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GENETIC AND BIOCHEMICAL CHARACTERIZATION OF THE E32DEL POLYMORPHISM IN HUMAN MESOTRYPSINOGEN

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Abstract

BACKGROUND/AIMS. Mesotrypsin is a minor pancreatic digestive enzyme that degrades dietary trypsin inhibitors in the gut. In this study, we tested the hypothesis that the E32del genetic variant of mesotrypsin might represent a risk factor for the development of chronic pancreatitis, as a result of enhanced degradation of pancreatic secretory trypsin inhibitor.

METHODS. We screened 97 German patients with chronic pancreatitis of alcoholic etiology and 109 healthy controls for the presence of the E32del variant and characterized the biochemical properties of E32del-mesotrypsinogen.

RESULTS. Higher allele frequency of the E32del variant was detected in the control population (25.7 % vs. 18.0 %), but the difference was not significant ($p=0.062$). Recombinant mesotrypsin-E32del exhibited normal catalytic activity, characteristic inhibitor-resistance and inability to activate pancreatic zymogens. Degradation of trypsin inhibitors was unaffected by the E32del genotype. Interestingly, mesotrypsinogen-E32del was biochemically distinguishable from mesotrypsinogen by its faster activation with bovine enterokinase, while activation by human enterokinase, trypsin or cathepsin B was unchanged.

CONCLUSION. The results classify mesotrypsinogen-E32del as a frequent polymorphic variant, which is not associated with chronic alcoholic pancreatitis.

Keywords

chronic pancreatitis; alcoholic pancreatitis; trypsinogen mutations; trypsinogen activation; SPINK1

Human mesotrypsinogen was first identified in 1978 and named after its intermediate isoelectric point and electrophoretic mobility by Rinderknecht et al. [1,2]. Compared to cationic and anionic trypsinogen, mesotrypsinogen represents a minor isoform, constituting only 3-10% of the total trypsinogen in human pancreatic juice [2-5]. Mesotrypsin was recognized early on for its remarkable resistance to natural polypeptide trypsin inhibitors, caused by the evolutionary selection of Arg198, which occupies the S2' subsite and sterically clashes with protein inhibitors [1,2,6-8]. Arg198 also interferes with substrate binding and mesotrypsin generally cleaves protein substrates with much lower activity than other trypsins [6-8]. We recently found that mesotrypsin rapidly hydrolyzed the reactive-site peptide-bond of the Kunitz-type soybean

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trypsin inhibitor (SBTI) and completely degraded the Kazal-type pancreatic secretory trypsin inhibitor (SPINK1; serine protease inhibitor Kazal-type 1) [8]. These results indicated that the physiological role of mesotrypsin is digestive degradation of dietary trypsin inhibitors. The observations also raised the possibility that mesotrypsin can contribute to the pathogenesis of pancreatitis by reducing the protective levels of SPINK1 in the pancreas. *In vitro*, mesotrypsinogen is robustly activated by the lysosomal cysteine protease cathepsin B [8], which is responsible for trypsinogen activation in experimental pancreatitis [9,10]. Should mesotrypsinogen get converted to mesotrypsin prematurely in the pancreas, the ensuing degradation of SPINK1 might represent an immediate risk factor for the development of pancreatitis.

To obtain genetic evidence for this model, we have set out to identify mesotrypsinogen mutations that are associated with chronic pancreatitis. Such mutations would be expected to cause increased mesotrypsin activity or stability (gain-of-function mutations). Alternatively, mutations that inactivate mesotrypsin or decrease its proteolytic stability (loss-of-function mutations) are expected to afford protection against chronic pancreatitis. The E32del mesotrypsinogen variant, in which Glu32 is deleted, was first described as the "b-form" of an alternatively spliced mesotrypsinogen cDNA cloned from human brain [11]. The brain cDNA used for cloning was heterozygous for this mutation and genomic sequencing of 2 additional individuals revealed one other heterozygote for E32del. Subsequently, Chen et al. (1999) found a heterozygous E32del carrier in 1 out of 6 hereditary pancreatitis patients tested for mesotrypsinogen mutations [12]. These data suggest that the E32del variant is probably quite common; however, its allele frequency has not been determined and its association with pancreatitis or other diseases has not been investigated. In the present study we compared the biochemical properties of wild-type and E32del-mesotrypsinogen, and measured the frequency of the E32del variant in healthy individuals and patients with alcoholic chronic pancreatitis.

PATIENTS AND METHODS

Patients and controls. The diagnosis of chronic alcoholic pancreatitis was based on the patient's history, and the presence of pancreatic duct dilatation, pancreatic calcifications, pseudocysts and pancreatic insufficiency. After obtaining written informed consent according to the ethical guidelines of the 1975 Declaration of Helsinki as revised in 1983, we investigated the E32del mutation in exon 2 of the *PRSS3* gene (protease, serine, 3 gene, encoding mesotrypsinogen) in 97 patients with chronic pancreatitis of alcoholic origin. As controls, we investigated 109 healthy blood donors. All subjects tested negative for the most frequent *SPINK1* (N34S) and *PRSS1* (N29I and R122H) gene mutations.

Sequencing and genotyping of the *PRSS3*-E32del polymorphism. Leukocyte DNA was extracted from anticoagulated blood specimens using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden/Germany). Exon 2 of the *PRSS3* gene was amplified with gene specific PCR primers Mesofw 5'-GGCTTGTTAAGGATTTCTAATTG-3' and Meso-rev 5'-AAGCCTGCAGGTGGTTTTGAC-3'. The length of the amplified section was 740 bp. To investigate the presence of the E32del polymorphism, PCR fragment length polymorphism analysis was used (Teich et al., 2002). After nested-PCR with the purified primary PCR product and the E32del-fw 5'-TGGGGGCTACACCTGTGAG-3' and E32delrev 5'-CCTGGTAGGGGAGAGAATT-3' primers, which directly flank the E32 codon, polyacrylamide gel electrophoresis was performed (Fig 1A). In case of homozygous wild-type and E32del genotypes, single products of 41 bp and 38 bp length were visible, respectively. In heterozygous samples both bands were detected simultaneously. To confirm the accuracy of the PCR fragment length polymorphism test, several homozygous (E32del n=6; wild-type n=10) and heterozygous (n=10) DNA preparations were sequenced with both the Meso-fw and Meso-rev primers.

Biochemical characterization of mesotrypsinogen. Expression and purification of mesotrypsinogen variants was carried out as described previously [8]. The E32del variant was constructed by oligonucleotide-directed site-specific mutagenesis using the 5'-GGGGAGAGAATTCTCGCAGGTGTATCCTC CAAC-3' antisense mutagenic primer. The *EcoR* I site within the primer sequence is underlined. The PCR product was digested with *Bam*H I and *EcoR* I, and cloned into the pTrap_PRSS3 expression plasmid, harboring the mesotrypsinogen gene under the control of the alkaline phosphatase promoter [8]. Alternatively, the PCR product was digested with *Xba* I and *EcoR* I and cloned into the pTrap-T7_PRSS3 expression plasmid, under the control of the T7 promoter [8]. Recombinant human mesotrypsinogen was expressed in two *E. coli* expression systems: in the SM138/pTrap expression host/plasmid system trypsinogen is constitutively secreted to the periplasmic space, whereas in the Rosetta(DE3)/pTrap-T7 expression system trypsinogen is produced as inclusion bodies which are subsequently solubilized and renatured *in vitro*. The mesotrypsinogen preparations were purified by ecotin-affinity chromatography and the concentrations of mesotrypsinogen solutions were calculated from the ultraviolet absorbance at 280 nm, using a theoretical extinction coefficient of $40,570 \text{ M}^{-1} \text{ cm}^{-1}$. The mesotrypsinogen preparations derived from the two bacterial expression systems were indistinguishable in their functional properties studied here. Biochemical assays and gel analyses were performed as reported by Szmola et al. (2003) in [8].

RESULTS

Genetic characterization of the E32del mesotrypsinogen variant. The E32del mutation was detected with 100% accuracy with the PCR fragment length polymorphism method; as judged by direct DNA sequencing of 26 PCR products. The E32del variant was present heterozygously in 35 of the 97 pancreatitis patients tested, whereas in the control group we found 6 homozygous and 44 heterozygous E32del carriers (Fig 1B). A two-tailed chi-square test revealed no statistically significant difference in the genotype distribution between patients and controls carrying the E32del variant (36.1 % vs 45.9 %; $\chi^2=2.03$, $df=1$, $p=0.154$). The difference in overall allelic frequencies was not significant either (E32del allele frequency for patients 18.0%, for controls 25.7%, $p=0.062$). The results of the genetic analysis clearly indicate that the presence of the E32del mesotrypsinogen variant is not a risk factor for alcoholic chronic pancreatitis.

Biochemical characterization of E32del mesotrypsinogen. To complement the genetic analysis, biochemical properties of recombinant E32del-mesotrypsinogen were also investigated.

Both wild-type and E32del-mesotrypsinogen were readily activated by enterokinase (enteropeptidase). Interestingly, compared to wild-type mesotrypsinogen, the E32del form was more rapidly activated by bovine enterokinase (Fig 2A) or its catalytic subunit (not shown). Human enterokinase activated both mesotrypsinogen forms 5-10-fold better (note the lower concentrations used), however, no difference was detected between the activation of wild-type and E32del-mesotrypsinogen (Fig 2A). Human cationic and anionic trypsin activated wild-type and E32del-mesotrypsinogen in an indistinguishable manner (Fig 2B). Finally, activation of E32del-mesotrypsinogen by cathepsin B showed the previously observed biphasic time-course [8]; which did not differ from that of wild-type mesotrypsinogen (Fig 2C).

The catalytic activity of the two mesotrypsin forms was measured by the cleavage of the *N*-CBZ-Gly-Pro-Arg-p-nitroanilide synthetic substrate. The determined K_M and k_{cat} values were essentially identical (mesotrypsin K_M $21 \pm 1 \mu\text{M}$; k_{cat} $123 \pm 2 \text{ s}^{-1}$; E32del-mesotrypsin K_M $19 \pm 0.9 \mu\text{M}$; k_{cat} $120 \pm 2 \text{ s}^{-1}$). Wild-type mesotrypsin did not activate chymotrypsinogen and E32del-mesotrypsin was similarly defective (not shown). Both mesotrypsin forms exhibited

characteristic resistance to soybean trypsin inhibitor or human SPINK1, with no measurable difference (Fig 3). Similarly, digestion of trypsin inhibitors was unaltered by the E32del mutation. Thus, wild-type and E32del-mesotrypsin cleaved the reactive-site peptide-bond of SBTI (Fig 4) and degraded SPINK1 (not shown) in a comparable fashion (see also Figs 6 and 7 in ref [8]).

Finally, the effect of the E32del mutation on the proteolytic stability of mesotrypsinogen was tested. Degradation of wild-type and E32del-mesotrypsinogen (2 μ M) by human cationic trypsin (0.2 μ M) at 37 °C, in 0.1 M Tris-HCl (pH 8.0) and 2 mM K-EDTA, exhibited comparable half-lives of 2.5 min and 3.5 min, respectively.

DISCUSSION

We recently proposed that human mesotrypsin might contribute to the development of pancreatitis through degradation of protective SPINK1 in the pancreas. Identification of genetic variants of mesotrypsinogen that are associated with chronic pancreatitis might lend support for this theory. The present study is such an attempt, where we report the genetic and biochemical characteristics of the E32del mesotrypsinogen variant.

First, we determined allelic frequency of the E32del variant in healthy blood donors, and found that E32del is a common genetic polymorphism (25.7 % allele frequency). Next, we have screened patients with alcoholic chronic pancreatitis for the presence of the E32del variant. The genetics of chronic alcoholic pancreatitis is poorly understood. On the one hand, only about 10 % of chronic alcoholics develop pancreatitis, suggesting a role for underlying genetic susceptibility. On the other hand, the “alcoholic pancreatitis gene” has escaped detection thus far, raising the possibility that multiple genes with relatively small individual contributions might determine overall susceptibility for the disease [13 and references therein]. In this regard, the N34S mutation of the *SPINK1* gene has been recently shown to increase the risk of pancreatitis in alcoholics approximately 3-fold [14,15]. Because mesotrypsin degrades SPINK1, it seemed reasonable to speculate that mutant mesotrypsin alleles might also confer some degree of susceptibility to alcoholic pancreatitis. However, we found that allele frequency of the E32del mesotrypsinogen variant in chronic pancreatitis patients was not significantly different (18 %; $p=0.062$) than in controls, indicating that the E32del allele is not a susceptibility gene for alcoholic pancreatitis.

Biochemical analysis of the E32del mesotrypsinogen variant provided further support for the conclusion of the genetic studies. The results indicated that E32del is a functionally innocuous mutation, which did not affect the catalytic properties, inhibitor resistance and inhibitor-digesting ability of mesotrypsin. Activation of mesotrypsinogen by human enterokinase, trypsin, or cathepsin B was also unaffected by the E32del mutation. Finally, degradation of wild-type and E32del-mesotrypsinogen by human trypsins were comparable.

Among the human trypsinogen genes, the E32del variant has been observed only in mesotrypsinogen. Interestingly, it is also present in the recently released genomic sequence of the chimpanzee (<http://www.genome.wustl.edu/projects/chimp>). It is unclear at this time whether the E32del is the only mesotrypsinogen variant present in the ape or the chimpanzee *PRSS3* gene is also polymorphic. In any event, the E32del variation in mesotrypsinogen appears to be highly primate-specific, because it is absent in all other vertebrate trypsinogens, which strictly contain 19 amino-acid residues between Cys30 and Cys48 in the primary amino-acid sequence (mesotrypsinogen numbering, starting from Met1).

In summary, the genetic and biochemical data are in perfect agreement and classify E32del-mesotrypsinogen as a frequent polymorphic variant, which does not predispose to chronic alcoholic pancreatitis.

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REFERENCES

1. Rinderknecht H, Renner IG, Carmack C, Friedman R, Koyama P. A new protease in human pancreatic juice. *Clin Res* 1978;26:112A.
2. Rinderknecht H, Renner IG, Abramson SB, Carmack C. Mesotrypsin: a new inhibitor-resistant protease from a zymogen in human pancreatic tissue and fluid. *Gastroenterology* 1984;86:681–692. [PubMed: 6698368]
3. Scheele G, Bartelt D, Bieger W. Characterization of human exocrine pancreatic proteins by twodimensional isoelectric focusing/sodium dodecyl sulfate gel electrophoresis. *Gastroenterology* 1981;80:461–473. [PubMed: 6969677]
4. Rinderknecht H, Renner IG, Carmack C. Trypsinogen variants in pancreatic juice of healthy volunteers, chronic alcoholics and patients with pancreatitis and cancer of the pancreas. *Gut* 1979;20:886–891. [PubMed: 533700]
5. Rinderknecht H, Stace NH, Renner IG. Effects of chronic alcohol abuse on exocrine pancreatic secretion in man. *Dig Dis Sci* 1985;30:65–71. [PubMed: 3965275]
6. Nyaruhucha CNM, Kito M, Fukuoka SI. Identification and expression of the cDNA-encoding human mesotrypsin(ogen), an isoform of trypsin with inhibitor resistance. *J Biol Chem* 1997;272:10573–10578. [PubMed: 9099703]
7. Katona G, Berglund GI, Hajdu J, Gráf L, Szilágyi L. Crystal structure reveals basis for the inhibitor resistance of human brain trypsin. *J Mol Biol* 2002;315:1209–1218. [PubMed: 11827488]
8. Szmola R, Kukor Z, Sahin-Tóth M. Human mesotrypsin is a unique digestive protease specialized for the degradation of trypsin inhibitors. *J Biol Chem* 2003;278:48580–48589. [PubMed: 14507909]
9. Saluja AK, Donovan EA, Yamanaka K, Yamaguchi Y, Hofbauer B, Steer ML. Cerulein-induced in vitro activation of trypsinogen in rat pancreatic acini is mediated by cathepsin B. *Gastroenterology* 1997;113:304–310. [PubMed: 9207291]
10. Halangk W, Lerch MM, Brandt-Nedelev B, Roth W, Ruthenbuerger M, Reinheckel T, Domschke W, Lippert H, Peters C, Deussing J. Role of cathepsin B in intracellular trypsinogen activation and the onset of acute pancreatitis. *J Clin Invest* 2000;106:773–781. [PubMed: 10995788]
11. Wiegand U, Corbach S, Minn A, Kang J, Müller-Hill B. Cloning of the cDNA encoding human brain trypsinogen and characterization of its product. *Gene* 1993;136:167–75. [PubMed: 8294000]
12. Chen J-M, Audrezet M-P, Mercier B, Quere I, Férec C. Exclusion of anionic trypsinogen and mesotrypsinogen involvement in hereditary pancreatitis without cationic trypsinogen gene mutations. *Scand J Gastroenterol* 1999;34:831–832. [PubMed: 10499487]
13. Whitcomb DC. Genetic predisposition to alcoholic pancreatitis. *Pancreas* 2003;27:321–326. [PubMed: 14576495]
14. Witt H, Luck W, Becker M, Bohmig M, Kage A, Truninger K, Ammann RW, O'Reilly D, Kingsnorth A, Schulz HU, Halangk W, Kielstein V, Knoefel WT, Teich N, Keim V. Mutation in the SPINK1 trypsin inhibitor gene, alcohol use, and chronic pancreatitis. *JAMA* 2001;285:2716–2717. [PubMed: 11386926]
15. Schneider A, Pfützer RH, Barmada MM, Slivka A, Martin J, Whitcomb DC. Limited contribution of the SPINK1 N34S mutation to the risk and severity of alcoholic chronic pancreatitis: a report from the United States. *Dig Dis Sci* 2003;48:1110–1115. [PubMed: 12822871]
16. Laskowski M Jr, Qasim MA. What can the structures of enzyme-inhibitor complexes tell us about the structures of enzyme substrate complexes? *Biochim Biophys Acta* 2000;1477:324–337. [PubMed: 10708867]

Figure 1.

Panel **A**. PCR fragment length polymorphism analysis. Two primers, which directly flank the E32 codon, were used for nested PCR. The products were separated on a polyacrylamide gel and stained with ethidium bromide. Homozygous wild-type samples yielded a single 41 bp PCR product (lanes 3, 4 and 8). In case of homozygous E32del genotype, one single product of 38 bp length was visible (lane 7). In heterozygous samples, both bands were detected simultaneously (lanes 2, 5 and 6). Lane 1, molecular weight markers. Panel **B**. Relative distribution of *PRSS3* genotypes in alcoholic pancreatitis patients (n=97) and controls (n=109). The actual numbers are indicated above the white (controls) and grey (patients) columns. Abbreviations: del=E32del; wt=wild-type.

Figure 2.

Activation of wild-type (PRSS3, empty symbols) and E32del-mesotrypsinogen (E32del, solid symbols) by enterokinase, human trypsins and cathepsin B. Aliquots (1 μ L) were withdrawn from the incubation mixtures at the indicated times and trypsin activity was measured with the synthetic substrate N-CBZ-Gly-Pro-Arg-p-nitroanilide. Trypsin activity was expressed as percentage of potential maximal activity. Panel **A**. Mesotrypsinogen samples at 2 μ M final concentration were incubated with a final concentration of 400 ng/mL bovine enterokinase (Biozyme Laboratories, San Diego, California) or 50 ng/mL human enterokinase (R & D Systems, Minneapolis, Minnesota) at 37 °C, in 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl₂ and 2 mg/mL bovine serum albumin. Although not shown, increased activation of E32del-mesotrypsinogen was also observed with the small catalytic subunit of bovine enterokinase (Invitrogen, Carlsbad, California). Panel **B**. Mesotrypsinogen (2 μ M) was activated with a final concentration of 0.2 μ M human cationic trypsin (cationic) or anionic trypsin (anionic) at 37 °C in 0.1 M Tris-HCl (pH 8.0), 2 mg/mL bovine serum albumin and 1 mM or 2.2 mM CaCl₂, respectively. Note that because of the higher Ca²⁺ concentration, the activation of mesotrypsinogen by anionic trypsin is more significant than previously observed [8]. Panel

C. Wild-type and E32del-mesotrypsinogen were activated at 2 μ M concentration with recombinant human cathepsin B (90 μ g/mL; generous gift from Paul M. Steed, Novartis Pharmaceuticals, Summit, New Jersey) at 37 °C in 0.1 M Na-acetate buffer (pH 4.0) in the presence of 1 mM dithiothreitol, 2 mg/mL bovine serum albumin, 1 mM K-EDTA, and 300 μ M benzamidine.

Figure 3.

Inhibition of human cationic trypsin (PRSS1), wild-type and E32del mesotrypsin (PRSS3) with soybean trypsin inhibitor (SBTI, **panel A**) and human pancreatic secretory trypsin inhibitor (SPINK1, **panel B**). Trypsins were incubated with the indicated concentrations of inhibitors at 22 °C in 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl_2 and 1 mg/mL bovine serum albumin for 10 min, in a volume of 100 μL . Residual trypsin activity was then determined with N-CBZ-Gly-Pro-Arg-p-nitroanilide. Cationic trypsin and mesotrypsin concentrations were 20 nM and 10 nM, respectively.

Figure 4.

Rapid cleavage of the reactive-site peptide-bond of SBTI by mesotrypsin. Panel **A**. SBTI (1.2 μM final concentration) was incubated with 10 nM concentration of human cationic trypsin (PRSS1), anionic trypsin (PRSS2), wild-type mesotrypsin (PRSS3) or E32del-mesotrypsin (PRSS3-E32del). As an additional control, bovine enterokinase used for the activation of the trypsinogens was also tested at 50 ng/mL final concentration. Incubations were performed for 20 min at 37 °C in 0.1 M Tris-HCl (pH 8.0) and 1 mM CaCl₂. Panel **B**. Comparative time-courses of SBTI (1.2 μM final concentration) cleavage by 10 nM wild-type mesotrypsin (PRSS3) and E32del-mesotrypsin (PRSS3-E32del). Samples were precipitated with 10 % trichloroacetic acid (final concentration), and subjected to reducing SDS-PAGE and Coomassie blue staining. Note that mesotrypsin-mediated cleavage of SBTI does not proceed to completion, but an equilibrium is established between the intact and cleaved forms, which is characteristic of canonical protease inhibitors [8,16]