Mast Cell–Restricted Tetramer-Forming Tryptases and Their Beneficial Roles in Hemostasis and Blood Coagulation

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KEYWORDS

• Mast cell • hTryptase-β • mMCP-6 • mMCP-7 • Fibrinogen
• Fibrin thrombin-dependent coagulation • Anaphylaxis

KEY POINTS

- Tryptase-β–dependent proteolysis of the α chain of hFibrinogen impairs its ability to form thrombin-dependent fibrin.
- The antithrombotic activity of hTryptase-β hinders the internal accumulation of life-threatening fibrin deposits and fibrin-platelet clots in tissues when activated mast cells (MCs) exocytose histamine and other vasopermeability factors which, in turn, induce vasodilation and edema of tissues.
- The anticoagulant activity of MC-restricted tryptases explains why there are 2 genes in mice and humans that encode similar tetramer-forming tryptases that can proteolytically damage fibrinogen, and why there is no endogenous protease inhibitor in normal blood that can rapidly inactivate these enzymes.
- The anticoagulant activity of tetramer-forming tryptases also explains the presence of hemorrhagic disorders in some patients with anaphylaxis or mastocytosis.
- Recombinant hTryptase-β could be a more effective and safer anticoagulant in the clinic than porcine-derived heparin oligosaccharides.
- C-terminal fragments of the α chain of hFibrinogen in blood and/or urine potentially could be biomarkers for the identification of patients who have undergone systemic anaphylaxis, have mastocytosis, or have an MC activation disorder.

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INTRODUCTION

Mast cells (MCs) are key effector cells in immediate hypersensitivity reactions because of their release of numerous proinflammatory mediators. The presence of too many MCs in tissues leads to mastocytosis (**Fig. 1**). However, the conservation of MCs in evolution and the failure to identify a human who lacks MCs suggests critical beneficial roles for these immune cells in our survival. In that regard, it has been demonstrated that the tetramer-forming tryptases exocytosed from activated MCs are needed for efficient host defense against certain bacterial, helminth, and virus infections.[1–6] Moreover, the loss of human immunodeficiency virus 1–infected hTryptase-β^+ MCs and their progenitors in patients with AIDS is now believed to be a contributing factor in their inability to combat opportunistic infections.[7–9]

Tetramer-forming tryptases are stored in the MC's secretory granules ionically bound to serglycin proteoglycans (SGPGs), which usually have heparin chains. These serine proteases are useful clinical and experimental biomarkers for mastocytosis, anaphylaxis, and the MC activation syndrome. Such markers can be measured by enzyme-linked immunosorbent assays and can be detected in peripheral blood for longer periods of time than other mediators exocytosed from activated MCs (eg, histamine and arachidonic acid metabolites).[10–12] The expression of hTryptase-β is also highly restricted to MCs. Despite its diagnostic value, the biological function of the hTryptase-β in plasma and blood has largely remained unknown.

The ability to form fibrin when the skin and other connective tissues are wounded is essential for preventing blood loss and the entry of pathogens into the body. Nevertheless, the formation of intravascular fibrin deposits and fibrin-platelet clots can have dangerous consequences. When the MCs in skin and other connective tissue sites degranulate, these immune cells quickly release the contents of their secretory granules, which include histamine and tetramer-forming tryptases bound to SGPGs that usually contain heparin glycosaminoglycans (GAGs). Histamine is the major

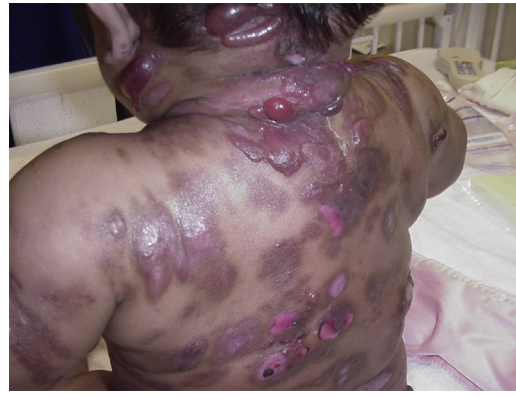

Fig. 1. Mastocytosis. The presence of an activating mutation in the tyrosine kinase receptor Kit/CD117 in the mast cell (MC)-committed progenitors of a mastocytosis patient eventually causes the accumulation of too many mature hTryptase-β^+ MCs in the skin and other connective tissues. The activation of these MCs and the exocytosis of their granule mediators can lead to numerous clinical problems, as occurred in the skin of this patient with bullous mastocytosis. Because the C-terminus of the α chain of hFibrinogen is preferentially cleaved by hTryptase-β, the identification of peptides derived from the protein's α chain in the circulation via an enzyme-linked immunosorbent assay could be of therapeutic valuable in the early identification of mastocytosis patients, as well as monitoring their treatment.

vasopermeability factor that induces the substantial edema that occurs at tissue sites where MCs degranulate, owing to its ability to bind to the H1 receptors on surfaces of vascular endothelial cells. This signaling reaction causes the redistribution of adhesion proteins and the loss of tight junctions between adjacent endothelial cells, which then allows the influx of fibrinogen and other circulating proteins into the inflammatory site.[13] Despite the rapid accumulation of fibrinogen, fibrin deposits are rare in the edema sites that form when cutaneous MCs degranulate. Recently, the authors uncovered a beneficial role for mouse and human tetramer-forming tryptases in anaphylaxis, namely, by preventing the formation of fibrin clots when vasodilation and plasma extravasation into a connective tissue occur following MC activation.[14] This vital antithrombotic function is mainly due to the ability of hTryptase-β to proteolytically damage fibrinogen before thrombin can convert the latter precursor protein to fibrin. The anticoagulant activity of hTryptase-β is an explanation for the hemorrhagic disorders that can occur in patients with anaphylaxis or mastocytosis. To understand the function of MC-restricted tetramer-forming tryptases, it is necessary to know the structural and biological characteristics of this family of serine proteases as outlined herein.

HUMAN MC TRYPTASES

It is now apparent that the hTryptase-β[15,16] preparations purified from the MCs isolated from human skin and lung biopsies in the 1980s by numerous groups consisted of a complex mixture of serine proteases derived from the *TPSAB1* and *TPSB2* genes on chromosome 16p13.3.[17] Miller and colleagues[18] isolated the first cDNA that originated from the human *TPSAB1* gene. When expressed, it was discovered that recombinant hTryptase-α had very little, if any, enzymatic activity, owing to an Asp^{246}/Gly^{246} mutation in its catalytic domain (**Fig. 2**).[19–21] Because one of the amino acids in the enzyme's propeptide also differed from that in pro-hTryptase-β, it was concluded

A Human TPSAB1 (GenBank GeneID 7177; Accession # NP_003285)

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  1 mlnllllalp vlasrayaap apgqalqrvg ivggqeaprs kwpwqvslrv hgpywmhfcg
 61 gslihpqwvl taahcvgpdv kdlaalrvql reqhlyyqdq llpvsriivh pqfytaqiga
121 dialleleep vvnsshvhtv tlppasetfp pgmpcwvtgw gdvdnderlp ppfplkqvkv
181 pimenhicda kyhlgaytgd dvrivrddml cagntrrdsc qgdsggplvc kvngtwlqag
241 vvswgegcaq pnrpgiytrv tyyldwihhy vpkkp
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B Human TPSB2 (GenBank GeneID 64499; Accession # NP_077078)

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  1 mlnllllalp vlasrayaap apgqalqrvg ivggqeaprs kwpwqvslrv rdrywmhfcg
 61 gslihpqwvl taahcvgpdv kdlaalrvql reqhlyyqdq llpvsriivh pqfytaqiga
121 dialleleep vnvsshvhtv tlppasetfp pgmpcwvtgw gdvdnderlp ppfplkqvkv
181 pimenhicda kyhlgaytgd dvrivrddml cagntrrdsc qgdsggplvc kvngtwlqag
241 vvswgegcaq pnrpgiytrv tyyldwihhy vpkkp
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Fig. 2. Allelic variations of the human *TPSAB1* (*A*) and *TPSB2* (*B*) genes. Based on the information at GenBank's Single-Nucleotide Polymorphism database, the Human Genome Consortium identified numerous mutations in the 2 genes that change the amino acids highlighted in yellow in the translated zymogens. Frame-shift or premature translational-termination mutations that cause the expression of truncated proteins are highlighted in blue. In each instance, the first 30 amino acids of the 275-mer zymogen (*red*) correspond to the signal/propeptide. The amino acids that comprise the mature domain of each processed tryptase are in black type. The arrowhead (*green*) points to the location of the Asp mutation that blocks the substrate-binding cleft of hTryptase-α.

that MCs might be unable to remove the propeptide from the defective zymogen, thereby causing the nonprocessed zymogen to be constitutively secreted rather than be targeted to the cell's secretory granules.[22]

The following year, Miller, Vanderslice, and their colleagues[23,24] isolated the first cDNAs from the human *TPSAB1* and *TPSB2* genes that encoded enzymatically active serine proteases, which they initially designated as hTryptase-β1, -β2, and -β3. Except for residue 132 that dictated how many *N*-linked glycans were bound to these proteases, the amino acid sequences of hTryptase-β1 and -β2 were identical. It therefore was not surprising that the 2 recombinant enzymes had identical substrate specificities when combinational peptide libraries were scanned.[1,19,25] Chromosome 16p13.3 is a recombination hot spot,[26] and many point mutations in the human *TPSAB1* and *TPSB2* genes have been identified by the Human Genome Consortium in recent years. Some of these mutations result in the expression of truncated proteases. Additional isoforms have been identified that are caused by alternate splicing of the precursor transcript and by differential posttranslational modification of the translated protein.[26,27] Most of the allelic isoforms of the *TPSAB1* and *TPSB2* genes that encode proteins with different amino acid sequences (see **Fig. 2**) have not been evaluated experimentally. Thus, investigators generally refer to any enzymatically active product of the human *TPSAB1* and *TPSB2* genes as hTryptase-β.

hTryptase-α is encoded by one of the most studied mutated alleles of the *TPSAB1* gene. Whereas hTryptase-α lacks enzymatic activity because of mutations in its propeptide[28] and catalytic domain,[19–21] the neutrophil serine protease gene family member azurocidin/CAP37 has bactericidal bioactivity even though it also lacks enzymatic activity.[29–31] It therefore is possible that hTryptase-α and other enzymatically inactive proteins encoded by some of the mutated alleles of the human *TPSAB1* and *TPSB2* genes noted in **Fig. 2** have bioactivity even though they lack enzymatic activity. Tryptase-α is constitutively secreted by MCs instead of being stored in the cell's secretory granules.[22] Many humans have the α allele in their *TPSAB1* gene.[32–34] Although it is common for a human to have one defective allele in their *TPSAB1* and/or *TPSB2* genes, no hTryptase-β-null human has been identified.[33,34] These genome data support the mouse data, which led to the conclusion that enzymatically active tetramer-forming tryptases normally have beneficial roles in mammals.

Human MCs also express hTryptase-δ and hTryptase-γ/transmembrane tryptase/Prss31, which originate from the respective *TPSD1* and *TPSG1* genes also located on chromosome 16p13.3. hTryptase-γ differs from hTryptase-β in that it is preferentially retained on the outer leaflet of the plasma membrane when MCs degranulate, owing to its unique C-terminal membrane-spanning domain.[35] Because of this feature, hTryptase-γ can regulate those cell types physically contacted in tissues by activated MCs. The substrate preference of recombinant hTryptase-γ[35,36] overlaps that of recombinant hTryptase-β,[1,25] and transgenic mice that lack the orthologues of these human enzymes have reduced inflammation in experimental arthritis, colitis, and chronic obstructive pulmonary disease.[37–40] β-Tryptases are the major enzymatically active proteases stored in the secretory granules of human MCs, and these tetramer-forming enzymes can diffuse away somewhat from activated MCs in tissues, in contrast to membrane-retained hTryptase-γ. It therefore has been proposed that the latter enzyme is less important than the former in the pathogenesis of anaphylaxis.[41]

MOUSE MC TRYPTASES

Mouse chromosome 17A3.3 contains 13 genes that encode tryptic-like serine proteases.[42] MCs express 4 of these enzymes, namely, mouse MC protease

(mMCP)-6/Tpsb2,[43] mMCP-7/Tpsab1,[44] mMCP-11/Prss34,[42] and transmembrane tryptase/tryptase γ/Prss31.[45] Like their human orthologues, mMCP-6/Tpsb2 and mMCP-7/Tpsab1 are tetramer-forming tryptases.[46] The amino acid sequences of BALB/c mMCP-6 and mMCP-7 are 75% identical, but mMCP-6 is more abundant in the constitutive MCs that reside in the peritoneal cavity than mMCP-7.[47] Mouse transmembrane tryptase/tryptase-γ/Prss31 also remains on the outer surface of activated MCs, like its human orthologue.[35,45,48] The connective-tissue MCs in every examined mouse strain express mMCP-6. By contrast, mMCP-7 and Prss31 are expressed in strain-dependent manners in wild-type (WT) mice.[45,49,50] For example, the MCs in BALB/c mice contain very little Prss31, presumably because of a defective promoter, whereas the MCs in C57BL/6 (B6) mice constitutively lack mMCP-7 owing to a splice-site mutation in its gene. However, the MCs in all examined WT mouse strains express at least 2 tryptases. Although mouse MCs and basophils express mMCP-11/Prss34, less is known about this soluble tryptase, as no human orthologue exists.[42,51]

SERGLYCIN PROTEOGLYCANS

The electron-dense secretory granules inside MCs contain protease-resistant SGPGs,[52–55] which contain highly sulfated heparin and/or chondroitin sulfate diB/E GAGs.[56–62] The GAG-attachment region of serglycin, with its Ser-Gly repeated amino acid sequence, cannot be cleaved by any known protease. This feature is biologically significant because the primary function of SGPGs in mammalian MCs is to package large amounts of enzymatically active neutral proteases in the cell's granules in such a way that does not result in appreciable autolysis of the bound proteases.[63–65] A positively charged surface is formed when each MC-restricted protease is properly folded. This face is recognized in the Golgi by the negatively charged GAGs attached to SGPGs.[66–70] The resulting zymogen-SGPG complex is then targeted to the secretory granule, where the removal of the propeptide takes place. SGPGs are therefore essential in the posttranslational processing and storage of histamine and varied peptidases in the secretory granules of MCs.

STRUCTURAL ANALYSIS OF MC TRYPTASES

The catalytic Ser-His-Asp triad of amino acids residing at the center of the active site of trypsin is also present in each MC tryptase. However, mice and human tetramer-forming tryptases have more restricted substrate preferences than trypsin and other serine proteases.[1,25,71,72] A comparison of the primary amino acid sequences of the mature domains of 223-mer trypsin and 245-mer mMCP-7 reveal 7 insertions and 2 deletions in the latter MC enzyme. Because of structural differences in the surface loops that form their active sites, the substrate-binding clefts of mMCP-7, mMCP-6, and hTryptase-β are more restricted than that in trypsin. The crystal structure of hTryptase-β2 also revealed that this serine protease has a unique doughnut-shaped structure.[70] The tetramer unit is formed by the interactions of specific Tyr and Pro residues in 6 loop segments of the monomers. A conserved Trp domain in the folded tryptase monomer is also essential in the formation of the tetramer unit.[46] The active site of each monomer faces toward the 50 Å × 30 Å central pore of the tetramer unit,[70] thereby sterically blocking the active site of each monomer. This structural feature explains why so few large-sized proteins are cleaved by hTryptase-β, mMCP-6, and mMCP-7. The presence of the associated SGPG provides stability to the 3-dimensional structure and also helps to restrict the enzyme's substrate specificity.[72,73] Although heparin-containing SGPGs are necessary to maintain the enzymatic activity

of the hTryptase-β tetramer, homotypic tetramers of enzymatically active mMCP-7 free of SGPGs have been detected in the plasma of V3 mastocytosis mice following systemic anaphylaxis.[69] mMCP-7 is therefore an exception in that it is not dependent on SGPGs once it is exocytosed from activated MCs.[69,71]

BIOACTIVITY OF MC TRYPTASES

Mouse and human tryptases are stored in the cell's acidic secretory granules as mature enzymatically active enzymes. The proteolytic activities of mouse and human tryptases are optimal at neutral pH. The low pH of the secretory granules is another way that MCs hinder the intracellular autolysis of their tryptases. When exocytosed, MC tetramer-forming tryptases cannot be inhibited efficiently by any protease inhibitor present in the normal blood of humans and mice, including α1-antitrypsin and α2-macroglobulin.[69,74]

The BALB/c V3 mastocytosis mouse contains large numbers of mMCP-6$^+$/mMCP-7$^+$ MCs in its spleen and liver.[75] Studies performed on these mastocytosis mice reveal that mMCP-6 and mMCP-7 are packaged in the MC's granules ionically bound to SGPGs.[69] However, after inducing systemic anaphylaxis, much of exocytosed mMCP-7 quickly dissociates from its SGPG in the neutral pH environment. By contrast, exocytosed mMCP-6–SGPG complexes remain intact for hours in the extracellular matrix close to degranulated MCs.

mMCP-7 homotypic tetramers dissociate from SGPGs at neutral pH because the SGPG-binding domain of this tryptase preferentially contains His residues[68] instead of Arg/Lys residues, as occurs in mMCP-6 and in hTryptase-β.[69] The amino acid has a positive charge in the MC's acidic granules, thereby allowing mMCP-7 to ionically recognize negatively charged SGPGs. The loss of this positive charge when mMCP-7 is exocytosed into the neutral pH environment of the extracellular matrix is the structural feature that allows mMCP-7 homotypic tetramers to more easily diffuse away from the activated MCs in sites of inflammation. Thus, substantial amounts of mMCP-7 were found in the circulation 15 minutes after systemic anaphylaxis was induced in V3 mastocytosis mice. The discovery that circulating mMCP-7 was enzymatically active confirmed the in vitro data that showed the resistance of tetramer-forming tryptases to inactivation by circulating protease inhibitors.[69] The retention of exocytosed mMCP-6 in the extracellular matrix close to tissue activated MCs suggests a local action, whereas the rapid dissipation of mMCP-7 suggests a more distant action. Although small amounts of hTryptase-β have been found in human blood after MC activation in systemic anaphylaxis,[11,76] most of this exocytosed enzyme is held in tissues for hours like mMCP-6 ionically bound to heparin SGPGs. Most of these exocytosed tryptase-SGPG macromolecular complexes eventually are endocytosed and proteolytically destroyed in the primary lysosomes of macrophages and other nearby cells in the inflammatory site.[77]

SUBSTRATE PREFERENCES OF MC TRYPTASES

Recombinant BALB/c mMCP-6 and mMCP-7 had similar, but distinct, substrate preferences when tested against Lys/Arg-containing combinational peptide libraries.[71,72] The substrate specificities of recombinant hTryptase-β more closely resembled that of recombinant mMCP-6. Recombinant mMCP-7 preferentially cleaved peptides after an Arg residue in the amino acid sequence Ser-Leu-Ser-Ser-Arg-Gln-Ser.[71] The C-terminus of the α chain of hFibrinogen contains such a sequence. Owing to the inward location of the active sites of the 4 monomers in the tetramer unit, very few candidate proteins are susceptible to tetramer-forming tryptases. The C-terminus of the α chain

of mouse and human fibrinogen are cleaved by mMCP-6, mMCP-7, and hTryptase-β because the exposed susceptible sequence extends out and away from the rest of the precursor plasma protein, thereby allowing it to pass through the central pore of the tetramer unit (Fig. 3).

The mMCP-6–susceptible peptides identified in the phage-display peptide library combinational studies revealed that this tryptase prefers substrates with more Pro, Lys, and/or Arg residues.[72] In contrast to mMCP-7, mMCP-6 and hTryptase-β remain ionically bound to SGPGs for hours after the macromolecular complexes are exocytosed from cutaneous MCs. This finding suggested that SGPGs and their covalently bound GAGs could influence the substrate specificities of their bound tryptases outside of the MC. A phage-display peptide library analysis using mMCP-6–heparin[72] and hTryptase-β–heparin[1,25] complexes showed that these complexes prefer substrates with the sequence Lys/Arg-Pro-X-Lys/Arg, where X may be up to 3 noncharged amino acids. The second Lys/Arg residue in the sequence is the P1 residue where the susceptible peptide is cleaved. Whether mMCP-6–chondroitin sulfate E and hTryptase-β–chondroitin sulfate E complexes have similar substrate preferences have not been evaluated.

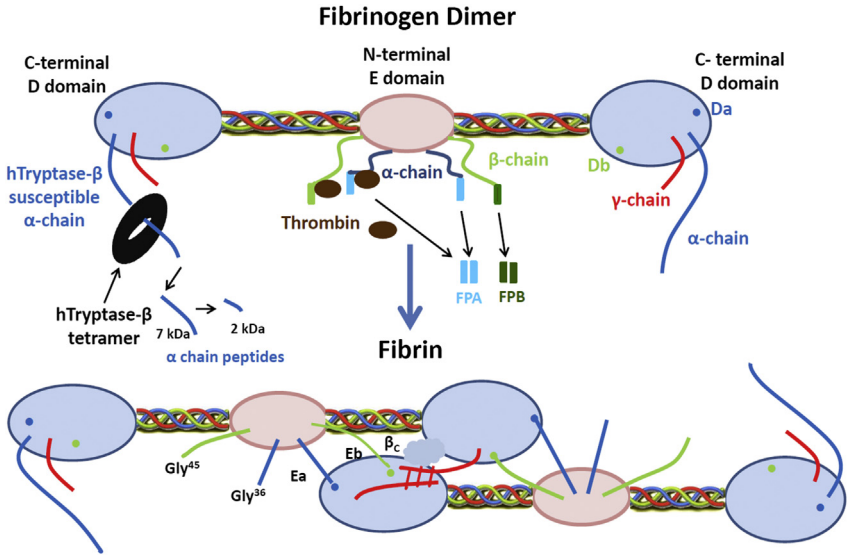

Fig. 3. Model of the structure of fibrinogen and its conversion to fibrin by thrombin, as well as its proteolytic damage by MC-restricted tetramer-forming tryptases. Native fibrinogen exists in blood and plasma as a head-to-head dimer that consists of 2 sets of 3 nonidentical polypeptide chains termed α (*blue*), β (*green*), and γ (*red*), which are joined together in the N-terminal E-domain by disulfide bridges. Each α chain in the dimer contains the inhibitory fibrinopeptide A (FPA, *light blue*) that is susceptible to thrombin (*brown*), which recognizes Arg-Gly sequences in the α and β chains. The proteolytic removal of FPA and then fibrinopeptide B (FPB, *dark green*) from the protein's β chain initiates fibrin assembly by exposing the Ea and Eb sequences in the N-terminal E-domain that recognize the respective Da and Db sequences in the C-terminal D-domain. Factor XIIIa cross-links Lys residues present in the γ chains of adjacent fibrin molecules to form stabilized fibrils. Less clear is the role of the C-terminus of the α chain in the conversion of fibrinogen to fibrin. However, this is the region of fibrinogen that is preferentially cleaved by tetramer-forming tryptases (*black doughnut*), resulting in the generation of a 7-kDa peptide that is further processed to a 2-kDa peptide.

Many in vitro studies have been carried out in attempts to identify those proteins that are preferentially cleaved by mouse and human tetramer-forming tryptases in the presence or absence of heparin. Although candidates have been identified, the data obtained in the in vitro studies carried out in the 1980s and 1990s are difficult to interpret today because the investigators at that time were unaware that their hTryptase-β preparations contained a complex mixture of functionally distinct enzymes (see **Fig. 2**). The possibility of heterotypic tetramers also was not considered, nor was the importance of the cofactor roles of chondroitin sulfate E–containing and heparin-containing SGPGs. Although the physiologic substrates of many of the identified hTryptase-β isoforms (see **Fig. 2**) remain to be determined, the in vitro data of pooled enzyme preparations identified several susceptible substrates, including high molecular weight kininogen, vasoactive intestinal peptide, pro-metalloproteinases, pro-urokinase, fibronectin, and complement factor C3.

THROMBIN-DEPENDENT CONVERSION OF FIBRINOGEN TO FIBRIN

Fibrinogen is the most important component of the coagulation system, and is a 340-kDa glycoprotein synthesized primarily in the liver. Fibrinogen is a major component of plasma, where it circulates at a concentration of 1.5 to 4.0 mg/mL. Because it also is an acute-phase protein, its circulating levels increase significantly in several infections and inflammatory diseases. Fibrinogen circulates in blood as a head-to-head dimer, with 2 external D-domains connected to one central E-domain by a coiled segment (see **Fig. 3**). Each ~170-kDa monomer is composed of 3 nonidentical α, β, and γ chains.[78–80] The γ chain is the largest, and its more extended C-terminus is not cross-linked to the other domains in the 3-chained protein. Conversion of fibrinogen to fibrin during vascular damage is a multistep process (see **Fig. 3**).[78] Initially, thrombin cleaves the N-termini of fibrinogen's α and β chains.[81,82] The newly formed N-terminal ends of these chains interact with complementary sequences in the neighboring fibrinogen molecules, resulting in double-stranded fibrils.[83,84] Fibrils also establish lateral junctions to create multiple-stranded fibrils.[85,86] The development of insoluble fibrin depends on the cross-linking activity of Factor XIIIa, which is a transglutaminase.[87,88] Less clear is the role of the large C-terminal domain of the α chain in the conversion process. Nevertheless, platelets express integrin $\alpha_{2b}\beta_3$, which recognizes fibrin, thereby creating the fibrin-platelet clot. Efficient blood coagulation does not occur when the plasma concentration of fibrinogen is less than 1 mg/mL, if its E- or D-domains are proteolytically damaged, or if the levels of enzymatically active thrombin are low.

HEPARIN-DEPENDENT ANTICOAGULATION

Sodium cromoglycate prevents MC degranulation, and Samoszuk and Corwin[89] noted that tumor-bearing BALB/c mice that were given the drug intraperitoneally contained lakes of blood in and around their tumors. These data and others suggested the presence of a potent anticoagulation factor in the MC's secretory granules. Heparin resides in that intracellular location, and some fragments of this MC-derived GAG and endothelial cell–derived heparan sulfate have anticoagulant activity in vivo (reviewed in Ref.[90]). These anticoagulant oligosaccharides catalyze the antithrombin-III/SERPINC1-dependent inactivation of thrombin by inducing a conformational change in the latter protease inhibitor, which causes the expulsion of residues Gly^{14} and Ser^{15} in the hinge region of the serpin at the base of its reactive-site loop. This change in the tertiary structure of serpin allows the serpin to accelerate the

inactivation of thrombin approximately 3,000-fold, ultimately resulting in the generation of less fibrin and a delayed blood-coagulation time.[91,92]

Commercial low molecular weight oligosaccharides generated from bovine or porcine heparin following a depolymerization step (eg, by exposure to crude heparin preparations to heparin lyase I) are routinely used pharmacologically to hinder blood coagulation in humans. Despite the value of these manufactured preparations, there is very little free heparin glycosaminoglycan or its low molecular weight oligosaccharides in the human body because of the preference of heparin-containing SGPGs for hTryptase-β and the other proteases stored in the secretory granules of human MCs. In addition, approximately 70% of the naturally occurring heparin purified from in vivo differentiated MCs has no anticoagulant activity. It is now believed that the endogenous anticoagulant physiologically relevant GAG at the blood-endothelium interface in the human body is endothelial cell–derived heparan sulfate from syndecan and glycipan proteoglycans rather than MC-derived heparin.[93] In support of this conclusion, MC-deficient[94] and heparin-deficient[63,64] mice do not constitutively have coagulation disorders. It therefore remained to be determined how MCs physiologically prevent fibrin deposition in edema tissue sites when these immune cells degranulate and release their vasopermeability factors.

ANTICOAGULANT ACTIVITY OF MC TRYPTASES

When the V3 mastocytosis BALB/c mouse underwent systemic anaphylaxis, substantial amounts of enzymatically active mMCP-7 was detected in the plasma within 15 minutes.[69] Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and N-terminal sequence analyses of the plasma from these mice revealed the presence of appreciable amounts of peptides that were derived from the C-terminus of the α chain of mFibrinogen. It was then shown that recombinant mMCP-7 was able to cleave the α chain of mouse and human fibrinogen in vitro, even if the digestion reactions were carried out in the presence of the diverse array of protease inhibitors in mouse serum or plasma.[71] These in vitro and in vivo data were unexpected because of the large size of the circulating fibrinogen dimer. The biological significance of the V3 mastocytosis data obtained in the 1990s also was not clear because mMCP-7-deficient WT B6 mice[49,50] constitutively had no defect in fibrinogen metabolism during an MC-dependent anaphylactic reaction.

Samoszuk and colleagues[95] concluded that the anticoagulant activity of the recombinant hTryptase-β–heparin complexes used in their in vitro study probably was due to the heparin component because its activity was inhibited by protamine and heparinase. Nevertheless, in support of the mMCP-7 data, Thomas and colleagues[96] reported that the α chain of hFibrinogen was highly susceptible in vitro to an undefined hTryptase-β in preparations from their human lung biopsies. No in vivo experiment was carried out to determine the physiologic relevance of the data published in this and other in vitro studies. Moreover, recombinant hTryptase-β was not used to eliminate the possibility that the fibrinogen-degrading activity originated from a minor allelic isoform of the *TPSAB1* or *TPSB2* gene (see **Fig. 2**).[97,98] Nevertheless, Thomas and colleagues[96] tentatively concluded that their enzymatic preparation preferentially cleaved the α chain of hFibrinogen at Arg^{591}.

SELECTIVE PROTEOLYSIS OF THE C-TERMINUS OF THE α CHAIN OF HUMAN FIBRINOGEN BY hTRYPTASE-β–HEPARIN COMPLEXES

Prieto-García and colleagues[14] showed that the α chain of hFibrinogen also was preferentially cleaved by a recombinant hTryptase-β–heparin complex in vitro at neutral

pH (Fig. 4). The generated fragment had a slightly lower molecular weight than the native α chain. Using an SDS-PAGE immunoblot approach, it was discovered that proteolysis selectively occurred in the C-terminus of the α chain. Tandem mass spectrometry (MS/MS) and C-terminal sequence analysis of the generated fragment revealed that hTryptase-β cleaved the α chain of hFibrinogen at Lys^{575}, 69 amino acids from its C-terminus.

These data were supported by the identification of a 7-kDa peptide in the SDS-PAGE immunoblot analysis of the digestion reactions. MS/MS analysis of the liberated peptide confirmed that it originated from the C-terminus of the α chain. The finding that the liberated peptide was only weakly recognized by an anti-hFibrinogen antibody in some assays indicated that hTryptase-β–heparin complexes subsequently cleaved the initially formed 69-mer peptide to smaller peptides.

PREFERENTIAL PROTEOLYSIS OF MOUSE FIBRINOGEN'S α CHAIN BY hTRYPTASE-β–HEPARIN, mMCP-6–HEPARIN, AND MMCP-6–SGPG COMPLEXES

In contrast to the results reported in the Prieto-Garcia and colleagues study,[14] Thomas and colleagues[96] concluded that hTryptase-β preferentially cleaved the α chain of hFibrinogen at Arg^{591}. A comparison of the amino acid sequences of the protein expressed in different species revealed that Arg^{591} resides in a 14-mer amino acid sequence that is not present in the corresponding α chain of mFibrinogen. Thus, if proteolysis preferentially occurred in humans at this nonconserved amino acid sequence, the α chain of mFibrinogen could not be cleaved by hTryptase-β or by its murine orthologue mMCP-6. That mFibrinogen was efficiently cleaved by both recombinant hTryptase-β–heparin and mMCP-6–heparin complexes in follow-up studies[14] raised questions as to where mouse and human tetramer-forming tryptases initially cleaved the α chain of fibrinogen.

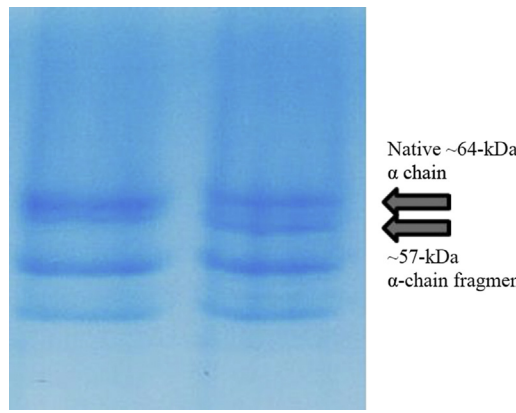

Fig. 4. The α chain of hFibrinogen is a preferred target of hTryptase-β-heparin complexes. hFibrinogen was incubated with a recombinant hTryptase-β-heparin complex, and the resulting digest was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the presence of β-mercaptoethanol to disrupt the protein's intrachain and interchain disulfide bonds. The position of the protein's native α chain and its prominent, slightly smaller fragment in the digest are highlighted. (*Adapted from* Prieto-Garcia A, Zheng D, Adachi R, et al. Mast cell restricted mouse and human tryptase-heparin complexes hinder thrombin-induced coagulation of plasma and the generation of fibrin by proteolytically destroying fibrinogen. J Biol Chem 2012;287(11):7837. © the American Society for Biochemistry and Molecular Biology; with permission.)

hTryptase-β and mMCP-6 are stored in MC secretory granules ionically bound to SGPGs instead of the GAGs used in aforementioned experiments. For this reason, the susceptibility of the α chain of mFibrinogen to the native mMCP-6–SGPG complexes present in interleukin (IL)-3/IL-33–developed mMCP-6^{+}/mMCP-7^{-} mouse bone marrow–derived MCs was evaluated. As occurred with the recombinant mMCP-6–heparin complex, the α chain of mFibrinogen was preferentially cleaved by a heat-sensitive neutral protease present in these cells bound to SGPG.

IN VITRO ANTICOAGULANT ACTIVITY OF mMCP-6–HEPARIN AND hTRYPTASE-β–HEPARIN COMPLEXES

The finding that α chains of mouse and human fibrinogen were rapidly cleaved by mMCP-6–heparin and hTryptase-β–heparin complexes in vitro (even if the digestions were carried out in the presence of serum protease inhibitors) raised the possibility that these MC mediators could hinder thrombin-dependent coagulation of plasma.[14] The clotting-time measurement in a fibrometer of plasma samples before and after incubation with tryptase-heparin complexes showed that the time required by thrombin to clot plasma was markedly delayed when plasma samples were exposed to the human or mouse tryptase-heparin complexes. This delay in the clotting time was longer than that induced by heparin at a dose of 100 μg, much higher than the amount present in the enzymatic complexes (0.5–4.5 μg). It therefore was concluded that the primary anticoagulant factor present in these complexes was the tryptase rather than heparin.

IN VIVO ANTICOAGULANT ACTIVITY OF mMCP-6–HEPARIN SGPG COMPLEXES

To confirm the aforementioned in vitro data, the anticoagulant activity of mMCP-6 was next investigated in vivo.[14] Like WT mice, mMCP-6-null B6 mice underwent a passive cutaneous anaphylaxis (PCA) reaction. However, more fibrin deposits accumulated in the skin of mMCP-6–deficient B6 mice 1 to 6 hours after these animals had been subjected to the PCA reaction than in the skin of the similarly treated WT B6 mice (**Fig. 5**). Thus, mMCP-6 has a critical role in preventing the accumulation of fibrin deposits in tissues when the cutaneous MCs in mice degranulate in response to the PCA reaction. The observation that the α chain of human and mouse fibrinogen were similarly cleaved by recombinant hTryptase-β–heparin complex at neutral pH suggested that the in vivo data from WT mice reflects what occurs in humans at inflammation sites.[14]

Less clear is why tryptase-treated plasma cannot clot efficiently. Loss of the C-terminal 69-mer domain in the fibrinogen's α chain could result in a structural change in those regions of the protein's γ and β chains that are recognized by the N-termini of the α and β chains formed when the precursor protein encounters thrombin. MS/MS and amino acid sequence analyses of hTryptase-β–heparin treated human fibrinogen failed to reveal proteolysis of the N-terminus of the protein's α chain. Nevertheless, the possibility that the enzyme damages the β chain of human fibrinogen was not ruled out. Whatever the mechanism, the MCs in mMCP-6^{-}/mMCP-7^{-} B6 mice have no defect in heparin expression.[4] Thus, the accumulated data suggest the primary way whereby activated MCs physiologically prevent fibrin formation in vivo is through their exocytosed tryptases proteolytically damaging fibrinogen.

CLINICAL IMPLICATIONS

The findings that mMCP-6, mMCP-7, and hTryptase-β are more effective anticoagulants than many heparin preparations have important clinical implications that change

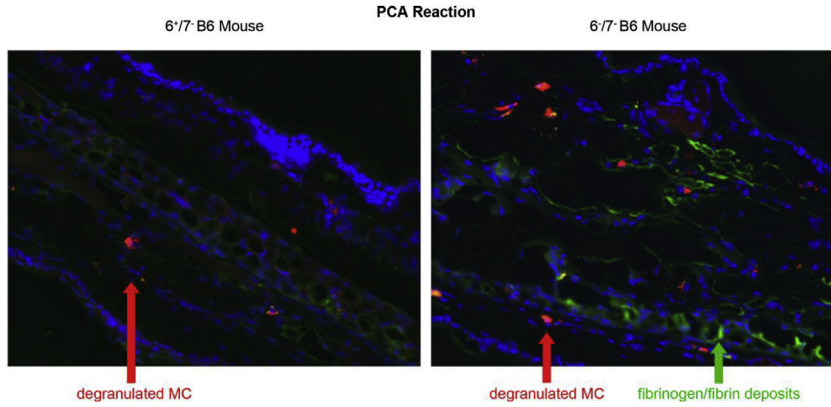

Fig. 5. Fibrin immunohistochemistry after inducing a passive cutaneous anaphylaxis (PCA) reaction. Anti-dinitrophenol (DNP)–immunoglobulin E was injected into the ears of a WT B6 mouse (*left panel*) and an mMCP-6-null B6 mouse (*right panel*). DNP-albumin was then injected into the tail veins of both sensitized animals. Six hours later, the animals were euthanized and processed for immunohistochemistry using fluorescein-labeled antimouse fibrinogen/fibrin antibody (*green color*; *arrow*). The tissue sections also were stained with Hoechst 33342, a dye that binds the DNA in the nucleus of cells (*blue*), as well as with Texas red–labeled avidin (*red color*; *arrows*), which recognizes heparin to identify MCs and their exocytosed protease-heparin complexes. The presence of these complexes outside of the activated MCs and the presence of edema that results from exocytosed histamine confirmed that the cutaneous MCs in both animal populations had degranulated. Six hours after the induction of the PCA reaction, the ear of the mMCP-6-null B6 mouse contained noticeably more fibrin (*green color*) than the corresponding ear of the treated WT B6 mouse. (*From* Prieto-Garcia A, Zheng D, Adachi R, et al. Mast cell restricted mouse and human tryptase-heparin complexes hinder thrombin-induced coagulation of plasma and the generation of fibrin by proteolytically destroying fibrinogen. J Biol Chem 2012;287(11):7842. © the American Society for Biochemistry and Molecular Biology; with permission.)

the paradigm as to how mouse and human MCs prevent blood coagulation and fibrin accumulation. Formation of fibrin deposits and fibrin-platelet clots internally could have devastating consequences in vivo. Thus, these data provide an explanation as to why no hTryptase-β–null human has been identified and why mammals possess 2 genes that encode MC-restricted serine proteases able to recognize fibrinogen. The findings also account for the apparent strong evolutionary pressure to prevent the expression of circulating protease inhibitors that could efficiently inactivate mouse and human tetramer-forming MC tryptases. The accumulated data raise the possibility that an hTryptase-β–null human cannot be born, because of an excess of fibrin deposits and/or fibrin-platelet clots in the developing fetus. Finally, the data explain why some pediatric patients with a diffuse cutaneous form of mastocytosis have excessive bleeding of their skin and gastrointestinal tract,[99,100] why some women have menstrual-like bleeding[101,102] shortly after they experience a severe anaphylactic event, and why some patients with systemic mastocytosis or anaphylactic shock show prolongation of activated partial thromboplastin time or prothrombin time with or without bleeding.[103–108]

FUTURE CONSIDERATIONS

The finding that mMCP-6, mMCP-7, and hTryptase-β are potent anticoagulants raise the possibility that the next generation of tryptase inhibitors that are more specific than

those currently available might be useful in hindering the bleeding abnormalities that sometimes occur in patients with systemic mastocytosis and/or anaphylaxis. The use of the recombinant hTryptase-β–heparin complexes[1,24,109] also might be a more effective way to prevent blood coagulation in the clinic in the prevention and treatment of thromboembolic disease, than by using heparin alone. Finally, the in vivo detection of the fragments released from hTryptase-β digestion of hFibrinogen (eg, the 2–7 kDa peptides derived from the C-terminus of fibrinogen α chain) possibly could be used as a biomarker to identify and evaluate the effectiveness of treatment of patients with mastocytosis (see **Fig. 1**) or the MC activation syndrome.[110–112] However, more investigations are needed to clarify the participation of different allelic isoforms of hTryptase-β in the coagulation system and the compensatory mechanisms that avoid bleeding as a frequent manifestation in mastocytosis and anaphylaxis. The role of MCs as reparative cells in thromboembolic events is another area of future investigation.

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