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From Acute to Chronic Pancreatitis: The Role of Mutations in the Pancreatic Secretory Trypsin Inhibitor Gene

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Summary

Pancreatic secretory trypsin inhibitor (PSTI) is a potent natural inhibitor of trypsin. We proposed the hypothesis that, if the function of the PSTI is impaired by its genetic mutation, trypsin may easily promote autodigestion causing pancreatitis and we performed a mutational analysis of the *PSTI* gene in patients with pancreatitis. Two exonic mutations (N34S and R67C) were thought to be associated with a predisposition to pancreatitis. The N34S mutation was co-segregated with two intronic mutations, IVS1-37T>C and IVS3-69insTTTT. Although we analyzed the function of the recombinant N34S protein, we could not demonstrate the loss of function of this protein. Intronic mutations, rather than N34S itself (IVS1-37T>C + N34S + IVS3-69insTTTT complex), may be associated with the decreased function of the PSTI. Alternatively, increased digestion of N34S *in vivo* may be applicable. As for R67C, the conformational alteration of the protein by forming intra-molecular or inter-molecular disulfide bonds with ^{67}Cys was strongly suggested. These results, along with the brand-new findings in PSTI knockout mice, suggest that the genetic mutation of the PSTI is one of the important mechanisms for predisposition to pancreatitis by lowering the trypsin inhibitory function.

Introduction

Inappropriate activation of trypsinogen within the pancreas leads to the development of pancreatitis. Once trypsin is activated, it is capable of activating many other digestive proenzymes. These activated pancreatic enzymes further enhance autodigestion of the pancreas. Trypsin also activates cells via the trypsin receptor. The trypsin receptor has recently become known as one of the protease activated receptors, namely PAR-2. Both acinar cells and duct cells express abundant PAR-2 [1].

Trypsin activity in the pancreas is mainly controlled by the pancreatic secretory trypsin inhibitor (PSTI), which is also known as serine protease inhibitor Kazal type 1 (SPINK1). The PSTI is synthesized in the acinar cells of the pancreas, acts as a potent natural inhibitor of trypsin in order to prevent the occurrence of pancreatitis. When trypsinogen is activated into trypsin in the pancreas, the PSTI immediately binds to trypsin to prevent further activation of pancreatic enzymes. The PSTI also blocks the further activation of pancreatic cells via the trypsin receptor, PAR-2 (Figure 1).

Several gene mutations in trypsinogen have been identified and are presumed to be pathogenic in patients with hereditary pancreatitis through the enhancement of intrapancreatic trypsin activity. The mutations lead to an 80% likelihood of developing pancreatitis. Although gene mutations in trypsinogen have been identified and are presumed to be pathogenic in patients with hereditary pancreatitis, no causative gene mutation was found in about 50% of the patients. Subsequently, we proposed the hypothesis that, if the function of the PSTI is

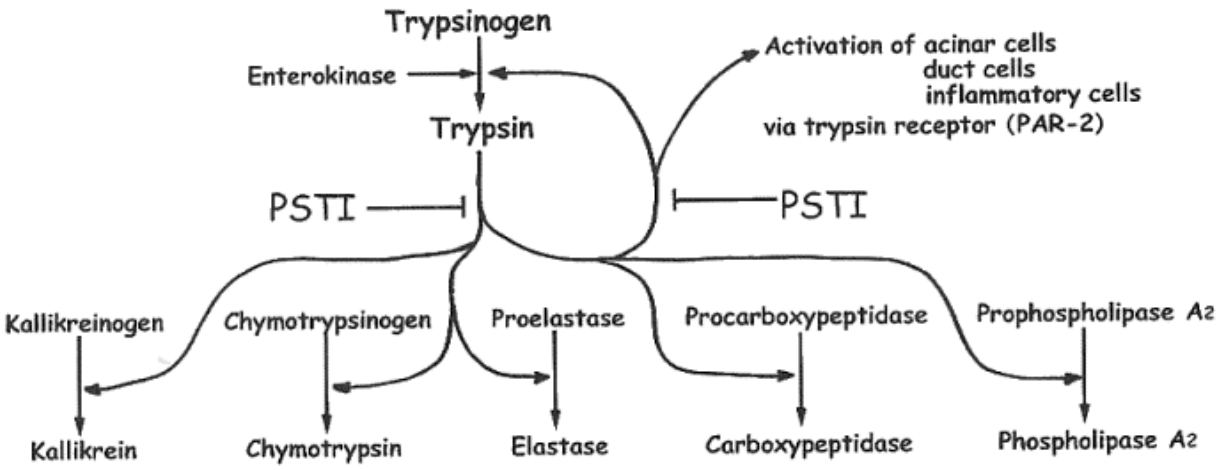

Figure 1. Activation pathways of proenzymes and PAR-2 by trypsin. Once trypsin is activated, it is capable of activating many other digestive proenzymes. Trypsin also activates pancreatic and inflammatory cells via PAR-2. The trypsin activity in the pancreas is mainly controlled by PSTI. When trypsinogen is activated into trypsin in the pancreas, PSTI binds immediately to trypsin to prevent further activation of pancreatic enzymes. (PAR: protease activated receptors; PSTI: pancreatic secretory trypsin inhibitor)

impaired by its genetic mutation, trypsin may easily promote autodigestion causing acute or chronic pancreatitis. Mutation of the *PSTI* gene may promote a predisposition to pancreatitis, by lowering the function of inhibiting trypsin activity. Five independent groups, including ours, started at approximately the same time and reported the mutational analysis of the *PSTI* gene in patients with pancreatitis [2, 3, 4, 5, 6, 7].

Mutational Analysis of the PSTI Gene in Familial and Juvenile Pancreatitis in Japan

All 4 exons of the *PSTI* gene and their flanking intronic regions were sequenced for 37 familial pancreatitis patients (24 families), 15 juvenile pancreatitis patients, 22 sporadic pancreatitis patients (15 acute and 7 chronic) and 33 healthy volunteers.

Three types of exonic mutations in the *PSTI* gene were observed. N34S was found in 6 familial pancreatitis patients (3 families) and 1 juvenile pancreatitis patient, and R67C was found in one familial pancreatitis patient and in one juvenile pancreatitis patient. It should be noted that the N34S mutation was co-segregated with two intronic mutations, specifically IVS1-37T>C and IVS3-69insTTTT (Table 1). The same set of mutations (N34S + IVS1-37T>C + IVS3-69insTTTT) observed in other countries was also observed in Japanese familial and juvenile pancreatitis patients.

There is considerable support for the idea that the N34S mutation leads to the development

Table 1. Summary of mutational analysis of *PSTI* in Japan.

Mutation	Familial Pancreatitis (n=74)	Juvenile Pancreatitis (n=30)	Sporadic Pancreatitis (n=44)	Healthy Volunteer (n=66)
IVS1-37T>C	8 (10.8%)	1 (3.4%)	0	0
Exon 3: N34S	8 (10.8%)	1 (3.4%)	0	0
IVS3-69insTTTT	8 (10.8%)	1 (3.4%)	0	0
Exon 4: R67C	1 (1.4%)	1 (3.4%)	0	0
Exon 4: 272C>T	2 (2.7%)	4 (13.3%)	6 (13.4%)	5 (7.6%)

PSTI: pancreatic secretory trypsin inhibitor

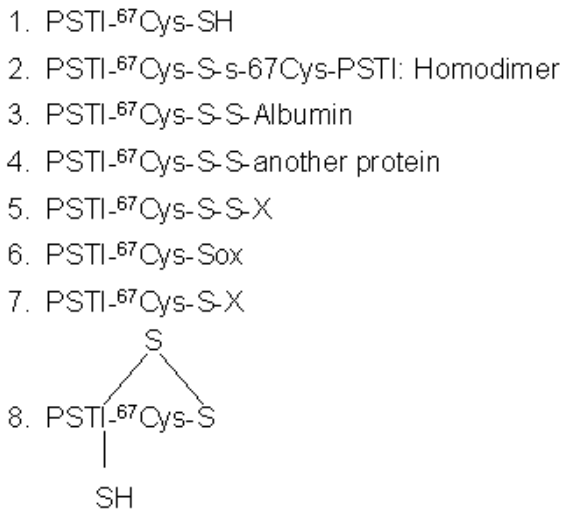

Figure 2. Predicted molecular forms of R67C.

of pancreatitis: a) based on theoretical computer analysis (Chou-Fosman and Robson-Garnier secondary structure prediction), it appears that the N34S mutation may affect the conformation of the nearby active site and then diminish the biological activity of PSTI [8]; b) the frequency of the N34S mutation in pancreatitis patients was considerably higher than that in non-pancreatitis subjects; c) the rate of association of pancreatitis in subjects with the homozygous N34S mutation was assumed to be high based on the data collected from recent reports (98%, 49/50). This high rate of association of pancreatitis in homozygous N34S subjects suggests that this mutation may be a recessive inherited trait.

The R67C mutation has not previously been discussed in reports from other countries. Hence, R67C may be a uniquely Japanese mutation. The mature PSTI protein has been reported to contain three intra-chain disulfide bonds: ^{32}C-^{61}C, ^{39}C-^{58}C and ^{47}C-^{79}C. The R67C mutation potentially forms a novel disulfide bridge between ^{67}Cys and any of the other Cys residues. ^{67}Cys may also form an intermolecular disulfide bond, such as PSTI homodimer or PSTI-albumin complex (Figure 2). Alternatively, ^{67}Cys may easily be oxidized, producing a modified molecular form or causing the destruction of acinar cells through endoplasmic reticulum stress.

We also found a 272C>T mutation in the 3' untranslated region of exon 4 in 1 patient with familial pancreatitis, 4 patients with juvenile pancreatitis, 3 patients with sporadic acute pancreatitis and 3 patients with sporadic chronic pancreatitis. This mutation, however, has been reported with high frequency even in healthy volunteers and apparently indicates a normal polymorphism (Table 1).

Functional Analysis of Recombinant PSTI Proteins with Amino Acid Substitution

We hypothesized that mutation of the *PSTI* gene may promote predisposition to pancreatitis, possibly by lowering the function of inhibiting trypsin activity. Based on the hypothesis, we performed a biochemical analysis of recombinant PSTI protein.

Trypsin inhibitory activity of recombinant protein was analyzed using human and bovine trypsin [9]. The activity of the PSTI protein with a point mutation of the most common type, N34S, was compared to that of the wild type. The function of the N34S PSTI remained unchanged under both normal alkali and acidic conditions as compared to the wild type PSTI (Figure 3). Calcium concentration did not affect the activity of recombinant PSTI. Trypsin susceptibility of the N34S protein did not increase either.

The interaction of recombinant N34S with

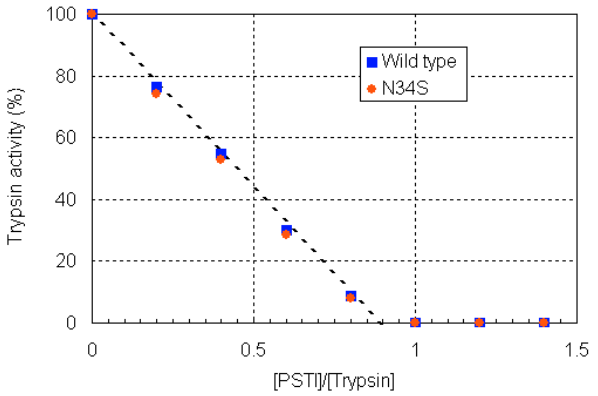

Figure 3. Inhibitory activity of recombinant PSTI proteins for human trypsin. The function of N34S PSTI (red circles) remained unchanged as compared to the wild type PSTI (blue squares). (PSTI: pancreatic secretory trypsin inhibitor)

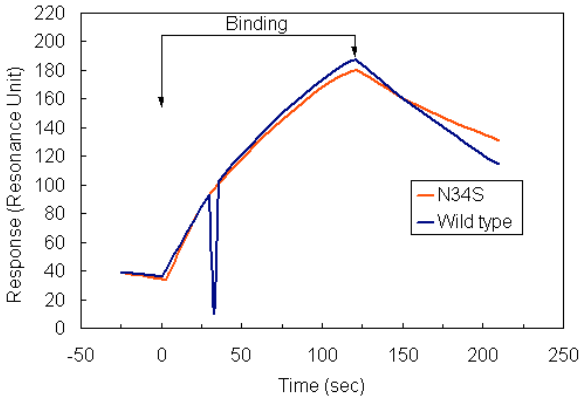

Figure 4. Binding affinity of recombinant PSTI proteins to human trypsin. The interaction of recombinant N34S with human trypsin was analyzed by using an SPR biosensor technique. The binding kinetics of N34S PSTI did not decrease as compared to the wild type PSTI. (PSTI: pancreatic secretory trypsin inhibitor; SPR: surface-plasmon-resonance)

human and bovine trypsin was also analyzed by using a surface-plasmon-resonance (SPR) biosensor technique [10]. The binding kinetics of the N34S PSTI protein to trypsin were compared to those of the wild type. The binding kinetics of the N34S PSTI did not decrease as compared to the wild type PSTI (Figure 4). These results along with enzymatic functional analysis suggest that other mechanisms than the conformational change of the PSTI by amino acid substitution may possibly underlie the predisposition to pancreatitis in patients with N34S. Enhanced digestion of N34S by enzymes other than trypsin and abnormal splicing may be applicable.

N34S is usually associated with two intronic mutations (i.e., IVS1-37T>C and IVS3-69insTTTT). Mutations in the intronic polypyrimidine tract, such as IVS3-69insTTTT which converts consecutive T5 to a T9 structure, sometimes result in a splicing abnormality. Hence, intronic mutations rather than N34S itself may be associated with the decreased function of the PSTI. The failure to exhibit function loss in the recombinant N34S PSTI protein supports this possibility.

As for R67C, there are many isoforms produced in the recombinant protein producing system, as suggested above. All these isoforms of R67C recombinant PSTI protein lost their reactivities with the anti-PSTI (wild type) antibody, suggesting the massive conformational alterations. As a result of the above-mentioned reasons, we could not purify recombinant R67C PSTI. R67C is possibly associated with the predisposition to pancreatitis.

Future Perspectives

Genetic mutations in the *PSTI* gene seem to promote a predisposition to pancreatitis, possibly by lowering the threshold for pancreatitis (Figure 5). To confirm the significance of the *PSTI* mutation, we are planning the following projects: a) transcriptional and translational analysis of the N34S mutation; b) processing analysis of R67C; c) analysis of *PSTI*-knockout mice.

Among these projects, we have recently succeeded in generating *PSTI*-knockout mice [11]. As to the heterozygous knockout mice, there was no alteration in the macroscopic and microscopic views of the pancreas nor was there any sign of pancreatitis. On the other hand, in the homozygous knockout mice of the *PSTI* gene, the pancreas had disappeared. There are two possibilities which may explain that phenomenon: a) failure of the pancreas to develop; b) autolysis of the pancreas. Because we found necrotic remnants of the pancreatic acinar cells in some siblings, the latter possibility may be applicable. These results

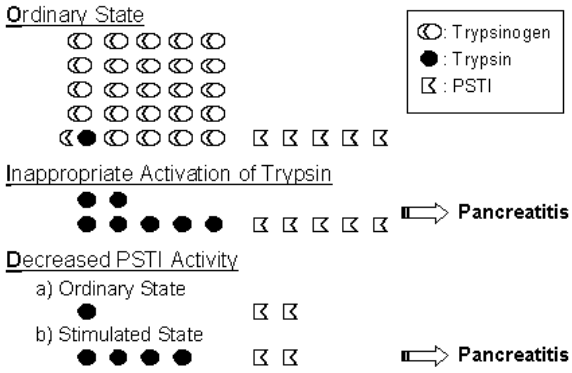

Figure 5. Intrapancreatic balance of trypsin and PSTI. Genetic mutations in the *PSTI* gene seem to promote a predisposition to pancreatitis, possibly by lowering the threshold for pancreatitis, as shown in the lowest situation. (PSTI: pancreatic secretory trypsin inhibitor)

also support the significance of the *PSTI* mutation.

Conclusion

Two exonic mutations (N34S and R67C) were thought to be associated with the predisposition to pancreatitis. The N34S mutation was co-segregated with two intronic mutations, IVS1-37T>C and IVS3-69insTTTT. Although we analyzed the function of recombinant N34S protein, we could not demonstrate the loss-of-function of this protein. Intronic mutations, rather than N34S itself (IVS1-37T>C + N34S + IVS3-69insTTTT complex), may be associated with the decreased function of the PSTI. Alternatively, increased digestion by enzymes other than trypsin may be applicable. As for R67C, the conformational alteration of the protein was strongly suggested. These results, along with the brand-new findings in *PSTI* knockout mice, suggest that the genetic mutation of the *PSTI* is one of the important mechanisms for predisposition to pancreatitis.

Keywords Acute Disease; Chromosome Disorders; Chronic Disease; Enzyme Activators; Genetic Diseases, Inborn; Mutation; Pancreatitis; Serine Proteinase Inhibitors; Surface Plasmon Resonance; Trypsin Inhibitor, Kazal Pancreatic; Trypsin; Trypsin Inhibitors; Trypsinogen

Abbreviations PAR: protease activated receptors; PSTI: pancreatic secretory trypsin inhibitor; SPINK1: serine protease inhibitor Kazal type 1; SPR: surface-plasmon-resonance

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