PhD thesis

Regulatory digestive proteases in the pathomechanism of chronic pancreatitis

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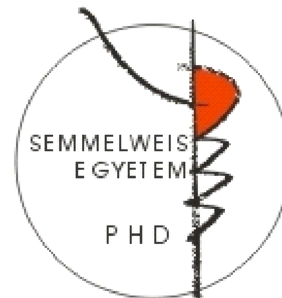

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I. INTRODUCTION

Chronic pancreatitis is a disease characterized by permanent destruction of the pancreatic parenchyma leading to maldigestion and diabetes mellitus. Unfortunately there is very little known about how to prevent or cure this debilitating disease to date. Recent progress in understanding the underlying causes came in 1996, when the genetic basis of the disease was firmly established: *Whitcomb and coworkers* identified a mutation in the gene encoding trypsinogen to associate with the disease phenotype. Hereditary pancreatitis is characterized by early onset episodes of acute pancreatitis with frequent progression to chronic pancreatitis and an increased risk for pancreatic cancer.

Animal models of experimental pancreatitis have long suggested that the initiating event in pancreatitis is premature activation of trypsinogen to active trypsin inside the acinar cells. However, direct evidence for this mechanism in human pancreatitis was lacking. Consequently, genetically determined pancreatitis, which includes classic hereditary pancreatitis and other forms of pancreatitis that are associated with trypsinogen or trypsin inhibitor gene mutations, emerged as the principal model of the human disease. Biochemical investigations suggested that mutations in the cationic trypsinogen gene upset the protease-antiprotease balance in the pancreas by promoting autoactivation of cationic trypsinogen to trypsin. Trypsin has the potential to activate the cascade of digestive enzymes prematurely within the pancreas, resulting in autodigestion of the organ. Consistent with the central pathophysiological role of trypsin, *loss-of-function* alterations in the trypsin inhibitor gene predispose to various forms of pancreatitis by lowering the protective levels of the inhibitor.

Elucidation of the pathomechanism of genetically determined pancreatitis provided valuable insight into the pathophysiology of human chronic pancreatitis. The central role of trypsin has prompted researchers for decades to understand the way trypsin activity is regulated within the pancreas.

II. AIMS OF THE STUDY

Inactivation of potentially harmful intrapancreatic trypsin by proteolytic degradation has been discussed as a possible protective mechanism against chronic pancreatitis. The aim of this work was to study and characterize biochemically two candidates, namely mesotrypsin and chymotrypsin C, to investigate their possible impact on pancreatic disease.

The characterization of mesotrypsin

Mesotrypsin is a minor trypsin isoform resistant to natural trypsin inhibitors in the human pancreatic juice. It was suggested that the inhibitor resistance of mesotrypsin was due to Arg^{198}. Despite our detailed structural knowledge, the biological function of mesotrypsin has remained mysterious.

Our specific aims were:

1. *To provide evidence that the inhibitor resistance of mesotrypsin is caused by Arg^{198}.*
2. *To analyze the potential of mesotrypsin to activate or degrade pancreatic zymogens.*
3. *To find the biological and pathological function of mesotrypsin.*
4. *To provide a biochemical basis for intrapancreatic mesotrypsinogen activation.*

The characterization of chymotrypsin C

Our preliminary observations suggested that chymotrypsin C may be the long-elusive digestive enzyme described by *Rinderknecht* (enzyme Y) responsible for trypsin degradation in the gut, and may serve as a protective protease in the pancreas to curtail premature trypsinogen activation.

Our specific aims were:

1. *To elucidate the role of chymotrypsin C in the degradation of human cationic trypsin.*
2. *To investigate the Ca^{2+} concentration dependence of the reaction.*
3. *To determine the sites of cleavage and the exact mechanism of trypsin degradation.*
4. *To prove that chymotrypsin C is identical to enzyme Y, the trypsinogen degrading activity from human pancreatic juice.*

III. METHODS

Construction of expression plasmids

First, we constructed expression plasmids harboring the genes encoding human digestive proteases: the cDNA for the enzymes was PCR-amplified and cloned into the pTrapT7 (trypsinogens) or pcDNA3.1 (chymotrypsinogens and elastases) plasmid. Mutations were introduced via site-directed mutagenesis into the enzymes using the overlap-extension PCR mutagenesis method.

Expression of digestive proenzymes

Expression of human trypsinogen isoforms was carried out in the *E. coli* Rosetta(DE3) strain. Cultures were harvested, inclusion bodies were isolated by sonication and *in vitro* refolding of trypsinogens was performed. Chymotrypsinogens and elastases were expressed in human embrionic kidney (HEK) 293T cells via transient transfection, conditioned media were collected.

Purification of the digestive proenzymes

Zymogens were purified using ecotin-affinity chromatography, utilizing the column-bound inhibitor ecotin. Exceptionally, a combination of ion-exchange and gel-filtration chromatography was used.

Protease activity assays

Enzymatic activity was measured with synthetic chromogenic substrates. The release of the yellow *p*-nitroaniline upon proteolytic cleavage was followed using a spectrophotometer.

Visualization of proteins

Gel electrophoresis was used to visualize proteins. Electrophoretic separation was performed under reducing conditions on Tris-glycine gels. To identify the N-terminal amino-acids, samples were transferred onto sequencing membranes and analyzed by N-terminal sequencing.

IV. RESULTS

The inhibitor degrading activity of mesotrypsin [I]

1. Direct experimental evidence for the role of Arg^{198} came from site-directed mutagenesis of Arg^{198} to Gly^{198} in mesotrypsin. The resulting R198G-mesotrypsin mutant became fully sensitive to soybean trypsin inhibitor and human pancreatic secretory trypsin inhibitor. **Therefore, Arg^{198} is solely responsible for the inhibitor resistance and restricted substrate recognition of human mesotrypsin.**
2. Pancreatic zymogens (chymotrypsinogen, proelastase, trypsinogen) were activated by mesotrypsin to their respective active enzymes at rates that are 500–1000-fold lower than activation by cationic or anionic trypsin. **The experiments convincingly rule out a possible role for mesotrypsin in promoting intrapancreatic zymogen activation.**
3. We analyzed the degradation of cationic and anionic trypsinogen by mesotrypsin. **The two major human trypsinogen isoforms were poor substrates for mesotrypsin, and dispute a protective role for mesotrypsin-mediated zymogen degradation in pancreatic physiology.**
4. Mesotrypsin rapidly cleaved the reactive site peptide bond of soybean trypsin inhibitor, whereas it completely and selectively degraded human pancreatic secretory trypsin inhibitor. **The findings suggest that premature activation of mesotrypsinogen in the pancreas might lower protective pancreatic secretory trypsin inhibitor levels and contribute to the development of pancreatitis.**
5. When activation of the three human trypsinogen isoforms by the pathological activator cathepsin B was compared, rapid activation of mesotrypsinogen was apparent, which was followed by slow degradation. **Cathepsin B can rather selectively activate mesotrypsinogen, and potentially initiate the mesotrypsin-mediated degradation of pancreatic secretory trypsin inhibitor.**

The trypsin degrading activity of chymotrypsin C [II]

1. Incubation of human cationic trypsin with chymotrypsin C at pH 8.0; 37 °C, in the presence of micromolar Ca^{2+} concentrations, resulted in rapid loss of trypsin activity. The specificity of chymotrypsin C in mediating trypsin degradation was striking as all other chymotrypsins and elastases tested were completely ineffective in this respect. **The results presented here support a putative defense mechanism, in which chymotrypsin C mediated trypsin degradation mitigates unwanted intrapancreatic trypsin activity.**
2. Trypsin degradation is initiated by selective cleavage of the Leu^{81}-Glu^{82} peptide bond within the Ca^{2+} binding loop, followed by trypsin-mediated autolytic cleavage of the Arg^{122}-Val^{123} peptide bond. **Chymotryptic cleavage of the Leu^{81}-Glu^{82} peptide bond and tryptic cleavage of the Arg^{122}-Val^{123} peptide bond are both essential for rapid trypsin degradation.**
3. Increasing the Ca^{2+} concentration progressively inhibited the degradation of cationic trypsin by chymotrypsin C, with essentially complete protection observed at 1 mM Ca^{2+}. Experiments utilizing a trypsin mutant with a distorted Ca^{2+} binding site confirmed that an intact Ca^{2+} binding site in trypsin is required for the protective effect of Ca^{2+} against chymotrypsin C. **Taken together, millimolar Ca^{2+} concentrations inhibited chymotrypsin C mediated cleavage after Leu^{81} by stabilizing the Ca^{2+} binding loop and thus protect against degradation.**
4. Chymotrypsin C was capable of degrading not only cationic trypsin, but also anionic trypsin and mesotrypsin, and the trypsin precursors cationic trypsinogen, anionic trypsinogen and mesotrypsinogen as well. **The observations presented seem fully consistent with the conclusion that chymotrypsin C is in fact enzyme Y, which contaminated the cationic trypsinogen preparations in *Rinderknecht's* experiments.**

V. SUMMARY

Genetic and biochemical evidence defines a pathological pathway in which a sustained imbalance between intrapancreatic trypsinogen activation and trypsin inactivation results in the development of chronic pancreatitis. Inactivation of intrapancreatic trypsin by proteolytic degradation has been highlighted as a possible protective mechanism against chronic pancreatitis for decades. In humans the inhibitor resistant mesotrypsin has been labeled a candidate for this function. Later, the presence of another unknown enzymatic activity referred to as enzyme Y was found effective in the degradation of pancreatic zymogens in human pancreatic juice.

Our results demonstrate that human trypsinogens are not degraded by mesotrypsin, and dispute a protective role for mesotrypsin-mediated trypsin degradation in pancreatic physiology. However, we identified a unique role for mesotrypsin in the degradation of trypsin inhibitors. Furthermore, we found that this distinctive enzymatic activity was endowed by the mesotrypsin-specific amino-acid Arg^{198}. The observations not only indicate a physiological role for mesotrypsin in the degradation of dietary trypsin inhibitors, but also suggest that premature activation of mesotrypsinogen could contribute to the pathogenesis of human pancreatitis by reducing the protective levels of pancreatic secretory trypsin inhibitor.

Furthermore, we identified chymotrypsin C as enzyme Y, the so far obscure trypsinogen-degrading activity from human pancreatic juice. Chymotrypsin C can specifically promote inactivation of all human trypsin and trypsinogen isoforms by selective cleavage of the Leu^{81}-Glu^{82} peptide bond within the Ca^{2+} binding loop. Our results support a defense mechanism against chronic pancreatitis, in which chymotrypsin C mediated trypsin degradation mitigates unwanted intrapancreatic trypsin activity.

VI. RELATED PUBLICATIONS

Papers

I. **Szmola R**, Kukor Z, Sahin-Toth M. (2003) Human mesotrypsin is a unique digestive protease specialized for the degradation of trypsin inhibitors. *J Biol Chem.* 278, 48580-48589. IF.: 6.482

II. **Szmola R**, Sahin-Toth M. (2007) Chymotrypsin C (caldecrin) promotes degradation of human cationic trypsin: Identity with Rinderknecht's enzyme Y. *Proc. Natl. Acad. Sci. U.S.A.* 104, 11227-11232. IF.: 9.643

Abstracts

Szmola R, Kukor Z, Sahin-Toth M. (2003) Cathepsin B preferentially activates mesotrypsinogen of the three human trypsinogen isoforms. *Pancreatology.* 3, 434.

Kukor Z, **Szmola R**, Sahin-Toth M. (2003) Human mesotrypsin rapidly degrades trypsin inhibitors. *Pancreatology.* 3, 434.

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Szmola R, Sahin-Toth M. (2006) The hunt for the mysterious Enzyme Y: A progress report. *Pancreas*. 33 (4), 500.

Szmola R, Ozsvari B, Sahin-Toth M. (2007) Chymotrypsin C regulates degradation of human cationic trypsin. *Gastroenterology*. 132 (4), Suppl 2, A-31.

Szmola R, Ozsvari B, Sahin-Toth M. (2007) Chymotrypsin C (caldecrin) promotes degradation of human cationic trypsin. *Pancreatology.* 7, 290.