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Biochemical models of hereditary pancreatitis

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Abstract

The past decade has witnessed remarkable progress in the genetics of chronic pancreatitis. Mutations of the *PRSS1* gene encoding cationic trypsinogen and the *SPINK1* gene encoding pancreatic secretory trypsin inhibitor were found in association with hereditary, familial or sporadic chronic pancreatitis; and the genotype - phenotype correlations have been characterized at the clinical level. Despite these accomplishments, our understanding of the molecular mechanism(s) through which PRSS1 and SPINK1 mutations cause chronic pancreatitis has remained sketchy. Pancreatitis-associated gene mutations are believed to result in uncontrolled trypsin activity in the pancreas. Thus, PRSS1 mutations would cause a gain of (trypsin) function, while SPINK1 mutations would result in the loss of a (trypsin inhibitor) function. However, experimental identification of the disease-relevant functional alterations caused by PRSS1 or SPINK1 mutations proved to be challenging, as results of biochemical analyses lent themselves to different interpretations. The present review focuses on PRSS1 mutations and summarizes the salient biochemical findings in the context of the mechanistic models that attempt to explain the connection between mutations and hereditary pancreatitis.

Human Trypsinogens and Pancreatitis-Associated Trypsinogen Mutations

The human pancreas produces the digestive pro-enzyme trypsinogen in three isoforms. On the basis of their relative isoelectric points and electrophoretic mobility, these are commonly referred to as cationic trypsinogen, anionic trypsinogen, and mesotrypsinogen. The isoenzymes are encoded by separate genes, the *PRSS1* (protease, serine, 1), *PRSS2* and *PRSS3* genes (for a recent review see [1] and references therein). Cationic trypsinogen (PRSS1) and anionic trypsinogen (PRSS2) make up the bulk of secreted trypsinogens in the pancreatic juice, while mesotrypsinogen (PRSS3) accounts for 2–10 % [2–6]. Typically, there is approximately twice as much cationic trypsinogen as anionic trypsinogen, but this ratio is reversed in chronic alcoholism or chronic pancreatitis [3,5]. The significance of the “isoform reversal” is unknown [7]. Human trypsinogens are synthesized as pre-pro-enzymes with a signal peptide of 15 amino acids, followed by the 8 amino acid long pro-peptide, the trypsinogen activation peptide. The signal-peptide is removed upon entry into the endoplasmic reticulum lumen and the pro-enzymes are packaged into zymogen granules and eventually secreted into the pancreatic juice. Physiological activation of trypsinogen to trypsin takes place in the duodenum by enteropeptidase (enterokinase), a highly specialized serine protease in the brush-border membrane of enterocytes. Trypsin can also activate trypsinogen, a process termed autoactivation, which in the duodenum may have a physiological role in facilitating zymogen activation, whereas inappropriate autoactivation in the pancreas might cause pancreatitis. Mutations in the *PRSS1* gene have been identified in patients with hereditary pancreatitis, familial or sporadic chronic pancreatitis (Table 1). In contrast, genetic variants of anionic trypsinogen (PRSS2) or mesotrypsinogen (PRSS3) have not been found in association with

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chronic pancreatitis [8–10]. To date, 22 *PRSS1* gene variants have been described, which affect 18 different amino-acid positions in trypsinogen, and result in 20 different amino-acid substitutions. When classified according to frequency of occurrence, R122H (~70 %), N29I (~25 %) and A16V (~4 %) are the three most prevalent mutations [11–14], whereas the large majority of genetic variants have been identified in very few patients or families (Table 1). Interestingly, the mutations are located in the N-terminal half of trypsinogen, and are clustered in the activation peptide (between Ala16 and Lys23), in the very N-terminal part of trypsin (between Asn29 and Val39) or in the longest peptide segment not stabilized by disulfide bonds (between Glu79 and Cys139).

Use of Recombinant Trypsinogen to Study Pancreatitis-Associated Mutations

In order to elucidate how mutations in cationic trypsinogen cause pancreatitis, biochemical analysis of the mutant proteins is essential. Pancreatic tissue or juice is not readily available in significant amounts from patients carrying PRSS1 mutations. In addition to limited availability, isolation of the heterozygously expressed mutant trypsinogen from the complex protein mixture poses another technical challenge. Therefore, heterologous recombinant expression has been the method of choice to generate wild-type and mutant trypsinogen proteins for biochemical analysis. The biochemical data discussed in this review were obtained on recombinant trypsinogen preparations expressed in *Escherichia coli* as denatured inclusion bodies, which were subsequently re-natured through an in vitro refolding procedure [15–17]. Re-folded trypsinogen was purified to homogeneity by affinity chromatography using immobilized ecotin [18]. Ecotin is a protease inhibitor with broad specificity isolated from the periplasm of *E. coli*. Cationic trypsin preparations produced recombinantly or isolated from pancreatic juice exhibit essentially identical catalytic activity, indicating that recombinant trypsinogen is a suitable experimental alternative to its native counterpart. However, subtle structural differences do exist between recombinant and native preparations, due to different post-translational processing in *E. coli* and pancreatic acinar cells. In *E. coli* cytoplasmic aminopeptidases can trim the N terminus of recombinant trypsinogens, whereas in human acinar cells trypsinogens get post-translationally modified on Tyr154. This modification was originally described as phosphorylation [19], but more recent data suggest that the modifying group is a sulfate [M. S.-T., unpublished observation]. Post-translational sulfation is absent in *E. coli*. Whether or not these structural differences between native and recombinant trypsinogen influence the effect of pancreatitis-associated mutations remains to be determined.

Phenotypic Alterations Caused by Pancreatitis-Associated Mutations

The biochemical investigations reviewed here examined the following aspects of trypsinogen function. (1) Trypsin activity, typically measured on the small peptide substrate N-CBZ-Gly-Pro-Arg-p-nitroanilide. (2) Trypsinogen activation by trypsin (autoactivation). (3) Trypsinogen activation by cathepsin B. (4) Auto-catalytic degradation of trypsin (autolysis). (5) Trypsin inhibition by SPINK1 (pancreatic secretory trypsin inhibitor). As listed in Table 2, trypsin activity or inhibitor binding was not affected by the mutations and the most frequently and consistently found phenotypic change was an increased propensity for autoactivation. Increased trypsin stability (i.e. decreased autolysis) was also observed with 3 mutations. Interestingly, cathepsin B mediated activation was either unaltered or decreased, indicating that increased susceptibility to cathepsin B does not play a role in hereditary pancreatitis. The interpretation of the biochemical data on the R122C mutant remains controversial, as discussed below.

From Biochemical Analysis of Mutant Proteins to Disease Mechanisms

There are three assumptions which guide the interpretation of experimental data on pancreatitis-associated mutations. (i) ***Mutations in the PRSS1 gene are the causative agents or risk factors of chronic pancreatitis***. This assumption is widely accepted today as fact and the association of the studied mutations with chronic pancreatitis is well documented by a decade of genetic investigation. However, in the strict sense, this notion only holds true for the relatively frequent mutations, where disease association could be convincingly established. In fact, for the majority of the reported mutations, the limited sample size did not allow a conclusive genetic analysis and we use inference to posit that these mutations have a causative relationship with chronic pancreatitis. (ii) ***The biochemical alterations discovered through analyzing recombinant trypsinogen mutants are relevant to the disease-mechanism***. Trypsinogen is highly amenable to in vitro biochemical investigations in which catalytic activity, activation and degradation can be studied on pure, homogenous preparations either from recombinant or native source. To date, these studies are the only source of functional information regarding the effect of the PRSS1 mutations and we rely on these data in our attempt to formulate a pathomechanistic model. On the other hand, it is important to remember that the mutations can potentially cause defects in the synthesis, intracellular transport or secretion of trypsinogen, and these cell-biological aspects have not been studied yet. (iii) ***There is a common mechanism through which the various mutations cause chronic pancreatitis.*** Consolidation of the sometimes conflicting biochemical data into a unifying model requires value judgments, which are based on the premise that a common pathogenic pathway exists. Although it remains formally possible that a number of different, mutation-specific mechanisms are responsible for hereditary pancreatitis, such a scenario seems unlikely. A logical and wishful extension of this assumption is that biochemical models developed through analysis of hereditary pancreatitis are widely applicable to all forms of chronic pancreatitis in humans. Nonetheless, some of the models reviewed below do not conform to this notion and imply multiple mechanisms in hereditary pancreatitis.

Model #1. Increased Trypsin Stability Causes Hereditary Pancreatitis (Whitcomb, 1996)

The R122H mutation was the first cationic trypsinogen variant identified by David Whitcomb in 1996 and still remains the most frequently found genetic alteration associated with hereditary pancreatitis [20]. To explain why the R122H mutation might cause pancreatitis, Whitcomb proposed that the Arg122-Val123 autolytic peptide bond in trypsin plays an important role in the degradation of prematurely activated trypsin in the pancreas. Destruction of this “failsafe mechanism” by the R122H mutation would increase intrapancreatic trypsin activity and eventually precipitate pancreatitis. Halangk et al. demonstrated that autodegradation of trypsin mitigates cathepsin B-mediated trypsinogen activation during cerulein-induced zymogen activation in isolated rat acini, suggesting that an autolytic failsafe mechanism might be operational in the mammalian pancreas [21]. Biochemical evidence supports the notion that Arg122 is important for autolysis of trypsin [15,16,22–26] and mutations of this amino-acid result in increased trypsin stability [16,23,26]. Because of its increased autolytic stability, R122H-trypsin was sometimes referred to as “supertrypsin”. However, more detailed biochemical analysis indicated that the R122H mutation results not only in increased trypsin stability [16] but also in increased zymogen stability [25,26] and increased autoactivation [16]. A weak trypsin-inhibitory activity associated with the Arg122 site is also lost in the R122H mutant [26]. Thus, the pleiotropic biochemical effect of R122H raises the possibility that the pathogenic alteration is unrelated to trypsin stability. More importantly, the model fails to explain how the other pancreatitis-associated PRSS1 mutations might work, as the majority of these do not affect trypsin stability (Table 2).

Model #2. Enhanced Trypsinogen Autoactivation is a Common Pathomechanism in Hereditary Pancreatitis (Sahin-Tóth and Tóth, 2000)

Indubitably, the most obvious limitation of the "supertrypsin model" was that it failed to offer a plausible and experimentally supported explanation for the mechanism of action of the N29I mutation. The N29I mutation is the 2nd most frequent PRSS1 variant that causes autosomal dominant hereditary pancreatitis with clinical and genetic features that are practically indistinguishable from those of the R122H-associated cases [11–13;27–29]. Therefore, it is very likely that the underlying pathomechanisms are identical. Biochemical characterization of the N29I mutation using recombinant trypsinogen found no effect whatsoever on trypsin or trypsinogen stability [15–17,24]. On the other hand, moderately increased autoactivation was observed by two independent laboratories in a handful of published studies [15–17,30]. Because increased autoactivation was observed with the R122H, N29I and N29T mutations, whereas N29I had no effect on trypsin stability, the logical conclusion was put forth that enhanced autoactivation is the common pathogenic mechanism of hereditary pancreatitis associated PRSS1 mutations [16]. This conclusion received very strong support from the analysis of a subset of mutations that alter the activation peptide [31,32]. Conceptually, these activation peptide mutations are very important, because they do not affect trypsin structure or function, indicating that pancreatitis-associated mutations exert their effect through altering the properties of the pro-enzyme trypsinogen and not the active enzyme trypsin. In this context, properties of the characterized mutants D19A, D22G and K23R confirmed that increased autoactivation of cationic trypsinogen is a relevant pathomechanism in hereditary pancreatitis [31,32]. To date, only mutation E79K seems to contradict this model, which was shown to decrease autoactivation significantly [33]. On the other hand, this mutant exhibited enhanced trans-activation of anionic trypsinogen.

Model #3. Cathepsin B is a Pathological Trypsinogen Activator in Hereditary Pancreatitis (Szilágyi, 2001)

A large body of data from various animal models of experimental pancreatitis indicates that one of the early events in pancreatitis is the intra-acinar premature activation of trypsinogen, and this process appears to be mediated by the lysosomal cysteine-protease cathepsin B [34 and references therein]. Intuitively, pancreatitis-associated mutations might cause pancreatitis by rendering trypsinogen more susceptible to activation by cathepsin B. This hypothesis was first described by Szilágyi et al. who found that the N29I mutant is activated faster by cathepsin B than wild-type trypsinogen [17]. A subsequent study, however, did not confirm this finding and demonstrated that activation of mutants N29I, N29T and R122H by cathepsin B is unaltered relative to wild-type trypsinogen [35]. Similarly, activation of mutants E79K, R122C or the double mutant N29I + N54S was unchanged [30,33,36]. Finally, experiments using synthetic analogs of the trypsinogen activation peptide revealed that activation of the D22G and K23R mutants by cathepsin B was actually decreased [37]. These results were also confirmed using full-length recombinant trypsinogens (M. S.-T., unpublished observations). Taken together, the evidence is overwhelming that increased activation of mutant trypsinogens by cathepsin B does not play a role in hereditary pancreatitis.

Model #4. Loss of Trypsin Function in Hereditary Pancreatitis (Lerch, 2002)

Mutation R122C was reported in 2001–2002 by three independent groups [36,38,39]. The recombinantly expressed R122C mutant preparation exhibited reduced activity, autolysis and autoactivation. This apparent loss of trypsin function was due to in vitro misfolding caused by the introduction of the extra unpaired cysteine. On the other hand, when the data were normalized to the active trypsinogen fraction, the biochemical phenotype became very similar

to that of R122H, exhibiting increased trypsin stability and increased autoactivation [36]. The complex properties of the R122C mutant were first interpreted as an example of a loss-of-function PRSS1 mutation associated with hereditary pancreatitis, suggesting that a trypsinogen mutation could cause pancreatitis by eliminating a trypsin-dependent protective mechanism [36]. Additional support for this hypothesis came from earlier studies indicating that trypsin autodegradation might play a protective role in cerulein-induced zymogen activation [21]. Although this model is provocative, the bulk of results from *in vitro* experiments using recombinantly expressed trypsinogen mutants remains inconsistent with it, as a loss of function could not be demonstrated for the large majority of mutations (Table 2).

Model #5. Chronic SPINK1 Depletion in Hereditary Pancreatitis (Sahin-Tóth, 2005)

The SPINK1-deficit model, first presented at the 5th International Symposium on the Inherited Diseases of the Pancreas, in Graz, Austria, is an extension of the autoactivation hypothesis stating that increased autoactivation is the common pathogenic change in mutant trypsinogens. We propose that because human cationic trypsinogen has an unusually high propensity for autoactivation, even at acidic pH, constant “physiological” autoactivation occurs during transit in the secretory pathway [see discussion in 40, 41]. Normally, this process is controlled by SPINK1, resulting in some degree of SPINK1 consumption. An increase in autoactivation induced by trypsinogen mutations would result in chronic depletion of SPINK1 and the resulting SPINK1 deficit would render the pancreas vulnerable to acute insults. In other words, trypsinogen mutations would exert their effect indirectly, via decreasing protective SPINK1 levels. It is noteworthy that within this model, loss-of-function mutations in the *SPINK1* gene or gain-of-function mutations in the *PRSS1* gene would have a similar impact on SPINK1 levels. There is some genetic evidence in support of this notion, as rare SPINK1 mutations that clearly cause loss of expression from the diseased allele seem to be associated with hereditary pancreatitis [42]. Finally, it appears likely that SPINK1 has a so far uncharacterized protective function in the pancreas, as homozygous genetic deletion of its ortholog in mice results in autophagic destruction of acinar cells [43]. It is intriguing to speculate that impairment of this novel function could play a role in hereditary pancreatitis.

SUMMARY

Association between mutations in cationic trypsinogen and hereditary pancreatitis has been convincingly documented. Biochemical analysis of the mutation-induced effects revealed a multifaceted picture, which resulted in the formulation of multiple pathomechanistic models. Extension of the biochemical studies to cellular and animal models is mandatory.

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Table 1

Pancretitis-associated genetic variants of human cationic trypsinogen. Only the documented affected carriers are listed; consult the appropriate references for more detailed pedigrees. For references see the Database of Trypsinogen and SPINK1 Variants at the Universität Leipzig ("http://www.uni-leipzig.de/pancreasmutation/").

Mutation	Frequency
−28delTCC	1 affected carrier
A16V	>25 affected carriers
D19A	1 affected carrier
D22G	2 affected carriers
K23R	2 affected carriers
N29I	>160 affected carriers
N29I+N54S	1 affected carrier
N29T	3 affected carriers
P36R	1 affected carrier
V39A	7 affected carriers
E79K	8 affected carriers
G83E	1 affected carrier
K92N	1 affected carrier
D100H	1 affected carrier
L104P	1 affected carrier
R116C	8 affected carriers
A121T	1 affected carrier
R122H (CAC)	>500 affected carriers
R122H (CAT)	3 affected carriers
R122C	5 affected carriers
V123M	1 affected carrier
C139F	1 affected carrier

Table 2

Functional properties of pancreatitis-associated cationic trypsinogen mutants. N.D. not determined. SPINK1, pancreatic secretory trypsin inhibitor. The R122C mutant exhibited different phenotypes depending on how trypsinogen concentrations were determined in the recombinant preparations. See text and references for experimental details.

	Trypsin activity	Autoactivation of trypsinogen	Activation by cathepsin B	Trypsin autolysis	SPINK1 binding	References
D19A	normal	increased	normal	normal	normal	[32,41]
D22G	normal	increased	decreased	normal	normal	[31,32,37]
K23R	normal	increased	decreased	normal	normal	[31,32,37]
N29I	normal	increased	normal	normal	normal	[15,16,17, 24,30,35]
N29I +N54S	normal	increased	normal	normal	normal	[30]
N29T	normal	increased	normal	decreased	N.D.	[15,35]
E79K	normal	decreased	normal	normal	normal	[33]
R122H	normal	increased	normal	decreased	N.D.	[16,23,25, 26,35]
R122C	decreased	decreased	decreased	decreased	N.D.	[36]
R122C	normal	increased	normal	decreased	N.D.	[36]