Mechanisms of polyamine catabolism-induced acute pancreatitis

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Abstract

Acute pancreatitis is an autodigestive disease, in which the pancreatic tissue is damaged by the digestive enzymes produced by the acinar cells. Among the tissues in the mammalian body, pancreas has the highest concentration of the natural polyamine, spermidine. We have found that pancreas is very sensitive to acute decreases in the concentrations of the higher polyamines, spermidine and spermine. Activation of polyamine catabolism in transgenic rats overexpressing SSAT (spermidine/spermine-N^1-acetyltransferase) in the pancreas leads to rapid depletion of these polyamines and to acute necrotizing pancreatitis. Replacement of the natural polyamines with methylated polyamine analogues before the induction of acute pancreatitis prevents the development of the disease. As premature trypsinogen activation is a common, early event leading to tissue injury in acute pancreatitis in human and in experimental animal models, we studied its role in polyamine catabolism-induced pancreatitis. Cathepsin B, a lysosomal hydrolase mediating trypsinogen activation, was activated just 2 h after induction of SSAT. Pre-treatment of the rats with bismethyl-spermine prevented pancreatic cathepsin B activation. Analysis of tissue ultrastructure by transmission electron microscopy revealed early dilatation of rough endoplasmic reticulum, probable disturbance of zymogen packaging, appearance of autophagosomes and later disruption of intracellular membranes and organelles. Based on these results, we suggest that rapid eradication of polyamines from cellular structures leads to premature zymogen activation and autodigestion of acinar cells.

Acute pancreatitis

The pancreas is an organ committed to active production, storage and secretion of digestive enzymes. Most of these enzymes are produced and stored as inactive zymogens, which are normally activated only after release into the duodenum. It is believed that in severe acute pancreatitis the tissue is affected by premature activation of these potentially harmful zymogens inside the acinar cells leading to pancreatic necrosis. Associated with pancreatic cell damage, extensive inflammation develops first locally in pancreas and later systemically. Systemic inflammatory response leading to multiple organ dysfunction syndrome accounts for the majority of the deaths in human acute pancreatitis [1]. In Western countries, acute pancreatitis is a common disease with increasing incidence, the main causes being related to biliary disease and alcohol. The treatment of severe acute pancreatitis is palliative, as no specific therapy is currently available. The overall mortality rate is up to 50% [2].

The exact mechanisms of the pathophysiology of acute pancreatitis are still relatively poorly known. Therefore, several experimental animal models have been created to study the mechanisms involved in the development of pancreatitis. The most commonly used animal models are the induction of oedematous pancreatitis with a secretagogue cerulein in mice or rats and the induction of necrotizing pancreatitis with infusion of taurodeoxycholate into biliopancreatic duct in rats. The severity of the disease is dependent on the doses used in both models. Whether these and other models mimic the human disease sufficiently to be suitable for use in evaluating therapy is not self-evident [3].

Role of polyamines in pancreas

Pancreas appears to be the richest source of spermidine in the mammalian body [4]. Although the reason for the requirement of high polyamine concentrations in pancreas is not known it is most likely related to the high activity of protein synthesis and the maintenance of tissue integrity. Cationic polyamines are generally thought to interact with negatively charged cellular components by binding to them with electrostatic forces. Polyamines thus play a role in major biological membrane functions as reviewed in [5]. In pancreas, polyamines have been localized in zymogen granules [6]. Enhanced polyamine biosynthesis has been observed in pancreatic cancer, and inhibition of ornithine decarboxylase with DFMO (difluoromethylornithine) retarded cell growth and increased apoptosis in pancreatic adenocarcinoma-derived cell lines [7]. Similarly, prolonged treatment with DFMO reduced spontaneous regeneration after cerulein-induced acute pancreatitis, indicating the importance of polyamines in pancreatic tissue repair [8]. We first observed the association

Figure 1 | Induction of pancreatic SSAT activity (A) and changes in the concentrations of polyamines (B) in SSAT transgenic rats with activated polyamine catabolism

Transgenic rats were treated with zinc (10 mg Zn/kg intraperitoneally) to induce the transgene expression. Tissue samples were taken at time points indicated. There were three rats per group. The results are expressed as means ± SD. One-way analysis of variance was used for statistical analysis. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ as compared with the animals at 0 h.
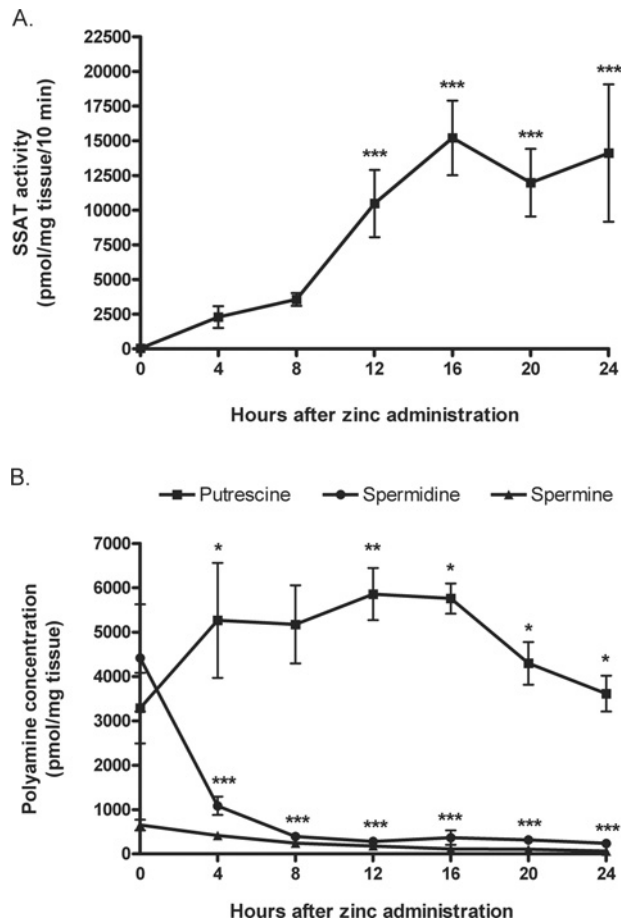
of changes in polyamine concentrations with pancreatic integrity when transgenic rats carrying metallothionein I promoter-driven SSAT gene were treated with a non-toxic dose of zinc to induce the transgene expression [9]. As shown in Figure 1, treatment of the rats with zinc resulted in huge induction of SSAT activity and rapid depletion of cellular spermidine and spermine pools with concomitant accumulation of putrescine, the end product of the polyamine back-conversion pathway completed by the action of PAO (polyamine oxidase). The appearance of N^1-acetylspermidine was also evident but its level remained below the level of the depleted spermidine pool (not shown). It should be noted that spermidine concentration was depleted by 80% of the normal concentration in just 4 h and the maximum depletion (90%) was achieved by 8 h (Figure 1B). First signs of developing pancreatitis, such as oedema and intraperitoneal accumulation

of ascitic fluid, were seen at 4 h, and fulminant severe pancreatitis, as demonstrated by oedema, inflammation and high percentage of necrosis, developed within 24 h after the treatment with zinc [9]. Similar outcome was obtained in mice carrying the same transgene and in mice with tetracycline-inducible SSAT expression [10]. Activation of pancreatic polyamine catabolism by a male infertility agent gossypol likewise resulted in acute pancreatitis in the SSAT transgenic rats [11]. Induction of SSAT and development of partial polyamine depletion in other, non-transgenic models of pancreatitis led us to conclude that activation of polyamine catabolism is a general phenomenon in the pathogenesis of acute pancreatitis [12].

We have used α-methylated polyamine analogues to further verify the causal relationship between polyamine depletion and development of pancreatitis in our model. These analogues are considered to be metabolically stable and at least partially fulfil the physiological role of the natural polyamines, as indicated by their capability to restore growth of polyamine-depleted cells [13,14] and early liver regeneration in transgenic rats with activated polyamine catabolism [15]. Although 1-methylspermidine and 1,12-bismethylspermine are resistant to SSAT-dependent acetylation the latter is converted into the former to some extent *in vivo* [16], which offers a possibility of replenishing the pools of both higher polyamines by administration of the spermine analogue. Pre-treatment of SSAT transgenic rats with methylspermidine before the activation of polyamine catabolism totally prevented acute pancreatitis, as judged by normalized plasma amylase activity and absence of histopathological changes [15]. Both analogues were also beneficial when administrated 4 h after induction, i.e. when severe polyamine depletion was already established and first signs of pancreatic damage were evident [12]. Development of damage was partially prevented, but the most notable finding was that these analogues dramatically protected the animals against the disease-associated mortality [12]. The cause of death of untreated rats with severe pancreatitis was in all likelihood associated with systemic complications due to haemoconcentration, as assessed by the haematocrit values which were fully normalized by treatment with bismethylspermine [12].

Trypsinogen activation in polyamine catabolism-induced acute pancreatitis

It is widely recognized among pancreatologists that the key early event in the onset of pancreatitis is premature trypsinogen activation that is at least partially mediated by lysosomal hydrolases [17]. This interaction is facilitated by the co-localization of these proteins in the same cellular compartment because of perturbed segregation of lysosomal hydrolases and digestive enzymes at the packaging phase in the Golgi complex, fusion of lysosomes with zymogen granules [18] or leakage of these enzymes into the cytoplasm [17]. A lysosomal hydrolase cathepsin B is known to activate trypsinogen and it has been used as a marker of lysosomal enzyme activation in co-localization studies. We analysed the pancreatic

Figure 2 | Activation of pancreatic cathepsin B and trypsinogen in SSAT transgenic rats with induced polyamine catabolism

Where indicated, 1,12-bismethylspermine (Me$_2$Spm) was injected (25 mg/kg intraperitoneally) 20 and 4 h before zinc (10 mg Zn/kg intraperitoneally). Tissue samples were taken at 2 and 4 h after zinc. The amount of cathepsin B was determined from pancreatic homogenates by immunoblotting with cathepsin B antibody (2 μg/ml) (Upstate). The blot shows 25 kDa, double-chain and 30 kDa, single-chain mature forms. Cathepsin B activity was measured with commercial kit (BioVision) and trypsin activity was determined using a synthetic substrate Z-Gly-Pro-Arg-p-nitroanilide (Sigma). Results were normalized to total protein amount in the homogenate and are expressed as fold change compared with the untreated control group. NA, not available.

	Control	2h		4h	
		Zn	Me$_2$Spm+Zn	Zn	Me$_2$Spm+Zn
Cathepsin B	1.0	2.7	1.6	3.8	1.2
Trypsin	1.0	NA	NA	10.7	3.1
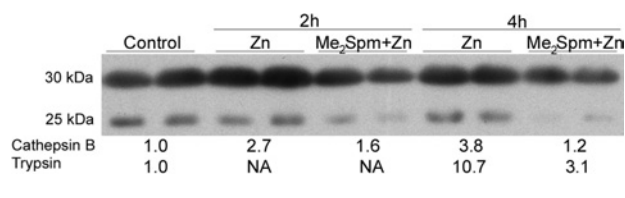
content of active cathepsin B protein as well as the relative enzymatic activity of cathepsin B at early stages of pancreatitis in SSAT transgenic rats. Figure 2 illustrates that both the amount of protein and the relative activity were elevated just 2 h and 4 h after administration of zinc. These increases were mostly prevented by pre-treatment of the rats with bismethylspermine before induction of pancreatitis, indicating that these changes were dependent on polyamine depletion. Pancreatic trypsin activity, the expected result of trypsinogen activation, increased in accordance with the activity of cathepsin B 4 h after induction of pancreatitis and was likewise prevented by pre-treatment with bismethylspermine (Figure 2).

The actual activation of intracellular trypsinogen was studied using pancreatic acinar cells isolated from syngenic and SSAT transgenic rats. With the aid of trypsin-specific substrate we detected zinc dose-dependent activation of trypsinogen in transgenic but not in syngenic cells, indicating the cells' response to the inducer of polyamine catabolism also in culture [12]. Pre-treatment of the animals with bismethylspermine before isolation of the cells completely blocked the trypsinogen activation by zinc, which further strengthens our view that polyamine depletion is an event upstream of trypsinogen activation in the course of pancreatitis.

The early changes in cellular structure of pancreas were investigated using transmission electron microscopy. As depicted in Figure 3, pancreatic ultrastructure was distinctly changed at 6 h after induction of pancreatitis. Compared with the untreated control animals (Figure 3A), activation of polyamine catabolism caused disorganization of endoplasmic reticulum and appearance of shrunken, partially degranulated zymogen granules (Figure 3B), swelling of Golgi apparatus and appearance of decondensed vacuoles (Figure 3C), and appearance of large autophagosomes containing fragments of cellular organelles and zymogen granules (Figure 3D). Mitochondrial damage was manifested as mitochondrial swelling

and destruction of the cristae (Figures 3B–3D). In addition, some parts of the pancreas exhibited whorls in endoplasmic reticulum, necrosis and interstitial oedema (results not shown). It should be noted that the earliest changes, such as dilatation of endoplasmic reticulum, swelling of Golgi apparatus and appearance of decondensed vacuoles, were observed as early as 1 h after induction of pancreatitis (results not shown). These observations clearly indicate that the integrity of cellular components was perturbed in relation to decreasing polyamine levels in the early stages of pancreatitis. Similar changes have also been characterized in other experimental models of acute pancreatitis [19–22]. Although it is not possible to detect the co-localization of lysosomes and zymogen granules with this standard method it is tempting to speculate that rupture of cellular membranes facilitates the direct contact of the proteins of different organelles.

Other mechanisms involved in acute pancreatitis

Reactive oxygen species are considered to play a critical role in the pathogenesis of experimental acute pancreatitis. Polyamines, on the other hand, have been shown to act as antioxidants [23,24]. In addition to general H$_2$O$_2$-producing mechanisms, our SSAT transgenic model has an intrinsic pathway for the enhanced production of potentially harmful molecules, as H$_2$O$_2$ and acetamidopropanal, a reactive aldehyde, are end products in the oxidative catabolism of polyamines catalysed by SSAT and PAO. The contribution of these products to the development of pancreatitis was ruled out by inhibition of PAO by MDL 72527 [9]. The inhibition of PAO in combination with activated SSAT did not protect pancreas from damage but actually resulted in even more severe pancreatitis in transgenic animals, while syngenic animals remained normal [9]. It is, however, possible that depletion of polyamines weakens the cellular defence against reactive oxygen species generated by mechanisms other than polyamine catabolism itself.

The damage of acinar cells causes local inflammatory response, which leads to systemic response in the severe cases of acute pancreatitis. Inflammatory mediators, such as pro-inflammatory cytokines, play a critical role in these processes [1]. Polyamines are immunomodulatory molecules, and spermine especially has been found to possess anti-inflammatory effects associated with regulation of cytokine expression [25,26] and macrophage activation [27]. It is thus possible that polyamine depletion resulting from activation of polyamine catabolism in our transgenic model leads to inhibition of counterregulatory mechanisms exerted by spermine, allowing the inflammatory processes to proceed. Histological examination of pancreas at early phases after induction of polyamine catabolism in SSAT transgenic rats showed little indication of local inflammation although the presence of inflammatory cells was clearly evident 24 h after induction [9,15]. A thorough analysis of inflammatory factors will eventually reveal their contribution to the pathogenesis of pancreatitis induced by activated polyamine catabolism.

Figure 3 | **Ultrastructural changes in the early phases of polyamine catabolism-induced acute pancreatitis**

Untreated control rats had normal pancreatic appearance with abundant and undilated rough ER (endoplasmic reticulum) and numerous electron dense ZG (zymogen granules) in the apical pole facing the lumen of the acinus (**A**). Specimens taken at 6 h after induction of pancreatitis showed dilatation of endoplasmic reticulum and shrunken, partially degranulated zymogen granules (**B**), and decondensed maturing zymogen granules (condensing vacuoles, CV) associated with swollen Golgi complex (G) (**C**). Mitochondrial damage (M) (**B**, **C**, **D**) and the appearance of large autophagosomes (arrow) were also evident (**D**). Magnifications: (**A**) 7500×; (**B**) 25 000×; (**C**) 20 000×; (**D**) 10 000×.
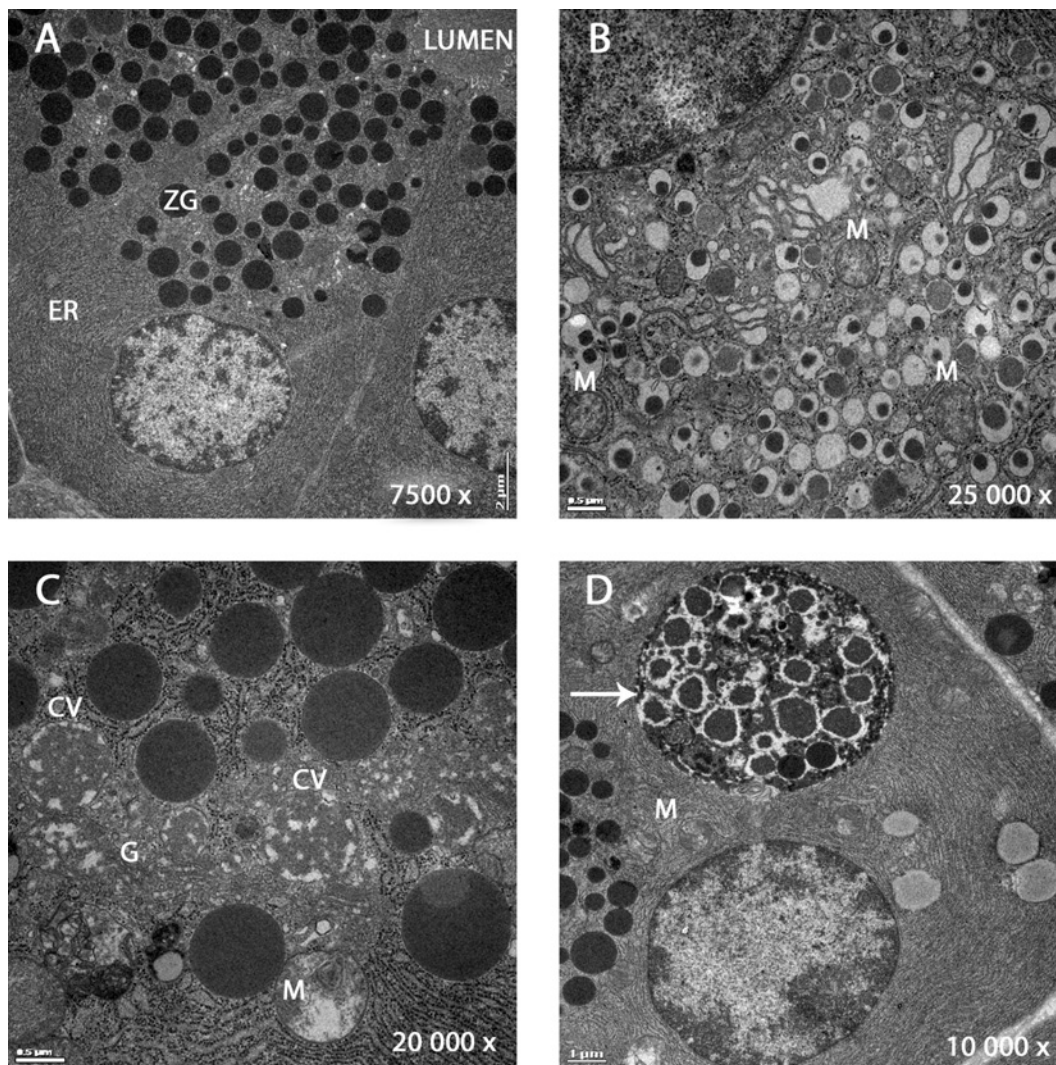
Conclusions

Activation of polyamine catabolism in SSAT transgenic model results in a very rapid and, in molar terms, massive disappearance of spermidine in particular. Trypsinogen activation in isolated acinar cells as well as pancreatic cathepsin B activation, increase in trypsin activity and disturbances in acinar cell structure were all seen within the first 4 h of induction of pancreatitis. These findings tend to suggest that polyamine depletion triggers the co-localization of lysosomal hydrolases and zymogens. This event is possibly facilitated by disruption of membrane structures due to polyamine depletion but the exact mechanism needs to be elucidated.

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