FINAL REPORT

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The role of pancreatic ductal secretion in the pathogenesis of acute pancreatitis

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1. Background

Pancreatic duct cells are responsible for **secreting** an alkaline, **HCO_3^- rich isotonic fluid**, which serves two important functions: to flush digestive enzymes and toxic agents down the ductal tree and to help neutralize gastric acid. The initial step of HCO_3^- secretion is the accumulation of HCO_3^- within the duct cell. This can occur by two mechanisms: (1) the forward transport of HCO_3^- by the Na^+/HCO_3^- co-transporter (NBC), and (2) diffusion of CO_2 into the duct cell which is then hydrated to carbonic acid by carbonic anhydrase, followed by the backward transport of protons via the Na^+/H^+ exchangers (NHEs) and H^+-pumps. HCO_3^- is then secreted across the apical membrane via Cl^-/HCO_3^- exchangers (**SLC26A3, DRA-down regulated in adenoma** and **SLC26A6, PAT-1-putative anion transporter 1**) and/or **cystic fibrosis transmembrane conductance regulator (CFTR)** Cl^- channels, which exhibit a finite permeability to HCO_3^-. The exact mechanism how the SLC26 exchangers and apical Cl^- channels produce a high HCO_3^- secretion is controversial. Nevertheless, the key role of CFTR in HCO_3^- secretion is emphasized, so the adequate localization of CFTR on the apical membrane of the pancreatic ductal epithelial cells is very important. **Na^+/H^+ exchanger regulatory factor-1 (NHERF-1)** is responsible for the formation of a multiprotein complex, which ensures the localization of CFTR on the apical membrane of pancreatic ductal cells. The role of NHERF-1 in the pancreas has not yet been investigated, despite the fact that CFTR, a key regulator of epithelial functions, is controlled by this scaffolding protein.

Acute pancreatitis is a sudden inflammation of the pancreas which usually develops either as a result of **gallstone disease** or moderate to heavy **ethanol consumption**. Key

events in the pathogenesis of pancreatitis include premature activation of trypsinogen and the activation of the proinflammatory transcription factor nuclear factor-κB (NF-κB) in pancreatic acinar cells. The disease can present in mild edematous and severe necrotizing forms, the latter of which can lead to **unacceptably high mortality**. Unfortunately, **no specific treatment** is available for acute pancreatitis and basically the main goal of therapy is supportive care.

Recent evidence has shown that besides acinar cells, **ducts cells may also have an important role in development of acute pancreatitis**.

2. Aims of the project

According to our preliminary experiments, both NHERF-1 and SLC26A6 (PAT-1) play important roles in regulating pancreatic ductal secretion (the secretion of both knock-out mice is deficient compared to their wild-type littermates). Our hypothesis was that pancreatic ductal cells have beneficial effects in protecting against the development of acute pancreatitis. Therefore, **the main aims of the project were to characterize the physiological and pathophysiological roles of NHERF-1 and PAT-1 in pancreatic ductal secretion**. In order to prove our hypothesis, we induced different types of acute necrotizing pancreatitis in wild-type and NHERF-1 or PAT-1 knock-out mice.

3. Results and discussion

It proved to be a great challenge to set up the L-arginine-induced acute pancreatitis model in mice. In BALB/c mice, intraperitoneal injection of 2×4 g/kg L-arginine resulted in a relatively low rate (around 15%) of pancreatic necrosis (Kui et al., 2015), whereas others have detected much higher rates (up to 55%). We suspected that this may be due to differences between mouse strains. Therefore, we administered various concentrations (5-30%, pH = 7.4) and doses (2×4, 3×3, or 4×2.5 g/kg) of L-arginine-HCl in BALB/c, FVB/n and C57BL/6 mice. The fate of mice in response to the intraperitoneal injections of L-arginine

followed one of three courses. Some mice (1) developed severe AP or (2) remained AP-free by 72 h, whereas others (3) had to be euthanized (to avoid their death, which was caused by the high dose of L-arginine and not AP) within 12 h., In FVB/n and C57BL/6 mice, the pancreatic necrosis rate (about 50%) was significantly higher than that observed in BALB/c mice using 2×4 g/kg 10% L-arginine, but euthanasia was necessary in a large proportion of animals. The intraperitoneal injection of lower L-arginine concentrations (e.g. 5-8%) in case of the 2×4 g/kg dose, or other L-arginine doses (3×3 or 4×2.5 g/kg, 10%) were better for inducing AP. We could not detect any significant differences between the AP severity of male and female mice. Taken together, when setting up the L-arginine-induced AP model, there are several important factors that are worth consideration such as the dose and concentration of the administered L-arginine-HCl solution and also the strain of mice.

NHERF-1 was shown to modulate the apical localization of CFTR in epithelial cells. The genetic deletion of NHERF-1 resulted in significantly lower CFTR expression in the apical membrane of mouse pancreatic ducts (Pallagi et al., 2014). This was accompanied by markedly lower HCO_3^- and fluid secretion in isolated pancreatic ducts and in anesthetized mice. NHERF-1 (and CFTR) expression also influenced the development of two acute necrotizing pancreatitis models (induced by cerulein or sodium taurocholate). In fact, the severity of pancreatitis, especially the extent of acinar cell death, was significantly higher in NHERF-1-knock-out vs. wild-type mice. Importantly, NHERF-1 deletion did not alter acinar bile acid or cerulein sensitivity, and no changes in leukocyte functions were detected. The observed effects on acute pancreatitis severity also seemed to be independent of trypsiongen and nuclear factor-κB activation, and intracellular Ca^{2+} signalling.

The importance of CFTR in acute pancreatitis was further highlighted by the complex set of experiments published in Gastroenterology by Maléth et al. (2015). Sweat Cl^- concentration was significantly increased (indicating decreased CFTR activity, as in the sweat gland, the anion channel mediates Cl^- absorption rather than secretion) in people who acutely abused alcohol vs age- and sex-matched healthy volunteers. Sobering up for a few days after alcohol abuse resulted in normalization of sweat Cl^- concentration suggesting a reversible effect. In alcoholics who did not drink alcohol for a week, sweat Cl^- concentration was higher than that in the control group, but lower than in the acutely intoxicated group

suggesting a chronic effect of ethanol. Furthermore, in patients with pancreatitis of alcoholic origin, CFTR expression was decreased at both mRNA and protein levels. We also evaluated the effects of ethanol and its metabolites on pancreatic ductal secretion in animal models and in a human pancreatic duct cells. Both basal and secretin-stimulated secretion was decreased by intraperitoneal injection of 1.75 g/kg ethanol and 750 mg/kg palmitic acid in mice. Ethanol administration markedly reduced expression of CFTR mRNA, the stability of CFTR protein at the cell surface, and also disrupted the folding of CFTR in the endoplasmic reticulum. Electrophysiological studies on native guinea pig pancreatic ductal epithelial cells showed that ethanol (10 and 100 mM) increased basal, but reversibly blocked, forskolin-stimulated CFTR currents (Judák et al., 2014). The inhibitory effect of ethanol was mimicked by its non-oxidative metabolites, palmitoleic acid ethyl ester (POAEE) and palmitoleic acid (POA), but not by the oxidative metabolite, acetaldehyde. Ethanol, POAEE and POA markedly reduced intracellular ATP (ATPi) levels which was linked to CFTR inhibition since the inhibitory effects were almost completely abolished if ATPi depletion was prevented. We proposed that ethanol causes functional damage of CFTR through an ATPi-dependent mechanism, which compromises ductal fluid secretion and likely contributes to the pathogenesis of acute pancreatitis.

In summary, the above mentioned results suggest that high concentrations of ethanol cause inhibition of apical Cl^-/HCO_3^- exchangers and CFTR. Ethanol inhibits both expression and function of CFTR. The effects of ethanol may be partly mediated by its fatty acid metabolites.

Obviously, one may wonder whether the results of the above mentioned animal experiments have any human relevance. Notably, we have shown in a small pilot study that intraluminal pancreatic ductal pH is lower in patients with biliary pancreatitis vs control patients (Takács et al., 2013). The decrease in ductal pH was greater in patients with biliary pancreatitis who had symptoms for longer time-periods before endoscopic retrograde cholangio-pancreatography. Taken together, these results indicate that HCO_3^- secretion is likely impaired in patients with biliary pancreatitis.

Unfortunately, we could not confirm the results of our preliminary *in vitro* experiments showing reduced pancreatic ductal secretion in PAT-1 knock-out vs wild-type

mice. In fact, there were no significant differences in basal and secretin-stimulated pancreatic ductal secretion of anesthetized wild-type and PAT-1 knock-out mice. Furthermore, no significant differences were observed between wild-type and PAT-1 knock-out mice in the severity of acute pancreatitis induced by caerulein, intraductal sodium-taurocholate-induced or L-arginine. Administration of cerulein, L-arginine or sodium taurocholate did not alter the extent of pancreatic NF-κB and trypsinogen activation in PAT-1 knock-out vs wild-type mice. Similarly, we could not find any significant differences in acinar Ca^{2+} signaling of PAT-1 knock-out and wild-type mice.

PAT-1 is also a reasonable candidate for a chronic pancreatitis susceptibility gene, which has not been investigated in CP patients so far. Sequencing of its entire coding region revealed four common variants: intronic variants c.23 + 78_110del, c.183-4C > A, c.1134 + 32C > A, and missense variant c.616G > A (p.V206M) which were found in linkage disequilibrium indicating a conserved haplotype (Balázs et al., 2015). The distribution of the haplotype did not show a significant difference between patients and controls in the two cohorts. A synonymous variant c.1191C > A (p.P397=) and two intronic variants c.1248 + 9_20del and c.-10C > T were detected in single cases. Overall, our data showed that PAT-1 variants do not alter the risk for the development of pancreatitis.

We contributed to a multi-centre study published in Nature Genetics which demonstrated that variants in *CPA1* are strongly associated with early-onset chronic pancreatitis (Witt et al., 2013).

We have published numerous review papers which have relevance to the topic of this proposal. The strengths and weaknesses, relevance to human disease, selection, and appropriate use of some commonly employed experimental acute pancreatitis models were discussed in The Pancreapedia: Exocrine Pancreas Knowledge Base (Hegyi et al., 2013). The central role of mitochondria in acute pancreatitis was highlighted by Maléth et al. (2013). We have also reviewed the role, function and pathophysiological relevance of potassium channels in pancreatic duct cells (Venglovecz et al. 2015). The physiological and pathophysiological functions of pancreatic ducts were described in two other articles (Hegyi & Rakonczay, 2015; Pallagi et al., 2015). In collaboration with Prof. Gábor Varga's group

(Semmelweis University), we compared similarities and differences in pancreatic and salivary chronic inflammation (Rakonczay et al., 2014).

Finally, I'd like to mention that the published results also contributed to the Ph.D. theses of two young researchers in the laboratory (Petra Pallagi and József Maléth).

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4. Publications related to the project

Balázs A, Ruffert C, Hegyi E, Hritz I, Czakó L, Takács T, Szepes Z, Németh BC, Gervain J, Izbéki F, Halász A, Kelemen D, Szmola R, Novák J, Crai S, Illés A, Vincze Á, Molnár Z, Varga M, Bod B, Farkas G Jr., Sümegi J, Szepes A, Dubravcsik Z, Lásztity N, Párniczky A, Hamvas J, Andorka C, Veres G, Szentkereszty Z, Rakonczay Z Jr., Maléth J, Sahin-Tóth M, Rosendahl J, Hegyi P, the Hungarian Pancreatic Study Group. Genetic analysis of the bicarbonate secreting anion exchanger SLC26A6 in chronic pancreatitis. Pancreatology 2015; 15: 508–513.

Hegyi P, Perides G, Steer ML, Rakonczay Z Jr. Commonly employed rodent models of experimental acute pancreatitis: Their strengths and weaknesses, relevance to human disease, selection, and appropriate use. The Pancreapedia: Exocrine Pancreas Knowledge Base, 2013, DOI: 10.3998/panc.2013.4.

Hegyi P, Rakonczay Z Jr. The role of pancreatic ducts in the pathogenesis of acute pancreatitis. Pancreatology 2015; 15(4 Suppl):S13-7.

Judák L, Hegyi P, Rakonczay Z Jr, Maléth J, Gray MA, Venglovecz V. Ethanol and its non-oxidative metabolites profoundly inhibit CFTR function in pancreatic epithelial cells which is prevented by ATP supplementation. Pflugers Arch Eur J Phys 2014; 466:549-62.

Katona M, Vizvári E, Németh L, Facskó A, Venglovecz V, Rakonczay Z Jr, Hegyi P, Tóth-Molnár E. Experimental evidence of fluid secretion of rabbit lacrimal gland duct epithelium. Invest Ophthalmol Vis Sci 2014; 55:4360-7.

Kemény LV, Schnúr A, Czepán M, Rakonczay Z Jr, Gál E, Lonovics J, Lázár G, Simonka Z, Venglovecz V, Maléth J, Judák J, Németh IB, Szabó K, Almássy J, Virág L, Geisz A, Tiszlavicz L, Yule DI, Wittmann T, Varró A, Hegyi P. Na^+/Ca^{2+} exchangers regulate the migration and proliferation of human gastric myofibroblasts. Am J Physiol Gastrointest Liver Physiol. 2013, 305:G552-63.

Kui B, Balla Z, Vasas B, Végh ET, Pallagi P, Kormányos ES, Venglovecz V, Iványi B, Takács T, Hegyi P, Rakonczay Z Jr. New insights into the methodology of L-arginine-induced acute pancreatitis. PLOS ONE 2015; 10:e0117588.

Kui B, Balla Z, Végh ET, Pallagi P, Venglovecz V, Iványi B, Takács T, Hegyi P, Rakonczay Z Jr. Recent advances in the investigation of pancreatic inflammation induced by large doses of basic amino acids in rodents. Lab Invest. 2014; 94:138-49.

Maléth J, Balla Z, Kui B, Balázs A, Katona M, Judák L, Németh I, Pallagi P, Kemény LV, Rakonczay Z Jr, Venglovecz V, Földesi I, Pető Z, Somorácz Á, Borka K, Perdomo D, Lukacs GL, Gray MA, Monterisi S, Zaccolo M, Sendler M, Mayerle J, Kühn JP, Lerch MM, Sahin-Tóth M, Hegyi P. Alcohol disrupts levels and function of the cystic fibrosis transmembrane conductance regulator to promote development of pancreatitis. Gastroenterology 2015; 148: 427-439.e16.

Maléth J, Rakonczay Z Jr, Venglovecz V, Dolman NJ, Hegyi P. Central role of mitochondrial injury in the pathogenesis of acute pancreatitis. Acta Physiol. 2013; 207:226-35.

Pallagi P, Hegyi P, Rakonczay Z Jr. The physiology and pathophysiology of pancreatic ductal secretion: the background for clinicians. Pancreas 2015; 44:1211-1233.

Pallagi P, Balla Z, Singh AK, Dósa S, Iványi B, Kukor Z, Tóth A, Riederer B, Liu YJ, Engelhardt R, Jármay K, Szabó A, Janovszky Á, Perides G, Venglovecz V, Maléth J, Wittmann T, Takács T, Gray MA, Gácser A, Hegyi P, Seidler U, Rakonczay Z Jr. The role of pancreatic ductal secretion in protection against acute pancreatitis in mice. Crit Care Med. 2014; 42:e177-88.

Rakonczay Z Jr, Vág J, Földes A, Nagy K, Nagy Á, Hegyi P, Varga G. Chronic inflammation in the pancreas and salivary glands - lessons from similarities and differences in pathophysiology and treatment modalities. Curr Pharm Des. 2014, 20:1104-20.

Takács T, Rosztóczy A, Maléth J, Rakonczay Z Jr, Hegyi P. Intraductal acidosis in acute biliary pancreatitis. Pancreatology. 2013; 13: 333-5.

Venglovecz V, Rakonczay Z Jr, Gray MA, Hegyi P. Potassium channels in pancreatic duct epithelial cells: their role, function and pathophysiological relevance. Pflugers Arch Eur J Phys. 2015; 46: 625-640.

Witt H, Beer S, Rosendahl J, Chen JM, Chandak GR, Masamune A, Bence M, Szmola R, Oracz G, Macek M Jr, Bhatia E, Steigenberger S, Lasher D, Bühler F, Delaporte C, Tebbing J, Ludwig M, Pilsak C, Saum K, Bugert P, Masson E, Paliwal S, Bhaskar S, Sobczynska-Tomaszewska A, Bak D, Balascak I, Choudhuri G, Reddy DN, Rao GV, Thomas V, Kume K, Nakano E, Kakuta Y, Shimosegawa T, Durko L, Szabó A, Schnúr A, Hegyi P, Rakonczay Z Jr, Pfützer R, Schneider A, Groneberg DA, Braun M, Schmidt H, Witt U, Friess H, Algül H, Landt O, Schuelke M, Krüger R, Wiedenmann B, Schmidt F, Zimmer KP, Kovacs P, Stumvoll M, Blüher M, Müller T, Janecke A, Teich N, Grützmann R, Schulz HU, Mössner J, Keim V, Löhr M, Férec C, Sahin-Tóth M. Variants in *CPA1* are strongly associated with early-onset chronic pancreatitis. Nat Genet. 2013; 45:1216-20.

5. Ph.D. disssertations related to the project

Maléth J. Crucial role of pancreatic ducts in the initiation and progression of pancreatitis, 2014.

Pallagi P. The effects of Na^+/H^+ exchanger regulatory factor 1 and trypsin on pancreatic ductal bicarbonate secretion, 2013.