 days after infection with *N. brasiliensis*

Adjacent sections were stained with Alcian Blue and Safranin O to demonstrate the presence of mast cells and the coincidence of enzyme activity in mast cells.

intestine were drastically altered during infection. The amount of gMCT mRNA correlated well with the number of mast cells, a gradual increase for 2 weeks and a subsequent decrease by 3 weeks (Figure 5b). Throughout the course of infection, enzyme-histochemically detectable tryptase activity was limited to mucosal mast cells as described below.

Enzyme histochemistry

By Alcian Blue and Safranin O staining, the number of gerbil intestinal mast cells did not change significantly on day 7 after infection, increased to reach a peak on day 14 and then decreased gradually for the next several weeks as described previously [21]. By enzyme-histochemical staining for tryptase, all mast cells in the intestinal mucosa showed strong positive activity regardless of the time of infection. These results are consistent with the kinetic changes in the amount of gMCT mRNA during infection (Figure 5b). Typical staining patterns are shown in Figure 6.

DISCUSSION

In the present study, we successfully cloned and sequenced gMCT cDNA and demonstrated the functionally active gMCT protein in mast cells of intestinal mucosa. From the deduced amino acid sequence of the gMCT cDNA, the sequence similarity with mMCP-7 (88.2%) was far higher than that with mMCP-6 (74.3%) or mast cell tryptases of other species. The gMCT cDNA had an extremely long 5' non-coding region like mMCP-7 [12] and a 'ccaa' sequence immediately upstream of the initiation ATG codon (Figure 3, doubly underlined), which may be a consequence of a mutation in the 3' acceptor site of the first intron, as in mMCP-7 [12]. The gMCT had a potential

N-glycosylation site, Asn²¹, which is found only in mMCP-7. Therefore, the gMCT reported here seemed to be an analogue of mMCP-7 rather than mMCP-6. While mMCP-6 is predominantly expressed in murine CTMC, mMCP-7 is thought to be rather a minor transcript. The mMCP-7 gene is transiently transcribed during the culture of bone marrow-derived immature mast cells but not transcribed in mature MMC *in vivo* [12]. Furthermore, the mMCP-7 gene was expressed exceptionally in the CTMC of Balb/c mice but not in other strains [13,40]. In contrast to mMCP-7, the gMCT gene was strongly expressed in MMC *in vivo*, suggesting that gMCT may play an important role in the intestinal mucosa of gerbil under various pathophysiological conditions. Whatever the reason for the different usage of the homologous genes, gMCT and mMCP-7, between the two species, the present results show that Mongolian gerbil could be a useful model for investigating the heterogeneity of MCPs.

Including the gMCT reported here, all known mast cell tryptases of various species have an activation peptide ending with the residue Gly, whereas all known mast cell chymases have an activation peptide ending with the residue Glu [11]. Therefore, tryptases and chymases have a distinct mechanism of activation [34]. Based on the rules of von Heijne [41], mMCP-6 and -7 each has a predicted cleavage site at residue -11 for the hydrophobic signal peptide followed by 10 amino acids of activation peptide [9,12]. Vanderslice et al. [34] proposed that the cleavage site of dog mast cell tryptase and dog mastocytoma protease was perhaps at Pro⁻¹¹ or Gln⁻⁴. However, all other known mast cell tryptases including gMCT, except for mMCP-6 and -7, do not have a defined cleavage site at residue -11 for a signal peptide, by the rules of von Heijne. Because Thr⁻⁴ for mMCP-7 and Gln⁻⁴ for mMCP-6 and mast cell tryptases of other species satisfy von Heijne's rules, the cleavage site for the signal peptide of all mast cell tryptases might be residue -4. The mature enzyme of all known mast cell tryptases has an N-terminus IVGG sequence, whereas that of mast cell chymase has IIGG [9]. The N-terminus of the mature enzyme deduced from gMCT cDNA also has IVGG. Therefore, in general, mast cell tryptases have a consensus sequence of GIVGG at residues -1 to 4, whereas mast cell chymase has the sequence EIIGG at the same position.

gMCT cDNA was cloned from gerbil small intestine and its mRNA was indeed strongly detected in the small intestine and to a far lesser extent in the skin and tongue. This weakly detected mRNA in the skin and tongue may represent the transcripts of an additional gMCT-like gene in addition to gMCT, because the probe used in this study could detect an additional gMCT-like gene product (Figure 4). Indeed, both mMCP-6 and -7 were detected in the CTMC of mice [9,12,13]. Since gerbil CTMC was enzyme-histochemically negative for tryptase activity [22], the gerbil CTMC might have a relatively low amount of tryptase or its activity might be inhibited by tryptase inhibitors, such as trypstatin [35,42,43] or α 1-macroglobulin [44]. Further cloning and sequencing of an additional gMCT-like gene from gerbil CTMC would confirm the presence of tryptase subtypes. Regardless of MCP subtypes, tryptases have not been detected in rodent MMC [9,12,22], though they are strongly expressed in both human MMC and CTMC [4,22]. In addition, the mRNA level as well as enzyme-histochemical activity of gMCT was drastically altered in parallel with the number of mast cells during the course of *N. brasiliensis* infection. In this regard, Mongolian gerbil would be a fascinating model for studying the biological role of human mast cell tryptase, especially that expressed in the intestinal and respiratory mucosa.

In conclusion, we have successfully cloned and sequenced gMCT cDNA and demonstrated the existence of functionally active gMCT protein in mast cells of the intestinal mucosa. Since

gMCT was preferentially expressed in MMC and its level drastically altered during infection, this enzyme activity may be critically important to the function of mast cells under various pathophysiological conditions.

The excellent technical assistance of Ms. Eri Ohno is gratefully acknowledged. We also thank Dr. H. Kataoka, Second Department of Pathology, Miyazaki Medical College, for constructive criticism and valuable discussion. This work was supported in part by a Grant-in-Aid for Encouragement of Young Scientists No. 06770160, from the Ministry of Education, Science and Culture, Japan, and by grants from the Ichiro Kanehara Foundation, Japan Research Foundation for Clinical Pharmacology and Uehara Memorial Foundation.

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